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Journal of Immunology 2009; 183:8119-8127; Prepublished online 18 November 2009;
doi: 10.4049/jimmunol.0901925
http://www.jimmunol.org/content/183/12/8119

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Matrix Metalloproteinase (MMP)-1 and MMP-3 Induce Macrophage MMP-9: Evidence for the Role of TNF-α and Cyclooxygenase-2

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Matrix metalloproteinase (MMP)-9 (gelatinase B) participates in a variety of diverse physiologic and pathologic processes. We recently characterized a cyclooxygenase-2 (COX-2)→PGE₂→EP4 receptor axis that regulates macrophage MMP-9 expression. In the present studies, we determined whether MMPs, commonly found in inflamed and neoplastic tissues, regulate this prostanoid-EP receptor axis leading to enhanced MMP-9 expression. Results demonstrate that exposure of murine peritoneal macrophages and RAW264.7 macrophages to MMP-1 (collagenase-1) or MMP-3 (stromelysin-1) lead to a marked increase in COX-2 expression, PGE₂ secretion, and subsequent induction of MMP-9 expression. Proteinase-induced MMP-9 expression was blocked in macrophages preincubated with the selective COX-2 inhibitor celecoxib or transfected with COX-2 small interfering RNA (siRNA). Likewise, proteinase-induced MMP-9 was blocked in macrophages preincubated with the EP4 antagonist ONO-AE3-208 or transfected with EP4 siRNA. Exposure of macrophages to MMP-1 and MMP-3 triggered the rapid release of TNF-α, which was blocked by MMP inhibitors. Furthermore, both COX-2 and MMP-9 expression were inhibited in macrophages preincubated with anti-TNF-α IgG or transfected with TNF-α siRNA. Thus, proteinase-induced MMP-9 expression by macrophages is dependent on the release of TNF-α, induction of COX-2 expression, and PGE₂ engagement of EP4. The ability of MMP-1 and MMP-3 to regulate macrophage secretion of PGE₂ and expression of MMP-9 defines a nexus between MMPs and prostanoids that is likely to play a role in the pathogenesis of chronic inflammatory diseases and cancer. These data also suggest that this nexus is targetable utilizing anti-TNF-α therapies and/or selective EP4 antagonists. The Journal of Immunology, 2009, 183: 8119–8127.

Matrix metalloproteinase (MMP)-9, a neutral endopeptidase, participates in diverse physiologic and pathologic processes (1–3). The degradation of extracellular matrix (ECM) components is commonly thought to be the principal role of MMP-9 in these diverse biological processes. However, in addition to degrading ECM, the biological actions of MMP-9 result from its ability to degrade and modify the activities of cytokines and chemokines, growth factors, and proteinase inhibitors (1, 2).

MMP-9 expression is low or absent in most normal tissues, and it is markedly elevated during inflammation, wound healing, and neoplasia (1). Major inducers include inflammatory cytokines, growth factors, LPS, and ECM components (1). In this regard, we previously demonstrated that macrophage MMP-9 expression induced by TNF-α, LPS, and ECM is dependent on enhanced cy-

cyclooxygenase-2 (COX-2) expression, increased PGE₂ synthesis, and PGE₂ binding to the EP4 prostanoid receptor (4, 5). MMP-9 expression was markedly reduced in macrophages isolated from COX-2-deficient mice or in wild-type macrophages treated with selective COX-2 inhibitors (4, 5). Likewise, induction of macrophage MMP-9 expression was blocked by selective EP4 antagonists or EP4 silencing (5). Taken together, these data point to the important role the COX-2→PGE₂→EP4 receptor axis plays in regulating macrophage MMP-9 expression.

MMP-9 is secreted as a zymogen (pro-MMP-9) and is activated through the proteolytic removal of its pro-domain by MMP-3 and indirectly by plasmin via the activation of pro-MMP-3 (6, 7). However, the role of MMP-3 and other MMP family members in the regulation of macrophage MMP-9 expression has not been explored. In studies reported herein, we determined whether MMPs commonly found in both inflamed and neoplastic tissues regulate macrophage MMP-9 expression by stimulating the COX-2→PGE₂→EP4 receptor axis. We found that macrophage exposure to MMP-1 or MMP-3 led to a marked increase in COX-2 expression and PGE₂ secretion, and subsequent induction of MMP-9. Proteinase-induced MMP-9 expression was blocked in macrophages preincubated with celecoxib or transfected with COX-2 small interfering RNA (siRNA). Likewise, proteinase-induced MMP-9 was blocked in macrophages preincubated with the EP4 antagonist ONO-AE3-208 or transfected with EP4 siRNA. Exposure of macrophages to MMP-1 and MMP-3 triggered the rapid release of TNF-α, which was blocked by MMP inhibitors. Furthermore, both COX-2 and MMP-9 expression were effectively blocked in macrophages preincubated with anti-TNF-α IgG or transfected with TNF-α siRNA. Thus, proteinase-induced MMP-9 expression by macrophages is dependent on the release of TNF-α,
induction of COX-2 expression, and PGE₂ engagement of EP4. Taken together, these data identify a novel regulatory mechanism whereby MMP-1 and MMP-3 contribute to the inflammatory process by liberating TNF-α and stimulating PGE₂ secretion via the induction of COX-2, which leads, in turn, to increased MMP-9 expression. The ability of select MMPs to regulate macrophage secretion of PGE₂ defines a nexus between MMPs and prostanoids that is likely to play a role in the pathogenesis of chronic inflammatory diseases and cancer.

