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

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Running title: Resolvins rescue human macrophages from smoke

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Abstract

Inflammation is a protective response to injury, but can become chronic, leading to tissue damage and disease. 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, contributing 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 exhaled breath condensate, 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. 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 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 (1). 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 Non-typeable *Haemophilus influenzae* and *Streptococcus pneumoniae* (42, 72). 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 (60, 72). 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 (60, 72). Despite this chronic inflammatory activation, patients with COPD are also more susceptible to bacterial and viral infections (42, 65), due at least in part to an impairment in macrophage phagocytic abilities; these defects in phagocytosis also lead to impaired clearance of apoptotic cells (20, 33, 38, 43, 47). 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.

In the past, the resolution of inflammation was thought to be passive. Resolution, however, is now known to be an active and dynamic process (7). Recent investigations have led to the discovery of specialized pro-resolving mediators (SPMs). These bioactive lipid mediators, endogenously produced, play a critical role in the active resolution of inflammation by counter regulating pro-inflammatory actions and promoting resolution...
pathways, and are not immunosuppressive (7, 12, 63). SPMs are formed by enzymatic oxygenation of polyunsaturated fatty acids. They are divided into families based on their metabolic pathway and structures, including lipoxins (LX), resolvins (Rv), protectins (PD), and maresins (MaR) (7). These small molecules are amenable to modification and act via unique receptors, including ALX and GPR32, and new modes of action that give them potential as novel therapeutics (2, 4, 11, 13, 22, 34, 36). Several studies have shown that SPMs are dysregulated in human diseases, and several chronic inflammatory diseases are hypothesized to be a result of a failure to resolve. 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, as well as the effect of SPMs on human macrophage function in general.

SPMs mediate some of their key actions through modulation of inflammatory signaling pathways, including the mitogen activated protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) families (2, 3, 50, 55, 57, 73). NF-κB proteins are involved in a number of cellular responses and are particularly important in promoting and regulating inflammation (18). Both canonical and non-canonical NF-κB pathways exist and several members of the alternative NF-κB signaling pathway, specifically RelB, possess anti-inflammatory abilities (5, 18, 68, 70). The actions of SPMs on these signaling pathways in cigarette smoke-exposed cells and human cells in general are of considerable interest, and study of these mechanisms would provide important new insight into SPMs actions. 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**

PGE₂, PGD₂, TxB₂, 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), 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 Ab (GX71225) was purchased from GeneTex (Irvine, CA). Antibodies to CD11b (560914) and CD14 (555398) and ELISA components for IL-6 (554543 and 554546) and TNF-α (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 ELISA kit (DY240) 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**

Exhaled breath condensate (EBC), 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. For EBCs, human volunteer breath was collected in a cold trap for 15 minutes, aliquoted in 250ul aliquots, and frozen at -80°C. 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. (15) or by EIA according to manufacturer’s protocol (20% cross reactivity with Lipoxin A4, 4.2% cross reactivity with 17(R) - Resolvin D1 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 (56). 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 supplemented RPMI 1640 media (supplemented with 1% FBS [to minimize SPM binding by serum components], anti-mycotic/anti-myotic) and 10ng/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.
Cigarette smoke extract preparation

Cigarette smoke extract (CSE) was prepared in RPMI 1640 as previously described by our lab (30). CSE was diluted to 30U (units/mL) unless otherwise described. Serum-free RPMI 1640 was used as a vehicle control.

ELISA and EIA

Macrophages were incubated with 1-100 nM of SPMs or vehicle (0.1% EtOH in PBS) for 24 hrs, followed by exposure to CSE. Supernatants were removed 24 hrs after CSE exposure and cytokines and lipid concentrations determined by ELISA (IL-6, IL-8, IL-10, TNF-α, TGF-β) or EIA (PGE₂, PGD₂, TxB₂) 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 hrs, 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 (50mM Tris-HCl, 2% SDS). Total protein was quantified by BCA (Thermo-Sci, Waltham, MA, 23225). Western blots were performed as described previously (40). 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 1hr, followed by incubation with 1μM H$_2$DCFDA (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 hrs. 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 0.5mM 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 were assessed by flow cytometry.

