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Dietary fatty acids amplify inflammatory responses to infection through p38 MAP kinase signaling

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Disclosures

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Author contributions

S.R, R.Z, D.X, P.M.H, J.B and B.G.O conceived and planned the experiments. S.R. and R.Z carried out the experiments. S.R, R.Z, P.M.H, J.C.H, B.G.O and L.G.W. contributed to the interpretation of the results. S.R. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Abstract

Obesity is an important risk factor for severe asthma exacerbations, which are mainly caused by respiratory infections. Dietary fatty acids, which are increased systemically in obese patients and are further increased after high fat meals, affect the innate immune system and may contribute to dysfunctional immune responses to respiratory infection. This study investigated the effects of dietary fatty acids on immune responses to respiratory infection in pulmonary fibroblasts and a bronchial epithelial cell line (BEAS-2B).

Cells were challenged with BSA-conjugated fatty acids (ω-6 PUFAs, ω-3 PUFAs or SFAs) +/- the viral mimic polyinosinic:polycytidylic acid (PolyI:C) or bacterial compound lipoteichoic acid (LTA) and release of pro-inflammatory cytokines was measured. In both cell types, challenge with arachidonic acid (AA) (ω-6 PUFA) and PolyI:C or LTA led to substantially greater IL-6 and CXCL8 release than either challenge alone, demonstrating synergy. In epithelial cells, palmitic acid (SFA) combined with PolyI:C also led to greater IL-6 release. The underlying signaling pathways of AA and PolyI:C -or LTA-induced cytokine release were examined using specific signaling inhibitors and immunoblotting. Cytokine production in pulmonary fibroblasts was prostaglandin-dependent, and synergistic upregulation occurred via p38 MAP kinase signaling, whereas cytokine production in BEAS2Bs was mainly mediated through JNK and p38 MAPK signaling. We confirmed these findings using rhinovirus infection, demonstrating that AA enhances rhinovirus-induced cytokine release.

This study suggests that during respiratory infection, increased levels of dietary ω-6 PUFAs and SFAs may lead to more severe airway inflammation and may contribute to and/or increase the severity of asthma exacerbations.

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Key words: viral infection; asthma exacerbations; dietary fatty acids; primary lung fibroblasts; obese asthma.

Introduction

More than two billion people around the world are overweight or obese with a body mass index (BMI) of 25kg/m^2 or more (1). This global epidemic is associated with many chronic diseases, including asthma. A number of epidemiological studies show that obesity is an important risk factor for asthma development, increasing the risk by 2.7 fold compared to normal body weight (2). However, the underlying mechanisms are still poorly understood.

Clinical studies suggest that asthma in obese individuals differs from the classical phenotype of the disease. The obese asthma phenotype is characterized by greater severity, poorer control and quality of life, and lack of atopy with neutrophilic inflammation being specifically reported in obese women (2-5). Obesity also increases the risk of exacerbations and obese patients are almost five times more likely to be hospitalised for asthma exacerbations, compared to lean patients (3, 6, 7). Higher BMI appears to particularly increase the risk of autumn/winter exacerbations in more severe forms of asthma (6).

The major cause of asthma exacerbations is respiratory infection with rhinovirus (RV) accounting for up to 80% of all exacerbations (8). Viral-induced exacerbations in asthma are associated with increased levels of interleukin (IL)-6, the neutrophil chemoattractant CXCL8 and neutrophilic inflammation (9, 10). There are some studies that have reported bacterial infection to also be related to asthma exacerbations (11, 12), however this relationship is less evident. The impact of obesity on immune responses to infections is not clear, however obesity is associated with more severe outcomes following respiratory infection (13, 14). Several studies have shown associations between obesity and hospitalization and mortality following infection with pandemic influenza A/H1N1 in 2009 (15, 16). A recent study by Campitelli *et al.* (2014) showed that obesity increases the risk of outpatient visits following respiratory infection, compared to a normal body weight (14).

The innate immune system is the first line of defence against pathogens and its aim is to rapidly clear

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the body of pathogens. To do so, the innate immune system triggers an immediate inflammatory response that induces migration and activation of immune cells into infected sites. However, an excessive inflammatory response may induce greater tissue damage than that caused by pathogens and can contribute to the cause and severity of exacerbations (8).

Obesity is the result of the continuous overconsumption of nutrients. The western diet contributes to obesity, being rich in saturated- (SFA) and ω-6 polyunsaturated fatty acids (PUFAs) and low in ω-3 PUFAs (17). It has been shown that the consumption of high fat meals leads to increased levels of circulating fatty acids and modulates the innate immune system, as shown by increases in the levels of CXCL8 as well as the proportion of neutrophils in the circulation and sputum (18, 19). Obesity itself is also associated with increased fatty acid levels (20). The serum levels of SFAs and ω-6 PUFAs are substantially higher than ω-3 PUFAs (21-23). ω-6 PUFAs and SFAs have predominantly been associated with pro-inflammatory effects and current evidence suggests that SFAs promote inflammation through the activation of a family of receptors involved in innate immunity, known as Toll-like receptors (TLRs) (24). Several studies suggest that ω-3 PUFAs have anti-inflammatory and immunosuppressive properties and may be beneficial in treating infectious diseases. ω-3 and ω-6 PUFAs act as bio-active molecules that are metabolized by cyclooxygenase (COX) and lipoxygenase (LOX) into prostaglandins and leukotrienes respectively, which have potential anti- and pro-inflammatory actions of their own (25).

An emerging hypothesis to explain why obese patients have more frequent and severe asthma exacerbations is dysfunctional innate immune responses to viral and/or bacterial respiratory infections. Increased levels of dietary fatty acids could potentially contribute to these dysfunctional immune responses.

The bronchial epithelium has always been considered as the primary site of infection. However, *in vivo* evidence shows infection also occurs in submucosal cells including pulmonary fibroblasts (26). This study investigated the effect of dietary fatty acids on primary human pulmonary fibroblasts (HPFs) and a bronchial epithelial cell line (BEAS-2B) *in vitro*, specifically examining the possible

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enhancement of respiratory infection through measuring release of inflammatory mediators involved in immune responses against infection.

Methods

Cell culture

HPFs were isolated from the parenchyma of lungs from patients undergoing lung transplantation or lung resection for thoracic malignancies, as previously described (27). Ethical approval for all experiments involving the use of human lung tissue was provided by the Sydney South West Area Health Service, and written informed consent was obtained. *Table 1* shows the patient demographics. We also used the bronchial cell line BEAS-2B (ATCC, Manassas, VA). Detailed methods for culture of HPFs and BEAS2B are provided in the *Online Supplements.*

Preparation of BSA-conjugated fatty acids

Stock solutions of 0.5M ω-3 polyunsaturated fatty acids (PUFAs) (docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and α-Linolenic acid (ALA)), saturated fatty acid (SFA) (palmitic acid (PA)) and 0.3M ω-6 PUFA (arachidonic acid (AA)) (Sigma Aldrich) were prepared in 100% EtOH and stored at -20°C. Working water-soluble solutions of 10mM were generated by incubating the fatty acids in 10% endotoxin and fatty acid-free BSA (Sigma Aldrich), as previously described by Gupta *et al.* (2012) (28).

Treatment of cells

The cells were challenged with DHA, EPA, ALA, PA or AA (100µM) or vehicle (EtOH/BSA/cell culture medium) 4 hours prior to stimulation with or without the viral mimic polyinosinic:polycytidylic acid (PolyI:C) (10µg/ml) or bacterial compound lipoteichoic acid (LTA) (10µg/ml)(Sigma Aldrich). All cells were incubated at 37°C with 5% CO_2 for 24 hours.

