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Investigating the Antibacterial and Immunomodulatory Effects of Resolvin D2 on Monocytes and Macrophages

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INVESTIGATING THE ANTIBACTERIAL AND IMMUNOMODULATORY EFFECTS OF RESOLVIN D2 ON MONOCYTES AND MACROPHAGES

by

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A Dissertation

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Abstract

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Doctorate in Cell Biology and Neuroscience

In the late phase of sepsis, immunosuppression occurs, where the host is unable to clear the pre-existing infection and is susceptible to secondary infections. It is believed that the ideal treatments for sepsis should attenuate immunosuppression so that the host can get back to homeostasis. Specialized Pro-resolving Mediators (SPMs) are endogenously-produced fatty acids that resolve infectious inflammation without being immunosuppressive. We hypothesize that an SPM – Resolvin D2 (RvD2) – can augment exhausted macrophage function during the immunosuppressive phase of sepsis. We developed a two-hit model to establish macrophage exhaustion in vitro, and found that RvD2 increased NF-κB activity, TNF-α release, and bacterial clearance in exhausted macrophages compared to controls. Toll-like receptor-2 (TLR2) inhibition abolished RvD2-mediated changes in exhausted macrophages. In a mouse sepsis model, splenic macrophage response to exogenous LPS was reduced compared to controls and was restored by in vivo administration of RvD2. However, if RvD2 was added to monocytes before differentiation to macrophages, RvD2 reduced LPS responses and increased bacterial clearance. The results showed that RvD2 can attenuate macrophage suppression in vitro and in vivo and that this effect was macrophage-specific.

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Chapter 1

Introduction

1.1 Sepsis

Sepsis is defined as a life-threatening infection that can lead to organ failure. It is the leading cause of death in U.S. hospitals, and its high mortality rate may be attributed to a lack of diagnostics and viable treatment regimens. The early phase of sepsis is characterized by immune overactivation, where release of pro-inflammatory mediators may lead to multi-organ dysfunction. In the late phase of sepsis, there is paradoxical immunosuppression, where the host is susceptible to opportunistic infections (See **Figure 1**). Sepsis management typically focuses on blunting the early, hyper-proinflammatory phase of sepsis, where the patient's tissues and organs are overwhelmed by a release of cytokines – a phenomenon known as cytokine release syndrome (CRS). CRS is a clinical syndrome that comprises the early phase of sepsis. CRS can be defined as a hyper-proinflammatory immune response to a stimulus leading to elevated levels of pro-inflammatory cytokines, inflammation, and organ dysfunction (Athale et al., 2022). There is significant overlap between this definition and the consensus definition of the early phase of sepsis; however, in sepsis, this early phase typically is followed by a late phase of immunosuppression (Athale et al., 2022).

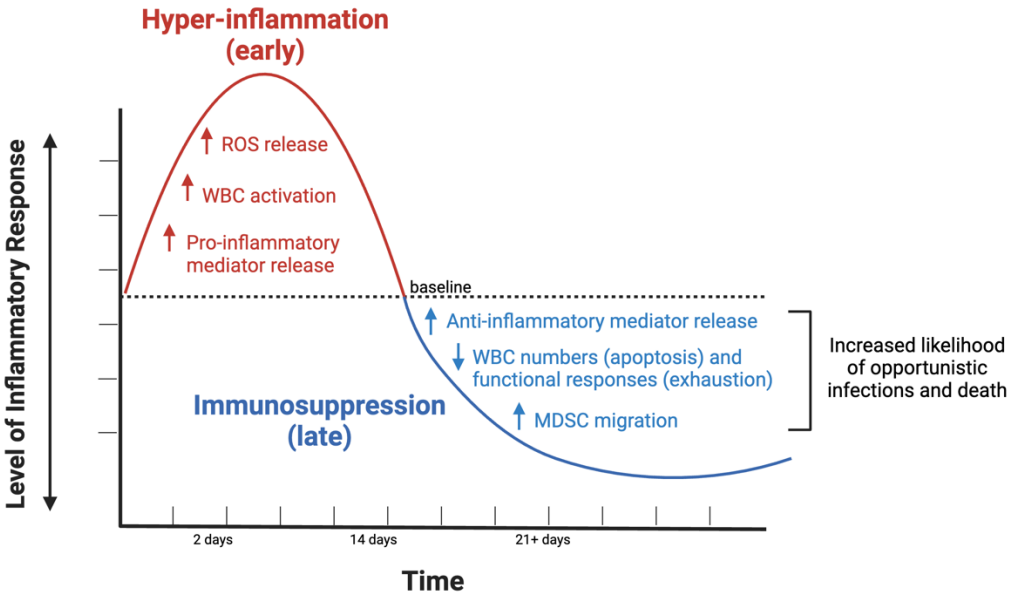


Figure 1. The Timeline of Sepsis. Sepsis is characterized by an early hyper-proinflammatory phase and a paradoxical late immunosuppressive phase in the host. If the host is unable to recover following the late phase, he or she is susceptible to an amalgam of medical conditions, including but not limited to multi-organ dysfunction, opportunistic infections, and potentially death. *Please note that information about figure sources can be found in Appendix B.*

Historically, there was very little focus on management of the late, immunosuppressive phase of sepsis. In this stage, the patient's immune system is considered to be hypoactive, as these deaths are attributed to the patient being unable to clear the original infection or to the acquisition of secondary infections (Liu et al., 2022). At present, there is no one treatment that has consistently been shown to reduce morbidity and mortality in sepsis patients, despite decades of research and hundreds of clinical trials (Marshall, 2014). Even though there have been recent advancements in antibiotic therapy, there is still a need for new, effective treatments for sepsis; however, before effective

modalities targeting the late phase of sepsis can be implemented, there needs to be a better understanding of the factors contributing to sepsis-induced immunosuppression.

1.2 The Innate Immune System and Response to Infection

Levels of defense against pathogens include host barriers and the human immune system (Esposito, 2021). Host barriers include physical, chemical, and biological obstacles that help prevent the entrance of pathogens. Physical barriers include the skin and mucous membranes, chemical barriers include pH and lysozymes, and biological barriers include the host's normal flora (Esposito, 2021). If these barriers are breached, then the pathogen will encounter the host's immune system (See **Figure 2**).

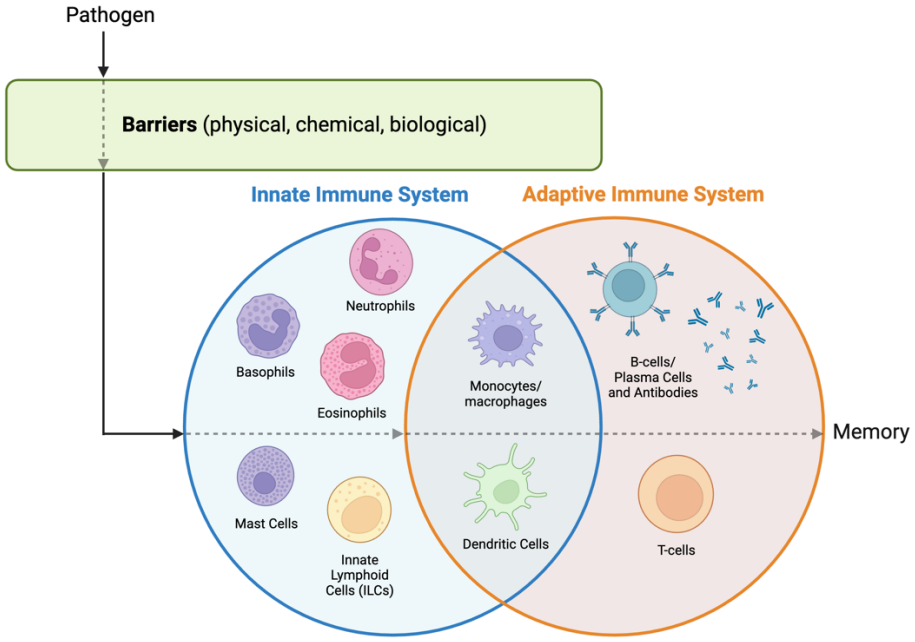


Figure 2. Host Barriers of Defense. Once a pathogen breaches the first line of defense (host barriers), it is then encountered by the innate immune system. Main cell players in the

innate immune system include neutrophils, basophils, eosinophils, mast cells, and innate lymphoid cells (ILCs; e.g., natural killer cells) (Chaplin, 2010; Esposito, 2021; Mazzurana et al., 2018). If the innate immune system is not successful at completely clearing this pathogen, the pathogen needs to contend with the adaptive immune system. Main cell players in the adaptive immune system include B cells, plasma cells, and T cells (Esposito, 2021). Monocytes/macrophages and dendritic cells are innate immune cells that are said to bridge both the innate and adaptive immune systems, as they are the main initiators of the adaptive immune system (Janeway et al., 2001). Once the adaptive immune system successfully removes the pathogen, immunological memory is established. Key: defense breach is depicted by a dotted arrow.

The human immune system can be subdivided into two systems: the innate immune system and the adaptive immune system. The innate immune response is quick-acting, does not discriminate, and does not develop memory, whereas the adaptive immune response is slow-acting, is highly specific, and develops memory (See **Table 1**) (Chaplin, 2010).

Table 1

The Innate vs. Adaptive Immune Systems

	Innate Immune System	Adaptive Immune System
Response Speed?	Quick	Slow (upon first exposure)
Nonspecific or Specific?	Nonspecific	Specific
Development of Traditional Immunologic Memory?	No	Yes

During infection, the immune system initiates a complex and tightly coordinated response not only to clear the infection from the host, but also to ensure it will be prepared the next time it encounters the same pathogen. The host's immune system utilizes a highly regulated balance of pro-inflammatory, anti-inflammatory, and resolution processes in order to achieve this. If one of these processes becomes dysregulated, the delicate homeostatic balance will become disrupted, ultimately leading to chronic disease and/or death.

The innate immune response acts first and is usually strong enough to clear an infection without triggering the adaptive immune response. A key feature of this immune system is that it must be able to differentiate between "self" and "non-self" so that it does not accidentally attack the body's own cells, thinking they are pathogens (Chaplin, 2010). It is able to perform such discrimination via recognition of pathogen-associated molecular patterns (PAMPs), conserved motifs found on a pathogen's surfaces, which help the immune system identify the pathogen as foreign, or "non-self" (Chaplin, 2010). Some examples of PAMPs are lipopolysaccharide (LPS) found in the outer membranes of gram-negative bacteria, peptidoglycans found in Gram-negative and Gram-positive bacterial cell walls, lipoteichoic acid from Gram-positive bacteria, single-stranded DNA and double-stranded RNA found in some viruses, and beta-glucans found in fungal cell walls (Chaplin, 2010). The innate immune system contains a series of pattern recognition receptors (PRRs), proteins that are able to recognize PAMPs; once activated, these PRRs elicit a series of cell signaling events that activate the cell's specific effector functions that will be helpful to rid the host of the invading pathogen – namely via the inflammatory response (Chaplin, 2010).

The inflammatory response is a crucial part of the body's innate immune system, and when it acts acutely, it is very effective at clearing an infection. The cardinal signs of inflammation include redness, heat, swelling, and pain (Zigterman and Dubois, 2022), and these are thought of as clinical manifestations of the protection offered by the innate immune system. The acute inflammatory response is dominated by neutrophils, white blood cells whose primary goals are to phagocytose the invading pathogen and to release pro-inflammatory mediators (Chen et al., 2018). They are able to travel to the site where the pathogen breached the first level of defense following the release of local mediators, such as histamine and nitric oxide, which both function to increase blood vessel vasodilation (Nedeva, 2021). Monocytes, which then differentiate into macrophages, are also recruited to the site of injury to assist with pathogen removal. Pro-inflammatory mediators are produced via activation of several signaling pathways, the most prevalent of which is the nuclear factor kappa light chain enhancer of activated B cells (NF-κB) pathway (Liu et al., 2017). The pro-inflammatory mediators produced downstream of this pathway include cytokines, chemokines, free radicals, and inflammatory lipid mediators (e.g., prostaglandins, leukotrienes) (See **Figure 3**). Many immune cells operate normally by utilizing the NF-κB pathway; thus, NF-κB status is an accepted marker for the state of an immune cell's functional capabilities (Yang et al., 2014). This concept will be highlighted again in the discussion of immune cell exhaustion later on.

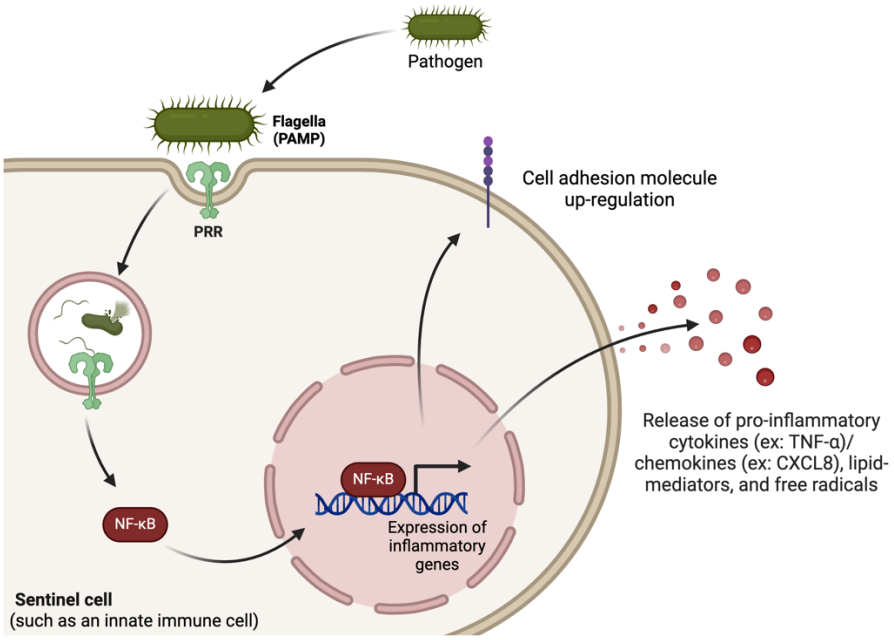


Figure 3. The Innate Immune Response to Infection. Activation of the innate immune system begins with sentinel cell recognition of "non-self." This usually occurs in the form of PAMP-PRR interactions. Many downstream signaling cascades can become triggered by this PAMP-PRR interaction (which are cell- and pathogen-specific), but one of the most important signaling pathways that becomes activated is the NF-κB pathway. The effector molecules produced via this pathway are pro-inflammatory cytokines/chemokines, lipid mediators, and free radicals (Liu et al., 2017).

NF-κB is a family of transcription factors which are ultimately responsible for regulating various genes involved in the inflammatory response. There are five protein members within the NF-κB family: p50, p52, p65, RelB, and c-Rel (Liu et al., 2017). A family of NF-κB regulatory (inhibitory) proteins, called IκB, sequester members of the NF-κB family into the cytoplasm (Liu et al., 2017). One mechanism of NF-κB activation involves ubiquitin-dependent degradation of IκB via phosphorylation (activation) of IKK (IκB kinase) complex (Liu et al., 2017). IKK complex becomes activated by various stimuli, including but not limited to cytokines, chemokines, and microbial components (such as PAMPs) (Liu et al., 2017). Once IκB is degraded, p50 and p65 rapidly translocate

to the nucleus and turn on/off regulatory inflammatory genes (Liu et al., 2017) (See **Figure 4**). Activation of the NF-κB pathway within innate immune cells such as macrophages involves detection of PAMPs via surface/intracellular PRRs (Liu et al., 2017). There are five families of PRRs, including the Toll-Like Receptor (TLR) family, which will be described below.

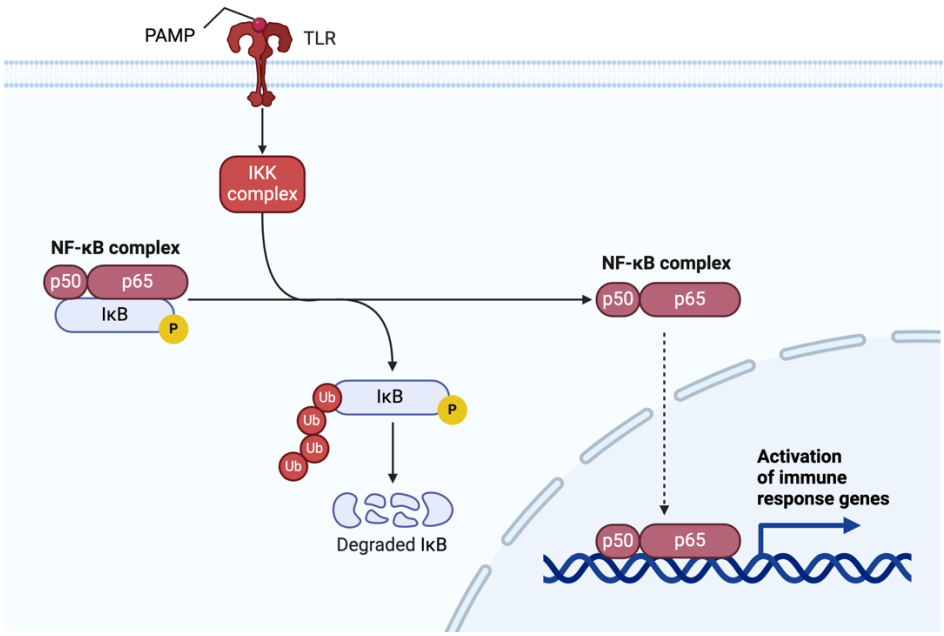


Figure 4. The NF-κB Pathway. Activation of the NF-κB pathway within innate immune cells involves recognition of microbial components (commonly PAMPs) by surface/intracellular PRRs (such as TLRs). The downstream signaling pathway that arises from the activation of PRRs by PAMPs ultimately leads to the translocation of active NF-κB family members into the nucleus. Once present in the nucleus, these components can regulate genes necessary for inflammatory processes.

TLRs are a type of PRR located on the surface of or within the cytoplasm of white blood cells and sentinel cells in the body. Normally, TLRs are inert unless acted upon by their ligands (Hug et al., 2018). TLRs allow the white blood cells and sentinel cells to sense

specific changes in their environment in the form of PAMPS or DAMPS (damage-associated molecular patterns; released by dying/damaged tissue and can be released if a microbe is causing harm to tissues), thereby allowing the cell to respond to that environmental change appropriately once activated – this is known as a PAMP-PRR interaction. Upon activation, TLRs homo- or hetero-dimerize, initiating a complex cell signaling cascade, ultimately culminating in the activation or inhibition of certain transcription factors, leading to downstream effector functions (Hug et al., 2018). If the TLR is located on an innate immune cell and becomes activated, the cell signaling cascade will ultimately lead to NF-κB activation (Hug et al., 2018).

Some TLRs span the cell's plasma membrane with the cell surface side being responsible for recognition of PAMPs/DAMPs and the cytoplasmic side, called the TIR domain (Toll-IL-1 receptor domain), being responsible for the initiation of the signaling cascade (Hug et al., 2018). If TLRs on innate immune cells are activated, the signaling cascade involves various adaptors that depend on the type(s) of TLR(s) that is (are) activated. While many adaptors exist and can function with various TLRs, two very common ones utilized are MYD88 (myeloid differentiation primary response protein 88) and TRIF (TIR domain-containing adaptor inducing IFN-β) (Hug et al., 2018). MYD88 is an adaptor utilized by all TLRs, whereas TRIF is an adaptor utilized specifically by TLR3 and TLR4 (Hug et al., 2018). Involved in this complex cell signaling cascade are Rho GTPase proteins, which are cell signaling proteins that play a regulation role in both the innate and adaptive immune systems (Manukyan et al., 2009). Previously, it was thought that PAMP-PRR interactions will *always* produce the same result; however, studies have

shown that the location of the TLR at the time of activation can actually change the signal output (Hug et al., 2018; Mukherjee et al., 2016).

TLR2 and TLR4 are two of the most important TLRs in terms of responding to infections, as they are excellent at recognizing PAMPs, initiating the proper innate immune response, and bridging the gap between the innate and adaptive immune systems if necessary (Mukherjee et al., 2016). TLR2 activation is dependent on MYD88 adaptor, as TLR2 hetero-dimerization triggers MYD88 binding, ultimately leading to downstream activation of NF-κB and production of cytokines (Hug et al., 2018). Importantly, pro- or anti-inflammatory cytokine production by the MYD88-dependent TLR2 activation pathway depends on which co-receptor TLR2 dimerizes with (Hug et al., 2018). While TLR4 can also act through MYD88 (i.e., MYD88-dependent TLR4 signaling), TLR4 activation can also trigger signal transduction pathways in a TRIF-dependent, MYD88-independent manner (Hug et al., 2018). LPS is a very potent activator of TLR4 homo-dimerization, as both MYD88-dependent *and* TRIF-dependent signal transduction pathways will become activated (Hug et al., 2018). The MYD88-dependent pathway leads to the translocation of NF-κB proteins and the production of pro-inflammatory mediators; the TRIF-dependent pathway also leads to the production of pro-inflammatory mediators as well as important anti-viral mediators called type I interferons (Hug et al., 2018) (See **Figure 5**). While these two TLRs, once activated, both produce similar downstream effectors and have similar cell signaling pathways, studies have shown that their total protein levels (both surface and intracellular receptor expression) can change in response to the same stimulus (Skinner et al., 2005). This is an important concept for not only understanding how the same TLR can mediate different effects across diverse cell types,

but also for understanding how the same PAMP does not always have the same effect across immune cells with the same TLR.

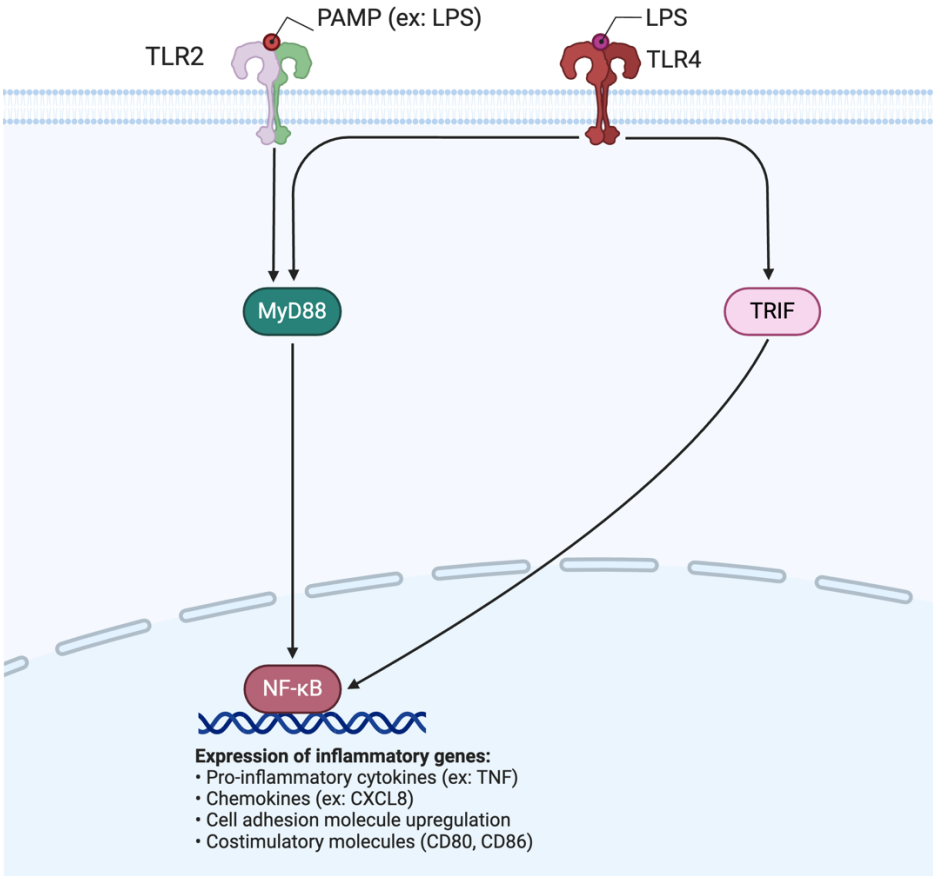


Figure 5. TLR2 and TLR4 Signaling Pathways. Upon activation, TLR2 and TLR4 homo- or hetero-dimerize, initiating a complex cell signaling cascade, ultimately culminating in the activation or inhibition of certain transcription factors. One transcription factor that lies downstream of TLR2 and TLR4's signaling pathways is NF-κB. NF-κB's activation leads to the production of cytokines and chemokines, upregulation of cell adhesion molecules, and presentation of co-stimulatory molecules (these molecules will be discussed in detail later when discussing the adaptive immune system).

Maintenance of the innate immune system relies on immunological homeostasis, whereby the host's pro- and anti-inflammatory responses balance one another in order to help return the host to homeostasis (Horwitz et al., 2019). Therefore, resolution circuits are activated in order to ensure that this response and other aspects of the innate immune response act acutely and do not persist, and these circuits function to reduce neutrophil activation and increase macrophage efferocytosis of apoptotic neutrophils (Kourtzelis et al., 2020; Serhan and Savill, 2005). This is a critical step in the acute inflammatory response – if this inflammatory response either persists for too long or fails to clear the pathogen, chronic inflammation can and will ensue (Chen et al., 2018). Because the innate immune system is always active within the host, it is typically not inhibited, but rather, it is regulated to help return the host to homeostasis; however, there are some ways in which the host can ensure its cessation as a backup mechanism (Rumpret et al., 2022). For example, in addition to increasing IL-10 (anti-inflammatory cytokine) levels and the activity of anti-inflammatory T cell subsets such as T-regulatory cells (Tregs), which both function to dampen the pro-inflammatory response, the innate immune system also modulates PRR expression on innate immune cells that favor inhibition rather than activation (Rumpret et al., 2022). These inhibitory PRRs (iPRRs) are said to fine-tune the degree of innate immune cell activation via inhibition of cell signaling pathways (Rumpret et al., 2022). The cell signaling pathways that will be explored in later chapters include NF-κB and TLR2 signaling pathways.

If this innate inflammatory process is not successful at eliminating the body of the infection, then the adaptive immune response will become activated. The adaptive immune response primarily comprises B and T lymphocytes and memory functions. The activation

of these lymphocytes leads to the production of antibodies (B cells) and increased macrophage signaling to clear pathogens (T cells).

1.3 The Adaptive Immune System and Response to Infection

The adaptive immune system is the last line of defense against invading pathogens. This system is triggered directly by T cell recognition of foreign invaders or by presentation of foreign antigen(s) to T cells by one of the three antigen presenting cells (APCs; monocytes/macrophages, dendritic cells, and B cells) (Nedeva, 2021). APCs contain major histocompatibility complex (MHC) class II (MHC-II) on their surfaces, and these molecules are required for MHC-restricted antigen presentation to T cells and the subsequent activation of the adaptive immune response (Cheadle, 1993; Jendro et al., 1991). From here, the activated T cells can help stimulate other immune cells (including other types of T cells), can activate B cells to produce class-switched antigen-specific antibodies, and can clear the invading pathogen (Nedeva, 2021). Finally, completion of this response allows for the development of immunological memory.

Overactivation of the adaptive immune system is commonly implicated in the development of autoimmune diseases (Petersone et al., 2018). This is likely because the adaptive immune response is robust and produces memory. With that being said, there are many regulatory molecules in place to ensure proper functioning and activation (i.e., that T and B cells will not be inappropriately activated). One such mechanism occurs during T and B cell development: positive and negative selection (i.e., central tolerance) (Xing and Hogquist, 2012). These selection processes occur during development to ensure that T and B cells both recognize foreign antigens but do not recognize them too strongly (Xing and

Hogquist, 2012). Once these lymphocytes "pass" these selection processes, they are able to circulate in the body; however, there are more selection mechanisms in place in the periphery that the host subjects the naïve lymphocytes to (i.e., peripheral tolerance) (Xing and Hogquist, 2012). For example, in order to ensure that activation of a naïve T lymphocyte is warranted, this activation process in the periphery requires two signals (Tai et al., 2018) (See **Figure 6**).

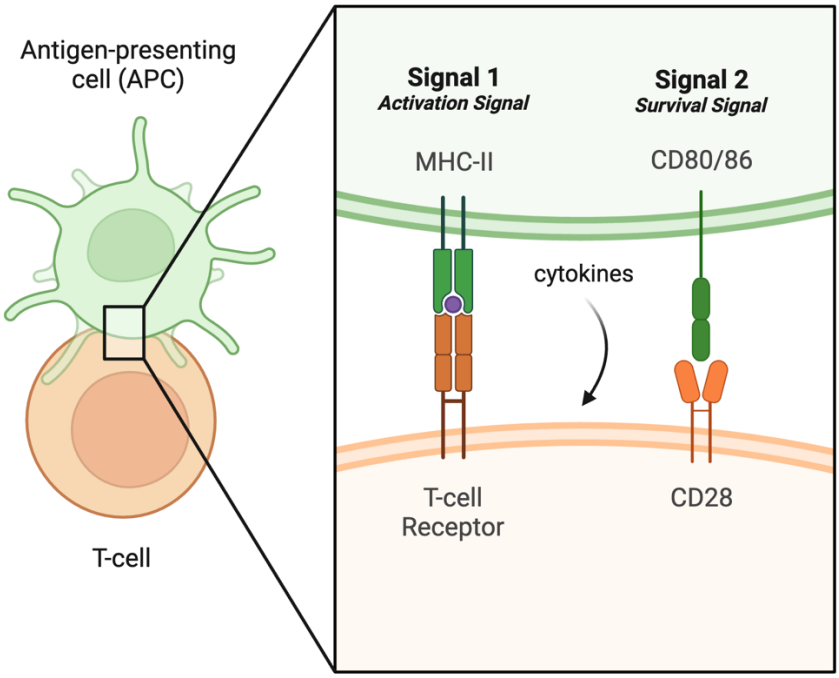


Figure 6. The Two-Signal Hypothesis of T Cell Activation. Activation of the adaptive immune system can begin with T cell activation by an APC. This schematic highlights the important concept of peripheral tolerance, or the two-signal hypothesis.

The first signal is the "activation signal," which comprises the interaction of the T cell receptor (TCR) on the T cell with an MHC-II molecule on an APC (Tai et al., 2018).

The second signal is the "survival signal," which comprises the interaction of the CD28 molecule on the T cell with a co-stimulatory molecule (CD80/86) on the APC (Tai et al., 2018). Once the T cell becomes activated, it is able to carry out its effector functions, depending on what is needed at that time for pathogen clearance (Tai et al., 2018). Typically, the T cell is "told" what is needed at that time for pathogen clearance via cytokines released by the APC onto the T cell (Tai et al., 2018). These effector functions include the additional release of cytokines, activation of B cells, direct destruction of the pathogen, or activation of other cells or T cell subtypes (Tai et al., 2018). Additionally, some white blood cells (mostly lymphocytes) have molecules expressed on their surfaces known as "immune regulatory molecules" or "checkpoints," which can modulate T cell functions (Sharma and Allison, 2015). These regulatory molecules include cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1), and programmed death ligand 1 (PD-L1) (Sharma and Allison, 2015). CTLA-4 and PD-1 are both located on the surfaces of T cells and act as halting mechanisms of T cell activation and function (Buchbinder and Desai, 2016). CTLA-4 interacts strongly with APC co-stimulatory molecules (i.e., signal two molecules), and this interaction inhibits T cell activation and functioning (Buchbinder and Desai, 2016). Similarly, PD-1 can interact with PD-L1 on the APC's surface, leading to T cell anergy and exhaustion (Buchbinder and Desai, 2016). The concepts of immune cell anergy and exhaustion will be discussed in detail later.

The adaptive immune response is maintained throughout the course of the host's lifetime via the development of immunological memory. Following an adaptive immune response, memory T cells and memory B cells are created; upon repeated exposure to the

same antigen in the future, these memory cells will be able to clear the pathogen quickly and efficiently (Janeway et al., 2001).

1.4 Specialized Pro-Resolving Mediators and Inflammation Resolution

Many studies have disproved the idea that inflammation resolution (also referred to as *catabasis*) is a passive process, whereby the mere cessation of the inflammatory response is adequate enough to return the body to homeostasis (Barnig et al., 2019; Basil and Levy, 2016). It is now widely accepted that inflammation resolution is an active process, whereby the body synthesizes endogenous pro-resolving compounds and activates or suppresses certain cells/mediators, ultimately resolving inflammation without being immunosuppressive (Serhan et al., 2000). These pro-resolving compounds are known as specialized pro-resolving mediators (SPMs) (Serhan et al., 2000). SPMs are a group of endogenously produced lipid mediators derived from fatty acids that work to resolve inflammation without being immunosuppressive (Abdulnour et al., 2016; Basil and Levy, 2016; Chen et al., 2020; Chiang et al., 2017; El Kebir et al., 2012; Seki et al., 2010; Serhan and Savill, 2005; Spite et al., 2009; Winkler et al., 2016). SPMs are produced mostly by cell-cell interactions between innate immune cells (Krishnamoorthy et al., 2018) and are enzymatically derived from arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) (Basil and Levy, 2016) (See **Figure 7**). SPMs are crucial for actively resolving acute inflammation following infection by interacting with their cognate receptors (i.e., receptor-ligand interactions) found on many cellular targets, including other immune cells (Basil and Levy, 2016; Krishnamoorthy et al., 2018; Serhan and Savill, 2005).

