Investigation of the Production, Distribution, and Trafficking of MMP-9 in Classically Activated Macrophages

by

Raed Hanania

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Cell and Systems Biology
University of Toronto

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Abstract

As major effector cells of the innate immune response, macrophages must adeptly migrate from blood to infected tissues. Endothelial transmigration is accomplished by matrix metalloproteinase (MMP)-induced degradation of basement membrane and extracellular matrix components. The classical activation of macrophages with LPS and IFN-γ causes enhanced microtubule stabilization and secretion of MMPs. Macrophages upregulate MMP-9 expression and secretion upon immunological challenge, and require its activity for migration during inflammatory response. However, the dynamics of MMP-9 production and intracellular distribution, as well as the mechanisms responsible for its trafficking, are unknown. Using immunofluorescent imaging, we localized intracellular MMP-9 to small Golgi-derived cytoplasmic vesicles that contain calreticulin and PDI, in activated macrophages. Vesicular organelles of MMP-9 aligned along stable subsets of microtubules and colocalized with the anterograde molecular motor protein, kinesin. We demonstrated a functional contribution of stable MTs in the enhanced trafficking of MMP-9 extracellularly, and showed that heterogeneity exists in macrophage cell populations with respect to MMP-9 production.
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<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>BFA</td>
<td>Brefeldin A</td>
</tr>
<tr>
<td>BM</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>BMDM</td>
<td>Bone marrow-derived macrophage</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation (ie CD14)</td>
</tr>
<tr>
<td>CLIP-170</td>
<td>Cytoplasmic linker protein 170</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase 2</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′-6-Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EP4</td>
<td>Prostaglandin E receptor</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK 1/2</td>
<td>Extracellular signal-regulated kinase 1/2</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>Fn</td>
<td>Fibronectin</td>
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<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
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<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HDAC6</td>
<td>Histone deacetylase 6</td>
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</table>
HSC     Hematopoietic stem cell
ICAM-1  Inter-cellular adhesion molecule 1
IFN-γ   Interferon-γ
IL      Interleukin
iNOS    Inducible nitric oxide synthetase
LAMP-1  Lysosome-associated membrane protein-1
LC3     Light chain 3
LPS     Lipopolysaccharide
Mac-1   Macrophage-1 antigen
MAP     Microtubule-associated protein
MAPK    Mitogen-activated protein kinase
MHCII   Major histocompatibility class II complex
MMP     Matrix metalloproteinase
MR      Mannose receptor
MT      Microtubule
MTOC    Microtubule organizing centre
PAMP    Pathogen-associated molecular pattern
PBS     Phosphate-buffered saline
PDI     Protein disulfide isomerase
PFA     Paraformaldehyde
PGE₂    Prostaglandin E₂
PKC     Protein kinase C
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>PM</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translational modification</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline/Tween 20</td>
</tr>
<tr>
<td>TIMPs</td>
<td>Tissue inhibitors of metalloproteinases</td>
</tr>
<tr>
<td>TIRF</td>
<td>Total internal reflection</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
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1 Introduction

1.1 Host Defense against Infection

To successfully infect a host, pathogenic microorganisms must first surpass many physical and chemical barriers. For example, the skin provides an effective mechanical barrier to invasion, and the enzymes and mucus of the eye, respiratory tract, and gastrointestinal tract provide potent chemical barriers that are directly antimicrobial. Hosts rely on their immune system for protection against the adverse effects of pathogens that successfully breach these barriers. The immune system constitutes a network of widely disseminated cells, tissues, and organs that recognize foreign substances and act to eliminate them (Prescott et al., 2005). To defend against invading microorganisms, the immune system takes action by utilizing two different types of responses. The innate immune response is non-specific and provides the first line of defense against any microorganism or foreign material encountered by the host (Tsirogianni et al., 2006; Kemper and Atkinson, 2007). If the innate immune system is unsuccessful in terminating the pathogen, it will activate the adaptive immune response. The adaptive immune system is antigen-specific and can recognize and ‘remember’ particular foreign agents (Iriti and Faoro, 2007).

1.1.1 Innate Immunity

Activation of the innate immune system is commenced by the recognition of pathogen-associated molecular patterns (PAMPs), a limited number of molecular sequences that are common to entire classes of pathogens (Carroll and Prodeus, 1998; Iwasaki and Medzhitov, 2004). PAMPs, such as lipopolysaccharides (LPS), flagellin, double stranded RNA and methylated DNA, are highly conserved due to their importance to pathogen survival (Akira et al., 2006).
These PAMPs are recognized by a small set of germline-encoded pattern recognition receptors (PRRs) found on the surface of host cells involved in immunity (Medzhitov and Janeway, 1997; Akira et al., 2006). There are many types of PRRs, and generally, these can be classified into one of three categories: secreted, endocytic, or signaling (Medzhitov and Janeway, 2000). Secreted PRRs include the mannose-binding lectin, a complement-activating serum protein that binds to the mannose and acetylglucosamine sugar groups found specifically on microbial surfaces (Turner and Hamvas, 2000). The mannose receptors (MRs) found on the surface of antigen-presenting cells (APCs), such as macrophages and dendritic cells (DCs), provide a well-characterized example of endocytic PRRs. Since mannose sugars are not expressed on the surface of host cells, MRs can easily identify pathogens by detecting the mannose sugars present on their surface (Blasi et al., 2005). Ligation of pathogen mannose sugars with host MRs promotes pathogen internalization and engulfment into lysosomes (Fraser et al., 1998).

Signaling PRRs are also found on the surface of APCs, and are capable of detecting multiple PAMPs (Takeda et al., 2003). Toll-like receptor (TLR) stimulation by PAMPs results in a cascade of signaling events that instigate an inflammatory response.

1.1.2 Inflammatory Response

Inflammation aims to coordinate the delivery of blood components, such as leukocytes, to the site of infection, a process that involves a complex network of chemical signals and cell interactions (Medzhitov, 2008; Ono, 2008). TLR recognition of PAMPs is initially carried out by tissue resident macrophage cells. This induces the expression of a variety of inflammatory mediators, including chemokines and cytokines, which provide directional cues for the movement of blood-derived leukocytes to the site of pathogen detection (Bochner, 2000; Dean
et al., 2008). Sensing the inflammatory signals, the endothelial lining of blood vessel walls becomes activated and expresses selectin on its surface. Selectin binds to leukocyte integrins and chemokine receptors to allow the specific extravasation of leukocytes and not erythrocytes (Medzhitov, 2008). Extravasation from blood into tissues involves the tethering, rolling, and adhesion of leukocytes to the endothelium, which is followed by intravascular crawling and transmigration through the sub-endothelial basement membrane and connective tissues (Wong et al., 2010). The initial steps of tethering and rolling are mediated by selectins, while adhesion is mediated by the use of integrins, and the final steps of crawling and transmigration are dependent on macrophage-1 antigen (Mac-1) and inter-cellular adhesion molecule 1 (ICAM-1) to seek desired sites of penetration (Ley et al., 2007). Neutrophils make up the first wave of cells to arrive at the site of inflammation, which attempt to clear the microbes by phagocytosis and by releasing the toxic contents of their granules, such as reactive oxygen species (ROS) (Tsirogianni et al., 2006). If the infection persists, circulating monocytes then enter the afflicted area and mature into macrophages and DCs that function to engulf the microbes (Tsirogianni et al., 2006; Lee and Kim, 2007). Macrophages are the focus of this study and will be discussed later. Internalized microorganisms are ultimately degraded by the APCs, and may be used to activate the adaptive immune response (Steinman and Hemmi, 2006).

1.1.3 Adaptive Immunity

Antigen-protein fragments of internalized pathogens are bound to major histocompatibility class II complexes (MHCII) in the APCs, and are shuttled to the cell surface for display to naïve T lymphocytes (Bryant and Ploegh, 2004). In response, the T cells are triggered to expand clonally and to activate naïve B cells to differentiate into antigen-specific antibody secreting
plasma cells (Horowitz et al., 1996; Pozzi et al., 2005; Boscardin et al., 2006). The antibodies function to coat the invading pathogen by binding to the appropriate surface antigen. Coated or opsonized pathogens are more easily identified by macrophages and are more readily phagocytosed (Blander, 2007).

1.2 Macrophages

Macrophages are major effector cells of the innate immune response that make up the predominant cell type in the inflammatory site after the neutrophils are spent (Ross and Pawlina, 2006). These mononuclear phagocytic leukocytes originate from hematopoietic stem cells (HSCs), which reside in the bone marrow. HSCs give rise to populations of daughter cells, one of which are the myeloid progenitors, which differentiate into monocytes upon entering the bloodstream (van Furth, 1980; Gordon et al., 1986). Monocytes transiently circulate the bloodstream and differentiate further upon dispersion to peripheral tissues, giving rise to tissue resident macrophages (Kumar and Jack, 2006).

There are many types of tissue resident macrophages and are given different names depending on their location. The type of macrophage produced is determined by the chemical and physical nature of the tissue matrix (Sudhakaran et al., 2007). Regardless of their location, the tissue resident macrophages are either stationary or constantly migrating throughout the tissue and function to detect and clear any particles or microorganisms (van Furth et al., 1972; Mantovani et al., 2004). The newly differentiated macrophages are initially in a resting state and display minimal oxygen consumption, protein synthesis, and cytokine production rates (Martinez et al., 2006). Once established in the tissues, macrophages may become activated from exposure to various signals, such as in cases of pathogen invasion, resulting in morphological, metabolic,
and physiological changes (Adams and Hamilton, 1984). Activated macrophages can more effectively recognize and destroy pathogens, present degraded antigen fragments to T cells, and produce a myriad of cytokines for the recruitment of additional leukocytes (Sweet and Hume, 1996; Gordon, 2003; Mantovani et al., 2004).

1.2.1 Macrophage Activation

Macrophage activation involves complex levels of signal transduction. Activation is possible through multiple pathways and can result in different structural and functional phenotypes depending on the immunological challenge (Van Ginderachter et al., 2006). Generally, macrophages can be either “alternatively” or “classically” activated. Alternative activation is accomplished by exposure to interleukin (IL)-4 or IL-13 and results in macrophages that are characterized by the secretion of low levels of IL-12 and IL-23, and high levels of IL-10. Although the main sources of IL-4 and IL-13 are not well defined, these macrophages are stimulated to play roles in tissue remodeling and angiogenesis or in tumor promotion (Gordon, 2003; Mantovani et al., 2007). Macrophage classical activation is induced by exposure to interferon-γ (IFN-γ) either alone or in conjunction with pathogenic stimuli, such as LPS (Mantovani et al., 2007). Classically activated macrophages are characterized by the secretion of high levels of IL-12 and IL-23, and low levels of IL-10, and function in the killing of intracellular pathogens and in tumor resistance (Goerdt et al., 1999; Van Ginderachter et al., 2006).

IFN-γ is a pro-inflammatory cytokine that is produced by the T cells and natural killer cells of the host in response to pathogens (Gordon, 2003). Binding of macrophage IFN-γ receptors with IFN-γ initiates signaling that enhances macrophage microbicidal activities such as pathogen recognition and antigen processing (Randal and Kossiakoff, 2001; Schroder et al., 2004).
Further, exposure of resting macrophage cells to IFN-γ enhances their sensitivity to activation by PAMPs such as LPS, and augments the magnitude and maintenance of the PAMP-induced inflammatory response (Gifford and Lohmann-Matthes, 1987; Hayes et al., 1991).

LPS is an essential component of the outer membrane of Gram-negative bacteria that provides an effective permeability barrier towards external stresses (Alexander and Rietschel, 2001). LPS is composed of three regions; the O side chain, the core polysaccharide, and the lipid A moiety. The lipid A region is often toxic and represents the major immunostimulatory component of LPS (Meng and Lowell, 1997; Alexander and Rietschel, 2001). Membrane-bound cluster of differentiation (CD)14 is the major cell surface binding protein for LPS on macrophages and acts to present LPS to the signal propagating TLR4 (Wright et al., 1990).

The synergistic effects of IFN-γ priming and LPS ligation on TLR4 cause the full classical activation of macrophage microbicidal abilities. Classically activated macrophages demonstrate increased cell spreading, enhanced phagocytosis and antigen presentation capacities, and amplified inducible nitric oxide synthetase (iNOS) expression for the production of nitric oxide (Wirth et al., 1985; Higginbotham et al., 1992; Gordon, 2003; Webster and Crowe, 2006; Binker et al., 2007b). Additionally, classical activation results in the enhanced production and secretion of matrix metalloproteinases (MMPs) to the cell exterior (Webster and Crowe, 2006), as well as increased levels of stable cytoplasmic microtubules (MTs) (Binker et al., 2007b; Khandani et al., 2007; Patel et al., 2009).
1.3 Microtubules

The eukaryotic cytoskeleton is made up of intermediate filaments, actin filaments, and MTs (Schliwa and van Blerkom, 1981). The MTs are rigid hollow rods approximately 25 nm in diameter that play important roles in basic cell functions such as mitosis, intracellular trafficking, and establishment of cell shape (Cooper and Hausman, 2007). MTs are equally important in specialized cell functions, such as migration, phagocytosis of pathogens, and antigen presentation (Allen and Aderem, 1996; Nogales, 2000; Billadeau et al., 2007; Khandani et al., 2007).