Determination of IL-6,CXCL8, GM-CSF and CCL5 levels

Levels of supernatant IL-6, CXCL8, granulocyte-macrophage colony-stimulating factor (GM-CSF) and

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chemokine (C-C motif) ligand 5 (CCL5) were measured using commercial ELISA kits according to the manufacturer's instructions (R&D Systems, Minnesota, USA).

Western blotting

Total protein concentrations were obtained using a bicinchoninic acid assay according to the manufacturer's instructions (Sigma-Aldrich). Cell lysates (10µg) were separated by SDS-PAGE on 10% gels and transferred to PVDF membranes. The membranes were incubated with rabbit monoclonal antibodies against total and phosphorylated NF-κB p65, p38 mitogen-activated protein kinase (MAPK) or stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) (all 1:1000, Cell Signaling Technology) and anti-mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5000, Merck Millipore, USA). Primary antibodies were detected with goat anti-rabbit or rabbit anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (DAKO, USA) and visualized by enhanced chemiluminescence (Image Station 4000MM; Kodak Digital Science, New Haven, CT). GAPDH served as the loading control. Detailed methods are provided in the *Online Supplements*.

Signaling pathway inhibition

HPFs and BEAS-2Bs were treated with inhibitors of p38 MAPK (SB239063, 3µM, IC_{50} = 44nM) (Tocris, Ellisville, MO, USA), JNK (SP600125, 10µM, IC_{50} = 40 nM for JNK-1 and 2 and 90 nM for JNK-3) (Calbiochem, San Diego, CA), COX (indomethacin, 10µM, IC_{50} = 0.23µM for COX-1 and IC_{50} = 0.63 µM for COX-2) (Sigma) and NF-κB (BAY-117082, 10µM, IC_{50} = 10µM) (Sigma) for 1 hour before stimulation with AA (100µM) with or without PolyI:C (10µg/mL) or LTA (10µg/mL).

Rhinovirus infection

Major group human RV serotype-16 (RV16) was a kind gift from Prof Johnston (imperial College UK). RV16 was grown in HeLa cells and infectivity titre was determined using a titration assay as previously described (29, 30). HPFs were unstimulated or treated with AA (100µM) 4 hours prior to

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infection with or without live RV16 at a multiplicity of infection (MOI) of 1. Plates were incubated at 37°C with 5% CO_2 for 24 hours.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism version 7 software (San Diego, CA). Comparisons of the data were carried out by one-way ANOVA with repeated measures followed by a Bonferroni post-test, where appropriate unless otherwise specified. A probability (*p*) value of less than 0.05 was considered significant.

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Results

Stimulation with AA and PolyI:C leads to greater cytokine release from fibroblasts.

To assess if dietary fatty acids modulate the response to viral infection, HPFs were challenged with 100µM of DHA, PA or AA prior to stimulation with PolyI:C (10µg/ml) and IL-6, CXCL8, GM-CSF and CCL5 release was measured. AA alone, but not DHA or PA, induced IL-6 and CXCL8 release ($n = 11$, $p<0.05$) (*Figure 1*). PolyI:C alone also induced IL-6 and CXCL8 release ($n = 11$, $p<0.05$). Challenge with the combination of AA and PolyI:C resulted in substantially greater IL-6 and CXCL8 release than AA alone ($n = 11$, $p<0.01$) (*Figure 1A and 1B)*. The effect of the combination of AA with PolyI:C on IL-6 and CXCL8 release was greater than the sum of the individual effects of AA and PolyI:C, demonstrating a synergistic effect. There was no interaction between DHA or PA and PolyI:C on IL-6 and CXCL8 release. None of the treatments induced GM-CSF release from the HPFs (*data not shown*). CCL5 was induced upon PolyI:C -challenge and interestingly AA and DHA suppressed PolyI:C-induced CCL5 release ($n = 5\text{-}8$, $p<0.05$) (*Figure 1C and 1F*).

Stimulation with AA and LTA leads to greater cytokine release from fibroblasts.

To evaluate whether there is an interaction between dietary fatty acids and bacterial infection, HPFs were treated with dietary fatty acids prior to stimulation with LTA (10µg/ml). LTA alone did not induce IL-6 or CXCL8 release (*Figure 2*). However, both IL-6 and CXCL8 release ($n = 11$, $p<0.05$) was greater upon challenge with the combination of AA and LTA compared to AA alone (*Figure 2A and 2B*), also demonstrating synergistic effects. Again, there was no interaction between DHA or PA and LTA. In addition, there was no induction of GM-CSF or CCL5 with any of these treatments (*data not shown*).

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ω-3 PUFAs do not suppress combined AA and PolyI:C-induced cytokine release.

The ω-3 PUFA DHA did not affect PolyI:C -induced IL-6 and CXCL8 release in HPFs. To confirm that ω-3 PUFAs do not affect IL-6 and CXCL8 release, we investigated the effects of the other ω-3 PUFAs ALA and EPA and found that these fatty acids also do not affect the response to PolyI:C (*See Supplementary Figure 1*). Because nutrients do not occur in isolation, and healthy diet consists of ω-3 PUFAs:ω-6 PUFAs in a 1:4 ratio (31), we next investigated the effects of DHA:AA, EPA:AA and ALA:AA in a 1:4 ratio on PolyI:C-induced cytokine release. There was no difference in the response to AA with or without DHA, EPA or ALA in combination with PolyI:C (*See Supplementary Figure 2*), showing that ω-3 PUFAs do not suppress AA and PolyI:C mediated inflammatory responses in HPFs.

Stimulation with AA and PolyI:C or LTA also leads to greater cytokine release from epithelial cells.

To explore whether other structural lung cells respond similarly to HPFs, we repeated selected experiments in the bronchial epithelial cell line, BEAS-2B. AA alone did not induce IL-6 or CXCL8 release from BEAS-2Bs, however AA in combination with PolyI:C resulted in greater IL-6 release ($n = 6$, $p<0.05$) than PolyI:C alone (*Figure 3A*), showing similar responses as in HPFs. PA in combination with PolyI:C also resulted in greater IL-6 release ($n = 6$, $p<0.05$) than PolyI:C alone *(Figure 3G).* DHA did not affect PolyI:C -induced IL-6 or CXCL8 release, but suppressed PolyI:C-induced CCL5 release ($n = 7$, p<0.05) (*Figure 3F*). Furthermore, LTA alone did not induce cytokine release from BEAS-2BS. However, the combination of AA and LTA resulted in significant IL-6 and CXCL8 release ($n = 6$, $p<0.01$) (*Figure 4A* and *4B*). There was no interaction between DHA or PA and LTA (*Figure 4C-F*).

p38 MAP kinase hyperactivation in pulmonary fibroblasts upon challenge with AA and PolyI:C

To investigate the mechanisms underlying the effects of AA- and combined AA and PolyI:C or LTA-induced IL-6 and CXCL8 release in HPFs, we used protein immunoblotting to investigate the activation of three main signaling pathways (p38, NF-κB and SAPK/JNK), all of which have been

