Aspirin-triggered Resolvin D1 reduces pneumococcal lung infection and inflammation in a viral and bacterial co-infection pneumonia model

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Abstract

Formyl peptide receptor 2 (Fpr2/ALX) coordinates the transition from inflammation to resolution during acute infection by binding to distinct ligands including serum amyloid A (SAA) and Resolvin D1 (RvD1). Here, we evaluated the pro-resolving actions of aspirin triggered-RvD1 (AT-RvD1) in an acute co-infection pneumonia model. Co-infection with *Streptococcus pneumoniae* and influenza A virus (IAV) markedly increased pneumococcal lung load and neutrophilic inflammation during the resolution phase. Fpr2/ALX transcript levels were increased in the lungs of co-infected mice, and immunohistochemistry identified prominent Fpr2/ALX immunoreactivity in bronchial epithelial cells and macrophages. Levels of circulating and lung SAA were also highly increased in co-infected mice. Therapeutic treatment with exogenous AT-RvD1 during the acute phase of infection (day 4-6 post pneumococcal inoculation) significantly reduced the pneumococcal load. AT-RvD1 also significantly reduced neutrophil elastase activity and restored total antimicrobial activity in bronchoalveolar lavage fluid of co-infected mice. Pneumonia severity, as measured by quantifying parenchymal inflammation or alveolitis was significantly reduced with AT-RvD1 treatment, which also reduced the number of infiltrating lung neutrophils and monocytes/macrophages as assessed by flow cytometry. The reduction in distal lung inflammation in AT-RvD1 treated mice was not associated with a significant reduction in inflammatory and chemokine mediators. In summary, we demonstrate that in the co-infection setting, SAA levels were persistently increased and exogenous AT-RvD1 facilitated more rapid clearance of pneumococci in the lungs, whilst concurrently reducing the severity of pneumonia by limiting excessive leukocyte chemotaxis from the infected bronchioles to distal areas of the lungs.
Abbreviations

Fpr2/ALX: Formyl peptide receptor 2 / LXA₄ receptor

SAA: serum amyloid A

AT-RvD1: Aspirin triggered-Resolvin D1

LXA₄: Lipoxin A₄

SPMs: specialised pro-resolving mediators

SP: *Streptococcus pneumoniae*

IAV: influenza A virus

SAL: saline

MPO: myeloperoxidase

BAL: bronchoalveolar lavage

BALF: bronchoalveolar lavage fluid

H&E: hematoxylin and eosin
Introduction

Colonisation of the upper respiratory tract with *Streptococcus pneumoniae* (*S. pneumoniae*, the pneumococcus) provides a reservoir for transmission of the pneumococcus within a population. Colonisation of the upper airways is normally confined to this compartment; however *S. pneumoniae* is a leading cause of bacterial pneumonia and invasive disease. There are multiple environmental and microbial factors that can facilitate dissemination of pneumococcus from the nasopharyngeal niche to the lower airways [1]. In particular, concurrent respiratory viral infections can transiently compromise host immunity to the pneumococcus [2], as well as increase the density of pneumococcal colonisation and severity of disease [3]. Influenza A virus (IAV) also triggers pneumococci to be released from biofilms in a more virulent form [4]. High mortality caused by pandemic strains of IAV has been directly associated with secondary bacterial pneumonia caused by common upper respiratory tract bacteria [5]. The rate of pneumococcal hospitalisations also coincides with the emergence of influenza pandemics [6].

People with underlying chronic lung disease such as chronic obstructive pulmonary disease (COPD) are particularly susceptible to respiratory infections including co-infection with bacterial and viral pathogens [7]. COPD is a major global cause of premature death [8], and respiratory infections commonly trigger acute exacerbations of COPD (AECOPD). Bacterial and viral co-infections during AECOPD are common and can result in more severe exacerbations associated with an increase in pulmonary inflammation [9]. We have previously shown that AECOPD associated with bacterial and viral co-infection results in significantly higher levels of systemic inflammation as determined by the acute phase reactant, serum amyloid A (SAA) [10]. Circulating levels of SAA markedly increase during acute infection where plasma concentrations can increase over 1000-fold. Both viral and bacterial respiratory infections increase circulating SAA levels that typically peak 3-5 days following
infection and the levels of SAA decline with clinical recovery [11]. In addition to increased circulating levels of SAA during acute infection, we have shown that IAV and the bacterial ligand lipopolysaccharide (LPS) potently stimulate de novo synthesis of SAA transcript in the lungs, where SAA synthesis peaked on day 5 and resolved to baseline levels by day 10 in IAV-infected mice [12].

SAA serves pleotropic roles during acute infection including opsonising Gram-negative bacteria to facilitate more efficient phagocytosis by neutrophils [13]. SAA is also a functional agonist for the Formyl peptide receptor 2 (Fpr2/ALX) where it stimulates leukocyte chemotaxis [14]. Furthermore, we have recently shown that local lung challenge with recombinant SAA potently stimulates neutrophilic inflammation via IL-17A dependent mechanisms [15, 16]. SAA can also promote neutrophil survival by suppressing the apoptotic machinery through activation of ERK and PI3K/Akt signaling pathways [17]. Hence, increased production of SAA during acute infection plays an important protective role involving stimulation of neutrophil and monocyte recruitment, phagocytosis and survival required for clearance of bacterial pathogens. This process is normally self-limiting with a decline in SAA production during the resolution phase of infection; however the persistent elevation of SAA may lead to accumulation of neutrophils that can result in excessive lung injury.

