Macrophages JAK-Dependent IL-10 Expression in Metalloproteinase-9 Is Modulated by Mediated Induction of Matrix IL-6

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IL-6–Mediated Induction of Matrix Metalloproteinase-9 Is Modulated by JAK-Dependent IL-10 Expression in Macrophages

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The mechanisms by which IL-6 contributes to the pathogenesis of chronic inflammatory diseases and cancer are not fully understood. We previously reported that cyclooxygenase-2 (Cox-2)–dependent PGE2 synthesis regulates macrophage matrix metalloproteinase (MMP)-9 expression, an endopeptidase that participates in diverse pathologic processes. In these studies, we determined whether IL-6 regulates the Cox-2→PGE2→MMP-9 pathway in murine macrophages. IL-6 coinduced Cox-2 and microsomal PGE synthase-1, and inhibited the expression of 15-hydroxyprostaglandin dehydrogenase, leading to increased levels of PGE2. In addition, IL-6 induced MMP-9 expression, suggesting that the observed proteinase expression was regulated by the synthesis of PGE2. However, inhibition of PGE2 synthesis partially suppressed IL-6–mediated induction of MMP-9. In the canonical model of IL-6–induced signaling, JAK activation triggers STAT and MAPK erk1/2-signaling pathways. Therefore, the ability of structurally diverse JAK inhibitors to block IL-6–induced MMP-9 expression was examined. Inhibition of JAK blocked IL-6–induced phosphorylation of STAT3, but failed to block the phosphorylation of MAPK erk1/2, and unexpectedly enhanced the ability of structurally diverse JAK inhibitors to block IL-6–induced MMP-9 expression without affecting the phosphorylation of STAT3. Thus, IL-6–induced MMP-9 expression is dependent on the activation of MAPK erk1/2 and is restrained by a JAK-dependent gene product. Using pharmacologic and genetic approaches, we identified JAK-dependent induction of IL-10 as a potent feedback mechanism controlling IL-6–induced MMP-9 expression. Together, these data reveal that IL-6 induces MMP-9 expression in macrophages via Cox-2–dependent and –independent mechanisms, and identifies a potential mechanism linking IL-6 to the pathogenesis of chronic inflammatory diseases and cancer.

Interleukin-6, a pleiotropic cytokine expressed by a variety of immune and nonimmune cells, plays an important role in the recruitment and survival of neutrophils and macrophages, regulation of CD4 T cell effector functions, angiogenesis, bone and cartilage metabolism, lipid metabolism, and the expression of acute-phase proteins (1, 2). Circulating levels of IL-6 are elevated in patients with cancer (3) and several chronic inflammatory diseases including rheumatoid arthritis (4) and cardiovascular disease (CVD) (5). In addition, adipose tissue is a major source of circulating IL-6, and levels are elevated in obese patients (6). Because of its multiple roles in the pathogenesis of inflammatory diseases and cancer (1, 2), IL-6 has emerged as a major target for therapeutic intervention (7, 8).

IL-6–induced biological responses are mediated by the membrane-bound IL-6R (IL-6Rα; CD126) (7, 9, 10). The IL-6–IL-6R complex engages transmembrane gp130 (IL-6Rβ; CD130), and the ternary complex dimerizes triggering the binding and phosphorylation of JAK, which then phosphorylates gp130, leading to the activation of STAT and MAPK signaling pathways. Despite the broad biologic activities of IL-6, surprisingly few cell types (e.g., monocyte/macrophages and hepatocytes) express membrane-bound IL-6Rα. In contrast, virtually all cell types express gp130, which can bind soluble IL-6–IL-6Rα complexes (i.e., trans signaling), thereby triggering STAT and MAPK signaling pathways.

