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Immunomodulatory Effects of Resolvin D2 in a Model of Infection

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IMMUNOMODULATORY EFFECTS OF RESOLVIN D2 IN A MODEL OF INFECTION

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ABSTRACT

Dysregulated hyperinflammatory host immune response to underlying bacterial infections is a characteristic of sepsis. In sepsis, bacteria often trigger abnormal hyperinflammatory responses which can cause multiple organ failure and if sustained can lead to an immunosuppressive phase where the host is susceptible to secondary infections caused by opportunistic bacteria like *Pseudomonas aeruginosa* (*P. aeruginosa*). In our studies, we used a 2-hit model of cecal ligation and puncture (CLP) followed by *P. aeruginosa* secondary lung infection to investigate cellular and molecular mechanisms in the beneficial action of resolvin D2 (RvD2). Resolvins of the D-series are a group of fatty acids known as Specialized Pro-resolving Mediators (SPMs), synthesized endogenously from docosahexaenoic acid (DHA) by different immune cells including neutrophils and macrophages during infection and inflammation. In CLP sepsis, we observed that production of RvD2 in spleens had returned to basal levels by 48 hours after CLP surgery. Administering RvD2 (100 ng/mouse, i.v.) in CLP mice at this time point where endogenous RvD2 synthesis is low enabled CLP mice to clear blood bacteria more efficiently at 24 hours after administration compared to saline controls. There was no significant difference in plasma cytokine production. To further understand cellular and molecular mechanisms, we used flow cytometry to identify different immune cells in spleen and found that RvD2 treatment increased splenic neutrophils ($Ly6G^+$) and myeloid-derived suppressor cells (MDSCs: $CD11b^+$ $Ly6G^+$ $Ly6C^+$) compared to vehicle treated mice. RvD2 treatment increased oxidative burst in splenic neutrophils but not

in splenic MDSCs compared to vehicle treated mice. These results provide evidence that RvD2 can expand splenic neutrophil and MDSC numbers to effect greater blood bacterial clearance even when given 48 hours after primary infection. When mice were challenged with *P. aeruginosa* (intranasally; 24 h after RvD2 treatment) we found that RvD2 increased bacterial clearance in lungs, increased alveolar macrophage numbers and reduced lung IL-23. These results suggest that late RvD2 administration boosted host defense to reduce infection and inflammation. This study provides insight into immunomodulatory effects of RvD2 in a 2-hit infection model of sepsis (Sundarasivarao et al., 2022).

INTRODUCTION

Immune system is one of the most complex systems whose primary goal is to protect our body from invading pathogens like bacteria, viruses, fungi, and parasites (Bennett et al., 2018). Our immune system can be divided into innate and adaptive immune systems based on their function. Innate immune system is considered as a rudimentary first line of defense from pathogens. However, the adaptive immune system is highly evolved since it can learn and create memory of the invading pathogen and generate an immune response specific to the pathogen. Innate immune system is made up of neutrophils, monocytes, macrophages, dendritic cells (DCs), and natural killer cells (NK). The adaptive immune system consists of T- and B-lymphocytes. Bone marrow and thymus are primary lymphoid organs. Bone marrow is the site of hematopoiesis where hematopoietic stem cells differentiate into different types of leukocytes. Spleen and lymph nodes are considered as secondary lymph organs where immune cells interact with each other, and other microorganisms (Bennett et al., 2018). Abnormal immune response to infection may lead to life-threatening conditions like sepsis. Throughout this section we will discuss more on how immune response is altered in sepsis while understanding basic functioning of our immune system.

1. Sepsis:

Sepsis is a life-threatening multiple organ dysfunction due to abnormal immune response to infection (Verdonk et al., 2017). According to centers for disease control and prevention (CDC) in US annually more than 1.7 million adults develop sepsis and

around 270000 die due to sepsis. Bacterial infections and viral infections including COVID-19 can cause sepsis. Sepsis can be divided into two distinct phases hyperinflammation and immunosuppression based on immune response (Figure 1).

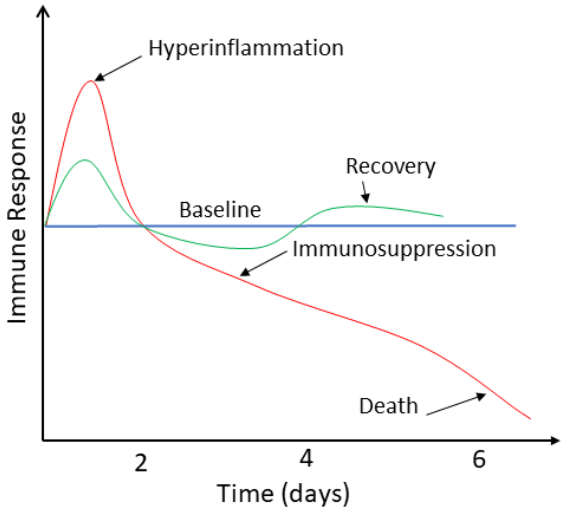

Figure 1: Different phases in sepsis based on immune response. Image adapted from (Das, 2014)

2. Hyperinflammation:

Hyperinflammation is a type of immune response marked by excessive inflammation due to infection, trauma (physical and chemical), ischemia, and by abnormal recognition of harmless self-antigens. Five pillars of inflammation are *calor* (fever), *rubor* (redness), *tumor* (swelling and oedema), *dolor* (pain), and *functio laesa* (loss of function) (Basil and Levy, 2015). Mostly, inflammation inhibits proliferation and propagation of different pathogens, and this immune response prevents further infection by other pathogens. However, bacteria, viruses, and other pathogens developed mechanisms to evade inflammatory response. Persistent inflammation or hyperinflammation can be damaging to host. Hence, our immune system developed an inflammation resolution program to resolve inflammation and restore tissue

homogeneity. During resolution of inflammation, specialized pro-resolving mediators (SPMs) are secreted. SPMs derived from polyunsaturated fatty acids (PUFA) and consists of resolvins, lipoxins, maresins, and protectins. Most of these SPMs function as immunoresolvents. However, they reduce inflammation without compromising host immune defense (Serhan and Levy, 2018). Various mechanisms by which SPMs reduce inflammation without compromising host defense remains unclear.

3. Immunosuppression:

Immunosuppression is a phase during which pro-inflammatory cytokines are downregulated and anti-inflammatory cytokines are upregulated (Das, 2014). This switch prevents activation of adaptive immune system and affects various anti-microbial functions of innate immune system as well. Recent research suggests that hyperinflammation precedes immunosuppressive phase in sepsis (Ono et al., 2018). Immunosuppressive phase in sepsis is characterized by apoptosis of various immune cells including NK cells, CD4+ T-cells, CD8+ T-cells, and B-cells. HLA-DR expression was also down-regulated resulting in significant decrease in antigen-presentation by cells (Winkler et al., 2017). Interestingly, during this immunosuppressive phase, specialized pro-resolving mediators were synthesized and secreted to mediate inflammation resolution. Resolvin D2 is a type of SPM synthesized at the site of infection by immune cells to resolve inflammation(Spite et al., 2009). Interestingly, resolvin D2 also improves host defense, bacterial clearance, and improves survival of septic mice model(Serhan and Levy, 2018). Underlying

mechanisms by which resolvin D2 improves host defense remains unclear and some of the mechanisms are explained in this dissertation.

4. Resolvins:

Resolvins are derived from eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Basil and Levy, 2015). E-series resolvins are RvE1and RvE2. D-series resolvins include RvD1, RvD2, RvD3, RvD4, RvD5, and RvD6 (Serhan and Petasis, 2011). In this dissertation we would be focusing on biological functions of resolvin D2 (RvD2). Resolvin D2 is derived from DHA by sequential lipoxygenation and epoxide hydrolysis. First DHA is converted into 17S-HpDHA (17S-hydroperoxy-4Z,7Z,10Z,13Z,15E,19Z-docosahexaenoic acid) by enzyme 15-Lipoxygenase (15-LOX) and then 17S-HpDHA is converted by leukocytes into an 7S,8S-epoxide-containing intermediate by 5-LOX (Figure 2). Finally, these intermediates transformed into various resolvins including resolvin D2(Spite et al., 2009). These enzymatic lipoxygenation reactions can occur within immune cells and also by transcellular biosynthesis (Serhan and Petasis, 2011). Eosinophils were found to be rich in 15-LOX and these cells can readily convert DHA into 17S-HpDHA which can further be converted into resolvin D2 by phagocytic polymorphic neutrophils (PMNs) (Spite et al., 2009). In a murine model of zymogen induced- peritonitis (Spite et al., 2009) showed that resolvin D2 reduced neutrophil infiltration into peritoneum by 70%, decreased Platelet-activating factor (PAF) mediated leukocyte adherence and leucocyte emigration, resolvin D2 reduced bacteria levels, reduced inflammatory cytokines, and improved survival of mice. Researchers found that resolvin D2 binds

to G-protein coupled receptor (GPR18) to exert various functions in cells (Chiang et al., 2017, Chiang et al., 2015). GPR18 receptor is expressed on different cells

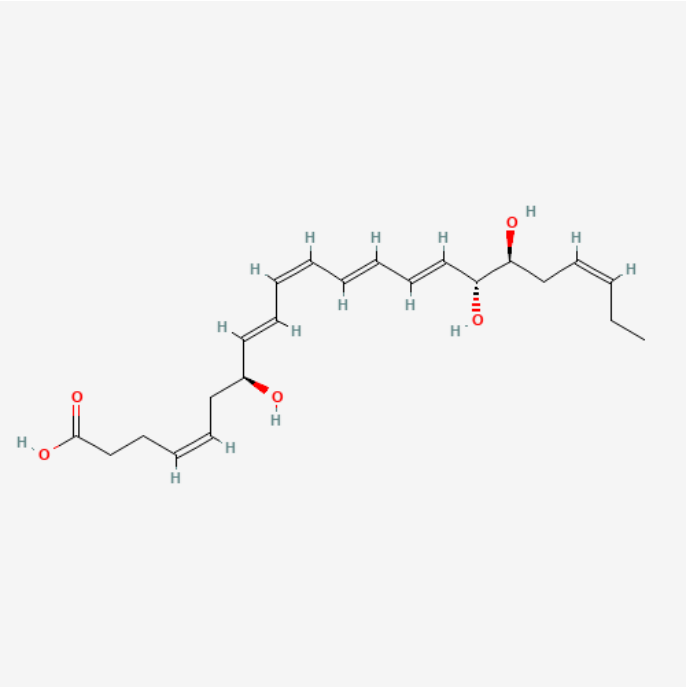

Figure 2: Structure of resolvin D2. Image Source: PubChem ID 11383310

including human PMNs, monocytes, and macrophages. However, downstream signaling after binding of resolvin D2 to GPR18 remains unclear. Other agonists that bind to GPR18 receptor include N-arachidonoyl glycine (NAGly), and Δ^9-tertrahydrocannabinol (THC). Interestingly, expression of GPR18 on human PMNs isolated from blood of sepsis patients was found to correlate with disease severity and outcome of sepsis (Zhang et al., 2019). Patients with sepsis had a smaller fraction of PMNs with GPR18 compared to healthy controls. Also, GPR18 expression was found to be mediated by TLR4 signaling among these subjects. Activation of TLR4 with LPS reduced GPR18 expression on PMNs from sepsis patients.

5. Neutrophils:

Polymorphic neutrophils (PMNs) represent 70% of all leukocytes and are important effector cells of innate immune system. Neutrophils are produced from hematopoietic stem cells (HSCs) in bone marrow. HSCs differentiate into multipotent progenitor cells (MPP) which further differentiate into granulocyte-monocyte progenitors (GMP). GMP are then primed to produce myeloblasts by granulocyte colony stimulating factor (G-CSF). Myeloblasts sequentially undergo stages of maturation into promyelocyte, myelocyte, metamyelocyte, band cell, and terminally into mature neutrophils (Von Vietinghoff and Ley, 2008). Osteoblasts and bone marrow stromal cells express CXCL12 a ligand that binds to CXCR4 receptor on neutrophils to prevent emigration of neutrophils into blood and other tissues. However, during infection, upregulation of G-CSF disrupts interaction between CXCL12 and CXCR4 resulting in migration of neutrophils out of bone marrow into blood (Summers et al., 2010, Rosales, 2018). Neutrophils in blood are mobilized to sites of infection and inflammation to perform various anti-microbial functions including phagocytosis, degranulation, release of neutrophil extracellular traps (NETs), oxidative burst, production of inflammatory cytokines, (Rosales, 2018) and production of inflammation resolving molecules (Serhan and Petasis, 2011). Neutrophils in blood are recruited to sites of infection and inflammation by a series of processes called Leukocyte adhesion cascade (Petri et al., 2008). During recruitment, endothelial cells express ligands such as P-selectin, E-selectin, VCAM-1, and ICAM-1. These ligands

bind to receptors expressed on neutrophils such as L-selectin, α4β1 integrin, CD11b integrin respectively. Specifically, CD11b firmly binds to intercellular adhesion molecule (ICAM-1) expressed on endothelial cells and emigrates to sites of infection and inflammation (Hajishengallis and Chavakis, 2013). Interestingly, CD11b expression in granulocytes was elevated in infants with infection and suggested to be a better diagnostic marker than C-reactive protein (CRP) in infants (Weirich et al., 1996, Qiu et al., 2019). After emigration of neutrophils from blood vessels, they move towards site of infection based on gradients of chemoattractants such as bacterial peptide *formyl-methionyl-leucyl-phenylalanine* (fMLP), and Complement C5a (Kolaczkowska and Kubes, 2013). Formyl peptide receptors such as FPR-1 that are highly expressed on neutrophils recognize fMLP and generate reactive oxygen species (ROS) in a NADPH oxidase (NOX)2-dependent manner (Jeong and Bae, 2020)

