EFFECT OF EXPOSURE OF RAW264.7 MACROPHAGES TO SALMONELLA TYPHIMURIUM COMPONENTS ON CELL VIABILITY, CYTOSKELETON RE-ARRANGEMENT AND CYTOKINE SECRETION

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

By

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ABSTRACT

Alyahya, Khalid Abdullah. M.S. Microbiology and Immunology Program, Wright State University, 2017. Effect of Exposure of Raw264.7 Macrophages to Salmonella typhimurium Components on Cell Viability, Cytoskeleton Re-arrangement and Cytokine Secretion

Innate immune system plays an important role in individual’s protection against pathogens and in activation of adaptive immune system. Utilizing RAW 264.7 murine macrophages as an innate immune response representative in this study, we analyzed the effect of invasive pathogen’s components (e.g. flagellin) on the arrangement of macrophage’s cytoskeleton, on viability of immune cells and on secretion of pro-inflammatory and anti-inflammatory cytokines and on fluorescence intensity of cytoskeleton after rearrangement. Additionally, we studied the similarity and differences between bacterial (Salmonella typhimurium) and synthetic TLR4 agonist (synthetic lipid-A) on viability, fluorescence intensity, cytokine secretion, and cytoskeleton rearrangements. Similarly, we studied the differences between TLR2 receptor agonist from gram-negative and the TLR2/6 receptor agonist from gram-positive bacteria. Flagellin at highest concentration (10 μg/ml) decreased the macrophages viability significantly and increased the tubulin fluorescence intensity significantly. S. typhimurium’s LPS and highest concentration of synthetic lipid A (10 μg/ml) decreased the cell viability dramatically. However, the intensity of microtubules was dramatically lower in the S. typhimurium’s LPS compared to synthetic lipid A at 5 and 10 μg/ml concentrations. Both bacterial and synthetic TLR4 agonists elevate fluorescence intensity of microfilaments significantly. TLR2/6 agonists from gram-positive bacteria decrease the cell viability more than TLR2 agonist from gram negative but not significantly; however, the fluorescence intensity of microtubules was significantly increased in TLR2 agonist in compared to TLR2/6 agonists at all
concentrations. *S. typhimurium* at a concentration (10^6 cell/ml) that activates TLR4 induced production of IL-6, IL-10, and TNF-α significantly. Additionally, di-acylated lipoprotein at 5μg/ml induced very high levels of IL-10 secretion compared to control.
HYPOTHESIS

Exposing RAW264.7 murine macrophages to pathogen components either bacterial (*S. typhimurium*) or synthetic (synthetic di-acylated lipoprotein and synthetic lipid A) will induce changes on viability of macrophages, cytokine secretion, and arrangement of cytoskeleton at 24 hours. Cytokine production is predicted to increase, especially TNF-α as pro-inflammatory cytokines in TLR4 agonist components. Since the secretion of anti-inflammatory cytokines increases after secretion of pro-inflammatory cytokines, the production of anti-inflammatory cytokines are predicted to be high. As macrophages are exposed to TLR4 agonists, macrophage’s cytoskeleton is expected to be elongated and is predicted to have higher fluorescence intensity after 24 hours because of converting un-polarized macrophages to polarized M1 macrophages. Moreover, cell viability is also estimated to decrease after exposure to pathogenic components such as LPS.
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INTRODUCTION

Salmonella enterica serovar typhimurium, which is resisted to multiple antibiotics (Rabsch W et al., 1987), is a pathogenic gram-negative bacteria, and it is considered a Zoonotic bacteria. The size of Salmonella typhimurium’s DNA is 4,857,432 base pairs in addition to 94,000 base pairs of virulence plasmid (McClelland et al., 2001). The gut is considered the site of infection in Salmonella typhimurium. S. typhimurium causes gastroenteritis and diarrhea for both animals and humans (Mccormick et al., 1995). The vast majority of people who are infected with S. typhimurium are healthy except a minority such as immune compromised patients, elderly, and very young individuals (Simon et al., 2009). The symptoms of gastroenteritis include abdominal pain, nausea, vomiting, fever, and diarrhea, and they usually last up to seven days. In mice, it causes a fatal disease, which has symptoms similar to Typhoid disease. Thus, mice are used as a model to study human typhoid fever (McClelland et al., 2001).

Macrophages, which differentiate from monocytes, are one of the first responders in the immune system. Macrophages are present in all different tissues of the human body. They are responsible for eliminating pathogens and clear the human body of cells in the damaged tissues and recruit additional macrophages and other immune cells to the site of infection. The elimination process is done by the phagocytic ability of macrophages. The engulfment of pathogens helps to activate adaptive immune response. Cytokines that are released by immune cells, in addition to the metabolites and some transcription factors, help to specialize the function of macrophages. Macrophages are able to differentiate into classical (M1) or alternative pathway (M2) based on the stimuli (Lavin et al., 2015).
Flagellin is a small helical protein and the main component of flagellum. The flagellum is responsible for the movement of microorganisms such as *S. typhimurium*. It moves rapidly clockwise or counterclockwise. The number of flagella is varied between bacterial cells and ranges from five to ten flagella. The length also varies and ranges between 5 to 15 μm (Zhao et al, 2014). The human body recognizes the flagella though TLR5 to initiate an innate immune response toward these pathogens (Kurnasov et al., 2012).

