Cell Viability, Cytoskeleton Organization and Cytokines Secretion of RAW 264.7 Macrophages Exposed to Gram-Negative Bacterial Components

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Cell Viability, Cytoskeleton Organization and Cytokines Secretion of RAW 264.7 Macrophages Exposed to Gram-Negative Bacterial Components

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science.

By

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ABSTRACT


Macrophages play an important role in innate immunity by controlling cellular responses. In this study, the effects of gram-negative bacterial components (Flagellin, lipoprotein, lipopolysaccharides (LPS), outer membrane proteins-A (OMP-A) and peptidoglycan) were determined on cell viability, morphology, cytoskeletal filament and cytokines secretion of murine RAW 264.7 macrophages at 24 hours. The effect of LPS, flagellin and peptidoglycan from gram negative bacteria on viability murine RAW 264.7 macrophages were evaluated using different concentrations (1, 5 and 10 μg/ml). Cells stimulated with LPS displayed ~ 2-fold decrease (P≤0.001) in cell viability compared to control cells at 24 hours whereas cells stimulated with flagellin displayed gradual and significant decrease (P ≤ 0.01; P ≤ 0.05) in cell viability with concentrations 5 and 10 μg/ml of this product. Effects of gram-negative bacteria components on the organization of F-actin and microtubule (tubulin) in murine RAW 264.7 macrophages were monitored via immunofluorescent staining to study distribution of cytoskeleton such as elongation cell with cells stimulated with LPS. By using ImageJ analysis, the fluorescent intensity of immunofluorescent images were quantified to evaluate F-actin and tubulin rearrangement. Cells stimulated with Flagellin, lipoprotein, LPS, OMP-A or peptidoglycan show morphological changes as elongation and flatten with some components from gram negative bacteria with different concentrations (1, 5 and 10 μg/ml). LPS at all concentrations displayed a significant increase (p≤0.001) in F-actin staining compared to control cells at 24 hours. The cells appeared flattened with irregular shapes compared to control cells appeared rounded. High level of TNF- α were secreted by murine RAW
264.7 macrophages exposed to LPS (2300 pg/ml) and flagellin (300 pg/ml) with 10 μg/ml concentration. These observations suggest that activation of TLRs leading to production of inflammatory cytokines such as TNF-α may account for our observed decreases in cell viability since LPS activates TLR4 and flagellin activates TLR5.
HYPOTHESIS

Exposure of macrophage to bacterial substances from gram-negative bacteria will induce changes in cell viability, cell morphology, immunofluorescent intensity of microtubules (tubulin) staining and microfilaments (F-Actin) staining at 24 hours as well as differences in cytokines secretion. These characteristics will accompany the transition of unstimulated RAW 264.7 macrophages to the pro-inflammatory phenotypes.
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Macrophage

Tissue Macrophages are heterogeneous immune cells that play a major role in response to inflammation in both innate and adoptive immunities. Macrophages are mononuclear cells that are derived from blood monocytes which in turn develop from bone marrow stem cells, passing through proliferation and differentiation cascades (Murray and Wynn, 2011). Colony Stimulating Factor (CSF-1) is a critical growth factor that regulates the proliferation and differentiation of mononuclear phagocytic cells (P. Roth and Stanley 1992; Martinez et al. 2006). The main function of macrophages is to defend and clear pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) as well as to repair damage tissues (Mills, 2012). Macrophages recognize different PAMPs and DAMPs through recognition receptors such as toll like receptors TLR, nucleotide-binding oligomerization domain NOD-like receptors, retinoic acid-inducible gene I RIG-1 family, lectins and scavenger receptors that are distributed throughout the cell membrane and cytoplasmic organelles. Macrophages respond to PAMPs and DAMPs through signaling pathways that lead to the release of a variety of cytokines and chemokines. Macrophages also activate adaptive immune B and T cells to eliminate any pathogens (Small et al., 1994). Macrophages have been morphologically and functionally grouped into three classes. Depending on signals present in the microenvironment, resting macrophages M0 are polarized to pro-inflammatory M1 macrophages or anti-inflammatory M2 macrophages.

M0 are polarized to pro-inflammatory M1 in response to intracellular pathogens, microbial products such as LPS, or pro-inflammatory cytokines such as IFN-y and IL-1. Pro-inflammatory M1 cells respond to inflammation by releasing different cytokines, chemokines, and effector molecules such as TNF-α, IL-6, CCL3 and iNOS. IL-4, IL-10 or IL-13 polarize M0 to anti-inflammatory M2 macrophages. M2 cells play major roles in maintaining tissue hemostasis and in modulating inflammation through multiple mediators such as TGF-β, IL4, IL10, and IL13. M2 elevates cellular metabolic activities, including phagocytosis, endocytosis, and tissue remodeling (Martinez et al.
Macrophage subtypes are important in controlling immune response by keeping the balance between pro-inflammatory and anti-inflammatory macrophage populations (Junliang et al., 2010; Cheng et al., 2000).

**Figure 1**: Macrophage polarization. M0 Resting cells are stimulated with PAMPs via TLR. LPS or IFN-γ polarized cells to M1 phenotype and secrete pro-inflammatory cytokines IL-1β, IL-6 and TNF-α. However, M2 anti-inflammatory phenotype is polarized by IL-4, IL-10 and IL-13 and release IL-10 that down-regulates M1 functions. (Remodified from Nakagawa, Y., & Chiba, K. 2014).