1.3.1 Microtubule Structure

Each MT rod is formed by the polymerization of α- and β-tubulin dimers into 13 linear protofilaments that are assembled around a hollow core. MTs are polar structures due to the isoform-alternating fashion of the tubulin heterodimers, with a fast-growing plus end and a slow growing minus end (Allen and Borisy, 1974; Amos and Klug, 1974; Nogales, 2000). MT growth is initiated at the centrosome, which functions as a MT organizing centre (MTOC), by γ-tubulin subunits that act as a scaffold for polymerization (Moritz et al., 1995). With minus ends oriented towards the MTOC, MT growth occurs outward at the plus ends towards the cell periphery (Nogales, 2000).

1.3.2 Microtubule Dynamics

Both α- and β-tubulin subunits bind guanosine triphosphate (GTP); however the GTP bound to β-tubulin is exchangeable and regulates polymerization (MacNeal and Purich, 1978). The β-tubulin GTP is hydrolyzed to guanosine diphosphate (GDP) during or shortly after polymerization and favors depolymerization due to the low binding affinity of tubulin for
adjacent molecules. MTs are dynamic as a result of GDP-bound tubulin subunits being continually lost and replaced by GTP-bound tubulin subunits (Mitchison and Kirschner, 1984; Erickson and O'Brien, 1992). Depending on the rate of GTP hydrolysis and addition of new tubulin, the MT cytoskeleton alternates between phases of elongation towards, and shrinkage away, from the cell periphery at the plus ends, as depicted in Figure 1. This process is termed “dynamic instability” (Mitchison, 1995; Desai and Mitchison, 1997). In instances when GTP-bound tubulin is added more rapidly than GTP is hydrolyzed, a GTP cap is retained at the plus end and the MT grows. However, in cases where GTP hydrolysis is greater than tubulin addition, the plus end GTP cap is hydrolyzed to GDP and the MT shrinks (Nogales, 2000). Dynamic instability is essential for MT functions, such as the remodeling necessary for mitosis, or the rapid intracellular changes necessary for many cellular processes to occur (Nogales, 2000).

MT dynamics can be influenced by the binding of MT-associated proteins (MAPs) to MT subunits (Walczak, 2000). MAPs serve several functions, including the provision of structural support to enhance MT stabilization as well as the severing of protofilaments, guiding of MT strands, and mediating interactions with other proteins in the cell (Maiato et al., 2004). MTs with bound stabilizing MAPs, such as MAP4 (Nguyen et al., 1997), show half-lives in the order of hours, compared with minutes observed in highly dynamic MTs (Schulze et al., 1987). As a result of their longevity, stable MTs may accumulate post-translational modifications (PTMs) such as the acetylation of α-tubulin on its lysine 40 residue (Piperno et al., 1987), as depicted in Figure 2. Therefore, subsets of stabilized MTs can be identified by the presence of acetylated-α-tubulin subunits, and are biochemically distinct from their dynamic counterparts (Piperno et al., 1987; Schulze et al., 1987; Bulinski and Gundersen, 1991). Although PTMs do not change the
**Figure 1. Schematic of MT growth and shrinkage.** The MT cytoskeleton is composed of α- and β-tubulin subunits. Each MT rod has a minus end anchored at the MT organizing centre, and a plus end oriented to the cell periphery. MTs are characterized by dynamic instability, in which the plus ends are constantly alternating between distinct phases of either shrinkage or elongation.
Figure 2. Schematic of MT posttranslational modification after stabilization. MTs bound with stabilizing MAPs demonstrate reduced dynamic instability and show longer half lives. As a result of their longevity, these MTs become posttranslationally modified by acetylation on the lysine 40 residues of α-tubulin subunits. Stabilized MTs can thus be identified by acetylation of their α-tubulin subunits.
intrinsic properties of MTs, acetylation has been shown to enhance the binding of plus end-directed anterograde MT motors (Reed et al., 2006).

1.4 Matrix Metalloproteinases

In order to carry out their function, it is important for macrophages to be able to migrate from blood, where they circulate as monocytes, to target tissues. As depicted in Figure 3, following endothelial transmigration, they must transverse the sub-endothelial basement membrane and the interstitial matrix composed mainly of collagen. This is accomplished by MMP-induced cleavage of cell surface molecules, such as L-selectin (Galon et al., 1998), and mainly by the degradation of basement membrane and extracellular matrix (ECM) components (Webster and Crowe, 2006).

MMPs comprise a family of 25 zinc-dependent endopeptidases that are members of the metzincin superfamily of proteases (Parks et al., 2004; Page-McCaw et al., 2007; Tomlinson et al., 2008). MMPs may be either secreted or presented on the extracellular surface, where they participate in ECM degradation, cell migration, and the processing of soluble factors (Sternlicht and Werb, 2001). Functionally, MMPs can be subdivided into three groups. The interstitial collagenases, such as MMP-13, show specificity for collagen type I, II, and III (Webster and Crowe, 2006). The stromelysins, such as MMP-3, preferentially cleave laminin (Webster and Crowe, 2006). Finally, the gelatinases, MMP-2 and MMP-9, process collagen type IV, a major component of the basal lamina (Timpl, 1989; Odaka et al., 2005).
Figure 3. Schematic of macrophage migration across blood vessel walls during an inflammatory response. In response to various inflammatory cytokines and chemokines, macrophages migrate from blood, where they circulate as monocytes, to target tissues during an inflammatory response. Transmigration through the endothelial basement membrane, composed mainly of laminin, and the sub-endothelial interstitial matrix, composed mainly of collagen, is largely accomplished by MMP-induced cleavage of these ECM components.
1.4.1 Matrix Metalloproteinase-9

Of particular importance to cell migration is MMP-9, also known as gelatinase B and 92-kDa type IV collagenase, due to its ability to degrade ECM components such as collagens and elastins (Min et al., 2002). Blocking of MMP-9 by monoclonal antibodies has been shown to inhibit the migration of bone marrow stem cells into the circulation (Pruijt et al., 1999), and MMP-9 has been shown to assist in monocyte migration (Watanabe et al., 1993; Rahat et al., 2006). Additionally, a role for MMP-9 in macrophage migration during inflammatory response was recently demonstrated in vivo, where inhibition of MMP-9 activation resulted in the abolished ability of macrophages to migrate to the elicited site of inflammation (Gong et al., 2008).

1.4.2 Structure of MMP-9

Although MMPs differ in the specificity of their substrates, they possess the same basic structure (Page-McCaw et al., 2007). Figure 4 demonstrates the structure of MMP-9. Starting from the amino-terminus, MMPs contain a signal peptide that directs them to the endoplasmic reticulum (ER), and is cleaved once the MMP arrives at the ER (Webster and Crowe, 2006). Next is the propeptide domain, a short stretch of 10 amino acids, which folds over the catalytic domain and acts to maintain enzyme latency. The propeptide is cleaved during MMP activation (discussed later) (Sternlicht and Werb, 2001). The active site of the catalytic domain contains a conserved zinc binding region common to all MMPs. However, the cleft at the active site varies between MMPs, offering specificity towards different substrates (Webster and Crowe, 2006). For example, the catalytic domain of MMP-9 contains three head-to-tail cysteine-rich repeats that are similar to the collagen-binding type II repeats of fibronectin (Fn). These Fn-like repeats
Figure 4. Structure of MMP-9. MMP-9 is produced with a signal peptide on its amino-terminus, which is cleaved upon arrival to the ER. MMP-9 contains a propeptide domain, that when attached, maintains enzyme latency by folding over the catalytic domain and interacting with the zinc ion that is responsible for MMP-9’s enzymatic activity. In the catalytic domain, MMP-9 contains fibronectin (Fn)-like repeats that mediate its selectivity for collagen and elastin. Additionally, the homopexin-like domain on the carboxy terminus, which is attached to the catalytic domain via a hinge region, also mediates substrate specificity.
are necessary for the binding and cleavage of collagen and elastin by MMP-9 (Murphy et al., 1994; Shipley et al., 1996). Most MMPs, including MMP-9, contain a carboxy-terminal homopexin-like domain that is attached to the catalytic domain through a hinge region. The homopexin domain and hinge region provide further specificity, and influence the binding of certain substrates (Alexander and Elrod, 2002).

1.4.3 Regulation of MMP-9 Expression

MMP-9 expression is typically absent or minimal in normal tissues, and is greatly enhanced during inflammation and wound healing (Opdenakker et al., 2001b). Macrophages do not constitutively express MMP-9; rather, LPS exposure has been shown to induce MMP-9 gene expression as well as increase the amount of biologically active MMP-9 in the cell culture supernatant (Rhee et al., 2007). MMP-9 expression in response to LPS is regulated by a number of different signaling pathways. The promoter of the MMP-9 gene contains binding sites for the transcription factors NF-κB, Sp1, and AP-1 (Van den Steen et al., 2002).

The NF-κB and Sp1 binding sites on the MMP-9 promoter have been implicated in activating MMP-9 gene expression in response to phorbol 12-myristate 13-acetate (PMA) and tumor necrosis factor-α (TNF-α) (Van den Steen et al., 2002). However, NF-κB activation has been proven particularly important in LPS-induced MMP-9 gene expression in macrophages (Rhee et al., 2007). LPS-induced increase of MMP-9 is mediated by TLR4 signaling (Mendes Sdos et al., 2009) and has been reported to occur via a Mitogen-activated protein kinase (MAPK) pathway, particularly involving extracellular signal-regulated kinase (ERK) 1/2 and p38, that activates NF-κB (Lai et al., 2003; Mendes Sdos et al., 2009).
The MAPK pathway, involving ERK 1/2, may be activated by Protein kinase C (PKC) in response to LPS, and has been reported to induce the expression of cyclooxygenase (COX)-2. The metabolism of arachidonic acid by COX-2 mediates its conversion to prostaglandin E2 (PGE2), and the subsequent binding of PGE2 to the prostaglandin E receptor (EP4) has been shown to cause cyclic adenosine monophosphate (cAMP) signaling that stimulates MMP-9 gene expression (Pavlovic et al., 2006; Steenport et al., 2009). Alternatively, the MAPK pathway, involving p38, may be activated by mitochondrial derived ROS, which stimulates AP-1 to induce MMP-9 gene expression (Woo et al., 2004).

1.4.4 Regulation of MMP-9 Activity: Zymogen Activation

While MMP activity is strongly regulated at the transcriptional level, MMP function is managed by recruitment of MMPs to the cell surface, where they are secreted as inactive zymogens (pro-MMPs) (Fridman et al., 2003). The propeptide domain of pro-MMPs contains a conserved cysteine residue whose sulfhydryl group interacts with the zinc ion of the active site. This interaction preoccupies the active site, making it unavailable for other MMP substrates (Sang et al., 1995). Activation of MMPs is known as the “cysteine switch,” in which the cysteine-zinc interaction is abrogated by removal of the propeptide domain (Van Wart and Birkedal-Hansen, 1990). Pro-MMP-9 activation is achieved by the action of extracellular proteases such as plasmin (Gong et al., 2008), other MMPs such as MMP-3 (Ogata et al., 1992), MMP-7 (von Bredow et al., 1998), and MMP-13 (Knauper et al., 1997), and LPS-associated serine proteinases (Min et al., 2002). Upon activation, MMP-9 is converted from a 92 kDa zymogen form to an 82 kDa active form (Fridman et al., 2003).
1.4.5 Regulation of MMP-9 Activity: Endogenous Inhibitors

Further regulation of MMP activity is offered by their natural inhibitors in vivo, the Tissue Inhibitors of Metalloproteinases (TIMPs). Vertebrates posses four known TIMPs (TIMP-1, -2, -3, and -4), that are capable of specifically inhibiting MMP activity in a 1:1 stoichiometric ratio (Visse and Nagase, 2003). Regulation of MMP activity by TIMPs is necessary to avoid excessive proteolysis and tissue damage (Lambert et al., 2004). All four TIMPs are capable of inhibiting all activated MMPs, however, with varying affinities (Baker et al., 2002; Van den Steen et al., 2002). Inhibition by TIMPs is accomplished by the non-covalent binding of the TIMP N-terminal domain with the MMP active zinc-binding site (Gardner and Ghorpade, 2003). TIMP-1 binds with high affinity to MMP-9, and interestingly, has been shown to also bind with pro-MMP-9 (Lambert et al., 2004). Binding of TIMP-1 with pro-MMP-9 has been shown to prevent its activation by MMP-3, suggesting an extra level of regulation. Activation of pro-MMP-9 is only possible when the concentration of MMP-3 exceeds that of TIMP-1, in which case TIMP-1 is displaced by MMP-3 (Ogata et al., 1995; Massova et al., 1998; Van den Steen et al., 2002).

1.5 Rationale and Objectives

Classical activation of macrophages is characterized by extensive vesicle trafficking and secretion events. Particularly, MMP-9 secretion is greatly enhanced upon activation (Rhee et al., 2007), and has been directly implicated in mediating macrophage migration during the inflammatory response (Gong et al., 2008). The mechanisms involved in regulating MMP-9 expression, as well as its extracellular activation and inhibition, have been extensively characterized (Van den Steen et al., 2002). Despite this, the dynamics of MMP-9 production and intracellular distribution after its expression, as well as the mechanisms involved in its
trafficking before its secretion, remain unclear. In the present work, we aim to characterize the intracellular sorting of MMP-9 in activated macrophage cells, as well as the mechanism by which it is secreted.