6. Myeloid-derived suppressor cells:

Myeloid-derived suppressor cells (MDSCs) are heterogenous population of immature myeloid cells that expand during infection and inflammation. Hematopoietic stem cells (HSC) in bone marrow differentiate into common myeloid progenitor cells and these cells differentiate into immature myeloid cells. These immature myeloid cells were found to suppress T-cell activation and function and hence they were called myeloid-derived suppressor cells (Schrijver et al., 2019). MDSCs in mice are divided into two major subsets depending on expression of CD11b, Ly6G, Ly6C. Granulocytic MDSCs (G-MDSCs) are identified as $CD11b^{+}$ $Ly6C^{low}$ $Ly6G^{+}$ and

Monocytic MDSCs are identified as $CD11b^{+}$ $Ly6C^{hi}$ $Ly6G^{-}$ (Bergenfelz and Leandersson, 2020). In humans Ly6G antigen is not present (Rosales, 2018) and hence MDSCs are identified by six different set of markers including CD11b, CD14, CD15, CD33, CD66b, and HLA-DR. Current literature defines human granulocytic MDSCs as $CD11b^{+}$ $CD14^{-}$ $CD15^{+}$ $CD33^{+}$ $CD66b^{+}$ $HLA\text{-}DR^{-}$, and human monocytic MDSCs as $CD11b^{+}$ $CD14^{+}$ $CD15^{-}$ $CD33^{+}$ $CD66b^{+}$ $HLA\text{-}DR^{-/low}$ (Damuzzo et al., 2015, Brandau et al., 2013, Favaloro et al., 2014, Keskinov and Shurin, 2015). Based on current knowledge, it is not clear if G-MDSCs and M-MDSCs are progenitors of mature neutrophils and monocytes respectively or a sub-population of mature neutrophils and monocytes and continued efforts to identify these MDSCs using flow cytometry are being undertaken to identify these cells more precisely (Solito et al., 2017, Mandruzzato et al., 2016).

MDSCs mediate immunosuppression through ROS/RNS production, depletion of L-arginine, secretion of anti-inflammatory cytokines such as IL-10 and TGF-β, proliferation of T-regulatory cells (T-regs) (Schrijver et al., 2019). However, although the release of ROS can be immunosuppressive, ROS released during early sepsis was found to be effective in phagocytosing and killing pathogenic *E.coli* and *Streptococci* during late sepsis (Derive et al., 2012, Leiber et al., 2017). MDSCs also limit availability of L-arginine to T-cells by producing arginase that metabolizes arginine into ornithine (Darcy et al., 2014, Bronte and Zanovello, 2005, Wijnands et al., 2015). In sepsis, *de novo* synthesis of arginine was impaired (Luiking et al., 2008), also arginine consumption by cells including MDSCs was increased and these mechanisms suppressed T-cell function and caused immunosuppression in sepsis

patients (Mathias et al., 2017). MDSCs were found to accumulate in distal organs during infection and in cancer (Schrijver et al., 2019). However, overall effect of increased MDSCs in distal organs such as spleen during infection is not clear.

7. Alveolar Macrophages:

Alveolar macrophages (AM) reside in airway lumen of lungs and are distinct from tissue resident macrophages that reside between the airway epithelium (Hussell and Bell, 2014a). Alveolar macrophages have ability to self-renew and repopulate airway lumen after depletion due to infection with influenza (Hashimoto et al., 2013, Ghoneim et al., 2013). In mice, alveolar macrophages can be distinguished from interstitial macrophages easily by markers such as CD11b, CD11c, and SiglecF. Alveolar macrophages express high amounts of CD11c and SiglecF but do not express CD11b. On the contrary interstitial macrophages express CD11b moderately but do not express both CD11c and SiglecF (Zaynagetdinov et al., 2013, Misharin et al., 2013). Depending on the type of signals alveolar macrophages receive in airway environment, alveolar macrophages are pro-inflammatory or anti-inflammatory in function. IL-1β, TNF-α, IFN-γ, TLR-2/TLR-4 ligands activate pro-inflammatory phenotype in alveolar macrophages. On the contrary, IL-10 and TGF-β prime alveolar macrophage into anti-inflammatory and prevents lung inflammation and both IL-10 and TGF-β are important components of healthy lung microenvironment (Morris et al., 2003). Alveolar macrophages caused inflammation in response to viral infections (Högner et al., 2013, Herold et al., 2008, Bem et al., 2010) and also limited excessive

inflammation in response to viral infections (Kim et al., 2008, Tate et al., 2010, Tumpey et al., 2005).

8. Cytokines

Cytokines are soluble proteins secreted by various immune cells. Based on their function, cytokines are broadly divided into pro-inflammatory and anti-inflammatory cytokines. Cytokines such as TNF-α, IL-6, IL-1β, IFN-γ, IFN-β, IL-17A, IL-23, IL-12, and MCP-1 are pro-inflammatory in function. Cytokines such as IL-10, and TGF-β are anti-inflammatory in function. Refer to Table 1 for detailed information on above mentioned cytokines in sepsis and other diseases adapted from (Liu et al., 2021).

Cytokine	**Source**	**Functions**
TNFα	Macrophages, Mast cells, NK cells, T- and B-Lymphocytes	• Activates neutrophils. • Increases vascular permeability. • Stimulates adhesion molecules.
IL-6	B- and T-lymphocytes, monocytes, fibroblasts, and endothelial cells	• Activates cellular and humoral responses. • Inhibits TNF and IL-1 production by macrophages.
IL-1β	Monocytes, Macrophages	• Mediates systemic effects of IL-1. • Affects IL-6 induced gene expression.

IFN-γ	Macrophages, Th1 cells, Tc cells, B cells, NK cells	• Promotes Th1 immune response. • Promotes secretion of Th1 associated cytokines.
IFN-β	Resolution phase macrophages	• Improves bacterial clearance. (Kumaran Satyanarayanan et al., 2019) • Promotes PMN apoptosis and efferocytosis. (Kumaran Satyanarayanan et al., 2019)
IL-17A	Th17 Cells	• Promotes lung inflammation during infection and injury. (Lei et al., 2016, Senoo et al., 2021, Ivanov et al., 2007, Morrow et al., 2019)
IL-23	$CD11c^{+}$ Alveolar Macrophages	• Promotes Th17 cell response. (Bosmann et al., 2013, Senoo et al., 2021, Ivanov et al., 2007, Morrow et al., 2019)
IL-12	Monocytes, Alveolar Macrophages, dendritic cells, B-cells	• Promotes neutrophil activation, phagocytosis, anti-microbial activities of neutrophils.(Gee et al., 2009)

		• Induces IFN-γ response. (Moreno et al., 2006)
MCP-1	Monocytes	• Potent chemoattractant of neutrophils and macrophages.(Wang et al., 2018) • Promotes inflammation. (Ramnath et al., 2008)
IL-10	Monocytes/macrophages, Th2 cells, B cells	• Inhibits cytokine production by macrophages and neutrophils. • Inhibits Th1 response.
TGF-β	Alveolar macrophages, broncho-epithelial cells, Eosinophils	• Regulates activity of alveolar macrophages (Hussell and Bell, 2014a)

9. Toll-like receptors:

Toll-like receptors (TLRs) are members of pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (Takeuchi and Akira, 2010). TLRs are expressed by immune cells and epithelial cells. PAMPs such as lipopolysaccharides (LPS), and Lipoproteins on the bacterial cell wall are recognized by TLR-4 and TLR-2 receptors. Recognition of these PAMPs activates MyD88-dependent signaling pathway in cells

finally leading to translocation of NF-κB into nucleus to transcribe various pro-inflammatory cytokine genes discussed earlier. Interestingly, in sepsis and murine sepsis models, activation, and expression of TLRs play important roles in inducing production of pro-inflammatory cytokines. For example, TLR-2 and TLR-4 are highly expressed in lungs of septic mice suggesting that excessive expression of TLRs may promote acute respiratory distress. (Bakopoulos et al., 2017, Kumar, 2020). Interestingly, *in vitro* studies on THP-1 monocytes showed that resolvin D2 decreased expression of TLR4 receptor post activation with LPS. Resolvin D2 reduced production of pro-inflammatory cytokines in LPS activated monocytes by induction of microRNA (miR-146a) (Croasdell et al., 2016a).

10. *Pseudomonas aeruginosa* virulence genes:

Pseudomonas aeruginosa is an opportunistic bacterium that causes pneumonia and ear infections in immunosuppressed and immunocompromised patients. During infection, a bacterial cell-density dependent chemical signaling called quorum sensing is initiated by bacteria (Wu et al., 2016b). Quorum sensing (QS) is a cell-cell communication through which bacteria get information about their own cell-density and adjust virulence gene expression to maximize effect on host (Rutherford and Bassler, 2012). Quorum sensing controls many functions including biofilm formation and virulence factor secretion (Williams and Cámara, 2009). Biofilm produced by *Pseudomonas aeruginosa* protects bacteria from various antibiotics (Thornton et al., 2021). Also recently, various virulence genes have been identified to contribute to this biofilm formation including *pqsA, rhlA, and mucA*. *pqsA* gene produces 2,4-

dihydroxyquinoline (DHQ) and 4-hydroxy-2-alkylquinoline (HAQs) which acts as a communication signal between cells (Lépine et al., 2007). *pqsA* gene also plays an important role in biofilm formation (Kang et al., 2017). Similarly, *rhlA* gene is for biosynthesis of Rhamnolipids by *Pseudomonas aeruginosa* (Zhu and Rock, 2008) and to produce surfactant (3-(3-hydroxyalkanoyloxy) alkanoic acid (HAA) that increase motility of *Pseudomonas* by providing suitable hydrophobic environment to swim (Déziel et al., 2003). *mucA* protein acts as an anti-sigma factor preventing sigma factor AlgU from transcribing genes. Downregulating *mucA* gene, allows free AlgU to bind to alginate genes and produce exopolysaccharide alginate (Li et al., 2019). Excessive alginate production is a key pathogenic factor increasing resistance of *Pseudomonas aeruginosa* to immune cells and antibiotic therapy especially in cystic fibrosis patients (May et al., 1991).

In this project we investigated various functions of resolvin D2 in an infection model in which mice undergoes peritonitis followed by secondary infection with *Pseudomonas aeruginosa* and investigated effects of resolvin D2 on various immune cells (neutrophils, myeloid-derived suppressor cells, alveolar macrophages) and on *Pseudomonas aeruginosa*. Although, downstream signaling after resolvin D2 binds to its receptor (GPR18) remains unclear, we analyzed cellular level changes of resolvin D2 mediated improvement of bacterial clearance and survival after secondary lung infection with *Pseudomonas aeruginosa.*

RATIONALE:

We observed that endogenous resolvin D2 levels returned to baseline 48 hours after cecal ligation and puncture surgery in our mice model of infectious peritonitis and secondary lung infection (Figure 3). Hence, we wanted to investigate if external administration of resolvin D2 can improve survival of mice after secondary infection with *Pseudomonas aeruginosa* and study cellular and molecular mechanisms underlying this reduction in mortality of our infection model.