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shown to play a role in prostaglandin or infection-mediated inflammatory responses (32, 33). Phosphorylation of p38 MAP kinase was increased after stimulation with AA alone (n = 8, p<0.01) and in combination with PolyI:C (p<0.001) or LTA (p<0.01), while PolyI:C and LTA alone did not affect p38 MAP kinase phosphorylation (*Figure 5A*). The combination of AA and PolyI:C led to greater phosphorylation of p38 MAP kinase than AA alone (n = 8, p<0.05), indicating hyperactivation of this pathway to be the mechanism by which synergism occurs. NF-κB phosphorylation was increased upon challenge with PolyI:C (n = 8, p<0.01) and LTA alone (n = 8, p<0.01), but not in combination with AA (*Figure 5B*). Phosphorylation of SAPK/JNK was increased upon challenge with AA in combination with PolyI:C (n = 7, p<0.05), but not with any other challenge (*Figure 5C*). Total p38 MAP kinase, NF-κB and SAPK/JNK did not change with any treatment (*Figure 5D-F).*

AA and PolyI:C or LTA-induced cytokine release in pulmonary fibroblasts is mediated via p38 MAP kinase signaling. To further investigate and confirm the mechanisms underlying the effects of AA- and combined AA and PolyI:C- or LTA-induced IL-6 and CXCL8 release, specific inhibitors were used to block p38 MAPK, JNK and NF-κB activation. Inhibition of p38 MAP kinase suppressed IL-6 and CXCL8 (n = 9-14, p<0.01) release induced by AA alone (*Figure 6A and 6B*), the combinations of AA and PolyI:C (*Figure 6E and 6F*) and AA and LTA (*Figure 6G and 6H*), but did not affect cytokine release induced by PolyI:C alone. Inhibition of JNK attenuated IL-6 release induced by PolyI:C alone (n = 10, p<0.0001) (*Figure 6C*) and AA combined with PolyI:C (n = 10, p<0.01) (*Figure 6E*). Inhibition of NF-κB only suppressed IL-6 release induced by AA in combination with LTA (n = 14, p<0.01) (*Figure 6G*).

Inhibition of COX suppresses AA and PolyI:C or LTA- induced cytokine release in pulmonary fibroblasts. AA is a bio-active molecule and is a precursor that is metabolized by COX to produce eicosanoids, including the prostaglandins (25, 34). Prostaglandins are known to play a key role in the generation of inflammatory responses. To investigate whether COX-mediated prostaglandins contribute to the induction of IL-6 and CXCL8 release, HPFs were pre-treated with the non-selective COX-inhibitor, indomethacin. We found that indomethacin (10^{-5}M) pre-treatment suppressed IL-6

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and CXCL8 release induced by AA alone (*Figure 6A and 6B*), AA in combination with PolyI:C (*Figure 6E and 6F*) and AA in combination with LTA (*Figure 6G and 6H*) (n = 9-14, p<0.01). However, indomethacin pre-treatment did not affect cytokine release induced by PolyI:C alone. Inhibition of COX and p38 MAPK were the only two pathways to inhibit both AA and PolyI:C- as well as AA and LTA-induced IL-6 and IL-8 release.

AA and PolyI:C or LTA-induced cytokine release in BEAS-2Bs is mainly mediated JNK and p38 MAPK signaling. The underlying mechanisms in BEAS-2Bs were also investigated and we found that phosphorylation of p38 MAP kinase was increased 30 minutes after stimulation with AA alone, and AA in combination with PolyI:C or LTA (n = 8, p<0.05) (*Figure 7A*). NF-κB phosphorylation was increased upon challenge with PolyI:C and LTA alone, and challenge with AA in combination PolyI:C (n = 7, p<0.05) (*Figure 7B*). Phosphorylation of SAPK/JNK was increased upon challenge with AA in combination with LTA (n = 7, p<0.05) (*Figure 7C)*.

In addition, inhibition of p38 MAP kinase or JNK suppressed IL-6 and CXCL8 release induced by PolyI:C alone, AA in combination with PolyI:C and AA in combination with LTA (n = 7, p<0.05) (*Figure 8*). Inhibition of NF-κB suppressed IL-6 and CXCL8 release induced by AA in combination with PolyI:C (n = 7, p<0.05) (*Figure 8A and 8B)*, and CXCL8 release induced by PolyI:C alone (p<0.05) (*Figure 8D*). Inhibition of JNK resulted in the greatest suppression of combined AA and PolyI:C- or LTA-induced IL-6 and CXCL8 release. Inhibition of COX did not suppress IL-6 or CXCL8 release in BEAS-2Bs. These results show that the responses in BEAS2Bs are different from HPFs and are mediated through NF-κB, JNK, p38 MAP kinase, but not COX signaling and suggest JNK signaling to be the dominant pathway.

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Infection with human RV16 leads to greater AA-induced cytokine release.

To ensure that our results with pathogen components are reflective of a live infection, we next assessed if the innate immune response to RV was modulated by AA. RV was chosen due to its high prevalence in the community and has been shown to be a major cause of asthma exacerbations (8). Challenge with AA (100µM) in combination with RV16 infection at MOI of 1.0 resulted in substantially greater IL-6 and CXCL8 ($p<0.05$) release than AA or RV alone from HPFs ($n = 9$) (*Figure 9A and 9B*) and BEAS2Bs ($n = 7$) (replicates) (*Figure 9C and 9D*). The effect of the combination of AA with RV16 on IL-6 and CXCL8 release was greater than the sum of the individual effects of AA and RV16 in both cell types, demonstrating synergistic effects.

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Discussion

Obese asthma patients have more frequent and severe exacerbations, which may be a result of excess dietary fatty acids enhancing the innate immune response to viral and/or bacterial infection. This study is the first to examine the effects of dietary fatty acids in this context.

This study demonstrates that the ω-6 PUFA AA in combination with the viral mimic PolyI:C results in greater IL-6 and CXCL8 release than either AA or PolyI:C alone. Interestingly, the effect of the combination on cytokine release was substantially greater than the sum of the individual effects of AA and PolyI:C which indicates that these effects are synergistic. We also examined the effects of dietary fatty acids on respiratory infection in epithelial cells. Interestingly, we found that epithelial cells are unresponsive to AA alone, but the combination with PolyI:C results in synergistic cytokine release. In addition, PA enhanced PolyI:C-induced cytokine release from epithelial cells. These results show that dietary fatty acids have different effects on different lung cells and suggest that increased levels of AA and PA during viral infection may lead to more severe airway inflammation.

The current study mainly focussed on the cytokines IL-6 and CXCL8, as these are crucial drivers of neutrophilic inflammation and are clinically important in both the pathogenesis of asthma and clinical outcomes in severe and obese asthma, including viral-induced exacerbations. However multiple cytokines and chemokines are important in driving granulocyte recruitment and activation in asthma. We also measured CCL5 (RANTES) and GM-CSF release. There was no induction of GM-CSF in both cell types. However, DHA reduced PolyI:C-induced RANTES in HPFs and BEAS2Bs. In addition, AA reduced PolyI:C-induced RANTES in fibroblasts, but not BEAS2Bs. We interpret these results as further evidence that the immune response in obese asthma is skewed towards neutrophilic inflammation. If there was GM-CSF induction, this would have been evidence of a granulocytic response, since it promotes the proliferation, differentiation, and activation of monocytes, neutrophils, eosinophils and dendritic cells and act as a cofactor for superoxide production and degranulation (35, 36). The suppression of CCL5 (RANTES) by AA further reinforces neutrophilic inflammation as it typically recruits monocytes, T cells, and eosinophils and has been

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associated with eosinophilic airway inflammation in asthma (37, 38).