Notwithstanding the significant progress in unraveling the many effects of IL-6 on immune and nonimmune cells, the mechanisms by which IL-6 contributes to the pathogenesis of chronic inflammatory diseases and cancer is not fully understood. In this regard, evidence derived from mouse models indicates that matrix metalloproteinase (MMP)-9 (type IV collagenase; gelatinase B), a family member of Zn2+-dependent neutral endopeptidases, participates in the pathogenesis of arthritis (11), airway disease (12), cancer (13, 14), and CVD (15–17). MMP-9 expression is low or absent in most normal tissues and is markedly elevated during inflammation, wound healing, and neoplasia (18–20). We and others have reported that macrophage MMP-9 expression is stimulated by PGE2 (21–29). Increased cyclooxygenase-2 (Cox-2)–dependent synthesis of PGH2 and subsequent isomerization to PGE2 by microsomal PGE synthase (mPGES)-1 (29–34), in combination with reduced catabolism by NAD+–dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH) (35, 36), are largely responsible for increased levels of PGE2 associated with inflammation. Consequently,
we determined whether IL-6 regulates the Cox-2→mPGES-1→PGE2→MMP-9 pathway in macrophages.

Results demonstrate that IL-6 induced MMP-9 expression in macrophages via Cox-2–dependent and –independent pathways. Because IL-6 can activate both JAK/STAT and MAPK pathways, we explored their roles in regulating MMP-9 expression. Inhibition of MAPKerk1/2 blocked IL-6–mediated induction of MMP-9. In contrast, inhibition of JAK led to a paradoxical increase in MMP-9 expression, which proved to be a consequence of diminished IL-10 levels. To the best of our knowledge, this is the first demonstration that IL-6 induces macrophage expression of MMP-9, which has been directly associated with the pathogenesis of chronic inflammatory diseases and cancer (18–20). In addition, these data suggest that JAK inhibitors have the potential to increase MMP-9 expression in macrophages via the inhibition of IL-10 expression.

Materials and Methods

Macrophages

Thioglycollate-elicited peritoneal macrophages were obtained from Swiss Webster mice by the method of Edelson and Cohn (37) as described previously (38). Mice were injected i.p. (3 ml/mouse) with 3% Brewer Thioglycollate Medium (DIFCO). Four days later, cells were harvested by lavage with cold Dulbecco’s PBS. Peritoneal cells were recovered by centrifugation and re-suspended in DMEM supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and 4 mM glutamine, and plated into 6-well (2 × 10⁶ cells/well) or 12-well plates (6 × 10⁵ cells/well). Cells were allowed to adhere for 4 h and then washed free of non-adherent cells. The murine macrophage cell line RAW264.7 (39) was obtained from American Type Culture Collection and maintained as adherent cultures in DMEM–10% FBS. RAW264.7 cells were mechanically harvested and plated into 12-well plates (6 × 10⁵ cells/well). For serum-free conditions, macrophages were grown in DMEM supplemented with antibiotics, glutamine, and 0.1% BSA (Sigma Aldrich) containing low levels of endotoxin (LE; <0.1 ng/ml; LE-BSA). Cellgro DMEM was obtained from Corning, and heat-inactivated FBS was obtained from Atlanta Biologicals. Antibiotics and glutamine were obtained from Life Technologies/Life Technologies. Macrophages were incubated with recombinant human IL-6 (Santa Cruz Biotechnology), recombinant mouse IL-10 (Biorbyt), rat monoclonal anti-mouse IL-10 or nonimmune rat IgG (R&D Systems) celecoxib (LC Laboratories), JAK Inhibitor 1 (Calbiochem/EMD Millipore), tofacitinib (Selleckchem), or U0126 (Cayman Chemical). All animal studies described in this report have been reviewed and approved by the Weill Cornell Medical College Institutional Animal Care and Use Committee.

Preparation of cell lysates

Cells lysates were electrophoresed in 8–16% polyacrylamide gels and proteins were transferred to a polyvinylidene fluoride membrane. The membrane was blocked in 5% BSA in TBST, washed in TBST, and incubated 18 h in blocking buffer containing rabbit anti-mouse phosphorylated STAT3 (Tyr705) or rabbit anti-human phosphorylated erk1/2 IgG (Cell Signaling Technology). Membranes were washed 3 × in TBST and incubated 1 h in blocking buffer containing affinity-purified goat anti-rabbit IgG HRP conjugated (1:3000; Bio-Rad Laboratories). The membranes were washed 3 × in TBST, and bound HRP was visualized using chemiluminescence (Pierce/Thermo Scientific). Membranes were then stripped in 0.1% SDS, 1.0% Tween 20, and 200 mM

