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RESEARCH PAPER

Diverse patterns of cyclooxygenase-independent metalloproteinase gene regulation in human monocytes

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BACKGROUND AND PURPOSE

Matrix metalloproteinase (MMP) production from monocyte/macrophages is implicated in matrix remodelling and modulation of inflammation. However, knowledge of the patterns and mechanisms of gene regulation of MMPs and their endogenous tissue inhibitors (TIMPs) is fragmentary. MMP up-regulation may be a target for cyclooxygenase (COX) and prostaglandin (PG) receptor inhibition, but the extent and mechanisms of COX-independent MMP up-regulation are unclear.

EXPERIMENTAL APPROACH

We studied MMP mRNA expression and selected protein levels in human peripheral blood monocytes before and after adhesion, upon stimulation with bacterial lipopolysaccharide (LPS), PGE_2 or forskolin and after culturing with monocyte colony-stimulating factor on plastic or human fibronectin for up to 7 days.

KEY RESULTS

Monocyte adherence for 2 h transiently up-regulated COX-2, MMP-1, MMP-7 and MMP-10 mRNAs, and persistently up-regulated MMP-2, MMP-9, MMP-14 and MMP-19 mRNAs. LPS, PGE_2 or forskolin selectively increased MMP-1, MMP-9, MMP-10, MMP-12 and MMP-14 mRNAs. LPS increased PGE_2 production through COX but up-regulated MMP levels independently of COX. Differential dependence on inhibition of p42/44 and p38 mitogen-activated protein kinases, c-jun N-terminal kinase and inhibitor of κB kinase2 paralleled the diverse patterns of MMP stimulation by LPS. Differentiation on plastic increased mRNA levels of MMP-7, MMP-9, MMP-12 and MMP-14 and TIMP-2 and TIMP-3 independently of COX; fibronectin accelerated MMP but not TIMP up-regulation.

CONCLUSIONS AND IMPLICATIONS

Adhesion, LPS stimulation and maturation of human monocytes lead to selective, COX-independent MMP and TIMP gene regulation, which is a potential target for selective inhibition by signalling kinase inhibitors.

Abbreviations

AP-1, activator protein-1; COX, cyclooxygenase; ECM, extracellular matrix; IκB, inhibitors of κB; JNK, c-jun N-terminal kinase; LPS, lipopolysaccharide; MAP kinase, mitogen activated protein kinase; MMP, metalloproteinase; NFκB, nuclear factor κB; PG, prostaglandin; SP-1, specificity protein-1; TIMP, tissue inhibitor of metalloproteinases; TLR, Toll-like receptor

Introduction

Matrix metalloproteinases (MMPs) play an important part in acute and chronic inflammation, partly by promoting turnover of the extracellular matrix (ECM) and partly by modulating the action of inflammatory mediators (Parks *et al.*, 2004). MMPs aid efficient invasion of inflammatory cells to sites of damage or infection, permit angiogenesis and prepare the tissue for deposition of new ECM – all actions that ultimately benefit tissue repair (Parks *et al.*, 2004). On the other hand, excessive accumulation of ECM can lead to fibrosis in such tissues as the liver (Smart *et al.*, 2001) and lungs (Taggart *et al.*, 2005). Conversely, excessive destruction of the ECM by MMPs can lead to adverse consequences including the damage to bone and cartilage in arthritis (Baker *et al.*, 2002) and to several ECM components in periodontal disease (Sorsa *et al.*, 2006). During atherosclerosis, destruction of the ECM can cause weakening of the arterial wall, leading to aneurysm formation (Shimizu *et al.*, 2006) or atherosclerotic plaque rupture (Newby, 2005) Hence, the ability to modulate MMP activity and consequently ECM turnover and inflammation could lead to effective preventive treatments for several diseases.

The MMPs are a family of 23 genetically related Zn^{2+}-dependent proteases (Nagase *et al.*, 2006). There are also four related tissue inhibitors of MMPs (TIMPs) that complex with and inhibit the active enzymes (Nagase *et al.*, 2006). Most MMPs are secreted as pro-forms, which are then activated by other proteases or chemical compounds in the ECM (Nagase *et al.*, 2006). The membrane-type MMPs are activated in the endosomal compartment and then remain anchored to the external membrane surface (Nagase *et al.*, 2006). MMPs cleave a variety of ECM substrates, and they have been divided somewhat arbitrarily into collagenases, gelatinases, elastases and stromelysins based on their preferred substrates, but specificities overlap (Nagase *et al.*, 2006). In addition, several MMPs can activate the pro-forms of other MMPs, and many MMPs also remodel cell surface and peri-cellular proteins, which in turn regulate the migration, proliferation and apoptosis of inflammatory and other cells (Parks *et al.*, 2004; Newby, 2006). Hence, the spectrum of MMPs and TIMPs expressed as well as their absolute levels determine the extent of ECM turnover and subsequent biological effects. However, only one previous study measured a wide range of MMPs and TIMPs in inflammatory cells (Bar-Or *et al.*, 2003), and this study provided limited information on MMP gene regulation.

MMP activity in monocytes is under transcriptional control by growth factors, matrix contacts and inflammatory mediators. Furthermore, up-regulation of several MMPs in response to adhesion and bacterial lipopolysacharride (LPS) can occur indirectly via the action of prostaglandin E_2 (PGE_2) produced via cyclooxygenase (COX) activation (see Newby, 2008). As a result, COX inhibitors (Shankavaram *et al.*, 2001; Ardans *et al.*, 2002; Khan *et al.*, 2004) and antagonists at the prostanoid EP_4 receptor (Cipollone *et al.*, 2005; Pavlovic *et al.*, 2006; nomenclature follows Alexander *et al.*, 2009) prevent MMP up-regulation experimentally and have therefore been proposed as therapies to reduce damage to the ECM caused during inflammation. However, COX-independent MMP-9 production was also reported in a one study (Zhang *et al.*, 1998), although the scope and relative magnitude of COX-dependent and -independent responses was not clarified. Also, to our knowledge, there have been no previous publications regarding the mechanisms underlying COX-independent regulation of MMPs.

We therefore set out to conduct a comprehensive study of MMP and TIMP expression in human monocytes in the presence and absence of COX inhibitors under conditions that mimic several processes likely to occur in the initial stages of inflammation. These conditions included monocyte adhesion, stimulation by LPS and differentiation to macrophages. We also sought to define mechanisms for any COX-independent regulation.