Materials and Methods

Macrophages

Thioglycollate-elicited peritoneal macrophages were obtained from Swiss Webster mice by the method of Edelson and Cohn (8) as described previously (9). 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 resuspended in DMEM supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and 4 mM glutamine, and plated into tissue culture flasks or multiwell plates. Cells were allowed to adhere for 4 h and then washed free of nonadherent cells. All tissue culture supplies were obtained from Invitrogen. The murine macrophage cell line RAW264.7 (10) was obtained from American Type Culture Collection and maintained as adherent cultures in DMEM-10% FBS. All animal studies described in this report have been reviewed and approved by the Weill Cornell Medical College Institutional Animal Care and Use Committee.

Matrix metalloproteinases

Human recombinant pro-MMP-1, active MMP-2, an active fragment of MMP-3, and active MMP-7 were obtained from Calbiochem. Human recombinant active MMP-9 was obtained from Oncogene. Before use, pro-MMP-1 was activated by incubation (2 h at 37°C) with 1 mM 4-aminophenylmercuric acetate (APMA) in 50 mM Tris (pH 7.5), containing 0.01% Triton X-100 and 5 mM CaCl₂ (11). The conversion of pro-MMP-1 to its active form is associated with a loss of the pro-domain (~10 kDa), which was verified by SDS-PAGE and Western blot (data not shown).

Preparation of cell lysates

Cell lysates utilized in the analysis of COX-2 were prepared by adding 1× SDS sample buffer directly to washed macrophage monolayers. In experiments designed to monitor levels of phosphorylated and total MAPKs, macrophages were lysed in TBS containing 2 mM EDTA, 1% Triton X-100, 10 μg/ml β-glycerophosphate, 1 mM sodium vanadate, 2 mM sodium pyrophosphate, 1 mM PMSF, and 10 μg/ml aprotinin. Lysates were centrifuged (14,000 × g) for 20 min at 4°C. The supernatants were recovered, normalized for protein, and mixed with SDS sample buffer with 2-ME and boiled 5 min. Equal amounts of cell lysates were applied to gels based on protein content.

Western blots

Cell lysates were electrophoresed in 4–15% polyacrylamide gels, and proteins were transferred to a polyvinylidene difluoride membrane. The membrane was blocked in 5% defatted milk in TBST, washed in PBS, and incubated 1 h in blocking buffer containing rabbit IgG raised against a TNF-α C-terminal peptide containing amino acids 584–598 of murine COX-2 (0.5 μg/ml; Cayman Chemical) or rabbit anti-phosphospecific p44/p42 MAPK (i.e., MAPKerk1/2) IgG (75 ng/ml; Cell Signaling Technology). The membranes were washed three times in TBST, and bound HRP was visualized utilizing chemiluminescence (HyGlo). In the case of membranes probed with anti-phosphospecific MAPKerk1/2, following visualization of bound HRP, the membranes were stripped in 62.5 mM Tris buffer (pH 6.7) containing 100 mM 2-ME and 2% SDS for 30 min at 50°C, washed, and probed for total MAPKerk1/2 (Cell Signaling Technology).

Macrophage conditioned media were electrophoresed in gradient gels and proteins were transferred to a polyvinylidene difluoride membrane. The membrane was blocked in placing blocking buffer for 1 h, washed in PBS, and incubated 2 h in blocking buffer containing rabbit anti-mouse MMP-9 IgG (0.2 μg/ml; Chemicon International) or incubated at 4°C overnight in blocking buffer containing rabbit anti-mouse TNF-α (2 μg/ml; Thermofisher Scientific). Membranes were washed twice in TBST and incubated 1 h in blocking buffer containing goat anti-rabbit IgG conjugated to HRP or goat anti-mouse IgG conjugated to HRP.

