INFLUENCE OF PHYSICOCHEMICAL PROPERTIES OF SILVER NANOPARTICLES ON MAST CELL AND MACROPHAGE UPTAKE AND ACTVATION: ROLE OF SCAVENGER RECEPTOR B1

by

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Silver nanoparticles (AgNPs) are widely used in consumer products and the medical field for their antimicrobial properties. Some studies reported toxic effects of AgNPs but cellular uptake of AgNPs and AgNP-induced inflammation pathways are still not fully understood. Mast cells and macrophages are essential players of innate and adaptive immune response against invading foreign materials. Mast cells and macrophages express various receptors to help in recognition and clearance of invading pathogens and foreign molecules. Scavenger receptors such as scavenger receptor B1 (SR-B1) expressed by different types of cells, play an important role in biomolecule transport such as cholesterol transport inside cells. In addition, SR-B1 has been reported to be a multi-ligand receptor and can interact with negatively charged molecules leading to uptake and activation of signaling pathways. The aim of this study was to investigate role of SR-B1 and influence of physicochemical properties of AgNPs on mast cell and macrophage AgNPs uptake and subsequent inflammatory responses. We assessed mast cell and macrophage uptake of different types of AgNPs that differ in shape, size, charge, coating, and suspension media. The physicochemical properties were found to influence levels of AgNPs uptake by mast cell and

macrophage. In addition, inflammatory responses of mast cells and macrophages were found to be influenced by the physicochemical properties of AgNPs. Furthermore, SR-B1 was found to mediate AgNPs cellular uptake and influence the inflammatory response following AgNPs exposure.

These findings have implications in understanding mechanisms of AgNPs toxicity. Further, these findings suggest that modifications of the physicochemical properties can help in targeting or avoiding specific receptors. Therefore, safer AgNPs can be produced and used in biomedical applications.

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DEDICATION

To my family to whom I owe for being the person I am

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AcLDL	Acetylated low-density lipoprotein
AgNPs	Silver nanoparticles
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BC	Biocorona
Blt2	Blocking Lipid Transporter-2 (scavenger receptor B1 inhibitor)
BMDMs	Bone marrow-derived macrophages
BMMCs	Bone marrow-derived mast cells
BSA	Bovine serum albumin
C/EBP	CCAAT/enhancer binding protein
C110	110 nm spherical silver nanoparticles suspended in citrate
C20	20 nm spherical silver nanoparticles suspended in citrate
C60	Fullerene contains 60 carbon atoms

CD206	Mannose receptor
CeO_2	Cerium oxide
CLRs	C-type lectin receptors
CNS	Central nervous system
CNTs	Carbon nanotubes
COX-2	Cyclooxygenase-2
CVA	Coxasckievirus
DCs	Dendritic cells
EGF-TM7	Epidermal growth factor seven-transmembrane receptor
ER	Endoplasmic reticulum
FcRs	Fc receptors
FcγRII	IgG receptor
FcεRI	High affinity IgE receptor
GM-CSF	Granulocyte-macrophage colony-stimulating factor

GPCRs	G protein-coupled receptors
HAS	Human serum albumin
HDL	High-density lipoprotein
IFN-γ	Interferon-gamma
IL	Interleukin
iNOS	Nitric oxide synthase
IRFs	Interferon regulatory factors
ITAMs	Immunoreceptor tyrosine-based activation motifs
JAK	Janus-activated kinase
KLF4	Krüppel-like factor 4
Lamp	Lysosome-associated membrane proteins
LAT	Linker of activated T cells
LDL	Low-density lipoprotein
LIMP	Lysosomal integral membrane protein

LOX	Lectin-like oxidized low-density lipoprotein receptor
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
M1	Classically activated macrophage
M2	Alternatively activated macrophage
MAPKs	Mitogen-activated protein kinases
MARCO	Macrophage receptor with collagenous structure
MCP6	Mast cell protease 6
M-CSF	Macrophage colony-stimulating factor
MC_T	Mast cell contains only tryptase
MC_{TC}	Mast cell contains both tryptase and chymase
MDMs	Monocyte-derived macrophages
MHC	Major histocompatibility complex
MSR	Macrophage scavenger receptor

MWCNTs	Multi-walled carbon nanotubes
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor-κB
NGF	Nerve growth factor
NK	Natural killer
NLRs	Nucleotide-binding oligomerization domain like receptors
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
NOS2	Nitric oxide synthase 2
NPs	Nanoparticles
OPN	Osteopontin
OSM	Oncostatin M
OVA	Ovalbumin
OxLDL	Oxidized low-density lipoprotein

P110	110 nm spherical silver nanoparticles suspended in polyvinylpyrrolidone
P20	20 nm spherical silver nanoparticles suspended in polyvinylpyrrolidone
P550	Nanoplates with optical resonance peak at specific wavelengths of 550 nm suspended in polyvinylpyrrolidone
P850	Nanoplates with optical resonance peak at specific wavelengths of 850 nm suspended in polyvinylpyrrolidone
PAMPs	Pathogen-associated molecular patterns
PC	Protein corona
PGE_2	Prostaglandin E2
PI3K	Phosphoinositide 3-kinase
PLC	Phosopholipase C
polyI:C	Polyinosinic–polycytidylic acid
PPARγ	Peroxisome proliferator-activated receptor γ
PRRs	Pattern recognition receptors

PVP	polyvinylpyrrolidone
RAW	RAW264.7 Macrophage cell line
RIG-I	Retinoic acid-inducible gene-I
RLRs	RETINOIC acid-inducible gene-I like receptors
ROS	Reactive oxygen species
SCF	Stem cell factor
SiO2	Silicon dioxide
SR-B1	Scavenger Receptor B1
SREC	Scavenger receptor expressed by endothelial cells
SRs	Scavenger receptors
SSC	Side scatter
STAT	Signal transducer and activator of transcription
SWCNTs	Single-walled carbon nanotubes
TGF	Transforming growth factor

T_H	T helper cells
TiO_2	Titanium dioxide
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
VCAM-1	Vascular cell adhesion, molecule-1
VEGF	Vascular endothelial growth factor
VLDL	Very low density lipoprotein

CHAPTER 1: INTRODUCTION

Nanoparticles

Nanotechnology is a fast-developing field that includes the production and use of nanoparticles (NPs). NPs are defined as particles that have at least one dimension between 1-100 nanometers (nm). Nanotechnology is expected to reach 1 trillion $US with more than 1,800 consumer products introduced to the market by 2015 compared to 54 products in 2005 (Vance et al., 2015). Due to their small size, NPs exhibit unique properties that differ from their bulk form. These properties include physical and chemical properties such as high tensile strength, low weight, and electrical conductivity which leads to increased use of NPs in different products such as paints, cosmetics, food storage, and nanomedicines (Ma et al., 2008, Vance et al., 2015).

Silver nanoparticles (AgNPs) are one of the most widely produced and used NPs due to their broad-spectrum antimicrobial properties (Levard et al., 2013, Dong et al., 2012). Currently, AgNPs can be found in more than 400 consumer products such as water filters, cosmetics, wound dressings, toothpaste and household products which make them the most popular type of NPs (Marambio-Jones and Hoek, 2010, Vance et al., 2015). This increase in use of AgNPs products raises concern of its effects on environmental and human health (Varner et al., 2010, Luoma, 2008, Levard et al., 2012, Liu et al., 2012, Reidy et al., 2013, Sharma, 2013, Stevenson et al., 2013, Unrine et al., 2012, Yu et al., 2013b, Wijnhoven et al., 2009).

Factors Affecting NPs Toxicity

The toxic effects caused by NPs can be influenced by different factors that should be measured and characterized to ensure sustainability and safe use of NPs. First, variation in size of

NPs can modulate the level of cellular uptake or interaction with biological systems (Nel et al., 2006). The smaller size of the particle has more biological activity and toxicity due to their ability to reach and translocate easier than larger particles in biological systems (Oberdörster et al., 2005). Second, surface area of the NPs is another important factor in which reduction in size of particles to the nanoscale leads to a significant increase in the surface to volume ratio resulting in an increase in the number of particles per mass unit that are exposed to tissues or cells (Warheit, 2004). Third, NPs can be found in different shape such as spheres, tubes, and needles. The shape of NPs can affect their mobility, diffusion, deposition, and adsorption kinetics in biological systems (Park et al., 2003, Radomski et al., 2005, Jia et al., 2005, Seaton and Donaldson, 2005). Fourth, chemical composition of NPs can affect their biological activity. NPs can be found as inorganic such as metal or metal oxides, or organic such as fullerenes. In addition, some NPs can be found as hybrids such as quantum dots where the core consists of metalloid crystalline and an inorganic outer shell (Hardman, 2006). Also, chemical purity of NPs can affect biological interaction with the NPs such as presence of Fe or Ni as contaminants found within carbon nanotubes which are left over from the synthesis process (Maynard et al., 2004, Pulskamp et al., 2007, Jia et al., 2005, Bianco et al., 2007). Fifth, surface chemistry can influence aggregation of NPs. The increase in surface to volume ratio increases interparticle attraction by London and van der Waals forces, and electrostatic interactions (Werth et al., 2003). Coating of NPs is a method to stabilize NPs and to prevent aggregate formation by adding surface groups such as -–COOH, and –OH (Dutta and Hofmann, 2004). Adding these surface groups can impact the toxic effect of the NPs and their behavior in the biological media (Sayes et al., 2004, Sayes et al., 2006, Borm et al., 2006, Knaapen et al., 2002, Schins et al., 2002, Yin et al., 2005). Sixth, surface charge of NPs due to formation of an electric charge when dispersed in media can influence toxicity (Cosgrove, 2010). This charge

can control the reaction of the NPs with cells such as phagocytosis, and the adsorption of biomolecules and ions (Powers et al., 2006, Schins et al., 2002, Hoet et al., 2004, Lockman et al., 2008, Malik et al., 2000, Nigavekar et al., 2004, Quintana et al., 2002). Seventh, NPs can form aggregates in solution, powder, and gas phase and that depending on their size, chemical composition and surface charge. In addition, aggregation can depend on the production, storage, and handling conditions. The aggregation affects the stability of NPs suspensions and can affect the level of the toxic effect of NPs (Duffin et al., 2002, Wilson et al., 2002, Wick et al., 2007). Eighth, surface crystal defects which occur when the periodic crystalline structure of a material is interrupted. At defected sites, atoms tend to be more reactive than atoms at normal sites. Due to their large surface area, NPs tend to have more defects sites compared to bulk materials (George et al., 2012). The presence of surface defects was found to increase NPs toxicity through disruption of biomolecules via direct interaction or by increased ROS production as it has been reported that AgNPs with surface defects have higher toxicity on fish gill cell line and zebrafish embryos compared to normal AgNPs (George et al., 2012).

Nanoparticles Protein Corona

Upon NPs reaching a biological fluid, they are exposed to biomolecules mainly proteins that form a corona around the NP termed the biocorona (BC) or protein corona (PC) (Maiorano et al., 2010, Walkey and Chan, 2012, Cedervall et al., 2007). In addition, NPs may interact with other biomolecules such as sugars, nucleic acids and lipids (Wan et al., 2015, Hellstrand et al., 2009). The PC gives a new identity to the NPs and may carry new properties different from the original NP which requires investigators to re-characterize the NP after forming the PC. Over time, the binding or adsorption of proteins on the surface is controlled by affinity interactions of proteins to the NPs surface and by affinity based protein to protein interactions (Monopoli et al., 2011). The

PC can be classified into hard and soft based on the exchange time of its composition (Monopoli et al., 2011). The hard corona is the first strong bound layer of proteins and it has long exchange time, whereas, the soft corona is the second layer of proteins which is not directly bound to the NP and only needs seconds to minutes for protein exchange (Milani et al., 2012). The composition of the PC is unique for each NP and it depends on the physicochemical properties of NPs (Pozzi et al., 2014, Lundqvist et al., 2008, Deng et al., 2009, Qiu et al., 2010, Capriotti et al., 2012, Pozzi et al., 2015). In addition, some biological factors can influence the composition of PC such as the protein source e.g. plasma vs serum or animals vs humans, incubation time, temperature and pH of the media (Tenzer et al., 2013, Pozzi et al., 2015, Mahmoudi et al., 2013, Hajipour et al., 2014). By adsorption of proteins on the surface of NPs, a protein denaturation can result due to a decrease in thermal stability leading to conformational changes of the adsorbed proteins (Gheshlaghi et al., 2008, Wangoo et al., 2008, Deng et al., 2011). The toxic effects of NPs could be increased after formation of PC which increases internalization of NPs by phagocytic cells such as macrophages (Saptarshi et al., 2013).

Importance of AgNP Physicochemical Properties in Toxicity

Due to their antimicrobial activity, AgNPs are the most widely used NP on the market which led to increased concern of the potential for the release of AgNPs into the environment and its effects on human health (Varner et al., 2010, Schluesener and Schluesener, 2013, Levard et al., 2012, Reidy et al., 2013, Wijnhoven et al., 2009). The most common side effect caused by AgNPs is argyria where the skin turned to a bluish color (Wadhera and Fung, 2005, Lansdown, 2004). Some studies have reported different types of toxicities of AgNPs such as oxidative stress and DNA damage (Awasthi et al., 2013, Haase et al., 2012, Lim et al., 2012, van Aerle et al., 2013, Johnston et al., 2010). The AgNPs undergo different transformations in the environment and

biological system that depend on the type of preparation of AgNPs, types of surface coating, and environmental conditions, (Kruszewski et al., 2011, Cunningham et al., 2013, El Badawy et al., 2010, George et al., 2012, Kim et al., 2013, Levard et al., 2013, Liu et al., 2010).

When AgNPs are introduced into biological media, the NPs undergo physical and chemical changes. This includes losing or displacing the surface coating. Surface coating agents such as citric acid, polyvinylpyrrolidone (PVP) or amino acids are attached to the AgNPs by noncovalent bonding in an equilibrium state and are used to maintain stability and solubility of the AgNPs. When AgNPs are dispersed in the biological medium, the surface coating undergoes the process of reestablishing equilibrium by losing some coating molecules or displacing the surface coating agent itself with other biological molecules or ions which resulted in a change in AgNPs stability (He et al., 2012a, George et al., 2012, Li et al., 2013b). Additional changes that occur include alteration in the aggregation and agglomeration state of the NPs. Losing or displacement of the surface agents leads to instability of the NPs and results in aggregation (Li et al., 2013b, Meyer et al., 2010). Another critical change is the release of silver ions after surface oxidation. The presence of silver atoms on the surface of AgNPs leads to interactions with oxygen molecules to produce silver oxide which can interact with redox active compounds to produce silver ions which can take place in biological media or inside the cell (Reidy et al., 2013, Yang et al., 2011, George et al., 2012, Kim et al., 2013, Liu et al., 2010, Ahamed et al., 2008, Bae et al., 2011, Bilberg et al., 2011). The transformation of AgNPs in biological media has also been reported to be influenced by the presence of sulfur ions, sulfur-containing compounds, dissolved oxygen, chloride ions, and light (Levard et al., 2012, Liu et al., 2012, Cunningham et al., 2013, Levard et al., 2013, Li et al., 2013b, Lankveld et al., 2010, Lee et al., 2007, Liu et al., 2011).

Mechanisms of AgNP Toxicity

As mentioned before, silver ions released when AgNPs are introduced to biological media could contribute to the toxic effects of the AgNPs therefore it is important for researchers to distinguish between the toxic effect that is caused by AgNPs or silver ions (Beer et al., 2012, van der Zande et al., 2012). AgNPs have been reported to inhibit cell proliferation via activating different signaling pathways after interacting with cell membrane proteins (Braydich-Stolle et al., 2010, Asharani et al., 2008b, Asharani et al., 2009, Gopinath et al., 2010). In addition, cellular uptake of AgNPs has been reported to cause mitochondrial dysfunction, and protein and DNA damage due to reactive oxygen species (ROS) generation (Haase et al., 2012, Roh et al., 2012, van Aerle et al., 2013, Li et al., 2013b, He et al., 2012b, Bressan et al., 2013, Chairuangkitti et al., 2013, Ahmadi and Branch, 2012, Carlson et al., 2008). Another reported toxicity mechanism of AgNPs and silver ions is their interaction with biomolecules that have sulfur or cysteine residues such as proteins (Ahamed et al., 2008, Lee et al., 2007, Choi et al., 2009, Asharani et al., 2009, Banerjee and Das, 2013, Kaur and Tripathi, 2011, Hou et al., 2013). The NP-protein interaction can modify the structure and the function of the adsorbed protein resulting in conformational changes in the structure of adsorbed protein as it has been reported in different studies conducted on different types of NPs such as gold NPs, titanium dioxide (TiO_2) NPs, and iron oxide NPs (Wangoo et al., 2008, Gheshlaghi et al., 2008, Mahmoudi et al., 2011). AgNPs found in different studies to interact with different types of proteins such as bovine serum albumin (BSA) and human serum albumin (HAS) and that resulted in induced and increase in the protein conformational entropy and decrease in α-helix content (Treuel et al., 2010, Treuel et al., 2012, Shannahan et al., 2013b, Gebauer et al., 2012, Chen et al., 2012b).

AgNPs have been reported to accumulate outside the mitochondria causing damage that disturbs the function of the respiratory chain leading to ROS generation and oxidative stress

(Bressan et al., 2013). In addition, the mitochondrial damage caused by AgNPs can lead to ROS generation and interrupt ATP synthesis which results in DNA damage (AshaRani et al., 2008a). Another study reported that AgNPs induced apoptosis in fibroblasts through ROS generation and C-Jun pathway activation which resulted from increased cytochrome c production (Hsin et al., 2008). Apoptosis can also be induced by AgNPs through G1 arrest and S phase block (Park et al., 2010).

The antimicrobial activity of AgNPs has been reported to be due to an interaction of AgNPs with bacterial membrane, penetration, and accumulation in cells and causing cytoplasm damage or binding to the DNA (Wigginton et al., 2010, Khan et al., 2011a, Khan et al., 2011b, Grigor'eva et al., 2013). In different types of mammalian cells, AgNPs have been reported to cause membrane damage, altering membrane permeability, apoptosis due to oxidative damage, increase calcium influx, reduce in mitochondrial membrane potential and reduce in cell viability (Baruwati et al., 2013, Chairuangkitti et al., 2013, Cheng et al., 2013).

AgNPs can be taken up and internalized by different type of cells and can be dependent on the size, shape, or incubation time (Yu et al., 2013a, Locht et al., 2011, Lu et al., 2010, Miao et al., 2010, Choi and Hu, 2008, Meyer et al., 2010). Different studies reported comparison of the AgNPs uptake by different types of cells at different concentrations, time points, and different coating including PC. These studies demonstrated the influence of physicochemical properties on cellular uptake and the resulting toxic effects (Haase et al., 2012, Kruszewski et al., 2011, Meyer et al., 2010, Choi and Hu, 2008, Miao et al., 2010, Yu et al., 2013a, Kruszewski et al., 2013, Monteiro-Riviere et al., 2013).

Mast Cell

Mast cells are derived from $CD13^{+}CD34^{+}CD117^{+}$ (also known as c-Kit) hematopoietic stem cells in the bone marrow (Metcalfe et al., 1997). Developing mast cells migrate to different tissues such as the skin and airways then differentiate in the presence of stem cell factor (SCF) which is a ligand for the c-Kit receptor (Brown et al., 2008b). In addition to SCF, several mediators contribute in mast cell growth and differentiation such as IL-3, IL-4, IL-6, IL-9, IL-10, nerve growth factor (NGF), Prostaglandin E2 (PGE_2), α4 integrins, and vascular cell adhesion molecule-1 (VCAM-1) (Wiener et al., 2004, Brown et al., 2008a, Abonia et al., 2006, Hallgren et al., 2007). Mast cells can survive for months or years and can proliferate in response to appropriate signals (Abraham and Malaviya, 1997, Padawer, 1974). Mast cells are found in most tissues such as brain, heart, lymph nodes and are mainly located at the host's interface with the environment such as skin and mucosa as they play an important role in the recognition of pathogens such as parasites and bacteria (Woodbury et al., 1984, Nawa et al., 1985, Yong, 1997, Ghanem et al., 1988, Weidner and Austen, 1991). Mast cells rapidly respond to environmental stimulation by degranulation and release of their preformed mediators, such as histamine, serotonin, and proteases as protective immune response and can synthesize *de novo* mediators including leukotrienes, prostaglandins, and cytokines to help promote adaptive immune responses (Brown et al., 2008a). Mast cells and their mediators are best known for their association with different pathological conditions such as asthma, allergy, and anaphylaxis (Brightling et al., 2002, Patterson and Suszko, 1971, Metcalfe et al., 2009). In addition, due to the presence of mast cells in different types of tissues, mast cells have been reported to contribute to different pathophysiological conditions in organs such as the brain and the cardiovascular system (Wilhelm et al., 2005, Zhuang et al., 1996, Bot et al., 2007, Dvorak, 1986, Ghanem et al., 1988, Gilles et al., 2003).

Paul Ehrlich in 1879 was the first to discover and describe the mast cell and named it as mastung (i.e. overfed) cells where their cytoplasmic granules showed a strong purple-red metachromasia after staining with methylene or toluidine blue (Ehrlich, 1879, Beaven, 2009). Mast cells are highly heterogeneous cells and can be found in different phenotypes (Abe et al., 1990, Vliagoftis and Befus, 2005). The heterogeneity of mast cells can be shaped based on the specific type of the host tissue or to the type of the invading pathogen (Abraham and John, 2010). Based on staining properties of their granules, rodent mast cells can be categorized into mucosal or connective tissue mast cell types (Abraham and John, 2010). Further, these two categories can be distinguished by several properties including granules composition, degranulation response to stimulation, and their proliferation ability (Metcalfe et al., 1997). Proteases are the main protein components of mast cell granules (Metcalfe et al., 1997). Tryptases and chymases are the protease components of mouse connective tissue mast cells granules and can be found to contain heparin (Metcalfe et al., 1997). Mouse mucosal mast cell granules contain only chymases and can be found to contain chondroitin sulphate (Metcalfe et al., 1997). Human mast cells can also be differentiate based on the protease types into mast cell containing only tryptase (MC_T) and mast cell containing both tryptase and chymase (MC_{TC}) (Welle, 1997, Irani et al., 1986). In addition to proteases content, human mast cells can be classified based upon their expression of complement component C5a receptor (C5aR)(CD88) (Oskeritzian et al., 2005). Mast cell protease content varies not only between mast cell subtypes but also within individual mast cells depending on the type of stimulus as it has been reported that protease expression levels can be modulated by IL-10 and IL-4 (Ghildyal et al., 1992, Toru et al., 1998).

Mast Cell Activation by FcRs

Mast cells express Fc receptors (FcRs) including FcγRII receptors and FcεRI which is the high-affinity receptor for IgE (Brown et al., 2008a, Abraham and John, 2010). Mast cells can bind to both IgG and IgE and become sensitized to antigens that have been encountered previously and that leads to receptor cross-linking by the antigen subsequently resulting in mast cell activation and degranulation (Kawakami and Galli, 2002, Woolhiser et al., 2001). This type of mast cell activation is most studied and well characterized in models of asthma and allergy. When FcεRI is crossed linked with the antigen-antibody complex, signaling events started by phosphorylation of FcεRI cytosolic domains, immunoreceptor tyrosine-based activation motifs (ITAMs), activation of phosopholipase C (PLC) resulting in the initiation of calcium signaling within the endoplasmic reticulum through store-operated calcium channels as well as an influx of extracellular calcium which leads to degranulation of mast cell (Metcalfe et al., 2009). Activation of mast cells via FcεRI and Toll-like receptors (TLRs) at the same time leads to an increase in activity of mitogen-activated protein kinases (MAPKs) and that results in synergistic effects on cytokine production by the mast cell (Qiao et al., 2006). Mast cell activation can be achieved via FcR signaling that is induced by bacterial superantigens such as *Staphylococcus aureus* protein A and *Peptostreptococcus magnus* protein L which both can bind to certain antibodies independent of antigen specificity (Genovese et al., 2000).

Mast Cell Activation by Endogenous Inflammatory Factors

Mast cell activation can be induced by different endogenous peptides such as neurotensin, substance P and endothelin 1 (Johnson and Krenger, 1992, Maurer et al., 2004). In addition, mast cells can be activated by complement components such as C5aR (Nilsson et al., 1996, Mullaly and Kubes, 2007). Further, complement components C3 and C4 have been reported to activate mast cells and that has been demonstrated using knock out mice models (Prodeus et al., 1997). Mast

cells also can be activated by C3a and C5a complement proteins by initiating signaling through activation of G protein-coupled receptors (GPCRs) expressed on the surface of mast cells resulting in calcium mobilization and Nuclear factor of activated T-cells (NFAT) activation (Ali et al., 2000). In addition, the C3 complement pathway can be activated following IgG binding to FcγRIII that is expressed on the surface of mast cells resulting in opsonization of pathogens and subsequent mast cell activation (Baumann et al., 2001). A newly recognized pathway of mast cell activation is through the ST2 receptor and IL-33. Importantly, it has been reported that mast cells are activated through IL-33 following nanoparticle exposure. (Katwa et al., 2012, Wang et al., 2011).