The production of specialized pro-resolving mediators (SPMs) during acute infection can also initiate essential molecular mechanisms that prevent excessive neutrophil recruitment. Resolvin D1 (RvD1) and its aspirin-triggered epimer (AT-RvD1) are generated from the essential ω-3 fatty acid, docosahexaenoic acid (DHA) and interact with two known human receptors, namelyFpr2 and DRV1/GPR32 to initiate organ protective properties. Since mice do not express DRV1/GPR32, Fpr2 represents the major receptor for RvD1 in mice. AT-RvD1 is a promising therapeutic target in bacterial pneumonia as it enhanced macrophage phagocytosis of Gram-negative Escherichia coli (E. coli) and
*Pseudomonas aeruginosa* (*P. aeruginosa*) and accelerated neutrophil efferocytosis during pneumonia to prevent excessive lung injury [18]. AT-RvD1 also promoted more rapid clearance of Gram-negative non-typeable *Haemophilus influenzae* (*H. influenzae*) by stimulating efferocytosis mediated by M2 skewed macrophages [19]. Here, we demonstrate that in our bacterial and viral co-infection model associated with increased neutrophilic inflammation, levels of circulating and lung tissue SAA remained markedly elevated during the resolution phase following pneumococcal infection (7 days post *S. pneumoniae* inoculation). Furthermore, delivery of exogenous AT-RvD1 during the acute phase of infection improved clearance of pneumococci in the lungs and reduced pulmonary consolidation caused by excessive recruitment of neutrophils and monocytes during co-infection.
Methods

Animals

Male C57BL/6J mice (8-10 weeks old) from the Animal Resources Centre (Perth, Australia) were housed at 22±1°C under normal 12h light/dark cycle and fed a standard chow and water \textit{ad libitum}. All experiments were approved by the Animal Ethics Committee of RMIT University (AEC #1509) and performed in compliance with the National Health and Medical Research Council (NHMRC) of Australia guidelines. In the co-infection experiment, mice were divided into 4 groups: SAL (saline), SP (\textit{S. pneumoniae}), IAV and SPIAV (co-infected). On day 0 of experiment, mice from SP and SPIAV groups were infected intranasally with \textit{S. pneumoniae} (serotype 19F, strain EF3030, $10^5$ CFU in 35μL saline) under light isoflurane anaesthesia. On day 1, mice from IAV and SPIAV groups were infected intranasally with influenza A virus (strain A/HKx31 (H3N2), $10^4$ PFU in 30μL saline). In separate experiments, SAL and SPIAV mice were treated intranasally with AT-RvD1 (17(R)-RvD1, Cayman Chemical, 100ng in 35μL saline) or vehicle (1% ethanol in equivalent volume of saline) once daily on day 4 and day 6. On day 7, mice were culled by intraperitoneal overdose of sodium pentobarbitone. BAL was performed via tracheotomy and total/differential BAL cell counts were determined as previously described [16]. Lung was perfused with ice-cold PBS to remove excess blood. The right lobe of the lung was immediately excised and kept on ice-cold PBS for flow cytometric analysis. The left lobe was fixed in 10% neutral-buffered formalin for histology and the rest was snap-frozen in liquid nitrogen prior to -80°C storage for further analysis.

Quantification of \textit{S. pneumoniae} and influenza A virus

Quantification of \textit{S. pneumoniae} in the BAL fluid (BALF) was performed by viable count, where serial dilutions were cultured overnight on selective agar (horse blood agar supplemented with 5μg/ml gentamicin). In addition, quantitative real time PCR (qPCR) was used to measure \textit{S. pneumoniae} and
influenza A virus in the lung tissue. Briefly, bacterial DNA and viral RNA were isolated by homogenising lungs in Trizol (Life Technologies) using a TissueLyser (Qiagen) in accordance to the manufacturer’s instructions. *S. pneumoniae* DNA qPCR was performed using a commercial kit from Qiagen (Microbial DNA qPCR Assay #330025) as per manufacturer’s instructions and bacterial load was determined by using standard curve generated from a known quantity of pneumococci. qPCR on polymerase A subunit (PA) gene of influenza A virus was performed using TaqMan® Fast Virus 1-Step Master Mix as previously described [20]. Viral load was determined by using a standard curve generated from a known quantity of influenza A virus.

**Reverse Transcriptase Quantitative PCR (RT-qPCR) for gene expression analysis**

RNA was purified from lung tissue using RNeasy kit (Qiagen), from which cDNA was prepared using High Capacity cDNA Kit (Life Technologies) as previously described [21]. RT-qPCR was performed using bioinformatically validated Taqman primers/probes (Life Technologies), namely glyceraldehyde phosphate dehydrogenase (*Gapdh*, Mm03302249_g1), Interferon-γ (*Ifng*; Mm01168134_m1), Fpr2 (*Fpr2*, Mm00484464_s1), SAA (*Saa1*, Mm00656927_g1), IL-6 (*Il6*, Mm00446190_m1), IL-1β (*Il1β*, Mm00434228_m1), CCL2/MCP1 (*Ccl2*, Mm00441242_m1) and CXCL2/KC (*Cxcl2*, Mm00436450_m1). The threshold cycle values (Ct) were normalized to a reference gene GAPDH and the relative fold change determined by the ΔΔCt value as previously described [21].