FIGURE 1. IL-6 induces Cox-2 and mPGES-1 expression, and inhibits 15-PGDH expression in RAW264.7 macrophages. A: Macrophages were incubated in DMEM–0.1% LE–BSA containing 0–100 ng/ml IL-6 or 10 ng/ml LPS. After 18-h incubation, total RNA and conditioned media were recovered. B: Macrophages were incubated 0–18 h in DMEM–0.1% LE–BSA containing 100 ng/ml IL-6 or 10 ng/ml LPS for 18 h. The RQ of Cox-2, mPGES-1, and 15-PGDH mRNAs was determined by quantitative RT-PCR as described in Materials and Methods. Levels of PGE2 in conditioned media were determined using ELISA. Target gene mRNA and PGE2 levels are expressed as the mean ± SD of three independent samples from a representative experiment.
glycine, pH 2.2 10 min at room temperature, washed, blocked for 1 h in blocking buffer, and incubated with rabbit anti-mouse STAT3 (1:2000; Cell Signaling Technology) or mouse anti-erkl/2 (KLH-synthetic peptide; 1:3000; Cell Signaling Technology).

Macrophage conditioned media were electrophoresed in gradient gels, and proteins were transferred to a polyvinylidene fluoride membrane. The membrane was placed in 5% dry defatted milk in TBST for 1 h, washed in TBST, and incubated 18 h in 3% blocking buffer containing rabbit anti-mouse MMP-9 IgG (1:2500; Abcam). Membranes were washed 2× in TBST and incubated 1 h in 3% blocking buffer containing goat anti-rabbit IgG conjugated to HRP (1:3000; Bio-Rad Laboratories).

**IL-10 knockdowns**

RAW264.7 macrophages were transfected with control small interfering RNA (siRNA; i.e., nonspecific [NS]) or IL-10 siRNA (Santa Cruz Biotechnology) using HiPerFect transfection reagent according to the manufacturer’s protocol (Qiagen). Cells were aliquoted into a 12-well (3 × 10^5 cells/well) plate containing siRNA transfection complexes and incubated 30 h. PCR and immunoassay was used to determine the extent of IL-10 knockdown.

**Real-time PCR analysis**

RNA was prepared using TRIzol reagent kits (Invitrogen). RNA (2 µg) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen) and random hexamers. The resulting cDNA diluted 10-fold was used for amplification. For real-time PCR analysis, the reaction volume was 5 µl and contained 1–2 µl of the diluted cDNA, 1× Gene Expression Assay (Life Technologies), 1× PerfeCTa FastMix II, Rox (Quanta) using the StepOne instrument (Applied Biosystems). The following Applied Biosystems Gene Expression Assays were used: actin (mm00607939_s1), Cox-2 (mm00478374_m1), MMP-9 (mm00442991_m1), mPGES-1 (mm00452105_m1), IL-6 (mm00446190_m1), 15-PGDH (mm00515121_m1), and IL-10 (mm00439614_m1). The comparative C_T (ΔΔC_T) method was used to determine the relative target quantity in samples. With the comparative C_T method, the StepOne software measures amplification of the target and endogenous control in the samples and in a reference sample. Measurements are normalized using the endogenous control and expressed as relative quantity (RQ).

**Determination of PGE_2 and IL-10 levels**

The concentrations of PGE_2 and IL-10 in macrophage conditioned media were determined using the PGE_2 enzyme immunoassay kit (monoclonal; Cayman Chemical) and IL-10 Quantikine Immunoassay (R&D Systems).
Statistics

RQs of target gene mRNA and PGE$_2$ or IL-10 levels are expressed as the mean ± SD of three independent samples from a single representative experiment. Each experiment was repeated two to three times. Where indicated, mean levels of mRNA, PGE$_2$, or IL-10 were compared using t test (e.g., comparing means of two groups) or single-factor ANOVA (e.g., comparing means of greater than two groups). After the determination of significant differences between the means of a given experiment, subsequent pairwise comparisons were performed using Newman–Keuls multiple-range testing (40).