RV is the most common cause of virus-induced exacerbations in both children and adults with asthma (8, 39). In the past, RV was considered as an upper respiratory pathogen only. However, *in vivo* studies have conclusively shown that RV can also replicate in the lower airways (26, 40) and can infect submucosal cells which include pulmonary fibroblasts and airway smooth muscle cells. We (41) and others (42) have shown that RV infects primary pulmonary fibroblasts inducing pro-inflammatory mediators including IL-6 and CXCL8 (41). Fibroblasts are located within the airway submucosa where airway blood vessels are found and are therefore directly exposed to constituents of tissue fluids (plasma) including dietary fatty acids, and are likely to be key cells in driving inflammatory responses to serum derived factors. As such, this study primarily focussed on pulmonary fibroblasts.

Viruses activate the innate immune response through activation of the molecular pattern recognition TLR 3,7 and 8 (43) via the activation of specific transcription factors including NF-κB and AP-1. PolyI:C is a synthetic analogue of double stranded RNA (dsRNA) and is known to activate TLR3 (44). TLR 7 and 8 detect ssRNA, while TLR 3 detects dsRNA which occurs when ssRNA viruses, including RV, replicate (43). We used an agonist for TLR3 rather than TLR 7 or 8 based on previous studies showing that RV induces cytokines via the activation of TLR3 and not TLR 7/8 in bronchial epithelial cells. Furthermore, we have previously shown that RV-induced cytokine release in fibroblasts is replication dependent (i.e the cells are detecting and responding only to dsRNA)(41)..

To confirm that AA increases RV-induced inflammation, we also assessed the response to AA in combination with RV16 infection. We found that challenge with AA in combination with RV16 infection results in substantially greater cytokine release than either AA or RV16 alone in HPFs and BEAS-2Bs.

Less is known about bacterial infections in asthma. Some bacterial pathogens are more frequently found in the airways of patients with asthma than in healthy patients (12), but their role in exacerbations is unclear. Studies have reported mycoplasmal infection in up to 25% of children with

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wheezing (11) and in 20% of asthmatic children requiring hospitalization due to exacerbations. However, not all studies confirm these findings (45). The current study also investigated the effect of dietary fatty acids on bacterial infection. To model bacterial infection, we challenged cells with the bacterial endotoxin LTA. We found that challenge with AA in combination with LTA results in greater IL-6 and CXCL8 release from pulmonary fibroblasts and epithelial cells than either alone. The effect of the combination on cytokine release was substantially greater than the sum of the individual effects of AA and LTA which indicates that these effects are synergistic. These results indicate that exposure to AA during bacterial infection may lead to more severe airway inflammation. There was no interaction between the other dietary fatty acids and LTA. Bacterial recognition is dependent on TLR 2 and 4. TLR4 mainly senses lipopolysaccharide (LPS), which is a major component of the outer membrane of gram-negative bacteria. TLR2 is the primary innate immune receptor for gram-positive bacteria. We used LTA, an important cell wall polymer found in gram-positive bacteria that has been shown to cause innate immune responses mediated through TLR2 (46).

LTA alone however, did not induce cytokine release from either HPFs or BEAS2Bs. Presumably, even though bronchial epithelial cells express toll like receptor (TLR)2, challenge with LTA is not potent enough to induce the production of IL-6 or CXCL8 in epithelial cells and a co-challenge is needed. Other studies have reported similar findings. Amstrong *et al.* (2004) found that LTA alone does not induce the production of CXCL8 or IL-6 (47) in bronchial epithelial cells and a low responsiveness of lung epithelial cells to LTA (small increase in CXCL8 release) was observed in another *in vitro* study (48).

Effects of dietary fatty acids on immune responses have been an area of interest for many years. However, there is a great deal of conflicting data. DHA and AA serve as important cell membrane components as well as precursors for biologic mediators with many effects, including numerous roles in immune function and inflammation. In general, ω-6 PUFAs and SFAs have predominantly been associated with pro-inflammatory effects, whereas the ω-3 PUFAs are associated with anti-inflammatory and immunosuppressive effects (25, 34). None of the ω-3 PUFAs (DHA, EPA and ALA)

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suppressed PolyI:C-induced cytokine release, or combined AA and PolyI:C-induced inflammatory responses in HPFs, suggesting that ω-3 PUFAs do not have anti-inflammatory effects in these lung cells. SFAs including PA initiate innate immune responses through activation of TLR2 and 4 in adipocytes and macrophages (24, 49, 50). These results have been replicated in human studies where within 4 hours of consumption of a high fat meal, innate immune responses are activated with increased TLR2, -4 and NF-κB activity in mononuclear and polymorphonuclear cells (51, 52). The non-responsiveness to PA in this study may be explained by the lack of functional TLR4 signaling in pulmonary fibroblasts. Lung mesenchymal cells do not express CD14, which acts as a co-receptor for TLR4 (53, 54). This is also the reason why we used LTA (TLR2 agonist) and not LPS (TLR4 agonist). SFAs have been shown to inhibit virus replication in a mouse model of chronic hepatitis B infection (55), however another mouse model study showed that SFAs increase bacterial load in *S. aureus* infection (56). Studies looking at effects of ω-6 PUFAs on inflammatory processes and infections have found conflicting data. A study in healthy men found that supplementation with ω-6 PUFAs significantly increased the number of circulating neutrophils and production of LPS-induced leukotriene B4 from leukocytes. Conversely, supplementation with AA did not affect *in vitro* secretion of TNFα by peripheral mononuclear cells, nor did it affect peripheral blood mononuclear cell proliferation and natural killer cell activity (57). A study by Jordao *et al.* (2008) found that AA enhanced bacterial killing of *Mycobacterium tuberculosis* in macrophages, but increased pathogen survival in a mouse model of tuberculosis (58). In the current study a clear pro-inflammatory effect of AA on bacterial and viral infection was observed.

To understand the underlying mechanisms involved in (synergistic) AA and PolyI:C- or LTA-induced IL-6 and CXCL8 production, signaling pathways were investigated. We observed activation of p38 MAPK upon challenge with AA alone and in combination with PolyI:C or LTA. Challenge with PolyI:C and LTA alone led to increased phosphorylation of NF-κB, while challenge with the combination of AA and PolyI:C caused increased phosphorylation of SAPK/JNK. AA in combination with PolyI:C led to greater phosphorylation of p38 MAP kinase than challenge with AA alone, indicating p38 MAP kinase

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signaling to be the mechanism by which synergism occurs. We further investigated and confirmed the underlying mechanisms involved in AA and PolyI:C- or LTA-induced IL-6 and CXCL8 release using specific signaling inhibitors at concentrations previously shown to be effective in human airway cells (26-28). SB239063 is a potent and selective inhibitor of p38 MAP kinase and displays specific and high-affinity binding (IC50 = 44nM) (59). It suppressed IL-6 and CXLC8 release induced by AA alone and the combinations of AA and PolyI:C or LTA. These data suggest that AA in combination with PolyI:C or LTA activates the p38 MAPK pathway, leading to both IL-6 and CXCL8 release. Inhibition of JNK with SP600125 suppressed IL-6 release induced by PolyI:C alone and in combination with AA, but did not affect CXCL8 release. Inhibition of NF-κB with BAY- 117082 partially suppressed IL-6 release induced by the combination of AA and LTA, again indicating the involvement of multiple pathways. These results, which showed that BAY-117082 suppressed AA and LTA-induced IL-6 but not CXCL8 release while SP600125 suppressed PolyI:C- and AA/PolyI:C-induced IL-6 but not CXCL8 release, are unexpected. Previous studies have shown IL-6 and CXCL8 transcription to be regulated by the same transcription factors: NF-κB, CREB protein, AP-1, and CCAAT/enhancer binding protein (C/EBP) (60, 61). However, it appears that in this study the dominant transcription factors regulating CXCL8 are different to those regulating IL-6 with p38 MAPK being the only common transcription factor for both cytokines.

