Specialized Pro-resolving Mediators Act on the Innate Immune System to Attenuate Pulmonary Inflammation

by

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Dedication

This dissertation is dedicated to Ned Ballatori, whose unwavering strength, integrity, and leadership were matched only by his kindness and the love he bore for his students.

Biographical Sketch

Amanda Croasdell was born in San Diego, California on October 14, 1988. She grew up in Albuquerque, New Mexico before attending college at Utah State University in Logan, Utah. She graduated *cum laude* in May 2010 with a Bachelor of Science degree in Biochemistry and a minor in English. In August 2010, she began doctoral studies in Toxicology at the University of Rochester and was named the Incoming Toxicology Scholar. Amanda joined the labs of Drs. Richard P. Phipps and Patricia J. Sime in May 2011. Amanda was awarded a Toxicology Training Fellowship from 2011-2013, a Predoctoral Fellowship from the PhRMA Foundation from 2013-2015, and a Lung Biology Training Fellowship from 2015-2016. She was also the recipient of a Clinical and Translational Science Institute pilot grant and the William F. Neuman Award of Exemplary Scholarship and Citizenship, both in 2014. Amanda received her Master of Science degree from the University of Rochester in December 2013.

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Croasdell A, Lacy SH, Owens K, Thatcher TH, Sime PJ, Phipps RP. *Resolvin D1 Dampens Pulmonary Inflammation and Promotes Nontypeable Haemophilus Influenzae Clearance.* Society of Toxicology Annual Meeting. Mar. 2016, New Orleans, LA.

Croasdell A, Thatcher TH, Lyda E, Kottmann RM, Sime, PJ, Phipps RP. *Resolvins Act Therapeutically to Promote a Pro-Resolving Phenotype in Human Macrophages*. Lung Biology Research Day. Oct 2015, Rochester, NY.

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Croasdell A, Hsiao H, Thatcher TH, Serhan CN, Sime PJ, Phipps RP. *Specialized Pro-resolving Mediators Act Through Macrophages To Attenuate Cigarette Smoke-Induced Inflammation.* American Thoracic Society International Meeting. May 2013, Philadelphia, PA.

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Finally, I owe immeasurable gratitude to my parents, my family, and Scott Lucchini for their love, encouragement, and optimism in my graduate career and life.

Abstract

Toxic stimuli, such as cigarette smoke, air pollution, and infection, can disrupt the normal functions of immune cells, resulting in dysregulated and excessive inflammatory responses. This leads to chronic inflammation and diseases such as chronic obstructive pulmonary disease (COPD). Recently discovered specialized pro-resolving mediators (SPMs) play a critical role in the active resolution of inflammation by suppressing pro-inflammatory actions and promoting resolution pathways. In the lung, macrophages are critical for mediating inflammation and promoting resolution, as these cells can produce pro- and anti-inflammatory cytokines, clear apoptotic neutrophils and bacteria, and promote wound healing. Based on their important role in mediating resolution, **I hypothesized that SPMs will act on monocytes and macrophages to attenuate pulmonary inflammation and promote resolution.**

Cigarette smoke is a major inducer of pulmonary inflammation, and acts on macrophages to promote pro-inflammatory cytokine release, activate NF-κB signaling, and impair phagocytosis. In my studies, human macrophages that were treated with D-series resolvins (RvDs) prior to cigarette smoke exposure had suppressed production of pro-inflammatory cytokines, enzymes, and oxidative stress. RvDs also restored cigarette smoke-induced defects in phagocytosis. These actions were mediated through modulation of NF-κB expression. SPMs can also dampen inflammatory responses due to other stimuli, such as lipopolysaccharide, which signals through activation of toll-like receptor 4 (TLR4). RvD2 dampened TLR4-driven cytokine production and reduced expression of

TLR4 and downstream molecules in THP-1 cells and human monocytes. These decreases were mediated by RvD2-induction of regulatory microRNAs.

The lung is particularly susceptible to bacterial infection, and COPD patients often experience exacerbations resulting from infection by pathogens such as Nontypeable *Haemophilus influenzae* (NTHi). Using a model of NTHi infection in C57BL/6 mice, RvD1 modified the immune cell profile and reduced pro-inflammatory cytokine production. Importantly, RvD1 treated mice had reduced NTHi bacterial load, with this clearance dose-dependently mediated by enhanced phagocytosis from M2 macrophages. RvD1 further improved the overall health of the mice by preventing weight loss, hypothermia, altered respiratory physiology, and hypoxemia. Overall, my data show that SPMs are effective in attenuating pulmonary inflammation in a variety of inflammatory and infection models through their actions on the innate immune response.

Contributors & Funding Sources

This work was supervised by a dissertation committee consisting of Drs. Richard P. Phipps (advisor), Patricia J. Sime (co-advisor), and M. Kerry O'Banion from the Department of Environmental Medicine and Dr. Terry W. Wright from the Department of Microbiology and Immunology. Chapters 2 and 4 have been published as listed in the Biographical Sketch. Chapter 3 has been submitted for publication and is in press. The work in this thesis was conducted by the author with the following exceptions: Drs. Romain Colas, Jesmond Dalli, and Charles Serhan generated and analyzed the mass spectrometry data in Fig. 2.1. Lindsay Zehr, Elizabeth Lyda and R. Matthew Kottmann assisted in bronchoalveolar lavage collection for Figs. 2.1-2.2 and Table 2.1. Ann Casey and the University of Rochester Medical Center Blood Bank provided phlebotomy assistance for Chapters 2 and 3. Jian Dong-Li provided the NTHi specimen used in Chapter 4. Parker Duffney, Shannon Lacy, Claire McCarthy, and Kristina Owens assisted in mouse specimen collection for Chapter 4. Samir Bhagwat, Frank Gigliotti, and Terry Wright provided training and equipment for the respiratory measures in Figs. 4.15-4.16. This work was supported by NIEHS T32ES007026, NIH P30ES01247, NIH R01HL120908, NIH T32HL066988, the National Center for Research Resources (UL1RR024160, 8UL1TR000042), and the PhRMA Foundation.

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List of Abbreviations

ALX/FPR2	lipoxin A_4 receptor
ANOVA	analysis of variance
BALF	bronchoalveolar lavage fluid
COPD	chronic obstructive pulmonary disease
Cox-2	cyclooxygenase-2
CSE	cigarette smoke extract
DHA	docosahexaenoic acid
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EPA	eicosapentaenoic acid
EtOH	ethanol
GPR	G-protein coupled receptor
HA	hyaluronan
IL	interleukin
LBP	LPS-binding protein
LPS	lipopolysaccharide
Mϕ	macrophage
MCP-1	monocyte chemoattractant protein-1
MD-2	lymphocyte antigen 96
miR	microRNA
MyD88	myeloid differentiation primary response gene 88

NF-κB	nuclear factor kappa-light-chain-enhancer of B cells
NTHi	Nontypeable *Haemophilus influenzae*
PG	prostaglandin
PMN	polymorphonuclear leukocyte
Poly I:C	polyinosinic:polycytidylic acid
ROS	reactive oxygen species
Rv	resolvin
SPM	specialized pro-resolving mediator
TAK1	transforming growth factor beta-activated kinase 1
TGF-β	transforming growth factor beta
TLR	toll like receptor
TNF	tumor necrosis factor
TRIF	TIR-domain-containing adapter-inducing interferon-β

Chapter 1

Introduction & Background[1]

[1]This work was published in part as "Croasdell A, Duffney PF, Kim N, Lacy SH, Sime PJ, Phipps RP. *PPAR-gamma and the Innate Immune System Mediate the Resolution of Inflammation.* PPAR Res. 2015;2015:549691. Epub 2015 Dec 2" and is reprinted with their permission.

Inflammation & Resolution

The hallmarks of inflammation were identified as far back as the 1st century, but the identification of and importance of the resolution phase is a much more recent development. Inflammation is broadly defined as the accumulation of fluid, plasma proteins, and white blood cells that is initiated by physical injury, infection, or a local immune response. Generally a protective response, inflammation occurs as a reaction to external forces (like twisting an ankle), toxic exposures, or pathogenic infections. Inflammation is characterized by four hallmarks: rubor/redness (due to vasodilation), calor/heat (due to production of pyrogens and increased blood flow), tumor/swelling (due to edema and cellular influx), and dolor/pain (due to production of molecules that sensitize nerve endings). These actions are broad ranging and occur to various extents in response to different stimuli. Initial inflammatory responses are mediated by the innate immune system, with secondary responses coming from the adaptive immune response.

While these key components of inflammation occur to mitigate invasive threats, if left unchecked inflammation results in loss of function, cellular dysregulation, and disease. Therefore, there exist endogenous, active mechanisms for the resolution of inflammation, which allow for dampening of pro-inflammatory responses and a shift to wound healing, repair, and a return to homeostasis. Pro-resolving actions are distinct from anti-inflammatory processes; while anti-inflammatory molecules and medications act to dampen and suppress pro-inflammatory cells and signals, resolution represents a phenotypic shift in immune cell function towards repair and homeostasis. The initiation, progression, and resolution of inflammation are characterized by several distinct phases.

First, there is an end to the production of pro-inflammatory cytokines and a cessation of inflammatory neutrophil influx. Second, neutrophils present at the inflammatory site undergo apoptosis. Third, macrophages demonstrate a phenotypic switch and enhanced efferocytosis of apoptotic cells; the second and third phases coincide with increased production of anti-inflammatory and pro-resolving molecules. Finally, there is a clearance of macrophages, promotion of wound healing, and tissue repair to mediate the end of the inflammatory response (1). These phases frequently overlap and the specific aspects of resolution can vary based on the inflammatory stimuli, organ location, and individual host characteristics (Fig. 1.1).

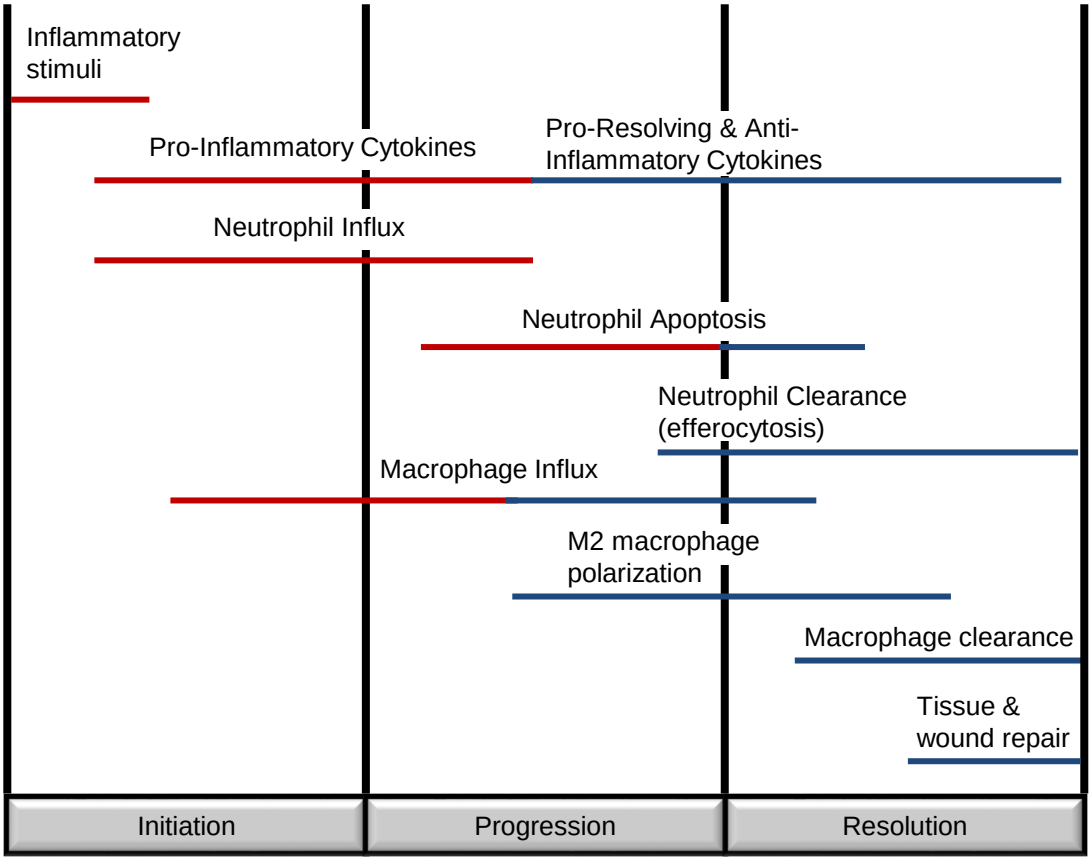

Fig. 1.1. Inflammation and resolution are active and dynamic processes. The initiation, progression, and resolution of inflammation are characterized by unique cellular signals and cell trafficking. Red lines indicate inflammatory events, while blue lines indicate resolving events.

Specialized Pro-Resolving Mediators Promote the Resolution of Inflammation

Resolution is mediated by many different proteins and lipids, which can be produced by a variety of cell types. Recently, particular interest has been paid to the role of endogenous, lipid-derived, specialized pro-resolving mediators (SPMs). A growing body of evidence shows that these mediators play a critical role in the active resolution of inflammation by suppressing pro-inflammatory actions and promoting alternative resolution pathways (1). SPMs are formed by enzymatic oxygenation of polyunsaturated fatty acids. Diets high in omega-3 and omega-6 fatty acids have long been thought to have anti-inflammatory benefits, and increases in omega supplements have demonstrated beneficial effects in several inflammatory disease states, including COPD (2-4). Arachidonic acid, an omega-6 fatty acid, is typically associated with the production of pro-inflammatory prostaglandins and thromboxanes via induction of cyclooxygenase 2 (Cox-2) and leukotrienes via the induction of 5-lipoxygenase. Arachidonic acid can also be metabolized via 15-lipoxygenase or 12-lipoxygenase to lipoxins, which have anti-inflammatory and pro-resolution properties. Lipoxin A_4 (LxA_4) was the first SPM discovered (5). Subsequently it was shown that two omega-3 fatty acids—eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)—can be metabolized into the resolvins of the E series (RvE), and resolvins of the D series (RvD), protectins (PD), and maresins (Ma), respectively (Fig. 1.2, Fig. 1.3) (6). These omega-3 derived SPMs can be produced through the actions of 5-lipoxygenase and 15-lipoxygenase; alternatively, aspirin-triggered epimers are produced in combination with Cox-2 activity (7).

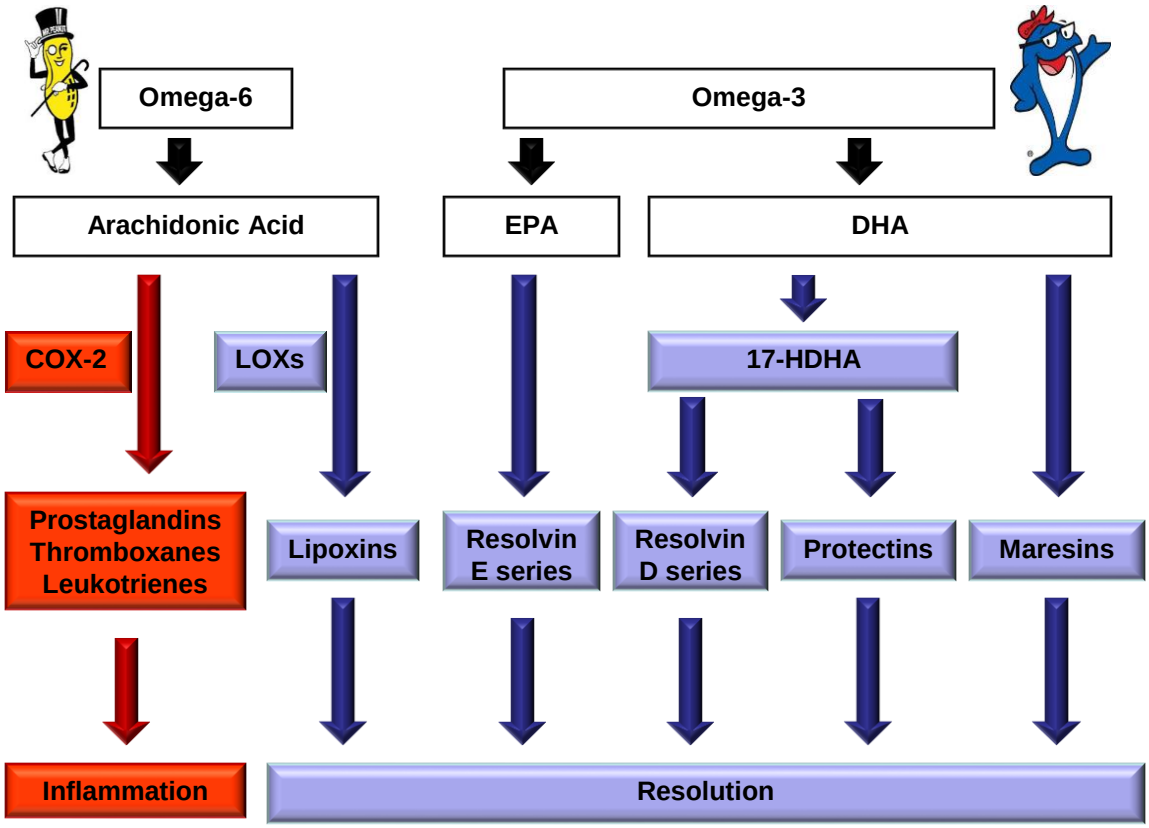

Fig. 1.2. Specialized pro-resolving mediators are metabolized from omega-3 and -6 fatty acids. Omega-6 fatty acids (such as arachidonic acid) and omega-3 fatty acids (such as EPA and DHA) are enzymatically metabolized to produce intermediates and lipid-derived mediators, including lipoxins, resolvins, protectins, and maresins.

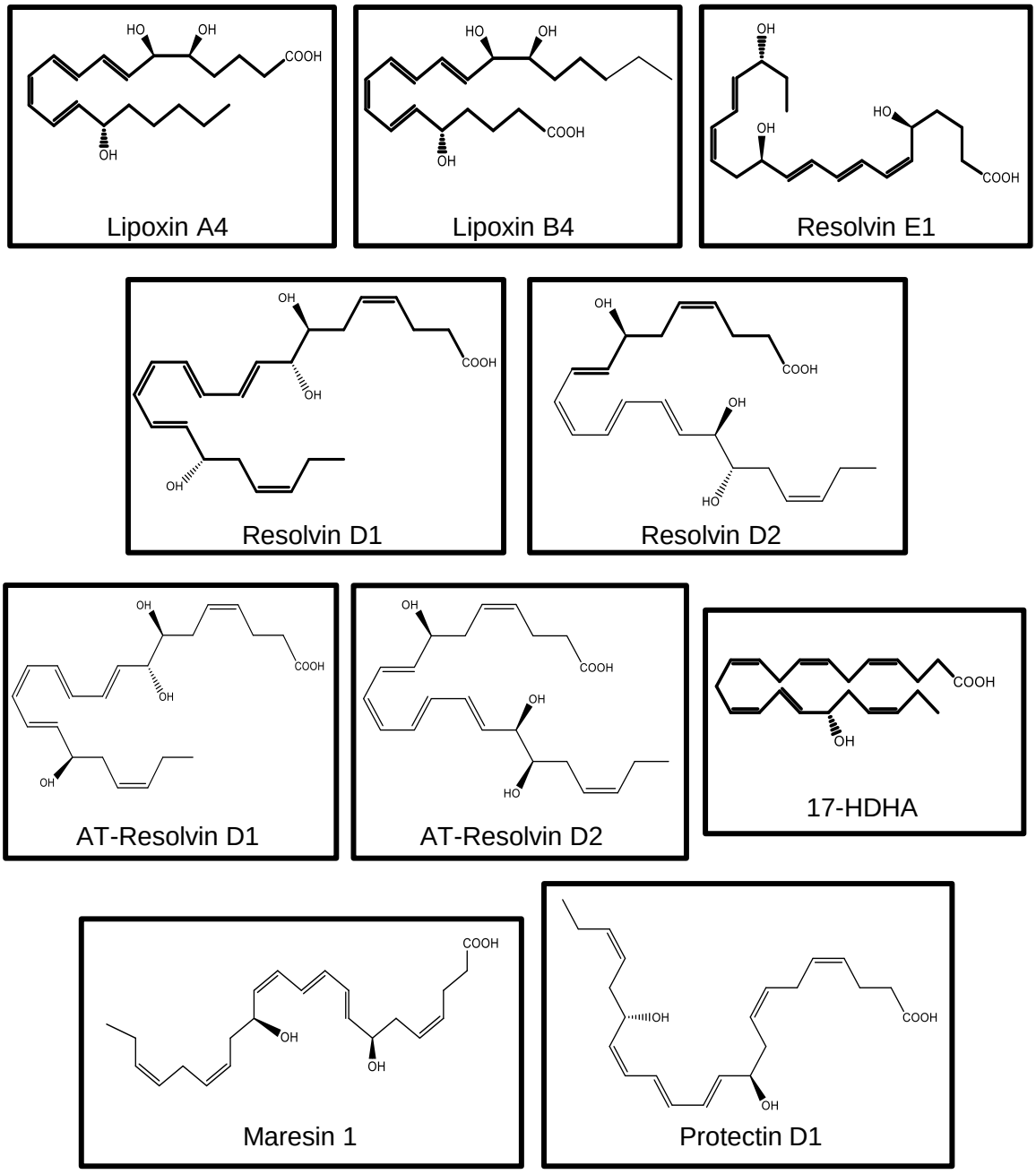

Fig. 1.3. Structures of select specialized pro-resolving mediators.

SPMs are synthesized by macrophages, platelets, neutrophils, epithelial cells, endothelial cells, and fibroblasts. SPMs are endogenously produced via transcellular synthesis, whereby one cell type converts the omega fatty acids to intermediate molecules, transfers these intermediates to a second cell type, and the second cell metabolizes the intermediate to the final SPM molecule. As an example, phospholipases cause release of fatty acids from membrane storage of multiple cell types, including arachidonic acid. Arachidonic acid is converted by 15-lipoxygenase by epithelial cells, macrophages, basophils, and other cells and then undergoes further hydrolysis by neutrophils and PMNs. Alternatively, neutrophils can produce leukotriene A, transfer LTA to platelets, and platelets then convert LTA to LxA_4. While much is still unknown about this process, it is hypothesized that intermediates are packaged into microparticles to facilitate transfer between cells. SPMs have been identified in nanomolar concentrations in the human lung, synovial fluid, and tonsils and can be produced by a number of cell types, including macrophages (1, 5, 8, 9).

Once synthesized and secreted, SPMs act through newly identified G-protein coupled receptors (GPCRs) to mediate their effects (Fig. 1.4) (10). Five primary receptors have been identified so far (11-13). G-protein coupled receptor (GPR) 32 and GPR18, previously orphan receptors, have been recently shown to bind RvD1 and RvD2, respectively (12, 14). These receptors have been identified on human leukocytes, including macrophages (12-14). Chemokine Receptor 23 (ChemR23) and leukotriene receptor B1 (BLT1) have both been shown to bind RvE1, with RvE1 binding with higher affinity to ChemR23 in all identified cells except neutrophils (15, 16). The most well

characterized receptor is the Lipoxin A_4 receptor/Formyl Peptide Receptor 2 (ALX/FPR2) (17). This receptor is a member of the formyl peptide receptor family and has been identified in neutrophils, monocytes, T and B cells, fibroblasts, and endothelial cells (17-23). While LxA_4 has the highest stereoselective binding for this receptor, ALX/FPR2 has been shown to bind other lipoxin derivatives, in addition to RvD1, RvD2, and other small inflammatory peptides (13, 14, 24, 25). For those SPMs that bind to multiple receptors (i.e. RvD1), there appears to be dose-dependency in receptor selectivity. In one study using human neutrophils, low dose RvD1 was sensitive to GPR32 blockade, while higher dose RvD1 was sensitive to ALX/FPR2 blockade (26). These receptors, particularly ALX/FPR2, may be promiscuous in their signaling mechanisms, with downstream effects dependent on ligand and cell-specificity. In monocytes particularly, ALX/FPR2 binding triggers intracellular calcium release, whereas in polymorphonuclear cells it releases arachidonic acid and blocks production of inositol 1,4,5-triphospate (18, 25).

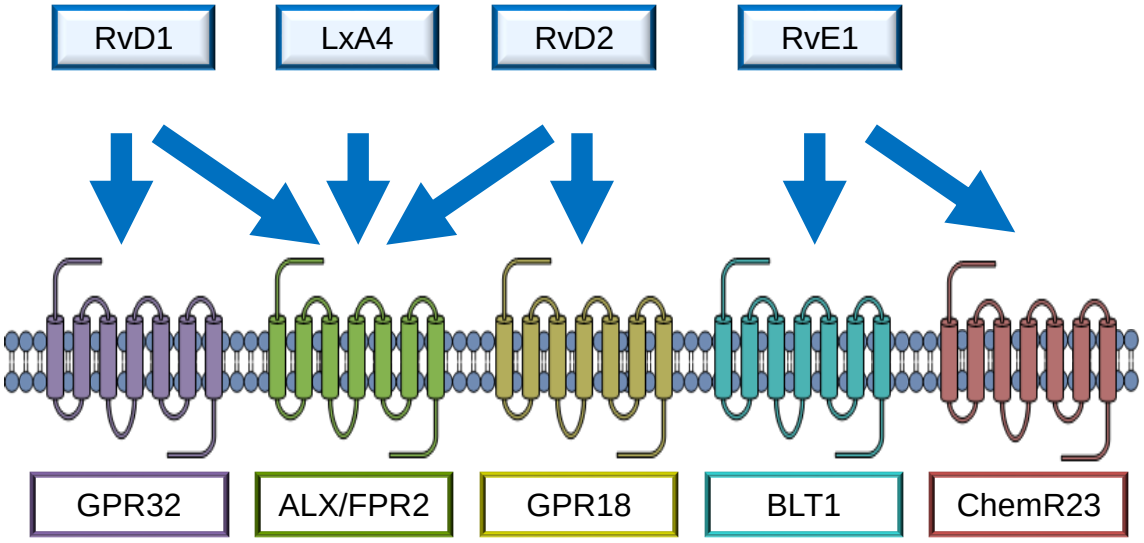

Fig. 1.4. Specialized pro-resolving mediators signal through GPCRs. Five canonical SPM receptors have been identified which specifically bind different SPMs. GPR32 (RvD1), ALX/FPR2 (LxA_4, RvD1, RvD2), GPR18 (RvD2), BLT1 (RvE1), and ChemR23 (RvE1) are all g-protein coupled receptors and have all been identified in human monocytes/macrophages.

Macrophages & the Role of Innate Immunity in Resolution

Many aspects of the resolution of inflammation are mediated by the innate immune system. The innate immune system is comprised of a collection of cells, including neutrophils, macrophages, dendritic cells, eosinophils, basophils, platelets, and natural killer cells. Neutrophils, platelets, and macrophages are the first responders to inflammatory stimuli and are also the first cells to begin to signal the resolution process. Macrophages and monocytes are critical leukocytes involved in multiple stages in the inflammatory process. These cells are responsible for initiating an immune response which triggers cytokine and chemokine production, phagocytosis of pathogens, killing of infected and injured cells, and activation of the adaptive immune response. Monocytes and macrophages play active roles in both acute and chronic inflammation, as well as in the resolution phase. During resolution, non-phylogistic macrophages are anti-inflammatory and play roles in phagocytizing apoptotic cells and bacteria (27).

Monocytes make up 4%-11% of white blood cells found in the bloodstream (28). These cells are bone marrow derived and circulate in the bloodstream, typically living for several days. Monocytes express higher CD14 and lower CD11b and MHC antigens than differentiated macrophages. Comparatively, macrophages can be derived from monocytes under inflammatory stimuli or may be resident in tissue or interstitial spaces. These cells are larger and contain more granules. Macrophages have greater heterogeneity than monocytes with a longer lifespan (ranging from several weeks to months, with rare cases of years). Furthermore, macrophages possess some self-renewal capacities, unlike monocytes.

Recent studies have begun to distinguish between two broad classes of macrophages: classically activated (M1) and alternatively activated (M2) macrophages (Fig. 1.5) (29). Classically activated macrophages are associated with a pro-inflammatory phenotype. These cells are activated by a number of traditional inflammatory stimuli, including interferon gamma (IFNγ) and lipopolysaccharide (LPS) (27, 30). M1 macrophages produce numerous pro-inflammatory cytokines and chemokines, have increased levels of inducible nitric oxide synthesis (iNOS), and demonstrate enhanced abilities to kill intracellular pathogens. There are many markers used to identify M1 markers, but several of the most common include iNOS, tumor necrosis factor alpha (TNFα), and CD80. M2 macrophages are characterized by their roles in the resolution phase of inflammation and in tissue repair. M2 macrophages are identified by increased expression of CD206 (mannose receptor-1, or MRC-1) and arginase-1 (Arg1). M2 macrophages can be further subdivided into M2a, M2b, and M2c, with different stimuli polarizing each group (27, 30). M2a macrophages, stimulated by IL-4 or IL-13, downregulate several apoptotic proteins, prevent pro-inflammatory cytokine release, and can promote fibrogenesis. M2b macrophages, stimulated by a combination of a classical pro-inflammatory molecule such as LPS or IL-1β along with an Fc-recognized immune complex, are not entirely pro-resolving, but produce a combination of pro- and anti-inflammatory cytokines and can prolong the adaptive immune response by stimulating Th2 cells. M2c macrophages are stimulated by IL-10, TGFβ, or glucocorticoids and are primarily characterized by decreased production of pro-inflammatory cytokines and increased production of IL-10 and TGFβ. However, there is still dispute about the

physiological relevance of each of these M2 subgroups, and thus M1 vs. M2 broad classifications are more established and commonly studied.

Further complicating these studies is the fact that macrophages are not permanently polarized, but can be shifted from one polarization state to another (30). There is a growing interest in the correlation between macrophage phenotype and macrophage origin, with some evidence that monocyte-derived macrophages are predisposed to an M1 phenotype and alveolar, or resident, macrophages are disposed to an M2 phenotype. Along with these major macrophage classes, there are numerous specialized macrophage groups identified by high or low expression of a particular cell surface marker, such as pro-resolving macrophages, which are characterized by low expression of CD11b (31, 32).

Many SPMs have been detected in human blood, serum, and lymphoid tissue, areas with high populations of monocytes and macrophages. Additionally, macrophages in different tissues (including liver, lung, and brain) assist in production of lipoxins, resolvins, protectins, and maresins. Maresins were first identified as macrophage-derived compounds and were named specifically for this cellular origin (33). SPMs have also been shown to affect multiple aspects of macrophage function. SPMs promote phagocytosis of apoptotic cells in mouse and human inflammatory disease models, a finding corroborated in our lab (34-37). In addition, SPMs have been shown to inhibit production of pro-inflammatory cytokines by several different inflammatory stimuli *in vivo* (38-40). In several different *in vivo* models of bacterial infection—including pneumonia, peritonitis, and microbial sepsis—resolvins enhanced bacterial clearance,

inhibited leukocyte trafficking, and resulted in an increased mouse survival rate (41-43). These findings are key in supporting the role of SPMs in not just acting as anti-inflammatory mediators, but enhancing the resolution capabilities of macrophages. LxA_4 and its synthetic analogs have even been shown to prevent apoptosis of monocytes and macrophages, allowing these cells to clear cellular debris and shift to a resolution phenotype (36, 44, 45). Many of these effects, though, have been demonstrated in mouse models or using mouse and human cells lines, and need further investigation using human cells. Additionally, there are variable responses between macrophages resident in different tissues; therefore, further studies specific to pulmonary inflammation are warranted.

SPMs have been shown to decrease production of pro-inflammatory cytokines, which may indirectly cause a shift in macrophage phenotype. Resolvins and lipoxins have been shown to promote M2 macrophages in mouse models, particularly in obesity and in cardiac models of inflammation (46-48). Furthermore, M2 macrophages may produce higher levels of SPMs (49). Additionally, our lab has shown that SPMs can promote M2 macrophages *in vivo*; alveolar macrophages (AMs) isolated from mice treated with RvD1 and subsequently exposed to cigarette smoke had increased levels of M2 mRNA markers arginase 1 (Arg1) and mannose receptor 1 (MRC1), as well as increased IL-10 production. M1 vs M2 phenotypes may also have differential expression of SPM receptors (50). However, the ability of SPMs to promote an M2 phenotype in human macrophages remains uncharacterized.

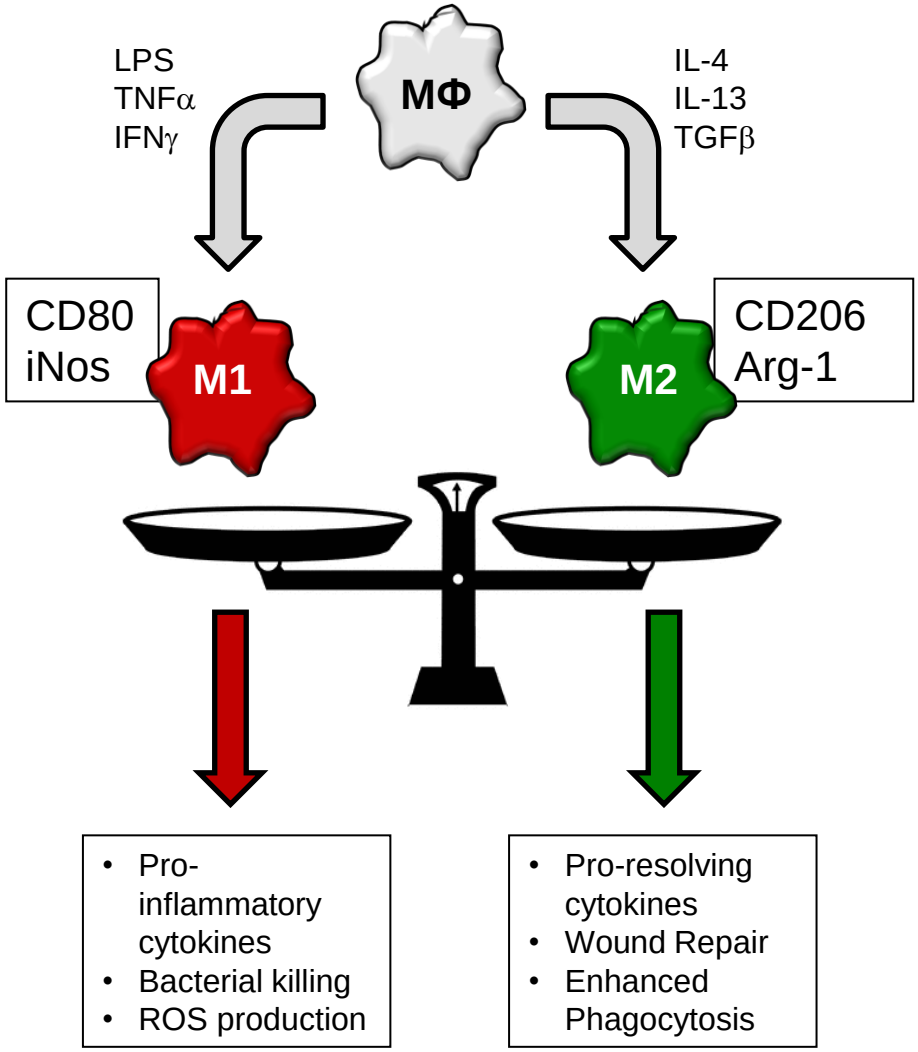

Fig. 1.5. Macrophages can be polarized to M1 (classical) or M2 (alternative) phenotypes. Pro-inflammatory stimuli such as LPS and IFNγ promote M1 cells, characterized by CD80 and iNOS expression. M1 macrophages produce pro-inflammatory cytokines and reactive oxygen species and activate other innate immune cells. Alternative stimuli such as TGFβ and IL-4 promote M2 cells, characterized by CD206 and Arg-1 expression. These cells have roles in phagocytosis and wound healing.

Cigarette Smoke & Pulmonary Inflammation

The lung is particularly susceptible to inflammation. Many toxicants, such as air pollution, cigarette smoke, and nanoparticles, are inhaled and directly target the airways. Many bacteria and viruses are also transmitted through respiratory droplets. Because of the structure of the respiratory system, these insults do not undergo first-pass metabolism or initial neutralization and can infiltrate to the bronchi, bronchioles, and alveoli. While the respiratory system has defenses (such as cilia that filter air in nasal passages, a mucociliary escalator to clear deposits, and high populations of resident immune cells) in place to prevent exposures, bacteria, virus, and particles can still cause damage and disease. In addition to outside stimuli, the lung is also a major target for autoimmune diseases, such as asthma.

A major source of pulmonary inflammation is cigarette smoke. Cigarette smoke is currently the leading cause of preventable mortality in the United States, accounting for 1 in 5 deaths, as well as over $193 billion in health care costs and loss of productivity annually (51). Cigarette smoke exposure is the major risk factor for chronic obstructive pulmonary disease (COPD) and can cause multiple cancers, including lung cancer, laryngeal cancer, and acute myeloid leukemia (51, 52). COPD is primarily comprised of chronic bronchitis (narrowing of the airways due to swelling) and emphysema (destruction of the alveoli), both of which have strong inflammatory components (52, 53). Cigarette smoke exposure is also associated with an increased susceptibility to bacterial infections, such as those from Nontypeable *Haemophilus influenzae* and *Streptococcus pneumoniae* (52). Despite the prevalence of COPD and other smoking-

associated diseases, there exist few therapeutics for these chronic diseases. Bronchodilators and steroids are the most commonly used treatments to alleviate COPD symptoms, however, they do not halt disease progression and have limited efficacy. Some studies suggest that steroids only alleviate symptoms of COPD patients with a co-morbidity of asthma. Additionally, use of steroids can lead to immunosuppression and thereby increase the risk of exacerbations resultant from viral and bacterial infections. With over 45 million smokers in the U.S. alone, better therapeutics are critically needed.

Underlying many cigarette smoke-induced disease states is chronic inflammation. Macrophage activation specifically has been implicated in the pathogenesis of diseases associated with smoking, such as COPD (52-55). Activation of macrophages by cigarette smoke promotes tissue destruction and mucus production (53, 56). Additionally, macrophages are increased in emphysematous lungs, and have increased proteinase activity, which could lead to alveolar damage (53, 56). Cigarette smoke exposure increases macrophage synthesis of pro-inflammatory cytokines and proteins along with altering levels of maintenance enzymes such as matrix metalloproteinases (54, 57-60). Cigarette smoke further acts as a major inducer of oxidative stress; macrophages are one of the cells that mediate reactive oxygen species production and are susceptible to oxidative damage. Additionally, smoke exposure can impair macrophage phagocytosis, resulting in impaired bacterial clearance *in vivo* (55, 58, 61-64).

New work from our lab shows that cigarette smoke itself and COPD can alter normal SPM signaling. Exhaled breath condensate from normal subjects or COPD patients revealed altered SPM profiles, with altered production of SPM intermediates and

reduced LxA_4 and RvD1 in COPD patients (65). In lung tissue taken from COPD and non-COPD biopsies, COPD lung samples demonstrated overexpression of ALX/FPR2 and GPR32, indicative of dysregulated receptor feedback loops in an attempt to compensate for impaired resolution (66). These results were mirrored in a mouse model of emphysema, wherein mice exposed to cigarette smoke for twelve weeks had elevated ALX/FPR2 expression (66). These data, in combination with dysregulation of SPM production and SPM receptor expression seen in other pulmonary disease models, highlight the critical contribution of these mediators to organ homeostasis.

Signaling Pathways Important in Resolution & Inflammation

There are many signaling pathways critical to both inflammation and resolution, and exposure to cigarette smoke, pathogens, and other toxic stimuli can promote, suppress, or otherwise alter these pathways. One critical family of proteins that regulates inflammatory responses is the NF-κB family. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a family of proteins with both classical and alternative signaling pathways (67). The NF-κB family consists of multiple key proteins, notably NF-κB1 (p105), NF-κB2 (p100), RelA (p65), RelB, and c-Rel. Active proteins are formed from the cleavage of p105 to p50 and p100 to p52 subunits, which then form heterodimers with p65, RelB, and c-Rel. In canonical NF-κB inflammatory signaling, the inhibitory IκBα protein is phosphorylated and degraded, leaving the active dimer of p65 and p50 to translocate into the nucleus and bind to target DNA response elements, resulting in the synthesis of inflammatory mRNAs and proteins (67). An alternative NF-

κB pathway, though, results from the formation of a RelB/p52 dimer (68). This complex can also translocate to the nucleus and bind to DNA response elements, but instead results in decreased inflammatory mediator production and anti-inflammatory effects (Fig. 1.6).

SPMs have been shown in several models to alter NF-κB signaling. RvD1 and LxA_4 have been shown to act through inhibition of NF-κB activation and decreased phosphorylation of MAPK proteins, specifically ERK and PI3K (38, 40, 44, 69). Docosahexaenoic and D-series resolvins are particularly potent at decreasing p65 phosphorylation and/or translocation in macrophages, dendritic cells, and other innate immune system players (48, 70, 71). Our lab has previously observed that NF-κB is downregulated by RvD1 treatment in fibroblasts and epithelial cells (37, 72). Despite this growing body of evidence regarding SPM regulation of NF-κB activity, fewer studies have tested for these effects in human cells, including macrophages. No work has yet been done evaluating the effects of SPMs on the alternative NF-κB pathway.

In addition to NF-κB, numerous other pathways contribute to inflammation, including MAP kinases, interferons, and toll-like receptors (TLRs). TLRs are one of the essential components of the immune response, and are particularly critical in the innate immune response to invading pathogens. More than a dozen TLRs have been identified which respond to a variety of pathogens, including bacteria, viruses, and fungi, and damage-associated molecular patterns (DAMPs). These TLRs act through conserved pathways, signaling through myeloid differentiation factor 88 (MyD88) or TIR-domain-containing adapter-inducing interferon-β (TRIF) to induce an inflammatory response

(73). Many of these TLRs are expressed by human monocytes and macrophages, which are primary mediators of pathogen-induced inflammation (74). In particular, monocytes highly express TLR4, the primary TLR responsible for responding to LPS. CD14 and LPS-binding protein are responsible for presenting LPS to the TLR4-myeloid differentiation 2 (MD-2) complex to initiate TLR4 signaling (73, 75, 76). Both the MyD88 and TRIF/TRAF signaling pathways activates NF-κB, AP-1, and interferon elements and promote pro-inflammatory cytokine production, cell activation, and anti-microbial and anti-viral responses (Fig. 1.7) (73, 75, 76). Dysregulation of TLR signaling can contribute to the pathogenesis of multiple diseases, including asthma, COPD, and cystic fibrosis (56, 77, 78). Despite this evidence, little work has been done regarding the efficacy of SPMs in regulating TLR expression.