Inhibition of SPM Receptors

Macrophages were treated with Boc-2 (1uM) or GPR32 (10ug/mL) for 30min prior to SPM treatment and CSE exposure as described above. Specificity for Boc-2 and GPR32 has been established previously (31, 45, 67). 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 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 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 exhaled breath condensate (EBC) and bronchoalveolar lavage fluid (BALF). Using LM-metabololipidomics (15), we identified by matching criteria and MS/MS spectra, bioactive LM, RvD1 and pathway markers 17-hydroxyl docosahexaenoic acid (17-HDHA) and 14-HDHA, in both healthy and COPD EBCs, (Fig. 1A, Table 1). 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. 1B-D). Additionally, COPD patients had decreased concentrations of RvD1 in both their BALF and serum (Fig. 1E-F). LxA4, 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 (52, 53). This is the first evidence that COPD patients have dysregulated levels of the omega-3 derived SPMs.
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. Smokers have increased susceptibility to bacterial infections, in part because of impaired macrophage phagocytic abilities (20, 38, 43, 47). 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. In initial studies we evaluated both 1-100nM concentrations of RvD1 and RvD2 in several donors. Both RvD1 and RvD2 dampened spontaneous production of IL-6 and TNFα, with the most consistent effect seen at 100nM (Fig. 2A-B). We next investigated the efficacy of 100nM 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 and enhanced phagocytic uptake of E. coli by unstimulated human alveolar macrophages (Fig. 2C-F).

We also examined COPD and non-COPD populations separately (Table 2). RvD1 and RvD2 were efficacious on both COPD and non-COPD alveolar macrophages, with stronger potency on non-COPD patients, demonstrating further their potential efficacy in humans as potential therapeutics (Table 2).

RvDs dampen cigarette smoke-induced production of pro-inflammatory cytokines and promote anti-inflammatory cytokines and M2 macrophage phenotype

In order to characterize the effects of SPMs on human macrophages, we conducted further experiments using macrophages derived from peripheral blood monocytes. Blood derived monocytes from multiple healthy human donors were isolated to >95% purity (Fig. 3A),
differentiated to macrophages, and incubated with 100 nM SPMs for 24 hrs. Based on our human lipid profiles, efficacy with alveolar macrophages, and screening of multiple SPMs and 17-HDHA (Fig. 3B-C), RvD1 and RvD2 were chosen for these studies; we used 1nM and 100nM of SPMs based on preliminary dose curve studies in blood-derived macrophages (data not shown) and our results with BAL macrophages (Fig. 2A-B). Following SPM treatment, cells were exposed to CSE for 24 hrs. Neither SPMs nor CSE at the indicated doses affected cell viability (Fig. 3D). CSE increased production of the pro-inflammatory mediators IL-6, IL-8, and TNF-α, cytokines important in propagating an immune response, attracting neutrophils, enhancing phagocytosis and promoting cell death. RvD1s completely attenuated IL-6 increases (Fig. 4A) and dose-dependently dampened IL-8. 1nM RvD1 and both doses RvD2 additionally dampened TNF-α expression (Fig. 4B-C). In addition to preventing pro-inflammatory cytokine release, resolvins promote a resolution phenotype in macrophages and the production of anti-inflammatory cytokines. CSE decreased production of the anti-inflammatory cytokine IL-10 (Fig. 4D), but this effect was not attenuated by SPMs. Conversely, RvD1 increased concentrations of total TGF-β, and RvD2 increased concentrations of both active and total TGF-β had an increased ratio of active/total TGF-β, an anti-inflammatory cytokine important in tissue repair (Fig. 4E-G). We hypothesized that the reduction in cytokines and increase in active TGFβ ratio might indicate a skewing of the macrophage phenotype away from a classically activate, pro-inflammatory “M1” phenotype and toward an alternatively activated, anti-inflammatory and pro-resolving “M2” phenotype (39). 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 a decreased CD206/CD80 ratio (Fig. 4H). 100nM RvD1 prevented this M1 skewing, while RvD2 dose-
dependently prevented M1 skewing and increased the alternative M2 phenotype over the vehicle
group (Fig. 4H).