COX-2 and TNF-α siRNA transfection

RAW264.7 macrophages were transfected with either COX-2, TNF-α, or nonspecific siRNA (Santa Cruz Biotechnology) according to the manufacturer’s protocol, which was modified as follows. Macrophages (T-75 flask) were washed three times with Dulbecco’s PBS to remove serum, and medium was replaced with antibiotic-free DMEM. The cells were mechanically harvested, recovered by centrifugation, and resuspended in antibiotic-free DMEM supplemented with 10% FBS. The cells (2 × 10⁶/well) were aliquoted into a 6-well plate and incubated overnight. The next morning, 9.1 μl of 10 μM siRNA was added to 152 μl of transfection medium (Santa Cruz Biotechnology) and incubated 5 min at room temperature. In another tube, 9.1 μl of the transfection reagent (Santa Cruz Biotechnology) was added to the transfection medium and incubated 5 min. The two tubes were combined and incubated 20 min to form the siRNA transfection reagent complex. Immediately before transfaction, macrophage medium was removed and replaced with 1.5 ml of antibiotic-free DMEM supplemented with 10% FBS. The plate was placed on a rocker plate in the laminar flow hood, and the transfection reagent complex was added dropwise. Cells were incubated 72 h at 37°C.

EP4 siRNA transfection

RAW264.7 macrophages were transfected with EP4 or nonspecific siRNA (Thermo Scientific/Dharmacon) utilizing the Accell siRNA delivery protocol. Macrophages were aliquoted into a 6-well plate (0.5 × 10⁶/well) in DMEM-10% FBS. The next day media were replaced with 1.5 ml of Accell delivery medium containing 1 μM siRNA, and cells were incubated for 72 h at 37°C.

RT-PCR

RNA was prepared using TRIzol reagent kits (Invitrogen). RNA (2 μg) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Roche Applied Science) and oligo(dT)16 primer. The resulting cDNA was then used for amplification. The primers and conditions for PCR analysis of murine COX-2, MMP-9, EP4, and actin have been previously described (5). Primers for murine TNF-α were obtained from R&D Systems. PCR products were electrophoresed in a 1% agarose gel with 0.5 μg/ml ethidium bromide and photographed under UV light. PCR conditions for the target genes were optimized to ensure that experimental samples, excluding exuberant positive controls, are in the linear range when scanned. Densitometric analysis of the scanned target mRNAs were determined utilizing Scion Image and normalized for levels of actin mRNA, which served as a loading control.

Determination of PGE₂ and TNF-α levels in macrophage conditioned media

The concentrations of PGE₂ in conditioned media were determined utilizing the STAT-PGE₂ ELISA kit (Cayman Chemical). Concentrations of TNF-α were determined utilizing the Legend Max ELISA kit for mouse TNF-α (BioLegend).

Cytokine Ab array

The presence of 18 cytokines released into conditioned media from macrophages incubated 1 h with 50 nM MMP-3 was monitored utilizing an Ab array according to the manufacturer’s instructions (Panomics). The assay is based on the sandwich ELISA method.

Evaluation of cellular toxicity

To rule out toxic effects of the inhibitors utilized in these studies, cellular morphology and/or protein content following incubation are regularly monitored. Additionally, we test for potential cytotoxicity by monitoring mitochondrial dehydrogenase activity utilizing a MTT assay kit (Sigma-Aldrich). Levels of mitochondrial dehydrogenase activity were not significantly reduced following exposure to any of the inhibitors over the time periods described herein.

Cytokines and chemokines

Recombinant murine TNF-α, MIG (CCL9), MIP-α (CCL3), INF-γ, and RANTES (CCL5) were obtained from R&D Systems.

Results

MMP-1 and MMP-3 induce macrophage COX-2 expression

In previous studies, we characterized a COX-2 → PGE₂ → EP4 receptor axis that regulates macrophage MMP-9 expression (5). In
the present study, we have determined whether MMP family members commonly found in inflamed and neoplastic tissues regulate expression of this prostanoid-EP receptor axis. For this purpose, RAW264.7 macrophages (10) and thioglycollate-elicited macrophages were incubated overnight with 50 nM active MMP-1, MMP-2 (gelatinase A), MMP-3, MMP-7 (matrilysin), or MMP-9. Incubation with 10 ng/ml LPS, a potent inducer of COX-2 expression by macrophages, served as a positive control (12, 13). Levels of COX-2 in cell lysates were prepared and levels of COX-2 and GAPDH were determined utilizing Western blot. A. Macrophages were dispersed into a 48-well plate (2 × 10⁶ cells/well). Following adherence, cells were washed three times with Dulbecco’s PBS and media were replaced with DMEM-0.1% low endotoxin (LE) BSA alone (Ctrl) or DMEM-0.1% LE-BSA containing 50 nM MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, or 10 ng/ml LPS. The next day cell lysates were prepared and levels of COX-2 and GAPDH were determined utilizing Western blot. B. Macrophages were dispersed into a 6-well plate (2 × 10⁶ cells/well). Following adherence, cells were washed. Media were replaced with DMEM-0.1% LE-BSA alone (Ctrl) or DMEM-0.1% LE-BSA containing 50 nM MMP-1, MMP-3, or 10 ng/ml LPS. After overnight incubation, total RNA was isolated, and mRNA levels for COX-2 and actin were determined utilizing RT-PCR. The data are representative of three separate experiments.