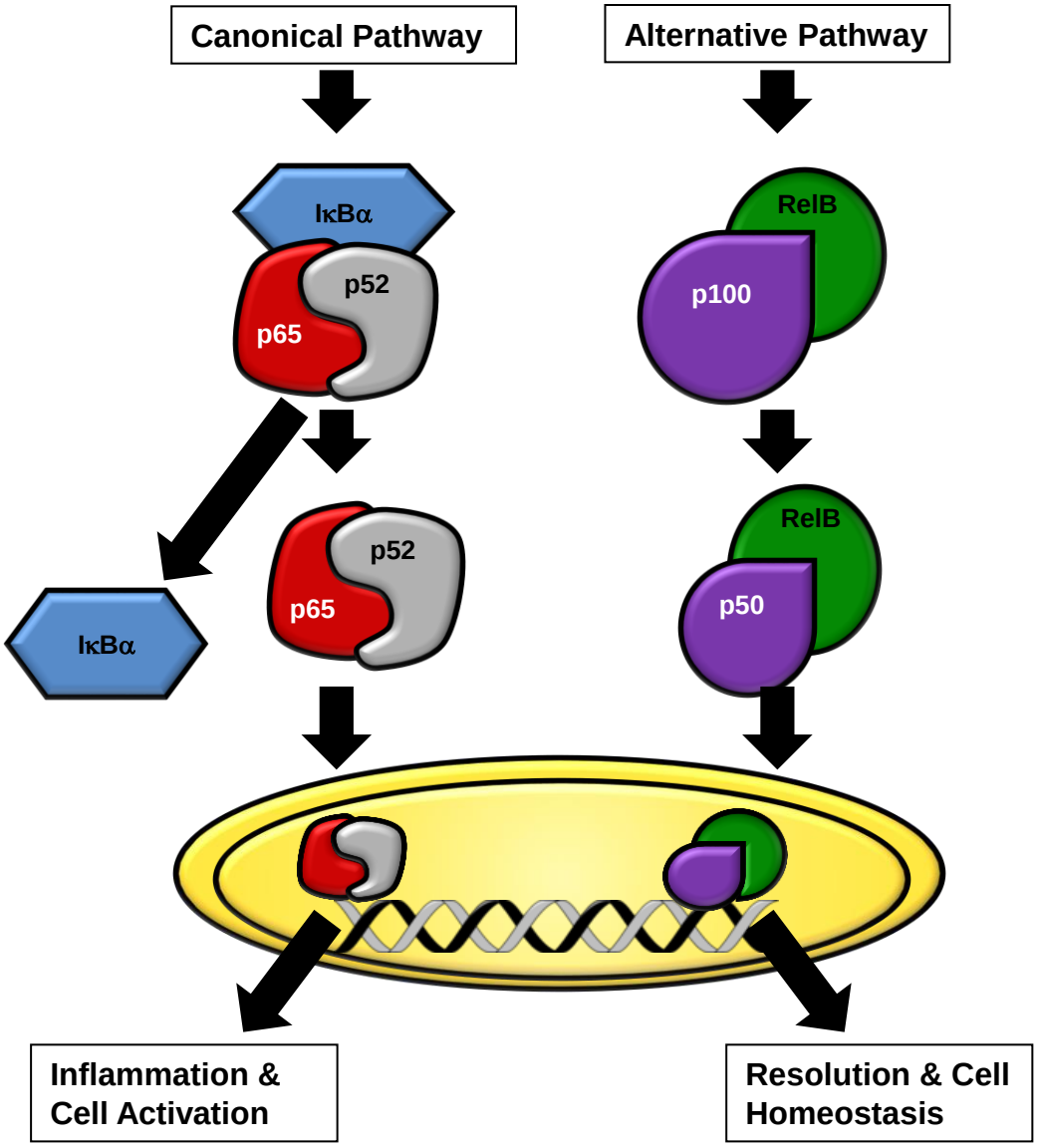

Fig. 1.6. Canonical and alternative NF-κB pathways contribute to inflammation and resolution. Pro-inflammatory stimuli activate the canonical NF-κB pathway. IκBα is phosphorylated, leading to its degradation and release of the p65/p52 complex. Phosphorylation of p65 and translocation into the nucleus leads to binding to NF-κB response elements, and subsequent initiation of inflammatory pathways. In contrast, phosphorylation of p100 allows for cleavage to p50, which forms an active complex with RelB. RelB/p50 similarly translocate to the nucleus and bind to NF-κB response elements, counteracting p65/p52 activation and/or promoting alternative anti-inflammatory pathways.

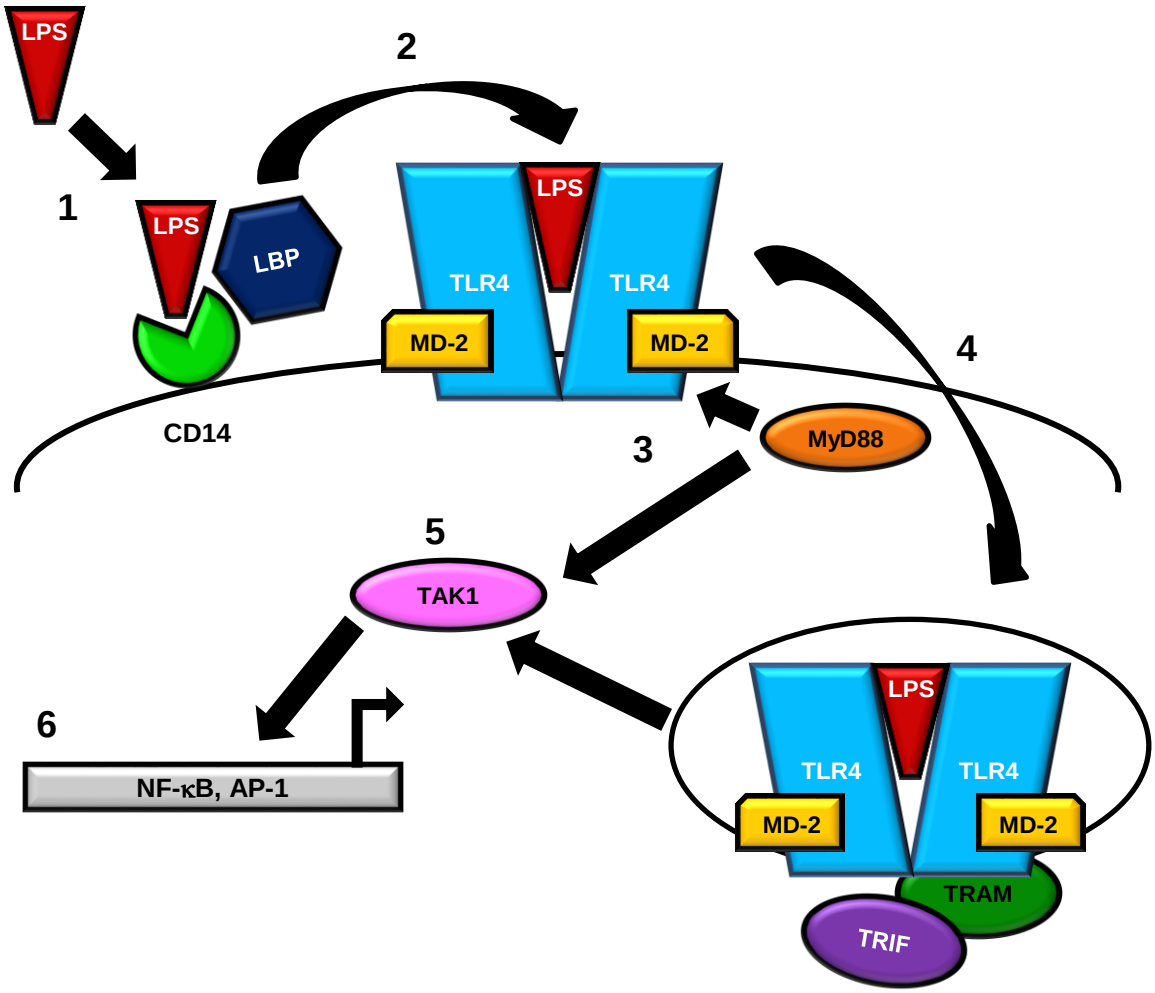

Fig. 1.7. LPS activates TLR4 signaling pathways. LPS is initially recognized by LPS-binding protein (LBP) and CD14 (1) and presented to the TLR4/MD-2 multimer. Upon TLR4 binding (2), MyD88 is recruited to the signaling complex (3). Additionally, LPS/TLR4/MD-2 can be internalized in endosomes, allowing for signaling through the TRIF/TRAF pathway (4). Both MyD88 and TRIF/TRAF act through activation of TAK1 (5), and subsequent activation of NF-κB, AP-1, and interferon signaling elements (6).

In summary, SPMs represent novel therapeutics with high clinical potential and important endogenous roles in promoting resolution. These eicosanoids act through cells of the innate immune system, among other cell types, to mediate their anti-inflammatory and pro-resolving effects. While a growing body of evidence supports the important contribution of SPMs to health and homeostasis, there still exist major knowledge gaps regarding their efficacy on human cells and in pulmonary diseases. In this thesis, I have sought to investigate the efficacy of SPMs in attenuating pulmonary disease by acting on the innate immune system. Chapter 2 of this work has focused on cigarette smoke as a primary inflammatory insult, and determined the ability of SPMs to decrease cigarette smoke-induced inflammation. Microbial insults are also a major source of inflammation and disease in the lung, and infectious exacerbations are prevalent in cigarette smoking associated diseases such as COPD. Chapters 3 and 4 have focused on SPM modulation of pathogen-responsive TLR4 signaling and SPM efficacy in a mouse model of NTHi infection to address questions regarding SPM use in infectious inflammation. We believe these are the first reports regarding SPM actions on macrophages exposed to cigarette smoke, bacterial mimetics, and live pulmonary infections and represent a next step towards use of SPMs as therapeutics in pulmonary disease.

Chapter 2

Resolvins Attenuate Inflammation and Promote Resolution in Cigarette Smoke-Exposed Human Macrophages[2]

[2] This work was published in part in the American Journal of Physiology: Lung, Cell, and Molecular Physiology as "Croasdell A, Thatcher T, Kottmann RM, Colas R, Dalli J, Serhan CN, Sime PJ, Phipps RP. *Resolvins Attenuate Inflammation and Promote Resolution in Cigarette Smoke-Exposed Human Macrophages*. Am J Physiol Lung Cell Mol Physiol. 2015 Oct 15;309(8):L888-901. Epub 2015 Aug 21" and is reprinted with their permission.

Abstract

Cigarette smoke causes multiple inflammatory diseases, which account for thousands of deaths and cost billions of dollars annually. Cigarette smoke disrupts the function of immune cells, such as macrophages, by prolonging inflammatory signaling, promoting oxidative stress, and impairing phagocytosis, which contributes to increased incidence of infections. Recently, new families of lipid derived-mediators coined "specialized pro-resolving mediators" (SPMs) were identified. These mediators play a critical role in the active resolution of inflammation by counter-regulating pro-inflammatory signaling and promoting resolution pathways. Here, we identified dysregulated concentrations of lipid mediators in bronchoalveolar lavage fluid and serum from COPD patients. In human alveolar macrophages from COPD and non-COPD patients, D-series resolvins decreased inflammatory cytokines and enhanced phagocytosis. To further investigate the actions of resolvins on human cells, macrophages were differentiated from human blood monocytes and treated with D-series resolvins followed by exposure to cigarette smoke extract. Resolvins significantly suppressed macrophage production of pro-inflammatory cytokines, enzymes, and lipid mediators and reduced protein carbonylation. Resolvins also increased anti-inflammatory cytokines, promoted an M2 macrophage phenotype, and restored cigarette smoke-induced defects in phagocytosis, highlighting the pro-resolving functions of these molecules. These actions were receptor dependent and involved modulation of both canonical and non-canonical NF-κB expression, with the first evidence for SPM action on alternative NF-κB signaling. These data show that resolvins act on human macrophages to attenuate cigarette smoke-induced inflammatory effects

through pro-resolving mechanisms, and provide new evidence of the therapeutic potential of SPMs.

Introduction

Cigarette smoke is the leading cause of preventable death, accounting for 1 in 5 deaths in the United States and nearly six million deaths annually worldwide, with mortality rates rising (51). Exposure to cigarette smoke causes many diseases, including chronic obstructive pulmonary disease (COPD, comprised of chronic bronchitis and emphysema) and an increased susceptibility to bacterial infections, such as those from Nontypeable *Haemophilus influenzae* and *Streptococcus pneumoniae* (53, 79). Chronic inflammation underlies most cigarette smoke-induced diseases. In particular, chronic activation of macrophages by cigarette smoke promotes tissue destruction and can lead to COPD (53, 56). Macrophages produce cytokines that stimulate excess mucus production and lead to chronic bronchitis. Additionally, macrophages are increased in emphysematous lungs, and have increased proteinase activity, reactive oxygen species (ROS) production, and secretion of inflammatory cytokines (53, 56). Despite this chronic inflammatory activation, patients with COPD are also more susceptible to bacterial and viral infections (79, 80), due at least in part to an impairment in macrophage phagocytic abilities; these defects in phagocytosis also lead to impaired clearance of apoptotic cells (62, 64, 81-83). Clearly, the underlying inflammatory mechanisms involved in cigarette smoke exposure and the progression of COPD are complex and inadequately addressed through the current standard treatments, which primarily involve bronchodilators and immunosuppressive steroids.

There is increasing interest in the regulation and dysregulation of the resolution component of inflammation, and how this may contribute to chronic disease. Several

studies have shown that SPMs are decreased in chronic diseases, and several chronic inflammatory diseases are hypothesized to be a result of a failure to resolve. Specific to lung diseases, reduced levels of lipoxins and resolvins have been detected in patients with asthma or with cystic fibrosis (84-86). In asthma, the levels of LxA_4 even correlate with disease severity, wherein severe asthmatics have further reduced levels compared to moderate asthmatics (84). Despite this evidence for the role of SPMs in chronic pulmonary diseases, there currently exists a large and important knowledge gap regarding the role of SPMs in COPD and whether SPMs can attenuate the effects of cigarette smoke in human macrophages.

SPMs mediate some of their key actions through modulation of inflammatory signaling pathways, including the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) family (15, 44, 69, 87-89). Cigarette smoke has been broadly shown to activate the canonical NF-κB family (90-92). In addition to increasing p65 phosphorylation, translocation, and activity, cigarette smoke also strongly affects RelB and alternative NF-κB signaling. In mouse lungs, cigarette smoke exposure results in a loss of RelB; similar results are seen in humans, where lung fibroblasts from smokers with and without COPD have decreased RelB expression (93, 94). Collectively, these studies highlight NF-κB as an important mechanistic target for cigarette smoke. Therefore, the actions of SPMs on these signaling pathways in cigarette smoke-exposed cells and human cells in general are of considerable interest. In the present work, we tested the hypothesis that SPMs attenuate cigarette smoke-induced inflammation via their pro-resolving and anti-inflammatory actions on human macrophages.

Materials & Methods

Materials

PGE_2, PGD_2, TxB_2, and RvD1 EIA kits, and all SPMs were purchased from Cayman Chemical (Ann Arbor, MI). Antibodies to RelB (4954S), p65 (4764), phospho-p65 (3033P), IκBα (4814), p100/p52 (3017), cleaved PARP (9541S), and β-tubulin (2146) were purchased from Cell Signaling (Danvers, MA). ALX/FPR2-specific antagonist Boc-2 was purchased from Genscript (Piscataway, NJ). A GPR32-neutralizing antibody (GX71225) was purchased from GeneTex (Irvine, CA). Antibodies to CD11b (560914) and CD14 (555398) and ELISA components for IL-6 (554543 and 554546) and TNFα (555212) were purchased from BD Biosciences (San Jose, CA). ELISA antibodies for IL-8 (M-801, M-802-B) were purchased from Endogen (Farmingdale, NY). IL-10 ELISA kit (430603) was purchased from Biolegend (San Diego, CA). TGF-β (DY240) and MCP-1 (MAB679, BAF279) ELISA components, Nrf2 antibody (MAB 3925), ALX/FRP2 antibody (R&D MAB3479) and GM-CSF (9023305) were purchased from R&D Systems (Minneapolis, MN). HO-1 antibody (OSA-110) was purchased from Stressgen (Farmingdale, NY). Actin antibody (CP-01) was purchased from Calbiochem (Darmstadt, Germany). Secondary western blot antibodies (115-035-146, 111-035-144) were purchased from Jackson laboratories (Bar Harbor, ME). PBS (14200-075) and RPMI 1640 (11875-119) were purchased from Gibco (Waltham, MA). Fetal bovine serum (SH30070.03HI) was purchased from Hyclone (Pittsburgh, PA).

Assessment of lipid mediator profiles in human samples

Bronchoalveolar lavage fluid (BALF), and human blood and serum were obtained from both male and female volunteer donors with informed written consent as approved by the University of Rochester Institutional Review Board and Office for Human Subjects Protection. BALF was obtained from COPD and non-COPD patients undergoing bronchoscopy at the University of Rochester Medical Center. All non-COPD patients are non-smokers; COPD patients are a mix of current and ex-smokers. BALF was strained through sterile gauze to remove mucus and then centrifuged for 5 mins at 425 x g to pellet cells. Lipid mediator concentrations were determined by mass spectrometry as described previously by Colas *et. al* (95) or by EIA according to manufacturer's protocol (20% cross reactivity with LxA_4, 4.2% cross reactivity with 17(R) – RvD1 and <1% reactivity for all other SPMs tested).

Human monocyte and macrophage isolation

Human peripheral blood mononuclear cells were obtained as previously described from male and female healthy, non-smoker donors (96). Briefly, whole units of blood (500 mL) were obtained from healthy consenting donors as approved by the University of Rochester Institutional Review Board and Office for Human Subjects Protection via venipuncture. Donors were non-smokers who were feeling in good health and who had not taken aspirin or other nonsteroidal anti-inflammatory drugs for 10 days before donation. Whole blood was centrifuged for 15 mins at 4°C at 500 x g and the buffy coat of white blood cells removed. Peripheral blood mononuclear cells (PBMCs) were isolated

by Ficoll-Paque (Amersham, 17-1440-03) gradient centrifugation and washed with 1X PBS. Monocytes were purified by incubation with CD14 conjugated DynaBeads according to manufacturer's protocol (Invitrogen, Grand Island, NY, 113-67D). To induce macrophage differentiation, monocytes were cultured in RPMI 1640 media (supplemented with 1% FBS, anti-mycotic/anti-myotic) and 10 ng/mL GM-CSF for 7 days. Blood derived macrophages were plated in 12-well plates at a concentration of 1 x 10^6 cells in serum-free RPMI 1640 for treatments unless otherwise described.

BALF was obtained as described above and the cell pellet was washed with PBS and resuspended in RPMI 1640. Alveolar macrophages were purified by adhesion (Fig. 2.1).

Cigarette smoke extract preparation

Cigarette smoke extract (CSE) was prepared in RPMI 1640 as previously described by our lab (37). In brief, smoke from two 1R3F research cigarettes was siphoned into 20 mL of serum-free RPMI 1640 media by continuous vacuum to make CSE. The solution was pH adjusted to pH 7, filtered using a 2 micron filter, and an OD value read by spectrophotometer at 320 nm wavelength. CSE was diluted to 30U (units/mL) unless otherwise described. Serum-free RPMI 1640 was used as a vehicle control.

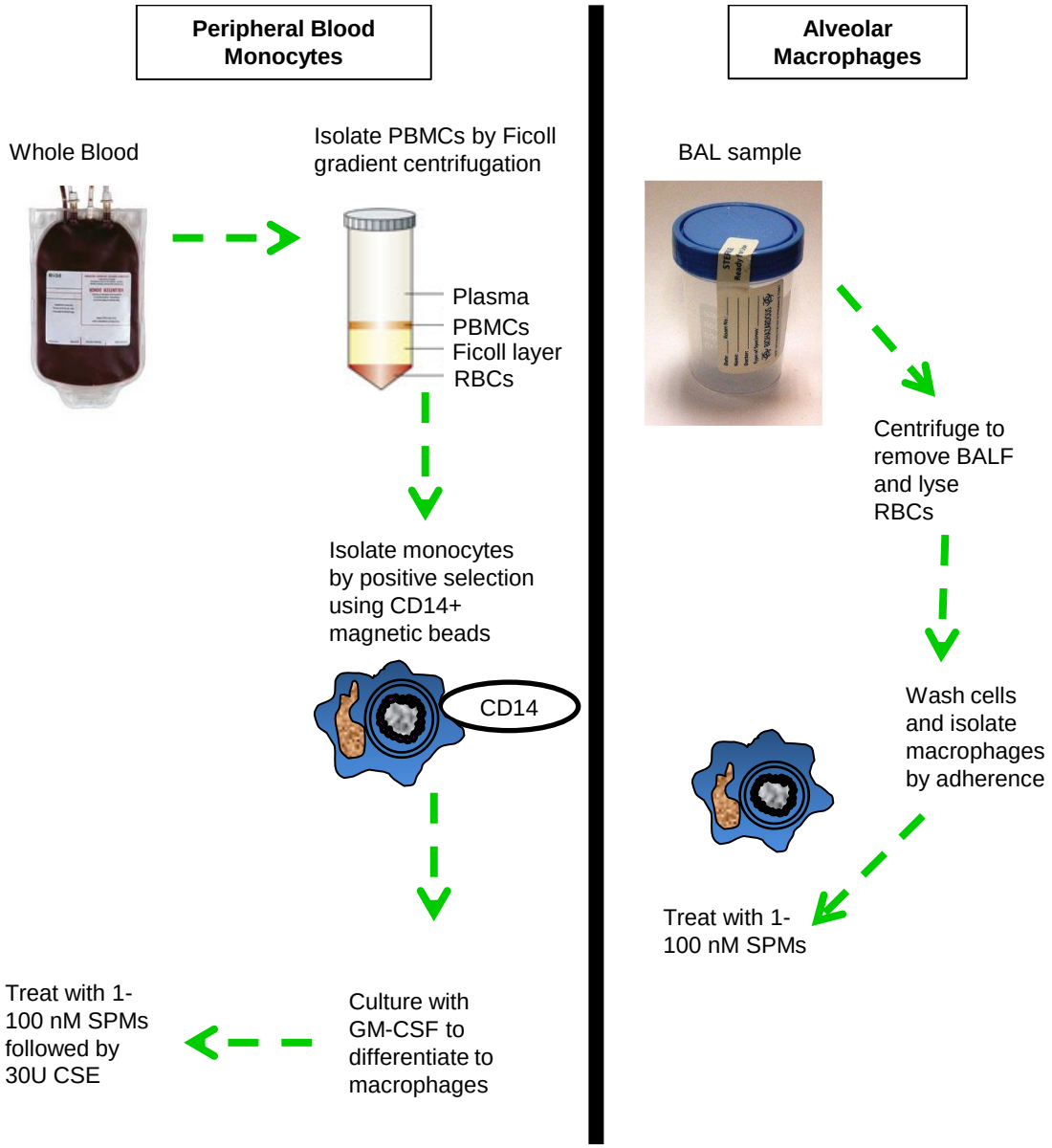

Fig. 2.1. Schematic of macrophage isolation. Macrophages were either differentiated from peripheral blood monocytes or isolated from bronchoalveolar lavage fluid. For blood-derived cells, PBMCs were isolated from whole blood of healthy human donors by Ficoll centrifugation. Monocytes were purified using CD14+ magnetic beads. Macrophages were differentiated with GM-CSF for 7 days prior to treatment with SPMs and CSE. For alveolar macrophages, cells were isolated from bronchoalveolar lavage fluid from human subjects by centrifugation. Macrophages were purified by adhesion prior to treatment with SPMs.

ELISA and EIA

Macrophages were incubated with 1-100 nM of SPMs or vehicle (0.1% EtOH in 1X PBS) for 24 hours, followed by exposure to CSE. Supernatants were removed 24 hours after CSE exposure and cytokines and lipid concentrations determined by ELISA (IL-6, IL-8, TNFα, MCP-1 TGF-β, IL-10) or EIA (PGE_2, PGD_2, TxB_2) according to manufacturer's protocols. Viability of the cells was determined by trypan blue exclusion. For alveolar macrophages, cells were incubated with SPMs for 24 hours, after which supernatants were removed and assayed for inflammatory mediators by ELISA.

Western blots and Oxyblot

Macrophages were treated as described above. Cells were washed twice with 1X PBS and lysed with CW Buffer (50 mM Tris-HCl, 2% SDS). Total protein was quantified by BCA (Thermo-Sci, Waltham, MA, 23225). Western blots were performed as described previously for cleaved-PARP, Cox-2, HO-1, Nrf2, and NF-κB family members (97). Briefly, equal amounts of proteins were loaded per lane prior to electrophoresis with 8-12% (w/v) SDS-PAGE gels. After transfer onto polyvinylidene difluoride membranes, immunoblots were probed with primary antibodies to target proteins followed by donkey anti-mouse or rabbit secondary antibodies. Immunoblots were visualized using ECL (WBKLS0500, Millipore). Antibodies against actin or β-tubulin were used as loading controls. Densitometric analysis was performed using ImageJ Software. For Oxyblot, samples were derivatized with a 1X 2,4-dinitrophenylhydrazine (DNPH) solution

followed by addition of a neutralization buffer and carbonylated proteins were detected according to the manufacturer's protocol (Millipore, Darmstadt, Germany, S7150).

Quantification of reactive oxygen species (ROS)

Macrophages were incubated with 1-100 nM SPMs or vehicle (0.01% EtOH in PBS) for one hour, followed by incubation with 1 μM H_2DCFDA, a marker of ROS, (Invitrogen, Waltham, MA, C400) for 30 min. The cells were washed and exposed to CSE. Quantification of ROS was determined by flow cytometry.

Determination of macrophage phagocytosis

Macrophages were treated with SPMs or vehicle (0.01% EtOH in PBS) for one hour prior to exposure to CSE for 2 hours. Cells were washed and incubated with FITC-labeled *Escherichia coli* (*E. coli*) (Molecular Probes, Waltham, MA, E13231) for one hour at a 1:5 cell:*E. coli* ratio. Cells were detached from plates using a release buffer (PBS with 0.5 mM EDTA) and resuspended in phosphate-azide-bovine serum albumin buffer (PAB) with trypan blue to quench any extracellular fluorescence. Macrophages were washed with PAB, fixed with 4% PFA, and stained with anti-CD14 and anti-CD11b antibodies. The percentage of *E. coli*$^+$ macrophages and *E. coli* mean fluorescent intensity (MFI) were assessed by flow cytometry.

Identification and Inhibition of SPM Receptors

Macrophages were treated with 10U or 50U CSE for 24 hours prior to staining with antibody against human ALX/FPR2. Expression of ALX/FPR2 was quantified by flow cytometry. In separate experiments, macrophages were treated with Boc-2 (1 μM) or GPR32 (10 ug/mL) for 30 min prior to SPM treatment and CSE exposure as described above. Specificity for Boc-2 and GPR32 has been established previously (26, 72, 98). Supernatants were removed and lysates were collected. ELISAs and EIA were performed as described above.

Statistical Analysis

Results are expressed as mean ± standard error (SEM). Statistical analyses on normally distributed data were performed using a student's t-test or one- or two-way analysis of variance (ANOVA) with Bonferroni's posttest correction for multiple comparisons using GraphPad Prism Software (San Diego, CA).

Results

COPD patients have dysregulated lipid mediator levels

The chronic inflammatory causes of COPD, namely airway destruction, mucus production, and cell infiltration, can be viewed as a failure to resolve inflammation. To explore this idea, we first investigated the levels of pro-resolving lipid mediators in COPD and non-COPD human bronchoalveolar lavage fluid (BALF). Using LM-metabololipidomics (95), we identified by matching criteria and MS/MS spectra bioactive SPMs and intermediates in both COPD and non-COPD subjects. Increases in HDHAs (14-HDHA, 17-HDHA) and hydroxyeicosatetraenoic acids (12-HETE, 15-HETE) from arachidonic acid were also identified in BALF of COPD patients, with hydroxyeicosapentaenoic acids (5-HEPE, 12-HEPE) trending towards an increase but not statistically different (Fig. 2.1A-C). These intermediates are important precursors to eicosanoids, including SPMs. Additionally, COPD patients had reduced concentrations of RvD1 in both their BALF and serum (Fig. 2.1D-E). LxA_4, a potential confounder due to crossreactivity in the ELISA, was not detected in our BALF and has been found at extremely low levels in human serum, making the likelihood of cross-reactivity very low (99, 100). This is the first evidence that COPD patients have dysregulated levels of the omega-3 derived SPMs.

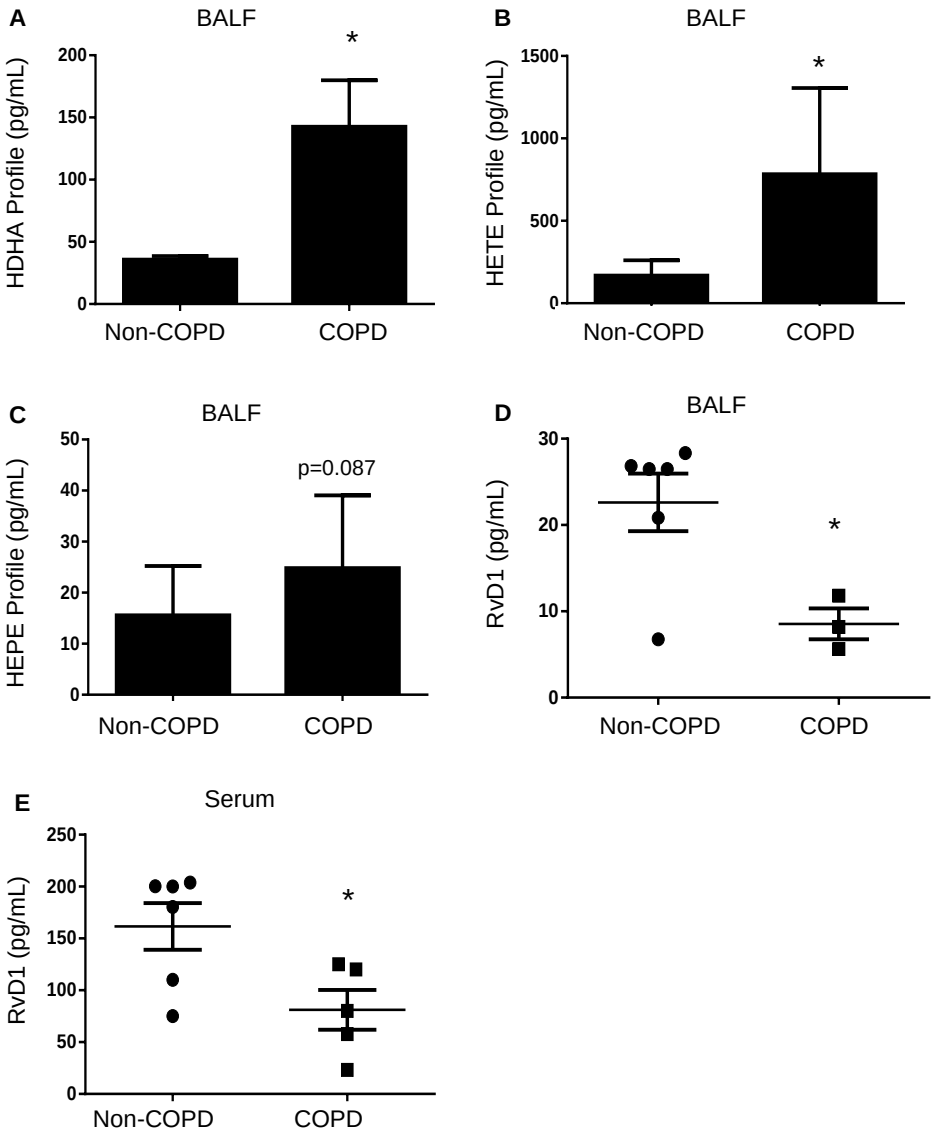

Fig. 2.2. Lipid mediators are dysregulated in patients with COPD. Lipid mediators were identified in BALF (A-C) of COPD and non-COPD human subjects by LM-metabololipidomics. COPD patients had increased concentrations of pathway markers HDHA (14-HDHA, 17-HDHA) and HETE (12-HETE, 15-HETE) in BALF as well as other dysregulated lipid signaling. RvD1 concentration in BALF (D) and serum (E) were also evaluated by EIA. Statistical significance was determined by t-test (*$p<0.05$ compared to non-COPD), n=3-6.

RvDs act on human alveolar macrophages to decrease inflammatory mediators and enhance phagocytosis

Macrophages are involved in the initiation, propagation, and resolution phases of inflammation and are major producers of inflammatory cytokines and lipid mediators. They are also the primary cell responsible for phagocytosis of apoptotic neutrophils, cell debris, and bacteria. The dysregulation of normal macrophage processes contributes to the progression of COPD and increases susceptibility to bacterial exacerbations (64, 81-83). Based on this key role of macrophages in mediating COPD pathology, we investigated whether or not SPMs could have a therapeutic impact on human alveolar macrophages isolated from bronchoalveolar lavage fluid. The efficacy of SPMs on macrophages from several subjects were initially screened using a dose range of 1-100 nM treatments with RvD1 or RvD2. Both RvD1 and RvD2 dampened spontaneous production of IL-6 and TNFα, with the most consistent effect seen at 100 nM (Fig. 2.3A-B). We next investigated the efficacy of 100 nM RvDs in both COPD and non-COPD patients. RvD1 and RvD2 dampened spontaneous production of TNFα, though not IL-8; RvD2 additionally dampened production of IL-6 (Fig. 2.3C-E). Along with dampening cytokine production, RvD2 enhanced phagocytic uptake of *E. coli* by unstimulated human alveolar macrophages (Fig. 2.3F). We also analyzed the effects of RvDs on COPD and non-COPD populations separately (Table 2.1). RvD1 and RvD2 were efficacious on both COPD and non-COPD alveolar macrophages, demonstrating further their potential as therapeutics in smoking-associated diseases (Table 2.1).

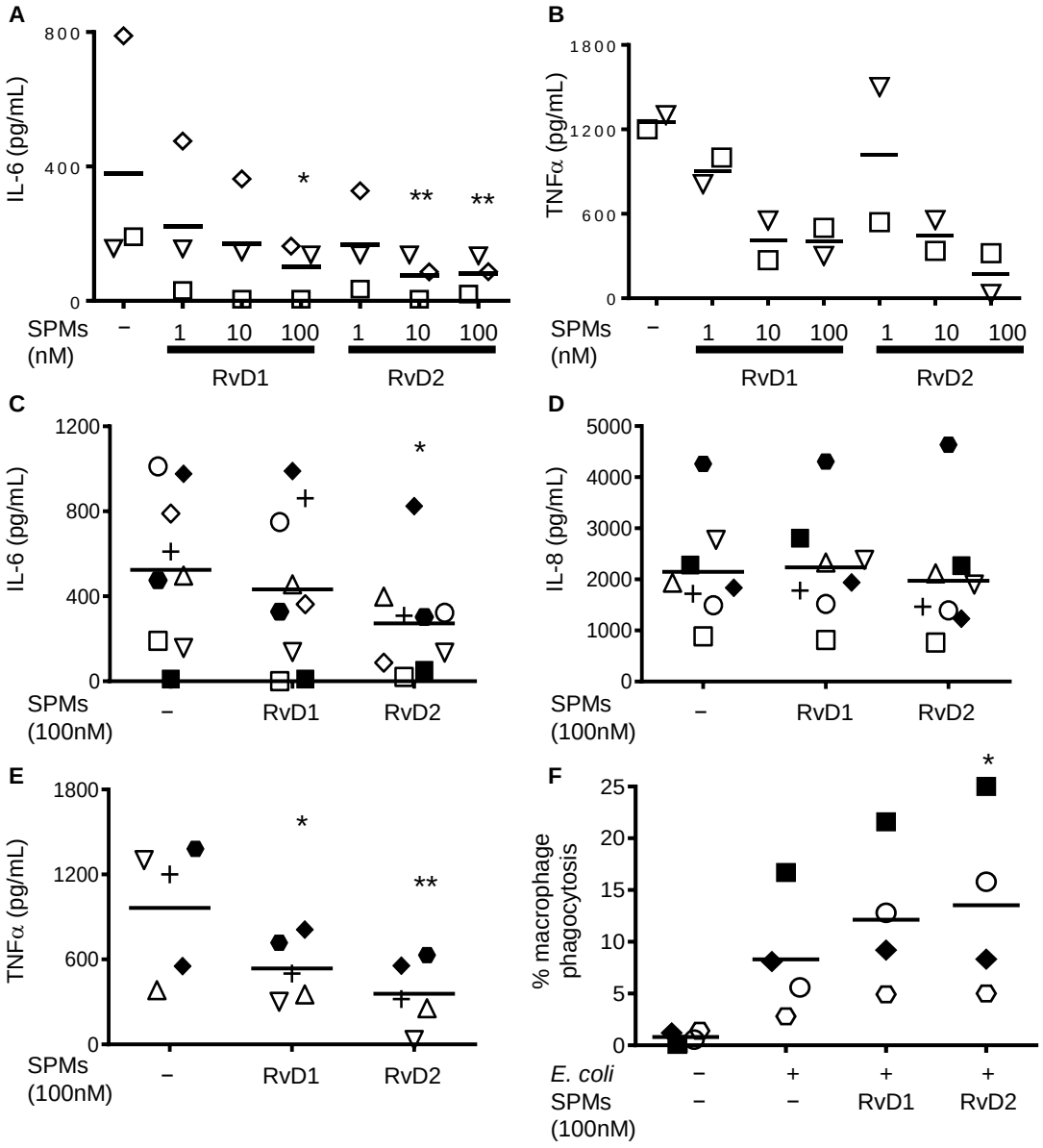

Fig. 2.3. SPMs dampen cytokine release and enhance phagocytosis in unstimulated alveolar macrophages from COPD and non-COPD patients. Alveolar macrophages from human subjects were incubated with 1-100 nM RvD1 or RvD2 for 24 hours. Supernatants were assessed for IL-6 (A) and TNFα (B) by ELISA. (C-F) Alveolar macrophages from COPD (closed symbols) and non-COPD (open symbols) human subjects were incubated with 100 nM RvD1 or RvD2 for 24 hours. Supernatants were assessed for IL-6 (C), IL-8 (D), and TNFα (E) by ELISA. Additionally, cells were incubated with fluorescently-labeled *E. coli* and phagocytosis was determined by flow cytometry (F). Each symbol represents an individual donor. Statistical significance was determined by one-way ANOVA (A-B) or paired t-test (C-F) (*$p<0.05$, **$p<0.01$, compared to vehicle alone), n=4-8.

	COPD			**Non-COPD**		
	Vehicle	RvD1	RvD2	Vehicle	RvD1	RvD2
IL-6	487 ± 279	442 ± 289	392 ± 228	543 ± 137	428 ± 137	212 ± 62*
IL-8	2792 ± 745	3017 ± 690	2712 ± 1007	1761 ± 307	1766 ± 289	1528 ± 234
TNFα	966 ± 414	764 ± 46†	592 ± 38†	961 ± 291	385 ± 60*	202 ± 87.6*
% phagocytosis	12.40	15.40†	16.66†	4.20	8.87†	10.40†
Values shown are mean ± SEM.*p<0.5 compared to vehicle in same disease group, †sample size insufficient for statistical analysis						

Table 2.1. SPMs dampen cytokine release and enhance phagocytosis in unstimulated alveolar macrophages from COPD and non-COPD patients. Alveolar macrophages from COPD and non-COPD human subjects were incubated with 100 nM RvD1 or RvD2 for 24 hours. Supernatants were assessed for IL-6, IL-8, and TNFα by ELISA. Additionally, cells were incubated with fluorescently-labeled *E. coli* and phagocytosis was determined by flow cytometry. Significance was determined by paired t-test (*p<0.05 compared to vehicle alone, †sample size insufficient for statistical analysis), n=2-5.

RvDs dampen cigarette smoke-induced production of pro-inflammatory cytokines and promote anti-inflammatory cytokines

In order to characterize the effects of SPMs on human macrophages, we conducted further experiments using macrophages derived from human peripheral blood monocytes. Blood derived monocytes from multiple healthy human donors were isolated to >95% purity (Fig. 2.4A), differentiated to macrophages, and incubated with 100 nM SPMs for 24 hours. We first assessed whether our dosages of cigarette smoke extract (CSE) and RvDs would impact cellular viability and/or promote macrophage apoptosis. At 30U of CSE and 100 nM of various SPMs, no differences in cellular viability as determined by trypan blue were observed (Fig. 2.4B). We additionally used cleaved-PARP as a marker of apoptosis. While CSE did slightly increase cleaved-PARP protein expression, these changes were not statistically significant nor did RvDs alter apopotosis (Fig. 2.4C-D). Therefore, we moved forward with nanomolar dosages of SPMs and 30U of CSE for further experiments.

Monocytes and macrophages are involved in the synthesis of numerous SPMs, and many eicosanoids can be produced from the intermediates we detected in human BALF. We screened several candidate SPMs and 17-HDHA on macrophages to determine which were the most efficacious at moderating cigarette smoke induced inflammatory effects. Several SPMs dampened CSE-induced IL-6 and IL-8, including the D-series resolvins (Fig. 2.5A-B). Based on this screening data, our human lipid profiles, and efficacy in our experiments with alveolar macrophages, RvD1 and RvD2 were chosen for future studies.