The generation of certain pro- and anti-inflammatory cytokines and bioactive lipid
mediators are regulated by the cyclooxygenase-2 (Cox-2) enzyme (46). Cigarette smoke can
increase expression of Cox-2 and elevated Cox-2 expression is associated with several smoking-
related diseases, including COPD and cancer (5, 37, 48, 51). CSE increased expression of Cox-2
in human macrophages and treatment with RvDs significantly attenuated Cox-2 expression (Fig.
5A-B). Macrophages produce lipid mediators, including Cox-2 regulated prostaglandins such as
prostaglandin E2 (PGE2) and prostaglandin D2 (PGD2) (29). Both PGD2 and PGE2 are important
prostaglandins with both pro- and anti-inflammatory roles (25, 27, 29, 32). At 24 hrs, CSE
significantly increased PGE2 production; this increase was dose-dependently diminished by
RvDs (Fig. 5C). CSE similarly increased concentration of PGD2, but in contrast to PGE2, RvD2
further potentiated this increase (Fig. 5D). We also evaluated the concentration of thromboxane
(Tx) B2, the inactive metabolite of TxA2, an important pro-thrombotic signaling molecule
involved in tissue injury and inflammation (44). TxB2 was unaffected by either CSE or RvD
exposure (Fig. 5E), indicating that RvDs may regulate specific lipid mediator pathways.

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 (19, 54). 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$_2$DCFDA in exposed macrophages. (Fig. 6A-B). RvD treatment, however, did not dampen this initial oxidative burst 20 min (data not shown) or 1 hour after CSE exposure (Fig. 6A-B). We additionally investigated RvD1 and RvD2 actions on carbonylated proteins; reactive oxygen species can induce the modification of native amino acids to carbonyl derivatives. RvDs did not significantly affect levels of carbonyl groups at 1 hour after smoke exposure (Fig. 6C-D). We next evaluated longer time points of CSE exposure to determine if RvDs were acting to shorten resolution time or reduce propagation of oxidative stress rather than preventing initiation. RvDs did not affect ROS at 24 hrs (data not shown), but 1nM RvD1 and 100nM RvD2 did act to decrease levels of carbonylated proteins (Fig. 6E-F), the first evidence for RvDs acting on protein carbonylation.

RvDs rescue cigarette smoke-induced defects in phagocytosis

Based on 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. 7A-B). RvDs incubation prevented these decreases and restored macrophage phagocytic abilities (Fig. 7A-B). In addition, CSE decreased the number of macrophages that phagocytized bacteria, a decrease which was rescued by RvDs (Fig. 7C).

Resolvin D1 signals through GPCRs to mediate pro-resolving effects

Resolvin D1 is known to signal through two receptors- ALX and GPR32; the receptors for RvD2 are currently unknown (4, 34, 45). We investigated the role of these receptors through the use of
an ALX inhibitor, Boc-2, and an anti-GPR32 neutralizing antibody. 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. 8A-B).