We used gelatin zymography and Western blotting analysis to monitor the changes in MMP-9 levels after activation and experimental manipulation. We also used indirect immunofluorescence to visualize endogenous MMP-9 distribution in activated cells, and to characterize the nature of its cellular compartments. This approach also allowed a direct comparison of MMP-9 trafficking in macrophages versus neutrophils. Using these techniques, we also investigated the maturation of MMP-9 through the biosynthetic pathway by using BFA to inhibit ER-to-Golgi transport. Previous work in our laboratory has characterized extensive MT stabilization during macrophage activation (Binker et al., 2007b; Khandani et al., 2007; Patel et al., 2009), and demonstrated that MT stabilization is pertinent to increased cell spreading and phagocytic capabilities (Binker et al., 2007b). We hypothesize that stabilized MTs also function as highways to mediate the extensive targeting of MMP-9 to the cell surface in activated macrophages. To determine a functional role of stable MTs in MMP-9 secretion, we monitored the levels of intracellular and extracellular MMP-9 when MT stabilization was dramatically enhanced using taxol or low-dose nocodazole, using immunofluorescence and Western blotting.
2 Materials and Methods

2.1 Reagents and Antibodies

Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from Wisent Inc. (St-Bruno, Quebec). Lipopolysaccharide from Salmonella enterica serotype Typhimurium, interferon-γ, nocodazole, and taxol were from Sigma-Aldrich Inc. (St. Louis, MO). Brefeldin A (BFA) from Penicillium brefeldianum, Alexa Fluor® phalloidin, and 4’-6-Diamidino-2-phenylindole (DAPI) were obtained from Invitrogen Canada Inc. (Burlington, Ontario). FuGENE HD was purchased from Roche Diagnostics (Laval, Quebec). Antibodies were obtained as follows. Rabbit polyclonal anti-MMP-9, rat polyclonal anti-CD11b (Mac-1), and mouse monoclonal anti-PDI were from Abcam Inc. (Cambridge, MA). Mouse monoclonal anti-α-tubulin and anti-acetylated tubulin were obtained from Sigma-Aldrich Inc. Mouse monoclonal anti-MAP4 and anti-GM130 were from BD Transduction Laboratories™ (San Jose, CA). Mouse monoclonal (SUK4) anti-kinesin and rat polyclonal (ID4B) anti-lysosome-associated membrane protein-1 (LAMP-1) were purchased from Developmental Studies Hybridoma Bank (Iowa, IA). Mouse monoclonal anti-ubiquitinylated proteins was from Enzo® Life Sciences (Plymouth, PA), and goat polyclonal anti-NFκB was from Santa Cruz Biotchenology Inc. (Santa Cruz, CA). Mouse monoclonal anti-KDEL was from Stressgen® (Ann Arbor, MI). Goat polyclonal anti-calreticulin was a gift from Dr. Michal Opas (University of Toronto, Toronto, ON). Cy™2-, Cy™3- and Cy™5-conjugated AffiniPure donkey anti-rat, -goat, -rabbit and -mouse IgG, and peroxidase-conjugated AffiniPure donkey anti-rabbit and -mouse IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA).
2.2 DNA Constructs and preparation

DNA constructs were gifts obtained as follows. CD63-GFP and mCherry vector were from Dr. Sergio Grinstein (The Hospital for Sick Children, Toronto, ON, Canada). Rab27a-CFP was from Dr. John Brumell (The Hospital for Sick Children, Toronto, ON, Canada). LC3-RFP was from Dr. Nicola Jones (The Hospital for Sick Children, Toronto, ON, Canada). Finally, TLR4-mCherry was from Dr. Luke O’Neill (Trinity College Dublin, Dublin, Ireland).

Plasmid DNA constructs were transformed into MAX Efficiency® DH5α™ Competent Cells (Invitrogen Canada Inc.) for propagation, and purified by EndoFree® Plasmid Maxi kit (QIAGEN Inc., Mississauga, Ontario), according to manufacturer’s instructions.

2.3 Cell Culture, mouse macrophage isolation, and transfection

The murine RAW264.7 macrophage cell line was obtained from the American Type Culture Collection (Manassas, VA) and maintained at 37 °C supplied with 5% CO2 in DMEM supplemented with 10% heat-inactivated FBS. For all experiments, RAW 264.7 cells were grown to 60-80% confluence in tissue culture 6-well plates (Starstedt Inc., Montreal, Canada), with or without 25 mm glass coverslips. Primary bone marrow-derived macrophages (BMDMs) were harvested from the femoral bones of C57BL/6 mice. Briefly, femoral bones were separated from surrounding tissues, and cut on both ends. BMDMs were obtained by femoral lavage with DMEM containing 10% heat-inactivated FBS, supplemented with penicillin and streptomycin (100 IU/ml and 100 μg/ml, respectively). Cells were grown in tissue culture 6-well plates at 37 °C with 5% CO2 for 5 days, to allow for differentiation into macrophages.
RAW 264.7 cells were transiently transfected using FuGENE HD (Roche Diagnostics) according to the manufacturer’s instructions. Briefly, for each 35 mm dish of a 6-well plate, 2 μg plasmid DNA and 3-6 μl FuGENE HD were diluted in 100 μl Opti-MEM® I Reduced Serum Media (Invitrogen Canada Inc.) for 25 minutes, added to cells of 70% confluence, and incubated overnight for plasmid expression.

2.4 Macrophage activation and pharmacological treatments

For classical activation of resting RAW 264.7 cells or differentiated murine BMDMs, the cells were washed twice with Phosphate-buffered saline (PBS) and cultured in FBS-free DMEM supplemented with 0.1 μg/ml LPS and 100 U/ml IFN-γ for 9 hrs, unless indicated otherwise. Inhibition of protein traffic from the ER to the Golgi apparatus was achieved by incubating resting RAW 264.7 cells with 5 μg/ml BFA in the presence of LPS/IFN-γ (0.1 μg/ml and 100 U/ml, respectively) for 9 hrs. For depolymerization of the MT cytoskeleton, RAW 264.7 cells were LPS/IFN-γ (0.1 μg/ml and 100 U/ml, respectively) activated for 6 hrs and incubated for another 3 hrs in the presence of 10 μM nocodazole. To increase MT stabilization by enhancing the rate of MT assembly and form depolymerization resistant MTs, RAW 264.7 cells were LPS/IFN-γ (0.1 μg/ml and 100 U/ml, respectively) activated for 8 hrs and incubated for another 1 hr in the presence of 0.1 μM taxol. Alternatively, to increase MT stabilization by suppressing MT dynamic instability and increasing the time MTs spend in a paused state, RAW 264.7 cells were LPS/IFN-γ (0.1 μg/ml and 100 U/ml, respectively) activated for 6 hrs and incubated for another 3 hrs in the presence of 0.1 μM nocodazole.
2.5 Gelatin zymography and densitometric analysis

Cell culture supernatants were collected and centrifuged at 12 000 x g for 10 minutes at 4 °C. Protein concentration was determined by Lowry Protein Assay (Bio-Rad Laboratories Ltd., Mississauga, Ontario), and samples were mixed in 20% 5x Laemmli sample buffer, lacking the reducing agent 2-mercaptoethanol, and incubated at RT for 15 minutes. Equal amount of protein was loaded and separated on 8% Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 0.1% Gelatin from bovine skin, type B (Sigma-Aldrich). After electrophoresis, gels were washed three times for 20 minutes in zymogram wash buffer (2.5% Triton X-100, 50 mM Tris-HCl, pH 7.5) to remove SDS, and incubated overnight at 37 °C in zymogram developing buffer (50 mM Tris-HCl, pH 7.5, 10 mM CaCl2, 5 μM ZnCl2, 150 mM NaCl). Gels were then stained with 0.25% Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories Ltd.) for 6 hrs, and destained twice for 30 minutes in 7% acetic acid, 40% methanol solution, and once for 1 hr in 0.7% acetic acid, 5% methanol solution. Gelatinolytic activity appeared as a clear band on a dark background. Gelatinolytic bands were measured by densitometry scanning of independent experiments using NIH Image J software (NIH, Bethesda, MD) to determine the relative fold increases of pixel intensities compared to resting cell culture supernatants.

2.6 Western blot and densitometric analysis

Cell culture supernatants were collected and centrifuged at 12 000 x g for 10 minutes at 4 °C. Cells were washed twice with cold PBS, and the total cell lysates were obtained by scraping cells in RIPA lysis buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.2, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 5mM EDTA) in the presence of protease and phosphatase inhibitor cocktails (Sigma-Aldrich Inc.), and centrifuged at 12 000 x g for 10 minutes at 4 °C.
concentration was determined by Lowry Protein Assay (Bio-Rad Laboratories Ltd., Mississauga, Canada), according to manufacturer’s instructions. Bovine serum albumin (BSA) (Sigma-Aldrich Inc.) was used at 0, 5, 10, 15, 20, and 25 μg/ml to construct standard protein concentration curves. Protein samples were mixed with 20% 5x Laemmli sample buffer and boiled for 10 minutes. Equal amount of protein was loaded and separated on 8% or 10% SDS-PAGE, and transferred to nitrocellulose membranes (Bio-Rad Laboratories Ltd.). Blots were blocked overnight at 4 °C in Tris-buffered saline/Tween 20 (TBST) containing 5% skim milk powder (BioShop Canada Inc., Burlington, Canada). The nitrocellulose membranes were exposed to the primary antibodies MMP-9 (1:1000), or acetylated-α-tubulin (1:1000), or α-tubulin (1:2000), in 1% skim milk/TBST for 1 hr at RT. After washing three times for 10 minutes in TBST, the blots were incubated with the relevant peroxidase-coupled secondary antibody (1:1000) in 1% skim milk/TBST for 1 hr at RT. Blots were washed three times for 10 minutes in TBST, and bound antibody was visualized on Bioflex® scientific imaging films (Clonex Corporation, Markham, Canada) by the Supersignal® West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL, USA), according the manufacturer’s instructions. Protein bands were measured by densitometry scanning of independent experiments using NIH Image J software to determine the relative fold increases of pixel intensities compared to resting or activated cell culture supernatants or lysates. Protein bands subjected to densitometric analysis were not saturated or over-exposed.

2.7 Immunostaining and fluorescent imaging

All washes were done three times with PBS, unless indicated otherwise. Cells were washed and fixed in 4% paraformaldehyde (PFA) (Canemco Inc., Lakefield, Quebec, Canada) in PBS for 20
minutes at RT. The cells were washed and permeabilized with 0.1% Triton X-100 in PBS containing 100 mM glycine for 20 minutes. For immunostaining of kinesin, cells were fixed in ice-cold 100% methanol for 10 minutes at -20 °C, and then washed 10 times with PBS. After fixation, cells were washed and blocked in 5% FBS/PBS for 1 hr at RT and incubated with the indicated primary antibodies in 1% FBS/PBS for 1 hr at RT using the following dilutions: MMP-9 (1:1000), acetylated-α-tubulin (1:5000), MAP4 (1:500), α-tubulin (1:1000), PDI (1:200), GM130 (1:200), LAMP-1 (1:4), ubiquitinated proteins (1:1000), NF-κB (1:100), KDEL (1:200), calreticulin (1:200), Mac-1 (1:1000), and kinesin (1:100). Excess primary antibody was washed off and the cells were incubated with the corresponding fluorochrome-conjugated secondary antibodies (1:1000) in 1% FBS/PBS for 1 hr at RT. Where indicated, secondary antibodies were incubated together with Alexa Fluor® phalloidin (1:500) to stain for the actin cytoskeleton. Cells were washed to remove excess secondary antibody and were mounted onto coverslips using Dako Fluorescent Mounting Medium (DakoCytomation, Carpinteria, CA). For nuclear staining, cells were washed twice with ddH2O and incubated for 10 minutes with DAPI before mounting.

For triple immunolabeling of MMP-9, acetylated-α-tubulin or MAP4, and α-tubulin, cells were PFA fixed, permeabilized, and FBS blocked as before. Briefly, the cells were incubated with primary antibodies to MMP-9 and acetylated-α-tubulin or MAP4, followed by incubation with the appropriate fluorochrome-conjugated secondary antibodies. The cells were then blocked in 5% FBS/PBS for 1 hr at RT and then incubated with primary antibody to α-tubulin, followed by incubation with the relevant fluorochrome-conjugated secondary antibody.

Cells were visualized under a 63x oil-immersion objective using an inverted Zeiss Axiovert 200M epifluorescent microscope using Axiovision software, or with an upright Zeiss LSM510 laser
scanning confocal microscope using the LSM510 Meta System (Zeiss, Thornwood, NY). Total internal reflection (TIRF) microscopy was used to visualize the basal cell area of cells where indicated, using an inverted Zeiss Axiovert 200M epifluorescent microscope equipped with an α-Plan-Fluor x 100/1.45 objective. Proteins were considered recruited to MMP-9 vesicles if they demonstrated the same punctate staining that exactly co-localized with MMP-9.

2.8 Quantification

To determine the average number of MMP-9 vesicles per activated macrophage cell, RAW 264.7 cells were activated (0.1 μg/ml LPS and 100 U/ml IFN-γ, for 9 hrs). The cells were fixed and immunostained for endogenous MMP-9, and imaged by epifluorescence. The number of discrete MMP-9 vesicles in each cell was counted for 100 macrophages and averaged.

For quantification of the total intracellular fluorescence intensity of MMP-9, RAW 264.7 cells were activated (0.1 μg/ml LPS and 100 U/ml IFN-γ, for 9 hrs) and treated with 10 μM nocodazole in the last 3 hrs, or left activated or resting. Cells were fixed and immunostained for MMP-9. Images were acquired using an inverted Zeiss Axiovert 200M epifluorescent microscope, using the same fluorescence intensity and exposure time between treatments and replicates. MMP-9 fluorescence intensity of 100 individual cells was measured using NIH Image J software, and the average relative fold increase of pixel intensities of nocodazole treated cells was calculated, compared with activated macrophage cells, and normalized for background fluorescence observed in resting cells.