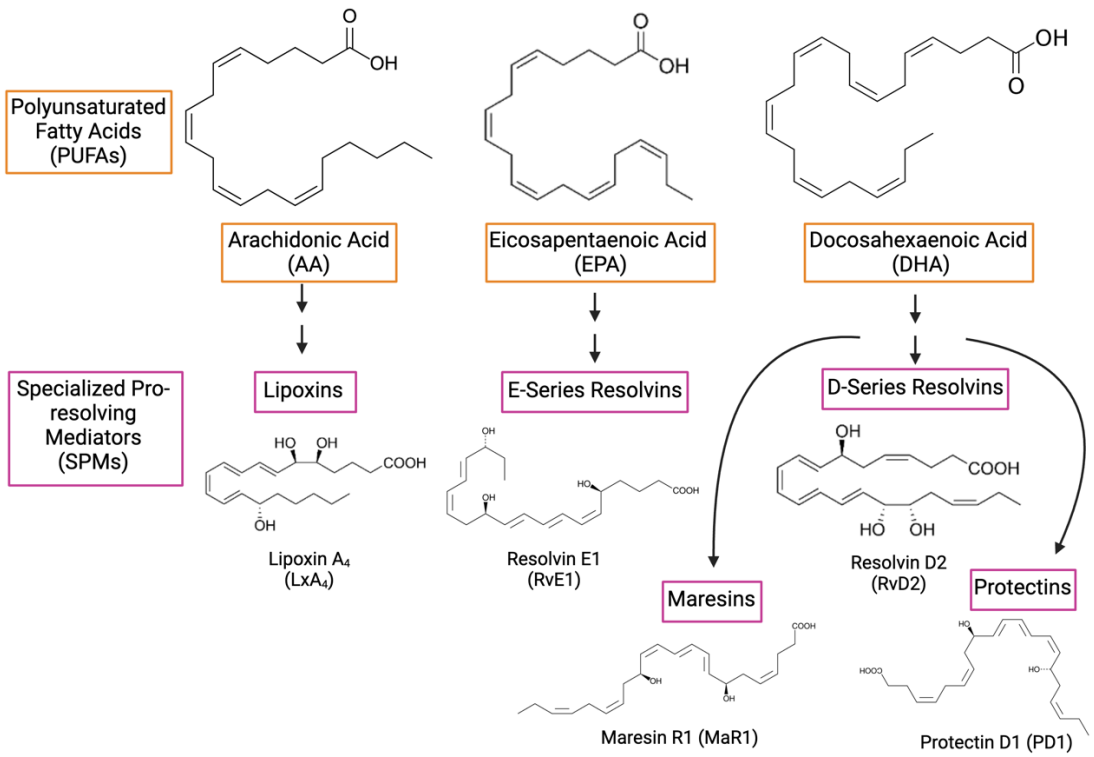


Figure 7. Endogenous SPM Biosynthesis. This schematic highlights the endogenous biosynthesis pathways of SPMs from their respective polyunsaturated fatty acids.

SPMs actively resolve inflammation in many ways. One way is by enhancing macrophage functions during inflammation by both increasing macrophage phagocytic and efferocytic abilities and dampening the macrophage-mediated release of pro-inflammatory cytokines (Gao et al., 2023; Serhan and Savill, 2005). SPM-mediated decreases in pro-inflammatory cytokine production work partly through regulation of the NF-κB pathway (Croasdell et al., 2016; Sham et al., 2018). For example, RvD2 increases expression of the NF-κB regulator molecule called single Ig IL-1R-related (SIGIRR) (Sham et al., 2018). Increased expression of SIGIRR decreases NF-κB-mediated TLR responses (Sham et al., 2018). RvD2 also decreases expression of TLR4, which similarly dampens NF-κB responses (Croasdell et al., 2016). However, some studies suggest that D-series resolvins

can also influence immune cell functions through additional signaling pathways, such as MAPK and PI3K/Akt, depending on the stimulus (Benabdoune et al., 2016; Gilbert et al., 2015; Ohira et al., 2010; Wang et al., 2011).

SPMs also reduce neutrophil activation and migration (Serhan and Levy, 2018; Serhan and Savill, 2005), while increasing their phagocytic ability (Jundi et al., 2021; Wu et al., 2009). Overall, they mediate inflammation resolution by acting on immune cells (mostly on neutrophils and macrophages) to limit tissue damage and promote healing (Basil and Levy, 2016). Defects in any SPM production pathway can lead to chronic inflammation and can contribute to immunopathology states, further highlighting the importance of endogenous SPM production in maintaining immune system homeostasis (Basil and Levy, 2016). Taken together, SPMs resolve acute inflammation and prevent chronic inflammation.

Resolvins are a type of SPM derived from EPA ("E-series resolvins") and DHA ("D-series resolvins") (Serhan and Levy, 2018), and their potent pro-resolving activities have been primarily studied in preclinical murine models in the context of early infection or sepsis (Seki et al., 2010; Spite et al., 2009; Walker et al., 2011). For example, Jundi et al. (2021) mapped resolution circuits in sepsis patients for two D-series resolvins, Resolvin D1 (RvD1) and Resolvin D2 (RvD2) (Jundi et al., 2021). They found from these studies that there is decreased bioavailability of endogenously produced SPMs present in these patients, ultimately leading to uncoupled inflammation resolution pathways in various immune cells (Jundi et al., 2021). When these septic patients were treated with ex vivo RvD1 and RvD2, their inflammation resolution pathways normalized (Jundi et al., 2021). Similarly, Dalli et al. (2017) reported that there was a measurable increase in SPM levels

in the blood of sepsis non-survivors (Dalli et al., 2017). Like the conclusions drawn in the article published by Jundi et al. (2021), the authors suggest this may likely reflect the dissociation of circulating SPMs and their receptors, leading to the uncoupling of inflammation resolution pathways (Dalli et al., 2017).

While there are many studies in the literature that focus on the roles of SPMs in early sepsis, there are very few studies that focus on the role of SPMs in late sepsis. An established and accepted model of late polymicrobial sepsis is the two-hit model of cecal-ligation and puncture (CLP) (hit one) followed by *Pseudomonas aeruginosa* secondary infection (hit two) (Richard S Hotchkiss et al., 2013; Muenzer et al., 2010; Sundarasivarao et al., 2022; Walker et al., 2022). This two-hit model enables the observation of how SPMs (administered late in an infection) may impact the host prior to a secondary bacterial lung infection (Sundarasivarao et al., 2022; Walker et al., 2022). In studies using this preclinical murine model, the administration of RvD2 increased secondary lung bacterial clearance, inflammation, and survival in mice after secondary infection (Sundarasivarao et al., 2022; Walker et al., 2022). While the exact mechanism of this protection is currently unknown, it can be postulated that the SPMs positively enhance the function of certain immune cells such as monocytes, macrophages, and neutrophils in order to help clear the infection *without* triggering excess inflammation. This possibility is further demonstrated by studies showing that SPM administration lowers the amount of antibiotics needed in order for the host to successfully clear the bacterial infection or bacteria-produced biofilm (Chiang et al., 2017, 2012; Thornton et al., 2023, 2021). Additionally, D-series resolvins were shown to directly increase macrophage phagocytic functions as a primary mechanism of clearing bacteria in late sepsis murine models (Walker et al., 2022), as well as in cystic fibrosis

murine models (Codagnone et al., 2018; Isopi et al., 2020). RvD2 was also reported to increase the number of monocytic myeloid-derived suppressor cells (M-MDSCs) in CLP mouse spleens compared with sham spleens, and this increase was associated with enhanced bacterial clearance and reduced mortality due to sepsis (Sundarasivarao et al., 2022). These studies provide evidence that SPMs may hold therapeutic value in the treatment of sepsis during the late, immunosuppressive phase.

Overall, the goal of inflammation resolution is to allow the body's tissues that have been harmed by a stimulus (e.g., pathogen, damaged tissue) or by the acute inflammatory response to the stimulus to return to homeostasis. Inflammation resolution is an active, coordinated process whereby certain molecular/cellular mechanisms take place in various immune cells in order to heal the harmed tissues (Barnig et al., 2019). One key event in this process is the recruitment and activation of macrophages to the site of infection (Barnig et al., 2019). Another key event in this process is the release of endogenous SPMs by various cells within the body. Expectedly, the loss of or the dysregulation of inflammation resolution mechanisms results in pathological chronic inflammation and disease (Barnig et al., 2019).

1.5 Mechanisms of Immunosuppression in Sepsis

Three hallmarks of sepsis-induced immunosuppression include: monocyte/macrophage exhaustion, lymphocyte apoptosis, and myeloid-derived suppressor cell (MDSC) migration (See **Figure 8**).

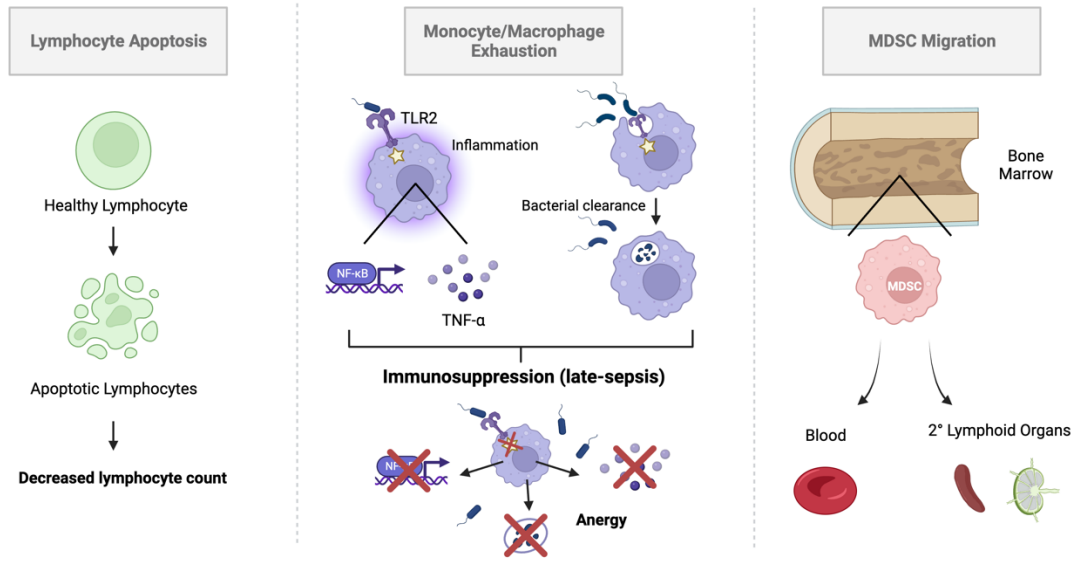


Figure 8. Mechanisms of Sepsis-Induced Immunosuppression. Illustration of three major mechanisms underlying the pathophysiology of sepsis-induced immunosuppression: lymphocyte apoptosis, monocyte/macrophage exhaustion, and migration of myeloid-derived suppressor cells (MDSCs). Key: TLR2: toll-like receptor 2; MDSC: myeloid-derived suppressor cell.

1.5.1 *Monocyte/Macrophage Exhaustion*

One hallmark of sepsis-induced immunosuppression is monocyte/macrophage exhaustion (Liu et al., 2022). Monocytes are circulating white blood cells derived from the bone marrow and are major players in the innate immune system. Their primary role is to detect changes in homeostasis (usually caused by an infection and/or tissue damage) and respond to these changes by replenishing the pool of macrophages that are present in the tissues (Yang et al., 2014). Once monocytes become activated, they turn into effector cells and produce cytokines, present antigens to adaptive immune cells, phagocytose, efferocytose, initiate inflammation, or resolve inflammation (Yang et al., 2014). They initiate inflammation by producing various pro-inflammatory cytokines, such as TNF-α and IL-6 (Duque and Descoteaux, 2014). In turn, these pro-inflammatory cytokines

function to enhance the inflammatory response in numerous ways, such as recruiting/activating other innate immune cells (i.e., neutrophils) (Jang et al., 2021; Rose-John et al., 2017). When monocytes initiate this process of tissue inflammation, they exit the blood and enter the tissues at the site of the inflammation, thereby becoming macrophages. Macrophages can either be recruited to a specific tissue (which is what happens during the inflammatory response), or they can exist as residents within the body's tissues. Macrophages can polarize into different subtypes depending on which subtype can best assist the host at that time. The previously accepted polarization classes are M0, M1-like, and M2-like; an M0 macrophage is a nonactivated tissue resident macrophage, an M1-like macrophage is a pro-inflammatory macrophage, and an M2-like macrophage is an anti-inflammatory macrophage (See **Figure 9**) (Yang et al., 2014). It is understood that classical M1-like macrophages have increased production of pro-inflammatory cytokines, enhanced antigen presentation, and enhanced phagocytosis capabilities (Lam et al., 2016). M2-like macrophages have diminished pro-inflammatory cytokine output and weakened antigen presentation, but enhanced phagocytic capabilities (Lam et al., 2016; Rőszer, 2015). These phenotypes can be artificially induced in vitro via monocyte stimulation, with LPS and IFN-γ leading to the development of a "classic" M1-like macrophage, and IL-4 leading to the development of a "classic" M2-like macrophage (Strizova et al., 2023; Watanabe et al., 2019). A non-classical but newly-reported class of macrophages are "pro-resolving" macrophages (Saas et al., 2020), and they will be discussed in detail below.

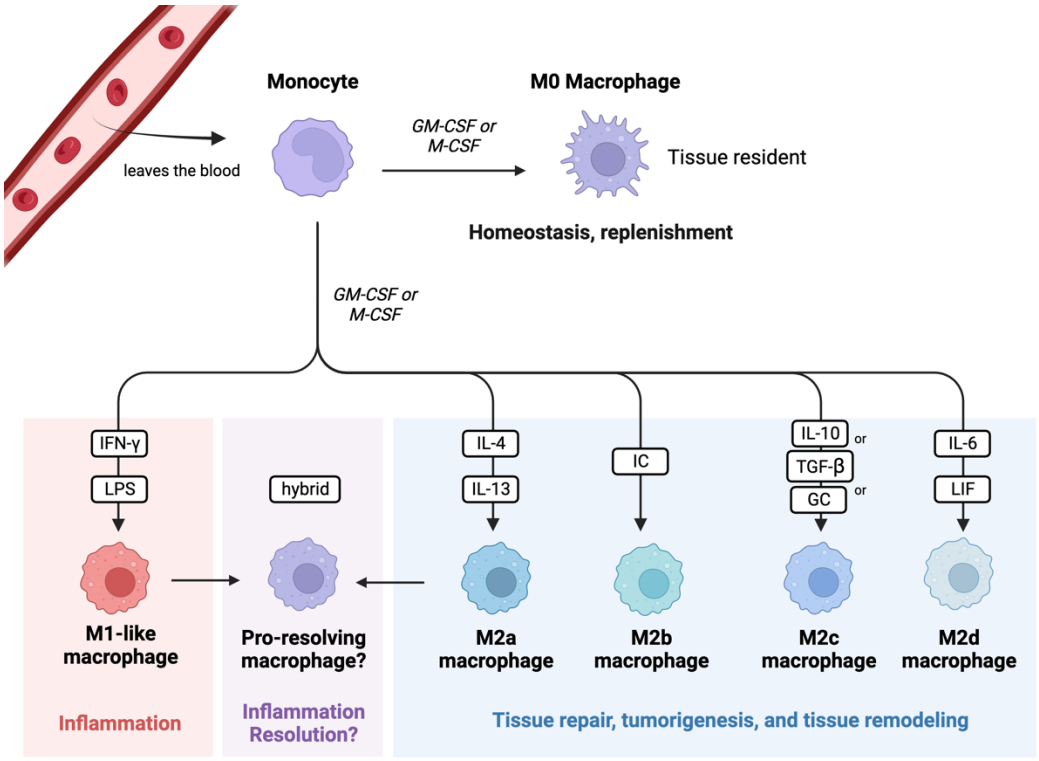


Figure 9. Monocyte Differentiation and Macrophage Polarization. This schematic highlights the classical monocyte differentiation scheme and polarization classes of macrophages. Each class has a unique role within the immune system. Key: GM-CSF: granulocyte macrophage colony-stimulating factor; M-CSF: macrophage colony-stimulating factor; LPS: lipopolysaccharide; IC: immune complex; GC: glucocorticoid; LIF: leukemia inhibitory factor.

M1-like macrophages are stimulated by IFN-γ and LPS; they are key initiators of the inflammatory response and are critical for the removal of bacteria (Sica and Mantovani, 2012). On the other hand, M2-like macrophages are highly phagocytic and are key drivers of tissue remodeling following damage, tumor progression via the production of angiogenic factors, and parasite removal (Sica and Mantovani, 2012). M2-like macrophages are also a main driver in the development of allergies. Additionally, the M2-like macrophage subtype can be further subclassified into M2a, M2b, M2c, and M2d (Yao et al., 2019; Zhang and Sioud, 2023). These subtypes vary in terms of what cytokines they

release, what surface markers they express, and what specific roles they play within M2-like macrophage responses. M2a macrophages are stimulated by IL-4 and IL-13; they enhance cell growth and tissue repair and enhance macrophage endocytosis (Yao et al., 2019; Zhang and Sioud, 2023). M2b macrophages are stimulated via immune complexes (IC); they are able to regulate the host's immune status by secreting both pro- and anti-inflammatory cytokines (Yao et al., 2019; Zhang and Sioud, 2023). M2b macrophages are also well known for their ability to promote tumor progression (Zhang and Sioud, 2023). M2c macrophages are stimulated by IL-10, TGF-β, or glucocorticoids (GCs); like M2b macrophages, they are highly immune-regulatory, but specialize in efferocytosis and phagocytosis of other apoptotic cells (Yao et al., 2019; Zhang and Sioud, 2023). M2d macrophages are stimulated by IL-6 and leukemia inhibitory factor (LIF); they promote angiogenesis and tumor progression via the release of vascular endothelial growth factors and dampening of the immune response (Yao et al., 2019; Zhang and Sioud, 2023).

While the M1/M2 paradigm is helpful for investigators studying these different capabilities of macrophages in vitro, many researchers find that limiting the description of macrophage phenotypes to only M1-like or M2-like is not clinically relevant, as this "tidy" polarization process does not occur at a tissue level (Guilliams et al., 2018; Nahrendorf and Swirski, 2016; Strizova et al., 2023; Watanabe et al., 2019; Xue et al., 2014). Current research explains that activated macrophages have high levels of plasticity and do not solely polarize into an M1-like or M2-like phenotype at the tissue level; rather, they adopt a phenotype that is highly dependent on both the environment they are in and their own ontogeny (Strizova et al., 2023; Watanabe et al., 2019). One such newly reported phenotype is the "pro-resolving" phenotype (Saas et al., 2020; Strizova et al., 2023; Vannella and

Wynn, 2017; Watanabe et al., 2019). These dynamic, pro-resolving macrophages function primarily to resolve inflammation so that the host can return to homeostasis, while simultaneously contributing to wound/tissue repair (Strizova et al., 2023; Watanabe et al., 2019). Interestingly, these pro-resolving macrophages are said to have qualities of the classic M1- and M2-like phenotypes, with aspects that contribute to both pro-inflammatory and anti-inflammatory pathways (Saas et al., 2020). Some studies even report that abnormal activation of this pro-resolving phenotype can lead to chronic organ dysfunction, highlighting their direct importance in the inflammation resolution process (Bah and Vergne, 2017; Cronan et al., 2016; Linke et al., 2017).

Monocytes/macrophages contribute to sepsis-induced immunosuppression in a number of ways. Monocytes/macrophages can modulate the expression of their immune regulatory markers, an action that has implications for other immune cells and physiological pathways (Zhang and Sioud, 2023). Changes in the expression of immune regulatory markers such as CTLA-4, PD-1, and PD-L1 can alter these interactions (Sharma and Allison, 2015). PD-1 (also referred to as CD279) is a transmembrane protein located on the surfaces of many immune cells; it is expressed in the highest concentration on activated T cells (Jiang et al., 2019). When PD-1 interacts with its ligand PD-L1, the T cell becomes anergic, or unresponsive to viable antigens (Alsaab et al., 2017; Karwacz et al., 2011; Song et al., 2014; Zhou et al., 2009). PD-L1 is expressed by certain APCs (such as monocytes/macrophages) in inflammatory scenarios as well as by tumor cells (Alsaab et al., 2017). Additionally, certain pro-inflammatory cytokines (especially IFN-γ) can trigger various signal transduction cascades within APCs (NF-κB pathway, PI3K/AKT pathway,

MAPK pathway, JAK/STAT pathway, WNT pathway, and Hedgehog pathway) leading directly to increased expression of PD-1/PD-L1 on their surfaces (Han et al., 2020).

Physiologically, the PD-1/PD-L1 pathway aids in controlling the degree of inflammation at the tissue level by deactivating T cells that are directly contributing to the inflammation so as to protect tissues from immune-mediated damage (Alsaab et al., 2017). Tumor cells overexpress PD-L1 in order to exist for longer periods of time without being "seen" by the adaptive immune system (Alsaab et al., 2017; Han et al., 2020). The PD-1/PD-L1 pathway has therefore become a major contributor to immunosuppression within the tumor microenvironment in many different types of cancers (notably melanoma and small-cell lung cancer) (Pardoll, 2012). Understanding this major contribution of PD-1/PD-L1 to cancer-induced immunosuppression has led to the development of a new class of cancer immunotherapies called checkpoint inhibitors (Pardoll, 2012; Sharma and Allison, 2015). Due to many parallels existing between cancer-induced immunosuppression and sepsis-induced immunosuppression (Liu et al., 2022), checkpoint inhibitors were tested in clinical trials of sepsis patients and cecal ligation and puncture (CLP) sepsis models (Hotchkiss et al., 2019; L. Yang et al., 2023). Hotchkiss et al. (2019) was the first to study the effects and safety profile of the anti-PD-1 immunotherapy agent nivolumab in a small clinical trial of sepsis patients (Hotchkiss et al., 2019). More recently, Yang et al. (2023) showed that blocking PD-L1 on monocytes led to a dramatic increase in survival of CLP-septic mice (L. Yang et al., 2023).

Additionally, many researchers have assessed the potential correlation between immune regulatory marker expression and outcomes due to sepsis. Ruan et al. (2020) found that there is an increased number of cells expressing both PD-1 and PD-L1 in CLP-septic

mice (Ruan et al., 2020). This finding was also true for humans, as there are reported increases of expression in both PD-1 and PD-L1 in human septic patients (Patera et al., 2016; Ruan et al., 2020; Wilson et al., 2018; Zhang et al., 2011). While changes in immune regulatory marker expression on monocytes/macrophages contributes to sepsis-induced immunosuppression, this finding is *not* specific to septic patients. As previously mentioned, cancer cells can implement similar changes in regulatory marker expression levels in order to evade the immune system. A more explicit example of monocytes/macrophages contributing to immunosuppression due to sepsis is through monocyte/macrophage exhaustion (Liu et al., 2022). The concept of exhaustion does not include PD-1/PD-L1 signaling; however, the end result of exhaustion is similar to PD-1/PD-L1's systemic anergic effects on immune cells.

Exhausted monocytes/macrophages are "hypoactive," meaning that they have a decreased capacity to perform their normal effector functions when stimulated (Biswas and Lopez-Collazo, 2009; Cavaillon and Adib-Conquy, 2006; Hoppstädter et al., 2019; Pradhan et al., 2021). These normal effector functions include presenting antigens via HLA-DR, phagocytosing, efferocytosing, and releasing pro-inflammatory cytokines (e.g., TNF-α, IL-6, IL-8) via the NF-κB pathway (Yang et al., 2014). Therefore, tracking cellular NF-κB status is an accepted way to track exhaustion (Cavaillon et al., 2001). As monocytes/macrophages from septic patients also have similarly-reduced capacities of these normal functions, it can be inferred that septic monocytes/macrophages are exhausted (Hotchkiss et al., 2013a). A recent study revealed that septic macrophages had decreased pro-inflammatory cytokine production in response to various ligands, including bacteria and LPS (Bick et al., 2022). In this study, the authors found that whole blood samples taken

from 61 septic patients (blood taken within 24h of meeting sepsis-3 criteria) had an overwhelming presence of inflammation (i.e., significantly increased levels of the pro-inflammatory cytokines TNF-α, IL-6, and IL-1β/α), yet white blood cells from these samples had depressed responses to both LPS and bacteria (Bick et al., 2022), providing evidence that septic white blood cells were similar to the "exhausted" phenotype. This hypoactive phenotype present in septic monocytes/macrophages can be recapitulated in vitro by repetitively challenging monocytes/macrophages with isolated, low-dose bacterial endotoxins such as LPS (Boomer et al., 2011; Hoppstädter et al., 2019).

It is important to briefly discuss here that monocyte/macrophage exhaustion is different from the phenomenon called endotoxin tolerance (ET). ET has many similar characteristics to exhaustion (Hotchkiss et al., 2013a), and it is defined as reduced immune cell functional responsiveness to the bacterial endotoxin LPS after previously encountering LPS (Biswas and Lopez-Collazo, 2009; Cavaillon and Adib-Conquy, 2006). Blackwell et al. (1997) examined ET via monocyte cytokine production; they discovered that tolerized monocytes had impaired activation of NF-κB (Blackwell et al., 1997), which is also observed in exhaustion. Tolerized monocytes/macrophages also exhibit a reduced capacity to react to subsequent LPS challenge; primarily of note is the drastic decrease in NF-κB-mediated TNF-α production upon LPS re-stimulation (Chan et al., 2005; Del Fresno et al., 2009; Deng et al., 2013; Dobrovolskaia and Vogel, 2002; Foster and Medzhitov, 2009; Hoogendijk et al., 2017). While monocytes/macrophages from late septic patients seem to have many aspects similar to monocytes/macrophages experiencing ET, the conventional understanding of ET does not encompass the fundamental characteristics of immunosuppression observed in septic patients (Pradhan et al., 2021). For example,

monocytes/macrophages experiencing ET have *enhanced* bacterial phagocytosis, as it has been theorized that ET monocytes/macrophages adopt a phenotype similar to M2-like macrophages (Biswas and Lopez-Collazo, 2009). However, exhausted monocytes/macrophages and monocytes from septic patients demonstrate *impaired* bacterial phagocytosis, even though the mechanism underlying this decline is not known (Huang et al., 2009; Zhou et al., 2022). For these reasons, the emerging literature more accurately describes septic monocytes/macrophages as *exhausted* rather than *tolerized* (Pradhan et al., 2021). However, the mechanism(s) underlying monocyte/macrophage exhaustion during the immunosuppressive stage of sepsis remains elusive.

Several possibilities for sepsis-induced monocyte/macrophage exhaustion include increased production of the anti-inflammatory cytokine IL-10, down-regulation of the NF-κB signaling pathway, decreased HLA-DR expression, and reduced lymphocyte signaling due to lymphocyte cell death (Cavaillon and Adib-Conquy, 2006; Hoogendijk et al., 2017; Hotchkiss et al., 2013a). Another possibility is the high mobility group box 1 (HMGB1) protein, which has been shown to increase macrophage pyroptosis (Chen et al., 2018; G. Yang et al., 2023; Wendan Zhang et al., 2023), decrease macrophage efferocytosis ability (Wang et al., 2022), and enhance the suppressive activity of an immunosuppressive cell population called myeloid-derived suppressor cells (MDSCs) (Parker et al., 2014).

To overcome monocyte/macrophage exhaustion, research teams have tried to use IFN-γ in order to stimulate monocyte activity. In one study performed by Döcke et al. (1997), the authors were able to increase HLA-DR expression in anergic monocytes, leading to sepsis resolution in eight of nine septic patients (Döcke et al., 1997). On the other hand, a more recent study showed that increased plasma IFN-γ levels were associated

with secondary *Candida* species infections in late-sepsis patients (Kim et al., 2020). Surprisingly, the authors also showed that IFN-γ reduced macrophage phagocytosis of zymosan particles (Kim et al., 2020). Together, these two contradictory studies suggest that IFN-γ cannot reliably be regarded as an appropriate treatment to reverse sepsis-induced monocyte/macrophage exhaustion.

1.5.2 *Lymphocyte Apoptosis*

Another hallmark of sepsis-induced immunosuppression is lymphocyte apoptosis (Liu et al., 2022). Before apoptosis can be discussed in more detail, it is important to mention that there are numerous types of regulated and unregulated cell death processes involving complex signaling cascades. The regulated and clinically-relevant processes include, but are not limited to, autophagy, ferroptosis, pyroptosis, necroptosis, and apoptosis (See **Figure 10**). Autophagy is a cellular stress response that can lead to the regulated cell death process by which autophagosomes collect cell waste products and deliver them to lysosomes for destruction (Shen et al., 2023). This process is mainly employed to maintain homeostasis within the cell and to manage lipid metabolism (Gao et al., 2022). Ferroptosis is a process of regulated cell death that is primarily caused by the accumulation of iron (Bertheloot et al., 2021). This accumulation leads to the development of reactive oxygen species (ROS), which contribute to both cell membrane rupture and lipid peroxidation (Gao et al., 2022). Pyroptosis is a process of regulated cell death whereby destruction of the cell membrane is triggered by the activation of a multi-protein complex called the inflammasome (Bertheloot et al., 2021; Tang et al., 2019). Necroptosis is a process of regulated cell death that eventually leads to an influx of ions, swelling of the

cell, and eventual breakdown of the membrane (Bertheloot et al., 2021). Due to many similarities between necroptosis and apoptosis (apoptosis is described in detail below), it has been suggested that necroptosis exists as a backup mechanism for apoptosis (Gao et al., 2022).

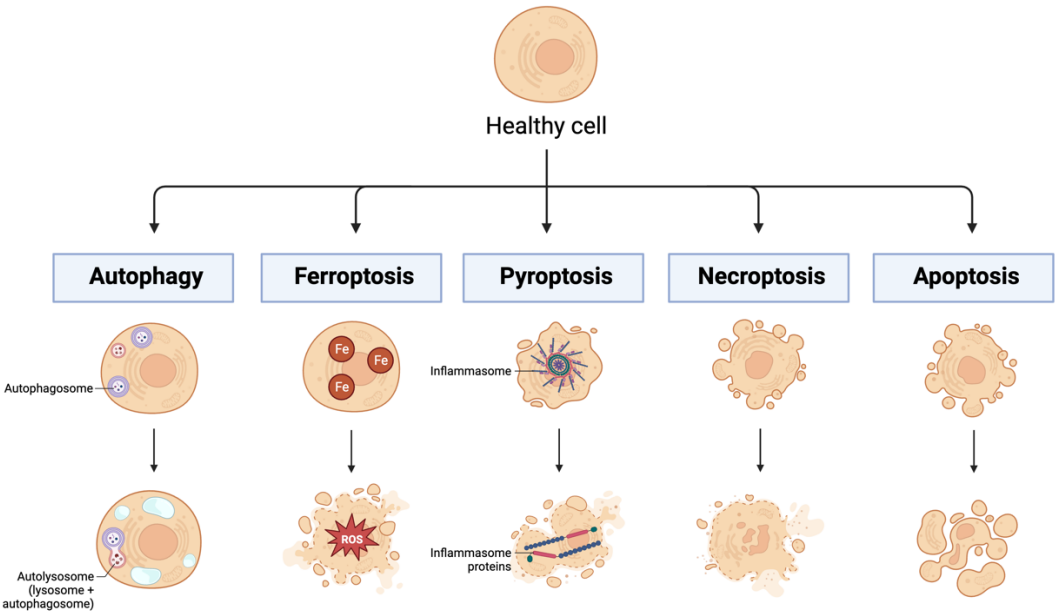


Figure 10. Mechanisms of Regulated Cell Death. This schematic highlights some important processes of regulated cell death or precursor processes of regulated cell death: autophagy, ferroptosis, pyroptosis, necroptosis, and apoptosis. Key: Fe: iron; ROS: reactive oxygen species.

Apoptosis, or programmed cell death, is an active cellular process that is essential for proper functioning and regulation of many cells within the body, including immune cells (Green and Llambi, 2015). As discussed earlier, apoptosis of faulty lymphocytes during and following T and B cell development is crucial for proper immune functioning.

There are two pathways that lead to lymphocyte apoptosis: “death by neglect” and “death by instruction” (Rathmell and Thompson, 2002). As previously mentioned, there are many mechanisms in place during lymphocyte development and activation to ensure proper performance and homeostasis within the population. If a lymphocyte fails to meet certain functional requirements, it will die via apoptosis (“death by neglect”). On the other hand, if a lymphocyte functions too aggressively, it will also die via apoptosis (“death by instruction”). These apoptosis pathways that regulate lymphocyte development and expansion are necessary to ensure there are no self-reactive and/or hyporesponsive lymphocytes present in the adaptive immune system’s repertoire.

A defining feature of apoptosis is that the programmed cell death event does not trigger inflammation: the process occurs neatly and “quietly.” This highly coordinated pathway involves two main groups of proteins that work with each other in concert to ensure that this organized process remains balanced: apoptosis activators and apoptosis regulators. There are two main pathways of apoptosis activation: the extrinsic pathway and the intrinsic pathway (Green and Llambi, 2015). (See **Figure 11**). Both pathways involve an apoptotic signal, which activates a protease called an initiator caspase, which in turn activates another protease called an executioner caspase (Green and Llambi, 2015). The two pathways differ in how the initiator caspase becomes activated, but the end result remains the same. The extrinsic pathway, also known as the death receptor pathway, involves receptor-ligand interactions (Green and Llambi, 2015). An example of a death receptor within this pathway is the Fas receptor; this death receptor interacts with its ligand, Fas ligand, and this interaction subsequently triggers apoptosis (Green and Llambi, 2015). The intrinsic pathway, also known as the mitochondrial pathway, involves the

mitochondrial release of cytochrome c into the cytoplasm, which carries out effector functions necessary for apoptosis completion (Tsujimoto, 1998). Two Bcl-2 family member proteins that assist in the activation of apoptosis (and hence are considered "pro-apoptotic" proteins) are Bax and Bak (Peña-Blanco and García-Sáez, 2018). When Bax and Bak interact/oligomerize, a channel within the mitochondrial membrane is formed; this leads to membrane destabilization and the subsequent release of cytochrome c into the cytoplasm (Peña-Blanco and García-Sáez, 2018). If Bax and Bak do not interact/oligomerize, the mitochondrial membrane remains stabilized, and there is no release of cytochrome c (Peña-Blanco and García-Sáez, 2018). Equally as important as apoptosis activation is the regulation of apoptosis by another protein within the Bcl-2 family called Bcl-2 (Green and Llambi, 2015). Bcl-2 is a regulatory protein within this family that functions to sequester Bax and Bak so that they cannot oligomerize (Green and Llambi, 2015), earning Bcl-2 its "anti-apoptotic" title (Tsujimoto, 1998). The level and location of Bcl-2, therefore, are good biomarkers to study changes in apoptosis within cells.