**RvD2 acts via modulation of NF-κB signaling pathways**

Resolvins can mediate their effects through canonical inflammatory pathways, including NF-κB signaling (9, 35, 55). To investigate SPM actions in human macrophages, we focused on the mechanistic actions of RvD2. Total expression of p65 was unaffected by CSE or RvD2. However, expression of phosphorylated p65 was increased 30 min after CSE exposure (Fig. 9A-B). This increase in phosphorylated p65 was dampened with RvD2. Phosphorylated IκBα expression were also increased with CSE and attenuated by RvD2 (Fig. 9C-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 (5, 40, 70). 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. 9E-F). This increase in p52 was reduced by RvD2, though p100 expression remained unchanged. Interestingly, CSE caused a dramatic loss of RelB expression, which was significantly prevented by RvD2 (Fig. 9G-H). 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.
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 chronic disease states in human blood derived and human alveolar macrophages. RvDs dampened 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 and pro-resolving actions, RvD1 and RvD2 promoted the production of anti-inflammatory cytokines and enhanced pro-resolving macrophage phagocytosis in both macrophage types. 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 SPM 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 (17, 21, 24, 69). Omega-3 and omega-6 derivatives were also detected in the urine of smoker and nonsmoker volunteers, with smokers having decreased concentrations of SPMs (61). The LM-metabololipidomics methodology used here can unambiguously identify over 50 pro- and anti-inflammatory bioactive lipid mediators and their pathway markers. Our analysis further demonstrates dysregulated levels of HDHA and HETE intermediates, as well as dampened concentrations of RvD1, indicating that COPD may certainly be seen as a “failure to resolve” inflammation, and that bioactive lipid mediators play a key role in this process. We have previously shown that expression of a key SPM receptor 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 (31). 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 (41, 64, 77). 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 and in future investigations. 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. They have been shown to not only reduce the production of pro-inflammatory proteins, but to induce a phenotypic shift in cells to promote anti-inflammatory effects and alternative M2 activation (7, 16, 49, 57, 71). Particularly in the context of an inflammatory microenvironment, the collective changes in these pro- and anti-inflammatory cytokines may have a combined potent effect and act in concert to promote resolution. 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 protein 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 exposure (23, 30, 62, 66). 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 was 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 multiple different underlying inflammatory conditions
than would be seen in a cell line. This further strengthens the role of SPM as potential
therapeutics in human smoking related diseases.

Alternatively activated macrophages that promote resolution and phagocytosis rather than
pro-inflammatory signaling have recently become a key area of interest and investigation (8).
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 IL-10
and have an increased ratio of M2/M1 macrophages. In addition, RvD treatment alone increased
expression of cyclooxygenase-2. While this enzyme is commonly categorized as pro-
inflammatory, (37, 51) Cox-2 can also produces precursors of SPMs (14, 27, 59), and can be
temporally regulated by SPMs (76). Furthermore, several prostaglandins can have either pro-
inflammatory or anti-inflammatory actions depending on concentration and cell type. PGD₂, 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 (25, 28). PGD₂ also counterregulates expression of PGE₂ and is converted to 15-
deoxyPGJ₂, which has pro-resolution properties that may result from inhibition of NF-κB,(26,
32, 58, 59). Although PGE2 is often considered a pro-inflammatory mediator, it also has anti-
inflammatory properties, including the ability to reduce IL-6 levels and suppress allergic airway
inflammation (6, 32, 37, 79). PGE2 also upregulates IL-10 (REF). 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 PGE₂ 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 most 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 (14). 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 24hrs, indicating that it takes some time for both carbonylated proteins to appear and for the RvDs to have an effect. 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, or to enhance clearance and degradation of oxidatively damaged proteins. More comprehensive studies are needed to fully elucidate the effects of RvDs as anti-oxidant molecules and the mechanisms through which they act.

There are several mechanistic pathways through which SPMs promote the resolution of inflammation. Human macrophages have been shown to have multiple SPM receptors, including ALX and GPR32 (34). We showed that Boc-2 and anti-GPR32 antibodies can attenuate the effects of RvD1, with the inhibitors/antibodies affecting different cytokine release. This implicates complimentary roles for the different RvD1 receptors. The receptor for RvD2 is currently unknown, and further investigations will be needed to uncover its binding partners.
Additionally, multiple inflammatory stimuli, including cigarette smoke, can induce activation of the NF-κB pathway; SPMs have additionally been shown to act by affecting expression of this pathway (9, 35, 55). We have shown here that in human macrophages RvD2 acts, in part, through preventing the phosphorylation and thereby degradation of IκBα and activation of p-65. 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 (5, 40, 70, 78). 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 (40). 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 signaling pathways. SPMs, including RvD1 and LxA4 have been shown to decrease cytokines through decreasing STAT3 signaling (10, 74, 75). Multiple SPMs have also been shown to decrease members of MAPK pathways (31, 50, 73). Clearly, the signaling mechanisms of resolution and multifaceted and bear extensive investigation by the field at large.