Mast Cell Activation

Within seconds to minutes following recognition of an invading pathogen, mast cells release preformed mediators and are one of the first cells to respond to an invading pathogen (Burke et al., 2008). Following recognition, mast cells produce certain cytokines and mediators depending on the type of pathogen and can also replenish its granule storage during an infection or after resolving the infection (Metcalfe et al., 1997, Burwen, 1982). In infections, mast cells are responsible for recognizing the invading organism. With help of dendritic cells (DCs), epithelial and endothelial cells that are located in different sites such as the skin or the gut, the host immune system becomes alerted. Mast cells express pattern recognition receptors (PRRs) such as TLRs that can directly recognize pathogen-associated molecular patterns (PAMPs) and become activated (Trinchieri and Sher, 2007). In addition to TLRs, mast cells express other receptors that play an important role in pathogen recognition such as FcRs which bind to pathogen-specific antibodies (Kawakami and Galli, 2002, Woolhiser et al., 2001). Based on the type of PAMPs, mast cell responses can vary as it has been reported that activation of TLR4 of rodent mast cells by lipopolysaccharide (LPS) resulted in cytokine production with no degranulation while activation

of TLR2 by peptidoglycan resulted in degranulation and cytokine production (Supajatura et al., 2002). Further, the previous study reported that activation of TLR4 and TLR2 resulted in production of cytokines including tumor necrosis factor (TNF), IL-6, and IL-13 while IL-1β was produced only in TLR4 activation whereas IL-4 and IL-5 only produced following TLR2 activation (Supajatura et al., 2002). Human mast cells also show different responses upon TLR4 and TLR2 activation by peptidoglycan and LPS where both induced T helper2 (T_H2)-type response while peptidoglycan alone induced histamine release but not LPS (Varadaradjalou et al., 2003). Mast cells can also be activated through the CD48 receptor that can detect pathogens such as *Mycobacterium tuberculosis* and *Staphylococcus aureus* (Malaviya et al., 1999, Munoz et al., 2003, Rocha-de-Souza et al., 2008).

Functions of Mast Cells in Innate Immunity

Innate immunity is the first line of immune defense that detects pathogen-associated molecular patterns that induce a toxic and inflammatory response (Medzhitov and Janeway, 2000). The innate immune system utilizes genetic memory to recognize the molecular patterns of common invading pathogens (Dempsey et al., 2003). Mast cells play a main role in immune cell recruitment to the site of infection. In blood vessels, mast cells release various mediators such as histamine, TNF, vascular endothelial growth factor (VEGF) and proteases that increase vascular permeability and produce edema at the site of infection (Boesiger et al., 1998, Sendo et al., 2003, Heltianu et al., 1982). Mast cells also produce different chemokines including CC-chemokine ligand 11 (CCL11; eotaxin) and CXC-chemokine ligand 8 (CXCL8; IL-8) that facilitate the recruitment of other cells such as eosinophils and natural killer (NK) cells (Burke et al., 2008). Neutrophil recruitment to inflamed locations following release of TNF and mast cell protease 6 (MCP6) by mast cells has been reported in different studies (Huang et al., 1998, Sutherland et al., 2008,

Biedermann et al., 2000). In addition, during viral infections, mast cells activated by virus-associated stimuli such as polyinosinic–polycytidylic acid (polyI:C) produce different chemokines that induces NK cell chemotaxis (Burke et al., 2008).

Following pathogen invasion, mast cells release products that affect pathogen activity such as cathelicidins which have bactericidal activity (Di Nardo et al., 2008). Furthermore, cathlicidin can be produced by mast cells in response to stimuli such as LPS (Di Nardo et al., 2003). Mast cells also help in killing bacteria following phagocytosis by releasing products such as ROS (Abraham and Malaviya, 1997). Mast cell products such as histamine, serotonin, and proteases play an important role in the process of pathogen elimination and removal from the body by communicating with other cells such as nerve cells and smooth muscle cells (Johnson and Krenger, 1992, Margulis et al., 2009, Pothoulakis et al., 1998, Klimpel et al., 1995). Further, mast cells help in the process of pathogen clearance from tissues such as nasal mucosa by promoting mucus production by epithelial cells (Bischoff, 2009).

Mast Cell Function in Adaptive Immunity

Adaptive immunity involves the process of body recognition of a pathogen's specific antigens and production of a specific response to destroy it (Dempsey et al., 2003). Adaptive immunity is responsible for generation of immunologic memory and regulation of host immune homeostasis following interactions between antigen-presenting cells and T and B lymphocytes (Bonilla and Oettgen, 2010). Mast cells activation results in a multitude of inflammatory cytokines being produced that contribute to and regulate the activation of other immune cells that are involved in adaptive immunity (McLachlan et al., 2003). In the infected tissues, mast cells produce TNF which increases E-selectin expression on local blood vessels which results in an increased influx of DCs (Shelburne et al., 2009). Mast cells also produce CCL20 which contributes to DC

precursor recruitment into the tissues (Galli et al., 2005). Furthermore, mast cells promote activation of epidermal Langerhans cells, a subset of DCs, following bacterial infection which results in increased DCs recruitment (Shelburne et al., 2009, Jawdat et al., 2006). Mast cell-derived histamine has been reported to modulate DC activation by promoting antigen uptake and cross-presentation and the upregulation of co-stimulatory molecules required for T cell activation (Caron et al., 2001, Amaral et al., 2007). Furthermore, mast cells have been reported to promote DCs to acquire a T_H2 cell-inducing property (Mazzoni et al., 2006). Mast cells also promote the recruitment of T cells as it has been reported that activation of TLR3 on mast cells leads to expression upregulation of CXCL10 (IP10) and CCL5 (RANTES) and an increase in $CD8^+$ T cell recruitment (Orinska et al., 2005).

Mast cells can act as antigen-presenting cells (APCs) and upregulate expression of major histocompatibility complex (MHC) class II and co-stimulatory molecules and they have been reported to interact with T cells (Metcalfe et al., 1997). Mast cells also act as APCs for MHC class I specific to $CD8^+$ T cells which results in $CD8^+$ T cell activation and proliferation that leads to in induced secretion of IL-2, interferon-gamma (IFN-γ), macrophage inflammatory protein-1, and granzyme B (Stelekati et al., 2009).

Mast cells during infection can modulate and promote the development of adaptive immunity to pathogens (Shelburne et al., 2009). Mast cells in models of allergy can modulate adaptive immunity following binding of antibodies to FcRs and sensitization (Metcalfe et al., 1997). In the presence of high-affinity pathogen-specific antibodies, the antibody cross-links FcRs on mast cells which results in a primary fast response that contributes to improving immunological memory formation which enhances protection against a pathogenic challenge (Metcalfe et al., 1997, Gurish et al., 2004, Knight et al., 2000).

In contrast, mast cells can be modulated by T cells by physical contact between both types of cells or by T cell products that induce mast cell degranulation such as CCL3 (MIPIα) and CCL2 (Mekori and Metcalfe, 1999). In addition, T cells can induce production of TNF and histamine by mast cells which indicates the presence of a feedback mechanism between both types of cells, and mast cell activity modulation by the adaptive immune system during infections (Mekori and Metcalfe, 1999).

Macrophages

Macrophages are important immune cells that play a critical role in host defense. Macrophages are phagocytic cells that engulf and help in removing foreign particles, pathogens, and cell debris. In addition, macrophages regulate organ homeostasis and play an important role in tissue remodeling. Macrophages are generally derived from circulating monocytes and show a high degree of heterogeneity which reflects the specialization of functions of macrophages in different anatomical locations (Gordon, 1986, Gordon, 1998). The growth and differentiation of macrophages depends on cytokines, such as macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), and interactions with stroma in haematopoietic organs (Gordon, 2003). Macrophage determination can be affected by different cytokines and factors such as IL-3, c-kit, TNF-family proteins, TNF-receptor related molecules, PU.1, and ETS-family (Gordon, 2003). Monocytes arise from common bone marrow myeloid progenitor cells then differentiate into macrophages under the influence of M-CSF and GM-CSF or into DCs under the influence of GM-CSF and IL-4 (Gordon, 2003, Alikhan and Ricardo, 2013, Lawrence and Natoli, 2011, Zhou et al., 2014). Monocytes distribute through the blood stream and enter all tissues of the body then differentiate to macrophages based on tissue-specific phenotype signals from the extracellular matrix and neighbor cells and their products which result in different

phenotypes of macrophages such as Kupffer cells in liver, alveolar macrophages in lungs, osteoclasts in bones, histocytes in interstitial connective tissue, and microglia in the central nervous system (CNS) (Gordon, 2003, Alikhan and Ricardo, 2013, Galli et al., 2011, Ricardo et al., 2008). Macrophage migration from the blood, endothelium, the interstitium and epithelium is controlled by adhesion molecules including integrins (such as β1 and β2), immunoglobulin-superfamily molecules (such as CD31), selectins and epidermal growth factor seven-transmembrane spanning (EGF-TM7)-type receptors which ae related to the F4/80(EMRI) antigen (Gordon, 2003, Stacey et al., 2000). The expression of macrophage genes can be influenced by cytokines such as transforming growth factor-β (TGF)-β, chemokines and growth factors where they are found to bind to proteoglycans (Gordon, 2003). While monocytes have a short life span where they undergo spontaneous apoptosis every day, macrophages are long-lived cells with a life-span range from months to years (van Wilgenburg et al., 2013, Fahy et al., 1999). Monocyte-derived tissue macrophages can re-enter the blood stream and differentiate into DCs which depends on different stimuli such as phagocytosis and leukotrienes (Randolph, 2001, Gordon, 2003). Under the influence of different inflammatory and immune stimuli, local macrophages get activated which also enhances recruitment of monocytes from bone-marrow which results in macrophage accumulation in the affected tissue (Gordon, 2003).

Macrophage Receptors

Macrophages express pattern recognition receptors (PRRs) on their surface including TLRs, C-type lectin receptors (CLRs), scavenger receptors (SRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) that help in recognizing danger signals and the presence of foreign substances or dead cells (Murray and Wynn, 2011). These receptors initiate inflammatory

signaling resulting in macrophage activation (Ivashkiv, 2011). Activated macrophages produce different chemokines, cytokines, and inflammatory mediators as a result of activation of different signaling pathways such as MAPK, nuclear factor-κB (NF-κB), and interferon regulatory factors (IRFs) (Ivashkiv, 2011). Phagocytosis and pathogens binding by macrophages are facilitated by different receptors such as mannose receptor (CD206) a type of PRR, or macrophage receptor with collagenous structure (MARCO). Other PRR receptors such as TLRs, RLRs, or NLRs are expressed on the surface or in the cytoplasm of macrophages and they mediate microbial product recognition which induces expression of transcriptional factors and leads to phagocytosis and macrophage activation (Murray and Wynn, 2011, Gordon and Taylor, 2005). Following macrophage activation, SRs which are type of PRR facilitate opsonization and removal of pathogens, dead cells, cell debris, and foreign molecules from the tissues and the circulation of the host (Gordon and Taylor, 2005, Murray and Wynn, 2011).

Macrophage Polarization

Macrophage functions and phenotypes can be defined by its polarization state and that depends on the type of stimulus (Kittan et al., 2013). Polarized macrophages can be divided into classically activated (M1) and alternatively activated (M2) which can be further subdivided into M2a, M2b, and M2c (Kittan et al., 2013, Lawrence and Natoli, 2011, Bannon et al., 2013). Macrophages can adapt to the type of the stimulus resulting in heterogeneity of macrophages based on the function needed in response to the activating signals (Wang et al., 2014, Lawrence and Natoli, 2011). In addition, bone marrow-derived macrophages (BMDMs) that are grown in the presence of GM-CSF resulted in differentiation into M1 type which can produce IL-12 while grown BMDMs in presence of M-CSF resulted in differentiation into M2 type that produce IL-10

(Takeuch and Akira, 2011). The polarized state of macrophages has been reported to be connected to the cytokines produced by T_H cells (Bannon et al., 2013).

The M1 macrophage phenotype has a high expression of MHC class II molecules and co-stimulatory CD80/86 molecules, whereas the M2 macrophage phenotype have high expression of CD206/ or CD163 (Tiemessen et al., 2007, Juhas et al., 2015). Different types of human and mouse tissue macrophages express the CD68 surface marker (Nucera et al., 2011). It has been reported that CD163 or CD68 in combination with pSTAT1 or RBP-j maybe used as markers for the M1 phenotype, while a combination of c-MAF with CD163 or CD68 may be used as markers for the M2 phenotype (Barros et al., 2013).

Macrophage Activation

Classical activation of macrophages through TLR stimulation by ligands such as LPS and some cytokines such as IFN-γ resulted in M1 phenotype (Banerjee et al., 2013). M1 activation produces certain pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-12, IL-23, chemokines such as CCL5, CCL8, CXCL2, and CXCL4, and upregulate nitric oxide synthase (iNOS) expression that promotes production of nitric oxide (NO) (Sica et al., 2015). In addition, different transcription factors involved in M1 macrophage activation include NF-κB, activator protein-1 (AP-1), PU.1, CCAAT/enhancer binding protein (C/EBP) α, STAT1, and IRF5 (Banerjee et al., 2013, Lawrence and Natoli, 2011). Furthermore, M1 macrophages increase in abundance during antigen presentation and pathogen removal (Sica et al., 2015, Espinoza-Jiménez et al., 2012).

Alternatively, activation of the M2 phenotype results in upregulation of markers such as inter alia arginase-1, FIZZ1 (RELMα), Ym1, mannose receptor, MHC class II, CD14 receptor,

CCL17, CCL22, and CCl24 (Locati et al., 2013, Takeuch and Akira, 2011, Gordon, 2003). The M2 phenotype plays a role in regulation of the immune response to parasites, allergic reactions, tissue remodeling, angiogenesis, and tumor progression (Takeuch and Akira, 2011). Different cytokines involved in directing macrophage activation towards the M2 phenotype include IL-4 and IL-13, as well as IL-33 (Wang et al., 2014, Locati et al., 2013, Kurowska-Stolarska et al., 2009). IL-4 and IL-13 are well established in their association with T_H2-type response and their effect on macrophages and other immune cells (Brombacher, 2000, Brubaker and Montaner, 2001, Montaner, 1999). Interestingly, IL-10 has similarity to a T_H2 cytokine and can be found to be co-induced with T_H2 cytokines during the immune response (Gordon, 2003). IL-10 is a multifunctional cytokine that can inhibit antigen-presenting cell function and induce and activate different genes (Moore et al., 2001, Gordon, 2003, Lang et al., 2002a, Lang et al., 2002b, Montaner, 1999). Recombinant IL-4 has been reported to upregulate expression of MHC class II proteins and increase activity of the mannose receptor (McInnes and Rennick, 1988, Stein et al., 1992). IL-4 also partially inhibits pro-inflammatory cytokines such as TNF as a response to LPS compared to IL-10 which is a potent inhibitor to these pro-inflammatory cytokines (Mokoena and Gordon, 1985, Gordon, 2003). IL-13 has been reported to exhibit the same effect as IL-4 on mouse macrophages, as well as moderately inhibit nitric oxide synthase 2 (NOS2) compared to potent inhibition by IL-10 (Doherty et al., 1993, Doyle, 1994). Same effects produced by IL-13 in human blood monocyte-derived macrophages (MDMs) but with higher expression of MHC class II and mannose receptor levels but not with IL-4 (de Waal Malefyt, 1993, Hart, 1999). In addition, IL-4 and IL-13 have been reported to increase endocytosis through activation of the mannose receptor (Montaner, 1999). Furthermore, IL-4 and IL-13 can induce arginase activity and inhibition of

NOS2 activation and NO release which results in increased collagen production which explains their involvement in fibrosis and granulomatous inflammation (Gordon, 2003).

IL-4 and IL-13 share a common receptor chain which produces similar effects on macrophages and other immune cells. However, the presence of different subunits on these receptors leads to different cells types responding to them (Gordon, 2003). IL-4 and IL-13 can activate signaling pathways including Janus-activated kinase (JAK) and signal transducer and activator of transcription (STAT) families (Shimoda, 1996, Takeda et al., 1996). M2 macrophage activation has been reported to be promoted by different transcription factors such as IRF4, C/EBP-β, Krüppel-like factor 4 (KLF4), STAT6, and peroxisome proliferator-activated receptor γ (PPARγ) (Lawrence and Natoli, 2011, Banerjee et al., 2013).

Interaction of Immune System with Nanoparticles

The immune system acts to protect the host from infections, invading pathogens, and unwanted foreign substances. Following internal or external encounters with invading pathogens or foreign substances, the immune system can be suppressed or overstimulated. NPs can be found as engineered or naturally-occurring in the environment and are being used extensively in different fields such as medicine and industry (Stern and McNeil, 2008). The host can be unintentionally exposed to NPs in work places that produce NP-containing products or intentionally exposed as in the use of nanomedicines. NPs are used in nanomedicine as delivery vehicles for therapeutic agents as well as in imaging studies. NPs can be made of lipids, metals/metal-oxides, silicon and silica, polymers, proteins, and carbon (Gentile et al., 2013, Paolino et al., 2014, Shen et al., 2014a, Xu et al., 2012, Grigor'eva et al., 2013, Shen et al., 2014b, Molinaro et al., 2013, Choi et al., 2011, Xu et al., 2013, Chen et al., 2005, Meng et al., 2013). Once NPs enter the biological system, phagocytic cells such as macrophages are the first to interact with NPs which may suppress or overstimulate

the immune system which can result in toxic effects. These effects include granuloma formation in lung and skin when animals are exposed to carbon nanotubes (CNTs) (Poland et al., 2008, Witzmann and Monteiro-Riviere). NPs are also reported to be accumulated primarily in the spleen and liver following intravenous injection (Kumar et al., 2010, Lee et al., 2010).

Immunosuppression can be a desired effect as it enhances treatment of allergic conditions and autoimmune diseases or it may be an unwanted effect where it may lower the host's defense against infections and cancer (Ryan et al., 2007, Balenga et al., 2006, Gomez et al., 2007, Wegmann et al., 2008, Basarkar and Singh, 2009, Kim et al., 2002, Choi et al., 2008, Sakai et al., 2006). It has been reported that CNTs can suppress B cell function through transforming growth factor produced by macrophages (Mitchell et al., 2009). Another study in breast cancer patients reported that paclitaxel bound to human serum albumin NPs showed fewer neutropenia incidents compared to the regular paclitaxel formulation (Stinchcombe et al., 2007, Higaki et al., 2005). In addition, in animal model studies, clodronate loaded in liposomes was used to deplete macrophages in order to protect animals' lungs from endotoxin effects (Gaca et al.).

Immunostimulation by NPs can include activating either the innate or the adaptive immune response. Activation of the complement system by NPs can lead to hypersensitivity reactions and anaphylaxis (Chanan-Khan et al., 2003, Szebeni, 2005, Szebeni et al., 2007, Szebeni et al., 2002). It has been reported that complement activation in tumor tissues induces tumor-associated immune cell activation resulted in stimulating cancer progression which may impact the use of nanoparticles in cancer diagnosis or treatment (Markiewski et al., 2008, Markiewski and Lambris). NPs have also been shown to enhance antigenicity of weak antigens or can act as antigens by themselves (Manolova et al., 2008, Reddy et al., 2007, Fifis et al., 2004). NPs can modulate the type of immune response by induction of release of different mediators and cytokines such as IFN-

γ or IL-4 (Mottram et al., 2007). NPs have been reported to induce production of particle-specific antibodies such as fullerene (C60) derivatives and polyamidoamine dendrimers (Braden et al., 2000, Chen et al., 1998, Lee et al., 2004).

Allergic reactions are common side effects of NPs which have been reported in different studies such as CNTs which can enhance allergic reactions induced by the allergen, ovalbumin (OVA), in mice (Nygaard et al., 2009). Occupational exposure of humans to dendrimers has been reported to play a role in development of allergic reactions such as epidermal necrolysis-like dermatitis in workers exposed to NPs in work places involved in handling of NPs (Toyama et al., 2008).

Cytokines produced by immune cells can be released in response to exposure to different type of NPs such as gold, colloids, dendrimers, polymers, and lipid NPs (Fifis et al., 2004, Mottram et al., 2007, Scholer et al., 2002, Scholer et al., 2001, Shvedova et al., 2005, Vallhov et al., 2006).

Interaction of Mast Cell with NPs

Due to the location of mast cells in tissues such as the skin and lungs, mast cells are exposed to the external environment which results in this critical cell type acting as sentinel cells in the host defense (Brown et al., 2008b). The rapid response of mast cells to the external stimuli results in mast cell activation and degranulation and release of pre-formed mediators or newly synthesized de novo mediators (Brown et al., 2008b). Mast cells are a main immune cell type that can interact with NPs because of their location in the host body and because of its role in the immune system response. Thus, understanding the mast cell-NP interaction is important for improved understanding of NP toxicity. Mast cells have been reported to be activated by different types of NPs such as TiO_2 NPs that can increase histamine release and activate mast cells via activation of

membrane L-type Ca^{2+} channels, can also increase ROS production and activate the PLC pathway which results in exacerbated allergic inflammatory responses with or without IgE sensitization. Based on the previous results, NP exposure may worsen inflammatory diseases associated with mast cells such as arthritis (Chen et al., 2012a). Mast cells can also be activated by cerium oxide (CeO_2) NPs and they have been reported to contribute to pulmonary inflammation, exacerbation of myocardial ischemia/reperfusion injury, and release of mediators such as IL-6, MIP-1α, TGF-β, and osteopontin (Wingard et al., 2011). In that same study, mast cell deficient mice did not show any inflammatory response (Wingard et al., 2011).

Mast cells are involved in allergic responses through activation of FcεRI receptor following binding with IgE resulting in the release of many mediators. Mast cells exposed to fullerene C(70)-tetraglycolic acid have been reported to inhibit degranulation and cytokine production, while C(70)-tetrainositol blocked only cytokine production in mast cells which suggested a new approach for treatment allergic diseases (Norton et al., 2010, Dellinger et al., 2010, Ryan et al., 2007). The previous findings showed that NPs can interfere with FcεRI signaling pathways and stabilize mast cells by blocking calcium release and ROS generation through initiation of signaling pathways such as extracellular signal regulated kinase 1/2 (ERK), p38-MAPK, linker of activated T cells (LAT), phosphoinositide 3-kinase (PI3K), and v-src (Norton et al., 2010). Multi-walled carbon nanotubes (MWCNTs) can activate mast cells and it has been reported that mast cell exposure to MWCNTs leads to production of IL-33 that activates mast cells and results in adverse pulmonary and cardiovascular responses in normal mice which were confirmed by testing mast cell deficient mice and ST2 knock out mice (Feng et al., 2011, Shannahan et al., 2012, Katwa et al., 2012).

The mechanisms of mast cell activation by NPs exposure are not well understood necessitating the need for further investigation of NP-mast cell induced toxicity and the underlying mechanisms. This may allow for design of better formulations of NPs with less side effects that can effectively be used in medicine and consumer products.

Interaction of Macrophages with NPs

Macrophages are important immune cells in host defense due to their phagocytic function which acts to engulf and remove pathogens and foreign substances from the body. Phagocytosis of NPs has been reported in different studies and can occur within a range of time from minutes to hours (Tsai et al., 2012, Geiser et al., 2008, Takenaka et al., 2006, Semmler-Behnke et al., 2007). NPs are likely to be taken up by different mechanisms such as micropinocytosis and pinocytosis (Geiser, 2010). In micropinocytosis, large vesicles ranging in size from 0.2-10 μm are formed spontaneously or following stimulation by growth factors resulting in particle internalization by macrophages and other cells (Geiser, 2010). Pinocytosis forms vesicles 100nm in diameter and can be divided in two types including clathrin-mediated pinocytosis and caveolae mediated pinocytosis (Geiser, 2010). Uptake and internalization of NPs with sizes up to 200nm in diameter have been reported to use clathrin-mediated pathways, whereas caveolae-mediated pathway is utilized for larger size NPs with diameter up to 500nm (Rejman et al., 2004). Upon NP internalization, the NPs can translocate to different locations and interact with different types of macrophages or other types of cells as it has been reported that NPs can pass the epithelial barrier into the interstitium and enter the blood circulation following inhalation exposure (Semmler-Behnke et al., 2007).

Macrophages can be activated following NP exposure resulting in production of different mediators such as pro-inflammatory cytokines, ROS, and nitric oxide. It has been reported that

MWCNTs can induce cyclooxygenase-2 (COX-2) through ERK 1/2 pathway activation and iNOS stimulation which was found to be ERK 1/2 independent (Lee et al., 2012). Interaction of silica NPs with macrophages has been reported to induce cytotoxicity and oxidative stress in murine alveolar macrophages (Gazzano et al., 2012).

NP size has been reported to play an important role in macrophage activation as different studies showed that NPs such as AgNPs and gold NPs can induce cytotoxicity and ROS generation in a size-dependent manner (Tsai et al., 2012, Carlson et al., 2008).

