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CCR1-Mediated STAT3 Tyrosine Phosphorylation and CXCL8 Expression in THP-1 Macrophage-like Cells Involve Pertussis Toxin-Insensitive G\(\alpha_{14/16}\) Signaling and IL-6 Release

Maggie M. K. Lee,*† Ricky K. S. Chui,*† Issan Y. S. Tam,‡ Alaster H. Y. Lau,‡ and Yung H. Wong*§

Agonists of CCR1 contribute to hypersensitivity reactions and atherosclerotic lesions, possibly via the regulation of the transcription factor STAT3. CCR1 was demonstrated to use pertussis toxin-insensitive G\(\alpha_{14/16}\) to stimulate phospholipase C\(\beta\) and NF-\(\kappa\)B, whereas both G\(\alpha_{14}\) and G\(\alpha_{16}\) are also capable of activating STAT3. The coexpression of CCR1 and G\(\alpha_{14/16}\) in human THP-1 macrophage-like cells suggests that CCR1 may use G\(\alpha_{14/16}\) to induce STAT3 activation. In this study, we demonstrated that a CCR1 agonist, leukotactin-1 (CCL15), could indeed stimulate STAT3 Tyr\(^{705}\) and Ser\(^{727}\) phosphorylation via pertussis toxin-insensitive G proteins in PMA-differentiated THP-1 cells, human erythroleukemia cells, and HEK293 cells overexpressing CCR1 and G\(\alpha_{14/16}\). The STAT3 Tyr\(^{705}\) and Ser\(^{727}\) phosphorylations were independent of each other and temporally distinct. Subcellular fractionation and confocal microscopy illustrated that Tyr\(^{705}\)-phosphorylated STAT3 translocated to the nucleus, whereas Ser\(^{727}\)-phosphorylated STAT3 was retained in the cytosol after CCR1/G\(\alpha_{14}\) activation. CCL15 was capable of inducing IL-6 and IL-8 (CXCL8) production in both THP-1 macrophage-like cells and HEK293 cells overexpressing CCR1 and G\(\alpha_{14/16}\). Neutralizing Ab to IL-6 inhibited CCL15-mediated STAT3 Tyr\(^{705}\) phosphorylation, whereas inhibition of STAT3 activity abolished CCL15-activated CXCL8 release. The ability of CCR1 to signal through G\(\alpha_{14/16}\) provides a linkage for CCL15 to regulate IL-6/STAT3-signaling cascades, leading to expression of CXCL8, a cytokine that is involved in inflammation and the rupture of atherosclerotic plaque.

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Chemokines are a large family of low-molecular-weight cytokines that are characterized by their ability to direct the migration of leukocytes from the bloodstream to sites of inflammation (1). Leukotactin-1 (CCL15) belongs to the CC subfamily, one of four chemokine groups (CXC, CC, C, and CX3C), as defined by their primary structures. CCL15 exerts its effect mainly via CCR1 (2, 3), which is a G protein-coupled receptor. In addition to mediating chemotaxis, CCL15 and CCR1 were shown to regulate hematopoiesis (4), angiogenesis (5), mast cell activation (6), and inflammatory diseases, including atherosclerosis (7, 8). CCR1 is expressed in myeloid progenitor cells (9) and endothelial cells (10), both of which are capable of proliferating and differentiating into mature cells. CCR1 knockout mice are defective in the trafficking and proliferation of myeloid progenitor cells (4).

Hematopoiesis and angiogenesis require transcriptional activation, which can be mediated by STAT3. STAT3 is involved in the cell proliferation induced by IL-6 (11), c-Kit–mediated stem cell factor (SCF)-independent proliferation in human leukemia cells (12), and vessel formation triggered by GM-CSF (13). Deletion of STAT3 is embryonic lethal (14). In addition, STAT3 acts as a negative regulator of inflammatory responses in hematopoietic cells. Tissue-specific deletion of STAT3 in macrophages enhances the production of inflammatory cytokines (15), whereas disruption of STAT3 during hematopoiesis leads to severe inflammatory bowel disease (16). In addition, expression of RANTES/CCL5 (a CCR1 agonist) is regulated, in part, by a transcription complex of STAT3 and NF-\(\kappa\)B (17).

Although chemokine receptors are typically characterized as G\(\alpha_i\)-coupled receptors, there is substantial evidence to suggest that chemokines may be able to stimulate STAT3 activity through pertussis toxin (PTX)-insensitive G proteins. CCR1 agonists were found to induce gene expression of the STAT-inducible protooncogene, c-fos (18). c-Fos expression and transcriptional activation is induced upon G\(\alpha_{16}\) activation (19), and constitutively active mutants of G\(\alpha_{14}\) and G\(\alpha_{16}\) were demonstrated to enhance the activity of STAT3 in cotransfection systems (20, 21). The coexistence of G\(\alpha_{14/16}\) and CCR1, as well as their demonstrated functional coupling in THP-1 macrophage-like cells (22–26), suggests that CCR1 may use G\(\alpha_{14/16}\) to stimulate STAT3. There is increasing evidence to support a role for STAT3 activation in CCR1-mediated cellular responses. In human macrophages and macrophage-derived foam cells, CCL15 promotes the release of matrix metalloproteinase (MMP)-9 (27), which is implicated in the progression of atherosclerosis and whose expression is regul-
lated by STAT3 (28). Moreover, CCR1 was shown to mediate IL-6 production in marrow stromal cells upon stimulation by human myeloma cells (29), whereas MCP-1/CCL2 (a CCR2 agonist) enhances IL-6 production in fibroblast-like synoviocytes from patients with rheumatoid arthritis, and the response is mediated, in part, via PTX-insensitive G proteins (30). In addition, in THP-1 macrophage-like cells, lipoprotein (31) and human placenta extracts (32) were demonstrated to promote the expression of IL-8 (CXCL8), which is capable of inducing MMP expression (33). Interestingly, both IL-6 (34) and CXCL8 (35) are STAT3-regulated cytokines, and we recently demonstrated that activation of Gα16 can lead to STAT3 activation and upregulation of CXCL8 via IL-6 autocrine signaling in HEK293 and human Jurkat T cells (36). Given the importance of STAT3 in hematopoiesis and its purported involvement in inflammatory diseases, we explored whether CCR1 can indeed induce STAT3 phosphorylation and the production of IL-6 and CXCL8 through Gα14/16-mediated signaling. Mapping of such a pathway will help to elucidate the intricate interplay between chemokines and cytokines in their regulation of complex diseases, such as atherosclerosis and rheumatoid arthritis.

