ROLE OF ADIPONECTIN IN MONOCYTIC MICROPARTICLE-INDUCED ENDOTHELIAL DYSFUNCTION

by

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A thesis submitted in conformity with the requirements for the degree of Master of Medical Science
Institute of Medical Science
University of Toronto

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2015

Abstract

Monocyte-derived microparticles (MPs) have been suggested to link cardiovascular risk factors to vascular injury. We hypothesized that adiponectin, an anti-inflammatory and vasculoprotective adipokine, limits the production and/or action of monocytic MPs on endothelial cells. Adiponectin attenuated MP release from untreated THP-1 monocytes and lipopolysaccharide (LPS)-treated conditions. Furthermore, peritoneal monocytes from Adipoq−/− mice generated significantly greater MPs than those from Adipoq+/+ littermates in the absence and presence of LPS. LPS-induced MP expression of NLRP3 inflammasome and its principal components, namely cleaved ASC, caspase-1, and IL-1β (pro- and cleaved), were all markedly attenuated by adiponectin treatment. HUVECs incubated with MPs from LPS-treated THP-1 cells exhibited an increase in the expression of VCAM-1; adiponectin inhibited this effect. The effects of adiponectin on MP release and molecular signaling occurred at least in part through modulation of the AMPK-Akt and NFκB pathways. The findings highlight another pleiotropic effect of adiponectin in limiting endothelial activation.
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List of Abbreviations

APC  Allophycocyanin
ABCA1  ATP Binding Cassette A1
Acrp30  Adipocyte Complement Related Protein of 30 kD
AdipoR1  Adiponectin Receptor 1
AdipoR2  Adiponectin Receptor 2
ADP  Adenosine diphosphate
AICAR  Aminoimidazole Carboxamide Ribonucleotide
AMPK  AMP-activated Protein Kinase
apMI  Adipose most abundant gene Transcript 1
Apo  Apolipoprotein
ATP  Adenosine Triphosphate
BAT  Brown Adipose Tissue
Ca$^{2+}$  Calcium Ion
CAD  Coronary Artery Disease
CHD  Coronary Heart Disease
CRP  C-Reactive Protein
CVD  Cardiovascular Disease
EDTA  Ethylenediaminetetraacetic Acid
ELISA  Enzyme-linked Immunosorbent Assay
EMP  Endothelial-derived Microparticle
eNOS  endothelial Nitric Oxide Synthase
FCM  Flow Cytometry
FITC  Fluorescein Isothiocyanate
FSC  Forward Scatter
GLUT-4  Glucose Transporter 4
GP  Glycoprotein
HDL  High-Density Lipoprotein
HMW  High Molecular Weight
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAM-1</td>
<td>Intercellular Adhesion Molecule-1</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin 1</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>IR</td>
<td>Ischemia-Reperfusion</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin Resistance</td>
</tr>
<tr>
<td>ISTH</td>
<td>International Society of Thrombosis and Hematology</td>
</tr>
<tr>
<td>IkB</td>
<td>Inhibitor Kappa B</td>
</tr>
<tr>
<td>Kda</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-Density Lipoprotein</td>
</tr>
<tr>
<td>LMP</td>
<td>Leukocyte-derived Microparticle</td>
</tr>
<tr>
<td>LMW</td>
<td>Low Molecular Weight</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein Lipase</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte Chemotactic Protein</td>
</tr>
<tr>
<td>MdFI</td>
<td>Median Florescent Intensity</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean Florescent Intensity</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial Infarction</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Matrix Metalloproteinase-9</td>
</tr>
<tr>
<td>MMW</td>
<td>Middle Molecular Weight</td>
</tr>
<tr>
<td>MP</td>
<td>Microparticle</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear Factor κappa B</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PECAM</td>
<td>Platelet-Endothelial Cell Adhesion Molecule</td>
</tr>
<tr>
<td>PerCP</td>
<td>Peridininchlorophyll protein</td>
</tr>
<tr>
<td>PFP</td>
<td>Platelet-free Plasma</td>
</tr>
<tr>
<td>PMP</td>
<td>Platelet-derived Microparticle</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome Proliferator Activated Receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>PPP</td>
<td>Platelet-poor Plasma</td>
</tr>
<tr>
<td>PPRE</td>
<td>Peroxisome Proliferator Response Element</td>
</tr>
<tr>
<td>PRP</td>
<td>Platelet-rich Plasma</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PSGL-1</td>
<td>P-Selectin Glycoprotein Ligand-1</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated Kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SSC</td>
<td>Side Scatter</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducers and Activators of Transcription Protein</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue Factor</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular Cell Adhesion Molecule-1</td>
</tr>
<tr>
<td>WAT</td>
<td>White Adipose Tissue</td>
</tr>
</tbody>
</table>
Introduction

Microparticles

Initially characterized as inert, dust-like particles (P. Wolf, 1967) the role of microparticles in different diseases has been highlighted by various studies over the last decade. Microparticles are small heterogeneous cell-derived vesicles, between 0.1 and 1.0 μm in size (Boulanger, 2010), that are shed from various cell types either spontaneously or in response to stimuli such as shear stress (Miyazaki et al., 1996), physiological agonists, pro-apoptotic stimulation and/or damage (Rautou, Vion, et al., 2011). Though microparticles are shed from cells under physiological conditions, many studies have linked higher levels of circulating MPs with diseases such as atherosclerosis (Boulanger, 2010), diabetes (Amabile, Rautou, Tedgui, & Boulanger, 2010), obesity (Simak & Gelderman, 2006), cancer (Owens & Mackman, 2011), and auto-immune disorders such as rheumatoid arthritis and sepsis (Owens & Mackman, 2011).

1.1. Microparticles and other Extracellular vesicles

There are three major extracellular vesicles released from the cells; exosomes, microparticles and apoptotic bodies (Thery, Ostrowski, & Segura, 2009). Microparticles are spherical vesicles with an intact lipid bilayer that are released from the cell under both physiological conditions and after cellular activation and apoptosis (Lynch & Ludlam, 2007). A crucial step in the formation of MPs is the loss of plasma membrane symmetry which results in expression of Phosphatidyl Serine (PS), a key marker for identification of MPs through flow cytometry, on the outer leaflet of the membrane (Simak, Gelderman,
Yu, Wright, & Baird, 2006). Due to high PS expression MPs are often characterised as being Annexin V positive, which is a protein that binds to PS (Rand et al., 2010). In addition to PS, MPs also express cell surface antigens which highlight their origin, for example, CD14 for monocytes, CD144 for endothelial cells, and CD162. Further, MPs have also been identified to carry proteins, transcription factors, lipids, RNA, microRNA, lipids and organelles (Mause & Weber, 2010).

Exosomes are smaller microvesicles, about 40 nm to 100 nm in diameter that are formed within the cytoplasmic compartment and stored intracellularly in multivesicular compartments until their release (Mathivanan, Ji, & Simpson, 2010). Unlike MPs, exosomes are released outside the cell when these multivesicular bodies fuse with the plasma membrane (Simpson, Jensen, & Lim, 2008). Similar to MPs, exosomes are intact vesicles having a lipid bilayer (Stoorvogel, Kleijmeer, Geuze, & Raposo, 2002). Due to a different mechanism of release, exosomes have very little or no PS expression and, therefore, cannot bind Annexin V (Simpson et al., 2008). However, they can be identified by exosome-specific antigens which include the membrane tetraspanin protein CD63, tumor susceptibility gene 101 (TSG101) and lysosomal associated membrane protein 1 (LAMP1) (Mathivanan et al., 2010). Similar to MPs, exosomes have also been showed to contain RNA, protein and miRNA (Simpson et al., 2008), and once released from the cell they are capable of causing signaling changes in target cell.

Apoptotic bodies are the largest of the microvesicles ranging from 1 μM to 4 μM in size (Sadallah, Eken, & Schifferli, 2011). They form exclusively during the late apoptosis stage of the cell and thus have a high PS expression (Elmore, 2007). Unlike MPs and
exosomes, apoptotic bodies have a permeable cell membrane and in addition to protein, RNA, miRNA contain significant nuclear material such as DNA (Gyorgy et al., 2011).

Table 1. Key differences between exosomes, microparticles and apoptotic bodies.

<table>
<thead>
<tr>
<th>Feature</th>
<th>Exosomes</th>
<th>Apoptotic Bodies</th>
<th>Microparticles</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Size</strong></td>
<td>50 – 100 nm</td>
<td>1000 – 4000 nm</td>
<td>100 – 1000 nm</td>
</tr>
<tr>
<td><strong>Origin</strong></td>
<td>Intracellular, Multivesicular Bodies</td>
<td>Cellular Fragments following Death</td>
<td>Blebbing of Plasma Membrane</td>
</tr>
<tr>
<td><strong>Detection</strong></td>
<td>Nanosight, Electron Microscopy, Western blot</td>
<td>Flow Cytometry, Electron Microscopy</td>
<td>Flow Cytometry, nanosight, Electron Microscopy</td>
</tr>
<tr>
<td><strong>PS Expression</strong></td>
<td>No/Low</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td><strong>Formation</strong></td>
<td>Constitutive and Cellular Activation</td>
<td>Late Apoptosis</td>
<td>Cellular apoptosis and apoptosis</td>
</tr>
<tr>
<td><strong>Characteristic features</strong></td>
<td>Annexin V binding &amp; cell-specific surface markers</td>
<td>Annexin V Binding, DNA and permeable membrane</td>
<td>LAMP1, CD63, TSG101</td>
</tr>
</tbody>
</table>

1.2. Microparticle Formation and Release

Microparticles are released from cells following the outward blebbing of the plasma membrane (Boulanger, 2010). Though there have been a number of studies on microparticles the exact mechanism of MP formation, remains unknown. It is believed that the loss of phospholipid symmetry and cytoskeletal arrangement are two key processes involved in the formation and release of MPs (Morel, Jesel, Freyssinet, & Toti, 2011).

The cytoskeletal rearrangement is essential for the outward blebbing of the plasma membrane. One of the key proteins governing this process is the cytoskeletal protein...
Regulators of actin polymerization have been described to control MP formation, for example, cytochalasin D, an inhibitor of actin polymerization, inhibits MP formation (Cauwenberghs et al., 2006). Inhibition of calcium dependent protease calpain, which is known to cleave talin and α-actin and has been shown to reduce MP formation in platelets and neutrophils (Yano et al., 1993). Recently, Rho-associated kinases ROCK-I and ROCK-II have been identified as key regulators of MP formation in endothelial cells. These kinases are regulated by caspase-3 and Rho-GTPase activity, and regulate cytoskeletal reorganization by controlling Myosin Light Chain Kinase activity. Rho-kinases have been shown to regulate MP release, for example, inhibition of Rho Kinases reduce EMP release (Coleman et al., 2001). Further studies have also highlighted that caspase-2 activity is an important regulator of ROCK-II, independent of ROCK-I identifying two separate pathways of MP release through ROCK (Sapet et al., 2006). To fully understand the role of ROCKs in MP formation, studies need to be performed in other cell types and with different stimuli. Though the complete pathway for leading to regulation of MP release has not been identified yet, these findings highlight that cytoskeletal arrangement plays a critical role in MP formation.

Externalization of Phosphatidylserine is the second most critical step in the formation of MPs (Morel et al., 2011). Under resting conditions, healthy cells maintain an asymmetric plasma membrane with negatively charged phospholipids, including phosphatidylserine (PS) reside on the inner leaflet (Zahra, Anderson, Stirling, & Ludlam, 2011). This membrane asymmetry is maintained mainly by three phospholipid transporter enzymes: flippases, floppases and scramblases. Cellular activation and/or apoptosis leads to
dysregulation of these enzymes leading to PS externalization that leads to MP formation and subsequent release (Bevers, Comfurius, Dekkers, & Zwaal, 1999). Flippases actively transport negatively charged phospholipids including PS and phosphatidylethanolamine (PE) against their electrochemical gradient towards the inner leaflet of the plasma membrane. Floppases, which work to counteract flippases, actively translocate PS to the outer membrane. Scramblases facilitate the diffusion of PS between both inner and outer membrane leaflets in an ATP-independent manner. In the case of MP formation via cellular activation, there is an increase intracellular calcium concentration that causes inactivation of flippase and activation of both scramblase and floppase (Morel et al., 2011). Studies in patients with Scott Syndrome, a hereditary condition where genetic defects in floppase lead to impaired PS externalization, have highlighted the importance of regulating these membrane enzymes. As a result these individuals have reduced ability to shed MPs and have lower circulating levels of platelet MPs (Albrecht et al., 2005; Toti, Satta, Fressinaud, Meyer, & Freyssinet, 1996). Similar results have also been replicated in a mouse model for Scott Syndrome. For example, these mice with ABCA1 (floppase) deficiency have reduced PS exposure and significantly lower circulating MPs than their littermate controls (Satta, Toti, Fressinaud, Meyer, & Freyssinet, 1997). PS externalization is believed to be a critical step in formation of MPs; however, recent data suggests that individual MP populations might not bind Annexin V suggesting a lack of PS on their surface. However, this could also be due to lower PS expression, which is below the threshold of current detection methods. Further studies are required to evaluate what stimuli control MP release and whether these stimuli dictate the level of PS expression on MP surface. For instance Zahra et al. (2011) have shown that endothelial
MPs released following apoptotic stimuli have a higher PS to endothelial marker (CD62E) expression ratio when compared to MPs released after cellular activation.

Recent reports of MP formation, independent of PS exposure, have implicated the role of lipid rafts in the formation of MPs. A recent study showed that increased cellular cholesterol levels upregulate MP release in monocytes (Davizon, Munday, & Lopez, 2010). Another study identified that platelet MPs have a higher cholesterol concentration than platelets themselves (Del Conde, Shrimpton, Thiagarajan, & Lopez, 2005).

The precise mechanism governing the release of MPs is an active area of research. Identification of pathways regulating MP release will help us target key signaling pathways that can be regulated to control MP release and also provide us with a deeper understanding of their potential functions.

1.3. **Sources of Microparticles**

In healthy individuals, circulating MPs are predominantly platelet derived, with endothelial and leukocyte derived MPs also present, but to a lesser extent (Rautou, Leroyer, et al., 2011). There are a number of other cells that contribute to circulating MPs such as red blood cells (Tissot, Rubin, & Canellini, 2010), podocytes (Burger et al., 2014), however their number is relatively small. Table 2 outlines the key surface markers for identification of MPs from different sources and the various stimuli for MP formation.
Table 2. Key MP surface markers and the specific triggers for MP release.

<table>
<thead>
<tr>
<th>Cell</th>
<th>MP Surface Markers</th>
<th>Stimuli Triggering Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet</td>
<td>CD31, CD40L, CD41a, CD42a, CD61, CD62P, CD63, CD107</td>
<td>Shear stress, exercise, pregnancy, CD40L, ADP, thrombin, collagen, apoptosis, A23187, calcium, TRAP, PMA, Cytochalasin D, LPS, epinephrine</td>
</tr>
<tr>
<td>T-Cell</td>
<td>CD3, CD4, CD8, CD11b, CD16, CD19, CD20, CD45, CD66b</td>
<td>Hepatitis C, storage, actinomycin D, PHA, etoposide, staurosporine, TNF-α</td>
</tr>
<tr>
<td>Monocyte</td>
<td>CD14, CD142, CD144, CD162</td>
<td>LPS, A23187, etoposide, TNF-α, Fas ligand</td>
</tr>
<tr>
<td>Endothelial Cell</td>
<td>CD31, CD34, CD51, CD54, CD62E, CD63, CD105, CD106, CD142, CD144, CD146</td>
<td>Shear stress, uremic toxins, CRP, Ang II, High Glucose, ROS, thrombin, IL-1α, TNF-α, LPS, PAI-1</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>CD11a, CD11b, CD15, CD16, CD35, CD49, CD59, CD62L, CD66b, CD142</td>
<td>PMA, Bacterial infection, ANCA, FMLP</td>
</tr>
<tr>
<td>Red Blood Cell</td>
<td>CD35, CD235a</td>
<td>A23187, pH, ATP depletion, decompression stress, diamide</td>
</tr>
</tbody>
</table>

1.4. Identification of Microparticles

Current microparticle research is limited to assessment of microparticles isolated from biological fluids, including plasma (Mause & Weber, 2010), urine (Smalley, Sheman, Nelson, & Theodorescu, 2008), cerebrospinal fluid (Hosseinzadeh et al., 2013), bronchi alveolar lavage (BAL) fluid (Mutschler, Larsson, Basu, Nordgren, & Eriksson, 2002), sputum (Porro et al., 2010), saliva (Berckmans et al., 2002) and synovial fluid (Berckmans, Sturk, van Tienen, Schaap, & Nieuwland, 2011). Most of the studies
regarding MPs involve assessing MPs in plasma that usually contain MPs from cells such as endothelial cells, leucocytes, platelets and erythrocytes (Morel et al., 2011). Recent studies have also highlighted the role of non-circulating MPs. For example, endothelial MPs have been shown to act as biological messengers carrying microRNA-126 onto vascular smooth muscle cells controlling their proliferation and neointimal formation (Jansen et al., 2013). Another study, isolated microparticles from atherosclerotic plaque and identified them as originating from different sources, mainly leukocytes (Rautou, Vion, et al., 2011). Though there is evidence that MPs are released within a number of tissues by multiple cells further investigation is required to identify, characterize and clarify the role of non-circulating microparticles.