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 RvDs pro-resolving effects on human alveolar macrophages particularly highlights the potential of these SPMs in dampening inflammation associated with chronic diseases, including COPD, as well as the novel identification of SPMs action on alternative NF-κB signaling. The dual anti-
inflammatory and pro-resolving actions, along with the non-immunosuppressive nature, 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.
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the time frame of the conducted research. Dr. Serhan is an inventor on patents [on resolvins and
their analogs] assigned to BWH and licensed to Resolvyx Pharmaceuticals for clinical
development. He was the scientific founder of Resolvyx Pharmaceuticals and owns equity (stock
currently of unknown value) in the company. His interests were reviewed and are managed by
the Brigham and Women’s Hospital and Partners HealthCare in accordance with their conflict of
interest policies.
References


17. de Batlle J, Sauleda J, Balcells E, Gomez FP, Mendez M, Rodriguez E, Barreiro E, Ferrer JJ, Romieu I, Gea J, Anto JM, and Garcia-Aymerich J. Association between Omega3 and Omega6


Figure Legends

Fig. 1. Lipid mediators are dysregulated in patients with COPD. Lipid mediators were identified in EBCs as shown with representative multiple reaction monitoring along with MS-MS spectra used for identification of RvD1 (A) and BALF (B-D) of COPD and non-COPD or healthy 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 (E) and serum (F) were also evaluated by EIA. Statistical significance was determined by T-test (*p<0.05 compared to non-COPD) n=3-6. Dotted arrows represent retention time of compounds below the limit of detection (LOD), LOD ~0.1 pg.

Table 1. Lipid mediators and pathway markers levels in human exhaled breath condensate from healthy volunteers and COPD patients. Human exhaled breath condensate were collected and immediately frozen. Lipid mediators (LM) were assessed using LM-metabololipidomics (see methods for details). Q1, M-H (parent ion) and Q3, diagnostic ion in the MS-MS (daughter ion). Results are expressed as pg/mL. Mean ± SEM of n = 7 for healthy volunteers, n = 10 for COPD patients. The detection limit was ~ 0.1 pg. *Below limits of detection, †p < 0.05 and ††p < 0.01 vs healthy volunteers.

Fig. 2. SPMs dampen cytokine release and enhance phagocytosis in unstimulated alveolar macrophages from COPD and non-COPD patients. (A-B) Alveolar macrophages from human subjects were incubated with 1-100nM RvD1 or RvD2 for 24hrs. Supernatants were assessed for
IL-6 (A) and TNF-α (B) by ELISA. (C-F) Alveolar macrophages from COPD (closed symbols) and non-COPD (open symbols) human subjects were incubated with 100nM RvD1 or RvD2 for 24 hrs. Supernatants were assessed for concentrations of IL-6 (C), IL-8 (D), and TNF-α (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.

**Table 2. 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 100nM RvD1 or RvD2 for 24 hrs. Supernatants were assessed for concentrations of IL-6, IL-8, and TNF-α by ELISA. Additionally, cells were incubated with fluorescently-labeled *E. coli* and phagocytosis was determined by flow cytometry. Data shown are the same as Fig. 2C-F, but the effects of RvDs were analyzed separately for COPD and non-COPD populations. Significance was determined by paired t-Test (*p<0.05 compared to vehicle alone, †sample size insufficient for statistical analysis), n=2-5.