Different studies showed the important role of alveolar macrophages in NP removal which correlates to the role of macrophages in pulmonary responses following NP exposure such as AgNPs and gold NPs (Takenaka et al., 2012, Haase et al., 2011). In the previous studies, AgNPs were reported to be internalized by macrophages within minutes while gold NPs were taken up by macrophages within 24 hours. In addition, alveolar macrophages have been reported to interact with and take up single-walled carbon nanotubes (SWCNTs) in a manner that was dependent on the type of NP coating and the surfactant used in the study (Kapralov et al., 2012, Ruge et al., 2011). Furthermore, macrophages in the pleural space have been reported to interact with CNTs which resulted in increased production of cytokines such as IL-1β, TNFα, IL-6 and the chemokine, IL-8 which can lead to mesothelioma induction (Murphy et al., 2012).

Scavenger Receptors

SRs were first described in 1979 by Drs. Michael Brown and Joseph Goldstein in their work investigating the uptake and internalization of low-density lipoprotein (LDL) by cells (Brown and Goldstein, 1979, Brown et al., 1979). Now SRs are recognized to have a wide range of ligands in addition to LDL such as endogenous proteins and lipoproteins, as well as, microbial

associated products such as LPS and lipoteichoic acid (LTA) (Pluddemann et al., 2011, Plüddemann et al., 2006). The mammalian SRs can be distributed into classes designated A to J, however the class C scavenger receptor is not included because it is only expressed in *Drosophila melanogaster* (Canton et al., 2013, PrabhuDas et al., 2014b). In a recent published report, it was proposed that a method for SR nomenclature be developed to provide clarity (PrabhuDas et al., 2014a). As an example, a member for SR class A would be designated as SR-A1, where SR stands for scavenger receptor followed by a hyphen, then a capital letter such as A to represent the class of SR followed by Arabic numeral to represent the type of molecule within the class. If there are alternate splice variants, then it would be designated by a dot and an Arabic numeral such as SR-A1.1 (PrabhuDas et al., 2014b). The previous report explained in detail the nomenclature for SR family members. In summary, class A of SR includes SR-A1 (also known as macrophage scavenger receptor 1 (MSR1), CD204, or SCARA1, SR-A1.1 (also known as SR-AII), SR-A1.2 (also known as SR-AIII), SR-A3 (also known as MSRL1, APC7, or SCARA3), SR-A4 (also known as COLEC12, SR C-type lectin (SRCL), SCARA4, or CL-P1), SR-A5 (also known as TESR, NET33, or SCARA5), SR-A6 (also known as MARCO, Ly112, or SCARA2). Class B of SR includes SR-B1 (also known as SR-BI, SCARB1, or CD36L1), SR-B1.1 (also known as SR-BII), SR-B2 (also known as CD36, SCARB3, FAT, GPIV, or PAS4), and SR-B3 (also known as SCARB2, lysosomal integral membrane protein 2 (LIMP2), CD36L2, or LGP85). Class D of SR includes SR-D1 (also known as CD68, gp110, SCARD1, Macrosialin, or lysosomal associated membrane protein 4 (LAMP4). Class E of SR includes SR-E1 (also known as lectin-like oxidized low-density lipoprotein receptor-1(LOX-1), SCARE1, CLEC8A, or OLR1), SR-E1.1 (also known as LOXIN), and SR-E2 (also known as Dectin-1 or CLEC7A). Class F of SR includes SR-F1 (also known as scavenger receptor expressed by endothelial cells I (SREC-I) or SCARF1), SR-F2 (also

known as SREC-II or SCARF2), and SR-F3 (also known as multiple EGF-like domains 10 (MEGF10), GM331, or EMARDD). Class G of SR includes only SR-G1 (also known as scavenger receptor for phosphatidylserine and oxidized low-density lipoprotein (SR-PSOX) or CXCL16). Class H of SR includes SR-H1 (also known as fasciclin, EGF-like, laminin-type EGF-like, and link domain-containing scavenger receptor-1 (FEEL-1) or STAB1) and SR-H2 (also known as FEEL-2 or STAB2). Class I of scavenger receptor includes SR-I1 (also known as CD163 or M130), SR-I1.1, SR-I1.2, and SR-I2 (also known as CD163B, CD163L1, or M160). Class J of SR includes SR-J1 (also known as receptor for advanced glycation end products (RAGE)) and SR-J1.1 (PrabhuDas et al., 2014b). Other molecules that have scavenging activity but are still not classified within the SR family include the LDL receptor family proteins such as LRP1, LRP2/megalin, and CD11b/CD18α (PrabhuDas et al., 2014b).

In addition to their scavenging activity, SRs act as lipid transporters and even act as chemokines (Shimaoka et al., 2004b, Gu et al., 1998, Kennedy and Kashyap, 2011). CD36 has been reported to be involved in atherosclerosis by inducing the formation of foam cells and also has been proposed to be involved in granulomatous conditions such as tuberculosis by its role in formation of multinucleated giant cells to prevent spreading of the pathogens (Helming et al., 2009, Martin et al., 2011a, Martin et al., 2011b). LIMP2, a member of SR class B, has been reported to be involved in transportation of β-glucocerebrosidase from the endoplasmic reticulum (ER) to the lysosomes. Therefore impairment in LIMP2 function can be correlated to neurodegenerative and renal disorders (Reczek et al., 2007, Blanz et al., 2010, Desmond et al., 2011). Feel-1 a member of SR class H has been reported to affect lysosomal delivery of chitinase-like protein which in turn affects macrophage mediated uptake and degradation of unwanted self-molecules and which can affect production of mediators by macrophages (Kzhyshkowska et al., 2006). Previous reports

suggested that SRs have important roles in the cellular functions including those within the cell, and on the cell surface as well as in the circulation.

The recognition of SRs to self-molecules such as endogenous proteins and non-self-molecules (PAMPs) led them to be classified as pattern recognition receptors (PRRs) (Krieger, 1997, Medzhitov and Janeway, 2002). The SR family shares some functional properties such as identifying and removing self-molecules including dead cells and damaged proteins or non-self-molecules including microorganisms and foreign substances, whereas their structures are very heterogeneous between classes and even among members of each class (Plüddemann et al., 2006, Mukhopadhyay and Gordon, 2004, Pluddemann et al., 2007, Suzuki et al., 1997, Taylor et al., 2005, Herrmann et al., 2012). Molecule removal induced by SRs can be carried out by simple endocytosis or can be produced through processes requiring signal transduction such as phagocytosis or micropinocytosis (Canton et al., 2013). The SR families can also be involved in other processes such as cellular adhesion and antigen presentation (Palani et al., 2011, Shimaoka et al., 2004a, Santiago-Garcia et al., 2003). Due to their various functions, SRs have been found to be involved in the maintenance of homeostasis and in the pathogenesis of different diseases (Canton et al., 2013). In addition, metabolism and inflammatory disorders have been linked to SRs for their involvement in innate immunity and their affinity for modified lipids, pathogens, and foreign molecules (Feng et al., 2011, Kzhyshkowska, 2010, Park et al., 2013, Podrez et al., 2007, Silverstein and Febbraio, 2009).

Scavenger Receptor B1

Scavenger Receptor B1 **(**SR-B1) is a cell surface glycoprotein with a large middle part of its structure located extracellularly and attached to the plasma membrane by transmembrane domains with short N- and C-terminal cytoplasmic domains (Figure 1.1) (Acton et al., 1994, Calvo

and Vega, 1993). SR-B1.1 is an alternatively spliced variant of SR-B1 mRNA that lacks the C-terminal cytoplasmic domain (Webb et al., 1997). SR-B1 is distributed in different cells and tissues including hepatocytes, platelets, macrophages, mast cells, endothelial cells, and epithelial cells (Brown et al., 2009, Dieudonne et al., 2012, Uittenbogaard et al., 2000, Kzhyshkowska et al., 2012). SR-B1 can bind to various ligands such as high-density lipoprotein (HDL) cholesterol, oxidized and acetylated LDL (OxLDL and AcLDL), very low density lipoprotein (VLDL), and phospholipids (Krieger, 2001). SR-B1 activity has been reported to be controlled by PDZK1 which is a PDZ containing protein primarily expressed in kidney, liver, and small intestine and can interact with the C-terminal resulting in signaling pathway activation such as Akt and MAPKs (Ikemoto et al., 2000, Kocher et al., 1998, Nourry et al., 2003, Mineo et al., 2003). The main function of SR-B1 is to mediate cellular HDL uptake as well as to mediate bidirectional flux of unesterified cholesterol and phospholipids between HDL and cells (Krieger, 2001). Therefore, SR-B1 can play important role in cholesterol metabolism which has been addressed by using a SR-B1 knock out mice model that was characterized by an increase in plasma total cholesterol, decreased cholesteryl ester stores, and reduced biliary cholesterol secretion (Rigotti et al., 1997b, Trigatti et al., 1999, Mardones et al., 2001, Huszar et al., 2000, Temel et al., 1997).

Role of Scavenger Receptors in Macrophage Polarization

SRs contribute in macrophage polarization and some SRs are found to be highly expressed in the M2 phenotype such as SR-A1 and CD36 as well as CD163 where its expression can be used as M2 phenotype marker (Van Gorp et al., 2010, Oh et al., 2012, Buechler et al., 2000, Weaver et al., 2007, Xu et al., 2007, Tippett et al., 2011). SR-A1 and CD36 can contribute to M2 generation by delivering signals to ER stress pathways, Jun N-terminal kinase (JNK), PPARγ pathways that are required for M2 generation (Oh et al., 2012). Increased expression of SR-A1 and CD36 has

been reported to enhance M2 function in removing molecules such as apoptotic cells (Todt et al., 2008, Ogden et al., 2005, Xu et al., 2006, Zizzo et al., 2012). CD36 is highly expressed in M2 macrophages and has an anti-inflammatory function but can also be found in M1 macrophages where it can induce production of pro-inflammatory cytokines following an interaction with TLRs (Triantafilou et al., 2006, Hoebe et al., 2005, Kennedy et al., 2011). On the other hand, CD163 can promote anti-inflammatory effects in M2 macrophages by inactivating pro-inflammatory molecules such as TNF-like weak inducer of apoptosis (Bover et al., 2007, Schaer et al., 2007, Philippidis et al., 2004). Therefore, SRs are highly expressed in M2 macrophages but they can also be expressed on other macrophage cell populations and can induce pro-inflammatory cytokine production by macrophages.

Scavenger Receptors and Immune System

SRs can recognize PAMP molecules in addition to scavenging modified lipoproteins. SRs have an important role in innate immunity through their action in phagocytosis and removal of pathogens (Mukhopadhyay and Gordon, 2004, Pluddemann et al., 2011).

Different ligands can bind to a single SR resulting in wide range of substances that are covered by SRs. SR-A1 and MARCO have been reported to bind to bacterial-associated molecules such as LPS, LTA, and CpG DNA resulting in uptake of the source pathogen (Suzuki et al., 1997, Mukhopadhyay and Gordon, 2004, Peiser et al., 2006, Thomas et al., 2000, Peiser et al., 2002). Furthermore, some ligands overlap among different SRs such as SR-A1 and MARCO that can bind to other ligands such as LPS which can induce the innate immune response (Pluddemann et al., 2009).

SRs can influence other PRRs such as TLRs and have an impact on their signaling. SR-A1 can influence TLR4 and induce phagocytosis of Gram-negative bacteria such as *Escherichia coli,* while interaction of SR-A1 with TLR2 can induce phagocytosis of Gram-positive bacteria such as *Staphylococcus aureus* (Amiel et al., 2009). Furthermore, the interaction of SR-A1 and TLR3 has been reported to enhance pathogen uptake and the inflammatory response (Mukhopadhyay et al., 2011). MARCO can also interact with TLRs as it has been reported that interaction of MARCO with TLR2 and CD14 in the process of recognition to *Mycobacterium tuberculosis* cell wall glycolipid results in pro-inflammatory cytokine production (Bowdish et al., 2009).

CD36 which is a member of class B SR family can interact with TLRs such as TLR2 and TLR6 in response to bacterial-associated molecules such as LTA resulting in cytokine production and infected cell internalization (Stewart et al., 2010, Triantafilou et al., 2006). CD36 also has been reported to be required for induction of mycobacterial infection and CD36 deficiency results in attenuated susceptibility to mycobacterial infection (Philips et al., 2005). SR-B1 has been reported to act as co-receptor for hepatitis C virus internalization inside the cell (Ploss et al., 2009, Dreux et al., 2009, Westhaus et al., 2013, Dao Thi et al., 2012). In addition, SR-B1 has been reported to play a role in pathogen survival such as *Chlamydia trachomatis* where this pathogen uses SR-B1 to provide lipids like phosphatidylcholine that are essential to its survival and inhibition of SR-B1 resulted in impaired pathogen replication (Cox et al., 2012). Also, SR-B1 has been reported to play important role in host defense during bacterial pneumonia where the investigators found that SR-B1$^{-/-}$ mice showed increased mortality, increased neutrophils recruitment, and cytokine production which were associated alteration in LPS clearance by cells (Gowdy et al., 2015). LIMP2 is another member of SR class B that has been reported to facilitate cell entry of different type of viruses such as enterovirus 71 and coxasckievirus (CVA) such as CVA7, CVA14, and CVA16 as well as

play an important role as an infection efficiency determinant which depends on viral uncoating by LIMP2 (Yamayoshi et al., 2009, Yamayoshi et al., 2012, Yamayoshi et al., 2013).

Interaction of Scavenger Receptors with Nanoparticles

Interaction of SRs with NPs has been reported in various studies. SR-A receptors such as SR-A1 have been reported to mediate amorphous silica NP uptake by macrophages (Orr et al., 2011). In addition, overexpression of SR-A1 in non-phagocytic cells resulted in cellular uptake of amorphous silica NPs and silencing expression of SR resulted in a reduction in amorphous silica NP uptake and pro-inflammatory cytokine production (Orr et al., 2011). SR-A1 also has been reported to contribute to cellular uptake of superparamagnetic iron oxide NPs following bacterial exposure which resulted in IL-10 inhibition, an increase in TNF-α production, modulation of TLR4 gene expression, and dysregulation of macrophage activation profiles which affect macrophage polarization from the M1 into the M2 phenotype (Kodali et al., 2013). Furthermore, superparamagnetic iron oxide NPs were found to impair antimicrobial function by macrophages in response to LPS exposure (Kodali et al., 2013). MARCO another member of SR class A has been reported to play a role in the interaction with NPs such as MWCNTs, superparamagnetic iron oxide NPS, and polystyrene nanoparticles (Chao et al., 2012, Hirano et al., 2008). In addition, MARCO was found to contribute to AgNP uptake where macrophages isolated from $MARCO^{-/-}$ mice showed less NP uptake and less toxic effects compared to macrophages isolated from wild type mice (Hamilton et al., 2014).

Fewer studies described the interaction of NPs and SRs class B. SR-B1 was found to contribute in synthetic layered silicate NP recognition and clearance by phagocytosis (Mortimer et al., 2014). Another study reported that chitosan NP as a non-viral delivery carrier of siRNA in HePG2 cell line resulted in SR-B1 silencing for up to 96 hours which demonstrated and interaction

between SR-B1 and NPs (Farid et al., 2014, Lin et al., 2012). Synthetic high density lipoprotein NPs also have been reported to interact with SR-B1 which have been suggested for use in treatment of B-cell lymphoma by inducing apoptosis (Yang et al., 2013). In addition, SR-B1 has been reported to facilitate paclitaxel encapsulated in HDL-NPs uptake by cancer cells which can be used to increase treatment efficiency (Mooberry et al., 2010).

Goal of Research and Statement of Hypothesis

AgNPs have been reported to induce toxicity such as oxidative stress and DNA damage. The influence of physicochemical properties of AgNPs on mast cell and macrophage activation has not been investigated. In addition, the mechanism by which the AgNPs bind to and interact with cells is still unclear.

The goal of this research was to evaluate mast cell and macrophage activation following AgNP exposure. Mast cells and macrophages have shown the ability to interact with different types of NPs and induce toxic effects, therefore it is important to understand the effect of the physicochemical properties of AgNPs and the nature and role of receptors involved in toxicity induction. Therefore, this project will provide information that has an impact on formulating nanomedicine products and consumer products exhibit low toxic profiles.

Specific Aims

This project is based on the main hypothesis which states that the scavenger receptor B1 (SR-B1) mediates mast cell and macrophage uptake of silver nanoparticles (AgNPs) and subsequent inflammatory responses. To test this hypothesis, three aims were developed as follows:

Aim 1: *Investigate Silver Nanoparticle (AgNP) uptake by mast cells and macrophages.*

This aim will evaluate uptake of AgNPs by bone marrow derived mast cells (BMMCs) and murine macrophages (RAW 264.7) cell line. Different methods will be used in this aim including enhanced darkfield hyperspectral imaging technology (Cytoviva) to image AgNPs in cells and to create hyperspectral profiles for mapping AgNPs within cells, flow cytometry, and inductively coupled plasma mass spectrometry (ICP-MS).

Aim 2: *Investigate mast cell and macrophage activation following AgNP exposure.*

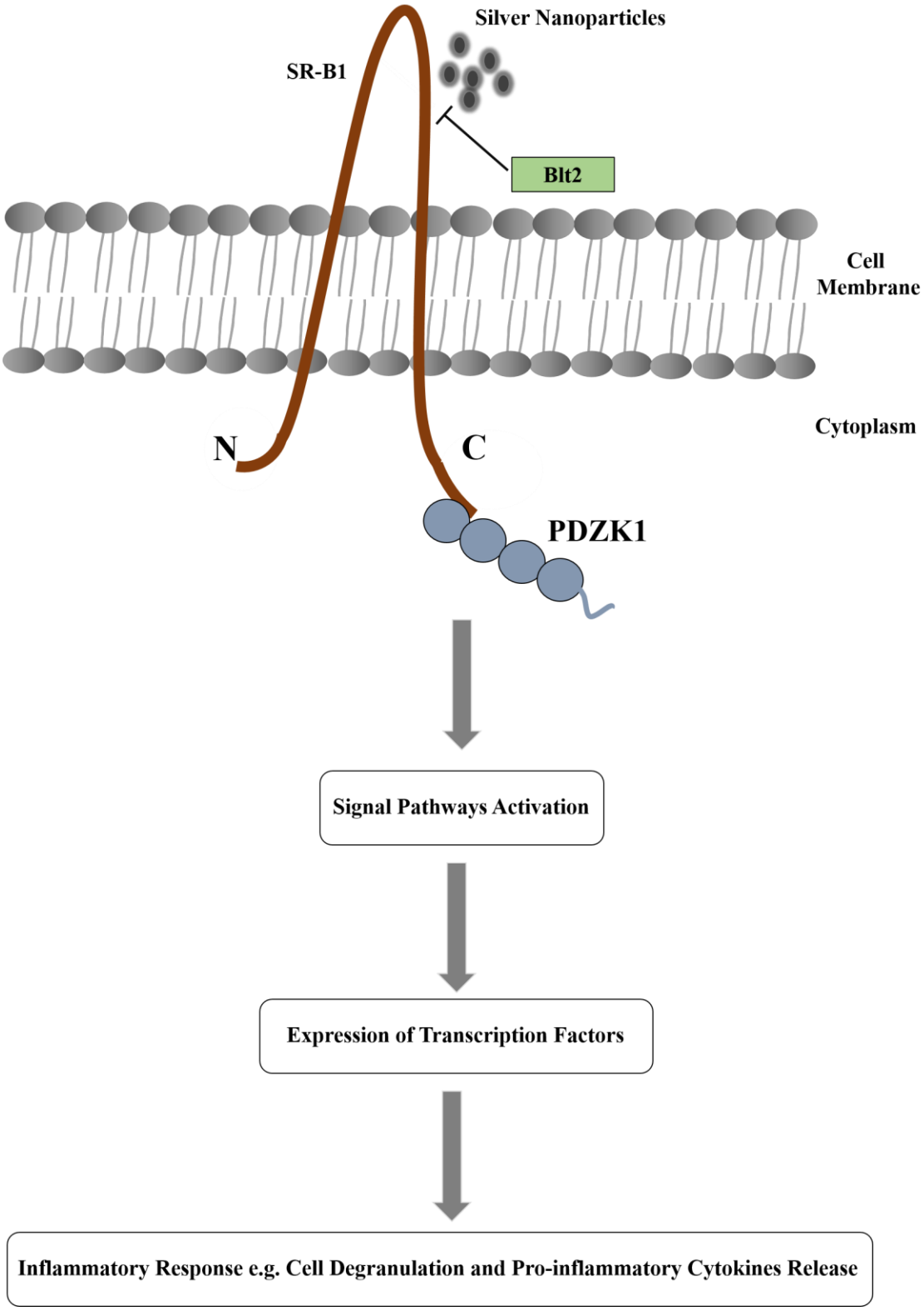
This aim will quantitatively evaluate cell activation following AgNP exposure. BMMC cytokine release and degranulation will be measured following AgNP exposure. In addition, PCR array, activation markers, and cytokine release will be evaluated in macrophages.

Aim 3: *Establish a role of the scavenger receptor B1 (SR-B1) in mast cell and macrophage uptake and activation following AgNPs exposure.*

This aim will focus on the role of SR-B1 in AgNP uptake and cellular activation by using an SR-B1 inhibitor 2-(2-butoxyethyl)-1-cyclopentanone thosemicarbazone (Blt2) in *in vitro* studies or by using Blt2 in an *in vivo* study in addition to SR-B1$^{-/-}$ mice.

Figure 1.1 Proposed pathway of silver nanoparticles binding to scavenger receptor B1 and subsequent inflammatory responses

Silver nanoparticles (AgNPs) interact with scavenger receptor (SR-B1) which is a protein with 2 transmembrane domains and intracellular N and C termini. PDZK1 is a PDZ containing protein can interact with the C-terminal resulted in signal pathways activation following AgNPs exposure. By using blocking lipid transporter-2 (Blt2) which is an SR-B1 inhibitor, the signal pathway activation following AgNPs exposure can be inhibited.


Silver Nanoparticles
SR-B1
Blt2
Cell Membrane
Cytoplasm
N
C
PDZK1
Signal Pathways Activation
Expression of Transcription Factors
Inflammatory Response e.g. Cell Degranulation and Pro-inflammatory Cytokines Release

CHAPTER 2: INFLUENCE OF PHYSICOCHEMICAL PROPERTIES OF SILVER NANOPARTICLES ON MAST CELL ACTIVATION AND DEGRANULATION

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Abstract

Silver nanoparticles (AgNPs) are increasingly being incorporated into products for their antimicrobial properties. This has resulted in increased human exposures and the possibility of adverse health effects. Mast cells orchestrate allergic immune responses through degranulation and release of pre-formed mediators. Little data exists on understanding interactions of AgNPs with mast cells and the properties that influence activation and degranulation. Using bone marrow-derived mast cells and AgNPs of varying physicochemical properties we tested the hypothesis that AgNP physicochemical properties influence mast cell degranulation and osteopontin production. AgNPs evaluated included spherical 20 nm and 110 nm suspended in either polyvinylpyrrolidone (PVP) or citrate, Ag plates suspended in PVP of diameters between 40-60 nm or 100-130 nm, and Ag nanowires suspended in PVP with thicknesses <100 nm and length up to 2 microns. Mast cell responses were found to be dependent on the physicochemical properties of the AgNP. Further, we determined a role for scavenger receptor B1 in AgNP-induced mast cell responses. Mast cell degranulation was not dependent on AgNP dissolution but was prevention by tyrosine kinsase

inhibitor pretreatment. This study suggests that exposure to AgNPs may elicit adverse mast cell responses that could contribute to the initiation or exacerbation of allergic disease.

Introduction

The applications of nanotechnology are rapidly expanding and revolutionizing many fields primarily through the incorporation of nanoparticles (NPs) into numerous biomedical and consumer products. In particular, silver nanoparticles (AgNPs) are one of the most utilized NPs due to their antimicrobial/fungal properties (Nocchetti et al., 2013, Dong et al., 2012, Levard et al., 2013). More than 300 globally available consumer products, such as wound dressings, IV bags, dermal creams, water filters, and many household products, incorporate AgNPs (Project, 2014). Indeed, the annual global production of AgNPs is estimated to be >55 tons (Piccinno et al., 2012). A direct interaction of end user and AgNP-based products increases the risk of possible exposure through Ag or Ag+ leaching out from these products and could possibly result in adverse health outcomes (Christensen et al., 2010). For instance, AgNPs used as coatings on surgical implants may enter into the systemic circulation and translocate into different organs such as the lung and/or liver (Rahman et al., 2009, Tang et al., 2009). More importantly, some food storage containers that use AgNP coatings that have been found to release nanostructured Ag into food due to an increase dissolution under high salt concentration (Echegoyen and Nerín, 2013). Animal studies have demonstrated that AgNP exposure results in hepatotoxicity and pulmonary inflammation (Sung et al., 2008, Tiwari et al., 2011). In addition, AgNPs have been reported to interact with immune cells and induce cytotoxicity through the generation of reactive oxygen species (Carlson et al., 2008, Nishanth et al., 2011). To date, limited research exists evaluating the ability of NPs to directly interact with immune cells involved in allergy such as mast cells and possibly resulting in or exacerbation of allergic disease.