Materials and Methods

Materials

The cDNAs encoding human CCR1, wild-type and constitutively active forms of Gα14, and STAT3, STAT3Y705F, STAT3S727A, and p-STAT3–TA–CREB were used as markers for cytosolic and nuclear compartments, respectively. The immunoblots were visualized with a chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ). Signal intensities of the immunoreactive bands were quantified using Image J software, version 1.38x (National Institutes of Health, Bethesda, MD).

Cell culture

U-937 and HEL cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 50 μg/ml streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate. THP-1 and U-937 cells were differentiated into macrophage-like cells by treatment with 20 nM PMA for 48 h. HEK293 cells were cultured in MEM supplemented with 10% FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin. Primary human mast cells were prepared from peripheral blood mononuclear cells (PBMCs) by dextran sedimentation and centrifugation at 16,000 × g for 30 min. Supernatants were treated with cycloheximide for 2 h or with STAT3, IL-6, or CXCL8-neutralizing Ab for 30 min and then incubated in the absence or presence of chemokines at 10 nM for specific durations at 37°C. Subsequently, cells were lysed in 100 μl lysis buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 5 mM EDTA, 40 mM NaP2O7, 1% Triton X-100, 1 mM DTT, 200 μM Na3VO4, 200 μM PMSF, 4 μg/ml aprotinin, and 0.6 μg/ml leupeptin) and then shaken at 4°C for 30 min. Supernatants were collected by centrifugation at 16,000 × g for 8 min. HEK293 cells were seeded on six-well plates at a density of 5 × 105 cells/well, serum-starved in MEM, and lysed in 250 μl lysis buffer. Protein concentration was determined by DC protein assay kit (Bio-Rad, Hercules, CA). Eighty micrograms of proteins of each lysate was resolved by 12% SDS-PAGE and transferred to nitrocellulose membrane by electroblotting. Abs against phosphorylated STAT3 were used in the respective membranes.

Cytokine detection

Culture supernatants from THP-1 macrophage-like cells or HEK293 stable cell lines stimulated with CCL15 were harvested, and the presence of cytokines, including basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), G-CSF, GM-CSF, IFNs (IFN-α, IFN-β, IFN-γ), IL-2, IL-4, IL-6, IL-10, CXCL8, and TNF-α, were analyzed with the Procarta cytokine assay kit (Affymetrix, Santa Clara, CA). Briefly, 50 μl Ab beads was added to the prewet 96-well microtiter plate. Then, 50 μl each standard or test sample was added to the wells, followed by 25 μl detection Ab

Luciferase reporter assay

After 24 h of transfection, cells were serum starved with 100 mg/ml PTX for 4 h and then stimulated in the absence or presence of 10 nM CCL15 for another 24 h. Subsequently, the assay medium (culture medium without FBS) was removed and replaced by 150 μl lysis buffer provided in the Luciferase Reporter Gene Assay Kit (Roche Applied Science, Penzberg, Upper Bavaria, Germany). The 6-well plate was shaken at 4°C for 30 min, and 25 μl lysis buffer was transfected to white 96-well microplates designed for luminescent work (Nunc, Roskilde, Denmark). An additional 25 μl lysis buffer and 25 μl luciferin substrate were added to each well to initiate the reaction. Luciferase activity was determined using a microplate luminometer LB960 (EG&G Berthold, Bad Wildbad, Germany) (20, 21).

Assay for STAT3 phosphorylation

HEL cells, as well as THP-1 and U-937 macrophage-like cells, were seeded at 1 × 106 cells in assay medium (culture medium containing 0.1% BSA instead of FBS) and cultured or not with 100 ng/ml PTX for 16 h. Cells were treated with cycloheximide for 2 h or with STAT3, IL-6, or CXCL8-neutralizing Ab for 30 min and then incubated in the absence or presence of chemokines at 10 nM for specific durations at 37°C. Subsequently, cells were lysed in 100 μl lysis buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 5 mM EDTA, 40 mM NaP2O7, 1% Triton X-100, 1 mM DTT, 200 μM Na3VO4, 200 μM PMSF, 4 μg/ml aprotinin, and 0.6 μg/ml leupeptin) and then shaken at 4°C for 30 min. Supernatants were centrifuged at 16,000 × g for 8 min. HEK293 cells were seeded on six-well plates at a density of 5 × 105 cells/well, serum-starved in MEM, and lysed in 250 μl lysis buffer. Protein concentration was determined by DC protein assay kit (Bio-Rad, Hercules, CA). Eighty micrograms of proteins of each lysate was resolved by 12% SDS-PAGE and transferred to nitrocellulose membrane by electroblotting. Abs against phosphorylated STAT3 were used for the recognition of their respective phosphorylations. Fluorograms were visualized with a chemiluminescence detection kit (Amerham Pharmacia Biotech, Piscataway, NJ). Signal intensities of the immunoreactive bands were quantified using Image J software, version 1.38 (National Institutes of Health, Bethesda, MD).

Confocal microscopy of STAT3 Tyr705 phosphorylation

CICR1/293 cells, CCR1/Gα14/293 cells, or HEK293 cells transiently transfected with wild-type or constitutively active forms of Gα14 were collected, and whole-cell lysates were subjected to nuclear/cytosol fractionation according to the manufacturer’s instructions (Artemis Pharmacia Biotech, Piscataway, NJ). Briefly, 1 × 106 cells was resuspended in cytosol extraction buffer and centrifuged at 16,000 × g for 5 min to collect the supernatant (cytosolic fraction). The resulting pellet was resuspended in nuclear extraction buffer and centrifuged again at 16,000 × g for 10 min to collect the supernatant representing the nuclear fraction. The concentration of protein was determined using a DC protein assay kit (Bio-Rad). Eighty micrograms of protein was separated in 12% SDS-PAGE and transferred to nitrocellulose membrane by electroblotting. Abs against caspase-3 and CREB were used as markers for cytosolic and nuclear compartments, respectively. Fluorograms were visualized with a chemiluminescence detection kit (Amerham Pharmacia Biotech).
mixture. The samples were then incubated for 30 min at 25°C with gentle shaking, followed by three rounds of gentle rinsing to wash away unbound Abs. Finally, 50 μl streptavidin-FITC was added and incubated for 30 min. The beads were then washed again and resuspended in 120 μl reading buffer. Analysis was performed with a Bio-Plex 200 system (Bio-Rad) using the Bio-Plex manager software (version 5.0). A minimum of 100 beads/region was analyzed. A curve fit was applied to each standard curve, according to the manufacturer’s manual, and sample concentrations were interpolated from the standard curves. For detection of CXCL8 release from human mast cells, cells cultured for ≥6 wk were counted and seeded in IMDM containing SCF overnight before sensitization with 0.5 μg/ml human myeloma IgE for 24 h. IgE-sensitized mast cells were then washed and resuspended in IMDM containing SCF. CCR1 was activated by preincubating the cells with 100 nM CCL15 for 10 min before challenging the sensitized cells with 0.25 μg/ml anti-human IgE (Sigma) for 6 h. Culture medium was harvested, and cells were removed by centrifugation at 4°C. The amount of CXCL8 released into the culture medium by human mast cells or CCL15-stimulated U-937 macrophage-like cells was measured by ELISA (BD).