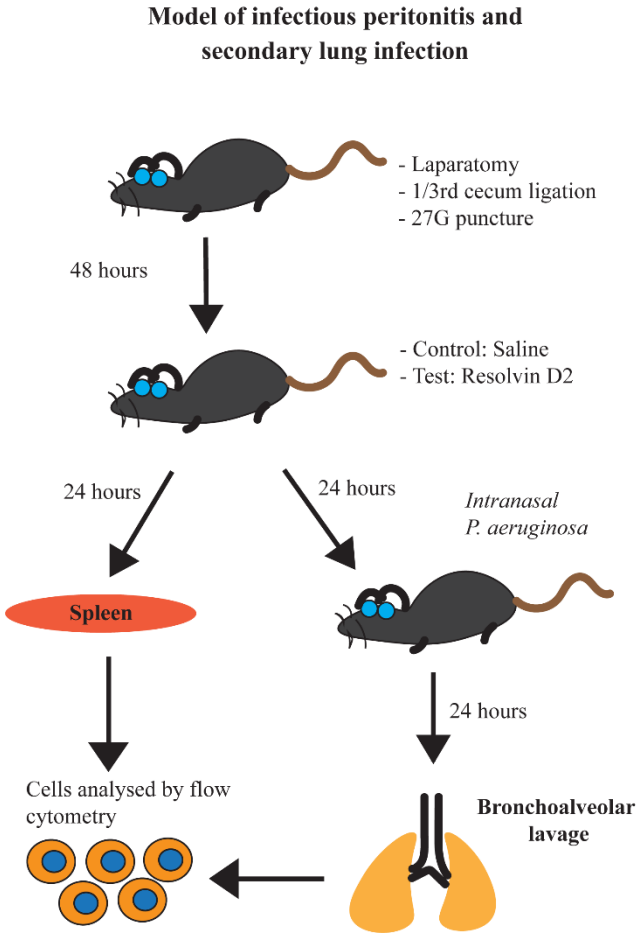

Figure 3: Model of infectious peritonitis and secondary lung infection: Schematic representation of our infection model. Image source - (Sundarasivarao et al., 2022)

MATERIALS AND METHODS:

1. Cecal-ligation and puncture + *Pseudomonas aeruginosa*:

CLP surgery was performed on male C57BL/6 mice (11-13 weeks old) using modified methods as previously published (Walker et al., 2022, Sundarasivarao et al., 2022). On the day of surgery, mice were anesthetized with isoflurane (+ O_2). A 1 cm-long midline incision was made in the abdomen to expose the cecum. The distal third of the cecum was ligated with 4.0 surgical silk. Using a 27-gauge needle, the cecum was punctured twice, through and through. A small drop of feces was extruded through the holes to ensure patency of the punctures. The cecum was placed back into the abdomen, which was then closed in 2 layers. For sham controls, ceca were removed but not ligated, nor punctured. Saline (8 mL/100 g; s.c.) was injected to replace any fluid loss during surgery. Buprenorphine-SR (1 mg/kg, s.c.) was injected. Mice were monitored every 12 hours. 48 h after surgery, CLP mice were anesthetized and injected with either RvD2 (100 ng/mouse) or saline vehicle via the tail vein. 24h later, mice were anesthetized with ketamine/xylazine (100/10 mg/kg, i.p.). Blood was obtained by intracardiac puncture into 1 ml syringes containing 0.05 M EDTA. The blood was centrifuged at 500 X g for 12 min. Plasma was stored at -75°C until analysis. Spleens were taken, cells were isolated and then analyzed by flow cytometry (Figure 3). To introduce mice with secondary infection with *Pseudomonas aeruginosa*, mice were anesthetized with isoflurane and *P. aeruginosa* (approx. 10^7 cells) (ATCC 27853) or saline vehicle was inoculated intranasally. Mice continued to be monitored every 8h. At 24h, post inoculation, mice were sacrificed. Tracheas

were cannulated using 23G tubing adaptors. Lungs were then lavaged with 1 ml PBS containing sodium citrate (0.38% final concentration).

2. Isolation and counting of total leukocytes:

Spleen: Whole spleen was passed through 70µm cell strainer into a petri dish containing 10mL of phosphate buffered saline (PBS). Then samples were transferred into a 50mL tube and centrifuged at 2000 RPM for 5min at 4°C. After centrifugation, supernatant was discarded, and pellet was resuspended in 5mL of ACK lysis buffer and incubated on ice for 5min to gently lyse red blood cells in all samples. After incubation, 30mL of ice-cold PBS was added to all tubes and centrifuged at 2000 RPM for 5min at 4°C. After centrifugation, supernatant was discarded, and cells were resuspended in 2mL of cell staining buffer and samples were stored in ice until further processing.

Bone Marrow: Mouse femur bones were cut and transferred into a petri dish containing 10mL Phosphate-buffered saline (PBS) containing 0.4% sodium citrate. Using 10mL syringe attached with 27-gauge needle femur bones were flushed with Hanks' balanced salt solution (HBSS) to extract cells from femur bone. Extracted cells were centrifuged and resuspended in 5mL of ACK lysis buffer for 5min on ice to perform red cell lysis. After lysis, cells were washed and resuspended in 2mL of HBSS for immunostaining.

Cell Counting: To count total number of cells using automated cell counter (COUNTESS II), 10µL of trypan blue stain was mixed well with 10 µL of cells using pipette and then 10µL was loaded onto cell counting cassette for counting. Gates were adjusted to exclude counting cells smaller than 5 µm (to exclude red blood cells)

and cells larger than 35 µm (to exclude cell aggregates). Shape gates were set to ellipse to include cells of different shapes (to include irregular shaped macrophages).

3. Immunostaining:

Approximately 1 million cells were aliquoted in separate tubes and Fc receptors are blocked using 1 µl of TruStain FcX (anti-mouse 16/32) antibody (Biolegend Cat# 101320) for 1 hour on ice. Then cells are washed with cell staining buffer and 1 µl of different antibodies including anti-CD11b (Biolegend Cat# 101207), anti-Ly6G (Biolegend Cat# 127610), and anti-Ly6C (Biolegend Cat# 128022) were added to tubes along with 1 µl of live/dead cell stain (Invitrogen Cat# L10119) and incubated for 30min on ice. After incubation cells were washed with cell staining buffer and resuspended in the same buffer. For flow cytometry compensation of multiple fluorophores, ABC anti-rat capture beads (Invitrogen Cat# A10389) and ArC amine reactive capture beads (Invitrogen Cat# A10628) were used for accurate separation of overlapping emission wavelengths of multiple fluorophores used in identification and characterization of cells in lungs.

4. Flow Cytometry:

Approximately, 100000 – 250000 cells were analyzed by flow cytometry (Model: Attune acoustic focusing cytometer by applied biosystems). Compensation beads were used to adjust PMT voltages and compensation set for multiple fluorophores. Cells were gated based on size (forward scatter) and granularity (side scatter), single population, live cells, and expression of receptors for CD11b, Ly6G, and Ly6C.

FlowJo software (version 10) was used to analyze flow cytometry data and GraphPad Prism software (version 9) was used to perform statistical analysis.

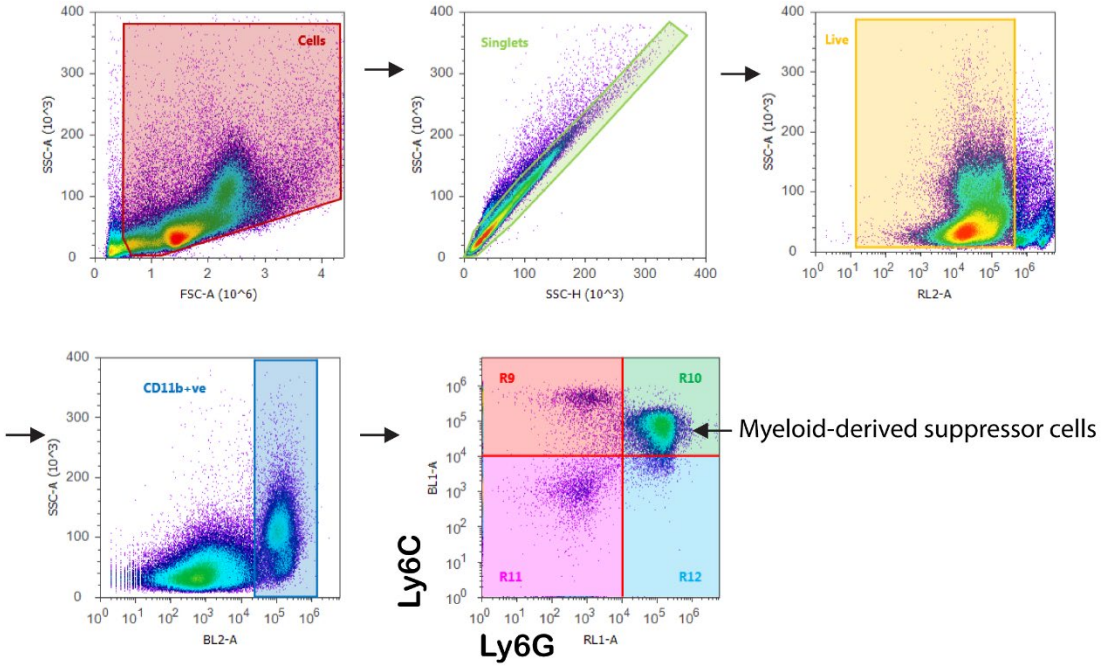

Figure 4: Cell density plots showing gating strategy for identifying myeloid-derived suppressor cells (MDSCs: $CD11b^+$ $Ly6G^+$ $Ly6C^+$) in spleen.

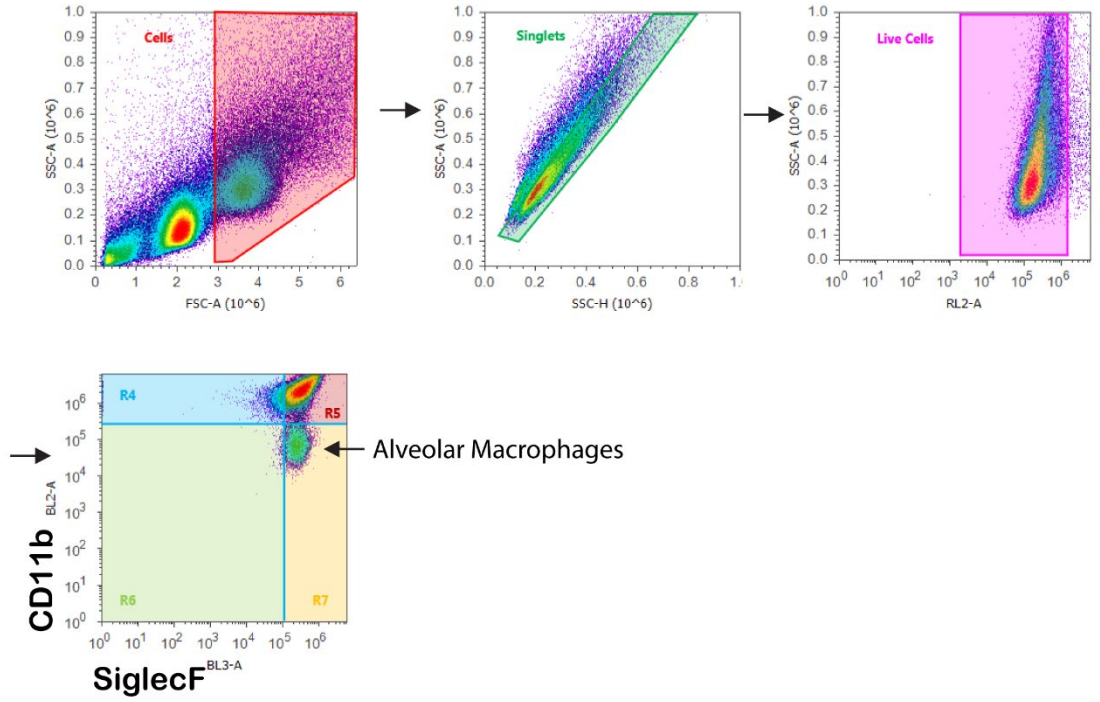

Figure 5: Cell density plots showing gating strategy for identifying alveolar macrophages ($CD11b^{-}$ $SiglecF^{+}$) in lungs.

5. Oxidative Burst Assay:

Splenic cells were isolated as described previously and approximately 1 million cells were blocked with 1 µL of TruStain FcX (anti-mouse 16/32) antibody (Biolegend, San Diego, CA) for 1 hr on ice. After blocking, 1µL of different antibodies including anti-CD11b, anti-Ly6G, and anti-Gr1 (BioLegend, San Diego, CA) were added to each tube together with 1 µL of live/dead cell stain (Invitrogen, Waltham, MA). Hanks' Balanced Salt solution was used for immunostaining, washing, and resuspending cells. Then 4 µL of dihydrorhodamine 123 (DHR; 5 mM) and 10 µL of N-formyl-Met-Leu-Phe (fMLP) bacterial peptide (10 mM) were added to cells,

resuspended in 500 µL of Hanks' Balanced Salt Solution (HBSS), incubated at 37 °C for 20 min and then immediately transferred to ice for 10 min to stop the reaction. Cells were washed twice with HBSS, and fluorescence intensity was measured by flow cytometry.

6. Quantification of cytokines by microbead immunoassay:

To measure concentration of different cytokines in blood, I used LEGENDplex mouse inflammation panel kit (Biolegend Cat# 740446). This panel includes cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-17A (IL-17A), interleukin-23 (IL-23), monocyte chemoattractant protein-1 (MCP-1), interferon-β (IFN-β), interferon-γ (IFN-γ), and Tumor Necrosis Factor-α (TNF-α). I used manufacturer's protocol to incubate capture beads with blood plasma samples of different groups of mice. Cytokines were measured based on the standard curve generated using LEGENDplex software and Graphpad Prism software (version 9) was used to plot graphs.

7. Sandwich Enzyme Linked Immunosorbent Assay:

To measure mouse latent TGF-β from lung lavage samples, I used LEGEND MAX Mouse latent TGF-β ELISA kit (Biolegend Cat# 433007 Lot# B341535). Kit was stored at 4°C until use. Frozen lung lavage samples were thawed on ice and centrifuged at 2500 RPM for 5 min at 4°C to pellet down cellular debris. After centrifugation, 150 µL of samples were transferred into new tubes and stored on ice. Using 0.69 mL assay buffer E, 20 ng/mL of stock solution was prepared and incubated at room temperature for 20min. After incubation, 7 standards with 4000

pg/mL, 2000 pg/mL, 1000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL TGFβ were made in separate tubes from 20 ng/mL stock solution. Assay buffer E was used as zero standard (0 pg/mL). Pre-coated ELISA plate was washed with 200 µL wash buffer for 2 times and the plate was dried firmly on paper towels. First 50 µL assay buffer E was added to all wells. Then 50 µL of standards/samples were added to standard/sample wells in duplicate. Plates were sealed with a plate sealer and wrapped in a aluminum foil and incubated at room temperature for 2 hours on a plate shaker rotating at 200 rpm. After incubation, plates were washed 4 times with 200 µL wash buffer and then incubated in 100 µL of mouse latent detection antibody at room temperature. After incubation, the plate was washed 4 times with 200 µL wash buffer and incubated for 30 min in avidin-HRP D solution at room temperature. After incubation the plate was again washed thoroughly 5 times with wash buffer and incubated for 20min in 100 µL of substrate solution D at room temperature in dark environment. After incubation, 100 µL of stop solution was added to stop the reaction. Appropriate PPE was worn to protect eyes, hand, and face while handling stop solution since it contains strong acid that can cause hazard. Then absorbance was recorded at 450 nm and 570 nm with correction for plate background.