Mast cells are found in most tissue types and play an important role in innate immunity, host defense and allergic disease (Brown et al., 2008a). Mast cells are well studied for their role in

allergic disease and activation through IgE and the high affinity IgE receptor (FcεRI) leading to the release of a variety of mediators including histamine, serotonin, and inflammatory cytokines such as TNF-α**,** osteopontin (OPN), and eosinophil chemoattractant factor as examples (Brown et al., 2008a). In addition, mast cells recognize pathogens through toll-like receptors and scavenger receptors (McCurdy et al., 2003, Medic et al., 2008). Recent animal studies have demonstrated that mast cells contribute to the inflammatory response following NP exposures. Specifically, it has been reported that mast cells are involved in lung inflammation and fibrosis following exposure to multi-walled carbon nanotubes (Katwa et al., 2012). In addition, mast cells have been shown to be involved in cerium oxide-induced alterations in vascular reactivity (Wingard et al., 2011). Even though mast cells appear to be central in the pathogenesis following NP exposure little research has been done assessing the direct interaction of NPs with mast cells. While mast cells are well-known to be involved in allergic conditions, it is currently unclear if NPs have the capacity to induce and/or promote an allergic disease state (Shannahan and Brown, 2014, Shannahan et al., 2012, Podila and Brown, 2013). One study reported that AgNPs can induce mast degranulation in mast the RBL-2H3 rat basophilic cell line, however, the study was focused on real-time live cell imaging of the degranulation process but not the influence of physicochemical properties of AgNPs (Yang et al., 2010b). In another study, researchers used mouse peritoneal mast cells to compare uptake of spherical Au and Ag NPs (Marquis et al., 2011). This study demonstrated that positively charged NPs were internalized more than negatively charged NPs while mast cell degranulation was decreased in cells exposed to negatively charged AgNPs. While mast cells are well-known to be involved in allergic conditions, it is currently unclear if NPs have the capacity to induce and/or promote an allergic disease state (Podila and Brown, 2013, Shannahan and Brown, 2014, Shannahan et al., 2012).

Scavenger receptors are well known for their role in recognizing and binding lipid molecules such as low-density lipoprotein (LDL) and high-density lipoprotein (HDL) (Goldstein et al., 1979, Krieger and Herz, 1994, Landschulz et al., 1996). Scavenger receptor B1 (SR-B1) is a multi-ligand receptor that preferentially binds lipid molecules and other negatively charged molecules (Krieger and Herz, 1994, Landschulz et al., 1996, Rigotti et al., 1997a). Furthermore, SR-B1 has been reported to recognize and bind with pathogens and NPs (Eyre et al., 2010, Mooberry et al., 2010). Many different types of cells express SR-B1 including epithelial cells, endothelial cells, and macrophages. Specifically, the cellular uptake of AgNPs by macrophages and subsequent apoptosis has been shown to be scavenger receptor dependent (Singh and Ramarao, 2012). Therefore it is likely that other cells, which express scavenger receptors on their surface such as mast cells, may interact with AgNPs similarly and this receptor interaction may mediate toxic responses.

In this study, we hypothesized that NP physicochemical properties such as size, shape, and surface coating will influence mast cell degranulation through interaction with SR-B1. To address this hypothesis, bone marrow derived mast cells were used to assess AgNP directed degranulation using AgNPs of differing size, shape and surface coating. Lastly, we evaluated the role of SR-B1 in the observed mast cell degranulation response to various AgNPs.

Materials and Methods

Silver Nanoparticles

20 and 110 nm spherical AgNPs either suspended in citrate (C20 and C110) or polyvinylpyrrolidone (PVP) (P20 and P110) were procured through the National Centers for Nanotechnology Health Implications (NCNHIR) and initially characterized by the National Characterization Laboratory at the National Cancer Institute. Two types of nanoplates with optical resonance peak at specific wavelengths of 550 nm and 850 nm suspended in PVP (P550 and P850), or Ag nanowires that are up to 2 μm suspended in PVP were purchased from NanoComposix at a concentration of 1 mg/ml. All AgNPs were negative for endotoxin contamination.

Silver Nanoparticle Characterization

The hydrodynamic size and Zeta potentials (ZetaSizer Nano, Malvern) of all AgNPs were characterized in N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES). All measurements were performed with 3 individuals samples at a concentration of 50 μg/ml. The size and shape of the AgNPs were confirmed via transmission electron microscopy (TEM, Hitach H7600). Size distribution analysis was performed using the freeware software Image J. A minimum of 100 particles per sample were counted by randomly surveying the entire TEM grid from multiple high magnification images. Image J was used to determine both area and Feret diameters (the greatest distance between two points on an objects boundary).

Reagents and Antibodies

SR-B1 inhibitor 2-(2-butoxyethyl)-1-cyclopentanone thosemicarbazone (Blt2) (Chembridge Corp., San Diego, CA, USA), Rat Lysosome-associated membrane proteins 2

(Lamp2) anti-mouse antibody (eBioscience Inc., San Diego, CA, USA), Imatinib (Cayman Chemical Company, Ann Arbor, MI, USA)

Cell Culture

Bone marrow-derived mast cells were cultured from femoral marrow cells of C57Bl/6 mice (Jackson Laboratories, Bar Harbor, ME). Bone marrow from 2 mice were used for each batch of mast cells. Cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml Primocin™ (Invivogen, San Diego, CA), 25 mM HEPES, 1.0 mM sodium pyruvate, nonessential amino acids (BioSource International, Camarillo, CA), 0.0035% 2-ME and 300 ng/ml recombinant mouse IL-3 (PeproTech, Rocky Hill, NJ). Mast cells were used following 4-6 weeks of culture at 37°C and 5% CO_2. All animal procedures were conducted in accordance with the National Institutes of Health guidelines and approved by the University of Colorado Denver Institutional Animal Care and Use Committee. All animals were treated humanely and with regard for alleviation of suffering. Cytotoxicity of AgNPs at concentrations used in this study were evaluated by MTS and LDH assays (Promega, Madison WI) and did not induce cytotoxicity effect compared to the control group (data not shown). Further these concentrations were based on the evaluation of other nanoparticles which have utilized similar concentrations as performed by the NIEHS Nano GO Consortium (Xia et al., 2013)

Enhanced Darkfield Imaging

$3x10^5$ cells were exposed to AgNPs at concentration of 50 μg/ml for 1 h then washed and spun on a glass slide using a Cytospin IV (Shandon Scientific Ltd., Cheshire, UK) at 300 rpm for 5 min. Cells were then fixed in 2% paraformaldehyde solution for 10 min at 37ºC then washed with phosphate-buffered saline (PBS) three times and mounted with DAPI staining ProLong®

(Life Technologies, Carlsbad, CA). Cells were then qualitatively assessed by enhanced darkfield microscopy (Cytoviva, Auburn, AL) for uptake of AgNPs by focusing on the DAPI stained nucleus.

Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)

$3x10^5$ cells were pretreated with or without the scavenger receptor B1 (SR-B1) inhibitor Blt2 at a concentration of 50 μM. After 30 min, cells were exposed to AgNPs at concentration of 50 μg/ml for 1 h. Following exposure, samples were collected and centrifuged for 10 min at 14,000 rpm (20,817 g) and washed three times with PBS to remove excess AgNPs. All samples were dissolved in 6 ml of 2% HNO_3. Subsequently, the Ag cellular concentration was determined with ICP-MS (X series II, Thermo Scientific) using an internal standard containing Li, Y, and In with a resolution of 0.1 ppb. All experiments were performed in triplicate from 3 individual batches of mature mast cells. Each batch of mast cells was grown from femoral bone marrow of 2 mice.

Mast Cell Degranulation

Bone marrow-derived mast cells were seeded at 5×10^4 cells/well in 96-well flat-bottom plates for assessment of degranulation by β-hexosaminidase release (Iwaki et al., 2005). For treated samples, all types of AgNPs were added at 0, 6.25, 12.5, 25, or 50 μg/ml for 1 h. In addition, 24 h prior to degranulation measurements a subset of cells were sensitized with 100 ng/ml mouse IgE anti-DNP (Sigma-Aldrich, St. Louis, MO) followed by addition of 100 ng/ml dinitrophenylated human serum albumin (DNP-HSA) (Sigma-Aldrich) for 1 h to generate a positive control. For experiments with Blt2, cells were pretreated with Blt2 at 50 μM for 30 minutes before being exposed to AgNPs. To assess the contribution of ionic Ag to β-hexosaminidase release, AgNPs were placed into HEPES for 1 h, spun at 3000 g for 10 min into

Amicon Ultra-15 Centrifugal Filter Units (EMD Millipore). Filtrate containing ionic Ag was used to treat cells. After 1 h incubation of either DNP-HSA or AgNPs at 37°C, *p*-nitrophenyl-*N*-acteyl-β-D-glucopyranoside (≥ 98%) was added to cell supernatants and lysates for 90 min as a chromogenic substrate for *N*-acetyl-β-D-hexosaminidase (Sigma-Aldrich) (Brown et al., 2007). The reaction was stopped with 0.4 M glycine. Optical density was measured at 405 nm using a Synergy™ HT Multi-Mode Microplate Reader (BioTek Instruments, Inc, Winooski, VT). β-hexosaminidase release was expressed as the percentage of total cell content after subtracting background release from unstimulated cells. All experiments were performed in triplicate from 3–6 individual batches of mature mast cells. Each batch of mast cells was grown from femoral bone marrow of 2 mice.

Lamp2 Expression

$3x10^5$ cells were treated with AgNPs, spun at 1200 rpm for 5 min and then suspended in 200 µl of PBS. Cells were then spun at 300 rpm for 5 min onto a glass slide using a Cytospin IV (Shandon Scientific Ltd., Cheshire, UK). Cells were then fixed in a 2% paraformaldehyde solution for 10 min at 37° C, washed with PBS and blocked in 0.1% Tween + 1% BSA for 30 min at 37° C. After removing blocking solution, a Lamp2 antibody (eBioscience Inc., San Diego, CA, USA) conjugated to FITC was added to slides at a dilution of 1:100 and incubated overnight at 4° C. Following incubation the slide was washed 3 times with PBS and mounted with DAPI staining ProLong® (Life Technologies, Carlsbad, CA). Cells were then assessed using a fluorescent microscope (Cytoviva) to evaluate Lamp2 expression.

Osteopontin Measurement

$2.5x10^5$ cells were pretreated with or without the scavenger receptor B1 (SR-B1) inhibitor Blt2 at a concentration of 50 µM. After 30 min, cells exposed to AgNPs at concentration of 50 µg/ml for for 24 h. OPN levels were measured in cell supernatant using a DuoSet ELISA kit (R&D Systems, Minneapolis, MN) in accordance with the manufacturer's instructions. Values are an average of three replicates and reported as pg/mL. All experiments were performed in triplicate from 3 individual batches of mature mast cells. Each batch of mast cells was grown from femoral bone marrow of 2 mice.

Statistical Analyses

All data are presented as mean ± SEM and were analyzed by one-way ANOVA, with differences between groups assessed using Bonferroni post hoc tests. Graphs and analysis were performed using GraphPad Prism 5 software (GraphPad, San Diego, CA). Differences were considered statistically significant at $p \leq 0.05$.

Results

AgNP Characterization

Hydrodynamic size and zeta potential were measured by dynamic light scattering in HEPES buffer that was used for the subsequent mast cell degranulation studies (Table 2.1). The hydrodynamic and TEM sizes were similar for the P20, C20, P110, and C110 samples since they are spherical nanoparticles unlike the P550, P850, and wires that have different morphology. It should be noted that the traditional dynamic light scattering technique is based on Einstein-Stokes relation that is apt only for spherical particles and may only provide an order of magnitude estimate for individual dimensions in different direction for non-spherical shapes. In our case, the hydrodynamic size for P550, P850, and wires in HEPES buffer clearly confirms their nanosize while TEM provides more accurate size data. We found that the C20 AgNPs have the smallest hydrodynamic size with an average of 26.61 nm while the Ag nanowires have largest hydrodynamic size with an average of 315 nm (n=3/group). All AgNPs had a negative zeta potential with the citrate suspended AgNPs displaying the most negative surface charge as compared to PVP suspended AgNPs. The shape and size was further confirmed by TEM images of AgNPs (Figure 2.1).

AgNP Uptake by Mast Cells

Enhanced darkfield imaging of BMMCs following 1 h exposure to AgNPs showed interaction and presence of particles within and on the surface of cells (Figure 2.2). The surface bound and internalized particles were quantified using ICP-MS analysis (n=3/group) (Figure 2.3). Following AgNP exposure for 1 h, we found that significant uptake of AgNPs by mast cells

occurred in all treated samples. However, pretreatment with the SR-B1 inhibitor significantly decreased the cellular uptake of P20 and P110 (Figure 2.3).

AgNP Induced Mast Cell Degranulation

Following a 1 h exposure to AgNPs, BMMC degranulation was evaluated through measuring β-hexosaminidase release into the supernatant (n=3/group) (Figure 2.4). P20 and C20 caused significant BMMC degranulation at concentrations of 25 and 50 μg/ml (Figure 2.4A). P110 and C110 were not found to cause BMMC degranulation compared to control (Figure 2.4B). P550 and P850 caused significant mast degranulation at all concentrations (Figure 2.4C). Exposure to Ag nanowires led to an increase in BMMC degranulation at concentrations of 12.5, 25, and 50 μg/ml (Figure 2.4D). P850 did not demonstrate a concentration-dependent increase in degranulation as shown by P20, C20, and Ag nanowires. To determine whether AgNP or Ag^+ ions were responsible for degranulation, BMMCs were exposed to Ag^+ ions for 1 h and degranulation was evaluated. Exposure to Ag^+ ions was not found to induce mast degranulation (Figure 2.4E).

To evaluate the role of SR-B1 in degranulation following AgNP exposure, BMMCs were pretreated with or without Blt2 at concentration of 50 μM for 30 min. In this experiment, we evaluated only AgNPs that elicited significant BMMC degranulation (>50%). Following a 1 h exposure to AgNPs at a concentration of 50 μg/ml, β-hexosaminidase was assessed. The degranulation of BMMCs exposed to P20, C20, and Ag nanowires was significantly reduced by pretreatment with the SR-B1 inhibitor Blt2 (Figure 2.4F).

To confirm our observation of AgNP directed BMMC degranulation measured by β-hexosaminidase release, we assessed lysosome-associated membrane proteins 2 (Lamp2) expression in BMMCs following AgNPs exposure (Figure 2.5). The expression of Lamp2

following exposure to the various AgNPs was found to match the results of the β-hexosaminidase assay. Lamp2 expression as measured by immunofluorescence (green) was observed in BMMCs exposed to P20, C20, P550, and Ag nanowires while expression of Lamp2 was low in samples treated with P110, C110, and P850 (Figure 2.5).

Osteopontin Release Following AgNP Exposure

Osteopontin (OPN) levels were measured in the supernatant following exposure to AgNPs (n=3/group) in order to assess AgNP-induced mast cell activation. Mast cells were pretreated with or without Blt2 at concentration of 50 μM for 30 min. Following inhibitor pretreatment, BMMCs were exposed to AgNPs at a concentration of 50 μg/ml for 24 h. OPN levels were increased in the supernatant of BMMCs exposed to all types of AgNPs except for P110 (Figure 2.6). Pretreatment with Blt2 was found to decrease OPN levels in the supernatant of BMMCs exposed to all types of AgNPs (Figure 2.6).

Pharmacological Inhibition of Mast Cell Activation by AgNPs

To begin to understand potential mechanisms of AgNP-induced degranulation we pretreated cells with imatinib, a tyrosine kinase inhibitor. BMMCs were pretreated with or without imatinib for 30 min at concentrations of 0.1, 1, 10, and 100 μM and then exposed to C20 at 50 μg/ml for 1 h (n=3/group). Following C20 exposure, β-hexosaminidase release was increased as observed previously, however, treatment with imatinib resulted in a concentration-dependent decrease in BMMC degranulation.

Discussion

Currently we lack sufficient knowledge regarding the ability of these NPs to induce and/or promote allergic disease. Recent studies by our laboratory have demonstrated that mast cells contribute to the inflammation and pathology induced following cerium oxide and carbon-based NP exposure in animal models (Katwa et al., 2012, Wingard et al., 2011). Our current study investigated the direct interactions of physicochemically distinct AgNPs on mast cell activation and degranulation. Further, we evaluated the role of SR-B1 in these AgNP-induced mast cell responses. In summary, this study determined that P20, C20, nanoplates and Ag nanowires could directly induce the degranulation of mast cells. Further this degranulation was driven by particle interactions through the SR-B1 receptor and not the result of dissolution of Ag^{+} ions. Through evaluation of our data we have provided insight into some of the physicochemical properties of NPs which could likely lead to adverse mast cell responses.

Our study evaluated mast cell responses to AgNPs that differed based upon size, shape, and surface coating. These AgNPs were specifically selected for the determination of NP physicochemical properties, which could influence interactions with mast cells and may initiate or promote allergic responses. Specifically, we selected four spherical AgNPs that differed based on size (20 nm or 110 nm) and surface coating (PVP or Citrate). Further we selected two plate-shaped AgNPs, which differed based upon size and resonance (P550 or P850) and were coated with PVP. Lastly we selected Ag nanowires that differed vastly from the other AgNPs based on their needle-like shape and high aspect ratio. Through the use of these selected AgNPs we were able to determine mast cell responses in terms of specific AgNP characteristics.

The internalization of NPs by mast cells has been reported in different studies. For instance silicon dioxide (SiO2) and titanium dioxide (TiO2) was found to be internalized by mast cells and

localized in the secretory granules (Maurer-Jones et al., 2010). Following internalization by mast cells, NPs have been reported to induce or suppress mast degranulation. For instance, mast cells have been shown to internalize fullerenes through nonspecific endocytosis causing an inhibition of degranulation (Ryan et al., 2007). In contrast, gold NPs have been reported to induce mast cell degranulation following internalization that was dependent on size, exposure time, and concentration (Huang et al., 2009, Marquis et al., 2009). Overall NP internalization and subcellular localization may play a major role in mast cell activation or suppression, however, limited data are available.

All of our selected AgNPs were found to be internalized following exposure albeit to varying degrees based upon their physicochemical differences. C20 and C110 were determined to have a more highly negative zeta potential compared to PVP suspended spherical AgNPs. However, PVP suspended particles were cell associated to a greater degree when compared to citrate suspended particles of the same size and shape. Such an observation concurs with the fact that PVP stabilized AgNPs are more prone to surface oxidation and subsequently interact strongly with biomolecules (e.g., serum albumin) (Podila et al., 2012). This suggests that uptake of these spherical AgNPs is not completely driven by charge but also the identity of the surface coating. Interestingly P110, P550, and Ag nanowires were found to have similar surface charges (-27.3, -29.6, -25.9 mV respectively) and the same PVP surface coating, however each were internalized at varying amounts. This demonstrates that uptake is also influenced by size and/or shape of the

NP. This was not unexpected as the influence of size and shape of NPs has been reported to play critical role in cellular uptake (Miethling-Graff et al., 2014). Lastly, P850 AgNPs were taken up more readily than P550 AgNPs. These plates have the same surface coating, shape but differ based upon size suggesting that size is a major factor in their internalization. Internalization of our selected AgNPs by mast cells is a complex process that seems to be dependent on a combination of physicochemical properties.

The primary objective of this study was to evaluate NP characteristics, which could possible promote allergic immune responses through direct interaction with mast cells resulting in degranulation and the release of inflammatory mediators. Exposure to spherical 20 nm AgNPs was found to induce mast cell degranulation whereas spherical 110 nm AgNPs did not. Further, exposure to P550 was found to induce degranulation to a greater degree than P850. Even though Ag nanowires and P110 spherical AgNPs existed with a similar surface charge and coating, Ag nanowires were found to induce degranulation whereas the spherical AgNPs did not. NPs with high aspect ratio such as (MWCNTs) have been reported to cause lysosomal damage and induce inflammasome activation that was dependent on NP length (Hamilton et al., 2013, Li et al., 2013a). Furthermore, TiO2 NPs with fiber structure have been found to induce cytotoxicity, reactive oxygen species (ROS) generation, increased expression of inflammatory cytokines, lysosomal damage, and induction of inflammasome activation (Li et al., 2013a, Hamilton et al., 2013). While in the present study the Ag nanowires reduced cellular uptake, this shape of AgNPs led to mast cell degranulation likely due to the high aspect ratio of the material (Champion et al., 2007, Champion and Mitragotri, 2006, Harris and Dalhaimer, 2012, Sunshine et al., 2014, Sharma et al., 2010). Overall these findings support a role for size and possibly shape in the degranulation response likely mediated through cell surface receptor recognition. Similar results have reported

that mast cells interact differently with different size and shape of same nanomaterial. For instance, carbon based NPs have been reported to induce mast cell activation as reported with multi-walled carbon nanotubes or can suppress mast cell activation as it has been reported with single-walled carbon nanotubes and fullerene C_{60} (Katwa et al., 2012, Umemoto et al., 2014, Huang et al., 2009, Ryan et al., 2007). Based on our current findings, mast cell degranulation is not entirely dependent on internalization of the NP. Specifically, both the citrate and PVP suspended spherical 110 nm AgNPs, while internalized by mast cells, were not found to induce degranulation. In comparison, the C20 AgNPs although not internalized to the same degree as the PVP suspended 20 nm AgNPs induce equivalent degranulation. Such an observation is intriguing in that the dissolution of C20 is expected to be much slower than P20 eliminating Ag+ as a possible cause for degranulation (Kittler et al., 2010). These findings are consistent with other reports that the uptake of NPs by mast cells causes a variety of mast cell responses including the suppression of mast cell degranulation and decreased reactive oxygen species generation while some have been shown to induce degranulation (Umemoto et al., 2014, Ryan et al., 2007, Dellinger et al., 2010). Our findings suggest cellular signaling initiated by size-dependent NP-cell surface receptor interactions facilitated mast cell degranulation that is not completely driven by NP uptake. Ultimately, through our assessment of physicochemically diverse AgNPs, we have demonstrated that some NPs can directly interact with mast cells inducing degranulation, which could induce and/or promote allergic disease *in vivo*.

Osteopontin is a secreted phosphoglycoprotein expressed by a variety of cell types and has been reported as a risk predictor in various diseases including cardiovascular and cancer such as mesothelioma that associated with asbestos exposure (Yang et al., 2008, Berezin and Kremzer, 2013, Pass and Carbone, 2009) . Mast cell activation following NP exposure leads to the release

different cytokines such as OPN and has been reported to be up-regulated following cerium oxide exposure in mice (Wingard et al., 2011). In our current study, we found that OPN is released from mast cells following exposure to all of the selected AgNPs even when NP exposures resulted in limited or no degranulation. These findings are similar to previous research conducted in our laboratory demonstrating that silica exposure also results in cytokine production with limited mast cell degranulation (Brown et al., 2007). This demonstrates that the *de novo* production and release of cytokines from mast cells following particle exposure may involve separate cell signaling pathways compared to degranulation. Our current study also demonstrates that inhibition of the scavenger receptor, SR-B1, reduces the production and release of OPN. Our findings also suggest that while certain NPs do not induce mast cell degranulation, these particles can significantly alter the release of mast cell derived cytokines thereby contributing to an inflammatory response absent of mast cell degranulation. Overall, these findings illustrate that further studies are needed to elucidate mast cell signaling mechanisms.

SR-B1 is well known for its role in the transport of lipid molecules into cells (Krieger and Herz, 1994, Landschulz et al., 1996, Rigotti et al., 1997a). However, it has also been reported that SR-B1 can recognize and bind to other molecules, specifically those that are negatively charged (Catanese et al., 2013, Baranova et al., 2005, Murao et al., 1997). In this study, all AgNPs carried a negative charge therefore interaction between SR-B1 and AgNPs was expected. Inhibition of SR-B1 was found to reduce the uptake of P20, P110, C110, and P850 AgNPs whereas uptake of other AgNPs was unaffected. Due to the range of surface charges exhibited by these AgNPs, SR-B1 recognition and SR-B1 mediated uptake does not appear to be strictly based on NP surface charge. Treatment with an SR-B1 inhibitor (Blt2) was found to reduce mast cell degranulation following exposure to P20, C20, P550, and Ag nanowires. However, SR-B1 inhibition did not

reduce degranulation of mast cells induced by P550 AgNPs or Ag nanowires to the same extent as P20 or C20 AgNPs, which were reduced to control levels. Comparatively, P20 and C20 AgNPs have a greater negative Zeta potential and smaller size than P550 and Ag nanowires. These findings suggest that exposure to P20 and C20 AgNPs induces mast cell degranulation through SR-B1 whereas P550 AgNPs and Ag nanowires induce degranulation primarily via other surface receptors or pathways. Overall our use of an SR-B1 inhibitor demonstrates that mast cell responses to AgNP exposure are to some degree mediated through SR-B1. These findings have safety implications for the design of NPs that do not interact with scavenger receptors may reduce the likelihood of unintended allergic responses mediated through mast cells.