Data analysis
The data shown in each figure represent mean ± SEM of determinations from three or more separate experiments. Statistical analyses were performed by ANOVA, followed by the Dunnett test or paired t test.

Results
CCR1/Go_{14/16}-mediated STAT3 phosphorylations at Tyr^{705} and Ser^{727} exhibit distinct temporal patterns
The coexistence of CCR1 (2, 25), Go_{14} (26), and Go_{16} (22, 25), as well as their functional coupling in THP-1 macrophage-like cells (23, 25), suggests that CCR1 may use Go_{14/16} to stimulate STAT3. We began the study by determining whether CCR1 can use PTX-insensitive G proteins to induce STAT3 Tyr^{705} and Ser^{727} phosphorylation in PMA-differentiated THP-1 cells. PMA-differentiated THP-1 cells have been widely used as an in vitro model of macrophage-like cells or CCL15-stimulated U-937 macrophage-like cells was measured by ELISA (BD).

![Figure 1](http://www.jimmunol.org/) CCL15-induced STAT3 phosphorylations at Tyr^{705} and Ser^{727} occur in a temporally distinct pattern. (A) PMA-differentiated THP-1 macrophage-like cells or HEL cells were stimulated in the absence or presence of 10 nM CCL15 for various durations (15 min–24 h) and with PTX pretreatment (100 ng/ml; 4 h). (B) PMA-differentiated THP-1 cells were transfected with Stealth Select RNAi against Go_{14} and Go_{16}, or Stealth RNAi negative control and stimulated in the absence or presence of 10 nM CCL15 for 6 h and with PTX pretreatment (100 ng/ml; 16 h). (C) HEK293 stable cell lines were stimulated in the absence or presence of 10 nM CCL15 for various durations (5 min–24 h). Phosphorylated STAT3 was detected by phospho-specific Abs, followed by subsequent quantification by densitometry. Numerical values shown above the immunoreactive bands represent mean relative intensities of STAT3 phosphorylation expressed as a ratio of the basal level (set as 1.0) from three or more separate experiments; they were increased significantly compared with the basal level. p < 0.05, Dunnett test.

Go_{14} and Go_{16} expression by siRNA completely abrogated the CCL15-mediated STAT3 Tyr^{705} phosphorylation (Fig. 1B). These results suggest that the PTX-insensitive portion of the CCR1-induced STAT3 Tyr^{705} phosphorylation in THP-1 cells was mediated via Go_{14} and Go_{16}.

To confirm the role of Go_{14} and Go_{16} in CCR1-induced STAT3 phosphorylation, we used previously established HEK293 cells (23, 26) stably expressing CCR1 alone (CCR1/293) or expressing CCR1 together with Go_{14} (CCR1/Go_{14}/293) or Go_{16} (CCR1/Go_{16}/293). Go_{14} and Go_{16} were prominently expressed in CCR1/ Go_{14}/293 and CCR1/Go_{16}/293 cells, respectively, but they were absent in HEK293 cells (23, 26). The expression of CCR1 was confirmed previously by both Western blotting analysis (26) and immunofluorescence microscopy (25), whereas the coupling of CCR1 to Go_{14} or Go_{16} was verified by agonist-induced phospholipase Cβ (PLCβ) stimulation (23). Thus, these cell lines were...
challenged with 10 nM CCL15 for various durations. In cells expressing CCR1 alone, CCL15 stimulated STAT3 Ser<sup>727</sup> phosphorylation transiently (15–45 min), whereas the STAT3 Tyr<sup>705</sup> phosphorylation level was unaffected by CCL15 during the entire duration of the experiment (Fig. 1C), indicating that CCR1 could not stimulate STAT3 phosphorylation at Tyr<sup>705</sup> via endogenous G proteins. The incorporation of G<sub>α<sub>14</sub></sub> or G<sub>α<sub>16</sub></sub> enhanced the CCL15-induced STAT3 Ser<sup>727</sup> phosphorylation response (Fig. 1C); the duration of the response was lengthened in CCR1/G<sub>α<sub>14</sub>/293</sub> cells. STAT3 Tyr<sup>705</sup> phosphorylation was also detected in cells coexpressing CCR1 and G<sub>α<sub>14</sub></sub> or G<sub>α<sub>16</sub></sub>, with stimulations of ~3.5–4-fold, but these events occurred much later, at >4 h (Fig. 1C).

The cell lines were then pretreated with PTX to eliminate possible G<sub>α</sub>-mediated STAT3 phosphorylation. Application of CCL15 for 15 min weakly stimulated Ser<sup>727</sup> phosphorylation of STAT3 in cells expressing CCR1 alone, which was sensitive to PTX treatment (Fig. 2A); PTX sensitivity suggests the involvement of endogenous G<sub>α</sub> proteins in HEK293 cells. Coexpression of G<sub>α<sub>14</sub></sub> or G<sub>α<sub>16</sub></sub> with CCR1 enhanced the CCL15-induced STAT3 Ser<sup>727</sup> phosphorylation, with the stimulations increased to ~2.5-fold (Fig. 2A). Ser<sup>727</sup> phosphorylation of STAT3 was resistant to PTX in CCR1/G<sub>α<sub>14</sub>/293</sub> and CCR1/G<sub>α<sub>16</sub>/293</sub> cells, demonstrating that CCR1 can mediate STAT3 Ser<sup>727</sup> phosphorylation via G<sub>α<sub>14</sub></sub> and G<sub>α<sub>16</sub></sub> because they are the only two PTX-insensitive G proteins known to be recognized by CCR1 (25, 26). To investigate CCR1/G<sub>α<sub>14/16</sub></sub>-stimulated STAT3 Tyr<sup>705</sup> phosphorylation, the cell lines were pretreated with PTX and stimulated with CCL15 for 6 h. In cells coexpressing CCR1 and G<sub>α<sub>14</sub></sub> or G<sub>α<sub>16</sub></sub>, CCL15-induced STAT3 Tyr<sup>705</sup> phosphorylation was resistant to PTX (Fig. 2A). Another CCR1 agonist, myeloid progenitor inhibitory factor-1 (CCL23), induced STAT3 phosphorylation at Ser<sup>727</sup> within 15 min, but STAT3 phosphorylation at Tyr<sup>705</sup> was detected 6 h after drug treatment, and the response was not sensitive to PTX (Fig. 2B). These results indicated that CCR1/G<sub>α<sub>14/16</sub></sub>-induced phosphorylation of STAT3 at Tyr<sup>705</sup> and Ser<sup>727</sup> have distinct kinetics. It should be noted that CCL15-induced STAT3 phosphorylation at Tyr<sup>705</sup> was a delayed response because it remained detectable at 6 h, even when the agonist was washed out 15 min after application (data not shown).