**Cytoskeleton of Macrophage**

The cytoskeleton of a macrophage is organized in a complex networks of protein fibers that give the cell its shape, support the cell, and allow cell movement as a whole; these fibers also facilitate the organelles’ movements in an organized fashion. A macrophage’s cytoskeleton is also a major player in the sliding movement toward target as well as in the endocytosis and phagocytosis processes that are required for these cells to achieve their proper function. The cytoskeleton provides an organized track for each cytokine and chemokine that is essential for the
orchestrated macrophage function. The cytoskeleton is differentiated according to the size and roll of the filament; there are three types of filaments: microfilaments (about 7 nm in diameter), microtubules (about 25 nm in diameter), and intermediate filaments (about 10 nm in diameter) (Jaffe and Hall 2005). Gram-negative bacterial pathogens impact a macrophage’s cytoskeleton by activating GTPases, which activate NF-kB; this in turn leads to increased gene expression of pro-inflammatory cytokines such as IL-1 and INF-y (Lee, 2000).

Microtubules are hollow cylinders composed of polymeric proteins formed by α- and β-tubulin dimers. The microtubule organization center (MTOC) arranges and nucleates microtubules Hervé and Bourmeyster, 2015). Microtubule-associated Rac1 and RhoA play a role in the polymerization of tubulin subunits and the distribution of ATP levels inside cells. Activating Rac1 and RhoA signaling controls actin dynamics and causes the dynamic microtubules to be unstable. Rho activity is stimulated by the depolymerization of microtubules after Rho GTPase is activated at the plasma membrane (Jaffe and Hall 2005). Dynamics of microtubules are modulated via several bacteria, which can play an important role of cell movement (Yoshida and Sasakawa 2003).

Microfilaments consist of linear polymers of double-stranded helical filamentous actin (F-actin). F-actin is formed by twisted subunits of a monomeric globular protein (G-actin). Microfilaments play a critical role in the dynamics of macrophages during infection with gram-negative bacteria. In response to infection, F-actin is involved in immune cell migration, chemotaxis, and phagocytosis. ATP molecules and Rho GTPases also play a role in the dynamics of microfilaments (Mattoo et al, 2007). Interactions between a pathogen with the cell membrane disturb the cytoskeleton of eukaryotic cells which is essential for phagocytosis and regulation of immune cell signaling. Rho GTPase is activated via many bacterial factors, which include virulence factors and toxins. This activation leads to the initiation of a cascade of immune cell signals (Jaffe and Hall 2005). Bacterial pathogens can survive the immune cell by manipulating Rho GTPase (Boquet and Lemichez, 2003).

Pathogen recognition receptors (PRRs)

The innate immune system recognizes and responds to pathogens by different immune surveillance mechanisms. Pathogen-associated molecular patterns (PAMPs) are identified by pathogen recognition receptors
PRRs on the plasma membrane of immune cells such as macrophages (Akira et al., 2006). Diverse PAMPs have been detected by various families of PRRs, such as C-type lectin receptors (CLRs), transmembrane receptors, Toll-like receptors (TLRs), RIG-I-like receptors (RLRs) and Nod-like receptors (NLRs) (Kumar et al., 2011). PRRs respond to PAMPs by creating an inflammatory response, in which chemokines, cytokines, and type-I interferons are released. These inflammatory cytokines attract other inflammatory cells, including innate and adaptive immune cells, to the site of infection (Takeuchi and Akira, 2010).

**Toll-like receptors**

Toll-like receptors (TLRs) play an important role in innate immunity. 13 TLRs detect certain PAMPs. TLRs induce the production of cytokines and chemokines and can also trigger NF-κB activation. TLRs are type-I transmembrane proteins that contain leucine-rich repeats (LRRs) on the extracellular domain and bind to corresponding PAMPs (Lemaitre et al., 1996). Some TLRs require co-receptors for ligand binding such as MD-2 for TLR4 when binding to LPS (Shimazu et al., 1999). The complex of TLR1/TLR2 is a heterodimer, while TLR5 has five loops that consist of 20 LRRs (Botos et al., 2011).

**Signaling of toll-like receptors (TLRs)**

In MyD88-dependent signaling pathway, interactions between TLRs on the cell surface and ligands of PAMPs activate a downstream signaling pathway. TIR-TIR domain interaction recruits one or more adaptor proteins. All TLRs, with the exception of TLR3, recruit the MyD88 adaptor protein that is essential for downstream signaling. IRAKs, TRAF6 and the TAK1 complex activate downstream TAB1, TAB2, TAK1 and MAP kinases. NEMO, IKKα and IKKβ make up the IKK complex that is activated by TAK1. NF-kB and IκBα are phosphorylated by IKKβ; phosphorylated NF-kB is then translocated to the nucleus of the cell. Finally, NF-kB and MAP kinases act as activators for pro-inflammatory cytokines IL-1 and INF-y. IKK complex is involved in the activation of ERK, P38, JNK, and MAP kinases (Mogensen, 2009).

In the MyD88-independent/TRIF-dependent signaling pathway, IRF3 and IRF7 are interferon regulatory factors that increase the expression of type I interferons. TLR3 and TLR4 recruit an adaptor protein TRIF which
activates downstream IRF3 through TBK1 and IKK activation, leading to the production of pro-inflammatory cytokines (Mogensen, 2009).

Figure 2: Toll-like receptor (TLR) pathways and activated signaling by PAMPs through MyD88 independent. phosphorylation is induced by IRAK after MyD88 recruit IRAK which associates with TRAF6 or TRAF3, leading to the activation of IKK complex or MKKs and resulting in the activation of NF-κB, CREB and AP1. (Remodified from O'Neil, et al 2013).