Mast cells can be activated through a variety of cell-surface receptor facilitated mechanisms including interactions with SR-B1, FCεRI, or c-Kit. Activation of these receptors leads to increased calcium flux, tyrosine kinase phosphorylation, and ultimately mast cell degranulation (Zhu et al., 2009, Canton et al., 2013) Imatinib is a therapeutic agent that inhibits the phosphorylation of tyrosine kinases thereby inhibiting downstream mast cell degranulation. We determined in this study, that imatinib treatment reduces mast cell degranulation following exposure to C20 AgNPs thereby demonstrating that the NP-induced mast cell degranulation can be therapeutically inhibited. These findings also suggest that there are downstream cell signaling events, which occur following NP cell-surface receptor interactions that require further investigation. These cellular signaling pathways are likely differentially induced based on NP physicochemical properties and concentrations.

Conclusion

This study demonstrates that mast cells can be directly activated by NPs, which may ultimately induce and/or promote an allergic immune response. Further, NP-induced mast cell degranulation is related to the physicochemical properties of the NP such as shape, size, and surface coating. This study implicates a role for SR-B1 in the degranulation of mast cells by NPs and suggests that possible allergic responses to NPs can be therapeutically treated by inhibition of tyrosine kinase phosphorylation. Future research needs to be performed to understand cell-signaling pathways, which control cytokine production and degranulation. Through an understanding of these mechanisms NPs can be formulated and utilized for numerous applications while mitigating unintended adverse health effects such as allergic immune responses.

Table 2.1 Characterization of AgNP

	P20	C20	P110	C110	P550	P850	Wires
Hydrodynamic Size (nm) (n=3/group)	28.75 ± 1.95	26.61 ± 4.09	111.5 ± 0.17	132.85 ± 2.05	37.54 ± 0.28	71.49 ± 2.59	315.75 ± 2.33
Zeta Potential (mV)	-39.9	-49.7	-27.3	-54.9	-29.6	-32.5	-25.9

Figure 2.1 Transmission electron microscopy (TEM) of AgNPs

Representative TEM images demonstrating AgNP shape and size. AgNPs of differing size and shape were evaluated including spherical 20 nm AgNPs suspended in either polyvinylpyrrolidone (P20) or citrate (C20), spherical 110 nm AgNPs suspended in either polyvinylpyrrolidone (P110) or citrate (C110), Ag plates suspended in polyvinylpyrrolidone of either 550 nm resonance (P550) or 850 nm resonance (P850), and Ag nanowires that were suspended in polyvinylpyrrolidone (Wires).

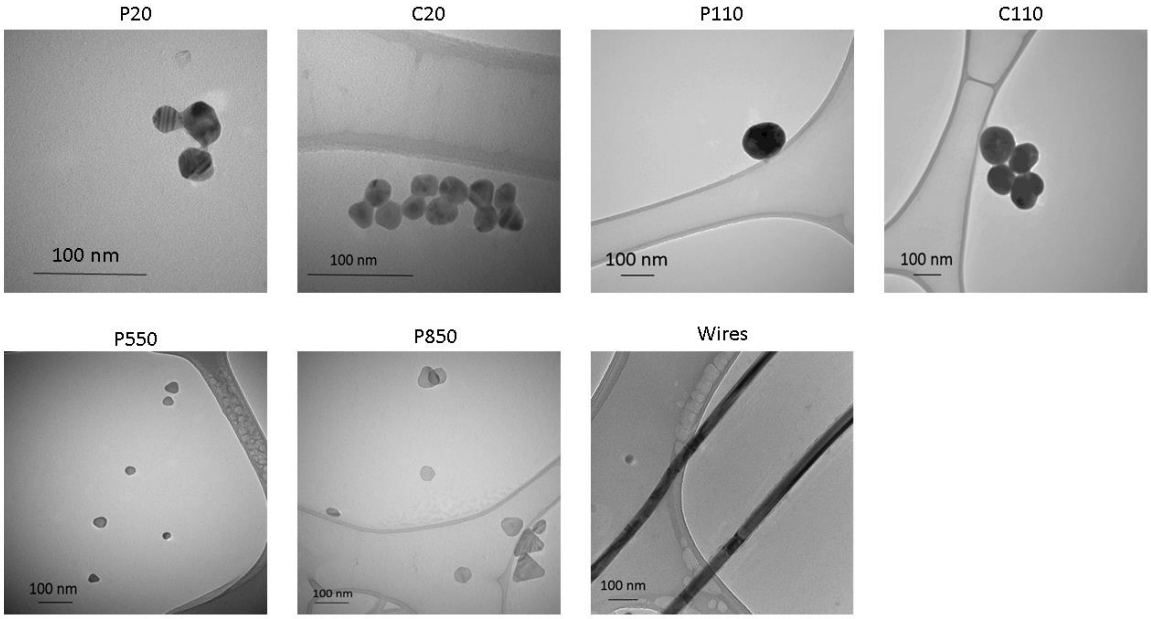

P20
100 nm
C20
100 nm
P110
100 nm
C110
100 nm
P550
100 nm
P850
100 nm
Wires
100 nm

Figure 2.2 Enhanced Darkfield Images of Control or AgNP Treated Bone Marrow-Derived Mast Cells (BMMCs)

Representative enhanced darkfield images of control or AgNP treated bone marrow-derived mast cells (BMMCs). AgNPs of differing sizes and shapes were evaluated including spherical 20 nm AgNPs suspended in either polyvinylpyrrolidone (P20) or citrate (C20), spherical 110 nm AgNPs suspended in either polyvinylpyrrolidone (P110) or citrate (C110), Ag plates suspended in polyvinylpyrrolidone of either 550 nm resonance (P550) or 850 nm resonance (P850), and Ag nanowires that were suspended in polyvinylpyrrolidone (Wires).

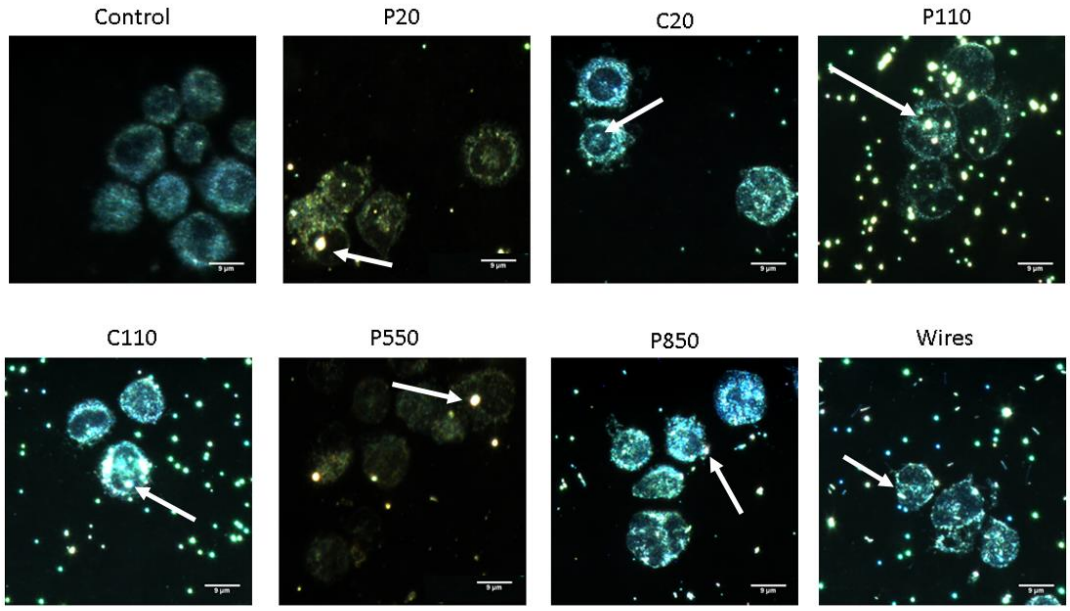

Control
P20
C20
P110
C110
P550
P850
Wires

Figure 2.3 ICP-MS Measurement of AgNP Uptake by Bone Marrow-Derived Mast Cells (BMMCs)

ICP-MS measurement of AgNP uptake by bone marrow-derived mast cells (BMMCs). Cells were pretreated with or without the scavenger receptor B1 (SR-B1) inhibitor Blt2 prior to AgNPs exposure. Values are expressed as mean ± SEM (n=3/group). * Significant difference from control group ($p \leq 0.05$). #Significant difference of Blt2 pretreated group compared to Blt2 untreated group ($p \leq 0.05$).

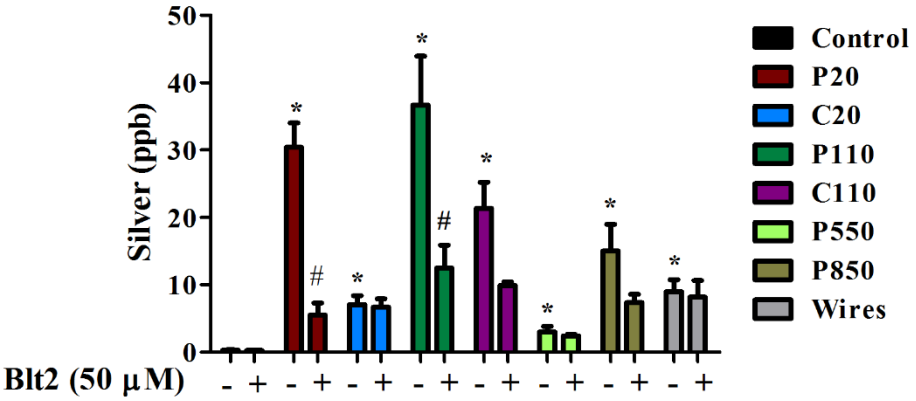

Silver (ppb)
50
40
30
20
10
0
Blt2 (50 µM)
- + - + - + - + - + - + - + - +
Control
P20
C20
P110
C110
P550
P850
Wires

Figure 2.4 Evaluation of Mast Cell Degranulation following AgNPs exposure

Mast cell degranulation was evaluated by measuring release of β-hexosaminidase into the supernatant 1 h following AgNP exposure. A) Bone marrow-derived mast cell (BMMC) degranulation following exposure to spherical polyvinylpyrrolidone (PVP) coated (red) or citrate coated 20 nm (blue) AgNPs. B) BMMC degranulation following exposure to spherical PVP coated (green) or citrate coated 110 nm (purple) AgNPs. C) 550 nm or 850 nm resonant AgNP plates. D) BMMC degranulation following exposure to PVP coated Ag nanowires. E) BMMC degranulation following exposure to Ag^+ ions. F) BMMC degranulation of samples pretreated with or without the scavenger receptor B1 (SR-B1) inhibitor Blt2. Values are expressed as mean ± SEM (n=3/group). *Significant difference from controlled group ($p \leq 0.05$). $^\#$Significant difference of Blt2 pretreated group compared to Blt2 untreated group ($p \leq 0.05$).

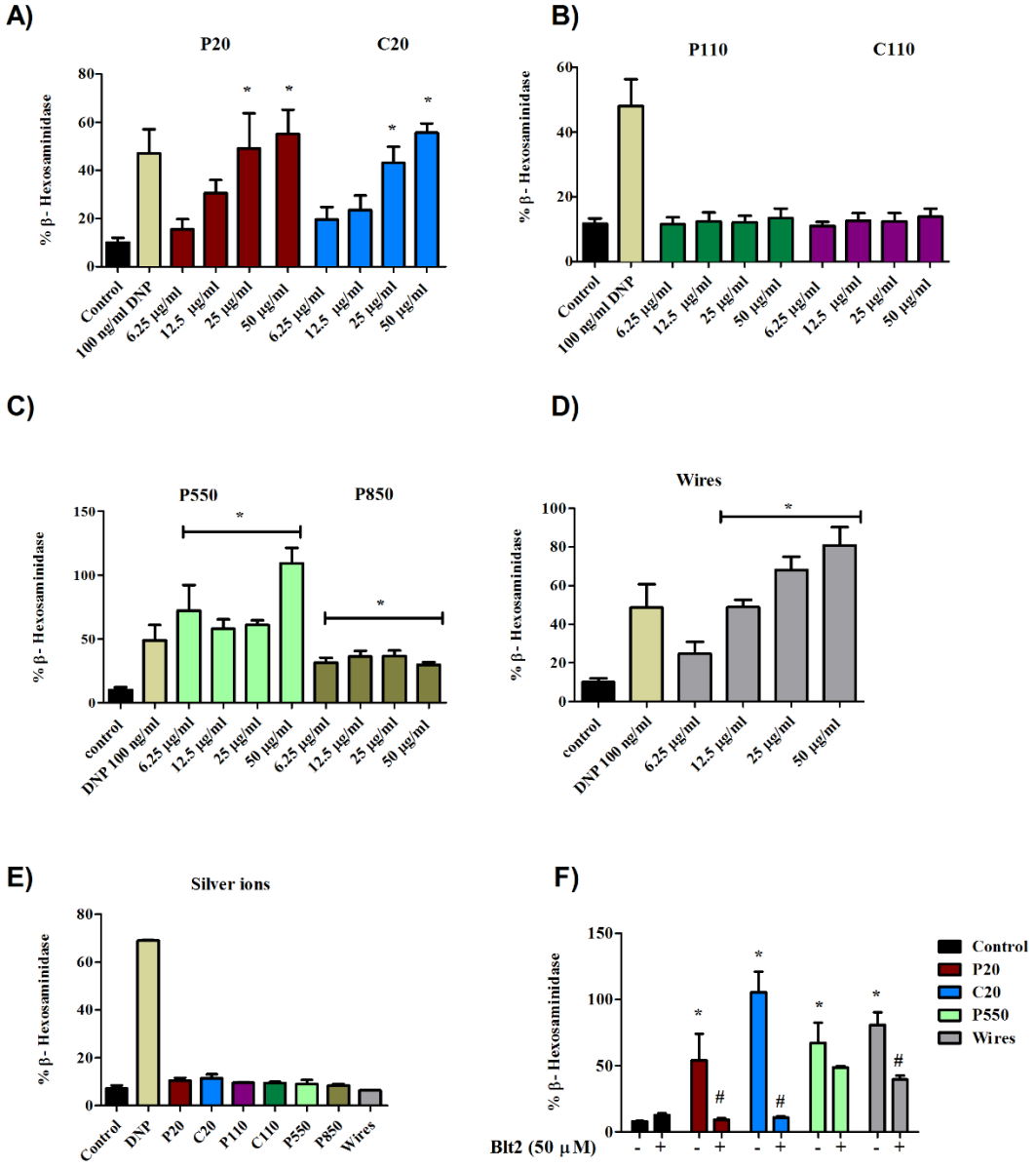

A)
P20
C20
% β- Hexosaminidase
Control
100 ng/ml DNP
6.25 µg/ml
12.5 µg/ml
25 µg/ml
50 µg/ml
B)
P110
C110
C)
P550
P850
control
DNP 100 ng/ml
D)
Wires
E)
Silver ions
DNP
P20
C20
P110
C110
P550
P850
Wires
F)
Blt2 (50 µM)
Control
P20
C20
P550
Wires

Figure 2.5 Evaluation of AgNPs-induced Lysosome-Associated Membrane Protein 2 (Lamp2) Expression

Lysosome-associated membrane protein 2 (Lamp2) expression (green) in bone marrow-derived mast cells (BMMCs) following AgNP exposure. BMMCs were collected 1 h following AgNP exposure, washed, spun onto glass slides and immunofluorescently stained with DAPI to identify the nucleus (blue) and Lamp2 to determine BMMC degranulation (green).

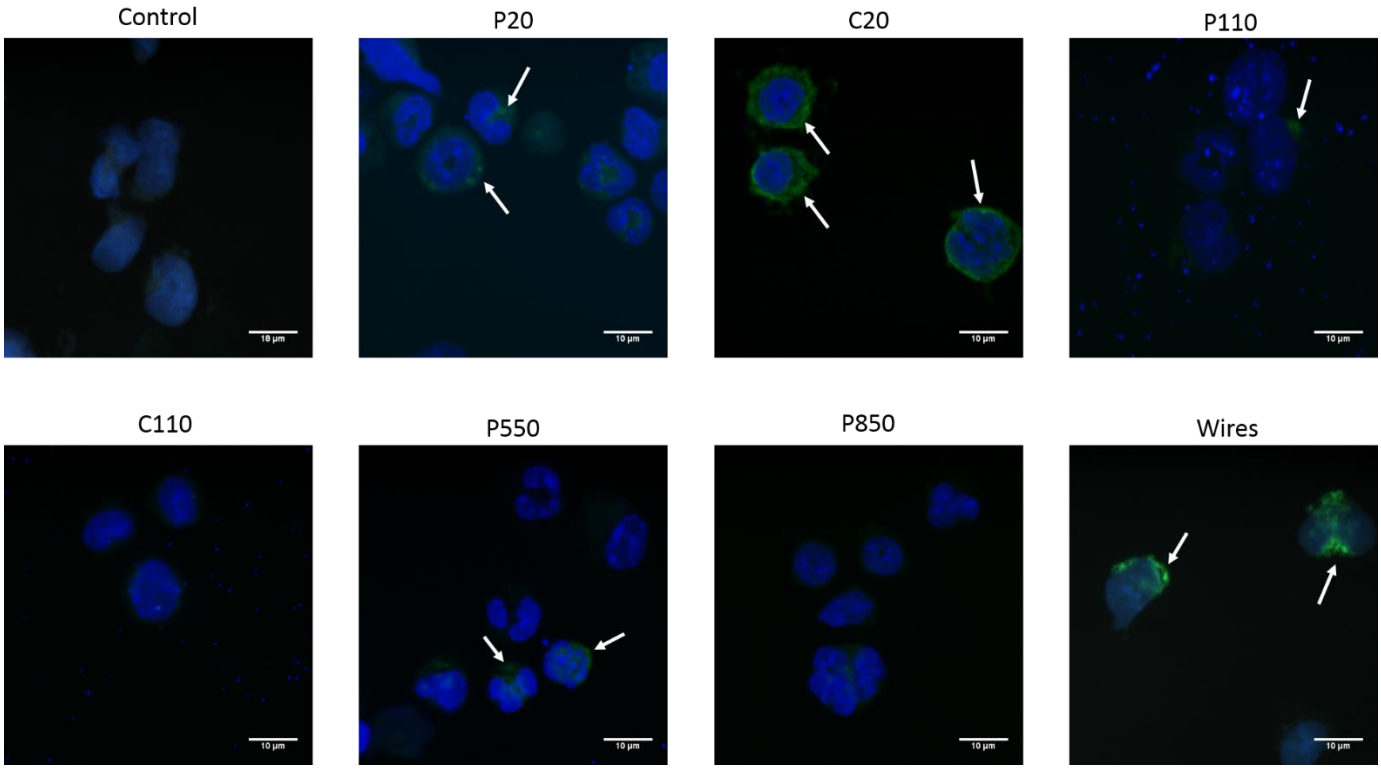

Control
P20
C20
P110
C110
P550
P850
Wires

Figure 2.6 Measurement of Osteopontin (OPN) level in Bone Marrow-Derived Mast Cells Following AgNPs Exposure

Osteopontin (OPN) levels were measured in supernatants by ELISA. Bone marrow-derived mast cells (BMMCs) were pretreated with or without the scavenger receptor B1 (SR-B1) inhibitor Blt2 before AgNPs exposure. Values are expressed as mean ± SEM (n=3/group). * Significant difference from controlled group ($p \leq 0.05$). #Significant difference of Blt2 pretreated group compared to Blt2 untreated group ($p \leq 0.05$).

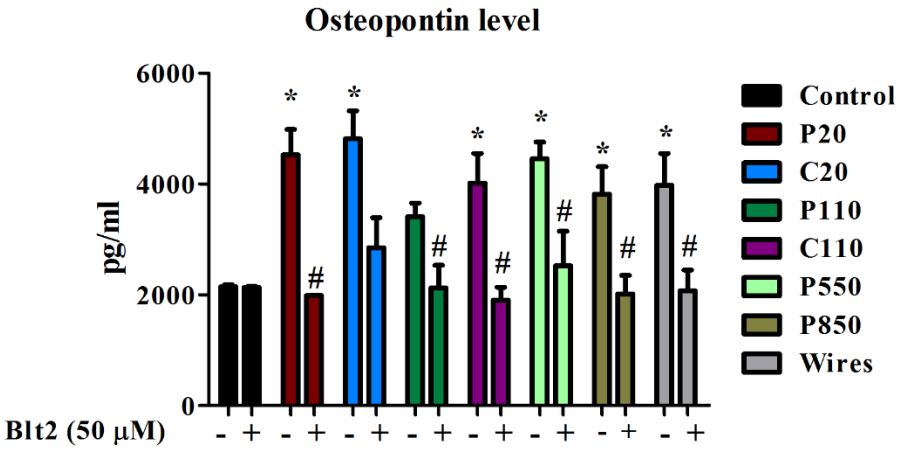
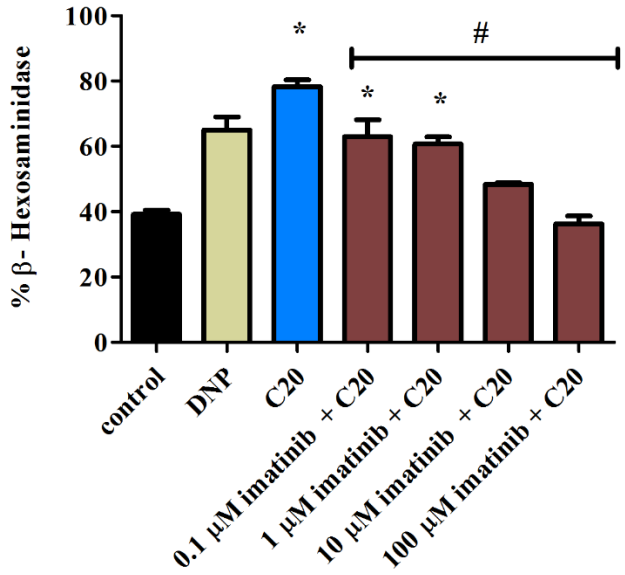

Osteopontin level
6000
4000
2000
0
pg/ml
Blt2 (50 μM)
- + - + - + - + - + - + - + - +
Control
P20
C20
P110
C110
P550
P850
Wires

Figure 2.7 Pharmacological Inhibition of Mast Cell Activation by AgNPs

Bone marrow-derived mast cell (BMMC) degranulation was evaluated by measuring release of β-hexosaminidase 1 h following exposure to 50 μg/ml of 20 nm citrate coated AgNPs (C20). Cells were pretreated with or without imatinib for 30 min at concentration of 0.1, 1, 10, 100 μM. *Significant difference from controlled group ($p \leq 0.05$). #Significant difference of imatinib pretreated group compared to imatinib untreated group ($p \leq 0.05$).


% β- Hexosaminidase
100
80
60
40
20
0
*
#
*
*
control
DNP
C20
0.1 μM imatinib + C20
1 μM imatinib + C20
10 μM imatinib + C20
100 μM imatinib + C20

CHAPTER 3: Scavenger Receptor B1 Facilitates Macrophage Uptake of Silver Nanoparticles and Cellular Activation

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Abstract

Due to increased use of silver nanoparticles (AgNPs) for their antimicrobial activity concerns have risen regarding potential adverse human health effects. Scavenger receptor B1 (SR-B1), a major receptor for high-density lipoprotein (HDL), is expressed by macrophages and has also been reported to play a role in recognition of negatively charged particles. We therefore hypothesized that SR-B1 mediates macrophage uptake of AgNPs and inflammatory activation. To test this hypothesis we exposed a mouse macrophage cell line RAW264.7 (RAW) and bone marrow-derived macrophages (BMDM) to 20 nm citrate-suspended AgNPs. To verify the role of the SR-B1 receptor we utilized a SR-B1 inhibitor (Blt2). *In vitro* studies demonstrated uptake of AgNPs and HDL coated AgNPs by macrophages which was significantly reduced following pretreatment with Blt2. Inflammatory cytokine arrays revealed that macrophages exposed to AgNPs up-regulated expression of *Tnf-α, Oncostatin m (OSM), Ccl4, Il17f, Ccl7*, and *Ccl2*, whereas *Il16* was found to be downregulated. Macrophage activation was observed following AgNP and HDL coated AgNP exposure as measured by OSM protein production and increased surface expression of CD86. These markers of activation were reduced with Blt2 pretreatment. The *in vitro* findings were confirmed *in vivo* through pulmonary instillation of AgNPs in mice. Pulmonary instillation of AgNPs resulted in a recruitment of inflammatory cells that were reduced

in SR-B1 deficient mice or following Blt2 pretreatment. This study suggests that SR-B1 plays a major role in cellular recognition of AgNPs and the induction of cell responses that could contribute to inflammation caused by AgNP exposure.