Once isolated, there are a number of techniques that can be used to detect and enumerate MPs. Flow cytometry is considered the gold standard for MP identification and enumeration. Flow cytometry not only identifies microparticles based on their size and cell surface markers but also allows for enumeration of microparticles. Flow cytometry measures individual cells or microparticles based on their light scattering properties as they pass through a microfluidic system (Lacroix, Robert, Poncelet, & Dignat-George, 2010). Using Annexin V as a marker for PS, we can detect the total number of microparticles in suspension. Additional antibodies for cell surface markers can be used to determine the cellular origin, in addition to enumeration, of the MPs. The flow cytometer has a number of advantages and disadvantages. It is readily available at most research institutions and allows for relatively rapid analysis of large number of samples. We can assess several cell surface markers at the same time with limited sample use. Further, once setup the flow cytometer offers reproducible and reliable detection and
enumeration of MPs with minimum variability (Brown, Feairheller, Thakkar, Veerabhadrappa, & Park, 2011; Shet et al., 2003; Simak & Gelderman, 2006). However, flow cytometry also has a number of limitations. Firstly, it is not very useful in detecting MPs sized between 100-300 nm. Assessment of MPs within these limits is often troublesome as background signals may give false positives, and it’s often impossible to distinguish between background, which include debris, and actual MP related signal (Lacroix, Robert, Poncelet, & Dignat-George, 2010). As a result, during the assessment of MPs we are unable to enumerate completely the total MP count in a sample. Another limitation is that identification of the cellular origin of MPs requires the use of antibodies. Though with the use of antibodies conjugated with different fluorophores we can identify MPs of different origins, antibodies are known to bind weakly to surfaces with relatively low antigen density, which is fairly standard on MP surface. Due to limited antigen, antibodies might not bind effectively resulting in the loss of signal or false negatives (Dolo et al., 1998; Fourcade et al., 1995; Peterson et al., 2008).

Another alternative method for detection of microparticles is the use of Enzyme-Linked Immunosorbent Assays (ELISAs). ELISAs quantify MPs using Annexin V or antibodies for cell surface markers to capture MPs and detect them based on a secondary antibody. Detection of MPs has been used by a number of studies, especially in the enumeration of platelet MPs, and its reproducibility and reliability is similar to flow cytometry. Further, unlike flow cytometry we can detect MP of all sizes by ELISA and it allows for semi-quantitative assessment of MP levels in biological samples (Osumi et al., 2001; Perez-Casal, Downey, Fukudome, Marx, & Toh, 2005). There are some disadvantages to this technique, however. Isolation of microparticles needs to be performed as analysis could
be due to false positive from apoptotic bodies which also present Annexin V on their surface. Unlike flow cytometry where MPs are identified ELISAs individually assesses MP in bulk. Moreover, ELISAs don’t allow us to distinguish relative change in cell surface markers on MPs or allow us to differentiate between MPs based on size and amount of antigen expressed per individual MP. Similar to ELISAs, functional assays measuring procoagulant or prothrombinase activity, especially for platelet microparticles have been used in studies to semi-quantitatively enumerate MPs (Al-Massarani et al., 2009). Though effective to analyze specific functional activity the ELISA method fails to provide information about any other effect of these MPs such as cellular activation or oxidative stress.

Recent technology has emerged for the quantification of microvesicles. Nanoparticle Tracking Analysis (NTA) enumerates MPs and identifies their size by measuring the Brownian motion of these microvesicles in suspension. NTA allows for visualization of individual MPs and unlike flow cytometry can identify MPs in the range of 50 - 1000 nm. Furthermore, it provides information on particle size distribution and relative concentrations per size. The NTA system though commercially available, is expensive and not readily available (Gercel-Taylor, Atay, Tullis, Kesimer, & Taylor, 2012; van der Pol, Coumans, Varga, Krumrey, & Nieuwland, 2013). More in-depth research is required to determine how reliable this system is in measuring cell surface markers.

Some other approaches including electron microscopy, atomic force microscopy have been used to characterize microparticles. Though they can provide information about MP phenotype, the enumeration of MPs through these techniques is not possible. The
different methods use to visualize MPs, and their advantages and disadvantages are outlined in Table 3.

**Table 3. Advantages and limitations of techniques in MP measurement** (Adapted from (Lacroix, Robert, Poncelet, & Dignat-George, 2010))

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<th>Bulk Quantification</th>
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<td>Atomic Force Microscopy</td>
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<td>Electron Microscopy</td>
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1.5. Monocytic Microparticles

In healthy humans, monocytes form 2-8% of the total circulating leukocyte population. Arising in the bone marrow these cells have an average lifespan of a few days. Monocytes take an active part in the immune response where they serve as precursors for cells such as macrophages (Geissmann, Manz, et al., 2010). Further, differentiation of monocytes is extramedullary, meaning it is done outside the bone marrow, usually within the tissue (Geissmann, Gordon, Hume, Mowat, & Randolph, 2010). For example in the case of atherosclerosis, monocytes infiltrate the endothelium and differentiate into a macrophage after being stimulated by the cytokines in the plaque microenvironment. In the absence of a differentiating signal, they retreat to lymph nodes where they are removed by the process of efferocytosis. It must also be noted that there are a number of different subsets of monocytes, characterised by their cell surface antigens, which are involved at various stages of disease (Ley, Miller, & Hedrick, 2011; Woollard & Geissmann, 2010). The exact mechanism for monocyte differentiation and recruitment has yet to be discovered.

Monocyte-derived MPs (mono-MPs) usually form a small percentage of total circulating MPs (Satta, Freyssinet, & Toti, 1997). However, their levels have been observed to be significantly higher in diseases such as sepsis (Meziani, Delabranche, Asfar, & Toti, 2010), atherosclerosis (Leroyer et al., 2008), diabetes (Omoto et al., 2002) and obesity (Goichot et al., 2006). In addition to the pathways discussed already, several different stimuli have been identified to stimulate the release of mono-MPs. Some of the stimuli responsible for mono-MP release include calcium ionophore (Cerri et al., 2006), LPS (Ben-Hadj-Khalifa-Kechiche et al., 2010), TNF-alpha (Eyre et al., 2011), Fas ligand (Terrisse et al., 2010), and etoposide (Mastronardi et al., 2011). Mono-MPs serve multiple
roles in physiological and pathological states and are important biochemical messengers inducing signaling cascades which aid processes like coagulation, endothelial activation and cellular recruitment.

1.5.1 Mono-MPs and Coagulation

Mono-MPs have been shown to play a critical role in regulating the coagulation pathway. Tissue factor (TF), which promotes coagulation, and activated protein C (APC) and thrombomodulin, which both favour anticoagulant activity, are expressed on the surface of Mono-MPs (Satta, Freyssinet, et al., 1997). In addition mono-MPs may also express endothelial cell protein C receptor (EPCR), which promotes the activation of APC via the thrombin-thrombomodulin complex (Meziani et al., 2010; Perez-Casal et al., 2005). Under resting and physiological conditions, TF expression on mono-MPs is negligible. Activation of monocyte by various stimuli, however, has been shown to increase the release of TF positive mono-MPs. For example, after treatment with lipopolysaccharide (LPS), there is a significant increase in procoagulant TF positive mono-MPs (Aras et al., 2004). This is clinically relevant as in sepsis TF expression is significantly higher than thrombomodulin that favors increased coagulation (Satta, Freyssinet, et al., 1997). MPs released from LPS treated monocytes, however, also express TF pathway inhibitor (TFPI) (Bajaj, Ghosh, & Bajaj, 2007). More studies need to be performed to evaluate the pathway governing the release of TF and other associated proteins on the mono-MP surface, especially considering that TF-positive mono-MPs constitute the second largest pool of thrombogenic MPs after platelet-derived MPs (PMPs) (Aharon, Tamari, & Brenner, 2008; Poitevin, Cochery-Nouvellon, Dupont, & Nguyen, 2007; Rauch et al., 2000).
TF positive mono-MPs can also promote coagulation via their interaction with a number of other cells. For instance TF positive mono-MPs are capable of transferring procoagulant activity to neutrophils (Egorina, Sovershaev, Olsen, & Osterud, 2008). Further, THP-1 derived mono-MPs express CD15 which allows P-selectin/P-selectin glycoprotein 1 (PSGL-1) dependent fusion with activated platelets (Rauch et al., 2000). This is important as within the thrombus microenvironment TF positive MPs bind activated platelets increasing the local concentration of TF which promotes fibrin deposition (Chou et al., 2004; Falati et al., 2003).

1.5.2 Mono-MPs and endothelial cells

Mono-MPs play a critical role in the regulation of vascular homeostasis. Recent studies have highlighted that mono-MPs released from cells in response to various stimuli activate endothelial cells, which can subsequently lead to endothelial dysfunction. One such study showed that incubation of human umbilical vein endothelial cells (HUVECs) with mono-MPs dysregulated endothelial function. Though the nitric oxide (NO) production and the generation of reactive oxygen species (ROS) such as superoxide was unaffected, these mono-MPs caused endothelial activation via activation of PI3K and ERK1/2 pathways. Endothelial activation was achieved by regulating nitration of several key endothelial cell proteins leading to increasing nitrosative stress (Lovren & Verma, 2013).

Another study showed that mono-MPs released from LPS treated THP-1 cells contained the pro-inflammatory cytokine, interleukin-1 beta (IL-1β) and its associated inflammasome components, mainly NLRP3, ASC, and caspase-1. These mono-MPs,
when incubated with HUVECs, showed increased endothelial activation by upregulating cell adhesion molecules on endothelial cells including Vascular Cell Adhesion Molecule-1 (VCAM-1), Intercellular Adhesion Molecule-1 (ICAM-1) and E-selectin. Mono-MPs caused changes in expression of these proteins via activation of the NFkB and ERK pathways (Wang et al., 2011). This could have significant implications for diseases such as diabetes, atherosclerosis and obesity where pro-inflammatory, inflammasome activating stimuli can cause the release of endothelial activating IL-1β rich mono-MPs.

Mono-MPs also promote endothelial dysfunction by affecting endothelial cell angiogenesis, apoptosis and thrombogenicity. The endothelial cell membrane plays in crucial role in coagulation as the integrity of membrane negatively regulates coagulation due to the presence of several anti-coagulant molecules on the surface. Mono-MPs, however, have been shown to increase endothelial cell apoptosis and thrombogenicity. Loss of cell membrane integrity coupled with increased tissue factor leads to procoagulant activity. Mono-MPs have also been shown to regulate angiogenesis as treatment of endothelial cells with mono-MPs from starved-, calcium ionophore- and endotoxin-treated monocytes caused induction of tube formation. However, it should be noted that this particularly study failed to distinguish whether the effect on endothelial cells was from mono-MPs or monocyte-derived exosomes (Aharon et al., 2008).

1.5.3 Effect on other cells

Mono-MPs have also been shown to interact with other cells. Mono-MPs expressing Human Leukocyte Antigen1 and 2 in the plaque microenvironment promote further lymphocyte activation (Mayr et al., 2009). Mono-MPs promote a pro-inflammatory state
within podocytes and affect podocyte specific secretion of vascular endothelial growth factor (VEGF), which regulates the permeability of the glomerular membrane. These findings have important implications for kidney inflammation that may lead to fibrosis and development of proteinuria (Eyre et al., 2011).

1.5.4 Mono-MPs in Sepsis

In a study comparing circulating microparticles between patients with septic shock and non-septic individuals, it was found that septic patients had a significantly higher level of TF-positive mono-MPs. High level of circulating TF, in addition to reduced anticoagulant proteins APC and anti-thrombin promoted a procoagulant state. Septic shock patients had an overall higher level of circulating MPs, including MPs derived from platelets, endothelial cells and leukocytes (Mostefai et al., 2008). Another study evaluating individuals with meningococcal sepsis observed increased TF-positive MPs in patient plasma. These CD14-positive mono-MPs were linked with increased plasma thrombin levels and in vitro findings confirmed that these MPs promoted thrombin generation (Nieuwland et al., 2000). These results further strengthen the link between inflammation and thrombosis, and the significant role of MPs.

1.5.5 Mono-MPs in Hypertension

Elevated circulating MPs have become a common theme in diseases related to vascular damage. These MPs are derived from different cells types including platelets, endothelial cells and leukocytes including monocytes. Studies highlighting mono-MPs have been limited so far. One study showed that hypertensive patients had high levels of CD14-
positive mono-MPs along with elevated platelet-derived MPs (Nomura, Inami, Shouzu, Urase, & Maeda, 2009). Another study evaluated microparticles in hypertensive patients with diabetes. In this study the authors showed that after administration of efonidipine, a calcium channel blocker, a significant decrease in circulating mono-MPs was seen, only in diabetic hypertensive patients (Nomura, Kanazawa, & Fukuhara, 2002). This study highlights that MPs in hypertensive patients maybe be a contributing factor to the risk of developing complications such as atherosclerosis

1.5.6 Diabetes

Levels of MPs in diabetes have long known to be elevated, due to increased pro-inflammatory signaling or high blood glucose levels. Consistently, levels of mono-MPs have been strongly linked with complications of diabetic retinopathy (Ogata et al., 2005; Ogata et al., 2006; Omoto et al., 2002). Diabetic patients often have elevated angiotensin II (Ang II) levels. One study highlighted that blocking Ang II receptors significantly reduced the release of mono-MPs, attenuating vascular damage in patients with type 2 diabetes. This presents as a novel strategy where Ang II receptor blockade in conjunction with statins might significantly reduce the risk of atherosclerosis in such patients when compared to statin therapy alone (Nomura, Shouzu, Omoto, Nishikawa, & Iwasaka, 2004).

1.5.7 Mono-MPs and atherosclerosis

Monocytes play a critical role in the development and progression of atherosclerosis. Many recent studies have as a result investigated the role of mono-MPs in
atherosclerosis. In one of these studies, researchers injected mono-MPs isolated from THP-1 cells into a ApoE/- mouse model. These mice showed accelerated atherosclerosis development with increased macrophage and T cell infiltration (Hoyer et al., 2012). Another study highlighted the role of mono-MPs in recruiting additional monocytes to the plaque microenvironment by transferring ICAM-1 to endothelial cells. Increased monocyte infiltration has been shown to be a critical step in the development of atherosclerosis, therefore, this MP-mediated ICAM-1 transfer may accelerate plaque progression (Rautou, Leroyer, et al., 2011). Mono-MPs have recently been shown to cause macrophage and foam cell apoptosis, which has been known to accelerate the development of atherosclerosis by increasing inflammation and plaque instability (Distler, Huber, et al., 2005; Huber et al., 2007). Another recent study isolated MPs from the human atherosclerotic plaques and showed an increased leukocyte MP count in plaque environment supporting these findings. Taken together these studies highlighted that mono-MPs recruit monocytes and macrophages to the plaque and led to higher apoptosis. Further, mono-MPs promote endothelial cell proliferation and angiogenesis that leads to remodelling of the plaque microenvironment contributing to the development of disease (Leroyer et al., 2008).
ADIPONECTIN

2.1. Gene and Expression

Adiponectin (APN), also termed as Acrp30 (adipocyte complement related protein of 30kDa) (Scherer, Williams, Fogliano, Baldini, & Lodish, 1995), AdipoQ (Hu et al., 1996), GBP28 (Gelatin Binding Protein 28) (Nakano, Tobe, Choi-Miura, Mazda, & Tomita, 1996) and apM1 (adipose most abundant gene protein 1) (Maeda et al., 1996), was discovered in the mid-1990s by four groups using different approaches.

