Exploration of Co-Toll-Like Receptor Signaling Cascades

Honors Thesis

Presented to the Department of Biology

Of Lafayette College

In Partial Fulfillment of Requirements

For Honors in the Degree of Bachelor of Science

Author: Kimberly Mae Manalang

Research Advisor: Dr. Robert A. Kurt

Thesis Committee: Dr. Michael Butler and Dr. Khadijah A. Mitchell

Fall 2020 - Spring 2021

BIOGRAPHICAL SKETCH

Kimberly's scientific journey began with the Howard Hugh Medical Institute (HHMI) funded Science Education Alliance-Phage Hunters Advancing Genomics and Evolutionary Science (SEA-Phages) Program. Her first semester of college was filled with class wide contaminations, learning several new techniques, and isolating her very own bacteriophage – Corazon. The following semester, she was honored to be one of two chosen to have her phage selected to be sequenced by the University of Pittsburgh. In the spring, she was able to annotate her phages whole genome and presented her findings at the annual SEA-Phages Symposium hosted by HHMI in the summer of 2018. That same summer, she participated in full time research at Weill Cornell Medical College under the supervision of a former Lafayette Alum, Dr.Barry Sleckman and his Co-PI, Dr.Jessica Tyler. She worked closely with a current MD/PhD student and it was this summer that confirmed her interest in research. She followed that summer by working in Professor Caslake Bio-Civil Engineering lab for a year before she switched to working in Professor Kurt's Lab with a heavy focus on Co-signaling cascades. The summer of 2019, she was able to go back to Cornell under the program "Gateways to the Laboratory" through the Tri-I MD/PhD summer program. There, she worked in a nanotechnology cancer lab at the Memorial Sloan Kettering cancer institute under Dr.Daniel Heller. This furthered her interest in research and pursing an MD/PhD. Kimberly wanted to further challenge herself and take on an Honors Thesis. Post-graduation, she will hone and refine her scientific skills in the south at the University of Birmingham Alabama (UAB). There, Kimberly will be working in the O'Neal Comprehensive Cancer Center under the supervision of Dr.Barry Sleckman to focus on DNA Damage and Repair mechanisms connection to cancer. To this, while it will be a long journey to become a Physician Scientist, Lafayette has taught Kimberly to ask herself "Cur non?"

Kimberly is **C**reative **U**niversal **R**esilient **N**imble **O**ptimistic **N**ifty

Table Of Contents

ABSTRACT

A hallmark discovery of Pattern Recognition Receptors (PRRs) in the field of immunology led to an understanding of how the immune system recognizes the variety of pathogens humans encounter on a daily basis. This recognition of pathogens is made possible by innate immune cells that provide a first line of defense that non-specifically, yet rapidly clear infections. Innate immune cells are able to recognize infections via a variety of Toll Like Receptors (TLRs), a type of PRR, that each recognize distinct pathogen-associated molecular patterns (PAMPs) that are unique to either bacteria, viruses, or parasites. Upon PAMP recognition, TLRs initiate a signaling cascade that results in activation of nuclear factor kappa B (NF-κB) and interferon regulatory factor (IRF), two master regulators required for further priming of the immune response. Despite the plethora of research behind TLR signaling cascades, it is unclear how the immune system responds to simultaneously activated TLRs as studies have shown both competitive and synergistic effects of co-signaling. Here, we investigated how human monocytes respond to treatment with TLR4 and TLR5 agonists as well as TLR4 and TLR7 agonists, alone and in combination. We found a competitive effect when cells were treated with both agonists at their highest concentration (5 ug/ml) over 72 hours. However, we also found a synergistic effect in IRF activation at the 24 hour mark, peaking at 0.05 ug/ml LPS and 5 ug/ml Flagellin which steadily declined as the concentration of the agonists increased. Our results demonstrate the diverse reactions the innate immune response is capable of creating with both time and dose dependent effects. We anticipate further exploration of co-toll like receptor signaling cascades can shed additional light on the complexity of the immune response to co-infections.

INTRODUCTION

The Complexity of Co-Infections

The immune system plays a large role in how viral and bacterial pathogens are recognized and dealt with. A pertinent feature of the immune system is that it is able to fight off a viral or bacterial infection in multiple ways in an attempt to eliminate it in a timely manner. When the innate immune response (the first line of the human defense system) is activated, cell signaling triggers activation of transcriptional factors that initiate release of inflammatory and antiviral factors to reduce bacterial and viral loads. However, how the immune system deals with co-infections is not as straightforward. For example, mice co-infected with influenza and *Legionella pneumophila* showed a higher inflammatory response when compared to mice infected with just one of these pathogens (Jamieson et.al., 2013) suggesting a synergistic or enhanced inflammatory response. Interestingly, the mice that were co-infected with both pathogens had greater mortality compared to mice infected with just one pathogen (Jamieson et al., 2013) suggesting that the enhanced immune response was not beneficial to the mice. COVID-19 is another example since bacterial co-infections increase the mortality rate from COVID-19 (Sharifipour et al., 2020), and COVID-19 patients who acquired secondary bacterial infections (e.g. bacterial pneumonia) have a more severe outcome (Feng et al., 2020). Thus, a comprehensive understanding of how single or multiple pathogens influence the inflammatory and antiviral response is needed.

Overview of TLRs

The immune system is capable of initiating two types of responses, innate and adaptive immunity. While the adaptive immune response is slow-acting and generates immunological memory, the innate immune response allows for a non-specific, yet rapid response to pathogens that enter the body (Parker et al., 2007). Once a pathogen enters the body, pattern recognition

receptors (PRRs) that are expressed on the surface of innate immune cells – such as macrophages, dendritic cells and neutrophils– can then recognize the foreign invader (Liu et.al., 2017). Toll-like Receptors (TLRs) are an important group of PRRs that are able to detect invading bacteria and viruses (T. Kawai & Akira, 2006; Mogensen, 2009). Specifically, TLRs recognize pathogen-associated molecular patterns (PAMPs) associated with bacteria and viruses (Okamoto et al., 2017). Overall, TLRs recognize components of bacterium and viruses that are needed for their own survival.

TLRs contain extracellular leucine-rich repeats that recognize PAMPs as well as transmembrane and cytoplasmic Toll/interleukin-1 receptor (TIR) domains (Akira & Takeda, 2004). TIR domains are required to trigger intracellular signaling (T. Kawai & Akira, 2006). The recruitment of TIR-domain-containing adaptors to TIR domains results in the initiation of TLR signaling (O'Neill & Bowie, 2007). As illustrated in figure 1, the end result of the TLR signaling cascades is to trigger the activation of nuclear factor kappa B (NF-κB) and interferon regulatory factor (IRF) which dictates the outcome of the innate immune response (Liu et al., 2017; Yanai et al., 2012). For example, the activation of NF-κB initiates the transcription of genes encoding pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α which can then start an inflammatory response, important for fighting bacterial infections (Liu et al., 2017; Zhang et al., 2017). The activation of IRF initiates the transcription of type I interferon (type I IFN) critical for fighting viral infections (Jefferies, 2019).

There are 5 known TIR-domain-containing adaptors required for the TLR signaling cascades : myeloid differentiation primary responses 88 (MyD88), MyD88-adaptor like protein (MAL/TIRAP), TIR domain containing adaptor protein inducing interferon-β (TRIF), TRIF-related adaptor molecule (TRAM), and sterile α- and armadillo-motif-containing protein (SARM)

(O'Neill & Bowie, 2007). The MyD88, MAL/TIRAP, TRAM, and TRIF adaptors promote TLR signaling cascades while SARM is used to regulate TLR3 and TLR4 by inhibiting TRIF-dependent signaling (O'Neill & Bowie, 2007). Notably, MyD88 is labeled as the universal adaptor with it being required for each TLR pathway except TLR3 (O'Neill & Bowie, 2007). MyD88 induces the activation of transcription factors such as NF-κB and IRF from several receptors either located at the plasma membrane or in endosomes (Deguine & Barton, 2014; O'Neill & Bowie, 2007). TRIF is used in TLR3 and TLR4 signaling to activate IRF transcription factors such as type I IFNs (McNab et al., 2015). In contrast, MAL/TIRAP and TRAM are recruitment adaptors; MAL/TIRAP is essential for the recruitment of MyD88 to TLR2 and TLR4 while TRAM is used only by TLR4 to recruit TRIF (O'Neill & Bowie, 2007).

In total, there are eleven known human TLRs (TLR1-TLR11) that use the TIR-domain-containing adaptors with TLR11 being non-functional (Lee et al., 2013). Different types of TLRs trigger different signaling cascades because fighting different pathogens requires different immune responses. For example, TLR4 and TLR5 can elicit an anti-bacterial response when they bind to PAMPs on bacteria (Figure 2). TLR4 is activated by binding to lipopolysaccharides (LPS) found on gram-negative bacteria such as *Escherichia coli (E. Coli)*, while TLR5 can be stimulated by a variety of bacteria binding to Flagellin (Park & Lee, 2013; Steiner, 2007). In contrast, TLR7 elicits an anti-viral response because it binds to single stranded RNA (ssRNA) present in many viruses (Petes et al., 2017). These advancements in unraveling the detailed signaling cascades for each TLR allowed for a better understanding of how the innate immune response is initiated when a pathogen enters the body

The Advantages and Disadvantages of Studying Single TLR Cascades

The understanding of each TLR has allowed for a better comprehension of how the immune system functions. In-depth knowledge of single TLR cascades allow the potential to manipulate pathways by inhibiting certain TLRs to reduce the risk for inflammatory diseases that cause overstimulation of the immune response (Gao et al., 2017). To further emphasize the impact of studying single TLR cascades, studies like Saito et al., (2016) shed light on the difference in immune response between the same TLRs in different species specifically humans and a model organism (e.g. mice). A proteomic study conducted by Koppenol-Raab et al. (2017) revealed a difference in signaling when comparing the single TLR cascades - TLR4, 7, and 2. The researchers observed that the activation of TLR4 was a more distinct response compared to TLR2 and TLR7, which elicited similar responses. This could be attributed to TLR4 having two adaptor pathways, MyD88 and TRIF, whereas TLR2 and TLR7 signaling only use the MyD88 pathway (Koppenol-Raab et.al., 2017). Similarly, Kaji et al. (2018) studied the activation of the single TLR cascades – TLR2, 3, 4, 5, 7, 9. Out of the 6 TLRs investigated only TLR3 and TLR5 enhanced IL-12 production. TLR signaling was also studied by Razonable et al. (2005), who found the single TLR cascades – TLR1 and 2 – were capable of inducing cellular activation. Sjoelund et al. (2014) also looked at TLR signaling comparing the outcome of TLR4, 2, and 7 signaling. The study found differences in activation between these TLRs, specifically, TLR4 and TLR2 signaling led to phosphorylation of proteins involved in phagocytosis but TLR7 activation did not elicit the same response. Similar to the Sjoelund et.al. (2014) study, Bösl et al. (2018) also observed a phosphorylation difference specifically a reduction in phosphorylation levels effecting MAPK signaling pathways. However, Bösl et.al. (2018) used different TLRs (TLR 2 and TLR 8) and also observed differences when investigating single versus multiple TLR stimulation. They detected that upon the co-stimulation of TLR2 and TLR8, there was a reduction in MAPK signaling activity

implying a competitive effect on the immune system at the cellular level (Bösl et.al. 2018). These data underscore two important points. First, most of what we know about TLR signaling comes from investigating single TLR signaling cascades, and second, the outcome of multiple TLR signaling cascades can differ from the outcome of single TLR signaling cascades. The information from single TLR studies, while useful, may not be representative of what really happens *in vivo* since innate immune responses are not elicited by a single TLR signaling cascade.

Studying multiple TLR signaling cascades may yield more useful information since in a normal situation multiple TLRs can be activated at the same time by a single bacteria that contains multiple PAMPs (Taro Kawai & Akira, 2010). For instance, *E.coli* has both LPS and Flagellin and *S.aureus* has peptidoglycan and lipoteichoic acids, so *E.coli* can initiate TLR4 and TLR5 signaling (Figure 1) and *S.aureus* can initiate TLR2 and TLR6 signaling (Parker et al., 2007). Likewise, a patient may be infected with more than one pathogen at a time, such as a patient with a viral respiratory infection who subsequently acquires bacterial pneumonia. In this case the viral genome would elicit TLR7 signaling while the bacteria could trigger TLR4 and/or TLR5 signaling; among others. As a result, in order to understand the innate immune response that would be elicited one would need to study what happens when multiple TLR are activated at the same time.

The Synergistic and Competitive Outcomes From Multiple TLR Cascades

The importance of studying multiple TLR signaling cascades is evident from a study conducted by Zhu et al. (2011). They proposed a comprehensive "tide" model in which a pathogen would activate multiple TLR signaling pathways which would result in co-stimulation or co-inhibition, and therefore upregulation or downregulation of the immune response. The "tide" refers to the complexity of the immune response as it can either rise or fall due to the interplay of the signaling cascades. The merits of studying co-signaling are emphasized again by a study focused

on the gram-negative bacteria, *Burkholderia pseudomallei (B.pseudomallei)* which was shown to activate both TLR2 and TLR4 (West et al., 2008). West et.al. (2008) found that, while TLR4 initiated a strong NF-κB response when activated by *B.pseudomallei*, TLR2 was dependent on co-stimulation with either TLR1 or TLR6 to create a similar level of response. This need for synergy was also evident in a study that found co-stimulation of either TLR2, TLR4, TLR9 (e.g., TLR2 and TLR4, TLR2 and TLR9, or TLR4 and TLR9) resulted in an increased inflammatory response when compared with signaling through a single TLR receptor (Rosenberger et al., 2014). Likewise, Bagchi et al. (2007) and Underhill (2007) reported a synergistic response was evident upon TLR2 and TLR4 co-signaling, and concluded the synergistic response was due to TLR activation of both Myd88 dependent (TLR2) and Myd88 independent (TLR4) signaling pathways. Kim et al. (2018) found the combination of TLR21 and TLR4 signaling resulted in enhanced production of IL1, IL-12 and nitric oxide (NO). Additionally, Bashir et al. (2019) observed a synergistic response, upregulation of IFNs, T helper type 1 (Th1), and T helper type 2 (Th2) cytokine responses, were evident following TLR2 and TLR3 co-stimulation. Similarly, He et.al. (2012) reported a stronger Th1 mediated immune response and enhanced NO production (He et al., 2007) occurred when TLR3 and TLR21 were both activated.

While some studies have demonstrated synergistic effects on cellular activation when TLRs are co-stimulated, Bösl et.al. (2018) and Jin et al. (2011), have noted a downregulation of immune responses resulted when certain TLRs were co-stimulated. Jin et.al. (2011) observed that while combining TLR4 and TLR2/6 had a synergistic effect on IL-6 production, TLR1/2 had the opposite effect (e.g. a competitive effect) and resulted in a decrease in IL-6 transcriptional activity. Franz & Kagan (2017) focused on understanding antagonistic antimicrobial activities and proposed that the more virulent a pathogen is then the more unpredictable the immune response would be. Franz

& Kagan (2017) speculated a few reasons for competitive immune responses, two that stood out were (1) different PRRs recognize the same PAMP yet induce different responses and (2) different PRRs can induce signals that counteract each other.

TLR5 and NAIP5 are examples of PRRs that recognize the same PAMP and yet induce different results. Flagellin can be sensed by TLR5 which results in activation of inflammatory cytokines but Flagellin is also sensed by NAIP5 and NAIP6– NLR family, apoptosis inhibitory proteins– which results in pyroptosis (Kofoed & Vance, 2012). The conditions under which a cell would elicit an inflammatory response or pyroptosis is unknown (Franz & Kagan, 2017).

As for PPRs that induce counteracting responses, examples include RLRs (retinoic acid-inducible gene-I-like receptors) and cytosolic PRRs which detect viral RNA. Upon detection of viral RNA, RLRs promote an antiviral response upregulating inflammatory chemokines and IRFs. But viral RNA is also detected by a cytosolic PRR called PKR (Protein Kinase R) which downregulates protein synthesis (Franz & Kagan 2017). These data further emphasize the diversity of responses that the immune response can elicit which may not always benefit the host.

Moreover, Kovarik et.al. (2016) reviewed type I IFN responses which include pro- and anti-inflammatory responses depending on the situation which adds to the complexity of immune signaling pathways. An example of type I IFNs creating immunosuppressive effects that would be beneficial to a host would be during an infection with *Streptococcus pyogenes (S.pyogenes),* a gram-positive bacterium. In this example type I IFNs suppress the transcription of IL-1β and prevent the lethal hyperinflammation that can result from too much IL-1β (Kovarik et al., 2016). However, this type I IFN signaling-mediated inhibition of IL-1β can blunt antimicrobial defenses and be detrimental for patients trying to fight *Mycobacterium Tuberculosis (M.tuberculosis)*

(Kovarik et.al., 2016). To this point, Jamieson et.al. (2013) reported that virus-induced IFNs can interfere with the immune response needed to eliminate a bacterial infection. Interestingly, Bösl et.al. (2018) observed both synergistic and competitive responses when co-stimulating TLR2 and TLR8 because this combination resulted in an increase in a T helper type 17 (Th17) mediated immune response and a decrease in a Th1 mediated immune response. Collectively, the diversity in immune responses evident upon single versus multiple TLR signaling cascades and the fact that multiple TLR signaling cascades can lead to increased or decreased immune responses indicates a need for further investigation.

Studying a single-signaling cascade can reveal important information about a specific signaling pathway and its constituent signaling components but it does not provide a full picture of the immune response that results when multiple TLRs interact in response to a single or multiple pathogens. Therefore, to create a more biologically holistic study and build off previous research on multiple TLR signaling cascades, my thesis focused on TLR4, 5, and 7 to explore the effects of TLR co-signaling cascades on the ability to initiate an innate immune response.

HYPOTHESIS & AIMS

Given that studies have highlighted both synergistic and competitive responses when multiple TLRs are activated, this study is focused on exploring co-TLR signaling to better understand the complexities of the innate immune response. I specifically focused on three TLRs -TLR4, TLR5, and TLR7, and the aims of this study were as follows:

(1) **Analyzed co-TLR4 and TLR5 signaling**. This would represent activation of two TLR signaling cascades by a bacterial infection.

(2) **Analyzed co-TLR4 and TLR7 signaling**. This would represent activation of two TLR signaling cascades by two different types of pathogens: a bacteria and a virus.

The **hypothesis** of my study was that **activation of NF-κB and IRF will decrease** when two TLR signaling cascades are initiated at the same time**.** To address this hypothesis, I used the human monocyte cell line THP-1. A monocyte cell line was chosen for this study because monocytes are innate white blood cells and are often one of the first types of cells to encounter pathogens (Bosshart & Heinzelmann, 2016; Chiu & Bharat, 2016). Additionally, this cell line contains NF-κB and IRF reporter systems, which allowed for the activation of multiple signaling cascades to be quantified. For TLR4 signaling, cells were treated with LPS. For TLR5 signaling, cells were treated with Flagellin. And, for TLR7, cells were treated with R848, an imidazoquinoline that triggers TLR7 signaling (Colak et.al., 2014). Based on the previous studies mentioned above I expected that there would be a decrease in activation levels of NF-κB and IRF upon co-signaling of multiple TLR.

MATERIALS AND METHODS

Cell Maintenance

The THP-1 cell line was purchased from InvivoGen (San Diego, CA). Aliquots of the cell line were stored in liquid N_2. Prior to the experiments, the THP-1 cells were thawed and maintained in 25cm^2 culture flasks (Corning LifeScience, Corning, NY) with 5ml complete RPMI (cRPMI) at 37℃ with 5% CO_2 and were split three times/week. Complete RPMI (cRPMI) contains RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (VWR LifeScience Sanborn, NY), glutamine (2mM, VWR LifeScience), penicillin (1000 U/mL), streptomycin (100 ug/mL, Lonza, Walkersville, MD), 1 x nonessential amino acids (Lonza), 2-mercaptoethanol (50 uM, Lonza), and sodium pyruvate (1mM, Lonza).