Methods

Monocyte purification and culture conditions

Monocytes were isolated from buffy coats from healthy blood donors, which were collected from National Blood Transfusion Service (Bristol, UK) or from heparinized blood of healthy volunteers under National Research Ethics Service approval 09/H0107/22 and 10/HO102/72, respectively. Mononuclear cells were isolated using Ficoll–Paque Plus and contaminating red blood cells were lysed with 150 mM ammonium chloride, 0.1% bovine serum albumin (BSA). Mononuclear cells were allowed to adhere in RPMI 1640 supplemented with 2 mM L-glutamine, 100 $IU \cdot mL^{-1}$ penicillin, 100 $\mu g \cdot mL^{-1}$ streptomycin and 1% heat-inactivated human male AB serum at $7 \times 10^5/cm^2$ for 1 h in a humidified atmosphere containing 5% CO_2 at 37°C. The COX inhibitor indomethacin and the COX-2-selective inhibitor NS-398 were included during adhesion and throughout the experiment when used. Non-adherent cells were removed with warm Dulbecco's phosphate buffered saline (DPBS), and the cells were transferred to RPMI containing 10% fetal bovine serum (FBS) for another hour. Where indicated, wells were pre-coated with human plasma fibronectin at 0.05 $mg \cdot mL^{-1}$ in DPBS by incubation for 1 h at room temperature and overnight at 4°C and then blocking with 10 $mg \cdot mL^{-1}$ fatty acid–free BSA for 2 h and two washes in DPBS. $CD16^-$ monocytes were purified from mononuclear cells by negative selection using MACS® monocyte isolation kit II according to the manufacturer's instructions. They were either lysed or adhered as described for mononuclear cells as required. Adherent monocytes were incubated in serum-free RPMI 1640 for 18 h, and samples were collected for RNA, cell lysates and conditioned medium. Monocyte maturation was

performed in RPMI 1640 containing 2% or 10% FCS (as indicated in the figure legends) and 20 ng·mL^{-1} monocyte colony-stimulating factor (MCSF) for 24 and 72 h followed by 18 h in serum-free RPMI 1640 to collect cell extracts and conditioned medium. RNA was extracted after 42 and 72 h and 7 days, respectively. Viability of the cells was confirmed by Trypan blue exclusion.

Real-time quantitative PCR

To quantify the steady-state concentration of mRNAs, total RNA was extracted using RNeasy Mini kit (Qiagen, Crawley, West Sussex, UK) according to the manufacturer's instructions, quantified using a Nanodrop TM 1000 Spectrophotometer (Thermo Fisher Scientific, Loughborough, Leicestershire, UK) and reverse transcribed using a QuantiTect Reverse Transcription Kit (100–200 ng RNA per reaction). A genomic DNA removal step was included, and the resulting cDNA was diluted 1:1 in 10 mM Tris–HCl, pH 8. Real-time quantitative PCR was performed in a Roche Light Cycler 1.5 (Roche Product Ltd, Welwyn Garden City, Hertfordshire, UK) to quantify the steady-state concentration of RNA using a Quanti Tect SYBR Green PCR Kit and primers as detailed in Table S1. The reaction contained 3.6–7.3 ng RNA and 0.5 μM primers. mRNA copy numbers were calculated using standard curves constructed using the respective PCR products eluted from agarose gels. All fragments were sequenced to confirm identity (Cogenics, Takely, UK).

Western blotting and zymography

Conditioned medium was collected and centrifuged at 270× *g* for 3 min to remove cellular debris and was concentrated as required using centrifugal filter units (Merck Chemicals, Nottingham, Nottinghamshire, UK). Cells were lysed in SDS buffer (2% SDS (w/v), 16% glycerol (v/v) and 50 mM Tris (pH 6.8). Protein concentration in lysates was measured using BCA Protein Assay Kit (Thermo Fisher Scientific) and was used to normalize loading of gels. Samples from the fibronectin experiments were normalized by cell numbers: DNA content in the cell lysates was measured using Picogreen (Molecular Probes, Invitrogen, Paisley, Renfrewshire, UK); cell numbers were calculated from a standard curve of human cell extracts. Cell extracts and conditioned medium were diluted fourfold in lysis buffer and reduced with 5% β-mercaptoethanol and then fractionated by PAGE and analysed by Western blotting as previously described (Chase *et al.*, 2002). Detection was performed using enhanced chemiluminescence (Merck Chemicals).

MMP-9 metalloproteinase activity in conditioned medium was evaluated by zymography as described (Chase *et al.*, 2002). Briefly, 7.5% polyacrylamide gels containing 2 mg·mL^{-1} gelatin were subjected to electrophoresis under non-reducing conditions. Following electrophoresis, SDS was removed by washing in 2.5% Triton X-100, and gels were incubated at 37°C for 18 h in 50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 10 mM Ca_2Cl, and 0.05% Triton X-100. A parallel gel was incubated without $CaCl_2$ in the presence of 10 mM EDTA. Gels were then stained in 0.2% Coomassie Brilliant Blue. Gelatinase activity was detected as clear bands on a dark background. Densitometric analysis of bands was performed using scanner and Quantity One software (Bio-Rad, Hemel Hempstead, Herts, UK).

PGE_2 production

Culture supernatants from cells incubated for 24 h in 2% FBS in RPMI 1640 were analysed for PGE_2 production according to manufacturer's instructions.

Statistical analysis

Means were compared by ANOVA followed by appropriate post tests. Normally distributed data are expressed as means ± s.e.mean. If any data were non-normally distributed, the whole data set was subjected to non-parametric (Mann–Whitney) testing. Alternatively, the data were log transformed and if normalized was then tested by Student's *t*-test with correction for multiple testing. Non-normally distributed data are expressed as means, and 95% confidence intervals were derived from the logarithmically transformed values.

Materials

Ficoll–Paque Plus (GE Healthcare, Little Chalfont, Buckinghamshire, UK) RPMI, FBS, DPBS (Lonza, Slough, Berkshire, UK) MACS monocyte isolation kit II (Miltenyi Biotec, Bisley, Surrey, UK). QuantiTect Reverse Transcription Kit and Quanti Tect SYBR Green PCR Kit (Qiagen, Crawley, West Sussex, UK), NS-398, SC514 and indomethacin (Cayman Chemicals, Cambridge Bioscience Ltd, Cambridge, UK) were suspended in DMSO except when otherwise stated. PD98059 (PD), SB20380 (SB) and SP600125 (SP) were obtained from Merck Chemicals and dissolved in DMSO. Recombinant human MCSF and Parameter PGE_2 immunoassay™ to measure PGE_2 release were from R&D Systems (Abingdon, Oxon, UK). Fatty acid–free BSA for cell treatments was obtained from Roche (Welwyn Garden City, Hertfordshire, UK). LPS (*E. coli* 026:B6) and all other reagents and primers were purchased from Sigma-Aldrich (Gillingham, Dorset, UK). The following antibodies were used: MAPKs phospho and total were from New England Biolabs (Hitchin, Hearts, UK), MMP-14 and GAPDH from Merck Chemicals, MMP-10 from R&D Systems, COX-2 and IκBα from Santa Cruz (Insight Biotechnology, Wembley, London, UK).

Results

MMP expression patterns in monocytes before and after adhesion to plastic for 2–18 h

Adhesion is an early step in the recruitment of monocytes to inflammatory foci and a prerequisite for their subsequent differentiation to macrophages. We mimicked this using the simplest protocol, namely adhesion to plastic in the presence of serum. Pooled buffy coats, a convenient source of large numbers of human primary monocytes and freshly drawn blood, yielded indistinguishable results (not shown). Non-adherent monocytes purified from buffy coats by negative selection on magnetic beads had widely varying copy numbers of mRNAs for MMPs and TIMPs (Figure 1, Table S2). Based on mRNA levels, MMP-8, -9, -14 and -19 were the most highly expressed in human monocytes, whereas MMP-1, MMP-2, MMP-7, MMP-10, MMP-12 and MMP-23 were expressed at very low levels. MMP-3 mRNA was undetectable. Interestingly, the mRNAs of TIMP-1 and TIMP-2 were 10-fold more abundant than any of the MMPs in non-adherent