The adiponectin gene ADIPOQ in humans is located on chromosome 3q27, and it encodes for a 244 amino acid long polypeptide (M. Takahashi et al., 2000). The human gene shares 84% protein sequence identity with the mouse adiponectin protein. The coding sequence contains two introns and three exons (M. Takahashi et al., 2000). The adiponectin gene codes for a secreted protein which is mainly synthesised in the white and brown adipose tissue, and has also been shown to have some expression in skeletal muscles, cardiomyocytes and endothelial cells (Kadowaki et al., 2006). Plasma adiponectin levels range from 0.5 - 30 μg/ml (Maeda et al., 1996), which is significantly higher than any of the other adipokines. Low serum adiponectin levels or hypoadiponectinemia is often associated with obesity, diabetes, hypertension, dyslipidemia and atherosclerosis, with circulating adiponectin being inversely linked with poor disease outcomes (Szmitko, Teoh, Stewart, & Verma, 2007). Plasma adiponectin levels correlate inversely with body mass index (BMI) and visceral fat accumulation (Arita et al., 1999; Ryo et al., 2004) and previous studies have also highlighted gender dependent difference in circulating adiponectin levels with women having higher values.
Although this may arise because women contain higher body fat and may be explained by the interference of female sex steroid hormones (Xu et al., 2005). Further, the elderly have higher levels of circulating adiponectin that may be partly explained by the reduced adiponectin clearance due to a decrease in kidney function with age (Sattar & Nelson, 2008).

Research has shown that the gene expression in both human and mice is regulated at both transcriptional and post-transcriptional stages with PPARγ (peroxisome proliferator activated receptor γ) being a key player in regulating expression (Kadowaki et al., 2006).

2.2. Adiponectin Protein

Adiponectin protein contains four distinct domains; a short signal sequence that promotes hormone secretion, a short variable region which is different for every species, a collagen-like fibrous domain which forms the amino terminal for the secreted protein and a globular complement factor C1q like domain which forms the carboxy-terminal (Yamauchi, Kamon, Ito, et al., 2003). Circulating adiponectin exists in four major forms; cleaved globular adiponectin, low molecular weight (LMW) trimer (180 kDa), a middle molecular weight (MMW) hexamer and high molecular weight (HMW) 12 to 18-mer adiponectin (360 kDa). The complex forms are generated by the formation of disulfide bonds within the collagen-like domain of different monomers (Tilg & Moschen, 2008; Waki et al., 2003).

Circulating adiponectin in plasma consists mainly of the HMW and LMW plasma form. The globular isoform, which resembles the structure of tumor necrosis factor-alpha (TNFα) has also been shown to circulate at very low levels in plasma (Pajvani et al., 2004). Previous studies have demonstrated that the different isoforms have distinct
cellular targets and activate different signalling cascades in target cells. The general consensus is that the HMW has pro-inflammatory effects whereas the LMW favors the anti-inflammatory signaling cascade in target tissues, however, further studies are required to establish this with every target cell type (Neumeier et al., 2006).

**Figure 1. The Protein structure of Adiponectin.** For activity adiponectin requires post-translational modifications such as hydroxylation and glycosylation. After modification adiponectin molecules are secreted in the globular form as a trimer (~90 kDa), and in full length forms as LMW hexamers (~180 kDa) and HMW isoforms (12–18-mers; >400 kDa). (Goldstein, Scalia, & Ma, 2009)
2.3. Adiponectin Receptors

Three receptors for adiponectin have been identified so far; AdipoR1, AdipoR2 and T-cadherin. AdipoR1 is expressed ubiquitously on most cells, however its major expression is in skeletal muscles. AdipoR2 is expressed mainly in the liver (Tilg & Moschen, 2006). Human and mice AdipoR1 and AdipoR2 gene share 96.8% and 95.25% homology respectively. Further both AdipoR1 and AdipoR2 share 66.7% amino acids with each other (Yamauchi, Kamon, Ito, et al., 2003). Both receptors have been found to be integral proteins containing seven transmembrane domains, however unlike similar G protein-coupled receptors their N-terminus is internal and the C-terminal is external (Buechler, Wanninger, & Neumeier, 2010; Tilg & Moschen, 2006).

AdipoR1 and AdipoR2 both bind globular and high molecular weight adiponectin but to varying affinities. AdipoR1 has a higher affinity for globular adiponectin whereas AdipoR2 has a higher affinity for HMW adiponectin (Yamauchi, Kamon, Ito, et al., 2003). T-cadherin was discovered recently as an adiponectin receptor although, it lacks an intracellular domain (Hug et al., 2004). Further studies need to be carried out to elucidate the mechanism by which T-cadherin affects adiponectin signaling.

Once stimulated both AdipoR1 and AdipoR2 increase activity of AMPK, PPARα ligand, increase fatty acid oxidation in skeletal muscles and liver, potentiate glucose uptake, and repress inflammation and oxidative stress which are critical to development and progression of cardiovascular diseases (Shetty, Kusminski, & Scherer, 2009). Several studies have highlighted AdipoR1 and AdipoR2 to be the key adiponectin receptors though they often have opposing effects on regulation of glucose and lipid metabolism.
AdipoR2-/- mice, are lean and protected from diet-induced increase in glucose tolerance. The opposite has been shown in AdipoR1 null mice. In one study, the loss of AdipoR1 reduced the ability of mice to increase glucose and lipid metabolism (Yamauchi et al., 2007). Further investigation is required to establish the complete pathophysiological role of these receptors in mammalian physiology.

Figure 2. Adiponectin signaling pathways. Globular adiponectin exists as a trimer, whereas full-length adiponectin exists as; a Low Molecular Weight trimer, a Middle Molecular Weight hexamer, and a High Molecular Weight multimer. Whereas full length adiponectin binds to AdipoR1/R2 with equal high affinity, globular adiponectin has a relatively weak affinity to AdipoR2. The downstream signaling molecules of these receptors include PPARα, AMPK, and p38 MAPK. Overexpression of AdipoR1/R2 in myocytes leads to adiponectin-mediated PPARα, AMPK and p38 MAPK activation, glucose uptake, and fatty-acid oxidation. A new class of adiponectin receptor, T-cadherin is identified but more research is required to elucidate the signalling pathway initiated by adiponectin’s binding to it. Adiponectin-mediated downstream metabolic effects such as lipid oxidation and glucose uptake appear to be regulated by interaction of AdipoR1 and APPL1. (Figure Taken from Kadowaki et al., 2006)
Adiponectin receptors interact with APPL1, an adaptor protein, which links its receptors to their downstream signaling molecules, the exact mechanism of interaction, however, is still unknown (Deepa & Dong, 2009). APPL1 is composed of a PH pleckstrin homology domain, a phosphotyrosine domain that binds the intracellular domain of adiponectin receptors to cause signaling changes and a leucine zipper motif. APPL1 plays a critical role in adiponectin dependent signaling such as regulation of insulin signaling in skeletal muscles and relaying anti-inflammatory effects of adiponectin in endothelial cells. Studies have shown that increased expression of APPL1 enhances and decreased expression of APPL1 reduces adiponectin mediated effects such as lipid oxidation and glucose uptake in skeletal muscle cells (Buechler et al., 2010; Deepa & Dong, 2009; Mao et al., 2006).

2.4. Adiponectin Signaling Pathways

Adiponectin signaling involves a number of key signaling pathways such as adenosine monophosphate-activated protein kinase (AMPK), mitogen-activated protein kinase (MAPK), peroxisome proliferator-activated receptors (PPARs), signal transducer activator of transcription (STAT3), mammalian target of rapamycin (mTOR), and nuclear factor kappa beta (NFκB) signalling pathways that regulate metabolism, insulin signalling and inflammation (Dalamaga, Diakopoulos, & Mantzoros, 2012; Tilg & Moschen, 2006).

2.4.1. Adiponectin and AMPK Activation

Adiponectin signaling causes phosphorylation and activation of AMPK, which plays a central role in mediating adiponectin’s effects. HMW is known to activate AMPK in skeletal muscle cells and liver cells whereas globular adiponectin activates AMPK in skeletal
AMPK is also known as the master regulator of cellular energy as it acts as the cell’s energy sensor (Canto et al., 2009, Hardie et al., 2010). AMPK is activated when the cellular levels of ATP are depleted and it serves to elevate ATP levels by increasing glucose uptake by promoting translocation of glucose transporters (GLUT4) to the cell surface and increased lipid oxidation. AMPK also reduces ATP consumption by decreasing energy dependent mechanisms such as gluconeogenesis, lipid and protein synthesis (Mihaylova and Shaw, 2011). Suppression of AMPK activity attenuates the adiponectin mediated increase in glucose metabolism and fatty acid oxidation, highlighting that these adiponectin dependent cellular metabolic changes are driven via AMPK activation (Mao et al., 2006). Further studies have emphasized that adiponectin suppresses lipid synthesis by suppressing expression of hepatic sterol regulatory element-binding protein (SREBP) 1c expression in an AdipoR1/AMPk dependent pathway (Awazawa et al., 2009). AMPK activation also allows for the regulation of other key adiponectin driven pathways including PPARα, mTOR and p53. Further, through its interactions with mTOR adiponectin inhibits cellular proliferation and, by its stimulation of p53, causes cell cycle arrest and initiation of apoptosis. (Fujisawa et al., 2008; Sugiyama et al., 2009).

In addition to acting as the energy sensor for cells, AMPK controls inflammation within the body. Inflammation is a critical for survival, however, if it becomes persistent it can aggravate metabolic disorders such as obesity, atherosclerosis and diabetes. Several studies have shown, through pharmacological or silencing of cells, that inhibition of AMPK leads to increased inflammation and its activation leads to suppression of inhibition. One of the pharmacological drugs targeting activation of AMPK, metformin, reduces systemic
inflammation and circulating levels of inflammatory factors such as C-reactive protein and IL-6 in metabolic syndrome. Further, *in vitro* studies where AMPK is activated by using AICAR have shown suppression of LPS-mediated inflammatory responses in different cells.

There have been several keys papers reporting the role of AMPK as a regulator of inflammation in immune cells. AMPK controls inflammation by primarily targeting the NFκB pathway via a number of downstream mediators which include Sirtuin 1 (SIRT1), Forkhead box O (FoxO) and tumor protein p53. Xue et al. (2012) demonstrated that omega 3 polyunsaturate fatty acids, in particular docosahexanoic acid, suppresses LPS-induced NFκB signaling in macrophages by deacetylating p65 through the AMPK/SIRT1 pathway. Recently, Lin et al. (2015) reported that curcumin improved cholesterol efflux in THP-1 macrophage-derived foam cells by increasing expression of ATP-binding cassette transporter 1 (ABCA1) in a AMPK/SIRT1 dependent manner. The study highlights that AMPK is critical in cholesterol homestasis which is disrupted in atherosclerosis. AMPK phosphorylates and activates p53 which has been shown to inhibit the NFκB signaling. Kawauchi et al. (2008), further showed that p53 regulates inflammation by decreasing glycosylation of IkB kinase-beta (IκKβ) which phosphorylates and subsequently inactivates the inhibitor of NFκB p65, IκB-alpha.

In innate immune cells, AMPK activation regulates inflammation and protects against the development of cardiovascular diseases. Vasametti et al. (2015) recently demonstrated that metformin suppresses the differentiation of monocytes into macrophages via AMPK-depandant STAT3 inhibition. Further, metformin significantly reduced monocyte infiltration and thereby suppressed Angiotensin-II-induced atherosclerotic plaque formation.
and aortic aneurysm formation in ApoE(-/-) mice. Similarly, Zeng et al. (2014) showed that sitagliptin, a dipeptidyl peptidase-4 inhibitor attenuated the progress of atherosclerosis by activation of the AMPK pathway. In this study ApoE(-/-) mice treated with sitagliptin exhibited reduced circulating levels of monocyte/macrophage related inflammatory cytokines MCP-1 and IL-6 and suppressed monocyte infiltration in plaques.

One of the key inflammatory pathways in monocytes and macrophages that has been implicated in the pathophysiology of atherosclerosis is the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome complex. Rajamaki et al. (2010) showed that in response to cholesterol crystal NLRP3 inflammasome complex is activated in macrophage resulting in the processing and secretion of mature IL-1β and IL-18, key inflammatory cytokines in the development and progression of atherosclerosis. Silencing of the NLRP3 inflammasome led to complete abrogation of cholesterol-mediated IL-1β secretion. Duewell et al. (2010) showed that when LDLr-/- mice were bone marrow transplanted with NLRP3 deficient bone marrow they had significantly reduced early atherosclerosis. These studies together highlight NLRP3 inflammasomes as potential therapeutic target for atherosclerosis.

In addition to regulation of NFκB recent work has demonstrated that AMPK signaling regulates the activation NLRP3 inflammasome. Bullon et al. (2015) showed that inhibition of AMPK signaling by compound C increased NLRP3 activation and increased circulating serum levels of IL-1β in mice. Further, in patients with fibromyalgia, administration of metformin reduced NLRP3 mediated inflammation in blood cells. Zhao et al. showed that the compound astragaloside IV reduced endoplasmic reticulum stress mediated NLRP3 inflammasome activation in endothelial cells in an AMPK dependant manner. AMPK
inhibitor compound C abrogated astragaloside IV-mediated suppression of ER stress and NLRP3 activation in these cells. Li et al. showed that curcumin attenuated glutamate mediated ER stress and activation of NLRP3 inflammasome in hippocampal cells via an AMPK dependant manner. These findings suggest that AMPK is important in regulation of the NLRP3 inflammasome, however, no study thus far has evaluated the role of AMPK in regulation of the inflammasome pathway in monocytes/macrophages.

2.4.2. Adiponectin and PPAR signaling

Adiponectin signaling regulates the activity of PPARs which are nuclear, ligand dependent transcription factors that play an important role in the regulation of lipid metabolism, cellular energy use, cell proliferation, cellular differentiation, apoptosis and inflammation. There are three major forms of PPARs (α, β, γ), each dependent on a distinct ligand and regulate different cellular processes (Delerive, Fruchart, & Staels, 2001). Adiponectin is known to regulate PPARα and PPARγ. PPARα is highly expressed in the liver, skeletal muscle, kidney, heart and vascular wall whereas PPARγ is expressed mainly in adipose tissue, intestines and macrophages (Tsuchida et al., 2005). Adiponectin has been shown to upregulate the expression of PPARα ligands resulting in enhanced fatty acid metabolism and increased energy consumption. These changes in energy metabolism are driven by peroxisome proliferator response elements (PPRE) in promoter regions of genes such as acyl-CoA oxidase and uncoupling protein (UCP) (Yamauchi et al., 2007). Though studies identifying signaling pathways dependent on adiponectin receptors have been conducted, not much is known about different isoforms of adiponectin and their activation of PPARs.
2.5. Protective Effects of Adiponectin on Endothelial Function

The protective effect of adiponectin on endothelial activation and inflammation, which are critical steps in the development of atherosclerosis, have previously been investigated. Ouchi and colleagues found that adiponectin reduced TNFα mediated increase in VCAM-1, ICAM-1 and E-selectin by suppressing the NFκB pathway (Ouchi et al., 2000). Further, overexpression of adiponectin in an atherosclerotic mouse model (ApoE-/-) causes reduced expression of VCAM-1 in the aorta region (Okamoto et al., 2002). Recently, it has been highlighted that adiponectin reduces TNF-α mediated endothelial activation in vitro, via caveolin mediated ceramidase recruitment and activation in an AdipoR1 dependent signaling mechanism. These findings are also supported by research studies conducted on adiponectin knockout (KO) mice. These mice exhibit increased VCAM-1 expression on endothelial cells, enhanced leukocyte rolling and an almost five fold increase in leukocyte adhesion in their microcirculation (. Wang et al., 2014). In other studies, these mice have also been shown to have an exacerbated acute lung injury after treatment with LPS which is accompanied by increased expression of IL-6 in alveolar endothelial cells when compared to their littermate controls (Nigro et al., 2013).

Endothelial nitric oxide synthase (eNOS) is critical for endothelial function. Adiponectin has been shown to stimulate production of nitric oxide in endothelial cells in an AMPK-endothelial nitric oxidase dependent manner. Adiponectin KO mice have also been shown to have reduced mRNA and protein expression of eNOS. This was further supported by studies demonstrating that adiponectin KO mice displayed salt-sensitive hypertension that was ameliorated by administration of recombinant adiponectin, in an eNOS
dependent manner. This study is supported by in vitro data where adiponectin has been shown to increase capillary-like tube formation, migration and reduce apoptosis in response to starvation, in an eNOS/AMPK dependent fashion (Ouchi et al., 2004). Adiponectin mediated eNOS activation is carried out by AMPK dependent phosphorylation of eNOS at Ser1177 (Ouchi et al., 2000) and Ser633 (Chen, Montagnani, Funahashi, Shimomura, & Quon, 2003). This effect has been shown by both globular and full-length adiponectin.