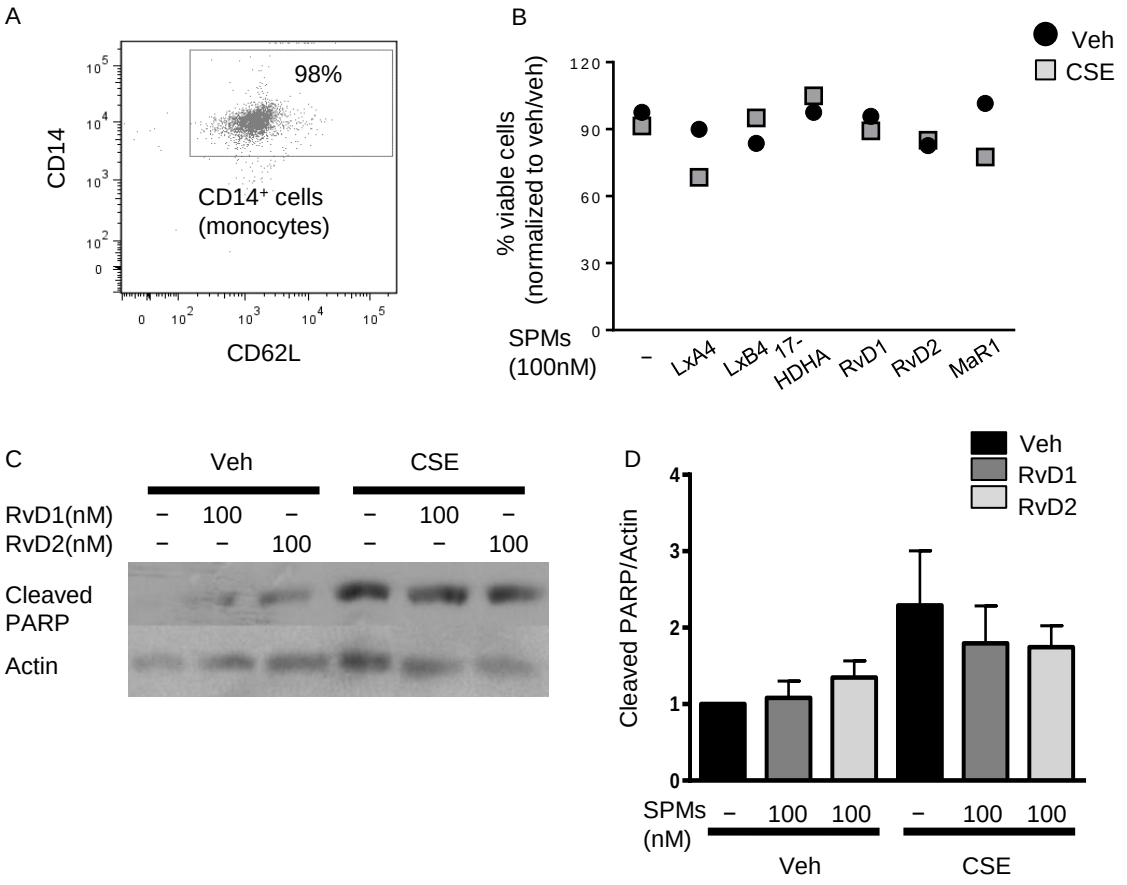

Fig. 2.4. Cigarette smoke extract and SPMs do not induce cell death. Human monocyte purity was confirmed by staining with anti-CD14 and anti-CD62 antibodies and assessed by flow cytometry (A). Monocytes were differentiated to macrophages and treated with 100 nM of the indicated SPMs for 24 hours. Viability was determined by trypan blue exclusion (B). Expression of cleaved-PARP was assessed by western blot (C-D, representative image show, n=4). Statistical significance was determined by two-way ANOVA with Bonferroni posttest. n=3-4, each symbol represents an individual donor.

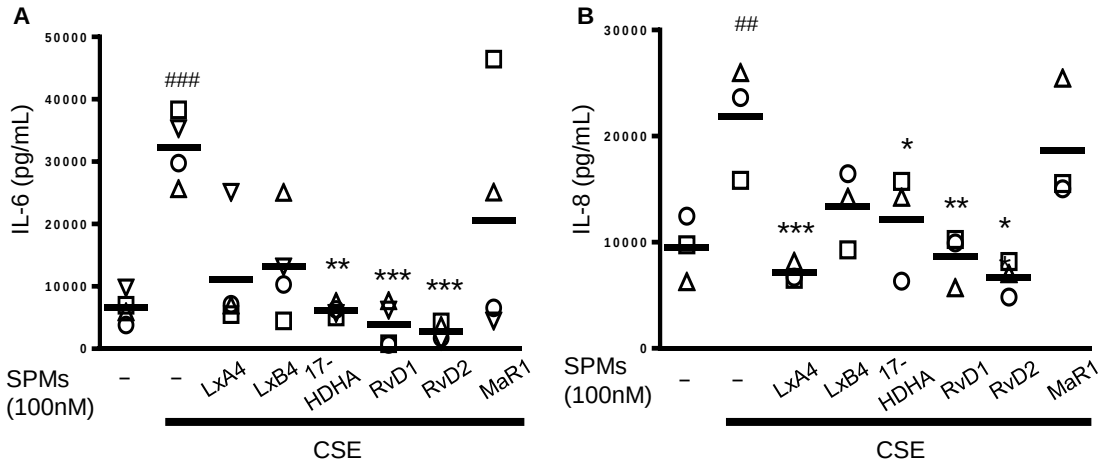

Fig. 2.5. **D-series resolvins are efficacious in reducing cigarette smoke extract-induced inflammatory mediator production.** Monocytes were differentiated to macrophages and treated with 100 nM of the indicated SPMs for 24 hours. Cells were subsequently exposed to CSE for 24 hours. Supernatant levels of pro-inflammatory cytokines IL-6 (B) and IL-8 (C) were evaluated by ELISA. Statistical significance was determined by two-way ANOVA with Bonferroni posttest ($^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to veh/veh, $*p<0.05$, $**p<0.01$, $***p<0.001$ compared to CSE alone), n=3-4, each symbol represents an individual donor.

We first evaluated additional doses of RvDs on pro- and anti-inflammatory cytokine production. CSE increased levels of the pro-inflammatory mediators IL-6, IL-8, TNFα, and MCP-1, cytokines important in propagating an immune response, attracting neutrophils, enhancing phagocytosis, and promoting cell death (Fig. 2.6A-D). RvDs completely attenuated elevated IL-6 (Fig. 2.6A) and dose-dependently decreased IL-8 (Fig. 2.6B). 1 nM RvD1 and both doses of RvD2 additionally dampened TNFα and MCP-1 expression (Fig. 2.6C-D). In addition to preventing pro-inflammatory cytokine release, resolvins promote the production of anti-inflammatory cytokines, including IL-10 and TGF-β. These cytokines induce alternative macrophages and aid in wound repair. RvD1 increased concentrations of total TGF-β, but not active or the ratio of active/total TGF-β (Fig. 2.7A-C). RvD2 increased concentrations of both active and total TGF-β and had an increased ratio of active/total TGF-β (Fig. 2.7A-C). CSE reduced production of the anti-inflammatory cytokine IL-10, but this effect was not attenuated by either RvD1 or RvD2 (Fig. 2.7D). Collectively, these results demonstrate that RvDs can selectively act to both decrease pro-inflammatory cytokines and to promote anti-inflammatory proteins.

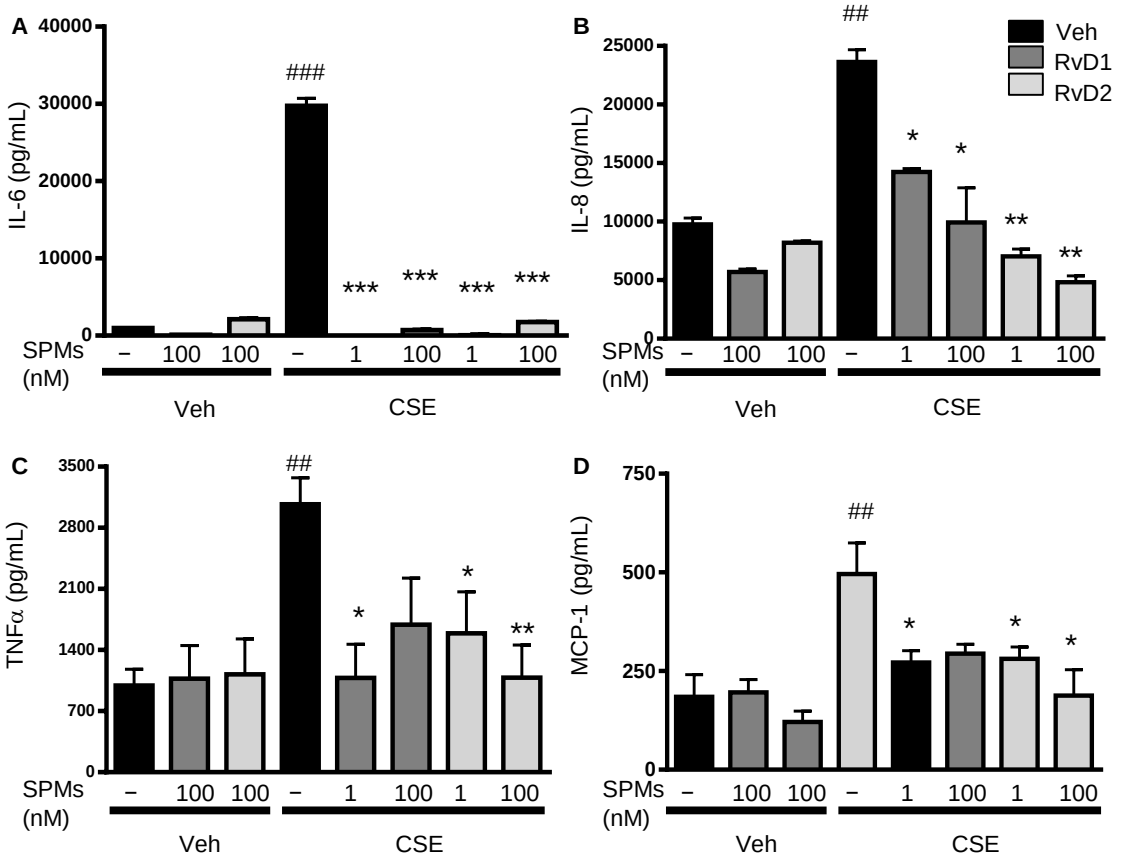

Fig. 2.6. Resolvins dampen cigarette smoke extract-induced increases in inflammatory cytokines and promote anti-inflammatory cytokine production. Blood derived macrophages were incubated with RvD1 or RvD2 for 24 hours prior to CSE exposure for 24 hours. RvDs reduced concentrations of pro-inflammatory [IL-6 (A), IL-8 (B), TNFα (C), MCP-1 (D)] cytokines and chemokines, as determined by ELISA. Statistical significance was determined by two-way ANOVA with Bonferroni posttest ($^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to veh/veh, *p<0.05, **p<0.01, ***p<0.001 compared to CSE alone), n=6 individual donors.

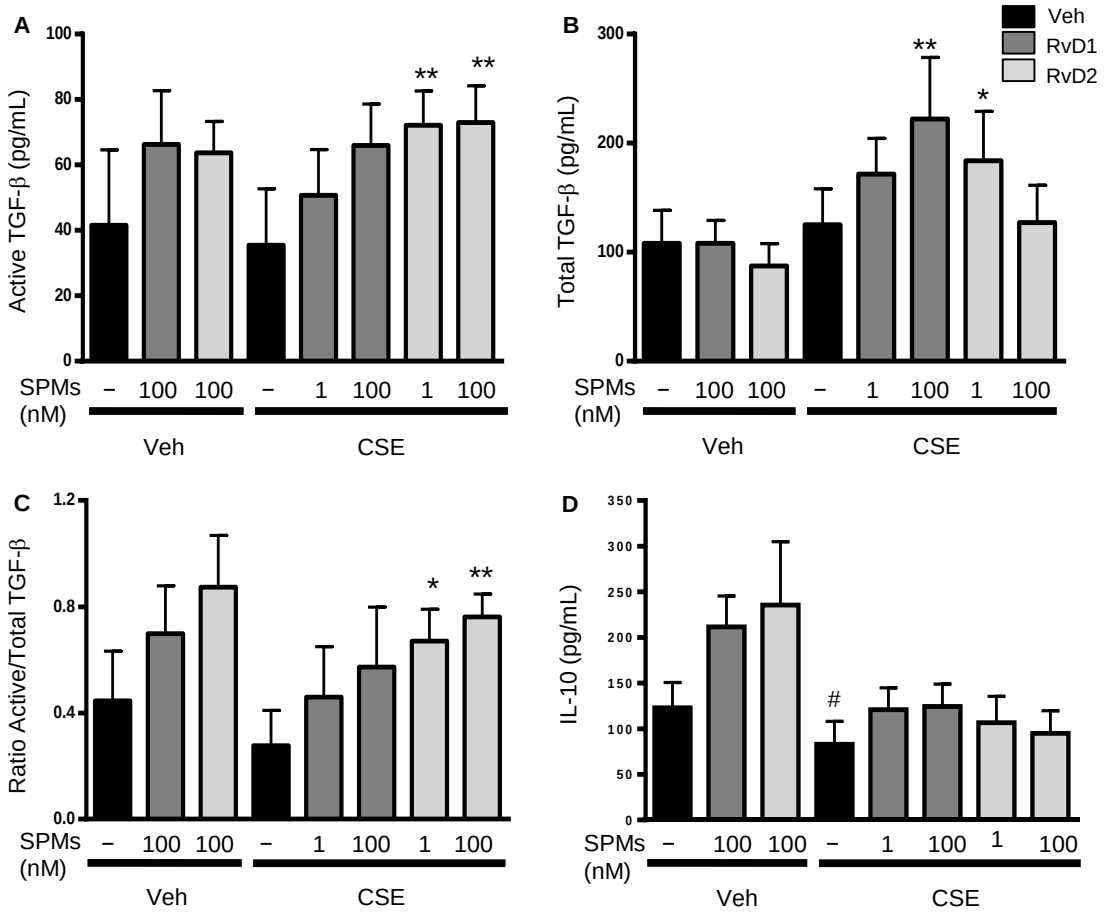

Fig. 2.7. Resolvins promote anti-inflammatory cytokine production. Blood derived macrophages were incubated with RvD1 or RvD2 for 24 hours prior to CSE exposure for 24 hours. RvDs increased active, total and the ratio of active/total TGF-β (A, B, C) and IL-10 (D) cytokines as determined by ELISA. Statistical significance was determined by two-way ANOVA with Bonferroni posttest ($^{\#}p<0.05$ compared to veh/veh, *$p<0.05$, **$p<0.01$ compared to CSE alone), n=6 individual donors.

The generation of certain pro- and anti-inflammatory cytokines and bioactive lipid mediators are regulated by the cyclooxygenase-2 (Cox-2) enzyme (101). Cigarette smoke has previously been shown to increase expression of Cox-2 in fibroblasts and elevated Cox-2 expression is associated with several smoking-related diseases, including COPD and cancer (102-105). CSE increased expression of Cox-2 in human macrophages and treatment with RvDs significantly attenuated Cox-2 expression (Fig. 2.8A-B). Macrophages further produce Cox-2 regulated prostaglandins, such as prostaglandin E_2 (PGE_2) and prostaglandin D_2 (PGD_2) (106). Both PGD_2 and PGE_2 are important prostaglandins with both pro- and anti-inflammatory roles (106-109). At 24 hours, CSE significantly increased PGE_2 production; this increase was dose-dependently diminished by RvDs (Fig. 2.8C). CSE similarly increased concentration of PGD_2, but in contrast to PGE_2, RvD2 further potentiated this increase (Fig. 2.8D). We also evaluated the concentration of thromboxane (Tx) B_2, the inactive metabolite of TxA_2, an important pro-thrombotic signaling molecule involved in tissue injury and inflammation (110). TxB_2 was unaffected by either CSE or RvD exposure (Fig. 2.8E), indicating that RvDs may regulate specific lipid mediator pathways.

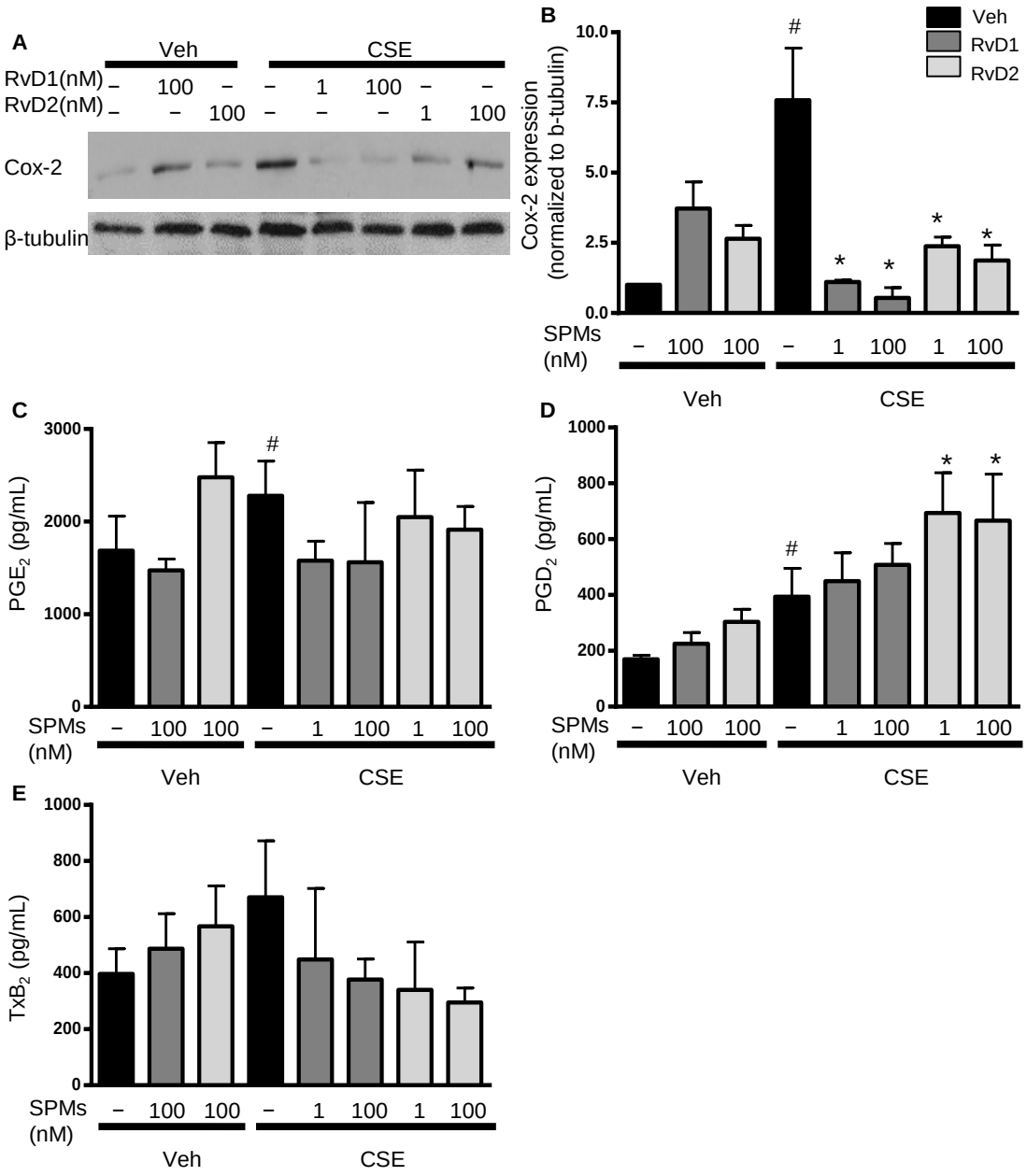

Fig. 2.8. RvDs prevent cigarette smoke extract-induced increases in Cox-2 and pro-inflammatory lipid mediators. Macrophages were incubated with RvD1 or RvD2 for 24 hours prior to CSE exposure for 24 hours. Cells were lysed and Cox-2 protein expression levels assessed by western blot (A-B, representative image shown, n=3). Supernatants from treated macrophages were also assessed for PGE_2 (C), PGD_2 (D), and TxB_2 (E) by EIA. Statistical significance was determined by two-way ANOVA with Bonferroni posttest ($^{\#}p<0.05$ compared to veh/veh, $^{*}p<0.05$ compared to CSE alone), n=5-6 individual donors.

RvDs dampen cigarette smoke-induced oxidative stress

In addition to production of inflammatory mediators, macrophages are important players in the regulation of oxidative stress. Cigarette smoke induces the generation of reactive oxygen species (ROS) and chronic smoke exposure leads to oxidative stress and tissue damage (111, 112). Little is known about the effect of SPMs on oxidative stress, particularly in human cells. To evaluate the ability of resolvins to attenuate oxidative stress we investigated several markers of oxidative damage. CSE increased reactive oxygen species as indicated by levels of H_2DCFDA in exposed macrophages (Fig. 2.9A-B). RvD treatment, however, did not dampen this initial oxidative burst at 20 min, one hour, or 24 hours after CSE exposure (Fig. 2.9A-B). Since we did not see any effect of RvDs on mitigating ROS production, we looked for markers of oxidative damage as potential endpoints. We investigated RvD1 and RvD2 actions on carbonylated proteins; reactive oxygen species can induce the modification of native amino acids to carbonyl derivatives. One hour after CSE exposure there is a slight but significant increase in carbonylated proteins (Fig. 2.10A-B). RvDs did not significantly affect levels of carbonyl groups one hour after smoke exposure (Fig. 2.10A-B). We also evaluated a longer CSE exposure to determine if RvDs were acting to shorten resolution time or reduce propagation of oxidative stress rather than preventing initiation. By 24 hours, CSE potently induced protein carbonylation (Fig. 2.10C-D). In contrast to ROS levels and early timepoints, certain doses (1 nM RvD1 and 100 nM RvD2) did act to decrease levels of carbonylated proteins, the first evidence for RvDs acting on protein carbonylation in human macrophages (Fig. 2.10C-D).

We were additionally curious to see if resolvins would affect any anti-oxidative pathways. At 24 hours post-CSE exposure, cigarette smoke increased expression of HO-1, an anti-oxidative enzyme. RvDs prevented this increase in HO-1, retaining homeostatic levels of enzyme expression (Fig. 2.11A-B). We also evaluated expression of Nrf2, a protein that regulates expression of other anti-oxidant proteins. Neither CSE nor RvDs altered total Nrf2 expression (Fig. 2.11C-D). Taken together, these data demonstrate a nuanced role for SPMs in mediating oxidative stress, wherein they may act at later time points to prevent propagation of oxidative damage and promote faster repair rather than preventing initial responses.

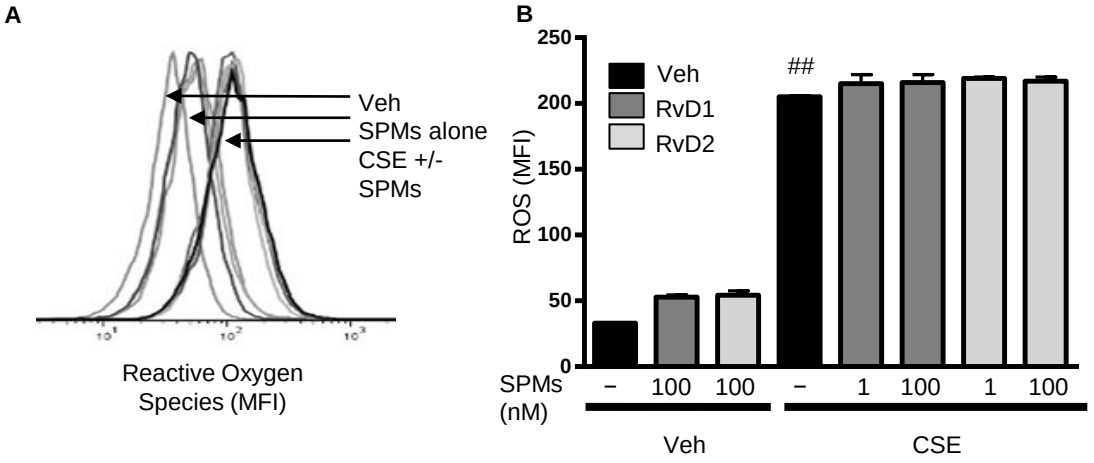

Fig. 2.9. Resolvins do not prevent induction of reactive oxygen species. Macrophages were incubated with RvD1 or RvD2 for 24 hours, following which cells were incubated with H_2DCFDA and exposed to CSE. One hour after CSE exposure ROS levels were determined by flow cytometry (A, B). Statistical significance was determined by two-way ANOVA with Bonferroni posttest ($^{\#\#}p<0.01$ compared to veh/veh), n=3, representative donor shown.

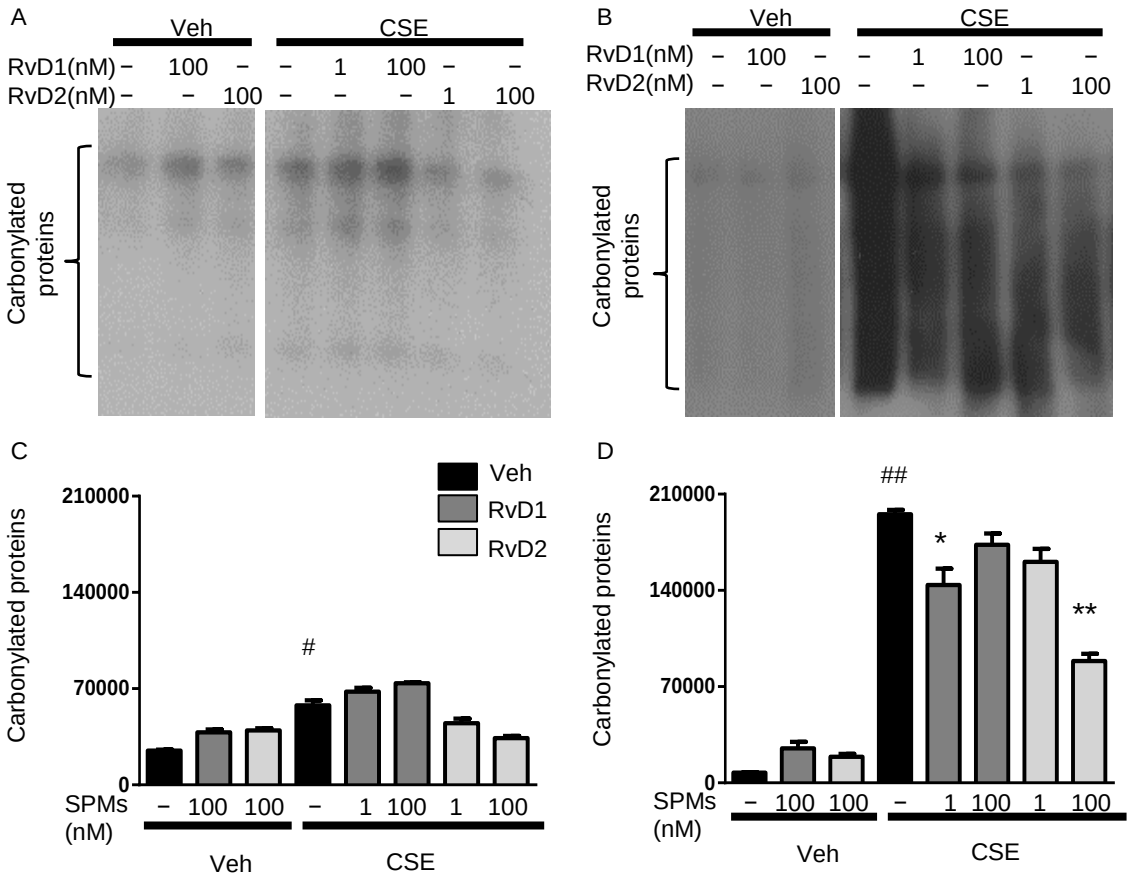

Fig. 2.10. Resolvins prevent accumulation of carbonylated proteins. Macrophages were incubated with RvD1 or RvD2 for 24 hours, following which cells were exposed to CSE. The presence of carbonylated groups in RvD and CSE exposed macrophages was assessed by Oxyblot at one hour (A-B) and 24 hours (C-D). Representative images are shown. Statistical significance was determined by two-way ANOVA with Bonferroni posttest ($^{\#}p<0.05$, $^{\#\#}p<0.01$ compared to veh/veh, $^{*}p<0.05$, $^{**}p<0.01$ compared to CSE alone), n=3 individual donors.

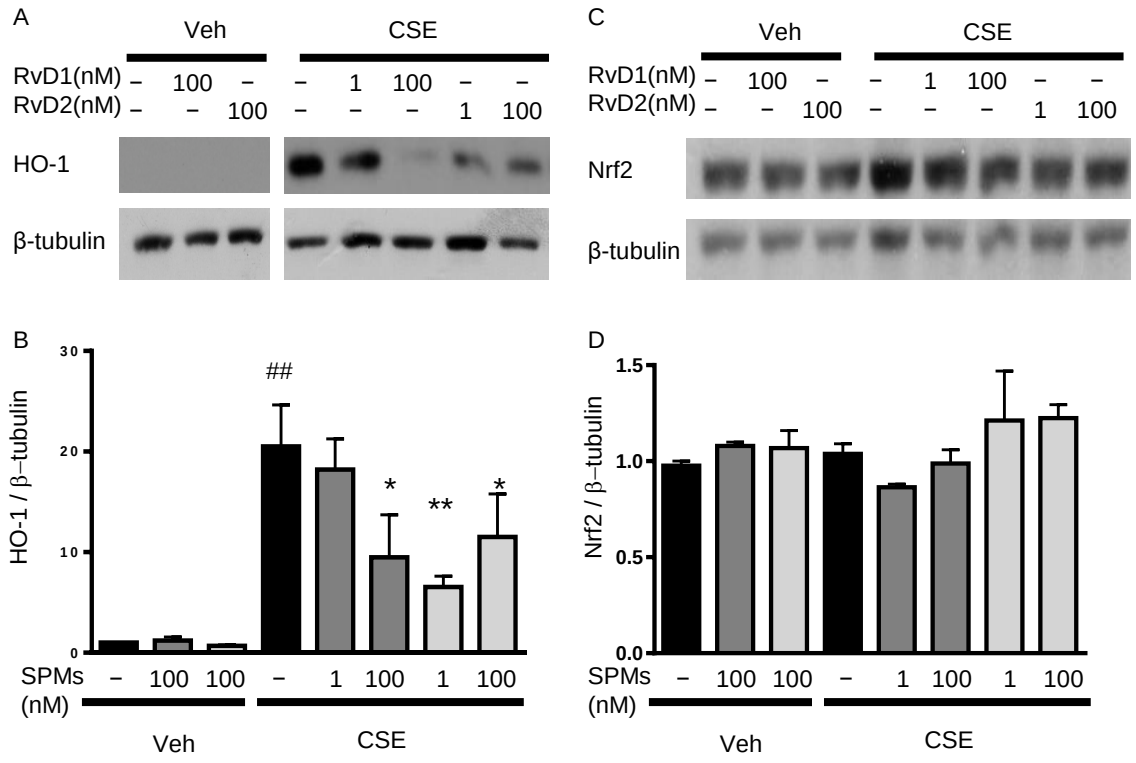

Fig. 2.11. Resolvins prevent cigarette smoke-induction of HO-1. Macrophages were incubated with RvD1 or RvD2 for 24 hours, following which cells were exposed to CSE. Protein expression levels of HO-1 (A-B) and Nrf2 (C-D) were assessed by western blot (representative images shown, n=3). Statistical significance was determined by two-way ANOVA with Bonferroni posttest ($^{\#\#}p<0.01$ compared to veh/veh, *p<0.05, **p<0.01 compared to CSE alone), n=3 individual donors.

RvDs rescue cigarette smoke-induced defects in phagocytosis

We hypothesized that the observed reduction in cytokines and increase in active/total TGF-β ratio might indicate a skewing of the macrophage phenotype away from a classically activated, pro-inflammatory "M1" phenotype and toward an alternatively activated, anti-inflammatory and pro-resolving "M2" phenotype (27). To assess this, we analyzed surface expression of CD80 (an M1 marker) and CD206 (an M2 marker) by flow cytometry. CSE alone drove alveolar macrophages toward a pro-inflammatory M1 macrophage profile, indicated by increases in $CD80^{+}/CD206^{-}$ cells and a decreased CD206/CD80 ratio (Fig. 2.12A-C). RvDs prevented this M1 skewing, decreasing the percentage of $CD80^{+}/CD206^{-}$ cells and increasing the percentage of $CD80^{-}/CD206^{+}$ cells (Fig. 2.12A-B). Overall, this resulted in RvDs rescuing the ratio of CD206/CD80 cells, and in the case of 100 nM RvD2, even increasing an M2 phenotype over vehicle levels (Fig. 2.12C).

Based on this increase in M2 macrophages and our data showing that SPMs enhanced phagocytosis in alveolar macrophages, we wanted to evaluate if these molecules could rescue or enhance cigarette smoke-induced phagocytic defects in blood-derived macrophages. Macrophages were incubated with RvD1 and RvD2 and exposed to CSE. CSE impaired uptake of fluorescently labeled *E. coli* (Fig. 2.13A-B). Incubation with RvDs prevented these decreases and restored macrophage phagocytic abilities (Fig. 2.13A-B). In addition to affecting the amount of bacteria taken up, CSE reduced the number of macrophages that phagocytized bacteria, which was rescued by RvDs (Fig. 2.13C).

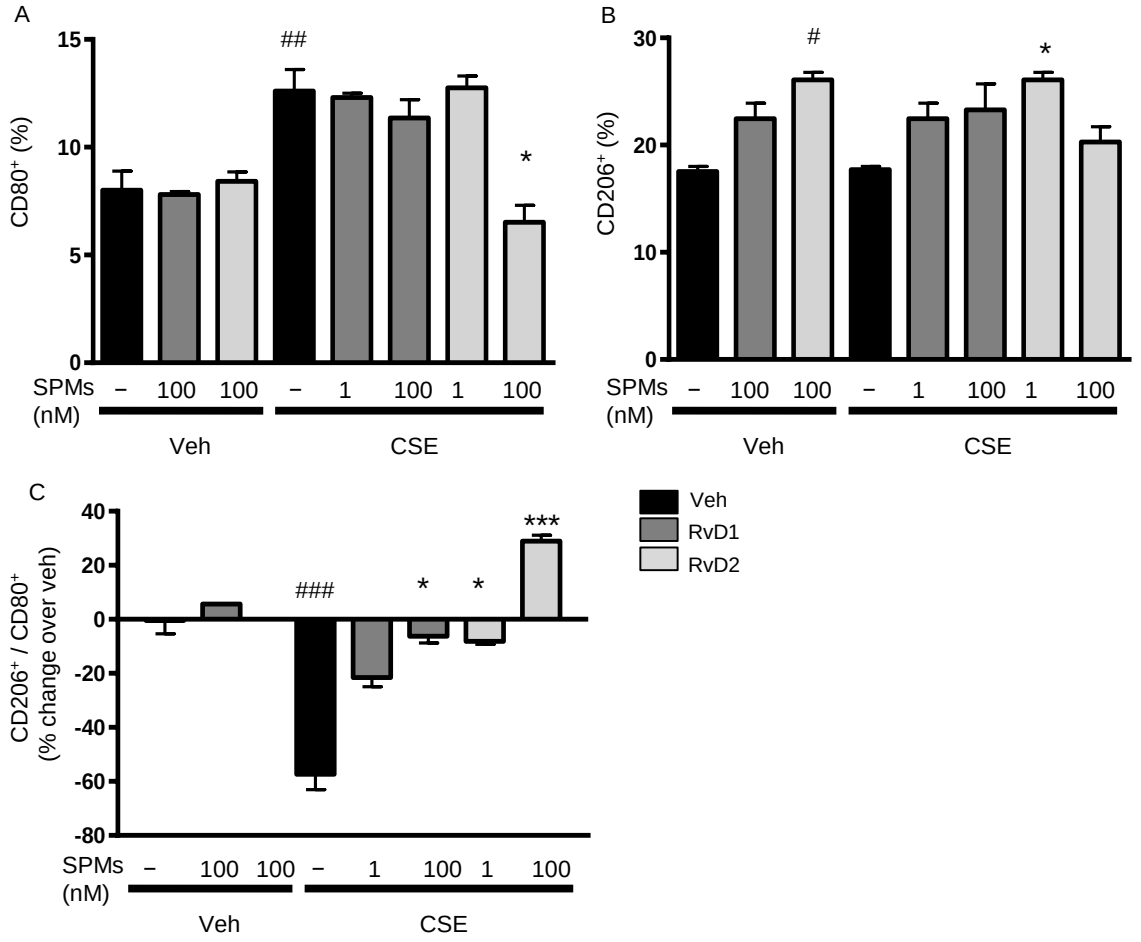

Fig. 2.12. Resolvins promote an M2 macrophage phenotype. Macrophages were incubated with RvD1 or RvD2 for 24 hours prior to CSE exposure for 24 hours. Cells were stained for CD80 (M1 marker) and CD206 (M2 marker) and cell populations were evaluated by flow cytometry. The percentage of $CD80^{+}CD206^{-}$ (A) and $CD80^{-}CD206^{+}$ (B) cells were quantified, as well as the change in CD206/CD80 ratio (C). Representative donor (out of 4 individual donors) is shown. Statistical significance was determined by two-way ANOVA with Bonferroni posttest ($^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to veh/veh, *p<0.05, ***p<0.001 compared to CSE alone), n=3 technical replicates.

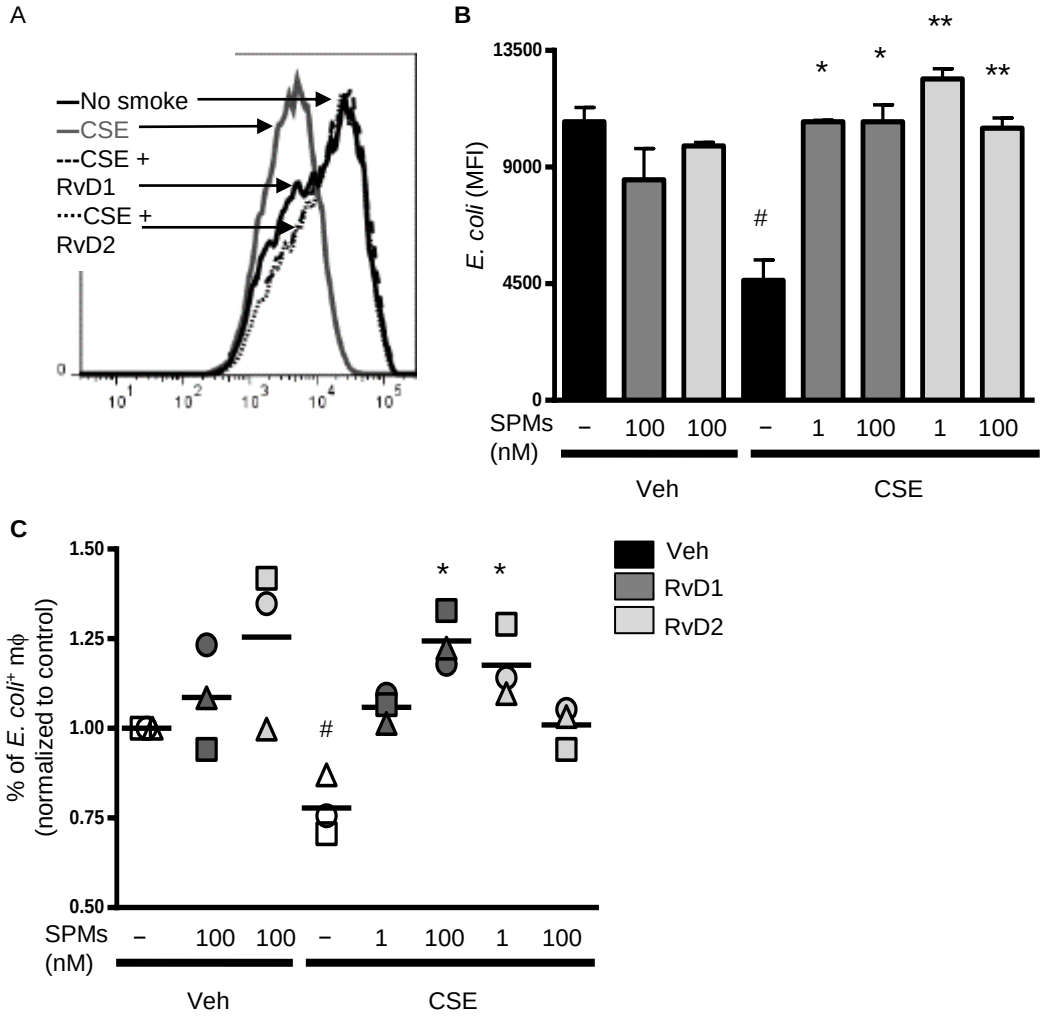

Fig. 2.13. Resolvins attenuate cigarette smoke extract-induced decreases in phagocytosis. Macrophages were incubated with RvD1 or RvD2 for 24 hours prior to CSE exposure for 24 hours. Cells were incubated with fluorescently-labeled *E. coli* and phagocytosis was determined by flow cytometry. Mean fluorescence intensity was quantified as a measure of *E. coli* uptake, with one representative donor shown (A, B, n=4). The percentage of macrophages that phagocytized *E. coli* was also quantified (C, each symbol represents an individual human donor). Statistical significance was determined by two-way ANOVA with Bonferroni posttest ($^{\#}p<0.05$ compared to veh/veh, *p<0.05, **p<0.01 compared to CSE alone).

Resolvin D1 signals through GPCRs to mediate pro-resolving effects

Resolvin D1 is known to signal through two receptors- ALX/FPR2 and GPR32; RvD2 can bind ALX/FPR2, but primarily signals through GPR18, which has recently been identified (12-14, 26). We wanted to evaluate if these receptors were altered in macrophages exposed to cigarette smoke, as their expression is dysregulated in COPD lungs. Cigarette smoke dose-dependently reduced expression of ALX/FPR2 in human monocytes (Fig. 2.14A-B). To investigate whether RvD1 was acting through its known receptors, we used available techniques, including an ALX/FPR2 inhibitor, Boc-2, and an anti-GPR32 neutralizing antibody, to block receptor expression. Boc-2 partially prevented RvD1's effects on IL-6, and completely blocked dampening of TNFα. Complementarily, anti-GPR32 blocked RvD1's effects on IL-6, and partially prevented dampening of TNFα (Fig. 2.14C-D).

