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CCR1-Mediated STAT3 Tyrosine Phosphorylation and CXCL8 Expression in THP-1 Macrophage-like Cells Involve Pertussis Toxin-Insensitive Go14/16 Signaling and IL-6 Release

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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 Go14/16 to stimulate phospholipase Cβ and NF-κB, whereas both Go14 and Go16 are also capable of activating STAT3. The coexpression of CCR1 and Go14/16 in human THP-1 macrophage-like cells suggests that CCR1 may use Go14/16 to induce STAT3 activation. In this study, we demonstrated that a CCR1 agonist, leukotactin-1 (CCL15), could indeed stimulate STAT3 Tyr705 and Ser727 phosphorylation via pertussis toxin-insensitive G proteins in PMA-differentiated THP-1 cells, human erythroleukemia cells, and HEK293 cells overexpressing CCR1 and Go14/16. The STAT3 Tyr705 and Ser727 phosphorylations were independent of each other and temporally distinct. Subcellular fractionation and confocal microscopy illustrated that Tyr705-phosphorylated STAT3 translocated to the nucleus, whereas Ser727-phosphorylated STAT3 was retained in the cytosol after CCR1/Go14 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 Go14/16. Neutralizing Ab to IL-6 inhibited CCL15-mediated STAT3 Tyr705 phosphorylation, whereas inhibition of STAT3 activity abolished CCL15-activated CXCL8 release. The ability of CCR1 to signal through Go14/16 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. The Journal of Immunology, 2012, 189: 5266–5276.

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 T 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-κB (17).

Although chemokine receptors are typically characterized as Gα-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 proto-oncogene, c-fos (18). c-Fos expression and transcriptional activation is induced upon Go16 activation (19), and constitutively active mutants of Go14 and Go16 were demonstrated to enhance the activity of STAT3 in cotransfection systems (20, 21). The coexpression of Go14/16 and CCR1, as well as their demonstrated functional coupling in THP-1 macrophage-like cells (2, 22–26), suggests that CCR1 may use Go14/16 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/CCR2 (a CCR2 agonist) enhances IL-6 production in fibroblast-like synoviocytes from patients with rheumatoid arthritis, and the response is mediated, in part, by 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 Gα14 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-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–14 and Gα16 was performed as described previously (25). HEK293 cells, CCR1/Gα14/293 and CCR1/Gα16/293 cells, or HEK293 cells transiently transfected with cDNAs encoding p-STAT3–TA–Luc, STAT3 or its phosphorylation-resistant mutants (500 ng) or wild-type or constitutively active forms of Gαi (625 ng) using Lipofectamine Plus reagent.

Luciferase reporter assay

After 24 h of transfection, cells were serum starved with 100 ng/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 was transferred 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 (BG&G Berthold, Bad Wildbad, Germany) (20, 21).

Assay for STAT3 phosphorylation

HEK cells, as well as THP-1 and U-937 macrophage-like cells, were seeded at 1 × 105 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 Static, IL-6, or CXCL8 medium. Aph for 30 min, 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 PMFS, 4 μg/ml ofophenin, and 0.6 μg/ml ofependin) 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 electrophobbing. Abs against phosphorylated STAT3 were used for the recognition of their respective phosphorylations. Fluorographs 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 1.38a (National Institutes of Health, Bethesda, MD).

Nuclear/cytosol fractionation

CCR1/293 cells, CCR1/Gα14/293 cells, or HEK293 cells transiently transfected with wild-type or constitutively active forms of Gαi were collected, and whole-cell lysates were subjected to nuclear/cytosol fractionation according to the manufacturer’s instructions. Briefly, 1 × 105 cells was resuspended in cytosol extraction buffer, 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 supernetant representing the nuclear fraction. The concentration of protein was determined using a DC protein assay kit (Bio-Rad). Eighty micrograms of proteins was separated in 12% SDS-PAGE and transferred to nitrocellulose membrane by electrophobbing. Abs against caspase-3 and CREB were used as markers for cytosolic and nuclear compartments, respectively. Fluorographs were visualized with a chemiluminescence detection kit (Amersham Pharmacia Biotech).

Confocal microscopy of STAT3 Tyr705 phosphorylation

CCR1/293 and CCR1/Gα14/293 cells grown on coverslips were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, labeled with rabbit phosphorylated Tyr705 STAT3 Ab and mouse STAT3 Ab, and stained with Alexa Fluor 555 anti-rabbit IgG and Alexa Fluor 488 anti-mouse IgG. Nuclei were stained with DAPI. Confocal microscopy was performed with a Zeiss LSM 510 META, and images were deconvoluted with LSM Image Browser Rel. 4.2 (Carl Zeiss, Oberkochen, Germany).

