

CCL11 blocks IL-4 and GM-CSF signaling in hematopoietic cells and hinders dendritic cell differentiation via suppressor of cytokine signaling expression

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Abstract: The chemokine eotaxin/CCL11 is an important mediator of leukocyte migration, but its effect on inflammatory cytokine signaling has not been explored. In this study, we find that CCL11 induces suppressor of cytokine signaling (SOCS)1 and SOCS3 expression in murine macrophages, human monocytes, and dendritic cells (DCs). We also discover that CCL11 inhibits GM-CSF-mediated STAT5 activation and IL-4-induced STAT6 activation in a range of hematopoietic cells. This blockade of cytokine signaling by CCL11 results in reduced differentiation and endocytic ability of DCs, implicating CCL11-induced SOCS as mediators of chemotactic inflammatory control. These findings demonstrate cross-talk between chemokine and cytokine responses, suggesting that myeloid cells tracking to the inflammatory site do not differentiate in the presence of this chemokine, revealing another role for SOCS in inflammatory regulation. *J. Leukoc. Biol.* 85: 289–297; 2009.

Key words: dendritic cells · monocytes/macrophages · chemokines · signal transduction

INTRODUCTION

Chemoattractant cytokines (chemokines) are a large family of secreted proteins that regulate migration and activation of a wide range of cell types including monocytes, macrophages, T cells, and platelets [1]. Chemokines produce their biological effect by interacting with specific seven-transmembrane G protein-coupled receptors on the surface of their target cells [2] and play an important role in attracting immune cells to sites of inflammation, while also influencing their response to the environment [3]. CCL11 is a prominent chemokine, which signals exclusively through CCR3 and is involved in the recruitment of eosinophils, macrophages, and dendritic cells (DCs) to the site of allergic inflammation [4, 5].

Cytokines regulate many cellular processes, including growth, proliferation, differentiation, and migration of leuko-

cytes and often signal through the Jak/STAT pathway [6]. GM-CSF is a multifunctional cytokine, which supports proliferation, survival, and differentiation of hematopoietic progenitor cells, as well as enhancing the inflammatory potential of mature cells. In fact, GM-CSF has physiological roles in mature macrophage activation and differentiation of both macrophages [7]. Studies about macrophages and neutrophils illustrated that GM-CSF could activate these cells to survive better, release inflammatory mediators, and kill certain organisms, thereby confirming the role of GM-CSF in enhancing innate immunity [8]. GM-CSF can signal through the Jak/STAT pathway with specific activation of Jak2, STAT3, and STAT5 [9, 10], which is controlled by the expression of the inhibitory proteins suppressor of cytokine signaling (SOCS)1, -2, and -3 [11, 12].

IL-4 is also a multipotent cytokine, acting on a spectrum of cell types to modulate host defense and immunity. IL-4 is classically described as a T_H2 cytokine, promoting humoral immunity and opposing T_H1-dependent inflammation, being released by CD4⁺ T_H2 T cells, and increasing MHC II expression on resting B cells [13]. However, it is increasingly documented that IL-4 is associated with proinflammatory type I immune responses, including its joint role with GM-CSF, in the differentiation of monocytes to DCs [14] and the modulation of macrophage activation [15, 16]. As with GM-CSF IL-4 signals through the Jak/STAT pathway, and its signal propagation is inhibited by SOCS1 and SOCS3 [17, 18].

SOCS are cytoplasmic proteins that complete a negative-feedback loop to attenuate signal transduction from cytokines that signal through the Jak/STAT pathway. There are eight members of the SOCS family (cytokine-inducible SH2-contain-

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Received July 1, 2008; revised September 22, 2008; accepted September 30, 2008.

doi: 10.1189/jlb.0708394

ing protein and SOCS1–7), each of which contains a central SH2 domain, a C-terminal 40-aa motif known as the SOCS box, and an N-terminal domain of variable length and sequence [19–22]. SOCS proteins are induced by a variety of stimuli including inflammatory cytokines such as IFN- γ , innate immune stimulatory factors such as LPS, and growth factors such as G-CSF [23, 24]. SOCS proteins inhibit signaling by binding membrane proximal intermediates, such as Jak kinases and tyrosine-phosphorylated residues, on the cytoplasmic portion of the receptors. Recent reports suggest that SOCS expression can be induced by chemoattractant stimulation. More specifically, SOCS1 and SOCS3 are induced by stromal cell-derived factor-1 α /CXCL12, and we have shown that SOCS1 is induced by IL-8/CXCL8 and fMLP [25–27]. Chemokines induce down-regulation of other chemotactic signaling pathways by a process known as nonspecific desensitization [28]. However, the role of chemokines in regulating other signaling pathways is poorly understood, but this study proposes that this cross-talk is prominent during inflammation and regulated by SOCS proteins.

Here, we demonstrate that CCL11 significantly impairs responses to IL-4 and GM-CSF in a range of hematopoietic cells. Furthermore, we show that CCL11 can hinder DC differentiation from monocytes, resulting in a down-regulation of DC endocytic capacity through the induction of SOCS, demonstrating a possible mechanism for the observed inhibition of GM-CSF- and IL-4-mediated responses. Therefore, our data suggest that CCL11-induced SOCS proteins can block IL-4 and GM-CSF signaling, thus controlling immune cell phenotypes at the site of allergic inflammation.

MATERIALS AND METHODS

Cell culture conditions

RAW264.7 macrophages were grown in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 250 U/ml penicillin, and 250 μ g/ml streptomycin. Cells were cultured at 37°C in 5% CO₂ and 95% humidity. Primary murine macrophages were cultured in OptiMEM, supplemented with 2 mM L-glutamine, 250 U/ml penicillin, and 250 μ g/ml streptomycin. For cytokine/chemokine stimulation, cells were treated with 20 ng/ml human IL-4, 20 ng/ml human GM-CSF, or 100 ng/ml human CXCL1, CXCL2, CXCL10, CXCL12, CCL2, CCL5, and CCL11 (Peptrotech, Rocky Hill, NJ, USA). Cells were treated with 100 ng/ml pertussis toxin (P. toxin; Sigma Chemical Co., St. Louis, MO, USA).