TLR agonist treatment

THP-1 cells were adjusted to 5.6 x 10^5 cells/ml, and 180 ul of cells were added to a 96 well round bottom plate (Corning LifeScience, Corning, NY). The cells were then treated with 3 different concentrations of LPS from InvivoGen (0.05 ug/ml, 0.5 ug/ml, 0.5ug/ml) +/- 3 different concentrations of Flagellin from InvivoGen (0.05 ug/ml, 0.5 ug/ml, 0.5ug/ml). Another group of cells were treated with 3 different concentrations of LPS from InvivoGen (0.05 ug/ml, 0.5 ug/ml, 0.5ug/ml) +/- 3 different concentrations of R484 from InvivoGen (0.05 ug/ml, 0.5 ug/ml, 0.5ug/ml). Hank's Balanced Salt Solution (HBSS, VWR LifeScience) was used as a control and to ensure each cell had 20 ul of respective agonists. The cells were incubated with the agonists at 37℃ with 5% CO_2 up to 72 hours before analysis. A separate 96 well round bottom round plate (Corning LifeScience) was used for each time point (2, 24, and 72 hours).

Detection of NF-κB Activation

To assess co-signaling between TLR4 and TLR5 as well as between TLR4 and TLR7, NF-

κB activation was measured. Supernatants from cells stimulated with the TLR agonists (LPS, Flagellin, and R848) were collected and 20 ul was added to each well in a 96 well flat bottom transparent plate (Corning LifeScience). Next, 180 ul of QUANTI-Blue (Invivogen) was added to the supernatants and the plate was incubated at 37°C, 5% CO_2. After a 1 hour incubation the absorbance was read with the spectrophotometer, i-Tecan Infinite 200 PRO (Tecan, Männedorf, Switzerland), at 620nm to determine NF-κB activation.

Detection of IRF Activation

To assess co-signaling between TLR4 and TLR5 as well as between TLR4 and TLR7, IRF activation was measured. Supernatants from cells stimulated with the respective TLR agonists (LPS, Flagellin, and R848) were collected and 10 ul was added to each well in a 96 well flat bottom white plate (Thermo Fisher Scientific, Waltham, MA). Next, i-Tecan Infinite 200 PRO (Tecan) was used to dispense 50 ul of QUANTI-luc (Invivogen) to the supernatants and luminescence was read with 100ms integration time to determine IRF activation.

Correlation Statistics

To look at correlations between different LPS concentrations (0ug/ml – 5ug/ml) with increasing concentrations of either Flagellin or R848, the Spearman's Rank Correlation Coefficient method was used. Refer to page 41 for the R-code for the Spearman's method.

RESULTS

TLR co-signaling displays a synergistic response for both NFκB and IRF activation when the data were normalized to untreated controls

Despite the plethora of research behind TLR signaling cascades, it is unclear how the immune system would respond to simultaneously activated TLRs as studies have shown both competitive or synergistic effects of co-signaling (Bösl et al., 2018; He et al., 2012). Over a 72 hour period, the THP-1 cells visually showed a difference between single and co-signaling activation (Figure 3). Wells with increasing concentrations of one agonist (either LPS, Flagellin or R848), had more pink or purple colored wells compared to blue wells. A darker blue color was observed with a higher concentration of each agonist, especially in the 5 ug/ml LPS X 5 ug/ml of either Flagellin/R848 (Figure 3). A darker blue color correlated with higher activation of NFκB. This synergistic response was seen when standardizing our data to our control that had neither agonist; 0 ug/ml LPS X 0 ug/ml Flagellin or R848. Out of all the experiments, there was no activation of NFκB or IRF at the 2 hour mark for either single or co-stimulation of LPS and Flagellin or LPS and R848 (Figures 4A-15A).

An upward trend of NFκB activation was shown when the cells were stimulated with LPS (TLR4) and Flagellin (TLR5). At the 24 hour mark, as concentrations of both LPS and Flagellin increased so did the NFκB activation (Figure 4B). This was further emphasized at the 72 hour mark with overall higher NFκB activation (Figure 4C). For example, at 24 hours NFκB activation was 3.5 +/- 0.53 when cells were treated with 0.5 ug/ml LPS and 5 ug/ml Flagellin. At the same treatment concentrations, NFκB activation was 4.7 +/- 0.77 at 72 hours. A synergistic response was also seen in IRF activation when stimulating with both LPS and Flagellin (Figure 5). While both the 24 hour and 72 hour time points had an upward trend, the 72 hour time point had an

overall higher IRF activation especially at the 5 ug/ml LPS and 5 ug/ml of Flagellin (Figure 5C). At the 24 hour mark when both agonists were at their highest concentration (5 ug/ml), IRF activation was 6.5 +/- 1.53 compared to the 72 hour mark which had an IRF activation of 15.0 +/- 3.73 (Figures 5B and 5C).

An upward trend for NFκB was also observed when the cells were stimulated with both LPS (TLR4) and R848 (TLR7). Similar to the co-signaling trends seen with LPS and Flagellin, both the 24 and 72 hour marks showed synergistic response but the responses at 72 hours were more prominent, a NFκB activation of 4.0 +/- 0.27 compared to 7.6 +/- 0.87 (Figures 6B and 6C). For IRF activation, there was a drastic difference in the response peaking at 20.0 +/- 5.17 at the 24 hour mark when the highest concentrations of both agonists were used, compared to 75.1 +/- 15.80 at the 72 hour mark (Figures 7B and 7C).

TLR co-signaling shows competitive inhibition for NFκB activation when the data were normalized within each dose of LPS

Notably, when standardizing NFκB activation within each dose of LPS (cells receiving no Flagellin or R848), the data suggested co-signaling resulted in competition between the signaling cascades.

While individual concentrations of LPS had an upward trend with increasing Flagellin, the overall NFκB activation level kept decreasing as the LPS concentration increased (Figure 8). Specifically, at the 24 and 72 hour marks, the NFκB activation peaked at 3.7 +/- 0.65 and 4.9 +/- 0.99 respectively and then steadily declined. When both agonists were at their highest concentrations (5 ug/ml), this co-stimulation resulted in the lowest NFκB activation in both the 24 hour with a NFκB activation of 1.6 +/- 0.09 and 72 hour mark with a NFκB activation of 1.3 +/-

0.04 (Figures 8B and 8C). This pattern was further emphasized when directly comparing single and co-signaling in figure 9.

Cells treated with LPS and R848, showed similar trends in NFκB activation to cells treated with LPS and Flagellin (Figure 10). In figure 11, the direct comparison highlighted the competitive response between single and co-signaling for LPS and R848. Moreover, these data further emphasized how NFκB activation continues to increase when comparing the NFκB activation induced by just R848 at the 24 hour and 72 hour marks. For example, NFκB activation peaked at 3.6 +/- 0.02 at the 24 mark (Figure 10B) while NFκB activation peaked at 7.5 +/- 0.89 at the 72 hour mark (Figure 10C).

TLR co-signaling led to both synergy and competitive inhibition for IRF activation when data were normalized within each dose of LPS

When LPS treated cells were stimulated with either Flagellin or R848, they showed a synergistic effect at the 24 hour mark but then made a steady decline as the concentration of LPS increased (Figures 12B and 13B). Unlike the NFκB activation trends observed between R848/Flagellin and LPS where the peak activation was seen with only one agonist stimulation (Figures 8 and 10), IRF activation peaked at the co-stimulation of 0.05 ug/ml LPS and 5 ug/ml of either Flagellin or R848 at the 24 hour mark (Figures 12B and 13B).

R848 displayed overall higher IRF activation compared to Flagellin

At the 72 hour mark for both R848 and Flagellin treatments, IRF activation peaked with the presence of just one signal (Figures 12C and 13C). This was further emphasized when directly comparing IRF activation after single and co-signaling (Figures 14 and 15). However, there is a drastic difference in scale between Flagellin versus R848. IRF activation peaked at 6.7 +/- 0.87 for Flagellin only stimulation and IRF activation at the highest concentration of LPS and Flagellin

was at 1.8 +/- 0.43 (Figure 14C). In contrast, IRF activation peaked at 33.7 +/- 2.22 for R848 only stimulation and at 14.2 +/- 6.51 when LPS and R848 were are the highest concentration at 72 hours (Figure 15C). Thus, R848 stimulated stronger IRF activation in human monocytes than Flagellin.

Over 12 hours, TLR co-signaling led to both synergy and competitive inhibition for IRF activation when data were normalized within each dose of LPS

To explore earlier time points, IRF activation was measured at 6 and 12 hours. When LPS treated cells were stimulated with Flagellin or R848, the same synergistic trend was observed at the 12 hour mark (Figures 16B and 17B). However, when LPS treated cells were treated with R848, synergy was observed at the same co-stimulation of 0.05 ug/ml LPS and 5 ug/ml of R848, but this response was not as prominent as the response at the 12 hour mark which peaked at 7.7 +/- 2.3 (Figure 17). Interestingly, at the 6 hour mark, when LPS treated cells were treated with Flagellin, IRF activation peaked at 2.1 +/- 0.98 when both agonists were at their highest concentration (Figure 16A). This was further emphasized when directly comparing single and co-signaling for IRF activation with LPS and Flagellin treated cells with synergy observed at the 6 hour mark, but competitive inhibition observed at the 12 hour mark (Figure 18). However, when directly comparing IRF activation of single and co-signaling of cells treated with both LPS and R848, competitive inhibition was observed by the decrease in IRF activation at both the 6 and 12 hour marks (Figure 19).

Over 12 hours, TLR co-signaling led to both synergy and competitive inhibition for IRF activation when data were normalized to untreated controls

When the data over a 12 hour period was normalized to neither agonist for both LPS treated cells with either Flagellin or R848, synergy and competitive inhibition was observed (Figures 20 and 21). Compared to previous data points over 72 hours, 12 hours did not show competitive

inhibition in IRF activation (Figures 5 and 7). Interestingly, the IRF activation peaked at two places instead of the one. At the 6 hour mark, the first peak was at 0.05 ug/ml of LPS and 5 ug/ml of Flagellin at 2.2 +/- 0.97 and the second peak showed higher IRF activation at the highest concentrations of LPS and Flagellin at 3.4 +/- 2.06 (Figure 20A). This same trend was seen at the 12 hour mark but a higher overall IRF activation was evident, with the highest peak evident at the highest concentration of LPS and Flagellin at 5.5 +/- 3.16 (Figure 20B). However, when LPS was co-stimulated with R848 at the 12 hour mark, there was only one peak at 8.2 +/- 0.34 at the 0.05 ug/ml LPS and 5 ug/ml R848 concentrations (Figure 21B). We see this same trend at the 6 hour mark but it was not as prominent and clear as the IRF activation at 12 hours (Figure 21).

Over 12 hours, TLR co-signaling displays a synergistic response for NFκB activation when the data were normalized to untreated controls

When LPS treated cells were stimulated with either Flagellin or R848, they showed a synergistic effect at 12 hours (Figures 22 and 23). While cells treated with LPS and Flagellin were observed to have a synergistic effect at each individual LPS dose, NFκB activation peaked at the highest concertation of each agonist at 2.3 +/- 0.66 at 6 hours compared to the 12 hour mark which peaked at the 0.05 ug/ml LPS and 5 ug/ml Flagellin co-stimulation at 3.2 +/- 0.03 (Figure 22). As for R848, the synergistic response was seen up to 0.5 ug/ml LPS but plateaued at 5 ug/ml LPS at 12 hours (Figure 23).

Over 12 hours, TLR co-signaling shows competitive inhibition for NFκB activation when the data were normalized within each dose of LPS

When standardizing NFκB activation within each dose of LPS (cells receiving no Flagellin or R848), the data suggested co-signaling resulted in competition between the signaling cascades earlier than the previous 24 and 72 hour time points (Figures 9-11).

While individual concentrations of LPS had an upward trend with increasing concentrations of Flagellin, the overall NFκB activation level kept decreasing as the LPS concentration increased (Figure 24). Specifically, at the 12 hour mark, the NFκB activation peaked at 2.9 +/- 0.04 and thus steadily decreased. When both agonists were at their highest concentrations (5 ug/ml), this co-stimulation resulted in the lowest NFκB activation at 1.8 +/- 0.43 (Figure 24). This pattern was further emphasized when directly comparing single and co-signaling in figure 25.

Cells treated with LPS and R848, showed similar trends in NFκB activation to cells treated with LPS and Flagellin (Figure 26). In figure 27, the direct comparison highlighted the competitive response between single and co-signaling for LPS and R848. Moreover, these data further emphasized how NFκB activation continued to increase when compare ng the NFκB activation at the 6 and 12 hour marks. For example, NFκB activation peaked at 1.5 +/- 0.22 at the 6 hour mark (Figure 26A) and 2.5 +/- 0.09 at the 12 hour mark (Figure 26B).

There is correlation between LPS doses when the data were normalized within each dose of LPS

The observed synergistic and competitive inhibition trends in figures 8 – 15 were all shown to have a correlation coefficient range of 0.8 – 1 ($r_s \geq 0.8$; Tables 1-8).

Over 12 hours, there is correlation between LPS doses when the data were normalized within each dose of LPS and normalized to untreated controls

Interestingly, at the 6 and 12 hour marks, the correlation coefficient range started to expand with a range of -0.4 to 1 ($r_s \geq -0.4$; Tables 9-13). Out of the 13 tables, table 13 was the only one with a negative correlation between LPS and R848 at the 6 hour time point in which a correlation coefficient of -0.4 was observed when comparing LPS 0 and LPS 5 (Figure 21A).

LPS only stimulation on NFκB and IRF activation showed no major difference between 48 and 96 hours at the highest concentration of LPS

To address a potential peak in either NFκB and IRF activation when stimulated with only LPS, another assay was conducted over 96 hours specifically at the 48 and 96 hour time points (Figures 28 and 29). However, when cells treated with different concentrations LPS (0.05, 0.5, and 5 ug/ml) were normalized to cells receiving no LPS, there was no huge difference shown between the 48 and 96 hour mark. If we focus on just the highest concentration of LPS (5 ug/ml), NFκB activation peaks at 5.3 +/- 0.92 and 5.7 +/- 0.45 at 48 and 96 hours respectively (Figure 28). There is only a 0.4 NFκB activation difference. Compared to the LPS at 0.5 ug/ml in which at the 48 hour time point, NFκB activation reached 3.2 +/- 0.93 (Figure 28A) whereas at the 96 hour time point, NFκB activation reached 2.1 +/- 0.30 (Figure 28B). This is a 1.1 difference in NFκB activation compared to the 0.4 that was seen at the highest LPS concentration.

For IRF activation, the same trend was observed (Figure 29). Again, if we focus on just the highest concentration of LPS (5 ug/ml), IRF activation peaks at 11.6 +/- 5.2 and 11.9 +/- 3.8 at 48 and 96 hours respectively (Figure 29). There is only a 0.3 NFκB activation difference. Compared to the LPS at 0.5 ug/ml in which at the 48 hour time point, IRF activation reached 4.2 +/- 2.3 (Figure 29A) whereas at the 96 hour time point, IRF activation reached 1.9 +/- 0.29 (Figure 29B). This is a 2.3 difference in IRF activation compared to the 0.3 that was seen at the highest LPS concentration.

LPS only stimulation on NFκB and IRF activation peaked at the 12, 48, and 96 hour time points

To further the investigation on a potential peak in either NFκB and IRF activation when stimulated with only LPS, data from all previous experiments with LPS only stimulation was used to compare NFκB and IRF activation at the 6, 12, 24, 48, 72, and 96 hour mark. (Figures 30 and 31). There are three points in which both NFκB and IRF activation peaks. For NFκB activation at the highest LPS concentration (5 ug/ml), the first peak is seen at the 12 hour mark at 2.6 +/- 0.34,

NFκB activation then decreased at the 24 hour mark, goes back up at the 48 hour mark at 5.3 +/- 0.92, then peaks again at the 96 hour at 5.7 +/- 0.45 (Figure 30). For IRF activation at the highest LPS concentration (5 ug/ml), the first peak is seen at the 12 hour mark at 4.3 +/- 1.05, IRF activation then decreased at the 24 hour mark, goes back up at the 48 hour mark at 11.6 +/- 5.16, then peaks again at the 96 hour at 11.9 +/- 3.8 (Figure 31).

Interestingly, at 0.05 ug/ml LPS at the 48 hour time point, NFκB and IRF activation peaked over the 96 hour period. At 0.05 ug/ml LPS, NFκB activation peaked at 3.2 +/- 0.93 and IRF activation peaked at 4.2 +/- 2.33 (Figures 30 and 31).

DISCUSSION

Synergistic and Competitive inhibition trends are observed when two TLRs are co-stimulated

I hypothesized that activation of NF-κB and IRF would decrease when two TLR signaling cascades were initiated at the same time**.** Interestingly, both synergy and competitive trends were observed over a 72 hour period depending on the dosage of LPS and either Flagellin or R848. We expected more competitive inhibition and thus a decrease in activation in both NF-κB and IRF activation because of the overlapping proteins involved in the signaling cascades. Figure 1 is a simplified version of the many proteins that influence NF-κB and IRF activation. TLR4 alone is able to activate both NF-κB and IRF by using both the MyD88-dependent and TRIF-dependent pathways respectively (Kawasaki & Kawai, 2014). Finding the balance on the production of inflammatory cytokines and type I IFNs is key when tackling problems like co-infections.

It was interesting to see how the activation of TLR4 either increased or decreased NF-κB and IRF activation when TLR5 or TLR7 were also stimulated. When the data were normalized to neither agonist, synergy within each LPS dose was observed with activation levels of both NF-κB and IRF usually peaking at the highest concentration of both agonists (Figures 4-7, 22-23). This did not support my hypothesis and instead suggested positive interactions between co-stimulated TLRs. However, upon normalizing the data to no Flagellin or no R848, a competitive inhibition response was observed in NF-κB activation as this emphasized the differences within each LPS dose over a 72 hour period (Figures 8-11, 24-27). In evaluating the data this way, my hypothesis was supported. While both perspectives contradict each other, they show something different. When normalizing the data to neither agonist, it shows the progression of how the different doses of each agonist effect the activation of NF-κB and IRF. The data showed that at

each LPS dose, all concentrations of Flagellin or R848 were able to initiate a higher response, the higher the dose. While normalizing the data to no Flagellin or no R848 showed how the overall activation was effected at specific LPS doses. To make it comparable to other LPS doses, setting the baseline to the NF-κB and IRF activation receiving either no Flagellin or no R848, accounts for the fact that higher agonist concentrations result in higher activation.

Time and Dose dependency effects on co-TLR stimulation

The innate immune system rapidly responds within a matter of minutes to hours when a foreign pathogen enters the body (Marshall et al., 2018). To simulate this rapid response, time points as early as 2 hours after incubation of treated cells were looked at. Unfortunately, no trends were observed for either co-stimulation of LPS and Flagellin or LPS and R848 at the two hour mark (Figures 4A-15A). These data suggest that 2 hours was not enough time for the human monocytes to be activated. However, the 6 hour mark was just enough to start seeing trends between LPS treated cells with either Flagellin or R848 (Figures 16A-27A).

Notably, at certain time points both synergy and competitive inhibition was observed depending on the dose of agonist. For instance, at the 6 hour mark IRF activation peaked at two points, the first time at 0.05 ug/ml LPS X 5 ug/ml Flagellin and the second time at 5 ug/ml LPS X 5 ug/ml Flagellin, showing a synergistic response twice in one time point (Figure 20A). This same trend was seen at the 12 hour mark but amplified (Figure 20B). This was the only time in this study that two synergistic responses occurred at one single time point. Interestingly, IRF activation in both co-stimulations experienced this dose dependent effect. The co-stimulation of LPS treated cells with either Flagellin or R848 at the specific dose of 0.05 ug/ml LPS X 5 ug/ml Flagellin or R848 was shown to have importance as IRF activation peaks at this point at the 6, 12, and 24 hour time points (Figures 12B, 13B, 16B, 17, 21B). These data imply that activation

of IRF at these time points and dose may be the most optimal to elicit the highest immune response.

While analysis of dose-dependent treatments is not reinventing the wheel, it is very important when creating a potential drug to be administered to patients especially for cancer immunotherapies (Wages et al., 2018). In a review, Kaczanowska et al. (2013) states that the most impressive antitumor responses are composed of a "mixture" of TLR agonists, specifically TLR4 and TLR7 agonists. This idea was previously emphasized by Adams (2009), in which TLR agonists - bacillus Calmette–Guerin (BCG; which stimulates TLR 2,3,4 and possibly 9) and imiquimod (TLR7) – are US FDA approved for cancer monotherapy.