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, CXCL8, and TNF-α, was analyzed using the Procarta cytokine assay kit (Alifymetrix, Santa Clara, CA). Briefly, 50 μl Ab beads was added to the twet 96-well microtiter plate. Then, 50 μl each standard or test sample was added to the wells, followed by 25 μl detection Ab
indicates that the increase in STAT3 phosphorylation at Tyr705 or Ser727 was not due to variations in the abundance of the protein. Similar kinetics of CCL15-induced STAT3 Tyr705 and Ser727 phosphorylation, we used previously established HEK293 cells (23, 26), which endogenously express CCR1 (40), Gα14, and Gα16 (20, 21). These results demonstrate that, in cells that endogenously express both CCR1 and Gα14/16, CCL15 can stimulate STAT3 Tyr705 and Ser727 phosphorylation in PTX-insensitive and temporally distinct pattern. To confirm the involvement of Gα14 and Gα16 in regulating CCR1-mediated signaling, THP-1 cells were transfected with Stealth Select RNAi against Gα14 and Gα16 or Stealth RNAi negative control. The amount of CCL15-stimulated U-937 macrophage-like cells was measured by ELISA (BD).
challenged with 10 nM CCL15 for various durations. In cells expressing CCR1 alone, CCL15 stimulated STAT3 Ser\textsuperscript{727} phosphorylation transiently (15–45 min), whereas the STAT3 Tyr\textsuperscript{705} 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\textsuperscript{705} via endogenous G proteins. The incorporation of Go\textsubscript{14} or Go\textsubscript{16} strengthened the STAT3 Ser\textsuperscript{727} phosphorylation response (Fig. 1C); the duration of the response was lengthened in CCR1/Go\textsubscript{14},293 cells. STAT3 Tyr\textsuperscript{705} phosphorylation was also detected in cells coexpressing CCR1 and Go\textsubscript{14} or Go\textsubscript{16}, 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\textsubscript{i}-mediated STAT3 phosphorylation. Application of CCL15 for 15 min weakly stimulated Ser\textsuperscript{727} phosphorylation of STAT3 in cells expressing CCR1 alone, which was sensitive to PTX treatment (Fig. 2A); PTX sensitivity suggests the involvement of endogenous G\textsubscript{i} proteins in HEK293 cells. Coexpression of Go\textsubscript{14} or Go\textsubscript{16} with CCR1 enhanced the CCL15-induced STAT3 Ser\textsuperscript{727} phosphorylation, with the stimulations increased to ∼2.5-fold (Fig. 2A). Ser\textsuperscript{727} phosphorylation of STAT3 was resistant to PTX in CCR1/Go\textsubscript{14}293 and CCR1/Go\textsubscript{16}293 cells, demonstrating that CCR1 can mediate STAT3 Ser\textsuperscript{727} phosphorylation via Go\textsubscript{14} and Go\textsubscript{16} because they are the only two PTX-insensitive G proteins known to be recognized by CCR1 (25, 26). To investigate CCR1/Go\textsubscript{14}16-stimulated STAT3 Tyr\textsuperscript{705} phosphorylation, the cell lines were pretreated with PTX and stimulated with CCL15 for 6 h. In cells coexpressing CCR1 and Go\textsubscript{14} or Go\textsubscript{16}, CCL15-induced STAT3 Tyr\textsuperscript{705} phosphorylation was resistant to PTX (Fig. 2A). Another CCR1 agonist, myeloid progenitor inhibitory factor-1 (CCL23), induced STAT3 phosphorylation at Ser\textsuperscript{727} within 15 min, but STAT3 phosphorylation at Tyr\textsuperscript{705} was detected 6 h after drug treatment, and the response was not sensitive to PTX (Fig. 2B). These results indicated that CCR1/Go\textsubscript{14}16-induced phosphorylation of STAT3 at Tyr\textsuperscript{705} and Ser\textsuperscript{727} have distinct kinetics. It should be noted that CCL15-induced STAT3 phosphorylation at Tyr\textsuperscript{705} 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/Go\textsubscript{14}16-mediated STAT3 phosphorylation can indeed lead to STAT3 transcriptional activity. CCR1/Go\textsubscript{14}293 cells were transfected with cDNAs encoding STAT3-driven luciferase reporter, pretreated with PTX, and challenged with 10 nM CCL15. Consistent with the results for STAT3 Tyr\textsuperscript{705} phosphorylation, CCL15 significantly stimulated the STAT3-driven luciferase activity (Fig. 2C). Similar results were obtained in STAT3-Luc-expressing CCR1/Go\textsubscript{16}293 cells (Fig. 2C). To investigate whether CCR1/Go\textsubscript{14}16-mediated Tyr\textsuperscript{705} and Ser\textsuperscript{727} phosphorylation of STAT3 can occur independently, CCR1/Go\textsubscript{14}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/Go\textsubscript{14}16-induced STAT3 Tyr\textsuperscript{705} phosphorylation was attenuated in STAT3Y705F-expressing cells but not in STAT3S727A-expressing cells (Fig. 2D, left panels). In contrast, CCR1/Go\textsubscript{14}16-mediated STAT3 Ser\textsuperscript{727} phosphorylation was abolished by STAT3S727A overexpression, whereas it was unaffected by STAT3Y705F overexpression (Fig. 2D, right panels). These results implied that CCR1/Go\textsubscript{14}16 activation STAT3 Tyr\textsuperscript{705} and Ser\textsuperscript{727} phosphorylations independently.