Introduction

The use of nanoparticles (NPs) has increased in the last decade due to their unique physical and chemical properties. Specifically, silver nanoparticles (AgNPs) have attracted high attention due to their antimicrobial and antifungal properties, which have led to their extensive usage in water filters, biomedical products, surgical tools, and wound dressing (Nocchetti et al., 2013, Dong et al., 2012, Levard et al., 2013). These incorporated AgNPs may potentially leach out of consumer products as Ag or Ag^{+} ions thereby posing a potential human health risk (Christensen et al., 2010). The use of AgNPs as drug carriers or in wound dressings and surgical implants could lead directly to their entrance into systemic circulation thereby distributing to a variety of organs including the liver, spleen, kidney and lungs (Rahman et al., 2009, Tang et al., 2009). Specifically, exposure to AgNPs has been found to induce hepatotoxicity and pulmonary inflammation (Tiwari et al., 2011, Sung et al., 2008). In addition, AgNPs have been reported to interact with macrophages and cause cytotoxicity through the generation of reactive oxygen species (Nishanth et al., 2011, Carlson et al., 2008).

Macrophages are well-known to be one the primary innate immune cells to remove foreign particles and pathogens through phagocytosis; scavenge apoptotic cells and cellular debris; and are involved in tissue remodeling (Gordon and Taylor, 2005, Wynn and Barron, 2010). Macrophages express various types of scavenger receptors that help in the removal of dead cells and foreign particles leading to cellular activation and the production of a variety of mediators that promote inflammation (Gordon and Taylor, 2005). It has been reported that macrophages readily interact with and phagocytose a number of different types of NPs such as zinc oxide, titanium dioxide, and AgNPs (Triboulet et al., 2014, Zhang et al., 2013, Singh and Ramarao, 2012).

Specifically, scavenger receptors have been found to mediate the uptake of AgNPs by macrophages and facilitate the apoptotic response (Singh and Ramarao, 2012).

Scavenger receptors were originally defined by their ability to bind modified low-density lipoprotein (LDL), however they recently have been found to interact with a variety of other substances including pathogens and foreign objects (Goldstein et al., 1979). The scavenger receptor family is subdivided, based on their structures and functions, into 8 classes designated A to I (Krieger, 1997, Murphy et al.). Scavenger receptor B1 (SR-B1) is a multi-ligand receptor that binds to high density lipoprotein (HDL) and other modified LDL molecules therefore facilitating lipid molecule uptake by cells (Krieger and Herz, 1994, Landschulz et al., 1996, Rigotti et al., 1997a, Rigotti et al., 1997b). SR-B1 is expressed on a variety of cell types including epithelial, endothelial, and macrophages (Duncan et al., 2002, Valacchi et al., 2011, Rigotti et al., 1997a). While the role of scavenger receptor A (SR-A) in NP recognition has been reported, little is known on how SR-B1 contributes to NP uptake and cellular responses (Orr et al., 2011, Chao et al., 2013, Aldossari et al., 2015).

In the current study, we hypothesized that SR-B1 on macrophages mediates uptake of AgNPs and subsequent cellular activation. To address this hypothesis, we utilized a mouse macrophage cell line RAW264.7 (RAW) and an inhibitor to SR-B1 to examine 20 nm AgNP uptake using inductively coupled plasma mass spectrometry (ICP-MS), flow cytometry and enhanced darkfield hyperspectral microscopy. In addition, we examined macrophage activation (cytokine production and CD86 expression) following exposure to AgNPs in the presence or absence of an SR-B1 inhibitor. Lastly, we performed an *in vivo* experiment in SR-B1$^{-/-}$ mice to examine macrophage uptake and inflammation following a pulmonary instillation of AgNPs.

Materials and Methods

Silver Nanoparticles and Protein Corona Formation

AgNPs with a diameter of 20 nm were procured from NanoComposix (San Diego, CA) by the NIEHS Centers for Nanotechnology Health Implications Research (NCNHIR) in a suspension of citrate ($C_6H_5O_7^{3-}$) at a concentration of 1 mg/ml. To form the protein corona (PC), AgNPs were incubated at 10°C for 8 h on a rotator in the presence HDL (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 2.5 mg/ml. After incubation AgNPs were centrifuged at 14,000 rpm (20,817 g) for 10 min at 4°C and resuspended to 1 mg/ml with DI water.

Silver Nanoparticle and Protein Corona Characterization

The size and shape of the AgNPs were confirmed via transmission electron microscopy (TEM, Hitach H7600). Size distribution analysis was performed using the freeware software Image J. A minimum of 100 particles per sample were counted by randomly surveying the entire TEM grid from multiple high magnification images. Image J was used to determine both area and Feret diameters (the greatest distance between two points on an objects boundary). The hydrodynamic size and zeta potentials (ZetaSizer Nano, Malvern) of AgNPs and HDL coated AgNPs were characterized in DI water. All measurements were performed with 3 individuals samples at a concentration of 50 µg/ml. Hyperspectral enhanced darkfield microscopy (Cytoviva, Auburn, AL) was utilized to characterize spectral shifts following association of HDL. Particles were loaded onto premium clean microscope slides and mean spectrums were created utilizing pixels with an intensity greater than 1000. Mean spectrums were then compared to AgNPs without HDL to determine changes due to the association of HDL. A red shift in spectrum following

incubation with protein is indicative of PC formation. These mean spectra were later used to identify AgNPs within macrophages to confirm uptake.

Cell Culture

Mouse macrophages (RAW264.7) were obtained from the American Type Culture Collection (ATCC) and cultured in DMEM media containing 10% FBS and 1.0% penicillin-streptomycin solution. Macrophages were maintained in flasks under standard conditions of 37°C and 5% CO_2. Bone marrow-derived macrophages (BMDM) were cultured from femoral marrow cells of C57Bl/6 mice (Jackson Laboratories, Bar Harbor, ME). Cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin-streptomycin, 1.0 mM sodium pyruvate, and 0.0035% 2-mercaptoethanol. Bone marrow cells were seeded in flasks at 3 x 10^7 cells/T75 in 20 ml of media overnight at 37°C for stromal cell elimination. At day 2, media was removed from cells and replaced with 20 ml of fresh media which contained 10 ng/ml macrophage colony-stimulating factor (M-CSF) (10 µg/ml stock, R&D). At day 4-5, an additional 5 ng/ml M-CSF was added to cells. At day 7-8, half of the media was replaced with fresh media and 5 ng/ml of M-CSF was added. This procedure of macrophage differentiation results in cells ready to use for experimentation between days 7 and 10 depending on health and confluency. All animal procedures were conducted in accordance with the National Institutes of Health guidelines and approved by the University of Colorado Denver Institutional Animal Care and Use Committee. All animals were treated humanely and with regard for alleviation of suffering.

Assessment of AgNP Cytotoxicity

RAW cells were grown to 90% confluency in 96 well plates (Costar) and were exposed to (0, 6.25, 12.5, 25, or 50 µg/ml) of AgNPs and AgNPs coated with HDL for 3 and 6 h in serum-

free media. The concentrations evaluated were selected based on previous in vitro examination of other nanoparticles (Xia et al. 2013). Changes in cell viability were assessed via the MTS assay (Promega, Madison, WI, USA) via manufacturer's instructions using a spectrophotometer (BioTek Synergy HT, BioTek, Winooski, VT, USA). Based on this dose response study a concentration of 50 µg/ml was utilized for all subsequent in vitro experiments. All experiments were performed in triplicate from 4 to 5 individual batches of RAW cells.

Cellular Uptake of Silver Nanoparticles

For ICP-MS determination of cellular uptake, $3x10^5$ RAW cells were pretreated for 30 min with or without an SR-B1 inhibitor 2-(2-butoxyethyl)-1-cyclopentanone thiosemicarbazone (Blt2) (Chembridge Corp., San Diego, CA, USA) at 50 uM and then exposed to AgNPs at a concentration of 50 µg/ml for 2 h. Following exposure, cells were washed three times with PBS to remove excess AgNPs. All samples were dissolved in 6 ml of 2% HNO_3. Subsequently, the Ag cellular concentration was determined by ICP-MS (X series II, Thermo Scientific) with a detection limit of 0.05 ppb using an internal standard containing Li, Y, and In. To assess AgNP uptake by flow cytometry, RAW cells were grown to 90% confluency in 24 well plates (Costar) and were pretreated for 30 min with either serum-free media (control) or Blt2 at 50 µM. Cells were then exposed to AgNPs at 50 µg/ml for 2 h. Following exposure, cells were washed with PBS and detached with 250 µl of trypsin. Trypsin was neutralized with an equal volume of media containing serum and macrophages were collected. Changes in uptake were assessed by alterations in side scatter shift through flow cytometry (Accuri C6 Flow Cytometer, BD Biosciences, San Jose, CA, USA). All experiments were performed in 3 individual batches of RAW cells

Enhanced Darkfield Hyperspectral Imaging

RAW cells ($3x10^5$) were grown on chamber slides and exposed to AgNPs at a concentration of 50 µg/ml for 2 h. Cells were then fixed in 2% paraformaldehyde solution for 10 min at 37°C, washed with PBS three times and mounted with ProLong® (Life Technologies, Carlsbad, CA). Cells were then qualitatively assessed by enhanced darkfield microscopy (Cytoviva, Auburn, AL) for uptake of AgNPs. Mean spectral profiles used to characterize AgNPs were utilized for spectral mapping of AgNPs within cells. Spectral mapping identifies pixels in images of AgNP-exposed macrophages that positively match the spectral profiles of AgNPs alone confirming the identity of the particle within the cell. Pixels that positively match AgNP spectral profiles are identified by being colored red in images.

PCR Array

A mouse inflammatory cytokine PCR array was utilized to identify markers of macrophage activation following AgNP exposure. BMDMs were grown and exposed to 50 ug/ml of AgNPs for 2 h and collected in Trizol. Total RNA was isolated using Direct-zol RNA MiniPrep (Zymo Research Corp., Irvine, CA, USA) via manufacturer's instructions and quantified via Nanodrop (Nanodrop 2000c Spectrophotometer, Thermo Scientific). cDNA synthesis was performed using RT² SYBR Green Mastermix and the RT^2 First Strand Kit (SABiosciences, Frederick, MD, USA). RT^2 Profiler PCR Array Systems (Cat# PAMM-011Z, SABiosciences; Frederick, Maryland, USA) of 84 genes encoding key inflammatory cytokines and chemokines were used. 96 well plates were prepared according to manufacturer's instructions and using a StepOnePlus Real-Time PCR System (ABI, Foster City, CA, USA)). The data analysis was based on the ΔΔCt method with normalization to glyceraldehyde 3-phosphate dehydrogenase. A web-based integrated PCR array expression analysis suite provided by SaBiosciences was used for data analysis. All experiments

were performed in triplicate from 6 individual batches of BMDM cells. Data from all 84 genes is available within Supplemental Table 1.

Oncostatin M Measurement

OSM levels were measured in the cell supernatant of $2.5x10^5$ RAW cells that were pretreated for 30 min with either serum-free media or Blt2 at 50 μM to evaluate the role of SR-B1 and then treated with AgNPs for 24 h. OSM levels were measured in the supernatant using a DuoSet ELISA kit (R&D Systems, Minneapolis, MN, USA) in accordance with the manufacturer's instructions. Values are an average of experiments that were performed in triplicate from 3 individual batches of RAW cells and reported as pg/mL.

CD86 Expression

RAW cells were grown to 90% confluency in 24 well plates (Costar) and pretreated for 30 min with either serum-free media or Blt2 at 50 μM. Cells were then exposed to AgNPs at 50 μg/ml for 6 h. Following exposure, cells were washed with PBS and detached with 250 μl of trypsin. Trypsin was neutralized with an equal volume of media with serum and cells were collected. Cells then were washed twice with staining buffer (Pharmingen™, BD Biosciences) and then suspended in 100 μl of staining buffer and 2 μl of Fc receptor antibody (eBioscience Inc., San Diego, CA, USA) for 10 min on ice. A CD86 antibody conjugated to PerCp-Cy5.5 (eBioscience Inc., San Diego, CA, USA) was then added at a concentration of 1:100 and incubated for 30 min at room temperature in the dark. Following incubation, cells were washed twice with staining buffer and finally suspended in 500 μl of staining buffer and $5x10^3$ events were assessed by flow cytometry Accuri™ C6 (BD Biosciences, USA)). All experiments were performed in 3 individual batches of RAW cells

Experimental Animals

C57BL/6J and Scarb1$^{-/-}$ (SR-B1$^{-/-}$) mice were acquired from Jackson Laboratories (Bar Harbor, ME, USA) at 4–10 weeks of age and breeding colonies were maintained at the University of Colorado. C57BL/6J mice were pretreated with PBS or 250 μg/kg body weight of Blt2 for 30 min prior to AgNP aspiration. C57BL/6 mice (n=3) (with or without Blt2 pretreatment) and SR-B1$^{-/-}$ mice (n=3) were exposed by oropharyngeal aspiration to AgNPs at a concentration of 2 mg/kg body weight. Two hours post-exposure, mice underwent *in situ* right lung bronchoalveolar lavage (BAL). The right lung was lavaged four times with the same aliquot of ice-cold Hanks balanced salt solution (HBSS) at 26.25 ml/kg body weight. Cells were then spun down onto microscope slides using a Cytospin IV (Shandon Scientific Ltd., Cheshire, UK). The collected bronchoalveolar lavage fluid (BALF) was used to qualitatively assess macrophage uptake by enhanced darkfield imaging (Cytoviva). Similar to the previous *in vitro* experiment, AgNPs were positively identified in macrophages via spectral mapping. In a separate experiment, C57BL/6J mice (n=3) pretreated with PBS or Blt2 for 30 min and SR-B1$^{-/-}$ (n=3) mice were exposed via oropharyngeal aspiration to AgNPs at a concentration of 2 mg/kg. BALF was collect 24 h post-exposure from the right lung for differential cell counts. The right lung was lavaged four times with the same aliquot of ice-cold HBSS at 26.25 ml/kg body weight. A total cell count on BALF samples was then performed and 20,000 cells from each sample were loaded on slides using a Cytospin IV (Shandon Scientific Ltd., Cheshire, UK). Slides were then prepared with a three-step hematology stain (Richard Allan Scientific, Kalamazoo, MI, USA) and differential cell counts were determined by morphology with evaluation of 300 cells per slide. All animal procedures were conducted in accordance with the National Institutes of Health guidelines and approved by the

University of Colorado Denver Institutional Animal Care and Use Committee. All animals were treated humanely and with regard for alleviation of suffering.

Statistical Analyses

All data are presented as mean ± SEM and were analyzed by one-way or two way ANOVA, with differences between groups assessed using Bonferroni post hoc tests. Graphs and analysis were performed using GraphPad Prism 5 software (GraphPad, San Diego, CA). Differences were considered statistically significant at $p \leq 0.05$.

Results

Silver Nanoparticle Characterization

Hydrodynamic size and zeta potential were measured using a ZetaSizer Nano (Malvern, Malvern UK). Citrate coated 20 nm AgNPs were found to have a hydrodynamic size of 19.13 nm which increased to 62.1 nm when a PC consisting of HDL formed on their surface (Table 3.1). Addition of HDL to AgNPs resulted in a decrease in AgNP surface charge (due to displacement of citrate groups) but increased hydrodynamic size as expected (Table 3.1). The size and spherical shape of AgNPs was confirmed through TEM imaging (Figure 3.1A). Enhanced darkfield hyperspectral microscopy was utilized to evaluate changes in the spectrum of AgNPs indicative of HDL PC formation. Hyperspectral images of AgNPs (Figure 3.1B) and AgNPs with an HDL PC (Figure 3.1C) were collected. Analysis of the mean spectrums generated from these particles demonstrated a red shift or shift to the right of the AgNP spectrum following association with HDL (Figure 3.1D) concurring with our previous results (Shannahan et al., 2014). This red shift is indicative of PC formation.

In Vitro Uptake of Silver Nanoparticles by Macrophages

AgNP internalization by macrophages was assessed by a variety of methods including ICP-MS, flow cytometry, and enhanced dark field hyperspectral microscopy. All experiments utilized a time point of 2 h and a AgNP concentration of 50 ug/ml. These parameters for the evaluation of uptake were selected based on preliminary experimentation which, demonstrated macrophage uptake of AgNPs had > 85% of cells viability up to 6 h with exposure to AgNPs at concentrations of 0, 6.25, 12.5, 25, or 50 μg/ml (Figure 3.2A and 3.2B). AgNP internalization was quantitatively measured through the use of ICP-MS. As expected RAW cell uptake of samples exposed to 50

μg/ml of AgNPs for 2 h had higher silver content compared to control samples (Figure 3.2C). Coating of particles with HDL led to a significant increase in AgNP uptake compared to uncoated AgNPs (Figure 3.2C). To assess the role of SR-B1, cells were pretreated with Blt2 at 50 uM for 30 min before AgNP exposure. Pretreatment with Blt2 resulted in a significant decrease in the internalization of AgNPs (Figure 3.2C). Macrophage uptake of AgNPs was further confirmed by flow cytometry via alterations in side scatter shift (SSC). Alterations in cellular SSC equate to changes in cellular granulation which is indicative of macrophage uptake of AgNPs (Suzuki et al., 2007). Macrophages that were exposed to 50 μg/ml of AgNPs + HDL for 2 h had a greater shift in SSC compared to control samples demonstrating increased uptake as observed with ICP-MS (Figure 3.2D). Pretreatment with Blt2 at 50 uM for 30 min before AgNP exposure resulted in a significant decrease in SSC of both the AgNP and AgNPs + HDL groups (Figure 3.2D). Finally, Blt2 pretreatment alone was not found to cause changes in SSC and appeared similar to control samples (Figure 3.2D).

Lastly, cellular uptake was qualitatively assessed through enhanced dark field hyperspectral imaging of RAW cells following a 2 h exposure to AgNPs. Macrophages exposed to AgNPs and AgNPs coated with HDL demonstrated the presence of NPs within and on the surface of cells (Figure 3.3B & 3.3D). Pretreatment with Blt2 at 50 uM for 30 min before exposure resulted in decreased AgNP uptake (Figure 3.3C & 3.3E). To confirm the presence of AgNPs in macrophages, the spectral profile of the AgNPs as shown in Figure 1 was mapped back to the exposed cells. Pixels that matched the AgNPs were highlighted in red (Figure 3.3, left panel) thus confirming the presence of AgNPs within the macrophages. Samples treated with HDL only or Blt2 alone did not show altered cellular morphology or the presence of particles (data not shown).

In Vitro Macrophage Activation by Silver Nanoparticles

PCR array analysis of BMDMs revealed that *Tnf-α, Osm, Ccl4, Il17f, Ccl7, and Ccl2* were up-regulated, whereas *Il16* was found to be down-regulated 2 hrs following AgNP exposure (Table 3.2). The inflammatory cytokine OSM was found to be highly induced following AgNP exposure and was selected as a novel marker of macrophage activation for subsequent studies. Further OSM was selected due to the high statistical significance compared to control cells versus TNF-α for example.

Oncostatin M protein levels were evaluated following AgNP exposure by ELISA (Figure 3.4A). Cells were pretreated with media or with Blt2 at a concentration of 50 μM for 30 min before a 24 h exposure to AgNP at 50 μg/ml in serum-free media. OSM protein levels significantly increased following AgNP exposure (Figure 3.4A). Further, exposure to HDL coated AgNPs caused a significant increase in OSM production compared to cells exposed to uncoated AgNPs (Figure 3.4A). Pretreatment with Blt2 was found to significantly decrease macrophage OSM production in response to both AgNP and AgNPs + HDL exposure (Figure 3.4A).

Macrophage activation was further evaluated by measuring the expression of the macrophage surface marker CD86 by flow cytometry following exposure to 50 μg/ml of AgNPs for 2h. Exposure to AgNPs and coated AgNP with HDL caused a significant increase in CD86 expression compared to control cells (Figure 3.4B). To evaluate the role of SR-B1, cells were pretreated with Blt2 for 30 min prior to AgNP exposure, which was found to decrease AgNP-induced expression of CD86 to control levels (Figure 3.4B). Furthermore, pretreatment with Blt2 caused a significant decrease in CD86 expression in cells exposed to AgNP coated HDL compared to cells exposed to AgNPs coated with HDL without Blt2 pretreatment (Figure 3.4B).

In Vivo Uptake of Silver Nanoparticles by Macrophages

To investigate the mechanism of AgNP cellular uptake in a physiological system, an *in vivo* experiment was performed using C57BL/6 and SR-B1$^{-/-}$ mice. C57BL/6 mice were pretreated with 50 µl of PBS or PBS with Blt2 at 250 µg/kg of body weight for 30 min followed by AgNP exposure at a concentration of 2 mg/kg body weight. At 2 h post-exposure bronchoalveolar lavage fluid (BALF) was collected and macrophage uptake was qualitatively determined by enhanced dark field microscopy. Macrophages from C57BL/6 mice demonstrated higher particle uptake compared to C57BL/6 mice pretreated with Blt2 (Figure 3.5). SR-B1$^{-/-}$ mice also demonstrated a reduction in macrophage uptake of AgNPs further supporting the role of SR-B1 mediated internalization (Figure 3.5). Lastly, the presence of AgNPs was again confirmed by hyperspectral mapping and highlighted in red as described above (Figure 3.5, left panel).

In Vivo Assessment of Pulmonary Inflammation

To investigate the role of SR-B1 in pulmonary inflammation *in vivo*, AgNPs were instilled in the lungs of C57BL/6J and SR-B1$^{-/-}$ mice. In addition, a subset of C57BL/6J mice were pretreated with 50 µl of PBS or PBS with Blt2 at 250 µg/kg of body weight for 30 min prior to AgNP exposure. At 24 h post exposure, BALF was collected and differential cell counts were assessed to understand the *in vivo* inflammatory response. BALF cell counts revealed a significant increase in total cells in C57BL/6J and SR-B1$^{-/-}$ mice that were exposed to AgNPs compared to control mice. However, mice pretreated with Blt2 and exposed to AgNPs showed significant reduction in total cell counts compared to C57BL/6J mice that were exposed to AgNPs. Further, SR-B1 -/- mice exposed to AgNPs demonstrated a reduction in BALF total cell counts compared to wild type. Lung macrophage numbers were the highest in C57BL/6J mice exposed to AgNPs whereas pretreated mice with Blt2 and exposed to AgNPs had a reduction in macrophages similar to control mice. Furthermore, we found a significant increase in neutrophilic influx in C57BL/6

mice treated with AgNPs that was significantly reduced in mice pretreated with Blt2 (Figure 3.6). Further supporting the role of SR-B1 in macrophage activation, SR-B1$^{-/-}$ mice demonstrated a reduction in neutrophil recruitment following AgNP exposure (Figure 3.6). Finally, mice treated with Blt2 only did not show any significant change BALF cell counts (data not shown).

Discussion

Macrophages form the first line of immune defense against invading pathogens and foreign particles and are likely to interact with and facilitate NP clearance. Currently there are limitations to our knowledge regarding how immune cells such as macrophages recognize NPs and the subsequent induction of inflammation. Recent studies have reported that different types of immune cells including mast cells and macrophages can interact with NPs resulting in an inflammatory response, oxidative stress, and pathology (Singh and Ramarao, 2012, Katwa et al., 2012, Wingard et al., 2011). The interactions that occur between NPs and cells can be influenced by a variety of factors such as the physicochemical properties of the NP and the characteristics of the cell type including cell surface receptor expression (Aldossari et al., 2015). Our current study investigated the role of SR-B1 in the recognition, uptake, and activation of macrophages by AgNPs. Through the use of pharmacological inhibition of SR-B1 and SR-B1 deficient mice, we demonstrated that SR-B1 contributes to macrophage uptake and activation by AgNPs. Further, by coating AgNPs with HDL, a known ligand of SR-B1, we were able to enhance macrophage uptake and activation. Taken together these *in vivo* and *in vitro* findings support the role of SR-B1 in the immune response that is induced by AgNP exposure.

Macrophages express a wide range of cell surface receptors including a variety of scavenger receptors. Specifically, SR-B1 is known to interact with negatively charged particles and mediates the uptake of lipoproteins such as HDL. In our current study we demonstrated that pharmacological inhibition of SR-B1 or use of a knockout mouse model reduced AgNP uptake by macrophages. This finding suggests that drug delivery systems, which utilize NPs that are not readily recognized by the SR-B1 receptor, may be able to more efficiently evade the immune system thereby increasing the amount of delivered drug to target sites. Overall macrophage uptake

and responses to AgNPs were not completely blocked through the inhibition of SR-B1. Therefore it is likely that other cell surface receptors such as scavenger receptor A, Toll-like receptors and others contribute to macrophage uptake and responses. However, our study does support a significant role for SR-B1 in the uptake of AgNPs by macrophages and their inflammatory response.