Isolation of murine primary macrophages

C57BL/6 mice were given peritoneal injections of 1 ml 2% (w/v) BIOgel beads (BioRad, Hercules, CA, USA). Elicited macrophages were harvested by lavage on Day 4 with ice-cold PBS/5 mM EDTA. Peritoneal exudates were pelleted and resuspended in prewarmed OptiMEM, supplemented with 2 mM L-glutamine, 250 U/ml penicillin G sodium, and 250 μ g/ml streptomycin sulfate. Cells were plated at 2×10^6 macrophage per 35 mm dish and cultured at 37°C, 5% CO₂, and 95% humidity. Two hours after initial plating, cells were washed twice in ice-cold sterile PBS, and the appropriate stimulation was added. All experiments were carried out according to Home Office guidelines under the appropriate project license following local ethical review.

Real-time RT-PCR and RT-PCR analysis

Total RNA was isolated from murine primary macrophages (2×10^6 cells) or monocyte-derived DCs (MDDCs; 10×10^6 cells) following the Trizol reagent manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). RT-PCR was per-

formed with 1 μ g total cell RNA using Moloney murine leukemia virus RT enzyme, according to the manufacturer's instructions (Promega, Madison, WI, USA). PCR amplification was performed using primer pairs specific for SOCS1 (forward primer 5'-GAGAGCTTCGACTGCCTCTT-3'; reverse primer 5'-AGGTAGGAGCTGCCAGTTCA-3'), SOCS3 (forward primer 5'-CTCAAGACCTTCAGCTCCAA-3'; reverse primer 5'-TTCTCATAGGAGTCCAGGTG-3'), and β -actin (forward primer 5'-GGACTTCGAGCAAGAGATGG-3'; reverse primer 5'-AGCACTGTGTGGCGTACAG-3'). The PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining.

Taqman real-time RT-PCR reactions were performed on a Rotorgene 3000 (Corbett Life Science, Sydney, Australia) using primer pairs and probe specific for murine SOCS1 (forward primer 5'-GCATCCCTTTAACC CGGTACT-3'; reverse primer 5'-ATAAGCGCGCCCCACTTAAT-3'; probe 5'-TGACTACCTGAGTTCCCTTCCCTTCCAGATCT-3'), SOCS3 (forward primer 5'-CACCTG-GACTCCTATGAGAAAGTGA-3'; reverse primer 5'-CCTCTGACCCCTTTT-GCTCCTTA-3'; probe 5'-CTGCCTGGACCCATTCCGGAGTTC-3'), SOCS5 (forward primer 5'-GAGAAGACGGCTTAGTATCGAAGAA-3'; reverse primer 5'-GAGAAGACGGCTTAGTATCGAAGAA-3'; probe 5'-TGGATC-CCCCTCCCAACGCAC-3'), and hypoxanthine guanine phosphoribosyl transferase (HPRT; forward primer 5'-GACCGGTCCCGTCATGC-3'; reverse primer 5'-TCATAACCTGGTTCATCATCGC-3'; probe 5'-ACCCGCAGTC-CCAGCGTCGTG-3'; Eurogentec, San Diego, CA, USA). Reactions were incubated for 2 min at 50°C, denatured for 10 min at 95°C, and subjected to 40 two-step amplification cycles with annealing/extension at 60°C for 1 min, followed by denaturation at 95°C for 15 s. Results are normalized to HPRT expression and presented as fold increase in mRNA expression compared with the level detected in untreated samples.

Immunoprecipitation and immunoblot analysis of protein induction

To monitor phosphorylation of STAT proteins in primary murine macrophages, human PBMCs, monocytes, or Day 4 MDDCs, 1×10^7 , were stimulated with the appropriate cytokines, including 20 ng/ml IL-4, 20 ng/ml GM-CSF, or 100 ng/ml CCL11, for the times indicated and harvested in lysis buffer supplemented with aprotinin (5 μ g/ml), leupeptin (5 μ g/ml), PMSF (1 mM), and Na₃VO₄ (1 mM). Murine macrophage lysates underwent immunoprecipitation with anti-STAT5 and -STAT6 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and were then analyzed by Western blot analysis for STAT phosphorylation using the antiphosphotyrosine antibody PY20 (BD Transduction Laboratories, Franklin Lakes, NJ, USA). The blots were then re-probed with antibodies directed against STAT5 and STAT6 (Santa Cruz Biotechnology). PBMC whole cell lysates were analyzed by Western blotting using specific phosphorylated (p)STAT6 (New England Biolabs, Boston, MA, USA) and pSTAT6 (Santa Cruz Biotechnology) antibodies. Monocytes and Day 4 MDDC whole cell lysates were analyzed by Western blotting using a specific pSTAT5 (Y694) antibody (New England Biolabs). The blots were then re-probed with STAT5 antibody (Santa Cruz Biotechnology). SOCS1 (Zymed, San Francisco, CA, USA), SOCS3 (Santa Cruz Biotechnology), and β -actin (Sigma Chemical Co.) levels were also analyzed in murine macrophages and human monocytes treated with 100 ng/ml CCL11 by Western blotting.

PBMC and monocyte isolation and the generation of DCs and macrophages

PBMCs and monocytes were isolated from 300 ml whole blood, obtained from healthy donors who had given informed consent, modified from a method described previously [29]. DCs were generated from monocytes for over 7 days, following the protocol described previously, and on Day 4, the culture medium was replaced with fresh medium containing IL-4 and GM-CSF. In certain experiments, CCL11 (100 ng/ml) was also added for 6 h before GM-CSF or IL-4 on Day 0, Day 4, or both days. If CCL11 was only added on Day 0, it was not replaced on Day 4 along with the cytokines.

If Day 4 MDDCs were to be analyzed, the cells were resuspended in fresh RPMI medium, supplemented with 10% FCS, 1% sodium pyruvate, 5×10^{-5} M 2-ME, 1% L-glutamine, 1% nonessential amino acids, 1% penicillin, and 1% streptomycin for 3 h before being treated with GM-CSF (20 ng/ml) and CCL11 (100 ng/ml), and intracellular signaling was analyzed by Western blotting or RT-PCR.

To differentiate the cells into macrophages, the isolated monocytes (10×10^6 cells) were cultured in the above medium for 7 days, while refreshing the medium on Day 4.