**NOD-like receptors (NLRs)**

NLRs consist of nucleotide-binding oligomerization domains of soluble proteins found in the cytoplasm and N-terminal effector domains. NLRs can be divided into four subfamilies: NLRA, NLRB, NLRC, and NLRP (Strober, 2006). CARDs, pyrin domains (PYDs), baculoviral inhibitor of apoptosis repeat (BIR) domains, or the transactivator domain (AD) are the effector domains in NLRs. PAMPs are detected by NLRs as well as TLRs, while DAMPs are
only detected by NLRs. Activation of NLR cytoplasmic domains leads to an inflammatory response, autophagy, or cell death. (Le Bourhis, 2007).

**NOD1 and NOD2 receptors**

NOD1 and NOD2 are intracellular receptors of innate immune cells. They are located in the cytosol of phagocytes. Peptidoglycan is recognized by NOD1 and NOD2 receptors expressed on epithelial cells and antigen presenting cells (APCs) such as macrophages. NOD1 and NOD2 consist of a C-terminal LRR domain and a central NOD which is a NACHT domain. They also contain an ATPase activity domain and an N-terminal domain such as CARDs or pyrin domain. In NOD1, the signal CARDs have C-terminal LRR, NACHT domain, and N-terminal effector domain. In NOD2, the two signal CARDs consist of C-terminal LRR, NACHT domain, and N-terminal effector domain. NOD1 has a high affinity for bacterial peptidoglycan and binds to D-γ-glutamyl-meso-DAP dipeptide (iE-DAP). NOD2 recognizes the muramyl dipeptide (MDP) that is found in all bacteria (Le Bourhis, 2007).

![Figure 3: NOD1 and NOD2 signaling pathways and interaction partners by peptidoglycan of bacteria. In the cytosol, peptidoglycan is recognized by NOD1 or NOD2. NF-κB signaling is activated after NOD1 or NOD 2 bind RIP2 leading to the expression of pro-inflammatory genes to secrete IL-8 and TNF-α (Remodified from Claes, A et al, 2015).](image)
Signaling of NOD1 and NOD2 receptors

When PAMPs bind to NOD1 and NOD2 receptors, the CARD domain of NOD1 and NOD2 is activated; this leads to downstream activation of serine-threonine kinase RIP2. cIAP1 and cIAP2 that polyubiquitinate RIP2 and activate downstream signaling. As a result, IKK complex phosphorylates I\(\kappa\)B\(\alpha\) through activation of TRAF6 and TAK1 complex which have been recruited via RIP2. This leads NF-\(\kappa\)B to translocate to the nucleus. Activation of TAK1 also leads to activation of MAP kinase, ERK, p38 and JNK. This pathway leads to active the transcription factor AP-1 and the phosphorylation of histones H3, giving the transcription factor ERK access euchromatin. p38 works with both JNK and ERK to control the transcription factors. As a result of the translocation of NF-\(\kappa\)B to the nucleus, there is increased gene expression of pro-inflammatory response cytokines and chemokines, including IL-1, IL-6 and IL-8. This also leads to the release of an antimicrobial peptide that kills the invading bacteria (Strober, 2006; Le Bourhis, 2007).

Gram-negative bacteria

In 1884, Christian Gram discovered gram negative bacteria by their unique red color when using the gram staining technique (Silhavy et al, 2010). Some gram negative bacteria are saprophytic harmless and living in the bodies of many animal species, growing in the microflora of these species at the expense of harmful pathogens. Many gram negative bacteria are harmful and cause devastating conditions such as food poisoning, skin infection, gastrointestinal infection, urinary tract infection, and genital tract infection among others. These infections have a high impact on both medical and socioeconomic statuses in the USA and around the world (Bos et al, 2007).

Structure of gram negative bacteria

Cell wall

Gram negative bacteria have cell walls 2-3nm thick containing 2-3 layers of interconnected peptidoglycan. Gram negative cell walls activate both innate and adaptive immune cells including macrophages in human. PAMPS from gram negative bacteria are recognized by pattern associated receptors of macrophages and other innate immune cells. This recognition triggers the release of many pro-inflammatory cytokines. Cell walls can also activate antibody
production from adoptive immune B cells. These antibodies are directed against cell wall antigens; the gram negative cell wall protects bacteria from osmotic lysis (Vomer, 2012).

**Cell membranes**

The outer membrane of gram negative bacteria is composed of a bilayer that is 7 nm thick and consists of phospholipids, lipoproteins, lipopolysaccharides (LPS), and proteins. The inner layer of the outer membrane is formed by phospholipids and lipoproteins and faces the peptidoglycan layer. The outer layer of membrane is formed by lipopolysaccharides. Lipid A is embedded in the lipid portion of the membrane while the polysaccharide portion extends outside the surface. The LPS portion of the outer membrane forms the endotoxin of the gram negative bacteria. The membrane contains many proteins that distinguish different gram negative bacteria from each other. This membrane also contains porins, which function as channels that allow transportation of nutrients into the bacteria. The periplasmic space separates the two layers and is formed from a gelatineous material that separates the outer membrane from the peptidoglycan. This space is around 15 nm thick and contains periplasmic binding proteins which help transport ATP-binding cassette (ABC system) as well as the transport of chemoreceptors for chemotaxis (Caroff and Karbian, 2003).