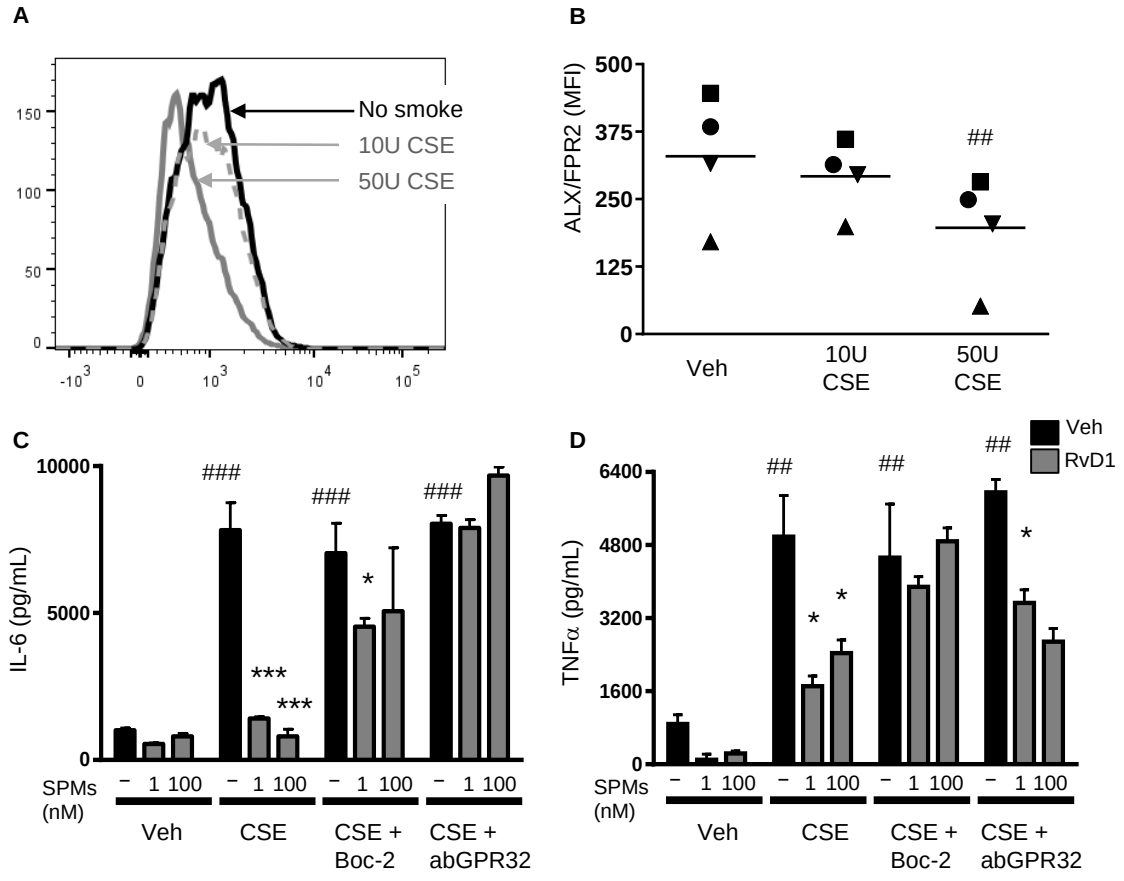

Fig. 2.14. Resolvin D1 signals through GPCRs to mediate pro-resolving effects. Macrophages were exposed to CSE for 24 hours and expression of ALX/FPR2 was assessed by flow cytometry. CSE dose-dependently reduced ALX/FPR2 expression (A-B, each symbol represents an individual donor). Macrophages were treated with Boc-2 or neutralizing anti-GPR32 antibody for 30 mins prior to incubation with RvD1 and RvD2 and CSE exposure. The effects of ALX/FPR2 and GPR32 inhibition were assessed by evaluating concentrations of IL-6 (C) and TNFα (D) by ELISA. Statistical significance was determined by two-way ANOVA with Bonferroni posttest ($^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to veh/veh, $^{*}p<0.05$, $^{***}p<0.001$ compared to CSE or CSE w/inhibitor for each respective group), n=3-4 individual donors.

RvD2 acts via modulation of NF-κB signaling pathways

In addition to acting through identified receptors, resolvins can mediate their effects through canonical inflammatory pathways, including NF-κB signaling (38, 71, 89). To investigate SPM actions in human macrophages, we focused on the mechanistic actions of RvD2 on the NF-κB signaling pathway. Total expression of p65 was unaffected by CSE or RvD2 (Fig. 2.15A-B). However, p65 activation is typically signified by increased phosphorylation or nuclear translocation. CSE did increase phosphorylation of p65 after 30 min of exposure (Fig. 2.15A-B). This increase in phosphorylated p65 was prevented by RvD2 treatment (Fig. 2.15A-B). Phosphorylation of another NF-κB member, IκBα, also contributes to inflammatory signaling as IκBα phosphorylation promotes degradation of this molecule. This frees p65 to form p65/p52 dimers and activate inflammatory genes. Similar to p65, CSE increased expression of phosphorylated IκBα, and these increases were dose-dependently prevented by RvD2 treatment (Fig. 2.15C-D).

Along with the canonical NF-κB signaling pathway, an alternative NF-κB signaling pathway exists which has several anti-inflammatory effects (68). Our lab has shown the importance of RelB in mediating anti-inflammatory responses in response to cigarette smoke (94, 97, 102). Thus, we decided to investigate several members of the alternative NF-κB signaling pathway. CSE had no effect on p100 expression, but increased expression of the cleaved p52 product (Fig. 2.16A-B). This increase in p52 was reduced by RvD2, though p100 expression remained unchanged (Fig. 2.16A-B). Interestingly, CSE caused a dramatic loss of RelB expression, which was significantly

prevented by RvD2 (Fig. 2.16C-D). These studies support changes in NF-κB expression as a possible mechanism for resolvins, and include evidence for the first time supporting the involvement of the alternative NF-κB pathway.

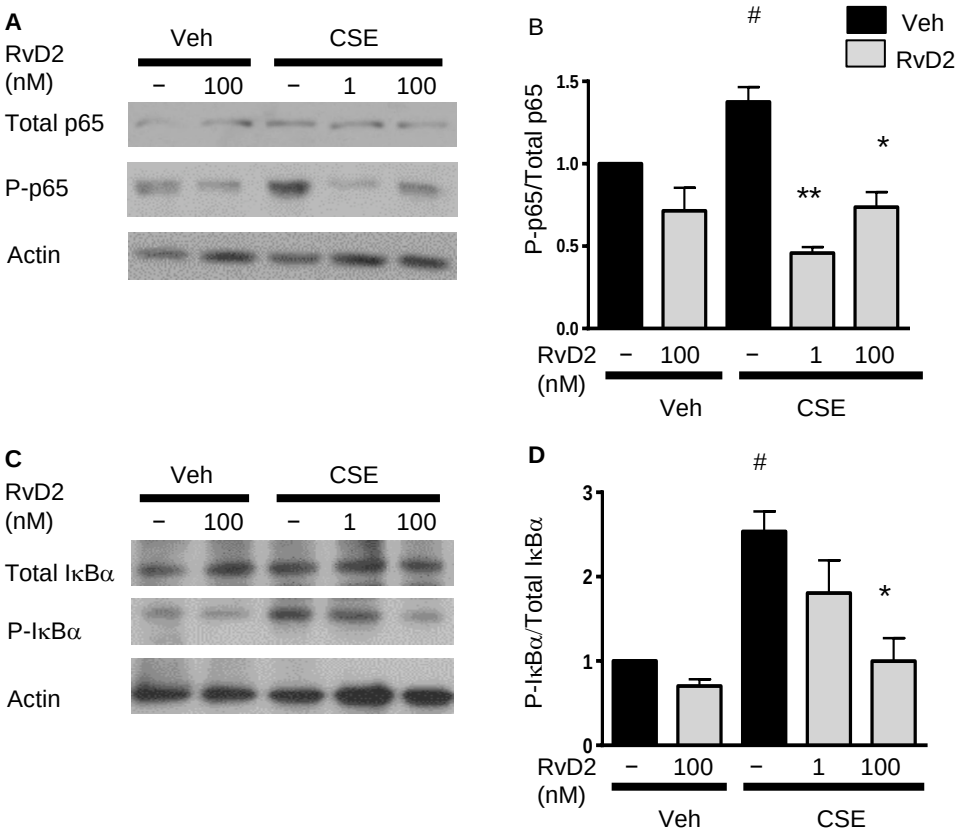

Fig. 2.15. Resolvin D2 dampens classical activation of the NF-κB pathway. Macrophages were incubated with RvD2 prior to CSE exposure. Protein expression levels of total and phosphorylated p65 (A, B) and total and phosphorylated IκBα (C, D), were determined by western blot and quantified by densitometry (representative images shown, n=4. Statistical significance was determined by two-way ANOVA with Bonferroni posttest ($^{\#}p<0.05$ compared to veh/veh, $^{*}p<0.05$, $^{**}p<0.01$ compared to CSE alone), n=4 individual donors.

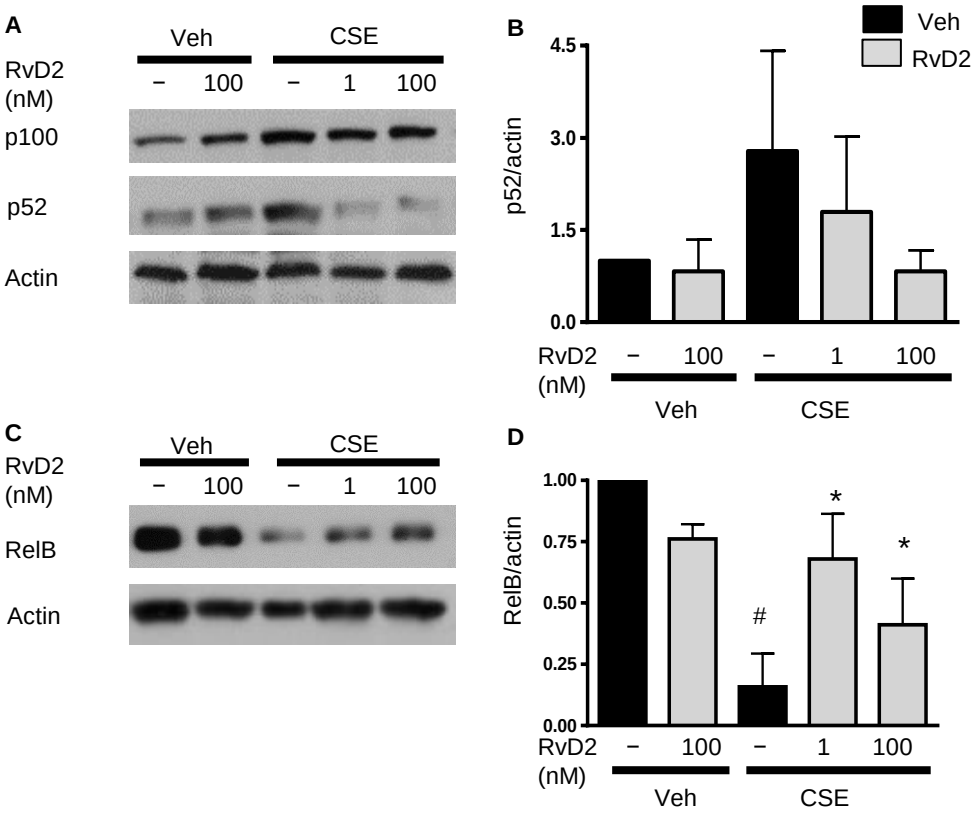

Fig. 2.16. Resolvin D2 dampens classical and promote alternative activation of the NF-κB pathway. Macrophages were incubated with RvD2 prior to CSE exposure. Protein expression levels of p100/p52 (A, B), and RelB (C, D) were determined by western blot and quantified by densitometry (representative images shown, n=4). Statistical significance was determined by two-way ANOVA with Bonferroni posttest ($^{\#}p<0.05$ compared to veh/veh, $^{*}p<0.05$ compared to CSE alone), n=4 individual donors.

Discussion

SPMs represent a novel class of lipid mediators with high clinical potential for treatment of inflammatory diseases. Here, we have shown that D-series resolvins dampen inflammatory effects induced by either cigarette smoke or resultant from smoking associated diseases in human blood derived and alveolar macrophages. RvDs reduced production of key pro-inflammatory cytokines, enzymes, and lipid mediators. RvDs also acted through anti-oxidant mechanisms to dampen the effects of cigarette smoke. In addition to these anti-inflammatory effects, RvD1 and RvD2 promoted the production of anti-inflammatory cytokines and enhanced pro-resolving macrophage phagocytosis in both alveolar and peripheral monocyte derived macrophages. We have presented evidence that these actions are mediated through changes in NF-κB expression. Taken together, these data present new evidence for the role of SPMs as novel therapeutics in attenuating human cigarette smoke-induced inflammation.

Previous studies have used targeted lipidomic analysis to evaluate the presence of a few particular lipids in COPD patients and have shown dysregulated levels of PGE_2, leukotriene B_4 and several isoprostanes (4, 113-115). Omega-3 and omega-6 derivatives were also detected in the urine of smoker and non-smoker volunteers, with smokers having decreased concentrations of SPMs (116). We have for the first time identified these lipid derivatives in human bronchoalveolar lavage fluid from COPD and non-COPD patients. We detected dysregulated levels of HDHA and HETE intermediates in BALF from COPD subjects, as well as lower concentrations of RvD1 in COPD serum, indicating that bioactive lipid mediators play a key role in the progression of this disease.

These data support the idea that chronic pulmonary diseases, and specifically COPD, occur in part due to a "failure to resolve" and produce appropriate eicosanoids to promote resolution and repair. We have previously shown that expression of a key SPM receptor (ALX/FPR2) is increased in COPD lungs, which correlates with the increases in HDHA and HETE shown here; these increases are likely a compensatory mechanism as cells attempt to respond to a chronic inflammatory stimulus (72). Alternatively, cigarette smoke may be dampening enzymatic activity that promotes SPM formation (resulting in decreased RvD1) while promoting autoxidation that contributes to increased HDHA and HETEs (117-119). In addition, there are multiple other SPMs, including other resolvins such as RvD3 and RvD5, which were not tested here; these SPMs may still play important roles in cigarette smoke associated diseases. These data together provide novel insight into endogenous inflammatory and resolving signals in the lung, as well as in the context of cigarette smoke exposure.

SPMs have both anti-inflammatory and pro-resolving effects, wherein they do not simply shut down inflammatory pathways, but rather promote a paradigm shift to activate resolution and repair mechanisms (6, 39, 46, 87, 120). This involves the reduction of pro-inflammatory cytokine and chemokine production with the simultaneous induction of anti-inflammatory cytokines. Changes in key cytokines may have strong biological consequences, but particularly in the context of an inflammatory microenvironment, the collective changes in cytokine regulation could have a combined potent effect and act in concert to promote resolution. Alternatively activated macrophages that promote resolution and enhance phagocytosis rather than pro-inflammatory signaling have

recently become a key area of interest and investigation (121). Here, we show that macrophages treated with RvDs not only have dampened inflammatory cytokine production, but also produce anti-inflammatory cytokines, such as TGF-β, and have an increased ratio of M2/M1 macrophages. These experiments are critical because they reinforce that resolvins are not only anti-inflammatory, but pro-resolving, and are not fully immunosuppressive, a crucial characteristic for their advancement as therapeutics. In addition to changes in cytokine production, SPMs have been shown to increase uptake of apoptotic neutrophils by human cells and to enhance mouse macrophage phagocytic abilities in a mouse model of acute lung injury (36, 37, 41, 43). Our studies here demonstrate for the first time that RvD1 and RvD2 prevent cigarette smoke-induced loss of human macrophage phagocytic abilities and promote increased uptake of bacteria. Additionally, we showed that RvDs enhanced phagocytosis in human alveolar macrophages from patients with chronic pulmonary diseases. In both cases, RvDs actions on macrophages were noted in cells from many different human donors. By using multiple donors we were able to account for inter-human variations and gauge the efficacy of these molecules across a broader human population with a greater range of underlying inflammatory conditions than would be seen in a cell line. The fact that efficacy was observed in multiple human donors points to a broad scope of eicosanoid actions, strengthening the therapeutic potential of these molecules.

RvDs are also capable of moderating production of other lipids and Cox-2. We observed that RvDs decreased CSE-induced Cox-2 and PGE_2 expression while promoting PGD_2 production. Interestingly, RvD treatment alone increased expression of Cox-2.

While this enzyme is commonly categorized as pro-inflammatory, (104, 105) Cox-2 also produces precursors of SPMs (108, 122, 123), and can be temporally regulated by SPMs (124). Furthermore, several prostaglandins can have either pro-inflammatory or anti-inflammatory actions depending on their concentration and/or what cell type they are acting on. PGD_2, for example, signals through two different G-protein coupled receptors (GPCRs) in macrophages with differential effects, which may account for increases in expression with both smoke and RvDs (109, 125). PGD_2 also counterregulates expression of PGE_2 and is converted to 15-deoxy-PGJ_2, which has pro-resolution properties that may result from inhibition of NF-κB (107, 123, 126, 127). Although PGE_2 is often considered a pro-inflammatory mediator, it also has anti-inflammatory actions, including reducing IL-6 levels and suppressing allergic airway inflammation (105, 107, 128, 129). PGE_2 also upregulates IL-10 (130). It is interesting in this context that RvD1 and RvD2 inhibit IL-6 production but do not upregulate IL-10; we cannot rule out the possibility that the absence of IL-10 is due to reduced PGE_2 production. The mechanisms by which SPMs regulate other lipids appear to be complicated and merit in depth investigation. Overall, Cox-2 is capable of producing a milieu of lipid mediators with counteracting and concentration-dependent actions, and investigation of this enzyme's role in resolution is an intriguing and ongoing area of research.

In addition to production of pro- and anti-inflammatory proteins, macrophages have key roles in oxidative stress and anti-oxidant activities. Oxidative stress is a hallmark of COPD and other smoking-associated diseases, and when left unchecked can cause excessive tissue damage. In our study, RvDs did not alter CSE-induced production

of reactive oxygen species (ROS). Other SPM precursors, such as docosahexaenoic acid (DHA), have been shown to reduce ROS induction in THP-1 cells, a monocytic cell line (122). This discrepancy may be due to a number of differences in methodology, as well as inherent differences between primary human cells and immortalized cell lines. We did observe changes in carbonylated proteins at 24 hours, indicating that it takes some time for carbonylated proteins to accumulate and thus to observe the effects of RvDs. Since RvDs have not been shown to be direct antioxidants, they may be acting to increase anti-oxidative proteins or enzymes, such as superoxide dismutase or glutathione, rather than to prevent ROS generation. They may also enhance clearance and degradation of oxidatively damaged proteins. Furthermore, while the anti-oxidant protein Nrf2 was unaffected, we observed that cigarette smoke increased HO-1 expression, possibly in a feedback loop. RvD treated cells did not show this same induction, which may indicate a lower level of oxidative stress that did not necessitate a compensatory induction of HO-1. Other studies have shown that SPMs can induce HO-1 activity, though these studies were done using macrophage cell lines or observed in the mouse system using different stimuli (122, 131, 132). There may also be temporal differences in HO-1 regulation, where RvDs upregulate expression earlier and thereby promote resolution more quickly. More comprehensive studies are needed to fully elucidate the effects of RvDs as anti-oxidant molecules.

There are several mechanistic pathways through which SPMs promote the resolution of inflammation. Human macrophages have been shown to have SPM receptors, including ALX/FPR2 and GPR32 (14). Expression of SPM receptors is often

dysregulated in chronic diseases (66, 133). Particularly regarding COPD, a disease in which ALX/FPR2 is dysregulated, no correlation has yet been made between receptor expression levels and disease severity. This poses an interesting area of research and could highlight a new usage for SPM receptors as biomarkers. Here, we have shown that acute cigarette smoke exposure dose-dependently decreases ALX/FPR2 receptor levels, which could be a contributing factor to failed resolution in smoking-associated diseases. While COPD patients have increased expression levels of ALX/FPR2, there may be an initial dampening, leading to reduced SPM efficacy, which long-term results in elevated ALX/FPR2 levels in an attempt to compensate for dysregulated resolution (66). We further showed that Boc-2 peptide and anti-GPR32 antibodies can attenuate the effects of RvD1, with blocking of each receptor affecting different cytokines. This implicates complementary roles for the different RvD1 receptors. GPR18, the receptor for RvD2, has very recently been identified, though whether RvD2 can also bind to other SPM receptors is largely unknown (12). Further investigations are needed to uncover if expression of GPR18 is altered with cigarette smoke exposure and/or in COPD progression.

In addition to acting through unique receptors, SPMs regulate activation and signaling of numerous inflammatory pathways, including the NF-κB pathway (38, 71, 89). We have shown here that, in human macrophages, RvD2 acts, in part, by preventing the phosphorylation and thereby degradation of IκBα. This in turn prevents the release of p65 and subsequent activation by phosphorylation. In addition, RvD2 acts through the non-canonical NF-κB pathway to mediate its effects. Alternative NF-κB signaling, and in

particular expression of RelB, has recently been shown by our lab to be anti-inflammatory (94, 97, 102, 134). Cigarette smoke-exposed mice have decreased expression of RelB in bronchoalveolar lavage fluid, and overexpression of RelB can attenuate the effects of cigarette smoke exposure in human fibroblasts *in vitro* (97). Our new data here shows that cigarette smoke exposure causes a loss of RelB expression in human macrophages and that this loss can be partially prevented by RvD2. This is the first evidence for SPMs altering alternative NF-κB signaling and presents a novel pathway through which these bioactive lipid mediators may act. There are also other signaling pathways that may play important roles in mediating SPM effects, including STAT3, CREB, and MAPK. SPMs, including RvD1 and LxA_4 decrease cytokines through decreasing STAT3 signaling (135-137). Multiple SPMs also act to decrease members of MAPK pathways (44, 69, 72). Clearly, the signaling mechanisms of resolution are multifaceted and represent a broad area for future studies.

In conclusion, we have shown that D-series resolvins effectively dampen and attenuate the effects of cigarette smoke exposure in human macrophages. Our data showing resolvins' pro-resolving effects on human alveolar macrophages particularly highlight the potential of these SPMs in dampening inflammation associated with human chronic diseases, including COPD. The identification of SPMs actions on alternative NF-κB signaling is also a novel finding that opens up multiple areas of investigation. The dual anti-inflammatory and pro-resolving actions of these bioactive lipid mediators makes them important candidates for treatment of inflammatory diseases, including those induced by cigarette smoke exposure. Future investigation of the mechanisms of action

and specific targets of these novel endogenous mediators will allow for translation into a clinical setting and development of SPMs as therapeutics.

Chapter 3

Resolvin D2 Decreases TLR4 Expression to Mediate Resolution in Human Monocytes

Abstract

Many inflammatory stimuli induce a response through activation of toll-like receptors (TLRs), a major family of receptors in innate immune signaling. Recent investigations have led to the discovery of endogenously produced, specialized pro-resolving mediators (SPMs); these lipid mediators play a critical role in the active resolution of inflammation. SPMs attenuate lipopolysaccharide-induced inflammation, but whether they alter early LPS signaling, including TLR4 induction, is unknown. We hypothesized that SPMs would dampen inflammatory endpoints in human monocytes by decreasing TLR4 expression. Human THP-1 cells and primary blood monocytes were treated with SPMs followed by LPS and other activators. LPS induced expression of ALX/FPR2 and GPR18, two primary SPM receptors. RvD2 most potently reduced LPS-induced cytokine levels, but not those stimulated by other TLR ligands. RvD2 further decreased expression of TLR4 and downstream signaling pathway markers (such as MyD88) in both human monocytes and THP-1 cells. In THP-1 cells, RvD2 further reduced expression of MD-2, a critical binding partner of TLR4 necessary for mediating LPS responsiveness. These effects were partially mediated through RvD2-induction of microRNA146a, and the actions of RvD2 were blocked by microRNA146a inhibition. These findings show that RvD2 attenuates LPS-induced inflammation through reductions in TLR4 and downstream signaling pathways. Modulation of TLR expression is a major area of therapeutic research, and provides a new area of SPM activity to investigate.

Introduction

Toll-like receptors (TLRs) are one of the essential components of the immune response, and mediate innate immune activation towards a variety of acute stimuli and invading pathogens. In particular, monocytes highly express TLR4, the primary TLR responsible for responding to lipopolysaccharide (LPS-the major cell wall component of gram-negative bacteria). TLR4 also allows signaling in response to non-microbial insults, such as hyaluronan fragments, resistin, and fibrinogen. These activators and LPS induce a strong pro-inflammatory response through initiation of the TLR4 signaling cascade. CD14 and LPS-binding protein (LBP) are responsible for presenting LPS to TLR4, which is complexed with myeloid differentiation factor-2 (MD-2) (73, 75, 76). A TLR4/MD-2 multimer forms and signals using both the MyD88 and TRIF/TRAF signaling pathways. These signals activate NF-κB, AP-1, and interferon elements and promote pro-inflammatory cytokine production, cell activation, and anti-microbial and anti-viral responses (73, 75, 76).

While the responsiveness to invading pathogens is an important protective response, prolonged activation of TLR4 can lead to numerous health complications (78, 138, 139). Exposure to LPS can lead to a feed forward loop, resulting in continual upregulation of TLR4 and amplified responses. Acutely, excessive TLR4 activation contributes to enhanced cell death, cytokine storm, systemic inflammatory responses, and septic shock (138, 140, 141). Chronically, TLR4 activation has also been implicated in a variety of diseases, including pneumonia, chronic obstructive pulmonary disease, and asthma (141-143). In studies using cigarette smoke and COPD models, excessive TLR4

activation leads to airway inflammation, but an absence of TLR4 signaling results in abnormal breakdown of alveolar structure, suggesting that the balance of TLR4 activation is critical for mitigating disease progression (77). Similar results have been observed in asthma models, wherein TLR4 expression is necessary to mount an allergic response, but the cytokine profiles/exacerbatory effects are dependent on the dose of LPS used to activate TLR4 (144). In several pulmonary disease states patients have altered TLR4 levels (reduced in asthmatics) or increased incidence of TLR4 polymorphisms (such as those found in COPD patients), further supporting a role for this particular TLR in disease progression (77, 145). Interestingly, a phenomenon termed "tolerance" occurs *in vitro* in response to LPS, where cells that undergo repeated LPS stimulations become tolerant to this activation and fail to produce pro-inflammatory cytokines or mount an immune response (146, 147). While the clinical existence and importance of tolerance is highly debated, it demonstrates the complexity of TLR4 regulation, and the importance of appropriate activation of TLR4 to avoid insufficient or excessive responsiveness.

There is a growing interest in the efficacy of SPMs in acute lung inflammation and against viral and bacterial infections. Many studies have used LPS as a bacterial mimetic to model microbial infections, and several of these studies (primarily in mice) have demonstrated efficacy of SPMs in attenuating LPS-induced inflammation. LPS triggers production of LxA_4 and SPM intermediates in mouse plasma, highlighting a potential feedback loop for resolution (148). LxA_4 and D-series resolvins can also dampen LPS-induced inflammation in mouse models of acute lung injury and acute injury (38, 40, 149, 150). RvD1 and other SPMs dampen pro-inflammatory cytokines and

cellular influx in mice exposed to LPS. The actions of these SPMs results in reduced tissue destruction and improved survival in mice (40, 149). These studies used different forms of LPS, and sometimes heat-inactivated *E. coli*, providing evidence that SPMs are acting on common mechanisms to mediate resolution. Despite these results showing that SPMs are effective at attenuating LPS-induced inflammation, very little is known about the effects of SPMs on TLR expression and signaling, especially in human cells. Based on the non-immunosuppressive nature of resolvins and their efficacy in acting on macrophages in other inflammatory models, we hypothesized that SPMs dampen TLR4 expression to attenuate LPS-induced inflammation.

Materials & Methods

Materials

RvD2 (10007279), RvD1 (10012554), and LxA_4 (90410) were purchased from Cayman Chemical (Ann Arbor, MI). Poly I:C (tlrl-pic) was purchased from Invivogen (San Diego, CA). Pam3CSK (NBP2-25297) was purchased from Novus Biologicals (Littleton, CO). LPS 0111:B4 (L4391), anti-TLR4 antibody (PRS3141), LPS 055:B5 (6529), hyaluronan (S0326) were purchased from Sigma (St. Louis, MO). Anti-TLR4 APC-conjugated antibody (17-9917-42) was purchased from eBioscience (San Diego, CA). Anti-MD-2 antibody (ab24182) was purchased from Abcam (Cambridge, United Kingdom). Anti-CD14 antibody (555398) and ELISA components for IL-6 (554543 and 554546) and TNFα (555212) were purchased from BD Biosciences (San Jose, CA). ELISA antibodies for IL-8 (M-801, M-802-B) were purchased from Endogen (Farmingdale, NY). Anti-actin antibody (CP-01) was purchased from Calbiochem (Darmstadt, Germany). Secondary western blot antibodies (115-035-146, 111-035-144) were purchased from Jackson ImmunoResearch (West Grove, PA). PBS (14200-075) and RPMI 1640 (11875-119) were purchased from Gibco (Waltham, MA). Primers for RT-PCR were ordered from Integrated DNA Technologies (Coralville, IA, see Table 3.1).

RT-PCR Primer Sequences		
18s	h18s F1	GGTCGCTCGCTCCTCTCCCA
	h18s R1	AGGGGCTGACCGGGTTGGTT
GPR18	hGPR18 F1	ACACAGACTTTTGATGGACAGG
	hGPR18 R1	AAGACAAGGGCTGCAATTTTG
GPR32	hGPR32 F1	GTGATCGCTCTTGTTCCAGGA
	hGPR32 R1	GGACGCAGACAGGATAACCAC
ALX/FPR2	hALXFPR2 F1	AGTCTGCTGGCTACACTGTTC
	hALX/FPR2 R1	AGCACCACCAATGGGAGGA
TLR2	hTLR2 F1	TGTGAAGAGTGAGTGGTGCAAGT
	hTLR2 R1	ATGGCAGCATCATTGTTCTCAT
TLR3	hTLR3 F1	CCTGGTTTGTTAATTGGATTAACG
	hTLR3 R1	GAGGTGGAGTGTTGCAAAGGTAGT
TLR4	hTLR4 F1	AGCTCTGCCTTCACTACAGAGACTT
	hTLR4 R1	GCTTTTATGGAAACCTTCATGGA
MyD88	hMyD88 Fr	AATCTTGGTTCTGGACTCGC
	hMyD88 R1	CAGAAGTACATGGACAGGCAG
TRIF	hTRIF F1	CTGGAACAGTGAATGGGTAGG
	hTRIF R1	GAAGGCGCTAGGAAGTGATG
TAK1	hTAK1 F1	GTAACACGTACACAGACCTCTC
	hTAK1 R1	GTGAAAACACTCAATGGGCC
LBP	hLBP F1	GAAGCCAGGAAAGGTAAAAGTG
	hLBP R1	GGGTAGAAGGTGTTAAGGATGT
MD-2	hMD-2 F1	CCCTGTATAGAATTGAAAAGATCCAAAG
	hMD-2 R1	TCAGATCCTCGGCAAATAACTTC
gp96	hGP96 F1	AAACGGGCAAGGACATCTC
	hGP96 R1	AAACCACAGCAAGATCCAAAAC
PRAT4A	hPRAT4A F1	CTCCTGGATTATAGCCTGCAC
	hPRAT4B R1	GTCTCGTTCCACAGCTCATAG

Table 3.1. Primer sequences for RT-PCR. All primers were used at a final concentration of 200 nM in PCR reactions.

RvD2 treatment & LPS exposure

For human monocytes, PBMCs were isolated as previously described (96). Human peripheral blood monocytes were further isolated by adherence to tissue culture dishes for 2 hours. Cells were treated with 1-100 nM of LxA_4, RvD1, or RvD2 for one hour prior to activation. Human monocytes were then treated with 20 ng/mL LPS (0111:B4), Poly I:C, or Pam3CSK. For assessment of GPR18, TLRs, MD-2, and MyD88 mRNA, one hour after activation cells were washed twice with 1X PBS and lysed with CW lysis buffer (50 mM Tris-HCl, 2% SDS). RNA was isolated as described below. Supernatants were collected 24 hours after exposure and cytokines (IL-6, IL-8, and TNFα) were assessed by ELISA according to manufacturer's protocols.

In separate experiments, THP-1 cells were plated in 12 well plates in serum-free media for 24 hours. Following serum starvation, monocytes were treated with 1-100 nM RvD2 or vehicle (0.1% EtOH in PBS) for 1 hour prior to exposure to 20 ng/mL LPS (0111:B4) or vehicle (1X PBS). To assess TLR4 activation with multiple stimuli, cells were additionally treated with LPS 055:B5 or hyaluronan. Following activation, THP-1 cells were washed twice with 1X PBS and lysed with CW Buffer (50mM Tris-HCl, 2% SDS).

Assessment of TLR4 & associated genes

RNA was isolated using the Qiagen miRNEASY kit (217004, Germantown, MD) according to manufacturer's protocols. RNA was quantified by Nanodrop Instrument and cDNA synthesized using a Biorad iScript kit according to manufacturer's protocols (170-

8891, Hercules, CA). MRNA levels of ALX/FPR2, GPR32, GPR18, TLR4, MyD88, TRIF, TAK1, LBP, MD-2, gp96, and PRAT4A were determined by semi-quantitative PCR using a CFX Connect Real Time Detection System from BioRad. CD14 and TLR4 were assessed by flow cytometry as previously described (65). Western blots were performed as previously described to detect TLR4 and MD-2 protein expression (65).

MiRNA evaluation and transfections

All miRNA reagents were purchased from Applied Biosystems Life Technologies (Grand Island, NY). Primers for hsa-miR-146a and 146b (4427975, 4427975) were used for cDNA and RNA synthesis. MiRNA specific cDNA was synthesized according to manufacturer's protocol using the Taqman MicroRNA Reverse Transcriptase Kit (4366595). MiRNA levels were assessed by PCR using the Taqman Universal PCR Master Mix (4304437).

For miR146a mimic and inhibitor experiments, THP-1 cells were plated in 12-well plates in RPMI 1640 with 1% FBS. Cells were transfected with 30 nM of a miR146a mimic (4464066) or a control mimic (4464058), or 20 nM of a miR146a inhibitor (4464084) or control inhibitor (4464076) for 24 hours using lipofectamine 2000 (11668-019, Invitrogen, Carlsbad, CA). Briefly, mimic/inhibitor or lipofectamine were added to separate tubes of 100μL/sample of Opti-Mem media and incubated at room temperature for 5min. Mimic/inhibitor and lipofectamine were then mixed in a 1:1 ratio and incubated at room temperature for an additional 20 min before addition of 200 μL lipofectamine mix to each sample. Following transfection, cells were washed with 1X PBS and then

treated as described above with RvD2 and LPS. The effects of miR146a mimic or inhibitor were assessed on IL-8, TNFα, and TLR4 expression.

Statistical Analysis

All experiments were repeated three to five times with THP-1 cells or with three to six human donors in independent experiments. Results are expressed as mean ± standard error (SEM). Statistical analyses on normally distributed data were performed using a t-test or one- or two-way analysis of variance (ANOVA) with Bonferroni's posttest correction for multiple comparisons using GraphPad Prism Software (San Diego, CA).

Results

RvD2 decreases LPS-induced cytokine production and TLR4 in human monocytes

Human monocytes and macrophages contribute to the synthesis of multiple SPMs, including D-series resolvins (151). In particular, LxA_4 and D-series resolvins (RvD1 and RvD2) have shown efficacy at attenuating inflammation and acting through monocytes/macrophages to mediate their effects (43, 65, 152-154). Along with producing both of these SPMs, monocytes and macrophages express their receptors. RvD1 signals through both ALX/FPR2 and GPR32, while GPR18 was recently identified as the RvD2 receptor (12, 14). LPS treatment of both THP-1 cells and human monocytes increased expression of ALX/FPR2 and GPR18, likely initiating a feedback loop; no changes were observed in GPR32 expression (Fig. 3.1A-F). These results, along with the known production of SPMs by human monocytes/macrophages, led us to next investigate whether candidate SPMs could alter inflammatory responses induced by LPS and other TLR ligands.

We tested several SPMs to determine if they could dampen LPS-induced inflammatory effects. Human monocytes were treated with 1-100 nM of LxA_4, RvD1, and RvD2 and exposed to LPS. Each SPM acted in a dose-dependent manner to reduce IL-6, IL-8, and TNFα levels (Fig. 3.2). The greatest anti-inflammatory effects were seen with RvD2, thus, we selected this SPM for further evaluation.

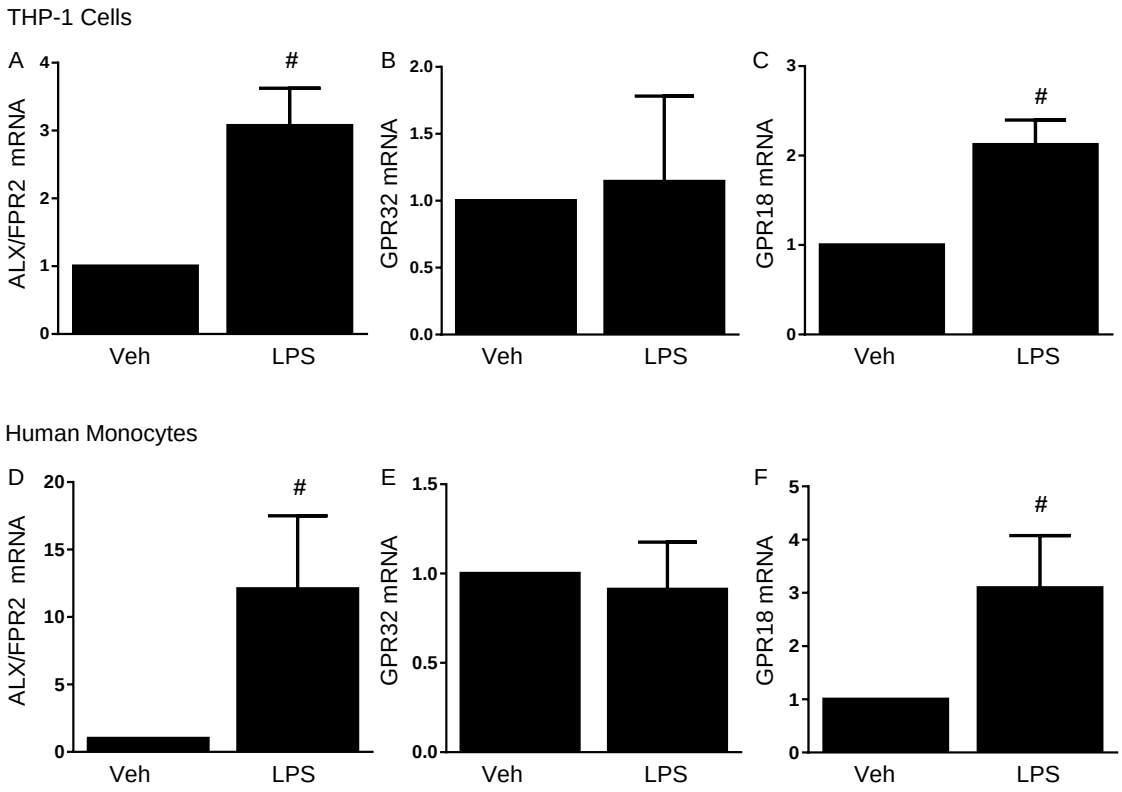

Fig. 3.1. ALX/FPR2 and GPR18 are increased with LPS treatment. THP-1 cells (A-C) and human blood monocytes (D-F) were treated with 20 ng/mL LPS. 4 hours after activation, levels of SPM receptors ALX/FPR2 (A, D), GPR32 (B, E), and GPR18 (C, F) were assessed by RT-PCR. Statistical significance was determined by t-test ($^{\#}p<0.05$ compared to veh), n=3-4 independent experiments or individual donors.

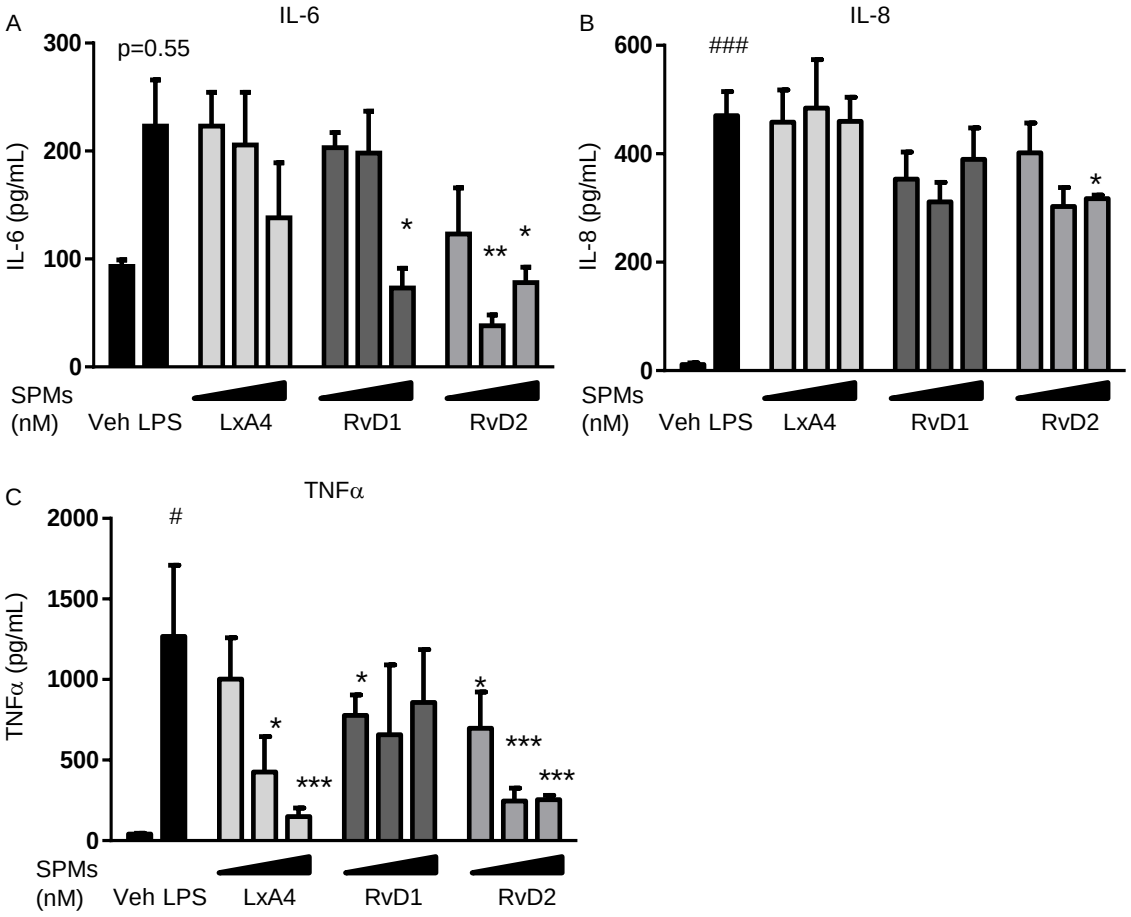

Fig. 3.2. RvD2 most potently dampens LPS-induced cytokine production by human monocytes. Human blood monocytes were treated with 1, 10, or 100 nM of LxA_4, RvD1, or RvD2 followed by 20 ng/mL LPS. 24 hours after activation levels of IL-6 (A), IL-8 (B), and TNFα (C) were assessed by ELISA. Statistical significance was determined by Two-way ANOVA ($^{\#}p<0.05$, $^{\#\#\#}p<0.0001$ compared to veh/veh, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared to veh/LPS) n=3-4 individual donors.