APPL1 has been identified as a critical mediator in adiponectin mediated activation of eNOS. Several rodent studies have highlighted that APPL1 expression in obese mice and diabetic rats has been linked with an impaired vasodilator response of the small mesenteric vessels (Cheng et al., 2007; Schmid et al., 2011). However, overexpression of an active AMPK can rescue eNOS activity and increase bioavailability of NO, even during the suppression of APPL1 suggesting that AMPK acts downstream of APPL1 and is directly responsible for both phosphorylation of eNOS at Ser and its interaction with heat shock protein (Cheng, Hashmi, Mao, & Zeng, 2008). Some other studies have also highlighted a possible involvement of phosphoinositide 3-kinase (PI3 Kinase) in AMPK mediated adiponectin-induced production of eNOS (Tan et al., 2004; Xi, Satoh, Kase, Suzuki, & Hattori, 2005).

The key feature of oxidative stress is the increased production of vascular ROS, resulting in the quenching of NO and activation of proinflammatory signaling pathways such as protein kinase C and NFkB (Schleicher & Friess, 2007). Adiponectin improves the redox state in human vessels by restoring eNOS coupling in endothelial cells (Margaritis et al., 2013). Adiponectin has been shown to decrease production of ROS induced in
endothelial cells by a number of stimuli including, high glucose (Ouedraogo et al., 2006), native and oxidized low density lipid (LDL) (Plant, Shand, Elder, & Scott, 2008) and palmitate (J. E. Kim et al., 2010). This effect of adiponectin is mediated through its regulation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.

Adiponectin has also been shown to reduce high glucose-mediated endothelial oxidative stress through cyclic AMP/protein kinase A dependent pathway (J. E. Kim et al., 2010; Ouedraogo et al., 2006). This was further, supported by in vivo studies highlighting that aortic rings in adiponectin KO mice showed higher levels of superoxide anion and peroxynitrite, key drivers of oxidative stress and that their levels were decreased after these mice were administered recombinant adiponectin (Cao et al., 2009).

The increased eNOS activity not only protects the cell by limiting ROS production but also by decreasing monocyte adhesion. Monocyte attachment mediated by increased expression of adhesion molecules (including intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin) during endothelial activation play a critical and early role in the inflammatory cascade leading to development of atherosclerosis (Kobashi et al., 2005). Adiponectin inhibits leukocyte adhesion to the endothelial surface by attenuating expression of VCAM-1 and E-selectin by increasing the bioavailability of NO (Ouedraogo et al., 2006). This adiponectin-mediated decrease in expression of cell surface adhesion molecules has also been observed in animal models of diabetes and atherosclerosis (C. J. Li et al., 2007). Adiponectin mediated inhibition of leukocyte adhesion can be reversed by inhibition of eNOS, highlighting the importance of eNOS signaling as a mediator of adiponectin’s anti-inflammatory actions on endothelial cells (Tan et al., 2004).
Another key signaling pathway by which adiponectin controls cell adhesion molecule expression is by its regulation of the NFκB pathway. Adenoviral delivery of adiponectin to aortic tissue in atherosclerotic ApoE-mice and atherosclerotic rabbits has been shown to reverse increase expression in cell adhesion molecules (Okamoto et al., 2002; Tan et al., 2004). These studies have demonstrated that adiponectin’s effects are mediated by its inhibition of protein kinase A-dependent kinase and NFκB via both AMP kinase-dependent and AMPK-independent mechanisms (Wu et al., 2007). Interestingly, short-term treatment of endothelial cells with globular adiponectin stimulates the NFκB pathway, thereby increasing expression of adhesion molecules and monocyte chemoattractant protein-1 (MCP-1) via activation of the sphingosine kinase signaling pathway (Kase et al., 2007). These contradictory findings may be due to different actions and of various isoforms of adiponectin or different incubation times used in various studies. Recent studies have supported this hypothesis as there is evidence that different oligomeric forms of adiponectin may have opposite functions concerning modulating NFκB activity in myocytes and monocytes (Tsao, Murrey, Hug, Lee, & Lodish, 2002). Adiponectin has also been shown to inhibit high glucose mediated IkB phosphorylation, NFκB activity, and production of C-Reactive Protein in human aortic endothelial cells in vitro (Devaraj, Torok, Dasu, Samols, & Jialal, 2008).

Adiponectin plays a critical role in the regulation of endothelial apoptosis. In atherosclerotic lesions, endothelial cells are turned over at a higher rate due to local apoptosis that aids in the development of atherosclerosis. These lesions can be induced by a number of risk factors such as high blood glucose, angiotensin II, palmitate, and oxidized LDL (J. E. Kim et al., 2010). High molecular weight adiponectin inhibits caspase
3 activity in HUVECs in an AMPK dependent signaling pathway (Kobayashi et al., 2004). In HUVECs, the globular domain of adiponectin has been shown to inhibit angiotensin II-induced apoptosis in a dose-dependent manner. Activation of AMP kinase via APPL1 regulates the protective effects of adiponectin against angiotensin II cytotoxicity on vascular cells (Zhi, Pengfei, Xiaoyi, & Genshan, 2014). Interaction between eNOS and Hsp90 has been implicated in adiponectin mediated regulation of apoptosis in endothelial cells. Further, globular adiponectin through its activation of PI3K/AKT and AMPK kinase signaling pathways, has been shown to increase NO and inhibit high glucose-induced apoptosis and oxidative stress in HUVECs (Xiao et al., 2011). The effects of adiponectin in terms of vascular endothelial damage and apoptosis via AMP kinase activation are dependent on binding of adiponectin to AdipoR1 on the cell surface (Zhao, Zhao, Yi, & Zhang, 2011; Zhi et al., 2014).

Endothelial progenitor cells (EPCs) play a critical role in endothelial repair after vascular injury (Szmitko et al., 2003). Dysfunction and/or low numbers of EPCs are associated with impaired endothelial function (Fadini, Sartore, Agostini, & Avogaro, 2007). Animal studies, as well as clinical studies, have shown that plasma adiponectin levels are positively correlated with the number of EPCs in subjects with coronary artery disease (Shibata et al., 2005). Adiponectin promote angiogenesis and subsequent repair by not only increasing the number of EPCs but also improving their function (Xu, Wang, Lam, & Vanhoutte, 2010; Zampetaki, Kirton, & Xu, 2008). In vivo studies using a hind limb ischemia model have shown that the angiogenic repair was impaired in adiponectin KO mice. However, adenoviral delivery of adiponectin reversed endothelial damage and increased EPC-mediated repair by increasing availability of EPCs from the bone marrow,
increased recruitment of EPCs to site of injury and by promotion of EPC differentiation and tube-like formation (Okamoto et al., 2002).

In diabetic mice, treatment with cobalt protoporphyrin, an inducer of heme oxygenase-1 (an antioxidant), leads to accelerated vascular repair by improving the function of EPCs as a result of upregulation of adiponectin (M. Li et al., 2008). This evidence highlights the importance of adiponectin in the prevention of atherosclerotic progression by endothelial renovation and angiogenesis. Adiponectin effect on EPC recruitment and function has been shown to be mediated by activation of AMP kinase (Sambuceti et al., 2009). In a diabetic mouse model adiponectin improves EPC function by activating AMPK, inhibiting p38 MAP kinase and decreasing expression of p16INK4A (a senescence marker) (Chang et al., 2010; Janzen et al., 2006). Further, eNOS has been identified as a key promoter of EPC recruitment, and a recent clinical study shows that adiponectin protects against EPC dysfunction in diabetic patients by enhancing eNOS production and signaling (Wegiel et al., 2014).

Globular adiponectin has also been shown to significantly increase endothelial cell proliferation, in vitro migration, and angiogenesis by the AMPK/Akt pathways through increased expression of MMP-2, MMP-9 and vascular endothelial growth factor. The effect of globular adiponectin on vascular endothelial growth factor appears to be mediated by AdipoR1 while the effect on MMP-2 and MMP-9 is mediated by AdipoR1 and AdipoR2. Further, globular adiponectin decreases glucose levels and CRP-induced angiogenesis in human microvascular endothelial cells, with a concomitant reduction in MMP-2, MMP-9, and vascular endothelial growth factor (Adya, Tan, Chen, & Randeva, 2012).
Clinical and experimental studies have highlighted the role of adiponectin deficiency in the development of various vascular diseases. Clinical studies have shown that adiponectin acts as an independent cardiovascular risk factor and hypoadiponectinemia being predictive of impaired endothelial function in both peripheral and coronary artery diseases. Increased carotid arterial intima-media thickness (IMT) is considered a risk factor for coronary artery disease and consistent with this, adiponectin levels are inversely related to carotid IMT under both physiological and disease state (Lo et al., 2006; Nilsson et al., 2006). Further, high plasma adiponectin levels are associated with lower risk of myocardial infarction in healthy men (Pischon et al., 2004). In the Framingham offspring study higher adiponectin levels correlated with protection from future cardiovascular disease events (Ai et al., 2011). In another study, hypoadiponectinemia has been associated with increased risk of cardiovascular outcomes in patients with end-stage renal failure (Zoccali et al., 2002).

Consistent with the clinical observations it has been shown that adiponectin KO mice are more likely to develop vascular diseases, highlighting the protective role of adiponectin in atherosclerotic disorders (Okamoto et al., 2002). Adiponectin KO mice have marked increased in intimal hyperplasia in response to vascular injury and impaired endothelial vasodilatation (Matsuda et al., 2002; Ouchi, Ohishi, et al., 2003). Further, adenoviral-mediated full-length adiponectin and transgenic overexpression of globular adiponectin has been shown to significantly reduce lesion formation, decrease expression of VCAM-
1 (class A scavenger receptor) and TNF-α mRNA in the aorta of atherosclerotic prone ApoE-deficient mice. These mice after treatment also showed improved endothelial function and reduction in blood pressure (Okamoto et al., 2002; Yamauchi, Kamon, Waki, et al., 2003). These findings were consistent with studies in atherosclerotic rabbits where treatment with adiponectin markedly reduced atherosclerotic plaque area as well as decreased expression of adhesion molecules, including VCAM-1 and ICAM-1 (Li et al., 2007).

The generation of an adiponectin/ApoE double KO mouse model demonstrated accelerated development of atherosclerosis. This was due to adiponectin’s ability to decrease chemokine production in macrophages resulting in reduced T cell accumulation in atherosclerotic plaques (Okamoto et al., 2008). In these mice, adiponectin also caused a marked increase in anti-inflammatory cytokine IL-10 and tissue inhibitor of metalloproteinase-1 (TIMP-1) in macrophages, which inhibits activity of matrix metalloproteinase and thereby suppressing matrix degradation and increasing plaque stability (Kumada et al., 2004).

The majority of experimental studies demonstrate a protective role of adiponectin in development of atherosclerosis. However, a recent study where adiponectin KO mice were crossed with atherosclerotic prone LDL receptor null mice, and mice with overexpressing adiponectin were crossed with ApoE−/− atheroprone mouse models no correlation of circulating adiponectin with inhibition of atherosclerosis was seen. These transgenic mice showed that adiponectin had no effect on plaque size or cholesterol accumulation in the aorta suggesting that adiponectin does not influence plaque development at advanced stages of atherosclerosis (Nawrocki et al., 2010). This study
confirmed a need to conduct further studies evaluating the role of adiponectin and its receptors in the development and progression of cardiovascular disease.

2.7. Anti-inflammatory Role of Adiponectin

Inflammatory conditions such as obesity, insulin resistance, and diabetes mellitus play a central role in the development and progression of vascular disease (Libby, Okamoto, Rocha, & Folco, 2010; Ouchi & Walsh, 2007, 2008). Adiponectin has been identified as a key anti-inflammatory molecule that confers vasculoprotection through a number of different mechanisms by its actions on various cell. C-reactive protein (CRP) an inflammatory cytokine and biomarker for inflammation (Yudkin, Stehouwer, Emeis, & Coppack, 1999), is an independent risk predictor of vascular disorders like coronary heart disease (Laaksonen et al., 2004; Ridker, 2003). CRP has also been shown to cause the release of inflammatory cytokines including TNF-α and activation of NFκB signaling pathway (Paffen & DeMaat, 2006). Adiponectin, by its activation of AMPK and inhibition of NFκB activity decreases CRP mRNA and protein in human aortic endothelial cells (Ouchi et al., 2000), and inhibit stimulation of NFκB signaling and TNF-α secretion from macrophages (Ouchi & Walsh, 2007). Adiponectin suppresses TNF-α-induced monocyte adhesion to human aortic endothelial cells, and reduces expression of cell surface adhesion molecules (Ouchi et al., 1999). Similarly, adiponectin attenuates VCAM-1 and ICAM-1 expression and activation of IL-8 and NFκB by reducing TNF-α expression in endothelial cells (Kobashi et al., 2005; Ouchi et al., 2000).

Previous studies have elucidated an inflammatory role for adiponectin in experimental liver disease. Adiponectin, by its suppression of TNF-α signaling, has shown to have
beneficial effects in mouse models of both alcoholic and non-alcoholic fatty liver disease. Delivery of adiponectin also improved liver function, reduced liver enzymes, decreased hepatomegaly and liver steatosis (Xu et al., 2003). In addition, these benefits of adiponectin have been shown to be mediated by AMPK signaling. Adiponectin significantly reduces liver fibrosis in an experimental carbon tetrachloride liver fibrosis model (Kamada et al., 2003). It has also been shown to protect against LPS induced liver injury in KK-Aγ obese mouse model (Masaki et al., 2004). Further, adiponectin protects from hepatotoxicity after concanavalin-A-induced liver damage in lipodystrophic transgenic mice. In this study adiponectin protected hepatocytes from TNF-α mediated cell death (Sennello et al., 2005). Considering the importance of liver in regulating tissue metabolism and inflammation, the role of adiponectin in liver injury needs further assessment.

In mouse models of viral myocarditis adiponectin can exert anti-inflammatory effects. Cardiomyocytes of diabetic leptin-deficient ob/ob mice showed decreased expression of adiponectin. The lack of inhibition of TNF-α due to decreased levels of adiponectin may explain the accelerated viral myocarditis in these mice (Takahashi et al., 2005). Further studies showed that delivery of recombinant adiponectin and adenoviral adiponectin expression in cardiac cells reduced myocardial damage and improved survival in experimental viral myocarditis mouse model (Kanda et al., 2007; T. Takahashi et al., 2006). Finally, cardiac allografts transplanted into adiponectin KO mice showed severe acute rejection compared to transplants in wild-type mice. The adiponectin KO mice exhibited significantly higher CD4+ and CD8+ T cell infiltration and markedly increased circulating levels of TNF-α and IFNγ (Okamoto et al., 2009).
Adiponectin has also been suggested to play a role in regulating inflammation during sepsis. Tsuchihashi and colleagues initially showed that in a rat cecal ligation perforation (CLP) sepsis model circulating levels of adiponectin are negatively correlated with plasma endotoxin and TNF-α levels (Tsuchihashi et al., 2006). Finding from our laboratory observed decreased survival in adiponectin KO mice when compared to littermate controls after experimental CLP, and this difference was reduced after administration of recombinant mouse adiponectin (Teoh et al., 2008). Similarly, a recent study by Uji et al. demonstrated that adiponectin KO mice subjected to CLP have increased circulating levels of inflammatory cytokines and greater hepatic injury compared to wild-type mice (Uji et al., 2010).

2.8. Adiponectin’s Effect on Immune Cells

2.8.1 T cell

The role of adiponectin in T cell function has not been studied extensively. Adiponectin has been shown to increase T cell apoptosis and reduce proliferation of antigen-specific T cells (Wilk et al., 2013). The effects of adiponectin on T cell studied so far have all been due to the actions of innate immune cells. Adiponectin reduced macrophage-mediated T cell recruitment and decreased allogeneic T cell response (Okamoto et al., 2008) whereas adiponectin treatment of DC caused a reduction in T cell proliferation and higher frequencies of T regulatory cells (Okamoto et al., 2008; Tsang et al., 2011, Tsan et al., 2005). Thus, the direct effect of adiponectin on T cells requires further investigation.
2.8.2 Natural Killer Cells

Natural Killer (NK) cells are widely effected by adiponectin in the circulation. Adiponectin reduces IL-2 mediated cytotoxicity in murine NK cells (K. Y. Kim et al., 2006) which is dependent on AMPK mediated inhibition of NFκB signaling and by down-regulation of IL-2 mediated increase in IFN-γ. Apoptosis in NK cells is reduced by adiponectin, a mechanism which occurs by inhibiting expression FasL (Fas ligand) and TRAIL (tumor necrosis factor related apoptosis inducing ligand), key inducers of apoptosis (K. Y. Kim et al., 2006). Further investigation by Wilk and colleagues (2013) demonstrated that adiponectin KO mice have elevated circulating NK cells, however, cytotoxic degranulation of NK cells was decreased in these mice.