Complexity of multiple TLR stimulations

TLRs are a very complex PRR in which they not only interact with each other but other PRRs in the immune system. There are a total of 11 human TLRs, 10 of which have a known function to them (Kawasaki & Kawai, 2014), so understanding how two different TLRs respond simultaneously is only the first step in understanding how complex not only the immune system is, but also the interplay between TLRs. In this study, TLR4 was a main reason why either the synergistic or competitive responses in the NF-κB and IRF activation occurred. However, it remains to be seen how activation of multiple TLRs (three, four, etc) at the same time may effect the response. The TLRs have the same goal of initiating an immune response, specially inflammatory cytokines or type I IFNs, but that simple goal gets complicated as more TLRs are activated (Figure 32). Figure 33 is a visual representation of how intertwined the TLRs on the cell surface and in the endosome are and how it all leads to either NF-κB or IRF activation. Unraveling these intricate and intertwined signaling cascades could lead to more predictable and

desirable outcomes when artificially stimulating multiple TLRs at once that can be used for a variety of future therapies and treating co-infections.

Issues with 6 and 12 hour Trials

Originally there were three trials for the 6 and 12 hour time points. However, due to a malfunction of the Tecan plate reader during the second trial of the LPS X R848 trials, it skewed some of the data too much to be used in the combined graphs; the result was large standard error bars. As for the second trial for LPS and Flagellin, month old cells were used in contingency with new cells causing a large discrepancy. Interestingly, the month old cells showed higher activation compared to the newer cells. For these reasons in the data produced the second trial were omitted for the 6 and 12 hour time points.

CONCLUSION & FUTURE STUDIES

It is important to note how complex the immune system is, not just innate immunity but also adaptative immunity and how they both play a role in clearing infections. As mentioned in the introduction, the innate immune system is a rapid first responder and thus an understanding of how foreign pathogens are recognized and dealt with is very important to moving towards better treatments. Increasing our understanding of co-TLR stimulation can shed light on co-infection and how our immune system responds to potentially being over stimulated.

While looking at two different TLRs being activated showed us the diverse effects co-stimulation has on NF-κB and IRF activation, the next step is to tackle three TLRs at the same time. Instead of cells being treated with just LPS and another agonist, all three- TLR4, 5, and 7 - should be evaluated. While it is common to study single TLRs and compare responses, co-TLR stimulated cell studies are infrequent, and even less so for more than three TLRs being stimulated at the same time. Co-infections such as bacterial infections happening the same time as a viral infection complicate immune responses and at times could be detrimental to host (Langford et al., 2020). Finding the most optimal time and dose in which the immune response is at its highest, would open doors to treating infections and understanding our very complex and diverse immune system.

Furthermore, this could open more doors to vaccine design in terms of triggering an optimal immune response with the possibility of limiting the number of vaccines that require more than one dose. For some inactivated vaccines, one dose is not enough to provide as much immunity as possible and thus a second dose is needed to increase immunity levels. For live vaccines, studies have shown that more than one dose is needed for everyone to develop the best immune response

(*CDC Understanding How Vaccines Work*, 2021). Utilizing studies that focus on finding optimal doses to initiate the best immune response are pertinent.

Lastly, another future step could be to look at the role of multiple TLRs stimulated at once and its effect on the adaptive immune response. The relationship between adaptive immune responses and TLRs is that TLRs control multiple dendritic cell functions and activate signals critical to the activation of the adaptive immune response (Iwasaki & Medzhitov, 2004). While there are studies - such as MacLeod & Wetzler (2007)- that look at the activation of TLRs and its connection to adaptive immune system, there is still more to understand how TLRs link the innate and adaptive immune system as well as manipulating them to have more desirable and predictable outcomes for future therapies and treatments.

ACKNOWLEDGEMENTS

This work was supported by Lafayette College and the honors program of the Biology department. I am especially grateful towards Dr. Robert Kurt for his mentorship, guidance, and patience throughout my 4 years at Lafayette. I started working with Dr.Kurt as a SEA-Phages TA in the Fall 2018 and remembered the early mornings filled with plate pouring activities to prep for our students. I am especially grateful for the opportunities outside of Lafayette that he brought to my attention. The opportunity to work with Dr. Barry Sleckman at Weill Cornell Medical College changed my trajectory of what I saw myself doing for the next three years at Lafayette. While I did not work underneath Dr.Sleckman during the summer of 2018, I will be working under him and the postdoctoral fellow Dr.Bo-Ruei Chen for the next two years. This connection would not have been possible without Dr. Kurt who exposed me to a whole different side of approaching my path as a prospective physician scientist.

I also wanted to thank Dr. Michael Butler and Dr. Khadijah A. Mitchell who are a part of my thesis committee who provide fruitful advice and mentorship. I have taken Molecular Genetics and Precision Medicine as well as TA'd for Dr. Mitchell. Being apart of her class has helped me understand and further emphasize my love for molecular genetics as well as medicine. She is an inspiration that has pushed me to be my best self in her classes. As for Dr. Butler, taking human physiology was filled with meaningful and life lasting advice that I took to heart.

Additionally, I want to thank Dr.Eric Ho who has helped me understand the best way to add statistics to my graphs and taught the proper code to test for correlation between non-linear data points.

REFERENCES

Adams, S. (2009). Toll-like receptor agonists in cancer therapy. *Immunotherapy*, *1*(6), 949–964. https://doi.org/10.2217/imt.09.70

Bagchi, A., Herrup, E. A., Warren, H. S., Trigilio, J., Shin, H.-S., Valentine, C., & Hellman, J. (2007). MyD88-dependent and MyD88-independent pathways in synergy, priming, and tolerance between TLR agonists. *Journal of Immunology (Baltimore, Md.: 1950)*, *178*(2), 1164–1171. https://doi.org/10.4049/jimmunol.178.2.1164

Bashir, K., Kappala, D., Singh, Y., Dar, J. A., Mariappan, A. K., Kumar, A., Krishnaswamy, N., Dey, S., Chellappa, M. M., Goswami, T. K., Gupta, V. K., & Ramakrishnan, S. (2019). Combination of TLR2 and TLR3 agonists derepress infectious bursal disease virus vaccine-induced immunosuppression in the chicken. *Scientific Reports*, *9*(1), 8197. https://doi.org/10.1038/s41598-019-44578-5

Bösl, K., Giambelluca, M., Haug, M., Bugge, M., Espevik, T., Kandasamy, R. K., & Bergstrøm, B. (2018). Coactivation of TLR2 and TLR8 in Primary Human Monocytes Triggers a Distinct Inflammatory Signaling Response. *Frontiers in Physiology*, *9*. https://doi.org/10.3389/fphys.2018.00618

Bosshart, H., & Heinzelmann, M. (2016). THP-1 cells as a model for human monocytes. *Annals of Translational Medicine*, *4*(21). https://doi.org/10.21037/atm.2016.08.53

Chiu, S., & Bharat, A. (2016). Role of monocytes and macrophages in regulating immune response following lung transplantation. *Current Opinion in Organ Transplantation*, *21*(3), 239–245. https://doi.org/10.1097/MOT.0000000000000313

Colak, E., Leslie, A., Zausmer, K., Khatamzas, E., Kubarenko, A. V., Pichulik, T., Klimosch, S. N., Mayer, A., Siggs, O., Hector, A., Fischer, R., Klesser, B., Rautanen, A., Frank, M.,

Hill, A. V. S., Manoury, B., Beutler, B., Hartl, D., Simmons, A., & Weber, A. N. R. (2014). RNA and Imidazoquinolines are sensed by distinct TLR7/8 ectodomain sites resulting in functionally disparate signaling events. *Journal of Immunology (Baltimore, Md. : 1950)*, *192*(12), 5963–5973. https://doi.org/10.4049/jimmunol.1303058

Deguine, J., & Barton, G. M. (2014). MyD88: A central player in innate immune signaling. *F1000Prime Reports*, *6*. https://doi.org/10.12703/P6-97

Feng, Y., Ling, Y., Bai, T., Xie, Y., Huang, J., Li, J., Xiong, W., Yang, D., Chen, R., Lu, F., Lu, Y., Liu, X., Chen, Y., Li, X., Li, Y., Summah, H. D., Lin, H., Yan, J., Zhou, M., … Qu, J. (2020). COVID-19 with Different Severities: A Multicenter Study of Clinical Features. *American Journal of Respiratory and Critical Care Medicine*, *201*(11), 1380–1388. https://doi.org/10.1164/rccm.202002-0445OC

Franz, K. M., & Kagan, J. C. (2017). Innate immune receptors as competitive determinants of cell fate. *Molecular Cell*, *66*(6), 750–760. https://doi.org/10.1016/j.molcel.2017.05.009

Gao, W., Xiong, Y., Li, Q., & Yang, H. (2017). Inhibition of Toll-Like Receptor Signaling as a Promising Therapy for Inflammatory Diseases: A Journey from Molecular to Nano Therapeutics. *Frontiers in Physiology*, *8*. https://doi.org/10.3389/fphys.2017.00508

He, H., Genovese, K. J., Nisbet, D. J., & Kogut, M. H. (2007). Synergy of CpG oligodeoxynucleotide and double-stranded RNA (poly I:C) on nitric oxide induction in chicken peripheral blood monocytes. *Molecular Immunology*, *44*(12), 3234–3242. https://doi.org/10.1016/j.molimm.2007.01.034

He, H., Genovese, K. J., Swaggerty, C. L., MacKinnon, K. M., & Kogut, M. H. (2012). Co-stimulation with TLR3 and TLR21 ligands synergistically up-regulates Th1-cytokine

IFN-γ and regulatory cytokine IL-10 expression in chicken monocytes. *Developmental and Comparative Immunology*, *36*(4), 756–760. https://doi.org/10.1016/j.dci.2011.11.006

Iwasaki, A., & Medzhitov, R. (2004). Toll-like receptor control of the adaptive immune responses. *Nature Immunology*, *5*(10), 987–995. https://doi.org/10.1038/ni1112

Jamieson, A. M., Pasman, L., Yu, S., Gamradt, P., Homer, R. J., Decker, T., & Medzhitov, R. (2013). Role of Tissue Protection in Lethal Respiratory Viral-Bacterial Coinfection. *Science*, *340*(6137), 1230–1234. https://doi.org/10.1126/science.1233632

Jin, J., Samuvel, D. J., Zhang, X., Li, Y., Lu, Z., Lopes-Virella, M. F., & Huang, Y. (2011). Coactivation of TLR4 and TLR2/6 Coordinates an Additive Augmentation on IL-6 Gene Transcription via p38 MAPK Pathway in U937 Mononuclear Cells. *Molecular Immunology*, *49*(3), 423–432. https://doi.org/10.1016/j.molimm.2011.08.026

Kaczanowska, S., Joseph, A. M., & Davila, E. (2013). TLR agonists: Our best frenemy in cancer immunotherapy. *Journal of Leukocyte Biology*, *93*(6), 847–863. https://doi.org/10.1189/jlb.1012501

Kaji, R., Kiyoshima-Shibata, J., Tsujibe, S., Nanno, M., & Shida, K. (2018). Short communication: Probiotic induction of interleukin-10 and interleukin-12 production by macrophages is modulated by co-stimulation with microbial components. *Journal of Dairy Science*, *101*(4), 2838–2841. https://doi.org/10.3168/jds.2017-13868

Kawai, T., & Akira, S. (2006). TLR signaling. *Cell Death & Differentiation*, *13*(5), 816–825. https://doi.org/10.1038/sj.cdd.4401850

Kawai, Taro, & Akira, S. (2010). The role of pattern-recognition receptors in innate immunity: Update on Toll-like receptors. *Nature Immunology*, *11*(5), 373–384. https://doi.org/10.1038/ni.1863

Kawasaki, T., & Kawai, T. (2014). Toll-Like Receptor Signaling Pathways. *Frontiers in Immunology*, *5*. https://doi.org/10.3389/fimmu.2014.00461

Kim, S., Kaiser, P., Borowska, D., & Vervelde, L. (2018). Synergistic effect of co-stimulation of membrane and endosomal TLRs on chicken innate immune responses. *Veterinary Immunology and Immunopathology*, *199*, 15–21. https://doi.org/10.1016/j.vetimm.2018.03.005

Kofoed, E. M., & Vance, R. E. (2012). NAIPs: Building an innate immune barrier against bacterial pathogens. NAIPs function as sensors that initiate innate immunity by detection of bacterial proteins in the host cell cytosol. *BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology*, *34*(7), 589–598. https://doi.org/10.1002/bies.201200013

Koppenol-Raab, M., Sjoelund, V., Manes, N. P., Gottschalk, R. A., Dutta, B., Benet, Z. L., Fraser, I. D. C., & Nita-Lazar, A. (2017). Proteome and Secretome Analysis Reveals Differential Post-transcriptional Regulation of Toll-like Receptor Responses. *Molecular & Cellular Proteomics: MCP*, *16*(4 suppl 1), S172–S186. https://doi.org/10.1074/mcp.M116.064261

Kovarik, P., Castiglia, V., Ivin, M., & Ebner, F. (2016). Type I Interferons in Bacterial Infections: A Balancing Act. *Frontiers in Immunology*, *7*. https://doi.org/10.3389/fimmu.2016.00652

Langford, B. J., So, M., Raybardhan, S., Leung, V., Westwood, D., MacFadden, D. R., Soucy, J.-P. R., & Daneman, N. (2020). Bacterial co-infection and secondary infection in patients with COVID-19: A living rapid review and meta-analysis. *Clinical Microbiology and Infection*, *0*(0). https://doi.org/10.1016/j.cmi.2020.07.016

Lee, H., Lee, S., & Lee, I.-H. C. and S. J. (2013, January 31). *Toll-Like Receptors: Sensor Molecules for Detecting Damage to the Nervous System*. Current Protein & Peptide Science. https://www.eurekaselect.com/108031/article

Liu, T., Zhang, L., Joo, D., & Sun, S.-C. (2017). NF-κB signaling in inflammation. *Signal Transduction and Targeted Therapy*, *2*, 17023. https://doi.org/10.1038/sigtrans.2017.23

MacLeod, H., & Wetzler, L. M. (2007). T Cell Activation by TLRs: A Role for TLRs in the Adaptive Immune Response. *Science's STKE*, *2007*(402), pe48–pe48. https://doi.org/10.1126/stke.4022007pe48

Marshall, J. S., Warrington, R., Watson, W., & Kim, H. L. (2018). An introduction to immunology and immunopathology. *Allergy, Asthma & Clinical Immunology*, *14*(2), 49. https://doi.org/10.1186/s13223-018-0278-1

McNab, F., Mayer-Barber, K., Sher, A., Wack, A., & O'Garra, A. (2015). Type I interferons in infectious disease. *Nature Reviews Immunology*, *15*(2), 87–103. https://doi.org/10.1038/nri3787

Mogensen, T. H. (2009). Pathogen Recognition and Inflammatory Signaling in Innate Immune Defenses. *Clinical Microbiology Reviews*, *22*(2), 240–273. https://doi.org/10.1128/CMR.00046-08

Okamoto, M., Tsukamoto, H., Kouwaki, T., Seya, T., & Oshiumi, H. (2017). Recognition of Viral RNA by Pattern Recognition Receptors in the Induction of Innate Immunity and Excessive Inflammation During Respiratory Viral Infections. *Viral Immunology*, *30*(6), 408–420. https://doi.org/10.1089/vim.2016.0178

O'Neill, L. A. J., & Bowie, A. G. (2007). The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. *Nature Reviews Immunology*, *7*(5), 353–364. https://doi.org/10.1038/nri2079

Park, B. S., & Lee, J.-O. (2013). Recognition of lipopolysaccharide pattern by TLR4 complexes. *Experimental & Molecular Medicine*, *45*(12), e66–e66. https://doi.org/10.1038/emm.2013.97

Parker, L. C., Prince, L. R., & Sabroe, I. (2007). Translational Mini-Review Series on Toll-like Receptors: Networks regulated by Toll-like receptors mediate innate and adaptive immunity. *Clinical & Experimental Immunology*, *147*(2), 199–207. https://doi.org/10.1111/j.1365-2249.2006.03203.x

Petes, C., Odoardi, N., & Gee, K. (2017). The Toll for Trafficking: Toll-Like Receptor 7 Delivery to the Endosome. *Frontiers in Immunology*, *8*. https://doi.org/10.3389/fimmu.2017.01075

Razonable, R. R., Henault, M., Lee, L. N., Laethem, C., Johnston, P. A., Watson, H. L., & Paya, C. V. (2005). Secretion of Proinflammatory Cytokines and Chemokines during Amphotericin B Exposure Is Mediated by Coactivation of Toll-Like Receptors 1 and 2. *Antimicrobial Agents and Chemotherapy*, *49*(4), 1617–1621. https://doi.org/10.1128/AAC.49.4.1617-1621.2005

Rosenberger, K., Derkow, K., Dembny, P., Krüger, C., Schott, E., & Lehnardt, S. (2014). The impact of single and pairwise Toll-like receptor activation on neuroinflammation and neurodegeneration. *Journal of Neuroinflammation*, *11*. https://doi.org/10.1186/s12974-014-0166-7

Saito, M., Arakaki, R., Yamada, A., Tsunematsu, T., Kudo, Y., & Ishimaru, N. (2016). Molecular Mechanisms of Nickel Allergy. *International Journal of Molecular Sciences*, *17*(2). https://doi.org/10.3390/ijms17020202

Sharifipour, E., Shams, S., Esmkhani, M., Khodadadi, J., Fotouhi-Ardakani, R., Koohpaei, A., Doosti, Z., & EJ Golzari, S. (2020). Evaluation of bacterial co-infections of the respiratory tract in COVID-19 patients admitted to ICU. *BMC Infectious Diseases*, *20*(1), 646. https://doi.org/10.1186/s12879-020-05374-z

Sjoelund, V., Smelkinson, M., & Nita-Lazar, A. (2014). Phosphoproteome Profiling of the Macrophage Response to Different Toll-Like Receptor Ligands Identifies Differences in Global Phosphorylation Dynamics. *Journal of Proteome Research*, *13*(11), 5185–5197. https://doi.org/10.1021/pr5002466

Steiner, T. S. (2007). How Flagellin and Toll-Like Receptor 5 Contribute to Enteric Infection. *Infection and Immunity*, *75*(2), 545–552. https://doi.org/10.1128/IAI.01506-06

Toll-Like Receptors Research Areas: R&D Systems. (n.d.). Www.Rndsystems.Com. Retrieved December 2, 2020, from https://www.rndsystems.com/research-area/toll--like-receptors

Toll-like receptors—The Eye of Innate Immunity. (n.d.). CUSABIO. Retrieved May 6, 2021, from https://www.cusabio.com/c-20915.html

Underhill, D. M. (2007). Collaboration between the innate immune receptors dectin-1, TLRs, and Nods. *Immunological Reviews*, *219*(1), 75–87. https://doi.org/10.1111/j.1600-065X.2007.00548.x

Understanding How Vaccines Work. (n.d.). 2.

Wages, N. A., Chiuzan, C., & Panageas, K. S. (2018). Design considerations for early-phase clinical trials of immune-oncology agents. *Journal for ImmunoTherapy of Cancer*, *6*(1), 81. https://doi.org/10.1186/s40425-018-0389-8

West, T. E., Ernst, R. K., Jansson-Hutson, M. J., & Skerrett, S. J. (2008). Activation of Toll-like receptors by Burkholderia pseudomallei. *BMC Immunology*, *9*, 46. https://doi.org/10.1186/1471-2172-9-46

Yanai, H., Negishi, H., & Taniguchi, T. (2012). The IRF family of transcription factors. *Oncoimmunology*, *1*(8), 1376–1386. https://doi.org/10.4161/onci.22475

Zhang, Q., Lenardo, M. J., & Baltimore, D. (2017). 30 Years of NF-κB: A Blossoming of Relevance to Human Pathobiology. *Cell*, *168*(1–2), 37–57. https://doi.org/10.1016/j.cell.2016.12.012

Zhu, Y., Yao, S., & Chen, L. (2011). CELL SURFACE SIGNALING MOLECULES IN THE CONTROL OF IMMUNE RESPONSES: A TIDE MODEL. *Immunity*, *34*(4), 466–478. https://doi.org/10.1016/j.immuni.2011.04.008

FIGURES

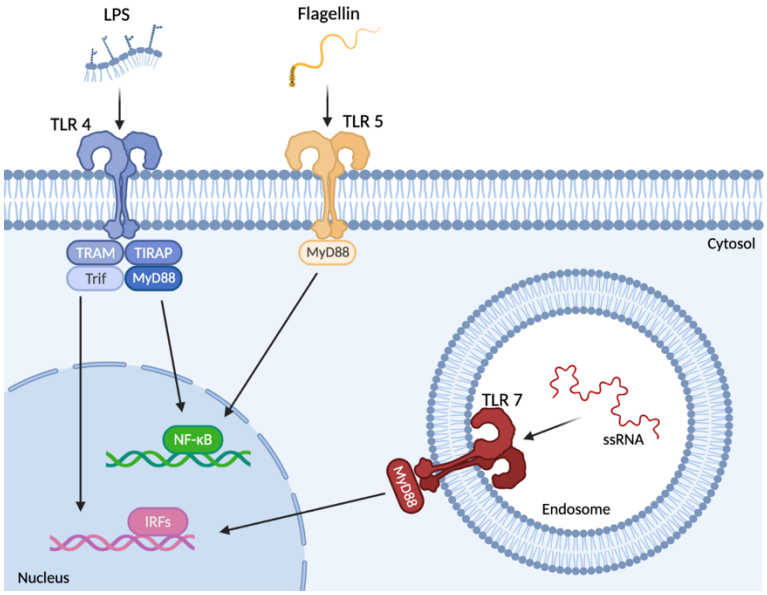

Figure 1 | Signaling Cascades of TLR4, TLR5, and TLR7. Intracellular pathways triggered by either LPS (TLR4), Flagellin (TLR5), and ssRNA (TLR7). Endosomal sensing of ssRNA induces production of IRFs. TLR4 and TLR5 are expressed on the cell surface, recognizing bacterial LPS and Flagellin respectively. Extracellular sensing of LPS and Flagellin causes downstream activation of NFκB and IRF.