Flow cytometry

The cells were harvested by scraping into the medium. After centrifugation for 5 min at 224G, the cells were washed twice in PBS and 5% AB serum and then stained at $+4^\circ\text{C}$ for 15 min with human CD80-PE, human CD1a-FITC, human IL-4R-FITC (eBioscience, San Diego, CA, USA), human CCR3-FITC, murine CCR3-allophycocyanin (APC), human GM-CSFR-PE, human IL-4R-PE (BD Biosciences), or the corresponding labeled isotype control antibodies. They were post-fixed in 1% paraformaldehyde and analyzed using a Coulter EPICS flow cytometer, which was calibrated daily; FlowCheck beads (Beckman Coulter, High Wickham, UK) were checked for each sample to ensure accurate reading of staining. The samples were gated on forward- and side-scatter and 1×10^4 cells collected. Results were analyzed using WinMDI software.

Endocytosis assay with FITC-dextran

On Day 7, 50 μL FITC-dextran (2 $\mu\text{g}/\mu\text{L}$; Sigma Chemical Co.) was added to DCs grown with/without CCL11 (added on Days 0 and 7). One-half was incubated at $+37^\circ\text{C}$ and one-half at $+4^\circ\text{C}$ for 15 min. After incubation, the cells were washed four times with cold PBS and analyzed by flow cytometry.

RESULTS

CCL11 uniquely induces SOCS1 and SOCS3 expression via $G_{\text{inhibitory (i)}}$ protein-coupled receptors

SOCS are induced by a number of chemotactic stimuli [25–27]; however, the effect of CCL11 upon SOCS expression has not been determined. Therefore, as CCL11 is known to be an inflammatory mediator for hematopoietic cells, we initially investigated its effect on SOCS1 and SOCS3 expression in primary murine macrophages. CCL11 was administered over an 8-h time course, and real-time RT-PCR was carried out to analyze SOCS mRNA levels. **Figure 1A** shows that following CCL11 stimulation, SOCS1 and SOCS3 mRNA expression was up-regulated at 6 h by four- and fivefold, respectively. CCL11-induced SOCS protein expression was also confirmed by Western blotting (Fig. 1A, insets). To determine if SOCS were induced directly by CCL11 rather than in an autocrine manner, unstimulated macrophages were treated with supernatant from macrophages stimulated with CCL11. No SOCS3 mRNA was induced in response to supernatant, ruling out the possibility of an autocrine factor being responsible for this induction and strongly indicating that CCL11 induces SOCS directly.

Having observed that CCL11 could up-regulate SOCS1 and SOCS3 expression in primary macrophages, we next analyzed whether this was specific to CCL11 alone. Murine macrophages were treated with CXCL1, CXCL2, CXCL10, CXCL12, CCL2, CCL5, CCL22, and CCL11 before SOCS1, SOCS3, and SOCS5 mRNA were measured relative to basal levels. Figure 1C shows that among a spectrum of chemokines, SOCS1 and SOCS3 mRNA only increased in response to CCL11. SOCS5 mRNA levels were relatively unchanged by any chemokine. Therefore, these results strongly indicate that among a range of chemokines, CCL11 is unique in its ability to induce SOCS1 and SOCS3 expression in macrophages.

Chemokines signal via G_i protein-coupled receptors; therefore, it was essential to determine if SOCS induction was mediated in this manner. Chemokine signaling is mediated predominantly through the P. toxin-sensitive G_i protein. P. toxin catalyzes the ADP ribosylation of the α subunit of the heterotrimeric G_i protein, thus preventing interaction with G protein-coupled receptors, which interferes with intracellular signaling. Therefore, primary murine macrophages were pretreated with P. toxin for 2 h before CCL11 treatment. P. toxin treatment blocked SOCS3 expression in response to CCL11, indicating that SOCS induction by CCL11 is mediated through G_i proteins (Fig. 1D). To confirm that murine macrophages express CCR3, cell-surface expression was analyzed by flow cytometry (Fig. 1E).

CCL11 blocks IL-4 and GM-CSF signaling in primary murine macrophages

At the site of inflammation, there are numerous mediators present, and their intracellular interactions are only beginning to be understood. Having observed that CCL11 could induce SOCS1 and SOCS3 in murine macrophages, we next investigated whether the induction of these inhibitory proteins by CCL11 would have an impact on the signaling of cytokines that are found at inflammatory sites, such as GM-CSF and IL-4. Therefore, primary murine macrophages were pretreated with or without CCL11 for 6 h to induce SOCS1 and SOCS3, observed previously before being exposed to a time course of GM-CSF or IL-4. Subsequently, the cell lysates were analyzed by Western blotting for STAT5 and STAT6 phosphorylation (**Fig. 2, A and B**). Pretreatment of the cells with CCL11 for 6 h inhibited GM-CSF- and IL-4-induced phosphorylation of STAT5 and STAT6, respectively. Densitometric analysis (calculated by normalizing each band to its β -actin control) confirmed a strong decrease in pSTAT6 and pSTAT5 signaling. In CCL11-pretreated cells, pSTAT6 and pSTAT5, after 5 min cytokine stimulation, decreased from a 83.6 to 4.7- and 3.4 to 1.2-fold change of stimulated/unstimulated band intensities, respectively.

Cell-surface IL-4R (Fig. 2A) and GM-CSFR (Fig. 2B) levels were analyzed after 6 h CCL11 treatment. CCL11 stimulation had no significant effect on IL-4 or GM-CSF receptor expression and is therefore not responsible for the observed inhibition of IL-4 and GM-CSF signaling. These data provide clear evidence for CCL11 blocking IL-4 and GM-CSF signal transduction and indicate the possible involvement of CCL11-induced SOCS in cross-talk between chemoattractant and cytokine signaling pathways.