2.8.3 Macrophages:

A role for adiponectin in modulating macrophage phenotype and function has previously been described. Adiponectin suppresses transition of macrophages to foam cells by inhibiting the expression of the macrophage scavenger receptor (Tian et al., 2009). Through interaction with calreticulin and CD91 adiponectin promotes the phagocytosis of dead cells by macrophages. Adiponectin, like other members of the collectin family, has also been shown to aid clearance of dead cells, by binding to apoptotic cell-associated molecular patterns. Therefore, persistent hypoadiponectinemia could significantly impair the clearance of cell apoptosis (Takemura, Ouchi, et al., 2007).

Adiponectin stimulates expression of anti-inflammatory cytokines IL-10 and IL-1 receptor antagonist in human monocytes, monocyte-derived macrophages, and dendritic cells (A. M. Wolf, Wolf, Rumpold, Enrich, & Tilg, 2004). These findings are consistent with clinical
studies where patients with coronary artery disease have reduced expression of adiponectin receptors on monocyte surface and have reduced adiponectin-mediated IL-10 secretion (Kollias et al., 2011). Adiponectin mediated IL-10 expression also increases expression of tissue inhibitor of metalloproteinases-1 (TIMP-1) expression in human monocyte-derived macrophages (Kumada et al., 2004). Clinical studies in patients with coronary artery disease also show similar results where adiponectin has an inverse relationship with MMP9 to TIMP-1 ratio, which is a significant and independent predictor of atherosclerotic plaque stability (M. Cheng et al., 2008).

Finally, recent studies have highlighted that adiponectin plays a critical role in the transition of resting macrophages to anti-inflammatory M2 phenotype rather than the pro-inflammatory M1 phenotype. Peritoneal macrophages from adiponectin KO mice have been shown to have reduced expression of M2 markers including, arginase-1, macrophage galactose N-acetyl-galactosamine specific lectin-1, and IL-10 and increased M1 markers, including TNF-α, IL-6, and monocyte chemoattractant protein-1 (Ohashi et al., 2010). Further studies determining the role of specific receptors in regulating macrophage phenotype are required to completely understand the mechanism of action of adiponectin on macrophages.

2.8.4 Monocytes

Very few studies have investigated the role of adiponectin on monocytes. LMW and HMW adiponectin have been shown to have different effects on THP-1 monocyte cell line. Both isoforms activate AMPK and reduce expression of macrophage scavenger receptor (MSR). HMW induces IL-6 secretion whereas LMW reduces LPS-mediated IL-6 release
and induces secretion of anti-inflammatory IL-10 by negatively inhibiting NFkB signaling (Neumeier et al., 2006). Globular adiponectin decreased expression of pro-inflammatory microRNA 146b-5p in circulating monocytes of obese subjects suggesting an anti-inflammatory role of adiponectin (Hulsmans, Van Dooren, Mathieu, & Holvoet, 2012).

In another study adiponectin treatment primed monocytes into M2 macrophage phenotype. Adiponectin treatment increased expression of M2 markers mannose receptor, alternative macrophage activation associated CC chemokine -1 by an AMPK/PPARα dependent pathway. This has important implications for patients with atherosclerosis, diabetes and obesity who have reduced circulating adiponectin levels (Lovren et al. 2010)

2.9. Controversial Role of Adiponectin in Regulation of Inflammation

Though a large number of clinical and basic science studies suggest that adiponectin has an inhibitory effect on or has an inverse relationship with inflammation, the literature also has some reports of adiponectin having no effect or even having a pro-inflammatory role in development of metabolic diseases.

HMW adiponectin has been shown to activate the NFκB pathway in both THP-1 and U937 monocyte cell line (Huagen et al., 2007, Neumeier et al., 2006). Huagen et al., (2007) showed that HMW from HEK293 cells and globular adiponectin expressed in E. coli. They showed that adiponectin increased activity of NFκB luciferase construct and induced expression of pro-inflammatory genes, and release of inflammatory cytokines. This effect, however, was not observed when cells were treated with mutated adiponectin which lost
its ability to oligomerize into HMW adiponectin. The study failed to rule out LPS contamination, however, their effects were shown to be independent of LPS-induced TLR4 signalling as suppression of TLR4 pathway did not affect adiponectin mediated NFκB translocation and activation. Neumeier et al., (2006) evaluated the contrasting effects of different adiponectin isoforms on monocytes. They observed that both HMW (derived from mouse myeloma cells) and LMW adiponectin (expressed in insect cells) caused phosphorylation of AMPK and led to increased monocyte apoptosis. Only HMW was shown to induce phosphorylation of NFκB and induce release of inflammatory IL-6. LMW adiponectin increased the release of anti-inflammatory cytokine IL-10 and led to reduced phosphorylation of NFκB after cells were stimulated with LPS.

A recent study by Cheng et al. (2012) highlighted that adiponectin induced the expression of pro-inflammatory genes in human macrophages and T cells. The authors reported that after treatment with full length adiponectin primary human macrophages do not polarize into either the classical (M1) or the alternative (M2 phenotype). Further, through PCR array data they showed that adiponectin primed primary macrophages exhibited a predominantly pro-inflammatory gene expression which resembles to that of the inflammatory classical M1 phenotype. These results are opposite to the results observed by our group and Ohashi et al. (2010) where adiponectin was shown to promote the transition of resting macrophages into the alternative M2 phenotype. It should be noted that the adiponectin used in all three sources was expressed and purified from different cell sources. Cheng et al. (2012) used full length adiponectin expressed in Human Embryonic Kidney 293 (HEK293) cell line, Lovren et al. (2010) used LMW adiponectin expressed in mouse myeloma cell line whereas Ohashi et al. (2010) used adiponectin
expressed in hamster ovary cells. Ohashi et al. (2010) failed to mention the isoform used in their experiments. Though the two studies suggesting anti-inflammatory role of adiponectin conducted in vivo and ex vivo studies using adiponectin mouse to back their findings it should be noted that there are a number of genes in the inflammatory pathway that are not conserved in both mice and humans. Though at first glance the results seem opposite Cheng et al. (2012) address in their discussion that the difference in results might be attributed due to the fact that adiponectin drives an initial wave of pro-inflammatory signalling which desensitizes the cell to further pro-inflammatory stimulus. This notion is supported by previous work from the same group (Folco et al., 2009) and others (Park et al., 2007 and Tsatsanis et al., 2005) where it has been shown that adiponectin causes the release of TNF-α in the cell leading to an initial pro-inflammatory signalling involving IL-6 which sensitizes the cell to future inflammatory signalling from adiponectin itself as well as other stimuli. Park and colleagues (2007) showed that macrophages pre-treated with globular adiponectin for 18 hours followed by 2 hour LPS treatment showed an initial increase in TNF-α expression followed by a decrease in expression and increase in IL-10 secretion which inhibited LPS mediated inflammatory effect.

There are a number of issues that might be contributing to the controversial reports in literature with regards to adiponectin’s modulation of inflammation. A number of these studies use recombinant human adiponectin which is derived from a number of different sources including E. Coli, Human Embryonic Kidney (HEK) cell lines or High Five (Hi5) insect cells. Not many studies have performed comparative analysis on adiponectin from different sources. The difference in results might be due to the fact that the recombinant
adiponectin from different cellular sources was used in similar experiments of different studies. Another key factor is the contamination of recombinant with endotoxin. Researchers need to assess and report endotoxin levels in recombinant adiponectin use in their studies. The pro-inflammatory effect of adiponectin observed in some studies, especially in those where adiponectin was expressed in E. coli, might be as a result of contamination with LPS. Other results may be attributed to the different isoforms used in these studies. For example, as mentioned earlier, et al. showed that different isoforms of adiponectin have contrasting effects on regulation of inflammation in monocytes. They showed that HMW adiponectin induced activation of pro-inflammatory pathway whereas LMW adiponectin inhibited NFκB mediated inflammatory pathway. Further studies to elucidate the effect of globular, trimeric and adiponectin of different molecular weights on different cell types need to be performed to help resolve this controversy. Further, differences in in vivo findings can also be attributed to the type of disease model or the strain of mice. For instance in the case of atherosclerosis some studies are performed with LDL receptor KO mice whereas others used the ApoE-/- mouse model. The development and pathophysiology of atherosclerosis in both these models is different and as a result may lead to different observations. Finally, it has been observed that adiponectin exerts its anti-inflammatory effects by first inciting inflammation which leads to desensitization of further inflammatory signalling and increase in expression of genes in the anti-inflammatory pathway.

Similar to the contrasting *in vitro* findings there are a number of recent clinical studies showing that adiponectin has no correlation with development of cardiovascular disease. Lindberg et al. (2013) reported findings from the Copenhagen City Heart Study (n=5624)
and found out that though lower level of circulating adiponectin are linked with higher cardiovascular risk factors, there was increased mortality and worse major adverse cardiovascular events in the group which had higher levels of adiponectin in circulation. The authors conclude that adiponectin seems to have a protective effect in healthy individuals and a negative effect in patients who are at risk of adverse cardiovascular outcomes or mortality.

Another recent study by Kizer and colleagues (2012) reported that higher mortality was observed in group with adiponectin higher than the cutoff levels of 12.4mg/L in subjects with no cardiovascular disease, heart failure or atrial fibrillation (n=3272). In group 2 (n=1030), subjects with cardiovascular disease but no heart failure or atrial fibrillation there was a direct relationship between adiponectin levels and mortality. Finally, in Group 3 (n=383) which included patients with heart failure and atrial fibrillation there was a positive correlation between plasma adiponectin levels and mortality. In another study by the same group (Kizer et al., 2013) they evaluated 3290 participants aged 65 years or older without any prevalent cardiovascular disease. They found that the HMW and total adiponectin showed a U-shaped relationship with cardiovascular disease. At levels upto 20mg/L adiponectin had an inverse relationship with disease outcomes but at levels above 20mg/L adiponectin was directly related with cardiovascular disease. However after inclusion of putative metabolic intermediates the inverse effects in the lower range were abrogated, however the direct relationship of plasma adiponectin with cardiovascular disease at higher levels (20mg/L) persisted. There was no difference observed in findings with HMW and total adiponectin levels.
Finally another major study, the Heart and Soul Study evaluated 981 patients with existing ischemic heart disease. They observed that higher adiponectin levels were associated directly with heart failure and mortality amongst patients (Beatty et al., 2012).

Though numerous studies have highlighted the anti-inflammatory and insulin sensitizing effect of adiponectin, these conflicting recent clinical findings show that much more research needs to be performed to understand the effects of adiponectin in the body. Most of the findings have been reported in diseased patients and we need more data from normal subjects to delineate the pathophysiological role of adiponectin in the body. We also have to consider that the clinical findings are observational in nature and may be as a result of unmeasured confounding factors. Further, high adiponectin levels could also be as a result of a compensatory mechanism in patients with severe cardiovascular disease. The role of adiponectin in cardiovascular disease is not understood completely and more work needs to performed at a basic science, translational and clinical level before we move towards the possibility of using adiponectin as a therapeutic target.
3. Rationale and Hypothesis

Atherosclerosis results from a complex interplay of immune activation, inflammation and dyslipidemia and currently, remains the number one cause of death and disability worldwide (Hansson & Hermansson, 2011; Libby et al., 2009). Reducing inflammation and subsequent endothelial activation by altering monocyte-endothelial cell (EC) interaction may yield a novel therapeutic approach to reduce the global burden of atherosclerosis (Weber & Noels, 2011). MPs can cause endothelial injury and increased circulating levels of MPs has been correlated with incidence of coronary artery disease (Rautou, Vion, et al., 2011; J. G. Wang et al., 2011). Further, collective evidence primarily supports the theory that adiponectin is vasculoprotective and reduces the risk of atherosclerosis, diabetes and obesity (Ouchi et al., 2000; Ouchi, Kihara, Funahashi, Matsuzawa, & Walsh, 2003; Takemura, Walsh, & Ouchi, 2007).

3.1. Hypothesis

Endothelial dysfunction is critical to the development and clinical course of atherosclerosis. We hypothesize that MPs released from activated monocytes stimulate the NLRP3 inflammasome leading to endothelial cell activation and the adipokine adiponectin limits endothelial cel activation, in part, through reducing monocytic MP production and modulation of inflammatory pathways to inhibit endothelial cell activation.
3.2. Specific Objectives

I. IN-VITRO STUDIES

1. Evaluate the effects of human recombinant adiponectin on the generation of mono-MP in LPS-treated THP-1 cells, and to establish a time and dose-response curve.

2. Evaluate the effects of adiponectin on LPS-induced NRLP3 inflammasome activation and NFκB up-regulation in mono-MPs.

3. To evaluate adiponectin signaling pathways that impact generation of mono-MP isolated from Adipoq−/− and Adipoq+/+ mice

4. To evaluate the effects of adiponectin on mono-MPs induced endothelial activation.

II. EX-VIVO STUDIES

1. To evaluate the effects of LPS treatment on MP production from peritoneal monocytes isolated in adiponectin KO (Adipoq−/−) mice, and their wild-type controls (Adipoq+/+).

III. TRANSLATIONAL STUDIES

1. To evaluate the relationship between plasma adiponectin levels and circulating mono-MPs in humans.
4. Materials and Methods

4.1. Materials


4.2. Microparticle Isolation from THP-1 Cells

THP-1 cells (1×10⁶/mL; ATCC TIB-202) were incubated with or without 5 µg/mL LPS for 24 h in the absence or presence of 10 µg/mL recombinant human adiponectin. Mono-
MPs were isolated by differential centrifugation. THP-1 cells in culture were centrifuged at 500 g for 5 min at room temperature to remove cells. The supernatant was removed and centrifuged at 1500 g for 15 min to pellet the cellular debris. The resultant supernatant was then centrifuged at 20,000 g for 30 min at 4°C to pellet mono-MPs. Isolated mono-MPs were washed with cold phosphate-buffered saline (PBS) and re-suspended in either annexin V binding buffer (BioLegend) or RIPA buffer (Sigma) for flow cytometry and Western blotting respectively.

4.3 Animal studies

All procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and approved by the St. Michael's Hospital Animal Care Committee. Male adiponectin knockout mice (Adipoq−/−) and their wild-type littermates (Adipoq+/+) (Ma et al., 2002) were sourced from the Jackson Laboratory.

Peritoneal cavities of Adipoq−/− and Adipoq+/+ mice were lavaged with ice-cold PBS and monocytes isolated by flow cytometric immunophenotyping were suspended in RPMI-1640. Samples from five mice were pooled, seeded at a density of 2 × 10^5 cells/mL in 6-well cell culture plates and incubated at 37°C under 5% CO₂. Non-adherent monocytes were removed 4 h later and the remaining monocytes either left untreated or exposed to 5 μg/mL LPS. Mono-MPs generated after 24 h of incubation under the previously detailed culture conditions were isolated and processed for further analysis.
4.4. Microparticle enumeration

THP-1 derived MPs were diluted with annexin V binding buffer and stained with an Annexin V, Alexa Fluor® 647 conjugate for 30 min at room temperature in the dark. Each sample was subsequently mixed with AccuCount Ultra Rainbow Fluorescent Particles (Spherotech; 30 μL) for enumeration of MPs using a Gallios cytometer (Beckman Coulter).

For enumeration of circulating microparticles, blood was collected in 0.129 mol/L trisodium citrate solution. Platelet-free plasma (PFP) was prepared within 30 minutes using 2 serial centrifugations (10’ × 1,500 g, 2’ × 13,000 g). PFP was stored at −80°C until further use. For the MP enumeration, 35 μL of PFP was incubated for 30 min at room temperature with 5 μL conjugated monoclonal antibodies (AnnexinV-Alexa647 [Invitrogen] and CD14-BV421 [BD Biosciences] or isotype and concentration-matched control antibodies [BD Biosciences]. Spherotech beads were added to determine absolute MP numbers. The PFP was then diluted in phosphate-buffered saline lacking Ca^{2+} and Mg^{2+}. Enumeration for mono-MPs was carried out on a high-sensitivity flow cytometer (Gallios Instrument, Beckman Coulter).