We also wanted to determine if SPM dampening of inflammation and inflammatory cytokine production was specific to LPS/TLR4 activation. To evaluate the potential efficacy of RvD2 in attenuating inflammation induced by different TLRs, human monocytes were treated with a range of TLR ligands and cytokine production was assessed. Ligands for TLR2, 3, and 4 were chosen based on the high expression level of these TLRs in human monocytes (73, 74). RvD2 failed to have a significant impact on IL-6, IL-8, or TNFα production induced by TLR2 (Pam3CSK) and TLR3 (PolyI:C) ligands. However, TLR4 (LPS)-induced cytokine production was significantly reduced (Fig. 3.3A-C).

Based on these data, we next evaluated the effect of RvD2 on expression of TLRs themselves. Similar to our observed cytokine effects, RvD2 did not dampen TLR2 or TLR3 mRNA expression (Fig. 3.4A-B). RvD2, however, did decrease TLR4 mRNA expression in human monocytes after stimulation with LPS (Fig. 3.4C). We further explored whether RvD2 would alter baseline expression of TLR4 or whether these effects were only seen with LPS activation. RvD2 did not alter basal TLR4 expression in these cells, and only attenuated LPS-induced TLR4 levels (Fig. 3.5A). Along with mRNA, we confirmed these changes by evaluating TLR4 protein expression by western blot. In similar fashion, LPS strongly upregulated TLR4 protein expression; RvD2 prevented this induction (Fig. 3.5B). RvD2 further decreased mRNA expression of MD-2, an important binding partner necessary for TLR4-mediated responses to LPS and MyD88, a downstream signaling molecule (Fig. 3.5C-D), indicating that these observed decreases in TLR4 also affect subsequent signals.

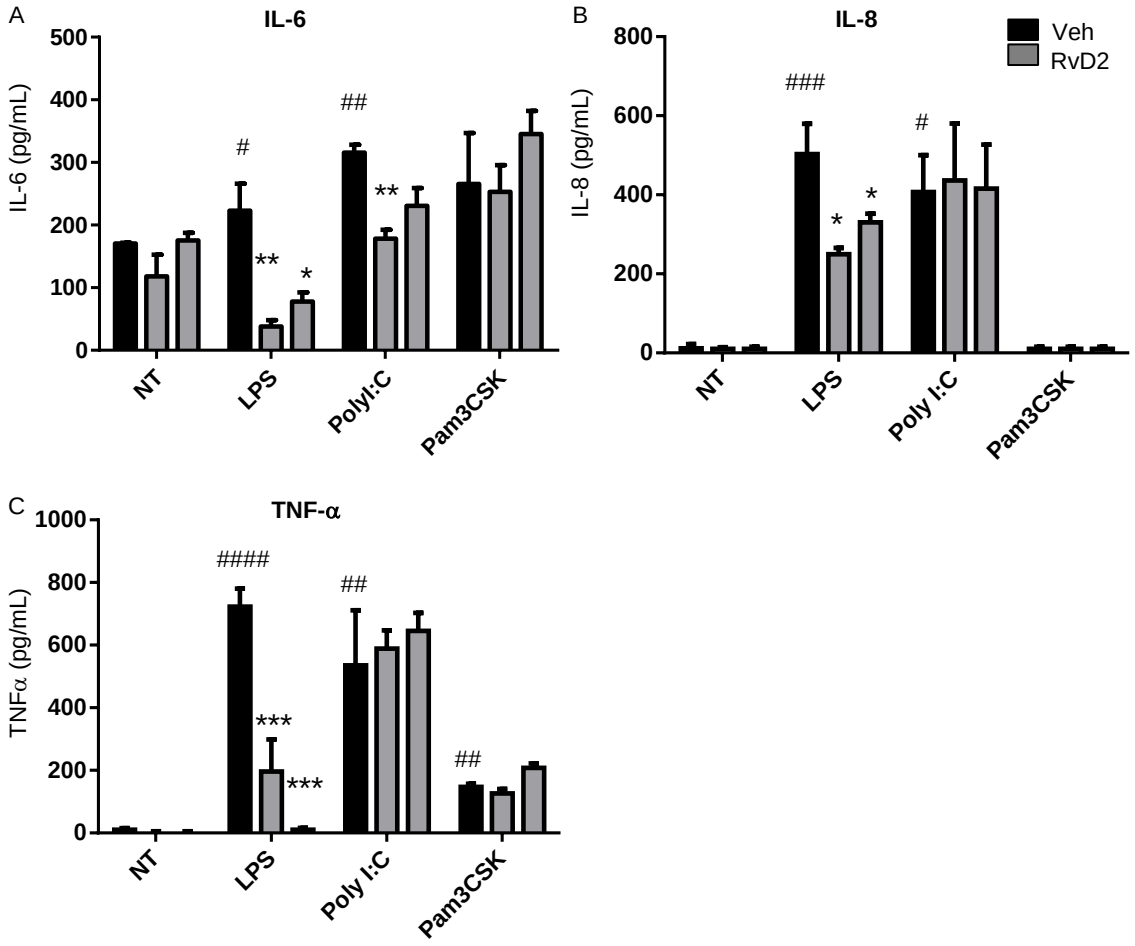

Fig. 3.3. RvD2 dampening of cytokine production is LPS-specific. Human blood monocytes were treated with 10 or 100 nM RvD2 followed by 20 ng/mL LPS, PolyI:C, or Pam3CSK. 24 hours after activation levels of IL-6 (A), IL-8 (B), and TNFα (C) were assessed by ELISA. Statistical significance was determined by two-way ANOVA with Bonferroni's posttest ($^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, $^{\#\#\#\#}p<0.0001$ compared to veh/veh, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared to veh/activator) n=3-4 individual donors.

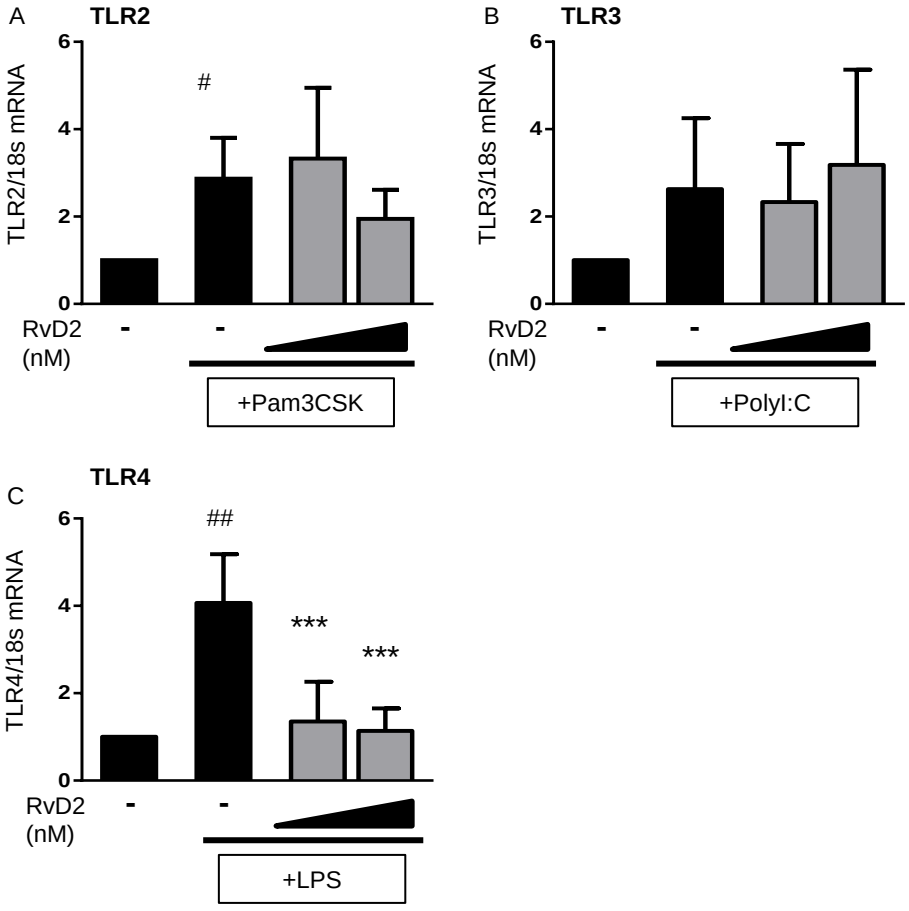

Fig. 3.4. RvD2 selectively decreases TLR4 in human monocytes. Human blood monocytes were treated with 10 or 100 nM RvD2 followed by 20 ng/mL LPS, PolyI:C, or Pam3CSK. TLR2 (A), TLR3 (B), and TLR4 (C) mRNA expression were determined 1 hour after activator stimulation by RT-PCR. Statistical significance was determined by two-way ANOVA with Bonferroni's posttest ($^{\#}p<0.05$, $^{\#\#}p<0.01$ compared to veh/veh, ***$p<0.001$ compared to veh/activator) n=4-5 individual donors.

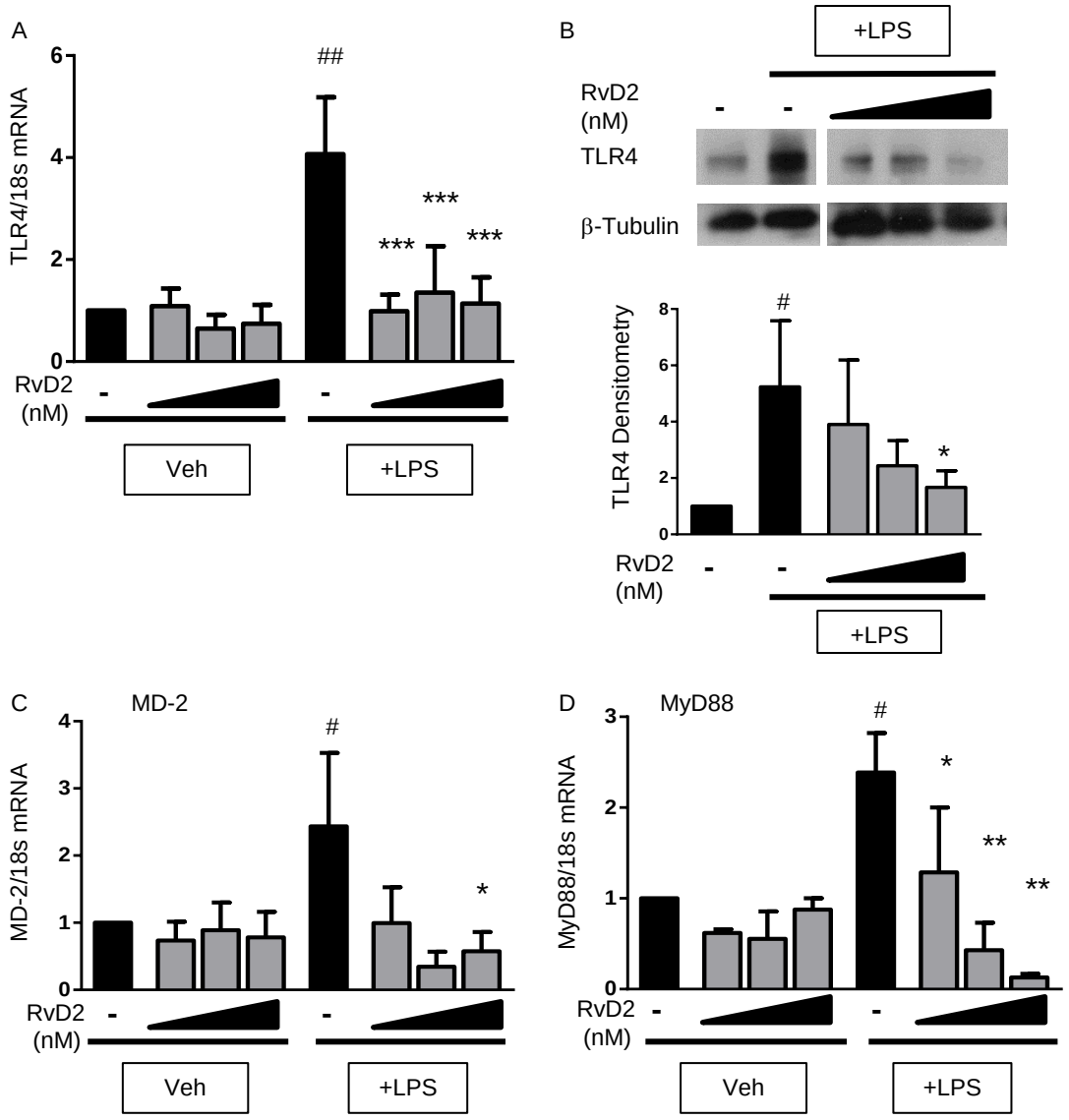

Fig. 3.5. RvD2 decreases LPS-induced TLR4 expression and downstream signaling molecules in human monocytes. Human blood monocytes were treated with 10 or 100 nM RvD2 followed by 20 ng/mL LPS. The effects of 1-100 nM RvD2 alone and with LPS treatment on TLR4 (D), MD-2 (F), and MyD88 (G) mRNA were assessed by RT-PCR. TLR4 protein expression (E) was determined by western blot (representative image shown, densitometry for n=4). Statistical significance was determined by two-way ANOVA with Bonferroni's posttest ($^{\#}p<0.05$, $^{\#\#}p<0.01$ compared to veh/veh, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared to veh/LPS) n=4-5 individual donors.

RvD2 decreases TLR4 and downstream signaling genes in THP-1 monocytes

To allow for more in depth studies, we used THP-1 cells, a human monocytic cell line, to further investigate the role of RvD2 in modulating TLR4 expression. THP-1 cells are a human monocytic cell line derived from a leukemia patient; they have been extensively characterized in the literature and used as an alternative to primary human monocytes and macrophages. LPS induced TLR4 mRNA expression in a time-dependent manner, with the strongest induction at 4 hours post-LPS exposure (Fig. 3.6A). RvD2 did not decrease TLR4 mRNA at 15 min or 1 hour, but significantly reduced mRNA at 4 hours post-exposure (Fig. 3.6A). We were curious if these reductions were specific to the form of LPS used in these studies. THP-1 cells were treated with several putative TLR4 ligands- two forms of LPS and hyaluronan all promoted TLR4 expression (Fig. 3.6B). RvD2 reduced TLR4 stimulated by all three of the ligands, indicating that these actions are not specific to a particular form of LPS or even to bacterial-derived ligands (Fig. 3.6B).

Similar to human monocytes, in THP-1 cells, RvD2 alone did not decrease basal TLR4 mRNA, but only decreased LPS-stimulated TLR4 (Fig. 3.7A). Along with mRNA, RvD2 reduced TLR4 protein expression (Fig. 3.7B). These changes were confirmed by specifically examining TLR4 surface protein expression by flow cytometry. Again, RvD2 dose-dependently reduced TLR4 surface expression, with the most potent effects observed at the 100 nM RvD2 dose (Fig. 3.7C-D).

We next investigated whether these changes in TLR4 expressed affected downstream signaling pathways. RvD2 can decrease NF-κB expression, the main endpoint of TLR4 signaling (65, 71). TLR4 is the only TLR to signal through both the MyD88 and TRIF signaling pathways, and indeed, we observed that LPS increased mRNA expression of both of these genes (Fig. 3.8A-B). RvD2 dose-dependently decreased expression of both MyD88 and TRIF after LPS-exposure (Fig. 3.8A-B). Additionally, RvD2 dampened LPS-induced decreases in TAK1, an intermediary gene that is downstream of both MyD88 and TRIF, which promotes NF-κB signaling (Fig. 3.8C). These data demonstrate that reductions in TLR4 have downstream functional effects, reducing multiple players in the signaling pathway to promote resolution.

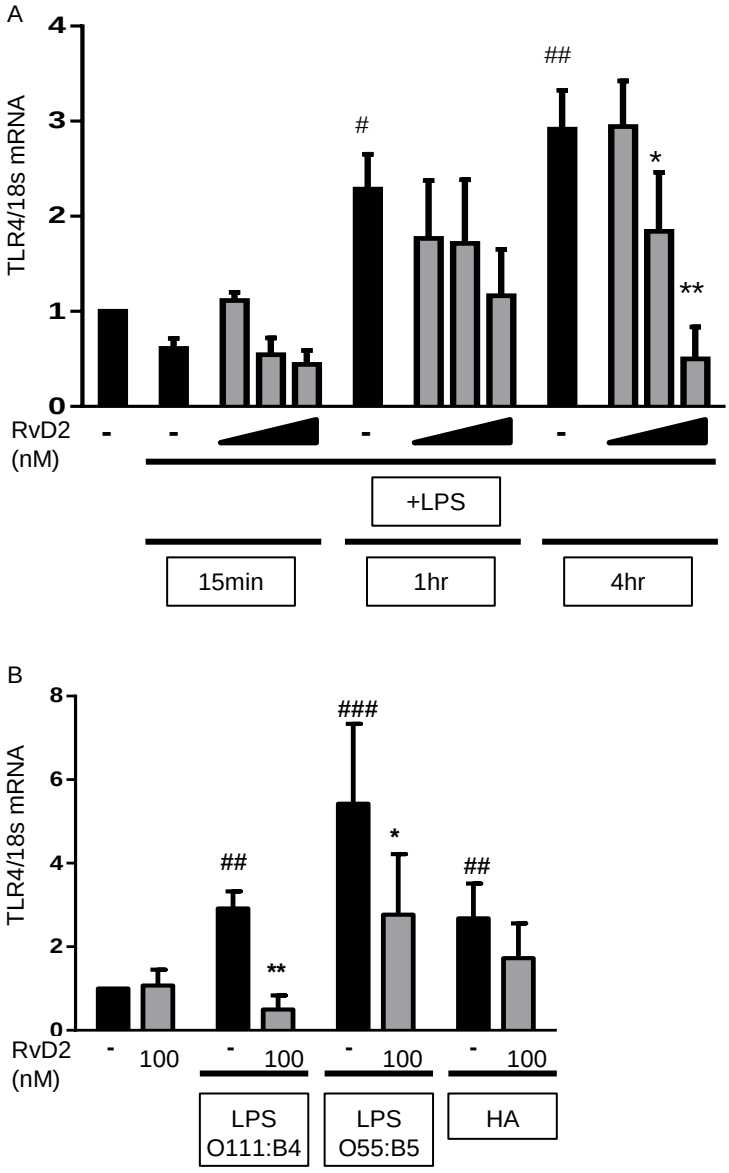

Fig. 3.6. RvD2 reduces TLR4 expression in a time-dependent manner in THP-1 monocytic cells. THP-1 cells were treated with 1-100 nM RvD2 followed by 20 ng/mL LPS. TLR4 mRNA was assessed 15 min, 1 hour, and 4 hours after LPS stimulation (A). THP-1 cells were also treated with 100 nM RvD2 followed by 20 ng/mL LPS (either 0111:B4 or 055:B5) or hyaluronan and TLR4 mRNA was assessed after 4 hours (B). Statistical significance was determined by two-way ANOVA with Bonferroni's posttest ($^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to veh/veh, *p<0.05, **p<0.01 compared to veh/activator) n=3-5 independent experiments.

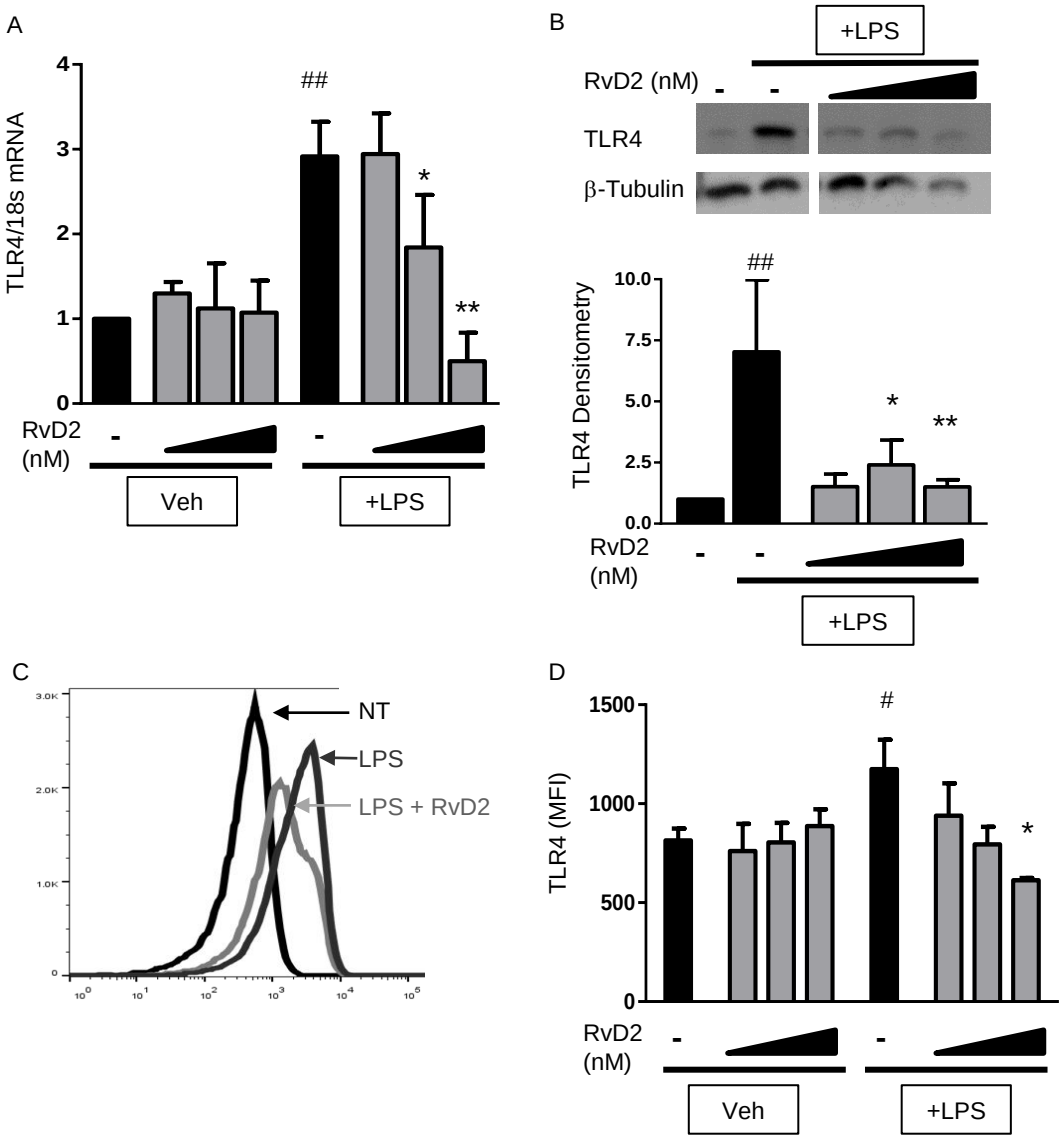

Fig. 3.7. RvD2 reduces TLR4 expression in THP-1 monocytic cells. THP-1 cells were treated with 1-100 nM RvD2 followed by 20 ng/mL LPS. The effects of 1-100 nM RvD2 alone and with LPS treatment on TLR4 mRNA were assessed 4 hours after activation (A). TLR4 protein expression (B) was determined by western blot (representative image shown, densitometry for n=4). TLR4 surface protein expression was evaluated by flow cytometry (C-D, representative donor shown). Statistical significance was determined by two-way ANOVA with Bonferroni's posttest ($^{\#}p<0.05$, $^{\#\#}p<0.01$ compared to veh/veh, $^{*}p<0.05$, $^{**}p<0.01$ compared to veh/LPS) n=3-5 independent experiments.

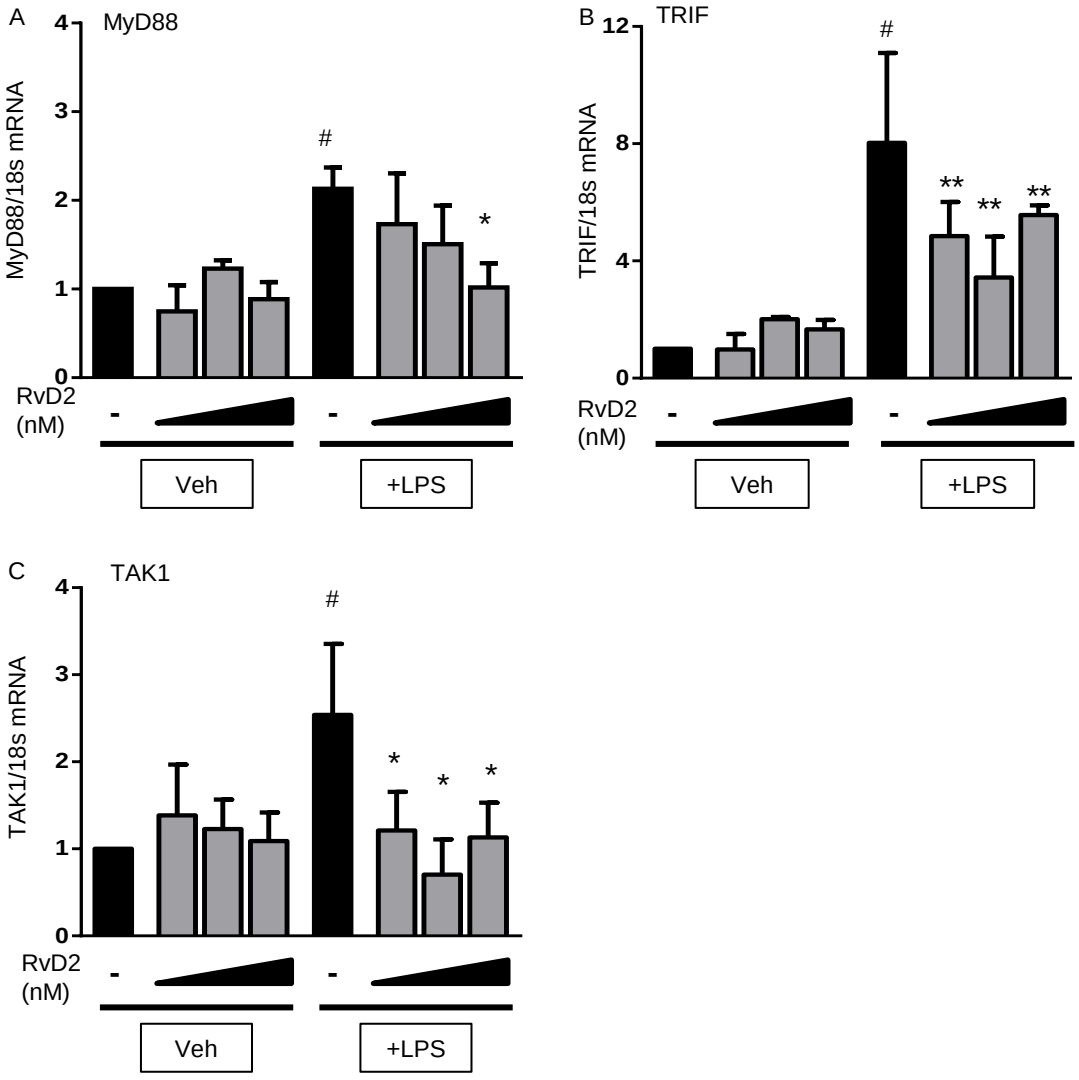

Fig. 3.8. RvD2 decreases mRNA expression of TLR4 downstream signaling molecules in THP-1 monocytic cells. Schematic of TLR4 downstream signaling (A). THP-1 cells were treated with 1-100 nM RvD2 followed by 20 ng/mL LPS for 4 hours. Expression of downstream signaling genes MyD88 (B), TRIF (C), and TAK1 (D) was determined by RT-PCR. Statistical significance was determined by two-way ANOVA with Bonferroni's posttest ($^{\#}p<0.05$ compared to veh/veh, $^{*}p<0.05$, $^{**}p<0.01$ compared to LPS/veh) n=3-5 independent experiments.

RvD2 decreases MD-2 expression in THP-1 cells

There are many additional signaling molecules necessary for mediating TLR4 responses to LPS. Two of these molecules, CD14 and LPS-binding protein (LBP), act to recognize LPS and present it to the TLR4 signaling complex. Neither CD14 nor LBP are necessary for cellular responsiveness to LPS, but they greatly increase the binding efficiency of LPS and TLR4, resulting in increased sensitivity (76). In our model, neither LPS nor RvD2 altered CD14 or LBP expression (Fig. 3.9A-B). In contrast, LPS strongly induced both mRNA and protein expression of MD-2 (Fig. 3.9C-D). MD-2 is a critical co-factor for LPS involved in TLR4 trafficking to the cell surface, formation of TLR4 homodimers, and LPS signaling. Furthermore, MD-2 is necessary for TLR4 responsiveness to LPS (75, 76, 155-157). RvD2 treatment dose-dependently reduced LPS-induced expression of MD-2 mRNA and protein (Fig. 3.9C-D).

Along with other proteins that aid in LPS signaling, multiple genes are involved in ensuring appropriate folding, glycosylation, and trafficking of TLR4 to the cell surface. Two main genes, gp96 and PRAT4A, are responsible for proper modification of TLR4 to allow for appropriate trafficking and activity (158-160). Neither LPS nor RvD2 altered expression of these trafficking proteins in THP-1 cells (Fig. 3.10A-B), indicating that the effects of RvD2 are not on the trafficking component of the TLR4 pathway. Overall, these data suggest that RvD2 specifically targets the TLR4/MD-2 complex, rather than altering LPS presentation or TLR4 trafficking to promote resolution and lower pro-inflammatory responses to LPS.

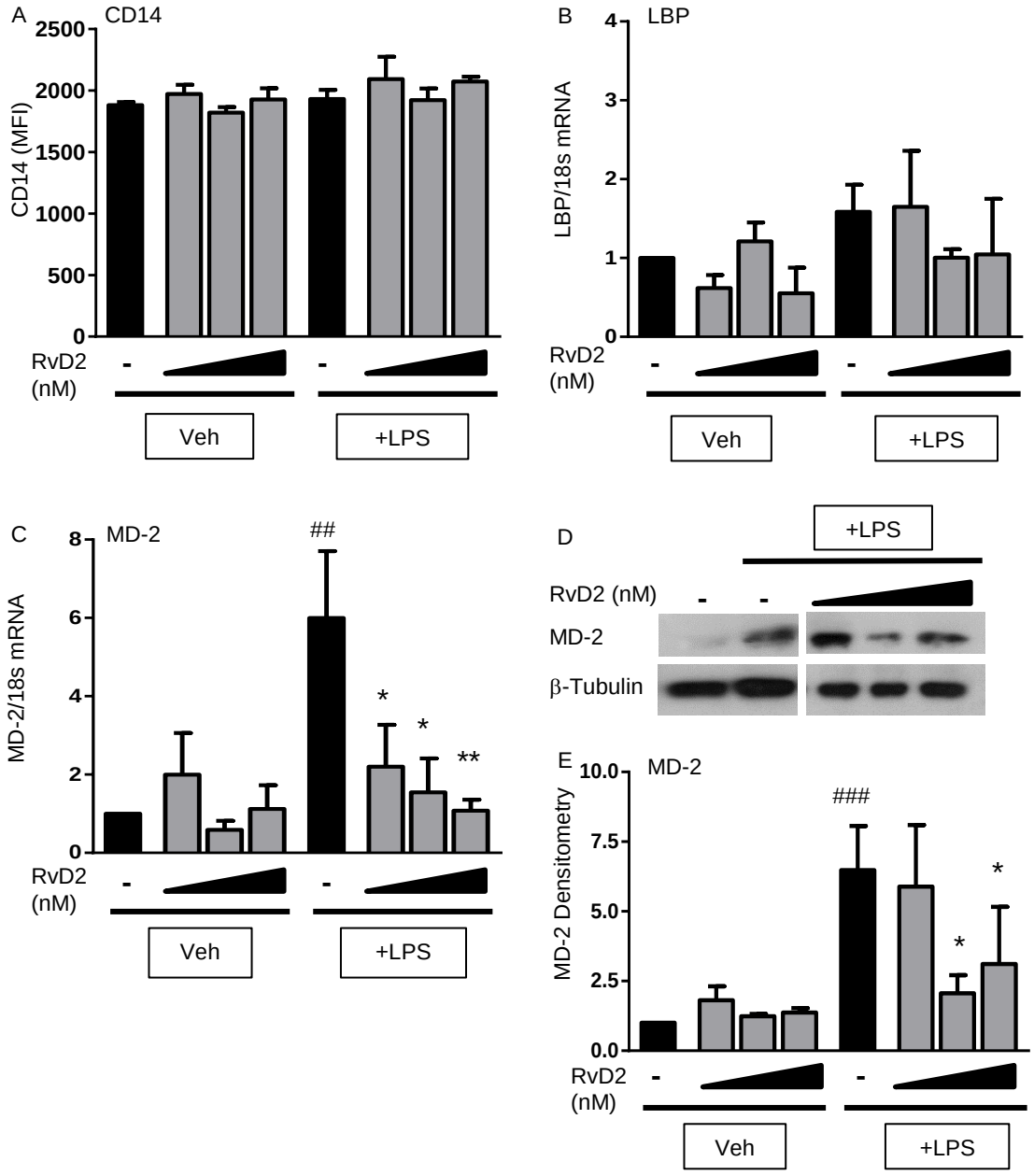

Fig. 3.9. RvD2 decreases expression of MD-2 in THP-1 monocytic cells. THP-1 cells were treated with 1-100 nM RvD2 followed by 20 ng/mL LPS for 4 hours. CD14 surface expression was determined by flow cytometry (A). LBP (B) and MD-2 (C) mRNA levels were determined by RT-PCR. MD-2 protein expression (D) was determined by western blot (representative image shown, densitometry for n=3). Statistical significance was determined by two-way ANOVA with Bonferroni's posttest ($^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to veh/veh, $^{*}p<0.05$, $^{**}p<0.01$ compared to veh/LPS) n=3-5 independent experiments.

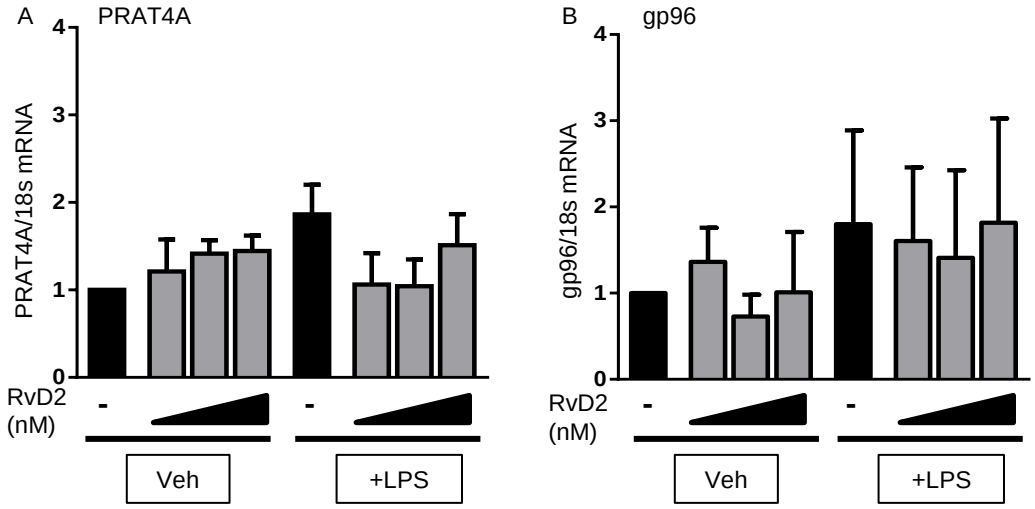

Fig. 3.10. RvD2 does not affect expression of trafficking proteins in THP-1 monocytic cells. THP-1 cells were treated with 1-100 nM RvD2 followed by 20 ng/mL LPS for 4 hours. PRAT4A (A), and gp96 (B) mRNA levels were determined by RT-PCR. Statistical significance was determined by two-way ANOVA with Bonferroni's posttest, n=3-5 independent experiments.

RvD2 increases miRNA146a to promote TLR4 degradation

TLR4 expression is regulated by a variety of genes and signaling pathways, providing a large number of possible targets which RvD2 could be acting upon. In particular, there is a growing body of literature regarding the regulation of TLR4 by microRNAs (miRs). Several miRNAs, including miR146a and miR146b, can negatively regulate TLR4 expression (161). We observed that RvD2 treatment of THP-1 cells followed by LPS activation increased expression of miR146a, but not miR146b, indicating one possible mechanism by which RvD2 decreases TLR4 expression (Fig. 3.11A-B). These findings were confirmed in human monocytes, where RvD2 dose-dependently increased miR146a but not miR146b following LPS treatment (Fig. 3.11C-D).

Overexpression of miR146a by transfection demonstrated similar outcomes as RvD2 treatment, wherein the miR146a mimic decreased TLR4 expression (Fig. 3.12A). This was seen both with miR146a mimic alone, which had a trending reduction in baseline expression, and with LPS stimulation. Additionally, we blocked miR146a expression by transfecting THP-1 cells with a miR146a inhibitor. Inhibition of miR146a resulted in strong LPS-induced increases in TLR4 (Fig. 3.12B). MD-2 expression was not altered by transfection with either a miR146a mimic or a miR146a inhibitor (data not shown). Furthermore, inhibition of miR146a partially blocked RvD2-induced decreases in TLR4 (Fig. 3.12B). Similar results were observed with LPS-induction of IL-8 and TNFα; miR146a mimic reduced LPS-induction of these cytokines, and inhibition of miR146a prevented RvD2-reductions of IL-8 and TNFα (Fig. 3.12C-D). Taken together,

these data demonstrate that RvD2 is acting in part by increasing miR146a expression to decrease TLR4 and mediating resolution.

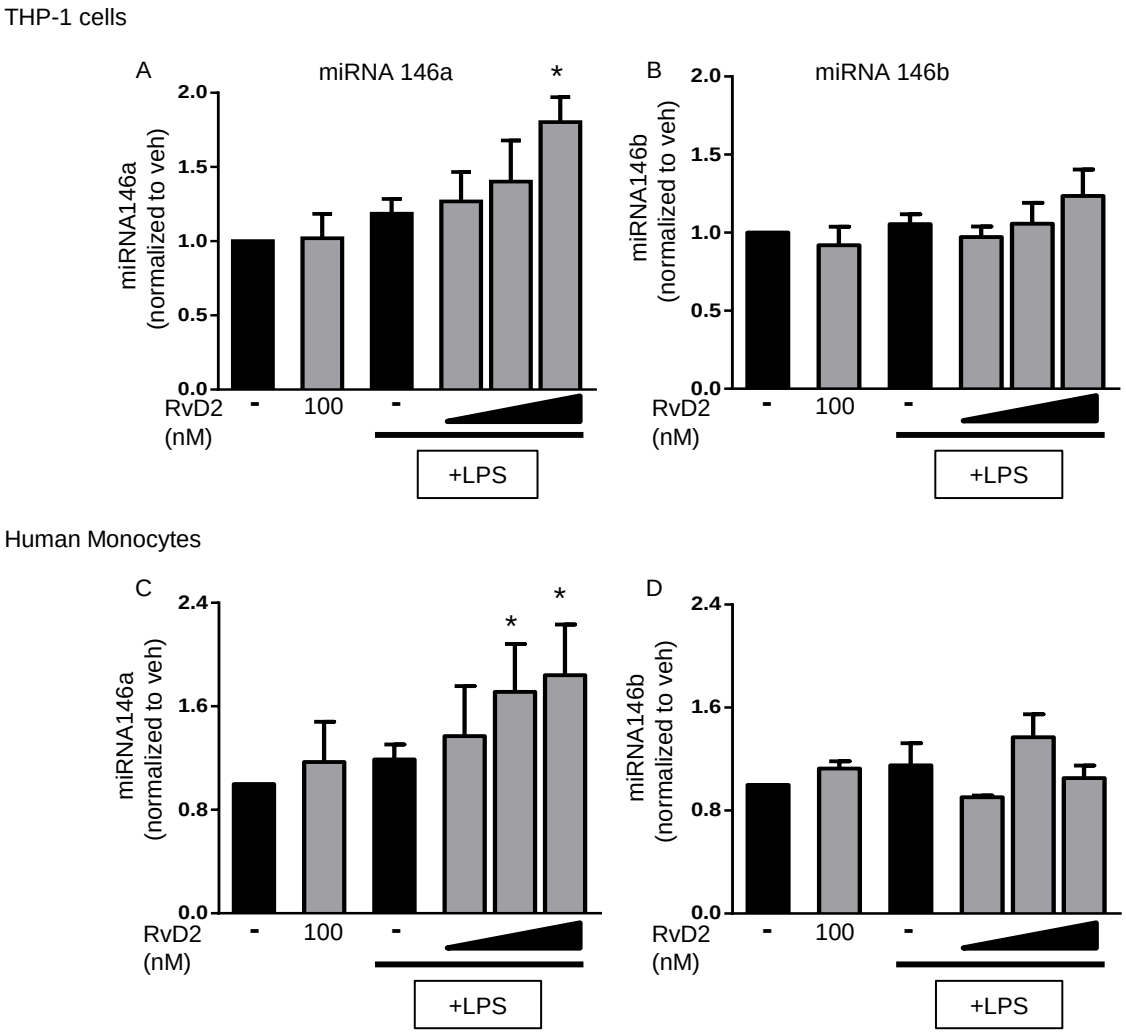

Fig. 3.11. RvD2 increases miR146a which decreases TLR4 expression. THP-1 cells or human monocytes were treated with 1-100 nM RvD2 followed by 20 ng/mL LPS for 4 hours. MiR146a and (A, C) and miR146b (B, D) expression levels were determined by RT-PCR for THP-1 cells (A-B) and human monocytes (C-D). Statistical significance was determined by two-way ANOVA with Bonferroni's posttest (*$p<0.05$ compared to veh/LPS) n=3-4 individual donors or independent experiments.