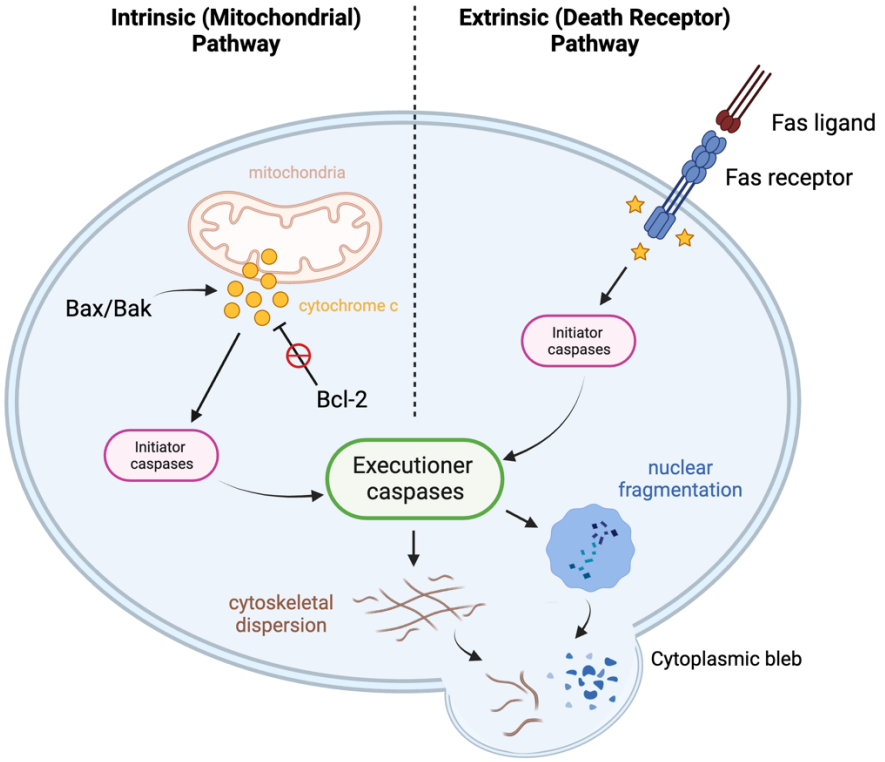


Figure 11. Apoptosis. This schematic demonstrates two pathways of apoptosis: the intrinsic (mitochondrial) pathway and the extrinsic (death receptor) pathway.

While some or all of the regulated cell death processes may be occurring in some way during the clinical course of sepsis, they are difficult to test for in a clinical setting. It is clear that cell death mechanisms are occurring in these patients, as one of the main features of late sepsis is a decreased white blood cell count, and more specifically, lymphopenia (Sheikh Motahar Vahedi et al., 2019). Of the many types of cell death described above, apoptosis of lymphocytes has been studied the most extensively in experimental settings of late sepsis (i.e., in CLP sepsis models).

Lymphopenia in late sepsis is partly caused by lymphocytes leaving the blood to enter tissues and participate in the inflammatory response; however, there are studies that suggest apoptosis of lymphocytes is the primary driver of lymphopenia observed in the late

phase of sepsis (Carrero and Unanue, 2006; Hotchkiss et al., 1999; Luan et al., 2015, 2014; Matsuda et al., 2010; Wang et al., 1994). While lymphocyte apoptosis is a normal physiologic process necessary for proper immune functioning, this process can become dysregulated or can be hijacked by microbes, leading to an unnecessary loss of lymphocytes. Microbes can induce apoptosis in lymphocytes and other types of white blood cells in order to artificially create a more permissive environment for their survival within the host cells (Carrero and Unanue, 2006). By triggering apoptosis within lymphocytes, microbes are able to thrive in the body because there are fewer immune cells capable of clearing them from the host. For example, *Bacillus anthracis*, the causative agent of anthrax, releases lethal toxin (LTx), which can lead to direct caspase activation within macrophages, deceptively triggering their cell death pathways (Van Hauwermeiren et al., 2022). In the CLP model of polymicrobial sepsis, there is ~10% increase in the number of apoptotic lymphocytes compared with control mice (Carrero and Unanue, 2006). Studies have shown that increased expression of Bcl-2 leads to increased survival of CLP-septic mice by approximately 40–50%, specifically through apoptosis prevention in splenic lymphocytes (Carrero and Unanue, 2006; Hotchkiss et al., 1999; Wang et al., 1994). Additionally, there are numerous accounts confirming apoptosis of lymphocytes in CLP-septic mice using specific transferase-mediated dUTP nick-end labeling (TUNEL) staining methods (Luan et al., 2015, 2014; Matsuda et al., 2010).

Another mechanism of lymphocyte suppression that is closely related to the apoptotic pathways is the PD-1/PD-L1 pathway that was described earlier. While this pathway does not lead to apoptosis of lymphocytes, it renders the lymphocytes inactive, producing a very similar outcome. While the application of checkpoint inhibitors (i.e., PD-

1 inhibitors) for sepsis-induced immunosuppression is still in its nascent stages, the enhancement of lymphocyte functionality in late sepsis may provide an exciting avenue for researchers moving forward.

In an attempt to target sepsis-induced immunosuppression, there have been several clinical trials whereby lymphocytes were targeted for restoration. In one study, recombinant GM-CSF or G-CSF was administered to late septic patients in order to increase their lymphocyte counts (Bo et al., 2011). These attempts initially seemed promising, but the beneficial effects did not persist long-term. In a similar clinical trial, IL-7 was administered to late septic patients in an attempt to increase their populations of T cells, but the patients did not improve long-term (Francois et al., 2018). In both of these instances, the failures were attributed to pathological migration and activation of MDSCs (Wendan Zhang et al., 2023). It was postulated that MDSCs suppressed T cell activity and proliferation, negating the beneficial action of the intervention.

1.5.3 *Myeloid-Derived Suppressor Cells (MDSCs)*

The third hallmark of sepsis-induced immunosuppression is increased myeloid-derived suppressor cell (MDSC) production/migration (Liu et al., 2022). MDSCs are a group of heterogenous, immature cells of the myeloid lineage that work to suppress the innate and adaptive immune systems (Veglia et al., 2021). MDSCs can be divided into two main populations based on their lineage (although there are others that have been reported (Goldmann et al., 2017)): monocytic MDSCs (M-MDSCs) and polymorphic MDSCs (PMN-MDSCs) (Bronte et al., 2016; Talmadge and Gabrilovich, 2013; Veglia et al., 2021). Both M-MDSCs and PMN-MDSCs are able to exert their inhibitory effects on the innate

and adaptive immune systems in similar ways, with the primary mechanisms being via the release of ROS, production of arginase-1, and release of IL-10 (Veglia et al., 2021). MDSCs exist in the bone marrow and are triggered to migrate into the blood and secondary lymphoid tissues via CXCL2 and IL-8 chemokine gradients (Veglia et al., 2021). Overall, MDSCs function as immunosuppressive cells, but their exact role and meaning behind their migration patterns in patients with sepsis remains to be seen.

There is some controversy regarding the significance of increased MDSC migration that is observed in septic patients (Brudecki et al., 2012; Cuenca et al., 2011; Delano et al., 2007; Mathias et al., 2017). Some studies report that the increase in MDSC migration out of the bone marrow in sepsis patients is partly responsible for the adverse clinical outcomes due to late sepsis (Landoni et al., 2016; Mathias et al., 2017; Tang et al., 2021; Wanying Zhang et al., 2023). In fact, Tang et al. (2021) suggests that inhibiting MDSCs would be one of the most effective treatments for sepsis-induced immunosuppression overall (Tang et al., 2021). In septic patients, having elevated numbers of MDSCs in the peripheral blood was associated in some patients with longer ICU hospital stays and a higher statistical likelihood of acquiring secondary infections (Wanying Zhang et al., 2023). In LPS-induced immunosuppression, MDSCs migrate from the bone marrow to the blood/secondary lymphoid tissues and inhibit the proliferation of lymphocytes (Landoni et al., 2016). Additional studies have shown that not only are MDSCs directly inhibitory on lymphocytes, but also their production of arginase-1 further disrupts T cell functions (Uhel et al., 2017). Similarly, MDSCs can indirectly cause immunosuppression by activating Tregs (Wanying Zhang et al., 2023). This anti-inflammatory T cell subset functions to

inhibit other innate and adaptive immune cells, primarily via the release of the anti-inflammatory cytokine IL-10 (Wanying Zhang et al., 2023).

Conversely, there are also reports that MDSC migration during late sepsis could actually contribute to long-term survival in sepsis patients (Arocena et al., 2014; Brudecki et al., 2012; Cuenca et al., 2011; Mathias et al., 2017; Sander et al., 2010). This paradoxical function of MDSCs was studied extensively in CLP-sepsis models. In one study, MDSCs derived from late septic mice were adoptively transferred into CLP mice early in the infection timeline, and these mice had decreased mortality compared with sham animals (Brudecki et al., 2012). In another study, mouse MDSC expansion was inhibited by pre-treatment with the chemotherapeutic gemcitabine, and survival to CLP was dramatically reduced in the gemcitabine treatment group compared with the sham animals (Cuenca et al., 2011). Similarly, one study found that inhibition of CLP mouse MDSC populations (by targeting an MDSC cell-surface receptor) led to decreased survival compared with the sham animals (Sander et al., 2010). In another infection model system, in vivo depletion of MDSCs in mice infected with *Trypanosoma cruzi* (the causative agent of Chagas disease) led to increased mortality compared with the sham animals (Arocena et al., 2014).

A study by Schrijver et al. (2022) provides a strong case for the expansion and migration of M-MDSCs in particular correlating with improved survival in sepsis patients (Schrijver et al., 2022). In this study, blood was taken from patients in eight ICUs with pneumonia secondary to sepsis (Schrijver et al., 2022). The authors found that patients with high levels of M-MDSCs overall had significantly reduced 90-day mortality rates and improved survival compared with patients with high levels of PMN-MDSCs (Schrijver et al., 2022). The contention is that due to the heterogeneity of MDSC populations, perhaps

overall, MDSCs play a dual role during sepsis, with each unique MDSC population evolving over time after sepsis onset (Brudecki et al., 2012; Hollen et al., 2019; Schrijver et al., 2022).

Overall, in sepsis patients, there is an elevation in MDSC numbers in the blood and the secondary lymphoid tissues. While a simple explanation would be that the migrating MDSCs are acting as the trigger for sepsis-induced immunosuppression, perhaps what is actually occurring here is that the host is attempting to correct the early hyper-proinflammatory phase of sepsis with MDSC migration. Due to unidentified dysregulation pathways in sepsis patients, this recruitment of MDSCs is not robust enough in these particular patients; therefore, the hyper-proinflammatory response persists until the late stage of sepsis sets in, and their presence then accentuates the immunosuppression. MDSC expansion and migration in sepsis appears to be a highly dynamic process, and these cells, conceivably, are malleable to the infection at hand. Although the complete consequences of increased MDSC production/migration in sepsis have not been fully elucidated, their presence in the blood as a biomarker of the immunosuppressive phase of sepsis cannot be disputed.

1.6 Biomarkers in Late-Phase Sepsis

One of the most difficult aspects of developing sepsis therapeutics is the heterogeneity of the disease. There have been decades of failed sepsis therapeutics in clinical trials that were based on promising preclinical research. The discrepancy likely lies in the understanding that there are many factors contributing to patient outcomes due to sepsis, and the challenge in moving toward successful interventions stems from the

variability of the immune system's actions. The way the immune system responds to infection can be influenced by the stage of the inflammatory timeline by which it is acting. Therefore, the therapeutics administered in sepsis rely heavily on specific biomarkers, which allow physicians to decipher the immune status of their patients. Then, the physicians can tailor treatments more accurately. For example, while common practice is to administer antibiotics in order to prevent detrimental outcomes due to sepsis, studies have shown that the antibiotics must be administered within the first hour of the sepsis diagnosis to provide any perceived benefit (Mayr et al., 2014; Rangel-Frausto, 1999); otherwise, administration of broad-spectrum antibiotics could actually facilitate the development of multi-drug-resistant infections, potentially causing an infection epidemic within the hospital (Mayr et al., 2014). Part of understanding the lack of favorable outcomes in sepsis due to antibiotic administration is appreciating that while microbial infections trigger sepsis, the main driver of sepsis is the patient's own immune system – not the infection itself. Since sepsis itself is a clinical diagnosis (i.e., there is no "one test" that can diagnose sepsis), many times it either goes undiagnosed or it is diagnosed outside the window of perceived benefit of antibiotic administration. Once this window passes, the risk of antibiotic resistance outweighs potential benefits (Mayr et al., 2014). The need for new, effective sepsis therapeutics will therefore depend heavily on quick thinking by the physician to test for specific biomarkers that could shed light on the patient's immune status, as well as what therapeutic would best be warranted for administration at that time. While there likely will not be a single treatment that will be equally effective for every single patient with sepsis, an ideal treatment would be one that supports the immune

system's intrinsic ability to combat the pathogen while also resolving the infectious inflammation before it further causes damage to the body (Tindal et al., 2021).

While there is no one test that definitively tells the physician that the patient has sepsis, there are certain reliable indictors that can shed light on a patient's immune status. These measurable indicators, or biomarkers, are reproducible, highly sensitive, and highly specific (Barichello et al., 2022). Some biomarkers that are indicative of the early, hyper-proinflammatory phase of sepsis are similar to typical markers of inflammation: increased acute-phase reactant protein levels and increased pro-inflammatory cytokine/chemokine levels (Barichello et al., 2022). According to the Third International Consensus Definition for Sepsis and Septic Shock, elevated lactate is included as a biomarker for sepsis diagnosis (Singer et al., 2016), as increased lactate production is correlated with increased mortality in sepsis (Andersen et al., 2013). Of note, one innate immune system protein called pentraxin 3 (PTX3) was recently assessed for its potential as a biomarker for early sepsis (Chen et al., 2021; Hamed et al., 2017; Jie et al., 2017; Lee et al., 2018). PTX3 is an acute-phase reactant that is highly expressed by phagocytes (such as monocytes/macrophages) following pro-inflammatory responses by the immune system (Davoudian et al., 2022). PTX3 expression was reported in many studies to be upregulated in septic patients compared with non-septic patients, and this increase correlated with clinical severity and unfavorable prognosis (Caironi et al., 2017; Chen et al., 2021; Davoudian et al., 2022; Hamed et al., 2017; Jie et al., 2017; Lee et al., 2018).

Observation shows that most of the accepted sepsis biomarkers are indicators of the early, hyper-proinflammatory stage of sepsis. This is because many of the sepsis therapeutics in development focus on blunting the infection-triggered hyper-

proinflammatory phase, with medications such as antibiotics, IL-6 inhibitors, and TNF-α inhibitors prevailing. The immunosuppressive stage of sepsis was not previously thought of as a potential area for therapeutic development, likely due to incomplete understanding of the underlying mechanisms leading to immunosuppression in sepsis. With that said, accurate diagnosis of the late, immunosuppressive stage via biomarkers is critical for the proper administration of therapeutics that could modulate one of the three main underlying sepsis-induced immunosuppressive mechanisms (See **Table 2**).

Table 2

Biomarkers of Late-Sepsis

Biomarker	Role and Correlation with Outcomes due to Late Sepsis
Decreased Lymphocyte Count	• Late-septic patients are reported to have a decreased lymphocyte count compared with non-septic patients. • The decrease is thought to be due to inappropriate activation of apoptosis pathways. • Correlated with a higher likelihood of developing secondary infections.
Increased MDSC Production/Migration	• Late-septic patients have higher levels of MDSCs in the blood and secondary lymphoid organs compared with non-septic patients. • While the purpose of increased MDSC production remains controversial, these cells are thought to contribute to worse outcomes due to sepsis due to their immunosuppressive and anti-inflammatory nature.
Increased IL-10	• Late-septic patients have elevated levels of IL-10 compared with non-septic patients. • Elevated anti-inflammatory responses suggest that the host will not be able to fight off a secondary infection. • Correlated with worse severity/worse clinical outcomes due to sepsis.
Changes in GPR18 Expression	• Late-septic patients have changes in GPR18 expression on their immune cells compared with non-septic patients. • These changes are attributed to SPM uncoupling in sepsis. • Increased GPR18 expression on monocytes and decreased GPR18 expression on neutrophils is correlated with an increase in mortality
Decreased HLA-DR Expression	• Late-septic patients have 70% less HLA-DR expression compared with non-septic patients. • Decreased HLA-DR expression suggests that the host will not be able to fight off a secondary infection. • Correlated with worse SOFA scores due to sepsis.

Note. Citations for decreased lymphocyte count: (Bo et al., 2011; Carrero and Unanue, 2006; Francois et al., 2018; Hotchkiss et al., 1999; Liu et al., 2022; Wang et al., 1994; L. Yang et al., 2023). Citations for increased MDSC production/migration: (Arocena et al., 2014; Brudecki et al., 2012; Cuenca et al., 2011; Eriksson et al., 2016; Hotchkiss et al.,

2013a; Landoni et al., 2016; Law et al., 2020; Liu et al., 2022; Mathias et al., 2017; Sander et al., 2010; Schrijver et al., 2022; Tang et al., 2021; Uhel et al., 2017; Wendan Zhang et al., 2023). Citations for increased IL-10: (Gogos et al., 2000; Liu et al., 2022; Wu et al., 2009). Citations for changes in GPR18 expression: (Jundi et al., 2021; Zhang et al., 2019; Zhao et al., 2023). Citations for decreased HLA-DR expression: (Berry et al., 2011; Hoogendijk et al., 2017; Liu et al., 2022; Venet and Monneret, 2018).

1.6.1 *IL-10*

Two potential biomarkers of sepsis-induced immunosuppression that were already discussed in this chapter include decreased lymphocyte numbers in the blood (due to apoptotic mechanisms) and increased numbers of MDSCs in the blood and secondary lymphoid organs. Related to the aspect of immune cell exhaustion discussed earlier, another valuable marker of late sepsis that has been widely accepted is elevated levels of IL-10 in the serum (Wu et al., 2009). Not only does the patient's IL-10 level correlate with his/her sepsis severity score, but it also has been reported to be a major risk factor for increased clinical severity (Gogos et al., 2000). Many immune cells (both innate and adaptive) produce IL-10, as do epithelial cells and keratinocytes (Iyer and Cheng, 2012). Physiologically, IL-10 has an important anti-inflammatory role in immune system homeostasis by balancing the pro-inflammatory response. IL-10 exerts its anti-inflammatory effect by inhibiting the release of pro-inflammatory cytokines, decreasing antigen presentation, and decreasing phagocytosis (Iyer and Cheng, 2012). While IL-10 is crucial for normal immune cell function, sustained release of IL-10 can lead to immunosuppression by artificially inhibiting the normal functioning of immune cells. This understanding contributes to elevated blood IL-10 levels acting as a possible marker of sepsis-induced immunosuppression.

1.6.2 *GPR18*

More recently, the transmembrane protein GPR18 has been identified as a potential biomarker of sepsis-induced immunosuppression. Elevations in GPR18, a G-protein-coupled receptor found on immune cells and most abundantly in the spleen, has only recently been considered a marker of late sepsis (Zhao et al., 2023). GPR18 is the receptor for the SPM RvD2 (Zhao et al., 2023). In sepsis patients, elevated GPR18 expression on monocytes correlates with a worse sepsis severity score (Jundi et al., 2021). Interestingly, low GPR18 expression on neutrophils correlates with increased severity and a worse prognosis due to sepsis (Zhang et al., 2019). Taken together, these data provide evidence that GPR18 expression in different cell types may provide a way of "fine-tuning" biomarkers in sepsis.

1.6.3 *HLA-DR*

Human leukocyte antigens (HLAs) are a group of genes in the MHC family that code for various proteins involved in the human immune system (Wang et al., 2007). HLA-DR is an MHC-II glycoprotein complex expressed on APCs (monocytes/macrophages, dendritic cells, and B cells) (Wang et al., 2007). Because HLA-DR is an MHC-II protein, it is required for adaptive immune system activation; therefore, researchers correlate increased expression of HLA-DR on APCs with a well-functioning immune system (Liu et al., 2022). Recent research has suggested that decreased HLA-DR expression can be a reliable biomarker for sepsis-induced immunosuppression for many reasons. For example, one study reported that septic patients express 70% less HLA-DR than non-septic patients, and this decrease is associated with an increase in the patient's sequential organ failure

assessment (SOFA) score (Berry et al., 2011). Similarly, another study found that decreased HLA-DR expression in immune cells within the bone marrow of septic patients was correlated with worse outcomes due to sepsis (Venet and Monneret, 2018). Another study found that following stimulation with LPS, immune cells with reduced expression of HLA-DR produce fewer pro-inflammatory cytokines than their immune cell counterparts with higher expression of HLA-DR (Hoogendijk et al., 2017); this phenomenon is suggestive of immune cell exhaustion. Taken together, the results of these studies suggest that decreased levels of HLA-DR can serve as a potential biomarker of one of the three major mechanisms underlying sepsis-induced immunosuppression (specifically monocyte/macrophage exhaustion).

1.7 Summary

Severe infection can lead to sepsis. Death by sepsis can occur during different stages of the sepsis timeline. Typically, infectious sepsis (i.e., sepsis triggered by an infectious agent) can lead to CRS, which is when a massive release of pro-inflammatory mediators leads to pathologic effects within the body. One such effect is life-threatening septic shock, which is characterized by hypotension and organ failure. If the patient is able to withstand this early stage of sepsis, they may be overwhelmed by the late stage of sepsis, which is characterized by the opposite phenomenon: immune suppression. During this late stage, mortality is increased because the host cannot fight against the presently-invading pathogens or any newly-acquired infections. While there have been promising advancements in antibiotic development and preclinical study designs and research techniques, there is still a great need for effective sepsis therapies. SPMs are a group of

inflammatory “resolvers” derived from essential fatty acids. We have previously shown that the SPM RvD2 enhances macrophage functions in vivo (Walker et al., 2022). However, the molecular mechanism of how RvD2 enhances macrophage functions is currently unknown.

During the acute stages of inflammation (i.e., the early phase of sepsis), pro-inflammatory mediators are produced via activation of the transcription factor NF-κB. Previous work in other labs (Basil and Levy, 2016; Brennan et al., 2021; Pan et al., 2022; Recchiuti and Serhan, 2012; Zhang et al., 2020) have shown that SPMs can reduce acute inflammatory response by down-regulating NF-κB. Effects of SPMs such as RvD2 on the immunosuppressive phase of sepsis have not been elucidated. Our central hypothesis is that RvD2 enhances macrophage inflammatory responses and antimicrobial activities in exhausted macrophages and these effects are correlated with NF-κB activity. We believe this to be the case as work by us and others have shown that resolvins resolve the acute inflammatory response by recruiting macrophages to the site of infection, by increasing macrophage efferocytic abilities, by decreasing neutrophil migration, and by increasing neutrophil apoptosis. Additionally, we have evidence showing that the actions of RvD2 on monocytes/macrophages is linked to changes in NF-κB activity. The objective of this thesis work was to elucidate the molecular mechanisms (including signaling pathways) of how RvD2 enhances monocyte/macrophage function during both stages of sepsis; however, there was more of a focus on RvD2’s effects on exhausted macrophages (present during the late phase of sepsis), as this has been historically understudied in comparison to sepsis’s early phase.

Three main mechanisms underlying sepsis-induced immunosuppression include monocyte/macrophage exhaustion, lymphocyte apoptosis, and MDSC migration. These mechanisms have one commonality: persistence of the early, hyper-proinflammatory phase of sepsis, which eventually leads to a late, immunosuppressive state. Early recognition of late-sepsis biomarkers, such as decreased lymphocyte count, increased MDSC production/migration, increased IL-10 levels, changes in GPR18 expression, and decreased HLA-DR expression, will help with targeted diagnoses and treatments by clinicians in the future. If an SPM such as RvD2 can attenuate immunosuppression, then it is an intriguing possible therapeutic to protect the patient from secondary infections and death due to sepsis-induced immunosuppression.

Chapter 2

Resolvin D2 (RvD2) Influences Transcription Factor NF-κB Activity and Monocyte/Macrophage Functions After Lipopolysaccharide (LPS) Stimulation

2.1 Introduction

Lipopolysaccharide (LPS), found in the outer membrane of Gram-negative bacteria, strongly stimulates monocytes and macrophages through surface and intracellular TLR4 (Meng and Lowell, 1997; Plevin et al., 2016). LPS is composed of a hydrophobic lipid region (Lipid A), an oligosaccharide core, and an O-Antigen chain (Plevin et al., 2016). LPS prompts immune cells such as monocytes/macrophages to release numerous pro-inflammatory mediators, such as IL-6 and TNF-α, via NF-κB (Meng and Lowell, 1997). These pro-inflammatory mediators have crucial roles in orchestrating the immune response to infections and injuries, but dysregulated or excessive production can also have harmful effects on the body. IL-8 is another pro-inflammatory mediator produced via activation of NF-κB that functions to recruit neutrophils to an area of infection, further increasing inflammation (Bickel, 1993).

While there are studies in the literature that focus on the roles of Specialized Pro-resolving Mediators (SPMs) such as Resolvin D2 (RvD2) in early sepsis (characterized by an overwhelming pro-inflammatory response), there are very few studies that focus on the roles of SPMs in late sepsis (characterized by an immunosuppressed phenotype of the host). An established and accepted model of late polymicrobial sepsis is the two-hit model of cecal-ligation and puncture (CLP) (hit one) and secondary lung *Pseudomonas aeruginosa* infection (hit two) (Hotchkiss et al., 2013a; Liu et al., 2022; Muenzer et al.,

2010; Walker et al., 2022). In studies using this model, late RvD2 administration (48h after CLP) increased survival after secondary infection by 30-40% (Sundarasivarao et al., 2022; Walker et al., 2022). The mechanism for these beneficial effects is partially due to increased macrophage phagocytic ability, which helps reduce bacterial load in these murine models (Chiang et al., 2017; Walker et al., 2022). However, the full effects of RvD2 on exhausted monocytes/macrophages has not been elucidated.

We and others have also shown that SPMs can decrease bacterial virulence by inhibiting quorum sensing, a common bacteria-driven mechanism of antibiotic resistance. Lipoxin A_4 (LxA_4), another type of SPM, has been shown to increase Ciprofloxacin's (fluoroquinolone antibiotic) efficacy for *Pseudomonas aeruginosa*, a common bacterial cause of sepsis in U.S. hospitals and among immunocompromised patients (Thornton et al., 2021; Wu et al., 2016). Additionally, some studies have demonstrated that LxA_4 exhibits direct antimicrobial activity against certain pathogens. For example, research has shown that LxA_4 can inhibit the growth of certain bacteria, including *Escherichia coli* (Ali et al., 2021), *Pseudomonas aeruginosa* (Thornton et al., 2023), and *Staphylococcus aureus* (Boff et al., 2020), as well as fungi like *Candida albicans* (Mei et al., 2021).

2.2 Rationale

According to the literature, incubating neutrophils with D-series resolvins such as RvD2 increases neutrophil functionality (Jundi et al., 2021; Thornton and Yin, 2021) and macrophage phagocytosis and efferocytosis (Gao et al., 2023; Sundarasivarao et al., 2022). In some instances, RvD2 has been reported to enhance these particular immune cell functions through NF-κB signaling (Li et al., 2020; Sham et al., 2018). However, other

studies suggest that D-series resolvins can also influence immune cell functions through additional signaling pathways, such as MAPK and PI3K/Akt, depending on the stimulus (Benabdoune et al., 2016; Gilbert et al., 2015; Ohira et al., 2010; Wang et al., 2011). In LPS-induced macrophage activation, the focus of the experiments in this chapter, a primary signaling pathway triggered is the NF-κB pathway (Meng and Lowell, 1997).

What is the timeline as to when in the monocyte-macrophage axis that RvD2 exerts this effect on cells: before monocyte differentiation into macrophages or after? Does RvD2 have the same effect on monocytes as it does on macrophages? In this chapter, we have designed several experiments to answer these questions and to explore if RvD2 mediates functional changes to monocyte/macrophage via NF-κB following LPS stimulation. Most importantly, we first wanted to establish in vitro models of acute inflammation and of macrophage exhaustion (as mentioned in **Chapter 1**, the latter is one of the accepted mechanisms underlying sepsis-induced immunosuppression). We established these scenarios by using LPS treatments (dubbed "hits"), and the specifics will be discussed in the methods section below. We were interested in establishing in vitro monocyte/macrophage *exhaustion* rather than *tolerance*, as we believe that monocyte/macrophage tolerance does not totally encompass all characteristics of immunosuppression observed in septic patients (as discussed in detail in **Chapter 1**).

In these studies, we used a monocyte reporter cell line that could help us discern NF-κB activation in real-time. RvD2 was first administered to monocytes prior to differentiation into macrophages as well as to fully-differentiated macrophages, but prior to incubation with one LPS hit or two LPS hits. Then, changes in NF-κB activity levels were assessed. Primarily, we were interested in the mechanism behind how RvD2

modulates monocyte/macrophage functionality when the cells encounter LPS (i.e., free endotoxin).

In this chapter, RvD2's stimulatory role on monocytes was also assessed, as it has been previously shown that LxA_4 can increase monocyte adhesion to laminin-coated plates (Maddox and Serhan, 1996) and to non-laminin-coated plates (Thornton et al., 2023). Additionally, there are numerous reports concluding that lipoxins display distinct stimulatory effects on human monocytes, encouraging cell migration and adhesion (Maddox and Serhan, 1996; Simões et al., 2010; Simões and Fierro, 2005). Monocyte adherence is an initial step towards differentiation into macrophages (Nielsen et al., 2020), as increased adherence can be thought of as an initial step of monocyte activation. Therefore, we designed experiments to study monocyte adherence following treatment with RvD2, as we were curious if RvD2 can directly activate monocytes and trigger their differentiation into macrophages.

2.3 Materials and Methods

2.3.1 RvD2 Synthesis

RvD2 was prepared by total organic synthesis as previously described with all spectroscopic data included (Rodríguez and Spur, 2004). Importantly, the synthesized RvD2 was compared with a commercial sample from Cayman Chemicals and found to be identical (Rodríguez and Spur, 2004). The purity of the compound was measured by HPLC-Mass Spectrometry and determined to have >98% purity (Rodríguez and Spur, 2004). 1 μg

of RvD2 was dissolved in 2.66 mL of sterile saline which had been bubbled with argon on days of experiments.

2.3.2 Monocyte Cell Cultures and Differentiation to Macrophages

All in vitro experiments used human THP-1 monocytes containing the NF-κB gene promoter coupled to a luciferase reporter (TIB-202-NFκB-LUC2™; ATCC: Manassas, VA, USA), which were cultured and maintained in RPMI 1640 with L-glutamine (Corning: Manassas, VA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Corning: Manassas, VA, USA), 0.05 mM 2-mercaptoethanol (VWR: Solon, OH, USA), 100 U/mL penicillin G (VWR: Solon, OH, USA), and 100 µg/mL streptomycin (VWR: Solon, OH, USA), and kept in an atmosphere of 37°C, 5% CO_2. THP-1 monocytes were seeded at 6 X 10^4 cells/well in 96-well plates (Corning, Manassas, VA, USA) in FBS-free and penicillin-/streptomycin-free RPMI. Plated cells were subsequently differentiated into macrophages with treatment of 100 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St. Louis, MO, USA) for 48h as previously described (Takashiba et al., 1999). After differentiation, the media was aspirated, the cells were washed with saline three times, and RPMI media was replenished. Phenotypic lineage of the THP-1 monocyte-derived macrophages was confirmed by imaging using a Keyence BZ-X710 Scope (Keyence: Itasca, IL, USA) and flow cytometry (see below).

2.3.3 Confirmation of Phenotypic Lineage of THP-1 Monocyte-Derived Macrophages Using Flow Cytometry

Approximately 1 X 10^6 cells were aliquoted into microcentrifuge tubes and Fc receptors were blocked using 1 µL of TruStain FcX anti-human antibody (BioLegend, Cat

#422301, San Diego, CA) for 30 min on ice. Next, cells were washed with cell staining buffer and 1 µL of CD14 antibody (BioLegend, Cat #301807, San Diego, CA) and 1 µL of CD11b antibody (BioLegend, Cat #101207, San Diego, CA), along with 1 µL of live/dead cell stain (Invitrogen, Cat #L10119, Waltham, MA) and incubated for 80 min on ice. After incubation, cells were washed with cell staining buffer and resuspended. Attune Acoustic Focusing cytometer (Applied Biosystems, Waltham, MA) was used to analyze samples. For flow cytometry compensation of multiple fluorophores, ABC anti-rat capture beads (Invitrogen, Waltham, MA) and ArC amine reactive capture beads (Invitrogen, Waltham, MA) were used for accurate separation of overlapping emission wavelengths of multiple fluorophores. Cells were gated based on size (forward scatter) and granularity (side scatter), single population, live cells, and CD14 and CD11b expression. FlowJo software (Version 10) was used to analyze flow cytometry data (**Figure 12**). The data show that there is a clear change in cell phenotype with a substantial increase in CD11b expression (an important protein in cell adhesion). Overall, the plots have clearly altered, indicating a change in phenotype.

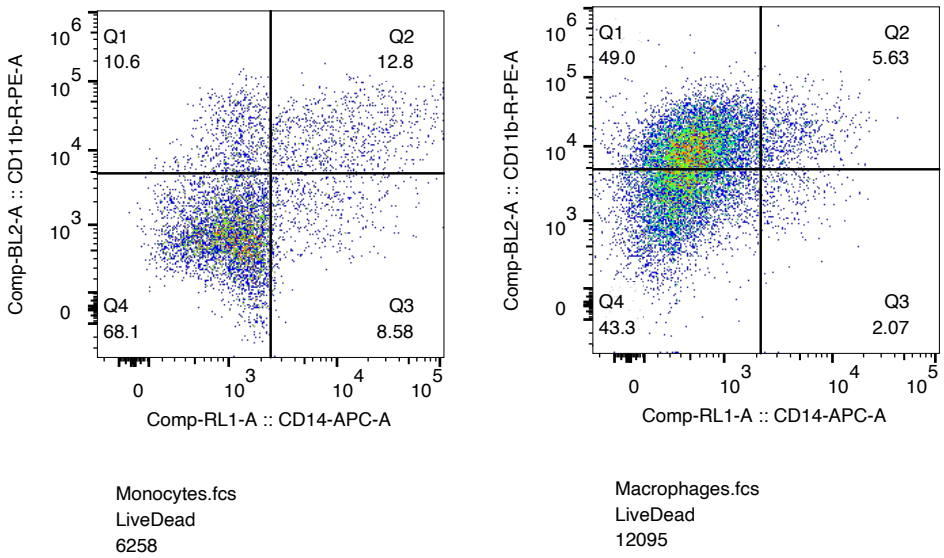


Figure 12. Confirmation of THP-1 Monocyte-Derived Macrophages with Flow Cytometry. THP-1 monocytes were seeded in a 96-well plate and stimulated with 100 ng/mL PMA for 48h. Following stimulation, the media and non-adherent cells were removed, and adherent cells were washed with PBS. Fresh RPMI media containing 10% heat-inactivated FBS was added for 24h. After 24h of rest, the media was removed, and the cells were washed with PBS. Serum-free RPMI was added, and the cells were taken for flow cytometry analysis. Flow cytometry analysis revealed that THP-1 monocyte-derived macrophages had increased cell surface expression of CD11b and CD14, which are two cell surface markers specific to macrophages (Starr et al., 2018). The increased presence of these particular cell surface markers confirms that 100 ng/mL of PMA was adequate and successful at differentiating THP-1 monocytes into macrophages.