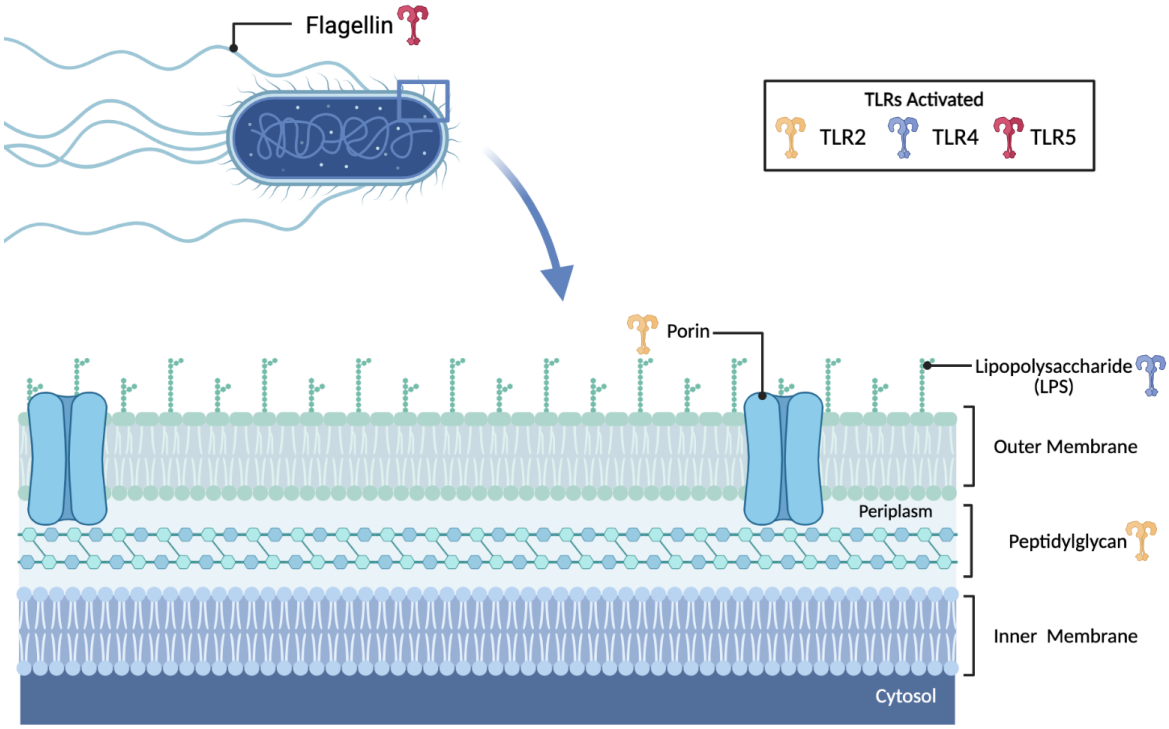

Figure 2 | An illustration of *Escherichia coli (E.Coli)* PAMPs. *E.Coli,* a gram negative bacterium, has the potential to activate three different Toll-like receptors (TLRs); TLR2 (yellow), TLR4 (blue), TLR5 (pink). TLR2 is depicted twice as porin and peptidylglycan can both simulate TLR2.

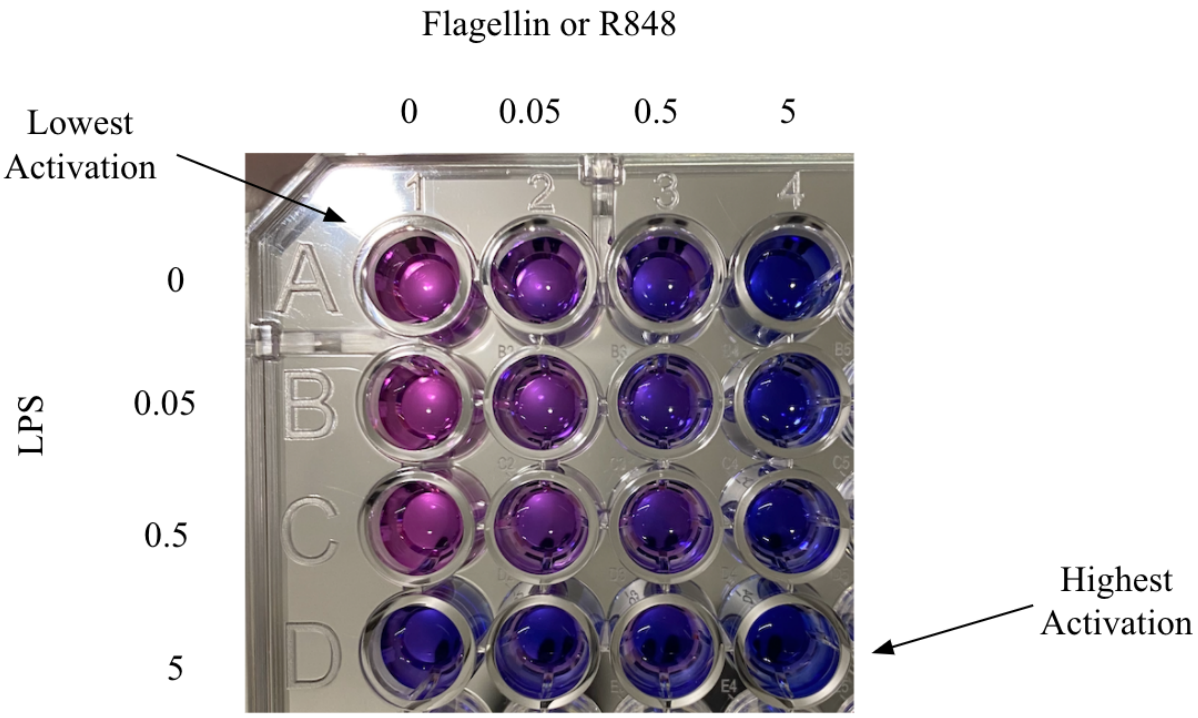

Figure 3 | Assay for NFκB activation at 72 hours. The color visually represents NFκB activation level. Pink = Low NFκB activation levels. Dark blue = High NFκB activation levels. Concentrations on x and y-axis refer to ug/ml.

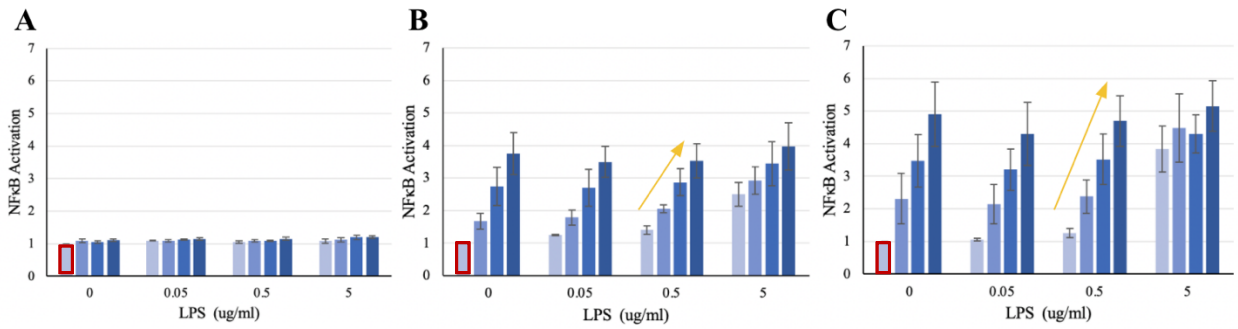

Figure 4 | The impact of LPS and Flagellin on NFκB activation. THP-1 cells were incubated with increasing concentrations of LPS and Flagellin respectively for **(A)** 2 hours **(B)** 24 hours and **(C)** 72 hours**.** All data represent the average and standard error of three separate experiments. All data show NFκB activation relative to cells without stimulation**;** 0 ug/ml LPS; 0 ug/ml Fla (outlined in red). The gradient of blue represents the concentration of Flagellin. (■) = 0 ug/ml of Fla; (■) = 0.05 ug/ml of Fla; (■) = 0.5 ug/ml of Fla; (■) = 5ug/ml of Fla. LPS: lipopolysaccharides; Fla: Flagellin; Yellow Arrows: show a synergistic response.

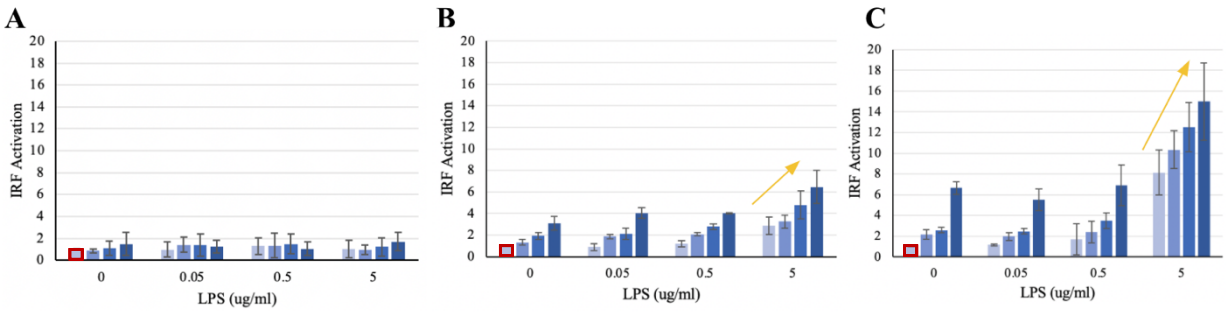

Figure 5 | The impact of LPS and Flagellin on IRF activation. THP-1 cells were incubated with increasing concentrations of LPS and Flagellin respectively for **(A)** 2 hours **(B)** 24 hours and **(C)** 72 hours**.** All data represent the average and standard error of three separate experiments. All data show IRF activation relative to cells without stimulation**;** 0 ug/ml LPS; 0 ug/ml Fla (outlined in red). The gradient of blue represents the concentration of Flagellin. (■) = 0 ug/ml of Fla; (■) = 0.05 ug/ml of Fla; (■) = 0.5 ug/ml of Fla; (■) = 5ug/ml of Fla. LPS: lipopolysaccharides; Fla: Flagellin; Yellow Arrows: show a synergistic response.

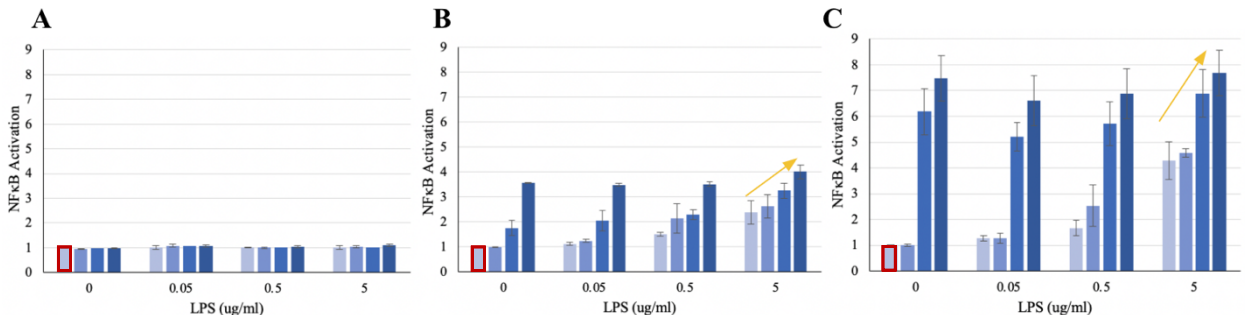

Figure 6 | The impact of LPS and R848 on NFκB activation. THP-1 cells were incubated with increasing concentrations of LPS and R848 respectively for **(A)** 2 hours **(B)** 24 hours and **(C)** 72 hours**.** All data represent the average and standard error of three separate experiments. All data show NFκB activation relative to cells without stimulation**;** 0 ug/ml LPS; 0 ug/ml R848 (outlined in red). The gradient of blue represents the concentration of R848. (■) = 0 ug/ml of R848; (■) = 0.05 ug/ml of R848; (■) = 0.5 ug/ml of R848; (■) = 5ug/ml of R848. LPS: lipopolysaccharides; Yellow Arrows: show a synergistic response.

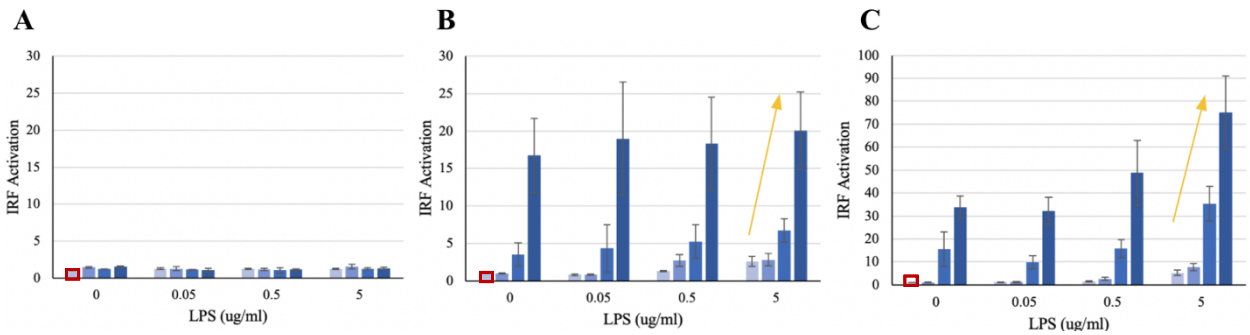

Figure 7 | The impact of LPS and R848 on IRF activation. THP-1 cells were incubated with increasing concentrations of LPS and R848 respectively for **(A)** 2 hours **(B)** 24 hours and **(C)** 72 hours**.** All data represent the average and standard error of three separate experiments. All data show IRF activation relative to cells without stimulation**;** 0 ug/ml LPS; 0 ug/ml R848 (outlined in red). The gradient of blue represents the concentration of R848. (■) = 0 ug/ml of R848; (■) = 0.05 ug/ml of R848; (■) = 0.5 ug/ml of R848; (■) = 5ug/ml of R848. LPS: lipopolysaccharides; Yellow Arrows: show a synergistic response.

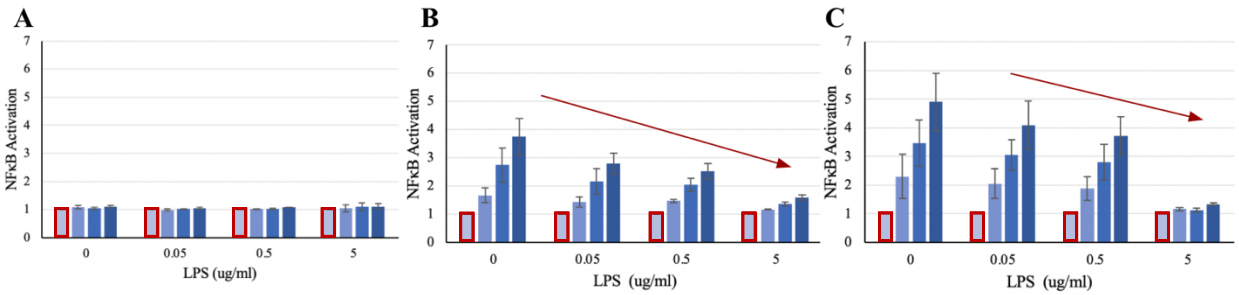

Figure 8 | The impact of LPS and Flagellin on NFκB activation. THP-1 cells were incubated with increasing concentrations of LPS and Flagellin respectively for **(A)** 2 hours **(B)** 24 hours and **(C)** 72 hours**.** All data represent the average and standard error of three separate experiments. All data show NFκB activation relative to cells receiving no Flagellin (0 ug/ml Fla) +/- LPS and Flagellin (outlined in red). The gradient of blue represents the concentration of Flagellin. (■) = 0 ug/ml of Fla; (■) = 0.05 ug/ml of Fla; (■) = 0.5 ug/ml of Fla; (■) = 5ug/ml of Fla. LPS: lipopolysaccharides; Fla: Flagellin; Red Arrows: show a competitive response.

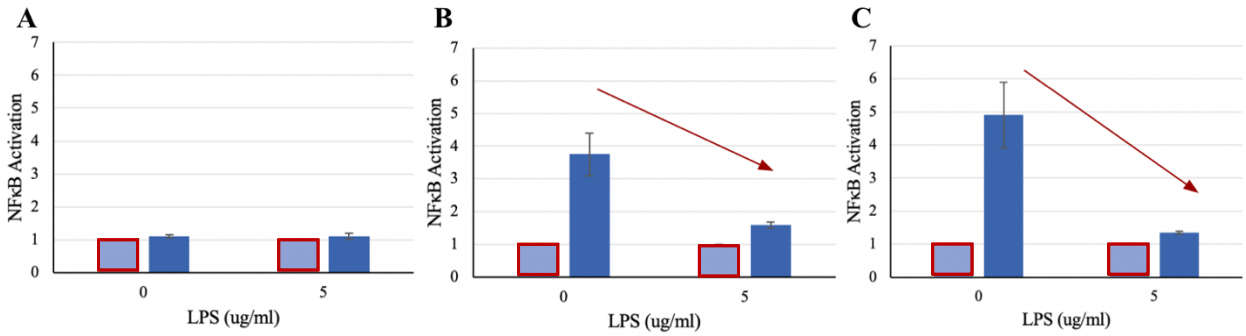

Figure 9 | The direct comparison of single VS co-signaling on NFκB activation. THP-1 were cells incubated with increasing concentrations of LPS and Flagellin for **(A)** 2 hours **(B)** 24 hours and **(C)** 72 hours**.** All data represent the average and standard error of three separate experiments. All data show NFκB activation relative to cells receiving no Flagellin (0 ug/ml Fla) +/- LPS and Flagellin (outlined in red). The gradient of blue represents the concentration of Flagellin. (■) = 0 ug/ml of Fla; (■) = 5ug/ml of Fla. LPS: lipopolysaccharides; Fla: Flagellin; Red Arrows: show a competitive response.

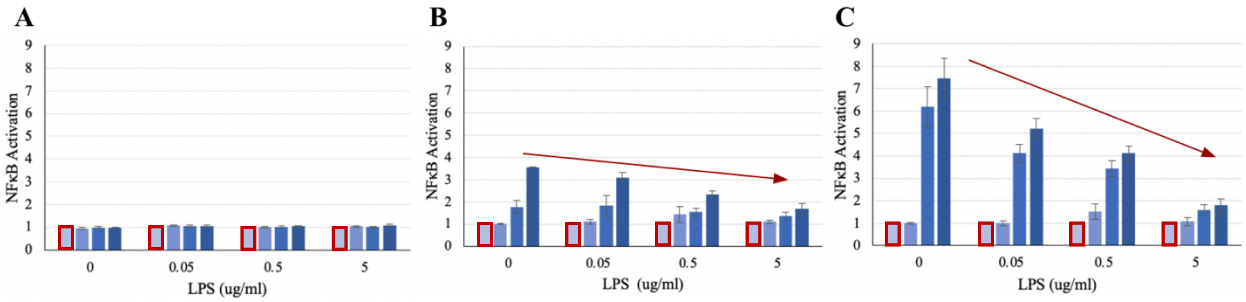

Figure 10 | The impact of LPS and R848 on NFκB activation. THP-1 cells were incubated with increasing concentrations of LPS and R848 respectively for **(A)** 2 hours **(B)** 24 hours and **(C)** 72 hours**.** All data represent the average and standard error of three separate experiments. All data show NFκB activation relative to cells receiving no R848 (0 ug/ml R848) +/- LPS and R848 (outlined in red). The gradient of blue represents the concentration of R848. (■) = 0 ug/ml of R848; (■) = 0.05 ug/ml of R848; (■) = 0.5 ug/ml of R848; (■) = 5ug/ml of R848. LPS: lipopolysaccharides; Red Arrows: show a competitive response.

