



Newby, A. C. (2016). Metalloproteinase production from macrophages - a perfect storm leading to atherosclerotic plaque rupture and myocardial infarction. *Experimental Physiology*, 101(11), 1327-1337. <https://doi.org/10.1113/EP085567>

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# Metalloproteinase production from macrophages – a perfect storm leading to atherosclerotic plaque rupture and myocardial infarction

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Running title: Metalloproteinases from macrophages

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A Keynote Lecture given at the International Society for Nephrology Forefronts Symposium 2015 in Shenzhen, China on October 14<sup>th</sup> 2015.

Key words

Macrophage, metalloproteinase, transcription, inflammation, atherosclerosis, plaque rupture, myocardial infarction

## Abstract

Matrix metalloproteinases (MMPs) produced from macrophages contribute to plaque rupture, atherothrombosis and myocardial infarction. New treatments could emerge from defining the mediators and underlying mechanisms. In human monocytes, prostaglandinE2 (PGE2) stimulates MMP production and inflammatory mediators such as TNF $\alpha$ , IL-1 and toll-like receptor ligands can act either through or independently of PGE2. Differentiation of human monocytes to non-foamy macrophages increases constitutive expression of MMPs-7, -8, -9, -14 and -19 and TIMPs-1 to -3 through unknown, PGE2-independent mechanisms. Human macrophages express more MMPs-1, -7, -9 and TIMP-3 and less MMP-12 and -13 than mouse macrophages. Inflammatory mediators working through AP-1 and NF- $\kappa$ B transcription factor pathways upregulate MMPs-1, -3, -10, -12 and -14 in human macrophages (MMP-9, -12 and -13 in mice) and studies with plaque tissue sections and isolated foam cells confirm this conclusion *in vivo*. Classical activation with GM-CSF upregulates MMP-12, whereas IFN $\gamma$  upregulates MMPs-12, -14 and -25 and downregulates TIMP-3 in human but not mouse macrophages. Alternative activation with IL-4 markedly stimulates the expression of only MMP-12 in humans and MMP-19 in mice. Anti-inflammatory cytokines, IL-10 and TGF $\beta$ , decrease production of several MMPs. Epigenetic upregulation of MMP-14 during foam cell formation or by GM-CSF occurs by decreasing miRNA-24. A 'perfect storm' caused by a combination of these mechanisms most likely promotes MMP-mediated macrophage invasion, tissue destruction and atherosclerotic plaque rupture.

## Introduction

Production of matrix metalloproteinases (MMPs) from macrophages contributes to destruction of the extracellular matrix (ECM) in a broad range of chronic inflammatory diseases. Atherosclerosis is a special case because lipoprotein particles trapped in the artery wall recruit monocytes that convert to foam-cell macrophages by engorging oxidised and other modified forms of these lipoproteins (Williams & Tabas, 1995). In advanced atherosclerosis, plaques consisting of amorphous lipid deposits with overlying, expanded connective tissue can obstruct the coronary and other conduit arteries, leading to stable ischaemic syndromes, including angina pectoris. Moreover, depletion of collagen and other ECM molecules from the core and fibrous cap overlying plaques can lead to loss of mechanical competence, culminating in rupture of the cap, thrombus formation on the exposed thrombogenic core and partial or complete occlusion of the lumen (Libby, 2013). Plaque rupture underlies the majority of myocardial infarctions (MIs) and strokes (Virmani *et al.*, 2006), which together constitute the principal cause of death in many advanced societies. Inhibiting MMP activity (Newby, 2012; Newby, 2015), or the mechanisms responsible for production of MMPs from macrophages (reviewed here), therefore represent viable targets for therapies to prevent MIs and strokes. The earlier literature relating to this topic was previously discussed exhaustively (Newby, 2008), and hence this article seeks to provide an update by emphasizing findings during the last seven years. These new insights suggest that multiple inflammatory mediators need to act in concert to raise a 'perfect storm' that provokes net destruction of the ECM leading to MIs and strokes.