**Myeloperoxidase, protein, elastase and ELISA assays**

Myeloperoxidase activity was assessed by homogenising ground lung tissue (50mg/mL) in extraction buffer (50mM potassium phosphate monobasic pH 6.0, 0.5% hexa-decyl-trimethyl ammonium bromide and 10mM EDTA). Following centrifugation, lung lysate was incubated with reaction buffer (50mM potassium phosphate monobasic pH 6.0, 0.167mg/mL o-Da [Fast Blue B, Sigma] and 0.005% H₂O₂)
and the change in absorbance 460 per minute (3min-2min) resulting from decomposition of H₂O₂ and subsequent oxidation of o-Da was measured using a CLARIOstar® plate reader. Total protein levels in the BALF were determined by a Pierce BCA Protein Assay Kit (Life Technologies). Neutrophil elastase (NE) activity in BALF as a marker for neutrophil activity was measured using an EnzChek® Elastase Assay Kit (Life Technologies). Briefly, 30μL of BALF was diluted with 60μL 1× Reaction Buffer in a 96-well plate, to which 10μL of 50μg/mL DQ elastin substrate was added. Reaction was protected from light and incubated at 37°C for 24h. Fluorescence was then measured using CLARIOstar® (Ex/Em 505/515nm). Lung tissue was homogenised in lysis buffer (150mM NaCl; 50mM Tris-HCl pH 7.4; 1% NP40, 10μL/mg protease inhibitor cocktail) using a TissueLyser (Qiagen) and lysates were collected for determination of protein lung levels. SAA levels in the lung lysate, serum and BALF were measured using a SAA Mouse ELISA Kit (Life Technologies) and RvD1 levels were determined using commercial ELISA Kit (Cayman Chemical) according to the manufacturer's instructions. The lung tissue lysate was also used to determine IL-1β and IL-6 protein levels in the lung using commercial ELISA assays (Life Technologies).

**S. pneumoniae susceptibility assay**

BAL fluid was centrifuged and cell-free aliquots were archived at -80°C and subjected to freeze-thaw cycle prior to use in this assay. Total antimicrobial activity in the BALF was determined using an *S. pneumoniae* susceptibility assay as previously described [22]. Briefly, 114μL of *S. pneumoniae* (3×10⁶ CFU/mL) cultured in Todd–Hewitt broth (THB, BD Biosciences) was incubated with 6μL of BALF from mice at 37°C for 4h in a 5% CO₂ incubator. Serial dilutions of the culture were incubated overnight on selective agar (horse blood agar supplemented with 5μg/ml gentamicin) and colonies were counted to assess bacterial growth rate the presence of BALF fluid.
Flow cytometric assessment of immune cells in lung

Single cell suspension preparation and flow cytometric analysis were performed as previously described [16]. Inhibition of non-antigen binding of immunoglobulins to Fc receptors was performed using a rat anti-mouse CD16/CD32 antibody (BD Biosciences). A strict gating strategy was used to determine different immune cell populations and propidium iodide was used to exclude dead cells. Neutrophils and macrophages were gated as single, live cells with intermediate or high expression of FSC-A and SSC-A, which displayed these cells as a distinct cluster compared to lymphocytes and myeloid cells. In addition, neutrophils were classified as CD45$^{Hi}$, F4/80$^{Low}$ and Ly6G$^+$ whereas macrophages were classified as CD45$^{Hi}$, F4/80$^{Hi}$ and Ly6G$^-$. For macrophage subpopulations, alveolar macrophages were classified as CD11b$^{Low}$, CD11c$^{Hi}$; exudative macrophages were classified as CD11b$^{Hi}$, CD11c$^{Hi}$; infiltrating macrophages were classified as CD11b$^{Hi}$, CD11c$^{Low}$. Lymphocytes were labelled with fluorochrome-conjugated antibodies against CD3 and CD8 used at pre-optimised dilutions in order to differentiate and quantify the number of CD3$^+$CD8$^+$ T cells. Cells were analysed on a BD FACSCanto II (BD Biosciences) and data was analysed using FlowJo software (FlowJo LLC, Oregon, US). All anti-mouse antibodies were purchased from BD Biosciences, namely FITC-conjugated CD45, PB-conjugated F4/80, PE/Cy7-conjugated CD11c, APC-conjugated CD11b, APC/Cy7-conjugated Ly6G.