FIGURE 1. MMP-1 and MMP-3 induce macrophage expression of COX-2. A, RAW264.7 macrophages or thioglycollate-elicited peritoneal macrophages were suspended in DMEM containing 10% FCS and dispersed into a 48-well plate (2 × 10⁶ cells/well). Following adherence, cells were washed three times with Dulbecco’s PBS and media were replaced with DMEM-0.1% low endotoxin (LE) BSA alone (Ctrl) or DMEM-0.1% LE-BSA containing 50 nM MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, or 10 ng/ml LPS. The next day cell lysates were prepared and levels of COX-2 and GAPDH were determined utilizing Western blot. B. Macrophages were dispersed into a 6-well plate (2 × 10⁶ cells/well). Following adherence, cells were washed. Media were replaced with DMEM-0.1% LE-BSA alone (Ctrl) or DMEM-0.1% LE-BSA containing 50 nM MMP-1, MMP-3, or 10 ng/ml LPS. After overnight incubation, total RNA was isolated, and mRNA levels for COX-2 and actin were determined utilizing RT-PCR. The data are representative of three separate experiments.

To determine whether MMP-induced COX-2 expression was dependent on their enzymatic activity, we compared the abilities of pro-MMP-1- and APMA-activated MMP-1 to induce COX-2 expression in RAW264.7 macrophages. As seen in Fig. 2 (top panel), in contrast to active MMP-1, incubation with pro-MMP-1 failed to induce COX-2 expression. Next, we determined whether two unrelated MMP inhibitors could block MMP-3-induced COX-2 expression. For this purpose, RAW264.7 macrophages were incubated with either a hydroxamic acid-based broad spectrum MMP inhibitor (GM6001) or an inhibitory peptide corresponding to the pro-domain of MMP-3 (MMP-3 inhibitor 1). As seen in Fig. 2 (bottom panel), MMP-3-induced COX-2 expression was completely blocked by either GM6001 or MMP-3 inhibitor 1. Taken together, these data demonstrate that the induction of COX-2 expression by MMP-1 and MMP-3 is dependent on their enzymatic activities.

MMP-1 and MMP-3 induce macrophage MMP-9 expression

Macrophage MMP-9 expression is regulated by COX-2-dependent synthesis of PGE₂ (4, 5). Therefore, we next determined whether pro-MMP-1- and APMA-activated MMP-1 to induce COX-2 expression in RAW264.7 macrophages. As seen in Fig. 2A, the levels of COX-2 and MMP-9 Ags in RAW264.7 lysates and conditioned media, respectively, were elevated following an overnight incubation with MMP-1 or LPS (positive control). The MMP-9 in conditioned media from MMP-1- and LPS-treated macrophages was exclusively in the pro-form (105 kDa), which is slightly larger than the human pro-form (92 kDa). In contrast, both pro (P; 105 kDa) and active (A; 95 kDa) MMP-9 were identified in cultures incubated with MMP-3. MMP-3-dependent activation of pro-MMP-9 was confirmed utilizing zymography (not shown) as previously described (4), and this is consistent with earlier reports indicating that pro-MMP-9 is activated extracellularly by MMP-3 (6, 7, 14, 15).
In a subsequent experiment, we examined the temporal relationship between MMP-3-induced COX-2, PGE₂, and MMP-9 expression (Fig. 3, A and B). Levels of COX-2 Ag were increased 4–6 h following the addition of MMP-3, and they were strongly elevated at 18 and 24 h. Levels of PGE₂ in media recovered from MMP-3-treated macrophages were markedly elevated at 18 and 24 h, as compared with PGE₂ levels in media recovered from control cells. Following the increase in PGE₂ levels, MMP-9 Ag in conditioned media of MMP-3-treated cells was strongly elevated at 24 h. In a separate experiment, levels of PGE₂ release by RAW264.7 macrophages incubated 18 h with media alone or media containing 50 nM MMP-1 or MMP-3 were compared. Media derived from control cells contained 107 pg/mg cell protein (average of duplicate wells). Following incubation with MMP-1 or MMP-3, PGE₂ levels increased to 661 and 905 pg/mg cell protein, respectively.