**Lipopolysaccarides (LPS)**

The main function of LPS is to protect gram-negative bacteria from environment attacks. LPS also contributes to stabilizing the outer membrane by keeping the negative charge on the outer membrane of the bacteria (Whitfield and Trent, 2014). Lipopolysaccharides consist of three different components in gram negative bacteria: a core of lipid A, an inner and outer core of polysaccharides, and O-antigen. Lipid A forms the hydrophobic endotoxin because of its high content of acyl chains (fatty acids); it also contains two glucosamines backbones that attach to the six acyl chains (Caroff and Karbian, 2003). The core oligosaccharide also includes sugars such as Kdo and heptose; Kdo is usually linked with lipid A. O-antigen is composed of repetitive subunits of glycosyl residues (2-8 monosaccharide moieties). The different composition of sugar moieties is the part of LPS that is targeted by host antibodies. Making O-antigen important for the activation of innate immunity (Erridge et al, 2002). LPS plays an important role in activating the innate immune system; LPS interacts with Toll-Like receptor 4 (TLR4) on the surface of macrophages.
LPS is expressed by gram negative bacteria bound to CD14 co-receptors on a macrophage surface. This complex activates TLR4-MD2 and leads to the downstream signaling activation that in turn releases the activated nuclear factor B (NF-B). The translocation of NF-kB to the nucleus leads to the release of pro inflammatory cytokines such as IL-1, IL-6, IL8 and TNF-α. Some of these cytokines polarize the cytoskeleton of the macrophage, leading to distribution in the morphology of the cell (Lu et al, 2008).

**Peptidoglycan**

Peptidoglycan is a protective layer in the bacterial membrane that covers the cytoplasmic membrane. It is composed of two alternating amino sugar residues, N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), which form the backbone of the structure that is cross-linked by peptides. Because of its rigidity, the peptidoglycan layer gives the cell its shape. However, it is also flexible and porous, allowing cell growth and permitting compounds to enter and exit from the cell (Young, 2011). Peptidoglycan from gram negative bacteria interact with plasma intracellular receptors of the immune cell called Nod-Like Receptors (NLRs). NOD1 binds to N-acetylglucosamine-N-acetylmuramyl tripeptide (GM tripeptide) or the dipeptide D-glutamine- diaminopimelic acid (D-Glu–DAP) found in the peptidoglycan of gram-negative bacteria. NOD2 reacts with peptidoglycan, which has the N-acetylmuramyl dipeptide (MDP), either in gram-negative bacteria or gram positive bacteria (Royet and Reichhart, 2003).

**Lipoprotein**

Lipoproteins of gram negative bacteria are formed from three fatty acids (N-Acyl-S-diacylglyceryl cysteine) which are modified by an unusual S-glycerylcysteine residue at the N-terminus in many lipoprotein Lp of Escherichia coli. N-terminus allows type II signal peptide sequences with a conserved lipid-modified cysteine residue from bacteria to attach onto the plasma membrane or outer membrane. Preprolipoprotein diacylglyceryl transferase (Lgt), prolipoprotein signal peptidase (Lsp) and apolipoprotein N-acyltransferase (Lnt) play important roles in the survival of enzymes required for the biosynthesis of bacterial lipoproteins in E. coli. A mature lipoprotein is localized within the cell wall of gram-negative bacteria by linking outer membrane with peptidoglycan (Nakayama et al, 2012). Bacterial lipoproteins play an important role in virulence and in inducing innate immunity via TLR2 in pattern
recognition receptors. TLR2 interacts with a bacterial lipoprotein that activates innate immune response through MHC-II in macrophages; this interaction is associated with an increase in production of cytokines such as IL-1beta, IL-12P40 and TNF-α by using MyD88-dependent signaling (Nakayama et al, 2012).

**OMP-A**

OmpA is complementary to outer membrane proteins in gram-negative bacteria. OMP-A is a highly conserved protein and contains two domains that are 325-residue, heat-modifiable proteins. In the outer membrane, the first domain of OMP-A contains an N-terminal transmembrane β-barrel domain, and in the periplasm, a C-terminal globular domain interacts with peptidoglycan in other domains (Wang, 2002). In many bacteria, the main function of OMP-A is to maintain in the structural integrity of the outer membrane along with murein lipoprotein and peptidoglycan-associated lipoprotein (Smith et al, 2007). During immune response, OmpA is a targeted adaptive immune defense and is a part of innate immunity. OMP-A plays an important role in activating antigen presenting cells (APCs) through TLR2. The acute-phase protein serum amyloid A optimizes OmpA, leading to enhanced phagocytosis by macrophages. (Confer and Ayalew, 2013). However, scavenger receptors, LOX-1 and SREC-I, mediate the ligands between OMP-A on gram-negative bacteria and TLR2 in macrophage (Egesten et al, 2008).

**Flagellin**

Flagellin is an extracellular locomotor and virulent organelle found on the bacterial surface of many gram negative bacteria. Flagella are motility systems that present on the surface of some gram negative bacteria. The flagellum is composed of 3 parts: a long hollow cylindrical helical flagellar filament, a short curved hook, and a basal body or flagellar motor which lies within the plasma membrane. The flagellum moves in a counter clockwise or clockwise direction and shifts bacterial movement utilizing the proton-motive force. The number of flagella range from 5-10 per bacterium, and length varies from 5 to 15 μm. Flagella are also used in gram negative bacteria as a sensory organ, helping the bacteria to attach to host cells such as immune cells. (Zhao et al, 2014). TLR5 on the surface of macrophages of the innate immunity system TLR5 allow the immune cells to recognize the flagellin protein forming the flagellum (Yoon et al, 2012). Flagellin ligands bind to TLR5, activating MyD88-dependent
signaling which leads to the activation of the pro-inflammatory transcription factor NF-κB in epithelial cells, monocytes, and dendritic cells. This activation lead to the release of pro-inflammatory cytokines such as TNF-α or IL-1B (Steiner, 2007).