## Involvement of matrix metalloproteinases in atherosclerosis

There are at least 23 MMP enzymes, most of which are secreted, except the six membrane-type MMPs that are inserted into or attached to the external membrane surface. The catalytic sites of MMPs may be blocked by all or at least some of the four tissue inhibitors of MMPs (TIMPs) (reviewed in detail elsewhere (Nagase *et al.*, 2006)). A structurally-similar active catalytic domain occurs also in some members of the disintegrin metalloproteinases (ADAMs) and in the ADAMs with thrombospondin domains (ADAM-TSs). MMPs have the ability to degrade a variety of ECM

components but also many other cell surface, secreted or ECM-sequestered substrates many of which regulate inflammation (Khokha *et al.*, 2013).

As summarized previously (Newby, 2012; Newby, 2015), the evidence that MMPs play pathological roles in atherosclerosis comes partly from rabbit and especially mouse models. However, the expression pattern of MMP mRNAs in human blood and mouse bone marrow macrophages isolated and classically activated under very similar conditions is quite divergent, with far more MMPs-1, -7, -9 and TIMP-3 in human macrophages but much less MMP-12 and -13 compared to mouse (Newby, 2015). This conclusion is reinforced by other studies of mouse bone marrow macrophages and Raw264.7 cells (Hald *et al.*, 2012; Murray *et al.*, 2013). Moreover, a comparison of unstimulated mouse and human blood monocytes and macrophages shows the same similarities and differences (Fig. 1). Differentiation of blood monocytes to macrophages greatly increases expression of MMPs in both mice and humans (Fig. 1) but levels of MMPs-1, -7, and -9 and TIMPs-1 and -3 are much higher in man (Huang *et al.*, 2012), whereas MMPs-12, -13 and -23 are much higher in mice (Tsaousi *et al.*, 2016). The high levels of MMP-12 and -13 expression in mice macrophages correspond with dramatic effects on atherosclerosis (Johnson *et al.*, 2011; Quillard *et al.*, 2011). However, MMP-12 (Scholtes *et al.*, 2012) and MMP-13 (Molloy *et al.*, 2004) have restricted expression in human atherosclerotic plaques, which invites caution over the clinical translation of the mouse studies. In the case of MMP-12 there are genome wide association studies (GWAS) studies supporting a causative role in strokes (Traylor *et al.*, 2014) but this is not the case for MMP-13. Conversely, MMP-7 is hardly expressed in mouse macrophages and has a modest impact on atherosclerosis (Johnson *et al.*, 2005) but could be more important in man (Fig. 1). Most recently, MMP-28 was shown to affect macrophage functions in mice (Ma *et al.*, 2013) but MMP-28 is not expressed actively in human monocytes or macrophages (Bar-Or *et al.*, 2003). Furthermore, the profound morphological differences and the need for high level transfer of fully active forms of MMPs to provoke plaque rupture in mice (Gough *et al.*, 2006; Liang *et al.*, 2006) discourage extrapolation to the human disease.

GWAS provides convincing evidence of a pathogenic role for MMP-12 (Traylor *et al.*, 2014) and the distantly-related ADAMTS-7 (Reilly *et al.*, 2011). For other MMPs and TIMPs only correlative evidence is available so far. For example, many MMPs and TIMPs are overexpressed in human atherosclerotic plaques compared to normal tissues (reviewed in detail elsewhere (Newby, 2005)). More persuasively, MMP-8, -9, -12 and -14 have been shown in biobank studies to associate with plaque morphologies suggesting vulnerability to rupture, whereas MMP-2 and TIMP-3 show negative association (Sluijter *et al.*, 2006; Peeters *et al.*, 2011; Scholtes *et al.*, 2012; Johnson *et al.*, 2014). Furthermore, at least MMP-8 and MMP-12 levels in plaques are risk factors for subsequent adverse cardiovascular events (Peeters *et al.*, 2011; Scholtes *et al.*, 2012). In future it may be possible to combine biochemical and genetic analyses for example in Mendelian Randomisation studies or by the identification of rare null mutations. In the meantime a causative role for MMPs in human plaque rupture is highly plausible but still a hypothesis.