8. RNA isolation from spleen tissue:

Mouse spleen removed 72hrs after CLP surgery was immediately frozen in liquid nitrogen and stored at -70°C until use. Frozen Spleen samples (30mg) were transferred into RNAlater™-ICE frozen tissue transition solution (ThermoFisher Cat # AM7030) and incubated overnight at 4°C to prevent mRNA degradation while

thawing. Next day, Spleen tissue was ground in liquid nitrogen using Mortar-Pestle and homogenized using Shredder columns (Qiagen Cat # 79654) and then RNA was purified using RNeasy Protect kit (Qiagen Cat # 74124). Purity and concentration of RNA was analyzed using nanophotometer by measuring Absorbances A260/280 (protein contamination) and A260/230 (buffer contamination) before proceeding to cDNA preparation.

9. cDNA preparation from RNA using reverse transcriptase PCR:

Approximately 2µg of total RNA was reverse transcribed using high-capacity cDNA reverse transcription kit (ThermoFisher Cat # 4368814) with RNase inhibitor (ThermoFisher Cat # N8080119). Concentration of cDNA was measured in nanophotometer after cDNA synthesis.

10.Quantitative real-time PCR:

qPCR was performed using Taqman probes from ThermoFisher (Cat # 4453320) (ref Table 1). 10 ng of cDNA was used for Taqman assay. Both Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin were used as endogenous controls. β-actin was used for relative quantitation since its variability between CLP (Control) and CLP RvD2 (Test) was less compared to GAPDH. Fold change was calculated using double-delta CT method as described (Livak and Schmittgen, 2001). Relative quantitation was performed against sham animals (animals which undergo laparotomy only) with beta actin as reference gene. Independent T-Test was used to investigate statistical difference between CLP saline and CLP RvD2 samples.

GENE	Probe Name	Amplicon length (nucleotides)	ThermoFisher Cat #
TLR2	Mm00442346_m1	69	4453320
TLR3	Mm01207404_m1	121	4453320
TLR4	Mm00445273_m1	87	4453320
TLR5	Mm07297422_m1	86	4453320
TLR7	Mm00446590_m1	125	4453320
TLR9	Mm00446193_m1	60	4453320
GAPDH	Mm99999915_g1	107	4453320
β-Actin	Mm00607939_s1	115	4453320

11.*Pseudomonas aeruginosa* culture:

Pseudomonas aeruginosa ATCC 27853™ (American Type Culture Collection, Manassas, VA, USA) was streaked zig-zag on a Tryptic soy agar plate (TSA; Ward's Scientific, Rochester, NY, USA) and incubated overnight at 37°C (Thornton et al., 2021). From the streaked plate, liquid cultures were made by swabbing bacteria with a wooden applicator stick and depositing into glass culture tubes with 3 mL LB broth (Luria-Bertani broth; Gibco: Gaithersburg, MD, USA). The cultures were incubated for 3 hours at 37°C with shaking (200 rpm) and pelleted by centrifuging for 6 min at 9100 g. LB broth was removed, and pellets were washed 3 times with LB broth. The OD600 was measured using Biotek Synergy H1 plate reader (Biotek, Winooski, VT,

USA). Then samples were diluted to correspond to 0.21A at OD600 which roughly corresponds to 10^7 cells in 25µL as determined by counting colony forming units (CFUs) next day after incubation overnight (19 – 20 hours) at 37°C.

12. Virulence gene expression analysis in *Pseudomonas aeruginosa*:

RNA EXTRACTION: RNA was extracted using Trizol Max Bacterial RNA isolation Kit (ThermoFisher Cat# 16096020) using kit protocol. RNA purity (Absorbance ratio A260/A280, A260/A230) and RNA concentration are measured using nanophotometer. RNA extracted was stored at -70^0C until further use.

REVERSE TRANSCRIPTION PCR: Approximately 1µg of RNA was reverse transcribed using High-Capacity cDNA reverse transcription kit (ThermoFisher Cat # 4368814).

QUANTITATIVE REAL-TIME PCR: Quantitative real-time PCR was performed by SYBR green method. Primer sequences for genes PqsA, RhlA, MucA and 16S rRNA were obtained from previously published paper (Magalhães et al., 2019) (Table. 2). 16S rRNA was used as endogenous control for relative gene quantification by $2^{-\Delta\Delta CT}$ method.

Table 2: Primer sequences for *Pseudomonas aeruginosa* genes

Oligo #	Oligo Name	Sequence - 5' to 3'	Length
1	16SrRNAForward	GGAGAAAGTGGGGGATCTTC	20
2	16SrRNAReverse	CCGGTGCTTATTCTGTTGGT	20
3	rhlAForward	GCGCGAAAGTCTGTTGGTAT	20
4	rhlAReverse	ATTTCCACCTCGTCGTCCTT	20
5	pqsAForward	ACCGCGAAGGACACACTATC	20
6	pqsAReverse	GGCAGGTAGGAACCAGAACC	20
7	mucAForward	CTGGACGAGGAGTTGGTGAT	20
8	mucAReverse	GCGTCTGTACAACCAGAACG	20

EXPERIMENTAL RESULTS

1. Resolvin D2 treatment increased Ly6G$^+$ neutrophils in spleen:

Neutrophils were identified based on expression of Ly6G, a 25 kD GPI-linked protein attached to plasma membrane (Lee et al., 2013). Using monoclonal antibody 1A8, we immunostained total splenic cells to identify neutrophils in mouse spleen. After immunostaining procedure, cells were analyzed by flow cytometer. All cells were selected to exclude debris, after then singlets were selected. Then live cells were negatively selected based on a cell permeable stain. Then Ly6G expressing live neutrophils were selected and counted. We found that there was a significant increase in Ly6G$^+$ splenic neutrophils in the CLP mice group that received resolvin D2 24 hours prior to analysis (Sundarasivarao et al., 2022) (Figure 6).

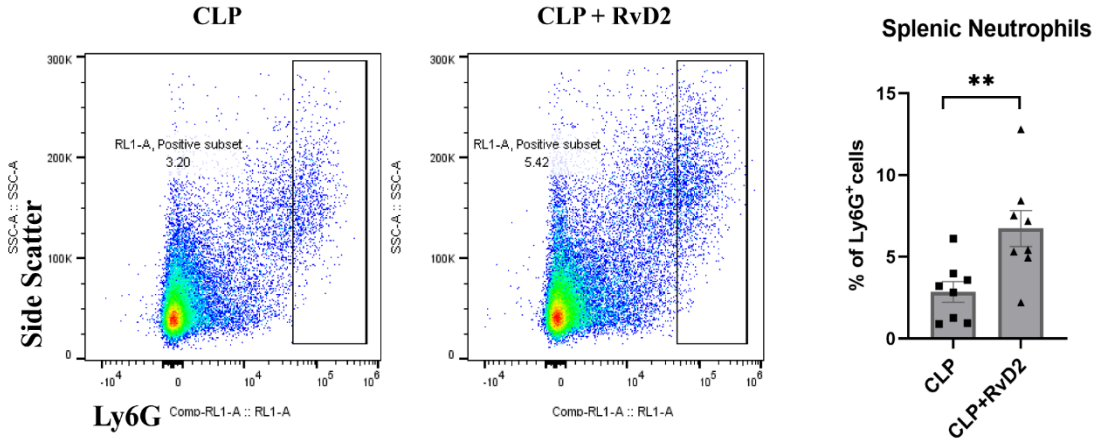

Figure 6: Splenic neutrophils count. Using flow cytometer, Ly6G+ splenic neutrophils were gated and counted based on side scatter and Ly6G expression. For n=8, **P<0.01 by independent t-test. All data are mean±S.E.M

2. Resolvin D2 treatment oxidative burst in CD11b^{+} Ly6G^{+} inflammatory neutrophils in spleen:

Sudden release of Reactive Oxygen Species (ROS) called oxidative burst is an anti-microbial host defense mechanism exhibited by neutrophils. We used cell permeable dihydrorhodamine 123 (DHR 123) to measure oxidative burst in neutrophils upon stimulation with bacterial peptide *N-formyl-Met-Leu-Phe* (fMLP). Bacterial peptide fMLP binds to FPR 1 receptor and stimulates production of hydrogen peroxide by mitochondria. Released hydrogen peroxide converted DHR123 into fluorescent rhodamine which was detected by flow cytometry (Chen and Junger, 2012).

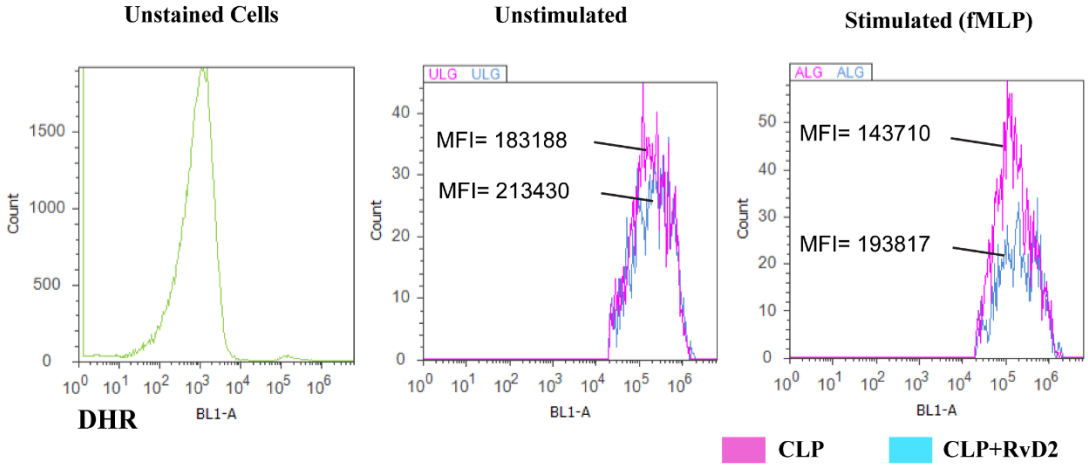

Figure 7: ROS production (DHR+ cells) in CD11b^{+} Ly6G^{+} splenic neutrophils was measured in unstimulated (basal) and fMLP stimulated cells. Histogram plots (Y-axis: count; X-axis: fluorescence intensity) shows oxidative burst in cells taken from CLP and CLP+RvD2 mice.

The amount of hydrogen peroxide released (oxidative burst) is directly proportional DHR MFI (median fluorescence intensity).

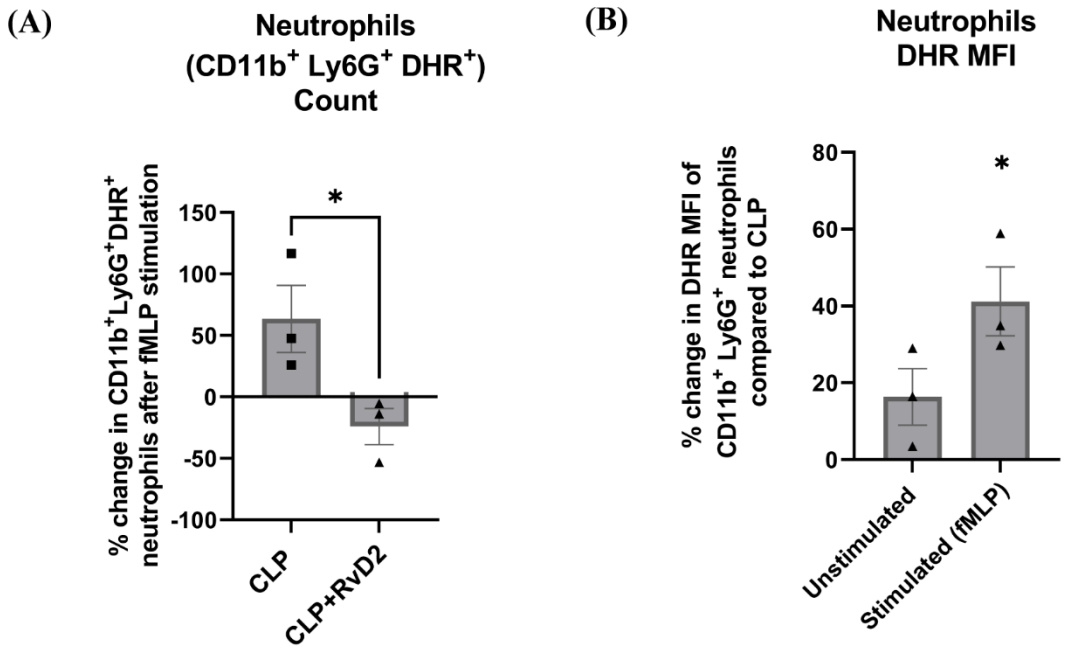

Figure 8: A. The percentage change in the number of the fMLP stimulated neutrophils ($CD11b^+$ $Ly6G^+$ DHR^+) was counted. RvD2 decreased the number of fMLP stimulated neutrophils. B. The percentage change in median fluorescence intensity (MFI) in $CD11b^+$ $Ly6G^+$ DHR^+cells (basal and stimulated) between cells taken from CLP + RvD2 treated mice and cells from CLP mice was measured. RvD2 increased the MFI of ROs producing splenic neutrophils. All data are mean ± S.E.M. *P<0.05 for n = 3 in all groups.