Lipopolysaccharide (LPS) is a major component in gram-negative bacteria. LPS is found mainly on gram-negative bacteria, but not all of them have LPS (Sutcliffe, 2010). LPS’s importance appears in protecting the bacterial cell from external environments. 75% of *S. typhimurium*’s outer membrane is covered with approximately 2 million LPS molecules (Whitfield and Trent, 2014). Lipopolysaccharides consist of lipid A and O-antigen. They stimulate the innate immune response by starting a cascade of actions inside macrophages after binding to TLR4 on the surface of leukocytes such as macrophages. LPS will lead to polarization of macrophages to form M1 phenotypes (Nakagawa et al., 2014).

Lipoproteins are potent activators of Toll like receptor 2 (TLR2). TLR2 forms a heterodimer with either TLR1 or TLR6. Triacyl lipoproteins of gram-negative bacteria activate TLR2-TLR1 heterodimer. On the other hand, diacylated lipoproteins of gram-positive bacteria activate TLR2-TLR6 heterodimer. Diacylated lipoproteins are composed of two diacylglycerol that are connected to each other through thioester bond (Rivet et al., 2011).

The cytoskeleton of human cells has three major components of filaments that play a role in supporting the cell to maintain the cell shape in addition to an important role in cellular activities, such as mitosis. These three types of cytoskeletons are microtubules, actin, also called
microfilaments, and finally intermediate filaments. The cytoskeleton is regulated in normal human cells. Actin can be seen as one single monomer that is called G-actin or in groups of G-actin bound together to form F-actin. The second major type is called microtubules, which are composed of $\alpha/\beta$ tubulin. Both of these two types, actin and microtubules, have an important role in the movement of the cells and mitosis. The third type is intermediate filament. 40 different proteins can form this type and can be divided into five categories. These five categories are desmin, vimentin, keratins, neurofilaments and laminin (Fife et al., 2014).
LITERATURE REVIEW

Macrophage:

Macrophages are one kind of leukocyte (white blood cells) that have astonishing phagocytic ability. Generally, they differentiate from monocytes in peripheral tissues. Macrophages have a wide range of receptors that dictate the cellular response, which plays a crucial role in innate and adaptive immune response (Murray & Wynn, 2011). So, depending on the environmental stimuli, the macrophage M0 will polarize to form either pro-inflammatory M1 or anti-inflammatory M2 macrophages.

M1 macrophages are polarized in response to LPS and INF-γ. As a result, pro-inflammatory M1 macrophages will respond to the stimulation and secrete pro-inflammatory cytokines, such as TNF-α and other effector molecules such as IL-1β, CCL3 and iNOS. Pro-inflammatory macrophages (M1) have a role in anti-tumor activity in addition to defending the body against microorganisms, such as bacteria (Kigerl et al., 2009) (Figure 1).

On the other hand, anti-inflammatory macrophages (M2) are polarized in response to interleukin-4 (IL-4), interleukin-10 (IL-10), or interleukin-13 (IL-13) (Reichard et al., 2015). M2 macrophages promote tissue remodeling and angiogenesis though secretion of anti-inflammatory cytokine (IL-10) (Kigerl et al., 2009) (Figure 1).
**Figure 1**: Polarization and activation of macrophages. Polarization of resting macrophages (M0) starts from environmental cytokines. First, polarization to M1 macrophages results after stimulation of M0 with LPS or INF-γ and become able to secrete pro-inflammatory cytokines, such as IL-1β, TNF-α, and IL-6 in order to respond to host defense. Second, Polarization to M2 results from activation of M0 with IL-4, IL-10, or IL-13 to secrete anti-inflammatory cytokines (IL-10) in order to have tissue remodeling and angiogenesis (Kigerl et al., 2009). (Modified from Nakagawa, Y. & Chiba, K., 2014).
Macrophage Cytoskeleton:

The cytoskeleton of macrophages has three different types of filaments and is arranged in the cell in complex network that helps the cell to maintain its structure, to move to the site of infection, and to phagocytose pathogens or dead tissues. The cytoskeleton can be classified into three groups, which have been categorized based on the diameter and role of filaments. The first cytoskeleton is the microtubule with approximately 25 nm in diameter. An intermediate filament is approximately 10 nm in diameter. A microfilament is the smallest cytoskeletal filament with approximately 7 nm in diameter (Streiblová et al., 1980).

Microtubules are arranged in a hollow cylinder and consist of polymers of $\alpha/\beta$ tubulin, which represent the fundamental components of microtubules that regulate the cell shape. $\alpha/\beta$ tubulin heterodimers are arranged from head to tail to form a protofilament. 13 of the protofilaments are connected together to form one microtubule (Howard & Hyman, 2007). The formation of microtubules involves polymerization of $\alpha/\beta$ tubulin. The initial polymerization requires the availability of $\gamma$-tubulin ring complexes ($\gamma$-TuRCs), which is found in the centrosome or microtubule organization center (MTOC) (Chaffey et al., 2003).

The movement of macrophages requires microtubule-associated motor proteins (MAPs) such as kinesin and dynein and consumes a huge amount of energy (GTP) in order to move for a few microns (Vale et al., 1992). Kinesin and dynein represent two important movements: plus-end directed movement and minus-end directed movement, respectively (Miki et al., 2005)(Pfister et al., 2006). In addition to maintaining the cell structure, microtubules form a mitotic spindle in order to divide chromosomes between two daughter cells (Fife et al., 2014).
Actin polymers and actin-binding proteins are the components of microfilaments. Microfilaments are formed by monomers of actin subunits, called G-actin. These monomers are arranged together to form a double-stranded helical structure called F-actin, and its diameter is around 6 nm. The building of G-actin to form F-actin is done through the hydrolysis of ATP (Fife et al., 2014). Microfilaments are crucially important in the case of infection. Microfilaments play a role in migration to the site of infection and in engulfment of pathogens (phagocytosis).