Results

IL-6 stimulates Cox-2 and mPGES-1 expression, and inhibits 15-PGDH expression in macrophages

We determined whether IL-6 coinduces Cox-2 and mPGES-1 expression in macrophages, leading to increased levels of PGE$_2$. Incubation with LPS, a potent inducer of Cox-2 and mPGES-1 in macrophages, served as a positive control (30, 31, 41). Levels of Cox-2 and mPGES-1 mRNA were determined using quantitative PCR. Incubation of RAW264.7 macrophages with IL-6 induced Cox-2 and mPGES-1 expression in a dose- and time-dependent manner (Fig. 1). Because reduced catabolism of PGs contributes to increased levels of PGE$_2$ in inflammatory foci, we also examined the effect of IL-6 on the expression of 15-PGDH, a key enzyme responsible for the catabolism of PGs (35, 36). In contrast with Cox-2 and mPGES-1 expression, IL-6 rapidly and potently inhibited 15-PGDH expression in macrophages (Fig. 1). Consistent with the IL-6–induced reciprocal changes in expression of genes directing the synthesis and degradation of PGE$_2$, levels of PGE$_2$ in media recovered from RAW264.7 cells increased in a dose- and time-dependent manner (Fig. 1).

To corroborate and extend these findings to primary macrophages, we examined the effect of IL-6 on the expression of Cox-2, mPGES-1, and 15-PGDH by macrophages recovered from thioglycollate-induced peritonitis (Fig. 2). After 18-h incubation with IL-6, levels of Cox-2 and mPGES-1 mRNA in elicited macrophages were markedly elevated, and levels of 15-PGDH mRNA were reduced. Likewise, levels of PGE$_2$ in media recovered from elicited macrophages treated with IL-6 were increased dramatically.

In these studies, murine macrophages were incubated with recombinant human IL-6, which binds and activates the murine IL-6R (42). To confirm that the observed alterations in Cox-2, mPGES-1, and 15-PGDH were due to IL-6 and not a potential contaminant, we preincubated human IL-6 with nonimmune IgG or neutralizing anti-human IL-6 IgG before incubation with macrophages. Anti–IL-6 IgG blocked IL-6–induced alterations in gene expression in murine macrophages (data not shown). Taken together, these data demonstrate that IL-6 stimulates macrophage expression of Cox-2 and mPGES-1, and inhibits the expression of 15-PGDH, leading to increased levels of PGE$_2$.

Cox-2–dependent PGE$_2$ synthesis plays a role in IL-6–mediated induction of MMP-9

Macrophage MMP-9 expression is regulated by Cox-2–dependent synthesis of PGE$_2$ (26–29). Thus, we examined whether the observed changes in expression of genes directing the synthesis and degradation of PGE$_2$ in response to IL-6 (Figs. 1, 2) were associated with changes in MMP-9 expression. Treatment of RAW264.7 macrophages with IL-6 resulted in a dose- and time-dependent increase in the levels of MMP-9 mRNA (Fig. 3A, 3B). The dose-dependent increases in MMP-9 mRNA were mirrored by an

FIGURE 4. Cox-2–dependent PGE$_2$ synthesis contributes to IL-6–induced MMP-9 expression. A, RAW264.7 macrophages were preincubated in DMEM–0.1% LE–BSA containing DMSO (vehicle control) or 5 μM celecoxib for 30 min, after which 100 ng/ml IL-6 was added. After 18-h incubation, conditioned media were recovered for determination of (A) PGE$_2$ and (C) MMP-9 levels using ELISA and Western blot, respectively. B, Total RNA was recovered and levels of MMP-9 mRNA were determined using quantitative RT-PCR. Levels of PGE$_2$ and MMP-9 mRNA are expressed as the mean ± SD of three independent samples from a representative experiment.

FIGURE 5. Opposing effects of JAK and MEK-1 inhibitors on IL-6–dependent MMP-9 expression. RAW264.7 macrophages were preincubated 30 min in DMEM–0.1% LE–BSA containing (A) 0–100 nM JAK Inhibitor 1, (B) 0–1.0 μM tofacitinib, or (C) 0–10 μM U0126, followed by the addition of 100 ng/ml IL-6. After 18-h incubation, total RNA and conditioned media were recovered. Levels of MMP-9 mRNA and MMP-9 in conditioned media were determined using quantitative RT-PCR and Western blot, respectively. MMP-9 mRNA levels are expressed as the mean ± SD of three independent samples from a representative experiment.
accumulation of MMP-9 in cellular conditioned media (Fig. 3A). Treatment with IL-6 similarly stimulated MMP-9 expression in elicited peritoneal macrophages ($p < 0.01$; Fig. 3C). IL-6–induced macrophage MMP-9 expression identifies a potential mechanism linking increased IL-6 levels to the pathogenesis of chronic inflammatory diseases and cancer (18–20).