Prostaglandins (PGs), including PGE_2, are COX metabolites of arachidonic acid. PGE_2 induces IL-6 release from bronchial epithelial cells and CXCL8 from lung mesenchymal cells (62, 63). To investigate whether COX-mediated prostaglandins contribute to IL-6 and CXCL8 release, we pre-treated pulmonary fibroblasts with indomethacin, which inhibits both COX-1 and COX-2. Indomethacin inhibited AA-induced IL-6 and CXCL8 release alone and in combination with PolyI:C or LTA. However, indomethacin did not affect cytokine release induced by PolyI:C alone. Our data suggest that IL-6 and CXCL8 production in HPFs is prostaglandin-mediated. We consider these effects to be mediated through COX-2 rather than COX-1, as COX-1 is responsible for constitutive production under basal conditions, whereas COX-2 is upregulated during inflammation and is

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responsible for PGE_2 biosynthesis at sites of inflammation (64). We consider the combination of the production of COX-mediated prostaglandins and the activation of transcription factors the most logical mechanism for synergistic cytokine release in HPFs.

We also investigated the underlying mechanisms in BEAS-2Bs and found that p38 MAP kinase signaling is involved in PolyI:C-, combined PolyI:C and AA- and combined LTA and AA-induced IL-6 and CXCL8 release in BEAS-2Bs. However, inhibition of COX did not suppress IL-6 or CXCL8 release in BEAS-2Bs, which is different from what we observed in fibroblasts. This is consistent with the lack of cytokine induction by AA in BEAS-2B. In addition, inhibition of JNK signaling resulted in the greatest suppression of combined AA and PolyI:C- or LTA-induced IL-6 and CXCL8 release in BEAS-2BS. These results suggest that there is a differential response in BEAS-2Bs compared to fibroblasts. Although the studies in this manuscript utilized primary human pulmonary fibroblasts, an important limitation of this study is that all studies were done *in vitro.* In future studies effects of dietary fatty acids on immune responses to infection will be investigated using an *in vivo* model.

In summary, this study demonstrates that exposure of HPFs and epithelial cells to ω-6 PUFAs causes an amplification of the inflammatory responses to viral and bacterial components, as measured by IL-6 and CXCL8 release. In HPFs the responses were prostaglandin dependent and mediated through p38 MAP kinase signaling, whereas the responses in BEAS-2Bs were mainly mediated through JNK and p38 MAP kinase signaling, suggesting that p38 MAPK inhibitors might be effective in obese asthmatics to prevent exacerbations. In epithelial cells exposure to PA also enhances the inflammatory response to viral infection. These results suggest that during respiratory infection, increased levels of dietary ω-6 PUFAs and SFAs may lead to more severe airway inflammation and might contribute to and/or increase the severity of asthma exacerbations in obese asthma patients.

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List of abbreviations

AA - arachidonic acid

ANOVA - Analysis of variance

BMI - body mass index

COX - cyclooxygenase

CCL5 - C-C Motif Ligand-5 (RANTES)

CXCL8 - chemokine (C-X-C motif) ligand 8

DHA - docosahexaenoic acid

GM-CSF - granulocyte-macrophage colony-stimulating factor

HPFs - human pulmonary fibroblasts

IL-6 - interleukin 6

LOX - lipoxygenase

LTA - lipoteichoic acid

MOI - multiplicity of infection

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

p38 MAPK - p38 mitogen-activated protein kinase

PA - palmitic acid

PGE2 - prostaglandin E2

PolyI:C - polyinosinic:polycytidylic acid

PUFA - polyunsaturated fatty acid

RV16 - human RV serotype-16

SAPK/JNK - stress-activated protein kinases/Jun amino-terminal kinases

SEM - standard error of the mean

SFA - saturated fatty acid

TLR - toll-like receptor

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Table 1. Summary of patient demographics

Donor #	Diagnosis	Age	Gender	Surgery	Experiment
1	Emphysema	61	F	explanted lung	fatty acids and bacterial/viral mimics
3	BOS	43	M	explanted lung	fatty acids and bacterial/viral mimics
4	COPD	60	F	explanted lung	fatty acids and bacterial/viral mimics
5	NSCLC	62	F	lung resection	fatty acids and bacterial/viral mimics
6	Ca adeno	60	F	lung resection	fatty acids and bacterial/viral mimics
7	Emphysema	65	F	explanted lung	fatty acids and bacterial/viral mimics
8	Healthy	65	M	explanted lung	fatty acids and bacterial/viral mimics
9	Healthy	41	F	explanted lung	fatty acids and bacterial/viral mimics
10	COPD	65	M	explanted lung	fatty acids and bacterial/viral mimics
11	Adeno ca	72	F	lung resection	fatty acids and bacterial/viral mimics
12	COPD	61	F	explanted lung	western blotting, inhibitors, fatty acids and bacterial/viral mimics
13	COPD	62	F	explanted lung	western blotting, inhibitors
14	Emphysema	65	F	explanted lung	western blotting, Inhibitors
15	Adeno ca	57	F	lung resection	western blotting, inhibitors
16	PAH	57	F	explanted lung	western blotting, inhibitors
17	IPF	67	M	explanted lung	western blotting
18	Adeno ca	76	F	lung resection	western blotting, inhibitors, RV16 infection
19	Adeno ca	64	F	lung resection	western blotting, inhibitors, RV16 infection
20	IPF	63	M	explanted lung	western blotting, inhibitors, RV16 infection
21	Emphysema	59	M	explanted lung	western blotting, inhibitors, RV16 infection
22	PAH	57	F	explanted lung	western blotting, inhibitors, RV16 infection
23	COPD	62	F	explanted lung	western blotting, inhibitors, RV16 infection
24	PAH	30	F	explanted lung	western blotting, inhibitors, RV16 infection
25	Emphysema	62	F	explanted lung	Inhibitors
26	Emphysema	59	M	explanted lung	Inhibitors
27	COPD	56	F	explanted lung	inhibitors
28	IPF	58	F	explanted lung	inhibitors
29	Emphysema	64	M	explanted lung	inhibitors
30	NSCLC, COPD	58	M	lung resection	inhibitors
31	IPF	64	M	explanted lung	inhibitors, RV16 infection
32	Emphysema	61	M	explanted lung	inhibitors, RV16 infection
33	COPD	69	F	explanted lung	mixed ω-6:ω-3 PUFAs, ALA and EPA
34	Interstitial pneumonitis	59	M	explanted lung	mixed ω-6:ω-3 PUFAs, ALA and EPA
35	IPF	64	M	explanted lung	mixed ω-6:ω-3 PUFAs, ALA and EPA
36	NSCLC	71	F	lung resection	mixed ω-6:ω-3 PUFAs, ALA and EPA
37	Adeno ca and COPD	75	F	lung resection	mixed ω-6:ω-3 PUFAs, ALA and EPA
38	IPF	63	F	explanted lung	mixed ω-6:ω-3 PUFAs, ALA and EPA
39	Squamous cell ca	65	M	lung resection	mixed ω-6:ω-3 PUFAs, ALA and EPA
40	Adeno ca	72	F	lung resection	mixed ω-6:ω-3 PUFAs, ALA and EPA
41	IPF	54	M	explanted lung	mixed ω-6:ω-3 PUFAs, ALA and EPA