**Pattern Recognition Receptor (PRR):**

The pattern recognition receptors help macrophages identify two different groups. Macrophages are able to detect pathogens or pathogen components through the pathogen associated molecular pattern (PAMP) and detect cellular damages through the damage associated molecular pattern (DAMP). Additionally, there are several different other classes that have been discovered, such as NOD-like receptors (NLRs), Toll like receptors (TLRs), RIG-I like receptors (RLRs), and DNA receptors (Kumar et al., 2011). The cell will respond the stimuli based on the stimulation of a receptor. The attack of *S. typhimurium* will be recognized by innate immune response through PAMP; as a result, macrophages will respond by secreting cytokines (pro-inflammatory cytokines), interferons (Type I) and chemokines, in addition to other responses (Takeuchi and Akira, 2010).
Toll like receptor:

Toll like receptors (TLRs) have a huge importance in recognizing pathogens. TLRs are able to differentiate between the self and the non-self. There are 13 different types of TLRs; humans have only 10 of these receptors while mice have 12 types of TLRs, which are TLRs from 1 to 13 except TLR10, which is only found in humans (Moresco et al., 2011). TLRs activate NF-κB and increase the production of cytokines and chemokines. TLRs have several Leucine-rich repeats (LRRs), which is a characteristic of the class I trans-membrane protein. Some of the TLRs require additional parts to detect the antigen, such as TLR4 requires MD-2 to attach to LPS (Shimazu et al., 1999). TLRs form homodimer except TLR2/1 and TLR2/6 (Moresco et al., 2011).

Signaling of toll like receptors (TLR):

The interaction of TLR with a Ligand (PAMP) stimulates MyD88 or TRIF. TLR5, TLR7, TLR8, and TLR9 stimulate MyYD88. However, TLR1/2 and TLR2/6 activate two adaptors, which are Mal and MyD88. TLR3 activates TRIF, which is down regulated by SARM. Lastly, TLR4 activates four different adaptors, which are MyD88, Mal, TRAM, and TRIF. After the activation of the TLR receptor, the death domain of MyD88 recruits IRAK4, which activates IRAK1 and IRAK2 through phosphorylation. As a result, TRAF6 will be activated. Then, TRAF6 activates TAK1, which is linked with TAB2, and both help ubiquitin NEMO from IKK complex and TRAF6 several times. The IKK complex is activated by TAK1 after phosphorylation of IKKβ. The activation of the IKK complex will lead to degradation of IκB
after phosphorylation. This will lead to the release and activation of NF-κB. Transcription factors AP1 and CREB will be activated through the activation of TAK1 to M KK3 and M KK6. All of these transcription factors (NF-κB, AP1, and CREB) will enter the nucleus and up-regulate the production of pro-inflammatory cytokines, such as TNF-α (Moresco et al., 2011).
Figure 2: Signaling of TLRs. Activation of TLR2/1, TLR2/6, TLR4, or TLR5 will activate MyD88. The death domain of MyD88 activates IRAK4, which activates IRAK1 and IRAK2 then TRAF6. TRAF6 activates TAK1, which activates three transcription factors (CREB, AP1 and NF-κB) (Adapted from Moresco et al., 2011 and O'Neill, et al 2013).
Salmonella enterica serovar typhimurium:

*S. typhimurium* is a gram-negative bacterium with a genomic DNA length of 4,857,432 base pairs in addition to an approximately 94,000 base pair virulence plasmid. *S. typhimurium* causes human and animal diseases. *S. typhimurium* causes typhoid like symptoms in mice, and this disease is life threatening (McClelland et al., 2001). On the other hand, *S. typhimurium* causes gastroenteritis to humans and cattle, and in a vast majority of cases it is not fatal. However, gastroenteritis can be lethal for elderly and immunocompromised individuals (Miao et al., 1999). *S. typhimurium* has more than one membrane, and several components, which could be represented as 50 percent of the phospholipid, 60 percent of the protein, and 90 percent of the lipopolysaccharide (LPS) of the total percentage of these components, present mainly in the outer membrane (Osborn et al., 1972).

The mechanism of gastroenteritis starts with eating contaminated meat. *S. typhimurium* colonizes in the intestine. So, *S. typhimurium* damages the intestinal microvilli, which facilitate *S. typhimurium* entry to the epithelium tissue. After penetration, the pathogen starts replication inside the epithelium cells. This activates inflammatory response. Now, sub-epithelial neutrophils migrate through the epithelium to attack the pathogen (McCormick et al., 1995).
Lipopolysaccharides:

LPS is essential for most gram-negative bacteria, especially in the interaction in the environment and in the stability of the outer membrane. Minorities of gram-negative bacteria have LPS that consists of lipid A only; however, the vast majority of LPS consists of two main components: lipid A that is attached to O linked polysaccharides through the core polysaccharides (Whitfield and Trent, 2014). The acylation and phosphorylation level of lipid A determines the strength of LPS (Mogensen, 2009). Gram-negative bacteria attach to negatively charged surfaces, such as glass with the help of LPS (Abu-Lail et al., 2003). As a result of availability of O-antigen, *S. typhimurium* polarizes M0 macrophages to form an M1 phenotype (Luo et al., 2016). Some researchers found that the effect of *S. typhimurium* cells is similar to the effect of LPS on the macrophage gene expression (Rosenberger et al., 2000).