Due to the role of SR-B1 in lipoprotein uptake and importantly that we have demonstrated the preferential association of lipoproteins with AgNPs following incubation in cell culture media, we decided to examine the influence of an HDL PC on macrophage responses to AgNPs (Shannahan et al., 2013a, Shannahan et al., 2013b). The formation of this PC is dependent on the physicochemical properties of the NP as well as the physiological environment. Addition of the PC influences the biological impact of the NP through modifying biodistribution, cellular uptake, activity, and toxicity (Podila and Brown, 2013). Through modifications in the identity of the PC it may be possible to precisely target NPs to specific cells and/or receptors. Binding of HDL with AgNPs was found to alter the physicochemical properties of AgNPs. Specifically, association of HDL with AgNPs increased their hydrodynamic size while decreasing surface charge. In our current study, upon coating AgNPs with HDL, macrophage uptake was increased. This increase in uptake was not charge driven as addition of HDL was found to reduce surface charge suggesting that the mechanism of this increased uptake was mediated through receptor recognition of HDL. Since HDL is a ligand for SR-B1 it is likely that an HDL PC increased the affinity of AgNPs for the SR-B1 receptor allowing for enhanced internalization. This finding also demonstrates that the recognition of NPs by immune cells is based to a degree on the identity of the PC. Further these findings suggest that individuals with serum rich in lipoproteins may have increased immune responses to AgNP exposure due to enhanced macrophage uptake. Additional studies have

demonstrated that addition of a complex corona (consisting of multiple proteins) often results in decreased uptake by cells (Monteiro-Riviere et al., 2013), however, our current study demonstrates increased uptake following association of AgNPs with an individual lipoprotein, HDL. It is likely that decreases in uptake caused by the addition of complex PC are merely a surface charge driven effect. When NPs however are coated with a receptor-specific protein this decrease in surface charge-driven uptake can be overcome through enhanced receptor affinity. Further this study demonstrates that production of NPs, which are coated with specific proteins or preferentially bind proteins in a biological system can be used to more efficiently target NPs.

Activation of macrophages following AgNP exposure led to an up-regulation of numerous chemokines and cytokines as it is indicated by our PCR array data. OSM was one of the most significantly upregulated genes and is a member of the gp130 cytokine family, which is secreted by several different immune cells (Kishimoto et al., 1995). The gp130 cytokine family has been reported to modulate cellular differentiation, immune, and inflammatory cell networks (Kishimoto et al., 1995). In addition, OSM has been reported to be upregulated following different types of particulate matter exposure (Erdely et al., 2012, Fujii et al., 2002). In our current study, OSM was found to be upregulated following AgNP exposure. This finding suggests that OSM may play a role in mediating AgNP-induced immune response, inflammation, and inflammatory cell recruitment at the site of exposure. Another marker of macrophage activation we utilized in our study was CD86, a costimulatory molecule, which has been studied for its role in immune responses (Orabona et al., 2004). CD86 is known to cause the induction of different inflammatory cytokines such as IL-6 via NF- κB signaling (Orabona et al., 2004). In addition, blockade or deletion of CD86 has been shown to reduce mortality and decrease pro-inflammatory cytokine production in promicrobial sepsis (Nolan et al., 2008). Furthermore, CD86 was found to be up-

regulated in immune cells following exposure to different types of NPs (Roy et al., 2014, Zhu et al., 2014). In our current study, we demonstrated that CD86 expression was up-regulated following AgNP exposure. This result indicates the induction of an inflammatory response through CD86 signaling which may lead to the production of pro-inflammatory cytokines via NF- κB signaling.

In this study, a variety of techniques were used to evaluate macrophage uptake of AgNPs including ICP-MS, changes in side scatter (SSC) by flow cytometry, and hyperspectral dark field microscopy. These methods were found to be in agreement demonstrating modifications in AgNP uptake via the use of SR-B1 inhibition as well as the addition of an SR-B1 ligand, HDL, to the AgNP surface. *In vitro* experimentation demonstrated the rapid (2 h) interaction and immune response following AgNP exposure. Induction of *in vitro* cellular uptake and activation in this study was confirmed *in vivo* by using SR-B1 knock out mice and mice pretreated with a SR-B1 inhibitor. Oropharyngeal instillation was utilized in our studies because this route of exposure allowed for the quickest assessment of a macrophage population *in vivo* for the mechanistic evaluation AgNP uptake and activation. In summary our *in vivo* findings confirmed our *in vitro* studies demonstrating a role for SR-B1 in macrophage uptake and activation.

Conclusion

In summary our current study demonstrates that SR-B1 partially mediates cellular uptake and activation following AgNP exposure. These findings have implications in understanding mechanisms of AgNP toxicity. In addition, our results demonstrate that association of biomolecules with NPs can influence not only their physicochemical properties but also their cellular distribution and effect. Further investigation of signaling mechanism pathways is needed for more understanding of AgNP toxicity. In conclusion our findings demonstrate that AgNPs can

be modified to avoid adverse immune effects and to increase their efficiency for biomedical applications.

Table 3.1 Characterization of AgNPs with and without Addition of an HDL Protein Corona

Nanomaterial	Hydrodynamic Size (nm)	Zeta Potential (mV)
20 nm AgNPs	19.13 ± 1.95	-35
20 nm AgNPs + HDL	62.10 ± 1.79	-8

Figure 3.1 Transmission electron microscopy (TEM) and Hyperspectral Enhanced Darkfield Images of AgNPs and AgNPs + HDL

A) Representative TEM images demonstrating AgNP shape and size. B) Hyperspectral enhanced dark field image of AgNPs. C) Hyperspectral enhanced dark field image of AgNP + HDL. D) Comparison of mean spectrums of AgNP (Black) and AgNP + HDL (Red) demonstrating a red shift in spectra indicative of association of HDL with the surface of AgNPs. Numbers above spectra are wavelengths of spectral peaks demonstrating the red shift.

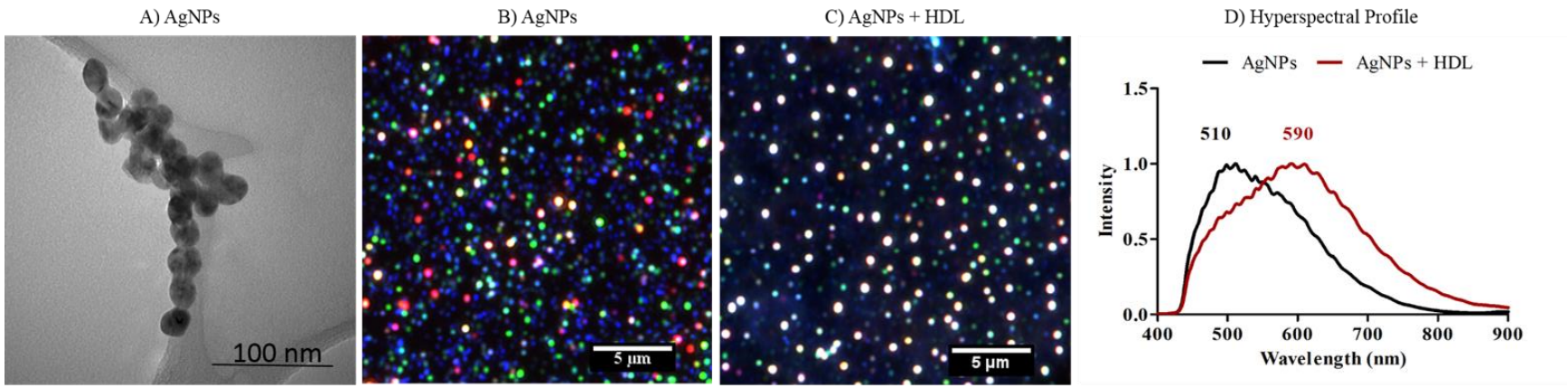

A) AgNPs
B) AgNPs
C) AgNPs + HDL
D) Hyperspectral Profile
100 nm
5 µm
5 µm
AgNPs
AgNPs + HDL
510
590
Intensity
1.5
1.0
0.5
0.0
400
500
600
700
800
900
Wavelength (nm)

Figure 3.2 Evaluation of AgNPs Cytotoxicity and Uptake by Macrophages

A) Cytotoxicity in mouse macrophages RAW264.7 (RAW) at 3 h following exposure to AgNP, AgNP + HDL. Cells were exposed to (0, 6.25, 12.5, 25, or 50 µg/ml) of AgNPs or AgNPs + HDL in serum-free media. B) Cytotoxicity in RAW cells at 6 h following exposure to AgNP, AgNP + HDL. Cells were exposed to (0, 6.25, 12.5, 25, or 50 µg/ml) of AgNPs or AgNPs + HDL in serum-free media. C) Total macrophage uptake of AgNPs as measured by inductively coupled plasma mass spectrometry (ICP-MS). RAW cells were exposed to 50 µg/ml AgNPs for 2 h with or without pretreatment with the scavenger receptor B1 (SR-B1) inhibitor, Blt2 (50 uM). D) Measurement of macrophage uptake of AgNPs assessed through alterations in side scatter (SSC) shift using flow cytometry measurement of $5x10^3$ events. RAW cells were exposed to 50 µg/ml AgNPs for 2 h with or without pretreatment with the scavenger receptor B1 (SR-B1) inhibitor, Blt2 (50 uM). Values are expressed as mean ± SEM (n=3/group). *Significant difference from control ($p \leq 0.05$). $^{\#}$Significant difference of Blt2 pretreated group compared to Blt2 untreated group ($p \leq 0.05$). $^{\$}$Significant difference between the AgNPs + HDL exposed group and the AgNP exposed group ($p \leq 0.05$).

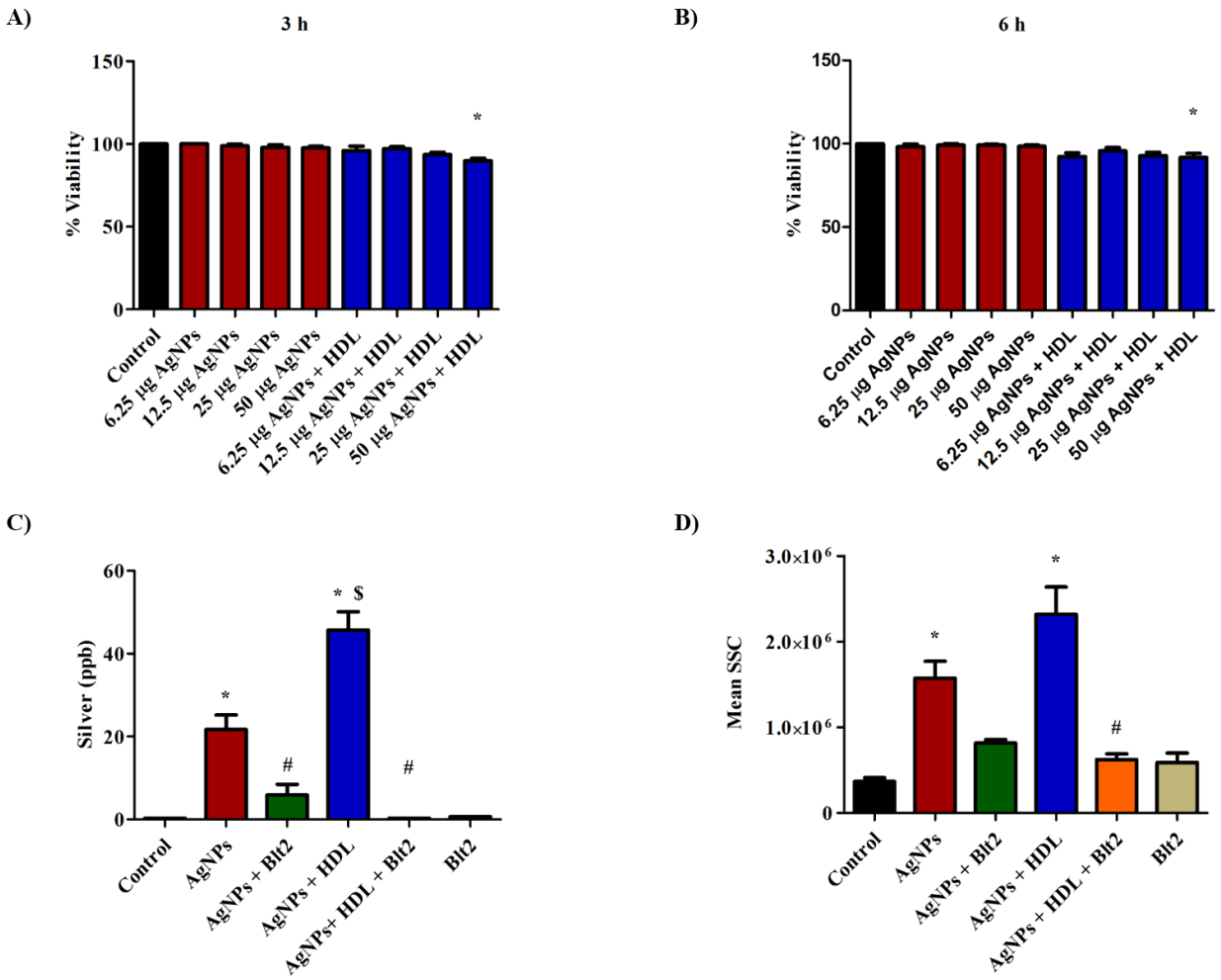

A)
3 h
% Viability
150
100
50
0
*
Control
6.25 µg AgNPs
12.5 µg AgNPs
25 µg AgNPs
50 µg AgNPs
6.25 µg AgNPs + HDL
12.5 µg AgNPs + HDL
25 µg AgNPs + HDL
50 µg AgNPs + HDL
B)
6 h
% Viability
150
100
50
0
*
Control
6.25 µg AgNPs
12.5 µg AgNPs
25 µg AgNPs
50 µg AgNPs
6.25 µg AgNPs + HDL
12.5 µg AgNPs + HDL
25 µg AgNPs + HDL
50 µg AgNPs + HDL
C)
Silver (ppb)
60
40
20
0
*
#
* $
#
Control
AgNPs
AgNPs + Blt2
AgNPs + HDL
AgNPs+ HDL + Blt2
Blt2
D)
Mean SSC
3.0×10⁶
2.0×10⁶
1.0×10⁶
0
*
*
#
Control
AgNPs
AgNPs + Blt2
AgNPs + HDL
AgNPs + HDL + Blt2
Blt2

Figure 3.3 Darkfield Imaging and Hyperspectral Mapping of RAW cells Following AgNPs and AgNPs + HDL Exposure

A) Darkfield image of control mouse macrophages RAW264.7 (RAW) (left panel), identification of AgNPs through hyperspectral mapping in RAW cells (right panel). B) AgNP uptake by RAW cells (left panel), hyperspectral mapping of AgNPs in RAW cells (right panel). C) RAW cells pretreated with scavenger receptor B1 (SR-B1) inhibitor (Blt2) then exposed to AgNPs (left panel), hyperspectral mapping of AgNPs (right panel). D) RAW cells exposed to AgNPs + HDL (left panel), hyperspectral mapping of AgNPs + HDL (right panel). E) RAW cells pretreated with Blt2 then exposed to AgNPs + HDL (left panel), hyperspectral mapping of AgNPs + HDL (right panel).

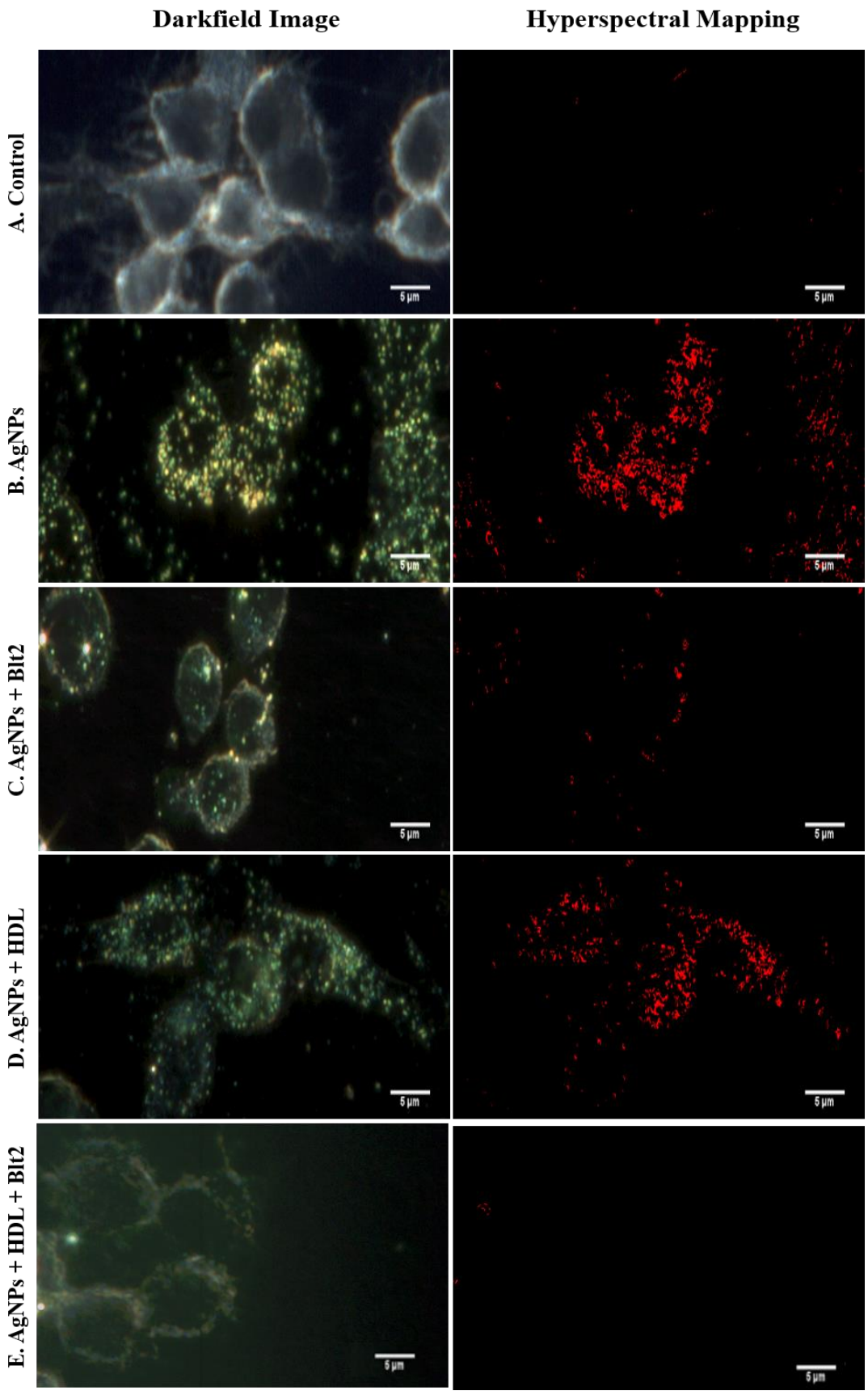

Darkfield Image
Hyperspectral Mapping
A. Control
B. AgNPs
C. AgNPs + Blt2
D. AgNPs + HDL
E. AgNPs + HDL + Blt2
5 µm

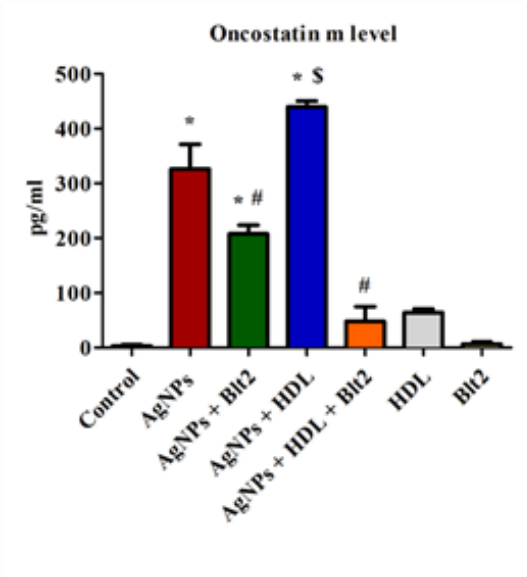
Table 3.2 Analysis of inflammatory cytokines and receptors from mice exposed to vehicle or AgNPs

Gene name	Gene Symbol	95% CI	Fold Up- or Downregulation of AgNPs vs. Control	*P* Value
Tumor necrosis factor	Tnf-α	(4.36, 14.61)	9.4842	0.037842
Chemokine (C-C motif) ligand 3	Ccl3	(2.98, 8.97)	5.9746	0.000642
Oncostain M	Osm	(3.86, 8.03)	5.9444	0.000065
Chemokine (C-C motif) ligand 4	Ccl4	(3.43, 7.31)	5.3717	0.000108
Interleukin 17F	Il17f	(1.73, 4.90)	3.3176	0.016831
Chemokine (C-C motif) ligand 7	Ccl7	(2.26, 3.76)	3.013	0.000001
Chemokine (C-C motif) ligand 2	Ccl2	(1.74, 2.92)	2.3313	0.000029
Glyceraldehyde-3-phosphate dehydrogenase	Gapdh	(1.00, 1.00)	1	N/A
Interleukin 16	Il16	(0.31, 0.52)	-2.39	0.000187

Figure 3.4 Evaluation of Oncostain M (OSM) and CD86 Expression Following AgNPs and AgNPs + HDL Exposure

A) Oncostatin M (OSM) supernatant protein levels 24 h post exposure. Mouse macrophages RAW264.7 (RAW) were exposed to 50 μg/ml of AgNPs for 24 h with or without pretreatment with scavenger receptor B1 (SR-B1) inhibitor, Blt2 (50uM). B) Mean fluorescence of CD86 expression measured by flow cytometry of $5x10^3$ events. RAW cells exposed to 50 μg/ml of AgNPs for 6 h with or without pretreatment with scavenger receptor B1 (SR-B1) inhibitor, Blt2 (50uM). Values are expressed as mean ± SEM (n=3/group). *Significant difference from control ($p \leq 0.05$). $^{\#}$Significant difference of Blt2 pretreated group compared to Blt2 untreated group ($p \leq 0.05$). $^{\$}$Significant difference between the AgNPs + HDL exposed group compared to the AgNPs exposed group ($p \leq 0.05$).

A)


Oncostatin m level
pg/ml
500
400
300
200
100
0
* $
*
* #
#
Control
AgNPs
AgNPs + Blt2
AgNPs + HDL
AgNPs + HDL + Blt2
HDL
Blt2

B)

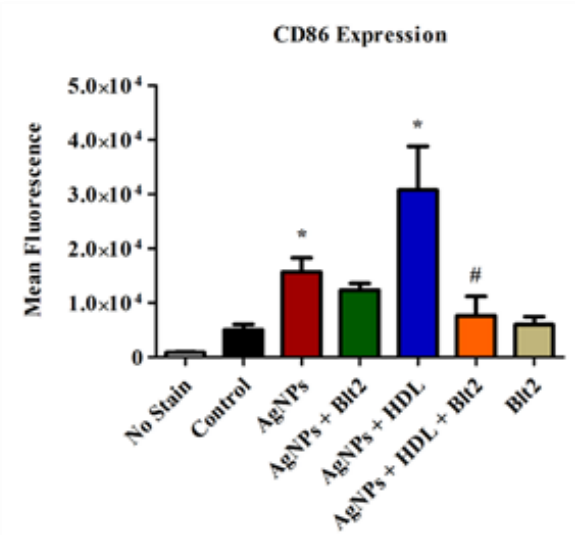

CD86 Expression
Mean Fluorescence
5.0x10⁴
4.0x10⁴
3.0x10⁴
2.0x10⁴
1.0x10⁴
0
*
*
#
No Stain
Control
AgNPs
AgNPs + Blt2
AgNPs + HDL
AgNPs + HDL + Blt2
Blt2

Figure 3.5 Evaluation Role of Scavenger Receptor B1 (SR-B1) in AgNPs uptake Following Oropharyngeal Aspiration

Macrophage uptake of AgNPs was evaluated *in vivo* using C57BL/6 and SR-B1$^{-/-}$ mice. C57BL/6J mice were pretreated with either PBS or Blt2 (250 µg/kg of body weight) for 30 min and then exposed to AgNPs at a concentration of 2 mg/kg body weight via oropharyngeal aspiration. Bronchoalveolar lavage fluid was collected 2 h post-exposure and macrophage uptake was qualitatively determined by enhanced dark field microscopy. Spectral mapping was utilized to positively identify pixels matching the spectrum of AgNPs. These pixels are shown in the right columns in red. A) Representative dark field image of macrophages collected from C57BL/6 mice exposed to AgNPs (left panel), and spectral mapping of AgNPs (right panel). B) Representative dark field image of macrophages collected from C57BL/6J mice pretreated with Blt2 and then exposed to AgNPs (left panel), and spectral mapping of AgNPs (right panel). C) Representative dark field image of macrophages collected from SR-B1$^{-/-}$ mice exposed to AgNPs (left panel), and spectral mapping of AgNPs (right panel).