CCL11 induces SOCS1 and SOCS3 in human hematopoietic cells and inhibits proinflammatory cytokine signaling

As IL-4 and GM-CSF mediate DC differentiation, we next investigated if their signal transduction could be blocked by CCL11 in human hematopoietic cells that may result in altered DC phenotypes. Before treating with CCL11, CCR3 levels were confirmed $\pm$ CCL11 in monocytes (**Fig. 3C**) and Day 4 DCs

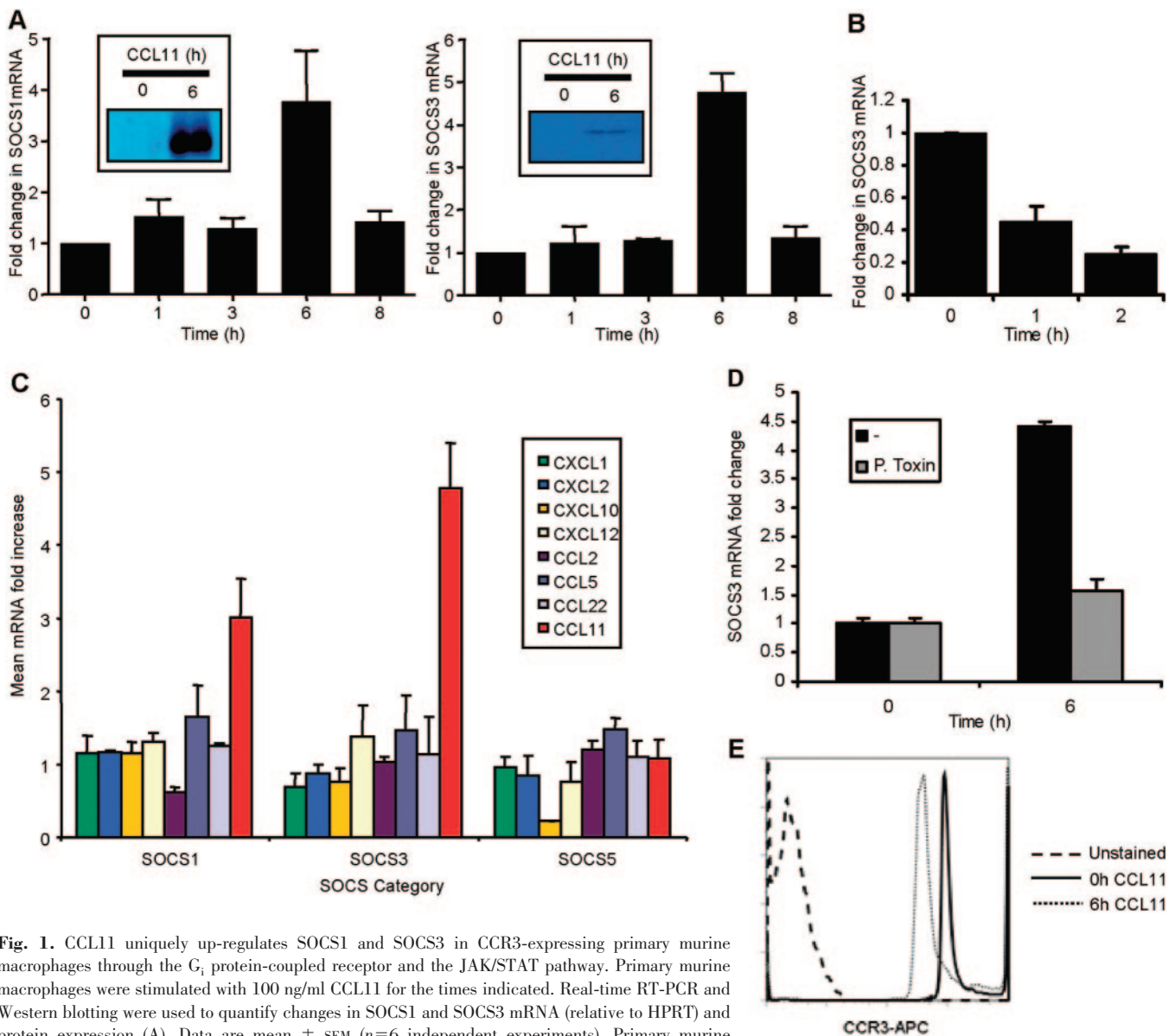


Fig. 1. CCL11 uniquely up-regulates SOCS1 and SOCS3 in CCR3-expressing primary murine macrophages through the G_i protein-coupled receptor and the JAK/STAT pathway. Primary murine macrophages were stimulated with 100 ng/ml CCL11 for the times indicated. Real-time RT-PCR and Western blotting were used to quantify changes in SOCS1 and SOCS3 mRNA (relative to HPRT) and protein expression (A). Data are mean $\pm$ SEM ($n=6$ independent experiments). Primary murine macrophages were treated with 100 ng/ml CCL11 for 4 h, and supernatant was added to fresh cells for 0, 1, and 2 h before SOCS3 mRNA levels were analyzed by real-time RT-PCR relative to HPRT ($n=3$ independent experiments; B). Primary murine macrophages were treated with 100 ng/ml CXCL1, CXCL2, CXCL10, CXCL12, CCL2, CCL5, CCL22, or CCL11 for 6 h. Real-time RT-PCR was used to quantify changes in SOCS1, SOCS3, and SOCS5 mRNA expression relative to HPRT (C; $n=3$ independent experiments). Primary murine macrophages were treated with/without 100 ng/ml P. toxin for 2 h before being stimulated with 100 ng/ml CCL11 for 6 h. Real-time RT-PCR was used to quantify changes in SOCS3 mRNA expression relative to HPRT (D; $n=3$ independent experiments). Primary murine macrophages were treated with/without 100 ng/ml CCL11 for 6 h before CCR3 levels were measured by flow cytometry ($n=3$ independent experiments; E).

(Fig. 3F). As SOCS1 and SOCS3 levels were induced upon CCL11 pretreatment in monocytes (Fig. 3A) and "Day 4" DCs (Fig. 3D) within 60 min, we next treated monocytes and Day 4 differentiating DCs with/without CCL11 before GM-CSF. We discovered that GM-CSF-induced STAT5 phosphorylation was reduced significantly upon CCL11 pretreatment of monocytes (Fig. 3A) and Day 4 DCs (Fig. 3C). Neither IL-4R nor GM-CSFR expression decreased significantly after 60 min CCL11 treatment in monocytes (Fig. 3C) or Day 4 DCs (Fig. 3F), indicating that the observed blockade of signal transduction was not a result of receptor internalization. Next, PBMCs were pretreated with CCL11 before being stimulated with/without IL-4. STAT6 signaling was analyzed by Western blotting, and

the results show that IL-4-induced STAT6 phosphorylation was reduced significantly upon pretreatment with CCL11 (Fig. 3G), further implicating SOCS proteins as possible mediators of the inhibition of CCL11. Therefore, these results strongly indicate that SOCS may be involved in the inhibition of cytokine signaling during DC differentiation of human monocytes.