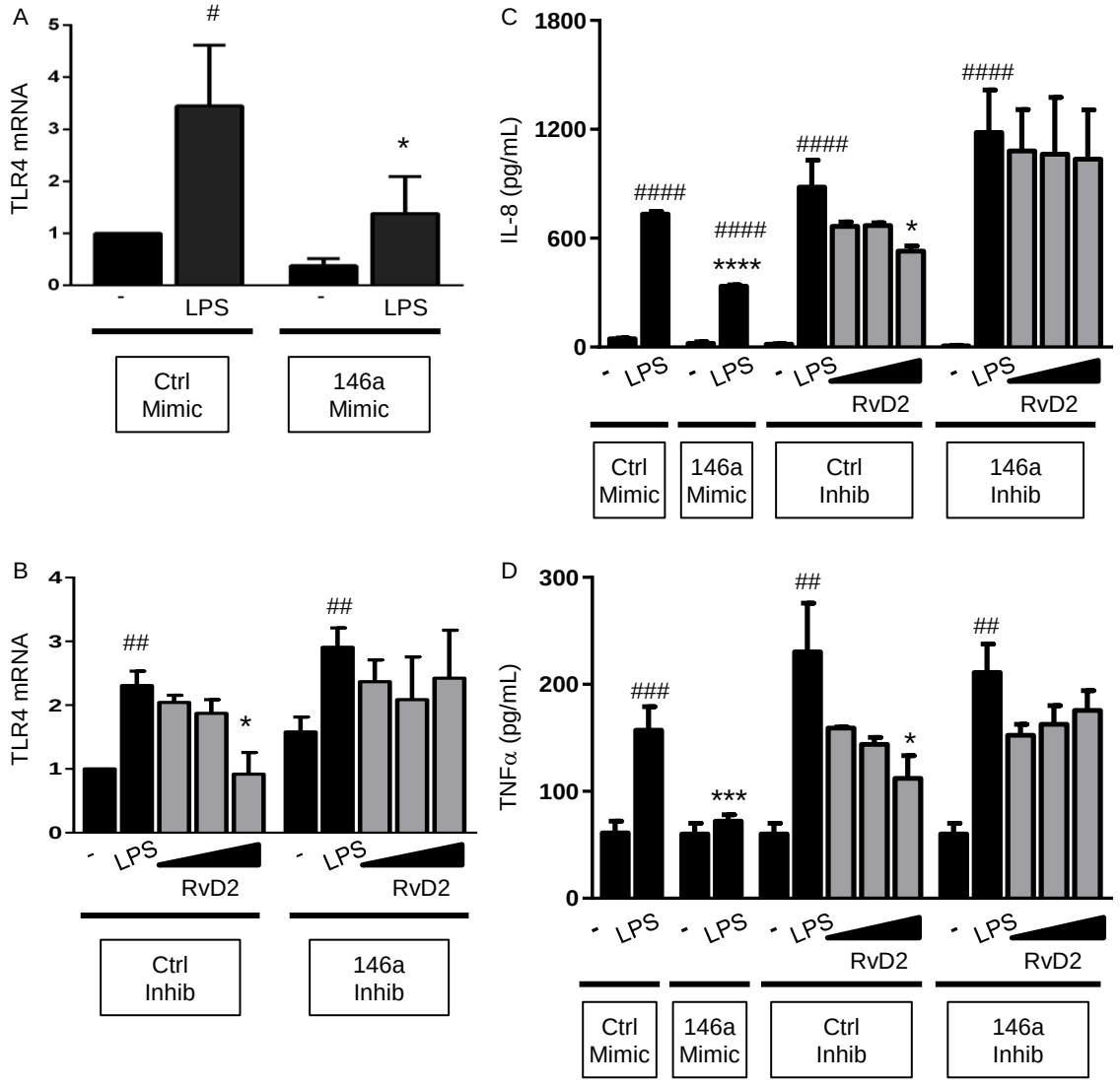

Fig. 3.12. RvD2's effects are attenuated with inhibition of miR146a. THP-1 cells were transfected with control or miR146a mimics or inhibitors prior to RvD2 and LPS treatment. THP-1 cells transfected with a miR146a mimic had reduced TLR4 expression compared to control transfection (A). Conversely, inhibition of miR146a partially blocked the effects of RvD2 in decreasing TLR4 expression (B). 146a mimic reduced IL-8 and TNFα cytokine production, while inhibition of miR146a prevented RvD2-reductions in these cytokines (C-D). Statistical significance was determined by two-way ANOVA with Bonferroni's posttest ($^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, $^{\#\#\#\#}p<0.0001$ compared to corresponding veh group, *p<0.05, ***p<0.001, ****p<0.0001 compared to Ctrl/LPS) n=3-5 independent experiments.

Discussion

SPMs represent a novel class of non-immunosuppressive small lipid molecules with high clinical potential for treatment of inflammatory diseases. Here, we have shown that SPMs, and most potently RvD2, dampen pro-inflammatory cytokine production induced by LPS in human monocytes. These actions are stimuli-dependent, as RvD2 only acted to decrease LPS-induced cytokines and TLR4 expression, and not TLRs or cytokines induced by Poly I:C or Pam3CSK. Along with human peripheral blood monocytes, THP-1 cells were similarly responsive to SPMs, and RvD2 reduced TLR4 expression in a time-dependent manner. RvD2 also decreased components of downstream TLR4 signaling pathways and selectively dampened MD-2 expression. These changes were mediated in part by RvD2 induction of miR146a, and inhibition of this microRNA blocked the efficacy of RvD2. Taken together, these data demonstrate that RvD2 dampens TLR4 expression to promote the resolution of LPS-induced inflammation.

We observed in our studies that LPS acutely increased expression of two SPM receptors, ALX/FPR2 and GPR18. While GPR18 is newly identified, it has important roles in mediating phagocytic and other inflammatory responses to *E. coli* (12). ALX/FPR2 has been more extensively studied, and has proven to be an important player in multiple models of inflammation. ALX/FPR2 demonstrates protective properties in sepsis models, highlighting that it contributes to resolution of microbial-stimulated inflammation (162). Blockade of ALX/FPR2 specifically negates the efficacy of RvD1 in response to LPS (150). It is curious that LPS induces GPR18 and ALX/FPR2 but does not affect GPR32 expression, however, given that RvD1 signals through both ALX/FPR2

and GPR32 there may be some redundancy between these two receptors, with a more prominent role for ALX/FPR2 in microbial models or with the doses of LPS used here.

The specificity of RvD2 regarding attenuation of only LPS-induced inflammation is interesting. There are multiple SPMs which each act through unique receptors, and what promotes production of one SPM over another is not fully understood. SPMs are derived from omega-3 and omega-6 fatty acids, precursors that can undergo "class switching" to produce both pro- and anti-inflammatory molecules in a temporal manner and under different stimuli (163). It is possible that other SPMs at different time points may promote resolution of inflammation initiated by other TLR ligands. There is some evidence for the link between SPMs and other TLRs in the literature. Activation of TLR7 with R-828 stimulates production of SPMs in mouse and human macrophages, which then act in a feedback loop to promote resolution of allergic airway inflammation (164). Interestingly, these studies were also stimuli dependent as LPS did not induce production of SPMs and lipoxygenase activity in this model. Additionally, activation of the lipoxin receptor (ALX/FPR2) using non-LxA_4 agonists decreases TLR2 activity in a model of allergic airway inflammation (165). Of note, supplementation with DHA and EPA reduced inflammation but did not alter TLR4 surface expression, an indication that precursors or non-SPM agonists for SPM receptors may act to dampen inflammation through other pathways (166). These studies underscore that SPMs and TLRs act to regulate expression of each other, and suggest that there may be some specificity regarding which SPMs may be efficacious in different diseases induced by TLR ligands.

In our studies, RvD2 acted specifically on LPS-induced inflammation to attenuate cytokine production and LPS expression. Notably, this decrease did not happen until several hours after LPS stimulation. This suggests that RvD2 did not fully block TLR4 responsiveness, but rather acted in a feedback mechanism to prevent excessive TLR4 activation. Eicosanoids, including both SPMs and pro-inflammatory mediators, produced in the early stages of inflammation can form feedback loops to promote the shift to anti-inflammatory lipids (124, 163). Excessive bacterial infiltration, of which LPS/TLR4 activation is a contributor, leads to septic shock, and prolonged LPS activation may contribute to tolerance phenomena and chronic inflammatory diseases, so shutting down these pathways to promote quicker resolution would be an advantageous therapeutic strategy. There is strong evidence that SPMs are not immunosuppressive and are strongly temporally regulated (151). Therefore, these molecules dampen excessive inflammation without fully blocking an immune response, an important characteristic for therapeutic use in pathogen-mediated disease.

Along with decreasing TLR4 expression, downstream signaling components are also decreased with RvD2 treatment. The observed decreases in MyD88, TRIF, and TAK1 confirm the functional effect of decreased TLR4 expression by highlighting that the downstream mediators are also decreased. Previously, our lab showed in epithelial cells that RvD1 can dampen TAK1 activation, highlighting another potential target for mediating resolution (72). Even further downstream from these signaling components, TLR4 eventually leads to activation of the NF-κB pathway. SPMs have also been shown in multiple models to act on the NF-κB pathway to mediate their effects (65, 71, 167,

168). RvD1 reduced NF-κB expression in mice with LPS-induced acute lung injury (40). Specifically in human macrophages, RvD2 dampens cigarette-smoke induced NF-κB expression and promotes alternative activation of RelB (65). SPMs may be directly acting to dampen NF-κB expression, which could then act as a feedback mechanism to decrease TLR4, and/or may decrease TLR4 through other mechanisms which then result in reduced NF-κB expression. TLR4 also acts on AP-1 signaling to promote inflammation, though there are discrepant results in the literature regarding whether SPMs upregulate or downregulate AP-1 signaling (69, 169, 170). In all cases, it is clear that SPMs modulate multiple stages of the TLR4 signaling pathway to mediate their anti-inflammatory effects.

RvD2 also decreased expression of MD-2, the first evidence for SPM regulation of this molecule. This was particularly interesting, as RvD2 did not affect expression of other genes involved in TLR4 trafficking (PRAT4A and gp96) or in LPS binding to TLR4 (CD14 and LBP). Therefore, RvD2 is not acting broadly to interrupt either of these processes, but more specifically on MD-2. CD14 and LBP greatly enhance TLR4 responsiveness to LPS and efficiency of signaling, but are not necessary for this response, as CD14 knockout mice can still respond to LPS (75, 171). In contrast, MD-2 is required for formation of TLR4 multimers necessary for signaling; MD-2 knockout mice display the same phenotype as TLR4 knockout mice (75, 155-157, 172). Curiously, in acute-phase responses, high concentrations of LBP have been shown to inhibit LPS recognition to help prevent septic shock, so there may actually be beneficial roles for this protein in inhibiting inflammation (173). Given the physical association of MD-2 to TLR4, it is possible that MD-2 is simply decreased as a result of less available TLR4 for binding.

Little is known about the mechanisms responsible for MD-2 regulation and degradation, but this protein is inducible and therefore must undergo some degree of regulation (174, 175). Further complicating this association is the fact that MD-2 has multiple TLR4 independent actions and is able to bind to molecules independent of TLR4 which then act as antagonists to canonical TLR4 signaling (176). Finally, while miR146a has not been shown to target MD-2, RvD2 may be upregulating other miRs to specifically decrease expression of MD-2 or target a molecule that regulates MD-2, independent of the miRs that act on TLR4. For instance, miR30a has been shown to target STAT1 (which induces MD-2), thereby decreasing MD-2 expression (175). Clearly, the regulation of MD-2 in regards to TLR4 signaling is still not fully understood, and the possible link between SPMs and MD-2 expression bears further investigation.

RvD2 dampens TLR4 expression at least partially through induction of miR146a. MicroRNAs both in general and specifically in regards to TLRs are an expanding area of research. Multiple miRs have been identified which target TLR4 or components of the TLR4 signaling pathway (161). These include miR146a, miR146b, miR155, miR132, miR27b, and more (161). Some of these miRs can also be induced by LPS or activation of TLR4 through other ligands, demonstrating a negative feedback loop and cyclic regulation of TLR4 signaling. MiR146a is one such example of a miR that is both regulated by and negatively regulates TLR4, MyD88, NF-κB, other TLRs, and a range of pro-inflammatory proteins, as well positively regulating RelB (134, 161, 177-181). The effects of miR146a regulation have been specifically demonstrated in macrophages. THP-1 cells that were exogenously transfected with a miR146a mimic had decreased

production of TNFα, IL-6, and IL-1β (179). Inhibiting miR146a similarly prevented miR146a induced shifts to resolution, and led to higher expression of cytokines and TLR4 (179). MiR146a also promotes M2 macrophage polarization, which is a common action for SPMs and often key mechanism by which they promote resolution (182). SPMs have been shown to induce several miRs in other inflammatory models, indicating that miR regulation may be a major target of SPMs actions (183, 184). Our data, however, are the first to show RvD2 induction of miR146a, uncovering a previously unknown possible mechanism for RvD2's actions.

Overall, our studies provide the first evidence of SPM regulation of TLR4 expression. The temporal regulation of TLR4 is particularly interesting, and highlights the non-immunosuppressive nature of RvD2. Furthermore, this is the first evidence for both SPM regulation of MD-2 and identification of miR146a as a new microRNA through which RvD2 may mediate its actions. This investigation brings to light new potential therapeutic targets to address septic shock and chronic diseases associated with LPS. RvD2 and SPMs have high therapeutic potential for addressing pathogen-mediated diseases, and these studies demonstrate the first evidence that they may act in novel ways to regulate TLR signaling, an important area of resolution research.

Chapter 4

Aspirin-Triggered Resolvin D1 dampens pulmonary inflammation and promotes clearance of Nontypeable Haemophilus influenzae[3]

[3] This work was published in part as "Croasdell A, Lacy SH, Thatcher T, Sime PJ, Phipps RP. *Resolvin D1 protects against Nontypeable Haemophilus influenza pulmonary infection.* J Immunol. 2016 Mar 15;196(6):2742-52. Epub 2016 Feb 3" and is reprinted with their permission.

Abstract

Nontypeable *Haemophilus influenzae* (NTHi) is a Gram-negative coccobacillus that frequently causes opportunistic bronchitis, pneumonia, and exacerbations in patients with underlying inflammatory diseases, such as COPD. In mice, NTHi is rapidly cleared, but a strong inflammatory response persists, suggesting that NTHi induces dysregulation of normal inflammatory responses and a failure to resolve. Lipid-derived specialized pro-resolving mediators (SPMs) play a critical role in the active resolution of inflammation. Importantly, SPMs lack the immune suppressive properties of classical anti-inflammatory therapies. In pilot studies using lipopolysaccharide (LPS) as a bacterial mimetic, we found that aspirin-triggered resolvin D1 (AT-RvD1) dampened cellular influx and pro-inflammatory cytokine production. Based on these findings, we hypothesized that AT-RvD1 would dampen NTHi-induced inflammation while still enhancing bacterial clearance. To test this, C57BL/6 mice were treated with AT-RvD1 and infected with live NTHi. AT-RvD1 treated mice had lower total cell counts and neutrophils in bronchoalveolar lavage fluid (BALF), and had earlier influx of macrophages. Additionally, AT-RvD1 altered temporal regulation of inflammatory cytokines and enzymes, with decreased KC at 6 hours and decreased IL-6, TNFα, and Cox-2 expression at 24 hours post-infection. Despite dampened inflammation, AT-RvD1 treated mice had reduced NTHi bacterial load, mediated by enhanced clearance of NTHi by macrophages and a skewing towards an M2 phenotype. Finally, AT-RvD1 protected NTHi-infected mice from weight loss, hypothermia, hypoxemia, and respiratory compromise. This research highlights the beneficial role of SPMs in pulmonary bacterial infections, and

provides the groundwork for further investigation into SPMs as adjunct therapies to reduce the need for antibiotics.

Introduction

Nontypeable *Haemophilus influenzae* (NTHi) is a Gram-negative, unencapsulated coccobacillus pathogen found in the upper respiratory tract. NTHi causes ear infections, bronchitis, and more invasive diseases such as bacteremia and pneumonia (185, 186). People with underlying lung inflammation, such as chronic obstructive pulmonary disease (COPD), cystic fibrosis, or an existing infection, are particularly susceptible to infectious exacerbations, which lead to high rates of hospitalization and worsening of symptoms; NTHi is a major cause of exacerbation (79, 187-189). NTHi is specifically the most common colonizing bacteria found in COPD patients, with NTHi bacterial load correlating to airway inflammation, symptom severity, and frequency of exacerbations (189-191). These infections are persistent and recurring, and a history of exacerbations is the single best predictor of further exacerbations (192). Several available vaccines targeting capsular polysaccharide are effective against *Haemophilus influenzae* type B (Hib), but these do not protect against unencapsulated NTHi, which continues to increase in incidence (193). Furthermore, there is a growing prevalence of strains with antibiotic resistance (193-195). NTHi infections are typically persistent, with some strains acquiring drug resistance or the ability to avoid opsonization and thereby "trick" macrophages to avoid phagocytosis, resulting in bacteria propagating and colonizing in the airways (196, 197). Multiple studies have shown that NTHi infections induce a strong and prolonged inflammatory response, characterized by influx of inflammatory cells, release of cytokines, activation of the NF-κB pathway and initiation of toll-like receptor (TLR) signaling pathways (198-201). In mice, NTHi bacteria are rapidly cleared, with

very low levels detected 24 hours after infection. Despite this rapid clearance, inflammation persists, indicating that this bacteria might induce a dysregulation of normal inflammatory responses and a failure to resolve.

There is growing interest in the roles, endogenously or exogenously (as therapeutics) that SPMs play in infection. SPMs have both anti-inflammatory and pro-resolving actions, and it is clear that they are not immunosuppressive. SPMs potently reduce inflammation and promote resolution in a variety of non-microbial models, including pulmonary diseases such as COPD, asthma, and fibrosis (37, 65, 87). Along with these pro-inflammatory non-microbial insults, several studies have begun investigating the actions of SPMs in bacterial infections or in models with bacterial surrogates as activators. Lipopolysaccharide (LPS) is often used as a surrogate for gram-negative bacteria, and certain SPMs, namely lipoxins and D-series resolvins, dampen LPS-induced inflammatory cell influx and pro-inflammatory cytokine production (38, 149, 168). SPMs have also shown efficacy in sepsis models by promoting resolution while enhancing bacterial clearance, and ligands for the LxA_4 receptor (ALX/FPR2) have a protective effect against experimental sepsis (43, 71, 162, 202-204). Indeed, SPMs can be used as an adjunct to antibiotics, and thereby lower antibiotic requirements or act synergistically with antibiotics for improved host responses (167, 205). There still exists, though, a major knowledge gap regarding the efficacy of SPMs in resolving pulmonary bacterial infections; this is especially critical given the susceptibility of the lung to infection. Based on the non-immunosuppressive nature of SPMs and their efficacy in other inflammatory lung models, we hypothesized that aspirin-triggered resolvin D1 (AT-

RvD1) would attenuate NTHi-induced lung inflammation without impairing bacterial clearance. Given the high incidence of NTHi and other bacterial lung infections, and their role in promoting exacerbations of COPD, this represents an important area of translational research with high clinical impact.

Materials & Methods

Materials

Mouse IL-6 (431401), IL-10 (431411) and TNFα (430901) ELISA kits and anti-F4/80 mouse BV421 antibody (123131) were purchased from BioLegend (San Diego, CA). Mouse CXCL1/KC ELISA kit (DY453-05) was purchased from R&D Systems (Minneapolis, MN). Collagenase (234155) was purchased from EMD Millipore (Billerica, MA). Anti-CD206 mouse Alexa Fluor 647 antibody (MCA2235A647T) and anti-Ly-6B.2 antibody (MCA771G) were purchased from AbD Serotec (Raleigh, NC). Anti-Ly-6G (Gr-1) mouse PE antibody (553128) was purchased from BD Pharmingen (San Jose, CA). LPS 0111:B4 (L4391), blood agar base (70133), hemin (H9039), and b-Nicotinamide Adenine Dinucleotide hydrate (NAD) (N3014) were purchased from Sigma (St. Louis, MO). Brain Heart Infusion (237500) and anti-CD80 mouse PerCP-Cy5.5 antibody (560526) were purchased from BD Bioscience (San Jose, CA). PGE_2 EIA kits and AT-RvD1 (7S,8R,17R-trihydroxy-4Z,9E,11E,13Z,15E,19Z-docosahexaenoic acid) were purchased from Cayman Chemical (Ann Arbor, MI). Secondary western blot antibodies (115-035-146, 111-035-144) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). PBS (14200-075) was purchased from Gibco (Waltham, MA).

LPS pilot experiments

Adult female C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME) and used at 8–10 weeks of age. All animal procedures were approved and supervised by the University of Rochester University Committee on Animal Resources (UCAR protocol number 2007-127). AT-RvD1 is a more stable analog of RvD1, and was used for all experiments. To determine the efficacy of AT-RvD1, mice were treated with 20 ng or 100 ng/mouse of AT-RvD1 in 40 μL 1X PBS by oropharyngeal aspiration and given a second dose 24 hours later, prior to exposure to 50 ng/mouse of LPS by oropharyngeal aspiration. 24 hours after LPS exposure mice were euthanized with IP injection of 100 mg/kg pentobarbital sodium plus 12.5 mg/kg phenytoin sodium (Euthasol, Virbac AH Inc., Fort Worth, TX). Bronchoalveolar lavage fluid (BALF) was collected as previously described (37). Differential BALF cell counts were obtained using Richard Allen 3-step staining according to manufacturer's protocol. Levels of IL-6, TNFα, and KC were determined by ELISA and levels of PGE_2 were determined by EIA. The right lung was homogenized in CW buffer (50mM Tris-HCL, 2% SDS) using a glass bead homogenizer (Next Advance Bullet Blender, Averill Park, NY) and Cox-2 protein was analyzed by western blotting (97).

NTHi culture and growth

NTHi strain 12 (clinical isolate) was used for all infections. To culture bacteria, NTHi glycerol stock was streaked on chocolate agar plates and grown at 37°C, 5% CO_2 for 24 hours. A single colony was inoculated into supplemented brain heart infusion broth

(BHI, hemin, NAD) overnight. The bacterial suspension was subcultured and then centrifuged to form a bacterial pellet. The NTHi pellet was resuspended in 1X PBS and the optical density measured via spectrophotometer at a 600 nM wavelength, using OD to determine colony forming units (CFUs) (1.00 OD = 1 x 10^9 CFUs).

In vivo treatment and exposures

Adult female C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME) and used at 8–10 weeks of age. All animal procedures were approved by the University of Rochester University Committee on Animal Resources (UCAR protocol number 2007-127). For initial dosing experiments, mice were infected by oropharyngeal aspiration (206) with 1 x 10^5-10^8 CFUs of NTHi in 40 μL 1X PBS or PBS vehicle and euthanized at 6-120 hours following infection. Mice were euthanized as described above.

To determine the efficacy of AT-RvD1, mice were treated with 20 ng or 100 ng/mouse of AT-RvD1 in 40 μL 1X PBS by oropharyngeal aspiration and given a second dose 24 hours later, prior to infection. As the average starting weight of the mice was 18.1±1.5 g (mean ± S.D.), this corresponded to doses of 1.1±0.1 mg/kg and 5.5±0.4 mg/kg. Mice were then infected with 1 x 10^6 CFUs of NTHi in 40 μL 1X PBS by oropharyngeal aspiration; 1X PBS was used as a vehicle control. Mice were euthanized 6-24 hours following NTHi infection. Mice were weighed immediately before NTHi infection and again immediately before euthanasia. Immediately prior to euthanasia, body temperature was measured using a rectal thermometer. Respiratory physiology was assessed using the Harvard Apparatus Small Rodent Plethysmograph. Oxygen saturation

of hemoglobin was measured using a tail clip and the Starr Life Science Corporations Mouse Oximeter according to manufacturer's protocols. After all biometric readings were obtained, the mice were euthanized and tissue harvested as described below.

Analysis of bronchoalveolar lavage fluid and lung tissue

Bronchoalveolar lavage fluid (BALF) was collected as previously described (37). The cranial lobe of the right lung was removed to assess bacterial load. The remainder of the right lung was flash frozen at -80°C for further analysis. Differential BALF cell counts were obtained using Richard Allen 3-step staining according to manufacturer's protocol. Total protein in BALF was determined by the bicinchoninic acid (BCA) colorimetric assay (Thermo-Sci, Waltham, MA, 23225). Levels of IL-6, TNFα, KC, and IL-10 cytokines in BALF were determined by ELISA and levels of PGE_2 were determined by EIA. The right lung was homogenized in CW buffer (50mM Tris-HCL, 2% SDS) using a glass bead homogenizer (Next Advance Bullet Blender, Averill Park, NY) and Cox-2 protein was analyzed by western blotting (97). In some experiments, the lungs were inflated and fixed with 10% neutral buffered formalin without undergoing lavage. Tissues were embedded with paraffin, sectioned (5μm), and stained either with H&E or for Ly-6B.2 (for neutrophils) as described previously (37).

Assessment of NTHi bacterial load

In preliminary experiments, the five lung lobes (i.e. left, cranial, medial, caudal, and accessory) were separated and homogenized in 1 mL of 1X PBS. Serial dilutions

were plated onto chocolate agar plates and NTHi was grown at 37°C, 5% CO_2. Colonies were counted 24 hours after plating. All further experiments used the cranial lung lobe for bacterial load assessment.

Assessment of NTHi bacterial clearance, efferocytosis, and macrophage phenotype

Analysis of efferocytosis was performed as previously described 6 and 24 hours after NTHi infection (37). To assess bacterial clearance and macrophage phenotype, mice were sacrificed 8 hours after NTHi infection. BALF was obtained or the right lung lobes were removed and digested with collagenase for 30 min. Cells were collected through a cell strainer and centrifuged at 7000 x g for 10 min. Red blood cells were removed using 1X ACK lysis buffer. Cells from BALF and digested lung were stained with antibodies against CD80, CD206, F4/80, CD11b, and Gr-1 for 30 min to identify cellular populations, macrophage phenotype, and bacterial clearance. For these experiments, NTHi was labeled with FITC antibody; 10 μL of a 10 mg/mL solution of FITC in DMSO was added to a suspension of NTHi and incubated in the dark for 30 min, followed by centrifugation and resuspension of the bacteria in 1X PBS as above. Following staining, cells were fixed with 4% paraformaldehyde (EMS, 15710) for 20 min. Fixed cells were then permeabilized and stained for intracellular Gr-1. Endpoints were assessed by flow cytometry.

Statistical Analysis

All experiments were performed on 4-9 mice/group and were repeated in a minimum of 2-4 independent experiments. Results are expressed as mean ± standard error (SEM). All data was normally distributed and parametric statistical analyses were performed using a t-test or one- or two-way analysis of variance (ANOVA) with Bonferroni's posttest correction for multiple comparisons using GraphPad Prism Software (San Diego, CA). No statistical significance was found between veh/veh vs. AT-RvD1 unless indicated.

Results

AT-RvD1 dampens LPS-induces inflammation in mice

We selected AT-RvD1 (a more stable, aspirin-triggered epimer of resolvin D1) as our candidate SPM for these studies based on our previously published *in vivo* work (37, 66). To first evaluate the efficacy of AT-RvD1 in mice, we used LPS as a bacterial surrogate and a model of pulmonary inflammation. LPS-induced acute lung injury is well characterized, and we previously observed that SPMs dampen LPS-induced inflammation *in vitro* (see Chapter 3). We therefore hypothesized that AT-RvD1 would be effective in dampening LPS-induced injury *in vivo*. Mice inoculated with LPS had increased total cell, neutrophil, and macrophage levels in bronchoalveolar lavage fluid (BALF) 24 hours after exposure (Fig. 4.1A-C). AT-RvD1 acted to dose-dependently attenuate total cell and neutrophil influx (Fig. 4.1A-B). Interestingly, AT-RvD1 did not dampen macrophage levels, which remained elevated in all LPS exposed groups (Fig. 4.1C).

Along with differential cell counts, we evaluated the production of inflammatory proteins. Among many other functions, the cytokines TNFα, IL-6, and KC (the mouse ortholog to IL-8) are responsible for bacterial killing and macrophage activation, acute phase reactions, and neutrophil influx, respectively. All three of these cytokines were elevated by LPS exposure (Fig. 4.2A-C). AT-RvD1 dose-dependently reduced cytokines, with the greatest reductions seen in KC (Fig. 4.2A-C). We also evaluated BALF levels of PGE_2, a pro-inflammatory prostaglandin enzymatically produced from arachidonic acid via Cox-2 activation. Both BALF PGE_2 and whole lung Cox-2 levels were elevated by

LPS and were dose-dependently attenuated by AT-RvD1 (Fig. 4.2D-E). These pilot experiments demonstrated the potential efficacy of AT-RvD1 *in vivo* and allowed us to move forward with a live bacteria model of pulmonary infection.

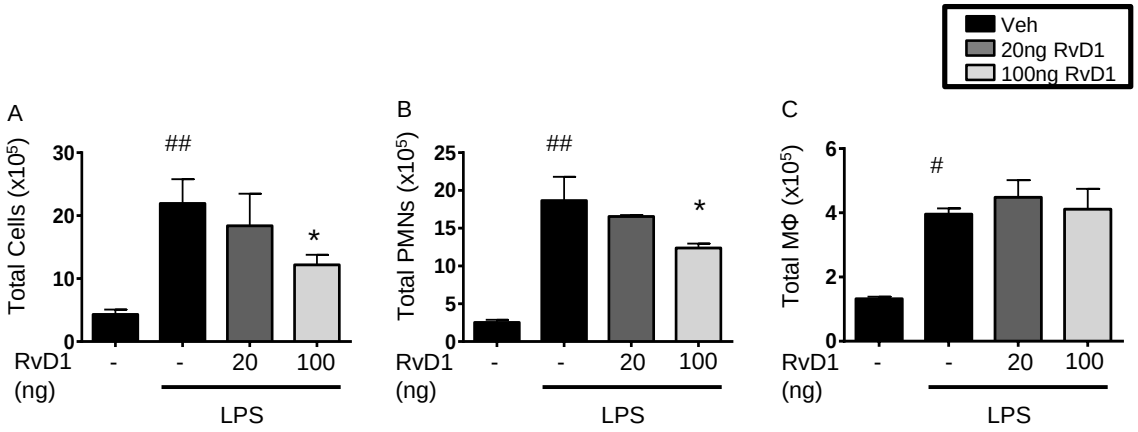

Fig. 4.1. AT-RvD1 attenuates LPS-induced cellular influx. Differential cell counts were determined from the BALF of mice inoculated with AT-RvD1 (20 or 100 ng/mouse, OA) and/or LPS (50 ng/mouse, OA). Total cell (A), neutrophil (B), and macrophage (C) counts were evaluated at 24 hours after LPS exposure. Statistical significance was determined by two-way ANOVA with Bonferroni's posttest for multiple comparisons ($^{\#}p<0.05$, $^{\#\#}p<0.01$ compared to veh/veh, $^{*}p<0.05$ compared to veh/LPS), n=3 mice/group.

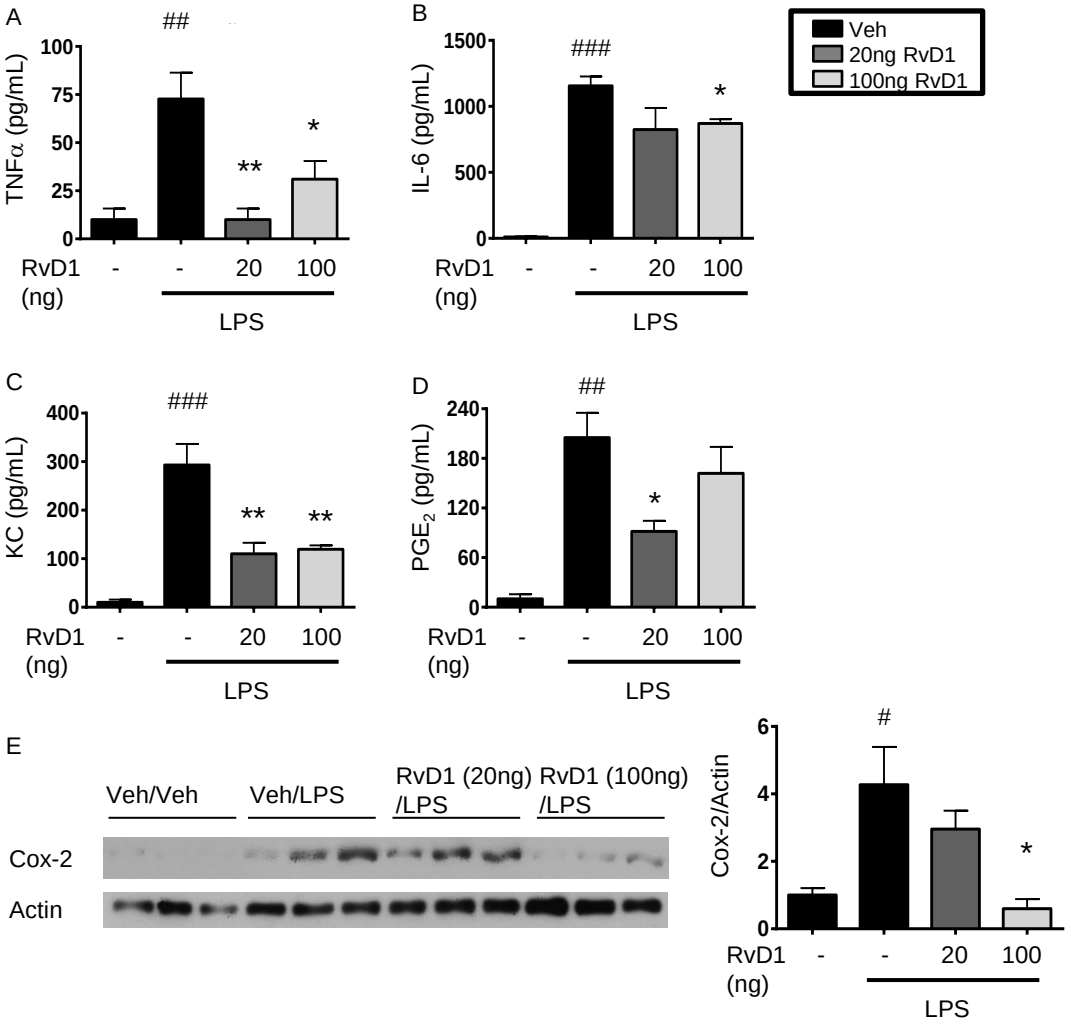

Fig. 4.2. AT-RvD1 decreases LPS-induced pro-inflammatory cytokines and Cox-2 expression. Bronchoalveolar lavage fluid was obtained from AT-RvD1 (20 or 100 ng/mouse, OA) and/or LPS (50 ng/mouse, OA) inoculated mice. Expression of TNFα (A), IL-6 (B), KC (C), and PGE_2 (D) was evaluated 24 hours after LPS exposure by ELISA or EIA. Protein expression of Cox-2 was determined by western blot (E). Statistical significance was determined by two-way ANOVA with Bonferroni's posttest for multiple comparisons ($^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to veh/veh, $*p<0.05$, $**p<0.01$ compared to veh/LPS), n=3 mice/group.

NTHi dose dependently induces persistent inflammation despite rapid bacterial clearance

To characterize the timing and magnitude of NTHi-induced inflammation and infection, we first infected mice with 1 x 10^5 - 1 x 10^8 CFUs NTHi and evaluated several key markers of inflammation. Instillation of 1 x 10^8 CFUs caused significant tissue damage and morbidity (>50% at 8 hours post-infection) and we were unable to recover lavage samples from these mice. At lower doses (1 x 10^5 - 1 x 10^7), NTHi increased the total number of inflammatory cells, neutrophils, and macrophages in BALF at both 6 and 24 hours post-infection in a dose-dependent manner (Fig. 2.3A-C). Total cell counts and neutrophils continued to accumulate past the initial 6hr response, though macrophage levels were similarly elevated at both time points.

Despite this rapid bacterial clearance and lack of bacteremia, mice infected with 1 x 10^6 CFUs of NTHi exhibited significant weight loss up to 96 hours post-infection, with recovery beginning at 48 hours (Fig. 4.4A). Mice additionally had increasing total inflammatory cell counts 48 hours post-infection; by 72 hours these elevated cell counts began to decrease, indicative of the initiation of resolution (Fig. 4.4B). These same results were reflected in differential cell counts, where both neutrophils and macrophages were elevated up to four days post-NTHi infection, with peak cell numbers at 24 hours post-infection (Fig. 4.4C-D).

We also evaluated bacterial distribution in the lung and timing of bacterial clearance. Mice possess five lung lobes of varying sizes (Fig. 4.5A). In pilot testing, mice were infected with 1 x 10^6 CFU of NTHi. Bacteria were distributed throughout the lung lobes in relatively equal proportion to the lobe size (Fig. 4.5B). To determine if NTHi

was being distributed to other areas of the body or remaining locally in the lung, we also evaluated bacterial load in the blood, liver, and spleen 6 hours after infection; no NTHi bacteria were detected in these fluids/organs (Fig. 4.5B). Based on these results, the right cranial lobe was used to evaluate bacterial load in future experiments to maintain consistency. Using our dose-range of NTHi ($1 \times 10^5 - 1 \times 10^7$ CFUs), we verified that NTHi is rapidly cleared, as has been previously published (82, 207). Bacterial CFUs reflected the infective dose at 6 hours; >98% of NTHi was cleared by 24 hours (Fig. 4.5C). For all subsequent experiments, an infective dose of 1×10^6 CFUs of NTHi was used. These data confirm that NTHi induces persistent and prolonged lung inflammation, despite rapid bacterial clearance.

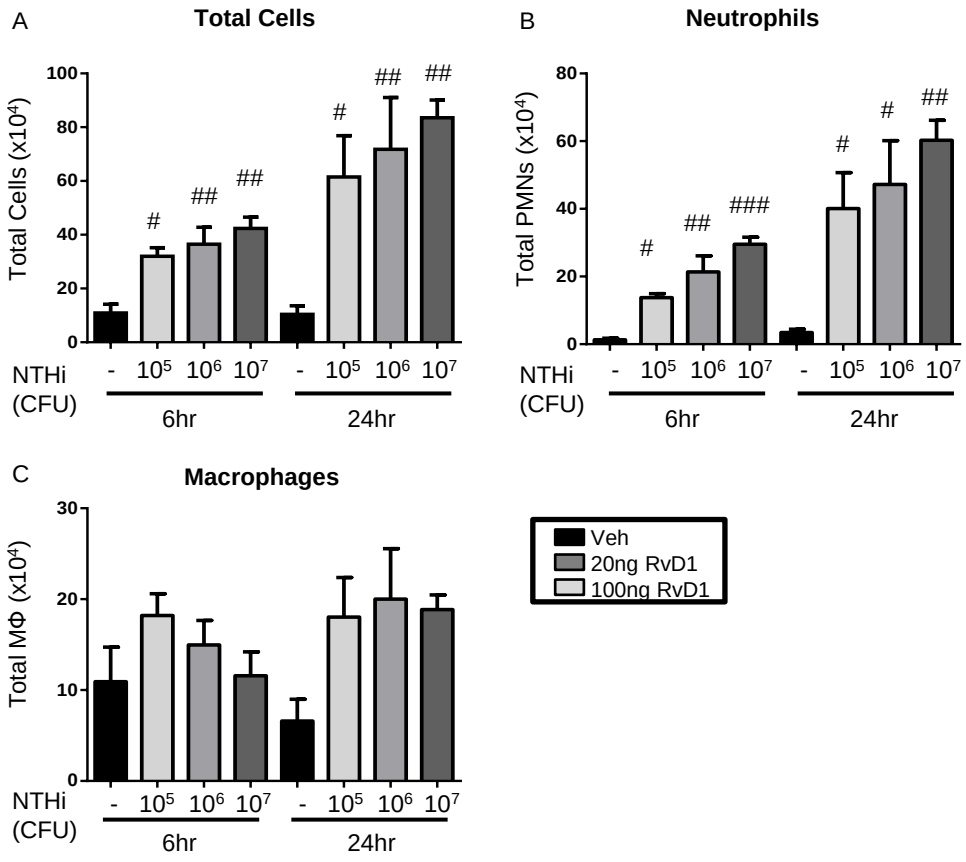

Fig. 4.3. NTHi dose-dependently induces cellular influx in C57BL/6 mice. Differential cell counts were determined from BALF of mice infected with 1 x 10^5- 1 x 10^7 CFUs NTHi. NTHi infection dose-dependently increased numbers of total cells (A), neutrophils (B), and macrophages (C) at 6 hours and 24 hours post-infection. Statistical significance was determined by two-way ANOVA with Bonferroni's posttest for multiple comparisons ($^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to veh/veh), n=5 mice/group.