Next, luciferase reporter assay was used to demonstrate that CCR1/G<sub>α<sub>14/16</sub></sub>-mediated STAT3 phosphorylation can indeed lead to STAT3 transcriptional activity. CCR1/G<sub>α<sub>14</sub>/293</sub> cells were transfected with cDNAs encoding STAT3-Tyr705F and STAT3-Ser727A, pretreated with PTX, and challenged with 10 nM CCL15. Consistent with the results for STAT3 Tyr<sup>705</sup> phosphorylation, CCL15 significantly stimulated the STAT3-driven luciferase activity (Fig. 2C). Similar results were obtained in STAT3-Luc–expressing CCR1/G<sub>α<sub>14</sub>/293</sub> cells (Fig. 2C). To investigate whether CCR1/G<sub>α<sub>14</sub></sub>-mediated Tyr<sup>705</sup> and Ser<sup>727</sup> phosphorylation of STAT3 can occur independently, CCR1/G<sub>α<sub>14</sub></sub>/293 cells were transfected with vector plasmid, wild-type STAT3 or its phosphorylation-resistant mutants (STAT3Y705F and STAT3S727A), pretreated with PTX, and then challenged with 10 nM CCL15 for 15 min or 6 h. CCR1/G<sub>α<sub>14</sub></sub>-induced STAT3 Tyr<sup>705</sup> phosphorylation was attenuated in STAT3Y705F-expressing cells but not in STAT3S727A-expressing cells (Fig. 2D, left panels). In contrast, CCR1/G<sub>α<sub>14</sub></sub>-mediated STAT3 Ser<sup>727</sup> phosphorylation was abolished by STAT3S727A overexpression, whereas it was unaffected by STAT3Y705F overexpression (Fig. 2D, right panels). These results implied that CCR1/G<sub>α<sub>14</sub></sub> activated STAT3 Tyr<sup>705</sup> and Ser<sup>727</sup> phosphorylations independently.

**CCR1/G<sub>α<sub>14</sub></sub> induces phosphorylation of STAT3 at Tyr<sup>705</sup> in nucleus and Ser<sup>727</sup> in cytosol**

As a latent cytoplasmic transcription factor, STAT3 is activated by cell surface receptors and translocates from cytosol to nucleus to regulate gene expression. The ability of CCL15 to stimulate STAT3 Ser<sup>727</sup> phosphorylation in the absence of Tyr<sup>705</sup> phosphorylation is intriguing, because the Ser<sup>727</sup> site is generally believed to enhance STAT3 transcriptional activity, which requires Tyr<sup>705</sup> phosphorylation (41). Nuclear/cytoplasmic fractionation was performed to investigate whether CCR1/G<sub>α<sub>14</sub></sub> activation can lead to the translocation of Tyr<sup>705</sup> or Ser<sup>727</sup>-phosphorylated STAT3 to the nucleus. Consistent with the results shown in Fig. 2A, the level of STAT3 Tyr<sup>705</sup> and Ser<sup>727</sup> phosphorylations could not be stimulated by CCL15 in PTX-treated CCR1/293 cells (Fig. 3A, upper right panels). Subcellular fractionation also indicated that no detectable change in STAT3 Tyr<sup>705</sup> or Ser<sup>727</sup> phosphorylation was observed in cytosolic or nuclear compartments. Coexpression of G<sub>α<sub>14</sub></sub> with CCR1/293 cells allowed CCL15 to stimulate STAT3

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**FIGURE 2.** CCL15-induced STAT3 phosphorylations at Tyr<sup>705</sup> and Ser<sup>727</sup> were independent. (A) HEK293 stable cell lines were stimulated in the absence (basal) or presence of 10 nM CCL15 for 15 min or 6 h, with or without PTX pretreatment (100 ng/ml; 6 h). Data represent mean ± SEM from at least three separate experiments. *p < 0.05, CCL15 stimulation versus no stimulation, Dunnett test. (B) HEK293 stable cell lines were stimulated in the absence (basal) or presence 10 nM CCL23 for 15 min or 6 h and with PTX pretreatment (100 ng/ml; 6 h). Three individual experiments showed similar results. (C) CCR1/G<sub>α<sub>14</sub>/293</sub> and CCR1/G<sub>α<sub>16</sub>/293</sub> cells were transfected with cDNAs encoding p-STAT3–TA–Luc (500 ng), pretreated with 100 ng/ml PTX for 4 h, and then stimulated in the absence (basal) or presence of 10 nM CCL15 for 24 h. Luciferase activity was analyzed and expressed as fold stimulation of the basal STAT3 activity for each cell lines. Data represent mean ± SEM of at least three experiments. *p < 0.05, CCL15 stimulation versus no stimulation, Dunnett test. (D) CCR1/G<sub>α<sub>14</sub>/293</sub> cells were transfected with cDNAs encoding vector plasmid STAT3, STAT3Y705F, or STAT3S727A (500 ng), pretreated with 100 ng/ml PTX for 16 h, and then stimulated in the absence (basal) or presence of 10 nM CCL15 for 15 min or 6 h. Three individual experiments showed similar results.
Tyr705 and Ser727 phosphorylation, even in the presence of PTX (Fig. 3A, lower right panels). Ser727 phosphorylation of STAT3 was increased after treatment with CCL15 for 15 min, and it gradually decreased to near basal levels by 6 h. Similar kinetics of CCL15-triggered STAT3 Ser727 phosphorylation were detected in the cytosolic compartment. However, CCL15-activated Ser727-phosphorylated STAT3 was not detectable in the nuclear compartments (Fig. 3A, lower panel). Tyr705 phosphorylation of STAT3 was elevated after treatment with CCL15 for 4 h and gradually increased to the maximal level by 6 h; a similar profile was observed in the nuclear compartment. However, CCL15-activated Tyr705-phosphorylated STAT3 was not detectable in the nuclear compartments (Fig. 3A, lower panel). Abs against caspase-3 and CREB were used as markers for the cytosolic and nuclear compartments, respectively. These results were further confirmed by confocal microscopy to reveal the subcellular localization of STAT3 Tyr705 phosphorylation. In line with the results illustrated in Fig. 3A, CCL15 did not elicit STAT3 Tyr705 phosphorylation in CCR1/293 cells (Fig. 4). However, the introduction of Ga14 to CCR1/293 cells supported the CCL15-induced STAT3 Tyr705 phosphorylation, which was primarily localized to the nucleus (Fig. 4). Thus, these results demonstrated that Tyr705-phosphorylated STAT3, but not Ser727-phosphorylated STAT3, was translocated to the nucleus after CCR1/Ga14 activation. We demonstrated previously that members of the Ga subfamily can induce STAT3 Tyr705 phosphorylation (37). Thus, we investigated whether the activation of Ga14 or Ga16 could translocate Tyr705-phosphorylated STAT3 into the nucleus. In line with the previous study, active mutants of Ga14 and Ga16 could induce STAT3 Tyr705 phosphorylation, which was primarily localized to the nucleus (Fig. 3B).