Histology and immunohistochemistry

The left lobe of lung fixed in 10% neutral-buffered formalin was processed, paraffin-embedded and sectioned at a thickness of 5μm. Histologic examination was performed on hematoxylin-eosin (H&E)–stained sections of the left lung using a slide scanner to capture the entire lobe (Olympus VS120). Airway inflammation in H&E sections were blindly graded and the average of five individual airways
was presented as previously described [23]. Parenchymal inflammation or pneumonia (area alveolar infiltrate minus airways/vessels) was graded using the following criteria across the entire lobe; 1= mild, inflammatory cells sporadically present in parenchyma; 2= moderate, single inflammatory lesion accounting for up to 10% lung parenchyma, 3= severe, multiple inflammatory lesions or pneumonia sites accounting for 10-50% lung parenchyma, 4= complete, multiple inflammatory lesions and diffuse consolidation or pneumonia accounting for >50% lung parenchyma. Immunohistochemical staining for Fpr2 was performed by incubating with Fpr2 antibody (NLS1878, Novus Biologicals, 1: 300) overnight at 4°C, followed by secondary antibody (biotinylated goat anti-rabbit IgG, Vector Laboratories, 1:200, 1h) and avidin-biotin horseradish peroxidase (HRP) complex (Vector Elite kit; Vector Laboratories, 1:200, 1h). Staining was developed in freshly prepared 3,3′-Diaminobenzidine (DAB) solution and sections were then counterstained in hematoxylin. Images were captured using the Olympus VS120 slide scanner.

**Data analysis**

Data are presented as the mean ± SEM. All data were statistically analysed using GraphPad Prism 6.0 (Graphpad, San Diego, CA). Where detailed and appropriate, two-tailed Students t-tests, one-way or two-way analyses of variance (ANOVA) with Dunnett or Bonferroni’s post-hoc tests were used. p <0.05 was considered to be statistically significant.
Results

Co-infection augments bacterial lung load and inflammation during resolution phase.

Schematic representation of the co-infection model is presented in Figure 1, where mice were inoculated intranasally with a 19F strain of *S. pneumoniae* (SP) or saline (SAL) on day 0, followed by influenza A virus (IAV) or saline infection (SAL) on day 1. To determine the chronicity of *S. pneumoniae* lung infection in this model, bacterial load in the BALF and the lung was assessed 7 days post pneumococcal inoculation (Figure 1B and C). In mice infected with *S. pneumoniae* alone, day 7 represents resolution of infection as residual pneumococcal numbers (<100 CFUs) remained in the BALF with none detected in the lung tissue. Co-infection with IAV resulted in markedly higher pneumococcal lung infection, evidenced by the high bacterial load in both the BALF and the lung (Figure 1B and 1C). IAV lung viral titres were not altered by co-infection (Figure 1D) and the increase in IFN-γ levels by IAV was not altered by co-infection (p > 0.05, Figure 1E).

Pneumonia caused by co-infection increases lung elastase activity and oedema.

The cellular inflammatory response to *S. pneumoniae* and/or IAV infection was assessed by analysing the bronchoalveolar lavage (BAL) compartment. The inflammatory response in mice inoculated with *S. pneumoniae* alone resolved by day 7 as no difference in leukocytes numbers was seen compared with saline-treated mice (Figure 2A-C). There was a significant increase in BAL macrophages in IAV-infected mice and this was not significantly altered by co-infection (Figure 2B). BAL neutrophil numbers were increased in IAV-infected mice on day 7, and this was further increased in co-infected mice by approximately ~100% (Figure 2C). Myeloperoxidase (MPO) activity was assessed on lung tissue and in concordance with BAL neutrophil counts, elevated MPO activity in the lungs of IAV-infected mice increased by ~80% in co-infection (Figure 2D). To assess neutrophil activation, neutrophil elastase (NE) released into the BALF was measured (Figure 2E). Similar to the MPO
activity results, IAV infection significantly increased NE activity, which was further elevated by SP co-infection by 20%. Consistent with increased NE proteolytic activity contributing to oedema during acute infection and inflammation, total BALF protein levels were increased in IAV-infected mice and this was significantly higher in co-infected mice (Figure 2F).

SAA and Fpr2 are markedly increased in the lungs of co-infected mice.

Expression of Fpr2 on lung tissue on day 7 following pneumococcal inoculation was determined by qPCR, where levels of Fpr2 were significantly elevated in co-infected mice relative to SAL or IAV treated mice (Figure 3A). Immunoreactive Fpr2 protein was detected by immunohistochemistry (Figure 3B); where modest Fpr2 immunoreactivity was mainly limited to bronchial epithelial cells in the lungs of saline treated control mice. In co-infected mice, Fpr2 immunoreactivity was much more prominent in bronchial epithelium and macrophages. We next evaluated expression of the inflammatory Fpr2 agonist, SAA. SAA mRNA levels were elevated by IAV-infection (4-fold, relative to SAL control) and this was further increased (9-fold relative to SAL control, p<0.05 vs IAV) by co-infection (Figure 4A). SAA protein levels in lung homogenate were also increased in IAV-infected mice compared to SAL control mice (Figure 4B, IAV; 6.7nmol vs. SAL; 0.22nmol/100mg tissue) and levels significantly increased further in co-infected mice (SPIAV; 26.2nmol/100mg, p<0.05 vs IAV). A similar pattern of SAA protein expression was detected in BALF and serum, where IAV increased SAA levels and this was further elevated in the co-infection setting (Figures 4C, D). In addition, levels of the specialized pro-resolving mediator, RvD1 were measured in the serum. Serum RvD1 levels were not altered by either single infections or co-infection on day 7 (Figure 4E, p > 0.05), and the ratio of SAA relative to RvD1 in serum was markedly elevated in co-infected mice (Figure 4F, p < 0.05). Levels of RvD1 in BALF and lung homogenate were below the limit of detection for this assay.
AT-RvD1 reduces pneumococcal lung load and alveolitis in co-infected mice.