\textbf{CCR1/Go\textsubscript{14}16 induces phosphorylation of STAT3 at Tyr\textsuperscript{705} in nucleus and Ser\textsuperscript{727} 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\textsuperscript{727} phosphorylation in the absence of Tyr\textsuperscript{705} phosphorylation is intriguing, because the Ser\textsuperscript{727} site is generally believed to enhance STAT3 transcriptional activity, which requires Tyr\textsuperscript{705} phosphorylation (41). Nuclear/cytosol fractionation was performed to investigate whether CCR1/Go\textsubscript{14}16 activation can lead to the translocation of Tyr\textsuperscript{705} or Ser\textsuperscript{727}-phosphorylated STAT3 to the nucleus. Consistent with the results shown in Fig. 2A, the level of STAT3 Tyr\textsuperscript{705} and Ser\textsuperscript{727} 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\textsuperscript{705} or Ser\textsuperscript{727} phosphorylation was observed in cytosolic or nuclear compartments. Coexpression of Go\textsubscript{14} with CCR1/293 cells allowed CCL15 to stimulate STAT3

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\caption{CCL15-induced STAT3 phosphorylations at Tyr\textsuperscript{705} and Ser\textsuperscript{727} 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; 16 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 of 10 nM CCL23 for 15 min or 6 h and with PTX pretreatment (100 ng/ml; 16 h). Three individual experiments showed similar results. (C) CCR1/Go\textsubscript{14}293 and CCR1/Go\textsubscript{16}293 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/Go\textsubscript{14}293 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.}
\end{figure}
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 cytosolic 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 Go14 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/Go14 activation. We demonstrated previously that members of the Gq subfamily can induce STAT3 Tyr705 phosphorylation (37). Thus, we investigated whether the activation of Go14 or Go16 could translocate Tyr705-phosphorylated STAT3 into the nucleus. In line with the previous study, active mutants of Go14 and Go16 could induce STAT3 Tyr705 phosphorylation, which was primarily localized to the nucleus (Fig. 3B).

**CCR1/Go14/16-mediated STAT3 Tyr705 phosphorylation involves protein synthesis**

Because CCR1/Go14/16-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-
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/G_{14/16}-induced STAT3 Tyr^{705} phosphorylation without affecting the cell viability, whereas no inhibitory effect was observed on Ser^{727} phosphorylation (Fig. 5A). The same inhibitory effect on CCL15-induced STAT3 Tyr^{705} phosphorylation was observed in PMA-differentiated THP-1 cells (Fig. 5B). CCL15-activated Ser^{727} 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 Tyr^{705} of an MEK1/2 kinase inhibitor (U0126) and its inactive analog (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 Tyr^{705} phosphorylation induced by CCR1/G_{14/16} may require the synthesis of STAT3 activators. We then investigated whether the conditioned medium from CCL15-stimulated CCR1/G_{14/293} or CCR1/G_{16/293} cells was sufficient to phosphorylate STAT3 at Tyr^{705}. Conditioned medium was collected from CCR1/G_{14/293} or CCR1/G_{16/293} cells stimulated with CCL15 for 15 min, 4 h, or 6 h, transferred to parental HEK293 cells, and incubated for 30 min; STAT3 Tyr^{705} phosphorylation was observed in the parental HEK293 cells treated with conditioned medium of CCR1/G_{14/293} or CCR1/G_{16/293} cells with 6 h of CCL15 treatment (Fig. 6A). Similarly, STAT3 Tyr^{705} 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 G_{14} subfamily members (G_{14a}, G_{16a}, G_{14b}, and G_{11b}) but not G_{14} (the PTX-insensitive G_{14} subfamily members) (Fig. 6C). These results indicated that there were cytokines or growth factors in the conditioned medium that could induce STAT3 Tyr^{705} phosphorylation.