To study changes in the number of MMP-9 containing cells after activation, RAW 264.7 cells were LPS/IFN-γ (0.1 μg/ml and 100 U/ml, respectively) activated for 3, 6, 9, or 12 hrs. The cells
were fixed and immunostained for endogenous MMP-9, and the number of cells containing at least one discrete MMP-9 vesicle was counted per 100 macrophages. Alternatively, RAW 264.7 cells were activated (0.1 μg/ml LPS and 100 U/ml IFN-γ, for 9 hrs) and treated with 10 μM nocodazole in the last 3 hrs, or left activated or resting. The average number of MMP-9 containing cells per 100 macrophages was tabulated.

To determine any effect of over-expressing the receptor TLR4 on the number of macrophage cells containing MMP-9, RAW 264.7 cells were transfected with TLR4-mCherry or mCherry vector as a control and activated (0.1 μg/ml LPS and 100 U/ml IFN-γ, for 9 hrs). The number of MMP-9-containing cells was tabulated per 50 transfected macrophages.

### 2.9 Statistical analysis

Experimental values are presented as mean ± SEM of the experimental triplicates. Microsoft Excel was used to perform Student’s t test evaluations of the data, with values of p < 0.05 considered significant.
3 Results

3.1 Characterization of MMP-9 Production and Distribution in Activated Macrophages

3.1.1 Analysis of the intracellular and extracellular levels of MMP-9 in classically activated RAW 264.7 cells

It has been reported that macrophages stimulated with LPS exhibit a time-dependent increase in MMP-9 expression and secretion (Rhee et al., 2007). However, the analysis of MMP-9 levels in LPS/IFN-γ activated RAW 264.7 cells remains poorly resolved. To begin, we examined the levels of MMP-9 released in the cell culture supernatant of serum-starved resting or LPS/IFN-γ (0.1 μg/ml and 100 U/ml, respectively) activated RAW 264.7 cells for 3, 6, 9, or 12 hrs, using gelatin zymography (Figure 5A, top) or Western blot analysis (Figure 5A, bottom). Macrophage activation was carried out under serum-free conditions to avoid non-specific stimulation by serum components, and because FBS was shown to partially inhibit LPS induced expression of MMP-9 (Lee et al., 2009). Densitometric scanning of zymograms and immunoblots indicated a steady increase of extracellular MMP-9 levels beginning 6 hrs or 9 hrs after activation, respectively (Figure 5B). It is important to notice that only the zymogen 92 kDa form of MMP-9 was detected. The absence of the active 82 kDa form in the cell culture supernatant may be a result of culture conditions, in which MMP-9 is diluted away from its activators in the media (Fridman et al., 2003). The detection of latent MMP-9 by gelatin zymography is possible by the artificial activation of MMP-9 on the zymogram, allowing proteolytic activity (Makowski and Ramsby, 1996). Although gelatin zymography is a common tool used to assess the levels of gelatinases, it is limited in its capacity to detect other cellular proteins (Makowski and Ramsby, 1996). Zymographic analysis was more sensitive than immunoblotting in detecting MMP-9 at an
earlier time of activation (Figure 5A, B); however, immunoblotting will be used for all subsequent analyses since it provides a more versatile venue for the detection of proteins other than MMP-9.

In order to study the intracellular nature of MMP-9 production and trafficking, it is important to determine a length of activation time that yields a substantial level of intracellular MMP-9. The total cell lysates of resting or LPS/IFN-γ (0.1 μg/ml and 100 U/ml, respectively) activated RAW 264.7 cells for 3, 6, 9, or 12 hrs were analyzed by Western blotting for MMP-9 and α-tubulin (Figure 5C). Probing for MMP-9 consistently revealed two bands of varying apparent molecular weights, one at 85 kDa, and another at 92 kDa. It was previously shown that the 85 kDa band represents an under-glycosylated precursor form of pro-MMP-9, while the 92 kDa band represents a fully glycosylated mature form that is secreted into the extracellular space (Olson et al., 2000). Densitometric analysis was performed on the upper 92 kDa band without consideration for the lower 85 kDa band (Figure 5D), as we are interested in studying the trafficking of this mature protein that is detected extracellularly. RAW 264.7 cells exhibited an increase over time, with the largest amount of intracellular 92 kDa MMP-9 obtained at 9 hrs after activation, followed by a drop at 12 hrs (Figure 5D). Since we do not detect the active form of MMP-9 (82 kDa) in the cell culture supernatant, and because we will not be considering the precursor form (85 kDa) in the cell lysate, the term “MMP-9” will be herein used to describe the 92 kDa zymogen detected both intracellularly and extracellularly.
Figure 5. Analysis of MMP-9 levels in the cell culture supernatants and total cell lysates of resting and LPS/IFN-γ activated RAW 264.7 cells. RAW 264.7 cells were stimulated with LPS/IFN-γ for 3, 6, 9, and 12 hrs or left unstimulated. After the indicated time points, the cell culture supernatants were collected and the cells were lysed and subjected to gelatin zymography or immunoblotting. Gelatin zymography or western blotting (A) and densitometric analysis (B) of macrophage cell culture supernatants for secreted MMP-9. Western blot (C) and densitometric analysis (D) of macrophage total cell lysates for intracellular MMP-9 and α-tubulin levels.
3.1.2 MMP-9 is processed through the biosynthetic pathway and packaged into discrete Golgi-derived vesicles

Resting RAW 264.7 cells immunostained for MMP-9 and imaged by epifluorescence do not demonstrate any intracellular MMP-9 (Figure 6A). RAW 264.7 cells (Figure 6B) and BMDM cells (Figure 6C) were activated and immunostained for MMP-9 and the Golgi marker GM130. Imaging of these cells by epifluorescence revealed MMP-9 staining that localized to the Golgi. Additionally, we found concentrated MMP-9 staining in discrete vesicular structures outside of the Golgi. In activated RAW 264.7 cells, we observed an average of 4.4 ± 0.6 vesicles per cell (Figure 6D).

In general, secreted proteins are transported from the ER to the Golgi and subsequently to the plasma membrane (PM) (Pfeffer and Rothman, 1987). To determine if MMP-9 follows this paradigm of protein transport, and to verify a Golgi-derived nature of MMP-9, RAW 264.7 cells were activated in the presence of BFA. BFA is a potent inhibitor of protein delivery from the ER to the Golgi apparatus (Nebenfuhr et al., 2002). Epifluorescent imaging of cells immunostained for MMP-9 and the ER marker protein disulfide isomerase (PDI) revealed an accumulation of MMP-9 in the ER (Figure 6E). To determine the effects of blocking ER to Golgi transport on MMP-9 production and secretion, immunoblotting for MMP-9 was performed on the cell culture supernatants and cell lysates of resting, and activated cells treated with BFA or not. Compared to activated cells, activated/BFA treated cells did not show MMP-9 in the cell culture supernatant (Figure 6F), indicating that BFA successfully halted its secretion. Additionally, the lysates of these cells revealed an intermediate form of MMP-9, with an apparent size of 89 kDa, and lacked the under-glycosylated (85 kDa) or fully glycosylated (92 kDa) forms (Figure 6F). This intermediate form may reflect a transient, immature form of MMP-9 that has been glycosylated.
Figure 6. MMP-9 is sorted in discrete Golgi-derived vesicles. Resting RAW 264.7 cells were fixed, immunostained for MMP-9, and imaged by epifluorescence (A). Alternatively, RAW 264.7 cells (B) and BMDM cells (C) were activated with LPS/IFN-γ (0.1 μg/ml and 100 U/ml, respectively for 9 hrs), fixed, immunostained for MMP-9 in green, GM130 in red, and imaged by epifluorescence. Scale bars, 10 μm. Arrows indicate colocalization of MMP-9 with GM130. The average number of MMP-9 vesicles observed per activated RAW 264.7 cell was tabulated (D). RAW 264.7 cells were activated (0.1 μg/ml LPS and 100 U/ml IFN-γ for 9 hrs) in the presence of 5 μg/ml BFA to inhibit ER to Golgi transport. Cells were fixed and analyzed for MMP-9 in green, and PDI in red by epifluorescence, Scale bar, 10 μm (E). Arrows indicate colocalization between MMP-9 and PDI. Alternatively, the cell culture supernatants were collected and the cells were lysed and subjected to immunoblotting for MMP-9 and α-tubulin (F).
in the ER and is missing glycosylation in the Golgi. Altogether, these results suggest that MMP-9 maturation requires processing in the ER as well as Golgi compartments, after which it is packaged into discrete vesicles.

3.1.3 MMP-9 vesicles found in activated macrophages differ from MMP-9 granules found in neutrophils

Unlike macrophages, neutrophils produce MMP-9 in the absence of immunological challenge, where it is stored in tertiary granules (Borregaard and Cowland, 1997). These tertiary granules have undergone extensive characterization (Kjeldsen et al., 1994), however, the identity of vesicles transporting MMP-9 in macrophage cells is unknown. We therefore sought to characterize the composition of the MMP-9-containing vesicular structures that we observed in activated macrophages. To begin, we wanted to determine if macrophage MMP-9 vesicles have a similar composition as neutrophil tertiary granules. It is well established that Rab27a, a GTPase involved in specialized vesicle trafficking, colocalizes with MMP-9 and regulates its secretion in neutrophils (Brzezinska et al., 2008). To test for a similar role in macrophages, we expressed CFP-tagged Rab27a in activated RAW 264.7 cells and immunostained for endogenous MMP-9. Epifluorescent imaging of these cells revealed a differential sorting of Rab27a and MMP-9 into separate vesicular subpopulations (Figure 7A). In addition to Rab27a, neutrophils pack the adhesion molecule Mac-1 in tertiary granules (Kjeldsen et al., 1994). Immunostaining of Mac-1 and MMP-9 in activated RAW 264.7 cells showed an absence of Mac-1 recruited in MMP-9 vesicles (Figure 7B). We also investigated the possible localization of CD63 to macrophage MMP-9 vesicles. Although CD63 is found predominately in primary granules, it has been shown to also colocalize with Mac-1 in neutrophils (Skubitz et al., 2000), and to associate with the membranes of macrophage phagolysosomes, secretory granules, and multivesicular
Figure 7. MMP-9 vesicles do not colocalize with Rab27a, Mac-1, and CD63. LPS/IFN-γ (0.1 μg/ml and 100 U/ml, respectively for 9 hrs) activated RAW 264.7 cells expressing Rab27a-CFP (A) or CD63-GFP (C), or immunostained for Mac-1 (B), all shown in red. Cells were analyzed for endogenous MMP-9, shown in green, by epifluorescence, Scale bars, 10 μm.
bodies (Rupani et al., 2004). RAW 264.7 cells expressing GFP-tagged CD63 were activated and immunostained for endogenous MMP-9 (Figure 7C). Epifluorescent imaging of these cells did not show recruitment of CD63 to the MMP-9 vesicles. All together, these results indicate that the composition of neutrophil MMP-9 tertiary granules is different than the MMP-9 vesicles we observe in activated macrophages.

3.1.4 MMP-9 is sorted into calreticulin- and PDI-containing vesicles that are not targeted for degradation in activated macrophages

We showed that activated macrophages distribute MMP-9 into vesicles that differ in composition from neutrophil tertiary granules. It was previously demonstrated that cells deficient in calreticulin, an ER calcium binding protein and chaperone, possess decreased MMP-9 levels and activity (Wu et al., 2007). We therefore wanted to determine if calreticulin associates with MMP-9 in activated macrophages. Epifluorescent imaging of cells immunostained for endogenous calreticulin and MMP-9 showed concentrated calreticulin staining recruited to every MMP-9 vesicle, as well as diffuse calreticulin staining localized to the ER (Figure 8A). We next wanted to determine if calreticulin was the only ER protein that was distributed into MMP-9 vesicles. Immunostaining and epifluorescent imaging of activated RAW 264.7 cells showed the same concentrated staining of PDI in MMP-9 vesicles, as calreticulin (Figure 8B). These results led us to investigate the possibility that the MMP-9 punctate structures we observe may actually be dilated pockets within the ER, since calreticulin and PDI are ER resident proteins (Opas et al., 1991; Turano et al., 2002). ER resident proteins are targeted and retained in the ER via a KDEL signal peptide. This sequence is thus found throughout the ER, on ER proteins that leak out towards the Golgi, and on recycling vesicles from the Golgi back to the ER (Munro and Pelham, 1987; Pelham, 1988; Tang et al., 1993).
Figure 8. Macrophage MMP-9 vesicles colocalize with the ER markers calreticulin and PDI, but not KDEL. RAW 264.7 cells were activated (0.1 μg/ml LPS and 100 U/ml IFN-γ, for 9 hrs) and fixed and immunostained for MMP-9, shown in green, and calreticulin (A) or PDI (B) or KDEL (C), shown in red, Arrows indicate colocalization between MMP-9 vesicles and calreticulin or PDI. Scale bars, 10 μm.
Immunostaining and epifluorescent imaging of activated RAW 264.7 cells revealed that MMP-9 vesicles were devoid of this KDEL sequence (Figure 8C), indicating that these vesicles are not contained within the ER, and are likely not transport or recycling vesicles between the ER and Golgi apparatus.