Figure 4: Structure of cell wall structure in gram-negative bacteria. Gram-negative bacteria contain two membranes which are outer membrane and inner membrane separated by peptidoglycan. Lipopolysaccharides is located in outer membrane and envelops the outer membrane. Lipoprotein is linked between outer membrane and peptidoglycan (Remodified from Lolis, and Bucala, 2003).
MATERIALS AND METHODS

Cell Line

The Cell line is RAW 264.7 murine macrophage (purchased from American Type Culture Collection ATCC, Manassas, VA). Culture petri plates 100mm x 20mm (from BD Biosciences) are used to culture RAW 264.7 murine macrophage with Dulbecco’s Modified Eagle’s Medium (DMEM; HyClone), Supplemented with 10% of fetal bovine serum (heat-inactivated) (from Fisher Scientific) and 1% of penicillin-streptomycin antibiotic (from MP Biomedical, LLC). At 37°C, RAW 264.7 murine macrophage were grown in a humidified 5%CO2 in incubator. Cells were splitting of the cells depends on confluency of the cell on the surface of petri plate by viewing cells under a microscope (approximately 2-3 times per a week).

Cytokine Measurements

After 24 hours, Exposure of gram-negative bacteria compounds onto RAW 264.7 macrophage release cytokines concentrations that have been collected and measured via Luminex Multiplex Immunoassays.

Stimulated Cells with Gram-Negative Bacterial Components

RAW 264.7 murine macrophages were grown in 12 well chambers (from Ibidi) to 70% confluency. RAW 264.7 murine macrophages were stimulated with peptides (1, 5 and 10 μg/ml) of LPS, peptidoglycan, lipoprotein, OMP-A and flagellin from gram negative bacteria. In each chamber, there were three wells for each concentration, and 3 wells with unstimulated cells as control. The chambers were incubated for 24 hours and the culture media were collected and frozen at -80 °C.
Cell Viability

At 70% confluency, the RAW 264.7 cells were then stimulated with peptide (1, 5 or 10 µg/ml) LPS, peptidoglycan, lipoprotein, OMP-A and Flagellin from gram negative bacteria. Unstimulated cells that served as control cells were incubated for 24 hours. After that, the cells, the control and each product (1, 5 and 10 µg/ml), were grown on 12-well plates for 24 hours. At 4°C, using centrifuge on new supernatant at 1500 rpm was made for 5 minutes. Then, solution of cell was aspirated and 1 mL of 10% DMEM medium was added to suspend the cell pellet. To determine the cell viability, trypan blue stain was added at a ratio of 1:2 the following equation was used to classify the cell viability: 

\[
\text{% Cell Viability} = \frac{\text{Total Viable Cells (Unstained)}}{\text{Total Cells (Viable + Dead)}} \times 100
\]

Figure 5: Trypan blue exclusion test (Hemocytometer method). Live cells appear colorless under microscope, whereas the dead cells stain blue.
Table 1: Cell viability calculations.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Equation</th>
</tr>
</thead>
</table>
| %Cell Viability | \[
\frac{\text{Total Viable cells (Unstained)}}{\text{Total cells (Viable + Dead)}} \times 100
\] |
**Immunofluorescent Staining**

On chambers, cells were washed with phosphate buffered saline (PBS) (3 times at 5 minutes each time). Next, (4%) paraformaldehyde was fixed the cells for 15 minutes. PBS was used to rinse the cells (3 times) (5 minutes). After that, 0.2% Triton diluted in PBS permeabilize the cells (5-10 minutes). One more time, PBS was used to rinse the cells (3 times at 5 minutes). The Blocking buffer (5% Goat serum, 3% BSA, and 0.05% tween) was used to block the cells for one hour preventing. Again, PBS was used to rinse the cells (3 times at 5 minutes). 3% BSA (1.5 mg of bovine serum albumin in 50 mL PBS) was used to dilute primary antibodies and cells were incubated at a temperature 4°C overnight. Next day, (1%) BSA (0.5 mg of bovine serum albumin in 50 mL PBS) was used to rinse the cells (3 times at 5 minutes). In dark, Texas Red-Phalloidin X on secondary antibody were added for 1–2 hours. (1%) BSA (0.5 mg of bovine serum albumin in 50 mL PBS) was used to rinse the cells (3 times at 5 minutes). Vectashield (H-1400) (from Vector Laboratories) was used to mount the stained cells onto the microscope slide by adding a drop in each well. The cells were evaluating on Olympus Epi-fluorescence microscope with a ‘spot’ digital camera.

**Image Analysis**

Image J (National Institutes of Health, http://imagej.nih.gov/ij/) was used to qualify the Immunofluorescent of cells after exposure. Image J quantifies regions of interest (qROI) of the fluorescent via several steps. First of all, the type of color is 8-bit image by opening original image → image → type → 8-bit image. After that, the background was subtracted to remove the pixel information from a continuous background. Also, the threshold 20 pixel values of black background for and 111 pixel values of bright saturated artifacts of image were adjusted. Finally, evaluating the image through analyze Particles by selecting analysis option then analyze the particles, so now the value total of the area and number of counted cells have been used to compare the images after stimulation via One-way ANOVA and T test in Sigma Plot 13.0 Software.
Figure 6: Steps of Image analysis in image j.
RESULTS

Cell viability of RAW 264.7 murine macrophage exposed to gram-negative components after 24 hours