## Monocyte and macrophage diversity in atherosclerosis

Production of monocytes and macrophages from myeloid precursors relies on the trophic effects of colony stimulating factor (CSF-1). Deletion of CSF-1 or blocking its receptor in mice prone to atherosclerosis greatly reduces plaque formation (Di Gregoli & Johnson, 2012). Similarly, depletion of monocytes and macrophages in the early stages of mouse atherosclerosis abolishes foam cell formation and reveals the accumulation of lipoprotein deposits in susceptible sites (Paulson *et al.*, 2010). These experiments establish that macrophages derived from circulating monocytes are

required to clear lipoprotein deposits retained in the ECM and that this leads to foam cell formation. Some contribution from macrophage proliferation (Robbins *et al.*, 2013) or from expansion of resident stem cell populations has also been debated (Nguyen *et al.*, 2012); and additional foam cells can be generated by transdifferentiation of resident vascular smooth muscle cells (VSMCs) (Shankman *et al.*, 2015). As reviewed previously (Newby, 2005), both macrophages and VSMCs elaborate MMPs and TIMPs. Moreover, MMPs and other proteases promote VSMC migration and proliferation so as to establish the fibrous cap of plaques. On the other hand, the high levels of many MMPs produced by macrophages (see below) may provoke destruction of the ECM causing plaque rupture.

At least two phenotypes of monocytes (Ly6Chi, CCR2hi, and Ly6CloCXCR3hi) and three phenotypes of human monocytes (CD14hiCD16lo, CD14dimCD16lo and CD14dimCD16hi) have been characterised (Ziegler-Heitbrock *et al.*, 2010). Despite performing different functions in relation to acute inflammation and patrolling behaviour, both monocyte phenotypes appear to contribute to atherosclerosis in mouse models (Combadiere *et al.*, 2008). Moreover they do not seem to give rise to different macrophage populations in plaques (Tacke *et al.*, 2007).

Differentiated macrophages adopt a host of different phenotypes. These were initially divided into pro-inflammatory, so-called classically activated or M1 type, or anti-inflammatory, so-called alternatively activated or M2 type. However, the M1/M2 dichotomy has more recently been replaced with more nuanced descriptions of phenotypes (Murray *et al.*, 2014) based on the activating mediators and their related signalling pathways some of which are illustrated in Fig. 2.

## Regulation of MMP and TIMP production from monocytes and macrophages

Binding of transcription factors of the activator protein-1 (AP-1) family to regulatory elements in the proximal promoters of many MMPs appears to be of central importance for their transcriptional regulation, and certainly contributes to their increased production during inflammation (Clark *et al.*, 2008). However, not all MMP promoters contain proximal AP-1 sites or even a TATA box, which is necessary for induced transcription of most genes (Clark *et al.*, 2008). Moreover, a plethora of other proximal transcription factors binding sites, including for specificity protein-1 (SP-1), nuclear factor- $\kappa$ B (NF- $\kappa$ B) and signal transducer and activator of transcription-1 (STAT-1), mediate inflammatory activation of several MMPs (Clark *et al.*, 2008). Synergy between activation of AP-1 and NF- $\kappa$ B is responsible for induction of several MMPs in a variety of cell types (reviewed in (Newby, 2005)), including macrophages. This may depend on a signalosome that brings together widely separated transcriptional activators including distal enhancer or suppressor elements (Glass & Natoli, 2015).

In order to influence MMP and TIMP expression therapeutically in plaques and other inflammatory foci, it would be valuable to identify the key mediators and also the underlying mechanisms. This review will attempt to synthesise the available information, in part by providing a searchable databases for monocytes (Supplementary Table 1) and macrophages (Supplementary Table 2).