We also investigated whether the MMP-9 punctate structures were vesicles of proteins targeted for intracellular degradation. Mammalian cells degrade cytoplasmic proteins and organelles mainly through the ubiquitin-proteosome or the autophagy-lysosome systems (Rubinsztein, 2006). The ubiquitin-proteosome pathway is typically responsible for the degradation of cytosolic proteins with short half-lives (Cuervo et al., 2004). It is also involved in ER quality control, in which misfolded proteins are translocated to the cytosol, where they are ubiquitinylated and targeted for degradation by proteosomes (Rubinsztein, 2006). To test for this kind of degradation, we immunostained activated RAW 264.7 cells for ubiquitinylated proteins and MMP-9 (Figure 9A). We found by epifluorescent imaging that the MMP-9 vesicles were not marked by ubiquitinylation, and were therefore likely not targeted for degradation by proteosomes. The autophagy-lysosome pathway is typically responsible for clearing of cytosolic proteins with relatively long half-lives (Cuervo et al., 2004). These proteins are enclosed within autophagosomes that fuse with lysosomes to form autophagolysosomes. Accordingly, the contents of the autophagolysosomes are degraded by the action of lysosomal acid hydrolases (Rubinsztein, 2006). To test for autophagic degradation, we expressed RFP-tagged light chain 3 (LC3), an autophagosome specific protein, in activated RAW 264.7 cells and immunostained for MMP-9. Additionally, we immunostained activated RAW 264.7 cells for lysosomal-associated membrane protein (LAMP)-1, a lysosomal membrane protein, and MMP-9. Epifluorescent
Figure 9. MMP-9 vesicles do not colocalize with markers of proteosomal, lysosomal or autophagic degradation. Some RAW 264.7 cells were transfected with RFP-tagged LC3. Cells were stimulated with LPS/IFN-γ (0.1 μg/ml and 100 U/ml, respectively for 9 hrs). Cells were fixed and immunostained for MMP-9 in green, and immunostained for ubiquitinated proteins (A) or analyzed for LC3-RFP (B) or immunostained for LAMP-1 (C) in red by epifluorescent microscopy. Scale bars, 10 μm.
imaging of these cells revealed that MMP-9 vesicles did not contain RFP-LC3 (Figure 9B) or LAMP-1 (Figure 9C). In summary, these results indicate that macrophage MMP-9 punctate structures are not targeted for intracellular degradation.

3.2 Vesicular Trafficking of MMP-9 in Activated Macrophages

3.2.1 Vesicular trafficking and secretion of MMP-9 is dependent on the MT cytoskeleton in activated macrophages

We observed that activated RAW 264.7 cells sort MMP-9 into calreticulin- and PDI-containing vesicles. We next wanted to investigate the intracellular means of MMP-9 vesicular trafficking and secretion in these cells. It has been well established that the cytoskeleton is involved in the trafficking of organelles and vesicles (Goode et al., 2000). A MT-dependent secretion for MMP-9 has been implicated in melanoma cells (Schnaeker et al., 2004), astrocytes (Sbai et al., 2010), and neurons (Sbai et al., 2008). To investigate its potential role in the trafficking of MMP-9 vesicles in activated macrophages, we immunostained activated RAW 264.7 cells for α-tubulin and MMP-9. Imaging of the basal cell area by total internal reflection (TIRF) microscopy revealed MMP-9 vesicles near the cell surface in close association with the MT cytoskeleton (Figure 10). Based on these results, we proceeded to elucidate a functional role of the MT cytoskeleton in MMP-9 secretion.

To test the MT dependence for MMP-9 secretion, RAW 264.7 cells were activated for 6 hrs prior to an additional 3 hrs in the presence of 10 μM nocodazole, or left activated or resting. Nocodazole is a pharmacological agent that binds to tubulin dimers and prevents assembly into MT strands. At high concentrations, the result is a net depolymerization of MTs in the cell over a short time frame (Liao et al., 1995). Epifluorescent imaging of α-tubulin revealed an efficient
Figure 10. MMP-9 vesicles localize on MT strands near the cell surface. RAW 264.7 cells were activated (0.1 μg/ml LPS and 100 U/ml IFN-γ, for 9 hrs), fixed, and immunostained for MMP-9 (green) and α-tubulin (red), Arrows indicates areas of colocalization between MMP-9 and the MT cytoskeleton. Scale bar, 10 μm.
disruption of the MT cytoskeleton in activated cells treated with nocodazole, compared with activated or resting cells (Figure 11A). The cell culture supernatants and total cell lysates of cells were collected and subjected to Western blotting (Figure 11B) and densitometric analysis (Figure 11C). Compared to activated cells, nocodazole-treated cells showed a statistically significant 1.8 ± 0.2-fold reduction of MMP-9 in the cell culture supernatant. Concurrently, these cells showed a significant 1.5 ± 0.2-fold increase in intracellular MMP-9 levels. In addition to serving as a loading control, the unchanged levels of α-tubulin confirmed that the disruption in MMP-9 secretion was a result of nocodazole-induced MT disorganization rather than a reduction of subunit levels. Epifluorescent imaging of cells immunostained for MMP-9 showed a tremendous accumulation of intracellular MMP-9 vesicles in activated cells treated with nocodazole, compared to activated cells (Figure 11D). A quantification of the number of MMP-9 vesicles in these cells proved ambiguous since it was difficult to distinguish individual structures. Therefore, to verify the accumulation of intracellular MMP-9, the total MMP-9 fluorescence intensity of cells was quantified using Image J Software. Activated/nocodazole-treated cells showed a statistically significant 1.6 ± 0.1-fold increase in intracellular MMP-9 fluorescence intensity compared to activated cells alone (Figure 11E). It has been shown that disruption of the MT cytoskeleton, such as that caused by nocodazole treatment, causes dispersal of the Golgi apparatus into fragments throughout the cell (Cole et al., 1996). To verify that the buildup of vesicles were not fragments of Golgi that contain MMP-9, we immunostained activated cells treated with nocodazole for GM130 and MMP-9 (Figure 12). These cells showed Golgi fragments positive for MMP-9, and an accumulation of other vesicles that were not Golgi-positive. Collectively, these results indicate that an intact MT cytoskeleton is necessary for the trafficking of MMP-9 to the cell exterior.
Figure 11. Disruption of the MT cytoskeleton reduces MMP-9 secretion in activated macrophages. RAW 264.7 cells were activated (0.1 μg/ml LPS and 100 U/ml IFN-γ, for 9 hrs) in the presence of 10 μM nocodazole for 3 hrs. Cells were fixed and immunostained for α-tubulin (green) and DAPI (blue), Scale bars, 10 μm (A). Alternatively, the cell culture supernatants were collected and the cells were lysed and the supernatants and lysates were subjected to Western blotting (B) and densitometric analysis (C) for MMP-9 and α-tubulin. * p < 0.05 compared to activated cells. Additionally, cells were fixed and analyzed for MMP-9 (green), phalloidin (red), and DAPI (blue), Scale bars, 10 μm (D), and the total fluorescence intensity of MMP-9 was measured by Image J Software (E). * p < 0.05 compared to activated cells.
Figure 12. Only a small fraction of accumulated MMP-9 vesicles in macrophage cells treated with nocodazole are within Golgi compartments. RAW 264.7 cells were activated (0.1 μg/ml LPS and 100 U/ml IFN-γ, for 9 hrs) in the presence of 10 μM nocodazole for 3 hrs. Cells were fixed and immunostained for MMP-9 (green) and GM130 (red), Scale bar, 10 μm.
3.2.2 The enhanced MT stabilization observed after macrophage activation mediates the efficient targeting of MMP-9 to the extracellular space

We have demonstrated a MT dependence for MMP-9 secretion in activated macrophage cells.

We reported previously that classical activation of macrophages results in enhanced levels of stable cytoplasmic MTs, and showed that this stabilization was important for increased cell spreading and phagocytic capabilities (Binker et al., 2007a; Patel et al., 2009). We next monitored the level of MT stabilization in RAW 264.7 cells activated (0.1 μg/ml LPS and 100 U/ml IFN-γ) for 9 hrs, using an anti-acetylated tubulin antibody (Piperno et al., 1987).

Epifluorescent imaging of RAW 264.7 cells immunostained for acetylated-α-tubulin and α-tubulin revealed a marked increase in the degree of MT acetylation, occurring in patches along individual MT strands, in activated cells compared to resting cells (Figure 13A). Immunoblotting for acetylated tubulin and total α-tubulin in resting and activated cells revealed an increase in acetylated tubulin levels after activation (Figure 13B). Densitometric analysis indicated that this was a statistically significant 3.7 ± 0.6-fold increase in activated cells, compared to resting cells (Figure 13C). We next wanted to determine whether the enhanced MT stabilization observed in activated macrophages was important for MMP-9 trafficking and secretion to the cell exterior.

To begin, we investigated whether MMP-9 vesicles were adjacent to stable cytoplasmic MTs. To visualize the subpopulation of stable MTs, activated RAW 264.7 cells were immunostained for acetylated tubulin or MAP4 (Patel et al., 2009), total α-tubulin, and MMP-9. Epifluorescent imaging showed that MMP-9 vesicles were often found on MT strands post-translationally modified with acetylation (Figure 14A), or bound to MAP4 (Figure 14B), thus indicating that these vesicles may be trafficked along these stabilized MTs.
Figure 13. Macrophages exhibit increased levels of stable cytoplasmic MTs after activation.
RAW 264.7 cells were activated (0.1 μg/ml LPS and 100 U/ml IFN-γ, for 9 hrs), fixed, and immunostained for acetylated tubulin and α-tubulin and imaged by epifluorescence, Scale bars, 10 μm (A). Arrows indicate patches of acetylation occurring along the length of MT strands. Alternatively, the cells were lysed and lysates were subjected to Western blotting (B) and densitometric analysis (C). * p < 0.05 compared to activated cells.
Figure 14. MMP-9 vesicles localize on a subpopulation of stable MTs marked by acetylation or MAP4. RAW 264.7 cells were stimulated with LPS/IFN-γ (0.1 μg/ml and 100 U/ml, respectively for 9 hours). Cells were fixed and immunostained for MMP-9 in green, α-tubulin in blue and acetylated tubulin (A) or MAP4 (B) in red, Insets show a higher magnification of MMP-9 vesicles and the MT cytoskeleton. Scale bars, 10 μm.
In order to elucidate a functional role of enhanced MT stabilization for MMP-9 secretion in activated macrophages, we investigated the effects of further exaggerating MT stabilization on the extent of MMP-9 secretion. To increase MT stabilization, taxol, a drug that enhances the rate and yield of MT assembly (Manfredi et al., 1982), was used. RAW 264.7 cells were activated for 8 hrs and incubated for another 1 hr in the presence of taxol at 0.1 μM, or left activated or resting. Epifluorescent imaging of cells immunostained for acetylated tubulin and total α-tubulin revealed completely stabilized MTs in activated/taxol-treated cells, indicated by acetylation along the entire lengths of MT strands, compared with the patches of acetylation observed in activated cells (Figure 15A). The cell culture supernatants and total cell lysates of these cells were analyzed by Western blotting. Compared to activated cells, the cell culture supernatants of activated cells treated with taxol showed increased MMP-9 levels, whereas the total cell lysates demonstrated reduced MMP-9 levels, and enhanced acetylated tubulin levels (Figure 15B). Following densitometric analysis, we observed a statistically significant increase in extracellular MMP-9 levels, 3.6 ± 0.3-fold higher in activated cells treated with taxol, compared with activated cells (Figure 15C). We also observed a statistically significant 2.9 ± 0.1-fold decrease, and a 2.6 ± 0.4-fold increase, in the intracellular levels of MMP-9 and acetylated tubulin, respectively, in activated cells treated with taxol, compared with activated cells alone (Figure 15C). Taxol has been shown to stabilize MTs by binding directly to individual strands (Manfredi et al., 1982), as well as by activating TLR4 in a similar way as LPS (Kawasaki et al., 2000). Although resting RAW 264.7 cells treated with taxol did not show any intracellular or extracellular MMP-9 by Western blotting (data not shown), we wanted to verify that the enhanced secretion was a result of enhanced MT stabilization and not a synergistic effect of LPS.
Figure 15. Activated macrophages treated with taxol to enhance MT stabilization show increased levels of MMP-9 secretion. RAW 264.7 cells were activated (0.1 μg/ml LPS and 100 U/ml IFN-γ, for 9 hrs) and treated with 0.1 μM taxol for 1 hr. Cells were fixed and immunostained for acetylated tubulin (red), α-tubulin (green), and DAPI (blue). Scale bars, 10 μm (A). Alternatively, the cell culture supernatants were collected and the cells were lysed and the supernatants and lysates were subjected to Western blotting (B) and densitometric analysis (C) for MMP-9, acetylated tubulin, and α-tubulin. * p < 0.05 compared to activated cells.
and taxol ligation on TLR4. Therefore we used an alternative way to enhance MT stabilization, by treating cells with a low dose of nocodazole (Vasquez et al., 1997).