We next explored the therapeutic effect of exogenous AT-RvD1 in our co-infection model, where co-infected mice were treated intranasally with AT-RvD1 or vehicle once daily on day 4 and day 6 as summarised in Figure 5A. On day 7, *S. pneumoniae* load was assessed in the BALF and the lung, demonstrating a significant 70% reduction in pneumococcal load in BALF (Figure 5B) and lung (Figure 5C) following AT-RvD1 treatment. AT-RvD1 did not significantly alter lung viral titres (Figure 5D), IFN-γ as determined by RT-qPCR (Figure 5E) or numbers of lung CD8+ T cells in co-infected mice, as determined by flow cytometry (Figure 5F). To assess airway and parenchymal inflammation/alveolitis, H&E stained lung sections were scored blinded as described in the methods sections using whole slide scanned images of the left lung lobe. Representative images of the entire lobe (left panels) and 200× magnification of areas of interest (right panels) are shown in Figure 6A. Quantitative evaluation of parenchymal inflammation or alveolitis demonstrated that AT-RvD1 significantly reduced pulmonary consolidation in co-infected mice (Figure 6B), whereas bronchial or airway inflammation was not significantly altered (Figure 6C).

Neutrophil staining of lung immune cells demonstrated that lung neutrophil numbers increased in co-infected mice and this was significantly reduced by AT-RvD1 treatment by approximately 50% (Figure 7A and B). The attenuated neutrophil influx after AT-RvD1 treatment was also consistent with a 30% reduction in MPO activity in the lung (Figure 7C) and 50% reduction in the neutrophil counts in the BALF (Figure 7D). Total neutrophil elastase activity in the BAL fluid of co-infected mice increased 2-fold relative to saline treated mice, and AT-RvD1 treatment significantly reduced elastase activity by approximately 13% (Figure 7E). In addition, the total antimicrobial activity was assessed by culturing *S. pneumoniae* in the presence of BAL fluid. The in vitro pneumococcal growth rate increased by 60% in cultures incubated with BAL fluid from co-infected mice relative to control mice (Figure 7F). The
BAL fluid from AT-RvD1 treated co-infected mice significantly reduced pneumococcal growth by approximately 20% (vs. vehicle treated co-infected mice, p < 0.05; Figure 7F).

In addition, analysis of the macrophage sub-populations (representative macrophage subsets shown in Figure 8A) revealed no changes in alveolar macrophages (Figure 8B), but a marked recruitment of exudative (CD11b^{Hi}, CD11c^{Hi}) and infiltrating monocytes/macrophages (CD11b^{Hi}, CD11c^{Low}) in the lungs of co-infected mice (Figure 8C and D). AT-RvD1 treatment did not alter alveolar macrophage (CD11b^{Low}, CD11c^{Hi}) or exudative macrophage numbers, but did result in ~50% reduction in infiltrating monocytes/macrophage numbers (Figure 8B-D). To determine whether AT-RvD1 reduced neutrophil/monocyte numbers through inhibition of inflammatory cytokines and chemokines, we measured IL-1β and IL-6 transcript and protein levels in the lungs. The data show that both inflammatory cytokines were markedly increased in co-infected lungs, but transcript or protein levels were not significantly reduced by AT-RvD1 (Supplementary Figure 1A-D). A very similar pattern of expression was seen for the neutrophil chemokine CXCL2 and the monocyte chemokine CCL2/MCP1, where elevated transcript levels were not significantly reduced by AT-RvD1 (Supplementary Figure 1E and F). In addition, elevated transcript levels of SAA and Fpr2 were not significantly reduced by AT-RvD1 (Supplementary Figure 1G and H).
Discussion

Infection with the 19F strain *S. pneumoniae* alone was self-limiting and resolved within 7 days. In contrast, acute viral (IAV) infection caused significant lung inflammation at day 7 and in the co-infection model, markedly compromised *S. pneumoniae* clearance, leading to a further significant increase in lung leukocytes. In this study, we demonstrate that global Fpr2 lung expression is significantly increased in co-infected mice. Murine Fpr2 is known to be expressed on neutrophils and monocytes/macrophages, and acute injury increases Fpr2 expression in normal bronchial epithelial cells via a COX-2-dependent manner [25]. Consistent with these findings, we observed prominent Fpr2 immunoreactivity in the bronchial epithelium and macrophages of co-infected mice. In addition, SAA protein was markedly elevated in the BAL fluid and serum of co-infected mice, where circulating SAA is predominately synthesised by the liver in response to the ‘spill over’ of inflammatory mediators from the infected/inflamed site. SAA is a potent chemotactic factor that mediates migration of leukocytes [14] and can also promote expression of pro-inflammatory mediators under *in vitro* [28] and *in vivo* conditions [12]. We have previously shown that SAA induces a pro-inflammatory macrophage phenotype [29] that stimulated acute neutrophilic lung inflammation in an Fpr2 dependent manner [16].