Lipoprotein:

Lipoproteins are crucially important to a bacterial cell. They contribute to the adhesion to host cells, colonization of bacterial cells, delivery of virulence factors, and play a role in antibiotic resistance. Gram-negative bacteria cannot live if there is any damage to lipoproteins. However, some gram-positive bacteria can live without a lipoprotein (Rivet et al., 2011). Lipoprotein has different forms based on the type of bacteria. Gram-positive bacteria have di-acylated lipoprotein, while gram-negative bacteria have tri-acylated lipoprotein. Lipoproteins are cell surface proteins and activate the TLR2 receptor on macrophages. Tri-acylated lipoproteins stimulate TLR2 and TLR1 heterodimer, and di-acylated lipoproteins stimulate TLR2 and TLR6
heterodimer. *S. typhimurium* has its lipoprotein, which is called Braun lipoprotein, and its size varies from 5 to 9 kilodaltons. This lipoprotein is formed by IppA and IppB genes, which are functional and play an important role in bacterial virulence (Fadl et al., 2005). Similar to LPS, Braun lipoproteins of *S. typhimurium* induce septic shock in the host (Tatiana et al., 2016).

**Flagellin:**

Flagellin is the main component of bacterial flagella, which represents the motility system, and has the ability to trigger the immune system. The ability of flagella to adhere to host cells is controversial (Wiedemann et al., 2014). Flagella form from three main components: the basal body, which is embedded in the bacteria lipid bilayer, the hook, and the filament. Filament is a hollow cylinder that is composed of flagellin. The number of flagella varies between bacterial cells and ranges from 5 to 10 flagella. TLR5 is the receptor that recognizes flagellin. The activation of TLR5 activates three transcription factors (NF-κB, CREB, and AP1), which leads to an increase in production of pro-inflammatory cytokines.
Figure 3: Cell wall structure of *Salmonella typhimurium*. *S. typhimurium* is a gram-negative bacteria and has three layers in the membrane (outer membrane, peptidoglycan, and cytoplasmic membrane). The outer membrane has LPS and is part of lipoprotein. The rest of the lipoprotein is connected to peptidoglycan. Flagellin is the main component of flagella (Modified from Daniel et al., 2014 and Lolis et al., 2003).
Materials and Methods

Cell line:

RAW264.7 murine macrophage cells (from American Type Culture Collection ATCC, Manassas, VA) collected from tumors of Abelson murine leukemia virus-induced tumors in adult male BALB/c mice. RAW 264.7 murine macrophages were cultured on a vented BioLite flask (Fisher Scientific, Pittsburgh, PA). The cells were split 4 to 6 times weekly and were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; HyClone), 10% Fetal Bovine Serum (FBS) (heat-sensitive) (purchased from Fisher Scientific), 10 mM HEPES buffer, and 1% of penicillin-streptomycin antibiotic (purchased from MP Biomedical, LLC). The cells were grown in 100% humidified air with 5% carbon dioxide in a 37° C incubator. The cells were split based on the confluency (60 to 57% confluency). Looking at the cells under the microscope helps in the estimation of confluence. Cells have the ability to adhere on the flask surface, so cell scrapers (Fisher Scientific, Pittsburgh, PA) were used to remove cells from the flask.

Selection of Time Frame:

Dr. Boehringer and his colleagues found that the level of pro-inflammatory and anti-inflammatory cytokine were high at 24 hours. Additionally, he indicated that the anti-inflammatory cytokines (IL-10) affect on the levels of pro-inflammatory cytokines (TNF-α) (Boehringer et al., 1999).
Cell Viability:

Cell viability has been used to split the cells into different passages, to apply a certain number of cells in each experiment, and to count the cells after 24 hours of treatment. Cell viability technique starts with scraping cells, then centrifuging the whole media and cells to form a pallet. Then, supernatant were collected to measure the levels of cytokines. The pellet is re-suspended in one milliliter of 10% FBS in DMEM. The cells are mixed using a micropipette. Then, a small amount of cell suspension is mixed with trypan blue (Fisher Scientific, Pittsburgh, PA) in a ratio 1:2 respectively. In order to count the cells, 10µl is taken from the mixture after mixing and placed onto the hemocytometer. Then, the cells are placed under the microscope at 10x magnification power. Dead cells take up [the dye and are stained blue. On the other hand, live cells are not stained with the dye (trypan blue) and appear colorless.

**Figure 4:** Trypan blue exclusion test (hemocytometer method). Three different colors of cells appear on the figure. Blue cells represent the counted dead cells that are stained with the Trypan blue dye. Colorless cells represent the counted live cells (do not stain). Red colored cells represent uncounted cells since they are outside the borders on two sides.
Table 1: Cell viability and cell counting equations. The table shows equations to find the percentage of viable cells and the counting of viable cells.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Cell Viability</td>
<td>[Viable cells/ Total cells (live and dead cells)] x 100</td>
</tr>
<tr>
<td>Counting of viable cells in ml</td>
<td>Average number of viable cells x Dilution Factor x 10000</td>
</tr>
</tbody>
</table>

Cytokine Measurement:

Cytokines were collected after 24 hours of treatment, stored at -80°C and used to measure the level of cytokines using Luminex Multiplex Immunoassays (R&D Systems). Multiplex Immunoassays (MIAs) have a high specificity and sensitivity technique that is able to detect and measure several cytokines in one sample. Thus, this technique is much faster than ELISA, which also has a high specificity and sensitivity (de Jager et al., 2009). The cytokine measurement was done in cooperation with Dr. David Cool’s laboratory at Wright State University.
Cell treatment:

Heat-killed *Salmonella enterica serovar typhimurium* (HKST) was purchased to be used to activate TLR2 and TLR4 based on the concentration. Flagellin from *Salmonella typhimurium* was purchased to activate TLR5. Synthetic di-acylated lipoprotein (Pam2CSK4) was purchased to activate TLR2/TLR6. Synthetic lipid A (MPLA) was purchased to activate TLR4. All treatments were purchased from InvivoGen (San Diego, CA). Additionally, all experiments were done in 24 hours. Concentrations used in the experiments are shown in Table 2.