2.3.4 Monocyte/Macrophage Treatments

The protocol for these studies is outlined in **Figure 13**. These studies were performed to evaluate the effect(s) of RvD2 on acute LPS stimulation (1-hit LPS) and on exhausted monocyte/macrophage responses (i.e., NF-κB activity, TNF-α production, and IL-8 production) to a 2nd hit of LPS. Briefly, THP-1 monocytes or THP-1 monocyte-derived macrophages were pre-incubated with RvD2 or saline vehicle for 1h at 37°C, 5% CO_2. Cells were then exposed to various treatments. These treatments included: 1) "one hit": 50 ng/mL of LPS (O111.B4 from *E. coli*; EMD Millipore Corporation: Burlington,

MA, USA) for 3h or 2) “two hits”: 50 ng/mL of LPS for 24h followed by an additional 2nd hit for 3h with 50 ng/mL of LPS. All studies were performed in triplicate.

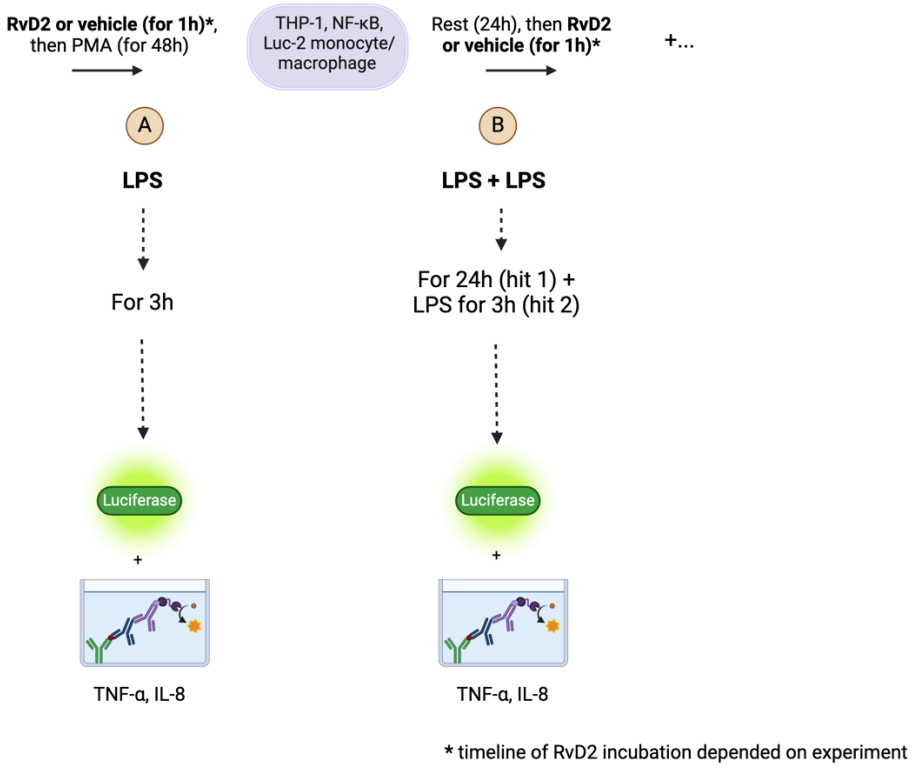


Figure 13. Methods for Monocyte/Macrophage Incubations. This schematic outlines the experiments conducted for RvD2 treatments on THP-1 monocytes prior to and following differentiation with PMA. In this chapter, cells were stimulated with free endotoxin (LPS).

2.3.5 Luciferase Assay

To elucidate the cellular mechanism by which RvD2 affects NF-κB activity following LPS stimulation, the THP-1 NF-κB-LUC2 (ATCC TIB-202-NFκB-LUC2™) cell line was used. In this cell line, the firefly luciferase gene, *luc2*, was placed under the control of the NF-κB promoter, such that NF-κB activity could be measured via luciferase

luminescence. Following treatments, Firefly Luc One-Step Glow Assay Kit (Pierce: Rockford, IL, USA) was used per the manufacturer's protocol. Briefly, the cells were lysed and incubated with D-Luciferin substrate for 15 min. The Biotek Synergy H1 plate reader was programmed to measure chemiluminescence with an integration time of 1 sec at 135 gain. In addition, THP-1 monocytes and THP-1 monocyte-derived macrophages that were not expressing luc2 were used as negative controls. To validate the assay, QuantiLum Recombinant Luciferase (Promega, Madison, WI, USA) in Firefly Luc One-Step assay buffer (Pierce, Rockford, IL, USA) with 1% bovine serum albumin (BSA; BioVision, Milpitas, CA, USA) was serially-diluted and luminescence measured every 5 min for a 30 min period. The data showed that the Pierce Firefly Luc One-Step Glow assay accurately detected luminescence from firefly luciferase, and that the luminescence of the diluted recombinant luciferase was stable for a 20 min duration (Wilson et al., 2021)

2.3.6 Cytokine Production Assays

The concentrations of human TNF-α (Thermo Fisher Scientific: Waltham, MA, USA) and human IL-8 (i.e., CXCL8) (RayBiotech: Peachtree Corners, GA, USA) were determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturers' instructions. Absorbance (450 nm) was read on a BioTek Synergy H1 plate reader (Biotek: Winooski, VT, USA).

2.3.7 Monocyte Adhesion Assay

To evaluate the effect of RvD2 on monocyte adherence, laminin coated 24-well plates (Corning, Manassas, VA, USA) were blocked with 0.1% bovine serum albumin

(BSA; BioVision, Milpitas, CA, USA) for 1h at 37°C, 5% CO_2 and washed with phosphate-buffered saline (PBS) before use. Briefly, THP-1 monocytes were seeded into laminin coated plates at 3 X 10^5 cells/well in FBS-free and penicillin-/streptomycin-free RPMI. RvD2 (1, 10, or 100 nM) or saline vehicle was added and incubated for 1h at 37°C, 5% CO_2. After incubation, non-adherent cells were removed by aspirating the media and washing with saline three times. FBS-free and penicillin-/streptomycin-free RPMI media was replenished, 10 μL of CellBrite Blue Cytoplasmic stain (Biotium, Fremont, CA, USA) was added to each well, and the cells were incubated for 20 min at 37°C, 5% CO_2. The stain was removed, the cells were washed with saline three times, and RPMI media was replenished. Adherent cells were then imaged using a Keyence BZ-X7010 (Keyence: Itasca, IL, USA). The number of adherent cells per well was quantified using ImageJ software. Briefly, each z plane was split into its component colors, but only the blue fluorescent channel (representing the area covered by adherent monocytes) was used. All z planes were stacked and converted to 8-bit grayscale. Using the Comstat2 plug-in, background noise was excluded from quantification. Using these merged z stacks, fluorescence intensity and/or % increased adhesion was quantified.

2.3.8 Statistical Analysis

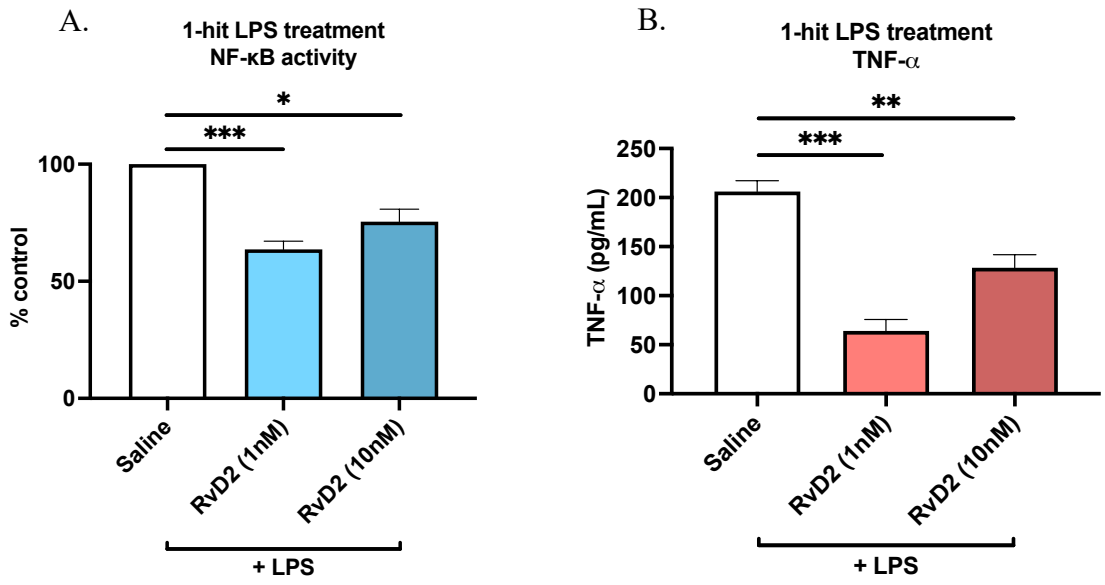
All analyses were performed using GraphPad Prism version 9.4.1 for Mac (GraphPad Software: San Diego, CA, USA). All data are expressed as mean ± SEM. For data sets that were normally distributed, data were subjected to one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons post-hoc test. For data sets that were not normally distributed (i.e., luciferase assays), data were subjected to Kruskal-

Wallis test with Dunn's multiple comparisons post-hoc test. Data from 1-hit vs. 2-hit LPS treatments were subjected to unpaired t-tests. A p value < 0.05 was taken as significant for at least three independent studies.

2.4 Experimental Results

2.4.1 RvD2 Modulated Macrophage Function After One LPS Administration

THP-1 macrophages were incubated with RvD2 (1 or 10 nM) or saline vehicle for 1h. The macrophages were then acutely stimulated with LPS for 3h. The results showed that RvD2 decreased NF-κB activity in macrophages following 3h treatment with LPS compared to the control (**Figure 14A**). In addition, RvD2 decreased TNF-α production by LPS-stimulated macrophages (**Figure 14B**) but did not influence IL-8 production (**Figure 14C**). These results suggest that RvD2 reduced macrophage inflammatory response to LPS in an NF-κB-dependent manner.



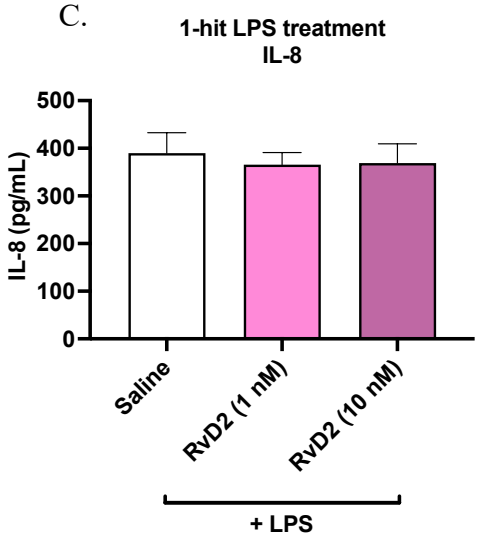


Figure 14. RvD2 Differentially Affected Acute Macrophage Responses (1-Hit LPS). THP-1 macrophages were treated with either saline vehicle or RvD2 (1 and 10 nM) for 3h. *A.* RvD2 reduced NF-κB activity compared to saline controls. Values are mean ± SEM. ***P < 0.001, *P < 0.05; n = 6. *B.* RvD2 dampened macrophage LPS-induced TNF-α production compared to saline controls. Values are mean ± SEM. ***P < 0.001, **P < 0.01; n = 3. *C.* IL-8 levels were not significantly different from saline controls following pre-incubation with RvD2. Values are mean ± SEM; n = 6.

2.4.2 Establishing Macrophage Exhaustion

THP-1 monocytes were differentiated into macrophages with PMA (100 ng/mL) for 48h and allowed to rest for an additional 24h. For 2-hit LPS treatments, macrophages were incubated with 50 ng/mL of LPS for 24h and then incubated again with 50 ng/mL of LPS for 3h. This 2-hit protocol is different from one 27h incubation with LPS. That scenario does not allow for observation of cell exhaustion since the cells are not induced to respond to a stimulus. Our 2-hit protocol remedies this, as it allows for observation of the stimulated macrophage's response to a subsequent LPS challenge (i.e., the 2nd hit). When THP-1 macrophages were incubated with 50 ng/mL of LPS for 24h without a 2nd LPS hit, the NF-κB activity remained elevated, similar to acutely-stimulated THP-1

macrophages, rather than to exhausted macrophages (**Figure 15A**). To ensure that the 2-hit LPS treatments effectively established macrophage exhaustion, macrophage functions were assessed. The results showed that 1-hit LPS treatments acutely increased NF-κB activity 11-fold, whereas NF-κB activity was increased only 6-fold after two LPS hits, a drop of 45% (**Figure 15B**). Similarly, TNF-α and IL-8 secretion were also significantly reduced after two LPS hits compared to one LPS hit (**Figures 15C** and **15D**, respectively). These results suggest that 2-hit LPS treatments caused a significant level of macrophage exhaustion in vitro.

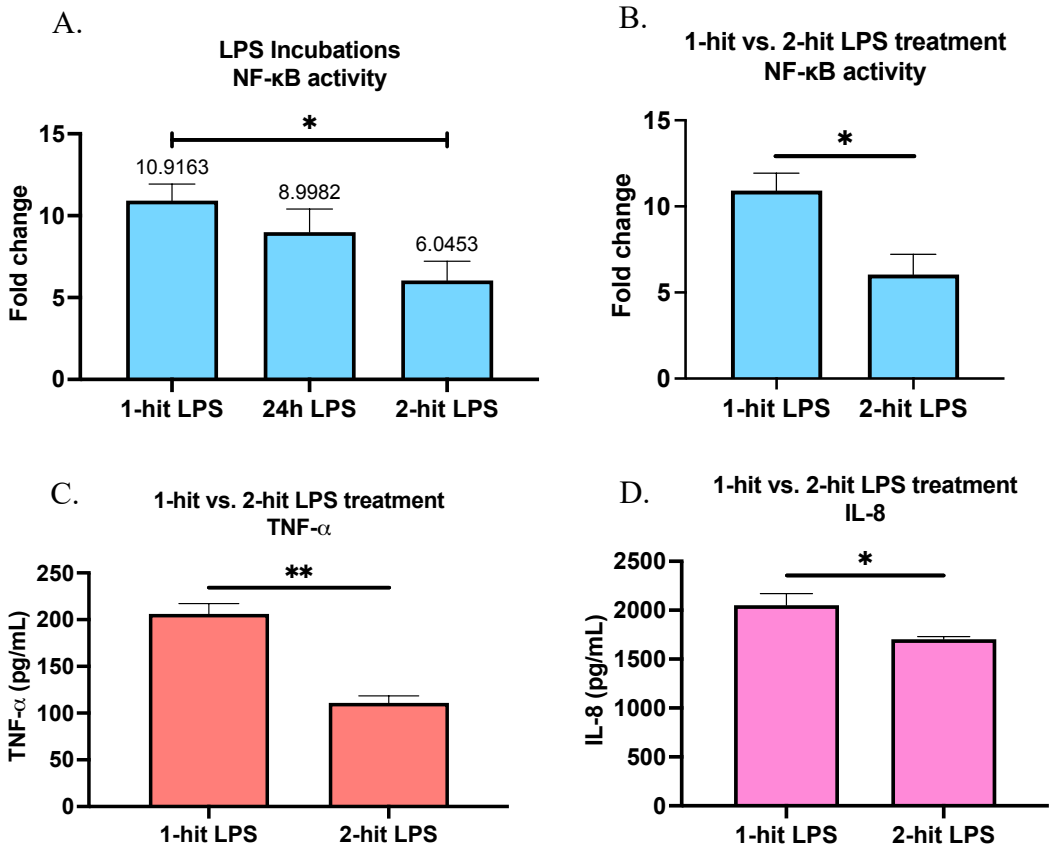


Figure 15. 2-Hit LPS Treatments Established Macrophage Exhaustion in vitro. *A.* Macrophages exposed one 3h LPS hit had 11-fold increased NF-κB activity compared to saline controls. Macrophages exposed to one 24h LPS hit had 9-fold increased NF-κB

activity compared to saline controls. Macrophages exposed to two LPS hits had 6-fold increased NF-κB activity compared to saline controls. Values are mean ± SEM. *P < 0.05; n = 3. *B.* Macrophages exposed to one LPS hit had 11-fold increased NF-κB activity compared to saline controls. In comparison, macrophages exposed to two LPS hits only increased NF-κB activity 6-fold compared to unstimulated controls. Values are mean ± SEM. *P < 0.05; n = 3. *C.* Macrophages exposed to one LPS hit had 61-fold increased TNF-α production. In comparison, macrophages exposed to two LPS hits only increased TNF-α production 15-fold compared to unstimulated controls. Values are mean ± SEM. **P < 0.01; n = 3. *D.* Macrophages exposed to one LPS hit had 4-fold increased IL-8 production. In comparison, macrophages exposed to two LPS hits had 3-fold increased IL-8 production compared to unstimulated controls. Values are mean ± SEM. *P < 0.05; n = 3.

2.4.3 RvD2 Attenuated Macrophage Exhaustion Following Two LPS Administrations

THP-1 macrophages were incubated with RvD2 (1 or 10 nM) or saline vehicle for 1h, followed by two LPS treatments to induce exhaustion. The results showed that RvD2 increased NF-κB activity (**Figure 16A**), TNF-α production (**Figure 16B**), and IL-8 production (**Figure 16C**) in exhausted macrophages compared to saline controls. These results suggest that RvD2 can attenuate macrophage suppression. Taken together, the results from **Figure 15** and **Figure 16** suggest that RvD2 modulates macrophage responses, where it reduces macrophage LPS-mediated acute inflammatory responses (1-hit LPS) but helps preserve macrophage functional activity during LPS-mediated exhaustion (2-hit LPS).

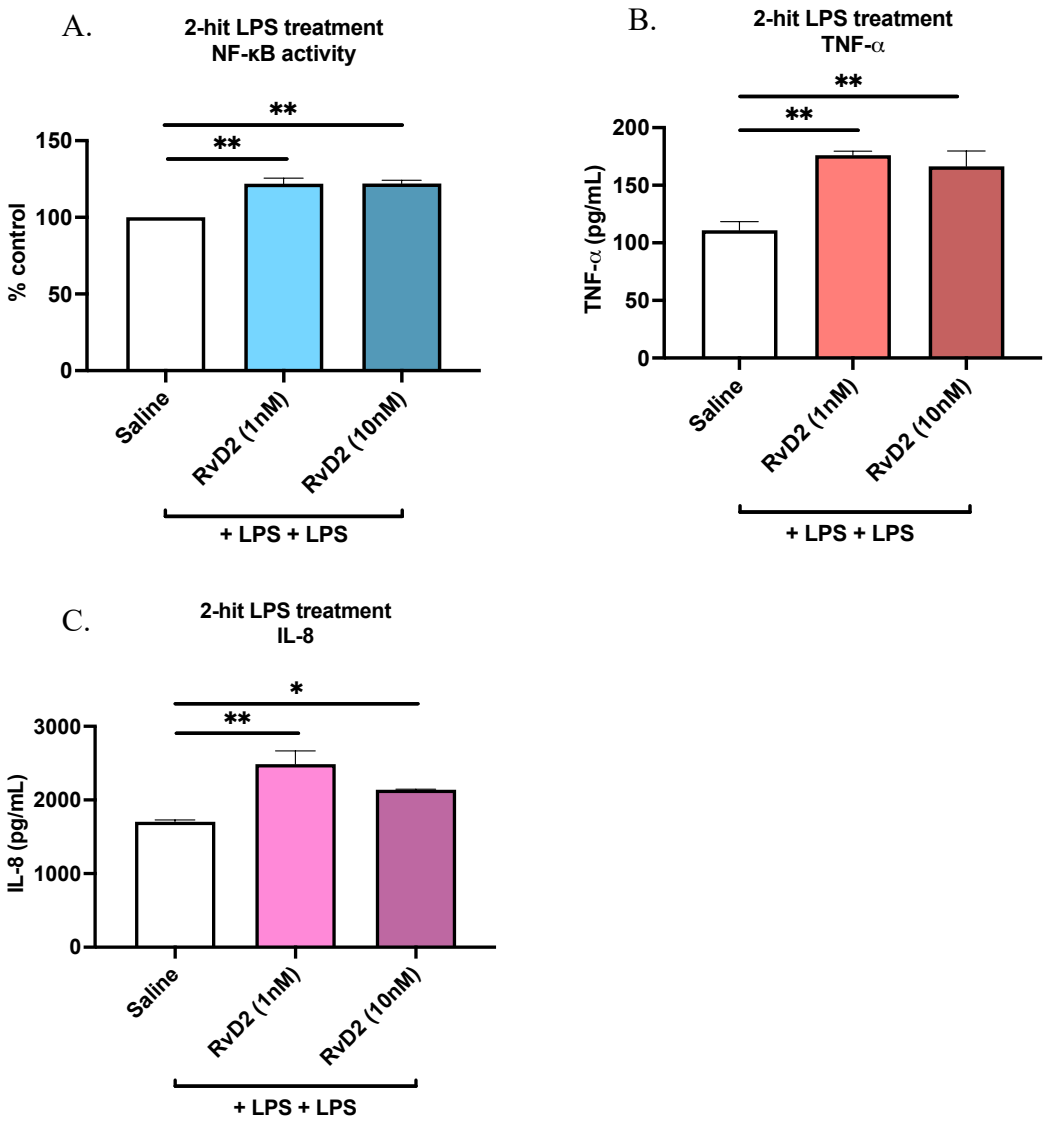
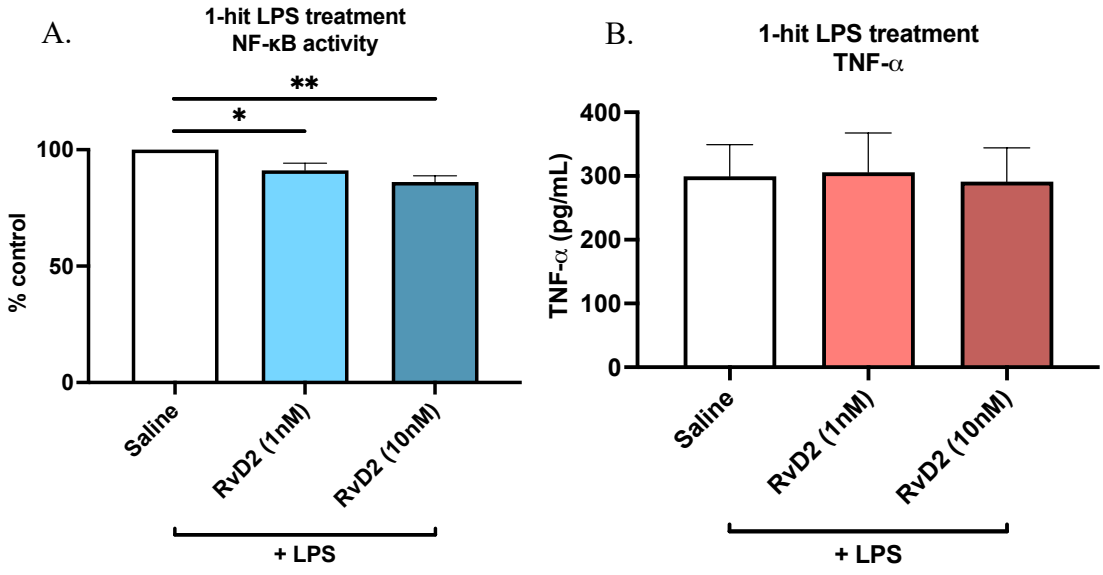


Figure 16. RvD2 Attenuated LPS-Mediated Macrophage Exhaustion in vitro. *A.* RvD2 increased NF-κB activity in exhausted macrophages compared to saline controls. Values are mean ± SEM. **P < 0.01; n = 6. *B.* RvD2 significantly increased TNF-α production by exhausted macrophages compared to saline controls. Values are mean ± SEM. ***P < 0.001; n = 6. *C.* RvD2 increased IL-8 production by exhausted macrophages compared to saline controls. Values are mean ± SEM. **P < 0.01, *P < 0.05; n = 3.

2.4.4 RvD2's Effects on THP-1 Monocytes Prior to PMA Differentiation

In these experiments, we investigated if the effects of RvD2 treatment on THP-1 monocytes (RvD2 administered to cells prior to differentiation with PMA) is different from RvD2 treatment on THP-1 monocyte-derived macrophages (RvD2 administered to cells after differentiation with PMA) following stimulation with LPS. THP-1 monocytes were plated and immediately treated with saline vehicle or RvD2 (1 or 10 nM) for 1h. Following this 1h incubation, the monocytes were then differentiated into macrophages with PMA (100 ng/mL) for 48h. After 24h of rest, the macrophages were incubated with LPS for 3h. The results showed that RvD2 significantly decreased macrophage NF-κB activity following a single LPS administration (**Figure 17A**). Interestingly, while there was no effect of RvD2 on macrophage TNF-α, RvD2 did decrease IL-8 production (**Figures 17B** and **17C**, respectively).



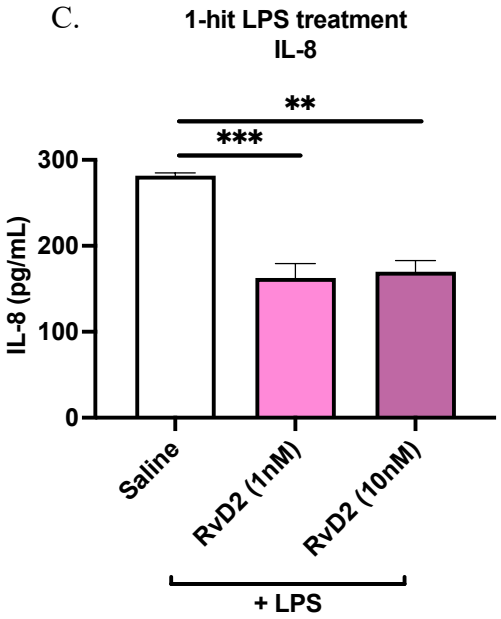


Figure 17. RvD2's Effects on THP-1 Monocytes when Given Before Differentiation to Macrophages. Saline vehicle or RvD2 was administered before THP-1 monocyte differentiation into macrophages. *A.* When given prior to monocyte differentiation into macrophages, RvD2 reduced 1-hit LPS-induced NF-κB activity compared to saline controls. Values are mean ± SEM. **P < 0.01, *P < 0.05; n = 6. *B.* When given prior to monocyte differentiation into macrophages, RvD2 did not alter LPS-induced TNF-α production compared to saline controls. Values are mean ± SEM; n = 3. *C.* When given prior to monocyte differentiation into macrophages, RvD2 reduced 1-hit LPS-induced IL-8 production compared to saline controls. Values are mean ± SEM. ***P < 0.001, **P < 0.01; n = 3.

2.4.5 RvD2's Effects on Exhausted Macrophages were Different if Given Before Differentiation

THP-1 monocytes were plated and immediately treated with saline vehicle or RvD2 (1 or 10 nM) for 1h. Following this 1h incubation, the monocytes were then differentiated into macrophages with PMA (100 ng/mL) for 48h. After 24h of rest, the macrophages were incubated with 2-hit LPS treatments. The results showed that when given to monocytes instead of differentiated macrophages, RvD2 significantly decreased NF-κB activity (**Figure 18A**), TNF-α production (**Figure 18B**), and IL-8 production

(**Figure 18C**). These results indicate that RvD2 was not uniform in its actions. RvD2's modulatory effects were dependent on whether the RvD2 was added to monocytes or to monocyte-derived macrophages.

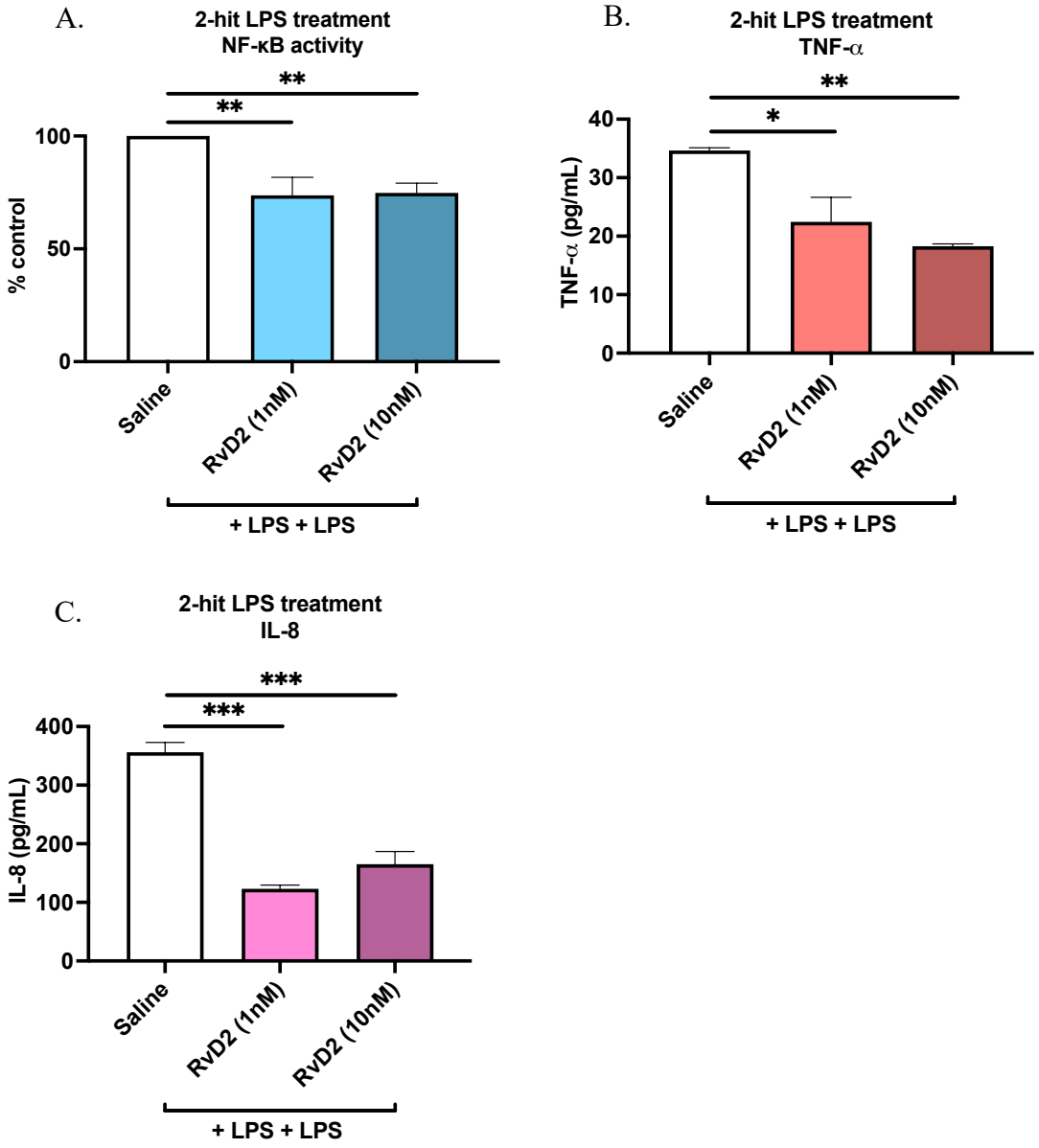


Figure 18. RvD2's Effects on Exhausted THP-1 Monocytes when Given Before Differentiation to Macrophages. *A.* When given prior to monocyte differentiation into macrophages, RvD2 reduced NF-κB activity compared to saline controls in LPS-induced exhausted (2-hit) macrophages. Values are mean ± SEM. **P < 0.01; n = 6. *B.* When given prior to monocyte differentiation into macrophages, RvD2 decreased TNF-α production

compared to saline controls in LPS-induced exhausted (2-hit) macrophages. Values are mean ± SEM. **P < 0.01, *P < 0.05; n = 3. *C.* When given prior to monocyte differentiation into macrophages, RvD2 decreased IL-8 production compared to saline controls in LPS-induced exhausted (2-hit) macrophages. Values are mean ± SEM. ***P < 0.001; n = 3.

2.4.6 RvD2 Increased Monocyte Adhesion

THP-1 monocytes were plated onto BSA-blocked laminin-coated plates and subsequently incubated with saline vehicle or RvD2 (1, 10, 100 nM) for 1h. Monocytes were stained with CellBrite Blue Cytoplasmic Membrane Dye according to the manufacturer's instructions. Wells were imaged to visualize and quantitate adherence to the plate. The fluorescence intensity results showed that increasing concentrations of RvD2 significantly increased adherence of monocytes to the wells (**Figure 19**). Since the first step of monocyte differentiation into macrophages is adhesion, this result suggests that RvD2 can directly trigger monocyte differentiation.