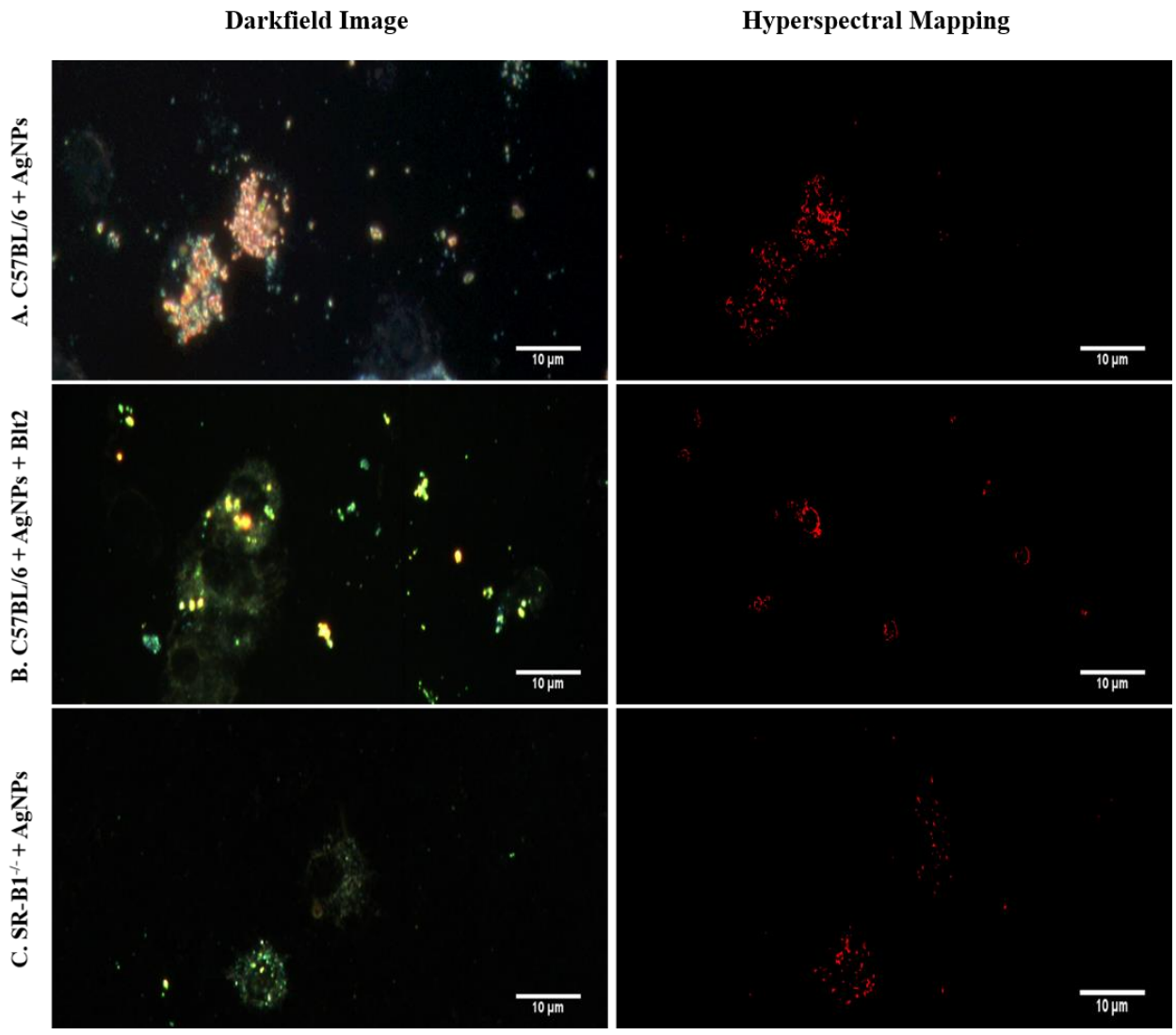
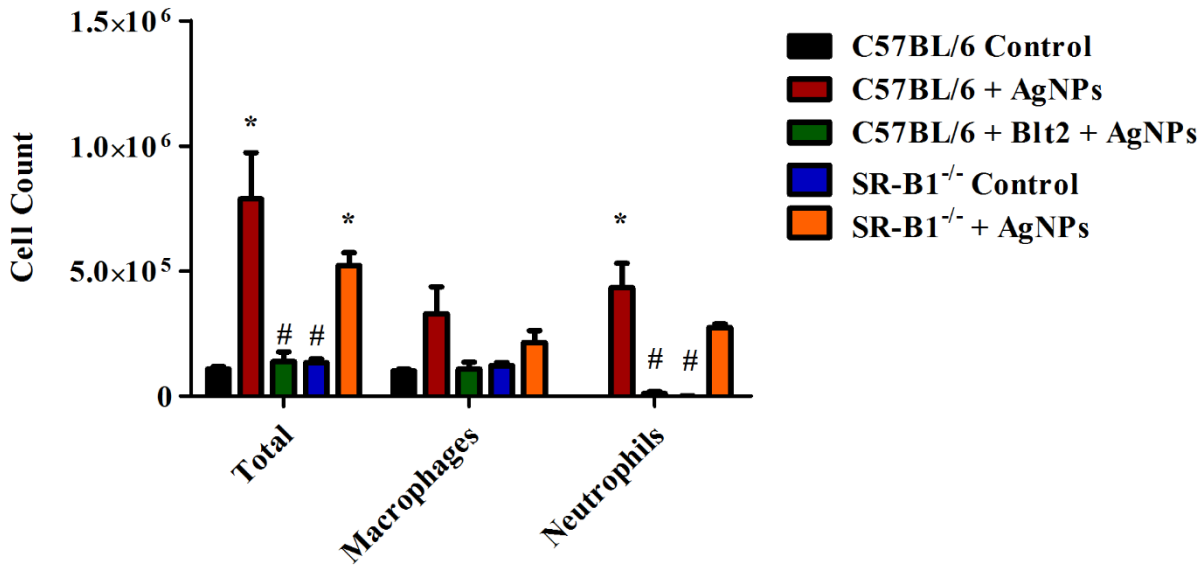

Darkfield Image
Hyperspectral Mapping
A. C57BL/6 + AgNPs
B. C57BL/6 + AgNPs + Blt2
C. SR-B1-/- + AgNPs
10 µm

Figure 3.6 Role of Scavenger Receptor B1 (SR-B1) in AgNPs-Induced Inflammation in Mice

Bronchoalveolar lavage fluid (BALF) differential cell counts from C57BL/6 or SR-B1$^{-/-}$ mice exposed to AgNPs. C57BL/6J mice were pretreated with either PBS or Blt2 (250 µg/kg of body weight) for 30 min prior to AgNP exposure. BALF was collected 24 h post-exposure to AgNPs. Values are expressed as mean ± SEM (n=3/group). *Significant difference from control ($p \leq 0.05$). $^{\#}$Significant difference compared to C57BL/6 mice exposed to AgNPs ($p \leq 0.05$).


Cell Count
1.5×10^6
1.0×10^6
5.0×10^5
0
*
*
*
# #
# #
Total
Macrophages
Neutrophils
C57BL/6 Control
C57BL/6 + AgNPs
C57BL/6 + Blt2 + AgNPs
SR-B1^-/- Control
SR-B1^-/- + AgNPs

Chapter 4: General Discussion

The present study was conducted to evaluate the influence of physicochemical properties of AgNPs on uptake and activation mast cells and macrophages and the role of SR-B1 following exposure to AgNPs.

In Aim 1, we evaluated the uptake of AgNPs uptake by mast cells and macrophages. Mast cells have been reported to internalize different types of NPs such as silicon dioxide (SiO2) and titanium dioxide (TiO2) which were found to be localized in the secretory granules (Maurer-Jones et al., 2010). NP internalization by mast cells can inhibit mast cell activation and degranulation as has been reported with fullerene exposure or can induce mast cell activation and degranulation as reported with gold NP exposure (Ryan et al., 2007, Huang et al., 2009, Marquis et al., 2009). Gold NPs were found to induce mast cell degranulation that was dependent on size, exposure time, and concentration (Marquis et al., 2009, Huang et al., 2009). Previous studies demonstrated the ability of the mast cell-NP interaction to mediate activation or suppression of the mast cell, however, limited data are available for mast cell interaction with AgNPs and the role of physicochemical characteristics of AgNPs.

In this study mast cells were exposed to AgNPs with different physicochemical characteristics including 20 and 110 nm spherical AgNPs either suspended in citrate (C20 and C110), or polyvinylpyrrolidone (PVP) (P20 and P110). In addition, two different sizes of nanoplates with optical resonance peaks at specific wavelengths of 550 nm and 850 nm suspended in PVP (P550 and P850), or Ag nanowires that were up to 2 μm long suspended in PVP (Figure 2.1) were utilized. All types of AgNPs were found to be internalized by mast cells in varying amounts based on their physicochemical differences (Figure 2.2 and 2.3). AgNPs suspended in citrate including C20 and C110 were found to have a more highly negative zeta potential compared

to spherical AgNPs that were suspended in PVP. AgNPs that were suspended in PVP were found to be more associated to cells compared to citrate suspended particles of the same size and shape (Table 2.1). This is likely due to PVP acting as stabilizer for AgNPs and being oxidized resulting in binding with biomolecules (Podila et al., 2012). Such an observation suggests that uptake is not completely driven by charge but also by the type of surface coating. Interestingly, AgNPs with the same coating agent and similar surface charge including P110, P550, and Ag nanowires were found to have varying internalization amounts which demonstrates that other NP properties including size and/or shape can influence their cellular uptake. These observations concur with other previous results that reported the influence of size and shape of NPs on their cellular uptake (Miethling-Graff et al., 2014). Furthermore, AgNPs including P550 and P850 that have the same surface coating and shape but differ in their size were found to be taken up differently where P850 had greater internalization by cells. Taken together, internalization of tested AgNPs by mast cells is a complex process that depends on a combination of different physicochemical factors.

Macrophages also can interact with NPs resulting in cellular activation and induction of an inflammatory response (Singh and Ramarao, 2012). Our current study tested macrophage uptake of C20 and C20 coated with HDL. AgNPs coated with HDL were used to investigate the role of SR-B1 in uptake since this receptor recognizes lipoprotein molecules. The physicochemical properties of AgNPs such as size and zeta potential were found to be affected after PC formation (Table 3.1 and Figure 3.1). AgNP internalization was demonstrated by different techniques including ICP-MS, flow cytometry, and hyperspectral dark field microscopy (Figure 3.2). In addition, alveolar macrophages were found to take up and internalize AgNPs in an *in vivo* study (Figure 3.3). AgNPs were found to be taken up by macrophages in *in vitro* experiments, whereas, HDL coated NPs had more internalization by macrophages demonstrating a potential role for the

SR-B1. The HDL coated AgNPs were found to have an increase in size and a decrease in their surface charge. The previous finding suggests the NP uptake was driven by changing AgNP identity which resulted in increased receptor recognition of AgNPs coated with HDL. These findings support a significant role of SR-B1 in the internalization of AgNPs by macrophages. Further, the higher uptake of HDL coated AgNPs suggests that individuals with serum rich in lipoproteins may display an increased immune response to AgNPs due to an increase in NP uptake by immune cells because of association of AgNPs with lipoprotein molecules. These findings suggest that the production of NPs, which are coated with key proteins preferentially bind receptors in a biological system and could potentially be used for improved tissue/cell targeting for NPs.

The focus of aim 2 was to evaluate mast cell and macrophage activation following AgNP exposure. Mast cells play an important role in innate immunity, host defense and allergic disease and can be found in most tissue types (Brown et al., 2008a). Mast cells acting through different receptors such as TLRs and SRs can recognize pathogens and foreign molecules resulting in inflammatory responses including degranulation and release of pro-inflammatory cytokines (Brown et al., 2007, Medic et al., 2008, McCurdy et al., 2003). Mast cells have been reported to play a role in the inflammatory response following NPs exposure such as MWCNTs (Wingard et al., 2011, Katwa et al., 2012). Limited data are available, however, that evaluate the role of physicochemical properties of AgNPs on mast cell activation.

Our current study investigated the direct interactions of AgNPs that have different physicochemical characteristics on mast cell activation and degranulation. While C110 and P110 did not induce mast degranulation, all other types of AgNPs induced mast cell degranulation (Figure 2.4). Although P550 and P850 have a similar shape and surface charge, P550 with a smaller size was found to induce more mast cell degranulation compared to P850. Furthermore, P110

shares the same surface charge and coating with Ag nanowires and P550, however, it did not induce mast cell degranulation. Interestingly, Ag nanowires displayed reduced cellular uptake, but induced mast cell degranulation which is likely due to the high aspect ratio particles. Similar NPs with high aspect ratio have been reported to induce immune cell activation (Sunshine et al., 2014, Hamilton et al., 2013, Li et al., 2013a, Sharma et al., 2010). These findings demonstrated the effect of size and possibly AgNP shape in mast cell degranulation which is likely mediated through cell surface receptor recognition. These findings are consistent with other reports that mast cells can interact differently based on different size and shape characteristics of the same nanomaterials such as CNTs (Huang et al., 2009, Katwa et al., 2012, Ryan et al., 2007, Umemoto et al., 2014).

Our current findings demonstrated mast cell degranulation is not entirely dependent on AgNPs internalization as C110 and P110 were found to be taken up by mast cells but did not induce mast cell degranulation. In addition, C20 and P20 induced a similar level of mast cell degranulation although the P20 AgNPs were found to be more heavily internalized by mast cells compared to C20. Finally, our current study suggests that lysosome-associated membrane protein 2 (Lamp2) should be considered as a candidate biomarker for identifying mast cell activation following AgNP exposure since we found Lamp2 upregulation correlated well with mast cell degranulation data (Figure 2.5). These findings of different responses by mast cells are consistent with other reports that mast cells can be induced or suppressed following NP exposure (Dellinger et al., 2010, Ryan et al., 2007, Umemoto et al., 2014). In addition, these findings demonstrated a direct interaction of mast cells with AgNPs that can induce mast cell degranulation which is not entirely driven by cellular internalization, therefore, this interaction can possibly occur *in vivo* and result in induction or promotion of allergic disease.

Activation of mast cells by NPs including MWCNTs and cerium oxide resulted in the release of cytokines such as osteopontin as previously reported by our laboratory (Katwa et al., 2012, Wingard et al., 2011). Osteopontin is a secreted phosphoglycoprotein produced by different types of cells and can be used as a risk predictor of various diseases such as cancer such as mesothelioma that developed following asbestos exposure (Berezin and Kremzer, 2013, Pass and Carbone, 2009, Yang et al., 2008). In our current study, we found osteopontin upregulation in mast cells following exposure to all selected types of AgNPs (Figure 2.6). The cytokine production by mast cells even in the absence of or limited degranulation supports similar previous findings by our laboratory which demonstrated that mast cell activation following silica exposure resulted in cytokine production with limited cell degranulation (Brown et al., 2007). This demonstrated that mast cell degranulation may involve different cell signaling pathways and that idea is further supported by a recent study conducted in our laboratory which demonstrated AgNP-directed mast cell degranulation involves activation of PI3K, PLCγ and an increase in intracellular calcium levels (Alsaleh et al., 2016).

Macrophage activation can be induced by NP exposure which results in upregulation of chemokine and cytokine expression, mitochondrial damage, and apoptosis (Singh and Ramarao, 2012). In our current study, we found upregulation of different cytokines and chemokines following exposure to AgNPs. Oncostatin M was one of the most significantly upregulated genes (Table 3.2). Oncostatin M is a member of the gp130 cytokine family and can be secreted by different types of immune cells (Kishimoto et al., 1995). The gp130 cytokine family has been reported to be involved in the modulation of different immune and inflammatory cell networks (Kishimoto et al., 1995). Specifically, oncostatin M has been reported to be upregulated in different types of cells such as macrophages and epithelial cells following particulate matter exposure

(Erdely et al., 2012, Fujii et al., 2002). In our current study, we found upregulation of the oncostatin M gene and protein levels which supports its involvement in the inflammation and inflammatory cell recruitment immune response induced by AgNPs exposure(Figure 3.4A). Furthermore, HDL coated AgNPs were found to induce higher levels of oncostatin M which indicates a potential role for the PC in induction of inflammatory response by macrophages.

The macrophage activation marker CD86 was found to be upregulated in our study. CD86 is a co-stimulatory molecule that has been studied for its role in immune responses (Orabona et al., 2004). In addition, CD86 is known to induce inflammatory cytokine production such as IL-6 through NF-κB signaling (Orabona et al., 2004). In our current study, we found CD86 expression was upregulated following AgNP exposure, whereas, AgNPs coated with HDL induced higher expression compared to exposure to AgNPs with no HDL coating thereby demonstrating a role for the PC in the inflammatory response induced by macrophages following NP exposure (Figure 3.4B). This finding concurs with other reports in which CD86 was found to be upregulated in immune cells following exposure to different types of NPs (Roy et al., 2014, Zhu et al., 2014). These findings suggest that AgNPs can induce macrophage activation through increasing CD86 signaling resulting in production of pro-inflammatory cytokines through the NF-κB signaling pathway.

In aim 3 we established a potential role of the scavenger receptor B1 (SR-B1) in mast cell and macrophage uptake and activation following AgNP exposure. SR-B1 is a multi-ligand receptor that preferentially interacts and facilitates uptake of lipoprotein molecules such as HDL and other negatively charged molecules. In addition, SR-B1 can interact with pathogens and NPs (Landschulz et al., 1996, Eyre et al., 2010, Mooberry et al., 2010).

In this current study, all different types of selected AgNPs were found to have a negative charge therefore they were hypothesized to interact with SR-B1. In mast cells, pretreatment with the specific SR-B1 inhibitor Blt2 was found to reduce the uptake of P20, P110, C110, and P850 AgNPs while C20, P550, and Ag nanowires found to be unaffected (Figure 2.3). This suggests that SR-B1 recognition of AgNPs does not appear to be dependent on NP surface charge due to the range of surface charge carried by each type of AgNPs. Mast cell degranulation was found to be reduced in pretreated cells with Blt2 following exposure to P20, C20, P550, and Ag nanowires (Figure 2.4). Inhibition of SR-B1 was found to reduce mast cell degranulation to control levels in cells exposed to P20 or C20. This suggests that P20 and C20 AgNPs induce mast cell degranulation via SR-B1 recognition whereas P550 AgNPs and Ag nanowires induce mast cell degranulation with possible contribution of other cell surface receptors.

Overall, by using the SR-B1 inhibitor we demonstrated that SR-B1 can modulate mast cell uptake of and activation by AgNPs following exposure. Our findings suggest that different characteristics of AgNPs may have an impact on designing safer NPs that have no, or at least very little, interaction with scavenger receptors to avoid activation of the inflammatory immune response by mast cells. Furthermore, our current study also demonstrates a role for SR-B1 in osteopontin release (Figure 2.6). Pretreatment with the SR-B1 inhibitor resulted in a reduction in the release of osteopontin which suggests AgNPs can induce production of mast cell cytokines and contribute in the mast cell inflammatory response in presence or absence of cell degranulation.

Finally, mast cells can be activated through signals from surface receptors such as SR-B1, FcεRI, or c-Kit which resulted in increased calcium flux, tyrosine kinase phosphorylation, and eventually degranulation of mast cells (Canton et al., 2013, Zhu et al., 2009). Inhibition of tyrosine kinase phosphorylation by a therapeutic agent such as imatinib leads to a reduction in mast cell

degranulation. In this study, we determined that C20 AgNPs-induced mast cell degranulation can be inhibited by pretreatment with imatinib (Figure 2.7). This suggests AgNP-induced mast cell degranulation can be inhibited therapeutically. In addition, this finding suggests that mast cell degranulation depends on the physicochemical properties of AgNPs which affect downstream cell signaling events such as activation of PI3K, PLCγ and an increase in intracellular calcium levels (Alsaleh et al., 2016).

Macrophage scavenger receptors have been reported to interact with NPs and influence NP internalization, cellular trafficking, and pro-inflammatory cytokine secretion (Orr et al., 2011). In our current study, we demonstrated a role for SR-B1 where AgNP uptake by macrophages was found to be reduced in cells pretreated with a SR-B1 inhibitor, while uptake of HDL coated AgNPs was reduced to control level in cells pretreated with Blt2 (Figure 3.2C&D and Figure 3.3). The partial inhibition of AgNP uptake through SR-B1 suggests that other cell surface receptors may contribute to AgNP uptake. In addition, uptake of AgNPs was found to be reduced in alveolar macrophages in animal studies using SR-B1 knockout mice or wild type mice pretreated with Blt2 (Figure 3.5). These findings suggest that utilizing NPs in therapeutics that are not recognized by SR-B1 may be able to avoid immune system activation and cellular uptake and thereby increase the amount of delivered drugs to target tissues.

The role of SR-B1 in macrophage activation was also supported by studies that demonstrated that the inhibition of SR-B1 resulted in a reduction in oncostatin M and CD86 expression following AgNPs exposure (Figure 3.4). In addition, formation of PC resulted in more inhibition by Blt2 of oncostatin M and CD86 expression compared to AgNPs with no HDL coating. This finding demonstrates the role of SR-B1 in lipoprotein uptake which supports the idea

that formation of PC could increase AgNP binding affinity and have an effect on biodistribution, cellular uptake, and cellular toxicity as it has been reported before (Podila and Brown, 2013).

Finally, the role of SR-B1 in the inflammatory response induced by AgNPs was confirmed in an *in vivo* study using SR-B1 knockout mice and mice pretreated with a SR-B1 inhibitor (Figure 3.6). We found an increase in total cell count and neutrophils in bronchoalveolar lavage fluid in wild type mice compared to SR-B1 knockout mice and mice pretreated with Blt2. These findings confirmed a role of SR-B1 in the inflammatory response following AgNP exposure.

Overall the data generated from this study has significantly contributed to our understanding of the influence of physicochemical properties of AgNPs on mast cell and macrophage activation. In addition, this study investigated role of SR-B1 in AgNP interactions with mast cells or macrophages. The physicochemical properties of AgNPs contributed to different immune responses, whereas, SR-B1 was found to partially mediate the interaction of AgNPs with immune cells. Future research is needed to understand and define the cell signaling pathways involved to gain additional insight into the mechanisms that underlie AgNP toxicity. In conclusion, our findings demonstrate that AgNPs can be formulated and modified to avoid or reduce their adverse effects and to increase their efficiency for biomedical applications.

Future studies

The interaction of NPs and physiological systems following exposure to NPs led to an increased effort to carry out research aimed to understand the molecular interactions and cellular uptake pathways such as scavenger receptors, and complement receptors that may participate in the toxicity associated with these substances (Patel et al., 2010, Yang et al., 2010a, Sahay et al., 2010). Differences in genetic background can affect this cellular-NP interaction through modifying

the expression of receptors involved in this interaction or by affecting T_H1-T_H2 and M1-M2 status (Murphy and Reiner, 2002). The immune response following exposure to NPs can be shifted towards a T_H1 or T_H2 response mediated by T helper cells leading to the production of specific cytokines and mediators to each response (Murphy and Reiner, 2002). The T_H1 response can polarize macrophages toward the M1 phenotype while T_H2 response has tendency to polarize macrophages toward the M2 phenotype (Mills et al., 2000, Gordon and Martinez, 2010). While the M1 and M2 phenotypes express different types of receptors, they can also share same receptors but with different levels of expression which can affect the level of cellular-NP interaction and clearance (Chinetti-Gbaguidi et al., 2011). Therefore, future studies should examine the effects of M1 versus M2 polarization on the response to NP exposure.

It has been reported that mice with different genetic backgrounds show different effects on NP clearance (Jones et al., 2013). The previous study reported that T_H1-biased strains such as C57BL/6 have slow NP clearance with high NP blood concentration while T_H2-biased strains such as BALB/c showed rapid NP clearance and that was mediated by immune cells including monocytes, macrophages, and granulocytes (Jones et al., 2013).

Another study that investigated pulmonary toxicity induced by AgNPs in different rat strains reported that the genetic background produced different inflammatory responses following NP exposure (Seiffert et al., 2015). The previous study reported that Brown-Norway rats exposed to NPs showed early bronchial hyperresponsiveness and rapid recruitment of neutrophils and eosinophils compared to exposed Sprague-Dawley rats (Seiffert et al., 2015). In addition, increased cytokine production in the lungs including IL-13 and CCL11 was found in Brown-Norway rats compared to Sprague-Dawly rats (Seiffert et al., 2015). The investigators explained the increase in inflammatory response in Brown-Norway rats might be due to an increase in T_H2 cytokine

production such as IL-10 and IL-13 in macrophages isolated from Brown-Norway rats and these finding was supported by an increase in IgE levels in bronchoalveolar lavage. Therefore, AgNPs are more likely to induce inflammatory responses and increase eosinophils in Th2-biased asthmatic subjects (Seiffert et al., 2015).

Based on the previous studies, the genetic background can play an important role in NP biodistribution and cellular interaction in different strains of mice and rats. Therefore, future studies should include efforts to assess the genetic background in the experimental design to have more understanding of the adverse effects and toxicity of NPs.

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