Resolvin D2 treatment increased oxidative burst in both unstimulated and stimulated $CD11b^+$ $Ly6G^+$ neutrophils in spleen (Sundarasivarao et al., 2022) (Figure 7). After stimulation with fMLP, neutrophils from CLP+RvD2 group significantly produced more ROS compared to neutrophils from CLP group (Sundarasivarao et al., 2022) (Figure 8.A). However, number of neutrophils producing ROS ($CD11b^+$ $Ly6G^+$ DHR^+) in CLP+RvD2 group was significantly less compared CLP group

(Sundarasivarao et al., 2022) (Figure 8.B). Therefore, prior resolvin D2 treatment improved efficiency of neutrophils to produce ROS in our model of infection.

3. Resolvin D2 treatment increased migration of $CD11b^+$ $Ly6G^+$ $Ly6C^+$ myeloid-derived suppressor cells from bone marrow to spleen:

Myeloid-derived suppressor cells (MDSCs) are a heterogenous population of undifferentiated cells of myeloid origin identified by expression of CD11b, Ly6C, and Ly6G receptors (Figure 9). Alternatively, MDSCs are also identified as $CD11b^+$ $Gr\text{-}1^+$ cells. 24 hours after resolvin D2 treatment in CLP model, there was a significant increase in $CD11b^+$ $Ly6G^+$ $Ly6C^+$ MDSCs (Sundarasivarao et al., 2022)

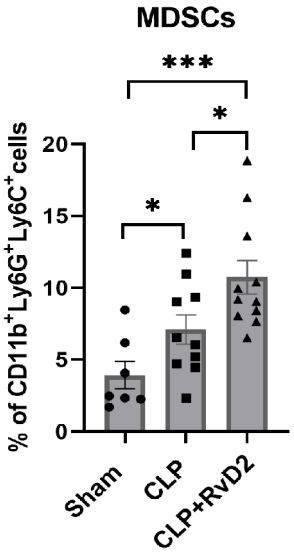

Figure 9: CLP mice were given vehicle saline or RvD2 48h after surgery. Mice were sacrificed 24h later and spleen samples were taken Splenic MDSC numbers were increased after CLP and RvD2 adminsitration increased the numbers even further. All data are mean ± S.E.M. *P<0.05, *** P < 0.001 for n = 7- 11 in all groups

(Figure 9). Some researchers reported that MDSCs produce ROS in a tumor environment (Ohl and Tenbrock, 2018). So, we investigated if resolvin D2 treatment enhanced ROS production in MDSCs upon stimulation with fMLP similar to the

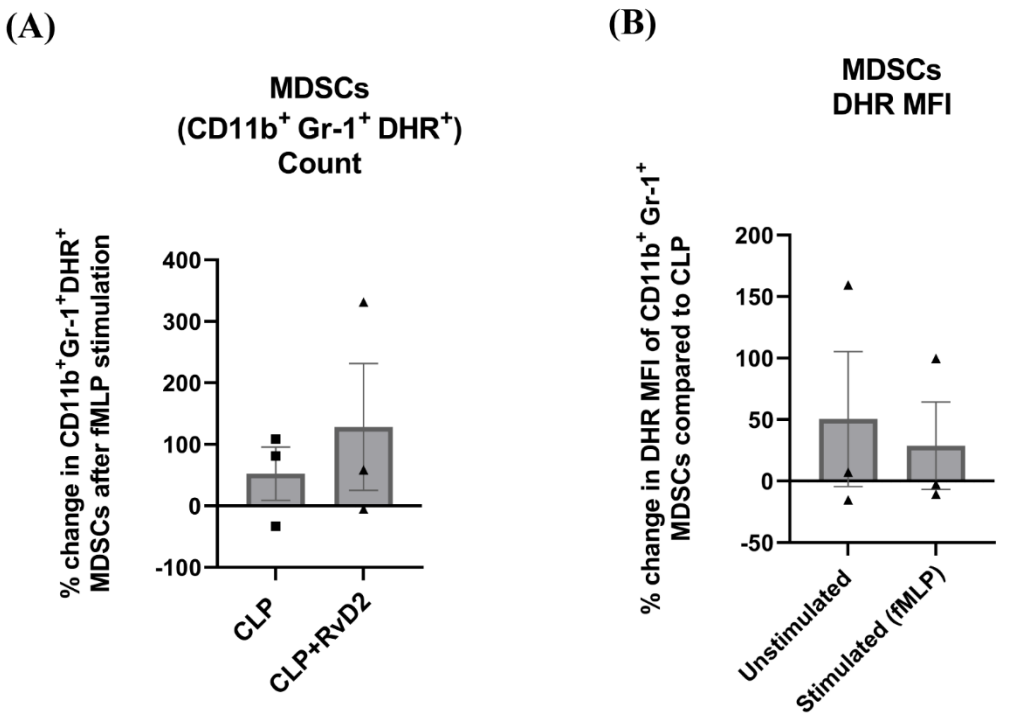

Figure 10: A. Percentage change in the number of fMLP stimulated ROS producing MDSCs ($CD11b^+$ $Gr\text{-}1^+DHR^+$) were counted from CLP and CLP + RvD2 mice. RvD2 had no effect on the number of MDSC stimulated cells. B. The percentage change in median fluorescence intensity (MFI) in ROS producing $CD11b^+$ $Gr\text{-}1^+DHR^+$cells (basal and stimulated) between CLP + RvD2 treated mice cells and cells from CLP mice was measured. RvD2 did not affect the MFI of ROS producing MDSCs.

effect we observed in neutrophils. Prior resolvin D2 treatment did not change number of $CD11b^+$ $Gr\text{-}1^+$ DHR^+ ROS producing MDSCs in CLP or CLP+RvD2 group (Sundarasivarao et al., 2022) (Figure 10.A). Also, resolvin D2 treatment did not significantly change ROS production in MDSCs upon stimulation with fMLP

(Sundarasivarao et al., 2022) (Figure 10.B). Hence resolvin D2 did not affect ROS production in MDSCs in our model of infection.

Since MDSCs originate from bone marrow, when we analyzed the number of MDSCs in resolvin D2 treated mice, we found a significant decrease in MDSCs in mice group that received resolvin D2 24 hours prior to analysis (Figure 11). Hence our results suggest that resolvin D2 directly increased migration of MDSCs from bone marrow to spleen in our infection model.

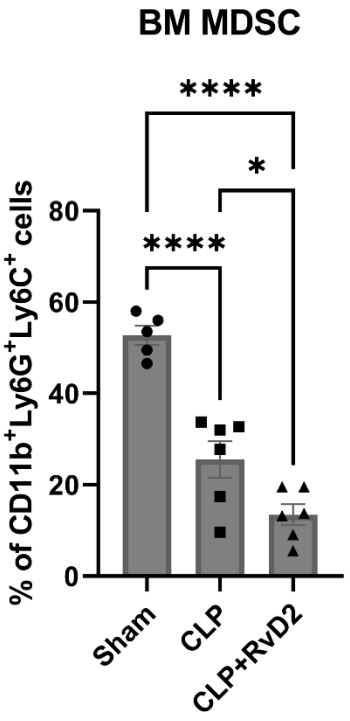

Figure 11: CLP mice were given vehicle saline or RvD2 48h after surgery. Mice were sacrificed 24h later and bone marrow cells were isolated and MDSCs in bone marrow were counted. MDSC numbers were increased after CLP and RvD2 adminsitration increased the numbers even further. All data are mean ± S.E.M. *P<0.05, *** P < 0.001 **** P<0.0001 for n = 5-6 in all groups. All data are mean±S.E.M.

4. Resolvin D2 treatment significantly increased expression of TLR 2:

Toll-like receptors are cell surface receptors that recognize bacteria, viruses, and microbial products including lipoproteins (TLR2), lipopolysaccharides (TLR4), unmethylated DNA (TLR9), Flagellin (TLR5), viral dsRNA (TLR3), and viral/bacterial ssRNA (TLR7) (Takeuchi and Akira, 2010).

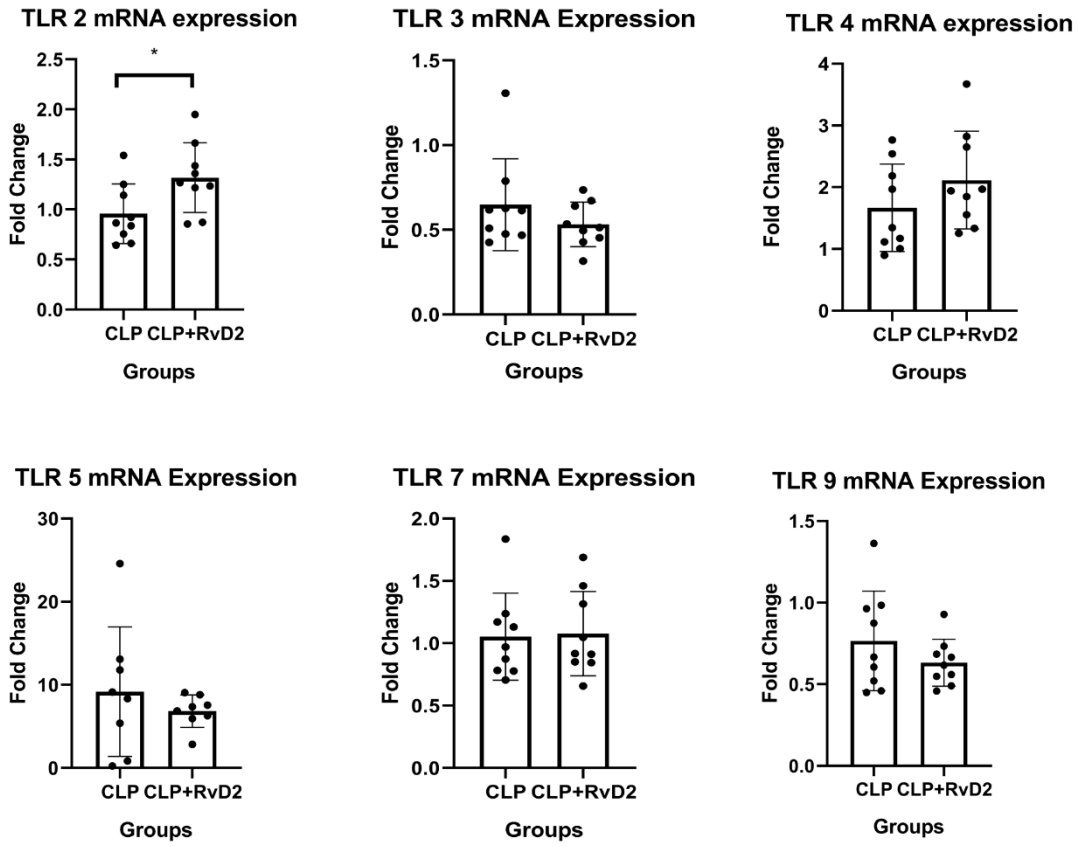

Figure 12: Gene expression of Toll-like receptors (TLRs) in spleens. 48 hours after CLP surgery was performed, CLP mice were given either vehicle saline or RvD2 (100 ng/mouse; i.v.). 24 hours after treatment, mice were sacrificed, and spleens removed for qPCR of TLRs. RvD2 treatment increased TLR-2 expression but no significant change in gene expression was observed in TLR3, TLR4, TLR5, TLR7, or TLR9. * $P < 0.05$, $n = 9$ for all groups. Error Bars represent mean ± S.D.

Resolvin D2 treatment increased gene expression of TLR2 24 hours after administration of resolvin D2 (Figure 12) (Walker et al., 2022). However, there was no significant change in TLR3, TLR4, TLR5, TLR7 gene expression (Figure 12) (Walker et al., 2022). However, compared to Sham animals CLP group of animals showed increase in gene expression of all toll-like receptors (TLRs) consistent with results reported by other labs in other tissues Lungs, Kidney, and Liver. (Aravanis et al., 2018, Bakopoulos et al., 2017, Krivan et al., 2019).

5. Resolvin D2 did not significantly change plasma cytokine levels 24 hours after administration.

There was no significant difference between untreated and treated CLP mice groups in blood plasma levels of various inflammatory cytokines (Figure 13) (Sundarasivarao et al., 2022). This suggested that blood bacterial clearance 24 hours after resolvin D2 treatment might be mediated directly through other cellular mechanisms and not through these pro-inflammatory cytokines.