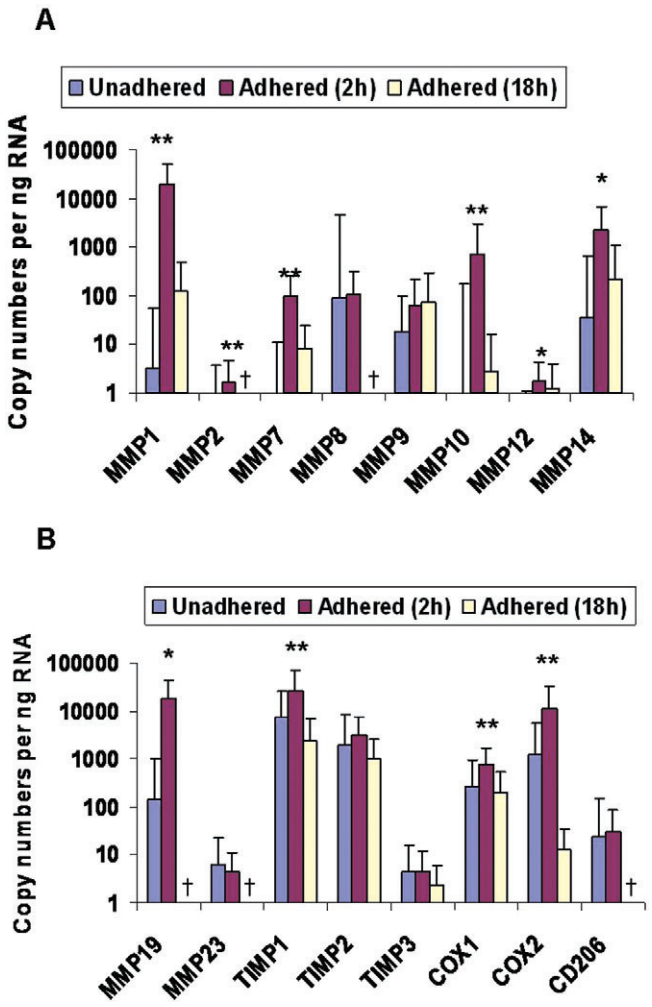

Figure 1

Copy numbers of mRNA per ng of RNA. Total RNA was extracted from monocytes negatively selected on magnetic beads ($CD16^-$, n = 4–8) or after adhesion of these same cells or $CD16^{+/-}$ mononuclear cells for 2 h (n = 8–13) or 18 h (n = 8–15). Cell viability was >90% after 2 h and >85% after 18 h. Complementary DNA was subjected to quantitative PCR and compared with standards to calculate copy numbers of mRNA per ng of RNA. (A) Results for several MMPs. (B) Results for further MMPs, TIMPs, COX isoforms and CD206. Values are means and upper 95 % confidence intervals. $^{**}P < 0.01$ 2 h adhesion versus non-adherent and 18 h adhesion; $^{*}P < 0.01$ 2 h versus non-adherent only (Mann–Whitney); † not determined. For more details see Table S2.

human monocytes, whereas TIMP-3 was 1000-fold less abundant than the other TIMPs. Adherence of negatively selected monocytes for 2 h increased mRNA levels of several MMPs (Table S2). Negative selection removes the minority (approximately 10%) $CD16^+$ population of blood monocytes. However, adhesion of non-selected mononuclear cells ($CD16^+$ and $CD16^-$) from buffy coats led to identical levels of MMP and TIMP mRNAs as in negatively selected cells (Table S2). Based on the pooled values from negatively selected and non-selected mononuclear cells, MMP-1 was induced approximately 6000-fold, MMP-2, 3-fold, MMP-7 from undetectable, MMP-10, 900-fold, MMP-12, from undetectable, MMP-14, 60-fold and MMP-19, 130-fold, whereas mRNA levels of MMP-8, MMP-9 and MMP-23 did not change significantly compared with non-adherent cells (Figure 1, Table S2). Copy numbers of mRNAs for TIMP-1 increased fourfold, but expression of TIMP-2 and TIMP-3 was unchanged. Based on mRNA levels, MMP-1, MMP-10, MMP-14 and MMP-19 became almost as abundant as TIMP-1 and TIMP-2 after adhesion. In monocytes adhered to plastic for 18 h, mRNA levels of MMP-1, MMP-7 and MMP-10, TIMP-1 and COX-1 and COX-2 declined significantly to values not statistically different from non-adherent monocytes (Figure 1, Table S2). Hence, adhesion caused a selective, transient up-regulation of the mRNAs for these proteins.

Previous studies have concluded that MMP up-regulation by adherent monocytes can be mediated indirectly by the production of PGE_2 mediated through COX. Consistent with this COX-1 and COX-2 mRNAs were expressed in non-adherent monocytes and were significantly up-regulated (fourfold and ninefold respectively) after adhesion for 2 h (Figure 1, Table S2). Despite these increases in COX mRNA levels, however, indomethacin (10 μM) had no effect on the mRNA levels of any of the MMPs or TIMPs we studied (Figure 2A).

Effects of LPS, PGE_2 and forskolin on mRNA expression after 18 h adhesion to plastic

Activation by Toll-like receptors (TLR) is also an early step in monocyte activation, especially at sites of inflammation. In order to model this mechanism, we designed experiments to investigate the scope of MMP and TIMP regulation by LPS, a TLR4 ligand, and its dependence on production of PGE_2. LPS strongly stimulated mRNA expression of MMP-1 (21-fold), MMP-10 (44-fold) and MMP-14 (13-fold) and to a lesser extent MMP-9 (2.3-fold) and TIMP-1 (3.1-fold) (Figure 2B). Interestingly, the mRNA levels of neither MMPs nor TIMPs were significantly reduced by indomethacin (25 μM) treatment (Figure 2A). These results implied, contrary to most previous literature, that MMP mRNA expression changes caused by LPS treatment were independent of PG synthesis. We concluded that under the condition of our experiments, the cells were either unresponsive to PGE_2, or the levels of PGE_2 produced were too low to affect MMP and TIMP production. However, we found that treating monocytes with a maximal concentration of PGE_2 (5 μM) increased MMP-1, MMP-9, MMP-10 and MMP-14 and TIMP-1 and slightly decreased TIMP-2 mRNA levels, showing that PGE_2 largely replicated the effects of LPS in our monocytes (Figure 2B). Concentration response studies (results not shown) showed that PGE_2 increased mRNA levels of MMPs at above 50 nM. Forskolin, a direct activator of adenylate cyclase, the downstream mediator of PGE_2, also affected MMP and TIMP-1 mRNA levels (Figure 2B) to the same or greater extent compared with LPS and PGE_2 (Figure 2A, B). The effects on MMP-9 protein were confirmed by zymography (inset in Figure 2B). Hence, we concluded that LPS, PGE_2 and cAMP elevation were able to stimulate expression of several MMPs and TIMP-1 in our preparations of monocytes.