Although nocodazole is typically used to depolymerize MTs, in vitro studies have shown that using nanomolar concentrations of nocodazole actually increases MT stabilization by suppressing MT dynamic instability and increasing the time MTs spend in a paused state (Liao et al., 1995; Vasquez et al., 1997). Immunofluorescent imaging of RAW 264.7 cells activated for 6 hrs prior to an additional 3 hrs in the presence of 0.1 μM nocodazole, immunostained for acetylated tubulin and α-tubulin, revealed a less elaborate but fully stabilized MT network, compared to activated cells (Figure 16A). The cell culture supernatants and total cell lysates of these cells were analyzed by Western blotting. Compared to activated cells, the cell culture supernatants of activated cells treated with low-dose nocodazole showed increased MMP-9 levels, whereas the total cell lysates possessed reduced MMP-9 levels, and enhanced acetylated tubulin levels (Figure 16B). Following densitometric analysis, we observed a statistically significant increase in extracellular MMP-9 levels, 1.7 ±0.2-fold higher in activated cells treated with low-dose nocodazole, compared with activated cells alone (Figure 16C). We also observed a statistically significant 1.7 ± 0.1-fold decrease, and a 3.2 ± 0.7-fold increase, in the intracellular levels of MMP-9 and acetylated tubulin, respectively, in activated cells treated with low-dose nocodazole, compared with activated cells alone (Figure 16C). All together, the enhanced secretion of MMP-9 observed in activated macrophages with pharmacologically-induced fully stabilized MT networks, suggests a function of enhanced MT stabilization observed after macrophage activation in the efficient trafficking of MMP-9 vesicles to the extracellular space.
Figure 16. Activated macrophages treated with low-dose nocodazole to enhance MT stabilization show increased levels of MMP-9 secretion. RAW 264.7 cells were activated (0.1 μg/ml LPS and 100 U/ml IFN-γ, for 9 hrs) and treated with 0.1 μM nocodazole for 3 hrs. Cells were fixed and immunostained for acetylated tubulin (red), α-tubulin (green), and DAPI (blue), Scale bars, 10 μm (A). Alternatively, the cell culture supernatants were collected and the cells were lysed and the supernatants and lysates were subjected to Western blot (B) and densitometric analysis (C) for MMP-9, acetylated tubulin, and α-tubulin. * p < 0.05 compared to activated cells.
3.2.3 MMP-9 vesicles may be trafficked along MTs by the anterograde molecular motor, kinesin

In concert with the MT cytoskeleton, organelle and vesicular trafficking is known to involve molecular motor proteins such as kinesins (Howard, 1996). We have previously demonstrated, in a large scale proteomic analysis of MT-associated proteins, that association of the kinesin KIF5B isoform with MTs is drastically increased after activation (Patel et al., 2009). We therefore wanted to see if KIF5B was involved in the vesicular transport of MMP-9. Activated RAW 264.7 cells immunostained for MMP-9 and kinesin using a KIF5B specific antibody, revealed that MMP-9 vesicles colocalize with kinesin, by confocal microscopy (Figure 17). Therefore, MMP-9 may be trafficking along stabilized MTs in a kinesin-dependent manner.
Figure 17. MMP-9 vesicles colocalize with the MT motor, kinesin. RAW 264.7 cells were activated with LPS / IFN-γ (0.1 μg/ml and 100 U/ml, respectively for 9 hours). Cells were fixed and immunostained for MMP-9 (green) and kinesin using a KIF5B specific antibody (red), and imaged by confocal laser scanning microscopy, Scale bar, 10 μm.
3.3 Production of MMP-9 by a Subpopulation of Activated Macrophage Cells

3.3.1 A subpopulation of macrophages is responsible for the production and secretion of MMP-9

Activation of BMDMs or RAW 264.7 cells with LPS/IFN-γ (0.1 μl/ml and 100 U/ml, respectively) and epifluorescent imaging for MMP-9 consistently revealed a subset of macrophage cells that contained MMP-9 vesicles (Figure 18A and B, respectively). We monitored the number of RAW 264.7 cells that were positive for MMP-9 vesicles at 3, 6, 9, or 12 hrs after activation (Figure 18C), and found an increase over time. At any of the given time points, only a fraction of cells demonstrated MMP-9 vesicles. The largest proportion was observed at 9 hrs post-activation, where 27.8 ± 3 % of cells contained MMP-9 vesicles. These results suggest that only some macrophages produce and secrete MMP-9, however, do not address the possibility that macrophages lacking MMP-9 vesicles at any given time may have contained MMP-9 previously.

We demonstrated that MMP-9 secretion is dependent on the MT cytoskeleton. We therefore examined whether we would observe an increase in the number of cells positive for MMP-9 when we inhibit MMP-9 secretion by depolymerization of the MT network. Epifluorescent imaging of RAW 264.7 cells activated for 6 hrs and incubated with nocodazole at 10 μM for another 3 hrs, revealed an accumulation of MMP-9 vesicles within each cell as before, however, a sub-population of MMP-9-containing cells still remained (Figure 19A). After quantification of the number of cells positive for MMP-9 vesicles, we did not observe a statistically significant difference between activated cells treated with nocodazole and activated cells alone (Figure 19B). Therefore, we can conclude that MMP-9 is in fact produced and secreted by a sub-
Figure 18. A Sub-population of activated macrophage cells contains MMP-9 vesicles. Activated (0.1 μg/ml LPS and 100 U/ml IFN-γ, for 9 hrs) BMDMs (A) and RAW264.7 cells (B) were fixed and immunostained for α-tubulin (red) and MMP-9 (green), and analyzed by epifluorescence. Scale bars 10 μm. Asterisks indicate cells containing MMP-9 vesicles. RAW 264.7 cells activated for 3, 6, 9, or 12 hrs, or left unstimulated, were analyzed for the average number of cells containing intracellular MMP-9 vesicles (C).
Figure 19. Blocking of MMP-9 secretion by disruption of the MT cytoskeleton does not reveal an increase in the number of MMP-9-containing macrophages. RAW 264.7 cells were activated (0.1 μg/ml LPS and 100 U/ml IFN-γ, for 9 hrs) in the presence of 10 μm nocodazole for 3 hrs. Cells were fixed and immunostained for MMP-9 (green) and α-tubulin (red), Scale bars, 10 μm (A). Asterisks indicate cells containing MMP-9 vesicles. The average number of cells containing MMP-9 vesicles was quantified per 100 resting, activated, and activated/nocodazole treated cells (B).
population of macrophage cells, since blocking MMP-9 secretion and not production using nocodazole, did not reveal more cells positive for MMP-9.

### 3.3.2 Aptitude for MMP-9 production in activated macrophages is not determined by TLR4 levels or the extent of NF-κB activation

MMP-9 production in macrophages is largely mediated by LPS ligation on TLR4 (de Kleijn and Pasterkamp, 2003), which initiates a signaling cascade that activates the transcription factor NF-κB to induce MMP-9 expression (Rhee et al., 2007). We therefore investigated whether MMP-9-containing macrophages were more responsive to LPS as a result of having sufficient TLR4 levels. To test this, we wanted to see if macrophages over-expressing TLR4 would be more likely to produce MMP-9 after activation. RAW 264.7 cells were transfected with mCherry tagged TLR4 or mCherry vector as a control, and immunostained for MMP-9 (Figure 20A and B, respectively). We observed by epifluorescence that most cells, either transfected with TLR4-mCherry or mCherry vector alone, did not contain MMP-9 vesicles. A quantification of the number of cells with MMP-9 vesicles did not reveal a statistically significant difference in cells over-expressing TLR4, compared with cells expressing mCherry vector (Figure 20C). These results suggest that increasing LPS responsiveness by overexpression of TLR4 does not affect the cells aptitude for MMP-9 production. We also investigated whether activated macrophages could produce MMP-9 as result of more efficient signal transduction downstream of TLR4, more specifically, by looking at NF-κB activation. NF-κB is typically found in the cytosol, where upon activation, such as that caused by LPS signaling, is translocated to the nucleus (Mendes Sdos et al., 2009). To verify NF-κB translocation to the nucleus after activation, and to compare NF-κB translocation between cells positive or negative for MMP-9, we immunostained resting and activated cells for NF-κB and MMP-9. Epifluorescent imaging of resting cells demonstrated
Figure 20. Over-expression of TLR4 does not confer MMP-9-producing capacities in activated macrophages. RAW 264.7 cells were transfected with mCherry (A) or TLR4-mCherry (B), shown in red, and stimulated with LPS/IFN-γ (0.1 μg/ml and 100 U/ml, respectively for 9 hrs) and immunostained for MMP-9 in green, Scale bars, 10 μm. The average number of transfected cells containing MMP-9 vesicles was quantified per 50 macrophages (C).
NF-κB localization to the cytosol, whereas activated cells showed both cytosolic and nuclear localization (Figure 21A and B, respectively). However, MMP-9-containing activated cells did not show a difference in nuclear localization of NF-κB, compared with activated cells lacking MMP-9 (Figure 21B). Altogether, these results suggest that macrophage responsiveness to LPS, as determined by its TLR4 levels, and its ability to activate NF-κB after stimulation with LPS/IFN-γ, are not factors explaining its ability to produce MMP-9.
Figure 21. Activated macrophage cells containing MMP-9 vesicles show the same NF-κB activation in response to LPS, as activated cells lacking MMP-9 vesicles. Resting (A) or activated (B) RAW 264.7 cells were fixed and immunostained for NF-κB (red) and MMP-9 (green) and analyzed by epifluorescence. Insets show these cells stained with DAPI (blue) to show the nucleus. Arrow indicates MMP-9-containing cell.
4 Discussion

The primary goal of this work was to characterize the distribution of MMP-9 in activated macrophages, and to elucidate the mechanism by which it is trafficked and secreted.

Macrophage migration during an inflammatory response requires MMP-9 as an extracellular protease to mediate the degradation of ECM components (Gong et al., 2008). Although a dependence of MMP-9 was demonstrated for macrophage migration, the dynamics of its production, distribution, and trafficking remain uncharacterized. We localized intracellular MMP-9 to distinct cytoplasmic vesicles that contained calreticulin and PDI, in macrophages stimulated with LPS and IFN-γ. Vesicular organelles of MMP-9 are aligned along stable subsets of MTs, showing colocalization with the molecular motor protein, kinesin. Therefore, these vesicles are likely to be intended for secretion at the plasma membrane. We demonstrated a functional contribution of stable MTs in the enhanced trafficking of MMP-9 extracellularly, and showed that heterogeneity exists in macrophage cell populations with respect to MMP-9 production. A summary of the composition of MMP-9 vesicles as well as their trafficking along stable MTs in activated macrophages is depicted in Figure 22.

4.1 Dynamics of MMP-9 production and secretion

Macrophage migration during an inflammatory response requires the targeted secretion of MMP-9 to the extracellular space, as well as its activation by extracellular proteases. Upon immunological challenge, macrophages respond by up regulating MMP-9 transcription, translation, and finally secretion (Webster and Crowe, 2006). A study by Rhee and colleagues demonstrated an upregulation of MMP-9 gene expression starting just 1 hr after LPS stimulation (Rhee et al., 2007). Another study by Woo and colleagues investigated extracellular
Figure 22. A model of MMP-9 sorting and trafficking in activated macrophages. Activated macrophages exhibit increased levels of stable cytoplasmic MTs with increased production and trafficking of MMP-9. Shown are MMP-9 vesicles being trafficked along stabilized MTs by the anterograde molecular motor, kinesin. MMP-9 is processed through the biosynthetic pathway, and is sorted in discrete vesicular organelles that contain calreticulin and PDI, and do not colocalize with markers of neutrophil tertiary granules or intracellular degradation.
MMP-9 levels after LPS exposure and demonstrated the detection of extracellular MMP-9 as early as 6 hrs after stimulation (Woo et al., 2004). Our work differs from the above analyses because our macrophage activation was achieved by LPS stimulation, in concert with IFN-γ, and because we analyzed the cell culture supernatants as well as the total cell lysates for MMP-9. We also monitored the changes in MMP-9 levels at a higher resolution, every 3 hrs after classical activation, rather than every 6 hrs (Woo et al., 2004; Rhee et al., 2007). In agreement with previous analysis (Woo et al., 2004), we found increasing levels of extracellular MMP-9, detected as early as 6 hrs after activation, by immunoblotting. However, our analysis of cell lysates, showing increasing MMP-9 levels that peaked at 9 hrs after activation with a subsequent drop at 12 hrs, is novel. Failure to replenish depleted MMP-9 pools, even while still under immunological challenge, suggests that MMP-9 may be negatively regulated. Extracellularly, MMP-9 has been shown to shed CD14, the adaptor molecule that mediates LPS-TLR4 ligation, thus decreasing sensitivity to LPS (Senft et al., 2005) and possibly reducing the downstream signalling responsible for MMP-9 induction. Alternatively, decreased endotoxin signalling may be due to LPS-induced expression of an alternatively spliced, soluble form of TLR4 that has been found to inhibit NF-κB activation (Iwami et al., 2000). Production of this truncated PRR has been suggested to provide a negative feedback mechanism for LPS signalling in macrophages (Iwami et al., 2000). Therefore, increased levels of extracellular MMP-9 and prolonged exposure to LPS may negatively regulate further induction of MMP-9 by shedding of CD14 and producing a truncated form of TLR4, respectively. Negatively regulating MMP-9 avoids its over-production, which is necessary for the maintenance of a healthy state. The dysregulation in MMP-9 secretion and activity has been implicated in numerous pathological
processes, including inflammation of the gastrointestinal tract, pulmonary tract, and joints (Van
den Steen et al., 2002).