**CCR1/Ga14/16-mediated STAT3 Tyr705 phosphorylation involves protein synthesis**

Because CCR1/Ga14/16-induced STAT3 Tyr705 phosphorylation occurred after prolonged treatment with CCL15 (Fig. 1), this may imply that protein synthesis of a STAT3 activator may be required. To address this possibility, HEK293 stable cell lines were pre-

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**FIGURE 3.** CCL15 induced STAT3 phosphorylations at Tyr705 in nucleus and Ser727 in cytosol. (A) CCR1/293 and CCR1/Ga14/293 cells were pretreated with 100 ng/ml PTX for 16 h and then stimulated in the absence (basal) or presence of 10 nM CCL15 for 15 min, 4 h, or 6 h. Total cell lysates (right panels) of HEK293 stable cell lines were fractionated into cytosolic (left panels) and nuclear fractions (middle panels). Three individual experiments showed similar results. (B) HEK293 cells were transfected with cDNA encoding wild-type and active mutant of Ga14 or Ga16. Total cell lysates (right panels) of transfected HEK293 cells were fractionated into cytosolic (left panels) and nuclear fractions (middle panels). Three individual experiments showed similar results.

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**FIGURE 4.** CCL15 induced STAT3 phosphorylation at Tyr705 in nucleus. CCR1/293 and CCR1/Ga14/293 cells were pretreated with 100 ng/ml PTX for 16 h and then stimulated in the absence (basal) or presence of 10 nM CCL15 for 15 min or 6 h. Cells were fixed, permeabilized, labeled with rabbit Tyr705-phosphorylated STAT3 Ab and mouse STAT3 Ab, and then stained with Alexa Fluor 555 anti-rabbit IgG for Tyr705-phosphorylated STAT3 detection (red) and Alexa Fluor 488 anti-mouse IgG for STAT3 detection (green). Nuclei were stained with DAPI (blue). Three individual experiments showed similar results. Original magnification ×63.
treated with cycloheximide (a transcription/translation inhibitor) for 2 h and then incubated with CCL15 for 15 min or 6 h. Cycloheximide treatment of cells prior to CCL15 treatment led to a complete abrogation of CCR1/Gα14/16-induced STAT3 Tyr705 phosphorylation without affecting the cell viability, whereas no inhibitory effect was observed on Ser727 phosphorylation (Fig. 5A). The same inhibitory effect on CCL15-induced STAT3 Tyr705 phosphorylation was observed in PMA-differentiated THP-1 cells (Fig. 5B). CCL15-activated Ser727 phosphorylation of STAT3 in HEK293 stable cell lines (Supplemental Fig. 1A) and THP-1 cells (Supplemental Fig. 1B) required ERK, as demonstrated by the use of an MEK1/2 kinase inhibitor (U0126) and its inactive analog (U0124). Therefore, the late and robust activation of STAT3 Tyr705 phosphorylation induced by CCR1/Gα14/16 may require the synthesis of STAT3 activators. We then investigated whether the conditioned medium from CCL15-stimulated CCR1/Gα14/293 or CCR1/Gα16/293 cells was sufficient to phosphorylate STAT3 at Tyr705. Conditioned medium was collected from CCR1/Gα14/293 or CCR1/Gα16/293 cells stimulated with CCL15 for 15 min, 4 h, or 6 h, transferred to parental HEK293 cells, and incubated for 30 min; STAT3 Tyr705 phosphorylation was observed in the parental HEK293 cells treated with conditioned medium of CCR1/Gα14/293 or CCR1/Gα16/293 cells with 6 h of CCL15 treatment (Fig. 6A). Similarly, STAT3 Tyr705 phosphorylation was detected in THP-1 cells treated with conditioned medium of THP-1 cells with 6 h of CCL15 treatment (Fig. 6B). The same phenomenon was observed when the conditioned medium was collected from HEK293 cells overexpressing active mutants of Gq subfamily members (Gqα14, Gqα16, Gqαq, and Gqα11) but not Gqα2 (the PTX-insensitive Gi subfamily members) (Fig. 6C). These results indicated that there were cytokines or growth factors in the conditioned medium that could induce STAT3 Tyr705 phosphorylation.

**FIGURE 5.** CCL15-induced STAT3 phosphorylation at Tyr705 required protein synthesis. (A) HEK293 stable cell lines were pretreated with 100 ng/ml PTX for 16 h and 50 μM cycloheximide for 2 h and then stimulated in the absence (basal) or presence of 10 nM CCL15 for 15 min, 2 h, or 6 h. (B) PMA-differentiated THP-1 macrophage-like cells were pretreated with 100 ng/ml PTX for 16 h and 50 μM cycloheximide for 2 h and then stimulated in the absence (basal) or presence of 10 nM CCL15 for 15 min, 2 h, or 6 h. Numerical values shown above the immunoreactive bands represent mean relative intensities of STAT3 phosphorylation expressed as a ratio of the basal level (set as 1.0) from at least three separate experiments; they were increased significantly compared with the basal level (p < 0.05, Dunnett test).