We then demonstrated that LPS increased PGE_2 levels after 24 h to 3.1 ± 0.9 nM from less than 0.2 nM (the limit of detection) in our monocytes (Figure 2C). Furthermore, PGE_2 production was reduced to 16 ± 8% of the value with LPS

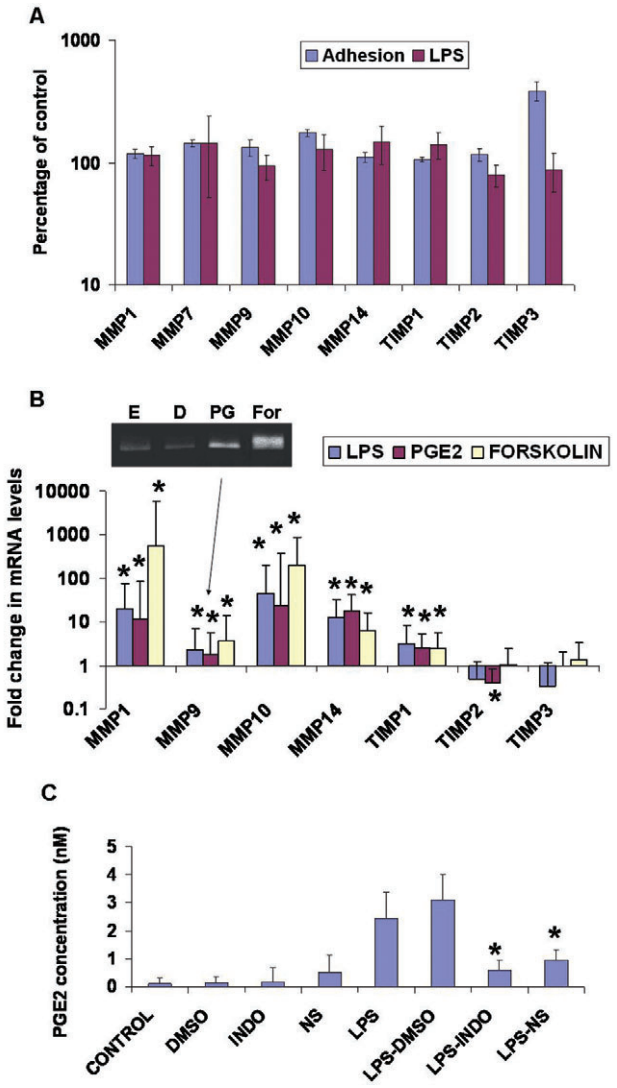

Figure 2

Effects of LPS and indomethacin on mRNAs for MMPs and TIMPs and PGE_2 levels. (A) Effect of addition of indomethacin during adherence (10 μM, $n = 3$) or LPS treatment (25 μM, $n = 6–10$) expressed as a percentage of the values without indomethacin. (B) Fold change in mRNA levels caused by adding 100 ng·mL^{-1} LPS, 5 μM PGE_2 or 50 μM forskolin to CD16$^{+/-}$ monocytes for 18 h in serum-free medium after 2 h of adhesion. Values are means and upper 95 % confidence intervals. *$P < 0.05$ versus no addition by ANOVA with Dunnett's post test ($n = 6–10$). Inset – protein levels of MMP-9 by zymography after treatment with ethanol (E), DMSO (D), PGE_2 (PG) or forskolin (For). (C) PGE_2 concentration was measured in conditioned media under the conditions shown above and in the presence of 100 ng·mL^{-1} LPS, 25 μM indomethacin (indo) or 10 μM NS-398 (NS) as shown. The final concentration of vehicle (DMSO) was 0.1% v/v. Values are means ± SEM. *$P < 0.05$ versus LPS. ANOVA with Dunnett's post test ($n = 3$).

alone by the non-selective COX inhibitor indomethacin and to 35 ± 12% by the COX-2 selective inhibitor, NS-398 (Figure 2C). Clearly, the concentrations of PGE_2 obtained under our experimental conditions were much lower than the concentration necessary to stimulate MMP expression in our preparations. Hence, the effects of LPS on MMP expression observed here were independent of COX and PGE_2. From our data, it is apparent that the COX- and PGE_2-independent LPS-mediated up-regulation of MMP-1, MMP-10, MMP-12 and MMP-14 mRNAs was as effective as maximal concentrations of added PGE_2. We therefore sought to investigate the mechanisms underlying this COX-independent regulation.

Effects of mitogen-activated protein kinase (MAP kinase) and inhibitor of κB kinase 2 (IKK2) inhibitors on LPS-induced MMP and TIMP expression

MMP gene promoters contain proximal activator protein-1 (AP-1) and stimulator protein-1 (SP-1) transcription factor binding sites that are known to be regulated by MAP kinases. Up-regulation of MMPs may also depend directly or indirectly on NF-κB (Vincenti and Brinckerhoff, 2002). We therefore investigated the ability of inhibitors of the ERK1/2 MAP kinase activator MEK (PD98059), p38 MAP kinase activity inhibitor (SB20380), c-jun N-terminal kinase (JNK) activity inhibitor (SP600125) and the IKK2 inhibitor (SC514) on adhesion or LPS-stimulated MMP and TIMP expression. To determine appropriate concentrations for our studies, we first showed that LPS treatment activated MAP kinases in adherent monocytes (Figure 3A, B). Adhesion also increased phosphorylation of ERK1/2, p38 and JNK (results not shown). PD98059 inhibited LPS-induced ERK1/2 phosphorylation (by 83 ± 6%) without effect on p38 or c-JUN phosphorylation (Figure 3A, B). SB20380 activated ERK1/2 and JNK phosphorylation (192 ± 36% and 151 ± 27, Figure 3A, B), which is in accord with previous reports (Birkenkamp *et al.*, 2000; Hall and Davis, 2002), but did not affect p38 phosphorylation as expected, since it regulates p38 activity but not its phosphorylation by MKK3/6 or c-JUN phosphorylation. SP600125 inhibited JNK phosphorylation (98 ± 1%, Figure 3A, B) via a partial inhibition of MKK4 and an inhibition of JNK autophosphorylation activity (Bennett *et al.*, 2001), without affecting p38 or ERK1/2 phosphorylation significantly. The ability of LPS to increase IKK2 activity was inferred from the resulting (49 ± 7%, $n = 3$) decrease in IκBα protein levels after 45 min (see Figure 3C) and the subsequent (5.2 ± 1.0 fold, $n = 5$) increase after 18 h in mRNA for I-κBα, which is transcribed by NF-κB to restore quiescence (Baeuerle and Baltimore, 1996). Adhesion for 2 h also increased IκBα mRNA levels (3.7 ± 1.2 fold, $n = 3$). Consistent with its known activities (Kishore *et al.*, 2003), SC514 inhibited the LPS induced decrease in IκBα protein back to 84 ± 9% ($n = 3$) of untreated controls (see Figure 3C) and reduced the increase in IκBα mRNA levels after adhesion or LPS treatment by 52 ± 3 and 74 ± 4% ($n = 5$), respectively.