CCL11 blocks DC differentiation and reduces functional endocytic capacity

Having observed CCL11-mediated SOCS induction in primary murine macrophages, human monocytes, and Day 4 DCs, we were interested in confirming the exact nature of a SOCS1 and SOCS3 increase in DCs and if these inhibitory proteins could

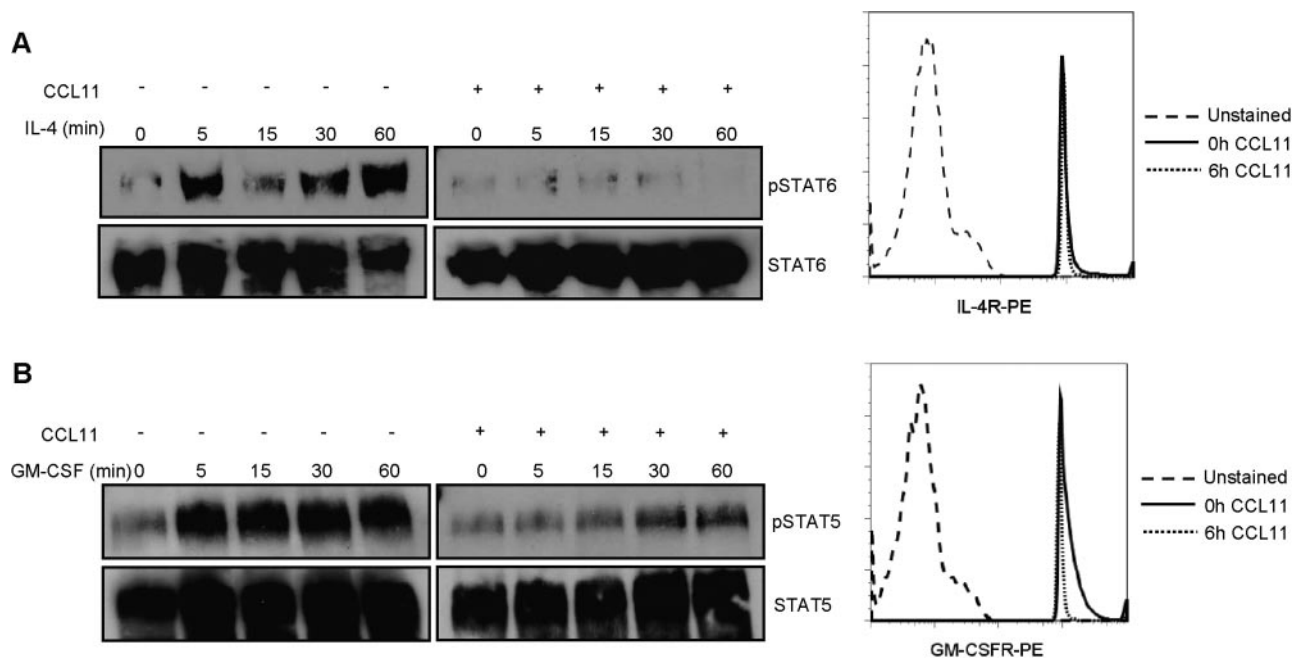


Fig. 2. CCL11 abrogates signal transduction of the proinflammatory cytokines IL-4 and GM-CSF. Primary murine macrophages were pretreated $\pm$ 100 ng/ml CCL11 for 6 h and stimulated with 20 ng/ml IL-4 or 20 ng/ml GM-CSF for the times indicated. Subsequent activation of STAT6 by IL-4 (A) and STAT5 by GM-CSF (B) was monitored by immunoprecipitation with specific STAT6 and STAT5 antibodies, respectively. The CCL11-treated and nontreated lysates were analyzed by PAGE using two separate gels in the same electrophoresis tank before being immunoblotted with specific antiphosphotyrosine or anti-STAT antibodies. IL-4R (A) and GM-CSFR (B) levels were analyzed by FACS in primary murine macrophages treated $\pm$ CCL11 (100 ng/ml) for 6 h. Data are representative of three independent experiments.

mediate an altered DC phenotype as a result of blocked cytokine signaling. Therefore, DCs generated from monocytes cultured in GM-CSF and IL-4 for 7 days were treated with CCL11 for 0–8 h before SOCS1 and SOCS3 levels were measured by RT-PCR. **Figure 4A** shows that SOCS1 and SOCS3 were up-regulated rapidly after 30 min of CCL11 treatment. SOCS1 levels remained high, and SOCS3 levels were more variable but remained above basal levels throughout. Taken together, these data show that SOCS1 and SOCS3 are up-regulated during DC responses to CCL11, strongly implicating these proteins in the CCL11-mediated inhibition of IL-4/GM-CSF signaling.

Monocytes are commonly differentiated *in vitro* to DCs using IL-4 and GM-CSF. Therefore, as CCL11 induced SOCS1 and SOCS3 in DCs and blocked IL-4 and GM-CSF signaling in macrophages and DCs, we investigated whether CCL11 could maintain a monocytes/macrophage phenotype even in the presence of mediators that induce DC differentiation. Consequently, we analyzed whether pretreatment with CCL11 to induce SOCS expression could affect GM-CSF/IL-4-mediated DC differentiation. To distinguish between DCs and monocytes/macrophages during the differentiation process, we analyzed the expression of established DC markers, CD1a and CD80. We isolated human monocytes from whole blood by Ficoll Paque gradient centrifugation (Fig. 4, B and C, Bars 1) and generated macrophages from monocytes cultured for 7 days (Fig. 4, B and C, Bars 2). DCs were produced by culturing monocytes in GM-CSF and IL-4 for 7 days (Fig. 4, B and C, Bars 3). In Figure 4, B and C, Bars 4–6, the monocytes were treated as in Bars 3 to generate DC cells but with the addition

of CCL11 6 h before GM-CSF and IL-4 treatment on Days 0 and 4 (Fig. 4, B and C, Bars 4) or only on Day 0 (Fig. 4, B and C, Bars 5) or only on Day 4 (Fig. 4, B and C, Bars 6). DC differentiation causes a significant rise in CD1a and CD80 levels compared with monocyte or macrophage phenotypes. However, with the addition of CCL11 on Days 0 and 4, the levels of CD1a and CD80 remained low (Fig. 4, B and C, Bars 4), indicating that the CCL11 could block GM-CSF/IL-4-induced differentiation, and a monocyte/macrophage phenotype was maintained. Interestingly, even if CCL11 were only added on Day 0 (Fig. 4, B and C, Bars 5) or Day 4 (Fig. 4, B and C, Bars 6), neither the CD1a nor CD80 levels rose to DC levels. These results clearly show that CCL11 inhibits the action of GM-CSF and IL-4 in mediating DC differentiation and maintains a monocyte/macrophage phenotype.