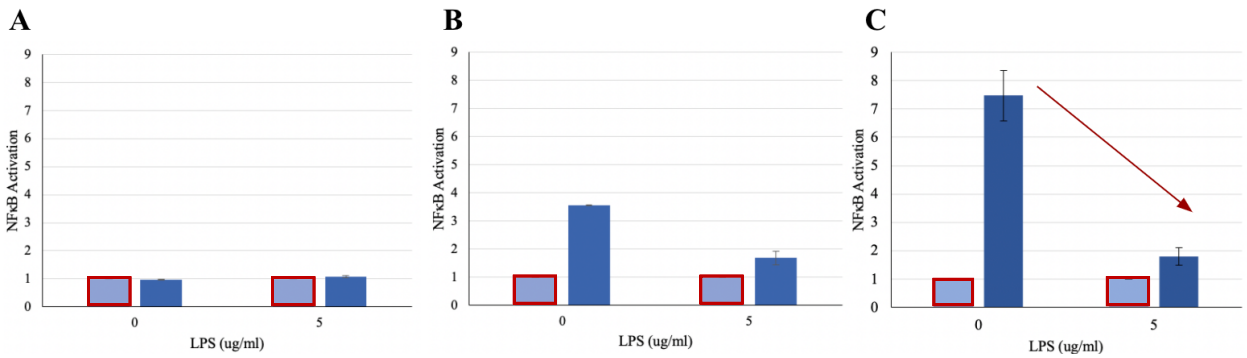

Figure 11 | The direct comparison of single VS co-signaling on NFκB activation. THP-1 were cells incubated with increasing concentrations of LPS and R848 for **(A)** 2 hours **(B)** 24 hours and **(C)** 72 hours**.** All data represent the average and standard error of three separate experiments. All data show NFκB activation relative to cells receiving no R848 (0 ug/ml R848) +/- LPS and R848 (outlined in red). The gradient of blue represents the concentration of R848. (■) = 0 ug/ml of R848; (■) = 5ug/ml of Fla. = 5ug/ml of R848. LPS: lipopolysaccharides; Red Arrows: show a competitive response.

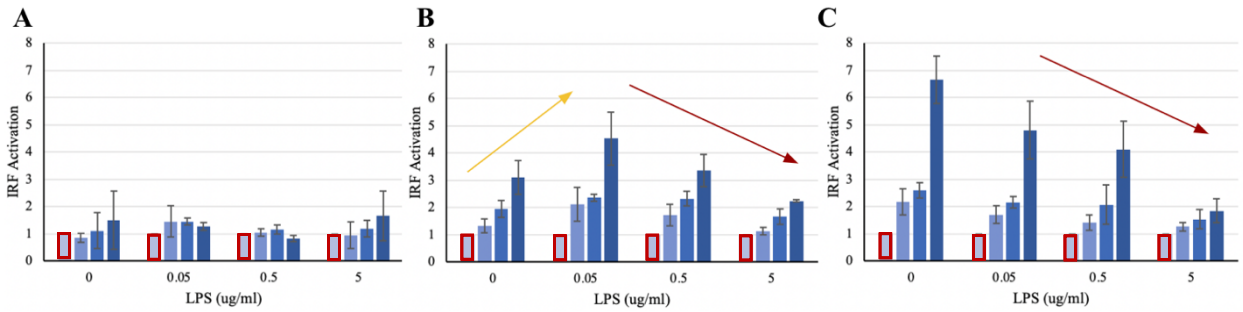

Figure 12 | The impact of LPS and Flagellin on IRF activation. THP-1 cells were incubated with increasing concentrations of LPS and Flagellin respectively for **(A)** 2 hours **(B)** 24 hours and **(C)** 72 hours**.** All data represent the average and standard error of three separate experiments. All data show IRF activation relative to cells receiving no Flagellin (0 ug/ml Fla) +/- LPS and Flagellin (outlined in red). The gradient of blue represents the concentration of Flagellin. (■) = 0 ug/ml of Fla; (■) = 0.05 ug/ml of Fla; (■) = 0.5 ug/ml of Fla; (■) = 5ug/ml of Fla. LPS: lipopolysaccharides; Fla: Flagellin; Yellow Arrows: show a synergistic response; Red Arrows: show a competitive response.

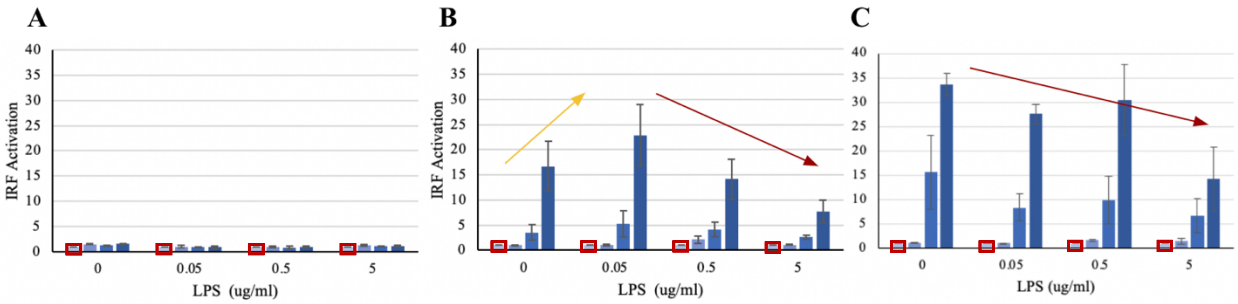

Figure 13 | The impact of LPS and R848 on IRF activation. THP-1 cells were incubated with increasing concentrations of LPS and R848 respectively for **(A)** 2 hours **(B)** 24 hours and **(C)** 72 hours**.** All data represent the average and standard error of three separate experiments. All data show IRF activation relative to cells receiving no R848 (0 ug/ml R848) +/- LPS and R848 (outlined in red). The gradient of blue represents the concentration of R848. (■) = 0 ug/ml of R848; (■) = 0.05 ug/ml of R848; (■) = 0.5 ug/ml of R848; (■) = 5ug/ml of R848. LPS: lipopolysaccharides; Yellow Arrows: show a synergistic response; Red Arrows: show a competitive response.

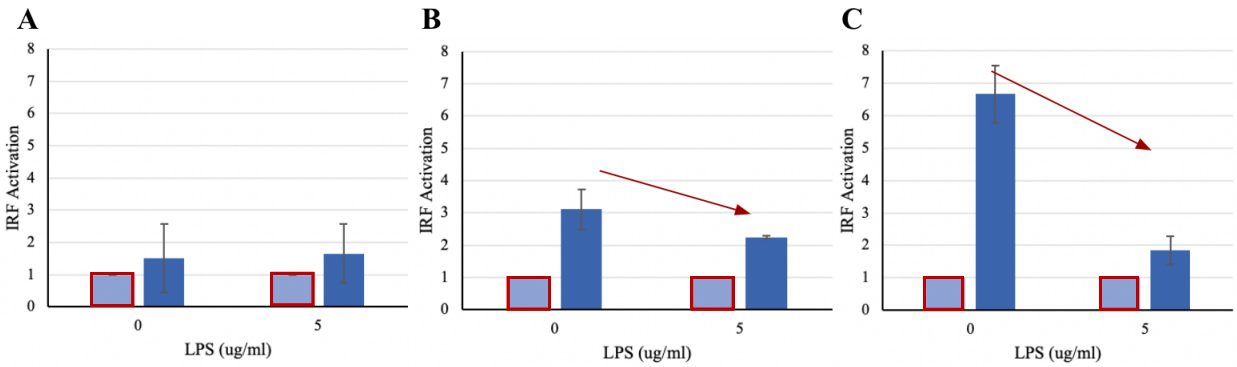

Figure 14 | The direct comparison of single VS co-signaling on IRF activation. THP-1 were cells incubated with increasing concentrations of LPS and Flagellin for **(A)** 2 hours **(B)** 24 hours and **(C)** 72 hours**.** All data represent the average and standard error of three separate experiments. All data show IRF activation relative to cells receiving no Flagellin (0 ug/ml Fla) +/- LPS and Flagellin (outlined in red). The gradient of blue represents the concentration of Flagellin. (■) = 0 ug/ml of Fla; (■) = 5ug/ml of Fla. LPS: lipopolysaccharides; Fla: Flagellin; Red Arrows: show a competitive response.

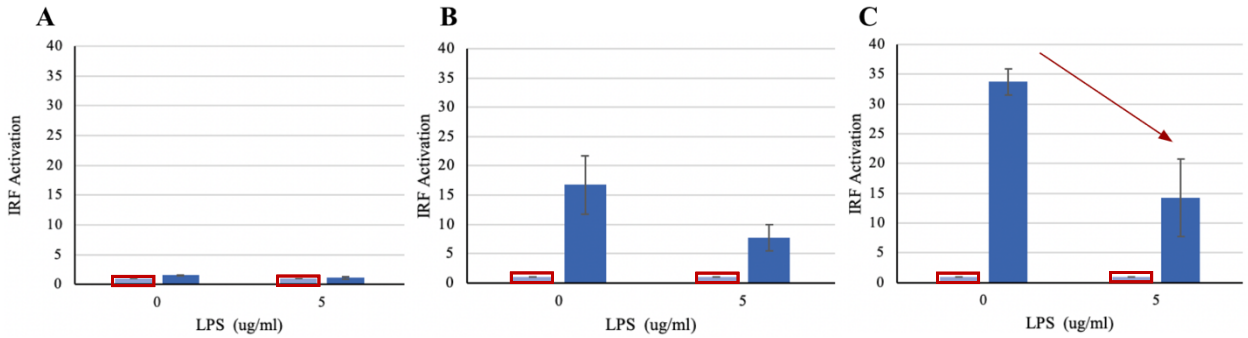

Figure 15 | The direct comparison of single VS co-signaling on IRF activation. THP-1 were cells incubated with increasing concentrations of LPS and R848 for **(A)** 2 hours **(B)** 24 hours and **(C)** 72 hours**.** All data represent the average and standard error of three separate experiments. All data show IRF activation relative to cells receiving no R848 (0 ug/ml R848) +/- LPS and R848 (outlined in red). The gradient of blue represents the concentration of R848. (▪) = 0 ug/ml of R848; (■) = 5ug/ml of Fla. = 5ug/ml of R848. LPS: lipopolysaccharides; Red Arrows: show a competitive response.

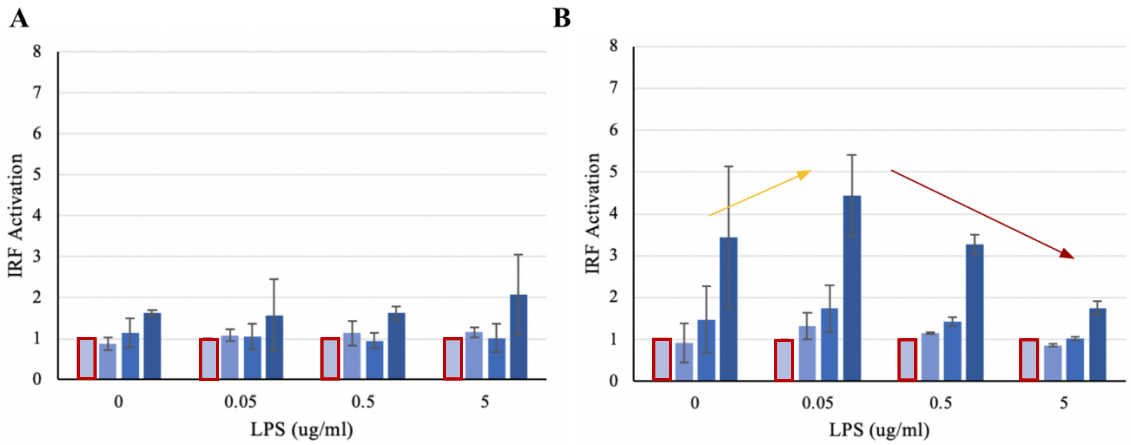

Figure 16 | The impact of LPS and Flagellin on IRF activation. THP-1 cells were incubated with increasing concentrations of LPS and Flagellin respectively for **(A)** 6 hours and **(B)** 12 hours**.** All data represent the average and standard error of two separate experiments. All data show IRF activation relative to cells receiving no Flagellin (0 ug/ml Fla) +/- LPS and Flagellin (outlined in red). The gradient of blue represents the concentration of Flagellin. (■) = 0 ug/ml of Fla; (■) = 0.05 ug/ml of Fla; (■) = 0.5 ug/ml of Fla; (■) = 5ug/ml of Fla. LPS: lipopolysaccharides; Fla: Flagellin; Yellow Arrows: show a synergistic response; Red Arrows: show a competitive response.

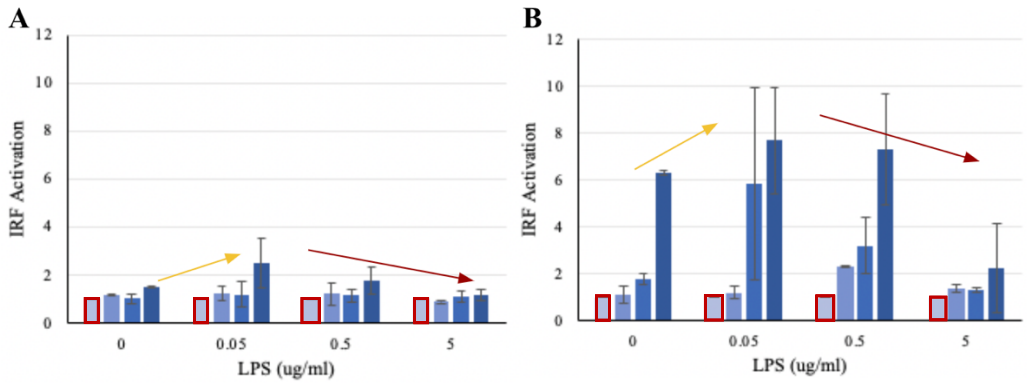

Figure 17 | The impact of LPS and R848 on IRF activation. THP-1 cells were incubated with increasing concentrations of LPS and R848 respectively for **(A)** 6 hours and **(B)** 12 hours. All data represent the average and standard error of two separate experiments. All data show IRF activation relative to cells receiving no R848 (0 ug/ml R848) +/- LPS and R848 (outlined in red). The gradient of blue represents the concentration of R848. (■) = 0 ug/ml of R848; (■) = 0.05 ug/ml of R848; (■) = 0.5 ug/ml of R848; (■) = 5ug/ml of R848. LPS: lipopolysaccharides; Yellow Arrows: show a synergistic response; Red Arrows: show a competitive response.

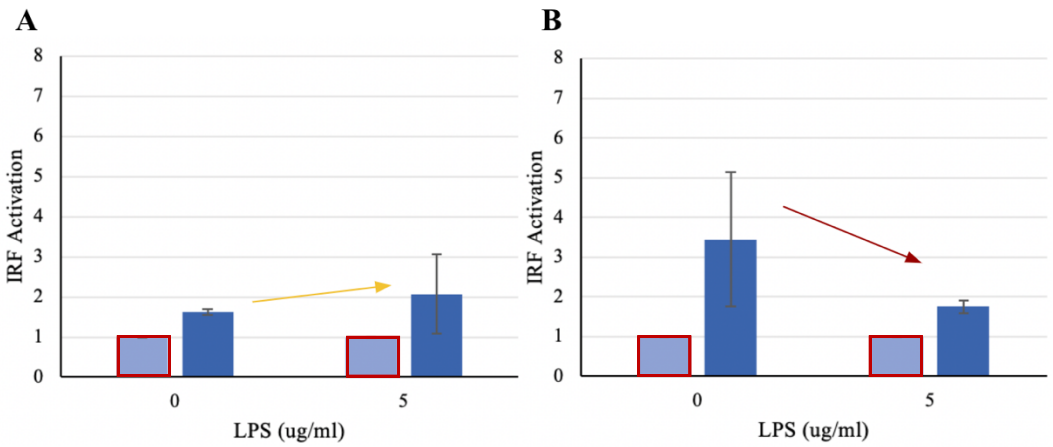

Figure 18 | The direct comparison of single VS co-signaling on IRF activation. THP-1 were cells incubated with increasing concentrations of LPS and Flagellin for **(A)** 6 hours and **(B)** 12 hours. All data represent the average and standard error of two separate experiments. All data show IRF activation relative to cells receiving no Flagellin (0 ug/ml Fla) +/- LPS and Flagellin (outlined in red). The gradient of blue represents the concentration of Flagellin. (■) = 0 ug/ml of Fla; (■) = 5ug/ml of Fla. LPS: lipopolysaccharides; Fla: Flagellin; Red Arrows: show a competitive response.

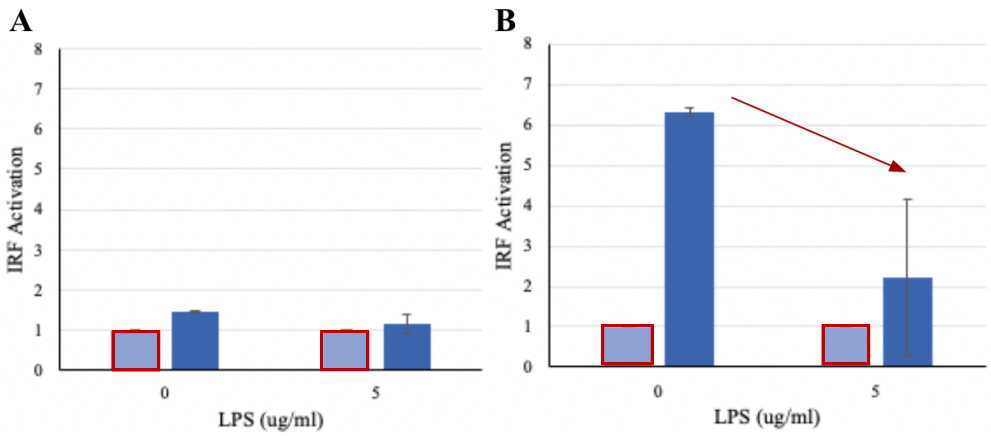

Figure 19 | The direct comparison of single VS co-signaling on IRF activation. THP-1 were cells incubated with increasing concentrations of LPS and R848 for **(A)** 6 hours and **(B)** 12 hours. All data represent the average and standard error of two separate experiments. All data show IRF activation relative to cells receiving no R848 (0 ug/ml R848) +/- LPS and R848 (outlined in red). The gradient of blue represents the concentration of R848. (▪) = 0 ug/ml of R848; (■) = 5ug/ml of Fla. = 5ug/ml of R848. LPS: lipopolysaccharides; Red Arrows: show a competitive response.

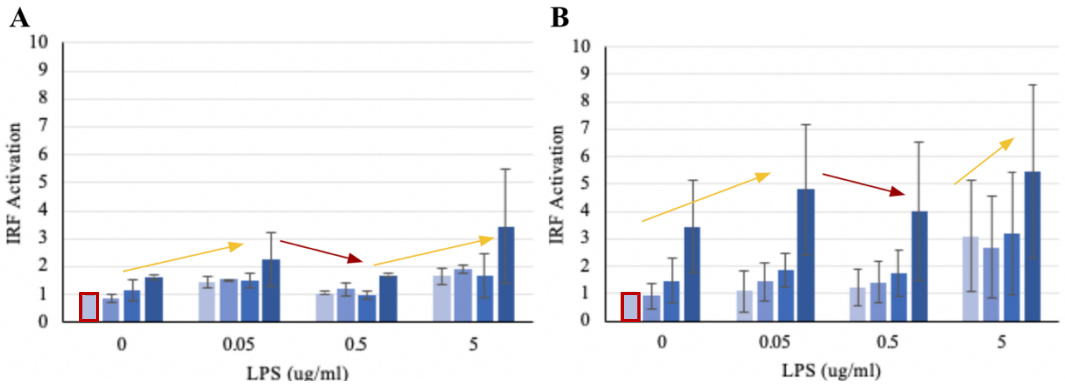

Figure 20 | The impact of LPS and Flagellin on IRF activation. THP-1 cells were incubated with increasing concentrations of LPS and Flagellin respectively for **(A)** 6 hours and **(B)** 12 hours. All data represent the average and standard error of two separate experiments. All data show IRF activation relative to cells without stimulation**;** 0 ug/ml LPS; 0 ug/ml R848 (outlined in red). The gradient of blue represents the concentration of Flagellin. (■) = 0 ug/ml of Fla; (■) = 0.05 ug/ml of Fla; (■) = 0.5 ug/ml of Fla; (■) = 5ug/ml of Fla. LPS: lipopolysaccharides; Fla: Flagellin; Yellow Arrows: show a synergistic response; Red Arrows: show a competitive response.