### ProstaglandinE2 and the cAMP pathway

ProstaglandinE2 (PGE2) mediates upregulation of at least MMP-1, -7, -9, -10 and -14, as well as TIMP-1 in undifferentiated human monocytes, as previously reviewed (Newby, 2008). PGE2-dependent MMP up-regulation has also been observed in human alveolar macrophages, mouse peritoneal macrophages and RAW264 cells (see Supplementary Tables 1 and 2). As shown in Fig. 2, action of inflammatory mediators or integrin-mediated binding to various ECM components activates phospholipase C, which releases arachidonic acid. This is transformed by the sequential

activity of cyclooxygenase (COX) and PGE2 synthase (PGES-1) to PGE2. COX-1 is constitutively expressed in human monocytes and COX-2 is rapidly upregulated by adherence or LPS (Reel *et al.*, 2011) or by TNF $\alpha$  together with GM-CSF (Zhang & Wahl, 2015). PGE2 acts specifically on EP4 receptors to stimulate cAMP formation, which then activates transcription through direct binding of cAMP response element binding protein (CREB) to the MMP-1 promoter or by enhancing the binding of NF- $\kappa$ B to the MMP-9 promoter (Lai *et al.*, 2003). Work from other cell types also identifies cross-talk with the mitogen activated protein kinases (MAPKs) (Gerits *et al.*, 2008) that could promote AP-1 binding (Fig. 2). Other activators of PGE2-dependent MMP production include extracellular MMP-1 or MMP-3, which can cleave active TNF $\alpha$  from the surface of mouse peritoneal macrophages, leading to MMP-9 secretion (Steenport *et al.*, 2009). Furthermore, TNF $\alpha$  generated in this way upregulates early growth response protein 1 (EGR-1), which induces mPGES-1 expression (Khan *et al.*, 2012). Exposure to Mycobacterium tuberculosis infection can also upregulate MMP-1 but not MMP-7 in a PGE2-dependent manner (Rand *et al.*, 2009).

### Differentiation of monocytes to macrophages

MMPs-2, -7, -9, -11, -12 and -14 and TIMPs-2 and -3 are selectively upregulated in human MCSF-differentiated macrophages, independently of COX, MAP kinases or NF- $\kappa$ B (Reel *et al.*, 2011), and MMP-8, -13, -19, -23 and -25 are also increased in mouse macrophages (Tsaousi *et al.*, 2016). Increased expression of MMP-14 has been ascribed to upregulation of the SAF-1 transcription factor (reviewed in (Newby, 2008)) but the other mechanisms remain to be clarified.

### Foam cell formation

The properties of foam cells formed from differentiated macrophages *in vitro* depends on the source of lipid (e.g. platelets, acetylated LDL, minimally or extensively oxidised LDL). Hence widely different results have been obtained suggesting stimulation, inhibition or little effect on levels of MMPs (Supplementary tables 1 and 2). Stimulation could result from (weak) action on toll like receptors (Lundberg & Yan, 2011), whereas inhibition of MMP-9 expression in U937 cells (Supplementary Table 1) resulted from formation of peroxisome proliferation activator receptor (PPAR $\gamma$ ) ligands. We found no effect of oxidised LDL on mRNA levels of MMPs and TIMPs but foam cells expressed more MMP-14 and less TIMP-3 protein, which implicated epigenetic mechanisms in part mediated by microRNA24 (Johnson *et al.*, 2014). *In vivo* results are also divergent. Rabbit granuloma foam cells showed increased MMP-1, -3, -12, -14 and decreased TIMP-3 expression (Supplementary Table 2), although mouse granuloma foam cells showed no changes (Thomas *et al.*, 2015) and peritoneal foam cells had decreased MMP-13 expression, owing to activation of the LXR nuclear receptor by the cholesterol pathway intermediate desmosterol (Spann *et al.*, 2012). So far **only the rabbit** studies investigated protein levels, which might vary despite similar mRNA expression if epigenetic mechanisms intervene (Johnson *et al.*, 2014).