We also investigated the functional endocytic capacity of cells to take up FITC-dextran if generated in IL-4 and GM-CSF, with or without CCL11 (added on Days 0 and 4). CCL11 inhibited the uptake of FITC-dextran and its accumulation compared with DCs grown with IL-4 and GM-CSF alone (Fig. 4D), indicating that these cells may have low endocytic ability. Furthermore, IL-4R and GM-CSFR levels, upon CCL11 treatment, were reduced, and GM-CSFR showed a significant decrease (Fig. 4, E and F), indicating that a block in GM-CSF/IL-4-mediated DC differentiation may have been in part a result of reduced cytokine receptor levels. Taken together, these findings demonstrate that DC differentiation and functional ability to endocytose are hindered by CCL11, and SOCS induction and cytokine receptor internalization by this chemokine mediate this controlled inhibition.

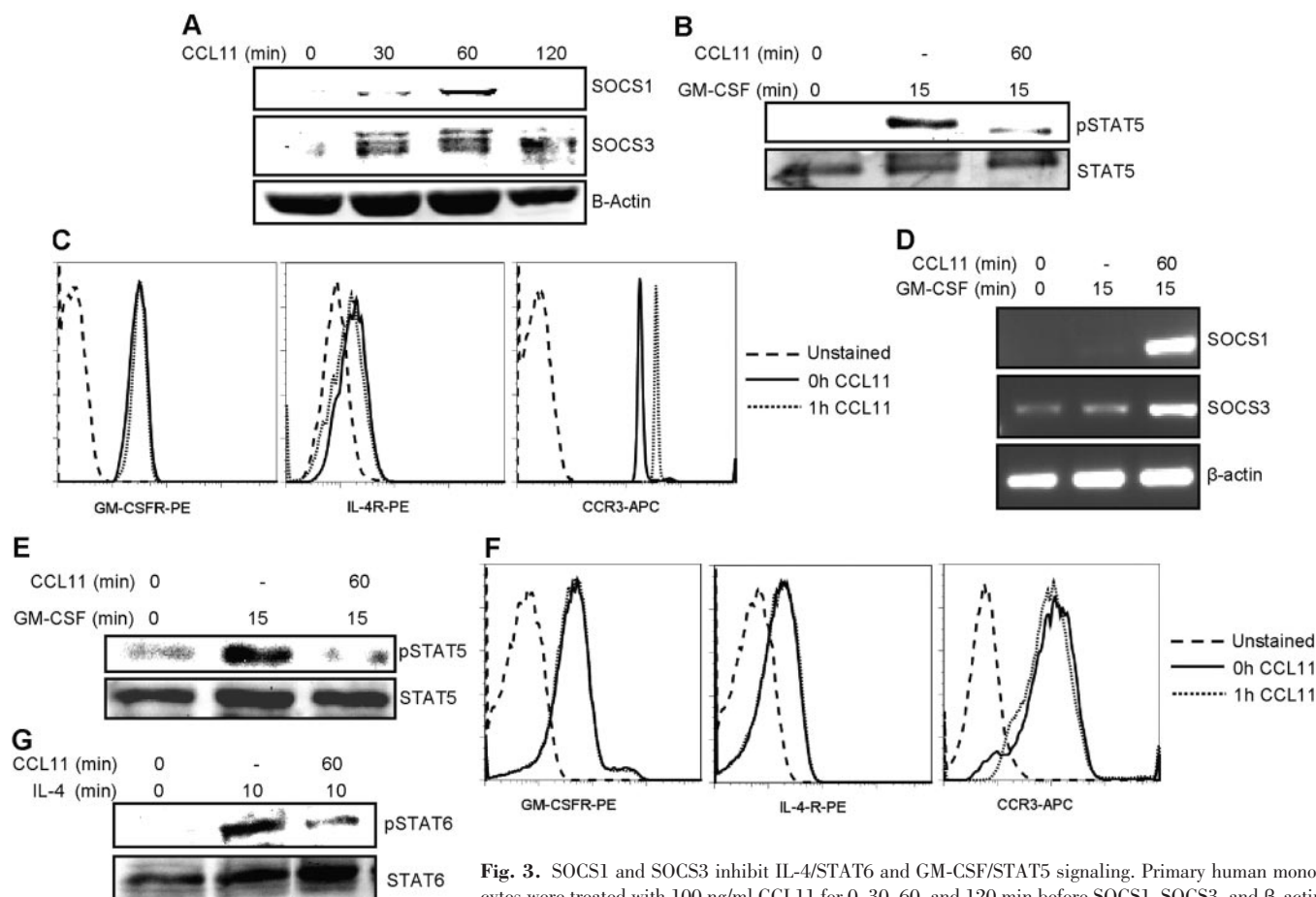


Fig. 3. SOCS1 and SOCS3 inhibit IL-4/STAT6 and GM-CSF/STAT5 signaling. Primary human monocytes were treated with 100 ng/ml CCL11 for 0, 30, 60, and 120 min before SOCS1, SOCS3, and β -actin levels were analyzed by Western blotting (A). Primary human monocytes were pretreated $\pm$ 100 ng/ml CCL11 for 60 min before being stimulated with 20 ng/ml GM-CSF for 15 min, before STAT5 phosphorylation was monitored by Western blotting whole cell lysates with specific antiphosphotyrosine or anti-STAT5 antibodies (B). Monocytes were treated with/without CCL11 (100 ng/ml) for 1 h, and GM-CSFR, IL-4R, and CCR3 levels were analyzed by flow cytometry (C). Day 4 DCs were pretreated $\pm$ 100 ng/ml CCL11 for 60 min before being stimulated with 20 ng/ml GM-CSF for 15 min, and RT-PCR was performed using 1 μ g total RNA using primers specific for SOCS1, SOCS3, and β -actin (D). Day 4 DCs were pretreated $\pm$ 100 ng/ml CCL11 for 60 min before being stimulated with 20 ng/ml GM-CSF for 15 min before STAT5 phosphorylation was monitored by Western blotting whole cell lysates with specific antiphosphotyrosine or anti-STAT5 antibodies (E). Day 4 DCs were treated with/without CCL11 (100 ng/ml) for 1 h, and GM-CSFR, IL-4R, and CCR3 levels were analyzed by flow cytometry (F). PBMCs were pretreated $\pm$ 100 ng/ml CCL11 for 60 min before being stimulated with 20 ng/ml IL-4 for 10 min. Subsequent STAT6 phosphorylation was monitored in PBMCs (G) by Western blotting whole cell lysates with specific antiphosphotyrosine or anti-STAT6 antibodies. All data are representative of three independent experiments.