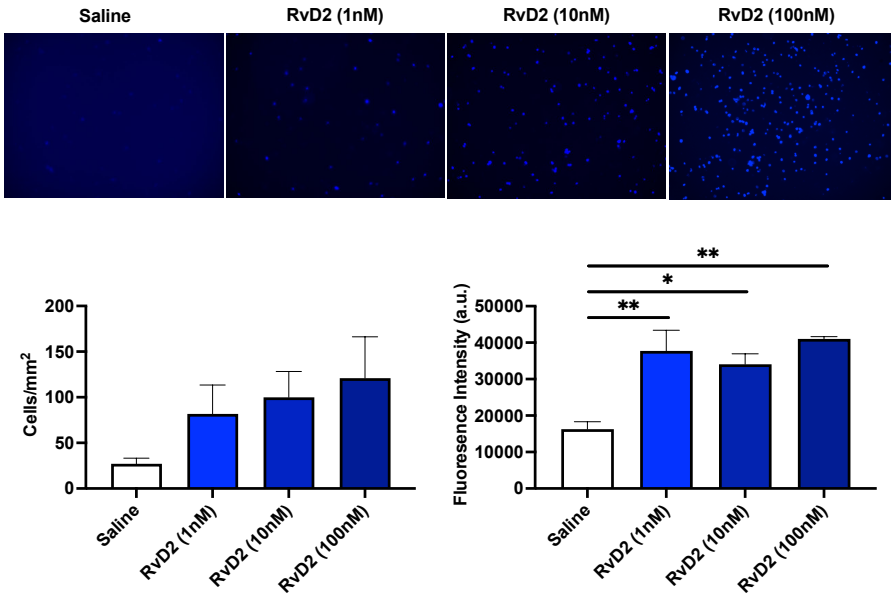


Figure 19. Pre-treatment of Monocytes with RvD2 Significantly Increased Their Adhesion to Cell Culture Plates. Fluorescently labeled THP-1 monocytes were treated with saline vehicle or RvD2 for 1h. RvD2 increased monocyte adhesion to laminin-coated plates compared to saline controls, as can be observed by quantifying fluorescence intensity. Values are mean ± SEM. $^{**}P < 0.01$, $^{*}P < 0.05$; n = 3.

2.5 Discussion

It is important to note that we tried to recapitulate an in vivo "scenario" of exhausted macrophage responses to LPS by using 2 doses of LPS rather than 1 high dose (100 – 1000 ng/mL) over a long period of time. We believe the latter does not provide a physiological cell model of exhaustion. In real life, the host gets infected by bacteria, and this small bacterial stimulus builds as the bacteria multiply. Similarly, our 2-hit LPS protocol is different from one 27h LPS incubation because the latter scenario does not allow for observation of cell exhaustion since the cells are not induced to respond to a stimulus. When THP-1 macrophages were incubated with 50 ng/mL of LPS for 24h without a second

LPS hit, their NF-κB activity remained elevated, similar to acutely-stimulated THP-1 macrophages, rather than to exhausted macrophages (**Figure 15A**).

The results from this chapter suggest that RvD2 can modulate macrophage functions in inflammation/infection, where it can reduce the acute inflammatory response to LPS (1-hit treatments) (**Figure 14**), but can increase effector responses in exhausted macrophages (2-hit LPS treatments) (**Figure 16**). Our results showing that RvD2 reduced macrophage inflammatory responses to LPS are consistent with work by us and others where RvD2 and other SPMs (such as LxA_4 and RvD1) were able to resolve inflammation in different in vivo models of inflammation/infection (Chiang et al., 2017; Codagnone et al., 2018; Spite et al., 2009; Walker et al., 2011; Zhuo et al., 2018). Interestingly, our results showed that in an acute inflammatory setting, RvD2 decreased NF-κB activity and TNF-α production, but IL-8 production was unaffected. There could be several explanations for this discrepant observation. First, the production of TNF-α and IL-8 can be regulated independently and therefore can be differentially expressed (despite their involvement in similar inflammatory processes) via different signaling pathways and transcription factors (Jundi and Greene, 2015; Osawa et al., 2002; Villarete and Remick, 1996). Second, negative feedback mechanisms may specifically regulate the production of one cytokine without affecting the other (Carballo et al., 1998; Lowin et al., 2020). Third and most convincingly, the kinetics of TNF-α and IL-8 production can differ (DeForge and Remick, 1991; Villarete and Remick, 1996). Studies have shown that they are transcribed at different times during the inflammatory response and/or their concentrations peak at different time points (DeForge and Remick, 1991). Additionally, there are studies reporting that transcription of IL-8 can be induced by changes in TNF-α levels (Kim et al., 2009;

O'Hara et al., 2009; Osawa et al., 2002). Perhaps in our experiments, a 3h LPS incubation was long enough to prompt changes in TNF-α production, but not long enough to induce changes in IL-8 production (either directly or indirectly).

Macrophage exhaustion in sepsis-induced immunosuppression is characterized by a reduced response to LPS stimulation, an inability to clear bacteria, and impaired antigen presentation (**Chapter 1**). There are several reports which show that impairment of the host's immune system to mount an inflammatory response to a secondary infection is responsible for the increased morbidity and mortality observed in sepsis (Cavaillon et al., 2001; Hotchkiss et al., 2013b; Jundi et al., 2021; Liu et al., 2022; López-Collazo and del Fresno, 2013; Muenzer et al., 2010; Pena et al., 2014). In addition, the host's ability to produce TNF-α (Liu et al., 2022) or IFN-γ (Fu and Wang, 2023) was reported to be beneficial in late-sepsis. In this chapter, we show that macrophages which were stimulated twice with LPS had decreased NF-κB activity, TNF-α production, and IL-8 production compared to cells that were stimulated once. This finding provides evidence of cell exhaustion similar to suppressed macrophages found in the late phase of sepsis. Here, RvD2 treatment increased NF-κB activity and TNF-α and IL-8 production, suggesting that RvD2 can augment effector responses in exhausted macrophages. These increased effector responses are biologically relevant, as they suggest that RvD2-treated exhausted macrophages are more active than exhausted macrophages given saline vehicle.

One interesting finding from our work is the observed difference if RvD2 was administered to THP-1 monocytes before differentiation and following differentiation to macrophages. When RvD2 was administered prior to differentiation with PMA, it led to decreased NF-κB activity, reduced TNF-α production, and reduced IL-8 production

regardless of whether cells were undergoing an acute inflammatory response (1-hit LPS) or were exhausted (2-hit LPS). One possibility for this effect is that RvD2 is priming and "pre-polarizing" the monocytes into an M2-like phenotype; therefore, following subsequent differentiation with PMA, regardless of the stimulus (1-hit LPS or 2-hit LPS), monocytes are already differentiated into M2-like macrophages, which have lowered inflammatory responses (Yunna et al., 2020) (See **Chapter 1**). Polarization of macrophages is highly dependent on their immediate environment, and repolarization can be triggered if necessary (Anand et al., 2023). During acute infection, M1-like macrophages dominate, and assist the host in clearing pathogens by triggering NF-κB-mediated inflammatory responses via the release of pro-inflammatory cytokines such as IL-6 and TNF-α (Yunna et al., 2020). Following cessation of infection, M2-like macrophages dominate, and their main functions are to repair tissue damage, decrease inflammation, and clear debris left from the infection/inflammatory response (Yunna et al., 2020). Our data suggest that RvD2 potentially polarizes monocytes into M2-like macrophages, and that this action is distinctly different from its effect on exhausted macrophages.

As discussed in **Chapter 1**, there is evidence that macrophages with a pro-resolving phenotype could exist (Saas et al., 2020). These pro-resolving macrophages are said to have qualities of the classic M1-like and M2-like phenotypes and contribute to both pro- and anti-inflammatory pathways (Saas et al., 2020). Considering this, our results suggest that when RvD2 is administered to macrophages after differentiation, the macrophages seem to become polarized to this pro-resolving phenotype, such that when exhausted (i.e., hit twice with LPS), the cells are able to increase relative NF-κB activity, TNF-α production, and IL-8 production.

Another interesting finding from this work is that RvD2 increased THP-1 monocyte adherence to laminin-coated plates, consistent with previous work performed using LxA_4 on laminin-coated plates (Maddox and Serhan, 1996). Under physiologic conditions, monocytes adhere to multiple basement membrane proteins such as laminin (Tobias et al., 1987). Additionally, one of the first steps of monocyte differentiation into macrophages is adherence (Nielsen et al., 2020). Taken together, our results suggest that RvD2 given to monocytes exerts a strong "pre-polarizing" effect which differentiates monocytes into adherent M2-like macrophages.

Chapter 3

Resolvin D2 (RvD2) Effects on Monocyte/Macrophage Functions After Bacteria Stimulation Are Not Dependent on NF-κB Activity

3.1 Introduction

Gram-negative sepsis, a severe and potentially life-threatening condition, arises from the systemic spread of bacterial infections caused by Gram-negative bacteria (DiPiro, 1990). These bacteria possess an outer membrane containing lipopolysaccharide (LPS) (i.e., endotoxin), which plays a significant role in triggering the body's inflammatory response. Among the various Gram-negative bacteria implicated in sepsis, *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* are notable pathogens frequently encountered in clinical settings (Holmes et al., 2021).

The pathogenesis of Gram-negative sepsis involves complex interactions between bacterial virulence factors and the host immune system. Upon invasion into the bloodstream, Gram-negative bacteria promptly activate immune cells such as monocytes and neutrophils. This activation triggers the release of pro-inflammatory cytokines, such as TNF-α and IL-6, initiating a cascade of inflammatory responses (as discussed in **Chapter 1**). This profound release of pro-inflammatory cytokines encompasses the early phase of sepsis and can lead to septic shock. Septic shock is characterized by profound hypotension and inadequate tissue perfusion, and is a critical stage of Gram-negative sepsis with a mortality rate of 30-50% (Hotchkiss et al., 2016).

Management of Gram-negative sepsis includes prompt administration of broad-spectrum antibiotics, fluid resuscitation, vasopressor support to maintain adequate blood

pressure, and targeted therapy aimed at attenuating the inflammatory response (Hotchkiss et al., 2016). Despite advances in medical care and antibiotic development, Gram-negative sepsis remains one of the leading causes of morbidity and mortality in the ICU even now in 2024 (CDC, 2024; Holmes et al., 2021; Plevin et al., 2016).

3.2 Rationale

Previously published work in our lab has assessed the antimicrobial ability of the Specialized Pro-resolving Mediators (SPMs) Resolvin D2 (RvD2) and Lipoxin A_4 (LxA_4). We have previously reported that alveolar macrophages were able to more effectively clear *Pseudomonas aeruginosa* bacteria in septic mice following CLP-sepsis if those mice were also given RvD2 (Sundarasivarao et al., 2022; Walker et al., 2022). We have also previously reported that LxA_4 has direct effects on *P. aeruginosa,* whereby it reduced biofilm formation and promoted Ciprofloxacin antibiotic efficacy in a static biofilm-forming system (Thornton et al., 2021). We also showed that LxA_4 increased monocyte ability to kill biofilm-associated *P. aeruginosa* (Thornton et al., 2023).

In this chapter, we designed several experiments to explore how RvD2's (and to a lesser extent, LxA_4's) effects on monocytes/macrophages did or did not differ when the cells encountered whole bacteria (instead of free LPS). We once again used a monocyte reporter cell line that helped us discern NF-κB activation in real-time. RvD2 (or LxA_4 in some experiments) were first administered to monocytes prior to differentiation into macrophages as well as to fully differentiated macrophages, and the cells were then incubated with *P. aeruginosa* bacteria. Changes in their NF-κB activity and in their ability to clear bacteria were assessed. We were interested in studying exactly how RvD2 exerted

its effect(s) by looking at mechanisms in vitro and by using *P. aeruginosa* as the stimulus. More specifically, in this chapter, we studied the relationship between bacterial clearance and NF-κB activity.

Additionally, in the experiments within this chapter, two concentrations of *P. aeruginosa* were utilized (denoted by "MOI"). MOI, or Multiplicity of Infection, is a method utilized to standardize in vitro bacterial/viral infections as best as possible (Bailer and Lieber, 2013). The MOI number reflects an estimated number of bacterial cells per every one non-bacterial cell. For example, an MOI of 15 (or 15:1) means that for every 1 monocyte/macrophage per well, there are around 15 *Pseudomonas aeruginosa* bacteria; an MOI of 5 (or 5:1) means that for every 1 monocyte/macrophage per well, there are around 5 *Pseudomonas aeruginosa* bacteria. The idea behind having two concentrations of *P. aeruginosa* in these experiments was to mimic two severities of infection: severe infections were represented with MOI 15 *P. aeruginosa* concentrations and moderate infections were represented with MOI 5 *P. aeruginosa* concentrations.

Determining which MOIs will best correlate to various infection severities is not a standardized process. It is recommended that MOIs should be determined by in-house pilot studies, as these numbers may differ depending on 1) what bacteria and host cells are being used for those experiments and 2) what host cell responses are being assessed (Bailer and Lieber, 2013). In general, one should aim for at least a 3-fold difference between MOIs in order to be analogous with different infection severities (Mistry et al., 2018), as this *should* lead to a statistical difference in the host cell response of interest. One should also consider not choosing an MOI that is too high, as that may overwhelm/kill the cells (Mistry et al., 2018).

3.3 Materials and Methods

3.3.1 RvD2 Synthesis

RvD2 was prepared by total organic synthesis as described in **Chapter 2**. 1 μg of RvD2 was dissolved in 2.66 mL of sterile saline which had been bubbled with argon on days of experiments.

3.3.2 LxA_4 Synthesis

LxA_4 was prepared by total organic synthesis as previously described (Thornton et al., 2021; Wu et al., 2016). The purity of the compound was measured by HPLC-Mass Spectrometry and determined to have >98% purity. 1 μg of LxA_4 was dissolved in 2.84 mL of sterile saline which had been bubbled with argon on days of experiments.

3.3.3 Monocyte Cell Cultures and Differentiation to Macrophages

All in vitro experiments used human THP-1 monocytes containing the NF-κB gene promoter coupled to a luciferase reporter (TIB-202-NFκB-LUC2™; ATCC: Manassas, VA, USA), which were cultured and maintained in RPMI 1640 with L-glutamine (Corning: Manassas, VA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Corning: Manassas, VA, USA), 0.05 mM 2-mercaptoethanol (VWR: Solon, OH, USA), 100 U/mL penicillin G (VWR: Solon, OH, USA), and 100 μg/mL streptomycin (VWR: Solon, OH, USA), and kept in an atmosphere of 37°C, 5% CO_2. THP-1 monocytes were seeded at 6 X 10^4 cells/well in 96-well plates (Corning, Manassas, VA, USA) in FBS-free and penicillin-/streptomycin-free RPMI. Plated cells were subsequently differentiated into

macrophages with treatment of 100 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St. Louis, MO, USA) for 48h as previously described (Takashiba et al., 1999). After differentiation, the media was aspirated, the cells were washed with saline three times, and RPMI media was replenished.

3.3.4 Monocyte/Macrophage Treatments

The protocol for these studies is outlined in **Figure 20**. These studies were performed to evaluate firstly the effect(s) of RvD2 on acute bacteria stimulation (1h *Pseudomonas aeruginosa* (ATCC 27853™) incubation) and on 2-hit LPS-mediated exhausted macrophage clearance of *P. aeruginosa* bacteria. Briefly, THP-1 monocytes or THP-1 monocyte-derived macrophages were pre-incubated with RvD2 or saline vehicle for 1h at 37°C, 5% CO_2. Cells were then exposed to various treatments. These treatments included: 1) *P. aeruginosa* at MOIs of 15:1 and 5:1 for 1h at 37°C and 2) 2-hit LPS treatments (50 ng/mL of LPS for 24h followed by an additional 2^{nd} hit for 3h with 50 ng/mL of LPS) followed by incubation with *P. aeruginosa* at MOIs of 15:1 and 5:1 for 1h at 37°C. All studies were performed in triplicate.

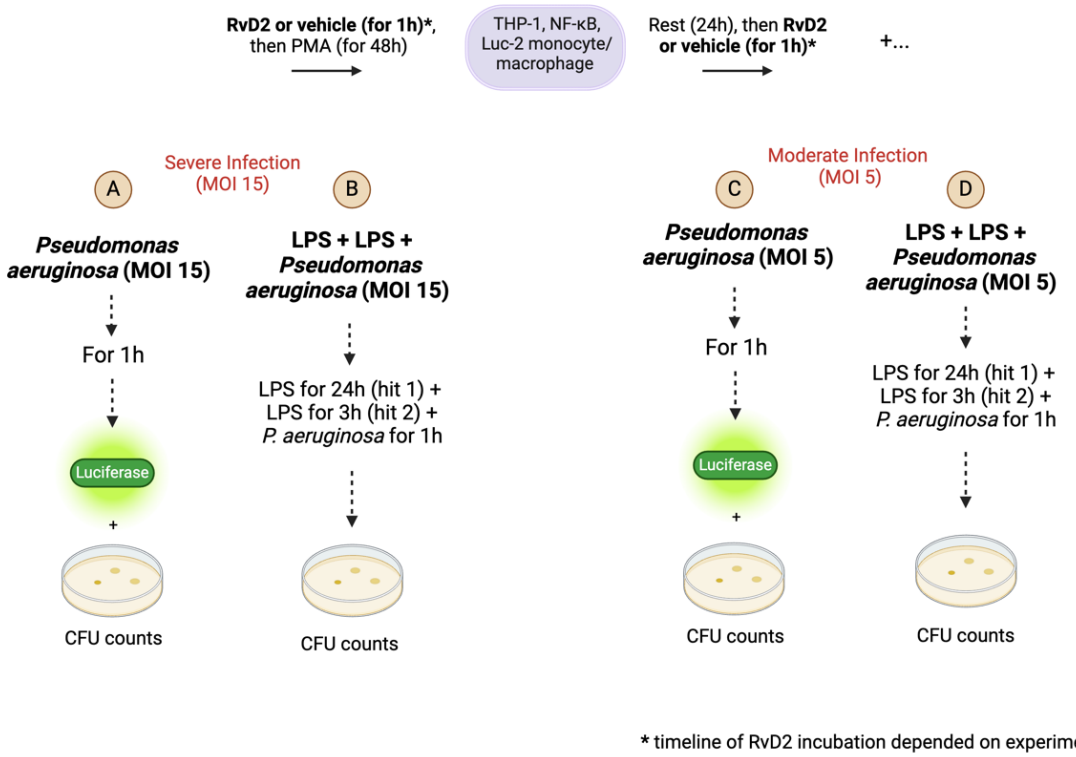


Figure 20. Methods for Monocyte/Macrophage Incubations. This schematic outlines the experiments conducted for RvD2 treatments on THP-1 monocytes prior to and following differentiation with PMA. In this chapter, cells were stimulated with whole bacteria at two concentrations (in order to replicate different infection severities).

3.3.5 Luciferase Assay

To elucidate the cellular mechanism by which RvD2 affects NF-κB activity following bacteria stimulation, the THP-1 NF-κB-LUC2 (ATCC TIB-202-NFκB-LUC2™) cell line was used. In this cell line, the firefly luciferase gene, *luc2*, was placed under the control of the NF-κB promoter, such that NF-κB activity could be measured via luciferase luminescence. Following treatments, Firefly Luc One-Step Glow Assay Kit (Pierce: Rockford, IL, USA) was used per the manufacturer's protocol. Briefly, the cells were lysed and incubated with D-Luciferin substrate for 15 min. The Biotek Synergy H1 plate reader

was programmed to measure chemiluminescence with an integration time of 1 sec at 135 gain.

3.3.6 Pseudomonas aeruginosa Bacterial Culture

P. aeruginosa (ATCC 27853™) was grown on tryptic soy agar (TSA; Ward's Scientific: Rochester, NY, USA) overnight at 37°C. Liquid cultures were inoculated by depositing *P. aeruginosa* colonies into Luria-Bertani broth (Gibco: Gaithersburg, MD, USA). The cultures were incubated for 5h at 37°C with shaking (180 rpm) and then centrifuged for 6 min at 9100 X g. Culture supernatants were removed and pellets were washed three times in M63 minimal medium. The cultures were diluted in M63 minimal medium to OD_{600} 0.1 using a BioTek Synergy H1 plate reader (Biotek: Winooski, VT, USA). To confirm MOI, cultures were serially-diluted in sterile saline, spread onto TSA plates, and incubated overnight at 37°C. Colony forming units (CFUs) were then counted by a blinded operator, and MOIs were subsequently calculated.

3.3.7 Infection of THP-1 Monocytes/Macrophages with Live P. aeruginosa and Assessment of Bacterial Clearance

THP-1 monocytes or THP-1 monocyte-derived macrophages were pre-incubated with RvD2, LxA_4, or saline vehicle as described above. Then, cells were treated with M63 minimal media (control) or M63 minimal media containing *P. aeruginosa* at MOIs of 15:1 and 5:1 as described above. After this incubation, supernatants were collected, serially-diluted using sterile saline, spread on TSA plates, and incubated overnight at 37°C. CFUs were then counted by a blinded operator to evaluate bacterial clearance.

3.3.8 Infection of Exhausted THP-1 Macrophages with Live P. aeruginosa and Assessment of Bacterial Clearance

THP-1 monocytes or THP-1 monocyte-derived macrophages were pre-incubated with RvD2, LxA_4, or saline vehicle as described above. Then, cells were incubated with 2-hit LPS treatments as described above. These two LPS hits were utilized with the intention of exhausting the macrophages. These 2-hit LPS-mediated exhausted macrophages were then incubated with M63 minimal media (control) or M63 minimal media containing *P. aeruginosa* at MOIs of 15:1 or 5:1 for 1h at 37°C. After this incubation, supernatants were collected, serially-diluted in sterile saline, spread onto TSA plates, and incubated overnight at 37°C. CFUs were then counted by a blinded operator to evaluate bacterial clearance.

3.3.9 Statistical Analysis

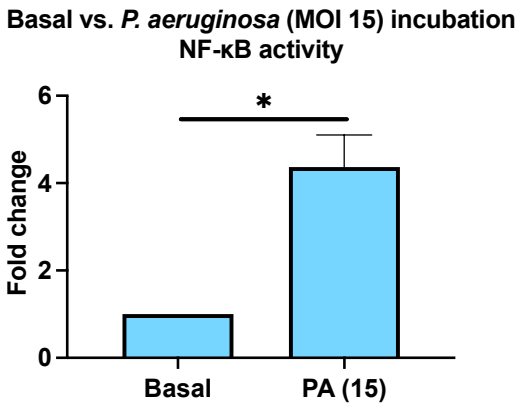
All analyses were performed using GraphPad Prism version 9.4.1 for Mac (GraphPad Software: San Diego, CA, USA). All data are expressed as mean ± SEM. For data sets that were normally distributed, data were subjected to one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons post-hoc test. For data sets that were not normally distributed (i.e., luciferase assays), data were subjected to Kruskal-Wallis test with Dunn's multiple comparisons post-hoc test. Data from basal vs. 2-hit LPS treatments and basal vs. *Pseudomonas aeruginosa* at MOI 15 were subjected to unpaired t-tests. A p value < 0.05 was taken as significant for at least three independent studies.

3.4 Experimental Results

3.4.1 RvD2 Did Not Modulate Macrophage NF-κB Activity After Acute Stimulation with Bacteria

THP-1 macrophages were incubated with RvD2 (1 or 10 nM) or saline vehicle for 1h. The macrophages were then acutely stimulated with *P. aeruginosa* (MOIs 15 and 5) for 1h. The results showed that *P. aeruginosa* (MOI 15) alone raised macrophage NF-κB activity 4-fold (**Figure 21A**), but RvD2 had no effect on *P. aeruginosa*-stimulated NF-κB activity (**Figures 21B** and **21C**, respectively). These results suggest that RvD2 does not influence macrophage inflammatory response to bacteria in an NF-κB-dependent manner.

A.



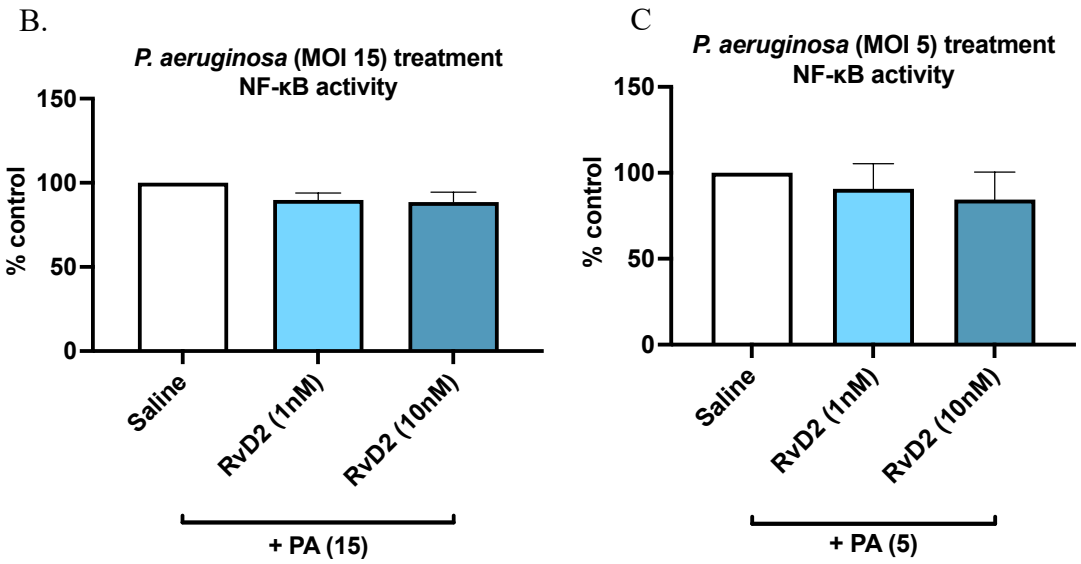


Figure 21. RvD2 Did Not Influence Macrophage Responses Following Acute Stimulation with Bacteria. *A.* Macrophages incubated with *P. aeruginosa* at MOI 15 (PA (15)) increased NF-κB by 4-fold compared to unstimulated controls (basal levels). Values are mean ± SEM. *P < 0.05; n = 3. *B.* THP-1 macrophages were incubated with *P. aeruginosa* at MOI 15 (PA (15)) for 1h. RvD2 did not significantly affect NF-κB activity compared to saline controls. Values are mean ± SEM; n = 3. *C.* THP-1 macrophages were incubated with *P. aeruginosa* at MOI 5 (PA (5)) for 1h. RvD2 did not significantly affect NF-κB activity compared to saline controls. Values are mean ± SEM; n = 3.

3.4.2 RvD2 Increased Macrophage Ability to Clear Bacteria

Despite the fact that RvD2 does not influence macrophage NF-κB activity following acute stimulation with bacteria (**Figure 21**), bacterial clearance was increased in RvD2-treated macrophages following acute stimulation with bacteria compared to saline controls (**Figures 22A** and **22B**). Interestingly, we found that LxA_4 did not have any effect on macrophage bacterial clearance compared to saline controls (**Figures 22C** and **22D**).

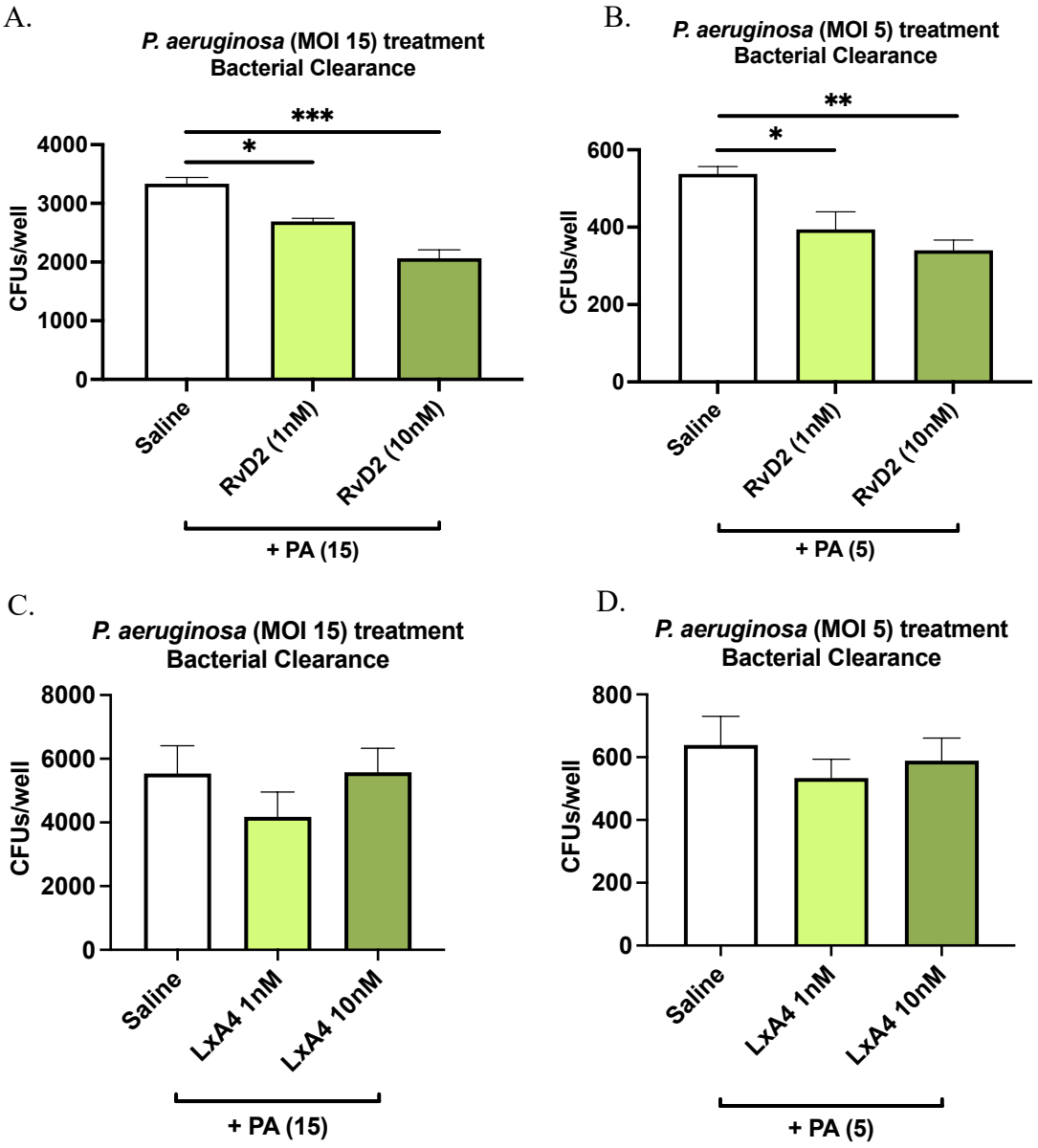


Figure 22. RvD2 Increased Macrophage Ability to Clear Bacteria. *A.* RvD2-treated macrophages were able to clear more bacteria (MOI 15) compared to saline vehicle-treated macrophages. Values are mean ± SEM. ***P < 0.001, *P < 0.05; n = 3. *B.* RvD2-treated macrophages were able to clear more bacteria (MOI 5) compared to saline vehicle-treated macrophages. Values are mean ± SEM. **P < 0.01, *P < 0.05; n = 3. *C.* Pre-treatment with LxA_4 did not affect macrophage ability to clear bacteria (MOI 15) compared to saline vehicle-treated macrophages. Values are mean ± SEM; n = 3. *D.* Pre-treatment with LxA_4 did not affect macrophage ability to clear bacteria (MOI 5) compared to saline vehicle-treated macrophages. Values are mean ± SEM; n = 3.

3.4.3 Further Establishing Macrophage Exhaustion

As discussed in **Chapter 2**, various macrophage functions were assessed as a means of observing successful induction of in vitro exhaustion. To build on this concept further, we measured the ability of exhausted macrophages to clear *P. aeruginosa* bacteria. The results revealed that after two LPS hits, THP-1 macrophage ability to clear bacteria was reduced compared to acutely-stimulated cells (**Figure 23**). These results confirm the findings in **Chapter 2**, whereby we concluded that our 2-hit LPS treatments caused a significant level of macrophage exhaustion in vitro.

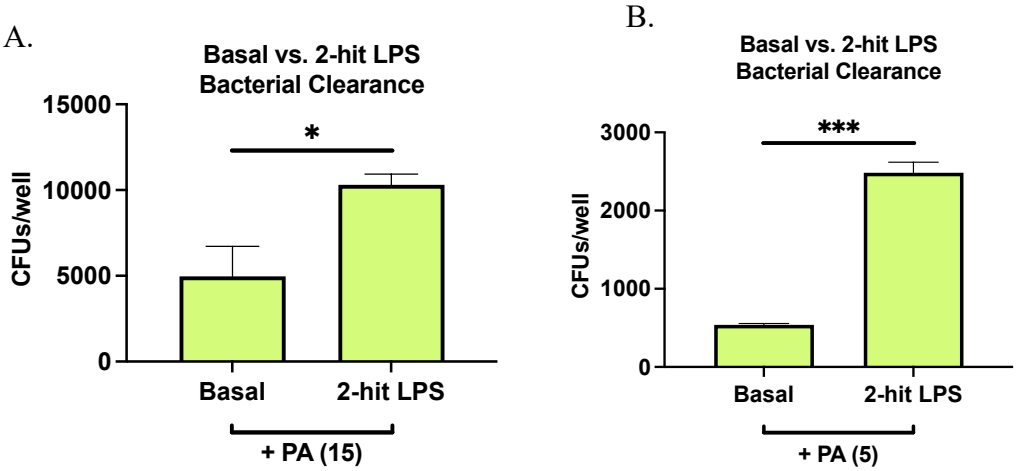


Figure 23. 2-Hit LPS Treatments Established Macrophage Exhaustion in vitro. *A.* On average, CFU counts on supernatants from wells containing macrophages that were not previously exposed to LPS (basal clearance) were approximately 5,000 CFUs/well. In comparison, CFU counts from wells containing macrophages that were exposed to 2-hit LPS treatments were approximately 10,000 CFUs/well. Values are mean ± SEM. *P < 0.05; n = 3. *B.* On average, CFU counts on supernatants from wells containing macrophages that were not previously exposed to LPS (basal clearance) were approximately 800 CFUs/well. In comparison, CFU counts from wells containing macrophages that were exposed to 2-hit LPS treatments were approximately 2,500 CFUs/well. Values are mean ± SEM. ***P < 0.001; n = 3.

3.4.4 RvD2 Increased Exhausted Macrophage Ability to Clear Bacteria

THP-1 macrophages were incubated with RvD2 (1 or 10 nM) or saline vehicle for 1h, followed by two LPS treatments to induce exhaustion as described above. The results showed that RvD2 increased bacterial clearance by exhausted macrophages compared to saline controls (**Figure 24**). These results suggest that RvD2 either augments macrophage functions during exhaustion or prevents macrophage exhaustion, partly by increasing their ability to clear bacteria.