### Classical macrophage activators and the AP-1/NF- $\kappa$ B pathway

The pro-inflammatory mediators TNF $\alpha$ , IL-1 $\beta$ , CD40L and pathogen associated molecular patterns that act at several toll-like receptors have been observed to stimulate MMP expression in both monocytes and macrophages (Supplementary Tables 1 and 2). Moreover, activation of TLR-2 was implicated directly in MMP-1 and MMP-3 production from isolated human plaque-derived cells examined *ex vivo* (Monaco *et al.*, 2009). These inflammatory mediators share the ability to activate the MAP kinases, extracellular related kinases 1/2 (ERKs1/2), p38 MAP kinase and c-jun N-terminal kinase (JNK), as well as phosphoinositide-3 kinase (PI3 kinase) and the inhibitor of  $\kappa$ B kinase2 (IKK2) that leads to activation of NF- $\kappa$ B (Fig. 2). Not surprisingly, therefore inhibitors of one or more these kinases generally reverse the effects of this broad class of inflammatory mediators (Supplementary Tables 1 and 2). However, the precise identity of the activating kinases seems to depend on the

MMP and the source of cells (Supplementary Tables 1 and 2). One complicating factor is the participation to greater or lesser degree of PGE2 derived from COX-2 (Fig. 2), induction of which requires all these kinases (Huang *et al.*, 2012). For example, inhibition of p38 reduced MMP-1 expression in a PGE2 dependent manner in human monocytes, whereas inhibition of ERKs1/2 decreased both MMP-1 and MMP-9 expression independently of PGE2 (Zhang & Wahl, 2015). In the absence of PGE2, upregulation of MMP-1 and -10 in human blood derived monocytes depended on ERKs1/2, JNK and IKK2 but not p38 MAP kinase (Reel *et al.*, 2011). The same was true for LPS induction of MMP-1, -3, -10, -12 and -14 in human macrophages but induction of MMP-25 required p38 (Huang *et al.*, 2012). Furthermore, specificity for the various activating kinases may depend on the specific inflammatory signal. For example, induction of MMP-1 in human alveolar macrophages by *M. tuberculosis* depends selectively on p38 MAP kinase (Rand *et al.*, 2009). Both basal and induced expression of many MMPs, especially MMPs-1, -3, -10 and -13 and TIMP-3 is reduced by inhibitors of PI3 kinase in human macrophages (Huang *et al.*, 2012). However, the basis for these effects is still not clarified. Other inflammatory mediators such as clusterin (Shim *et al.*, 2011) or complement component C5a (Speidl *et al.*, 2011), or homophylic interactions of CD147 (EMMPRIN) or with its ligand cyclophilin A (Yang *et al.*, 2008) also employ MAP kinases, PI3 kinase, IKK2 and the resultant activation of AP-1/NF- $\kappa$ B signalling to upregulate MMPs (Fig. 2). To confirm that these signalling pathways contribute to increased MMP expression in human atherosclerotic plaques, we demonstrated co-localisation of activated NF- $\kappa$ B with MMP-1 and MMP-10 (Huang *et al.*, 2012).