DISCUSSION

Our findings demonstrate that CCL11 uniquely induces SOCS1 and SOCS3 and a blockade in GM-CSF and IL-4 signaling. We also show that treatment of monocytes or differentiating DCs with CCL11 reduces IL-4/GM-CSF-mediated DC differentiation and the functional ability to endocytose antigen. These data suggest that CCL11 is a potent inhibitor of GM-CSF and IL-4 inflammatory responses in primary hematopoietic cells and blocks the differentiation of monocytes to functional DCs through the induction of SOCS1 and SOCS3.

Although CCL11 has recently been accepted to attract numerous cell types, including DCs [5], the exact nature of its effect on macrophages is far from clear. Along with IL-4, GM-CSF has important roles in macrophage biology [32]; therefore, we examined whether CCL11 treatment of primary murine macrophages affected responses to IL-4 or GM-CSF. The phosphorylation of STAT5 and STAT6 upon stimulation with GM-CSF and IL-4, respectively, has been studied

extensively [29], and our data demonstrate that this phosphorylation was inhibited strongly in primary macrophages pretreated with CCL11. IL-4 and GM-CSF potentiate macrophage inflammation [30–33], and GM-CSF induces macrophage growth and differentiation [34]. All of these responses must be controlled or abrogated at the allergic inflammatory site, and the inhibitory signals of CCL11 may allow the cells to respond to inflammatory stimuli with the appropriate strength, thus avoiding detrimental and overzealous consequences. CCL11 may also inhibit these intracellular pathways to potentiate its own signal transduction and thus, generate an appropriate response to allergic stimuli. Therefore, it seems that CCL11 may be at the top of an inhibitory hierarchy, which often exists in the world of chemoattractant intracellular signaling [35, 36] and allows inflammatory events to progress effectively in a controlled manner. This overall inhibition shows that the macrophage is desensitized to these cytokines after CCL11 treatment and suggests a wider regulatory role for CCL11 apart from