The effects of the MAP kinase and IKK2 inhibitors were then measured on MMP and TIMP expression. Indomethacin was present throughout the LPS treatment to inhibit any contribution from PGs. These series of experiments were expanded to include measurements of MMP-7 and COX-1 and COX-2 mRNAs. LPS significantly increased expression of MMP-1, MMP-7, MMP-9, MMP-10 and MMP-14, TIMP-1, COX-2 mRNAs but not TIMP-2, TIMP-3 or COX-1 (Figure 4). The results were similar to the series of experiments shown in Figure 2B conducted in the absence of indomethacin. The

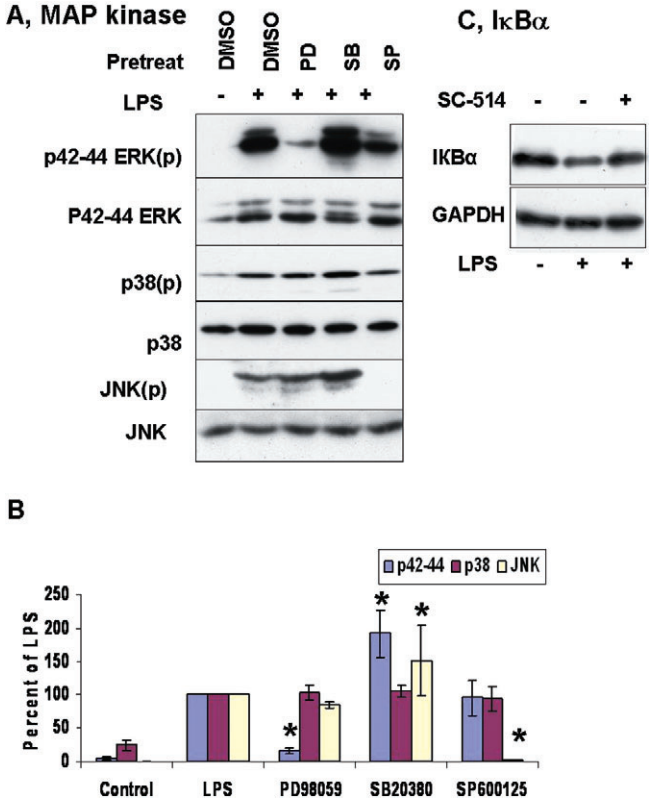

Figure 3

Effects of inhibitors of MAP kinase and IKK2 on kinase activities. Adherent monocytes were pre-treated with the inhibitors for 45 min before addition of 100 ng·mL^{-1} LPS in serum-free medium. MAP kinase inhibitors: PD98059 (PD), SB20380 (SB) and SP600125 (SP) were all added at 10 μM and the IKK2 inhibitor SC514 at 40 μM in 0.1% v/v DMSO. (A) Effects of MAP kinase inhibitors on LPS (100 ng·mL^{-1})-stimulated ERK, p38 MAP kinase and JNK phosphorylation by Western blotting. LPS was added for 45 min. (B) Densitometry of Western blots represented in panel A (n = 4–6). *P < 0.05 versus LPS alone. (C) A representative of three similar Western blots for IκBα showing depletion by LPS and restoration by 40 μM SC514.

increases in MMP-9, MMP-10, MMP-14 and COX-2 protein were confirmed by Western blotting or zymography (inset).

As illustrated in Figure 5A–D, the MEK inhibitor, PD98059, significantly inhibited MMP-1, MMP-10, MMP-14 and COX-2 expression after adhesion or LPS treatment (Figure 5A). The only consistent effect of the p38 inhibitor, SB20380, between adhesion and LPS treatment was to decrease COX-2 expression (Figure 5B). SB20380 reduced MMP-7 but increased MMP-10 expression after LPS treatment alone (Figure 5B). The JNK inhibitor, SP600125, consistently reduced MMP-1, and MMP-10, and COX-2 but increased TIMP-3 mRNA levels on adhesion and LPS treatment; MMP-14 was reduced only after adhesion (Figure 5C). SP600125 slightly increased MMP-7 mRNA levels after adhesion and MMP-9 mRNA levels after LPS treatment. Protein expression studies confirmed the small effects of PD98059 and SP600125 on MMP-9 and much more pronounced effects on MMP-10 protein levels (Figure S1A). The IKK2 inhibitor, SC514, also consistently decreased MMP-1, MMP-10 and

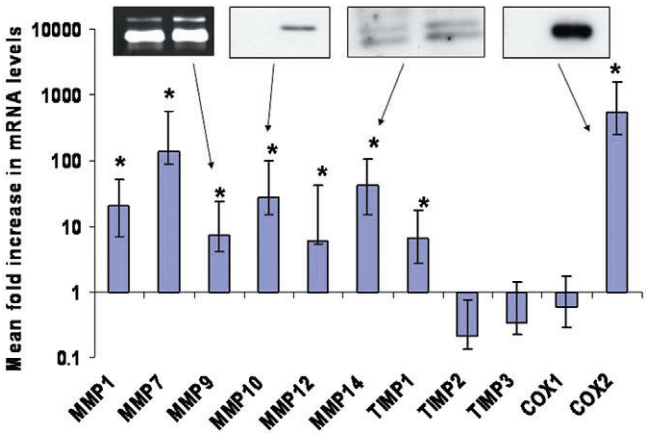

Figure 4

LPS effects in the presence of indomethacin. Stimulation of mRNAs (as fold increase) by 100 ng·mL^{-1} LPS for 18 h with 25 μM indomethacin present. Values are means and upper 95 % confidence intervals. *P < 0.05 versus no addition of LPS. ANOVA with Dunnett's post test on log-transformed data (n = 6–10). Inset – protein levels with (right-hand lanes) and without (left-hand lanes) LPS measured by Western blotting or zymography for MMP-9.

MMP-14 and COX-2 mRNA levels after adhesion and LPS treatment. Taken together our results clearly showed regulation of MMP-1, MMP-10 and MMP-14 depended on ERK1/2 and JNK MAP kinases and IKK2, but the other MMP and TIMP genes we studied did not.

MMP and TIMP expression during differentiation on plastic or fibronectin

After entering the blood vessel wall, monocytes differentiate to macrophages. To model this transformation, we studied monocytes from buffy coats during adhesion to plastic with M-CSF for 7 days. As summarized in Table S2, no differences were seen between un-fractionated and CD16- monocytes (Table S2). To confirm differentiation, we showed that the marker gene CD206 did not change on adhesion (Figure 1, Table S2) but was dramatically increased 108-fold during culture with M-CSF (Table S2). Differentiation with M-CSF for 7 days led to a fall in MMP-1 (332-fold), MMP-10 (190-fold), TIMP-1 (7.5-fold) and COX-2 (353-fold) mRNA levels but an increase in expression of MMP-7 (126-fold), MMP-9 (139-fold), MMP-12 (4.4-fold) and MMP-14 (3.5-fold) and of TIMP-2 (2.8-fold) and especially TIMP-3 (347-fold) (Table S2). MMP-8 and MMP-23 and COX-1 mRNA levels were not significantly changed during differentiation (Table S2). More detailed time-courses compared monocytes adhered either to plastic or fibronectin, as a model for the intimal extracellular matrix, as measured over the first 72 h. These independent experiments confirmed that mRNA levels of MMP-1 (Figure 6A) declined during differentiation on plastic or fibronectin, although the initial increase after 2 h was significantly attenuated by fibronectin. By contrast, expression of MMP-7 (Figure 6B), MMP-9 (Figure 6C), MMP-12 (Figure 6D) and MMP-14 (Figure 6E) increased progressively. Binding to fibronectin accelerated the increase in expression of MMP-7,