**Table 2: Treatments used in experiments.** The table shows each treatment with its pattern recognition receptor (PRR), the concentrations used in the experiments, and the company that treatments were purchased from.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PRR</th>
<th>Concentrations</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Unstimulated</td>
<td>No treatment</td>
<td>No company</td>
</tr>
<tr>
<td>HKST</td>
<td>TLR2</td>
<td>$10^4$ Cell/ml</td>
<td>InvivoGen</td>
</tr>
<tr>
<td>HKST</td>
<td>TLR4</td>
<td>$10^6$ Cell/ml</td>
<td>InvivoGen</td>
</tr>
<tr>
<td>Flagellin from <em>S. typhimurium</em></td>
<td>TLR5</td>
<td>1, 5, and 10 µg/ml</td>
<td>InvivoGen</td>
</tr>
<tr>
<td>Synthetic di-acylated lipoprotein</td>
<td>TLR2/TLR6</td>
<td>1, 5, and 10ng/ml</td>
<td>InvivoGen</td>
</tr>
<tr>
<td>Synthetic lipid A</td>
<td>TLR4</td>
<td>1, 5, and 10 µg/ml</td>
<td>InvivoGen</td>
</tr>
</tbody>
</table>
**Immunofluorescence Staining:**

RAW264.7 murine macrophage cells were seeded in 12-well chamber slides (Ibidi USA, Fitchburg, WI). Approximately 6000 cells were seeded in each well, and the maximum volume for each well in the chamber was 250µl. After attaching the cells in the chamber, the treatment was added to each well and was based on the concentrations in Table 2. After adding the treatments, the chamber was incubated for 24 hours in a humidified condition with 5% Carbon Dioxide at 37°C. After 24 hours, treatments were removed, and wells were washed gently three times with chilled Phosphate Buffer Saline (PBS). 100µl of 4% Paraformaldehyde for fixation was added after washing. The fixation step lasted for 15 minutes at room temperature before washing the cells again with PBS. Then, 100µl of 0.2% Triton as permeabilizer were added to the cells, and I waited for ten minutes before washing. Note that the permeabilization step depends on the target of the fluorescent antibody. If the target is inside the cells, the permeabilization step is recommended. However, if the target is outside the cells, permeabilization step is not required. After washing the wells three times, 150µl of blocking buffer were added. The blocking step lasts for 30 minutes. The washing step after removing the blocking buffer was done three times with 1% Bovine Serum Albumin (BSA).

The α/β-tubulin antibody (primary antibody) was added to the wells. The α/β-tubulin antibody was left overnight at -40°C in the refrigerator. The α/β-tubulin antibody was then removed, and cells were washed three times with 1% BSA. Next, the fluorescent antibody (secondary antibody) for the α/β-tubulin antibody was added and left for 60 minutes at room temperature. Note that fluorescent antibodies are light sensitive, and fluorescent probes can be
affected by the bulb’s light. So, all fluorescent steps have to be done in a dark place. Then, removing the secondary antibody and washing with 1% BSA was applied. After the washing step, Texas red-phalloidin was applied to the cells and left for 60 minutes. The washing with 1% BSA was applied after removing phalloidin. The silicon that holds the samples was removed, and the slide was left to dry. Next, the mounting agent was applied on the wells, and the cover slip covered the cells. The cells were imaged on the ACCU-SCOPE EXC-350 microscope with scopeLED fluorescence illuminator at 40x magnification.

Immunofluorescence staining for CD11b, a macrophage marker, followed the same technique in order to determine the classification of macrophages.

**Table3: Antibodies used in experiments.** The table shows antibodies used in immunofluorescence experiments.

<table>
<thead>
<tr>
<th>Detection</th>
<th>Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>α/β-tubulin (Primary Tubulin Antibody)</td>
<td>1:50</td>
<td>Cell Signaling Technology, Danvers, MA</td>
</tr>
<tr>
<td>Anti-Rabbit IgG (H+L), F (ab’) 2 Fragment (Alexa Fluor 488 conjugate)(Secondary Tubulin antibody)</td>
<td>1:250</td>
<td>Cell Signaling Technology, Danvers, MA</td>
</tr>
<tr>
<td>Texas Red-Phalloidin</td>
<td>1:40</td>
<td>Cell Signaling Technology, Danvers, MA</td>
</tr>
<tr>
<td>Anti- CD11B [M1/70]</td>
<td>5μg/ml</td>
<td>Abcam, San Francisco, CA</td>
</tr>
</tbody>
</table>
Selecting Fields using fluorescence microscope

In order to take images for the immunofluorescence, the field has to be specified for all wells. This step will minimize the bias in the results.

Figure 5: selected fields in each well.

Image Analysis:

The analysis of the images of the Immunofluorescence cells after exposure was done using ImageJ software (National Institutes of Health, http://imagej.nih.gov/ij/). In order to quantify the region of interest (qROI), several steps were followed. First, the colored image was opened using ImageJ through File → Open → select the image. Second, the picture was converted to black and white by going to Image → type → 8-bit. The next step was subtracting the background, which is an essential step for removing the pixel information from a continuous background. Subtracting the background can be done through Image → Process → Subtract background. After subtracting the background, the isolation of the threshold is done. Isolating the threshold is an important step to identify and control the exclusion region, which contains a black background (range from zero to 20 pixels on histogram) and bright-saturated artifacts.
(range from 111 to 255 pixels on histogram). In order to isolate the threshold, open the image ➔ adjust ➔ threshold. The final step is analyzing the image. The program quantifies region of intensity (qROI) by counting the cells and measuring the intensity in the each image. Then, the software divides the total area of intensity on the number of cells to find the average size of the area in the image. Analyzing the image is done after subtracting the threshold through image ➔ analyze ➔ analyze particles. Skipping one of the previous steps leads to unspecific data. After collecting data, statistical analysis of the images was done using SigmaPlot software.