Importantly, SAA can oppose the organ protective and pro-resolving actions of LXA₄, RvD1 and their aspirin-triggered epimers that facilitate the resolution of inflammation via FPR2/ALX in the absence of SAA [12, 25, 26]. Our approach to reducing excessive neutrophil and monocyte inflammation in co-infected mice was to therapeutically deliver exogenous AT-RvD1 during the acute phase of infection. Fpr2 is the main receptor for AT-RvD1 in mice, which reduces neutrophilic inflammation in response to Gram negative infection [18, 19]. Our data demonstrate that AT-RvD1 potently reduces neutrophil and monocyte numbers in the lungs of co-infected mice, thereby significantly limiting the degree of
pneumonia. The organ protective actions of RvD1 analogs include inhibition of neutrophil migration along a chemotactic gradient [30]; hence AT-RvD1 can directly oppose leukocyte movement from the infected bronchi into the distal lung. Interestingly, our histological findings show that parenchymal inflammation or alveolitis is reduced by AT-RvD1, whereas bronchial inflammation was not significantly reduced at this time point, suggesting that alveolar inflammation was cleared more rapidly than bronchial inflammation. We also observed that AT-RvD1 did not significantly reduce expression of the leukocyte chemokines CXCL2 or MCP-1, nor did it significantly reduce SAA or Fpr2 levels in co-infected mice. Hence, our data strongly suggest that AT-RvD1 primarily halts neutrophil and monocyte migration from the infected bronchioles into the distal alveoli by directly opposing chemotaxis towards inflammatory chemokines.

In addition, AT-RvD1 also stimulated pneumococcal lung clearance, hence would limit the spread of this pathogen into the distal airways and therefore, limit alveolitis exacerbated by co-infection. We found that neutrophil elastase activity was highest in co-infected mice and this was associated with a significant reduction in global antimicrobial activity in the BAL fluid, as the in vitro pneumococcal growth rate was highest in bacterial cultures spiked with co-infected BAL fluid. Excessive neutrophil elastase is known to compromise immunity to bacteria by degrading antimicrobial peptides in the BAL compartment leading to impaired bacterial clearance [24]. Importantly, AT-RvD1 significantly reduced neutrophil elastase activity and concurrently restored anti-microbial activity in the BAL fluid of co-infected mice. Our data are consistent with the known actions of AT-RvD1, which can stimulate the production of the anti-bacterial peptide lipocalin 2, thereby enhancing bacterial clearance in the lung [18].
We also evaluated the anti-viral CD8$^+$ T cell response in AT-RvD1 treated mice. Consistent with IFN-γ levels, viral infection in co-infected mice increased the number of CD8$^+$ T cells in the lung and this response was not altered by AT-RvD1. Hence, we clearly demonstrate that AT-RvD1 does not compromise viral immunity, but does reduce pneumonia by limiting neutrophil and monocyte chemotaxis and improving bacterial clearance. Acute bacterial infections will also promote the robust recruitment of blood monocytes to restore lung macrophage numbers through maturation of recruited blood monocytes [31]. However, excessive monocyte recruitment can stimulate a secondary phase of neutrophil migration that perpetuates acute lung injury [34]. Since AT-RvD1 potently reduced monocyte recruitment in co-infected mice, this will also reduce the secondary phase of neutrophil recruitment.

Acute immune responses to bacteria and viruses will also promote macrophage phenotypic heterogeneity in the lung [31]. Inoculation of mice with pandemic IAV strains can dramatically deplete resident alveolar macrophages in a temporal manner [32] with gradual restoration of lung macrophages through self-renewal proliferative mechanisms [33]. Significantly, this temporal depletion of lung alveolar macrophages can create a window of opportunity for pathogens such as S. pneumoniae to cause marked increase in pneumococcal outgrowth and lethal pneumonia [32]. At our resolution time-point (day 7), we observed robust recruitment of infiltrating monocytes and increased numbers of exudative macrophages in co-infected mice. Furthermore, exudative macrophages were not reduced by AT-RvD1 in our model. This lung macrophage population is particularly important to resolution processes as RvD1 stimulates exudative macrophages to clear apoptotic neutrophils [18].

In summary, our findings demonstrate that co-infection leads to a significant increase in lung oedema associated with excessive neutrophil elastase activity and as a consequence, levels of the acute phase
reactant SAA are markedly elevated in the BAL and lung compartment. Since SAA can directly target Fpr2 to stimulate neutrophilic inflammation, AT-RvD1 was administered locally into the lungs to initiate resolution of inflammation. AT-RvD1 promoted resolution of inflammation by improving pneumococcal lung clearance, which is compromised in the co-infection setting. Furthermore, AT-RvD1 initiated organ protective effects by limiting exuberant neutrophil and monocyte recruitment. AT-RvD1 represents a novel therapeutic option to reduce the burden of bacterial pneumonia that is exacerbated as a consequence of acute viral infections.

Clinical Perspectives

- Uncontrolled and persistent bacterial infection in the lungs of people suffering from acute influenza infection is a key element that promotes severe pneumonia and mortality. Hence, there is a need to develop novel therapies that can concurrently reduce damaging lung inflammation and improve bacterial lung clearance in the co-infection setting.
- In the present study, Serum amyloid A (SAA) levels were markedly increased in the lungs of co-infected mice. Since SAA is a functional inflammatory agonist for the Formyl peptide receptor 2 (Fpr2), we have evaluated the therapeutic efficacy of an alternative Fpr2 pro-resolution agonist. Aspirin-triggered resolvin-D1 significantly reduced pneumococcal lung load and severity of pneumonia in co-infected mice.
- This study provide novel insight into the mechanisms that drive excessive inflammation in the co-infection setting and for the first time, identify AT-RvD1 as an important therapeutic agent in this poly-microbial setting.