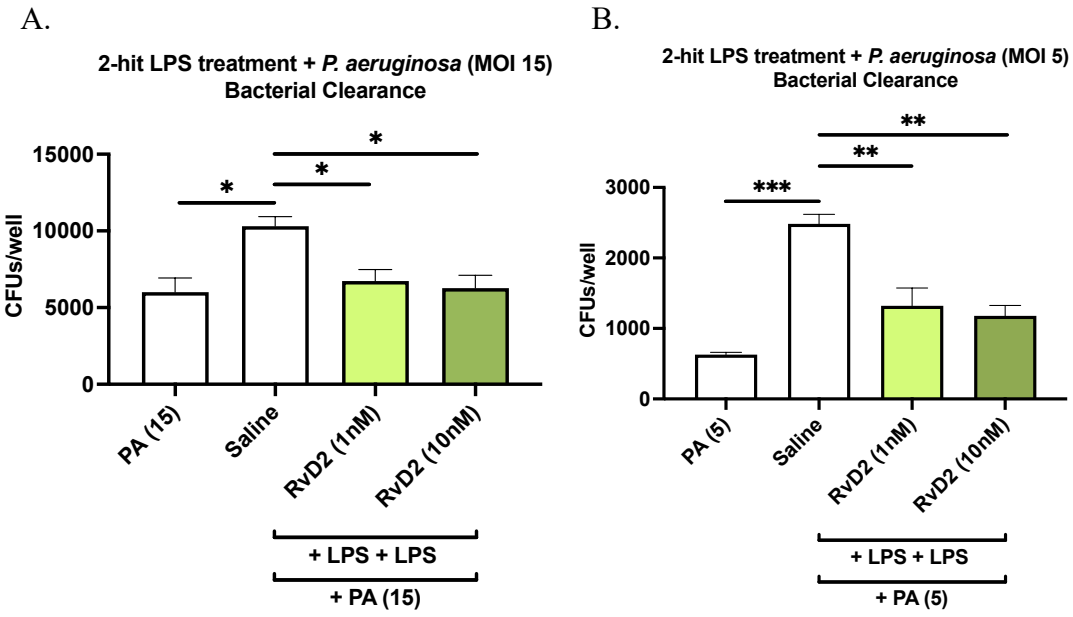
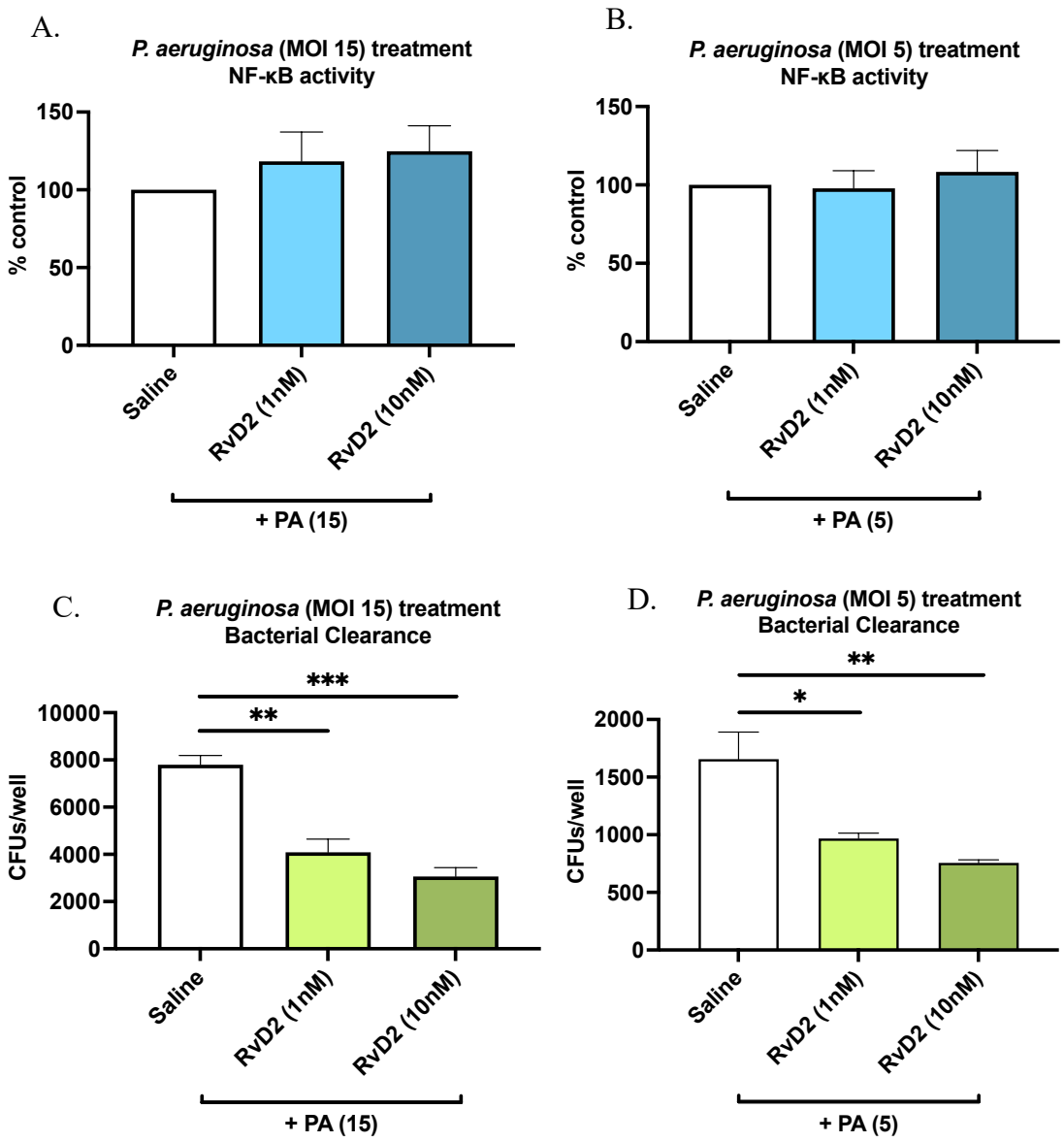


Figure 24. RvD2 Attenuated LPS-Mediated Macrophage Exhaustion in vitro. *A.* RvD2-treated exhausted macrophages were able to clear more *P. aeruginosa* at MOI 15 (PA (15)) compared to saline vehicle-treated exhausted macrophages. Values are mean ± SEM. *P < 0.05; n = 3. *B.* RvD2-treated exhausted macrophages were able to clear more *P. aeruginosa* at MOI 5 (PA (5)) compared to saline vehicle-treated exhausted macrophages. Values are mean ± SEM. ***P < 0.001, **P < 0.01; n = 3.

3.4.5 RvD2's Effects on THP-1 Monocytes Prior to PMA Differentiation

In these experiments, we investigated if the effects of RvD2 treatment on THP-1 monocytes (RvD2 administered to cells prior to differentiation with PMA) following bacteria addition are different from RvD2 treatment on THP-1 monocyte-derived macrophages (RvD2 administered to cells after differentiation with PMA) following bacteria addition. THP-1 monocytes were plated and immediately treated with saline vehicle or RvD2 (1 or 10 nM) for 1h. Following this 1h incubation, the monocytes were then differentiated into macrophages with PMA (100 ng/mL) for 48h. After 24h of rest, the macrophages were incubated with *P. aeruginosa* at MOIs 15:1 and 5:1 for 1h. The results showed that RvD2 had no effect on macrophage NF-κB activity following incubation with *P. aeruginosa* (**Figures 25A** and **25B**). On the other hand, RvD2 increased bacterial clearance of *P. aeruginosa* (**Figures 25C** and **25D**). Interestingly but consistently, LxA_4 did not increase bacterial clearance of *P. aeruginosa* (**Figures 25E** and **25F**). These results suggest that RvD2 and LxA_4 do not possess the same bacterial clearance properties.


A.
P. aeruginosa (MOI 15) treatment
NF-κB activity
% control
150
100
50
0
Saline
RvD2 (1nM)
RvD2 (10nM)
+ PA (15)
B.
P. aeruginosa (MOI 5) treatment
NF-κB activity
% control
150
100
50
0
Saline
RvD2 (1nM)
RvD2 (10nM)
+ PA (5)
C.
P. aeruginosa (MOI 15) treatment
Bacterial Clearance
***
**
CFUs/well
10000
8000
6000
4000
2000
0
Saline
RvD2 (1nM)
RvD2 (10nM)
+ PA (15)
D.
P. aeruginosa (MOI 5) treatment
Bacterial Clearance
**
*
CFUs/well
2000
1500
1000
500
0
Saline
RvD2 (1nM)
RvD2 (10nM)
+ PA (5)

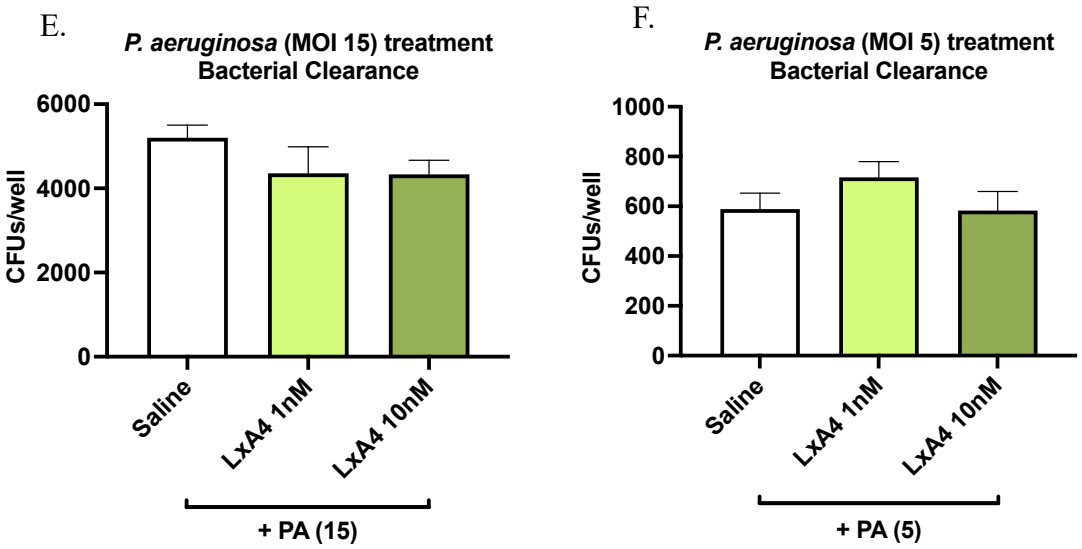


Figure 25. RvD2's and LxA_4's Effects on THP-1 Monocytes when Given Before Differentiation to Macrophages. *A. P. aeruginosa* at MOI 15 (PA (15)) was given to cells treated with saline vehicle or RvD2. When given prior to monocyte differentiation into macrophages, RvD2 had no effect on NF-κB activity compared to saline controls. Values are mean ± SEM; n = 6. *B. P. aeruginosa* at MOI 5 (PA (5)) was given to cells treated with saline vehicle or RvD2. When given prior to monocyte differentiation into macrophages, RvD2 had no effect on NF-κB activity compared to saline controls. Values are mean ± SEM; n = 3. *C.* When given prior to monocyte differentiation into macrophages, RvD2 increased bacterial clearance (MOI 15) compared to saline vehicle-treated macrophages. Values are mean ± SEM. ***P < 0.001, **P < 0.01; n = 3. *D.* When given prior to monocyte differentiation into macrophages, RvD2 increased bacterial clearance (MOI 5) compared to saline vehicle-treated macrophages. Values are mean ± SEM. **P < 0.01, *P < 0.05; n = 3. *E.* When given prior to monocyte differentiation into macrophages, LxA_4 did not significantly affect bacterial clearance (MOI 15). Values are mean ± SEM; n = 3. *F.* When given prior to monocyte differentiation into macrophages, LxA_4 did not significantly affect bacterial clearance (MOI 5). Values are mean ± SEM; n = 3.

3.4.6 RvD2 Increased Exhausted Macrophage Ability to Clear Bacteria when Given Before Differentiation

THP-1 monocytes were plated and immediately treated with saline vehicle or RvD2 (1 or 10 nM) for 1h. Following this 1h incubation, the monocytes were then differentiated into macrophages with PMA (100 ng/mL) for 48h. After 24h of rest, the macrophages were incubated with 2-hit LPS treatments, followed by 1h *P. aeruginosa* treatment (at MOIs 15:1

and 5:1). The results showed that even when given to monocytes instead of differentiated macrophages, RvD2 still significantly increased their ability to clear bacteria (**Figure 26**). These results are different from LPS-induced inflammatory responses (**Chapter 2**), where RvD2 given before differentiation had significantly different effects compared to RvD2 given to differentiated macrophages.

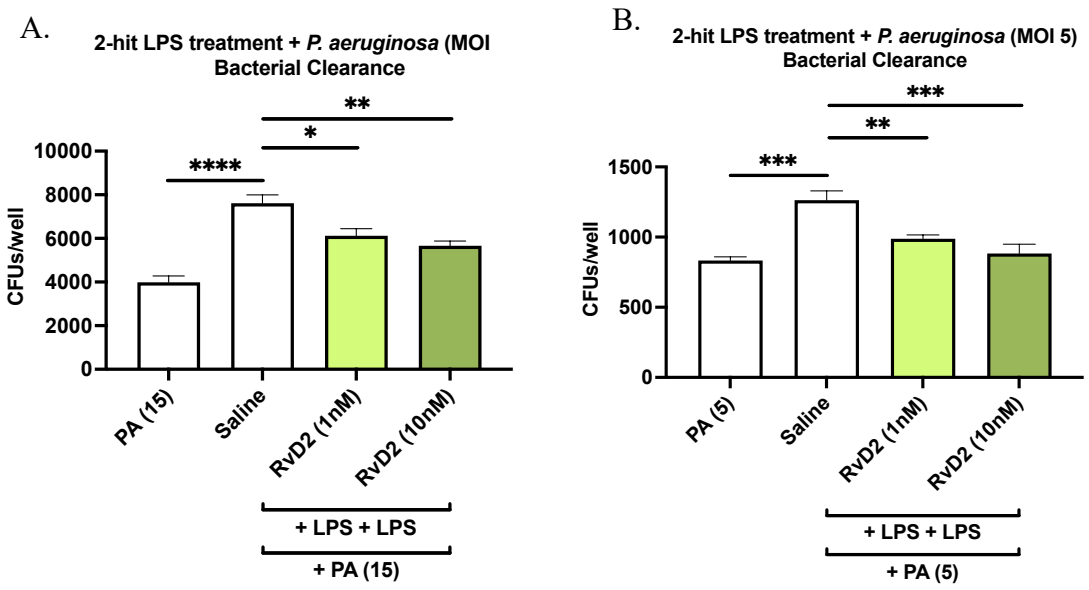


Figure 26. RvD2's Effects on Exhausted THP-1 Macrophages when Given Before Differentiation to Macrophages. *A.* When given prior to monocyte differentiation into macrophages, RvD2-treated exhausted (2-hit) macrophages were able to clear more bacteria (MOI 15) compared to saline vehicle-treated exhausted macrophages. Values are mean ± SEM. ****P < 0.0001, **P < 0.01, *P < 0.05; n = 4. *B.* When given prior to monocyte differentiation into macrophages, RvD2-treated exhausted (2-hit) macrophages were able to clear more bacteria (MOI 5) compared to saline vehicle-treated exhausted macrophages. Values are mean ± SEM. ***P < 0.001, **P < 0.01; n = 4.

3.5 Discussion

Our work showed that while macrophage-mediated bacterial clearance was significantly increased by RvD2-treated cells, this increase was not dependent on NF-κB activation (**Figure 21)**. This is consistent with other reports which show that RvD2's enhancement of macrophage-mediated bacterial phagocytosis acts through the STAT3 pathway instead of the NF-κB pathway (Chiang et al., 2017). On the other hand, investigators have reported that RvD1 increased human macrophage phagocytosis of *E. coli* (Palmer et al., 2011) through a TLR4-dependent increase in TNF-α production, which enhanced phagocytosis. Our results are not consistent with this study, as increases in *P. aeruginosa* clearance were not associated with any alteration in NF-κB activity by RvD2. Taken together, our results are consistent with the notion that RvD2-mediated increase in bacterial clearance acts through an NF-κB-independent signaling pathway.

One interesting finding from these studies is the fact that LxA_4 did not improve bacterial clearance by macrophages. This result aligns with a previous study which reported that LxA_4 did not impact macrophages' phagocytic ability when administered before bacterial addition, but did enhance macrophage phagocytosis when given simultaneously (Prescott and McKay, 2011). Additionally, this result differs from reports by several investigators showing that LxA_4 can increase macrophage phagocytosis of apoptotic neutrophils (Godson et al., 2000). The mechanism for this increased action is thought to be via a direct effect on macrophage redistribution of myosin IIA and cdc42 (Reville et al., 2006). Therefore, it could be plausible that LxA_4 does improve macrophage-mediated bacterial clearance, but this improvement occurs through NF-κB-independent mechanisms that stem from cytoskeletal rearrangements. Perhaps in this in vitro setting, the cell culture

plates did not provide a dynamic enough environment for these required rearrangements to successfully take place. Overall, the variance in results may stem from LxA_4's role in promoting inflammation resolution before exerting its antimicrobial effects.

RvD2's effects on monocyte/macrophage effector responses differed if they encountered free LPS (**Chapter 2**) or bacteria-bound LPS (i.e., whole bacteria) (**Chapter 3**). Considering this discrepancy from a teleological standpoint, the distinct effects of RvD2 in these scenarios could be attributed to the varying and specific immune cell requirements during that particular infection. These requirements are dictated by the local conditions in which the body encounters bacterial LPS (Palmer et al., 2011). For example, during a bacterial infection, monocytes/macrophages may encounter free LPS more frequently if the bacteria have already been killed by host defense mechanisms, as LPS shedding is a characteristic of dead bacteria (Palmer et al., 2011). In other words, LPS shedding could be an indication to the monocytes/macrophages that most of the bacteria causing the infection are dead. In this local condition, further TNF-α production may be harmful, and the immune system may favor inflammation resolution (Palmer et al., 2011). Our results from **Chapter 2** support this concept, whereby RvD2 decreased monocyte/macrophage inflammatory responses to acute (1-hit) LPS administration. Conversely, however, in the context of whole bacteria, RvD2 may trigger monocytes/macrophages to favor swift clearance of bacteria. Perhaps in these settings (and depending on the severity of infection), monocytes/macrophages are more apt to assume their role as antigen presenting cells (APCs) rather than as inflammation-inducers, so that they can facilitate activation of an adaptive immune response (Palmer et al., 2011). The monocytes/macrophages, in the meantime, may still want to clear the bacteria without further causing inflammation, and

they increase their ability to phagocytose in an NF-κB-independent mechanism. The cellular mechanism(s) by which macrophages differentiate their responses to the two different stimuli has not been elucidated.

Chapter 4

Resolvin D2 (RvD2) Acts Through TLR-Mediated Signaling that Leads to Monocyte/Macrophage Reprogramming

4.1 Introduction

Toll-like receptor (TLR) signaling through NF-κB is a crucial pathway in the immune response. When a TLR recognizes a pathogen-associated molecular pattern (PAMP), it activates intracellular signaling cascades that lead to the activation of transcription factors such as NF-κB (Hug et al., 2018). NF-κB regulates the expression of cytokines, chemokines, and other molecules that recruit immune cells to the site of infection and enhance their activity against pathogens (Liu et al., 2017).

TLR2 is a type of pattern recognition receptor (PRR) and plays a critical role in detecting and responding to microbial infections, shaping both innate and adaptive immune responses, and influencing the outcome of host-pathogen interactions (Oliveira-Nascimento et al., 2012). TLR2 binding to specific damage associated molecular patterns (DAMPs) and/or PAMPs is crucial for mediating both the inflammatory response and clearance of various infections (Chen et al., 2016; Oliveira-Nascimento et al., 2012; Reba et al., 2014). Excessive TLR2 signaling through NF-κB, however, may hyperactivate the inflammatory response, causing tissue damage (Cowardin et al., 2016; Lima et al., 2015). On the other hand, TLR2 agonism has also been reported to cause immunosuppression (Netea et al., 2004; Nguyen et al., 2020).

4.2 Rationale

Just as monocytes undergo a form of reprogramming when they differentiate into macrophages, macrophages can also "repolarize" from one type of macrophage to another type of macrophage (i.e., from an M1-like macrophages to an M2-like macrophage) depending on external stimuli (van Dalen et al., 2018). This repolarization process is driven by transcription factor NF-κB upstream signaling cascades such as TLRs (van Dalen et al., 2018). Can RvD2 specifically affect this controlled process of macrophage repolarization via a TLR-mediated pathway, leading to macrophage reprogramming?

RvD2 reduced TLR4 expression in human macrophages (Croasdell et al., 2016), and RvD1, specifically, reduced LPS-mediated inflammatory responses in macrophages through a TLR4-mediated mechanism (Palmer et al., 2011). As seen in **Chapter 2**, RvD2 reduced NF-κB activity and TNF-α production, suggesting that the reduction in TNF-α was through NF-κB signaling.

Additionally, we have previously shown that late administration of RvD2 in the CLP model of polymicrobial sepsis increased splenic cell TLR2 gene expression (Walker et al., 2022). This increase was associated with improved primary bacterial clearance from circulation and better clearance of *P. aeruginosa* secondary lung infection (Sundarasivarao et al., 2022; Walker et al., 2022).

In this chapter, we designed several experiments with the goal of identifying if and when RvD2 plays an active role in macrophage repolarization, with a particular focus on the role of TLR2. In other words, we wished to determine if RvD2-mediated actions on the TLR2 pathway leads to functional macrophage reprogramming. RvD2 and a selective TLR2 inhibitor, MMG-11, were administered to fully differentiated macrophages prior to

incubation with free LPS and whole bacteria, and then changes in NF-κB activity, TNF-α production, and bacterial clearance were assessed. We also assessed if there were any differences in RvD2-mediated reprogramming if the cells were acutely-stimulated (i.e., 1-hit LPS or 1h *P. aeruginosa* treatment) or were exhausted (2-hit LPS-induced).

4.3 Materials and Methods

4.3.1 RvD2 Synthesis

RvD2 was prepared by total organic synthesis as described in **Chapter 2**. 1 μg of RvD2 was dissolved in 2.66 mL of sterile saline which had been bubbled with argon on days of experiments.

4.3.2 Monocyte Cell Cultures and Differentiation to Macrophages

All in vitro experiments used human THP-1 monocytes containing the NF-κB gene promoter coupled to a luciferase reporter (TIB-202-NFκB-LUC2™; ATCC: Manassas, VA, USA), which were cultured and maintained in RPMI 1640 with L-glutamine (Corning: Manassas, VA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Corning: Manassas, VA, USA), 0.05 mM 2-mercaptoethanol (VWR: Solon, OH, USA), 100 U/mL penicillin G (VWR: Solon, OH, USA), and 100 μg/mL streptomycin (VWR: Solon, OH, USA), and kept in an atmosphere of 37°C, 5% CO_2. THP-1 monocytes were seeded at 6 X 10^4 cells/well in 96-well plates (Corning, Manassas, VA, USA) in FBS-free and penicillin-/streptomycin-free RPMI. Plated cells were subsequently differentiated into macrophages with treatment of 100 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-

Aldrich, St. Louis, MO, USA) for 48h as previously described (Takashiba et al., 1999). After differentiation, the media was aspirated, the cells were washed with saline three times, and RPMI media was replenished.

4.3.3 TLR2 Inhibition

TLR2 receptors were inhibited with 25 μM of the selective TLR2 inhibitor MMG-11 (ethyl 5-[2-oxo-2-(2,3,4-trihydroxyphenyl)ethyl]furan-2-carboxylate; Vitas M, Hong Kong, China). For 1-hit experiments, MMG-11 was given simultaneously with RvD2 for 1h. For 2-hit experiments, MMG-11 was given 1h before the 2nd LPS hit.

4.3.4 Monocyte/Macrophage Treatments

The protocol for these studies is outlined in **Figure 27**. These studies were performed to determine if TLR2 is involved in RvD2's effects on macrophages. Briefly, RvD2 (1 or 10 nM) ± MMG-11 (25 μM) or saline vehicle was added to THP-1 monocyte-derived macrophages for 1h at 37°C, 5% CO_2. Cells were then exposed to various treatments. These treatments included: 1) "one hit": 50 ng/mL of LPS (O111.B4 from *E. coli*; EMD Millipore Corporation: Burlington, MA, USA) for 3h, 2) *Pseudomonas aeruginosa* (ATCC 27853™) at Multiplicity of Infection (MOI) of 15:1 for 1h, 3) "two hits": 50 ng/mL of LPS for 24h followed by an additional 2nd hit for 3h with 50 ng/mL of LPS, and 4) 2-hit LPS treatment followed by incubation with *P. aeruginosa* at MOI of 15:1 for 1h. All studies were performed in triplicate.

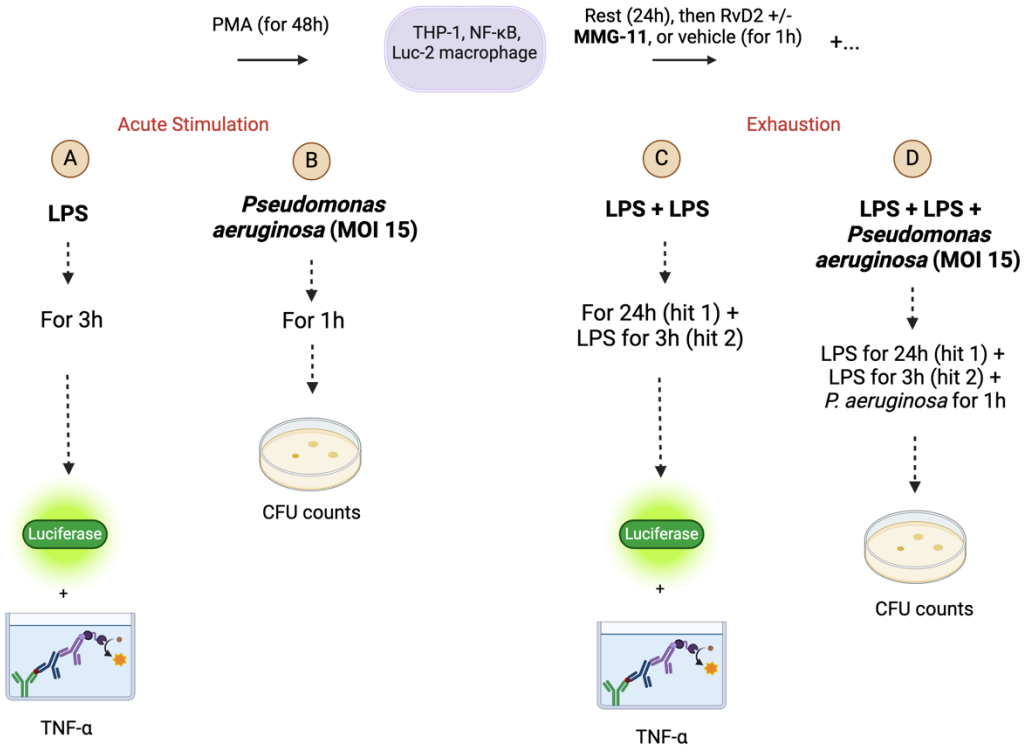


Figure 27. Protocol for Macrophage Incubations. This schematic outlines the experiments conducted for RvD2 treatments on THP-1 monocyte-derived macrophages following differentiation with PMA. In this chapter, cells were stimulated with both free endotoxin (LPS) as well as with whole bacteria.

4.3.5 Luciferase Assay

To elucidate the cellular mechanism by which MMG-11 affects RvD2-mediated changes in NF-κB activity, the THP-1 NF-κB-LUC2 (ATCC TIB-202-NFκB-LUC2™) cell line was used. In this cell line, the firefly luciferase gene, *luc2*, was placed under the control of the NF-κB promoter, such that NF-κB activity could be measured via luciferase luminescence. For 1-hit LPS treatments, THP-1 monocyte-derived macrophages were pre-incubated with RvD2 ± MMG-11 or saline vehicle as described above. For 2-hit LPS treatments, THP-1 monocyte-derived macrophages were pre-incubated with RvD2 or saline vehicle for 1h prior to the 1st LPS hit. MMG-11 was added to the wells 1h before the

2nd LPS hit. Following treatments, Firefly Luc One-Step Glow Assay Kit (Pierce: Rockford, IL, USA) was used per the manufacturer's protocol. Briefly, the cells were lysed and incubated with D-Luciferin substrate for 15 min. The Biotek Synergy H1 plate reader was programmed to measure chemiluminescence with an integration time of 1 sec at 135 gain.

4.3.6 Cytokine Production Assay

The concentration of human TNF-α was determined by enzyme-linked immunosorbent assay (ELISA; Thermo Fisher Scientific: Waltham, MA, USA) according to the manufacturer's instructions. Absorbance (450 nm) was read on a BioTek Synergy H1 plate reader (Biotek: Winooski, VT, USA).

4.3.7 Pseudomonas aeruginosa Bacterial Culture

P. aeruginosa (ATCC 27853™) was grown on tryptic soy agar (TSA; Ward's Scientific: Rochester, NY, USA) overnight at 37°C. Liquid cultures were inoculated by depositing *P. aeruginosa* colonies into Luria-Bertani broth (Gibco: Gaithersburg, MD, USA). The cultures were incubated for 5h at 37°C with shaking (180 rpm) and then centrifuged for 6 min at 9100 X g. Culture supernatants were removed and pellets were washed three times in M63 minimal medium. The cultures were diluted in M63 minimal medium to OD_{600} 0.1 using a BioTek Synergy H1 plate reader (Biotek: Winooski, VT, USA). To confirm MOI, cultures were serially-diluted in sterile saline, spread onto TSA plates, and incubated overnight at 37°C. Colony forming units (CFUs) were then counted by a blinded operator, and MOI was subsequently calculated.

4.3.8 Infection of THP-1 Macrophages with Live P. aeruginosa and Assessment of Bacterial Clearance

THP-1 monocyte-derived macrophages were pre-incubated with RvD2 (1 or 10 nM) ± MMG-11 (25 μM) as described above. Then, the cells were incubated with M63 minimal media (control) or M63 minimal media containing *P. aeruginosa* at MOI of 15:1 for 1h at 37°C. After this incubation, supernatants were collected, serially-diluted in sterile saline, spread onto TSA plates, and incubated overnight at 37°C. CFUs were then counted by a blinded operator to evaluate bacterial clearance.

4.3.9 Infection of Exhausted THP-1 Macrophages with Live P. aeruginosa and Assessment of Bacterial Clearance

THP-1 monocyte-derived macrophages were incubated with RvD2 (1 or 10 nM) ± MMG-11 (25 μM) and 2-hit LPS treatments as described above. These two LPS hits were utilized with the intention of exhausting the macrophages. These 2-hit LPS-mediated exhausted macrophages were then incubated with M63 minimal media (control) or M63 minimal media containing *P. aeruginosa* at MOI of 15:1 for 1h at 37°C. After this incubation, supernatants were collected, serially-diluted in sterile saline, spread onto TSA plates, and incubated overnight at 37°C. CFUs were then counted by a blinded operator to evaluate bacterial clearance.

4.3.10 Statistical Analysis

All analyses were performed using GraphPad Prism version 9.4.1 for Mac (GraphPad Software: San Diego, CA, USA). All data are expressed as mean ± SEM. For data sets that were normally distributed, data were subjected to one-way ANOVA with

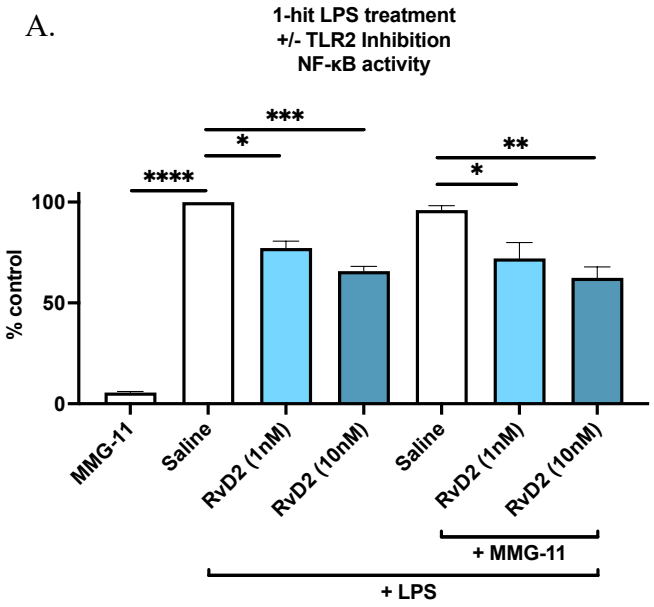
Tukey's multiple comparisons post-hoc test. For data sets that were not normally distributed (i.e., luciferase assays), data were subjected to Kruskal-Wallis test with Dunn's multiple comparisons post-hoc test. Data from (-) MMG-11 vs. (+) MMG-11 treatments were subjected to unpaired t-tests. A p value < 0.05 was taken as significant for at least three independent studies.

4.4 Experimental Results

4.4.1 TLR2 Inhibition Differentially Affected Acute LPS-Stimulated Macrophage Activity

In 1-hit LPS experiments, THP-1 macrophages were incubated with RvD2 (1 or 10 nM), the selective TLR2 inhibitor MMG-11 (25 μM), and saline vehicle for 1h. Then, the cells were incubated with LPS (50 ng/mL) for 3h. The results showed that TLR2 inhibition did not affect LPS-induced NF-κB activity (**Figure 28A**). RvD2 reduced NF-κB activity and TLR2 inhibition had no effect on this reduction (**Figure 28A**). The results similarly showed that RvD2 reduced TNF-α production and TLR2 inhibition had no effect (**Figure 28B**). However, the results showed that the RvD2-mediated increase in bacterial clearance was eliminated with TLR2 blockade (**Figure 28C**). Taken together, these results suggest that in acute inflammatory stimulation with LPS (1-hit LPS treatments), TLR2 inhibition does not influence RvD2's effects on macrophage NF-κB-dependent inflammatory activity.

A.