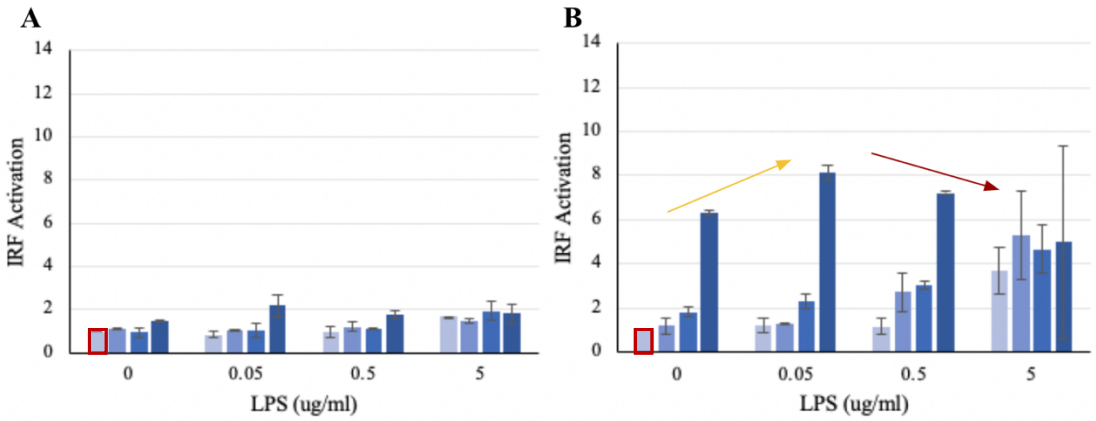

Figure 21 | The impact of LPS and R848 on IRF activation. THP-1 cells were incubated with increasing concentrations of LPS and R848 respectively for **(A)** 6 hours and **(B)** 12 hours. All data represent the average and standard error of two separate experiments. All data show IRF activation relative to cells without stimulation**;** 0 ug/ml LPS; 0 ug/ml R848 (outlined in red). The gradient of blue represents the concentration of R848. (■) = 0 ug/ml of R848; (■) = 0.05 ug/ml of R848; (■) = 0.5 ug/ml of R848; (■) = 5ug/ml of R848. LPS: lipopolysaccharides; Yellow Arrows: show a synergistic response; Red Arrows: show a competitive response.

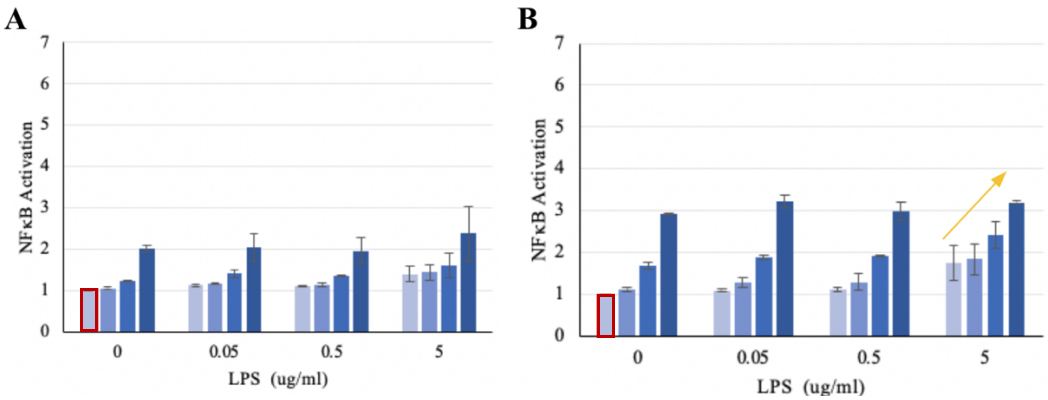

Figure 22 | The impact of LPS and Flagellin on NFκB activation. THP-1 cells were incubated with increasing concentrations of LPS and Flagellin respectively for **(A)** 6 hours and **(B)** 12 hours. All data represent the average and standard error of two separate experiments. All data show NFκB activation relative to cells without stimulation**;** 0 ug/ml LPS; 0 ug/ml Fla (outlined in red). The gradient of blue represents the concentration of Flagellin. (■) = 0 ug/ml of Fla; (■) = 0.05 ug/ml of Fla; (■) = 0.5 ug/ml of Fla; (■) = 5ug/ml of Fla. LPS: lipopolysaccharides; Fla: Flagellin; Yellow Arrows: show a synergistic response.

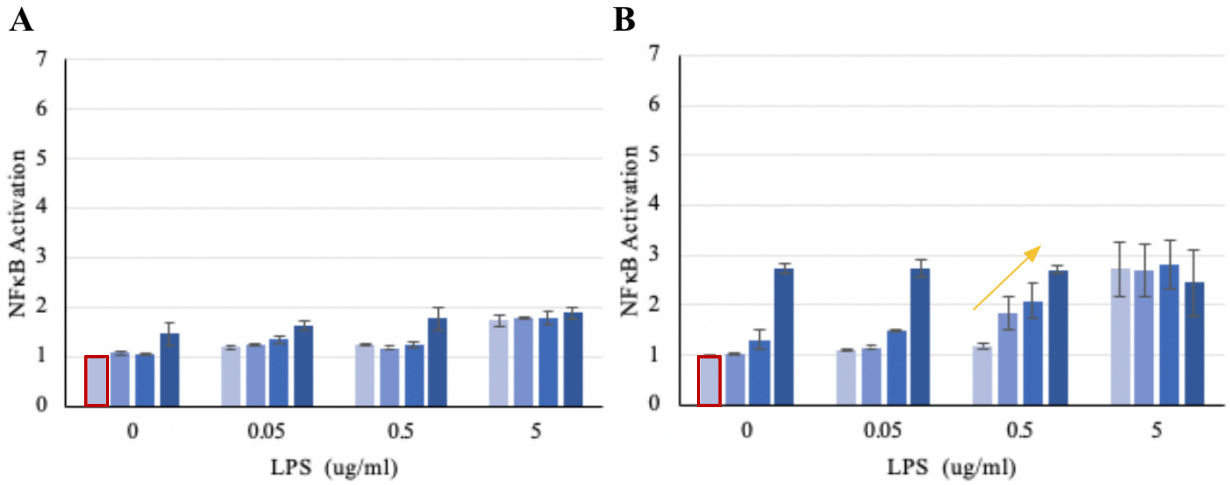

Figure 23 | The impact of LPS and R848 on NFκB activation. THP-1 cells were incubated with increasing concentrations of LPS and R848 respectively for **(A)** 6 hours and **(B)** 12 hours. All data represent the average and standard error of two separate experiments. All data show NFκB activation relative to cells without stimulation**;** 0 ug/ml LPS; 0 ug/ml R848 (outlined in red). The gradient of blue represents the concentration of R848. (■) = 0 ug/ml of R848; (■) = 0.05 ug/ml of R848; (■) = 0.5 ug/ml of R848; (■) = 5ug/ml of R848. LPS: lipopolysaccharides; Yellow Arrows: show a synergistic response.

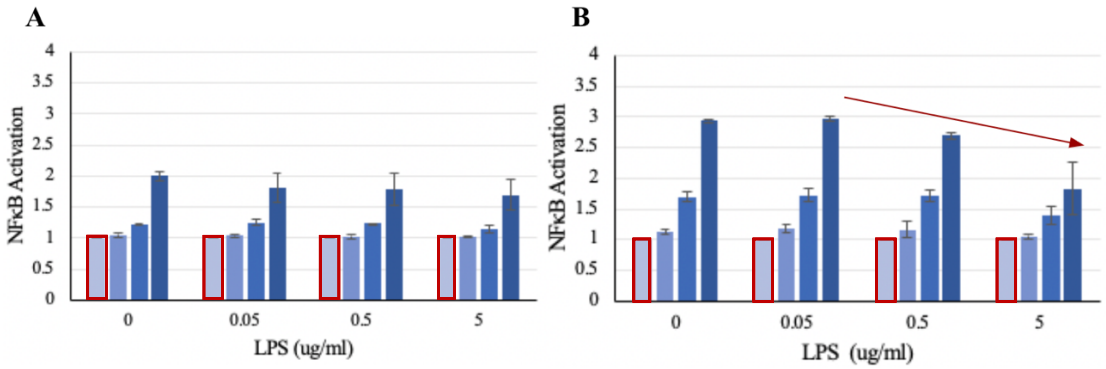

Figure 24 | The impact of LPS and Flagellin on NFκB activation. THP-1 cells were incubated with increasing concentrations of LPS and Flagellin respectively for **(A)** 6 hours and **(B)** 12 hours. All data represent the average and standard error of two separate experiments. All data show NFκB activation relative to cells receiving no Flagellin (0 ug/ml Fla) +/- LPS and Flagellin (outlined in red). The gradient of blue represents the concentration of Flagellin. (■) = 0 ug/ml of Fla; (■) = 0.05 ug/ml of Fla; (■) = 0.5 ug/ml of Fla; (■) = 5ug/ml of Fla. LPS: lipopolysaccharides; Fla: Flagellin; Red Arrows: show a competitive response.

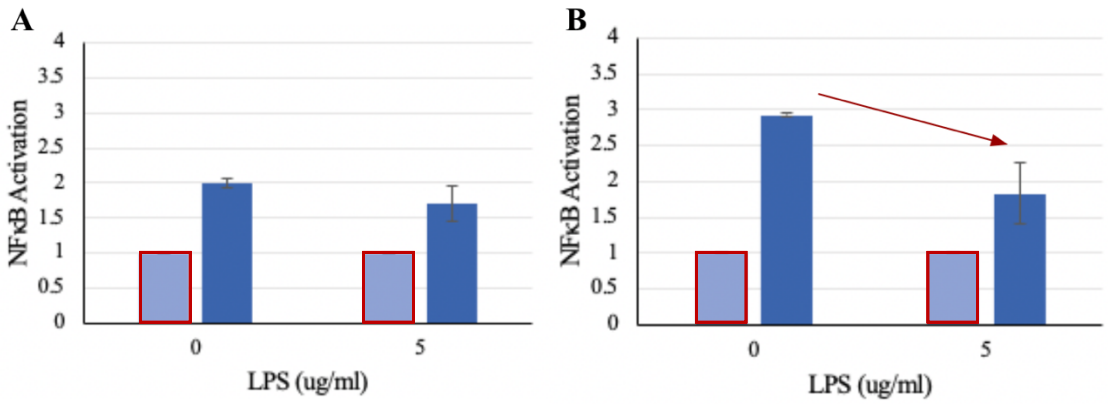

Figure 25 | The direct comparison of single VS co-signaling on NFκB activation. THP-1 were cells incubated with increasing concentrations of LPS and Flagellin for **(A)** 6 hours and **(B)** 12 hours. All data represent the average and standard error of two separate experiments. All data show NFκB activation relative to cells receiving no Flagellin (0 ug/ml Fla) +/- LPS and Flagellin (outlined in red). The gradient of blue represents the concentration of Flagellin. (▢) = 0 ug/ml of Fla; (■) = 5ug/ml of Fla. LPS: lipopolysaccharides; Fla: Flagellin; Red Arrows: show a competitive response.

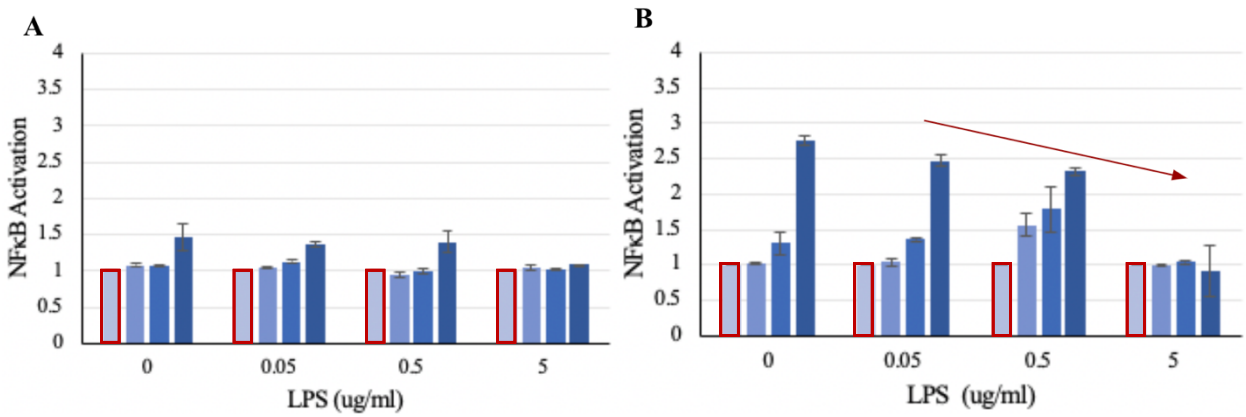

Figure 26 | The impact of LPS and R848 on NFκB activation. THP-1 cells were incubated with increasing concentrations of LPS and R848 respectively for **(A)** 6 hours and **(B)** 12 hours. All data represent the average and standard error of two separate experiments. All data show NFκB activation relative to cells receiving no R848 (0 ug/ml R848) +/- LPS and R848 (outlined in red). The gradient of blue represents the concentration of R848. (▪) = 0 ug/ml of R848; (▪) = 0.05 ug/ml of R848; (▪) = 0.5 ug/ml of R848; (▪) = 5ug/ml of R848. LPS: lipopolysaccharides; Red Arrows: show a competitive response.

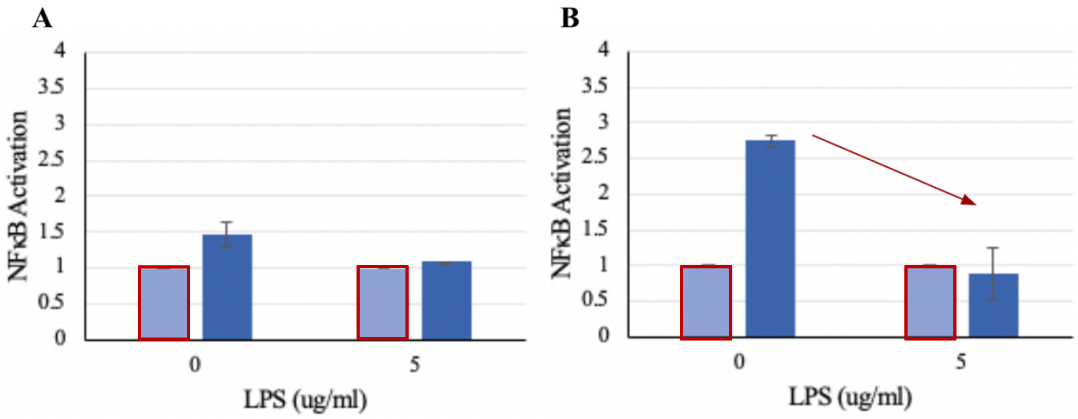

Figure 27 | The direct comparison of single VS co-signaling on NFκB activation. THP-1 were cells incubated with increasing concentrations of LPS and R848 for **(A)** 6 hours and **(B)** 12 hours. All data represent the average and standard error of two separate experiments. All data show NFκB activation relative to cells receiving no R848 (0 ug/ml R848) +/- LPS and R848 (outlined in red). The gradient of blue represents the concentration of R848. (■) = 0 ug/ml of R848; (■) = 5ug/ml of Fla. = 5ug/ml of R848. LPS: lipopolysaccharides; Red Arrows: show a competitive response.

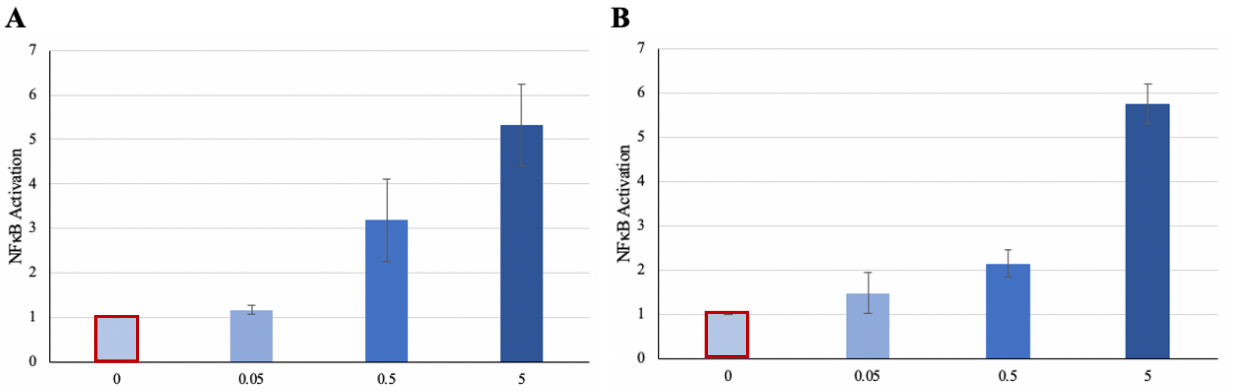

Figure 28 | The impact of LPS on NFκB activation. THP-1 cells were incubated with increasing concentrations of LPS for **(A)** 48 hours and **(B)** 96 hours. All data represent the average and standard error of three separate experiments. All data show NFκB activation relative to cells receiving no LPS (0 ug/ml LPS) (outlined in red). The gradient of blue represents the concentration of R848. (■) = 0 ug/ml of LPS; (■) = 0.05 ug/ml of LPS; (■) = 0.5 ug/ml of LPS; (■) = 5ug/ml of LPS. LPS: lipopolysaccharides.

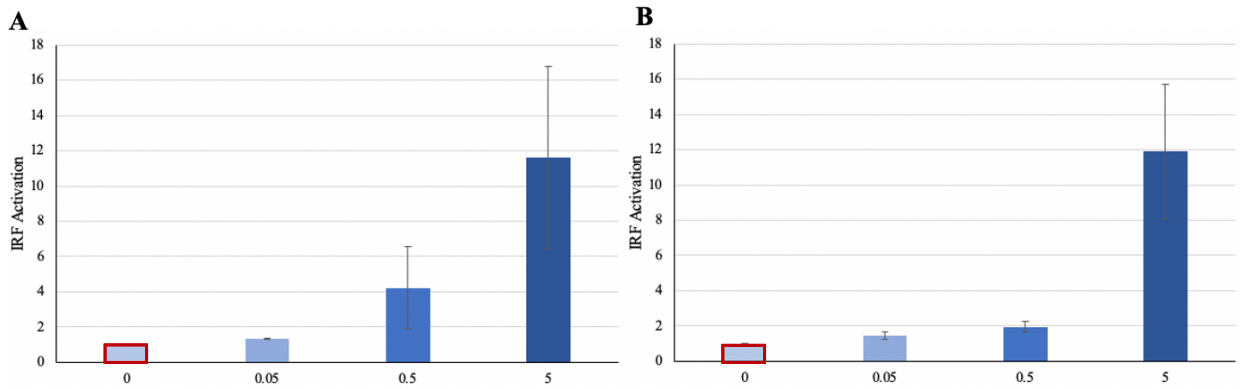

Figure 29 | The impact of LPS on IRF activation. THP-1 cells were incubated with increasing concentrations of LPS for **(A)** 48 hours and **(B)** 96 hours. All data represent the average and standard error of three separate experiments. All data show IRF activation relative to cells receiving no LPS (0 ug/ml LPS) (outlined in red). The gradient of blue represents the concentration of R848. (■) = 0 ug/ml of LPS; (■) = 0.05 ug/ml of LPS; (■) = 0.5 ug/ml of LPS; (■) = 5ug/ml of LPS. LPS: lipopolysaccharides.