### Interferons and the JAK/STAT pathway

Earlier studies demonstrated profound inhibitory effects of IFN $\gamma$  on MMPs-1, -3, -9, and -12 and TIMP-1 production from human monocytes and macrophages (Supplementary Tables 1 and 2). On the other hand, IFN $\gamma$  acting through the JAK/STAT pathway can upregulate MMP-12, -14 and -25 and suppresses TIMP-3 mRNA expression in human macrophages (Huang *et al.*, 2012). The effect on MMP-25 may be especially interesting in view of its ability to modulate the activity of several chemokines (Marco *et al.*, 2013). Given that many human plaques contain IFN $\gamma$ , induction of MMP-14 and suppression of TIMP-3 could promote the invasive and destructive MMP14<sup>+</sup>TIMP-3<sup>-</sup> macrophage phenotype that we detected in rabbit and human foam cells (Johnson *et al.*, 2008). Effects of IFN $\gamma$  appear to be very different in human and mouse macrophages (Hayes *et al.*, 2014), which complicates the interpretation of the mouse models. Nevertheless, inhibition of mouse macrophage MMP-9 production by IFN $\gamma$  correlated with slower ECM degradation and thrombus resolution in wild type compared to IFN $\gamma$  knockout mice in a model of deep vein thrombosis (Nosaka *et al.*, 2011). Deletion of TGF $\beta$  receptors in T-lymphocytes, which promotes polarization to the Thelper1 phenotype that releases IFN $\gamma$  also decreased MMP-9 expression in atherosclerotic mouse aortas (Ovchinnikova *et al.*, 2009). On the other hand, MMP-13 was increased, suggesting that IFN $\gamma$  from Thelper1 cells can promote as well as inhibit expression of different MMPs. In other experiments, deletion of all T and B cells (Hayes *et al.*, 2014) or just Thelper1 cells (Tsaousi *et al.*, 2016) did not affect MMP or TIMP expression in mouse foam cells from subcutaneous granulomas or in atherosclerotic plaques. Consequently, the evidence for stimulatory effects of IFN $\gamma$  on MMP expression is stronger in humans than mice.

### IL-6 and GM-CSF

As illustrated in Fig. 2, IL-6 activates JAK1 and STAT-3, MAP kinases and PI3 kinases (Schaper & Rose-John, 2015), which may account for its upregulation of MMPs (Supplementary Tables 1 and 2). Interestingly, induction of MMP-9 in mouse macrophages by IL-6 is independent of COX-2 (Kothari *et al.*, 2014).

GM-CSF signals through the CSFR2 complex to activate JAK2 and STAT-5 as well as MAP kinases and PI3 kinases (Broughton *et al.*, 2012). Hence the transcriptional programme initiated by GM-CSF is unique, although it replicates some aspects of both the classical and alternative paradigms. GM-CSF is especially associated with upregulation of MMP-12 (Supplementary Table 1), which occurs through activation of the proximal AP-1 site. Why this direct action of GM-CSF is selective for MMP-12 over other MMPs with proximal AP-1 sites is unclear. GM-CSF can also induce TNF $\alpha$  secretion leading to the upregulation of other MMPs (Zhang *et al.*, 1998). Given this and the fact that GM-CSF can be upregulated by oxidised LDL and several inflammatory mediators (Di Gregoli & Johnson, 2012), GM-CSF-stimulated and classically-activated macrophage phenotypes are probably an overlapping *in vivo*. Despite this, in mice exposed to cigarette smoke, neutralisation of GM-CSF selectively decreases MMP-12 but not MMP-9 activity in lung macrophages (Vlahos *et al.*, 2010). GM-CSF increases MMP-14 protein expression and activity independently of changes in mRNA expression but because micro-RNA24 is decreased, which relieves an inhibitory effect on protein translation (Di Gregoli *et al.*, 2014). These observations are particularly interesting because there is evidence for distinct populations of M-CSF and GM-CSF macrophages in human plaques that may make different contributions to plaque stability (Di Gregoli & Johnson, 2012). Indeed, GM-CSF action might also account for the harmful MMP14<sup>+</sup>TIMP-3<sup>-</sup> macrophage phenotype (Johnson *et al.*, 2008)

### Hypoxia

Most macrophages in atherosclerotic plaques are in a chronic state of hypoxia (Sluimer *et al.*, 2008). Hypoxia increases expression of MMP-7 (Supplementary Table 2). Transcriptomic data from hypoxic macrophages indicates that, MMPs-1, -3, -10 and -12 are also significantly upregulated, perhaps secondarily to increased production of IL-1 $\alpha$ , $\beta$  (Fang *et al.*, 2009). Pathways through hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) (Lee *et al.*, 2012), HIF-2 $\alpha$  (Yang *et al.*, 2010) and JAK2/STAT-3 have been implicated (Gao *et al.*, 2015).