Author Contribution

Steven Bozinovski and Hao Wang conceived and conducted the study. Hao Wang, Steven Bozinovski, Desiree Anthony and Selcuk Yatmaz performed the experiments. Catherine Satzke, Odilia Wijburg,
Ross Vlahos, Bruce Levy and Desiree Anthony assisted in data analysis and interpretation. Steven Bozinovski and Hao Wang wrote the paper. All co-authors provided intellectual input and critical editing to the paper.

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Figure legends

Figure 1. Co-infection increases pneumococcal lung infection.

(A) Schematic representation of the experimental protocol and the timing of inoculation with *S. pneumoniae* (SP) and/or influenza A virus (IAV). Mice were infected intranasally with SP (*S. pneumoniae*, serotype 19F/strain EF3030, 10^5 CFU) and/or IAV (influenza A virus, strain HKx31, H3N2, 10^4PFU). On day 7, mice were culled and outcomes were measured. SP load was assessed in both bronchoalveolar lavage fluid (BALF) and lung tissue. (B) Freshly collected BALF was serially diluted and incubated on horse blood agar supplemented with 5μg/ml gentamicin overnight at 37°C and colony forming units (CFUs) were recorded from highest-countable dilution (n = 6 – 8). *p<0.05, two-tailed Students t-tests. (C) Lung tissue that had been snap frozen was homogenised and microbial DNA extracted, and qPCR performed to quantify SP levels. (D) qPCR on viral RNA extracted from lung tissue was performed to quantify IAV levels. (E) Taqman qPCR was performed on lung mRNA extraction for assessment of IFNγ gene expression. n = 5 – 10, *p<0.05, one way ANOVA vs. SAL.

Figure 2. Co-infection increases neutrophilic inflammation and oedema caused by IAV.

(A) Total cells from bronchoalveolar lavage (BAL) were assessed by viability counting and cytospins were prepared for the assessment of (B) macrophages and (C) neutrophils numbers. (D) Neutrophil myeloperoxidase (MPO) activity in lung tissue was measured as an indicator for neutrophil numbers in the lung tissue. (E) Neutrophil elastase activity in the BALF was measured as a marker for neutrophil degranulation and (F) total proteins in the BALF was measured to assess degree of lung edema in the lung. n = 5 – 8, *p<0.05, one-way ANOVA compared to SAL or IAV as indicated.
Figure 3. Co-infection increases Fpr2 transcript expression and immunoreactivity in the lungs.

(A) Formyl peptide receptor 2 (Fpr2) transcript levels were assessed in the lungs by Taqman RT-qPCR demonstrating a significant increase in co-infected mice. (B) Fpr2 immunoreactivity was determined by immunohistochemistry on lung sections from saline (SAL) and co-infected (SPIAV) mice, demonstrating prominent bronchial epithelial staining and the presence of Fpr2+ lung macrophages in co-infected mice. The black arrows indicate lung macrophages. *p<0.05, one-way ANOVA compared to SAL or IAV as indicated.

Figure 4. Co-infection markedly increased circulating and tissue serum amyloid A (SAA) levels.

(A) Gene expression of SAA was measured in the lung by Taqman RT-qPCR. (B) SAA protein in the lung homogenate lysate, (C) BALF and (D) serum was measured by ELISA. (E) RvD1 levels in the serum was measured by ELISA. (F) The relative ratio of SAA/RvD1 was determined for the matching serum samples. n = 6 – 10, *p<0.05, one-way ANOVA compared to SAL or IAV as indicated.

Figure 5. AT-RvD1 treatment reduces bacterial load in the lung.

(A) Mice were infected with S. pneumoniae (SP) on day 0 and influenza A virus (IAV) on day 1 as described in Figure 1A. On day 4 and day 6, mice were treated intranasally with AT-RvD1 (SPIAV-RvD1) or vehicle (SPIAV-Veh) once daily. On day 7, mice were culled and outcomes were measured. (B) Freshly collected bronchoalveolar lavage fluid (BALF) was serially diluted and incubated on horse blood agar supplemented with 5μg/ml gentamicin overnight at 37°C and colony forming units (CFUs) were recorded from highest-countable dilution. (C) Lung tissue that had been snap frozen was homogenised and microbial DNA qPCR was carried out to quantify SP levels. (D) qPCR on viral RNA extracted from lung tissue was performed to quantify IAV levels. (E) Taqman qPCR was performed on lung mRNA extraction for assessment of IFNγ gene expression. (F) CD8+ T cells in the lungs were
determined by flow cytometry, demonstrating an increase in CD8+ numbers in co-infected mice that was not altered by AT-RvD1. n = 5 – 12, *p<0.05, two-tailed Students t-tests.

**Figure 6. AT-RvD1 treatment ameliorates degree of pneumonia in the lung.**

(A) Representative images of whole slide scan and 200× optical zoom using H&E stained lung sections. Degree of parenchymal inflammation (B) and airway inflammation (C) was scored blinded as detailed in the methods section. n = 10-12, *p<0.05, two-tailed Students t-tests.