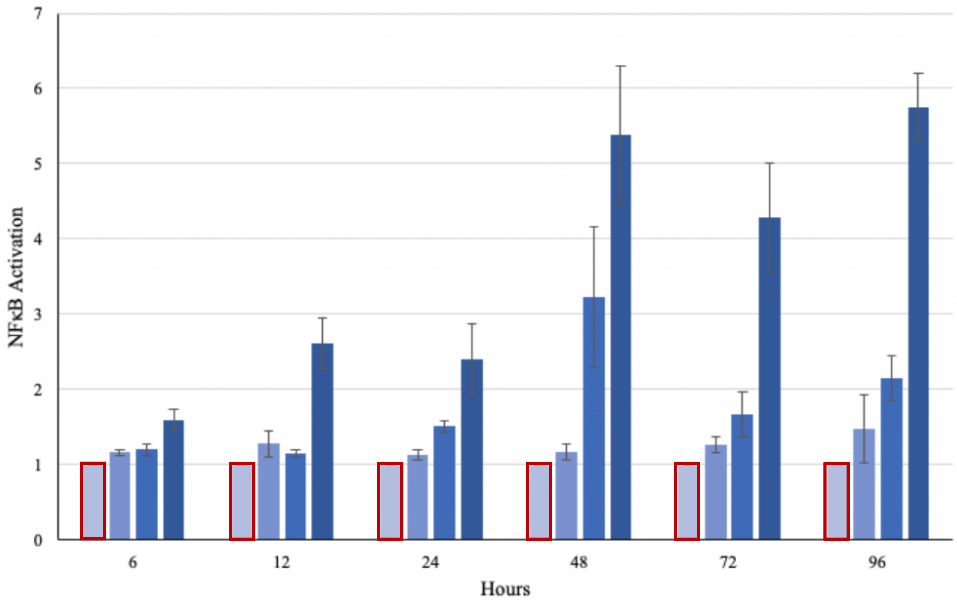

Figure 30 | The impact of LPS on NFκB activation. THP-1 cells were incubated with increasing concentrations of LPS for 6, 12, 24, 48, 72, and 96 hours. All data represent the average and standard error of three separate experiments. All data show NFκB activation relative to cells receiving no LPS (0 ug/ml LPS) (outlined in red). The gradient of blue represents the concentration of R848. (■) = 0 ug/ml of LPS; (■) = 0.05 ug/ml of LPS; (■) = 0.5 ug/ml of LPS; (■) = 5ug/ml of LPS. LPS: lipopolysaccharides.

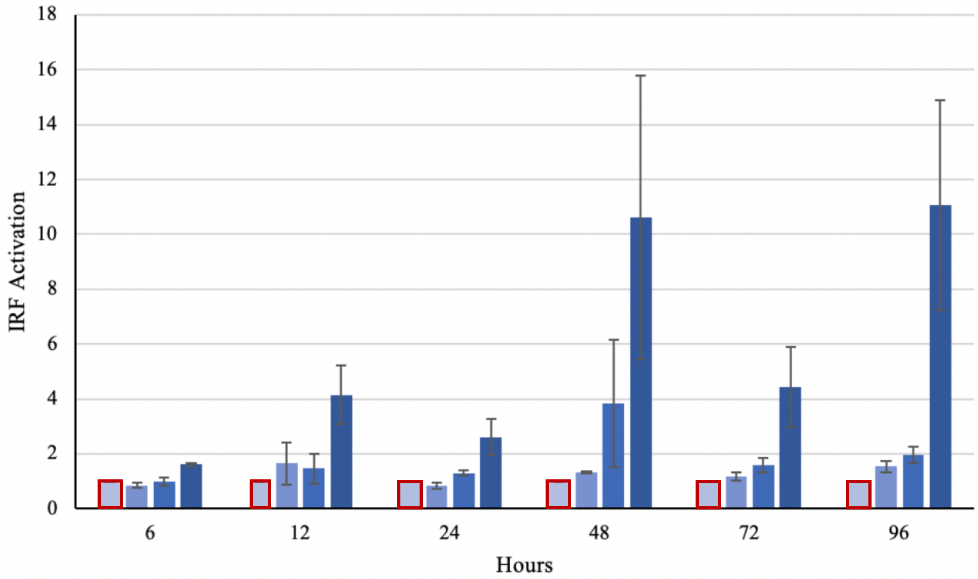

Figure 31 | The impact of LPS on IRF activation. THP-1 cells were incubated with increasing concentrations of LPS for 6, 12, 24, 48, 72, and 96 hours. All data represent the average and standard error of three separate experiments. All data show IRF activation relative to cells receiving no LPS (0 ug/ml LPS) (outlined in red). The gradient of blue represents the concentration of R848. (■) = 0 ug/ml of LPS; (■) = 0.05 ug/ml of LPS; (■) = 0.5 ug/ml of LPS; (■) = 5ug/ml of LPS. LPS: lipopolysaccharides.

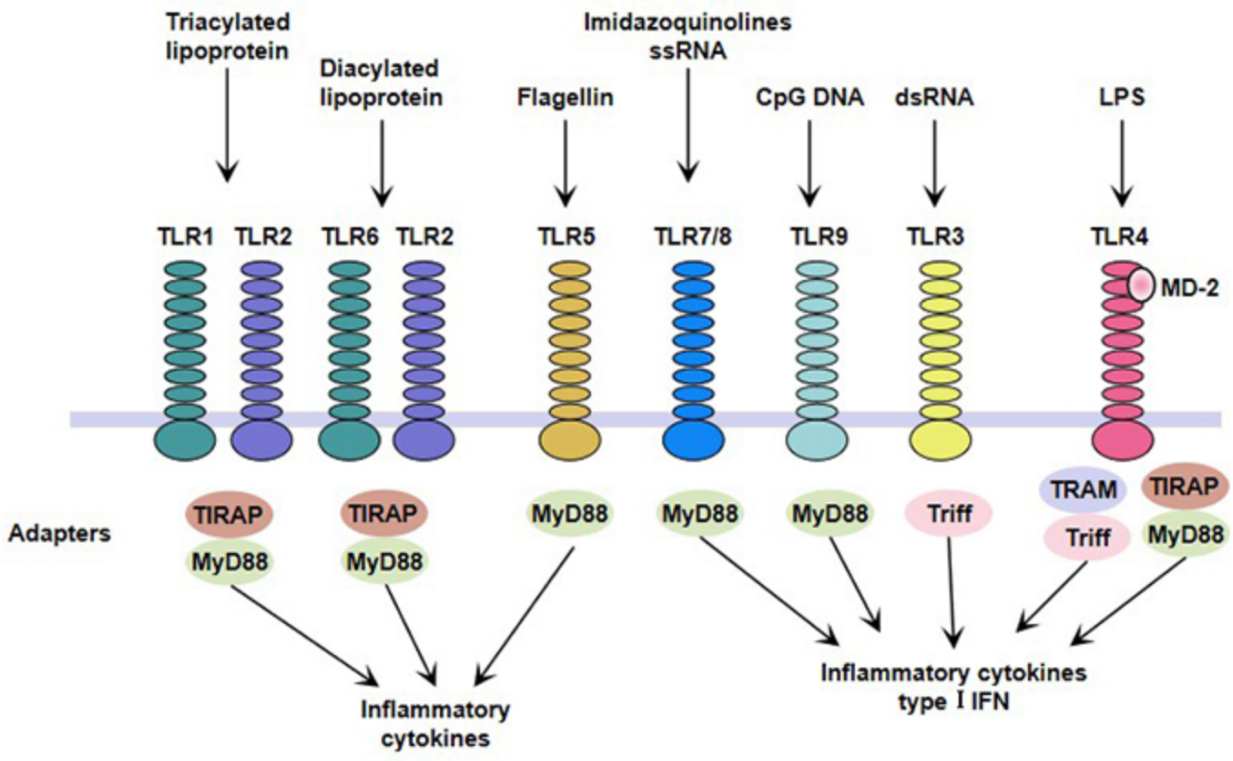

Figure 32 | TLRs 1-9 with their respective adaptors and signaling outcomes. Referenced from Toll-like Receptors--The Eye of Innate Immunity, 2021.

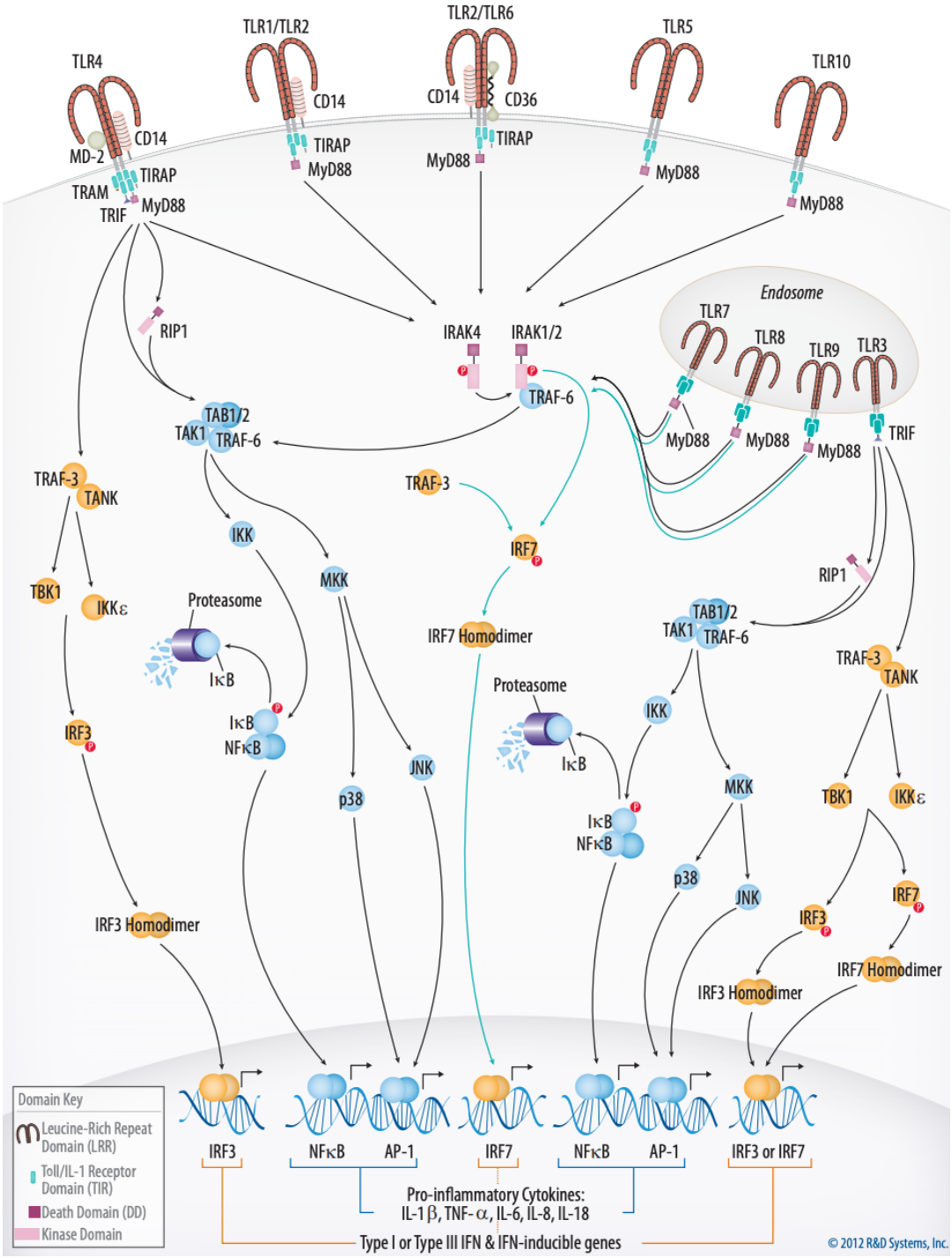

Figure 33 | Complexity of TLR signaling. Referenced from R&D System Inc, 2021.

TABLES

Table 1 | LPS and Flagellin NF-κB Correlation Data using Spearman's Correlation. Used RStudio and Figure 8 data to compare different concentration of LPS (0 ug/ml – 5ug/ml) with their respective increasing Flagellin concentrations. Spearman's correlation coefficient of ranges from -1 to 1 with .00-.19 "very weak" and .80-1.0 "very strong" and the negative sign as negative correlation.

24 Hours		72 Hours	
Type of Correlation	**Correlation #**	**Type of Correlation**	**Correlation #**
LPS 0 X LPS 0.05	1	LPS 0 X LPS 0.05	1
LPS 0 X LPS 0.5	1	LPS 0 X LPS 0.5	1
LPS 0 X LPS 5	1	LPS 0 X LPS 5	0.8
LPS 0.05 X LPS 0.5	1	LPS 0.05 X LPS 0.5	1
LPS 0.05 X LPS 5	1	LPS 0.05 X LPS 5	0.8
LPS 0.5 X LPS 5	1	LPS 0.5 X LPS 5	0.8

Table 2 | LPS and Flagellin NF-κB Correlation Data using Spearman's Correlation. Used RStudio and Figure 9 data to compare different concentration of LPS (0 ug/ml – 5ug/ml) with their respective increasing Flagellin concentrations. Spearman's correlation coefficient of ranges from -1 to 1 with .00-.19 "very weak" and .80-1.0 "very strong" and the negative sign as negative correlation.

24 Hours		72 Hours	
Type of Correlation	**Correlation #**	**Type of Correlation**	**Correlation #**
LPS 0 X LPS 5	1	LPS 0 X LPS 5	1

Table 3| LPS and R848 NF-κB Correlation Data using Spearman's Correlation. Used RStudio and Figure 10 data to compare different concentration of LPS (0 ug/ml – 5ug/ml) with their respective increasing R848 concentrations. Spearman's correlation coefficient of ranges from -1 to 1 with .00-.19 "very weak" and .80-1.0 "very strong" and the negative sign as negative correlation.

24 Hours		72 Hours	
Type of Correlation	**Correlation #**	**Type of Correlation**	**Correlation #**
LPS 0 X LPS 0.05	0.8	LPS 0 X LPS 0.05	0.8
LPS 0 X LPS 0.5	0.8	LPS 0 X LPS 0.5	0.8
LPS 0 X LPS 5	0.8	LPS 0 X LPS 5	0.8
LPS 0.05 X LPS 0.5	1	LPS 0.05 X LPS 0.5	1
LPS 0.05 X LPS 5	1	LPS 0.05 X LPS 5	1
LPS 0.5 X LPS 5	1	LPS 0.5 X LPS 5	1

Table 4 | LPS and R848 NF-κB Correlation Data using Spearman's Correlation. Used RStudio and Figure 11 data to compare different concentration of LPS (0 ug/ml – 5ug/ml) with their respective increasing R848 concentrations. Spearman's correlation coefficient of ranges from -1 to 1 with .00-.19 "very weak" and .80-1.0 "very strong" and the negative sign as negative correlation.

24 Hours		72 Hours	
Type of Correlation	**Correlation #**	**Type of Correlation**	**Correlation #**
LPS 0 X LPS 5	1	LPS 0 X LPS 5	1

Table 5 | LPS and Flagellin IRF Correlation Data using Spearman's Correlation. Used RStudio and Figure 12 data to compare different concentration of LPS (0 ug/ml – 5ug/ml) with their respective increasing Flagellin concentrations. Spearman's correlation coefficient of ranges from -1 to 1 with .00-.19 "very weak" and .80-1.0 "very strong" and the negative sign as negative correlation.

24 Hours		72 Hours	
Type of Correlation	**Correlation #**	**Type of Correlation**	**Correlation #**
LPS 0 X LPS 0.05	1	LPS 0 X LPS 0.05	1
LPS 0 X LPS 0.5	1	LPS 0 X LPS 0.5	1
LPS 0 X LPS 5	1	LPS 0 X LPS 5	1
LPS 0.05 X LPS 0.5	1	LPS 0.05 X LPS 0.5	1
LPS 0.05 X LPS 5	1	LPS 0.05 X LPS 5	1
LPS 0.5 X LPS 5	1	LPS 0.5 X LPS 5	1

Table 6 | LPS and R848 IRF Correlation Data using Spearman's Correlation. Used RStudio and Figure 13 data to compare different concentration of LPS (0 ug/ml – 5ug/ml) with their respective increasing R848 concentrations. Spearman's correlation coefficient of ranges from -1 to 1 with .00-.19 "very weak" and .80-1.0 "very strong" and the negative sign as negative correlation.

24 Hours		72 Hours	
Type of Correlation	**Correlation #**	**Type of Correlation**	**Correlation #**
LPS 0 X LPS 0.05	1	LPS 0 X LPS 0.05	1
LPS 0 X LPS 0.5	0.8	LPS 0 X LPS 0.5	1
LPS 0 X LPS 5	0.8	LPS 0 X LPS 5	1
LPS 0.05 X LPS 0.5	0.8	LPS 0.05 X LPS 0.5	1
LPS 0.05 X LPS 5	0.8	LPS 0.05 X LPS 5	1
LPS 0.5 X LPS 5	1	LPS 0.5 X LPS 5	1

Table 7 | LPS and Flagellin IRF Correlation Data using Spearman's Correlation. Used RStudio and Figure 14 data to compare different concentration of LPS (0 ug/ml – 5ug/ml) with their respective increasing Flagellin concentrations. Spearman's correlation coefficient of ranges from -1 to 1 with .00-.19 "very weak" and .80-1.0 "very strong" and the negative sign as negative correlation.

24 Hours		72 Hours	
Type of Correlation	**Correlation #**	**Type of Correlation**	**Correlation #**
LPS 0 X LPS 5	1	LPS 0 X LPS 5	1

Table 8 | LPS and R848 IRF Correlation Data using Spearman's Correlation. Used RStudio and Figure 15 data to compare different concentration of LPS (0 ug/ml – 5ug/ml) with their respective increasing R848 concentrations. Spearman's correlation coefficient of ranges from -1 to 1 with .00-.19 "very weak" and .80-1.0 "very strong" and the negative sign as negative correlation.

24 Hours		72 Hours	
Type of Correlation	**Correlation #**	**Type of Correlation**	**Correlation #**
LPS 0 X LPS 5	1	LPS 0 X LPS 5	1

Table 9 | LPS and Flagellin IRF Correlation Data using Spearman's Correlation. Used RStudio and Figure 16 data to compare different concentration of LPS (0 ug/ml – 5ug/ml) with their respective increasing Flagellin concentrations. Spearman's correlation coefficient of ranges from -1 to 1 with .00-.19 "very weak" and .80-1.0 "very strong" and the negative sign as negative correlation.

6 Hours		12 Hours	
Type of Correlation	**Correlation #**	**Type of Correlation**	**Correlation #**
LPS 0 X LPS 0.05	0.4	LPS 0 X LPS 0.05	0.8
LPS 0 X LPS 0.5	0.2	LPS 0 X LPS 0.5	0.8
LPS 0 X LPS 5	0.4	LPS 0 X LPS 5	1
LPS 0.05 X LPS 0.5	0.8	LPS 0.05 X LPS 0.5	1
LPS 0.05 X LPS 5	1	LPS 0.05 X LPS 5	0.8
LPS 0.5 X LPS 5	0.8	LPS 0.5 X LPS 5	0.8

Table 10 | LPS and R848 IRF Correlation Data using Spearman's Correlation. Used RStudio and Figure 17 data to compare different concentration of LPS (0 ug/ml – 5ug/ml) with their respective increasing R848 concentrations. Spearman's correlation coefficient of ranges from -1 to 1 with .00-.19 "very weak" and .80-1.0 "very strong" and the negative sign as negative correlation.

6 Hours		12 Hours	
Type of Correlation	**Correlation #**	**Type of Correlation**	**Correlation #**
LPS 0 X LPS 0.05	0.8	LPS 0 X LPS 0.05	1
LPS 0 X LPS 0.5	0.8	LPS 0 X LPS 0.5	1
LPS 0 X LPS 5	0.2	LPS 0 X LPS 5	0.8
LPS 0.05 X LPS 0.5	1	LPS 0.05 X LPS 0.5	1
LPS 0.05 X LPS 5	0.4	LPS 0.05 X LPS 5	0.8
LPS 0.5 X LPS 5	0.4	LPS 0.5 X LPS 5	0.8

Table 11 | LPS and Flagellin IRF Correlation Data using Spearman's Correlation. Used RStudio and Figure 18 data to compare different concentration of LPS (0 ug/ml – 5ug/ml) with their respective increasing Flagellin concentrations. Spearman's correlation coefficient of ranges from -1 to 1 with .00-.19 "very weak" and .80-1.0 "very strong" and the negative sign as negative correlation.