### Anti-inflammatory pathways

Priming with IL-4 inhibitors expression of MMP-1, MMP-9 and TIMP-1 in monocytes and macrophages (Supplementary Tables 1, 2), perhaps owing to overexpression of suppressor of cytokine signalling (SOCS) proteins. However, consistent with previous work in mouse macrophages (Supplementary Table 2), we found that IL-4 selectively increases MMP-12 in human monocyte derived macrophages (Huang *et al.*, 2012). MMP-25 and TIMP-3 were also up-regulated (Huang *et al.*, 2012) but the mechanisms responsible remain unclear. IL-10 also antagonises the upregulation of MMP-1 and MMP-9, but unlike IL-4 it increases expression of TIMP-1 (Supplementary Tables 1 and 2). Again the intermediary action of SOCS proteins appears reasonable but remains to be fully documented. IL-10, in particular, is abundant in atherosclerotic plaques and therefore most likely exerts a physiological dampening effect on MMP activity. TGF $\beta$  inhibits MMP-12 production in human monocytes (Supplementary Table 1). However, TGF $\beta$  can both stimulate and inhibit MMP-2 and MMP-9 secretion from mouse peritoneal macrophages (Ogawa *et al.*, 2011). Upregulation of MMP-9 by TGF $\beta$  has been recently ascribed to stimulation of PI3K leading to activation of AP-1 transcription factors (Haidar *et al.*, 2015). Activation of several anti-inflammatory nuclear hormone receptors inhibits MMP production (see Supplementary Tables 1 and 2). For example, PPAR $\alpha$  selectively inhibits IL-1 $\beta$  induced MMP-12 production by direct binding to components of the AP-1 complex (Souissi *et al.*, 2008), whereas both PPAR $\alpha$  and PPAR $\gamma$  inhibit MMP-9 secretion from human macrophages (Supplementary Table 2). PPAR $\gamma$  agonists protect against the macrovascular complications of diabetes (Dormandy *et al.*, 2005) and inhibition of MMP activity could play an important part in this action. Statins, the mainstay of atherosclerosis prevention, have also been

shown to inhibit the expression of a broad range of MMPs by both transcriptional and post-transcriptional mechanisms (reviewed in (Newby, 2008)).

## Conclusions: the combined action of multiple mediators causes MMP up-regulation and plaque rupture

Animal and human data supports the concept that an excess of MMP over TIMP production from macrophages and foam cells contributes to atherosclerotic plaque growth and rupture. In rabbit and mouse models, several MMPs promote plaque progression and affect plaque morphology in ways consistent with greater vulnerability to rupture. Furthermore, foam cell macrophages in subcutaneous granulomas or atherosclerotic plaques actively express several MMPs that are also secreted by non-foamy macrophages. Adaptive immunity seems to have little impact on macrophage polarization and increasing levels of MMPs in mice, implying a more prominent role for innate immune mechanisms, including the production of CSFs, inflammatory cytokines and toll-like receptor ligands. Even so MMP activity must be tightly regulated because overexpression of high levels of fully activated MMPs is needed to provoke plaque disruption in mice. The importance of specific MMPs may be over or under emphasized in mice, where they are more or less abundant, compared to man (see Fig. 1). Hence studies in human cells and tissues should be given primary importance, especially if genetic approaches at a population level (such as that for MMP-12) can be developed to give clearer indications of causality.