Activation of MAPKerk₁/₂ plays a causal role in MMP-induced COX-2 expression and MMP-9 synthesis

We previously have shown that the activation of MAPKerk₁/₂ is a determinant of ECM-induced COX-2 expression and MMP-9 synthesis (4, 5). Therefore, we determined whether exposure of cells to MMP-1 or MMP-3 led to the activation of MAPKerk₁/₂. As seen in Fig. 4A, the levels of phosphorylated MAPKerk₁/₂ in macrophages were elevated 20–30 min following the addition of active MMP-1 or MMP-3, and they were strongly elevated at 24 h. Levels of PGE₂ release by RAW264.7 macrophages incubated 18 h with media alone or media containing 50 nM MMP-1 or MMP-3 were compared. Media derived from control cells contained 107 pg/mg cell protein (average of duplicate wells). Following incubation with MMP-1 or MMP-3, PGE₂ levels increased to 661 and 905 pg/mg cell protein, respectively.

MMP-induced MMP-9 synthesis is dependent on COX-2

To examine the role of COX-2 in proteinase-induced expression of MMP-9, macrophages were preincubated with the selective

FIGURE 3. MMP-1 and MMP-3 stimulate macrophage expression of PGE₂ and MMP-9. A, RAW264.7 macrophages (48-well plate; 2 × 10⁶ cells/well) were incubated in DMEM-0.1% LE-BSA alone (Ctrl) or DMEM-0.1% LE-BSA containing 50 nM active MMP-1, MMP-3, or 10 ng/ml LPS. Cells were incubated overnight, conditioned media collected, and lysates were prepared. Levels of MMP-9 in conditioned media and COX-2 and GAPDH in lysates were determined by Western blot. B, RAW264.7 macrophages were incubated with 50 nM MMP-3 for 0–24 h, following which levels of MMP-9 in conditioned media and COX-2 and GAPDH in lysates were determined. C, RAW264.7 macrophages were incubated in DMEM-0.1% LE-BSA alone or media containing 50 nM MMP-3 for 0–24 h. Levels of PGE₂ in conditioned media were determined utilizing ELISA. The data in each panel are representative of three separate experiments.

FIGURE 4. MMP-induced macrophage expression of MMP-9 is MAPKerk₁/₂- and COX-2-dependent. A, RAW264.7 macrophages (48-well plate; 2.5 × 10⁵/well) were cultured in DMEM-0.1% LE-BSA for 24 h. Cells were then incubated with 50 nM MMP-1 or MMP-3 for 5–60 min, and lysates were prepared. Levels of phosphorylated and total MAPKerk₁/₂ were determined utilizing Western blot. B, RAW264.7 macrophages (48-well plate; 2.5 × 10⁵/well) were cultured in DMEM-0.1% LE-BSA for 24 h. Cells were then incubated with media alone (Ctrl) or media containing 50 nM MMP-1, MMP-2, MMP-3, MMP-7, or MMP-9 for 30 min, and lysates were probed for phosphorylated and total MAPKerk₁/₂. C, RAW264.7 macrophages (48 well plate; 2.5 × 10⁵/well) were preincubated with U0126 (10 μM; MEK-1 inhibitor) or celecoxib (5 μM; COX-2 inhibitor). Following an overnight incubation with 50 nM MMP-1, MMP-3, or 10 ng/ml LPS, levels of MMP-9 in conditioned media and COX-2 and GAPDH in lysates were determined by Western blot. The entire experiment was repeated twice with similar results.
COX-2 inhibitor celecoxib. Pretreatment with 5 μM celecoxib blocked MMP-induced MMP-9 expression (Fig. 4C), which was restored with the addition of PGE2 (not shown). The observation that celecoxib also attenuates COX-2 expression is consistent with the previously reported positive feedback loop whereby PGE2 stimulates COX-2 expression by engaging the EP4 receptor and triggering a Ras-MAPK signaling cascade (5, 16).

As an alternative strategy to examine the role of COX-2 in MMP-induced expression of MMP-9, RAW264.7 macrophages were transfected with COX-2 siRNA. As observed in Fig. 5A, levels of COX-2 Ag in cells transfected with nonspecific siRNA were increased following incubation with MMP-1, MMP-3, or LPS, whereas reduced levels of COX-2 were observed in MMP- or LPS-treated macrophages transfected with COX-2 siRNA. When conditioned media were examined for MMP-9 Ag, MMP-1, MMP-3, and LPS treatment led to a large increase in MMP-9 expression, which was markedly attenuated in macrophages in which COX-2 expression was knocked down.