1-hit LPS treatment
+/- TLR2 Inhibition
NF-κB activity
% control
100
50
0
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*
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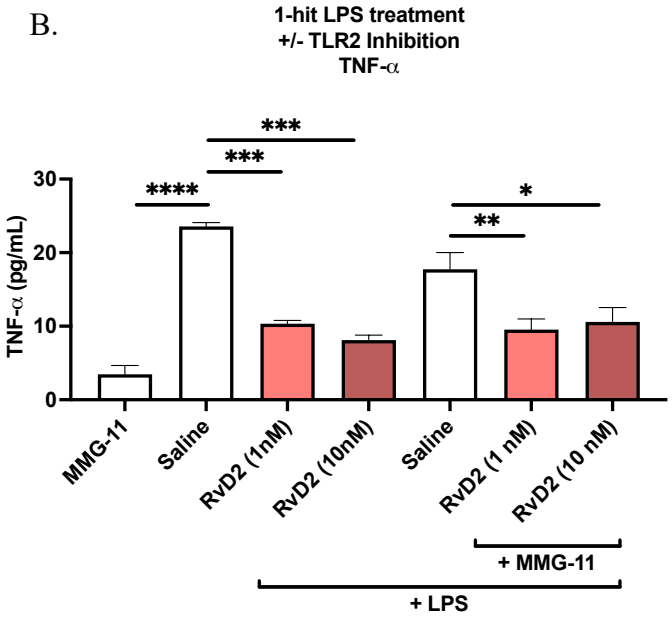
*
MMG-11
Saline
RvD2 (1nM)
RvD2 (10nM)
Saline
RvD2 (1nM)
RvD2 (10nM)
+ MMG-11
+ LPS

B.


1-hit LPS treatment
+/- TLR2 Inhibition
TNF-α
TNF-α (pg/mL)
30
20
10
0
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***
****
*
**
MMG-11
Saline
RvD2 (1nM)
RvD2 (10nM)
Saline
RvD2 (1 nM)
RvD2 (10 nM)
+ MMG-11
+ LPS

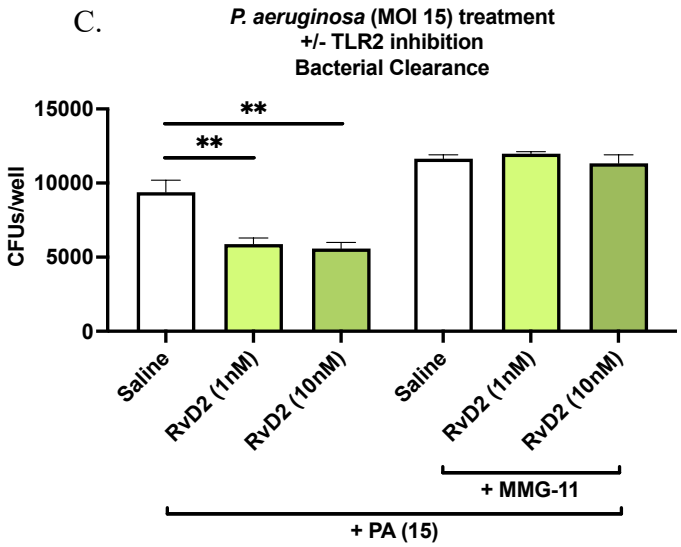


Figure 28. TLR2 Inhibition Modulated RvD2's Effects in Acute Inflammatory Settings. *A.* TLR2 blockade had minimal effects on baseline NF-κB activity. Acute (1-hit) LPS treatment increased NF-κB activity 19-fold compared to vehicle. RvD2 reduced NF-κB activity compared to saline controls. In this acute LPS-induced inflammatory setting, reduction in macrophage NF-κB activity by RvD2 was not affected by TLR2 blockade. Values are mean ± SEM; ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05; n = 6. *B.* Acute (1-hit) LPS treatment increased TNF-α production 20-fold compared to vehicle. RvD2 reduced TNF-α production compared to saline controls. In this acute LPS-induced inflammatory setting, reduction in macrophage TNF-α production by RvD2 was not affected by TLR2 blockade. Values are mean ± SEM; ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05; n = 3. *C.* RvD2 increased bacterial clearance of *P. aeruginosa* at MOI 15 (PA (15)) by acutely-stimulated THP-1 macrophages. TLR2 blockade abolished RvD2-mediated increase in bacterial clearance. Values are mean ± SEM. **P < 0.01; n = 3.

4.4.2 TLR2 Inhibition Did Not Affect Baseline Macrophage Functions

In 2-hit LPS experiments, the macrophages were incubated with RvD2 (1 or 10 nM) or saline vehicle for 1h. They were then incubated with LPS (50 ng/mL) (hit one) for 24h. Then, the macrophages were incubated with or without MMG-11 (25 μM) for 1h, followed by a 3h incubation with LPS (50 ng/mL) (hit two). The results showed that MMG-11 incubation with LPS-induced exhausted macrophages did not lead to a significant

difference in NF-κB activity (**Figure 29A**), TNF-α production (**Figure 29B**), or bacterial clearance (**Figure 29C**) compared to exhausted macrophages without TLR2 inhibition.

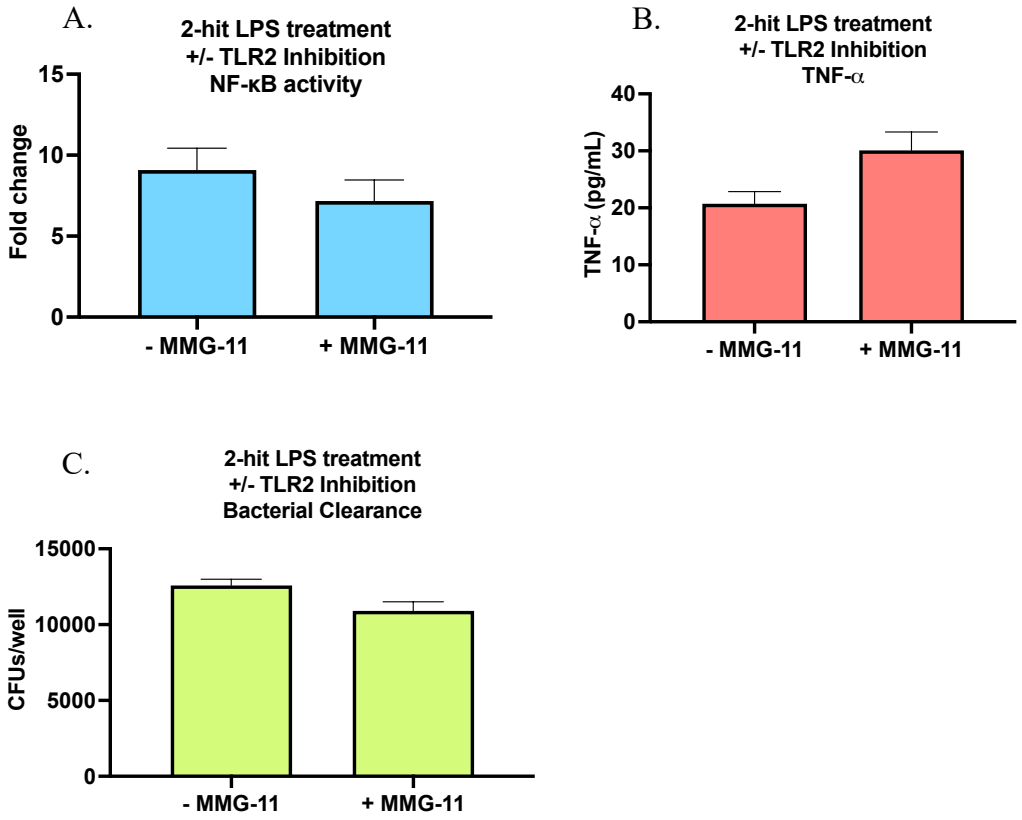
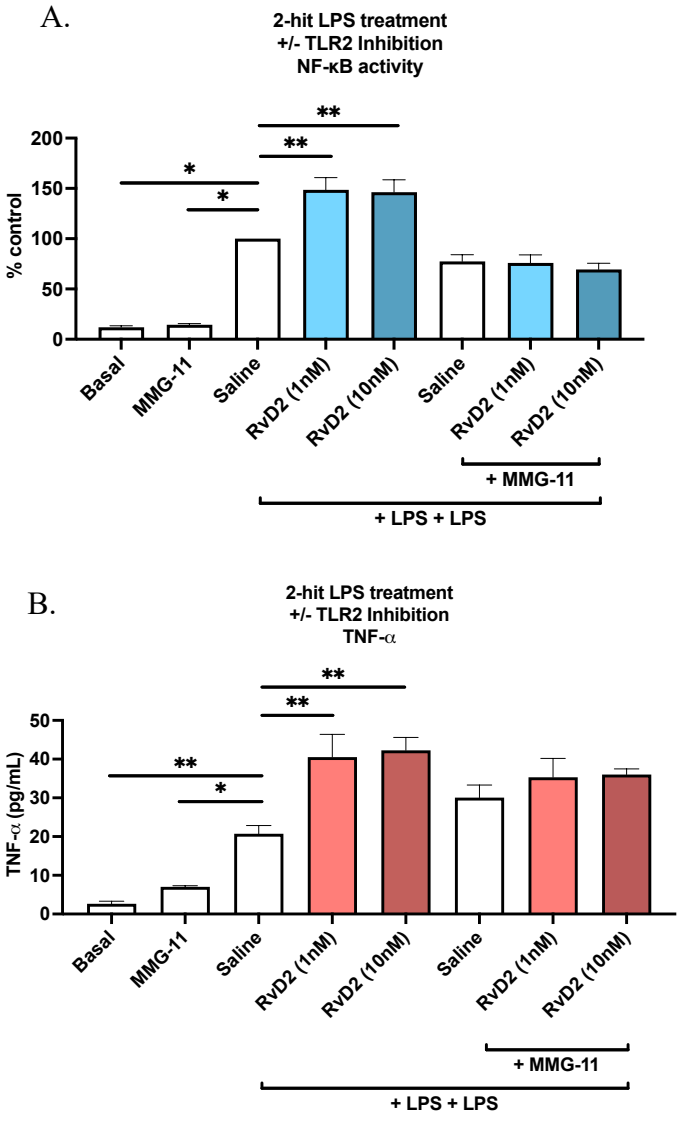


Figure 29. TLR2 Inhibition Did Not Influence Effector Functions by Exhausted Macrophages. Macrophages were subjected to 2-hit LPS. 1h before the 2nd hit of LPS, 25 μM of MMG-11, a TLR2 inhibitor, was added to the macrophages. *A.* TLR2 blockade had no significant effect on saline controls for NF-κB activity. Values are mean ± SEM; n = 6. *B.* TLR2 blockade had no significant effect on saline controls for TNF-α production. Values are mean ± SEM; n = 3. *C.* TLR2 blockade had no significant effect on saline controls for bacterial clearance. Values are mean ± SEM; n = 3.

4.4.3 TLR2 Inhibition Abolished RvD2-Mediated Protection from 2-Hit LPS-Induced Macrophage Exhaustion

Macrophages were incubated with saline vehicle or RvD2 (1 or 10 nM) for 1h. They were then incubated with LPS (50 ng/mL) (hit one) for 24h. Then, the macrophages were

incubated with MMG-11 (25 μM) for 1h, followed by a 3h incubation with LPS (50 ng/mL) (hit two). The results revealed that RvD2's ability to maintain normal macrophage function (via activation of NF-κB) in exhausted cells was abolished when TLR2 was blocked (**Figure 30A**). Similarly, the results showed that in exhausted macrophages, RvD2's modulatory effects on TNF-α release (**Figure 30B**) and on bacterial clearance (**Figure 30C**) were eliminated with TLR2 blockade.



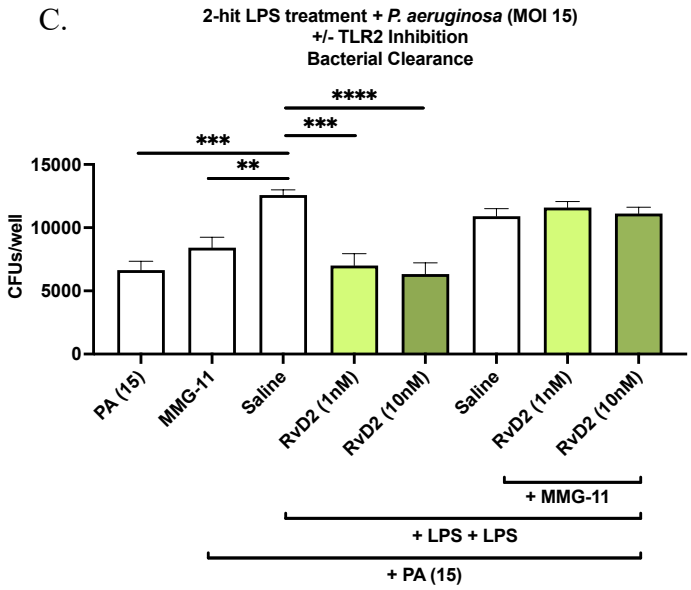


Figure 30. RvD2's Modulatory Effects on Exhausted Macrophages were Abolished with TLR2 Inhibition. Macrophages were subjected to 2-hit LPS. 1h before the 2nd hit of LPS, 25 μM of MMG-11, a TLR2 inhibitor, was added to the macrophages. *A.* Exhausted macrophages exposed to two LPS hits had 8-fold increased NF-κB activity compared to unstimulated controls. RvD2 increased NF-κB activity compared to saline controls. TLR2 blockade abolished RvD2 effects on NF-κB activity in these exhausted (2-hit LPS) macrophages compared to saline controls. Values are mean ± SEM % 2-hit LPS + saline control; **P < 0.01; n = 6. *B.* Exhausted macrophages exposed to two LPS hits had 19-fold increased TNF-α production compared to unstimulated controls. RvD2 increased TNF-α production compared to saline controls. TLR2 blockade abolished RvD2 effects on TNF-α production in these exhausted (2-hit LPS) macrophages compared to saline controls. Values are mean ± SEM. **P < 0.01. *P < 0.05; n = 3. *C.* 2-hits of LPS reduced bacterial clearance compared to macrophages not previously exposed to LPS (basal clearance; PA (15) only). RvD2 increased bacterial clearance compared to saline controls. TLR2 blockade abolished RvD2 effects on bacterial clearance in these exhausted (2-hit LPS) macrophages. Values are mean ± SEM. ****P < 0.0001, ***P < 0.001, **P < 0.01; n = 3.

4.5 Discussion

Our results indicate that TLR2 inhibition had no effect on RvD2-induced changes in NF-κB activity under acute inflammation conditions with a single LPS treatment. This provides evidence that RvD2-induced effects on acute inflammation were not mediated through TLR2 signaling. RvD2-mediated decrease in inflammatory response, however, did

not lead to a continued reduction in macrophage activity. In fact, with a subsequent LPS hit, cells that had been treated with RvD2 had an increased response to LPS and were still able to clear bacteria better than saline vehicle-treated cells. Overall, our results suggest that in acute inflammatory responses, TLR2 plays a part in RvD2-mediated increase in bacterial clearance but does not play a role in RvD2-mediated reduction in acute (1-hit) LPS-stimulated macrophage responses.

Our results showing that a TLR2 antagonist abolished the enhanced macrophage response to LPS in exhausted macrophages is consistent with the notion that RvD2 improves LPS responses by exhausted macrophages through a TLR2-mediated mechanism. One interesting observation from our TLR2 studies is that TLR2 inhibition blunted RvD2's protective effect on NF-κB activity in exhausted macrophages, but not in acutely-stimulated macrophages. This suggests that TLR2 does not play a direct role in LPS-induced macrophage responses, yet does play a direct role in RvD2-induced protection from exhaustion. Taken together, the results from this chapter suggest that TLR2-mediated effects of RvD2 are dependent on the activation state/phenotype of the macrophage (i.e., acutely-stimulated vs. exhausted) as well as the type of the stimulus (LPS or bacteria).

Chapter 5

Resolvin D2 (RvD2) Attenuates Sepsis-Mediated Macrophage Exhaustion in vivo

5.1 Introduction

The gold standard of inducing murine polymicrobial sepsis is by a procedure called cecal-ligation and puncture (CLP) (Dejager et al., 2011). Ligation of mouse ceca results in the production of inflammation and necrotic factors, which contributes to the immunosuppression observed after the procedure (Dejager et al., 2011). CLP-induced polymicrobial sepsis serves as a valuable model for studying sepsis because it initiates infection by a broad range of bacteria, including both Gram-positive and Gram-negative strains. This procedure also triggers hyper-inflammation, leading to the gradual release of pro-inflammatory cytokines over an extended period, as opposed to the sudden release of inflammatory mediators which is observed in acute infections (Dejager et al., 2011). It is important to note that the CLP model of sepsis is subject to variability in terms of infection severity due to factors such as the type of mouse/batch of mouse used, the duration of ligation, and the procedural differences (Rittirsch et al., 2009). For this reason, in the experiments in this chapter, we conducted repeated measurements of various cellular and molecular changes before drawing conclusions.

During CLP-induced polymicrobial sepsis, several cellular changes occur as a result over a period of time. Acutely, the procedure triggers the activation of various immune cells, including neutrophils and macrophages, which can lead to the production of pro-inflammatory mediators (such as IL-6 and TNF-α) (Dejager et al., 2011). Over the

course of the next 48h, there is a shift towards immunosuppression, when the immune cells become overworked, and eventually, exhausted (Dejager et al., 2011).

Overall, the cellular changes occurring in a mouse experiencing CLP reflect a complex interplay between the host immune response and the invading pathogens, leading to early inflammation and tissue damage, and a later immunosuppression. Studying these cellular changes is crucial for understanding the pathophysiology of sepsis, especially from a clinical standpoint. While Specialized Pro-resolving Mediators (SPMs) have been studied extensively in the setting of acute inflammation/infections, there are not many studies focusing on their roles in sepsis, where there is a complex interplay of pro- and anti-inflammatory responses. Resolvin D2 (RvD2) has been reported to increase survival of CLP-septic mice by increasing bacterial clearance (i.e., decreasing bacterial load) in CLP-induced polymicrobial sepsis (Chiang et al., 2017, 2015; Serhan et al., 2000; Spite et al., 2009; Sundarasivarao et al., 2022; Walker et al., 2022). Additionally, it has been previously reported that when LxA_4 is administered prior to CLP-sepsis, murine peritoneal macrophages have a reduction in NF-κB activity levels (Walker et al., 2011).

5.2 Rationale

It is important to remember that the cell line utilized in the in vitro experiments in earlier chapters is an immortal human monocyte cell line derived from the peripheral blood of a patient with acute myeloid leukemia (AML; a type of blood cancer). Studies have shown that despite being an immortal cell line, THP-1 cells still behave similarly to monocytes/macrophages in healthy patients, and are likewise an excellent monocyte/macrophage proxy (Bosshart and Heinzelmann, 2016). However, we find that it

is important to recapitulate our in vitro work in non-transformed monocytes/macrophages, such as macrophages from mice. It is also important to consider that differentiating monocytes to macrophages in vitro with PMA may be analogous to investigating inflammatory macrophages, which have differentiated from blood monocytes on entry into the infected tissue, but may not be analogous to looking at resident macrophage function in vivo.

In this chapter, we designed experiments with the goal of assessing the effects of in vivo administration of RvD2 on septic splenic macrophages. We performed CLP-sepsis surgery on mice using methods previously described by our lab (Yin et al., 1999; Walker et al., 2022; Sundarasivarao et al., 2022). Briefly, mice were given RvD2 48h after CLP-sepsis surgery. 24h after this, the mice were sacrificed, and the splenic macrophages were isolated and cultured. At this time point (72h after CLP), we believe that the splenic macrophages are “exhausted” and therefore will have a decreased ability to produce pro-inflammatory cytokines following subsequent immune stimulation. The cells were then incubated with lipopolysaccharide (LPS) for 4h (in order to stimulate them), and NF-κB activity was determined by quantitating downstream cytokine production (IL-6, TNF-α, and IL-10).

5.3 Materials and Methods

5.3.1 RvD2 Synthesis

RvD2 was prepared by total organic synthesis as described in **Chapter 2**. 1 μg of RvD2 was dissolved in 2.66 mL of sterile saline which had been bubbled with argon on days of experiments.

5.3.2 Cecal Ligation and Puncture (CLP) Surgery

The protocol for these studies is outlined in **Figure 31**. CLP surgery was performed on C57BL/6 mice (11-13 weeks old) using modified methods as previously published (Sundarasivarao et al., 2022). Mice were anesthetized with isoflurane (+ O_2), a midline incision was made in the abdominal wall, and the cecum was exposed. The distal 1/3rd of the cecum was ligated with 4.0 surgical silk. The cecum was then punctured twice, through and through, with a 27-gauge needle. The cecum was re-placed into the abdomen, and the incision was closed in 2 layers. For sham controls, ceca were isolated but not ligated nor punctured. Saline (8 mL/100g; s.c.) was injected to replace any fluid loss during surgery. Buprenorphine extended release (Ethiqa-XR, 3.25 mg/kg, s.c.) was injected. 48h after surgery, CLP mice were anesthetized and injected intravenously with either RvD2 (100 ng/mouse) or saline vehicle via the tail vein. 24h after this, the mice were euthanized, and the spleens were taken for analyses.

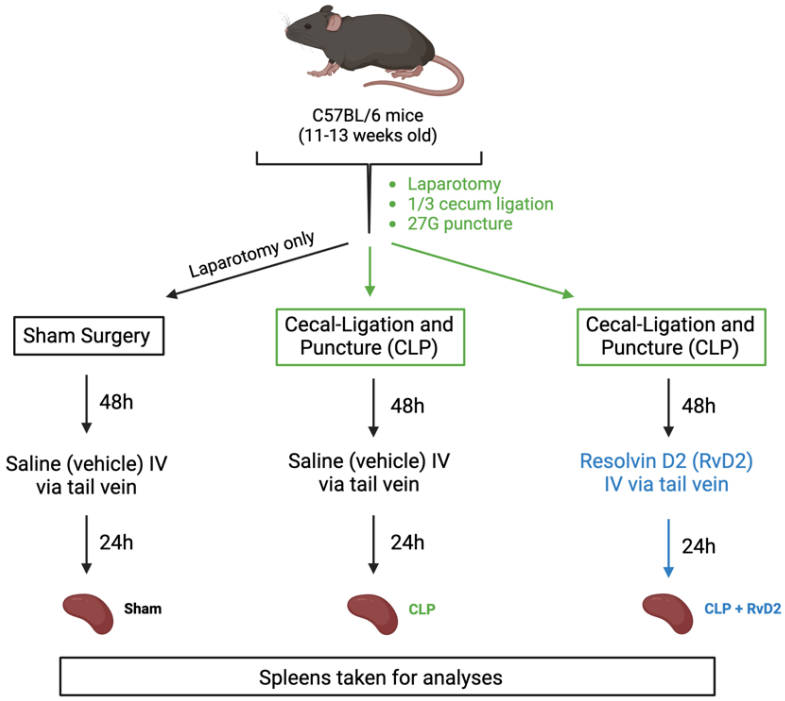


Figure 31. CLP Surgery Protocol. This schematic outlines the methods conducted for murine CLP-sepsis experiments and the control/experimental groups.

5.3.3 Isolation of Splenic Macrophages

Isolation of macrophages from spleens was performed as previously reported (Sundarasivarao et al., 2022). Briefly, whole spleens were passed through a 70 µm cell strainer placed inside a petri dish containing 10 mL ice-cold PBS (with 0.38% sodium citrate). Spleens were homogenized using the flat end of 3 mL syringes. The homogenate was centrifuged at 600 X g for 8 min at 4°C and supernatant discarded. Cells were then resuspended in 5 mL of ACK cell lysis buffer (Life Technologies: Grand Island, NY, USA) and incubated on ice for 5 min. After incubation, cells were centrifuged at 600 X g for 8 min at 25°C and supernatant discarded. Cells were then resuspended in RPMI 1640 containing L-glutamine, 10% heat-inactivated fetal bovine serum, 0.05 mM 2-

mercaptoethanol, 100 U/mL penicillin G, and 100 µg/mL streptomycin. Suspended cells (1.5 mL) were seeded into 12-well plates (Corning: Manassas, VA, USA) and incubated for 90 min at 37°C, 5% CO_2. Preliminary work had ascertained that 1.5 mL of this suspension provided 5-8 X 10^5 macrophages. We also confirmed this after each experiment by counting adherent macrophages (detailed below). Following incubation, the media was aspirated, and wells were washed 3 times with sterile saline (to remove nonadherent cells), and RPMI media was replenished. Cells were then incubated with or without 50 ng/mL LPS (LPS O111.B4 from *E. coli*) for 4h at 37°C, 5% CO_2. Following incubation, supernatants were collected and stored at -70°C. Cells were scraped from wells using cell scrapers following the addition of 250 µL of 5 mM EDTA (Promega: Madison, WI, USA) in 1X ice-cold PBS solution, and placed on ice for 3 minutes. Cells were collected into microcentrifuge tubes and counted in a Countess cell counter (Countess II FL; Invitrogen: Waltham, MA, USA). Gate sizes were adjusted to count cells between 15 and 25 µm.

5.3.4 Cytokine Production Assays

The concentrations of murine IL-6, TNF-α, and IL-10 were determined by enzyme-linked immunosorbent assay (ELISA; Thermo Fisher Scientific: Waltham, MA, USA) according to the manufacturers' instructions. Absorbance (450 nm) was read on a BioTek Synergy H1 plate reader (Biotek: Winooski, VT, USA).

5.3.5 Flow Cytometry Staining and Analysis

Approximately 1 X 10^6 cells were aliquoted in microcentrifuge tubes and Fc receptors were blocked using 1 µL of TruStain FcX (anti-mouse CD16/32) antibody

(BioLegend, Cat #15660, San Diego, CA) for 30 min on ice. Next, cells were washed with cell staining buffer and 1 µL of TLR2 antibody (BioLegend, Cat #148604, San Diego, CA) along with 1 µL of live/dead cell stain (Invitrogen, Cat #L10119, Waltham, MA) and incubated for 80 min on ice. After incubation, cells were washed with cell staining buffer and resuspended. Attune Acoustic Focusing cytometer (Applied Biosystems, Waltham, MA) was used to analyze samples. For flow cytometry compensation of multiple fluorophores, ABC anti-rat capture beads (Invitrogen, Waltham, MA) and ArC amine reactive capture beads (Invitrogen, Waltham, MA) were used for accurate separation of overlapping emission wavelengths of multiple fluorophores. Cells were gated based on size (forward scatter) and granularity (side scatter), single population, live cells, followed by expression of TLR2. FlowJo software (Version 10) was used to analyze flow cytometry data.

5.3.6 Statistical Analysis

All analyses were performed using GraphPad Prism version 9.4.1 for Mac (GraphPad Software: San Diego, CA, USA). All data are expressed as mean ± SEM. Spleen weight data was subjected to two-way analysis of variance (ANOVA) with Šídák's multiple comparisons post-hoc test. All other data was subjected to one-way ANOVA with Bonferroni's multiple comparisons post-hoc test. A p value < 0.05 was taken as significant for at least six independent studies.

5.4 Experimental Results

5.4.1 Murine Spleen Sizes Did Not Significantly Affect Macrophage Numbers

Following treatments (delineated in **Figure 31**), mouse spleen weights were removed and processed to isolate the macrophages. Prior to processing, the spleens were weighed. The results revealed that there was no significant difference in the spleen sizes (in g) of mice between different groups and different sexes (**Figure 32**). Since it is plausible that bigger spleens would have more cells (and therefore, more macrophages), these results suggest that the observed differences in macrophage cytokine production could not be attributed to spleen size.

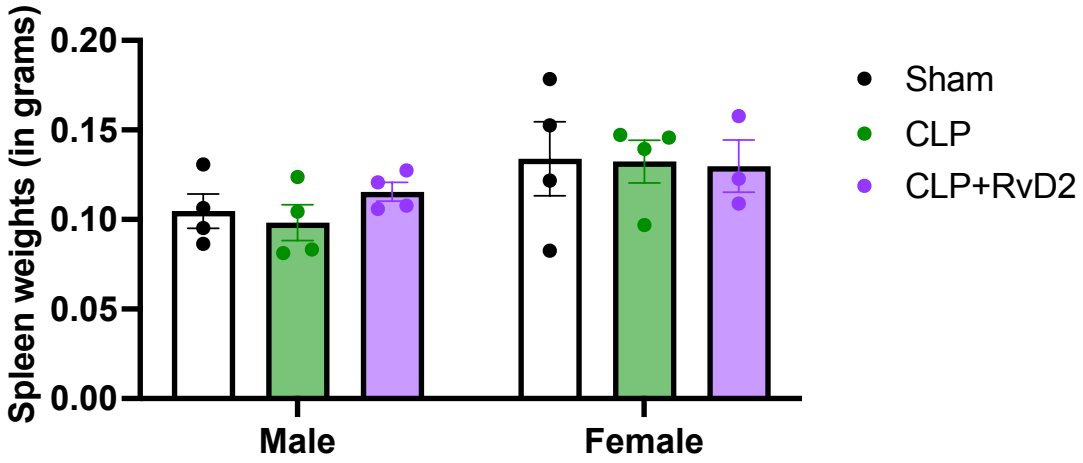


Figure 32. Murine Spleen Weights. Following removal and prior to processing, the spleens had excess fat removed and were then weighed. We found that there was no significant difference in the spleen sizes (in g) of mice between different groups (sham vs. CLP vs. CLP+RvD2) and between different sexes (male vs. female). n=7-8 per group.

5.4.2 RvD2 Attenuated Macrophage Exhaustion in vivo

In these studies, we investigated the in vivo effect(s) of RvD2 on LPS-stimulated splenic macrophages after CLP-induced infectious peritonitis. Mice were administered either an IV infusion of RvD2 or saline 48h after CLP surgery. The mice were sacrificed 24h after these injections. Macrophages were plated from the harvested spleens and subsequently treated with 50 ng/mL of LPS for 4h. Cytokine analysis revealed that pro-inflammatory cytokines IL-6 (**Figure 33A**) and TNF-α (**Figure 33B**) were decreased in CLP mice compared to sham controls, suggesting that the CLP macrophages were exhausted similar to our in vitro experiments. Pre-incubation with RvD2 increased the production of pro-inflammatory cytokines IL-6 and TNF-α. This result confirms our in vitro results from **Chapter 2**, whereby RvD2 attenuated macrophage exhaustion by maintaining and/or augmenting their pro-inflammatory responses to acute challenge. Cytokine analysis also revealed that the anti-inflammatory cytokine IL-10 (**Figure 33C**) was increased in CLP mice compared to sham controls, also suggesting that the CLP macrophages were exhausted, as elevated IL-10 is a biomarker of immunosuppression (**Chapter 1**). Interestingly, pre-incubation with RvD2 further increased the production of IL-10 compared to sham mice (**Figure 33C**). This result suggests that RvD2-treated macrophages are unique, in that they can increase production of both pro-inflammatory and anti-inflammatory cytokines in times of suppression.

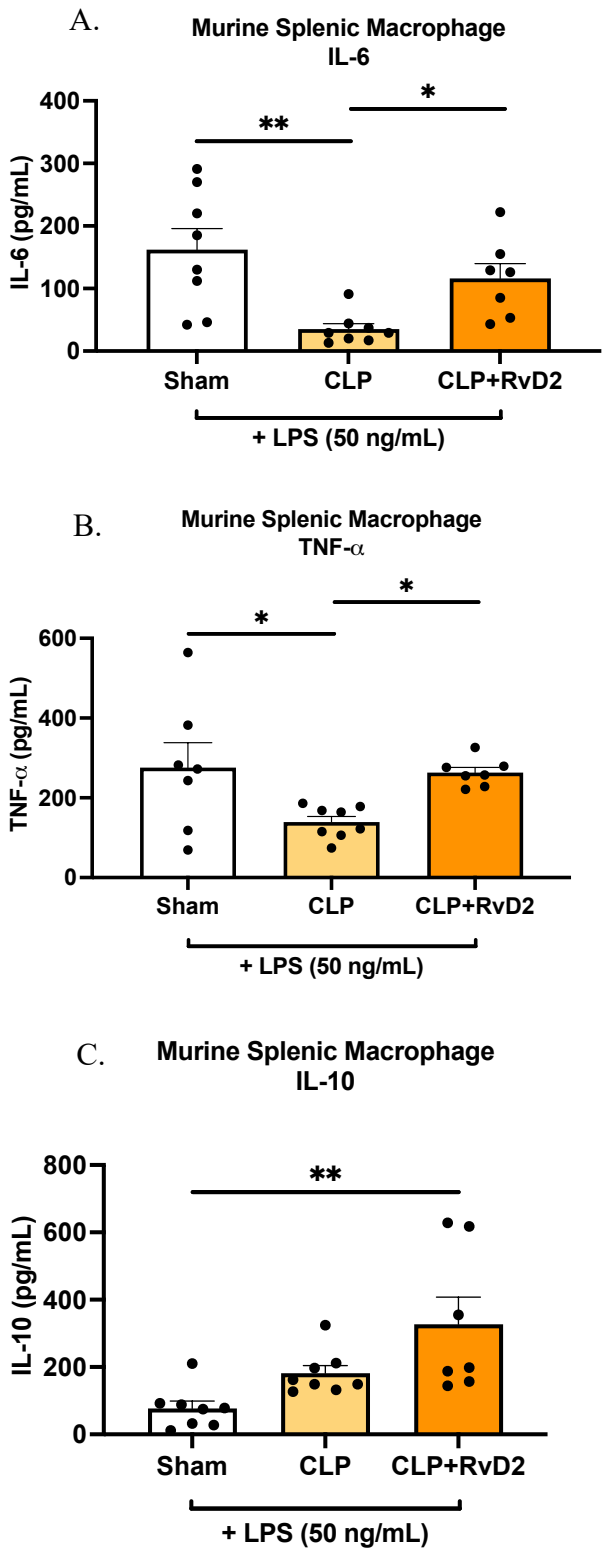


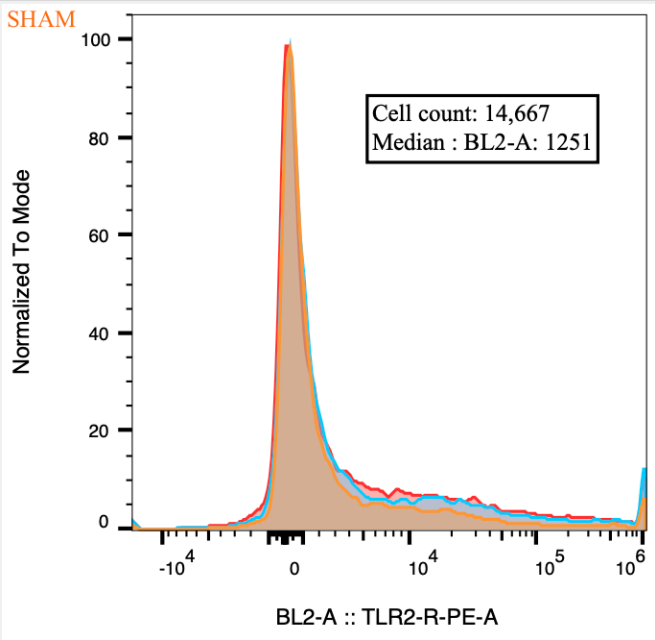
Figure 33. RvD2 Attenuated Macrophage Exhaustion in vivo. CLP surgery was performed on mice. RvD2 (100 ng/mouse) or saline was infused intravenously 48h after surgery. 24h after injections, mice were euthanized, and spleens were isolated. *A.* Following stimulation

with 50 ng/mL LPS, splenic macrophages from CLP mice produced less IL-6 compared to sham animals, and RvD2 increased IL-6 production. Values are mean ± SEM. **P < 0.01, *P < 0.05; n = 7-8 per group. *B.* Following stimulation with 50 ng/mL LPS, splenic macrophages from CLP mice produced less TNF-α compared to sham animals, and RvD2 restored TNF-α production. Values are mean ± SEM. *P < 0.05; n = 7-8 per group. *C.* Following stimulation with 50 ng/mL LPS, splenic macrophages from mice that were infused with RvD2 had increased IL-10 production compared to sham mice. Values are mean ± SEM. **P < 0.01; n = 7-8 per group. *Note*: each circle represents one mouse.