Flow cytometry assays were standardized using a blend of fluorescent size-calibrated beads (100, 300, 500, and 900 nm [Megamix-Plus prototype, Biocytex, Marseille, France]) (Lacroix, Robert, Poncelet, Kasthuri, et al., 2010). To augment the resolution of MP detection from background noise and similarly sized particles, the flow cytometer was set up in the “W2” mode. As shown in Fig. 1A, the lower and upper limits of the MP gate
were defined using 300 nm and 900 nm beads, respectively (Robert et al., 2009). Data obtained from flow cytometry was analyzed using Kaluza® Flow Analysis Software.

4.5. Nanoparticle tracking analysis (NTA)

MP suspensions were analyzed using a NanoSight LM10 instrument (NanoSight, Amesbury, UK). Briefly, a monochromatic laser beam (405 nm) was applied to the dilute suspension of vesicles. A video of 60 s duration was taken with a frame rate of 30 frames/s, followed by particle movement analysis by Nanoparticle Tracking Analysis software. Basing on the principles of Brownian motion, the velocity of particles is used to calculate particle size by applying the two-dimensional Stokes–Einstein equation.

4.6. Transmission Electron Microscopy

Mono-MPs, isolated according to the previously mentioned method, were re-suspended in PBS, deposited onto Formvar-copper-coated grids then stained with 2% phosphotungstic acid, washed and air-dried before being imaged on a Hitachi H-7000 transmission electron microscope. Digital images were recorded using high-resolution camera AMT XR-60 and mono-MP diameters quantified using AMT Image Capture for TEM.

4.7. Western Blotting

Mono-MP and THP-1 (whole cell, cytoplasmic and nuclear) lysates were separated on Novex 4-20% Tris-glycine gels (Invitrogen) and electrotransferred onto nitrocellulose membranes. Membranes were probed with primary antibodies directed against Akt (1:2000), p-Akt (1:2000), AMPK (1:1000), p-AMPK (1:1000), ASC (1:500), caspase-1
(1:500), GAPDH (1:2500), IκBα (1:1000), p-IκBα (Ser32/36) (1:1000), IL-1β (1:1000), NFκB p65 (1:1000), and NLRP3 (1:1000). Immunoblots were subsequently incubated with the appropriate horseradish peroxidase-associated secondary antibody before the ensuing signals were visualized by enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific). Signal intensity was quantified using the ImageJ software (NIH) and normalized using either the stain-free method or against the corresponding GAPDH signal.

4.8. VCAM-1 and ICAM-1 expression

MPs were collected from the culture media of THP-1 cells maintained for the preceding 24 h with no stimulant, LPS (5 µg/mL), LPS (5 µg/mL) +adiponectin (10 µg/mL) or LPS (5 µg/mL) +Bay 11-7082 (10 µM). Serum-starved human umbilical vein endothelial cells (HUVECs; Lonza) were overlaid with mono-MPs (20 µg/mL) from one of the four groups and kept at 37°C. HUVECs were collected 2 h later and incubated in the dark with APC-anti-CD106 (VCAM-1) (1:100) and PE-anti-CD54 (ICAM-1) (1:100) for 40 min at 4°C. HUVECs that stained positive for 4’,6-diamidino-2-phenylindole were identified as live cells. VCAM-1 and ICAM-1 levels in these cells were assessed by flow cytometry.

4.9. Apoptosis Assay

Floating cells from the media of THP-1 cultures were washed with cold PBS and centrifuged at 500 g for 5 min at room temperature. The resultant pellets were re-suspended in Cell Staining Buffer (BioLegend), stained with annexin V-FITC (5 µg) and propidium iodide (PI, 5 µg) for 15 min at room temperature, and washed before flow
cytometry analysis. Annexin V-positive, PI-negative cells were defined as early apoptotic
cells; Annexin V-positive, PI-positive cells were defined as late apoptotic cells.

4.10. Statistical Analysis

Results are presented as means±SE. Differences between two groups were compared
with the Student's t-test. Intergroup comparison of means was performed by ANOVA.
Significance was set at $P<0.05$. 
5. RESULTS

5.1. Transmission Electron Microscopy

As mentioned, at present, size and morphology are the key criteria for distinguishing MP population from other microvesicles, including exosomes and apoptotic bodies. There are a number of methods, including differential centrifugation, employed to isolate MPs therefore we sought to establish that the microvesicle population we have isolated is, in fact, rich in MPs. To this aim, we first performed transmission electron microscopy on mono-MP isolated from cultured THP-1 cells to establish size and morphology. The mono-MPs from untreated cells and LPS (5 μg/mL for 24 hours) treated THP-1 cells were isolated, fixed, stained and imaged by AMT XR-60 camera at 20 000 × magnification. The micrographs in Figure 1 show that our suspension was rich in double-membrane vesicles, and the size of the vesicles is within the pre-established size range of 100-1000 nm. Though on the basis of electron micrographs we cannot definitively say that LPS caused an increase in size of vesicles, the images showed a general trend where mono-MPs from LPS stimulated cells were larger than those isolated from untreated cells.

5.2. Nanosight Tracking Analysis

Though Electron microscopy shows us the presence of MP in our preparation it is not quantitative. Even flow cytometry, the modern standard to establish mono-MP phenotype is unable to distinguish between vesicles and background flow cytometry noise below 300 nm. So to directly visualize and quantify the relative size of MPs from our preparation we used nanoparticle tracking analysis (NTA). NTA allows detection of nanoparticles as small as 10 nm and measures not only size and size distribution but also the concentration on
a particle to particle basis. Our results (Figure 2) show that the majority of the vesicle population in our preparation lies within the range of 100 nm to 1000 nm. Further, NTA confirms our observations from TEM images, that MPs from LPS (5 µg/mL) treated cells have on average a larger diameter (184 nm) than MPs released from untreated cells (164 nm). We also assessed the concentration of MPs released from both untreated THP-1 cells and cells treated with LPS 5 µg/mL. The concentration of vesicles released from cells treated with LPS was 2.5 fold higher than MPs released from untreated cells. Overall, the NTA analysis confirms that our chosen method of ultracentrifugation to isolate microvesicles from cell culture is rich in MPs.

5.3. LPS Induces Release of Mono-MPs

Under resting (untreated) conditions, THP-1 cells have been shown to produce MPs. We evaluated the dose-dependent relationship of mono-MP release from THP-1 cells after LPS stimulation. We incubated THP-1 cells in culture for 24 hours with LPS 1-10 µg/mL. MP release was assessed by flow cytometry, with MPs identified as Annexin V positive events with a particle size between 300 to 900nm. As shown in Figure 4, we observed a dose-dependent increase in the release of Annexin V-positive MPs from LPS stimulated THP-1 cells compared to untreated cells. In addition to MP marker, we also labeled MPs with CD14, a cell surface marker for monocytes. CD14 showed a similar change in expression following LPS stimulation, however, the relative levels of CD14 positive MPs was lower than Annexin V positive MPs owing to the relatively low CD14 expression on THP-1 monocytes. Based on our results and findings in the literature we chose a LPS concentration of 5 µg/mL.
5.4. Adiponectin Inhibits Mono-MP Release from THP-1 cells in vitro

Recent studies from our lab have established adiponectin to be an anti-inflammatory molecule. We have shown that adiponectin primes human monocytes to an anti-inflammatory M2 phenotype rather than the inflammatory M1 phenotype. Since inflammatory signaling pathways increase MP production, we hypothesized that adiponectin will reduce mono-MP generation. To see the effect of adiponectin on mono-MP production THP-1 cells were incubated with or without LPS 5 µg/mL in the absence or presence of 10 µg/mL adiponectin for 24 hours. Adiponectin significantly inhibited mono-MP production in both untreated and LPS stimulated cells (Figure 5, p<0.05), suggesting adiponectin disrupted cellular signaling responsible for MP release in monocytes.

5.5. Adiponectin Does Not Affect THP-1 Apoptosis

MPs are also released from the cell in response to cellular activation and pro-apoptotic signaling. To rule out that the decrease in MP release was due to an anti-apoptotic effect of adiponectin on monocytes we measured THP-1 cell apoptosis following adiponectin treatment. To this aim, we stimulated THP-1 cells with or without LPS 5 µg/mL in the absence or presence of 10 µg/mL adiponectin for 24 hours. We then used Annexin V/Propidium Iodide (PI) staining to detect apoptosis. Early apoptotic cells are Annexin V positive whereas late apoptotic/necrotic cells are positive for both Annexin V and PI. As suggested by Figure 6 our results showed that THP-1 cells treated with LPS 5 µg/mL showed a significant increase in total, early and late apoptosis when compared to
untreated cells. However, no significant difference in apoptosis were observed between untreated cells and those treated with adiponectin 10 µg/ml and between groups treated with LPS 5 µg/mL and cells stimulated with both LPS 5 µg/mL and adiponectin 10 µg/mL. The results suggest that Adiponectin-induced inhibition of MP release is not due to its regulation of monocyte apoptosis, rather due to some other signaling pathway within THP-1 cells.

5.6. Adiponectin Deficiency Promotes Release of Mono-MPs From Peritoneal Monocytes

To support our in vitro findings that adiponectin inhibits MP release we used an ex vivo model to study the effects of adiponectin. To this aim we isolated peritoneal monocytes from 8-week old adiponectin knockout (Adipoq<sup>−/−</sup>) and their littermate control (Adipoq<sup>+/+</sup>) mice, according the methods outlined earlier. The cells were then treated with LPS 5 µg/mL for 24 hours. Mono-MP release from peritoneal monocytes with or without treatment with LPS 5 µg/mL was then assessed by their positive staining for Annexin V (AlexaFlour 647), Ly6C-hi (PE-Cy7) and Cd11b (BV421) using flow cytometry. Annexin V as stated earlier is a marker for MPs, and Ly6C-hi and Cd11b were used to identify mono-MPs. Our results (Figure 7) show that peritoneal monocytes from Adipoq<sup>−/−</sup> mice produced significantly more mono-MPs than cells from Adipoq<sup>+/+</sup> mice after LPS stimulation and under basal conditions. These results provide further support our in vitro data that suggests protective effects of adiponectin in vascular inflammation via its actions on mono-MP release.
5.7. Adiponectin Reverses LPS activation of NLRP3 inflammasome in Mono-MPs

MPs from LPS-treated THP-1 cells have been shown to contain pro-inflammatory molecules such as Interleukin-1 beta (IL-1β) and other inflammasome components including Nod like Receptor Protein 3 (NLRP3), caspase-1 and Adaptor Protein Apoptosis-Associated Speck-Like Protein Containing CARD (ASC). These proteins have been linked to vascular dysfunction in many studies. We sought to find out the effect of adiponectin treatment on the contents of mono-MPs. Levels of IL-1β, NLRP3, cleaved caspase-1 and ASC were all enriched in Mono-MPs from LPS-treated THP-1 cells compared to Mono-MPs from untreated cells (Figure 8 and 9). Treatment of THP-1 cells with Adiponectin reduced the expression of IL-1β, NLRP3, cleaved caspase-1 and ASC. The data was normalized using Criterion TGX Stain-Free gels, and the results are shown in the graphs below.

The inflammasome proteins are closely linked to the NFκB signaling pathway. So we sought to detect the presence of proteins from NFκB pathway in the MPs. Western blot analysis (Figure 10) also showed a decrease in IκBα and NFκB levels in Mono-MPs after treatment with adiponectin with LPS. This increase was however attenuated after treatment of adiponectin.

The results show that adiponectin not only inhibits the release of pro-inflammatory mono-MPs but also regulates the contents of mono-MPs, which induce downstream signaling changes in other cells.
5.8. Adiponectin Attenuates Lipopolysaccharide Mediated NFκB Translocation in THP-1 cells

NFκB pathway is one of the primary inflammatory pathways activated by Lipopolysaccharides and has also been implicated in development and pathogenesis of atherosclerosis, diabetes and obesity. It is also responsible for modulation of NLRP3 inflammasome pathway. One of the primary steps in activation of NFκB pathway is the phosphorylation and subsequent translocation of the nuclear transcription factor p65 from the cytoplasm to the nucleus. We wanted to investigate if adiponectin regulates the release and packaging of mono-MP via modulation of the NFκB pathway. To this aim we incubated THP-1 cells with LPS 5 µg/mL, both LPS 5 µg/ml and adiponectin 10 µg/mL and LPS 5 µg/mL and the NFκB specific inhibitor Bay11-7082 10 µg/mL for 24 hours. We then isolated nuclear and cytoplasmic fractions and performed western blot. THP-1 cells incubated with LPS alone demonstrated greater nuclear translocation of NFκB p65 (Figure 11). The LPS mediated translocation, however, was significantly inhibited with co-incubation of adiponectin, which was similar to the effect of the NFκB specific inhibitor Bay11-7082.

The results show that the adiponectin attenuates pro-inflammatory signaling within THP-1 cells via regulation of the NFκB pathway.

5.9. Adiponectin Inhibits NFκB Translocation by Preventing Phosphorylation of IκBα

There are a number of ways by which the cells regulates NFκB pathway, including phosphorylation, degradation, and ubiquitination. IκBα is one of the subunits of NFκB
protein complex, and it also regulates its activity. It binds to the nuclear localization signal of NFκB causing the protein to remain in the cytoplasm. During an inflammatory response, IκBα gets phosphorylated and subsequently gets dissociated allowing NFκB p65 and p50 components to move freely into the nucleus and initiate a signaling cascade.

We hypothesized that adiponectin attenuates translocation of NFκB p65 into the nucleus by inhibiting LPS mediated phosphorylation of IκBα. Our results (Figure 12) show that LPS stimulation led to significant phosphorylation of IκBα. However, co-incubation of adiponectin significantly reduced LPS mediated phosphorylation. Similar, yet stronger inhibition was seen after co-incubation of THP-1 cells with LPS and Bay11-7082. The results highlight that adiponectin modulates NFκB signaling by regulating phosphorylation of IκBα.

5.10. Adiponectin Exerts Anti-inflammatory Effect via Activation of AMPK/Akt pathway

To identify the mechanism by which adiponectin exerts its anti-inflammatory effects we looked at the activity of AMPK and Akt, downstream targets of adiponectin signaling pathway. AMPK plays a central role in the adiponectin signaling pathway and has been shown to control downstream signaling cascades leading from adiponectin signaling. Our results indicate that adiponectin causes activation of both AMPK (Figure 13) and Akt (Figure 14) by initiating their phosphorylation. Our results suggest that adiponectin mediated mono-MP release appears to be linked to the AMPK/Akt cascade as well as the NFκB-IκB signaling module.
5.11. Adiponectin Reverses Activation of Endothelial Cells by Mono-MPs

Circulating adiponectin levels have been inversely linked with endothelial dysfunction. To investigate if adiponectin protects endothelial cells against mono-MP mediated injury we incubated HUVECs with mono-MPs (25 ng/mL) with or without adiponectin (10 µg/mL) for 4 hours. We observed that adiponectin prevented endothelial activation by inhibiting expression of VCAM-1 and ICAM-1 in endothelial cells (Figure 16). Further, we showed that adiponectin suppressed NFκB activation in HUVECs while activating the ERK pathway. These findings suggest that adiponectin protects endothelial cells from mono-MP mediated injury by regulation of the NF-κB pathway.

5.12. No significant relationship Between Circulating Mono-MPs with Plasma Adiponectin Levels

Some studies have shown that circulating MPs are elevated in disease state. To determine if the increase in mono-MPs in circulation is correlated with plasma adiponectin levels we took samples from patients with coronary artery disease and matched ten samples for age, sex, and BMI. Samples from patients with diabetes were not used for this study. We assessed total circulating MPs by Annexin V staining, total myeloid MPs by CD45 positive staining and total mono-MPs by looking at MP population that was positive for Annexin V, CD45 and Cd11b. We observed no significant differences between the different groups (Figure 17).
Figure 1. Transmission electron micrographs of mono-MPs. MPs were isolated from the media of THP-1 cells by serial centrifugation. Samples were fixed and examined under Hitachi H-7000 TEM at a magnification (A) ×20,000 and (B) ×40,000. Bar represents 500 nm in each case.
Particle Concentration x10^8/mL

A

Untreated
Mean: 164 nm

B

LPS-treated
Mean: 184 nm
Figure 2. Nanoparticle Tracking Analysis (NTA) conducted with Nanosight Microscope.