Finally, the diverse biologic actions of PGE2 are mediated by the EP1–4 family of prostanoid receptors (17, 18). Earlier studies have demonstrated that PGE2-dependent MMP-9 expression was mediated via engagement of EP4 (5, 19). Thus, we examined the ability of a selective EP4 antagonist ONO-AE3-208 to block MMP-induced MMP-9 expression. Preincubation of macrophages with 50 nM ONO-AE3-208 blocked MMP-3-induced MMP-9 expression (Fig. 5B). To corroborate these findings, RAW264.7 macrophages were transfected with EP4 siRNA. The level of EP4 mRNA in cells transfected with EP4 siRNA was reduced ~95% when compared with cells transfected with nonspecific siRNA (Fig. 5C, left panel). When transfected cells were exposed to proteinases, MMP-1, MMP-3, and LPS (positive control) failed to significantly induce COX-2 or MMP-9 expression by macrophages transfected with EP4 siRNA (Fig. 5C, right panel). Thus, the induction of MMP-9 expression by selected MMPs is causally linked to MAPKerk1/2-dependent stimulation in COX-2 expression and subsequent PGE2 engagement of EP4.

MMP-dependent release of TNF-α stimulates macrophage COX-2 expression

Proteinases can affect cellular function by releasing tethered growth factors (20–22), cytokines (23, 24), or fragments of extracellular matrix (25–27) or by activating protease-activated receptor family members (28, 29). To determine whether MMP-induced COX-2 expression resulted from the release or activation of a soluble factor, conditioned media from macrophages exposed to MMP-3 overnight were recovered and lysates were prepared. Conditioned media alone or media to which MMP-3 inhibitor 1 was added (to block residual MMP-3 activity) were added to naïve macrophages and incubated overnight. Levels of COX-2 and GAPDH were monitored by Western blot. The data are representative of three separate experiments. Conditioned media recovered from RAW264.7 macrophages (6-well plate; 10 ng/ml, incubated with MMP-3 for 1 h, were collected and analyzed for the presence of the indicated cytokines and growth factors utilizing Ab array. C, RAW264.7 macrophages (24-well plate; 2.5 × 10^5/well) were incubated in DMEM-0.1% LE-BSA containing 50 nM of the indicated MMPs alone or in the presence of the broad-spectrum MMP inhibitor GM6001 (50 nM) overnight. The next day conditioned media were recovered and levels of TNF-α were determined by Western blot. The data are representative of two separate experiments.

mRNA in cells transfected with EP4 siRNA was reduced ~95% when compared with cells transfected with nonspecific siRNA (Fig. 5C, left panel). When transfected cells were exposed to proteinases, MMP-1, MMP-3, and LPS (positive control) failed to significantly induce COX-2 or MMP-9 expression by macrophages transfected with EP4 siRNA (Fig. 5C, right panel). Thus, the induction of MMP-9 expression by selected MMPs is causally linked to MAPKerk1/2-dependent stimulation in COX-2 expression and subsequent PGE2 engagement of EP4.

MMP-dependent release of TNF-α stimulates macrophage COX-2 expression

Proteinases can affect cellular function by releasing tethered growth factors (20–22), cytokines (23, 24), or fragments of extracellular matrix (25–27) or by activating protease-activated receptor family members (28, 29). To determine whether MMP-induced COX-2 expression resulted from the release or activation of a soluble factor, conditioned media from macrophages exposed to MMP-3 overnight were recovered, treated with MMP-3 inhibitor 1, and transferred to naïve cells. As seen in Fig. 6a (left panel), levels of COX-2 expression were elevated in macrophages incubated overnight with MMP-3. The ability of MMP-3 to induce
COX-2 expression in macrophages coincubated with MMP-3 inhibitor 1 was noticeably reduced. Similar results are seen in Fig. 2 (upper panel). Likewise, conditioned media recovered from cells following an overnight incubation with MMP-3 stimulated COX-2 expression by naive macrophages (Fig. 6A, right panel). The ability of conditioned media to up-regulate COX-2 expression in naive cells was relatively unaffected by the addition of MMP-3 inhibitor 1. These data indicate that exposure of cells to MMP-3 released or activated a soluble factor that was responsible for MMP-induced COX-2 expression.