Longitudinal imaging studies lead to the striking conclusion that most vulnerable plaques go on to heal rather than rupture (Van Mieghem *et al.*, 2006). Hence ulceration of human plaques is a relatively rare outcome that, just like any other accident, most probably occurs because of an unusual combination of adverse circumstances. Plaque rupture most likely results from a 'perfect storm' caused by the synergistic local effects of multiple inflammatory mediators acting together in a hypoxic environment combined with the loss of inhibitory signals from nuclear hormone receptors, TGF $\beta$  and IL-10. The potential mediators of MMP overproduction include IL-1 $\beta$ , which can be produced in plaques in response to oxidised LDL (Williams & Tabas, 1995) and as a result of inflammasome activation by cholesterol crystals (Dewell *et al.*, 2010; Rajamaki *et al.*, 2010). The ongoing CANTOS clinical trial will examine the causal role of IL-1 in unstable coronary disease (Dinarello *et al.*, 2012). Other pro-inflammatory mediators, including TNF $\alpha$ , GM-CSF and IL-6, which stimulate macrophages through different signalling pathways (Fig. 2) have the potential to induce MMPs synergistically. Suppression of TIMP-3 expression by foam cell formation, INF $\gamma$  or GM-CSF could be a further significant factor. Toll-like receptor ligands, the most effective stimulators of MMP production *in vitro*, are also present in the atherosclerotic plaques (Lundberg & Yan, 2011). Conversely, anti-inflammatory treatments including, importantly, the use of statins currently provide the best approach to reducing MMP activity in plaques and therefore preventing plaque rupture. In future it is likely that more selective treatments will be developed. These should be aimed at inhibiting excess production of specific MMPs, especially the collagenases MMP-1 (Libby, 2013) and MMP-8 (Ye, 2015) and MMP-12 (Traylor *et al.*, 2014), whilst preserving the activity of those MMPs, including MMP-9, that are primarily involved in vascular repair (Newby, 2005). The widely different regulation of different MMPs in human macrophages that recent studies have so clearly emphasized (Huang *et al.*, 2012) provide strong encouragement for such an approach.

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## Acknowledgements

The author's work is supported by the British Heart Foundation grant CH95001 and the National Institute for Health Research Bristol Cardiovascular Biomedical Research Unit.

## Legends to Figures

Figure 1 Comparison of MMP and TIMP expression in human and mouse macrophages

Human (Huang *et al.*, 2012) and mouse (Tsaousi *et al.*, 2016) blood monocytes were differentiated to macrophages for 10-14 days in the presence of M-CSF. Total RNA was extracted and mRNA levels were measured by quantitative reverse transcription polymerase chain reaction using standards to derive copy numbers of transcripts per ng RNA. Differences greater than 100 fold are noted with arrows.

Figure 2 Simplified pathways of MMP and TIMP induction.

Binding of ligands to integrins (INT), toll-like receptors (TLR) and receptors for interferons (IFN), IL-1 (IL1R), TNF (TNFR), PGE2 (EP4), IL-6 (GP130), GM-CSF (CSFR2) provide the initial signals. These interact with signal transduction pathways (shown in outline only). An integrative network activates the phosphoinositide-3 kinase (PI3K), extracellular related kinases 1/2 (ERKs), p38 MAP kinase and c-jun N-terminal kinase (JNK), as well as the inhibitor of  $\kappa$ B kinase2 (IKK2). These lead together to activation of the activator protein-1 (AP-1) and nuclear factor- $\kappa$ B (NF- $\kappa$ B) transcription factors that directly induce several MMPs and TIMP-1. Insulin response factors (IRFs) are also induced by this pathway and also through activation of janus kinase 2 (JAK2), which can cause release of IFN $\alpha$ , $\beta$ , leading to autocrine actions. Other autocrine pathways are triggered by the production of arachidonic acid (AA) from the action of phospholipaseA2 (PLA2). This is converted to prostaglandin E2 (PGE2) by the consecutive action of cyclooxygenase-2 (COX-2) and prostaglandin E synthetase-1 (PGES1). Autocrine action on EP4 receptors triggers cAMP production and activation of the cAMP response element binding protein (CREB) transcription factor, which induces MMPs further. Activation of janus kinases JAKs at the GP130 and CSFR2 receptors leads to nuclear translocation of signal transducer and activator of transcription-3 (STAT-3) and STAT-5, respectively. Production of TNF $\alpha$  through these pathways provides additional possibilities for autocrine feedback.