The size distributions of the microparticles were determined using a Nanosight NTA 2.3 instrument. MP size distribution for mono-MP isolated by serial centrifugation from (A) untreated THP-1 cells and LPS stimulated cells (B). Mono-MPs from untreated THP-1 cells had a slightly smaller size than those derived from LPS treated THP-1 cells. (C) Quantification of MPs shows that LPS stimulated cells produced about 2.5 times more MPs.
Figure 3. LPS promotes the production of mono-MPs from THP-1 cells in vitro. Mono-MPs isolated from THP-1 cells in culture after being stimulated with LPS at increasing concentration showed that the release of mono-MPs followed a dose-response curve. N = 3 independent experiments *P<0.05 versus control.
Fig. 4: Adiponectin decreases MP production by LPS-stimulated THP-1 cells. Mono-MPs generated from THP-1 cells after stimulation with LPS (5 µg/ml) and/or adiponectin (10 µg/ml) were isolated and microparticles were quantified using Beckman Gallios flow cytometer instrument using the MP gates established earlier. MPs were identified as events with positive Annexin-V signal which identifies Phosphatidyl Serine, a key marker for MPs. The number of mono-MPs generated by LPS-stimulated THP-1 cells was ~3.5-folds greater than that by quiescent THP-1 cells. THP-1 cells treated with both LPS (5 µg/ml) and adiponectin (10 µg/ml) exhibited significantly less annexin V-positive events when compared with those exposed to LPS (5 µg/ml) only. THP-1 cells treated with adiponectin (10 µg/ml) only showed a decrease in MPs detected by flow cytometry. N = 5 independent experiments *P<0.05 versus control, †P<0.05 vs. LPS only group.
**Fig. 5: Adiponectin does not affect THP-1 monocytes apoptosis.** THP-1 cells were treated with 5 µg/mL LPS in the presence or absence of 10 µg/mL adiponectin for 24 hours. Flow cytometry was performed to differentiate dead and apoptotic cells from living cells. Briefly, cells under different conditions were washed and stained with Annexin V- Alexaflour 647 and PI and then analyzed using the Beckman Gallios flow cytometer instrument. The combination of Annexin V-FITC and Propidium Iodide allowed for the distinction between early apoptotic cells (Annexin V-FITC positive), late apoptotic and/or necrotic cells (Annexin V-FITC and Propidium Iodide positive), and viable cells (unstained). THP-1 cells treated with LPS (5 µg/ml) and co-stimulation with LPS (5 µg/ml) and Adiponectin (10 µg/ml) showed significantly increased apoptosis when compared to untreated cells. However, no significant difference was observed between cells treated with or without adiponectin (10 µg/ml). *P<0.05 vs. control. N = 3 independent experiments.
Fig. 6: Monocytes from Adipoq^{-/-} (vs. Adipoq^{+/+}) mice produce more mono-MPs in the presence of absence of LPS stimulation. Peritoneal monocytes were isolated from Adiponectin knockout (Adipoq^{-/-}) mice and their littermate controls (Adipoq^{+/+}). Monocytes were cultured and mono-MPs were isolated after 24 hours from untreated cells and cells treated with 5 µg/ml LPS. To identify mono-MPs, flow cytometry was conducted with mono-MPs being identified as Annexin V positive, Cd11b positive and Ly6C-hi positive events. In the absence of LPS stimulation, monocytes from Adipoq^{-/-} mice produced double the number of AV+/Cd11b+/Ly6C-hi MPs compared to those from Adipoq^{+/+}. The same was true for LPS-stimulated monocytes from the two groups. *P<0.05 vs. control, †P<0.05 vs. LPS only group.
Fig. 7: Adiponectin inhibits LPS-stimulated increases in IL-1β and inflammasome proteins. Mono-MPs were isolated from THP-1 cells treated with or without 5 µg/mL LPS in the presence or absence of 10 µg/mL adiponectin for 24 hours. MPs isolated were lysed and subjected to western blotting. MPs isolated from LPS (5 µg/ml) showed a significant increase in NLRP3 and IL-1β protein levels. MPs isolated from cells co-treated with LPS (5 µg/ml) and adiponectin (10 µg/ml) showed a significant decrease in inflammatory protein content in comparison to LPS only treated group. Representative western blots and quantitative analysis are presented. Stain-free blots served as loading controls N = 3 independent experiments.

*P<0.05 vs. control, †P<0.05 vs. LPS only group.
Fig. 8: Adiponectin inhibits LPS-stimulated increases in caspase-1 and ASC inflammasome proteins. MPs isolated from LPS (5 µg/ml) showed a significant increase in caspase-1 and ASC protein levels. MPs isolated from cells co-treated with LPS (5 µg/ml) and adiponectin (10 µg/ml) showed a significant decrease in inflammatory protein content in comparison to LPS only treated group. Representative western blots and quantitative analysis are presented. Stain-free blots served as loading controls N = 3 independent experiments.

*P<0.05 vs. control, †P<0.05 vs. LPS only group.
Untreated LPS LPS+
NFKB/GAPDH Ratio

0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4

Adiponectin

†*

IKBα (39kD)

NFkB (65kD)

+                  +                  +
-                  -                  -
Ladder
Adiponectin

LPS

0.0 0.5 1.0 1.5 2.0

IκBα/GAPDH Ratio

Untreated LPS LPS+
Adiponectin

†*
Fig. 9: Adiponectin reverses LPS activation of NFκB in monocytic microparticles. Western blotting for NF-κB and IκBα were performed on lysates from mono-MPs recovered from 5 μg/mL LPS-stimulated THP-1 cells (in the presence or absence of 10 μg/mL adiponectin). Mono-MPs from cells treated with LPS had an increased expression of inflammatory proteins NFκB and IκBα, which was significantly inhibited after treatment with adiponectin (10 μg/ml). GAPDH served as the loading control. N = 3 independent experiments. *P<0.05 vs. control, †P<0.05 vs. LPS only group.
Adiponectin LPS Bay 11-7082

- + + + LPS
- - + - Adiponectin
- - - + Bay 11-7082

Relative nuclear/cytoplasmic NFκB

Untreated LPS LPS + Adiponectin LPS + Bay 11-7082
Figure 10. Adiponectin inhibits LPS mediated translocation of NFκB p65 in THP-1 cells.

A) Representative western blots for nuclear and cytoplasmic NFκB and B) semi-quantitative analyses for relative nuclear to cytoplasmic levels of NFκB in THP-1 cells cultured in the absence or presence of LPS, adiponectin and Bay 11-7082. LPS treatment caused significant translocation of p65, which was inhibited by simultaneous treatment of THP-1 cells with adiponectin. GAPDH served as the loading control. N=3 independent experiments. *P<0.05 vs. the treatment-naïve group; †P<0.05 vs. the group treated with LPS only.
Adiponectin

**P-IκBα**

**IκBα**

**GAPDH**

- **LPS**
- **Adiponectin**
- **Bay 11-7082**

![Graph showing relative p-IκBα levels](image)

- Untreated
- LPS
- LPS + Adiponectin
- LPS + Bay 11-7082

Relative p-IκBα
Figure 11. Adiponectin modulates LPS mediated activation of IκBa in THP-1 cells. A) Representative western blots, and B) semi-quantitative analyses for relative phosphorylation of IκBa in THP-1 cells cultured in the absence or presence of LPS, adiponectin and Bay 11-7082. LPS treated cells showed increased phosphorylation of IκB, which was significantly reduced following adiponectin treatment. GAPDH served as the loading control. N=3 independent experiments. *P<0.05 vs. the treatment-naïve group; †P<0.05 vs. the group treated with LPS only.
**Graph:**

- **X-axis:** Untreated, LPS, LPS + Adiponectin
- **Y-axis:** Relative p-AMPK/AMPK Ratio

**Legend:**
- Black bar: Untreated
- Red bar: LPS
- Green bar: LPS + Adiponectin

**Notes:**
- *: Indicates a significant difference compared to untreated controls.
- †: Indicates a significant difference compared to LPS treatment alone.

**Western Blot Images:**

- **Top:** P-AMPK
- **Middle:** AMPK
- **Bottom:** GAPDH

**Legend:**
- -: Untreated
- +: LPS, LPS + Adiponectin

**Markers:**
- Adiponectin
- LPS
Figure 12: Adiponectin exerts its anti-inflammatory effect by activation of AMPK. THP-1 cells were incubated with LPS 5 µg/ml with or without adiponectin 10 µg/ml for 24 hours. The cells were lysed, and western blot was carried out to assess activation of downstream pathways. Treatment with adiponectin caused significant phosphorylation of AMPK in THP-1 cells when compared to groups with untreated cells. LPS alone did not activate AMPK significantly. N = 3 independent experiments. *P<0.05 vs. control.
Figure 13: Adiponectin exerts its anti-inflammatory effect by activation of Akt. THP-1 cells were incubated with LPS 5 µg/ml with or without adiponectin 10 µg/ml for 24 hours. The cells were lysed, and western blot was carried out to assess activation of downstream pathways. Treatment with adiponectin caused significant phosphorylation of Akt in THP-1 cells when compared to groups with untreated cells. LPS alone did not activate Akt significantly. N = 3 independent experiments. *P<0.05 vs. control.
Figure 14. Adiponectin attenuates THP-1 derived mono-MP-induced endothelial activation. Quantitative flow cytometry results for VCAM-1 levels in HUVECs incubated with mono-MPs released by THP-1 cells maintained for 24 h in the absence and presence of LPS, adiponectin and Bay 11-7082. LPS derived mono-MPs caused significant increase in expression of VCAM-1. HUVECs treated with mono-MPs derived from THP-1 cells treated with both LPS and adiponectin had significantly less VCAM-1 expression than the cells treated with LPS derived mono-MPs. N=3 independent experiments.

*P<0.05 vs. the untreated group; †P<0.05 vs. LPS MPs only group
Figure 15: Adiponectin reverses activation of endothelial cells by mono-MPs. Mono-MPs (37.5 µg/ml), isolated from THP-1 cells after treatment with LPS (5 µg/ml), were incubated with HUVECs for 4 hours. Lysates collected from HUVECs after treatments were used to carry out western blot to assess for cell adhesion markers VCAM-1 and ICAM-1. HUVECs showed significantly increase in expression of VCAM-1 and ICAM-1 after treatment with mono-MPS, which was inhibited by the presence of adiponectin (10 µg/ml). N=2 independent experiments.
<table>
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<th>Baseline Characteristics</th>
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<th>High Adiponectin</th>
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**Table 1. Patients Characteristics.** Characteristics of patients from whom blood samples were used for assessment of correlation between circulating mono-MPs and plasma adiponectin levels.
Figure 16. Effect of adiponectin on circulating mono-MPs in patients with established coronary artery disease. A) Total MPs identified by positive staining for Annexin V, B) Total Myeloid derived MPs identified by positive staining for Annexin V and
CD45 and C) Total mono-MPs identified by positive staining for Annexin V, CD45 and Cd11b were assessed by flow cytometry. The groups with low adiponectin did not show any significant difference in MP number from the group with high circulating adiponectin levels.
6. Discussion

Cardiovascular diseases (CVD), according to the World Health Organization, account for almost one-third of the total worldwide mortality (Dahlof, 2010). Coronary artery disease and cerebrovascular diseases, which arise from atherosclerosis account for most of the mortality and morbidity resulting from CVDs (Hansson, 2005; Libby et al., 2009). The endothelium plays a critical and regulatory role in the maintenance of vascular homeostasis and endothelial cell activation plays an early and permissive role in the development of diabetes and obesity-induced endothelial dysfunction (Gimbrone & Garcia-Cardena, 2013; Weber & Noels, 2011). Several studies have determined that circulating levels of adiponectin are inversely associated with the risk of endothelial dysfunction, diabetes, obesity and myocardial infarction (Iwashima et al., 2004; Kojima et al., 2003; Ouchi et al., 2000; Shimabukuro et al., 2003). Further, adiponectin limits inflammation and the progression of atherosclerosis (Okamoto et al., 2002). Through the interference of NFκB activation, adiponectin decreases monocyte adhesion to endothelial cells by down-regulating VCAM-1, ICAM-1 and E-selectin levels (Ouchi et al., 1999; Ouchi et al., 2000). Adiponectin treatment also suppresses the formation of foam cells and the development of atherosclerotic plaques (Yamauchi, Kamon, Waki, et al., 2003). The present study adds to the already published work on the protective effects of adiponectin by delineating a novel mechanism of its protection against endothelial activation. We report that adiponectin, via activation of AMPK, Akt and NFkB pathways, suppressed the release of pro-inflammatory mono-MPs and limits their deleterious effects on endothelial cells.
Over the last decade, it has become increasingly evident that what was once thought of “dust particles” (P. Wolf, 1967), MPs act as key signaling molecules transferring information from parent cell to target cell. MPs transfer information in the form of cell surface receptors, mRNA, miRNA, protein and cytokines to not only other cells in the microenvironment but also to distant organs using the extensive circulatory system (Mause & Weber, 2010). The release of MPs is tightly regulated by cellular processes as well as external signaling molecules. Recently reported in vitro and in vivo findings suggest that MPs shed from monocytes may be key player in the pathogenesis of endothelial dysfunction and accordingly atherosclerosis (Hoyer et al., 2012; J. G. Wang et al., 2011). Other studies suggest that increased circulating levels of MPs in individuals with atherothrombosis, diabetes, hypertension, angiogenesis and inflammation (Amabile et al., 2010; Boulanger, 2010; Feng et al., 2010; Tushuizen, Diamant, Sturk, & Nieuwland, 2011; VanWijk, VanBavel, Sturk, & Nieuwland, 2003) contribute to the pro-inflammatory environment that culminates in endothelial derangement. Our results showing increased mono-MP production following LPS stimulation and then causing endothelial activation, together with findings demonstrating accelerated atherosclerosis in mono-MP-treated ApoE⁻/⁻ mice (Hoyer et al., 2012), support the contention that mono-MPs aid in development of atherosclerosis.

As mentioned earlier, elevated mono-MPs have already been linked closely with increased incidence of atherosclerosis, diabetes, and obesity. Similarly, adiponectin has been associated with cardiometabolic diseases. However the relationship is an inverse one, with a circulating adiponectin level representing a healthier state. Furthermore, as mentioned adiponectin limits inflammation and the progression of atherosclerosis
Considering the effect of adiponectin on monocytes and other myeloid cells, it's possible to hypothesize that adiponectin might control the release of pro-inflammatory mono-MPs and its downstream effects.

Our findings clearly demonstrate that adiponectin regulates mono-MP release, as treatment of THP-1 cells with adiponectin decreased mono-MP release both under basal conditions and after LPS stimulation. As mentioned in the introduction, there are two key drivers of MP production: cellular activation and apoptosis. To rule out that the suppression of mono-MP release was due to the effect of adiponectin on apoptosis, we investigated the effect of adiponectin on THP-1 apoptosis. Our results showed that adiponectin did not cause a significant increase or reduction in THP-1 apoptosis under basal conditions and after stimulation with LPS. These findings confirmed that the suppression of MP release was independent of apoptosis and probably due to adiponectin-mediated cellular signaling changes.

To further support our findings, we performed ex vivo experiments, by isolating peritoneal monocytes from Adipoq−/− and Adipoq+/+ mice. We demonstrated that monocytes from Adipoq−/− mice released more MPs than those from Adipoq+/+ littermates in the absence and presence of LPS stimulation, supporting our in vitro data that adiponectin regulates the release of mono-MPs.

Inflammasomes are multi-protein complexes that trigger immune responses. Extensive studies have highlighted that in response to cholesterol crystal and other key pro-inflammatory signaling molecules, monocytes activate the inflammasome complex, resulting in the release of pro-inflammatory cytokine IL-1β (Duewell et al., 2010; Rajamaki et al., 2010). Further LDLR−/− atherosclerotic mouse model with selective KO of
inflammasome protein NLRP3 showed significantly reduced development of atherosclerosis, highlighting a critical role of NLRP3 inflammasome in development and progression of atherosclerosis (Duewell et al., 2010). Wang and colleagues (2011) previously reported the presence of inflammasome components and IL-1β in mono-MPs released from LPS-stimulated THP-1 cells. We confirm that LPS treatment is associated with the enhanced presence of caspase-1, NLRP3, ASC and IL-1β in mono-MPs from THP-1 cells. Further, we highlighted that there was a significant reduction in levels of these proteins after simultaneous treatment with LPS and adiponectin. These mono-MPs also had reduced expression of both NF-κB and IκB-α, key pro-inflammatory signaling molecules. These results highlight the role of adiponectin as an anti-inflammatory signaling molecule as it not only protects from mono-MP induced injury by regulating their release but also by affecting the expression of key pro-inflammatory signaling proteins within mono-MPs.