In contrast with earlier studies where MMP-1– and MMP-3–induced Cox-2 expression and increased PGE2 synthesis preceded elevated MMP-9 expression (28), IL-6–induced MMP-9 expression parallels the expression of both Cox-2 and mPGES-1 (Figs. 1, 3). These findings raise the possibility that the increased synthesis of PGE2 may not be solely responsible for the induction of MMP-9. However, because macrophage MMP-9 expression is stimulated by PGE2 (26–29), IL-6–induced reciprocal changes in the expression of genes directing the synthesis and degradation of PGE2 may contribute to MMP-9 expression. Therefore, we examined the effect of the selective Cox-2 inhibitor celecoxib on IL-6–induced PGE2 levels and MMP-9 expression in macrophages. The levels of PGE2 in conditioned media recovered from macrophages incubated with IL-6 (18 h) were increased >20-fold, as compared with control macrophages (Fig. 4A). Pretreatment with celecoxib completely inhibited IL-6–induced PGE2 synthesis, but led to only a modest reduction in IL-6–mediated induction of MMP-9 mRNA ($p < 0.05$; Fig. 4B) and protein (Fig. 4C). These data indicate that IL-6 stimulates MMP-9 expression by both Cox-2–dependent and –independent mechanisms.

**IL-6–induced MMP-9 expression is MAPKerk1/2 dependent**

In the canonical model of IL-6–induced signaling, binding of IL-6/IL-6R to gp130 triggers the activation of the JAK and subsequent phosphorylation of several tyrosines within the cytoplasmic tail of gp130 creating binding sites for STAT and SHP2. The latter is believed to be critical for the activation of MAPKerk1/2 (7, 9, 10). Thus, inhibition of JAK activity ought to block both STAT and MAPKerk1/2 signaling pathways. Therefore, we examined the effect of two selective inhibitors of JAK on IL-6–induced MMP-9 expression (Fig. 5). Unexpectedly, pretreatment of macrophages with JAK Inhibitor 1 (1–100 nM) augmented IL-6–mediated induction of MMP-9 mRNA and MMP-9 protein in cellular conditioned media (Fig. 5A). Likewise, pretreatment with tofacitinib (0.1–1 μM), a structurally distinct JAK inhibitor, augmented IL-6–mediated induction of MMP-9 (Fig. 5B). In contrast, pretreatment with the MEK1 inhibitor U0126 (1–10 μM) led to a dose-dependent inhibition of IL-6–induced MMP-9 expression (Fig. 5C). The failure of JAK inhibitors to block IL-6–induced MMP-9 expression was unexpected. Thus, we monitored their ability to inhibit the phosphorylation of STAT3 and MAPKerk1/2. As anticipated, pretreatment with JAK Inhibitor 1 (100 nM) or tofacitinib (1 μM) completely blocked IL-6–induced phosphorylation of STAT3, whereas IL-6–induced phosphorylation of MAPKerk1/2
was unaffected (Fig. 6C). Pretreatment with U0126 blocked IL-6–induced phosphorylation of MAPKerk1/2 (Fig. 6B) but had no effect on IL-6–induced phosphorylation of STAT3 (Fig. 6A). Although these data are unexpected based on the established model of IL-6–induced signaling, several investigators have reported that diverse JAK inhibitors block STAT activation without inhibiting MAPKerk1/2 activation (43–47); however, the mechanism(s) underlying JAK-independent activation of MAPKerk1/2 remains undefined at this time.