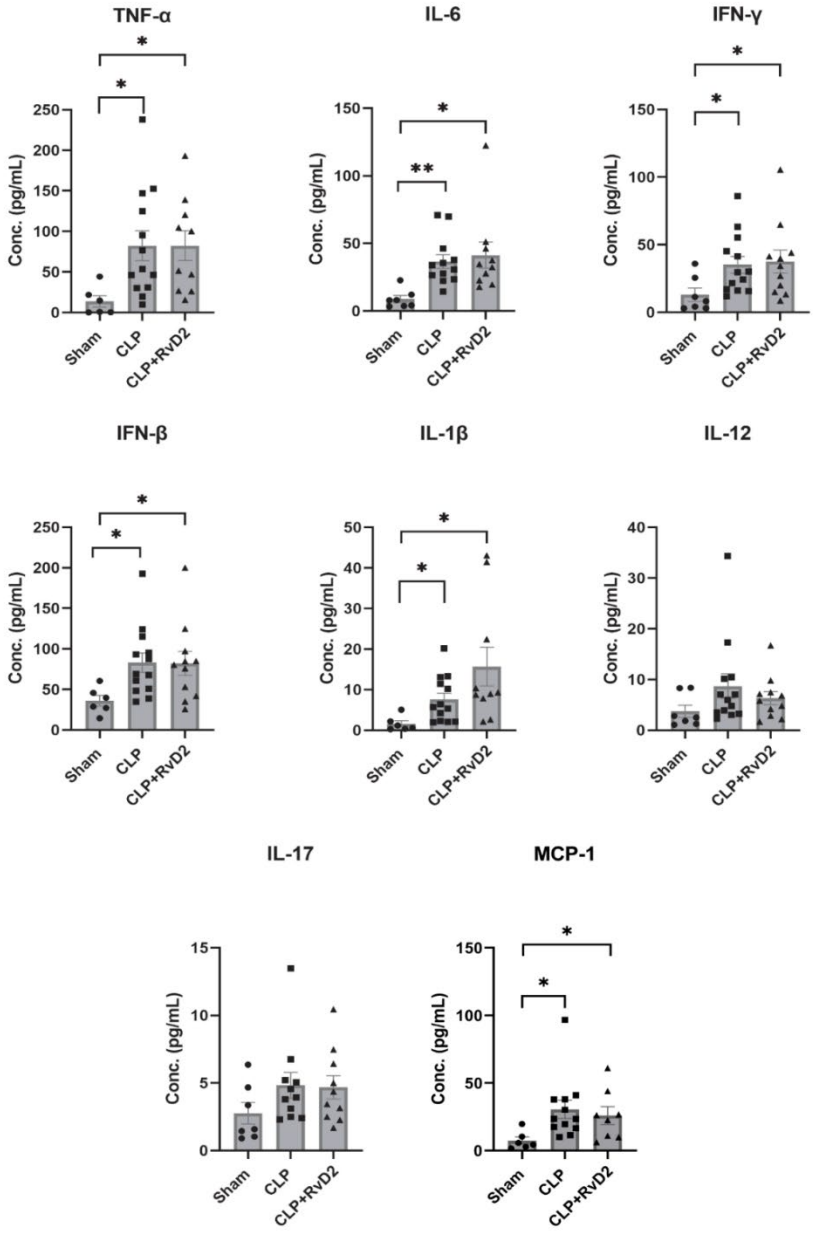

Figure 13: Sham or CLP surgery was performed on mice. CLP mice were given vehicle saline or RvD2 48h after surgery. Mice were sacrificed 24h later and blood sample were taken. Using flow cytometry, cytokines including TNF-α, IL-6, IFN-γ, IFN-β, IL-1β, IL-12, IL-17, and MCP-1 were quantified using bead-based immunoassay. CLP increased plasma levels of cytokines, but RvD2 did not change levels further. * $P < 0.05$, ** $P < 0.01$ for $n = 6 – 14$ in all groups. All data are mean±S.E.M

6. Resolvin D2 treatment improved bacterial clearance in blood within 24 hours.

Resolvin D2 treatment in our infection model significantly reduced bacteria in blood 24 hours after receiving treatment (Figure 14) (Sundarasivarao et al., 2022).

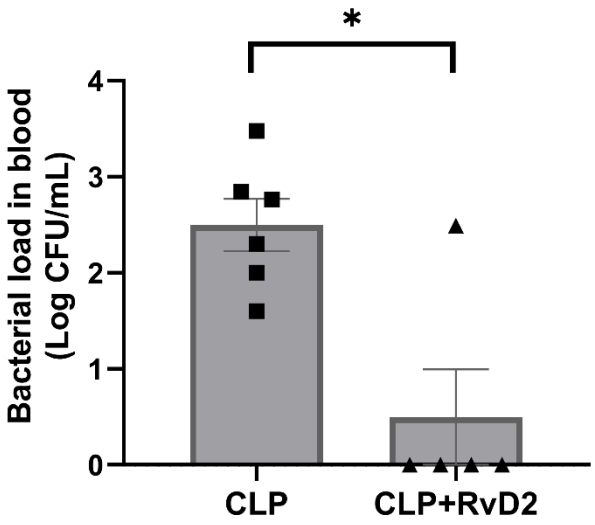

Figure 14: CLP mice were given vehicle saline or RvD2 48h after surgery. Mice were sacrificed 24h later and blood sample were taken. Samples were serially diluted, plated on TSA plates and colony forming units (CFUs) were counted 24 hours after plating. RvD2 reduced the blood bacteria load. * $P < 0.05$, for n = 5 -6 mice in each group. Data are mean±S.E.M

7. Resolvin D2 treatment prior to secondary infection with *Pseudomonas aeruginosa* infection prevents decrease of alveolar macrophages in lungs.

Alveolar macrophages are macrophages that reside in the alveolar space inside lungs. Alveolar macrophages express SiglecF receptor and do not express CD11b cell surface receptor unlike interstitial macrophages in lungs (Hussell and Bell, 2014a).

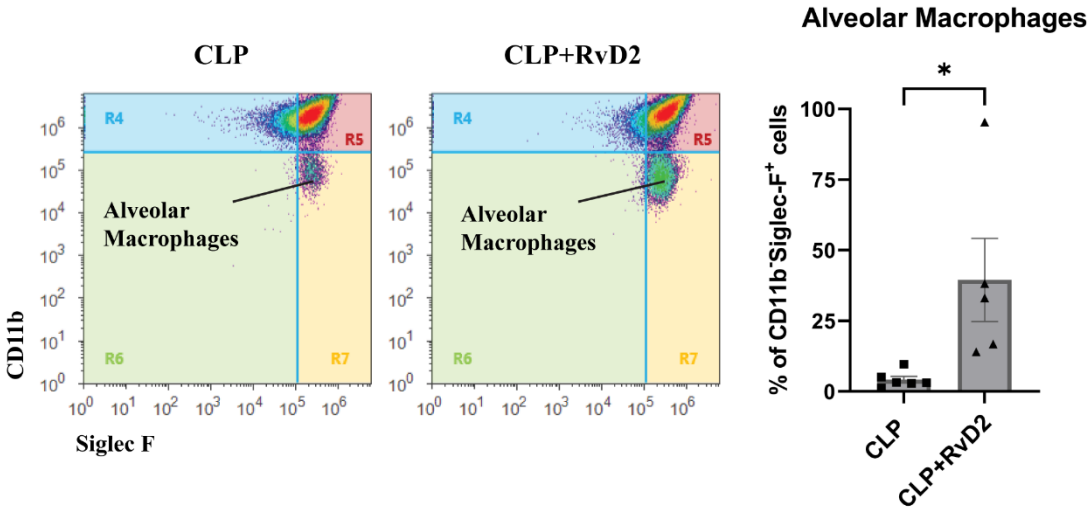

Figure 15: CLP surgery was performed on mice. Mice were given vehicle saline or RvD2 48h after surgery. 24h after injections, mice were intranasally administered with *P. aeruginosa* to cause secondary lung infection. 24h after secondary infection, mice were sacrificed and lungs lavaged. Using flow cytometry, alveolar macrophages ($CD11b^-$ Siglec F^+) were counted in lung lavage. Data are mean ± S.E.M. * $P < 0.05$ for $n = 5$ for both groups.

Hence alveolar macrophages can be identified as $CD11b^-$ $SiglecF^+$ cells in murine lungs. There was a decrease in alveolar macrophage count 24 hours after *Pseudomonas aeruginosa* infection in untreated CLP mice when compared to healthy sham control (Figure 15) (Sundarasivarao et al., 2022). However, prior resolvin D2

treatment of CLP mice prevented this decrease of alveolar macrophages ($CD11b^{-}$ $SiglecF^{+}$) which are essential for clearing neutrophils from lungs and maintaining anti-inflammatory environment of alveoli (Hussell and Bell, 2014b).

8. Prior resolvin D2 treatment improved *Pseudomonas aeruginosa* clearance in lungs within 24 hours:

Resolvin D2 treatment also decreased lung bacterial load (Figure 16) (Sundarasivarao et al., 2022). Therefore, decrease in lung bacterial load was mediated through increase in alveolar macrophages that can clear bacteria though phagocytosis and clear neutrophils through efferocytosis.

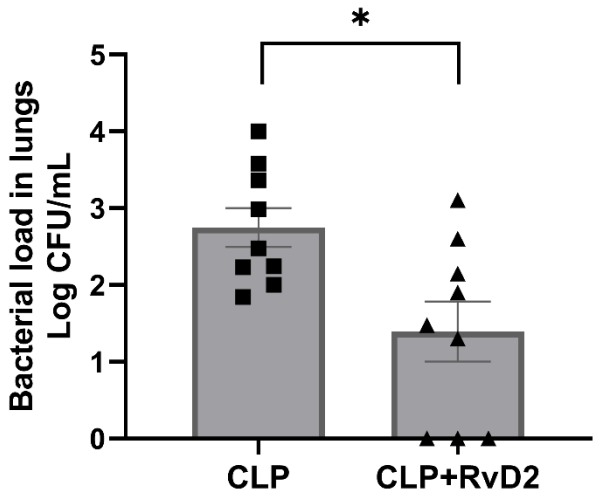

Figure 16: CLP surgery was performed on mice. Mice were given vehicle saline or RvD2 48 hours after surgery. 24 hours after injections, mice were given *P. aeruginosa* intranasally to cause secondary infection. 24 hours after the secondary infection, mice were sacrificed, and lungs were lavaged. Lavage fluid was serially diluted and plated on TSA plates. RvD2 administration reduced lung bacteria load. Data are mean ± S.E.M. for n = 9 in all groups.

9. Cytokine levels in lungs 24 hours after secondary lung infection with Pseudomonas aeruginosa:

I reported earlier (Figure 13) that there was no change in plasma cytokine levels 24 hours after resolvin D2 treatment (that is prior to secondary lung infection). 48 hours after resolvin D2 treatment and 24 hours after *P. aeruginosa* secondary lung infection, I measured cytokine levels in lung lavage and found that there was no significant difference in TNF-α, IL-6, IL-10, IL-17A, MCP-1, IFN-β, IFN-γ, and IL-1β (Figure 17) (Sundarasivarao et al., 2022). Interestingly, IL-23 was significantly reduced compared to untreated control group (CLP) (Figure 17) (Sundarasivarao et al., 2022). Also, there was no significant difference in TGF-β levels between CLP and CLP+RvD2 groups (Figure 18).

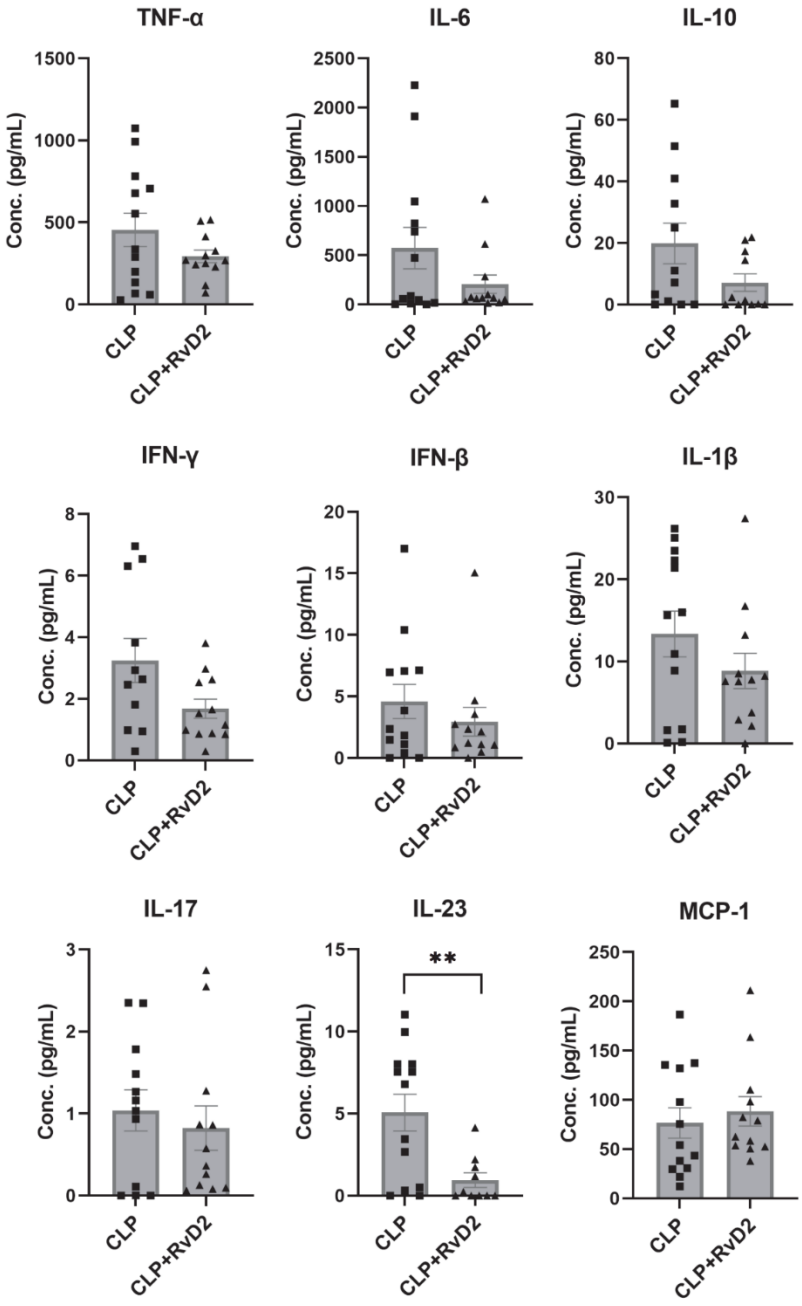

Figure 17: CLP surgery was performed on mice. Mice were given vehicle saline or RvD2 48h after surgery. 24h after injections, mice were given *P. aeruginosa* intranasally. 24h after the 2nd hit of p. aeruginosa, mice were sacrificed and lungs lavaged. Using flow cytometry, cytokines in lungs including TNF-α, IL-6, IL-10, IFN-γ, IFN-β, IL-1β, IL-17, IL-23, and MCP-1 were quantified by bead-based immunoassay. RvD2 signficantly reduced lung lavage fluid IL-23 levels. Data are mean ± S.E.M. ** $P < 0.01$ for n = 10 – 12 in all groups.