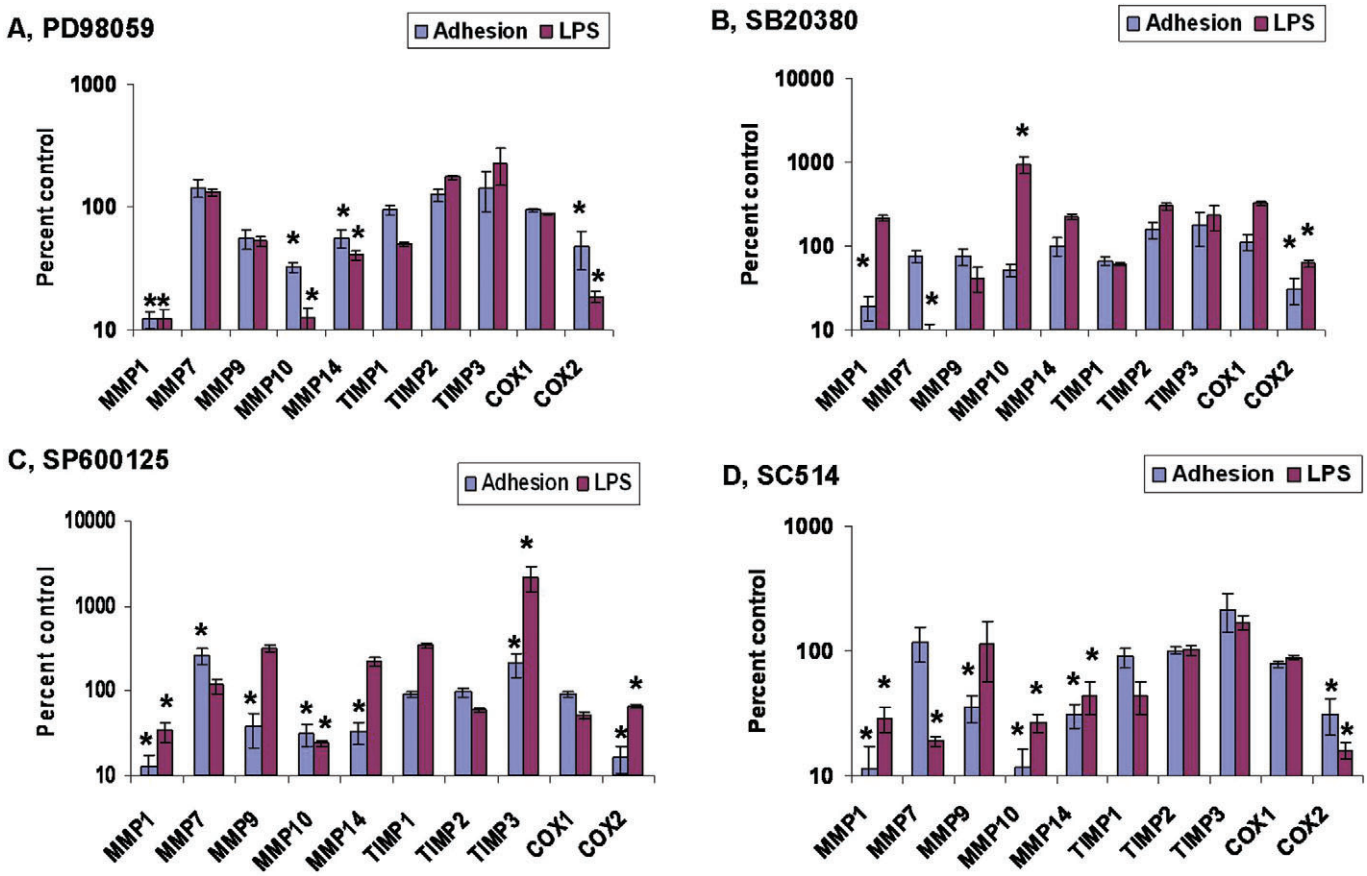

Figure 5

Effects of MAP kinase and IκB kinase2 inhibitors. Total mononuclear cells were pre-treated in suspension for 20 min at 37°C with the inhibitors shown or vehicle before adhering in 1% AB serum followed by 10% FBS always in the presence of the inhibitors for 2 h (adhesion). Alternatively, adherent monocytes were pre-treated in serum-free medium with the inhibitors for 45 min before addition of 100 ng·mL^{-1} LPS for 18 h in serum-free medium (LPS). (A) Levels of mRNA with 10 μM PD98059 present. *P < 0.05 versus adhesion or addition of LPS alone. ANOVA with Dunnett's post test (n = 5). (B) As above with 10 μM SB20380. (C) As above with 10 μM SP600125. (D) As above with 40 μM SC514.

MMP-9 and MMP-14 (Figure 5B, C, E), but the effect on MMP-12 was not statistically significant (Figure 6D). Increased expression of MMP-12 and MMP-14 appeared delayed compared with MMP-7 and MMP-9. The effect of fibronectin on MMP-9 was confirmed at the protein level by zymography (Figure S1B). In agreement with the mRNA data (Figure 6C), MMP-9 protein was significantly increased to 154 ± 12% (P < 0.05, n = 3) after 42 h of adhesion to fibronectin compared with plastic. Expression of TIMP-1 declined progressively (Figure 6F), while TIMP-2 (Figure 6G) and especially TIMP-3 (Figure 6H) progressively increased; TIMP expression was not modified by fibronectin. None of the increases in MMP or TIMP mRNA expression were decreased by indomethacin or NS-398. For example, MMP mRNA expression was 72 ± 16% (n = 6) in the presence of indomethacin and 200 ± 73% in the presence of NS-398 (n = 5). Protein expression of MMP-9 was also unaffected by indomethacin (96 ± 7%, n = 5; Figure S1C) or NS-398 (180 ± 21%, n = 5; not shown in Figure S1C). Hence, we concluded that the increased expression of MMPs and TIMPs during maturation of monocytes to macrophages is also independent of COX under the conditions we used. The expression of MMP, TIMP and COX-1 mRNAs were not affected by incubation of differentiated macrophages with inhibitors of ERK1/2, p38 or JNK MAP kinases for the final 48 h (Table S3). By contrast, inhibition of ERK1/2 and JNK decreased COX-2 mRNA levels by approximately 50% (Table S3).

Discussion

We studied the expression of a wide range of MMPs and TIMPs in monocytes after adhesion, treatment with LPS and differentiation into macrophages, thus mimicking events that occur early in inflammation. We then investigated the potential mechanisms responsible for the diverse pattern of MMP and TIMP regulation. Our study used human primary blood-derived monocytes rather than a cell line, thus allowing for a cautious extrapolation to inflammatory events *in vivo*.

In agreement with previous work (Bar-Or *et al.*, 2003), the most abundant MMP transcripts in adherent monocytes were MMP-1, MMP-7, MMP-8, MMP-9, MMP-10, MMP-14 and MMP-19. Interestingly, however, our data demonstrated that MMP-1, MMP-7 and MMP-10 were almost absent in