Although IL-6 is one of the most highly induced NF-κB–dependent cytokines (48), NF-κB is not generally reported to mediate IL-6–dependent signaling and gene expression (7, 9, 10). However, it has been reported that in addition to canonical JAK/STAT signaling, IL-6 induces the accumulation of unphosphorylated STAT3, which forms a complex with unphosphorylated NF-κB that can translocate into the nucleus (49). Therefore, we examined the effect of two cell-permeable NF-κB activation inhibitors on IL-6–induced MMP-9 expression. Preincubation of RAW264.7 macrophages with several concentrations of NF-κB Activation Inhibitor I (CAS 545380-34-5) or II (CAS 749886-87-1) failed to block or attenuate IL-6–induced MMP-9 expression (data not shown). Taken together, our data demonstrate that IL-6–induced MMP-9 expression in macrophages is dependent on the activation of MAPKerk1/2 and is paradoxically enhanced by inhibition of JAK.

**ToFacitinib inhibition of IL-6–induced IL-10 enhances MMP-9 expression**

The observation that IL-6–induced MMP-9 expression in macrophages was enhanced by JAK inhibition suggested that a JAK

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**FIGURE 8.** IL-10 potently suppresses IL-6–induced MMP-9 expression in macrophages. **A** RAW264.7 macrophages were preincubated 1 h in DMEM–0.1% LE–BSA containing 0–100 ng/ml recombinant mouse IL-10, after which 100 ng/ml IL-6 was added, and incubated 18 h. **B** Macrophages were preincubated 1 h in DMEM–0.1% LE–BSA containing nonimmune (n) IgG or anti–IL-10R, followed by 1-h incubation with 10 ng/ml IL-10, and an additional 18-h incubation with 100 ng/ml IL-6. **C** Macrophages were incubated in DMEM–0.1% LE–BSA containing 100 ng/ml IL-6 for 6 h, after which 10 ng/ml IL-10 was added and incubated for 18 h. After the recovery of total RNA and conditioned media, levels of MMP-9 mRNA and MMP-9 in conditioned media were determined using quantitative RT-PCR and Western blot, respectively. Levels of MMP-9 mRNA are expressed as the mean ± SD of three independent samples of a representative experiment.

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**FIGURE 9.** Inhibition of IL-10 activity or expression enhances IL-6–induced MMP-9 expression in macrophages. **A** RAW264.7 macrophages were incubated in DMEM–0.1% LE–BSA alone (Ctrl) or media supplemented with 100 ng/ml IL-6, IL-6, and 2 μg/ml anti-murine IL-10 IgG, or IL-6 and normal IgG for 18 h. After the recovery of total RNA, levels of MMP-9 mRNA were determined using quantitative RT-PCR, and levels of MMP-9 in conditioned media were determined by Western blot. **B** Macrophages transfected with NS or IL-10 siRNA received DMEM–0.1% LE–BSA (Ctrl) or media containing 100 ng/ml IL-6, and were incubated 18 h. Total RNA was recovered, levels of MMP-9 mRNA were determined using quantitative RT-PCR, and levels of IL-10 in conditioned media were determined using ELISA. Data are the mean ± SD of three independent samples of a representative experiment.
dependent gene product serves to limit IL-6–induced MMP-9 in macrophages. In this regard, the proinflammatory properties of LPS are limited, in part, by the JAK-dependent induction of IL-10, a potent anti-inflammatory cytokine (46), which is also reported to inhibit MMP-9 expression (50–53). Therefore, we examined whether IL-6 stimulated IL-10 expression in macrophages. As shown in Fig. 7A and 7B, levels of IL-10 mRNA in macrophages and IL-10 in conditioned media were increased in a dose-dependent manner. Likewise, IL-6 induced IL-10 expression in a time-dependent manner (Fig. 7C, 7D). When compared with control cells, levels of IL-10 mRNA are increased >60-fold in as little as 2 h and >1000-fold at 18 h. Levels of IL-10 in the conditioned media recovered from cells incubated with IL-6 were first elevated at 4 h (124 ± 90 pg/ml, mean ± SD; n = 3) and increased to 2031 ± 65 pg/ml at 18 h. Importantly, IL-6–induced IL-10 expression was inhibited, in a dose-dependent manner, by JAK Inhibitor 1 or tofacitinib (Fig. 7E, 7F).