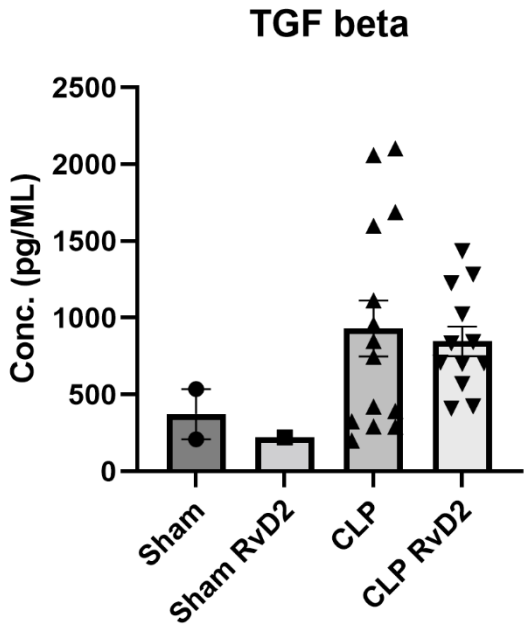

Figure 18: CLP surgery was performed on mice. Mice were given vehicle saline or RvD2 48h after surgery. 24h after injections, mice were given *P. aeruginosa* intranasally. 24h after the 2nd hit of *P. aeruginosa*, mice were sacrificed and lungs lavaged. Using Sandwich ELISA, mouse latent TGF-β was quantified in sham, sham+RvD2, CLP, CLP+RvD2 groups. All data mean±S.E.M for n=1-14.

10.Resolvin D2 treatment reduced expression of virulence genes in *Pseudomonas aeruginosa*:

Virulence genes *pqsA, rhlA, mucA* in *Pseudomonas aeruginosa* have unique functions and together they contribute to the virulence of the pathogen and help in biofilm formation that protects pathogen from immune cell attack.

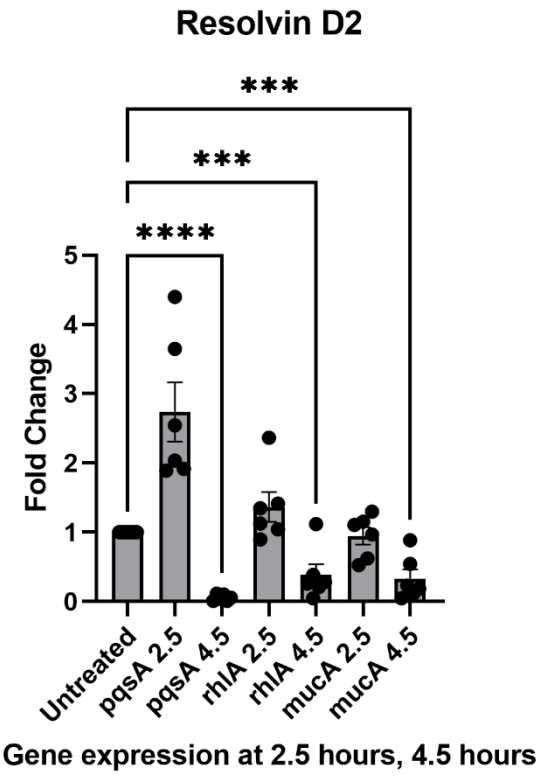

Figure 19: Effect of SPMs on *Pseudomonas aeruginosa* virulence genes. 1nM of resolvin D2 was added directly to cells and incubated for 2.5 and 4.5 hours and gene expression of *pqsA, rhlA,* and *mucA* were measured using quantitative real-time PCR. Resolvin D2 significantly reduced expression of all the genes compared to untreated samples. Data are Mean ± S.E.M for n=3 independent experiments. ***P<0.001, ****P<0.0001 by ONE-WAY ANNOVA for n=3.

We found that treating *Pseudomonas* with SPM Resolvin D2 at an effective concentration of 1nM decreases expression of virulence genes *pqsA*, *rhlA*, and *mucA* at 4.5 hours post treatment (Figure 19) (Thornton et al., 2021).

11. Resolvin D2 treatment improved survival of mice in our infection model:

Survival of mice in our two-hit infection model is dependent on multiple factors including reduction of hyperinflammation caused by peritonitis, clearance of fecal bacteria in blood by neutrophils in spleen, and clearance of *Pseudomonas aeruginosa* from lungs. Resolvin D2 treatment prior to secondary infection with *Pseudomonas*

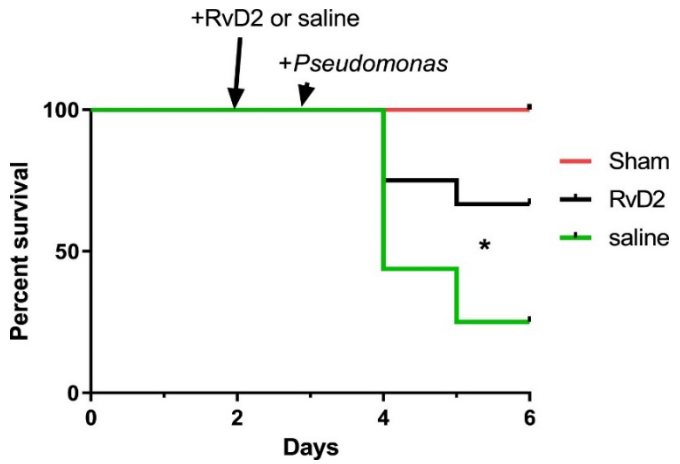

Figure 20: Cecal-ligation puncture (CLP) surgery was performed on CD1 mice. 48 hours after surgery, either RvD2 (100 ng/mouse, i.v.) or saline was injected into CLP mice. *Pseudomonas aeruginosa* was inoculated into the mice intranasally to cause secondary infection. Mice were monitored for 3 days after inoculation. CLP mice which were prior treated with RvD2 had greater survival compared to CLP mice administered saline. * $P < 0.05$ for n = 16 for CLP controls, n = 16 for CLP = RvD2, n = 9 for sham.

aeruginosa improved survival of mice compared to control group (Figure 17) (Walker et al., 2022).

DISCUSSION:

These studies provided us evidence on how resolvin D2 modulates immune response during infection and inflammation to improve survival of our 2-hit mice model of infectious peritonitis and secondary lung infection. Mice in general are resistant to *Pseudomonas aeruginosa* infection and murine models need some manipulation to trigger inflammation (Bayes et al., 2016, Thomsen et al., 2022, van Heeckeren and Schluchter, 2002). Cecal-ligation and puncture (CLP) procedure prior to administration of *Pseudomonas aeruginosa* makes mice more susceptible to lung infection due to underlying inflammation (peritonitis) and active infection (due to leakage of fecal bacteria into sterile peritoneum). Also, ligation of cecum produces necrotic factors contributing to the overall immunosuppression that follows the CLP procedure (Dejager et al., 2011). CLP induced polymicrobial sepsis is a good model to study sepsis because, this procedure induces infection by a wide spectrum of bacteria consisting of both gram-positive and gram-negative bacteria, triggers hyperinflammation, releases moderate amount of pro-inflammatory cytokines over a long period instead of sudden release of cytokines over a short period of time observed during acute infection (Dejager et al., 2011). However, much variability in severity can be introduced into this model due to the batch of mice used for the procedure, length of cecum ligated, or by the procedure itself which makes this model more complex (Rittirsch et al., 2009). Hence, we repeatedly measured various cellular and molecular level changes to make definitive conclusions.

During the first 48 hours after CLP procedure, constant leakage of fecal bacteria into sterile peritoneum triggered peritonitis. This peritonitis represents hyperinflammatory phase in our model. During this hyperinflammatory phase, we observed elevated cytokines in CLP group compared to sham group. Also, measurement of liver tissue cytokines such as IL-6, IL-10, MIP-2 at 72 hours after CLP indicated that by the end of 72h hours mice had reached post-inflammatory phase. So, this was a correct time to administer a 2^{nd} hit to the CLP mice. After resolvin D2 was administered, we observed an increase in neutrophils ($Ly6G^+$) and myeloid-derived suppressor cells (MDSCs) ($CD11b^+$ $Ly6G^+$ $Ly6C^+$) in spleen. Increase in splenic neutrophils post CLP procedure was reported by other labs and found to be due to upregulation of extramedullary myelopoiesis (Sengupta et al., 2020). Migration and accumulation of MDSCs were also observed in chronic bacterial infections (Janols et al., 2014, Uhel et al., 2017) and viral infections (Jeisy-Scott et al., 2011). Bacteria and viruses evade immune response by exploiting MDSCs immunosuppressive function (Peñaloza et al., 2019). Also, MDSCs prevent T-Cell activation by depleting cystine and cysteine (Srivastava et al., 2010), inducing T-regs (Huang et al., 2006), and through secretion of H_2O_2, TNF-α, NO, TGF-β (Monu and Frey, 2012). Increased number of MDSCs in spleen may effectively suppress T-Cell activation thereby preventing tissue damage due to excessive inflammation.

Prior RvD2 treatment, also increased free radical generation (DHR^+ MFI) in $CD11b^+Ly6G^+$ splenic neutrophils upon fMLP stimulation. We also observed significant reduction in $CD11b^+$ $Ly6G^+$ DHR^+ cells. Since excessive production of intracellular ROS can lead to cell death, reduction in ROS positive splenic neutrophil

count in CLP RvD2 group might be due to cell death by ROS producing neutrophils. However, free radical production in MDSCs was not altered and, we did not see any significant change in MDSC count. This provided evidence that although MDSCs produce ROS, resolvin D2 treatment and fMLP stimulation did not alter ROS production in MDSCs.

Interestingly, resolvin D2 treatment increased splenic TLR2 expression in 24 hours, but no significant difference was observed in TLR4, TLR3, TLR5, TLR7, and TLR9. Although *in vitro* resolvin D2 reduced TLR4 expression in THP-1 cells (Croasdell et al., 2016b), we did not find any significant difference in TLR4 expression in spleen 24 hours post treatment. Since toll-like receptors (TLRs) are important cell surface receptors that detect pathogen-associated molecular patterns (PAMPs), increased expression of TLR2 significantly increases TLR2 stimulation by LPS, lipoteichoic acid, and peptidoglycans from bacterial cell wall (Walker et al., 2022). However, this increase in TLR2 expression by resolvin D2 treatment did not significantly affect plasma levels of IL-6, IL-10, and MIP-2 (Walker et al., 2022). Also, resolvin D2 treatment did not significantly alter IL-6, IL-10, MIP-2 cytokines in liver 24 hours after treatment. However, liver IL-10 and MIP-2 levels were increased in CLP group compared to sham but no difference in IL-6 level was observed. This result provided evidence that mice were in post-inflammatory state.

Resolvin D2 treatment did not significantly alter plasma cytokine levels of TNF-α, IL-6, IFN-γ, IFN-β, IL-1β, IL-12, IL-17, and MCP-1 in CLP mice 24 hours post treatment. However, TNF-α, IL-6, IFN-γ, IFN-β, IL-1β, and MCP-1 cytokine levels

were elevated in CLP compared to sham. This indicates that CLP procedure induces a low-grade sustained elevation of pro-inflammatory cytokines in our model similar to a chronic infection. At this time point, plasma levels of IFN-β which suppresses pro-inflammatory cytokine release (Kasper and Reder, 2014), was higher in CLP mice than sham controls while IL-12 one of whose functions is to help differentiate CD4 T-cells into TH1 cells (Athie-Morales et al., 2004), was not significantly increased. Similarly, IL-17 a pro-inflammatory cytokine involved in chronic autoimmune disorders (Kuwabara et al., 2017) was not increased. RvD2 treatment did not alter the levels of any these cytokines. These results suggested that at this late time point after CLP, the overall state of the adaptive immune system was immunosuppressed. Since, resolvin D2 did not significantly alter pro-inflammatory cytokine levels, we concluded that resolvin D2 given 48h after initial CLP surgery improved bacterial clearance in blood by directly improving oxidative burst function of neutrophil to easily kill and phagocytose bacteria without affecting systemic inflammation.