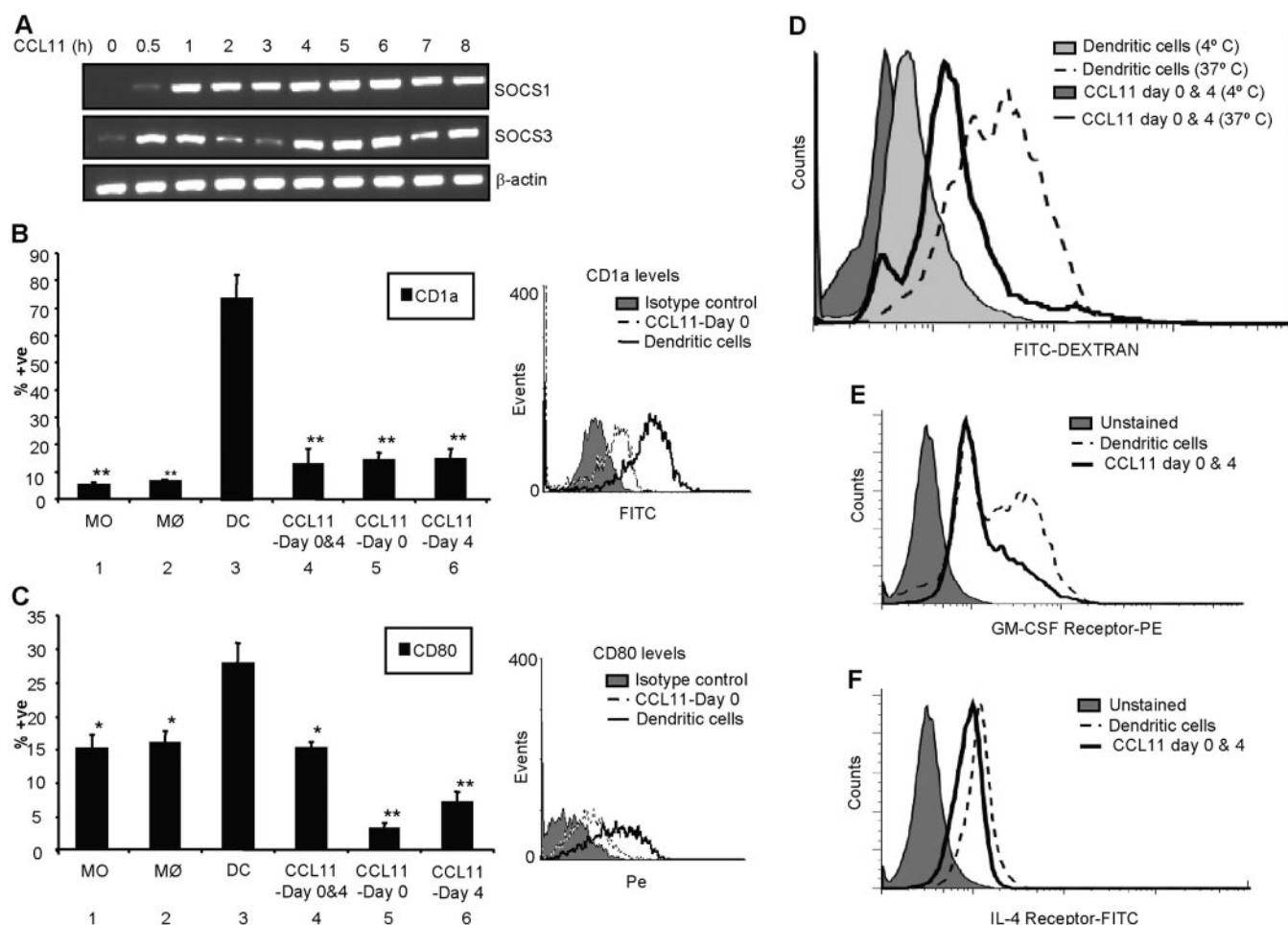


Fig. 4. CCL11 induces SOCS1 and SOCS3 expression and inhibits the differentiation and functional endocytic capacity of MDDCs without reducing GM-CSFR or IL-4R levels. RT-PCR was performed using 1 µg total RNA from Day 7 human MDDCs treated with 100 ng/ml CCL11 for the times indicated using primers specific for SOCS1, SOCS3, and β-actin (A; $n=3$ independent experiments). MDDCs were separated and cultured as described in Materials and Methods. MO, Monocytes isolated from whole blood; MØ, monocytes were cultured without cytokines for 7 days and differentiated into macrophages; DC, monocytes were differentiated into DCs after 7 days with the addition of 100 U/ml IL-4 and 50 ng/ml GM-CSF on Days 0 and 4; CCL11-Day 0&4, monocytes were cultured as with DCs with the addition of 100 ng/ml CCL11 for 6 h before 50 ng/ml GM-CSF and 100 U/ml IL-4 treatment on Days 0 and 4; CCL11-Day 0, monocytes were cultured as with DCs with the addition of 100 ng/ml CCL11 6 h before 50 ng/ml GM-CSF and 100 U/ml IL-4 on Day 0; CCL11-Day 4, monocytes were cultured as with DCs with the addition of 100 ng/ml CCL11 for 6 h before 50 ng/ml GM-CSF and 100 U/ml IL-4 on Day 4. Cells were harvested before CD80 and CD1a expression was measured by flow cytometry. Cells were normalized to isotype control, and the bars show percentage positivity $\pm$ SEM. Significance was measured against DC category; *, $P < 0.05$, and **, $P < 0.01$ ($n=3$). Histogram inlays represent corresponding data for CD1a (B) or CD80 (C) obtained from the isotype control (filled histogram), DCs (dark line), or CCL11-Day 0 cells (broken line). On Day 7, DCs and CCL11-Day 0&4 were incubated with 50 µL FITC-dextran (2 µg/µL) at +4°C and +37°C for 10 min and subsequently analyzed by FACS (D). Primary human monocytes were treated with/without 100 ng/ml CCL11 for 6 h on Days 0 and 4 before GM-CSFR (E) and IL-4R (F) levels were measured by flow cytometry ($n=3$ independent experiments).

chemotaxis, while also demonstrating a novel mechanism of inflammatory control.

It was important to determine whether the inhibitory effect of CCL11 could also be observed in human immune cells and particularly, in cell types involved in allergic inflammation, namely DCs. We observed that indeed pretreatment with CCL11 could functionally block IL-4/GM-CSF-mediated human DC differentiation, thereby sustaining a monocyte/macrophage phenotype, and that this resulting phenotype had the reduced ability to endocytose FITC-dextran. DCs have been found to be essential at the site of allergic inflammation, but these are thought to have reached terminal differentiation before they arrive at the inflammatory site [37–39]. Therefore, the generation of new DCs at the site of allergic inflammation

may not be required, and our results suggest a blockage in DC differentiation by CCL11 signaling. This inhibition of DC differentiation and reduction in ability to endocytose antigen at the target inflammatory area may thus maintain effector monocyte/macrophage populations, which are essential cellular components in the inflammatory response. This increased monocyte/macrophage presence may permit a strong, innate response to the inflammatory stimuli and along with terminally differentiated DCs, would also generate a directed adaptive response.

SOCS proteins act to inhibit cytokine-mediated responses, and there is growing evidence that they are regulated by other signaling pathways, such as that of bacterial components, insulin, and chemoattractants [25, 26, 40]. In fact, we have

reported previously that CXCL8 and fMLP induce the expression of SOCS1 and found this to functionally inhibit G-CSF signaling in primary human neutrophils [27]. We have also discovered, among a spectrum of chemoattractants, that CCL11 stands out as an exceptionally strong regulator of SOCS1 and SOCS3 in macrophages and DCs. CCL11 has been documented to signal through a number of pathways [41], and our data indicate a novel signaling mechanism that induces SOCS proteins via G_i proteins.

It has been documented that GM-CSF and IL-4 signals are controlled by SOCS [11, 12, 17, 18], and we have shown strong inhibition of IL-4 signaling by SOCS1 and SOCS3 in macrophages and PBMCs and a definite block in GM-CSF signal transduction in monocytes and differentiating DCs upon the up-regulation of SOCS1 and SOCS3 by CCL11. IL-4R and GM-CSFR cell-surface levels were reduced and may therefore be partially responsible for reduced intracellular signaling. Therefore, these results demonstrate strongly that CCL11-induced SOCS and cytokine receptor expression may have strong inhibitory effects on primary immune cell signaling and also block DC differentiation signals from GM-CSF and IL-4. Collectively, our results demonstrate that CCL11-induced SOCS proteins and reduced cytokine receptor levels may control allergic immune responses mediated by IL-4 and GM-CSF, such as macrophage activation and DC differentiation.

In summary, having found that SOCS expression could be induced by CCL11, we were able to demonstrate that this up-regulation had a functional effect on IL-4- and GM-CSF-induced DC differentiation and their ability to endocytose antigen. This provides further evidence for cross-talk between chemokine and cytokine signaling pathways and has important implications for SOCS proteins in the regulation of allergic inflammation. These findings may have relevance in allergic pathologies, such as asthma and atopic dermatitis, where CCL11 plays a vital role in recruiting cells of the immune system, and cytokines, such as IL-4 and GM-CSF, are known to promote allergic inflammation [42, 43]. It will be interesting to determine whether other chemokines, especially those others of the eotaxin family that signal through CCR3, display similar actions through SOCS in other cell types and to further elucidate the pathways involved in CCL11-mediated SOCS expression. Further study of the role of SOCS proteins in allergic pathology may reveal new targets for future therapies designed to prevent the detrimental effects of allergic inflammation.

ACKNOWLEDGMENTS

The Wellcome Trust and the British Heart Foundation (D. R. G. lab) and the Health and Personal Social Services, Research and Development Office, Recognized Research Group (J. A. J. lab), supported this work. We thank Dr. Kate Liddiard for the gift of murine thymus and activation-regulated chemokine-luciferase vector, Prof. A. Yoshimura for SOCS1 and SOCS3 vectors, Dr. Clare McCoy for densitometry analysis, Dr. Edward Lavelle and Miss Fiona Sharp for murine peritoneal lavage, and Drs. J. Burrows and J. Elliott for critically reviewing this manuscript.

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