Next, we determined the effect of exogenous IL-10 on IL-6–induced MMP-9 expression. When macrophages were pretreated (1 h) with as little as 1 ng/ml IL-10, subsequent exposure to IL-6 failed to induce MMP-9 expression (Fig. 8A). The IL-10–dependent inhibition of MMP-9 expression was blocked when macrophages were preincubated with neutralizing anti–IL-10R IgG (Fig. 8B). These data clearly demonstrate that pre-exposure of macrophages to IL-10 potently inhibits the ability of IL-6 to induce MMP-9 expression. In addition, IL-10 effectively turned off previously upregulated MMP-9 expression. As shown in Fig. 8C, levels of MMP-9 mRNA in macrophages incubated with IL-6 for 18 h were increased ~3-fold, whereas levels of MMP-9 mRNA in cells incubated with IL-6 for 6 h followed by the addition of IL-10 were similar to control macrophages at 18 h. Likewise, levels of MMP-9 in conditioned media of cells incubated 6 h with IL-6 before the addition of IL-10 were reduced >50%, as compared macrophages treated with IL-6 alone (Fig. 8C). Together, these data demonstrate that exogenously added IL-10 is a potent inhibitor of IL-6–induced MMP-9 expression by macrophages.

To directly test whether endogenously synthesized IL-10 inhibits IL-6–induced MMP-9 expression, we treated macrophages with IL-6 in the presence of anti-mouse IL-10 IgG or normal IgG. Levels of MMP-9 mRNA in macrophages incubated with IL-6 were increased 5.8-fold over control cells (p < 0.05; Fig. 9A). In the presence of anti–IL-10, levels of MMP-9 mRNA increased to 14-fold (p < 0.01). Likewise, IL-6–induced MMP-9 levels in macrophage conditioned media were increased in the presence of anti–IL-10 IgG (Fig. 9A). To corroborate these findings, we transfected macrophages with either NS-siRNA or IL-10 siRNA, and treated them with IL-6 for 18 h. Levels of IL-10 in macrophage conditioned media were significantly (p < 0.01) reduced in IL-6–treated cells transfected with IL-10 siRNA (Fig. 9B). As predicted, levels of IL-6–induced MMP-9 mRNA were significantly (p < 0.01) increased in cells transfected with IL-10 siRNA. Taken together, these data demonstrate that IL-6–induced IL-10 serves to limit MMP-9 expression, and administration of JAK inhibitors, which selectively block IL-10 expression, led to increased MMP-9 expression (Fig. 10).

**Discussion**

In this study, we have examined the regulatory role of IL-6 on macrophage MMP-9 expression, which participates in diverse physiologic and pathologic processes. Similar to other MMP family members, the degradation of extracellular matrix components is generally thought to be the primary role of MMP-9. However, in addition, MMP-9 modifies the activities of cytokines and chemokines, growth factors, and proteinase inhibitors (18–20). In these studies, we demonstrate that IL-6 rapidly and robustly stimulated an increase in MMP-9 mRNA levels in RAW264.7 macrophages and elicited peritoneal macrophages, which led to an accumulation of MMP-9 in their conditioned media. These data reveal that IL-6, recognized as a critical mediator of inflammation, induces macrophage expression of a metalloproteinase which is directly associated with the pathogenesis of chronic inflammatory diseases and cancer (18–20).

We and others have reported that macrophage MMP-9 expression is stimulated by PGE2 (21–29). PGE2–dependent proteinase expression is dependent on the EP4 prostanoid receptor and subsequent MAPK\(^{\text{erk}1/2}\) activation (27, 28). Because it is well established that increased levels of PGE2 associated with inflammation depend on the coupling of Cox-2 and mPGES-1 expression (30–33, 41, 54–59), as well as reduced catabolism by 15-PGDH (35, 36), we examined whether IL-6 regulates the expression of these genes in macrophages. Results demonstrate that IL-6 coinduced Cox-2 and mPGES-1 expression, and inhibited the expression of 15-PGDH. Consistent with these changes in gene expression, levels of PGE2 in macrophage conditioned media were increased.