In general, SPMs reduce neutrophil transmigration, promote neutrophil apoptosis but increase phagocytic ability (Sekheri et al., 2020, El Kebir et al., 2012, Bannenberg et al., 2005, Serhan et al., 1995, Papayianni et al., 1996, Sun et al., 2007).This attenuation of neutrophil migration is an important part of the inflammation resolution process to aid the host in restoring homeostasis and avoiding chronic inflammation. With the reduction in neutrophil migration to the site of injury, it is thought that a major mechanism by which neutrophils can aid in bacterial clearance is through an increase in phagocytic ability. Indeed, we have previously shown that

Lipoxin A4 (LxA4) can increase blood neutrophil phagocytic ability and RvD2 can increase lung alveolar monocyte/macrophage phagocytic ability (Walker et al., 2022, Wu et al., 2015, Wu et al., 2016a).

The spleen plays a major role in bacterial clearance and immune regulation where splenic macrophages aid in bacterial clearance by phagocytosis and together with dendritic cells present antigen to initiate T-cell activation, differentiation and expansion as part of the adaptive immune response (Prendergast et al., 2018, Barker et al., 2002). In addition, splenic B-cells can also act as antigen presenting cells for the production of antibodies as another part of the adaptive immune response. There has however been very little work done on splenic neutrophils. In their seminal publication, Kubes and co-authors (Deniset et al., 2017) reported that mature and immature neutrophils were important for bacterial clearance in the spleen. In addition, *in vitro* studies have shown that RvD2 can restore the directionality of neutrophils after burns (Kurihara et al., 2013) . Our results showing both an increase in number and an increase in free radical production coupled with a significant decrease in blood bacteria load, is consistent with the notion that splenic neutrophil accumulation improved blood bacteria clearance. The reduction in blood bacteria load together with any significant increase in plasma cytokines indicated RvD2 may directly affect cell function without changing inflammatory signaling. Taken together, our results suggest that RvD2 given as late as 48h after CLP-induced infection increased blood bacteria clearance at least partly through an increase in splenic neutrophil number and activity.

The role of MDSCs in infectious disease is unclear. This heterogenous population of cells has been shown to be increased in various infectious disease models such as CLP-induced sepsis (Delano et al., 2007, Brudecki et al., 2012), *Porphyromonas gingivalis* infection (Su et al., 2017), and *Trypanosoma cruzi* infection (Arocena et al., 2014). In these disease models the MDSCs have been shown to suppress T-cell proliferation (Mathias et al., 2017), inhibit ConA-induced splenocyte proliferation and suppress adaptive immunity through the Programmed Cell Death 1 receptor (PD1)/Programmed Cell Death Receptor 1 Ligand (PDL1) axis (Ruan et al., 2020). MDSCs obtained from early (3 days) CLP-sepsis appeared to express pro-inflammatory cytokines and nitric oxide while those isolated late (day 12) in sepsis expressed anti-inflammatory cytokines (IL-10) and TGFβ. Interestingly adoptive transfer of late MDSCs into CLP mice early in disease infection development, decreased overall mortality and vice versa (Brudecki et al., 2012). In the *Trypanosoma cruzi* infection model *in vivo* depletion of MDSCs actually led to increased pro-inflammatory cytokine production and mortality (Arocena et al., 2014). These latter reports highlight the heterogeneity and activity of these cells during infection. In this study, we show that there is an increase in MDSCs in the spleen of RvD2 treated mice. These cells do not appear to be significantly pro-inflammatory as there was no significant increase in plasma cytokine levels. The free radical production from these cells was not increased and circulating plasma cytokine levels were not altered but blood bacteria load was significantly reduced. These results suggest a mechanism by which RvD2 promotes host defense, is to increase bacterial clearance by increasing splenic MDSC number. Furthermore, although RvD2

increased the migration of MDSCs from the bone marrow to the spleen, it remains unclear as to whether RvD2 has direct modulatory actions on the MDSCs. This now is an ongoing area of research in the Yin lab.

RvD2 treatment prior to secondary infection with *P. aeruginosa* improved bacterial clearance from lungs. Interestingly, there was a decrease in IL-23 levels but no significant difference in other pro-inflammatory cytokines was observed. RvD2 treatment also increased non-inflammatory alveolar macrophage numbers. These results together suggested that not only did RvD2 play an important role in resolution of acute infection/inflammation but it also actively prevented chronic inflammation directly by reducing IL-23 levels in lungs.

We also measured cytokines levels of TNF-α, IL-6, IL-10, IFN-γ, IFN-β, IL-1β, IL-17, IL-23, and MCP-1 24 hours after *Pseudomonas aeruginosa* administration. Interestingly, we found that IL-23 was significantly reduced 24 hours after *Pseudomonas aeruginosa* administration. IL-23 is involved in T-cell differentiation to the Th-17 phenotype (Gaffen et al., 2014) and has been implicated in the inflammatory processes of several autoimmune disorders such as psoriasis, inflammatory bowel disease and arthritis (Ghoreschi et al., 2021, Moschen et al., 2019, Li and Tsokos, 2021). On the other hand, IL-23 deficient mice were unable to clear *Klebsiella pneumoniae* lung infections (Happel et al., 2005) . These reports suggested that IL-23 was essential for bacterial clearance but sustained high amounts of IL-23 may lead to chronic inflammatory disorders. Decreased IL-23 in RvD2 treated group suggested that RvD2 lowered the possibility of chronic infection through an IL-23-dependent mechanism within the adaptive immune system. This

finding was consistent with previous studies where both RvD1 and RvD2 modulated T-cell responses (Chiurchiù et al., 2016).

Bacterial clearance in the lung during infection is dependent on alveolar macrophage activity and depletion of alveolar macrophages reduces lung bacterial clearance (Allard et al., 2018, Ghoneim et al., 2013). We and others showed that resolvins enhance lung bacterial clearance by increasing macrophage activity (Walker et al., 2022, Codagnone et al., 2018, Isopi et al., 2020). In this study we found that non-inflammatory alveolar macrophage numbers are low 24h after *P. aeruginosa* administration in CLP but prior resolvin D2 treatment significantly increased their numbers. Alveolar macrophage numbers have been reported to have been decreased after influenza (Ghoneim et al., 2013) or *P. carinii* infections (Lasbury et al., 2003). This decrease was associated with a decrease in subsequent pathogen clearance. The decrease in alveolar macrophages may in part be due to macrophage apoptosis which is a process which helps in bacterial killing (Aberdein et al., 2013) but excessive macrophage amounts of apoptosis may leave the host unable to clear bacteria efficiently. Taken together increased non-inflammatory alveolar macrophage numbers in mice with RvD2 administration and reduced IL-23 preventing excessive inflammation, provides evidence for improved bacterial clearance and inhibition of chronic inflammatory response.

SUMMARY AND CONCLUSIONS

In this study, we uncovered some underlying functions of resolvin D2 in a model of infection that captures different phases of sepsis. We showed how resolvin D2 improved oxidative burst in splenic neutrophils and also increased neutrophils in spleen to improve killing of bacteria in spleen. Also, resolvin D2 improved accumulation of MDSCs in spleen to prevent excessive inflammation in spleen. Also, we showed evidence that resolvin D2 improved migration of MDSCs from bone marrow to spleen to actively prevent excessive inflammation in spleen. Increased anti-microbial activity of splenic neutrophils and immunosuppression by MDSCs in spleen improved spleen-mediated bacterial clearance in blood. Also, we showed that resolvin D2 increased alveolar macrophages and reduced pro-inflammatory cytokine IL-23 which are indicators of increased bacterial clearance and reduced inflammation respectively. Resolvin D2 synthesized by immune cells not only affected host immune system but also directly affected virulence gene expression in *Pseudomonas aeruginosa*. All these immunomodulatory and anti-microbial functions of resolvin D2 improved survival in our mouse model of infection. However, downstream signaling of resolvin D2 binding to its receptor GPR18 remains unclear. Lack of understanding of the resolvin D2-GPR18 signaling pathway significantly raised more questions. Despite those questions, it is evident that resolvin D2 modulates immune response by preventing hyperinflammation and simultaneously improving host defense through anti-microbial activity. This makes resolvin D2 a possible ideal therapeutic candidate

where both persistent hyperinflammation and infection are an issue as observed in medical conditions like sepsis.

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ABBREVIATIONS:

AM	Alveolar macrophages
ATCC	American type culture collection
ACK	Ammonium-Chloride-Potassium
ANNOVA	Analysis of variance
CLP	Cecal ligation and puncture
CD	Cluster of differentiation
CDC	Center for disease control and prevention
COVID-19	Coronavirus disease – 19
CXCL	C-X-C motif containing chemokine ligand
CXCR	C-X-C motif containing chemokine receptor
CRP	C-reactive protein
cDNA	Complementary deoxyribonucleic acid
CT	Cycle threshold
CFU	Colony forming unit
DC	Dendritic cells
DHA	Docosahexaenoic acid
DAMP	Damage-associated molecular pattern
DHQ	Dihydroxyquinoline
DHR	Dihydroxyrhodamine
DNA	Deoxyribonucleic acid
EPA	Eicosapentaenoic acid

EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
fMLP	Formyl-methionine-leucyl-phenylalanine
FPR	Formyl peptide receptor
GPR18	G-protein coupled receptor 18
GMP	Granulocyte Monocyte Progenitor
G-CSF	Granulocyte colony stimulating factor
G-MDSC	Granulocyte myeloid-derived suppressor cell
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPI	Glycosylphosphatidylinositol
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
HAQ	4-hydroxy-2-alkylquinoline
HAA	(3-(3-hydroxyalkanoyloxy) alkanoic acid
HRP	Horseradish peroxidase
HBSS	Hank's balanced salt solution
IL	Interleukin
ICAM	Intercellular adhesion molecule
IFN	Interferon
Ly6	Lymphocyte antigen 6 complex
LPS	Lipopolysaccharide
LB	Luria Bertani

LxA4	Lipoxin A4
LOX	Lipoxygenase
MDSC	Myeloid-derived suppressor cell
MPP	Multipotent progenitors
M-MDSC	Monocytic myeloid-derived suppressor cell
MCP-1	Monocyte chemoattractant protein-1
MyD88	Myeloid differentiation primary response 88
miR	Micro ribonucleic acid
MFI	Median fluorescence intensity
MIP-2	Macrophage inflammatory protein 2
NK	Natural killer cells
NIAID	National institute of allergy and infectious disease
NAGly	N-arachidonylglycine
NETs	Neutrophil extracellular traps
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa light chain enhancer of B cell
OD	Optical density
PUFA	Polyunsaturated fatty acids
PMN	Polymorphic neutrophil
PRR	Pattern recognition receptor
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline

PMT	Photomultiplier tube
PPE	Personal protective equipment
PA	*Pseudomonas aeruginosa*
PD-1	Programmed cell death protein 1
QS	Quorum sensing
RvD2	Resolvin D2
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
RPM	Revolutions per minute
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SPM	Specialized pro-resolving mediator
SiglecF	Sialic acid-binding Ig-like lectin F
SEM	Standard error of the mean
SD	Standard deviation
ssRNA	Single-stranded ribonucleic acid
THC	tetrahydrocannabinol
TLR	Toll-like receptor
TGF-β	Transforming growth factor β
T-reg	Regulatory T cells
Tc Cell	Cytotoxic T cell
TNF-α	Tumor necrosis factor α

TSA	Tryptic soy agar
VCAM	Vascular cell adhesion molecule
17S-HpDHA	(17S-hydroperoxy-4Z,7Z,10Z,13Z,15E,19Z-docosahexaenoic acid)

ATTRIBUTES:

Ceca-ligation and puncture surgeries were performed by Dr. Kingsley Yin (KY) and Jean Walker (JW) and mice monitoring after surgery was performed by Prem Yugandhar Kadiyam Sundarasivarao (PYKS), Julianne Thornton (JT), and Rachael Wilson (RW). Resolvin D2 required for all the experiments was synthesized by Dr. Bernd Spur and Dr. Ana Rodriguez. Tail vein injections and intranasal administration of *Pseudomonas aeruginosa* was performed by Dr. Kingsley Yin and Jean Walker. Isolation of cells, Immunostaining, measurement of cytokines, RNA extraction, cDNA preparation, nanospectrophotometer measurements, real-time PCR experiments, oxidative burst assays, ELISA and flow cytometric analysis of cells and cytokines were done by me (PYKS). Also, analysis of flow cytometric data, development of gating strategy, and preparation of figures including Figures (1,3,4,5,6,7,8,9,10,11,12,13,15,17, and 18) were done by me (PYKS). Figures (3,4,5,6,7,8,9,10,13,14,15,16,17) are all published in (Sundarasivarao et al., 2022) and Figure 12 was published in (Walker et al., 2022). Figure 11 and Figure 18 are unpublished.

Figure 2 was taken from NIH database of chemical structures https://pubchem.ncbi.nlm.nih.gov/compound/Resolvin-D2

For the work on virulence gene expression, JT cultured *Pseudomonas aeruginosa* and performed treatments and I extracted RNA, prepared cDNA, measured purity of RNA preparation using nanospectrophotometer, performed real-time PCR, and completed data analysis for Figure (19). Also, part of the data corresponding to expression of

pqsA and rhlA in Figure 19 was published in (Thornton et al., 2021). Bacterial clearance in blood and lungs, and mice survival were calculated by Dr. Kingsley Yin and Jean Walker. Also, the figures (14,16,20) were made by Dr. Kingsley Yin. Figures 14 and 16 were published in (Sundarasivarao et al., 2022) and Figure 20 was published in (Walker et al., 2022).