BOS: Bronchiolitis obliterans syndrome, COPD: chronic obstructive pulmonary disease, Adeno ca: adenocarcinoma, NSCLC: non-small cell lung carcinoma, PAH: pulmonary arterial hypertension, IPF: idiopathic pulmonary fibrosis, F: Female, M: Male

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Figure legends

Figure 1. Greater cytokine release with combined arachidonic acid and PolyI:C challenge, than either alone Human primary pulmonary fibroblasts were unstimulated or challenged with ω-6 polyunsaturated fatty acid (PUFA) arachidonic acid (AA) (*n* = 8-11) (patients) (A-C), ω-3 PUFA docosahexaenoic acid (DHA), (*n* = 5-9) (patients) (D-F) or saturated fatty acid (SFA) palmitic acid (PA), (*n* = 3-9) (patients) (G-I) in 0.1% BSA-DMEM (100µM) for 4h with or without the viral mimic polyinosinic:polycytidylic acid (PolyI:C) (10µg/ml) for another 24h. Cell free supernatants were collected and IL-6 (A, D, G), CXCL8 (B, E, H) or CCL5 (C, F, I) release was measured using ELISA. All data are represented as mean ± standard error of the mean. All challenges are compared to control and challenges with PolyI:C are compared to their respective challenge without PolyI:C and challenge with PolyI:C alone, using a one-way ANOVA with a Bonferroni post-test. Significance is represented as * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) or **** ($p<0.0001$).

Figure 2. Greater cytokine release with combined arachidonic acid and LTA challenge, than either alone in Human primary pulmonary fibroblasts were unstimulated (control) or challenged with ω-6 polyunsaturated fatty acid (PUFA) arachidonic acid (AA), (*n* = 11) (patients) (A, B), ω-3 PUFA docosahexaenoic acid (DHA), (*n* = 10) (patients) (C, D) or saturated fatty acid (SFA) palmitic acid (PA), (*n* = 9) (patients) (E, F) in 0.1% BSA-DMEM (100µM) for 4h prior with or without the bacterial compound lipoteichoic acid (LTA) (10µg/ml) for another 24h. Cell free supernatants were collected and IL-6 (A, C, E) and CXCL8 (B, D, F) release was measured using ELISA. All data are represented as mean ± standard error of the mean. All challenges are compared to control and challenges with LTA are compared to their respective challenge without LTA and challenge with LTA alone, using a one-way ANOVA with a Bonferroni post-test. Significance is represented as * ($p<0.05$) or *** ($p<0.001$).

Figure 3. Greater IL-6 release with combined arachidonic acid or palmitic acid and PolyI:C challenge than either alone in BEAS-2Bs. The human bronchial epithelial cell line BEAS-2B was unstimulated (control) or challenged with ω-6 polyunsaturated fatty acid (PUFA) arachidonic acid (AA) (*n* = 6-7) (replicates) (A-C), ω-3 PUFA docosahexaenoic acid (DHA) (*n* = 6-7) (replicates) (D-F) or saturated fatty acid (SFA) palmitic acid (PA) (*n* = 6-7) (replicates) (G-I) in 0.1% BSA-DMEM (100µM) for 4h prior with or without the viral mimic polyinosinic:polycytidylic acid (PolyI:C) (10µg/ml) for another 24h. Cell free supernatants were collected and IL-6 (A, D, G), CXCL8 (B, E, H) and CCL5 (C, F, I) release was measured using ELISA. All data are represented as mean ± standard error of the mean. All challenges are compared to control and challenges with PolyI:C are compared to their respective challenge without PolyI:C and challenge with PolyI:C alone, using a one-way ANOVA with a Bonferroni post-test. Significance is represented as * ($p<0.05$), ** ($p<0.01$) or *** ($p<0.001$).

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Figure 4. Greater cytokine release with combined arachidonic acid and LTA challenge, than either alone in BEAS-2Bs. The human bronchial epithelial cell line BEAS-2B was unstimulated (control) or challenged with ω-6 polyunsaturated fatty acid (PUFA) arachidonic acid (AA) (*n* = 6) (replicates) (A,B), ω-3 PUFA docosahexaenoic acid (DHA) (*n* = 6) (replicates) (C, D) or saturated fatty acid (SFA) palmitic acid (PA) (*n* = 6) (replicates) (E, F) in 0.1% BSA-DMEM (100µM) for 4h prior with or without the bacterial compound lipoteichoic acid (LTA) (10µg/ml) for another 24h. Cell free supernatants were collected and IL-6 (A, C, E) and CXCL8 (B, D, F) release was measured using ELISA. All data are represented as mean ± standard error of the mean. All challenges are compared to control and challenges with LTA are compared to their respective challenge without LTA and challenge with LTA alone, using a one-way ANOVA with a Bonferroni post-test. Significance is represented as * ($p<0.05$) or ** ($p<0.01$).

Figure 5. Activation of p38 MAP kinase, NF-κB and SAPK/JNK upon challenge with AA and PolyI:C or LTA. Primary human pulmonary fibroblasts (*n* = 7-8) (patients) were unstimulated (control) or challenged with arachidonic acid (AA) (100µM), the viral mimic polyinosinic:polycytidylic acid (PolyI:C) (10µg/ml), the bacterial compound lipoteichoic acid (LTA) (10µg/ml), AA (100µM) in combination with (PolyI:C) (1ng/mL) or AA (100µ) in combination with LTA for 30 minutes, before whole cell lysates were collected and p38 mitogen-activated protein (MAP) kinase (A), NF-κB p65 (B) or JNK (C) phosphorylation was assessed by western blotting. Total p38 MAP kinase (D), NF-κB p65 (E) or JNK (F) was also assessed. All values were normalized to GAPDH (housekeeping protein), detected on the same blots. Data are expressed as fold increase of control, mean ± standard error of the mean. All challenges are compared to control and challenges with PolyI:C or LTA are compared to their respective challenge without PolyI:C or LTA and challenge with PolyI:C or LTA alone, using a one-way ANOVA (Fisher's LSD test). Significance is represented as *($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) or **** ($p<0.0001$). Representative western blots of phosphorylated and total p38 MAPK, NF-κB p65 and JNK are shown under each graph.

Figure 6. Inhibition of cyclooxygenase (COX) or p38 MAPK suppresses cytokine release, induced arachidonic acid alone and in combination with viral or bacterial surrogates from fibroblasts. Primary human pulmonary fibroblasts *(n = 9-14)* (patients) were treated with or without the cyclooxygenase (COX) inhibitor indomethacin (10^{-5}M), the p38 mitogen-activated protein (MAP) kinase signaling inhibitor SB239063 (3µM), the NF-κB inhibitor BAY-117082 (1µM) or the c-Jun N-terminal kinase (JNK) inhibitor SP600125 (10µM) for 60 minutes before challenge with arachidonic acid (AA) (100µM) (A, B), the viral mimic polyinosinic:polycytidylic acid (PolyI:C) (10µg/ml) (C, D), AA (100µM) in combination with PolyI:C (E, F) or AA (100µM) in combination with LTA (10µg/mL) (G, H). Cell free supernatants were collected after 24h and IL-6 (A, C, E, G) and CXCL8 (B, D, F, H) release was measured using ELISA. All data are represented % cytokine release ± standard error of the mean. All treatments with inhibitor are compared to their respective control in the absence of the inhibitor using one-way ANOVA with a Bonferroni post-test. Significance is represented as ** ($p<0.01$), *** ($p<0.001$) or **** ($p<0.0001$).