**Figure 6: Image analysis.** Steps of processing images using ImageJ.
Statistical Analysis:

All experiments were done at least three times. Then, data were collected to study each experiment’s results (Immunofluorescence staining test for β tubulin and F-actin, cell viability test, and cytokine analysis test). By using SigmaPlot 12.0 software, statistically significant results were calculated using One-Way ANOVA.
RESULTS

Cell viability of RAW264.7 murine macrophages exposed to bacterial and synthetic components after 24 hours.

TLR4 agonist:
RAW 264.7 murine macrophages showed significantly lower percentages of viability after exposure to heat killed *S. typhimurium* (10^6 bacterial cell/ml), which activates TLR4 receptors (figure 6A). On the other hand, synthetic TLR4 agonists (synthetic lipid A) exhibited non-significant decreases in the viability compared to the control, except with 10 μg/ml concentrations as shown in figure 6B.

TLR2 agonist:
In this group, RAW246.7 cells show a lowering in the cell viability with all groups, which are *S. typhimurium* (10^4 bacterial cells/ml) and different concentrations (1, 5 and 10 ng/ml) of di-acylated lipoprotein; however, none of the groups express lowering at significant levels in compared to the control.

TLR5 agonist:
In TLR5 agonist group, RAW 256.7 macrophage cells exhibit gradual decreases in the level of cell viability with different concentrations of flagellin (1, 5 and 10 μg/ml). A 10 μg/ml concentration of flagellin showed significant lowering in the level of cell viability in comparison to the control and to the flagellin at 1 μg/ml of concentration.
Fluorescence intensity and morphological changes of RAW264.7 murine macrophages exposed to bacterial and synthetic components after 24 hours.

Macrophages (M0) served as a control in all experiments. Macrophages were treated with several treatments, such as *S. typhimurium* (10^6 and 10^4 cell/ml) and flagellin (1, 5 and 10μg/ml). Treatments that activate TLR4 receptors (10^6 S. typhimurium/ml and synthetic lipid A at different concentrations) activate M0 un-polarized macrophages and convert them to M1 polarized macrophages that demonstrate flattened and elongated cells, as shown in figure 9A.

**Microtubules (α/β Tubulin)**

In figure 9B, synthetic lipid A (5 and 10 μg/ml) exhibited significantly higher levels of fluorescence intensity than *S. typhimurium* (10^6 cell/ml). Moreover, 10 μg/ml of synthetic lipid A showed a significantly higher level of intensity than the control (M0). In the case of TLR2 agonists, *S. typhimurium* (10^4 cell/ml) exposure led to significantly higher levels of fluorescence intensity than synthetic di-acylated lipoprotein at all concentrations (1, 5, and 10 ng/ml), as shown in figure 10B. Flagellin at all concentrations (1, 5, and 10 μg/ml) led to higher levels of fluorescence intensity than the control. Only the highest level of flagellin (10 μg/ml) induced significantly higher levels of intensity, as shown in figure 11B.

**Microfilaments (F- actin)**

As shown in figures 12B and 14B, all TLR4 and TLR5 agonists induced significantly higher levels of fluorescence intensity than the control. Similarly, TLR2 agonists induced higher levels of fluorescence intensity than the control (M0); however, it was not at significant levels, as shown in figure 13B.
*S. typhimurium* surface components, synthetic di-acylated lipoprotein, and synthetic lipid A cause changes in macrophage’s cytokine production after 24 hours.

**IL-1β**

There was a gradual increase in IL-1β production in all treatments with increase the concentration of treatments. However, there was not any significant result as shown in figure 15.

**IL-6 and TNF-α**

*S. typhimurium* at high concentration (10⁶ cells/ml) increased significantly compared to all different groups, as shown in figure 16 and figure 18.

**IL-10**

Figure 17 demonstrated that *S. typhimurium* at high concentration (10⁶ cells/ml) increased significantly compared to all different groups, except synthetic di-acylated lipoprotein at 5 and 1 ng/ml.
I- Cell Viability

A - TLR4 agonists

A

![Graph showing cell viability](image)

B

![Graph showing cell viability](image)
Figure 7: Cell viability of RAW264.7 murine macrophages after 24 hours of exposing to TLR4 agonist components: Cell viability of RAW264.7 cells exposed to; A) heat killed *S. typhimurium* (10^6 cells/ml), B) Synthetic TLR4 agonist, and C) merge data from figure 6A and 6B. Each bar represented by the mean value ± standard error (SE) of three separate experiments.
B - TLR2 agonists

A

Percentage of Viable Cells

Control

Bacterial TLR2 agonist

B

Percentage of Viable Cells

Control

Synthetic TLR2/6 agonist 1 ng/ml

Synthetic TLR2/6 agonist 5 ng/ml

Synthetic TLR2/6 agonist 10 ng/ml
Figure 8: Cell viability of RAW264.7 murine macrophages after 24 hours of exposing to TLR2 agonist components: Cell viability of RAW264.7 cells exposed to; A) heat killed *S. typhimurium* (10^4 cells/ml), B) Synthetic TLR2/6 agonist, and C) merge data from figure 7A and 7B. Each bar represented by the mean value ± standard error (SE) of three separate experiments.
Figure 9: Cell viability of RAW264.7 murine macrophages after 24 hours of exposing to TLR5 agonist component: Cell viability of RAW264.7 cells exposed to flagellin from *S. typhimurium*. Each bar represented by the mean value ± standard error (SE) of three separate experiments.
II- Cytoskeleton