**FIGURE 6.** Conditioned medium from CCL15-stimulated CCR1/G_{14/293} cells, CCR1/G_{16/293} cells, and THP-1 macrophage-like cells induced STAT3 phosphorylation at Tyr^{705}. 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 G_{14a}, G_{16a}, G_{14b}, G_{11b}, or G_{14c} (G) 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 at 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 G_{14} subunits, three individual experiments showed similar results.

**IL-6, but not CXCL8, is required for CCR1/G_{14/16}-mediated STAT3 Tyr^{705} 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/G_{14/293} and CCR1/G_{16/293} cells. After 4 h of drug incubation, CCL15 significantly stimulated IL-6 production in CCR1/G_{14/293} and CCR1/G_{16/293} 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/G_{14/293} cells, indicating that CCR1 used G_{14a} and G_{16a} to stimulate IL-6 expression. CXCL8, a proinflammatory CXC chemokine, was also detected in the conditioned medium of CCR1/G_{14/293} and CCR1/G_{16/293} cells with a 4-h treatment with CCL15. CCL15 induced CXCL8 production by 22.5-fold in CCR1/G_{14/293} cells, whereas the production was increased 12-fold in CCR1/G_{16/293} cells (Fig. 7B). No significant CCL15-induced CXCL8 secretion was detected in CCR1/G_{16/293} 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- or CXCL8-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-mediated STAT3 Tyr705 phosphorylation in macrophages, U-937 macrophage-like cells were treated with IL-6-neutralizing Ab for 30 min before the addition of CCL15. PMA-differentiated U-937 macrophage-like cells were pretreated with 100 ng/ml PTX 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<sup>705</sup> phosphorylation was inhibited by IL-6–neutralizing Ab, illustrating the requirement for IL-6 in the phosphorylation of STAT3 at Tyr<sup>705</sup>.

**STAT3 Tyr<sup>705</sup> phosphorylation is necessary for CCR1/Go<sub>14/16</sub>-mediated CXCL8 expression**

Because the expression of cytokines could be regulated by STAT3 activation, we investigated whether STAT3 Tyr<sup>705</sup> phosphorylation was required for CCR1/Go<sub>14/16</sub>-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<sup>705</sup> phosphorylation in both CCR1/Go<sub>14/16</sub>/293 and CCR1/Go<sub>16/293</sub> cells (Fig. 9A), but it did not affect Ser<sup>727</sup> phosphorylation of STAT3 in these cells. The level of STAT3 phosphorylations at Tyr<sup>705</sup> and Ser<sup>727</sup> 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 Go<sub>14</sub>, but no inhibitory effect on CCL15-activated IL-6 production was observed (Fig. 9B, 9C); similar results were obtained with CCL15-treated CCR1/Go<sub>16/293</sub> 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<sup>705</sup> phosphorylation but not CCL15-triggered STAT3 Ser<sup>727</sup> phosphorylation (Fig. 10A). The requirement of STAT3 Tyr<sup>705</sup> phosphorylation was also demonstrated in CCL15-stimulated CXCL8 production (Fig. 10B), but not CCL15-activated IL-6 release (Fig. 10B), in THP-1 cells. In U-937 cells, application of Stattic attenuated CCL15-mediated STAT3 Tyr<sup>705</sup> phosphorylation (data not shown) and CXCL8 release (Fig. 10C). Pretreatment with IL-6–neutralizing Ab also abolished CCL15-induced CXCL8 production (Fig. 10C), showing the involvement of STAT3 Tyr<sup>705</sup> 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 Stattic, 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 Tyr\textsuperscript{705} 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 Ser\textsuperscript{727} phosphorylation has not been documented. By examining the role of G\textsubscript{α14/16} in transfected HEK293 cells, native HEL cells, and native THP-1 and U-937 macrophage-like cells, this study provided evidence that STAT3 Tyr\textsuperscript{705} phosphorylation is not a prerequisite for Ser\textsuperscript{727} phosphorylation, and IL-6 autocrine signaling is apparently involved in CCR1/G\textsubscript{α14/16}-mediated STAT3 Tyr\textsuperscript{705} 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 Tyr\textsuperscript{705} and Ser\textsuperscript{727} are most likely to be contributed by CCR1.