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Figure 7. Activation of p38 MAP kinase, NF-κB and SAPK/JNK upon challenge with AA and PolyI:C or LTA in BEAS-2Bs. The human bronchial epithelial cell line BEAS-2B (*n* = 4-8) (replicates) was unstimulated or challenged with arachidonic acid (AA) (100µM), the viral mimic polyinosinic:polycytidylic acid (PolyI:C) (10µg/ml), the bacterial compound lipoteichoic acid (LTA) (10µg/ml), AA (100µM) in combination with (PolyI:C) (1ng/mL) or AA (100µM) in combination with LTA for 30 minutes, before whole cell lysates were collected and p38 mitogen-activated protein (MAP) kinase (A), NF-κB p65 (B) or JNK (C) phosphorylation was assessed by western blotting. Total p38 MAP kinase (D), NF-κB p65 (E) or JNK (F) was also assessed. All values were normalized to GAPDH (housekeeping protein), detected on the same blots. Data are expressed as fold increase of control, mean ± standard error of the mean. All challenges are compared to control and challenges with PolyI:C or LTA are compared to their respective challenge without PolyI:C or LTA and challenge with PolyI:C or LTA alone, using a one-way ANOVA (Fisher's LSD test). Significance is represented as *(p<0.05) or ** (p<0.01). Representative western blots of phosphorylated and total p38 MAPK, NF-κB p65 and JNK are shown under each graph.

Figure 8. Inhibition p38 MAPK or JNK suppresses cytokine release, induced by AA in combination with PolyI:C or LTA from BEAS-2Bs. The human bronchial epithelial cell line BEAS-2B (*n* = 7) (replicates) was treated with or without the cyclooxygenase (COX) inhibitor indomethacin (10^{-5}M), the p38 mitogen-activated protein (MAP) kinase signaling inhibitor SB239063 (3µM), the NF-κB inhibitor BAY-117082 (1µM) or the c-Jun N-terminal kinase (JNK) inhibitor SP600125 (10µM) for 60 minutes before challenge with AA (100µM) in combination with the viral mimic PolyI:C (A, B), PolyI:C alone (C, D) or AA (100µM) in combination with LTA (10µg/mL) (E, F). Cell free supernatants were collected after 24h and IL-6 (A, C, E) and CXCL8 (B, D, F) release was measured using ELISA. All data are represented as % cytokine release ± standard error of the mean. All treatments with inhibitor are compared to their respective control in the absence of the inhibitor using one-way ANOVA with a Bonferroni post-test. Significance is represented as *(p<0.05) and ** (p<0.01) or *** (p<0.001) or **** (p<0.0001).

Figure 9. Greater cytokine release with combined arachidonic acid and human rhinovirus 16 challenge, than either alone in human pulmonary fibroblasts and BEAS-2Bs. Human primary pulmonary fibroblasts (*n* = 9) (patients) **(A, B)** or BEAS-2Bs (*n = 7)* (replicates) **(C, D)** were unstimulated (control) or challenged with arachidonic acid (AA) in 0.1% BSA-DMEM (100µM) for 4h prior to infection with human rhinovirus serotype-16 (RV16) at a multiplicity of infection (MOI) of 1 for another 24h. Cell free supernatants were collected and IL-6 **(A, C)** and CXCL8 **(B, D)** release was measured using ELISA. All data are represented as mean ± standard error of the mean. All challenges RV16 are compared to their respective challenge without RV16 and RV16 alone, using a one-way ANOVA with a Bonferroni post-test. Significance is represented as * (p<0.05), ** (p<0.01) or *** (p<0.001).

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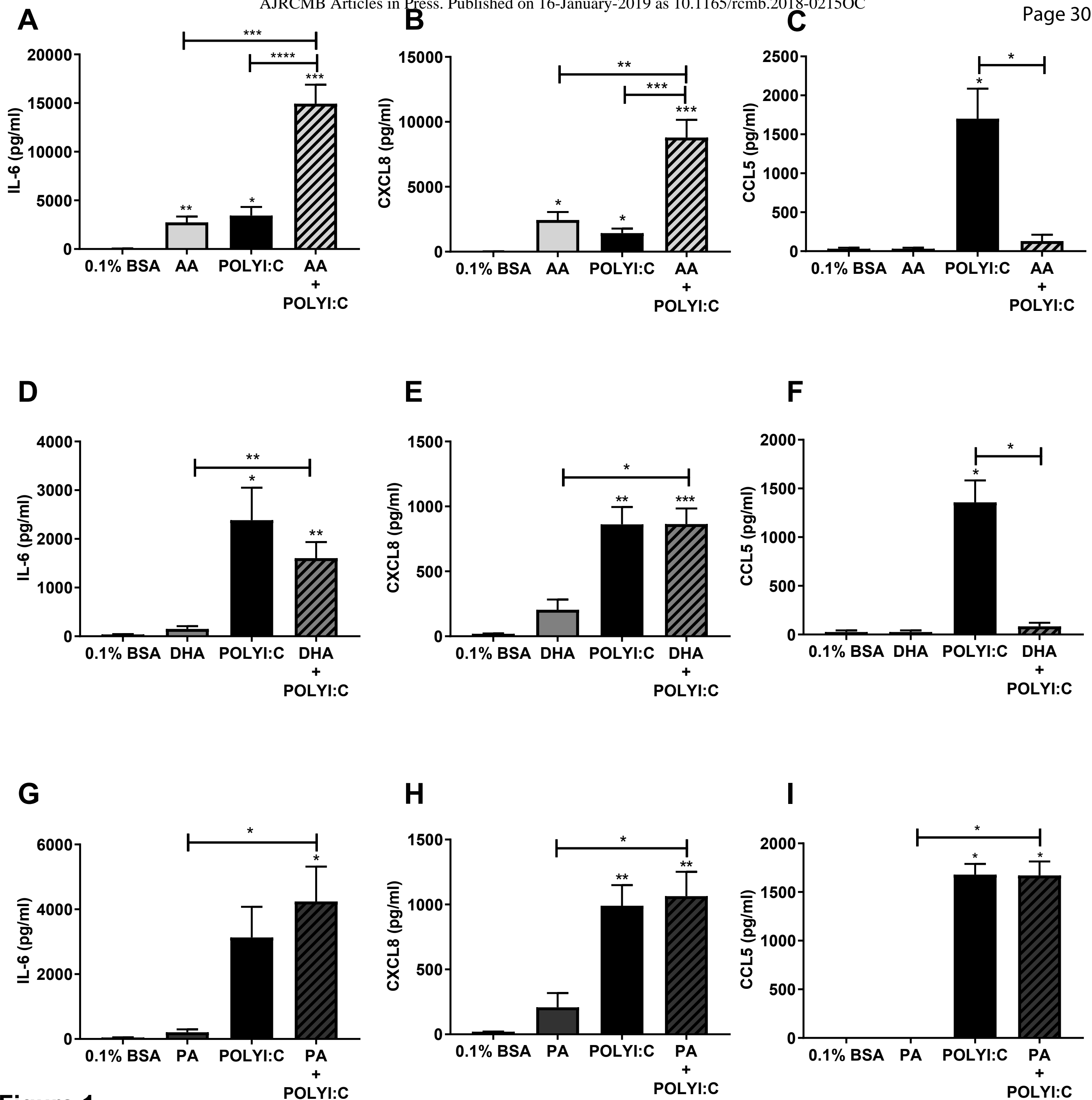