In an effort to identify the factors released by macrophages following exposure to MMP-3, the presence of 18 cytokines was monitored utilizing Ab array. As seen in Fig. 6B, 1 h of treatment with MMP-3 resulted in a substantial increase in levels of TNF-α previously reported to be a potent stimulator of COX-2 (5, 30) and MMP-9 expression by macrophages (5, 31). To corroborate and extend the Ab array data, conditioned media were recovered from RAW264.7 macrophages treated with MMP-1 and MMP-3 for 18 h, and probed for levels of TNF-α utilizing Western blot. Recombinant murine TNF-α (25 ng/lane) was utilized as a standard. When compared with the standard TNF-α, media samples recovered from MMP-treated macrophages contained relatively high levels of TNF-α (Fig. 6C, upper panel). In contrast, TNF-α was not detected in media recovered from cells incubated overnight with MMP-1 or MMP-3 in the presence of the MMP inhibitor GM6001 (Fig. 6C, lower panel). Finally, to obtain a quantitative measure of MMP-induced TNF-α release, media were recovered from RAW264.7 macrophages (48-well plate; 2.5 × 10^7/well) treated with 50 nM MMP-1, 50 nM MMP-3, or 10 ng/ml LPS (positive control) for 18 h and probed for levels of TNF-α utilizing ELISA. Media from control cells contained 698 ± 109 pg/ml (mean ± SEM; n = 3). Following exposure to MMP-1 or MMP-3, levels increased 25-fold (17,555 ± 6,544) and 34-fold (23,767 ± 1,574), respectively. TNF-α levels in media from cells incubated with LPS increased 160-fold (114,333 ± 1,828).

Next, we utilized a neutralizing rat monoclonal anti-murine TNF-α IgG to determine whether MMP-induced release of TNF-α was responsible for inducing COX-2 and MMP-9 expression in RAW264.7 macrophages. For this purpose, conditioned media recovered from cells incubated with MMP-1 or MMP-3 were preincubated 2 h with 20 µg/ml anti-TNF-α IgG or normal IgG before transferring to naive cells. As shown in Fig. 7, conditioned media derived from MMP-1-treated cells stimulated COX-2 and MMP-9 expression in naive macrophages. Preincubation of the conditioned media with anti-TNF-α IgG blocked its ability to induce COX-2 and MMP-9 expression; in contrast, normal IgG had no effect. Likewise, the addition of MMP-3 conditioned media to naive macrophages stimulated their COX-2 and MMP-9 expression. The addition of MMP-3 inhibitor I or normal IgG to the conditioned media recovered from MMP-3-treated macrophages had no effect on its ability to induce COX-2 or MMP-9 in naive cells; in contrast, preincubation with anti-TNF-α IgG suppressed its ability to stimulate COX-2 and MMP-9 by naive macrophages.

To directly test the role of TNF-α in proteinase-induced MMP-9 expression, RAW264.7 macrophages were transfected with either TNF-α siRNA or nonspecific siRNA. The level of TNF-α mRNA in cells transfected with TNF-α siRNA was reduced ~87% when compared with cells transfected with nonspecific siRNA (Fig. 8, left panel). When TNF-α siRNA-transfected cells were exposed to proteinases, MMP-1, MMP-3 failed to significantly induce COX-2 or MMP-9 expression. Importantly, COX-2 and MMP-9 expression were restored in these TNF-α siRNA-transfected cells by the addition of exogenous TNF-α (Fig. 8, right panel).
FIGURE 8. TNF-α silencing blocks MMP-induced MMP-9 expression. RAW264.7 macrophages transfected with nonspecific (NS) or TNF-α siRNA were suspended in DMEM-0.1% LE-BSA and aliquoted into a 24-well plate. Following adherence, cells received media alone (Ctrl) or media containing 50 nM MMP-1, MMP-3, or LPS (10 ng/ml) and were incubated overnight. Additionally, cells transfected with TNF-α siRNA were incubated with MMP-1, MMP-3, or LPS in the presence of exogenous TNF-α (50 ng/ml). Levels of MMP-9 in conditioned media and COX-2 and GAPDH in lysates were determined by Western blot. Levels of TNF-α mRNA were determined utilizing RT-PCR. The data are representative of two separate experiments.