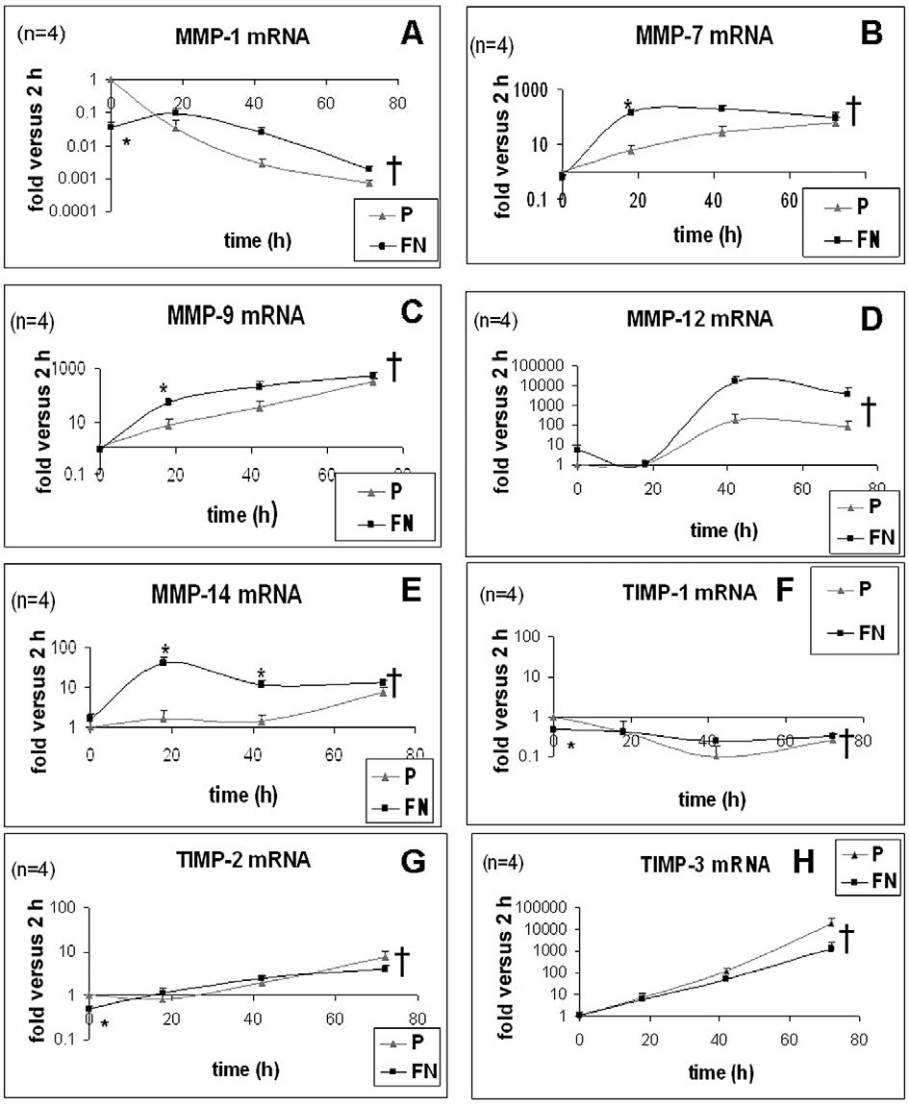

Figure 6

Time course of changes in MMP and TIMP mRNAs during $CD16^{+/-}$ monocyte differentiation on plastic and fibronectin. Fold changes in MMP and TIMP mRNAs levels relative to values for 2 h adhesion. Values are means and upper 95 % confidence intervals. *$P < 0.05$ fibronectin versus plastic. †$P < 0.05$ both fibronectin and plastic versus 2 h adhesion. ANOVA with Dunnett's post test on log-transformed data ($n = 4$). (A) MMP-1, (B) MMP-7, (C) MMP-9, (D) MMP-12, (E) MMP-14, (F) TIMP-1, (G) TIMP-2, (H) TIMP-3.

non-adhered monocytes, and that the MMPs were rapidly induced by adherence. Hence, only MMP-8, MMP-9, MMP-14 and MMP-19 mRNAs were constitutively expressed at high levels in non-adhered monocytes, which presumably reflect circulating monocytes. Adhesion rapidly increased mRNAs for several MMPs including MMP-1 and MMP-10 that could aid monocyte migration into areas of inflammation. No previous study has, to our knowledge, addressed the impact of monocyte subsets on the profiles of MMP expression observed. We found similar patterns of expression when using mixed $CD16^+$ and $CD16^-$ and negatively selected $CD16^-$ monocytes (Table S2), and therefore, no evidence of selective up-regulation of MMPs in the minority $CD16^+$ population. All the results we observed were dominated by the majority $CD16^-$ population.

The ability of LPS to increase expression of MMP-1, MMP-9 and MMP-14 and TIMP-1 has been previously reported (Shankavaram *et al.*, 2001; Ardans *et al.*, 2002). However, no previous study has simultaneously studied a large number of MMPs and TIMPs. Our study showed that LPS treatment of monocytes up-regulated two collagenases, MMP-1 and MMP-14 (a membrane-type MMP), a gelatinase (MMP-9), two stromelysins, MMP-7 and MMP-10, and the metalloelastase, MMP-12. Among the TIMPs, only TIMP-1 was up-regulated, which implies a strong increase in the MMP/TIMP balance. The substrate components of the MMPs up-regulated by LPS include most constituents of the ECM, which suggests a potentially high destructive power of recently recruited monocytes activated by bacterial products at a site of an infection.

	Effect of adhesion	Induced by LPS	Adhesion depends on ERK	LPS depends on ERK	Adhesion depends on JNK	LPS depends on JNK	Adhesion depends on IKK2	LPS depends on IKK2	Effect of differentiation
COX2	8.62	553.70	0.47	0.18	0.16	0.65	0.31	0.16	0.00
MMP1	6326.93	20.33	0.12	0.11	0.13	0.28	0.11	0.29	0.00
MMP10	896.65	27.42	0.32	0.11	0.31	0.24	0.12	0.27	0.01
MMP14	61.91	41.83	0.56	0.40	0.32	2.11	0.31	0.44	3.45
MMP7	From 0	132.59	1.43	1.30	2.60	1.03	1.17	0.19	126.99
MMP9	3.45	7.23	0.55	51.62	0.37	1.40	0.35	1.15	139.51
TIMP1	3.61	6.53	0.95	0.49	0.92	3.41	0.89	0.44	0.13
TIMP2	1.65	0.22	1.25	1.73	0.95	0.59	1.01	1.02	2.78
TIMP3	1.01	0.34	1.42	1.58	2.09	13.96	2.14	1.68	347.62

Figure 7

Summary of changes in MMP, TIMP and COX-2 mRNA levels. The table summarizes fold changes relative to the controls shown (see other figures for details). Red and blue numbers show increases and decreases, respectively.

From our results, differentiation into mature macrophages had a very different effect on MMP and TIMP expression compared with adherence or LPS treatment (see Figure 7). Expression of the collagenases MMP-1 and its activator MMP-10 (Saunders *et al.*, 2005), as well as COX-2, which were increased by adhesion and LPS treatment, were greatly reduced during maturation. Conversely, the gelatinases MMP-2 and MMP-9, the stromelysin MMP-7 and the metalloelastase MMP-12 were all greatly increased by LPS and differentiation (Figure 1, Table S2). Since MMP-10 is a preferred activator of MMP-1 (Saunders *et al.*, 2005), it appears that mature, unstimulated macrophages may have a reduced capacity to remodel fibrillar collagen but an increased capacity to turn over other ECM components. However, this increased potential for matrix turnover could be counteracted by increased TIMP-2 and TIMP-3 expression, the latter effect having already been reported (Fabunmi *et al.*, 1998). Interestingly, TIMP-3 expression was not up-regulated by adhesion or LPS, but cellular differentiation caused an increase greater than for any other of the genes examined. Binding of TIMP-3 to the ECM would tend to encapsulate mature macrophages and protect the ECM from further proteolysis.

Fibronectin is an abundant component of the ECM that is synthesized by macrophages in culture. Indeed, fibronectin is one of the most up-regulated genes during macrophage differentiation (Lehtonen *et al.*, 2007). Fibronectin is also increased during inflammation *in vivo* (Stecher *et al.*, 1986, Feaver *et al.*, 2010). Fibronectin was previously shown to up-regulate expression of MMP-9 and MMP-14 in human monocytes (Jacob *et al.*, 2002; Matias-Roman *et al.*, 2005). We found here that culturing on fibronectin actually accelerated expression of MMP-7, MMP-9 and MMP-14 during differentiation without affecting final expression levels after 4 days. Fibronectin did not influence TIMP expression. Hence, binding to fibronectin would be expected to increase the ratio of MMPs to TIMPs and favour ECM degradation in the early stages of monocyte to macrophage transformation *in vivo*.