**Fig. 3. D-series Resolvins are efficacious in reducing cigarette smoke extract-induced inflammatory mediator production.** 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 hrs. Cells were subsequently exposed to CSE for 24 hrs. Supernatant levels of pro-inflammatory cytokines IL-6 (B) and IL-8 (C) were evaluated by ELISA. Viability was determined by trypan blue.
Fig. 4. 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 hrs prior to CSE exposure for 24 hrs. RvDs reduced concentrations of pro-inflammatory [IL-6 (A), IL-8 (B), TNF-α (C)] and increased concentrations of anti-inflammatory [IL-10 (D), TGF-β (E, F)] cytokines and chemokines, as determined by ELISA. Statistical significance was determined by two-way ANOVA with Bonferroni posttest (#p<0.05, ##p<0.01, ###p<0.001 compared to veh/veh, n.s, *p<0.05, **p<0.01, ***p<0.001 compared to CSE alone), n=6 individual donors.

Fig. 5. RvDs prevent cigarette smoke extract-induced increases in cyclooxygenase-2 and pro-inflammatory lipid mediators. Macrophages were incubated with RvD1 or RvD2 for 24 hrs prior to CSE exposure for 24 hrs. Cells were lysed and Cox-2 protein expression levels assessed by Western blot. Image shown (A) is representative of three donors (B). 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.

Fig. 6. Resolvins prevent propagation of oxidative stress but not induction. Macrophages were incubated with RvD1 or RvD2 for 24 hrs, following which cells were incubated with
H$_2$DCFDA and exposed to CSE. One hour after CSE exposure ROS levels were determined by flow cytometry (A, B). The presence of carbonylated groups in RvD and CSE exposed macrophages was also assessed by Oxyblot at one hour (C-D) and 24 hrs (E-F, images are representative of 3 donors). (##p<0.01 compared to veh/veh, *p<0.05, **p<0.01 compared to CSE alone), n=3-4 individual donors.

**Fig. 7. Resolvins attenuate cigarette smoke extract-induced decreases in phagocytosis.**

Macrophages were incubated with RvD1 or RvD2 for 24 hrs prior to CSE exposure for 24 hrs. 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).

**Fig. 8. Resolvin D1 signals through GPCRs to mediate pro-resolving effects.** 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 and GPR32 inhibition were assessed by evaluating concentrations of IL-6 (A), TNF-α (B), and PGE2 by ELISA and EIA. Statistical significance was determined by two-way ANOVA with Bonferroni posttest (#p<0.05, ##p<0.01, ###p<0.001 compared to veh/veh, n.s, *p<0.05, **p<0.01, ***p<0.001 compared to CSE or CSE w/inhibitor for each respective group), n=3 individual donors.
Fig. 9. 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 total and phosphorylated p65 (A, B) total and phosphorylated IκBα (C, D), p100/p52
(E, F), and RelB (G, H) were determined by Western blot and quantified by densitometry (n=4
individual donors, representative images shown). 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).
<table>
<thead>
<tr>
<th>Lipid mediators levels (pg/mL)</th>
<th>DHA bioactive metabolome and pathway markers</th>
<th>Lipid mediators levels (pg/mL)</th>
<th>EPA bioactive metabolome and pathway markers</th>
<th>AA bioactive metabolome and pathway markers</th>
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<tbody>
<tr>
<td>Q1</td>
<td>Q3</td>
<td>Healthy volunteers</td>
<td>COPD patients</td>
<td>RvD1</td>
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<tr>
<td>RvD2</td>
<td>375</td>
<td>141</td>
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<td>RvD3</td>
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<td>173.4 ± 25.1</td>
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*below limit of detection (0.1pg), †p<0.05, ††p<0.01 vs healthy volunteers
Table 2. Comparison of alveolar macrophages from COPD and non-COPD patients.

<table>
<thead>
<tr>
<th></th>
<th>COPD</th>
<th>RvD1</th>
<th>RvD2</th>
<th>Non-COPD</th>
<th>RvD1</th>
<th>RvD2</th>
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<tbody>
<tr>
<td>IL-6</td>
<td>487 ± 279</td>
<td>442 ± 289</td>
<td>392 ± 228</td>
<td>543 ± 137</td>
<td>428 ± 137</td>
<td>212 ± 62*</td>
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<tr>
<td>IL-8</td>
<td>2792 ± 745</td>
<td>3017 ± 690</td>
<td>2712 ± 1007</td>
<td>1761 ± 307</td>
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<td>1528 ± 234</td>
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<tr>
<td>TNFa</td>
<td>966 ± 414</td>
<td>764 ± 60</td>
<td>592 ± 385</td>
<td>961 ± 291</td>
<td>385 ± 60*</td>
<td>202 ± 87.6*</td>
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<tr>
<td>% phagocytosis</td>
<td>12.40</td>
<td>15.40†</td>
<td>16.66†</td>
<td>4.20</td>
<td>8.87†</td>
<td>10.40†</td>
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</tbody>
</table>