RAW 264.7 murine macrophages (M0) were exposed to LPS, flagellin or Peptidoglycan in concentrations of 1, 5 or 10 μg/ml. The cell viability was measured after 24 hours using trypan blue staining assay. Macrophages exposed to LPS showed ~ 2-fold decrease (P≤0.001) in cell viability at all concentrations compared to control cells as shown in Figure 7. Also, macrophages displayed a gradual decrease in variability with high concentrations of flagellin and showed a significant decrease with 10 μg/ml of flagellin (P ≤ 0.01) compared to control cells. Also, there was a significant decrease between M0 with 5 μg/ml flagellin (P ≤ 0.05) compared to control cells as shown in Figure 8. However, macrophages exposed to Peptidoglycan showed no differences in viability of the cells as shown in Figure 9. In general, RAW 264.7 macrophages showed the highest response to LPS compared to flagellin or peptidoglycan of gram-negative bacteria.

Cell morphology

Morphological changes exposing to gram-negative bacterial compounds on RAW 264.7 murine macrophage comparing to control cells

M0 macrophages served as the control and appeared rounded after 24 hours. Macrophages were stimulated with gram-negative bacterial components, flagellin, lipoprotein, LPS, OMP-A and peptidoglycan, with different concentrations 1, 5 or 10 μg/ml. Morphologically, the cells appeared flattened and elongated with LPS from gram-negative bacterial.
Gram-negative bacterial components trigger tubulin cytoskeleton rearrangement in RAW 264.7 murine macrophage

At concentration of 5 and 10 μg/ml, the flagellin caused dispersed staining of the microtubules throughout the cell cytoplasm of RAW 264.7 macrophage as shown in Figure 10 A. The microtubule continued to show staining at lower concentrations with 5 and 1 μg/ml as compared to the control cells. At a concentration of 5 μg, bacterial lipoprotein, OMP-A, and peptidoglycan stimulated the macrophages to microtubule aggregation to one pole of the cell as shown in Figure 11A, 13A, 14A. In contrast, the 10 μg/ml concentration of lipoprotein caused the RAW264.7 cells to aggregate within 24 hours as shown figure11A. LPS expose to RAW264.7 cells at the three concentrations. Elongation of the cells’ morphology microtubule appeared throughout the cell as shown in Figure 12 A.

Gram-negative bacterial components trigger F-actin cytoskeleton rearrangement in RAW 264.7 murine macrophage

Exposure of flagellin at concentration of 10 μg/ml, F-actin shows low staining whereas 1 and 5 μg/ml show higher level of staining on RAW 264.7 macrophage as shown in Figure 15 A. At concentration of 5 and 10 μg/ml, bacterial lipoprotein caused aggregation one to one pole of the cell as shown Figure16 A. At the three concentrations, LPS caused flattened and extended irregular shapes of the cell morphology to express F-actin around the cells as shown in Figure 17 A. At concentration of 5 and 10 μg/ml, OMP-A displayed enlarged and flattened cells to express F-actin as shown in Figure 18 A. Peptidoglycan caused flattened cells at concentrations of 10 μg/ml for some cells as shown in Figure 19.

Gram-negative bacterial components stimulate the production of cytokines by RAW 264.7 murine macrophage

Pro-inflammatory cytokine IL-1b production increased gradually when the cells were exposed to LPS, flagellin or peptidoglycan at all consternations 1, 5 and 10 μg/ml. Similarly, the macrophages stimulated with OMP-A at 5 μg/ml showed a slight increase in IL-1b compared to the control cells. Macrophages responded to lipoprotein
by releasing IL-1b, but there is no difference between levels of IL-1b in the three concentrations 1, 5 and 10 μg/ml as shown in Figure 7. Secretion of pro-inflammatory cytokine IL-10 decreased with cells that were exposed to LPS, OMP-A, flagellin, lipoprotein and peptidoglycan in all concentrations compared to IL-1b, IL-12 and TNF-α productions as shown in Figure 21. IL-12 production increased gradually with the cells that were exposed to LPS, which IL-12 increased slightly when macrophages were stimulated to other gram-negative bacterial compounds at concentrations 1, 5 and 10 μg/ml as shown in Figure 22. TNF-α increased highly with cells that were exposed to LPS, OMP-A, flagellin, lipoprotein and peptidoglycan in 1, 5 and 10 μg/ml as shown in Figure 24. However, stimulated macrophage with LPS released the highest level of TNF-α compared to cells that exposed other gram-negative bacterial components as shown in Figure 23.
Table 4: Cytokines secretion after exposure of gram-negative bacterial components to RAW 264.7 murine macrophage after 24 hours.

<table>
<thead>
<tr>
<th>Bacterial compounds</th>
<th>Cytokines</th>
<th>Concentration</th>
<th>LPS</th>
<th>OMP-A</th>
<th>Flagellin</th>
<th>Lipoprotein</th>
<th>Peptidoylycan</th>
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<tr>
<td></td>
<td>IL-1b</td>
<td>Control</td>
<td>47.80</td>
<td>78.41</td>
<td>34.54</td>
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<td></td>
<td>pg/ml</td>
<td>1 µg/ml</td>
<td>121.20</td>
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<td></td>
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<td>5 µg/ml</td>
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<td>113.60</td>
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<td>10 µg/ml</td>
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<td>79.18</td>
<td>136.60</td>
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<td>IL-10</td>
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<td>IL-12</td>
<td>Control</td>
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Figure 7: Cell viability of RAW 264.7 murine macrophage exposed to LPS from gram-negative bacteria in concentrations (1.5 and 10 μg/ml) compared to control cells at 24 hours. Each value represents the mean ± standard error (SE) of 3 separate experiments. ***, p<0.001. (n=3)