**Figure 7. AT-RvD1 (RvD1) treatment reduced neutrophilic inflammation in the lung.**

Single cell suspensions were prepared from lung homogenates. Flow cytometric analysis was performed and (A) representative flow cytometry dot plots from lung homogenates gated on neutrophils (F4/80−Low, Ly6G+). Neutrophil numbers (B) in the lung demonstrated a marked increase in co-infected mice and a significant reduction with AT-resolvin-D1 treatment. (C) Increased neutrophil myeloperoxidase (MPO) activity in the lung (D) and increased BAL neutrophil numbers were also reduced by AT-RvD1 treatment. (E) Neutrophil elastase activity in the bronchoalveolar lavage (BAL) fluid was also assessed, demonstrating an increase in co-infected mice that was reduced by AT-RvD1. (F) Total antimicrobial activity in the BAL fluid was determined using the *S. pneumoniae* susceptibility assay as described in the methods section. *In vitro* bacterial growth was increased in the presence of BAL fluid from co-infected mice (vs. SAL), and bacterial growth was significantly lower in cultures containing BAL fluid from AT-RvD1 treated mice (vs. SPIAV-Veh). *p<0.05, one-way ANOVA compared to SPIAV-Veh as indicated.
Figure 8. AT-RvD1 treatment reduces infiltrating monocyte recruitment.

(A) representative flow cytometry dot plots from lung homogenates gated on macrophage subsets including alveolar macrophages classified as CD11b^{Low}, CD11c^{Hi}; exudative macrophages classified as CD11b^{Hi}, CD11c^{Hi}; infiltrating macrophage classified as CD11b^{Hi}, CD11c^{Low}. Macrophage subset numbers of (B) alveolar macrophages, (C) exudative macrophages and (D) infiltrating monocytes/macrophages were determined, demonstrating a significant reduction in infiltrating monocytes as a result of AT-RvD1 treatment (SPIAV-RvD1), *p<0.05, one-way ANOVA compared to SPIAV-Veh as indicated.
References


Figure 1

A

SP (19F) 10^5 CFU  IAV (HKx31) 10^4 PFU

0  1  2  3  4  5  6  7 days

B

Total SP in BALF (CFU)

C

Bacterial lung load (CFU/100mg)

D

Virus lung titres (PFU/100mg)

E

IFN/β/GAPDH

SAL  SP  IAV  SPIAV
Figure 2

A

Total BALF cells (x10^6/mL)

B

BALF macrophages (x10^5/mL)

C

BALF neutrophils (x10^6/mL)

D

MPO activity (% SAL)

E

Elastase activity (% SAL)

F

BALF protein (% SAL)
Figure 3

A

![Graph showing Fpr2/GAPDH levels for different conditions: SAL, SP, IAV, SPIAV.](image)

B

![Immunohistochemical images showing differences in expression between SAL and SPIAV conditions for ISO and Fpr2.](image)
Figure 5

A

SP (19F) 10^3 CFU
IAV (HKx31) 10^4 PFU

RvD1 (100ng) or Vehicle
RvD1 (100ng) or Vehicle

0 1 2 3 4 5 6 7 days

B

Total SP in BALF (CFU)

C

Total SP in lung (CFU/100mg)

D

Lung viral titres (PFU/100mg)

E

IFNγ/GAPDH

F

CD8 T cells (x10^6/100mg tissue)
Figure 6

A

SAL

SPIAV Veh

SPIAV RvD1

B

C

Parenchymal Inflammation

Airway Inflammation

*
Figure 7

A

<table>
<thead>
<tr>
<th>SAL</th>
<th>SPIAV - Veh</th>
<th>SPIAV - RvD1</th>
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</thead>
<tbody>
<tr>
<td>Neutrophils</td>
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<td>19.4</td>
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<tr>
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<td></td>
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<tr>
<td>Ly6G</td>
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</tr>
</tbody>
</table>

B

- **Lung neutrophils**
  - SAL: 1
  - Veh: 2
  - RvD1: 3

C

- **MPO activity**
  - SAL: 100
  - Veh: 200
  - RvD1: 150

D

- **BALF neutrophils**
  - SAL: 5
  - Veh: 10
  - RvD1: 20

E

- **Elastase activity**
  - SAL: 50
  - Veh: 200
  - RvD1: 150

F

- **Total SP**
  - SAL: 5
  - Veh: 10
  - RvD1: 15

* indicates statistical significance.
Figure 8

A

SAL

SPIAV - Veh

SPIAV - RvD1

CD11c

CD11b

B

C

D

Alveolar macrophages (x10^9/100mg tissue)

Exudative macrophages (x10^9/100mg tissue)

Infiltrating monocytes (x10^9/100mg tissue)

*
Supplementary Figure 1. AT-resolvin D1 does not reduce expression of inflammatory and chemokine mediators.

Gene expression of (A) IL-1β, (C) IL-6, (E) CXCL2, (F) MCP1, (G) SAA1 and (H) Fpr2 was determined by Taqman RT-qPCR in mouse lungs from saline (SAL) or co-infected (SPIAV) mice treated with either vehicle (Veh, open bar) or AT-resolvin D1 (RvD1, black bars). The same lungs were assessed for (B) IL-1β and (D) IL-6 protein levels in lung lysate. n = 6 – 10, two-way ANOVA analysis was used.