**FIGURE 10.** IL-6–mediated induction of MMP-9 is modulated by JAK/STAT-dependent IL-10 expression in macrophages. IL-6, heretofore recognized as a marker of CVD, induces macrophage expression of MMP-9, which is directly associated with the pathogenesis of vascular diseases. IL-6 stimulation of MMP-9 expression was dependent on the activation of MAPK\(^{\text{erk}1/2}\), and partially dependent on the synthesis of PGE2, which we have previously shown to bind EP4 and activate MAPK\(^{\text{erk}1/2}\). IL-6 also induced IL-10 expression, which potentially suppressed MMP-9 expression. Small-molecule inhibitors of JAK blocked IL-6–induced IL-10 expression and augmented MMP-9 expression. Likewise, inhibition of IL-10 expression using siRNA or engagement of the IL-10R using anti–IL-10 led to enhanced MMP-9 expression.
Reduced MMP-9 expression is dependent on the activation of JAK and subsequent activation of STAT and SHP2 (60, 61). Phosphorylated STAT dimerizes and translocates to the nucleus to regulate STAT-dependent genes, whereas the phosphorylation of SHP2 plays an important role in the activation of MAPKerk1/2 and MAPK-dependent gene expression (7, 9). Thus, we examined the ability JAK inhibitors to block MMP-9 expression. The inhibition of JAK predictably blocked IL-6–induced activation of STAT3, but failed to block the activation of MAPKerk1/2, and unexpectedly enhanced MMP-9 expression. In contrast, inhibition of MEK-1 blocked IL-6–induced phosphorylation of MAPKerk1/2 and MMP-9 expression without affecting the phosphorylation of STAT3. Thus, IL-6–induced MMP-9 expression is dependent on the activation of MAPKerk1/2 and restrained by a JAK-dependent gene product. Although these data were unexpected based on the established model of IL-6–induced signaling, several investigators have reported that diverse JAK inhibitors, including the highly specific tofacitinib and ruxolitinib, effectively block STAT activation without inhibiting MAPKerk1/2 activation (43–47). Although the mechanism(s) underlying JAK-independent activation of MAPKerk1/2 remains undefined at this time, the binding of Src kinase family member HCK to gp130, distal to the SHP2 docking site, reportedly plays a role in IL-6–induced MAPKerk1/2 activation (62).

Using pharmacologic and genetic approaches, we identified IL-10 as the IL-6–induced JAK-dependent suppressor of MMP-9 expression in macrophages. First, the robust induction of IL-10 expression by IL-6 was completely blocked by JAK Inhibitor 1 and tofacitinib. Second, as little as 1 ng/ml exogenous IL-10 increased when cells were incubated in the presence of neutralizing anti–IL-10 IgG. Third, IL-6–induced MMP-9 expression in macrophages was significantly increased when IL-10 null mice were treated with celecoxib, which reportedly blocks IL-10 expression. The inhibition of obliterative airway disease development in murine tracheal allografts by anti–IL-10 IgG. Fourth, IL-6–induced MMP-9 expression was completely blocked IL-6–induced MMP-9 expression. Third, IL-6–induced MMP-9 expression in macrophages was significantly increased when cells were incubated in the presence of neutralizing anti–IL-10 IgG. Fourth, IL-6–induced MMP-9 expression was significantly increased in macrophages in which IL-10 expression was enhanced by macrophages recovered from IL-10 null mice (46). Taken together, JAK inhibitors have the potential to paradoxically increase expression of inflammatory mediators under defined circumstances by inhibiting IL-10 expression.

In these studies, we report that IL-6 induced macrophage expression of MMP-9 via Cox–2-dependent and –independent pathways, and required the activation of MAPKerk1/2 (Fig. 10). Insomuch that MMP-9 plays a causal role in the pathogenesis of chronic inflammatory diseases and cancer, these data reveal a direct mechanism by which elevated IL-6 can contribute to these diseases. Current strategies to block IL-6–induced inflammation use anti–IL-6Rα (i.e., tocilizumab), soluble gp130, and small-molecule inhibitors of JAK (i.e., ruxolitinib and tofacitinib) (7). As reported in this article, the use of JAK inhibitors appears to have the unintended consequence of blocking IL-6–induced IL-10 expression, resulting in increased MMP-9 expression in macrophages.

Disclosures

The authors have no financial conflicts of interest.

References