Figure 8: Cell viability of RAW 264.7 murine macrophage exposed to flagellin from gram-negative bacteria in concentrations (1.5 and 10 μg/ml) compared to control cells at 24 hours. Each value represents the mean ± standard error (SE) of 3 separate experiments. *, p<0.05; **, p<0.010. (n=3)
Figure 9: Cell viability of RAW 264.7 murine macrophage exposed with peptidoglycan from gram-negative bacteria in concentrations (1, 5 and 10 μg/ml) compared to control cells at 24 hours. Each value represents the mean ± standard error (SE) of 3 separate experiments. (n=3)
Figure 10: A) Tubulin immunofluorescent images of RAW 264.7 murine macrophage. Macrophages cells show morphological changes and cytoskeleton immunostaining at 24 hours exposing to flagellin from gram-negative bacteria (Scale bar =20 μm). B) Tubulin immunofluorescent intensity for control and (1,5 and 10 μg/ml) of flagellin from gram-negative bacteria following 24 hours. Each value represents the mean ± standard error (SE) of 3 separate experiments. (n=3)
Figure 11: A) Tubulin immunofluorescent images of RAW 264.7 murine macrophage. Macrophages cells show morphological changes and cytoskeleton immunostaining at 24 hours exposing to lipoprotein from gram-negative bacteria (Scale bar =20 μm). B) Tubulin immunofluorescent intensity for control and (1, 5 and 10 μg/ml) of lipoprotein from gram-negative bacteria following 24 hours. Each value represents the mean ± standard error (SE) of 3 separate experiments. *, p<0.05; **, p<0.010. (n=3)
Figure 12: A) Tubulin immunofluorescent images of RAW 264.7 murine macrophage. Macrophages cells show morphological changes and cytoskeleton immunostaining at 24 hours exposing to LPS from gram-negative bacteria (Scale bar =20 μm). B) Tubulin immunofluorescent intensity for control and (1,5 and 10 μg/ml) of LPS from gram-negative bacteria following 24 hours. Each value represents the mean ± standard error (SE) of 3 separate experiments. **, p<0.010; ***, p<0.001. (n=3)
Figure 13: A) Tubulin immunofluorescent images of RAW 264.7 murine macrophage. Macrophages cells show morphological changes and cytoskeleton immunostaining at 24 hours exposing to OMP-A from gram-negative bacteria (Scale bar =20 μm). B) Tubulin immunofluorescent intensity for control and (1, 5 and 10 μg/ml) of OMP-A from gram-negative bacteria following 24 hours. Each value represents the mean ± standard error (SE) of 3 separate experiments. *, p<0.05. (n=3)
Figure 14: A) Tubulin immunofluorescent images of RAW 264.7 murine macrophage. Macrophages cells show morphological changes and cytoskeleton immunostaining at 24 hours exposing to peptidoglycan from gram-negative bacteria (Scale bar =20 μm). B) Tubulin immunofluorescent intensity for control and (1,5 and 10 μg/ml) of peptidoglycan from gram-negative bacteria following 24 hours. Each value represents the mean ± standard error (SE) of 3 separate experiments. (n=3)
Figure 15: A) F-actin immunofluorescent images of RAW 264.7 murine macrophage. Macrophages cells show morphological changes and cytoskeleton immunostaining at 24 hours exposing to Flagellin from gram-negative bacteria (Scale bar =20 μm). B) F-actin immunofluorescent intensity for control and (1,5 and 10 μg/ml) of flagellin from gram-negative bacteria following 24 hours. Each value represents the mean ± standard error (SE) of 3 separate experiments. (n=3)
Figure 16: A) F-actin immunofluorescent images of RAW 264.7 murine macrophage. Macrophages cells show morphological changes and cytoskeleton immunostaining at 24 hours exposing to lipoprotein from gram-negative bacteria (Scale bar =20 μm). B) F-actin immunofluorescent intensity for control and (1, 5 and 10 μg/ml) of lipoprotein from gram-negative bacteria following 24 hours. Each value represents the mean ± standard error (SE) of 3 separate experiments. **, p<0.010. (n=3)
Figure 17: A) F-actin immunofluorescent images of RAW 264.7 murine macrophage. Macrophages cells show morphological changes and cytoskeleton immunostaining at 24 hours exposing to LPS from gram-negative bacteria (Scale bar =20 μm). B) F-actin immunofluorescent intensity for control and (1, 5 and 10 μg/ml) of LPS from gram-negative bacteria following 24 hours. Each value represents the mean ± standard error (SE) of 3 separate experiments. *** p<0.001. (n=3)
Figure 18: A) F-actin immunofluorescent images of RAW 264.7 murine macrophage. Macrophages cells show morphological changes and cytoskeleton immunostaining at 24 hours exposing to OMP-A from gram-negative bacteria (Scale bar =20 μm). B) F-actin immunofluorescent intensity for control and (1,5 and 10 μg/ml) of OMP-A from gram-negative bacteria following 24 hours. Each value represents the mean ± standard error (SE) of 3 separate experiments. *, p<0.05. (n=3)
Figure 19: A) F-actin immunofluorescent images of RAW 264.7 murine macrophage. Macrophages cells show morphological changes and cytoskeleton immunostaining at 24 hours exposing to peptidoglycan from gram-negative bacteria (Scale bar =20 μm). B) F-actin immunofluorescent intensity for control and (1,5 and 10 μg/ml) of peptidoglycan from gram-negative bacteria following 24 hours. Each value represents the mean ± standard error (SE) of 3 separate experiments. (n=3)
Figure 20: Graph shows RAW 264.7 murine macrophage have been respond to gram-negative bacterial components (LPS, OMP-A, Flagellin, Lipoprotein, Peptidoglycan) by releasing IL-1b.