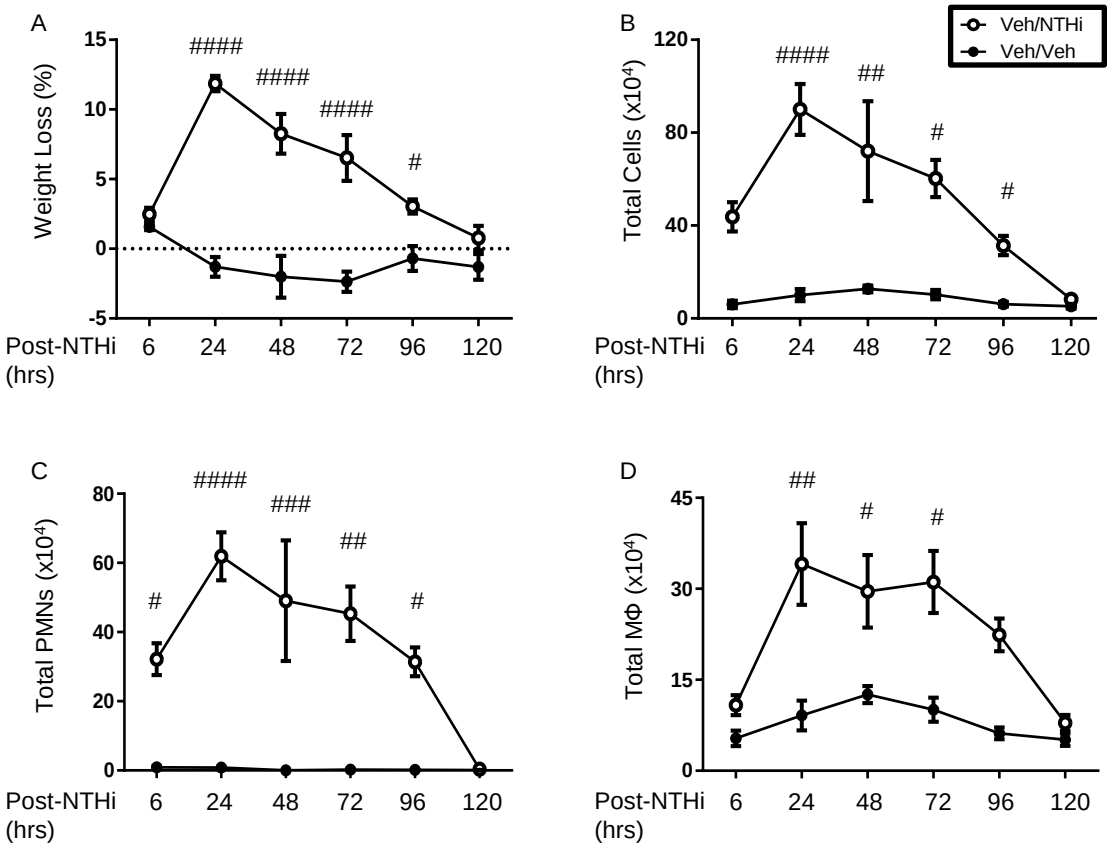

Fig. 4.4. NTHi infection results in prolonged weight loss and cellular influx in C57BL/6 mice. Body weights and differential cell counts from BALF were obtained from mice infected with 1 x 10^6 CFUs NTHi over the course of 5 days. NTHi infected mice had persistent weight loss (A). Mice also had prolonged levels of total inflammatory cells (B), neutrophils (C), and macrophages (D) up to four days post-infection. Statistical significance was determined by two-way ANOVA with Bonferroni's posttest for multiple comparisons ($^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, $^{\#\#\#\#}p<0.0001$ compared to veh/veh at each time point), n=5 mice/group.

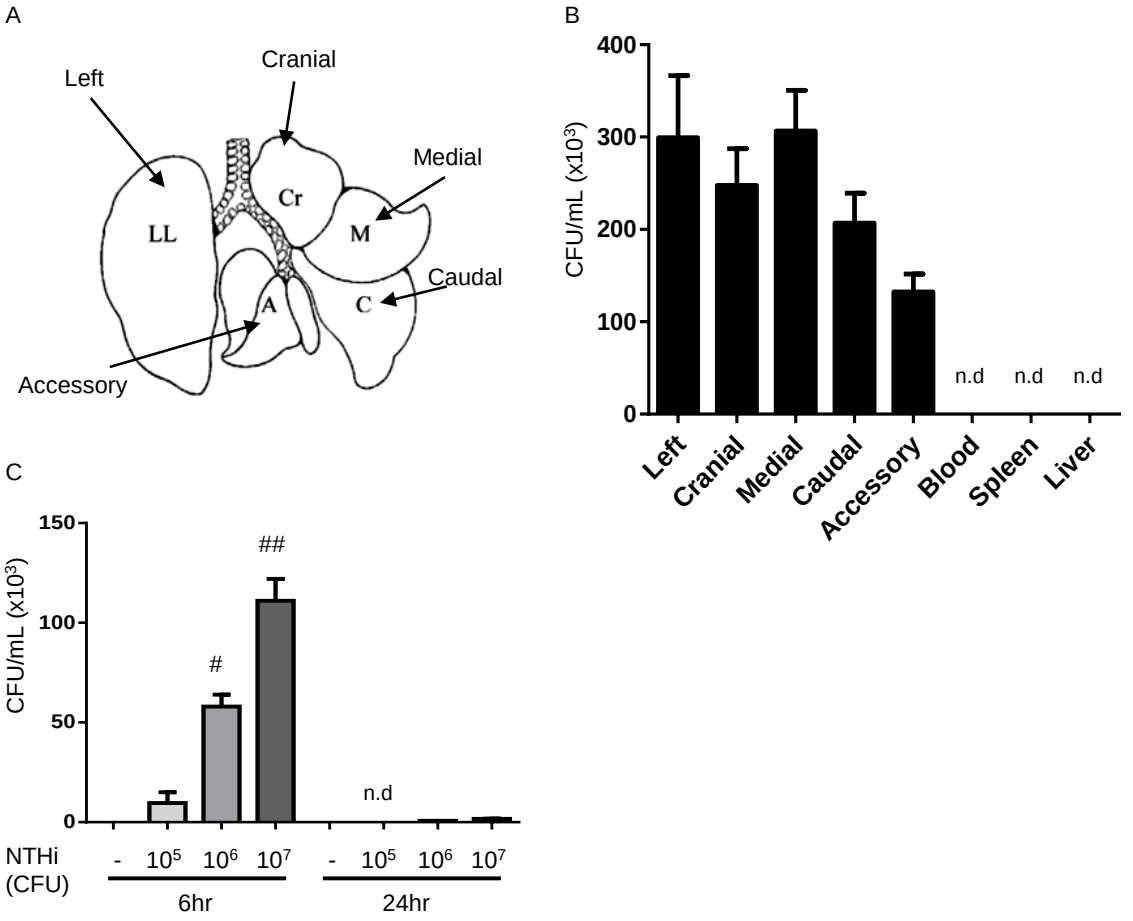

Fig. 4.5. NTHi is equally distributed in lung lobes and cleared within 24 hours. Bacteria colony counts were obtained from lungs of mice infected with 1 x 10^5-10^7 CFUs NTHi. Mice have five distinct lung lobes (A), and NTHi was distributed equally between lung lobes relative to lobe size at 6 hours post-infection (B). No bacteria was detected in the blood, liver, or spleen of infected mice. NTHi infected mice also had dose-dependent bacterial burden present in the lung at 6 hours, with >90% of bacteria cleared by 24 hours for all doses (C). Statistical significance was determined by two-way ANOVA with Bonferroni's posttest for multiple comparisons ($^{\#}p<0.05$, $^{\#\#}p<0.01$ compared to veh/veh, n.d. = not detected), n=3-5 mice/group.

AT-RvD1 alters the NTHi-induced inflammatory cell profile and promotes efferocytosis

We next evaluated whether AT-RvD1 could attenuate excessive NTHi-induced inflammation. To assess this, differential cell counts were obtained from BALF of mice treated with AT-RvD1 and infected with 1 x 10^6 CFUs of NTHi. AT-RvD1 did not dampen initial NTHi-induced cell infiltration at 6 hours, but AT-RvD1 treated mice had significantly reduced total BAL cell counts at 24 hours post-infection (Fig. 4.6A, D). AT-RvD1 treated mice also had decreased total neutrophil (PMN) numbers at both 6 and 24 hours (Fig. 4.6B, E). In contrast, AT-RvD1 increased the total number of macrophages at 6 hours (Fig. 4.6C). However, at 24 hours NTHi infected mice showed increased total levels of macrophages; AT-RvD1 treated mice had no difference in total macrophage counts compared to NTHi infected and vehicle treated mice by 24 hours (Fig. 4.6F).

Histological sections of lung tissue were also evaluated for inflammation and neutrophil influx. H&E staining revealed inflammatory cell infiltrates in the lungs of infected mice at both 6 hours and 24 hours (Fig. 4.7A). This cellular influx was reduced at 6 hours and was almost entirely absent at 24 hours in the lungs of AT-RvD1 mice, correlating with differential cell counts in BALF (Fig. 4.7A). We also stained for neutrophils using Gr-1 immunohistochemistry to specifically evaluate neutrophil influx. NTHi infected mice had increased neutrophil (i.e., Gr-1^+) staining at 6 and 24 hours after infection (Fig. 4.7B). These increases were strongly attenuated by AT-RvD1 treatment at both time points (Fig. 4.7B). These histological findings correlated with the cell counts obtained from BALF and provide further evidence for the anti-inflammatory effects of AT-RvD1.

We hypothesized that reduced neutrophils counts were due to enhanced efferocytosis. Macrophages were isolated from BALF or from collagenase digested lung tissue and their uptake of neutrophils was evaluated by flow cytometry. Macrophages obtained by these two different methods demonstrated unique results. AT-RvD1 treated mice had increased uptake of apoptotic neutrophils at 6 hours in macrophages from BALF (largely alveolar macrophages) but no differences at 24 hours post-infection (Fig. 4.8A-B). In macrophages obtained by lung digestion (i.e. a mixed population), on the other hand, AT-RvD1 only increased neutrophil uptake at 24 hours (Fig. 4.8C-D). These differences may indicate phagocytic specificity by different macrophage populations or simply differences due to technical preparation; overall AT-RvD1 promotes increased efferocytosis to clear apoptotic neutrophils and reduce the number of inflammatory cells present in infected lungs.

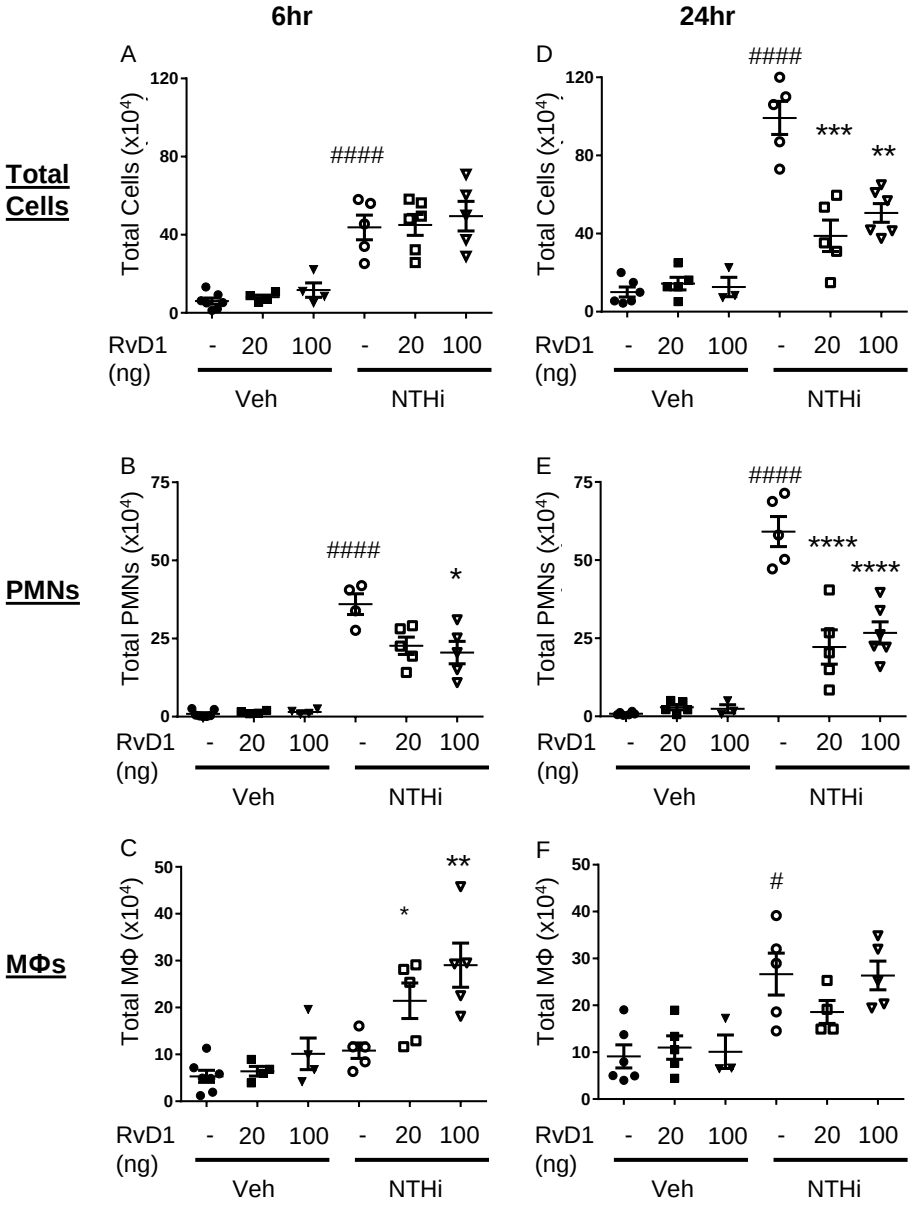

Fig. 4.6. AT-RvD1 shifts the profile of inflammatory cell influx. Differential cell counts were determined from BALF of AT-RvD1 (20 or 100 ng/mouse, OA) and/or NTHi (1 x 10^6 CFUs, OA) inoculated mice. Total cell (A, D), neutrophil (B, E), and macrophage (C, F) counts were evaluated at 6 hours (A-C) and 24 hours (D-F) post-infection. Statistical significance was determined by two-way ANOVA with Bonferroni's posttest for multiple comparisons ($^{\#}p<0.05$, $^{\#\#\#\#}p<0.0001$ compared to veh/veh, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$ compared to veh/NTHi), n=5-8 mice/group.

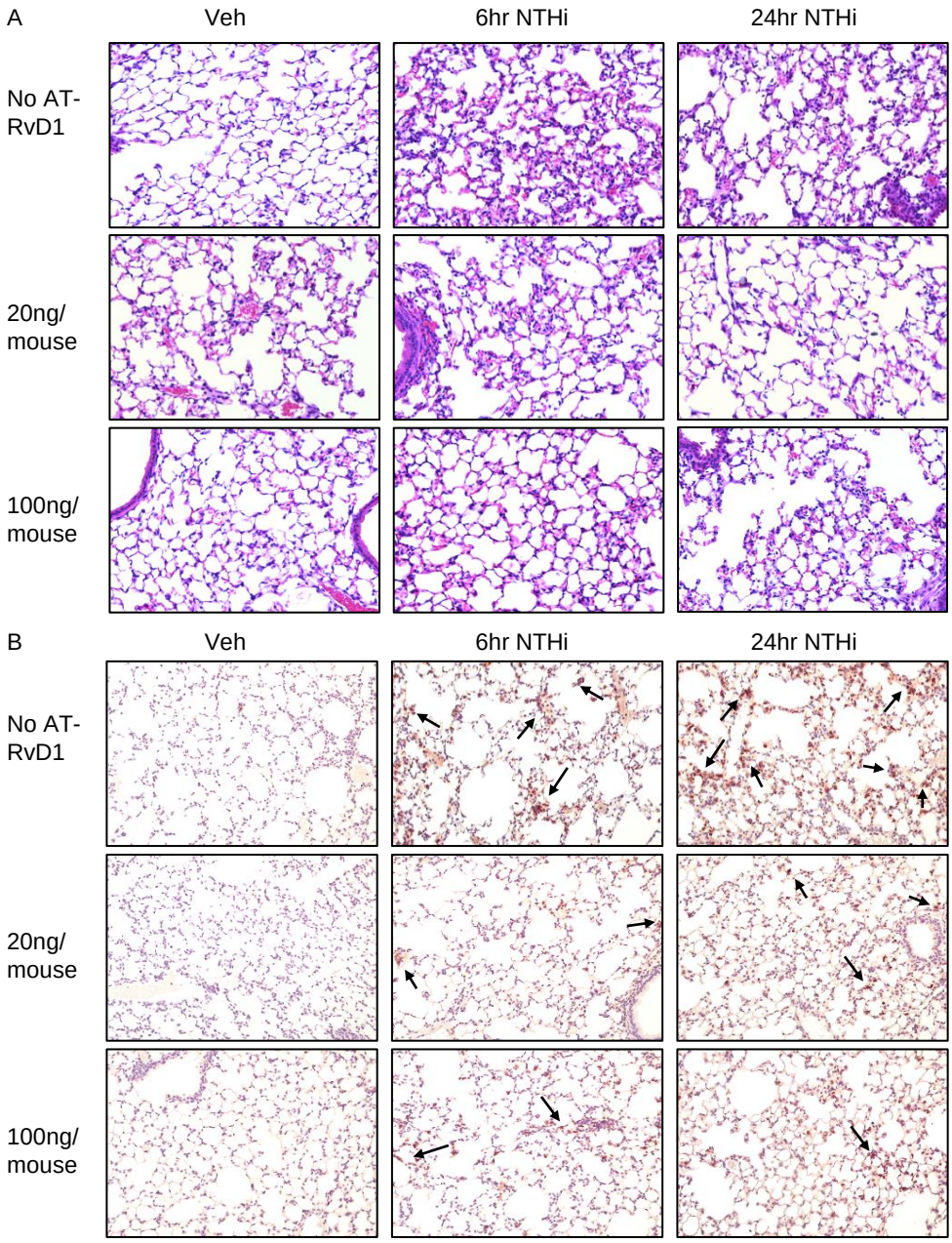

Fig. 4.7. AT-RvD1 decreases inflammation and neutrophil influx. Histological sections were taken from AT-RvD1 (20 or 100 ng/mouse, OA) and/or NTHi (1 x 10^6 CFUs, OA) inoculated mice at 6 hours and 24 hours post-infection. Sections were stained with H&E to reveal general inflammation (A). Separate sections were immunostained for Gr-1 to highlight neutrophils (brown stain) and counterstained with hemotoxylin (B). Images shown are at a 20x magnification.

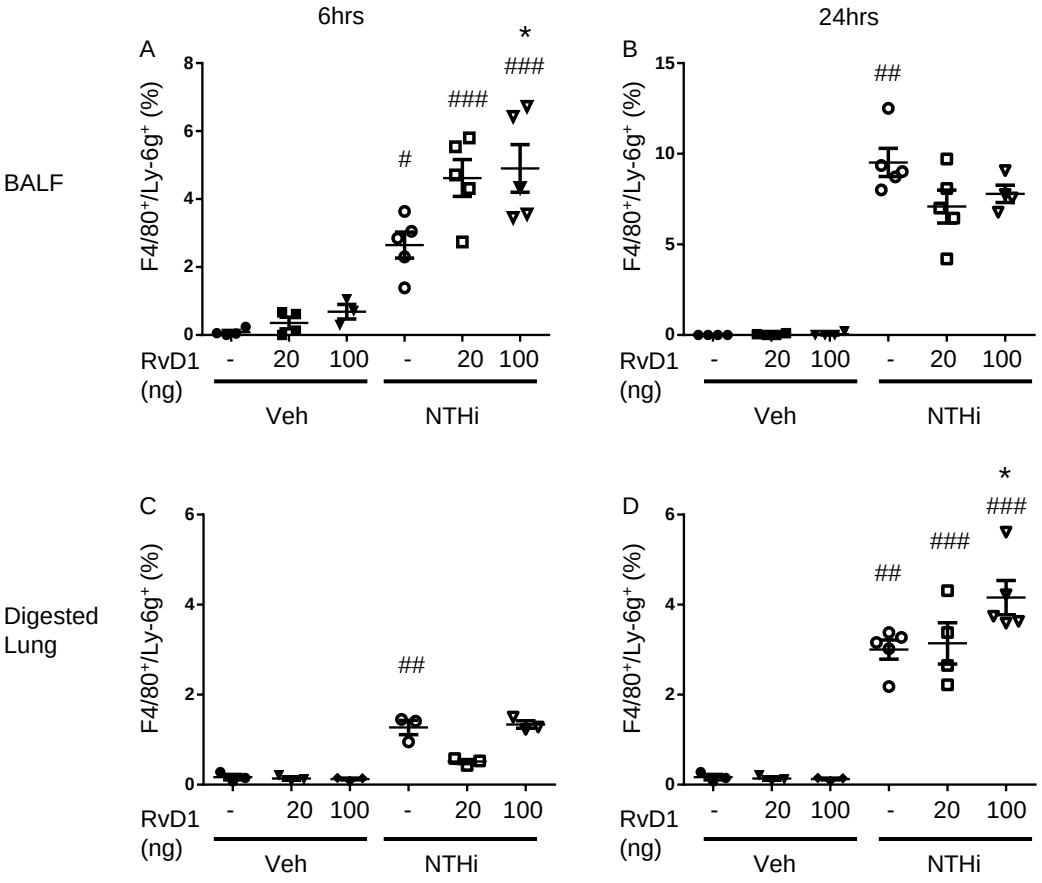

Fig. 4.8. AT-RvD1 decreases neutrophil influx and promotes enhanced efferocytosis. BALF cells or cells from collagenase digested lung tissue were obtained from AT-RvD1 (20 or 100 ng/mouse, OA) and/or NTHi (1 x 10^6 CFUs, OA) inoculated mice at 6 hours and 24 hours post-infection. Efferocytosis from BALF (A-B) and collagenase digestion (C-D) at 6 hours (A, C) and 24 hours (B, D) was evaluated by flow cytometry. $F4/80^+/Ly6G^+$ cells represent macrophages with ingested neutrophils. Statistical significance was determined by two-way ANOVA with Bonferroni's posttest for multiple comparisons ($^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to veh/veh, $^{*}p<0.05$ compared to veh/NTHi), n=5-8 mice/group.

AT-RvD1 temporally dampens Cox-2 expression and pro-inflammatory cytokines

In addition to inflammatory cell influx, NTHi infected mice had significantly more total protein in their BALF at both 6 and 24 hours, indicative of edema and inflammation (Fig. 4.10A). Due to this elevated protein content, we evaluated the presence of pro-inflammatory prostaglandins, enzymes, and cytokines. We first evaluated the effect of AT-RvD1 on prostaglandin E_2 (PGE_2) and cyclooxygenase-2 (Cox-2) expression. Cox-2 plays multiple roles in the inflammatory process and catalyzes production of PGE_2, a prostaglandin with important roles in bacterial infections. NTHi induced strong Cox-2 expression at both 6 hours and 24 hours post-infection (Fig. 4.10B-C). AT-RvD1 did not attenuate early NTHi induced Cox-2 expression at 6 hours, but dose-dependently dampened expression at 24 hours. (Fig. 4.10B-C). Interestingly, AT-RvD1 in the absence of NTHi infection increased Cox-2 expression at 24 hours (Fig. 4.10B-C). PGE_2 was also increased by NTHi, with trending decreases with AT-RvD1 treatment (Fig. 4.10D).

NTHi also induced pro-inflammatory cytokines IL-6, TNFα, and KC as early as 6 hours post-infection (Fig. 4.11A-C). AT-RvD1 did not alter IL-6 or TNFα production at 6 hours, but did reduce the levels of KC (Fig. 4.11A-C). At 24 hours, AT-RvD1 attenuated production of both TNFα and IL-6 levels (Fig. 4.11A-B). KC had returned to near-background levels at 24 hours and there were no further reductions with AT-RvD1 (Fig. 4.11C). Along with pro-inflammatory cytokines, we evaluated the effect of NTHi and AT-RvD1 on the anti-inflammatory cytokine IL-10. At 6 hours, NTHi reduced IL-10 expression, which was restored by AT-RvD1 treatment (Fig. 4.11D). Taken together,

these data reflect the temporal regulation abilities of AT-RvD1 in reducing inflammation and promoting resolution through modulation of cytokine production.

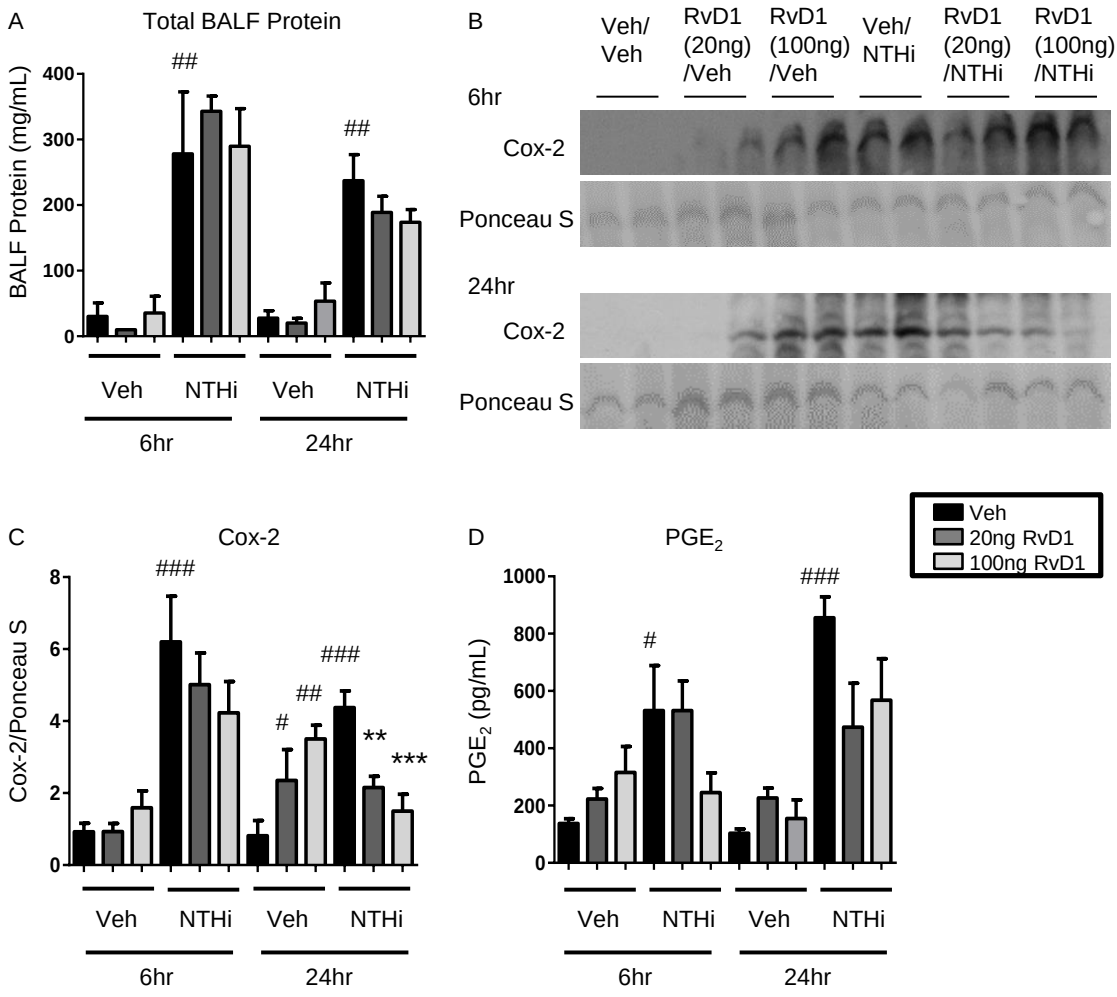

Fig. 4.9. AT-RvD1 dampens expression of Cox-2. Total protein in BALF of AT-RvD1 (20 or 100 ng/mouse, OA) and/or NTHi (1 x 10^6 CFUs, OA) inoculated mice was measured by BCA at 6 hours and 24 hours post-infection (A). Cox-2 expression was evaluated in lung homogenates by western blot (B, C, representative blots shown, densitometry on n=4-5 mice/group). PGE_2 levels in BALF were measured by EIA (D). Statistical significance was determined by two-way ANOVA with Bonferroni's posttest for multiple comparisons ($^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to veh/veh, $^{**}p<0.01$, $^{***}p<0.001$ compared to veh/NTHi), n= 4-6 mice/group.

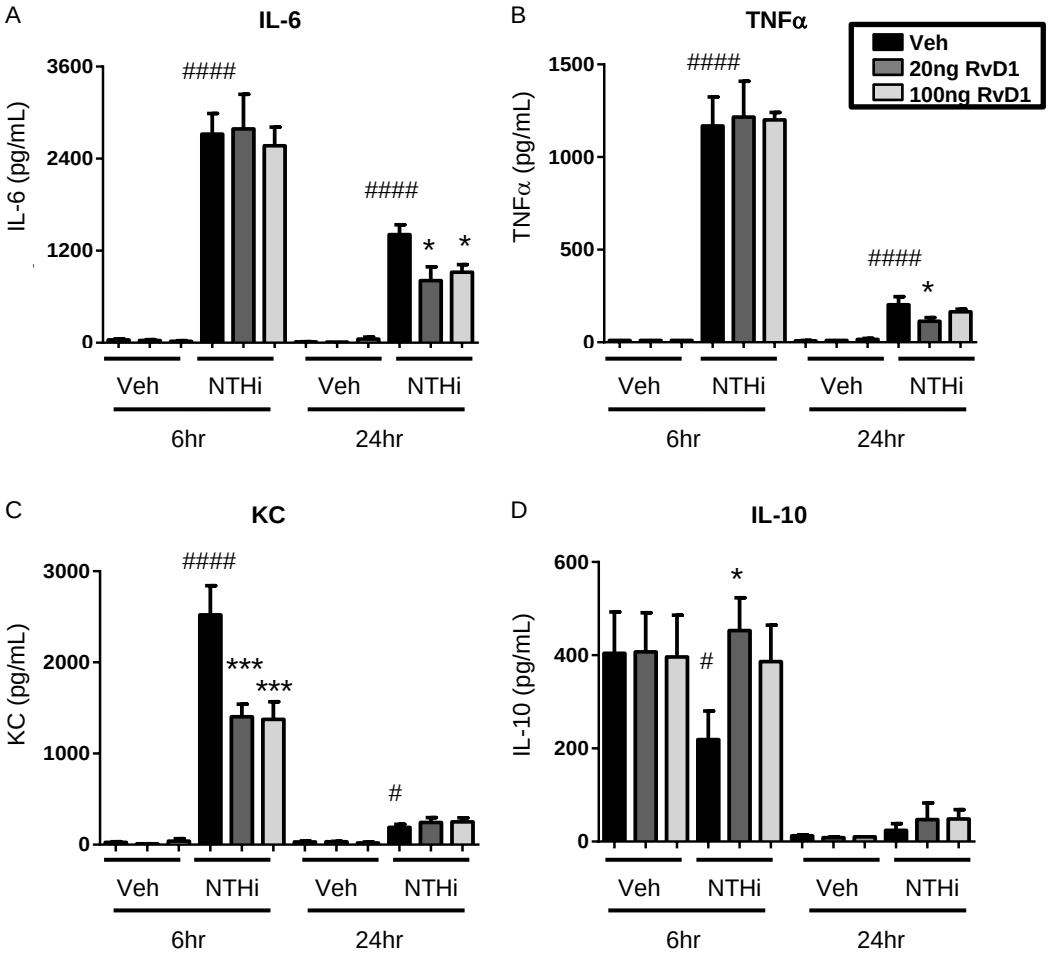

Fig. 4.10. AT-RvD1 dampens production of pro-inflammatory cytokines. Levels of pro-inflammatory cytokines were measured in BALF of AT-RvD1 (20 or 100 ng/mouse, OA) and/or NTHi (1 x 10^6 CFUs, OA) inoculated mice at 6 hours and 24 hours. BALF was assessed for IL-6 (A), TNFα (B), KC (C), and IL-10 (D) by ELISA. Statistical significance was determined by two-way ANOVA with Bonferroni's posttest for multiple comparisons ($^{\#}p<0.05$, $^{\#\#\#\#}p<0.001$ compared to veh/veh, *p<0.05, ***p<0.001 compared to veh/NTHi), n= 4-6 mice/group.

AT-RvD1 promotes bacterial clearance

A defining characteristic of AT-RvD1 is its ability to dampen inflammation without being immunosuppressive. To evaluate this property, we examined the effect of AT-RvD1 treatment on pulmonary bacterial burden. AT-RvD1 treated mice displayed dose-dependent decreases in bacterial colonies, consistent with bacterial improved clearance; this effect was evident as early as 6 hours and was statistically significant by 8, 12, and 24 hours (Fig. 4.11). AT-RvD1 did not directly act to kill NTHi, as colony counts on chocolate agar plates streaked with an NTHi or NTHi plus AT-RvD1 suspension were not significantly different.

SPMs have been shown in non-microbial models of inflammation to increase the phagocytic abilities of innate immune cells (65, 72, 95, 154). In particular, SPMs promote the alternative M2 macrophage phenotype to mediate enhanced phagocytosis. We hypothesized that reductions in bacterial CFUs with AT-RvD1 treatment were due to polarization of macrophages to an M2 phenotype which thereby mediated enhanced clearance of NTHi. Compared with those of NTHi-infected mice not treated with AT-RvD1, the lung macrophages of NTHi-infected mice treated with AT-RvD1, whether obtained by bronchoalveolar lavage or collagenase digestion of lung tissue, had a reduced ratio of pro-inflammatory M1 macrophage markers (e.g., CD80) to pro-resolving M2 macrophage markers (e.g., CD206) (Fig. 4.12A, 4.13A). This indicates a shift towards alternative M2 macrophages in AT-RvD1-treated, NTHi-infected mice. We further evaluated phagocytosis of NTHi by M1 macrophages, M2 macrophages, and neutrophils, as both macrophages and neutrophils are known to contribute to NTHi clearance. Mice

were infected with fluorescently labeled NTHi and double stained macrophages and neutrophils were analyzed by flow cytometry. In cells obtained from BALF, AT-RvD1 treatment induced no difference in NTHi phagocytosis by $CD80^+$ macrophages (M1) or by neutrophils, but $CD206^+$ macrophages (M2) had a dose-dependent increase in NTHi uptake with AT-RvD1 treatment (Fig. 4.12B-D). Similar results were observed in cells obtained from collagenase digestion of lung tissue, wherein no differences were observed in $CD80^+$ or neutrophil phagocytosis between infection groups, but $CD206^+$ macrophages had enhanced bacterial uptake (Fig. 4.13B-D). These data demonstrate a role for M2 macrophages in clearing NTHi bacteria and provide one mechanism by which AT-RvD1 promotes the resolution of lung inflammation and infection.

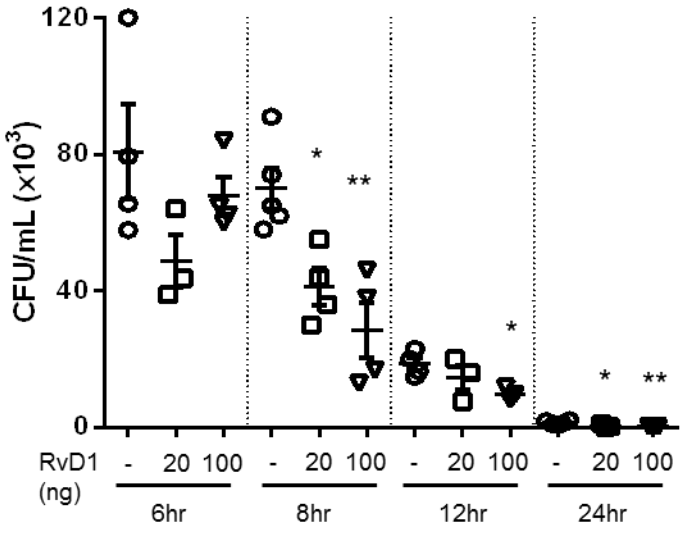

Fig. 4.11. AT-RvD1 treated mice have decreased NTHi bacterial burden. Colony counts were assessed in lung homogenates 6-24 hours post-infection with 1 x 10^6 CFUs of NTHi. Statistical significance was determined at each time point by one-way ANOVA with Bonferroni's posttest for multiple comparisons (*$p<0.05$, **$p<0.01$ compared to veh/NTHi), n=3-6mice/group.

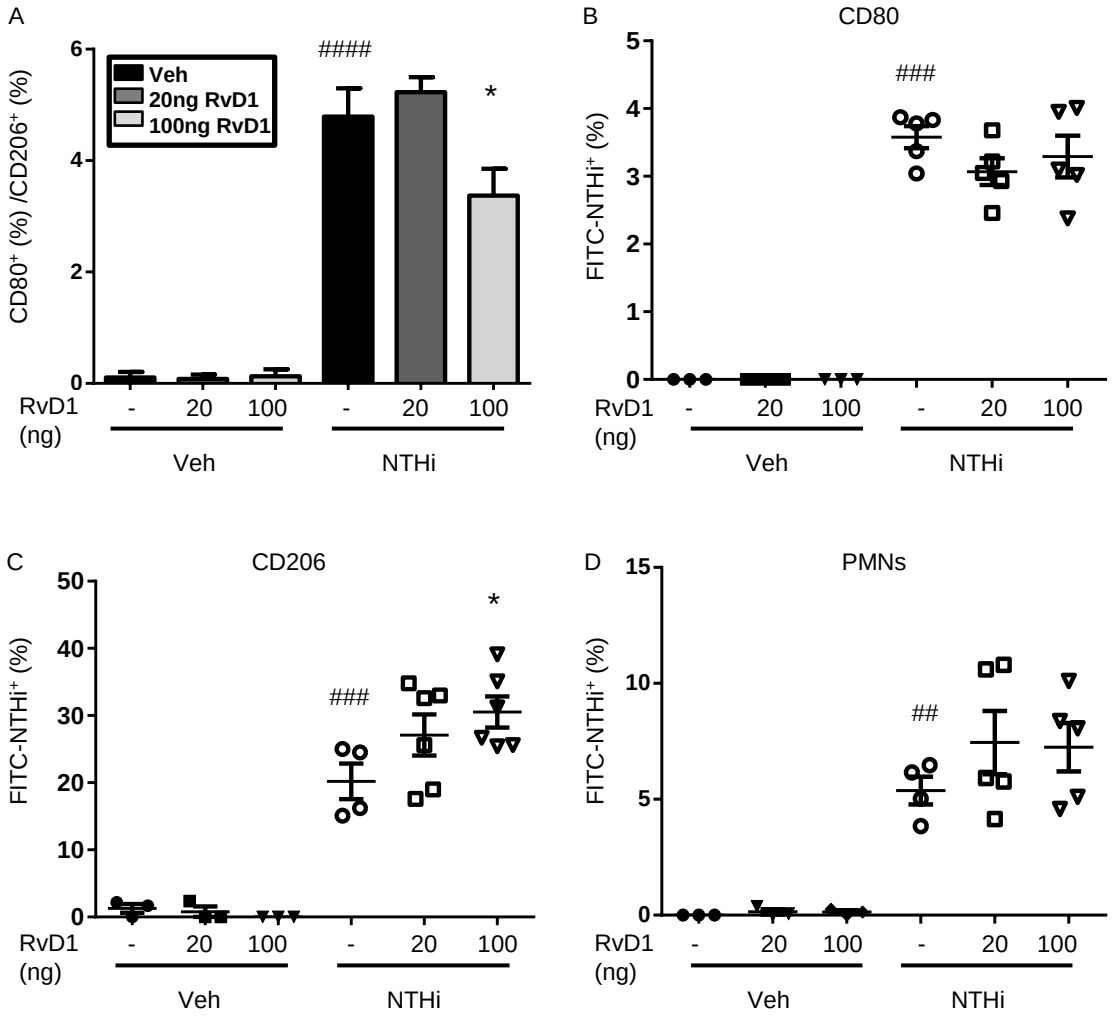

Fig. 4.12. AT-RvD1 promotes M2 macrophages in cells obtained from bronchoalveolar lavage to mediate NTHi clearance. Mice were treated with AT-RvD1 (20 or 100 ng/mouse, OA) and/or NTHi (1 x 10^6 CFUs, OA) for 8 hours. Macrophages and neutrophils were obtained from BALF. Macrophage phenotype was assessed by flow cytometry (A). Uptake of NTHi by $CD80^+$ macrophages, $CD206^+$ macrophages, and neutrophils was also assessed by flow cytometry (B-D). Statistical significance was determined by two-way ANOVA with Bonferroni's posttest for multiple comparisons ($^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, $^{\#\#\#\#}p<0.0001$ compared to veh/veh, $^*p<0.05$, compared to veh/NTHi), n=3-6 mice/group.

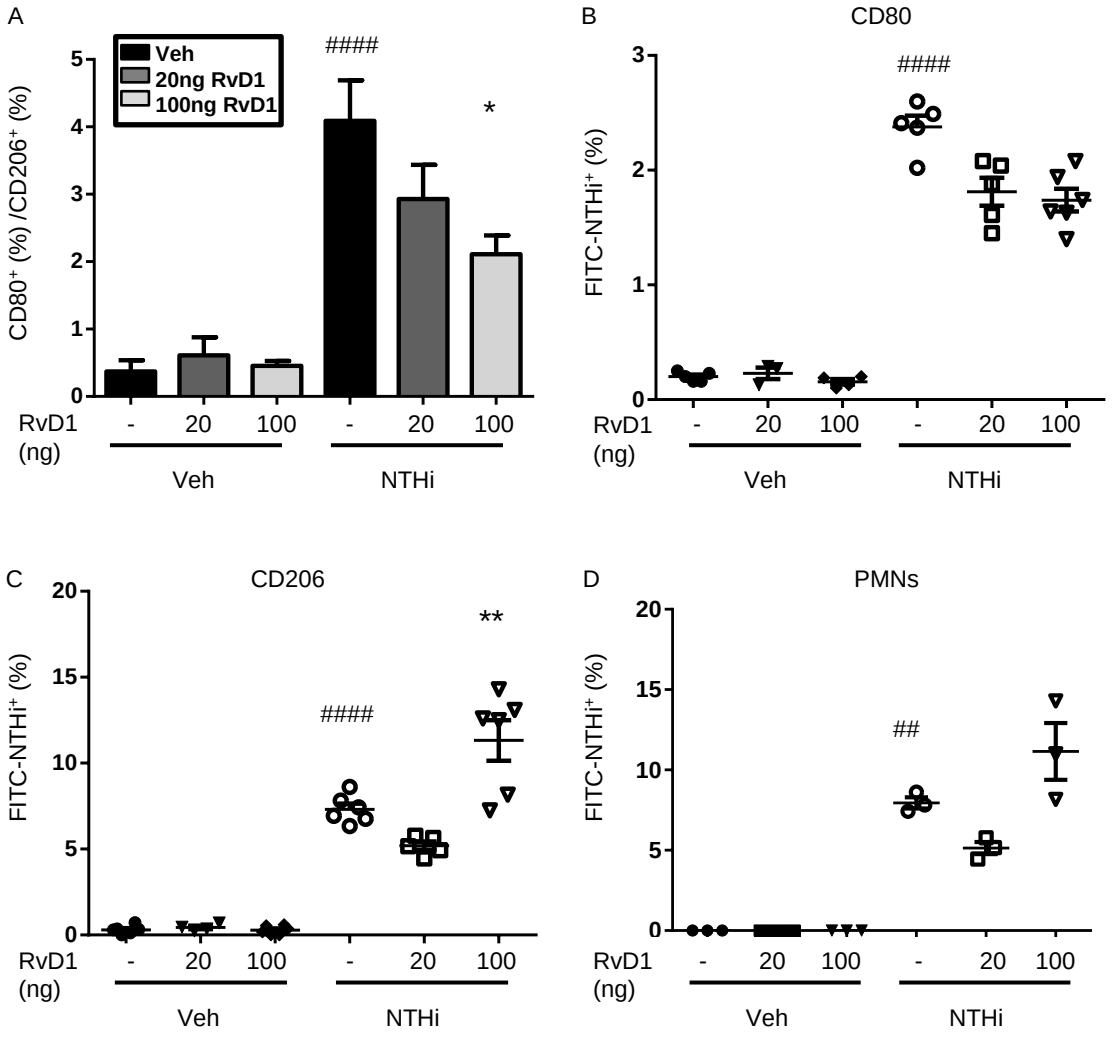

Fig. 4.13. AT-RvD1 promotes M2 macrophages in cells obtained from lung digestion to mediate NTHi clearance. Mice were treated with AT-RvD1 (20 ng or 100 ng/mouse, OA) and/or NTHi (1 x 10^6 CFUs, OA) for 8 hours. Macrophages and neutrophils were obtained from lungs digested with collagenase. Macrophage phenotype was assessed by flow cytometry (A). Uptake of NTHi by $CD80^+$ macrophages, $CD206^+$ macrophages, and neutrophils was also assessed by flow cytometry (B-D). Statistical significance was determined by two-way ANOVA with Bonferroni's posttest for multiple comparisons ($^{\#\#}p<0.01$, $^{\#\#\#\#}p<0.0001$ compared to veh/veh, $^*p<0.05$, $^{**}p<0.01$ compared to veh/NTHi), n=3-6 mice/group.