**FIGURE 6.** Conditioned medium from CCL15-stimulated CCR1/Gα14/293 cells, CCR1/Gα16/293 cells, and THP-1 macrophage-like cells induced STAT3 phosphorylation at Tyr705. Conditioned medium from HEK293 stable cells that were pretreated with 100 ng/ml PTX for 16 h and then stimulated in the absence (basal) or presence of 10 nM CCL15 for 15 min, 4 h, or 6 h (A), THP-1 cells that were pretreated with 100 ng/ml PTX for 16 h and then stimulated in the absence (basal) or presence of 10 nM CCL15 for 15 min, 2 h, or 6 h (B), or HEK293 cells transfected with cDNA encoding wild-type and active mutant of Gα14, Gα16, Gαq, or Gα11 (C) was collected, transferred to HEK293 cells (A, C) or THP-1 cells (B) and incubated for 30 min. Numerical values shown above the immunoreactive bands represent mean relative intensities of STAT3 phosphorylation expressed as a ratio of the basal level (set as 1.0) from at least three experiments; they were increased significantly compared with the basal level (p < 0.05, Dunnett test). For HEK293 cells transfected with cDNA encoding wild-type or active mutant of Gα subunits, three individual experiments showed similar results.

**IL-6, but not CXCL8, is required for CCR1/Gα14/16-mediated STAT3 Tyr705 phosphorylation**

Many cytokines and growth factors are either STAT3 activators or their transcriptions are regulated by STAT3 activation; they include bFGF, EGF, G-CSF, GM-CSF, IFN-α, IFN-β, IFN-γ, IL-2, IL-4, IL-6, CXCL8, and TNF-α. Multiplex cytokine detection analysis was performed to screen for the aforementioned STAT3 activators or STAT3-regulated cytokines or growth factors in the conditioned medium of CCL15-stimulated CCR1/Gα14/293 and CCR1/Gα16/293 cells. After 4 h of drug incubation, CCL15 significantly stimulated IL-6 production in CCR1/Gα14/293 and CCR1/Gα16/293 cells in a PTX-insensitive manner, with maximal stimulations of 11- and 8-fold, respectively (Fig. 7A). No significant CCL15-induced IL-6 secretion was observed in CCR1/293 cells, indicating that CCR1 used Gα14 and Gα16 to stimulate IL-6 expression. CXCL8, a proinflammatory CXC chemokine, was also detected in the conditioned medium of CCR1/Gα14/293 and CCR1/Gα16/293 cells with a 4-h treatment with CCL15. CCL15 induced CXCL8 production by 22.5-fold in CCR1/Gα14/293 cells, whereas the production was increased 12-fold in CCR1/Gα16/293 cells (Fig. 7B). No significant CCL15-induced CXCL8 secretion was detected in CCR1/293 cells. In contrast, CCL15 treatment was unable to stimulate bFGF, EGF, G-CSF, GM-CSF, IFN-α,
IFN-β, IFN-γ, IL-2, IL-4, or TNF-α expression in all three cell lines. The CCL15-stimulated THP-1 cells also produced IL-6 and CXCL8 in 2 and 6 h, respectively (Fig. 7C, 7D). Moreover, the reduction in Ga14 and Ga16 expression by siRNA completely abrogated CCL15-mediated CXCL8 production in THP-1 cells (Fig. 7E). This demonstrates the capability of CCL15 to express IL-6 and CXCL8 via Ga14 and Ga16 in a native cellular environment. We further investigated whether IL-6 expression was required for CCR1/Ga14- or Ga16-mediated STAT3 Tyr705 phosphorylation. CCR1/Ga14/293 cells were pretreated with IL-6-neutralizing Ab for 30 min before the addition of CCL15. CCR1/Ga14-induced STAT3 Tyr705 phosphorylation could be inhibited by IL-6-neutralizing Ab (Fig. 8A), showing the requirement for IL-6 in the phosphorylation of STAT3 at Tyr705. The same inhibitory effect of IL-6-neutralizing Ab on STAT3 Tyr705 phosphorylation was observed in CCR1/Ga14/293 cells (Fig. 8A). However, pretreatment with CXCL8-neutralizing Ab did not affect Tyr705 phosphorylation of STAT3 in HEK293 cells overexpressing CCR1 and Ga14 or CCR1 and Ga16 (Fig. 8B), demonstrating that CXCL8 expression was not involved in CCR1/Ga14 or Ga16-mediated STAT3 Tyr705 phosphorylation. The STAT3 Tyr705 phosphorylation level was unaffected by CCL15 after the addition of IL-6- or CXCL8-neutralizing Ab to CCR1/293 cells. The requirement of IL-6, but not CXCL8, for CCL15-induced STAT3 Tyr705 phosphorylation was also illustrated in THP-1 cells (Fig. 8C). To further confirm the involvement of IL-6 in CCL15-induced STAT3 Tyr705 phosphorylation in macrophages, U-937 macrophage-like cells were treated with 100 ng/ml PTX for 16 h and 10 μg/ml IL-6-neutralizing Ab for 30 min and then stimulated in the absence (basal) or presence of 10 nM CCL15 for 6 h. (D) PMA-differentiated U-937 macrophage-like cells were pretreated with 100 ng/ml PTX for 16 h and 10 μg/ml CXCL8-neutralizing Ab for 30 min and then stimulated in the absence (basal) or presence of 10 nM CCL15 for 15 min or 6 h. (C) PMA-differentiated THP-1 macrophage-like cells were pretreated with 100 ng/ml PTX for 16 h and 10 μg/ml IL-6–neutralizing Ab for 30 min and then stimulated in the absence (basal) or presence of 10 nM CCL15 for 15 min or 6 h. (D) PMA-differentiated U-937 macrophage-like cells were pretreated with 100 ng/ml PTX for 16 h and 10 μg/ml IL-6–neutralizing Ab for 30 min and then stimulated in the absence (basal) or presence of 10 nM CCL15 for 6 h. Numerical values shown above the immunoreactive bands represent mean relative intensities of STAT3 phosphorylation expressed as a ratio of the basal level (set as 1.0) from at least three separate experiments; they were increased significantly compared with the basal level (p < 0.05, Dunnett test).