Our study clearly demonstrated that CCL15-stimulated CCR1 is capable of inducing PTX-insensitive STAT3 phosphorylations at Tyr\textsuperscript{705} and Ser\textsuperscript{727} only in the presence of G\textsubscript{α14} or G\textsubscript{α16}. Moreover, the chemokine-induced phosphorylations of STAT3 at Tyr\textsuperscript{705} and Ser\textsuperscript{727} occurred independently (Fig. 2D) and in temporally distinct manners (Fig. 1), with Tyr\textsuperscript{705}-phosphorylated STAT3 translocated to the nucleus and Ser\textsuperscript{727}-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, 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, 8C, 8D).

We showed previously that activation of G\textsubscript{α14/16} 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 Tyr\textsuperscript{705} phosphorylation within 15 min in HEK293 cells. In contrast, CCR1/G\textsubscript{α14/16}-mediated Tyr\textsuperscript{705} 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 Tyr\textsuperscript{705} phosphorylation is not unique to CCR1; it has been similarly observed with G\textsubscript{α16}-coupled melatonin MT\textsubscript{1} and MT\textsubscript{2} receptors (36). Moreover, distinct temporal patterns of STAT3 phosphorylations at Tyr\textsuperscript{705} and Ser\textsuperscript{727} have been documented. In murine macrophage-like RAW 264.7 cells, STAT3 Ser\textsuperscript{727} phosphorylation induced by LPS can be observed at 5 min, whereas STAT3 Tyr\textsuperscript{705} phosphorylation requires 2 h of treatment (54). Likewise, two groups demonstrated that angiotensin II induces delayed STAT3 Tyr\textsuperscript{705} phosphorylation in rat aortic smooth muscle cells (55) and rat cardiomyocytes (56).

The induction of IL-6 and CXCL8 by CCR1/G\textsubscript{α14/16} 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/G\textsubscript{α14/16} 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 Tyr705 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 AMP 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 involved in the recruitment of human NK cells to sites of early viral infection by mast cells (61) and metastasis of colon cancer (62). The release of CXCL8 activates CXCR2, which is coexpressed with CCR1 in macrophages (39) and human mast cells (63, 64), leading to STAT3 Tyr705 phosphorylation (65). Hence, the production of CXCL8 mediated by CCR1/Gα14/16 may create another loop of STAT3 activation, which synergizes with the effect on CXCL8 secretion.

Although the Ser727 site is generally believed to enhance the transcriptional activity of Tyr705 phosphorylated STAT3 (41), recent studies demonstrated that STAT3 phosphorylation at Ser727 alone can trigger a variety of biological activities. STAT3 Ser727 phosphorylation is essential for postnatal survival and growth, as well as thymocyte proliferation, as shown in knock-in mice with alanine substituted for Ser727 in STAT3 (66). A recent study in mouse pro-B cells showed that mitochondrial STAT3 Ser727 phosphorylation is important in regulating the oxidative phosphorylation of mitochondria via association with complex I/II of the electron transport chain (67). We conducted mitochondria/cytosol fractionation to examine whether CCR1/Gα14 activation can modulate STAT3 Ser727 phosphorylation in the mitochondria; our preliminary data showed that the basal level of mitochondrial Ser727-phosphorylated STAT3 in CCR1/Gα14/293 cells was increased compared with cells expressing CCR1 alone. This tends to suggest that the basal cellular ATP level may be higher in CCR1/Gα14/293 cells. The elevated basal level of mitochondrial Ser727-phosphorylated STAT3 was apparently unaffected by CCL15 treatment. Because there are no data on chemokines regulating cellular metabolism, further investigation is warranted to examine the biological function of CCR1/Gα14/16-mediated STAT3 Ser727 phosphorylation.

In summary, IL-6 autocrine signaling is apparently involved in CCR1/Gα14/16-mediated STAT3 Tyr705 phosphorylation, which is required for the subsequent production of CXCL8. In turn, CXCL8 may activate CXCR2, leading to further STAT3 Tyr705 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.