A- $\alpha/\beta$ Tubulin

1- TLR4 agonist

A

Control

Bacterial TLR4 agonist

Synthetic TLR4 agonist 10µg/ml

Synthetic TLR4 agonist 5µg/ml

Synthetic TLR4 agonist 1µg/ml
Figure 10: Immunofluorescence images using α/β tubulin antibody and quantification of tubulin reorganization of both control cells and cells treated with TLR4 agonists. A) Immunofluorescence images of un-polarized macrophages (Control) and polarized macrophages (RAW264.7 macrophages exposed to heat killed *S. typhimurium* and synthetic TLR4 agonist at different concentrations (1, 5 and 10 µg/ml)) after 24 hours using α/β tubulin antibody. B) Quantification of the tubulin reorganization in each group. In the bar graph, each value represents the mean ± standard error (SE) of three separate experiments. (Scale bar =20 µm)
2- TLR2 agonist

A

Control  Bacterial TLR2 agonist

Synthetic TLR2/6 agonist 10ng/ml  Synthetic TLR2/6 agonist 5ng/ml

Synthetic TLR2/6 agonist 1ng/ml
Figure 11: Immunofluorescence images using α/β tubulin antibody and quantification of tubulin reorganization of both control cells and cells treated with TLR2 agonists. A) Immunofluorescence images of M0 (control) and RAW264.7 macrophages after 24 hours of exposing to TLR2 agonist of heat killed *S. typhimurium* and TLR2/6 agonist of gram-positive bacteria at different concentrations (1, 5 and 10 ng/ml) using α/β tubulin antibody. B) Quantification of the tubulin reorganization in each group. In the bar graph, each value represents the mean ± standard error (SE) of three separate experiments. (Scale bar =20 µm)
2- TLR5 agonist

A

Control

Fla. 10µg/ml

Fla. 5µg/ml

Fla. 1µg/ml
Figure 12: Immunofluorescence images using α/β tubulin antibody and quantification of tubulin reorganization of both control cells and cells treated with TLR5 agonist. A) Immunofluorescence images of M0 (control) and RAW264.7 macrophages after 24 hours of exposing to flagellin from *S. typhimurium* at different concentrations (1, 5 and 10 µg/ml) using α/β tubulin antibody. B) Quantification of the tubulin reorganization in each group. In the bar graph, each value represents the mean ± standard error (SE) of three separate experiments. (Scale bar =20 µm)
B- F-actin

1- TLR4 agonist

A

Control

Bacterial TLR4 agonist

Synthetic TLR4 agonist 10µg/ml

Synthetic TLR4 agonist 5µg/ml

Synthetic TLR4 agonist 1µg/ml
Figure 13: Immunofluorescence images using F actin antibody and quantification of microfilaments reorganization of both control cells and cells treated with TLR4 agonist. A) Immunofluorescence images of un-polarized macrophages (Control) and polarized macrophages (RAW264.7 macrophages exposed to heat killed S. typhimurium and synthetic TLR4 agonist at different concentrations (1, 5 and 10 μg/ml) after 24 hours using F actin antibody. B) Quantification of microfilaments reorganization in each group. In the bar graph, each value represents the mean ± standard error (SE) of three separate experiments. (Scale bar =20 μm)
2- TLR2 agonist

Control

Bacterial TLR2 agonist

Synthetic TLR2/6 agonist 10ng/ml

Synthetic TLR2/6 agonist 5ng/ml

Synthetic TLR2/6 agonist 1ng/ml
Figure 14: Immunofluorescence images using F-actin antibody and quantification of microfilaments reorganization of both control cells and cells treated with TLR2 agonist. A) Immunofluorescence images of M0 (control) and RAW264.7 macrophages after 24 hours of exposing to TLR2 agonist of heat killed *S. typhimurium* and TLR2/6 agonist of gram-positive bacteria at different concentrations (1, 5 and 10 ng/ml) using F actin antibody. B) Quantification of microfilaments reorganization in each group. In the bar graph, each value represents the mean ± standard error (SE) of three separate experiments. (Scale bar =20 µm)
A

3- TLR5 agonist
Figure 15: Immunofluorescence images using F-actin antibody and quantification of microfilaments reorganization of both control cells and cells treated with TLR5 agonist. A) Immunofluorescence images of M0 (control) and RAW264.7 macrophages after 24 hours of exposing to flagellin from *S. typhimurium* at different concentrations (1, 5 and 10 µg/ml) using F-actin antibody. B) Quantification of microfilaments reorganization in each group. In the bar graph, each value represents the mean ± standard error (SE) of three separate experiments. (Scale bar =20 µm)
Figure 16: Levels of IL-1β. Bar graph shows the levels of IL-1β secreted from RAW264.7 macrophages after exposure to S. typhimurium components, synthetic lipid A, and di-acylated lipoproteins.
Figure 17: Levels of IL-6. Bar graph shows the levels of IL-6 secreted from RAW264.7 macrophages after exposure to *S. typhimurium* components, synthetic lipid A, and di-acylated lipoproteins.
Figure 18: Levels of IL-10. Bar graph shows the levels of IL-10 secreted from RAW264.7 macrophages after exposure to *S. typhimurium* components, synthetic lipid A, and di-acylated lipoproteins.
**Figure 19: Levels of TNF-α.** Bar graph shows the levels of TNF-α secreted from RAW264.7 macrophages after exposure to *S. typhimurium* components, synthetic lipid A, and di-acylated lipoproteins.
Figure 20:  **Cell type conformation test.** Macrophage cells stained positively with CD11b, which is a macrophage marker. (Scale bar =20 µm)
**Figure 21: Flattened and elongated M1 macrophages:** RAW 264.7 macrophages show morphological changes after 24 hours of exposing to TLR4 agonists. (Scale bar =20 µm)
DISCUSSION