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FIGURE 7. CCL15 induced IL-6 and CXCL8 production. HEK293 stable cell lines (A, B) or PMA-differentiated THP-1 macrophage-like cells (C, D) were stimulated in the absence (basal) or presence of 10 nM CCL15 for various durations (15 min–24 h) and with PTX pretreatment (100 ng/ml; 4 h). (E) PMA-differentiated THP-1 cells were also transfected with Stealth Select RNAi against Ga14 and Ga16 or Stealth RNAi negative control and then were stimulated in the absence or presence of 10 nM CCL15 for 6 h and with PTX pretreatment (100 ng/ml; 16 h). Conditioned medium was collected, and the protein concentration of IL-6 (A, C) or CXCL8 (B, D, E), was measured by multiplex cytokine-detection analysis. Data represent mean ± SEM of at least three separate experiments. *p < 0.05, CCL15 stimulation versus no stimulation, Dunnett test.

FIGURE 8. IL-6, but not CXCL8, was required for CCL15-induced STAT3 phosphorylation at Tyr705. (A) HEK293 stable cell lines were pretreated with 100 ng/ml PTX for 16 h and 10 μg/ml IL-6–neutralizing Ab for 30 min and then stimulated in the absence (basal) or presence of 10 nM CCL15 for 6 h. (B) HEK293 stable cell lines were pretreated with 100 ng/ml PTX for 16 h and 10 μg/ml CXCL8-neutralizing Ab for 30 min and then stimulated in the absence (basal) or presence of 10 nM CCL15 for 15 min or 6 h. (C) PMA-differentiated THP-1 macrophage-like cells were pretreated with 100 ng/ml PTX for 16 h and 10 μg/ml IL-6–neutralizing Ab for 30 min and then stimulated in the absence (basal) or presence of 10 nM CCL15 for 6 h. (D) PMA-differentiated U-937 macrophage-like cells were pretreated with 100 ng/ml PTX for 16 h and 10 μg/ml IL-6–neutralizing Ab for 30 min and then stimulated in the absence (basal) or presence of 10 nM CCL15 for 6 h. Numerical values shown above the immunoreactive bands represent mean relative intensities of STAT3 phosphorylation expressed as a ratio of the basal level (set as 1.0) from at least three separate experiments; they were increased significantly compared with the basal level (p < 0.05, Dunnett test).
CCL15-induced STAT3 Tyr\(^{705}\) phosphorylation was inhibited by IL-6-neutralizing Ab, illustrating the requirement for IL-6 in the phosphorylation of STAT3 at Tyr\(^{705}\).

**STAT3 Tyr\(^{705}\) phosphorylation is necessary for CCR1/\(\alpha_{14/16}\)-mediated CXCL8 expression**

Because the expression of cytokines could be regulated by STAT3 activation, we investigated whether STAT3 Tyr\(^{705}\) phosphorylation was required for CCR1/\(\alpha_{14/16}\)-mediated IL-6 or CXCL8 production using STAT3 inhibitor V (Stattic), which selectively inhibits the activation, dimerization, and nuclear translocation of STAT3 (44). Application of Stattic abolished CCL15-stimulated STAT3 Tyr\(^{705}\) phosphorylation in both CCR1/\(\alpha_{14/293}\) and CCR1/\(\alpha_{16/293}\) cells (Fig. 9A), but it did not affect Ser\(^{727}\) phosphorylation of STAT3 in these cells. The level of STAT3 phosphorylations at Tyr\(^{705}\) and Ser\(^{727}\) was unaffected by CCL15 after pretreatment of Stattic in CCR1/293 cells. CCL15-mediated CXCL8 secretion was abrogated by Stattic in HEK293 cells overexpressing CCR1 and \(\alpha_{14}\), but no inhibitory effect on CCL15-activated IL-6 production was observed (Fig. 9B, 9C); similar results were obtained with CCL15-treated CCR1/\(\alpha_{16/293}\) cells. No significant CCL15-induced IL-6 and CXCL8 secretion was detected in CCR1/293 cells pretreated with Stattic. For THP-1 cells, pretreatment with Stattic also diminished CCL15-induced STAT3 Tyr\(^{705}\) phosphorylation but not CCL15-triggered STAT3 Ser\(^{727}\) phosphorylation (Fig. 10A). The requirement of STAT3 Tyr\(^{705}\) phosphorylation was also demonstrated in CCL15-stimulated CXCL8 production (Fig. 10B), but not CCL15-activated IL-6 release (Fig. 10B), in THP-1 cells. Pretreatment with IL-6-neutralizing Ab also abolished CCL15-induced CXCL8 production (Fig. 10C), showing the involvement of STAT3 Tyr\(^{705}\) phosphorylation and IL-6 in CCL15-triggered CXCL8 release. The putative mechanism is depicted in Fig. 11.

Lastly, to test whether chemokine-induced CXCL8 can be observed in other hematopoietic cells, we used isolated human mast cells.
cells to examine the ability of CCL15 to stimulate the production of CXCL8. Costimulation with CCR1 enhanced anti-IgE–induced mast cell activation and degranulation (45). In the unstimulated state, human mast cells did not release CXCL8 at detectable levels. Sensitization of human mast cells by human myeloma IgE and anti-human IgE resulted in the release of CXCL8; this response was enhanced significantly in the presence of 100 nM CCL15 (Fig. 10D). Anti-IgE–induced CXCL8 production in mast cells was completely abolished in the presence of Statc, suggesting the involvement of STAT3 (data not shown). Pretreatment with IL-6–neutralizing Ab diminished CCL15–mediated CXCL8 release (Fig. 10D), illustrating the requirement of IL-6 in CCL15–activated CXCL8 production.

Discussion

Although activation of STAT3 Tyr705 phosphorylation was shown to be mediated by several chemokines, including CCL5, CCL2, and SDF-1α (CXCL12) (18, 46–48), the biochemical linkage between heterotrimeric G protein activation and STAT3 in this pathway remains poorly defined. Several reports suggest that chemokines induce receptor dimerization, resulting in the activation of the JAK–STAT pathway (reviewed in Ref. 49). However, CCR1 oligomerization has not been unequivocally established. In addition, the ability of chemokine receptors to regulate STAT3 Ser727 phosphorylation has not been documented. By examining the role of G14 or G16 in transfected HEK293 cells, native HEL cells, and native THP-1 and U-937 macrophage-like cells, this study provided evidence that STAT3 Tyr705 phosphorylation is not a prerequisite for Ser727 phosphorylation, and IL-6 autocrine signaling is apparently involved in CCR1/G14/G16–mediated STAT3 Tyr705 phosphorylation, which is required for the subsequent CXCL8 production. Although CCL15 stimulated both CCR1 and CCR3 endogenously expressed in THP-1 cells (50), CCR3 is downregulated upon maturation of the monocytes to macrophages, whereas the expression of CCR1 remains unaffected (39). Thus, in the differentiated THP-1 and U-937 cells used in the current study, CCL15–mediated STAT3 phosphorylations at Tyr705 and Ser727 are most likely to be contributed by CCR1.