Figure 21: Graph shows RAW 264.7 murine macrophage have been respond to gram-negative bacterial compounds (LPS, OMP-A, Flagellin, Lipoprotein, Peptidoglycan) by releasing IL-10.
Figure 22: Graph shows RAW 264.7 murine macrophage have been respond to gram-negative bacterial compounds (LPS, OMP-A, Flagellin, Lipoprotein, Peptidoglycan) by releasing IL-12.

Figure 23: Graph shows RAW 264.7 murine macrophage have been respond to gram-negative bacterial compounds (LPS, OMP-A, Flagellin, Lipoprotein, Peptidoglycan) by releasing TNF-a.
Figure 24: Graph shows RAW 264.7 murine macrophage have been respond to gram-negative bacterial compounds (OMP-A, Flagellin, Lipoprotein, Peptidoglycan) by releasing TNF-α.
DISCUSSION AND FUTURE STUDIES

In this study, responses of murine RAW 264.7 macrophages to substances displayed on the outer surfaces of gram-negative bacteria: LPS, lipoprotein, peptidoglycan, outer membrane protein A (OMP-A) and flagellin were studied. These materials did not cause a dramatic alteration in cell morphology except for LPS; LPS-stimulated cells appeared flattened and elongated (as shown in figure 25).

![Figure 25](image_url)

Figure 25: RAW 264.7 murine macrophage exhibited morphological changes after interaction with LPS through TLR4.

The main objective of this study was to examine the cytoskeleton reorganization of RAW 264.7 macrophages exposed to these gram-negative substances. The first response of LPS-stimulated macrophages is their migration to the sites of the infection (Duque and Descoteaux, 2015). Tubulin filaments and F-actin were examined, and the amounts of fluorescent intensity staining of the tubulin and F-actin microfilaments were estimated by quantifying, using Image J analysis. LPS exerted the greatest effects on rearrangement of the cellular actin as the cells became flattened and irregularly shaped compared to the rounded morphology displayed by uninfected macrophages as
shown in Figure 12A and 17A and the graphs show high level of tubulin and F-actin that are increased compared to the control as shown in Figure 12B and 17B. Also, OMP-A caused an effect on the cytoskeleton, as noted by the F-actin staining, at 5 μg/ml as shown in Figure 18 while the lipoprotein decreased staining of F-actin at 10 μg/ml compared to that seen in unstimulated control cells as shown in Figure 16.

Upon exposure to LPS, innate immune system responds using the TLR4 pathway in macrophage. At downstream signaling, lipid A is a portion of LPS that generate the signaling to secrete pro-inflammatory cytokines such as TNF-α and IL1β (Katsuaki et al., 2016). Significant reductions in cell viability compared to unstimulated control cells were seen at 24 hours after exposure to 1, 5 and 10 μg/ml of LPS. Similar to the observation of Zhuang (1997), decreased cell viability was seen in cells stimulated with LPS compared to unstimulated cells after 24 hours. These observations suggest that activation of TLRs leading to production of inflammatory cytokines such as TNF-α may account for these decreases in cell viability; LPS activates TLR4 as shown in Figures 7 and 23.

Hayashi et al. (2016) found that fagellin from Gram-negative bacteria stimulates RAW 264.7 macrophages through TLR5. Triggering of the TLR5 receptor activates NF-kB and stimulates TNF-α production. In this study, none of the other substances tested caused decreases in cell viability except LPS and flagellin. Flagellin activates TLR5 as shown in Figures 8 and 24. Others have noted the decreased viability of cells expressing TNF-α (Wang et al., 2011).

In future studies, one of our limitation on this study is the cell line which is RAW 264.7 murine macrophage so it would be beneficial to study the effect of gram-negative bacterial components on another cell line such as J774A.1 macrophage phenotypes and human peripheral blood macrophages to compare the results of those cell line. It would be helpful to study the effects on viability, cytoskeleton filaments and cytokines expression on macrophages at 4, 8, 16 hours after interaction with gram-negative bacterial components with 1,5 and 10 μg/ml to understand the outcomes of the primary and immediate stages of infection. Also, it would be beneficial to expose RAW264.7 macrophages to flagellated and deflagellated E. coli to study the role of flagella on cell viability and secretion of cytokines from macrophage after exposing to those bacteria. Based on the results of this study, we predict that
deflagellated E. coli will induce less TNF-a than flagellated E. coli leading to reduced cell death and increase in cell viability.

It also would be beneficial to study the morphological changes and reorganization of RAW 264.7 macrophage by measuring RhoA Rac1 and Cdc42 proteins in exposed cells to LPS and assess cytoskeletal proteins in exposed macrophages through PPRs by using immunoblotting approaches after regulatory proteins affect the activation state of these cycling Rho molecules such as GEFs, GAPs and GDIs. Finally, it would be interesting if GEF exchange activity will be used to determine the rate of exchange of GTP of RAW 264.7 macrophages exposed to LPS from gram-negative bacteria by using GTP fluorescent conjugate. Levels of RhoA Rac1 GTPases proteins will be increased after the exposure of RAW 264.7 macrophage with LPS. Also, exposure of RAW 264.7 macrophages to LPS should exhibit a high rate of phosphorylation of GTP leading to cellular signaling by TLR4.
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