AT-RvD1 promotes improved health outcomes in NTHi-infected mice

Along with dampened inflammation and enhanced bacterial clearance, we investigated whether AT-RvD1 could improve the general health of infected mice. NTHi-infected mice exhibited a trend toward weight loss as early as 6 hours after infection, with significant and dramatic (up to 15%) weight loss at 24 hours (Fig. 4.14A-B). Mice also exhibited a significant 1.5°C drop in body temperature 6 hours post-infection; normal body temperature was restored by 24 hours (Fig. 4.14C-D). AT-RvD1 treatment reduced weight loss at 24 hours, with only 7-11% weight loss compared to 12-15% in non-treated NTHi-infected mice (Fig. 4.14B). AT-RvD1 also dose-dependently protected the mice from hypothermia at 6 hours, thereby preventing hypothermia (Fig. 4.14C).

NTHi infection also altered normal respiratory physiology. NTHi-infected mice had decreased tidal volume as early as 6 hours post-infection, with increased respiratory rates and further decreased tidal volume at 24 hours, indicative of respiratory distress (Fig. 4.15A-D). AT-RvD1 dose-dependently rescued these alterations to respiratory physiology; both doses of AT-RvD1 restored tidal volume, while 100 ng/mouse of AT-RvD1 fully restored respiratory rates 24 hours after infection (Fig. 4.15A-D). NTHi infected mice also exhibited a dramatic drop (down to 91%) in the O_2 saturation of hemoglobin, comparable to that seen in humans with severe respiratory improvements (Fig. 4.16A-B). AT-RvD1 dose-dependently attenuated this drop, enabling mice to maintain normoxia (Fig. 4.16A-B). These results demonstrate the strong therapeutic potential of AT-RvD1 against NTHi infection.

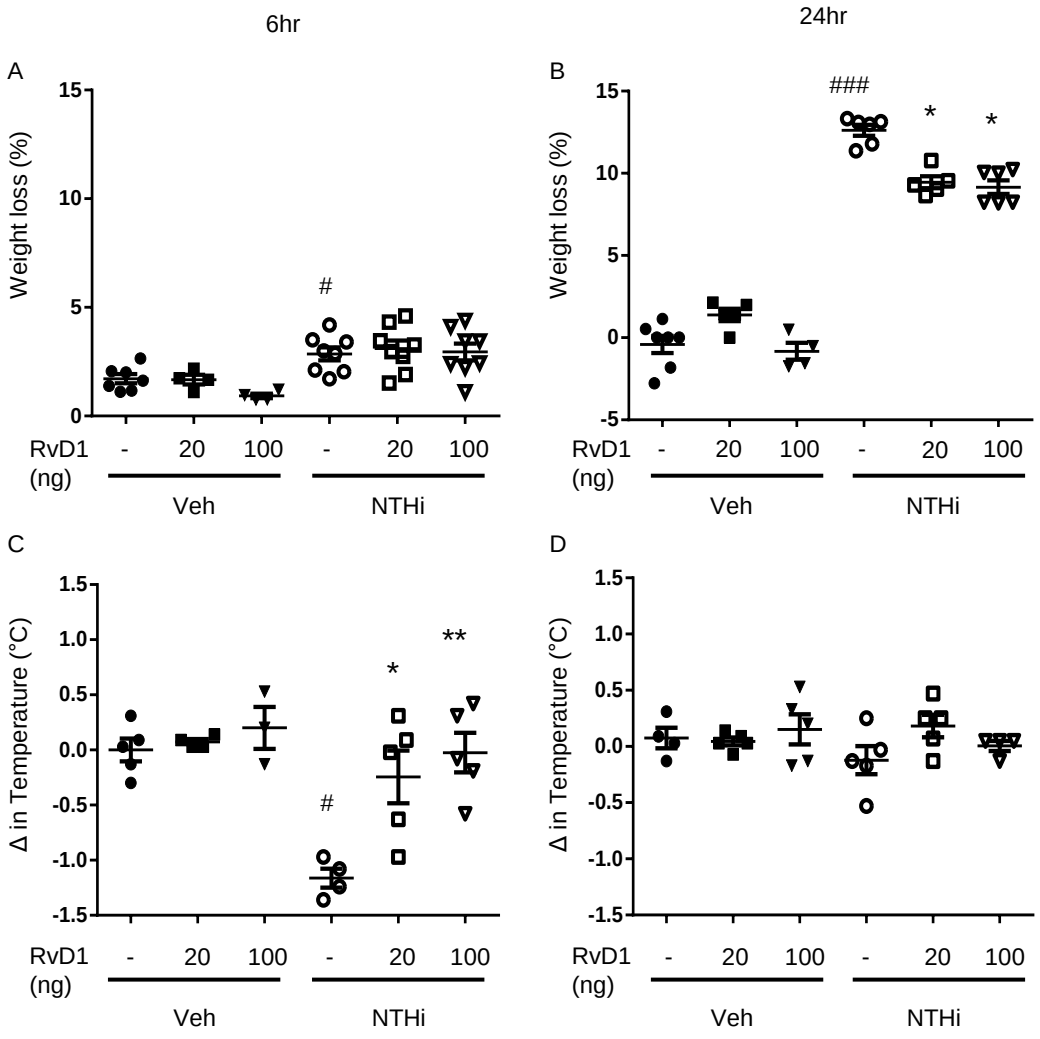

Fig. 4.14. AT-RvD1 dampens NTHi-induced weight loss and hypothermia. Mice inoculated with AT-RvD1 (20 or 100 ng/mouse, OA) and/or NTHi (1 x 10^6 CFUs, OA) were weighed immediately before infection and at time of sacrifice at 6 and 24 hours (A-B). Additionally, core body temperature was recorded prior to euthanasia at 6 and 24 hours (C-D). Statistical significance was determined by two-way ANOVA with Bonferroni's posttest for multiple comparisons ($^{\#}p<0.05$, $^{\#\#\#}p<0.001$ compared to veh/veh, $*p<0.05$, $**p<0.01$ compared to veh/NTHi), n=3-6 mice/group.

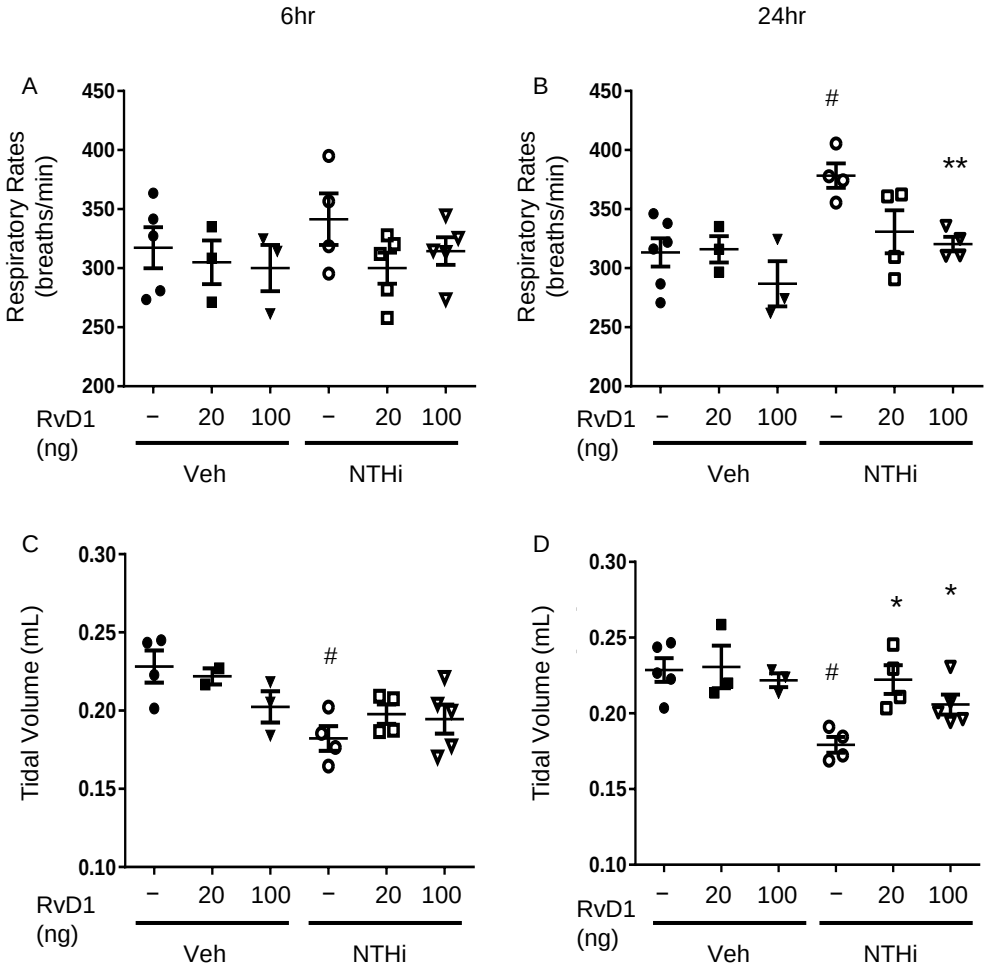

Fig. 4.15. AT-RvD1 improves respiratory health in NTHi infected mice. Respiratory outcomes were measured for mice inoculated with AT-RvD1 (20 or 100 ng/mouse, OA) and/or NTHi (1 x 10^6 CFUs, OA) at 6 and 24 hours post-infection. Respiratory rates (A-B) and tidal volume (C-D) were assessed using a small rodent plethysmograph. Statistical significance was determined by two-way ANOVA with Bonferroni's posttest for multiple comparisons ($^{\#}p<0.05$ compared to veh/veh, $^{*}p<0.05$, $^{**}p<0.01$ compared to veh/NTHi), n=3-5 mice/group.

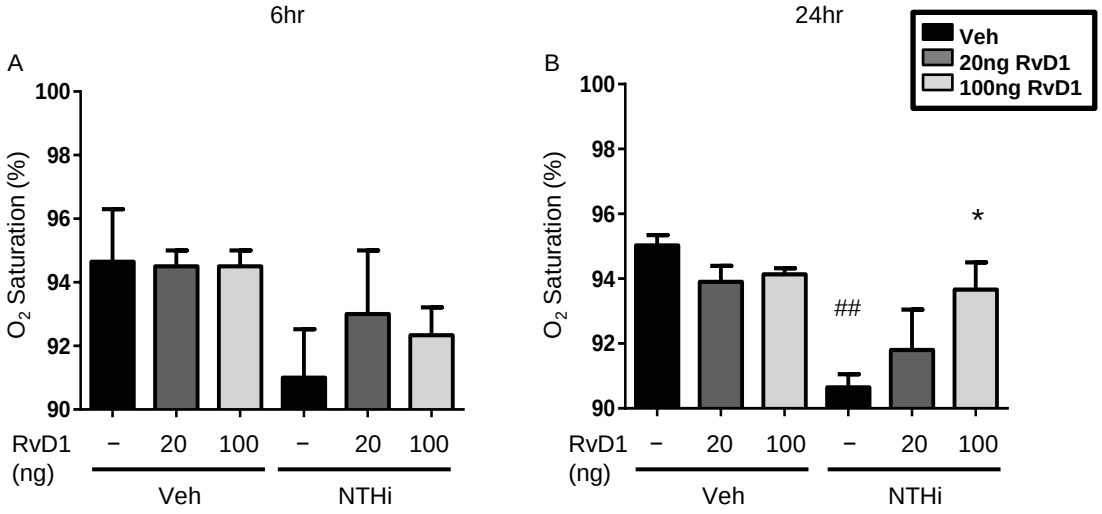

Fig. 4.16. AT-RvD1 prevents hypoxemia in NTHi infected mice. Respiratory outcomes were measured for mice inoculated with AT-RvD1 (20 or 100 ng/mouse, OA) and/or NTHi (1 x 10^6 CFUs, OA) at 6 and 24 hours post-infection. O_2 saturation of hemoglobin (A-B) was assessed using a mouse oximeter. Statistical significance was determined by two-way ANOVA with Bonferroni's posttest for multiple comparisons ($^{\#\#}p<0.01$ compared to veh/veh, $^{*}p<0.05$ compared to veh/NTHi), n=3-6 mice/group.

Discussion

SPMs represent a novel class of non-immunosuppressive small lipid molecules with high clinical potential for treatment of inflammatory diseases. Here, we have shown that AT-RvD1 is effective at dampening LPS-induced acute lung injury. AT-RvD1 is further effective at dampening live NTHi-induced lung inflammation without impairing bacterial clearance. AT-RvD1 treated mice had reduced inflammatory cell influx into the lung, with a higher macrophage:neutrophil ratio, and increased efferocytosis compared to NTHi infected mice with no SPM treatment. AT-RvD1 attenuated levels of pro-inflammatory cytokines and Cox-2 expression while preventing decreases in anti-inflammatory cytokines. Critically, these anti-inflammatory effects did not impair bacterial clearance, and AT-RvD1 treated mice had fewer NTHi CFUs in their lungs. This decreased bacterial burden was mediated by an increase in M2 macrophages, which had enhanced bacterial phagocytosis. Overall, AT-RvD1 treated mice had less weight loss, reduced hypothermia, improved respiratory physiology, including a smaller drop in O_2 saturation, indicating less hypoxemia. These effects underscore the clinical therapeutic potential of AT-RvD1 for NTHi infection.

In our pilot experiments with LPS, we observed *in vivo* effects comparable to the *in vitro* effects described in chapter 3 of this thesis. AT-RvD1 dampened neutrophil influx, but did not reduce macrophage levels. This correlates with our published studies and others' work highlighting the role of macrophages in resolution, and provides the first evidence that they may be an important player in our models of mouse pulmonary inflammation. We furthermore saw reductions in the pro-inflammatory cytokines TNFα,

IL-6, KC, and PGE_2 in BALF, as well as dose-dependent reductions in Cox-2 expression from lung homogenates. Interestingly, the most potent AT-RvD1 dose varied between these inflammatory markers. For example, both doses of AT-RvD1 showed similar efficacy at reducing KC levels, but 20 ng/mouse more potently decreased PGE_2 and 100 ng/mouse more potently decreased Cox-2 expression. These results provide an important reminder about the nuances of SPM dosing, and indicate that more studies are needed to fully elucidate the dose/response relationships between SPMs and receptor binding in order to broaden their therapeutic use.

Regarding our NTHi model, in each of our observed endpoints, all NTHi infected mice mounted an initial inflammatory response, but the AT-RvD1 treated mice had faster resolution of inflammation. At 6 hours post-infection, in the early stages of bacterial clearance, AT-RvD1 dampened KC, a cytokine that promotes neutrophil recruitment, contributing to reduced neutrophil counts in AT-RvD1 treated lungs. However, AT-RvD1 did not dampen levels of IL-6 and TNFα until 24 hours. These cytokines are important for initial inflammatory responses, with TNFα particularly contributing to bacterial phagocytosis and killing. This same effect was seen regarding inflammatory cell influx, wherein AT-RvD1 did not halt inflammatory cell influx, but shifted the cellular profile to promote an early increase in macrophages and a faster clearance of neutrophils. This regulation of cytokines at different time points is key for several reasons. First, AT-RvD1 does not broadly block all cytokine signaling, distinguishing SPMs from other strictly anti-inflammatory therapies. This non-immunosuppressive nature is further seen as SPMs increase macrophage influx, enhance efferocytosis, and increase the phagocytic abilities

of macrophages (32, 37, 65, 149, 154, 167). Second, AT-RvD1 decreases in KC levels early on demonstrate that SPMs can preferentially regulate early cytokine release to jump-start resolution. Third, endogenous resolution of NTHi-induced inflammation is slow, occurring days after bacterial clearance. AT-RvD1, however, enhances natural resolution, more rapidly clearing apoptotic cells and dampening pro-inflammatory cytokine release. These data highlight that SPMs do not suppress immune cell function, but instead promote a shift in macrophage functional activities to speed the resolution process.

Along with temporal regulation of pro-inflammatory cell influx and cytokines, SPMs are also capable of shifting cytokine production to promote enhanced levels of anti-inflammatory and/or pro-resolving cytokines. SPMs are derived from omega-3 and omega-6 fatty acids, precursors that can undergo lipid class switching to eicosanoids in a temporal manner and under different stimuli (163). This is highlighted by our results with Cox-2; while AT-RvD1 partially inhibited the increased expression of Cox-2 in NTHi-treated mice at 24hr, it paradoxically increased Cox-2 expression in vehicle-treated mice. Cox-2 is capable of producing both pro- and anti-inflammatory mediators and resolvins have been shown to increase Cox-2 under homeostatic conditions in human macrophages (65, 108). Therefore, AT-RvD1 may be acting to promote other SPMs or prostaglandins to maintain homeostatic conditions in the absence of an inflammatory response, but dampening NTHi-induced Cox-2 expression to prevent production of pro-inflammatory mediators. Furthermore, PGE_2 continues to increase at 24 hours, in contrast to other cytokines, despite no change in mPGES1 (data not shown). While Cox-2 expression is

lower at 24 hours than at 6 hours, Cox-2 in veh/NTHi mice is still significantly elevated above veh/veh mice, which could account for the increased PGE_2. Furthermore, PGE_2 has been shown to have both pro- and anti-inflammatory actions. Thus, late stage PGE_2 could be acting in an anti-inflammatory manner or may simply be the result of accumulated prostaglandins. While further in-depth investigations are needed to fully elucidate the role of Cox-2 in inflammation and SPM mediated resolution, Cox-2 is critical in producing various eicosanoids in a temporal manner. These distinct lipid mediator profiles produced at different stages of inflammation may mediate the progression from initiation of inflammation through to resolution and can reprogram immune cells to promote resolution.

In our studies, some actions of AT-RvD1 are dose-dependent (such as phagocytosis and efferocytosis), while other effects are similar at the 20 ng and 100 ng/mouse doses (such as reductions in inflammatory cells and cytokines). Different components of the resolution process may be more sensitive to AT-RvD1's actions (such as dampening of inflammatory cytokines compared to promotion of pro-resolving phagocytosis), and it is possible that dose-dependent changes in cytokines and inflammatory cells would have been seen at doses below 20 ng/mouse. Because different SPMs act through different receptors (12, 14, 24), it is also possible that a combination of SPMs would have stronger pro-resolving effects than higher doses of single SPMs. Such combination studies will be an important next step toward development of SPMs as clinical therapies.

The non-immunosuppressive properties of SPMs are most clearly demonstrated by their ability to mediate macrophage phenotypic switching. We observed an increase in the proportion of M2 macrophages to M1 macrophages with AT-RvD1 treatment. These changes were observed in both macrophages from BALF and from lung digestion, two methods which can yield different mixes of alveolar, interstitial, and monocyte-derived macrophages. That changes were observed with both macrophage preparations, and that studies have shown SPMs promote M2 macrophages in humans and mice, points to a common mechanism for SPM regulation of macrophage phenotype across different macrophage groups. M2 macrophages are known to promote resolution, and a number of studies have demonstrated their enhanced phagocytic capabilities (30, 32, 46, 208). Moreover, multiple chronic inflammatory diseases have been linked to a skewed M1/M2 profile, with a deficiency in M2 macrophages presumably causing phagocytic and resolution deficiencies (208, 209).

Chronic obstructive pulmonary disease patients in particular have deficient phagocytosis, and NTHi is a common pathogen known to induce COPD exacerbations (79). NTHi can avoid opsonization and clearance, allowing for persistent colonization in the human lung. In our study, AT-RvD1 increased bacterial clearance from the lung through enhanced macrophage phagocytosis. While the specific mechanisms by which AT-RvD1 acts to enhance phagocytosis are unknown, this ability has been observed in other models (37, 65, 95, 154). Additionally, AT-RvD1 treated mice may have decreased bacterial burden because of more effective macrophage killing of bacteria. While SPMs have not been found to have direct antibacterial properties, they can increase

internalization of bacteria, TNFα expression, and ROS production to mediate killing (149, 167). Although we have not yet demonstrated that AT-RvD1 or other SPMs can improve phagocytosis and bacterial killing in the context of chronic lung inflammatory disease, we recently reported that AT-RvD1 attenuates cigarette smoke-induced emphysema in a mouse model, with reductions in inflammatory signaling and oxidative stress (66). Future studies that evaluate SPMs in a setting of infection following chronic lung disease will be important to understand the translational potential of these compounds. We are also very interested to determine if SPMs are equally efficacious in a model of chronic recurrent infection; i.e., would SPMs be effective if given during a third or fourth NTHi infection after several rounds of untreated infection?

AT-RvD1 may be acting through a number of signaling pathways to mediate its effects. As described above, Cox-2 can regulate the production of pro- and anti-inflammatory signals, including SPM production. NTHi has been shown to activate the NF-κB and MAPK pathways to promote inflammation (200). SPMs have also been shown to act on the NF-κB pathway to mediate their effects (65, 71, 167, 168). Moreover, NTHi induces expression of TAK-1, which leads to enhanced NF-κB expression; TAK-1 is negatively regulated by cylindromatosis (CYLD), a primary negative regulator for NTHi induced inflammation overall (210, 211). Our lab has shown in small airway epithelial cells that AT-RvD1 can dampen TAK-1, highlighting another potential target in NTHi infections (72). Many different signaling molecules exist which are regulated by both NTHi and SPMs, and these candidate markers represent an interesting area for future mechanistic studies.

The pro-resolving actions of AT-RvD1 yielded markedly improved respiratory physiology in treated mice in this study. NTHi-infected mice demonstrated elevated respiratory rates and decreased tidal volumes; importantly, these alterations were accompanied by dramatic decreases in oxygen saturation of hemoglobin, highlighting an important clinical consequence of impaired lung physiology. Remarkably, AT-RvD1 treatment significantly improved lung physiology, resulting in improved oxygen saturation of hemoglobin in NTHi-infected mice. These results were presumably a result of reduced cellular influx, cytokine production, and edema in the lung, allowing for clearer airways and better oxygen exchange. The ability of AT-RvD1 to protect against NTHi-induced weight loss was also likely multifactorial; since treated mice had improved respiratory physiology and thermal homeostasis, and thus lower metabolic demand for basic functions such as breathing and thermoregulation, they were less susceptible to weight loss. It is also possible that mice in better overall health had greater motivation to move to obtain food and water in their cages, and had reduced need to huddle together for warmth; however, NTHi-induced decreases in water consumption or movement were not assessed in our model and would require additional testing. These are the first observed effects of NTHi on respiratory rates, oxygen saturation, and temperature in mice, and the first indication that SPMs can act to improve these health markers in a model of a live bacterial lung infection.

These data provide novel evidence in a pulmonary model that SPMs can act in a pro-resolving manner to dampen inflammation and improve lung physiology while still enhancing bacterial clearance. These unique properties open up a wide range of

therapeutic opportunities for AT-RvD1, particularly as an alternative therapeutic in chronic inflammatory conditions such as COPD, where infective exacerbations are common. They further support the use of SPMs as adjuvant therapies to antibiotics, lowering antibiotic need. AT-RvD1 has high therapeutic potential in these health settings, and these studies are a first step into investigating this novel and critical area of resolution research.

Chapter 5

Perspectives & Future Directions

SPMs Act on Multiple Stages of the Inflammatory Process

SPMs can act on multiple stages of the inflammatory process to promote resolution. Importantly, macrophages are major players at many of these stages and are critical in promoting resolution. After initiation of inflammation, SPMs act to decrease pro-inflammatory cytokine production (by fibroblasts, epithelial cells, and macrophages) and neutrophil recruitment. SPMs can then aid in promoting neutrophil apoptosis, while simultaneously increasing macrophage recruitment to the site of injury. These monocytes and macrophages, as well as tissue resident macrophages, produce anti-inflammatory cytokines and inhibit additional neutrophil influx and inflammatory platelet/neutrophil interactions. SPMs promote an M1 to M2 macrophage transition and enhanced phagocytosis and efferocytosis. Taken together, SPMs act on innate immune signaling to promote the resolution of inflammation (Fig. 5.1) (151).

Our findings clearly demonstrate a role for macrophages in mediating pulmonary inflammation and responding to SPM treatment. In addition to the general role of macrophages described above, we demonstrated specific macrophage actions in shifting from pro-inflammatory to anti-inflammatory cytokine production and in mediating neutrophil clearance. Macrophages are further responsible for phagocytosis, which is enhanced with SPM treatment. Our data reflect the ability of SPMs to act in multiple inflammatory settings, with both a toxic inflammatory stimuli (cigarette smoke) and microbial insults (LPS and NTHi infections). We further highlighted that SPMs act on established inflammatory signaling pathways, namely the TLR4 and NF-κB families, to mediate their pro-resolving effects.

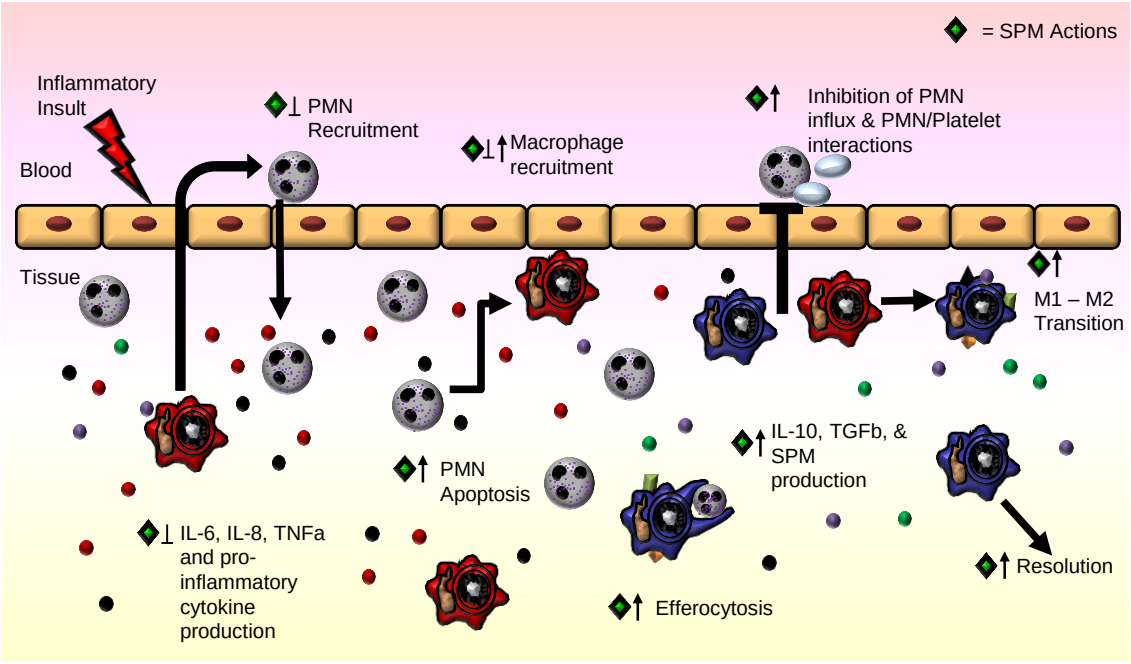

Fig. 5.1. SPMs act on macrophages to attenuate inflammation and promote resolution. SPMs (denoted by green diamond) play roles in all stages of inflammation. Early on, SPMs decrease neutrophil recruitment and pro-inflammatory cytokine production. SPMs then act to promote neutrophil apoptosis and efferocytosis, and a shift to anti-inflammatory cytokine production. Finally, macrophages move to an M2 phenotype and tissue repair is initiated to return to homeostasis.

SPMs Act in a Distinct Pro-Resolving Manner

A critical characteristic of SPMs is that they are pro-resolving rather than simply anti-inflammatory. We demonstrated in Chapters 2 and 4 that SPMs increase macrophage cell influx and M2 activation in response to both cigarette smoke and infection, rather than decreasing cellular influx overall and/or entirely dampening activation. Chapter 3 demonstrated a similar phenomenon, wherein RvD2 reduced prolonged TLR4 activation but did not suppress initial signaling or basal expression levels. These same pro-resolving effects are seen in other cell types with multiple activation states. In observing the adaptive immune response, 17-HDHA increased B-cell antibody production, leading to an enhanced response to viral infection and indicating SPMs may act as vaccine adjuvants (96, 212). SPMs can alter T-cell signaling as well, with increased production of Tregs and a shift from Th1 to Th2 responses with MaR1 and LxA_4, respectively (213, 214). Unpublished data from our lab further shows that platelets treated with MaR1 have decreased cellular activation, while still maintaining their hemostatic functions. In contrast, though, an RvD1 analogue inhibited DC maturation and impaired DC-mediated immune responses (215). In short, SPMs pro-resolving functions are distinct from anti-inflammatory ones, with cell-type dependent effects.

Specific to macrophages, a critical component of the pro-resolving phenotype is enhanced phagocytosis. We observed in both our human macrophages and our NTHi mouse model (Chapters 2 and 4) that resolvins enhanced phagocytic uptake of bacteria. In Chapter 2, this increase rescued cigarette smoke-induced defects in phagocytosis. Similar results were previously seen with macrophages from cigarette smoke-exposed

mice (37). Several other studies have also evaluated SPMs' abilities to enhance phagocytosis, and this collection of data demonstrates SPM efficacy in taking up latex beads and bacteria regardless of opsonisation (14, 41, 65, 87, 167). While the detailed mechanisms by which SPMs can enhance phagocytosis are unknown, the variety of insults for which SPMs are efficacious points to common mechanisms rather than specific phagocytic pathways (i.e., complement mediated uptake). Given that enhanced phagocytosis is used as a benchmark for identifying new SPMs in many recent publications, this is clearly a critical characteristic for resolution molecules.

In observing these pro-resolving properties, resolution intervals provide a useful benchmark. A resolution interval is the amount of time it takes for an inflammatory system to go from peak neutrophil counts to a 50% reduction as resolution is occurring. Resolution indices provide a quantifiable measurement of early resolution, allow for comparison between different models, and help assess whether SPMs can speed natural resolution. For example, two different published studies injected mice intraperitoneally with SPMs and zymosan, with one study giving SPMs 5 min before zymosan and the second study giving SPMs concurrent or 12 hours after zymosan. The use of resolution indices allows us to compare pre-, concurrent, and post-treatment; a lipoxin analog was more effective given 12 hours after zymosan (Ri=10 hours compared to 12 hours with pre-treatment), whereas RvE1 was most effective given concurrently (Ri= 12 hours pre, 8 hours concurrent, and 11 hours post-treatment) (216, 217). This sort of comparative analysis is important for identifying common trends between studies and designing potential treatment regimens.

While we observed potent SPM effects on macrophages alone, it is important to remember that these cells do not exist in isolation and many other cells contribute to the lung environment. Previous work in our lab focused on the roles of structural cells (fibroblasts and epithelial cells) in attenuating cigarette smoke or TLR ligand induced inflammation and similarly observed that these mediators could dampen inflammatory effects in these cell types (37, 72). The pulmonary system is comprised of many branches of bronchi, bronchioles, and alveolar structures, decreasing in size as the respiratory tract descends into the lungs. These lung areas also have varying cellular compositions. Because of these differences in diameter and cell makeup, different areas of the lung are differentially susceptible to inflammatory stimuli, and may therefore be more or less responsive to SPM treatments. While SPMs act through some common inflammatory signaling pathways and have demonstrated potency in many inflammatory models, each toxic stimuli activates unique genes and every SPM is not effective in every situation. Furthermore, SPMs are synthesized by transcellular synthesis, requiring multiple cell types to produce a final SPM product (6, 218). It is possible that SPM success *in vivo* is dependent on activating synthesis of additional eicosanoids, and if the cell types present in a particular region of the lung do not possess the proper machinery there may be no or limited therapeutic benefits.

SPMs Act through Multiple Inflammatory Signaling Pathways

Critical to exploring the endogenous role of SPMs and for moving these molecules forward as therapeutics is understanding the signaling mechanisms through

which SPMs act. In Chapter 2, we identified that D-series resolvins act through dampening canonical NF-κB signaling, reducing p65 and IκBα phosphorylation. Other SPMs have additionally been shown to dampen NF-κB signaling, highlighting the critical importance of this pathway in mediating resolution and inflammation (38, 65, 71, 170, 202). We presented for the first time that RvD2 increased expression of RelB in the alternative NF-κB pathway. This is a novel finding, as a link between SPMs and RelB has not previously been identified. RelB is a known anti-inflammatory play, and acts to attenuate cigarette smoke-induced inflammatory effects in human fibroblasts (97, 134, 181). Furthermore, RelB acts through regulation of certain miRNAs, including miR146a, to suppress Cox-2 and inflammatory mediator production (134, 181).

We demonstrated in Chapter 3 that RvD2 also increased miR146a expression in LPS-stimulated monocytes. SPM regulation of miRNAs is a new and growing area of investigation, and this is the first evidence for regulation of miR146a specifically. In addition to NF-κB signaling, miR146a is linked to several other inflammatory pathways, including TLRs (147, 219). In contrast to the body of literature showing SPM regulation of canonical NF-κB signaling, very little data exist evaluating upstream inflammatory pathways like TLRs. We presented data in Chapter 3 that SPMs can modulate TLR4 expression, resulting in decreased inflammation. SPMs acted not only on TLR4 itself, but on its important co-factor, MD-2. It is interesting that MD-2 (which is necessary for TLR4/LPS responses), but not other co-factors or trafficking proteins (which enhance signaling efficiency but are not necessary), is altered with SPM treatment, underscoring that these mediators selectively act on inflammatory signaling pathways. SPMs are

efficacious in multiple models of TLR-ligand induced inflammation, including Chapter 4 of this thesis which used LPS and NTHi infections as inflammatory stimuli; NTHi triggers TLR2 and TLR4 activation. Given this broad efficacy across multiple insults, future investigations into other TLR pathways may generate additional targets in both TLR and miRNA signaling for SPM actions.

SPMs in Chronic Disease

One limitation of our studies that remains to be addressed is the efficacy of SPMs in chronic disease settings. The vast majority of work in this field has employed models of acute inflammation, but many chronic lung diseases have underlying inflammatory causes and dysregulated SPM signaling, as outlined in Chapter 1. Understanding the role of SPMs in chronic inflammation is especially important given the lack of therapeutics for chronic lung diseases. Options range from no therapeutics that halt disease progression (as is the case in idiopathic pulmonary fibrosis) to largely immunosuppressive therapies like corticosteroids that leave patients immunocompromised and susceptible to infections and infectious exacerbations (as is the case in COPD). In Chapter 4, we highlight that SPMs are effective at attenuating inflammation and simultaneously enhancing bacterial clearance of NTHi. This was a first step using an acute model, but future work would evaluate SPM efficacy against repeated bacterial infections and a dual-insult model of chronic cigarette smoke exposure and NTHi infection to mimic the pathology of COPD exacerbations in humans. Our lab has demonstrated some SPM efficacy against COPD. First, Chapter 2 of this thesis

demonstrated that alveolar macrophages from human subjects with COPD or non-COPD lung pathologies are responsive to *ex vivo* treatment with SPMs. Second, previously we have employed a mouse model of emphysema, and shown that SPMs can prevent airspace enlargement, oxidative stress, and cell death (66). These data provide some early evidence that SPMs may be effective against chronic pulmonary injury, with more work needed to fully evaluate this potential.

Dose-Responsiveness & SPM Receptors in Mediating Resolution

Given that SPMs may need to be used frequently to be useful in chronic disease situations, it is important to consider dose-response effects and receptor sensitivity issues. It is unknown whether humans can become tolerant to SPMs, or reach receptor saturation. We have shown throughout this thesis that SPMs generally have no effect in the absence of inflammatory stimuli, indicating that there are important microenvironment contexts for their effects. Furthermore, we demonstrated repeatedly in this dissertation that higher doses of SPMs are not always the most efficacious. The effective dose of SPMs can depend on the SPM used and the cell type or organism, which are important considerations as these molecules more forward as therapeutics.

Furthermore, the identified SPM receptors are not exclusive to their SPM targets, and SPMs can bind to multiple receptors. We demonstrated in Chapter 2 that blockage of RvD1 binding to GPR32 and ALX/FPR2 prevented RvD1 dampening of cigarette smoke-induced cytokines, and that the effect of receptor inhibition was different depending on the observed cytokine. We also observed in Chapter 3 that LPS increased expression of

certain SPM receptors. While multiple SPMs were efficacious in dampening LPS-induced cytokines, there was not necessarily a correlation between altered receptor expression and SPM potency. We have hypothesized that some of the deleterious or less potent effects of higher doses of SPMs may be due to off-target binding effects or could be due to saturation of receptors, activating some of their other pathways. This is particularly concerning regarding ALX/FPR2. While this receptor most strongly binds LxA_4 in all identified cells (except neutrophils), it is host to a wide variety of mediators, including pro-inflammatory leukotrienes, which are also derived from arachidonic acid (13). We have additionally seen that SPM receptors are dysregulated with chronic disease (66, 153). This implies that for patients who already have underlying inflammatory conditions, the same doses may not be efficacious because of disrupted receptor signaling.

Concerns in Therapeutic Development of SPMs

Further compounding the chronic vs. acute efficacy question is the stability of SPMs. These mediators have a short half-life and are highly sensitive to oxidation. While this clearly represents a therapeutic challenge, SPMs are quite amenable to modification. Acetylated Cox-2 reactions can produce aspirin triggered version of D-series resolvins and lipoxins, which are more resistant to biological inactivation by oxidoreductases (7). Our work in Chapter 4 was conducted using aspirin-triggered RvD1, a more stable epimer of the RvD1 commonly produced by DHA metabolism. This particular epimer has been used in multiple *in vivo* studies and has displayed long term efficacy (7, 137,

203, 220). There also exist various resolvin and lipoxin analogues which have been used in the literature and demonstrated anti-inflammatory effects (45, 47, 204, 215, 217).

Our studies here have demonstrated efficacy of SPMs when used singly to attenuate inflammation. A major unexplored area of SPM research is the use of combinations of SPMs as therapeutics. It is important to consider what is known about SPM receptor binding in determining combination therapies. For example, since RvD1 and RvE1 have different primary receptors, with no RvD1 binding to ChemR23 and no RvE1 binding to GPR32 detected, these two RvDs may work well together as a combination therapy. However, using LxA_4 or RvD2 in conjunction with RvD1 could lead to competition as these SPMs all have some affinity for ALX/FPR2, and therefore could have reduced efficacy when used together. There is limited evidence that RvD1 and RvD2 do not act in an additive or synergistic manner, which could be due to their structural similarity (153).

There also exists significant literature regarding the use of omega-3 supplements, DHA/EPA treatments, and intermediate molecules. In Chapter 2 we observed that use of the 17-HDHA intermediate moderately reduced cigarette smoke-induced expression of IL-6 and IL-8. While we did not evaluate EPA or DHA precursors in our macrophage studies presented here, it is possible that we could see altered results had we treated our cells with DHA instead of a purified resolvin compound. DHA could allow for production of multiple SPMs to aid in resolution, but may also result in reduced efficacy if the proper cellular machinery or components are not present to allow for metabolism. It is also not clear if any of the SPM intermediates are themselves capable of binding to

SPM receptors. Overall, there are multiple important considerations regarding SPM stability and receptor binding, which should be further studied as these mediators progress in their therapeutic development.

Future Directions

Several particular issues are important as these molecules move into clinical use. Two modified resolvin molecules are already in clinical trials (151, 221). RX-10045, an analog of RvE1, is in late stage clinical trials for topical use with dry eye. RX-10001 is also being investigated in early stage clinical trials for oral administration and use in asthma and irritable bowel disease. These two molecules demonstrate that the therapeutic potential for SPMs is genuine, as the movement of RX-10045 past toxicity testing highlights that the endogenous nature of these mediators may aid in their tolerability. For further SPM candidates to move forward as therapeutics, it will be important to consider the structure of the SPM analog, the dose used (as discussed above), and route of administration. In Chapter 4, we used oropharyngeal aspiration to preferentially target the lung, but SPMs have not yet been widely studied in inhalation models. A comparative pilot study from our lab showed similar efficacy in attenuating acute cigarette smoke induced inflammation with inhalation and injection of AT-RvD1, highlighting that targeted organ dosing can still have systemic effects and systemic dosing can reach target organ areas. Many epidemiological studies also use oral omega-3 supplements (and in a few cases, oral administration of purified SPMs) and have seen protective effects, so oral dosing may be a potential avenue for therapeutic SPM use (2, 4, 217).

We have herein presented novel data regarding the efficacy of SPMs, primarily D-series resolvins, in attenuating pulmonary inflammation by acting on monocytes/macrophages. These data underscore the powerful role that monocytes/macrophages play in mediating not only initial inflammatory responses, but resolution as well. Importantly, our work provides support for the use of SPMs as therapeutics in inflammatory lung diseases with a high risk of infection, a critical area with few therapeutic options. It will be crucial to continue evaluating these SPMs in infective pulmonary models, as well as in chronic or recurrent lung diseases. Continuing to understand the role of SPMs in attenuating inflammation will broaden their potential for therapeutic use, and may uncover new roles in underserved pulmonary diseases for which there is little therapeutic hope.

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