Our study clearly demonstrated that CCL15–stimulated CCR1 is capable of inducing STAT3 in THP-1 and THP-1 cells. Thus, the chemokine-induced STAT3 phosphorylations at Tyr705 and Ser727 occurred independently (Fig. 2D) and in temporally distinct manners (Fig. 1). With Tyr705–phosphorylated STAT3 translocated to the nucleus and Ser727–phosphorylated STAT3 remaining in the cytosol (Fig. 3A). Apparently, CCR1–mediated STAT3 activation is a delayed response that requires the induction of IL-6 (Fig. 8A, 8C, 8D). In THP-1 cells, U-937 cells, and HEK293 cells, the biological consequence of CCR1–mediated STAT3 activation is the upregulation of yet another chemokine, CXCL8 (Figs. 9C, 10B, 10C). Fig. 11 shows a putative model of CCR1/G14/16–mediated STAT3 Tyr705 and Ser727 phosphorylation, in which the present findings are incorporated with established pathways. Upon binding of CCL15 to CCR1, G14 or G16 becomes activated and, in turn, stimulates PLCβ, which hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol, subsequently leading to activation of the PKCα/β–Src pathways. The coupling of tetratricopeptide repeat 1 to G16 (51) activates Ras and MEK/ERK, which then induces STAT3 Tyr705 phosphorylation (20, 21). Upon prolonged pretreatment with CCL15, activated MEK/ERK may phosphorylate transcription factors, such as Sp-1 (52), leading to the translocation of transcription factors to the nucleus, upregulation of IL-6, and transcriptional activation of IL-6. The secreted IL-6 induces STAT3 phosphorylation at Tyr705 in an autocrine fashion, resulting in dimerization and nuclear translocation of Tyr705–phosphorylated STAT3 and subsequent CXCL8 transcription.

We showed previously that activation of G16 by a variety of G protein–coupled receptors, including the δ-opioid, C5a, formyl peptide, and opioid receptor–like receptors (20, 21, 53), can lead to STAT3 Tyr705 phosphorylation within 15 min in HEK293 cells. In contrast, CCR1/G14/16–mediated Tyr705 phosphorylation of STAT3 was only detected after prolonged drug pretreatment. This discrepancy is puzzling, especially because the receptors were expressed in the same cellular background. Although we do not have a plausible explanation, it should be noted that delayed STAT3 Tyr705 phosphorylation is not unique to CCR1; it has been similarly observed with G16–coupled melanotin MT1 and MT2 receptors (36). Moreover, distinct temporal patterns of STAT3 phosphorylations at Tyr705 and Ser727 have been documented. In murine macrophage–like RAW 264.7 cells, STAT3 Ser727 phosphorylation induced by LPS can be observed at 5 min, whereas STAT3 Tyr705 phosphorylation requires 2 h of treatment (54). Likewise, two groups demonstrated that angiostatin II induces delayed STAT3 Tyr705 phosphorylation in rat aortic smooth muscle cells (55) and rat cardiomyocytes (56). Fukuda and coworkers (57) also showed that the delayed STAT3 Tyr705 phosphorylation stimulated by angiostatin II is mediated by autocrine/paracrine secretion of IL-6.

The induction of IL-6 and CXCL8 by CCR1/G14/16 signaling has several implications. IL-6 is a potent inflammatory cytokine that directly activates STAT1 and STAT3. The production of IL-6 in CCL15–stimulated THP-1 cells and transfected HEK293 cells implied that CCR1/G14/16 could also stimulate STAT1, which is in
line with our previous study showing that the constitutively active mutant of Gα16 can enhance the activity of STAT1 (19). Our demonstration of CCR1/Gα14/16-mediated STAT3 Tyr175 phosphorylation leading to subsequent CXCL8 production is in agreement with the report that CXCL8 expression is transcriptionally upregulated by STAT3 in human melanoma cells (58). In addition, it was reported that JAK/STAT3 is involved in thrombin-induced CXCL8 secretion in human dermal fibroblasts (59). CXCL8 was reported to activate MMP expression (33) and suppress tissue inhibitor of metalloproteinases expression (60), and these regulations by CXCL8 are responsible for the rupture of atherosclerotic plaques. In human macrophages and macrophage-derived foam cells, CCL15 is capable of inducing the production of MMP-9 (27), whose expression is regulated by STAT3 (28), whereas CXCL8 is apparently unaffected by CCL15 treatment. Because there are no apparent requirements for the STAT3 branches required for the subsequent production of CXCL8. In turn, CXCL8 may activate CXCRL2, leading to further STAT3 Tyr175 phosphorylation, which synergizes with the effect of CCR1 agonists. To our knowledge, this study represents the first demonstration of chemokine-stimulated CXCL8 production via CCR1/Gα14/16/STAT3-signaling pathways. It remains to be determined whether autocrine signaling of IL-6/STAT3/CXCL8 by CCR1/Gα14/16 plays a role in the progression of diseases, such as atherosclerosis.

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Disclosures
The authors have no financial conflicts of interest.

References

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Supplemental figure legends

Fig. S1. ERK signaling was involved in CCL15/Gα14/16-mediated STAT3 Ser\textsuperscript{727} phosphorylation. CCR1/Gα\textsubscript{14}/293 and CCR1/Gα\textsubscript{16}/293 cells (A) or THP-1 macrophage-like cells (B) were pretreated with 100 ng/mL PTX for 16 h and then 10 μM each of U0126 or U0124 for 30 min. Afterwards, cells were stimulated in the absence (basal) or presence of 10 nM CCL15 for 15 min. Data represents mean ± SEM of determinations from three or more separate experiments. * CCL15-induced STAT3 Ser\textsuperscript{727} phosphorylation was significantly inhibited as compared to the vehicle control (p < 0.05, Dunnett’s test).
Fig. S1

A

[Graph showing STAT3 phosphorylation in response to CCR1/Gα_{14} and CCR1/Gα_{16} with Basal and CCL15 conditions.

B

[Graph showing STAT3 phosphorylation in THP-1 cells in response to CCL15 with Basal and CCL15 conditions.]