Perhaps most interestingly, our new findings demonstrate that the magnitude and scope of COX-independent MMP regulation is much greater than previously appreciated. Indeed, up-regulation of MMP-1, MMP-7, MMP-9, MMP-10, MMP-12 and MMP-14 in human monocytes by adhesion, LPS treatment or differentiation to macrophages with MCSF can all occur by COX-independent mechanisms. This finding appears to contradict much previous work, which convincingly demonstrated the existence of a PG-dependent pathway of MMP up-regulation in human and mouse monocytes, even though a COX-independent regulation of MMP-9 and TIMP-1 by cytokines had been reported in one publication (Zhang *et al.*, 1998). In our study, we confirmed previous results that LPS induced COX-2 and PGE_2 production (Ardans *et al.*, 2002; Khan *et al.*, 2004). Furthermore, we also confirmed previous observations that MMP up-regulation can be mimicked by adding PGE_2 (Corcoran *et al.*, 1992; Shankavaram *et al.*, 2001) or forskolin (Pavlovic *et al.*, 2006), a direct activator of adenylate cyclase. Hence, the whole pathway from LPS to PGE_2 and from PGE_2 to MMP up-regulation is present in monocytes. However, we found that MMP expression could not be blocked by a non-selective COX inhibitor under the conditions of our experiments, which is in contrast to previous work (Shankavaram *et al.*, 2001; Ardans *et al.*, 2002; Khan *et al.*, 2004). This apparent disagreement was resolved by showing that PGE_2 levels did not accumulate sufficiently to trigger MMP induction in our experiments. Previous work showed that at least 10–100 nM PGE_2 is required to elicit an effect on MMP up-regulation even in monocytes co-stimulated with other agonists (Corcoran *et al.*, 1992; Shankavaram *et al.*, 2001; Khan *et al.*, 2004). Our dose–response studies were in broad agreement, whereas only 3 nM PGE_2 accumulated under the conditions of our experiments (Figure 2C). These conditions therefore revealed the existence of COX-independent pathways capable of up-regulating MMPs and TIMP-1 at least as effectively as the PGE_2-dependent pathways. Both COX-dependent and -independent pathways are likely to be impor-

tant *in vivo*, since the local concentrations of PGE_2 could be much higher close to a focus of inflammation than in our *in vitro* cultures. However, prostaglandin-independent pathways of monocyte and macrophage MMP production would remain active during treatment with COX inhibitors *in vivo*, and this would limit the effectiveness of these agents in treating the adverse ECM remodelling that occurs during inflammation. Such concerns have gained added importance in the wake of clinical trial data showing no benefit or harm from COX-2 inhibitor treatment in the context of symptomatic cardiovascular disease (Cipollone *et al.*, 2008).

In attempting to identify mechanisms for the COX-independent up-regulation of MMPs, we noted that the promoters of MMP-1, MMP-7, MMP-9, MMP-10 and MMP-12 and TIMP-1 all have essential, proximal AP-1 sites that bind Fos and Jun family transcription factors (Benbow and Brinkerhoff, 1997). Moreover, MAP kinases have been extensively implicated in AP-1 transcription factors and MMP up-regulation in other cell types (Vincenti and Brinckerhoff, 2002; Chakraborti *et al.*, 2003). Furthermore, a role for NF-κB in up-regulation of some MMPs has been clearly identified, including in monocyte/macrophages (Chase *et al.*, 2002; Vincenti and Brinckerhoff, 2002). Interestingly, adhesion and LPS-induced, COX-independent up-regulation of different MMPs and TIMPs showed a diverse pattern of dependence on activation of MAP kinases and NF-κB (see Figure 7). The first pattern was exemplified by MMP-1 and –10 (and COX-2) that were strongly up-regulated by adhesion and LPS treatment but decreased during maturation. Expression of these was strongly dependent on activation of ERK1/2, JNK and IKK2 (Figure 7). Up-regulation of MMP-10 only by the p38 MAP kinase inhibitor SB20380 (Figure 5B) could be explained by its ability to increase ERK1/2 phosphorylation (Birkenkamp *et al.*, 2000; Hall and Davis, 2002), as we confirmed (Figure 3A). The pattern of regulation of MMP-14 showed some similarity to MMP-1 and MMP-10, although the proximal promoter of the MMP-14 gene depends on an SP-1 rather than an AP-1 binding site (Lohi *et al.*, 2000). MMP-14 was up-regulated in response to adhesion dependent on ERK1/2, JNK and IKK2 activity and by LPS dependent on ERK1/2, and IKK2 activity only (Figure 7). The biggest difference was that MMP-14 expression increased rather than decreased during differentiation (Figure 7). MMP-7, MMP-9 and TIMP-1 showed another pattern; they were up-regulated by adhesion, LPS treatment and differentiation largely independently of ERK1/2, JNK and IKK2 (Figure 7). MMP-7 only showed a dependence on p38 MAP kinase activity after LPS treatment (Figure 7). Finally, TIMP-2 and TIMP-3 expression was not increased by adhesion or LPS and was independent of MAP kinases or IKK2. None of the MAP kinase inhibitors affected MMP or TIMP production from un-stimulated mature macrophages (Table S3). This was expected in the case of MMP-1 and MMP-10, which were already down-regulated after differentiation, but it underlines the fact that distinct pathways must be responsible for the up-regulation of MMP-14 by LPS and differentiation.

In summary, we found that adhesion and LPS-treatment of $CD16^-$ and $CD16^{+/-}$ monocytes selectively increased the steady-state mRNA levels of MMP-1, MMP-7, MMP-9, MMP-10 and MMP-14 to a greater degree than TIMPs, implying a shift in the protease/anti-protease balance in favour of matrix degradation. Differentiation into macrophages down-regulated MMP-1 and MMP-10 but increased MMP-7, MMP-9, MMP-12 and MMP-14 and TIMP-3 mRNAs. The effects on MMP and TIMP during adhesion, LPS treatment and differentiation were independent of PGE_2 production through COX. Only MMP-1, MMP-10 and MMP-14 up-regulation by adhesion and LPS treatment depended on ERK1/2, JNK or IKK2 activation. These results may have important implications for the pharmacological inhibition of MMP production and hence the mitigation of ECM degradation during acute and chronic inflammation.

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Conflict of interest statement

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Effects on MMP-9 and MMP-10 protein secretion. (A) A representative zymogram for MMP-9 (n = 3) and

Western blot for MMP-10 (n = 2) showing the effects of LPS with and without MAP kinase inhibitors. Conditioned medium on each lane was from 1.5 and 21 μg of cellular protein respectively. (B) A representative zymogram for MMP-9 (n = 3) showing the effect of fibronectin during differentiation for the times shown. Fresh serum-free medium was added for the last 18 h. Conditioned medium in each lane originated from 1500 cells. (C) As in (B) but showing the effect of 25 μM indomethacin present throughout differentiation on plastic.

Table S1 Primers used for qRT-PCR

Table S2 Copy numbers of messenger RNA per ng of total RNA

Table S3 Effects of mitogen activated protein kinase inhibitors on messenger RNA levels in microphages differentiated from human peripheral blood monocytes