6 Hours		12 Hours	
Type of Correlation	**Correlation #**	**Type of Correlation**	**Correlation #**
LPS 0 X LPS 5	1	LPS 0 X LPS 5	1

Table 12 | LPS and Flagellin IRF Correlation Data using Spearman's Correlation. Used RStudio and Figure 20 data to compare different concentration of LPS (0 ug/ml – 5ug/ml) with their respective increasing Flagellin concentrations. Spearman's correlation coefficient of ranges from -1 to 1 with .00-.19 "very weak" and .80-1.0 "very strong" and the negative sign as negative correlation.

6 Hours		12 Hours	
Type of Correlation	**Correlation #**	**Type of Correlation**	**Correlation #**
LPS 0 X LPS 0.05	0.4	LPS 0 X LPS 0.05	0.8
LPS 0 X LPS 0.5	0.2	LPS 0 X LPS 0.5	0.8
LPS 0 X LPS 5	0.4	LPS 0 X LPS 5	1
LPS 0.05 X LPS 0.5	0.8	LPS 0.05 X LPS 0.5	1
LPS 0.05 X LPS 5	1	LPS 0.05 X LPS 5	0.8
LPS 0.5 X LPS 5	0.8	LPS 0.5 X LPS 5	0.8

Table 13 | LPS and R848 IRF Correlation Data using Spearman's Correlation. Used RStudio and Figure 21 data to compare different concentration of LPS (0 ug/ml – 5ug/ml) with their respective increasing R848 concentrations. Spearman's correlation coefficient of ranges from -1 to 1 with .00-.19 "very weak" and .80-1.0 "very strong" and the negative sign as negative correlation.

6 Hours		12 Hours	
Type of Correlation	**Correlation #**	**Type of Correlation**	**Correlation #**
LPS 0 X LPS 0.05	0.6	LPS 0 X LPS 0.05	1
LPS 0 X LPS 0.5	0.8	LPS 0 X LPS 0.5	1
LPS 0 X LPS 5	-0.4	LPS 0 X LPS 5	0.4
LPS 0.05 X LPS 0.5	0.9	LPS 0.05 X LPS 0.5	1
LPS 0.05 X LPS 5	0.3	LPS 0.05 X LPS 5	0.4
LPS 0.5 X LPS 5	0	LPS 0.5 X LPS 5	0.4

LIST OF ABBREVIATIONS

PRRs = Pattern Recognition Receptors

TLRs = Toll-Like Receptors

LPS = Lipopolysaccharides

PAMPs = Pathogen-Associated Molecular Patterns

NF-κB = Nuclear Factor kappa B

IRF = Interferon Regulatory Factor

TIR = Toll/interleukin-1 receptor

Type I IFN = Type I interferon

MyD88 = Myeloid differentiation primary responses 88

MAL/TIRAP = MyD88-adaptor like protein

TRIF = TIR domain containing adaptor protein inducing interferon-β

TRAM = TRIF-related adaptor molecule

SARM = Sterile α- and armadillo-motif-containing protein

E.Coli = *Escherichia coli*

ssRNA = Single stranded RNA

NAIP = NLR family, apoptosis inhibitory proteins

RLR = Retinoic acid-inducible gene-I-like receptors

PKR = Protein Kinase R

S.pyogenes = *Streptococcus pyogenes*

Th17 = T helper type 17

APPENDIX: R - Codes

```
#IRF - Standardized to No Fla - 24Hours

def <- tibble(x = c(1,2,3,4), #LPSConc
              c1 = c(1,1.334555454,1.94454628,3.10907424),#Fla0-5XLPS0
              c2 = c(1,2.1127703,2.3650875,4.5386200),#Fla0-5XLPS0.05
              c3 = c(1,1.73366261,2.32256838,3.3487841),#Fla0-5XLPS0.5
              c4 = c(1,1.13049013,1.668841502,2.242202419)#Fla0-5XLPS5
)

cor12=cor(def$c1,def$c2,method="spearman")
cor13=cor(def$c1,def$c3,method="spearman")
cor14=cor(def$c1,def$c4,method="spearman")
cor23=cor(def$c2,def$c3,method="spearman")
cor24=cor(def$c2,def$c4,method="spearman")
cor34=cor(def$c2,def$c4,method="spearman")

#IRF - Standardized to No Fla - 72Hours

def <- tibble(x = c(1,2,3,4), #LPSConc
             c1 = c(1,2.168071798,2.59423443,6.661680718),#Fla0-5XLPS0
             c2 = c(1,1.7084465,2.15073833,4.80283520),#Fla0-5XLPS0.05
             c3 = c(1,1.4188764,2.07092770,4.096638994),#Fla0-5XLPS0.5
             c4 = c(1,1.269683167,1.534027395,1.840766138)#Fla0-5XLPS5
)

cor12=cor(def$c1,def$c2,method="spearman")
cor13=cor(def$c1,def$c3,method="spearman")
cor14=cor(def$c1,def$c4,method="spearman")
cor23=cor(def$c2,def$c3,method="spearman")
cor24=cor(def$c2,def$c4,method="spearman")
cor34=cor(def$c2,def$c4,method="spearman")

#IRF - Standardized to No R848 - 24Hours

def <- tibble(x = c(1,2,3,4), #LPSConc
              c1 = c(1,0.99273255,3.50436046,16.7198401),#R8480-5XLPS0
              c2 = c(1,0.991235,5.2532866,22.8821209),#R8480-5XLPS0.05
              c3 = c(1,2.1151038,4.0701858,14.1510387),#R8480-5XLPS0.5
              c4 = c(1,1.08514796,2.57705192,7.707844779)#R8480-5XLPS5
)

cor12=cor(def$c1,def$c2,method="spearman")
cor13=cor(def$c1,def$c3,method="spearman")
cor14=cor(def$c1,def$c4,method="spearman")
cor23=cor(def$c2,def$c3,method="spearman")
cor24=cor(def$c2,def$c4,method="spearman")
cor34=cor(def$c2,def$c4,method="spearman")
```

```
#IRF - Standardized to No R848 - 72Hours

def <- tibble(x = c(1,2,3,4), #LPSConc
              c1 = c(1,1.1340606,15.68980908,33.7054302),#R8480-5XLPS0
              c2 = c(1,1.0652513,8.4302901,27.634748),#R8480-5XLPS0.05
              c3 = c(1,1.67972636,9.9119506,30.580275),#R8480-5XLPS0.5
              c4 = c(1,1.47454018,6.71423651,14.2379431)#R8480-5XLPS5
)

cor12=cor(def$c1,def$c2,method="spearman")
cor13=cor(def$c1,def$c3,method="spearman")
cor14=cor(def$c1,def$c4,method="spearman")
cor23=cor(def$c2,def$c3,method="spearman")
cor24=cor(def$c2,def$c4,method="spearman")
cor34=cor(def$c2,def$c4,method="spearman")


#IRF - Direct Comparison- 24Hours - L+F

def <- tibble(x = c(1,2), #DirectLPSConc
              c1 = c(1,3.109074244), #Fla0&5 X LPS0
              c2 = c(1,2.242202419), #Fla0&5 X LPS5
)

cor12=cor(def$c1,def$c2,method="spearman")

#IRF - Direct Comparison- 72Hours - L+F

> def <- tibble(x = c(1,2), #DirectLPSConc
              c1 = c(1,6.661680718), #Fla0&5 X LPS0
              c2 = c(1,1.840766138), #Fla0&5 X LPS5
)

cor12=cor(def$c1,def$c2,method="spearman")

#IRF - Direct Comparison- 24Hours - L+R

def <- tibble(x = c(1,2), #DirectLPSConc
              c1 = c(1,16.71984012), #R8480&5 X LPS0
              c2 = c(1,7.707844779), #R8480&5 X LPS5
)

cor12=cor(def$c1,def$c2,method="spearman")

#IRF - Direct Comparison- 72Hours - L+R

def <- tibble(x = c(1,2), #DirectLPSConc
              c1 = c(1,33.7054302), #R8480&5 X LPS0
              c2 = c(1,14.23794318), #R8480&5 X LPS5
)

cor12=cor(def$c1,def$c2,method="spearman")
```

```
#NFkB - Standardized to No Fla - 24Hours

def <- tibble(x = c(1,2,3,4), #LPSConc
              c1 = c(1,1.67211381,2.74288343,3.748286715),#Fla0-5XLPS0
              c2 = c(1,1.4303342,2.16337930,2.7973841),#Fla0-5XLPS0.05
              c3 = c(1,1.4717779,2.0502351,2.521730883),#Fla0-5XLPS0.5
              c4 = c(1,1.1683845,1.374000872,1.587294945),#Fla0-5XLPS5
)

cor12=cor(def$c1,def$c2,method="spearman")
cor13=cor(def$c1,def$c3,method="spearman")
cor14=cor(def$c1,def$c4,method="spearman")
cor23=cor(def$c2,def$c3,method="spearman")
cor24=cor(def$c2,def$c4,method="spearman")
cor34=cor(def$c2,def$c4,method="spearman")


#NFkB - Standardized to No Fla - 72Hours

def <- tibble(x = c(1,2,3,4), #LPSConc
              c1 = c(1,2.3048106,3.46770333,4.904727109), #Fla0-5XLPS0
              c2 = c(1,2.0495227,3.050517,4.09705646), #Fla0-5XLPS0.05
              c3 = c(1,1.8888428,2.79625322,3.7268732), #Fla0-5XLPS0.5
              c4 = c(1,1.1663675,1.119711761,1.342849376) #Fla0-5XLPS5
)

cor12=cor(def$c1,def$c2,method="spearman")
cor13=cor(def$c1,def$c3,method="spearman")
cor14=cor(def$c1,def$c4,method="spearman")
cor23=cor(def$c2,def$c3,method="spearman")
cor24=cor(def$c2,def$c4,method="spearman")
cor34=cor(def$c2,def$c4,method="spearman")


#NFkB - Standardized to No R848 - 24Hours

def <- tibble(x = c(1,2,3,4), #LPSConc
              c1 = c(1,0.99639839,1.7658955,3.56046546), #R8480-5XLPS0
              c2 = c(1,1.101867,1.8119931,3.0898254), #R8480-5XLPS0.05
              c3 = c(1,1.423778,1.531705,2.343963142), #R8480-5XLPS0.5
              c4 = c(1,1.0996418,1.357093347,1.67629386) #R8480-5XLPS5
)

cor12=cor(def$c1,def$c2,method="spearman")
cor13=cor(def$c1,def$c3,method="spearman")
cor14=cor(def$c1,def$c4,method="spearman")
cor23=cor(def$c2,def$c3,method="spearman")
cor24=cor(def$c2,def$c4,method="spearman")
cor34=cor(def$c2,def$c4,method="spearman")
```

```
#NFkB - Standardized to No R848 - 72Hours

def <- tibble(x = c(1,2,3,4), #LPSConc
              c1 = c(1,0.9974493,6.18683841,7.47672490), #R8480-5XLPS0
              c2 = c(1,1.002015,4.1164196,5.2207437), #R8480-5XLPS0.05
              c3 = c(1,1.51746,3.4291280,4.114082619), #R8480-5XLPS0.5
              c4 = c(1,1.0695900,1.608317,1.794149184) #R8480-5XLPS5
)

cor12=cor(def$c1,def$c2,method="spearman")
cor13=cor(def$c1,def$c3,method="spearman")
cor14=cor(def$c1,def$c4,method="spearman")
cor23=cor(def$c2,def$c3,method="spearman")
cor24=cor(def$c2,def$c4,method="spearman")
cor34=cor(def$c2,def$c4,method="spearman")


#NFkB - Direct Comparison- 24Hours - L+F

def <- tibble(x = c(1,2), #DirectLPSConc
              c1 = c(1,3.748286715), #Fla0&5 X LPS0
              c2 = c(1,2.242202419), #Fla0&5 X LPS5
)

cor12=cor(def$c1,def$c2,method="spearman")

#NFkB - Direct Comparison- 72Hours - L+F

def <- tibble(x = c(1,2), #DirectLPSConc
              c1 = c(1,4.904727109), #Fla0&5 X LPS0
              c2 = c(1,1.342849376), #Fla0&5 X LPS5
)

cor12=cor(def$c1,def$c2,method="spearman")


#NFkB - Direct Comparison- 24Hours - L+R

def <- tibble(x = c(1,2), #DirectLPSConc
              c1 = c(1,3.560465426), #R8480&5 X LPS0
              c2 = c(1,1.676293869), #R8480&5 X LPS5
)

cor12=cor(def$c1,def$c2,method="spearman")

#NFkB - Direct Comparison- 72Hours - L+R

def <- tibble(x = c(1,2), #DirectLPSConc
              c1 = c(1,7.476724908), #R8480&5 X LPS0
              c2 = c(1,1.794149184), #R8480&5 X LPS5
)

cor12=cor(def$c1,def$c2,method="spearman")
```

```
#IRF - 6 Hours - L+F - Figure 16

def <- tibble(x = c(1,2,3,4), #LPSConc
              c1 = c(1,0.88262910,1.1408450,1.633802817), #Fla0-5XLPS0
              c2 = c(1,1.0825082,1.0495049,1.5775577), #Fla0-5XLPS0.05
              c3 = c(1,1.1345291,0.9551569,1.62780269), #Fla0-5XLPS0.5
              c4 = c(1,1.1558073,1.014164306,2.076487252) #Fla0-5XLPS5

)

cor12=cor(def$c1,def$c2,method="spearman")
cor13=cor(def$c1,def$c3,method="spearman")
cor14=cor(def$c1,def$c4,method="spearman")
cor23=cor(def$c2,def$c3,method="spearman")
cor24=cor(def$c2,def$c4,method="spearman")
cor34=cor(def$c2,def$c4,method="spearman")


#IRF - 12 Hours - L+F - Figure 16

def <- tibble(x = c(1,2,3,4) #LPSConc
              c1 = c(1,0.91847826,1.47554347,3.44565217), #Fla0-5XLPS0
              c2 = c(1,1.326633,1.741206,4.4422111), #Fla0-5XLPS0.05
              c3 = c(1,1.15625,1.43526785,3.276785714), #Fla0-5XLPS0.5
              c4 = c(1,0.86439195,1.03062117,1.756780402) #Fla0-5XLPS5

)

cor12=cor(def$c1,def$c2,method="spearman")
cor13=cor(def$c1,def$c3,method="spearman")
cor14=cor(def$c1,def$c4,method="spearman")
cor23=cor(def$c2,def$c3,method="spearman")
cor24=cor(def$c2,def$c4,method="spearman")
cor34=cor(def$c2,def$c4,method="spearman")

#IRF - Direct Comparison- 6 hours - L+F - Figure 18

def <- tibble(x = c(1,2), #DirectLPSConc
              c1 = c(1,1.633802817), #Fla0&5 X LPS0
              c2 = c(1,2.076487252), #Fla0&5 X LPS5
)

cor12=cor(def$c1,def$c2,method="spearman")

#IRF - Direct Comparison- 12 hours - L+F - Figure 18

def <- tibble(x = c(1,2), #DirectLPSConc
              c1 = c(1,3.445652174), #Fla0&5 X LPS0
              c2 = c(1,1.756780402), #Fla0&5 X LPS5
)

cor12=cor(def$c1,def$c2,method="spearman")
```

```
#IRF - 6 Hours - L+F - Figure 20

def <- tibble(x = c(1,2,3,4) #LPSConc
              c1 = c(1,0.8826291,1.14084507,1.633802817), #Fla0-5XLPS0
              c2 = c(1.422535,1.53990,1.49295,2.24413),#Fla0-5XLPS0.05
              c3 = c(1.046948,1.1877934,1,1.704225352), #Fla0-5XLPS0.5
              c4 = c(1.657276,1.91549,1.680751,3.4413145) #Fla0-5XLPS5

)

cor12=cor(def$c1,def$c2,method="spearman")
cor13=cor(def$c1,def$c3,method="spearman")
cor14=cor(def$c1,def$c4,method="spearman")
cor23=cor(def$c2,def$c3,method="spearman")
cor24=cor(def$c2,def$c4,method="spearman")
cor34=cor(def$c2,def$c4,method="spearman")


#IRF - 12 Hours - L+F - Figure 20

def <- tibble(x = c(1,2,3,4) #LPSConc
              c1 = c(1,0.918478,1.475543478,3.445652174), #Fla0-5XLPS0
              c2 = c(1.08152,1.434782,1.88315,4.8043), #Fla0-5XLPS0.05
              c3 = c(1.21739,1.407608,1.74728,3.989130),#Fla0-5XLPS0.5
              c4 = c(3.105978,2.684782,3.201086,5.456521) #Fla0-5XLPS5

)

cor12=cor(def$c1,def$c2,method="spearman")
cor13=cor(def$c1,def$c3,method="spearman")
cor14=cor(def$c1,def$c4,method="spearman")
cor23=cor(def$c2,def$c3,method="spearman")
cor24=cor(def$c2,def$c4,method="spearman")
cor34=cor(def$c2,def$c4,method="spearman")


#IRF - 6 Hours - L+R - Figure 17

def <- tibble(x = c(1,2,3,4) #LPSConc
              c1 = c(1,1.145921,0.9992055,1.461864407),#R8480-5XLPS0
              c2 = c(1,1.2138830,1.1786469,2.4866102),#R8480-5XLPS0.05
              c3 = c(1,1.1991869,1.122967,1.7311991),#R8480-5XLPS0.5
              c4 = c(1,0.8608728,1.09119496,1.162092624)#R8480-5XLPS5

)

cor12=cor(def$c1,def$c2,method="spearman")
cor13=cor(def$c1,def$c3,method="spearman")
cor14=cor(def$c1,def$c4,method="spearman")
cor23=cor(def$c2,def$c3,method="spearman")
cor24=cor(def$c2,def$c4,method="spearman")
cor34=cor(def$c2,def$c4,method="spearman")
```

```
#IRF - 12 Hours - L+R - Figure 17

def <- tibble(x = c(1,2,3,4) #LPSConc
              c1 = c(1,1.1103839,1.7792321,6.325635296),#R8480-5XLPS0
              c2 = c(1,1.20139,5.86119,7.690191),#R8480-5XLPS0.05
              c3 = c(1,2.3056442,3.1778073,7.3157565),#R8480-5XLPS0.5
              c4 = c(1,1.3590738,1.312534184,2.227751612)#R8480-5XLPS5

)

cor12=cor(def$c1,def$c2,method="spearman")
cor13=cor(def$c1,def$c3,method="spearman")
cor14=cor(def$c1,def$c4,method="spearman")
cor23=cor(def$c2,def$c3,method="spearman")
cor24=cor(def$c2,def$c4,method="spearman")
cor34=cor(def$c2,def$c4,method="spearman")


#IRF - 6 Hours - L+R - Figure 21

def <- tibble(x = c(1,2,3,4) #LPSConc
              c1 = c(1,1.1428571,0.9340659,1.450549451),#R8480-5XLPS0
              c2 = c(0.83516,1.04395,1.04395,2.18681),#R8480-5XLPS0.05
              c3 = c(0.9780,1.208791,1.120879,1.73626),#R8480-5XLPS0.5
              c4 = c(1.67032,1.472527,1.945054,1.813186),#R8480-5XLPS5

)

cor12=cor(def$c1,def$c2,method="spearman")
cor13=cor(def$c1,def$c3,method="spearman")
cor14=cor(def$c1,def$c4,method="spearman")
cor23=cor(def$c2,def$c3,method="spearman")
cor24=cor(def$c2,def$c4,method="spearman")
cor34=cor(def$c2,def$c4,method="spearman")


#IRF - 12 Hours - L+R - Figure 21

def <- tibble(x = c(1,2,3,4) #LPSConc
              c1 = c(1,1.1794687,1.8183776,6.307250538), #R8480-5XLPS0
              c2 = c(1.20818,1.3086,2.31371,8.16080), #R8480-5XLPS0.05
              c3 = c(1.16511,2.71572,3.03158,7.21895), #R8480-5XLPS0.5
              c4 = c(3.671213,5.26561,4.669059,4.97200), #R8480-5XLPS5

)

cor12=cor(def$c1,def$c2,method="spearman")
cor13=cor(def$c1,def$c3,method="spearman")
cor14=cor(def$c1,def$c4,method="spearman")
cor23=cor(def$c2,def$c3,method="spearman")
cor24=cor(def$c2,def$c4,method="spearman")
cor34=cor(def$c2,def$c4,method="spearman")
```