5.4.3 Preliminary Data May Suggest that Surface Expression of TLR2 was Decreased in CLP Mice

In these very preliminary studies, we investigated the effect of sepsis induction on TLR2 surface expression of splenic macrophages after CLP-induced infectious peritonitis. Mice were subjected to a sham surgery (laparotomy only) or to CLP surgery. The mice were sacrificed 72h later. Macrophages were plated from the harvested spleens. Cells were scraped, counted, and stained as described above, and then processed for flow cytometry analysis. Flow cytometry analysis revealed that sham mice had a median TLR2 expression level of 1251 (**Figure 34A**), whereas CLP mice had a median TLR2 expression of 239 (**Figure 34B**), a drop of 81%. Previously, we have shown that in vivo administration of RvD2 improves survival of CLP mice, promotes inflammation resolution in CLP-sepsis, and leads to a significant increase in TLR2 gene expression in splenocytes (Sundarasivarao et al., 2022; Walker et al., 2022). With that being said, we hypothesize that the beneficial effects of RvD2 on CLP mice could be due to an increase in TLR2 total protein levels (both intracellular and surface expression). While there is not enough data to draw conclusions from these studies, this is an interesting avenue that will be explored further in the lab (implications and future directions are discussed further in **Chapter 6**).

A.



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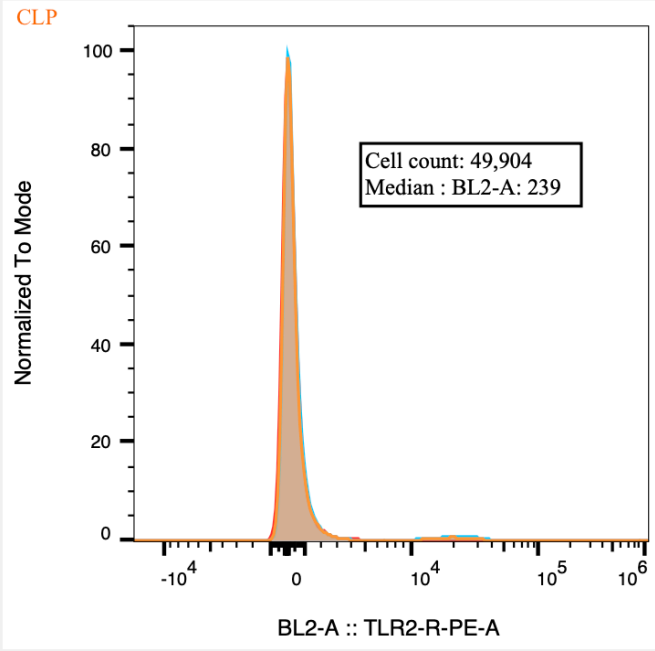

Figure 34. Preliminary Data May Suggest that Surface Expression of TLR2 was Decreased in CLP Mice. *A.* Sham mice were subjected to laparotomy only (sham) surgery. 72h later, the spleens were removed, and the macrophages were isolated and stained for TLR2 expression; n = 1. *B.* CLP mice were subjected to CLP surgery. 72h later, the spleens were removed, and the macrophages were isolated and stained for TLR2 expression; n = 1. While true conclusions cannot be drawn from these data due to them being underpowered, this work will provide an interesting starting point for future experiments within the lab.

5.5 Discussion

In these studies, splenic macrophages taken from septic mice had significantly diminished LPS-induced responses compared to sham controls, and RvD2 administration attenuated this macrophage suppression. These in vivo results completely support our in vitro studies.

These studies primarily focused on murine CLP-induced peritoneal infections leading to sepsis. CLP is considered a strong method for studying polymicrobial sepsis for several reasons. First, CLP mimics the complex nature of polymicrobial infections commonly encountered in clinical settings. Unlike other models that use a single microbial strain, CLP induces sepsis by introducing a mixture of bacteria from the gastrointestinal tract into the peritoneal cavity, closely resembling the diverse microbial populations observed in humans. Second, this model allows researchers to study the cascade of events that occur during the development and progression of sepsis, including the release of pro-inflammatory cytokines, activation of immune cells, and eventual immunosuppression. Third, CLP is a well-established and standardized model with reproducible outcomes across different laboratories and animal species. Standardized protocols for CLP surgery ensure consistency in experimental procedures, making it easier to validate experimental findings.

In these studies, we decided to isolate splenic macrophages for a few reasons. For one, the spleen plays a significant role in both clearing bacteria from the body and regulating the immune system (Tiron and Vasilescu, 2008). Splenic macrophages in particular are known for their role in clearing bacteria from the body via phagocytosis, while working alongside other immune cells such as dendritic cells to present antigens and

initiate the activation, differentiation, and expansion of lymphocytes (Barker et al., 2002; Prendergast et al., 2018).

Spite et al. (2009) found that in microbial sepsis induced by CLP, RvD2 significantly reduced both the local and systemic bacterial load, while enhancing macrophage phagocytosis in the peritoneal cavity (Spite et al., 2009). In the aforementioned study, the RvD2 was administered very early (1h after CLP surgery). In contrast, we administered RvD2 48h after CLP surgery, when there was clearly a reduction in the LPS-induced macrophage response. RvD2 significantly enhanced this response (**Figures 33A** and **33B**).

One interesting finding from this work is that while RvD2-treated exhausted splenic macrophages increased production of pro-inflammatory cytokines following LPS stimulation compared to exhausted splenic macrophages not previously exposed to RvD2, RvD2-treated macrophages also had significantly increased production of the anti-inflammatory cytokine IL-10 (**Figure 33C**). As described in **Chapter 1**, IL-10 is a potent anti-inflammatory cytokine that limits the host immune response to pathogens (Iyer and Cheng, 2012). This seemingly-contradictory role of RvD2 may actually give credence to the idea that unique, pro-resolving macrophages could exist. As discussed in **Chapter 2**, these pro-resolving macrophages are said to have qualities of the classic M1-like and M2-like phenotypes (**Chapter 1**) and contribute to both pro- and anti-inflammatory pathways (Saas et al., 2020). In **Chapter 2**, our results suggested that when RvD2 is administered to macrophages following differentiation with PMA, the macrophages seem to become polarized to this pro-resolving phenotype, such that when exhausted (i.e., hit twice with LPS), the cells are able to increase relative NF-κB activity, TNF-α production, and bacterial

clearance. Here, our results suggest that when RvD2 is administered to mice 48h after CLP-sepsis induction, the splenic macrophages seem to also become polarized to this pro-resolving phenotype, such that they can increase production of both pro-inflammatory cytokines as well as anti-inflammatory cytokines following subsequent stimulation with LPS. While the exact role of these pro-resolving macrophages is currently unknown in physiologic settings, it can be inferred that RvD2-treated macrophages are highly plastic and can undergo phenotype switching in response to changing environmental cues. This high level of plasticity would allow these macrophages to dynamically modulate their function and tailor their response to different stages of infection, tissue repair, or inflammation resolution (Bonnefoy et al., 2018).

While no conclusions can be drawn from the flow cytometry studies at this point (due to the studies being preliminary and therefore underpowered), we believe this is an exciting avenue for future studies on TLR2 protein levels. As mentioned in **Chapter 4**, we have previously shown that RvD2 increases gene expression of TLR2, and this increase was associated with improved primary bacterial clearance from circulation and better clearance of *P. aeruginosa* secondary lung infection (Sundarasivarao et al., 2022; Walker et al., 2022). One thing to keep in mind is that increased gene expression does not necessarily mean increased protein production. Therefore, future flow cytometry studies can shed light on TLR2 protein levels following these in vivo experiments. Since TLR2 is found intracellularly as well as on the surface of macrophages, future flow cytometry studies may want to stain for both intracellular TLR2 and cell-surface TLR2 within/on macrophages.

Interestingly, recent studies have found that TLR2 can be released (i.e., shed) by macrophages following treatment with glucocorticoids and LPS, potentially leading to glucocorticoid- and LPS-induced immunosuppression (Hoppstädter et al., 2019). Since glucocorticoids can polarize macrophages to anti-inflammatory, M2-like macrophages (**Figure 9**), perhaps macrophages shed TLR2 to act as "decoy receptors" in times of overwhelming immune stimulation (Hoppstädter et al., 2019). Other possibilities for TLR2 shedding are that shedding can regulate TLR2 signaling by competing with membrane-bound TLR2 for ligand binding or by altering the availability of TLR2 on the cell surface. The concept of TLR2 shedding could be further studied in our lab by performing TLR2 ELISAs on the supernatants of wells containing murine splenic macrophages plated from in vivo CLP experiments. Considering these findings, it is worth reflecting on the potential interplay between the mechanisms described and the actions of RvD2. In certain instances (such as overwhelming immune stimulation), TLR2 shedding may occur as an adaptive immune mechanism as described above. Since we have previously shown that RvD2 can increase murine TLR2 gene expression in splenocytes (Walker et al., 2022) and this upregulation is correlated with better outcomes due to sepsis, it is plausible that this action by RvD2 can override the infection-mediated shedding of TLR2 by macrophages. Perhaps RvD2-mediated enhancement of TLR2 gene expression can compromise the "loss" of TLR2 from macrophages via shedding. Investigating the effects of RvD2 on TLR2 shedding and its implications on immune regulation could provide valuable insights into the intricate mechanisms governing inflammation resolution during sepsis.

Chapter 6

Summary and Conclusions

The studies in this dissertation were designed to investigate the antibacterial and immunomodulatory properties of Resolvin D2 (RvD2) on monocytes and macrophages. In **Chapters 2 and 3**, we investigated the immunomodulatory role of RvD2 on THP-1 monocyte and monocyte-derived macrophage activation and pro-inflammatory cytokine secretion in different models of in vitro infection (free LPS and whole bacteria). Studies in **Chapter 4** focused on the signaling mechanism underlying the differences in monocyte/macrophage activation state/phenotype. In **Chapter 5**, we assessed RvD2's role in a mouse model of sepsis and measured LPS-induced IL-6, TNF-α, and IL-10 secretion. In this chapter, we will summarize the significance of these findings in the sepsis field. Furthermore, the limitations of these data and future directions of this work will also be discussed below.

6.1 Sepsis-Induced Immunosuppression

Characteristics of macrophage exhaustion in sepsis-induced immunosuppression include a reduced response to LPS stimulation, an inability to clear bacteria, and impaired antigen presentation (Pena et al., 2014). There are several reports which show that impairment of the host's immune system to mount an inflammatory response to a secondary infection is responsible for the increased morbidity and mortality observed in sepsis (Cavaillon et al., 2001; Hotchkiss et al., 2013a; Jundi et al., 2021; Liu et al., 2022; López-

Collazo and del Fresno, 2013; Muenzer et al., 2010; Pena et al., 2014). In addition, the host's ability to produce TNF-α (Liu et al., 2022) or IFN-γ (Fu and Wang, 2023) was reported to be beneficial in late-sepsis.

6.2 Resolvin D2 (RvD2) Influences Transcription Factor NF-κB Activity and Monocyte/Macrophage Functions After Lipopolysaccharide (LPS) Stimulation

Using THP-1 monocyte-derived-macrophages, we showed that RvD2 given 1h before an acute (1-hit) LPS treatment reduced NF-κB activity and TNF-α production (**Chapter 2**), but increased clearance of *P. aeruginosa* (**Chapter 3**). Macrophages which were stimulated twice with LPS had decreased NF-κB activity, TNF-α production, IL-8 production, and *P. aeruginosa* clearance compared to cells that were stimulated once. This finding provides evidence of cell exhaustion similar to suppressed macrophages found in the late phase of sepsis. Here, RvD2 treatment increased NF-κB activity, TNF-α production, IL-8 production, and *P. aeruginosa* clearance. These results suggest that RvD2 can modulate macrophage functions in inflammation/infection, where it can reduce the acute inflammatory response to LPS (1-hit treatments) but can increase effector responses in exhausted macrophages (2-hit LPS treatments). These increased effector responses are biologically relevant, as they suggest that RvD2-treated exhausted macrophages are more active than exhausted macrophages given saline vehicle.

Our results showing that RvD2 reduced macrophage inflammatory responses to LPS (**Chapter 2**) while increasing macrophage ability to clear *P. aeruginosa* (**Chapter 3**) are consistent with work by us and others where RvD2 and other SPMs (such as LxA_4 and RvD1) were able to resolve inflammation in different in vivo models of inflammation/infection. In addition, RvD2 reduces TLR4 expression in human

macrophages (Croasdell et al., 2016) and RvD1 reduces LPS-mediated inflammatory responses by macrophages through a TLR4-mediated mechanism (Palmer et al., 2011). As RvD2 reduced NF-κB activity and TNF-α production in our studies, the results suggest that the reduction in TNF-α was through NF-κB signaling. RvD2-mediated decrease in inflammatory responses, however, did not lead to a continued reduction in macrophage activity. In fact, with a subsequent LPS hit, cells that had been treated with RvD2 had an increased response to LPS and were still able to clear bacteria better than saline vehicle-treated cells. These results suggest that RvD2 can directly attenuate LPS-induced suppression of macrophages.

One interesting finding from **Chapter 2** and **Chapter 3** is the observed difference if RvD2 was administered to THP-1 monocytes before differentiation and following differentiation into macrophages. When RvD2 was given prior to PMA administration, there was decreased NF-κB activity, reduced TNF-α production, and increased bacterial clearance regardless of whether cells were acutely stimulated (1-hit LPS) or were exhausted (2-hit LPS). One possibility for this effect is that RvD2 is priming and pre-polarizing the monocytes into an M2-like phenotype; therefore, following subsequent differentiation with PMA, regardless of the stimulus (1-hit LPS or 2-hit LPS), monocytes are already differentiated into M2-like macrophages, which have lowered inflammatory responses and increased bacterial clearance (Yunna et al., 2020). Polarization of macrophages is highly dependent on changes within their immediate environment, and repolarization can be triggered if necessary (Anand et al., 2023). During acute infection, M1-like macrophages dominate, and assist the host in clearing pathogens by triggering NF-κB-mediated inflammatory responses via the release of pro-inflammatory cytokines such

as IL-6 and TNF-α (Yunna et al., 2020). Following cessation of infection, M2-like macrophages dominate, and their main functions are to repair tissue damage, decrease inflammation, and clear debris left from the infection/inflammatory response (Yunna et al., 2020). Our data suggest that RvD2 polarizes monocytes into M2-like macrophages and that this action is distinctly different from its effect on exhausted macrophages. An interesting finding from **Chapter 2** is that RvD2 increased THP-1 monocyte adherence to laminin-coated plates. This is consistent with previous work performed using LxA_4 (Maddox and Serhan, 1996; Thornton et al., 2021). Under physiologic conditions, monocytes adhere to multiple basement membrane proteins such as laminin (Tobias et al., 1987). Additionally, one of the first steps of monocyte differentiation into macrophages is adherence (Nielsen et al., 2020). Taken together, our results suggest that RvD2 exerts a strong pre-polarizing effect on monocytes and can differentiate monocytes into adherent M2-like macrophages.

6.3 Resolvin D2 (RvD2) Effects on Monocyte/Macrophage Functions After Bacteria Stimulation Are Not Dependent on NF-κB Activity

Our work showed that while macrophage-mediated bacterial clearance was significantly increased by RvD2-treated cells, this increase was not dependent on NF-κB activation (**Chapter 3**). This is consistent with other studies which reported that RvD2's enhancement of macrophage bacterial phagocytosis acts through the STAT3 pathway instead of the NF-κB pathway (Chiang et al., 2017). On the other hand, investigators have reported that RvD1 increased human macrophage phagocytosis of *E. coli* (Palmer et al., 2011) through a TLR4-dependent increase in TNF-α production, which enhanced phagocytosis. Our results are not consistent with this study, as increases in *P. aeruginosa*

clearance were not associated with any alteration in NF-κB activity by RvD2. RvD2-mediated increase in macrophage bacterial clearance, however, was dependent on TLR2 (**Chapter 4**). Taken together, our results are consistent with the notion that RvD2-mediated increase in bacterial clearance acts through a TLR2-dependent, NF-κB-independent signaling pathway.

6.4 Resolvin D2 (RvD2) Acts Through TLR-Mediated Signaling that Leads to Monocyte/Macrophage Reprogramming

TLR2 is a type of pattern recognition receptor (PRR) (Oliveira-Nascimento et al., 2012). TLR2 binding to specific damage associated molecular patterns (DAMPs) and/or pathogen associated molecular patterns (PAMPs) is crucial for mediating both the inflammatory response and clearance of various infections (Chen et al., 2016; Oliveira-Nascimento et al., 2012; Reba et al., 2014). Upon activation, TLRs homo- or hetero-dimerize; this initiates a complex cell signaling cascade which leads to downstream cell-specific effector functions (Hug et al., 2018). TLR2 activation by PAMPs such as LPS, lipoteichoic acid, and peptidoglycan leads to NF-κB activation (Hug et al., 2018). Excessive TLR2 signaling through NF-κB, however, may hyperactivate the inflammatory response, causing tissue damage (Cowardin et al., 2016; Lima et al., 2015). On the other hand, TLR2 agonism has also been reported to cause immunosuppression (Netea et al., 2004; Nguyen et al., 2020).

We have previously shown that late administration of RvD2 in the CLP model of polymicrobial sepsis increased splenocyte TLR2 gene expression (Walker et al., 2022). This increase was associated with improved primary bacterial clearance from circulation and better clearance of *P. aeruginosa* secondary lung infection (Sundarasivarao et al., 2022;

Walker et al., 2022). Our results showing that a TLR2 antagonist abolished the enhanced macrophage responses to LPS by exhausted macrophages is consistent with the notion that RvD2 improves LPS responses by exhausted macrophages through a TLR2-mediated mechanism. The enhanced functions of LPS-induced exhausted THP-1 macrophages was abolished by TLR2 inhibition, which is consistent with the notion that these beneficial actions of RvD2 were TLR2-dependent.

In contrast, our results indicate that TLR2 inhibition had no effect on RvD2-induced changes in NF-κB activity under acute (1-hit) LPS stimulation (**Chapter 4**). These results provide evidence that RvD2-induced effects on acute LPS stimulation were not mediated through TLR2 signaling. TLR2 inhibition, however, abolished RvD2-mediated increase in bacterial clearance (**Chapter 4**). These results suggest that in acute inflammatory responses, TLR2 plays a part in RvD2-mediated increase in bacterial clearance but does not play a role in RvD2-mediated reduction in acute (1-hit) LPS-induced macrophage responses. Taken together with our results showing RvD2's effect on 2-hit LPS-induced exhausted macrophages, these results suggest that TLR2-mediated effects of RvD2 are dependent on the activation state/phenotype of the macrophage (i.e., acutely-stimulated vs. exhausted) as well as the type of the stimulus (LPS or bacteria).

6.5 Resolvin D2 (RvD2) Attenuates Sepsis-Mediated Macrophage Exhaustion in vivo

In these in vivo studies, splenic macrophages taken from septic mice had significantly diminished LPS-induced responses compared to sham controls, and RvD2 administration attenuated this macrophage suppression. These results were completely

consistent with our in vitro data showing that RvD2 increased LPS-induced production of pro-inflammatory cytokines by exhausted macrophages.

Interestingly, RvD2-treated macrophages also produced significantly increased levels of IL-10, an anti-inflammatory cytokine. These results further provide evidence that RvD2-treated macrophages seemingly polarize into pro-resolving macrophages, as they have qualities of both M1-like and M2-like macrophages. Perhaps septic macrophages treated with RvD2 exhibit higher-than-normal plasticity, enabling them to switch phenotypes in response to their direct environment. This flexibility would empower these macrophages to adjust their activities dynamically, catering to various stages of infection and inflammation resolution.

6.6 Limitations and Future Directions

One limitation of these studies is that the investigation of the NF-κB response in THP-1 monocytes/macrophages was limited to the analysis of a transformed cell line. Although immortal cell lines provide many practical benefits, their transformed features may influence the functionality of NF-κB. On the other hand, there are still limitations if we were to obtain and culture primary monocytes/macrophages for this study. For example, there are restrictions in terms of their lifespan, cell numbers, and heterogeneity within the population among various human donors (Sharif et al., 2007). Many studies have concluded that despite being an immortal cell line, THP-1 cells behave similarly to monocytes/macrophages from humans and are likewise an excellent monocyte/macrophage proxy for in vitro work (Bosshart and Heinzelmann, 2016; Prehn et al., 1992; Reyes et al., 1999; Schreiber et al., 2006; Sharif et al., 2007). Importantly, we

used this cell line to investigate the effects of RvD2 on basic effector functions of macrophages (LPS responses, bacterial clearance) and are not using this cell line for drug screening studies or comprehensive immunological experiments (Bosshart and Heinzelmann, 2016; Prehn et al., 1992; Reyes et al., 1999; Schreiber et al., 2006; Sharif et al., 2007).

While our in vivo work assessed polymicrobial sepsis, our in vitro work was limited to Gram-negative infections (*Pseudomonas aeruginosa*). While Gram-negative bacterial infections are one of the most common causes of hospital-acquired sepsis (CDC, 2018) and can likewise provide valuable insights into specific aspects of sepsis pathogenesis, one limitation of these studies was that the broader spectrum of microbial pathogens (i.e. Gram-positive infections) was not investigated. Different pathogens elicit distinct host immune responses, which may vary in intensity, duration, and nature. We cannot rule out the possibility that the results from these studies may be pathogen-specific, as important insights into host responses triggered by other types of pathogens, including Gram-positive bacteria, fungi, or viruses, may be different.

One future direction of this work can be to assess total protein levels of TLR2 (including intracellular levels as well as surface levels), and to ascertain how TLR2 expression changes in response to pre-treatment with RvD2. Previous work in the lab has shown that splenic TLR2 gene expression was increased following in vivo infusion with RvD2 (Walker et al., 2022) and very preliminary flow cytometry data has shown that TLR2 surface expression was decreased on CLP animal splenic macrophages (**Chapter 5**).

6.7 Summary

In summary, we developed a clinically-relevant in vitro model of LPS-mediated macrophage exhaustion (rather than tolerance), which represents macrophages in the immunosuppressive stage of sepsis. We showed that RvD2 can modulate the host defense by directly acting on monocytes and macrophages in various ways. RvD2's differential actions depend on the context by which LPS is administered to the cells and if the cells were administered LPS or whole bacteria. In an acute LPS-mediated inflammatory setting, RvD2 reduced NF-κB activity and TNF-α production, while increasing bacterial clearance. In an immunosuppressed setting, RvD2 preserved/enhanced macrophage functionality via increased NF-κB activation, TNF-α production, and bacterial clearance. These actions of RvD2 were abolished by TLR2 inhibition (See **Figure 35**). Interestingly, the attenuation of macrophage suppression was absent if the RvD2 was given to monocytes before differentiation to macrophages, suggesting that these effects of RvD2 on the exhausted macrophages is dependent on whether the RvD2 was given before or after differentiation. To the best of our knowledge, there are no therapies which can directly attenuate macrophage immunosuppression. We believe that further studies into this action of RvD2 is warranted and is important in order to develop new therapies for sepsis.

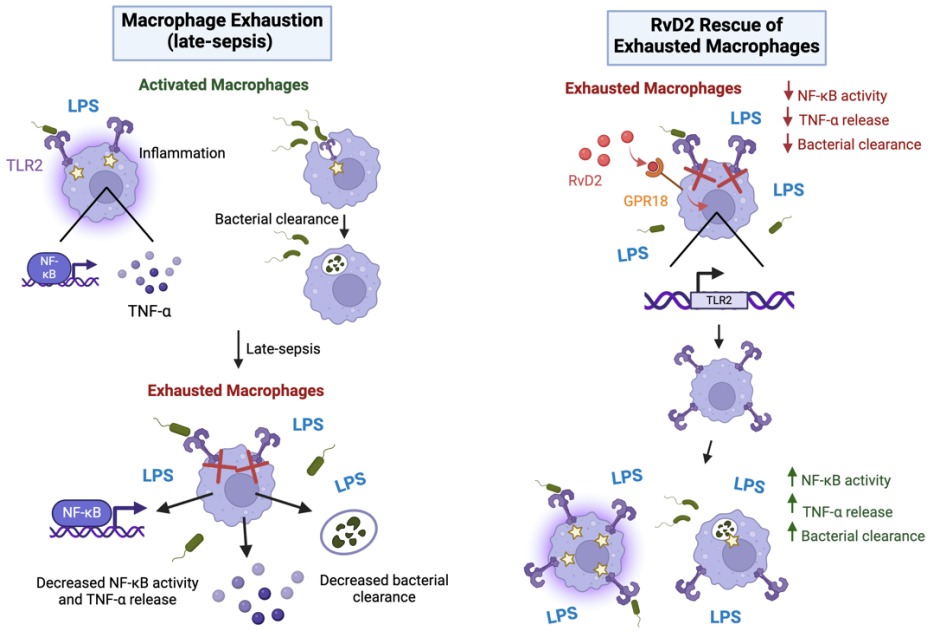

Figure 35. RvD2 Attenuates LPS-Induced Macrophage Exhaustion. This schematic summarizes the main findings from this thesis work. The late phase of sepsis is characterized by immunosuppression, which can be recapitulated in vitro with low-dose, repetitive LPS hits, and in vivo with CLP (for at least 72h). One hallmark of this sepsis-induced immunosuppression is monocyte/macrophage exhaustion. The SPM RvD2 can attenuate exhaustion by increasing macrophage NF-κB activity, TNF-α production, and bacterial clearance in a TLR2-dependent manner.

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Appendix A

List of Abbreviations

AA: arachidonic acid

AML: acute myeloid leukemia

ANOVA: analysis of variance

APC(s): antigen presenting cell(s)

BSA: bovine serum albumin

CFU(s): colony forming units

CLP: cecal-ligation and puncture

CRS: cytokine release syndrome

CTLA-4: cytotoxic T-lymphocyte-associated protein 4

CXCL2: chemokine (C-X-C motif) ligand 2

DAMPs: damage-associated molecular patterns

DHA: docosahexaenoic acid

ELISA: enzyme-linked immunosorbent assay

EPA: eicosapentaenoic acid

ET: endotoxin tolerance

FBS: fetal bovine serum

G-CSF: granulocyte colony-stimulating factor

GC: glucocorticoids

GM-CSF: granulocyte macrophage colony-stimulating factor

HLA(s): human leukocyte antigen(s)

HMGB1: high mobility group box 1

IC: immune complexes

IFN-γ: interferon-gamma

IKK: IκB kinase

IL-4: interleukin 4

IL-6: interleukin 6

IL-7: interleukin 7

IL-8: interleukin 8

IL-10: interleukin 10

IL-13: interleukin 13

ILC(s): innate lymphoid cell(s)

iPRRs: inhibitory pattern recognition receptors

IV: intravenous

LIF: leukemia inhibitory factor

LPS: lipopolysaccharide

LTx: lethal toxin

LxA_4: Lipoxin A_4

M-MDSC(s): monocytic myeloid-derived suppressor cell(s)

MAPK: Mitogen-Activated Protein Kinase

MDSC(s): myeloid-derived suppressor cell(s)

MHC: major histocompatibility complex

MHC-II: major histocompatibility complex class II

MOI(s): Multiplicity of Infection(s)

MYD88: myeloid differentiation primary response protein 88

NF-κB: nuclear factor kappa light chain enhancer of activated B cells

PA (15): *Pseudomonas aeruginosa* at MOI 15

PA (5): *Pseudomonas aeruginosa* at MOI 5

PAMP(s): Pathogen-associated molecular pattern(s)

PBS: phosphate-buffered saline

PD-1: programmed cell death protein 1

PD-L1: programmed death ligand 1

PI3K/Akt: phosphoinositide-3-kinase/Ak strain transforming

PMA: phorbol-12-myristate-13-acetate

PMN-MDSC(s): polymorphic myeloid-derived suppressor cell(s)

PRR(s): pattern recognition receptor(s)

PTX3: pentraxin 3

PUFA(s): polyunsaturated fatty acid(s)

ROS: reactive oxygen species

RvD1: Resolvin D1

RvD2: Resolvin D2

s.c.: subcutaneously

SIGIRR: single Ig IL-1R-related

SOFA: sequential organ failure assessment

SPM(s): Specialized Pro-resolving Mediator(s)

TCR: T cell receptor

TGF-β: transforming growth factor beta

TIR: Toll-IL-1 receptor

TLR(s): toll-like receptor(s)

TLR2: toll-like receptor 2

TLR4: toll-like receptor 4

TNF-α: tumor necrosis factor alpha

Tregs: Regulatory T cells

TRIF: TIR domain-containing adaptor inducing IFN-β

TSA: tryptic soy agar

TUNEL: transferase-mediated dUTP nick-end labeling

Appendix B

Attributes

Chapter 1: Introduction

Figure 1. Made with BioRender.com by CMP.

Figure 2. Made with BioRender.com by CMP.

Table 1. Made with BioRender.com by CMP.

Figure 3. Made with BioRender.com by CMP.

Figure 4. Made with BioRender.com by CMP.

Figure 5. Made with BioRender.com by CMP.

Figure 6. Made with BioRender.com by CMP.

Figure 7. Adapted from (Sandhaus and Swick, 2021). Made with BioRender.com by CMP.

Figure 8. Made with BioRender.com by CMP.

Figure 9. Made with BioRender.com by CMP.

Figure 10. Made with BioRender.com by CMP.

Figure 11. Made with BioRender.com by CMP.

Table 2. Made with BioRender.com by CMP.

Chapter 2: Resolvin D2 (RvD2) Influences Transcription Factor NF-κB Activity and Monocyte/Macrophage Functions After Lipopolysaccharide (LPS) Stimulation

Figure 12. Data collection, analysis, and figure by CMP and LDA.

Figure 13. Made with BioRender.com by CMP.

Figure 14. Data collection, analysis, and figure by CMP.

Figure 15. Data collection, analysis, and figure by CMP.

Figure 16. Data collection, analysis, and figure by CMP.

Figure 17. Data collection, analysis, and figure by CMP.

Figure 18. Data collection, analysis, and figure by CMP.

Figure 19. Data collection, analysis, and figure by CMP.

Chapter 3: Resolvin D2 (RvD2) Effects on Monocyte/Macrophage Functions After Bacteria Stimulation Are Not Dependent on NF-κB Activity

Figure 20. Made with BioRender.com by CMP.

Figure 21. Data collection, analysis, and figure by CMP.

Figure 22. Data collection, analysis, and figure by CMP.

Figure 23. Data collection, analysis, and figure by CMP.

Figure 24. Data collection, analysis, and figure by CMP.

Figure 25. Data collection, analysis, and figure by CMP.

Figure 26. Data collection, analysis, and figure by CMP.

Chapter 4: Resolvin D2 (RvD2) Acts Through TLR-Mediated Signaling that Leads to Monocyte/Macrophage Reprogramming

Figure 27. Made with BioRender.com by CMP.

Figure 28. Data collection, analysis, and figure by CMP.

Figure 29. Data collection, analysis, and figure by CMP.

Figure 30. Data collection, analysis, and figure by CMP.

Chapter 5: Resolvin D2 (RvD2) Attenuates Sepsis-Mediated Macrophage Exhaustion in vivo

Figure 31. Made with BioRender.com by CMP.

Figure 32. Data collection by CMP, KY, and JMW; analysis and figure by CMP.

Figure 33. Data collection by CMP, KY, and JMW; analysis and figure by CMP.

Figure 34. Data collection, analysis, and figure by CMP and LDA.

Figure 35. Made with BioRender.com by CMP.

Key:

CMP: Cristina M. Padovani

LDA: Likhitha Dandu

KY: Kingsley Yin

JMW: Jean M. Walker

Appendix C

List of Publications

1. **Padovani, C.M.**, Wilson, R.M., Rodriguez, A., Spur, B.W., Yin, K., 2024. Resolvin D2 attenuates LPS -induced macrophage exhaustion. The FASEB Journal. 38:e23569. https://doi.org/10.1096/fj.202302521R

2. **Padovani C.M.**, Yin K., 2024. Immunosuppression in Sepsis: Biomarkers and Specialized Pro-Resolving Mediators. Biomedicines. 12(1):175. https://doi.org/10.3390/biomedicines12010175

3. Thornton, J.M., **Padovani, C.M.**, Rodriguez, A, Spur, B.W., Yin, K., 2023. Lipoxin A4 promotes antibiotic and monocyte bacterial killing in established *Pseudomonas aeruginosa* biofilm formed under hydrodynamic conditions. The FASEB Journal. 37:e23098. https://doi:10.1096/fj.202300619R