The NFκB signaling cascade has been strongly linked with the development of inflammation in diabetes, obesity and atherosclerosis (Gil, Maria Aguilera, Gil-Campos, & Canete, 2007; Ho & Bray, 1999; Wilson et al., 2000) and has also been identified as a critical regulator of NLRP3 inflammasome priming in myeloid cells (Bauernfeind et al., 2009). Active NFκB signaling has been identified in human and mice atherosclerotic plaques (Gareus et al., 2008; Monaco & Paleolog, 2004). More importantly, the nuclear localization of NFκB p65 has been observed in mononuclear cells in patients with coronary artery disease and unstable angina (Real et al., 2010). Consistent with earlier findings we report that LPS stimulation led to an increased levels of nuclear localization of NFκB p65 protein in THP-1 cells. This was accompanied by enhanced phosphorylation
of IκBα. IκB proteins act as key inhibitors of NFκB signaling. In the absence of pro-inflammatory signaling, IκBα remains unphosphorylated and binds to p65 component of NFκB, keeping it localised to the cytoplasm. In the presence of pro-inflammatory signaling in response to LPS, IκBα becomes phosphorylated and releases p65, allowing its translocation into the nucleus leading to transcriptional activation resulting in the pro-inflammatory signaling cascade. In line with our hypothesis, we observed that adiponectin treatment significantly reduced phosphorylation of IκBα and resulting decrease in nuclear localization of NFκB p65. Similar results were observed in THP-1 cells treated with LPS and Bay 11-7082, a selective inhibitor of NFκB signaling. The results lend strong support to the notion that adiponectin acts as an anti-inflammatory molecule and exerts its actions on THP-1 cells through modulation of the NFκB signaling cascade.

AMPK has emerged as one of the critical regulators of cellular metabolism. Recent studies have highlighted that the metabolite sensing kinase AMPK through its suppression of the NFκB-pro-inflammatory signaling cascade is of paramount importance for the maintenance of long-term health and survival (Salminen, Hyttinen, & Kaarniranta, 2011). Adiponectin has been shown to exert its metabolic and anti-inflammatory effects via activation of AMPK (Canto & Auwerx, 2010; Mihaylova & Shaw, 2011). Further, AMPK and the serine/threonine specific protein kinase Akt have been shown to be critical regulators of metabolism in diabetes, with disruption in cross-talk leading to dysfunctional glucose metabolism (Schultze, Hemmings, Niessen, & Tschopp, 2012). We showed that adiponectin treatment of LPS-stimulated THP-1 cells resulted in an increase phosphorylation and thereby activation of both AMPK and Akt. These findings, in addition
to a regulatory role for AMPK in metabolism, highlight that adiponectin regulates mono-
MP release by modulation of the AMPK/NF-κB axis.

A number of studies have highlighted that MPs from different cells, including endothelial
cells, platelets, monocytes, adipocytes, promote endothelial dysfunction in a disease
state (Boulanger, 2010; Lovren & Verma, 2013; Mause & Weber, 2010). Endothelial
activation is a critical early step leading to endothelial dysfunction that is a key
distinguishing feature of cardiovascular diseases. Usually endothelial activation results
from activation of the NFκB system and results in the upregulation of cell signaling
molecules VCAM-1, ICAM-1 and E-selectin that aid the recruitment of circulating
leukocytes by enhancing their adhesion to the endothelium (Gareus et al., 2008; Kempe,
Kestler, Lasar, & Wirth, 2005). Similar to previous findings (J. G. Wang et al., 2011), we
observed that mono-MP; released after stimulation of pro-inflammatory signaling such as
that by LPS, caused endothelial activation by upregulating VCAM-1 and ICAM-1. As
highlighted earlier adiponectin modulates mono-MP release and contents, which would
suggest a decrease in endothelial activation. Our results indeed determined that mono-
MPs, released after concomitant treatment of THP-1 with LPS and adiponectin, caused
significantly less activation of endothelial cells, as measured by cell surface VCAM-1
expression, when compared to mono-MPs released from THP-1 cells treated with LPS
alone. Further, to confirm that these effects are mediated via regulation of NFκB signaling,
mono-MPs from THP-1 cells treated with LPS and NFκB inhibitor Bay 11-7082 showed
significant reduction in both VCAM-1 and ICAM-1 expression on endothelial cells.

Recent studies have highlighted that the levels of circulating monocyte–derived MPs are
increased in patients with coronary artery disease (Aharon et al., 2008; Boulanger, 2010;
Having identified adiponectin as a key regulator of mono-MP release we investigated a possible correlation between levels of plasma adiponectin levels and circulating mono-MPs in patients with stable, pre-established coronary artery disease. To our surprise, our results showed no significant differences between patients with low and high adiponectin levels. We evaluated total MPs in circulation by Annexin V positive staining, myeloid-specific microparticles were further identified by CD45 positive staining. Further, the monocyte-specific population was identified as being positive for Annexin V, CD45 and CD11b. No significant differences were observed in any of the groups. Though the results are not reflective of our in vitro and ex vivo findings, it should be noted that a larger sample size should be studied before making any concrete conclusion. In our present study, we used a small patient size of ten serum samples for each group. Any difference in such a small group of patients can be attributed to a number of factors that are highlighted in the limitations section.
7. Conclusion

In the current study we have provided significant advances in our knowledge of MPs in both physiological and pathological conditions. Current literature suggests a causal link between increased circulating levels of MPs and a number of diseases, especially inflammatory diseases. Though we know that MPs act as conveyors of biological messages between different cells and act as biomarkers of disease, the possibility of modulating release and packaging of MPs presents as an exciting therapeutic option for cardiovascular diseases.

Our findings support our hypothesis that adiponectin acts an anti-inflammatory molecule while highlighting a novel mechanism by which adiponectin exerts its vasculoprotective effects. Adiponectin inhibits mono-MP release from THP-1 cells and peritoneal monocytes. The latter is evident by increased production of mono-mps by peritoneal monocytes from Adipoq−/− mice in comparison to their littermate controls. This effect of adiponectin on mono-MP release is independent of cellular apoptosis that is a key driver of MP release in all cell types.

In addition to controlling the release of mono-mps, adiponectin also regulates the packaging of these pro-inflammatory MPs. The inflammasome pathway has been established as a key pathway in inflammatory diseases such as atherosclerosis, diabetes and obesity. Adiponectin modulates the expression of inflammasome complex components, including NLRP3, caspase-1, ASC and IL-1β and inhibits packaging of these inflammatory molecules with the MP double membrane. Further, adiponectin also
suppresses the expression of NFκB and IκB proteins within MPs reducing the inflammatory burden of these vesicles.

This reduction of pro-inflammatory mediators in mono-MPs was driven in part by the adiponectin dependent activation of AMPK and Akt pathway. Activation of adiponectin pathway leads to decreased phosphorylation of IκBα, a key regulator of NFκB pathway. THP-1 monocytes treated with adiponectin had significantly less p65 nuclear localization, leading to a reduction in pro-inflammatory signaling within the cell.

Adiponectin protected endothelial cells from microparticles-induced injury. Further, mono-MPs isolated from THP-1 cells treated with both adiponectin and LPS cause significantly less activation of endothelial cells, as measured by surface VCAM-1 expression, when compared to mono-MPs isolated from THP-1 cells treated with LPS alone. These findings highlight that the modulation of mono-MPs by adiponectin, both in number and packaging, reduce endothelial activation.

Human blood samples from patients with established coronary artery disease showed no significant relation between circulating mono-MPs and plasma adiponectin levels, which can be attributed to the small sample size.
8. Limitations

The study has a few limitations. The study evaluates the effect of adiponectin on mono-MP production. However, we have only used low molecular weight (LMW) adiponectin in our experiments. Circulating adiponectin consists of different forms including HMW. Therefore, the role of LMW adiponectin in relation to other isoforms need to be evaluated. Further, though THP-1 cells mimic most of the effects of monocytes, the effect of adiponectin on mono-MP needs to be evaluated with peripheral blood monocytes. Also, ex vivo studies have been used to evaluate mono-MP production in Adipoq\textsuperscript{-/-} mice, in vivo studies such as those evaluating circulating mono-MP in Adipoq\textsuperscript{-/-} compared with their littermate controls need to be performed. We unsuccessfully tried to optimize an assay for circulating mono-MPs in mouse blood, but not having pre-established markers for MPs in mouse blood limited our efforts.

We showed that adiponectin phosphorylates AMPK and Akt, however, more functional data highlighting the activity of these kinases needs to be performed. Further, downstream effects of AMPK pathway need to be assessed in detail. Lastly, experiments silencing AMPK or Akt through viral or pharmacological means need to be performed to confirm the involvement of these proteins.

One of the limitations of this study has been the use of THP-1 cells as a model for human peripheral blood monocytes. Though THP-1 has been used extensively as a model for human monocytes the findings of this study need to be replicated using human peripheral blood monocytes.
One of the major limitations in the detection, enumeration and use of MPs for treatment is the lack of technology to completely distinguish between different extracellular vesicles. We have used a number of different techniques to establish that our preparation of microvesicles was rich in MPs, rather than exosomes or apoptotic bodies. However, we do recognize that our preparation might be contaminated with exosomes or apoptotic bodies.

We did not observe any significant changes in our human study which can be attributed to a few limitations. The sample size for the study was not large enough to compensate for the variability. The samples were not fresh which affected quantification, as thawing is known to cause an increase phosphatidylserine expression that gives false positive Annexin V staining (Lacroix, Robert, Poncelet, & Dignat-George, 2010).

9. Future Directions

From originally being identified as dust particles to their role of biological messengers, our understanding of the role that microparticles play in (patho) physiological state has come a long way. Initially known to be released only by platelets we now know that MPs are released by most cells of the body and play an important role in disease development and progression.

A number of recent studies have given us a glimpse of the mechanisms regulating the release of MPs. Though the mechanisms seem diverse, the degree of consistency and selectivity of MP constituents suggest that the release of MPs is highly regulated. Similar to other membrane related events, studies have highlighted the involvement of cytoskeleton and calcium signaling, however, precise signaling molecules orchestrating
these events still seem elusive. Understanding the pathways of regulation of MP release is important to understand their role, especially in disease states. Future studies will have to make use of proteomics to not only identify key molecular targets for regulation of MP release but also to enhance our understanding of what regulates packaging of MPs in different cells after various stimuli. Our understanding of key signaling pathways regulating MP release and packaging will allow us to work towards new strategies to prevent and treat cardiovascular disease.

Another key issue is to find out whether MPs are causative of cardiovascular diseases or a consequence of disease. A number of recent studies such as by Wang et al. and Hoyer et al. (2012) seem to suggest that mono-MPs promote endothelial dysfunction or accelerate the development of atherosclerosis respectively. In vivo and in vitro finding have suggested that MPs play a critical role in inflammation, however in disease states such as obesity and atherosclerosis increased MPs could also be caused by the inflammation that accompanies these diseases. Future studies need to work towards establishing whether the increase in MPs in disease is causal or just a mere correlation.

We and others have shown that microparticles cause signaling changes in target cells, although further studies need to investigate the interaction of MPs with different cell types and the downstream signaling changes that follow within these cells both under physiological conditions and under stress or disease state. With respect to vascular diseases such as atherosclerosis, research into signaling in target cells needs to be performed in an in vivo setting to see how circulation (flow) affects MP derived signaling. Further, studies need to focus on cells from different locations, as cells react in a different
manner. For example, human coronary artery cells might be affected differently by mono-
MPs than the human umbilical vein endothelial cells used in our experiments.

Establishing a protocol for MP enumeration takes a lot of time and effort. There have been
a number of groups working towards standardizing MP isolation, enumeration and
assessment. However, the standards vary in every publication. Future efforts are required
to establish a universal protocol and evaluation standard by which publications are
judged. Further, more work needs to be done to quantify and establish markers for MPs
from mouse blood. Currently, there is not much research published with respect to
circulating MPs in mice or rats. Since rodent models are being used extensively in
research, more research relating to MPs in vivo will help us understand the mechanisms
modulating MP release and function.

Adiponectin has been shown to play a protective role in the development of
cardiometabolic diseases. It has also emerged as a key biomarker of cardiovascular
disease. A number of animal studies have shown the potential of adiponectin as a
therapeutic however the work has not been translated to clinical studies. Though the
general consensus is that adiponectin has a protective role one recent study used
adiponectin transgenic mice and atherosclerotic ApoE<sup>-/-</sup> and LDLr<sup>-/-</sup> mice to show that
there is no correlation between plasma adiponectin levels and atherosclerosis (Nawrocki
et al., 2010). These findings challenge clinical studies where adiponectin has been
consistently shown to be inversely related to the risk of obesity and cardiovascular
disease. These results beg the question whether the effects of adiponectin in vivo are the
same as those in human. Further, understanding of adiponectin in relation to
atherosclerosis, especially at different stages of atherosclerosis needs to be researched to come up with a definitive answer.

More research needs to be performed to evaluate the effects of different isoforms of adiponectin on various cell types. The literature is full of contradictory findings with respect to adiponectin’s modulation of the inflammatory cascade. These results are based on different cells types and the use of different isoforms. Therefore, efforts should be made to delineate the mechanisms by which different isoforms of adiponectin mediate their effects on different cells, especially those that play a key role in the development and progression of cardiometabolic diseases. Further, the exact role of adiponectin receptor signaling in different cells need to be evaluated in vitro and in vivo using mouse models such as using adiponectin receptor KO mice and making tissue-specific KOs of adiponectin receptors. Currently, three receptors for adiponectin have been identified, however, there is very little that we know about T-cadherin. These studies can be extended by crossing these KO mice with models of atherosclerosis, diabetes and obesity to investigate the role of adiponectin.

Low levels of adiponectin in patients have a causal role in the development of cardiometabolic diseases such as diabetes, obesity and atherosclerosis. Understanding mechanisms driving adiponectin mediated changes within cells will help us counteract this. For instance, it has been proposed that strategies targeting increased adiponectin receptor expression may help patients at risk of these diseases. One such idea is the development of PPARα agonist, which is a downstream protein activated by adiponectin. One agonist, Wy-14,643 has been shown to increase expression of AdipoR1 and AdipoR2 in WAT (Yamauchi, 2014). Another mechanism would be to activate AdipoRs,
using small-molecule compounds and activating antibodies against AdipoRs. A number of recent studies have highlighted novel compounds (Ikada-Iwabu, 2013). However, none of them have been shown to be useful in different models of disease. Further studies investigating new methods to activate adiponectin-induced signaling pathway can help us overcome the effects of decreased circulating adiponectin levels in high-risk population. There has been a lot of research presented relating to elevated circulating MPs in disease state. However, most of these studies show mere correlation. Future studies need to investigate the predictive nature of MPs in disease by detecting and enumerating MPs in prospective studies. Overall, advancement in our understanding of the constituents, generation, and targeting of MPs will be of great benefit. The potential for MPs to become either drug targets or drug carriers is an exciting concept that opens many lines of investigation and will ultimately benefit critically ill patients.
References:


Figure 3. Construction of MP gate using size-calibrated fluorescent beads ranging from 100-nm to 900nm. (A) Selection of bead subsets using the Beckman Gallios instrument with a set Side Scatter (SSC-A) threshold of 200. Gate A identifies the bead population and (B) construction of a MP gate with an upper limit of approximately 1 µm. Background noise from the bead mix is shown in the left gate of the scatter plots.
5.3. Characterization of MPs Using Flow Cytometry Beads

Flow cytometry is the current standard and most commonly used method for characterizing MPs both in terms of size and surface receptor expression. Therefore, we sought to phenotype MPs using highly sensitive flow cytometer, the Beckman Coulter Gallios. Some studies in the literature have highlighted that the Gallios is one of flow cytometers which better calibrated to study MPs. As mentioned in the methods section, MP gate was established using calibrated fluorescent beads sized between 0.3 to 0.9 μm and mono-MPs isolated from THP-1 cells in culture were prepared as mentioned in the methods and stained with the appropriate reagents. Though the current consensus is that MPs have a size between 100 nm to 1000 nm, modern flow cytometry machines cannot resolve signal below a threshold of 300 nm. Therefore, all studies involving flow cytometry will be looking at events within the range of 300 nm to 900 nm. Our results (Figure 3) confirmed that most of the population of the sample was within the desired range of 300 nm-1000 nm. In addition to size, the flow cytometer also allows us to phenotype MPs by measuring the expression of the surface antigen Annexin V, which labels Phosphatidyl Serine (PS), a marker for MPs. Our results showed that most of the events in our gated population were PS positive, therefore confirming the identity of these vesicles as MPs.