MMP-9 is produced as an inactive zymogen, 92 kDa in size, that becomes activated extracellularly by cleavage of its propeptide domain, yielding an 82 kDa protein (Fridman et al., 2003). Proteolytic cleavage of the propeptide domain is possible through the plasmin-plasminogen cascade in vivo (Gong et al., 2008). Additionally, macrophages themselves have been shown to secrete activating factors such as other MMPs (Ogata et al., 1992; Knauper et al., 1997; von Bredow et al., 1998). Despite this, our analysis of MMP-9 in the cell culture supernatants by Western blotting or gelatine zymography failed to detect its active form. Finding only pro-MMP-9 does not necessarily reflect macrophage intent to maintain enzyme latency after secretion, but rather may be a result of cell culture conditions in vitro. Secreted MMPs may be diluted in the cell culture supernatant away from their activators, thus preventing the activation of enough protein to be detected by immunoblotting (Fridman et al., 2003). Additionally, the cell surface association of MMP-9 after secretion is short-lived, and would therefore not favour its possible activation by other surface associated enzymes (Fridman et al., 2003). On the contrary, the detection of the active form of MMP-2 is more likely since it is capable of better surface association compared with MMP-9, and therefore has more access to its activating factors (Butler et al., 1998).

4.2 Maturation of MMP-9 through the biosynthetic pathway

MMP-9 is a heavily glycosylated glycoprotein, where 15% of its mass is contributed by posttranslational glycosylation (Opdenakker et al., 2001a). Although the function of such glycosylation has not been investigated for MMP-9, it has been suggested to regulate substrate
targeting for membrane type 1-MMP (Wu et al., 2004). Our analysis of macrophage cell lysates for MMP-9 by immunoblotting revealed two discrete intracellular forms, an under-glycosylated 85 kDa precursor, and a fully glycosylated, 92 kDa zymogen. These forms, of varying apparent molecular weights, have been previously described (Olson et al., 2000) and are commonly detected in cells lysates (Fridman et al., 2003). However, we have demonstrated for the first time an intermediate 89 kDa form of MMP-9 that appears in activated cells treated with BFA, by immunoblotting. BFA inhibits ER to Golgi transport (Nebenfuhr et al., 2002), and we demonstrated that activated cells treated with BFA show MMP-9 localized to the ER by immunofluorescence. Therefore, we can attribute any glycosylation of the 85 kDa form of MMP-9 in these BFA treated cells to the ER. This provides insight into the glycosylation patterns that MMP-9 undergoes through the biosynthetic pathway, whereby the ER is responsible for maturing MMP-9 from its 85 kDa underglycosylated form to its 89 kDa intermediate glycosylated form. Additionally, we can infer that full glycosylation of the 89 kDa form to the 92 kDa form occurs in the Golgi apparatus. In activated cells, not treated with BFA, we observe substantial amounts of the 85 kDa and 92 kDa forms, and do not detect the 89 kDa intermediate. This suggests two things: first, newly synthesized MMP-9 peptides, whether in the cytosol or held in the ER, may not be immediately processed in the ER; second, once committed to moving from the ER to the Golgi, the 85 kDa form is rapidly processed and the 89 kDa form is a transient intermediate. If such processing occurred slowly, we would expect to see a smear of bands between the 85 kDa and 92 kDa bands of MMP-9 by Western blotting.
4.3 Macrophage versus neutrophil MMP-9 vesicles

Although MMP-9 originates from the same gene in neutrophils and in peripheral blood monocytes (Opdenakker et al., 2001a), we showed by immunofluorescent imaging that macrophages pack MMP-9 into vesicles that differ in composition from neutrophil gelatinase granules.

Our observation that macrophage vesicles were not positive for Rab27a, a GTPase involved in the regulation of exocytic neutrophil granules (Brzezinska et al., 2008), may be due to differences in the dynamics of MMP-9 production in macrophages versus neutrophils. Neutrophils make up the first wave of leukocytes to arrive at the site of inflammation, and as such, need to be able to migrate without hesitation (Van den Steen et al., 2002). Unlike macrophages, neutrophils produce and pre-pack MMP-9 in tertiary granules to allow for rapid release upon stimulation, without the need for transcription and translation (Van den Steen et al., 2002). Therefore, release of neutrophil MMP-9 likely relies on posttranslational control of vesicle trafficking, whereas release of macrophage MMP-9 relies on transcriptional induction and subsequent secretion. We speculate that macrophage MMP-9 vesicles do not require regulation by Rab27a since the vesicles are produced only when the macrophage is immunologically challenged. On the other hand, the pre-packed MMP-9 granules in neutrophils are held within the cell, and become mobilized using Rab27a upon stimulation (Brzezinska et al., 2008).

Neutrophils have been shown to sort the adhesion molecule Mac-1 into MMP-9 granules (Kjeldsen et al., 1994). However, we observed that macrophage MMP-9 vesicles were devoid of Mac-1. This may be reflective of differences in control strategies employed by macrophages.
and neutrophils, in the regulation of MMP-9 activity. Neutrophils do not produce TIMP-1, and as a result, are unable to control MMP-9 activity by forming MMP-9/TIMP-1 complexes (Opdenakker et al., 2001a). Conversely, macrophages have been shown to produce TIMP-1 and are able to form these complexes (Webster and Crowe, 2006). Co-packing of molecules within the same exocytic organelles ensures their synchronous targeting at the cell surface through space and time. Packaging of the adhesion molecule Mac-1 with MMP-9 in neutrophils thus ensures that MMP-9 will only be released at sites of firm attachment with endothelial components (Kjeldsen et al., 1994), a control measure likely needed by neutrophils since they cannot provide control by TIMPs.

4.4 Analysis of macrophage MMP-9 vesicles

Having demonstrated a structural difference between neutrophil and macrophage MMP-9-containing vesicles, we next investigated their identity in macrophages. We showed that the normally ER resident proteins, calreticulin and PDI, are sorted into MMP-9 vesicles in activated macrophages. Our observations showing that these vesicles were not positive for markers of proteosomal, lysosomal, and autophagic processing suggest that they are likely not targeted for intracellular degradation. ER resident proteins are targeted and retained in the ER via a KDEL signal sequence that is recognized by KDEL receptors within the ER and Golgi compartments. KDEL proteins that make their way to the Golgi apparatus are recognized by KDEL receptors and are transported back to the ER (Munro and Pelham, 1987; Pelham, 1988). We did not observe KDEL staining localized to MMP-9 vesicular organelles in activated macrophages, which confirmed that these punctate structures were not expanded pockets within the ER, nor were they anterograde ER-to-Golgi vesicles, or retrograde Golgi-to-ER vesicles.
There is growing evidence that some ER proteins, such as calreticulin (Nash et al., 1994) and PDI (Turano et al., 2002), can be detected and function in non-ER locations. The exact means by which these proteins may evade the ER retention machinery is not well understood, however, some theories have been proposed. KDEL proteins may escape the ER in situations where KDEL receptors become saturated, or in cases where ER proteins become complexed with other macromolecules, or possibly when their KDEL signal sequence is removed (Johnson et al., 2001; Turano et al., 2002). In order to be incorporated into MMP-9 vesicles, calreticulin and PDI must have therefore bypassed the KDEL signal mechanism. Our observation that MMP-9 vesicles were devoid of KDEL staining thus implies that the calreticulin and PDI contained in these vesicles do not possess KDEL sequences. This is in support of the last theory for ER-escape, in which ER resident proteins can reside outside of the ER by loss of their KDEL retention signals. The mechanism by which this occurs remains unclear (Turano et al., 2002).

It has been suggested that binding of calreticulin with glycoproteins in the ER serves as a signal for PDI recruitment, which is responsible for forming disulphide bonds between the thiols of cysteine residues (Michalak et al., 1999). Our observation that MMP-9 vesicles contain both calreticulin and PDI may thus be explained by the cooperative roles of these two chaperones. Additionally, calreticulin has been shown to directly associate with PDI in a zinc-dependent manner (Baksh et al., 1995). We therefore speculate that the zinc ion found in the catalytic domain of MMP-9 (Webster and Crowe, 2006) may provide a structural ingredient necessary for the presence of both calreticulin and PDI in MMP-9 vesicles of activated macrophages.

Calreticulin has been described in non-ER compartments such as the acrosomal vesicles of sperm cells and the cytotoxic granules of T-cells (Nash et al., 1994). In the granules of T-cells,
calreticulin was shown to be associated with perforin, which becomes activated in the presence of calcium. It was suggested that calreticulin acts as a molecular chaperone, and being a calcium binding protein, was thought to also regulate the levels of free calcium in these granules (Nash et al., 1994). Interestingly, the presence of calcium was shown to be important for MMP-9 stability, in which treatment with trypsin in the presence of calcium resulted in zymogen activation, whereas trypsin treatment in the absence of calcium resulted in the degradation of MMP-9 (Bu and Pourmotabbed, 1995). It is possible that the calreticulin we observe in MMP-9 vesicles of activated macrophages may function as a molecular chaperone that maintains favourable calcium levels important for MMP-9 stability.

Although PDI has not been described in vesicular organelles outside of the ER, PDI has been reported to be secreted extracellularly and to associate with the cell surface via electrostatic interactions (Terada et al., 1995). In leukocytes, this interaction has been implicated in the regulation of cell adhesion, in which the reducing activity of PDI prevents L-selectin conformational changes that promote its degradation (Bennett et al., 2000). While a role for PDI in cell adhesion was demonstrated extracellularly (Bennett et al., 2000), calreticulin has been implicated in modulating cell adhesion by binding to the cytoplasmic tail of α-integrin and possibly regulating its function (Michalak et al., 1999). Since both calreticulin and PDI have been implicated in mediating cell adhesion (Michalak et al., 1999; Bennett et al., 2000), we speculate that their trafficking together with MMP-9 ensures their synchronous arrival at the PM, which may function to control MMP-9 targeting to sites of ECM attachment.

It was demonstrated previously that neutrophils traffic Mac-1 into MMP-9 granules (Kjeldsen et al., 1994), and we have shown that macrophages direct calreticulin and PDI into MMP-9
vesicles. Mac-1, calreticulin, and PDI have each been implicated in mediating leukocyte adhesion (Kjeldsen et al., 1994; Michalak et al., 1999; Bennett et al., 2000). Therefore, co-packing of these molecules with MMP-9 thus mediates their synchronous targeting to the cell surface to sites of ECM attachment. However, the reason for choosing to traffic MMP-9 with Mac-1 in neutrophils rather than with calreticulin and PDI, as in macrophages, is unclear. PDI and calreticulin are involved in maintaining selectin conformation (Bennett et al., 2000) and regulating integrin function (Michalak et al., 1999), respectively. Selectins and integrins are involved in the early steps of tethering and adhesion during the process of leukocyte extravasation (Ley et al., 2007). We suggest that macrophages, which are capable of regulating MMP-9 extracellular activity by TIMPs (Webster and Crowe, 2006), can afford to secrete MMP-9 early in the extravasation process while concurrently modulating selectins and integrins using PDI and calreticulin, respectively. On the other hand, neutrophils, which are incapable of TIMP production (Opdenakker et al., 2001a), traffic MMP-9 with Mac-1, a molecule involved in the late steps of extravasation, during tight adhesion and intravascular crawling (Ley et al., 2007). We speculate that these cells prefer to traffic MMP-9 with an adhesion molecule involved in the later stages of extravasation since they cannot regulate MMP-9 activity with TIMPs, and therefore would ensure that MMP-9 is only secreted at desired sites of endothelial penetration.

4.5 Evaluation of MT disruption on MMP-9 secretion

Secretion of MMP-9 has been shown to be dependent on MTs in several cell types (Schnaeker et al., 2004; Sbai et al., 2008; Sbai et al., 2010). We found that the same was true for macrophages, where disruption of the MT network resulted in decreased MMP-9 secretion with concurrently increased cytoplasmic MMP-9 levels, detected by Western blotting. However,
MMP-9 secretion was not completely halted in nocodazole treated cells, as some protein was still detected extracellularly. Vesicular secretion involves the docking and fusion of vesicular organelles with the PM, followed by the release of their contents to the extracellular space (Brumell et al., 1995). Vesicular fusion with the PM was shown to be dependent on the underlying actin cytoskeleton (Eitzen, 2003). We observed a tremendous accumulation of randomly distributed MMP-9 vesicles in activated cells treated with nocodazole by immunofluorescence, and it has been shown that the actin cytoskeleton is unaffected by nocodazole treatment (Sun et al., 1999). Therefore, we speculate that the modest amount of MMP-9 detected in the supernatants of activated cells treated with nocodazole is a result of random fusion and secretion of the dispersed vesicles via the actin cytoskeleton. Since we were interested in studying MMP-9 trafficking and not fusion with the PM, we did not investigate the effects of disrupting the actin cytoskeleton on MMP-9 secretion.