Values shown are mean ± SEM. *p<0.5 compared to vehicle in same disease group, †sample size insufficient for statistical analysis
Figure 1.

A Exhaled Breath Condensate

Docosahexaenoic Acid derived Resolvins

Arachidonic Acid derived Lipoxins

Arachidonic Acid derived Leukotrienes and Prostanoids

C BALF

HETE Profile (pg/mL)

Non-COPD COPD

D BALF

HBP Profile (pg/mL)

Non-COPD COPD

p=0.087

E BALF

RvD1 (pg/mL)

Non-COPD COPD

F Serum

RvD1 (pg/mL)

Non-COPD COPD
Figure 2.

A

B

C

D

E

F

IL-6 (pg/mL)

RvD1

−

SPMs (100nM) RvD2

% macrophage phagocytosis

E. coli

SPMs (100nM) − RvD1 RvD2

IL-8 (pg/mL)

RvD1

−

SPMs (100nM) RvD2

TNF-α (pg/mL)

RvD1

−

SPMs (100nM) RvD2

IL-6 (pg/mL)

RvD1

−

SPMs (100nM) RvD2

TNF-α (pg/mL)

RvD1

−

SPMs (100nM) RvD2

E. coli

SPMs (100nM) − RvD1 RvD2

* ** **
Figure 4.
Figure 5.

Histograms showing the effects of Vehicle (Veh) and CSE on Cox-2 expression, PGD2, PGE2, and TxB2 levels. The y-axes represent the relative expression of Cox-2, and the x-axes represent the concentration of SPMs (nM) in Vehicle (Veh) and CSE conditions.

A) Western blot analysis of Cox-2 and β-tubulin expression.

B) Cox-2 expression (relative to β-tubulin) levels in Vehicle (Veh) and CSE conditions.

C) PGD2 levels in Vehicle (Veh) and CSE conditions.

D) PGE2 levels in Vehicle (Veh) and CSE conditions.

E) TxB2 levels in Vehicle (Veh) and CSE conditions.
Figure 6.
Figure 7.

A

![Graph showing the effects of different treatments on E. coli counts.](image)

B

![Bar graph showing E. coli counts (MFI) with different SPM concentrations.](image)

C

![Scatter plot showing the normalized percentage of E. coli+ cells.](image)
Figure 8

**A**

IL-6 (pg/mL) vs. SPMs (nM) treated with Veh, CSE, CSE + Boc-2, or CSE + abGPR32.

**B**

TNF-α (pg/mL) vs. SPMs (nM) treated with Veh, CSE, CSE + Boc-2, or CSE + abGPR32.
**Figure 9.**

(A) Total p65, P-p65, and Actin images for different RvD2 concentrations and treatments (Veh, CSE).

(B) Graph showing P-p65/Total p65 values for RvD2 concentrations and treatments (Veh, CSE).

(C) Total IκBα, P-IκBα, and Actin images for different RvD2 concentrations and treatments (Veh, CSE).

(D) Graph showing P-IκBα/Total IκBα values for RvD2 concentrations and treatments (Veh, CSE).

(E) p100, p52, and Actin images for different RvD2 concentrations and treatments (Veh, CSE).

(F) Graph showing p52/Actin (normalized) values for RvD2 concentrations and treatments (Veh, CSE).

(G) RelB and Actin images for different RvD2 concentrations and treatments (Veh, CSE).

(H) Graph showing RelB/Actin (normalized) values for RvD2 concentrations and treatments (Veh, CSE).