Discussion
Genetic evidence indicates that MMP-9 participates in the recruitment of stem cells and tissue revascularization, skeletal growth plate angiogenesis and ossification, choroidal neovascularization following injury, tumor angiogenesis, dermal-epidermal separation in bullous pemphigoid, resolution of allergic inflammation, degradation of collagen and fibrin, ventricular enlargement and collagen accumulation following myocardial infarction, and the pathogenesis of occlusive and aneurysmal vascular diseases (2, 33, 34). The activity of this pleiotropic proteinase is regulated at three levels: gene expression and pro-enzyme activation and inhibition (1, 2). The proteolytic activation of pro-MMP-9 is currently understood to take place in the fluid phase and is catalyzed by MMP-3 (6, 7, 35). However, the ability of MMP-3 and/or other MMP family members to regulate MMP-9 expression has not been explored. Results of experiments reported herein define a complex mechanism by which extracellular MMP-1 and MMP-3 stimulate macrophage MMP-9 expression via the liberation of TNF-α and subsequent induction of COX-2, as well as PGE2 engagement of EP4 receptor. PGE2, a member of the prostanooid family of autacoids, is an important mediator of the inflammatory response (36). PGE2 can stimulate vasodilation and increase permeability, pain, fever (37, 38), and tissue destruction/remodeling via enhanced proteinase expression (4, 39–42). Synthesis of PGE2 requires the liberation of arachidonic acid from membrane phospholipids, COX-1 and/or COX-2-dependent generation of PGH2, and its isomerization to PGE2 by members of the PGE synthase family (36). In most cells, Cox-1 expression is constitutive, whereas COX-2 expression is induced in response to a variety of inflammatory mediators (43, 44). As reported here, levels of COX-2 mRNA and Ag in macrophages were selectively increased following exposure to catalytically active MMP-1 and MMP-3. Two mechanisms proposed for regulating COX-2 expression may be relevant in MMP-induced COX-2 expression: activation of MAPK superfamily pathways (45–48), and a PGE2-dependent positive feedback loop (5, 16, 49). Results of experiments reported herein suggest that both pathways are important. A causal role of activated MAPKerk1/2 in MMP-induced COX-2 expression is supported by the observation that MMP-1 and MMP-3 trigger MAPKerk1/2 activation in macrophages, and preincubation of cells with a MEK-1 inhibitor blocks MMP-induced COX-2 expression. Likewise, the failure of MMPs to induce COX-2 expression in macrophages pretreated with celecoxib or transfected with EP4 siRNA is consistent with the hypothesis that PGE2-dependent positive feedback loops regulate/amplify COX-2 expression.

The release of TNF-α is a well-defined mechanism by which proteinases regulate cell function. Pro-TNF-α, tethered to the cell membrane by its NH2-terminal domain, is efficiently processed to its mature form and released by membrane-associated TNF-α converting enzyme (i.e., TACE) (50, 51), which is a member of the ADAMs (a disintegrin and metalloproteinase) family of metalloproteinases (i.e., ADAM17) (52). In addition to TACE/ADAM17, several members of the MMP family are reported to process pro-TNF-α, although less efficiently (23, 24). Studies reported herein confirm the observation that MMP family members can process pro-TNF-α. We demonstrate that the levels of TNF-α in media recovered from macrophages incubated with active MMP-1 or MMP-3 were elevated as compared with control cells. The observed release of TNF-α provides a mechanism whereby these proteinases induce COX-2-dependent MMP-9 expression. We and others have reported that macrophage COX-2 (5, 30) and MMP-9 expression (5, 31) are stimulated by TNF-α, and knockdown of COX-2 expression blocks TNF-α-induced MMP-9 expression (5). Moreover, as reported herein, the ability of conditioned media recovered from macrophages incubated with MMP-1 or MMP-3 to induce COX-2 and MMP-9 was blocked by neutralizing anti-TNF-α IgG. Furthermore, proteinases failed to induce either COX-2 or MMP-9 in macrophages in which TNF-α expression was knocked down utilizing siRNA. Thus, MMP-1-and MMP-3-induced COX-2 and MMP-9 expression by macrophages is dependent on the release of TNF-α (Fig. 9).

MMP family members are potent proinflammatory and immunoregulatory molecules that are implicated in the pathogenesis of chronic inflammatory diseases and cancer (2, 53, 54). Results of experiments reported here define a complex mechanism by which collagenase-1 and stromelysin-1, commonly found in both inflamed and neoplastic tissues, stimulate macrophage MMP-9 expression via the liberation of TNF-α and subsequent modulation of the COX-2→PGE2→EP4 receptor axis (Fig. 9). The ability of MMPs to induce COX-2-dependent MMP-9 expression by macrophages defines a nexus between MMPs and prostanooids that is
likely to play a role in the pathogenesis of chronic inflammatory diseases and cancer. These data also suggest that this nexus is targetable utilizing anti-TNF-α therapies and/or selective EP4 antagonists.

Acknowledgments
The authors thank Dr. Takayuki Maruyama (ONO Pharmaceutical) for supplying the EP4 antagonist ONO-AE3-208.

Disclosures
The authors have no financial conflicts of interest.

References