In this study, RAW 264.7 murine macrophages were utilized to analyze the effect of invasive pathogen components: lipid A, flagellin, diacylated lipoprotein, and heat-killed Salmonella typhimurium (10^4 cell/ml and 10^6 cell/ml) were studied. None of these treatments caused dramatic changes on cell morphology, except TLR4 agonists (10^6 S. typhimurium/ml and lipid A). TLR4 agonists stimulate macrophages to form a M1 polarized phenotype. This form looks flattened and elongated, as shown in figure 21.

In addition to the morphology, TLR4 agonists exerted the highest level of microtubules (α/β-tubulin) intensity compared to TLR2 agonists and TLR5 agonists because of the polarization of macrophages to M1 phenotype. S. typhimurium showed significant decrease in the level of microtubules intensity than synthetic lipid A. This could be a result from the non-specific number of LPS on the S. typhimurium. Similarly, S.typhimurium exhibited a higher level of tubulin intensity than synthetic di-acylated lipoprotein. TLR5 showed a significant result compared to control (M0). This could result from impurity (endotoxin) of the sample, as mentioned in the product sheet. The results of fluorescence intensity of RAW264.7 macrophages after activation of TLR5 in this study are different than the study performed by Ali Alshehri, my lab mate. Alshehri’s TLR5 agonist is expected to be highly purified flagellin and the flagellin used in this study, which has an endotoxin, thus, less purity.

The highest levels of fluorescence intensity in microfilaments (F-actin) appeared to be with all TLR4 and TLR5 treatments. This is probably due to the stimulation of TLR4 receptors, which promote the migration of macrophage cells. The migration of cells that is stimulated with TLR4 agonists is done through microfilaments, which increased significantly after treatment.
with LPS (Lee et al., 2016). Additionally, the intensity of microfilament results from the change of distribution of actin from soluble actin to filamentous form, and the organization of microtubules allows redistribution of actin towards the leading edge.

A recent study has shown that level of Nitric Oxide (NO) plays an important role in cell viability (Kiemer et al., 2002). Dr. Kiemer and his colleagues show the inhibition of NO levels lead to significant increase in cell viability compared to LPS treated macrophages. Additionally, LPS treated cells induce the production of NO in RAW 264.7 macrophages (Reis et al., 2011). So, in this study, reactive oxygen species (ROS) and TNF-α contribute in macrophages’ death and explain the significant decrease in cell viability (figure 7C) (Schachtele et al., 2010). Results of cell viability show what is expected; however, using other techniques such as cytotoxicity and flow-cytometry will increase the accuracy of the results.

Similarly, the significant levels of IL-6 showed to be with the same group (10^6 S. typhimurium). IL-6 was significantly higher than all different treatments. IL-6 is one of the pro-inflammatory cytokines that is secreted after inflammation. IL-6 has both activities (pro-inflammatory and anti-inflammatory properties). The anti-inflammatory response is important in recovery and wound healing (Fernando et al., 2014). The increase in levels of IL-6 is similar to the results of a recent study with other gram-negative bacteria (Waters et al., 2013).

The significant increase in the secretion of IL-10 after exposing macrophages to S. typhimurium (10^6 cell/ml) and di-acylated lipoprotein (5μ g/ml) is the induction stage to the formation of the M2 macrophages (Reichard et al., 2015). The high level of IL-10 in TLR2 and TLR4 was indicated in other articles (Bzowska et al., 2012; Waters et al., 2013). This phenotype
promotes tissue remodeling and angiogenesis though secretion of anti-inflammatory cytokine (IL-10) (Kigerl et al., 2009) as shown in figure 1.
FUTURE STUDIES

In future studies, it would be helpful to study the NO levels in macrophage cells stimulated by TLR5, TLR4 and TLR2 agonists and its direct relationship with cell viability. It is expected to have high levels of NO in cells treated with LPS compared to other treated groups. Additionally, future research will use a Nitric Oxide inhibitor, such as α-lipoic acid (Kiemer et al., 2002), in different concentrations in infected mice, in order to find the appropriate concentration to remove pathogens from a host body. Researchers will utilize different cell lines, other than RAW 264.7 murine macrophages, such as J774A.1 macrophages and U937, and compare the results with results in the study.

Furthermore, previous a study shows the levels of pro-inflammatory cytokines (TNF-α) increase early, while anti-inflammatory cytokines (IL-10) increase later (Reynoso et al., 2017). Both peaked at 24 hours, so it will be helpful to study the levels of cytokines at 48 and 72 hours. It is expected that the levels of IL-10 will continue to increase and the levels of TNF-α will decrease sharply. Using whole bacteria mimicked the interaction between bacteria and macrophages in vivo. Whole bacteria were used to find the differences between TLR2 agonists. For future research, it will be useful to use extracted bacterial surface components to examine the differences between TLR2/1 and TLR2/6 in cell viability, cell morphology, fluorescence intensity, and cytokine production. In addition to studying the TLR2 agonists, studying the differences between synthetic (synthetic lipid A) and biological (LPS) is of interest.
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