In our assays, the amount of extracellular MMP-9 detected by immunoblotting may have been underappreciated since collection of the cell culture supernatants was followed by washing of the cells, to remove extracellular proteins that may contaminate the cell lysate fractions. Therefore, any secreted MMP-9 that was weakly associated with the cell surface may have been washed away and remained undetected. This may explain why the changes of MMP-9 levels in the cell lysates of nocodazole treated cells did not correspond perfectly to the changes observed in the cell culture supernatants, where a greater reduction was observed outside the cells than that accumulated inside the cells.
4.6 Evaluation of enhanced MT stabilization on MMP-9 secretion

Our laboratory has previously demonstrated that macrophage activation results in enhanced levels of stable cytoplasmic MTs (Binker et al., 2007b; Patel et al., 2009), and have shown that such stabilization mediates macrophage cell spreading and phagocytic capabilities (Binker et al., 2007b). Finding MT dependence for MMP-9 secretion, and keeping in mind that macrophage activation results in enhanced MT stabilization as well as MMP-9 secretion, we speculated that the rapid MT stabilization observed after macrophage activation may also function in mediating the efficient and extensive targeting of MMP-9 to the cell exterior. Directly testing for the effects of MT stabilization after activation on MMP-9 secretion was not possible for two reasons. First, although resting macrophages possess basal amounts of stable MTs, assessed by acetylated tubulin levels, these resting cells do not produce or secrete any MMP-9. Consequently, we cannot correlate a fold change of MMP-9 secretion with a fold change of MT stabilization after macrophage activation because there is a concomitant induction of MMP-9 production. Second, there is currently no means of globally inhibiting MT stabilization, and so we cannot compare the extent of MMP-9 secretion in normal activated cells to those without stable MTs. Therefore, we compared the ability of normal activated cells to secrete MMP-9 with that of activated cells with pharmacologically-induced fully stabilized MT networks. In this case, the production of MMP-9 is the same, but the level of stabilization is different, and so we can contribute any differences in MMP-9 secretion to differences in the level of MT stabilization.

Exaggerating MT stabilization by enhancing the rate of MT assembly and forming depolymerization resistant MTs using taxol (Manfredi et al., 1982), or by suppressing MT
dynamic instability and increasing the time MTs spend in a paused state using low-dose nocodazole (Liao et al., 1995; Vasquez et al., 1997), resulted in enhanced secretion of MMP-9 compared with activated cells alone. Dynamic MTs show half-lives in the order of minutes compared with hours in stabilized MT strands (Schulze et al., 1987). The trafficking of an MMP-9 vesicle along a dynamic MT strand is thus likely to be interrupted by depolymerization. We envisage that more efficient trafficking of MMP-9 to the cell exterior is possible along stable MTs because these have less catastrophe events (Schulze et al., 1987), thus providing a stable track that likely extends entirely to the final destination (Reed et al., 2006).

The pharmacological agents, taxol and nocodazole (at a low dose), stabilize the MT cytoskeleton by different mechanisms. Although the resulting effect of these agents was similar, some differences were observed in the changes of acetylated tubulin levels, and the extent of increased MMP-9 secretion. Low-dose nocodazole treated cells demonstrated higher folds of increase in the level of acetylated tubulin from activated cells alone, compared with that seen in taxol treated cells. It is important to notice that acetylation is a marker of stabilized MTs, and that the longer a MT is intact, the more it will accumulate posttranslational acetylation (Schulze et al., 1987). In our assays, nocodazole was administered for 3 hrs, whereas taxol was for 1 hr. Although both treatments caused stabilization, low-dose nocodazole treated MTs were stabilized for longer and therefore accumulated more modification. On the other hand, taxol treated cells demonstrated a greater fold increase of extracellular MMP-9 from activated cells alone, compared with low-dose nocodazole treated cells. This may be explained by the mechanism by which each agent acts to cause stabilization. Low-dose nocodazole treatment ‘freezes’ existing MTs and stabilizes them (Vasquez et al., 1997), whereas taxol
stabilizes existing MTs as well as promotes the nucleation and stabilization of new MT strands (Manfredi et al., 1982). Therefore taxol results in more elaborate stabilized MT networks, and potentially more tracks for which MMP-9 can be trafficked along.

4.7 Kinesin: a potential mediator of MMP-9 transport along stable MTs

Our laboratory has previously demonstrated in a large scale proteomic analysis of MT-associated proteins, that kinesin recruitment to MTs is greatly enhanced after macrophage activation (Patel et al., 2009). Generally, posttranslational modifications do not change the intrinsic properties of MTs. However, in addition to being a marker for stable MTs, acetylation of MTs has been shown to enhance the binding and motility of kinesin (Reed et al., 2006). We showed that kinesin colocalizes with MMP-9 vesicles by confocal microscopy. We therefore speculate that the increase in acetylated MTs after activation promotes enhanced kinesin binding on stable MT subsets, which can then efficiently traffic MMP-9 to the cell exterior.

Although the general mechanism by which α-tubulin subunits become acetylated has not been defined, the acetyltransferase elongator complex has been suggested to carry out this function in neurons (Creppe et al., 2009). On the other hand, the ability of histone deacetylase 6 (HDAC6), a member of class II deacetylases, to remove acetyl groups from tubulin subunits has been well established (Zilberman et al., 2009). Inhibition of HDAC6 activity has been shown to increase MT acetylation and reduce MT dynamic instability (Zilberman et al., 2009).

Interestingly, inhibition of SIRT1, a NAD-dependent histone deacetylase, has been shown to increase MMP-9 secretion (Nakamaru et al., 2009). We speculate that SIRT1 inhibition may result in the same increase in tubulin acetylation as described during inhibition of HDAC6.
(Zilberman et al., 2009), and that the enhanced MMP-9 secretion observed during SIRT1 inhibition (Nakamaru et al., 2009) may result in increased kinesin binding and subsequent trafficking of MMP-9. Therefore, in addition to enhancing kinesin binding with MTs (Reed et al., 2006), we suggest that MT acetylation may play a functional role in kinesin-dependent MMP-9 trafficking along stable MT strands.

4.8 Heterogeneity in populations of macrophage cells

We demonstrated heterogeneity in macrophage populations with respect to their MMP-9-producing capabilities. Production of MMP-9 in macrophages is TLR4-dependent (Mendes Sdos et al., 2009), and occurs mainly through a MAPK pathway, particularly involving ERK1/2 and p38, which activates the NF-κB transcription factor for the induction of gene expression (Lai et al., 2003; Mendes Sdos et al., 2009). Cells over-expressing TLR4 did not show a greater aptitude for MMP-9 production compared with control cells, suggesting that a sufficient level of TLR4 is not a limiting factor in macrophages ability to produce MMP-9. Additionally, MMP-9-containing cells showed the same pattern of NF-κB activation as control cells, in terms of NF-κB translocation to the nucleus, suggesting that signaling downstream of TLR4 is similar in cells containing or lacking MMP-9. MMP-9 induction is also thought to be regulated by other pathways, such as COX-2 signaling (Pavlovic et al., 2006), and although we did not investigate this, alterations in these other pathways may account for the varying capacity for MMP-9 production in macrophages.

General heterogeneity in macrophage populations is well recognized (Van Ginderachter et al., 2006). In vivo, macrophage variation may arise from exposure to different stimuli or immunological challenges (Van Ginderachter et al., 2006), and from differences in the degree of
maturation (Adams and Hamilton, 1984). Such differences may account for the subpopulation of MMP-9-containing BMDM cells we observed, considering that these cells may have varied stimulation history and maturity levels. Although heterogeneity in terms of MMP-9 production has not been demonstrated in RAW 264.7 cells, the existence of heterogeneity in this cultured cell line has been demonstrated (Pace et al., 1994). A subpopulation of RAW 264.7 cells was shown to express iNOS in response to LPS, while priming with IFN-γ increased the percentage of these iNOS-expressing cells (Pace et al., 1994).

4.9 Future directions and implications

Macrophage migration during the inflammatory response requires the controlled and efficient secretion of MMP-9 to the extracellular space. Our work provides a mechanism for MMP-9 trafficking in macrophages stimulated with LPS and IFN-γ, whereby increased MT stabilization mediates efficient MMP-9 secretion. Additionally, we provide for the first time a characterization of intracellular MMP-9 maturation and sorting in these cells. Packing the ER chaperone proteins calreticulin and PDI within MMP-9 vesicles provides new insight into possible chaperoning roles of these proteins outside of the ER in macrophages. These chaperones have been implicated in aiding cell adhesion. Demonstrating their localization within MMP-9 vesicles provides insight into the means by which these cells may co-target MMP-9 to sites of endothelial attachment.

MMP-9 vesicles were observed in activated RAW 264.7 cells in close proximity to stable subsets of MTs using epifluorescent imaging. Accordingly, we demonstrated a role for MT stabilization in MMP-9 secretion using Western blotting analysis. Further confirmation of the role of stable MTs in the secretion of MMP-9 may be achieved by live epifluorescent imaging of GFP-tagged
MMP-9 in concert with RFP-tagged cytoplasmic linker protein 170 (CLIP-170), a plus-end MT tracking protein (Arnal et al., 2004). We have previously shown that CLIP-170 accumulates along the length of stable MTs in activated macrophage cells (Binker et al., 2007b). Therefore, in these activated cells, CLIP-170 allows for the visualization of stable MTs, and so we can determine if MMP-9 is preferentially trafficked along these stable MT subsets. Live epifluorescent imaging of cells expressing RFP-tagged kinesin and GFP-tagged MMP-9 will provide confirmation of kinesin’s role in the trafficking of MMP-9 vesicles. We speculated that acetylation of the lysine 40 residue of α-tubulin subunits may provide a signal that mediates the trafficking of MMP-9 along stable MTs. We can determine a functional role of acetylation by live epifluorescent imaging of mCherry-tagged K40A-α-tubulin, a chimera that is incapable of being acetylated, and GFP-tagged MMP-9 to investigate the effects of reduced tubulin acetylation on the vesicular trafficking of MMP-9.

As major players of innate immunity, macrophages are endowed with the specialized capacity for cell migration, phagocytosis of pathogens, and antigen presentation to T cells (Gordon, 2003). MMP-9 is an important player mediating macrophage migration, where thioglycollate elicited macrophages have been suggested to be the main source of MMP-9 in mouse peritoneal cavities (Gong et al., 2008). Despite its role during the inflammatory response for migration, excessive or unregulated production and secretion of MMP-9 results in its contribution to pathological processes (Van den Steen et al., 2002). Excessive secretion of MMP-9 by macrophages was shown to cause ECM degradation that induces plaque rupture in the cephalic arteries of atherosclerosis mouse models (Gough et al., 2006). Additionally, patients with emphysema demonstrated alveolar macrophages as the most abundant
inflammatory cell present in the lung, which were shown to secrete elevated levels of MMP-9, compared with the cells of control patients (Finlay et al., 1997). Our investigation of the mode by which MMP-9 is trafficked in activated macrophages may thus provide pertinent insight for the regulation of excessive MMP-9 secretion in disease states, such as atherosclerosis and emphysema. As such, unwarranted MT stabilization in macrophages involved in such processes may account for the elevated secretion of MMP-9. An investigation of the level and role of stable MTs in macrophages contributing to atherosclerosis and emphysema may provide applicable treatments to prevent the excessive targeting of MMP-9 to the ECM.

In addition to mediating leukocyte migration during inflammation, MMP-9 has been implicated in tumor cell invasion and metastasis (Himelstein et al., 1994). Elevated levels of MMP-9 has been demonstrated in various cancers, including that of bladder tumors, squamous cell carcinomas of the lung, skin cancers, and colon cancers (Van den Steen et al., 2002). It was shown that MMP-9 functions in the proliferation of cancer cells by association with the cell surface through CD44, where the association of MMP-9 with CD44 was linked with increased tumor invasion (Yu and Stamenkovic, 1999). Additionally, it was suggested that interactions between the cytoskeleton and CD44 mediates oncogenic signaling in cancer cells, up-regulating its invasive properties, which includes increased activity of MMP-9 (Bourguignon et al., 1998).

Although increased MMP expression has been demonstrated in cells from various cancers, there is growing evidence showing a concurrent increase in the stromal cells and inflammatory cells that surround tumors (Van den Steen et al., 2002). A study by Itoh and colleagues monitored the ability of melanoma cells and Lewis lung carcinoma cells implanted in MMP-9-deficient mice to metastasize (Itoh et al., 1999). MMP-9 deficient mice demonstrated
significantly reduced metastatic colonies compared with control mice, suggesting an importance of MMP-9 contribution from non-cancer cells for the metastasis of cancer cells. Of these ‘non-cancer’ cells, macrophages have been implicated in playing a role in tumor metastasis, such as the tissue resident macrophages in skin cancer which demonstrate enhanced MMP-9 expression (Pyke et al., 1992). Additionally, macrophages near the tumor edge of colon cancer patients were positive for MMP-9 expression (Illemann et al., 2006). The discovery of increased MMP-9 expression in macrophages located near tumors has implications in cancer invasion. Therefore, in addition to demonstrating an understanding of how macrophages mount an inflammatory response, our characterization of MMP-9 trafficking in these cells may also provide insight into the mechanisms by which macrophages may mediate cancer metastasis.

4.10 Conclusions

We have characterized the dynamics of MMP-9 production and distribution in activated macrophages, and demonstrated for the first time MMP-9 vesicular organelles that contain the ER proteins calreticulin and PDI. Stabilization of the MT cytoskeleton is uniquely rapid and predominant in activated macrophages, whereas other animal cells have largely dynamic MTs (Desai and Mitchison, 1997). We have previously demonstrated that macrophage activation results in enhanced MT stabilization, and that this mediates increased cell spreading and phagocytic capabilities (Binker et al., 2007b). This work has implicated such stabilization in the efficient and extensive targeting of MMP-9 to the extracellular space. We obtained preliminary results that implicate kinesin as the molecular motor responsible for the anterograde transport of MMP-9 vesicles, and suggested that such transport may be regulated by enhanced MT
stabilization. Finally, we presented data demonstrating heterogeneity that exists within populations of macrophage cells with respect to their ability to produce MMP-9. Ultimately, this work aids in our understanding of the molecular mechanisms responsible for the migration of activated macrophages to sites of infarcted tissues, to mount an immune response.
References