Figure 1

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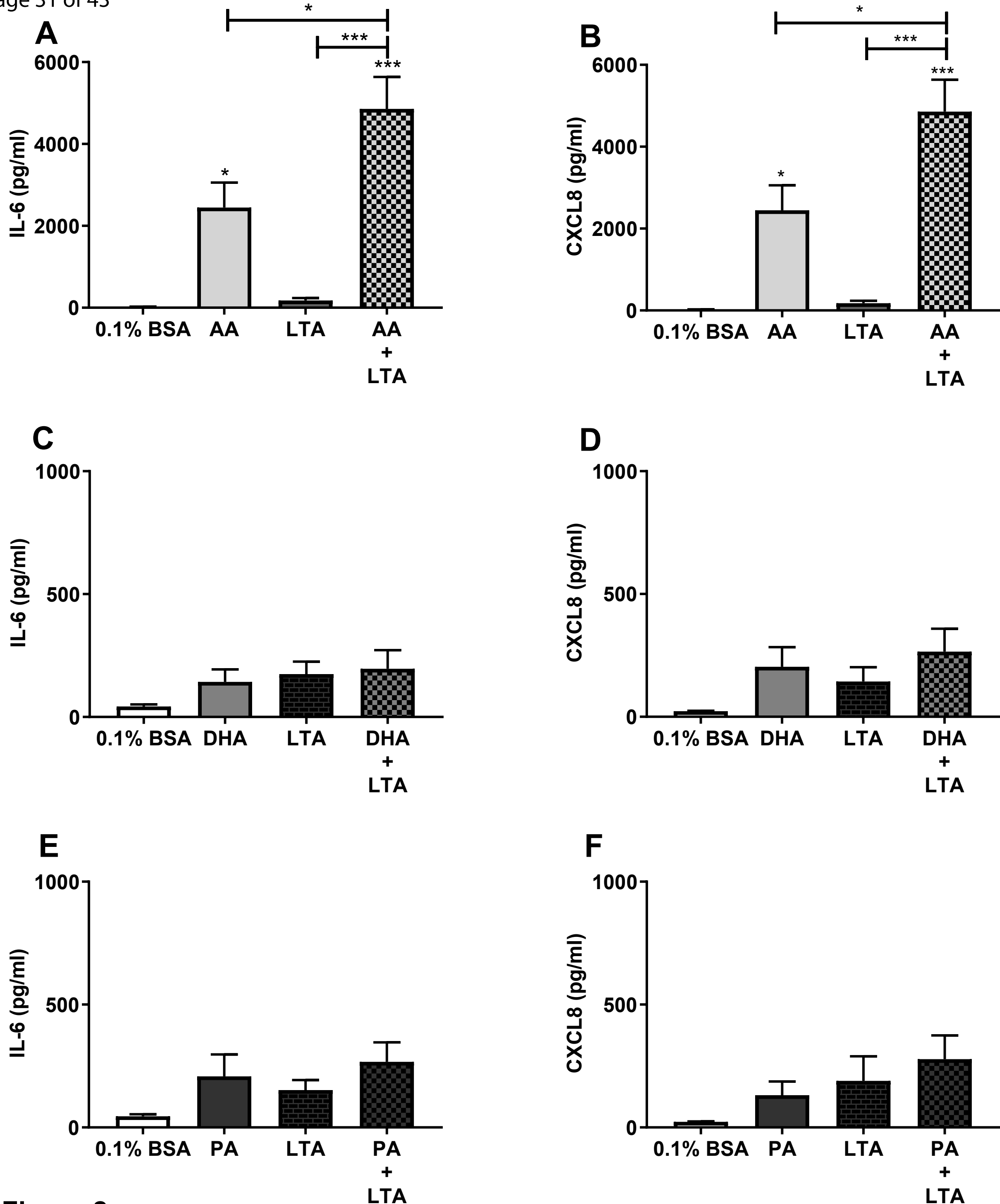

Figure 2

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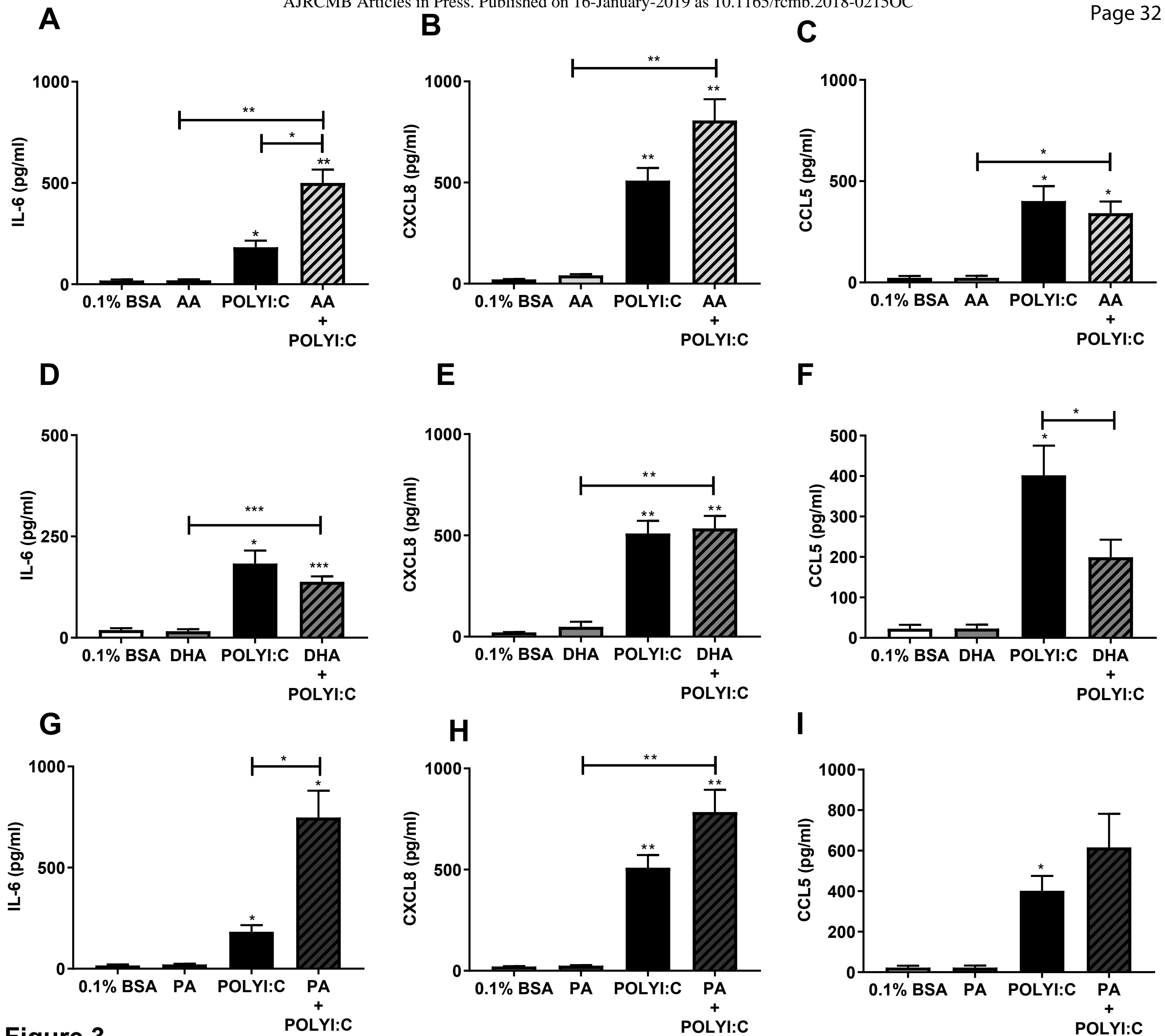

Figure 3

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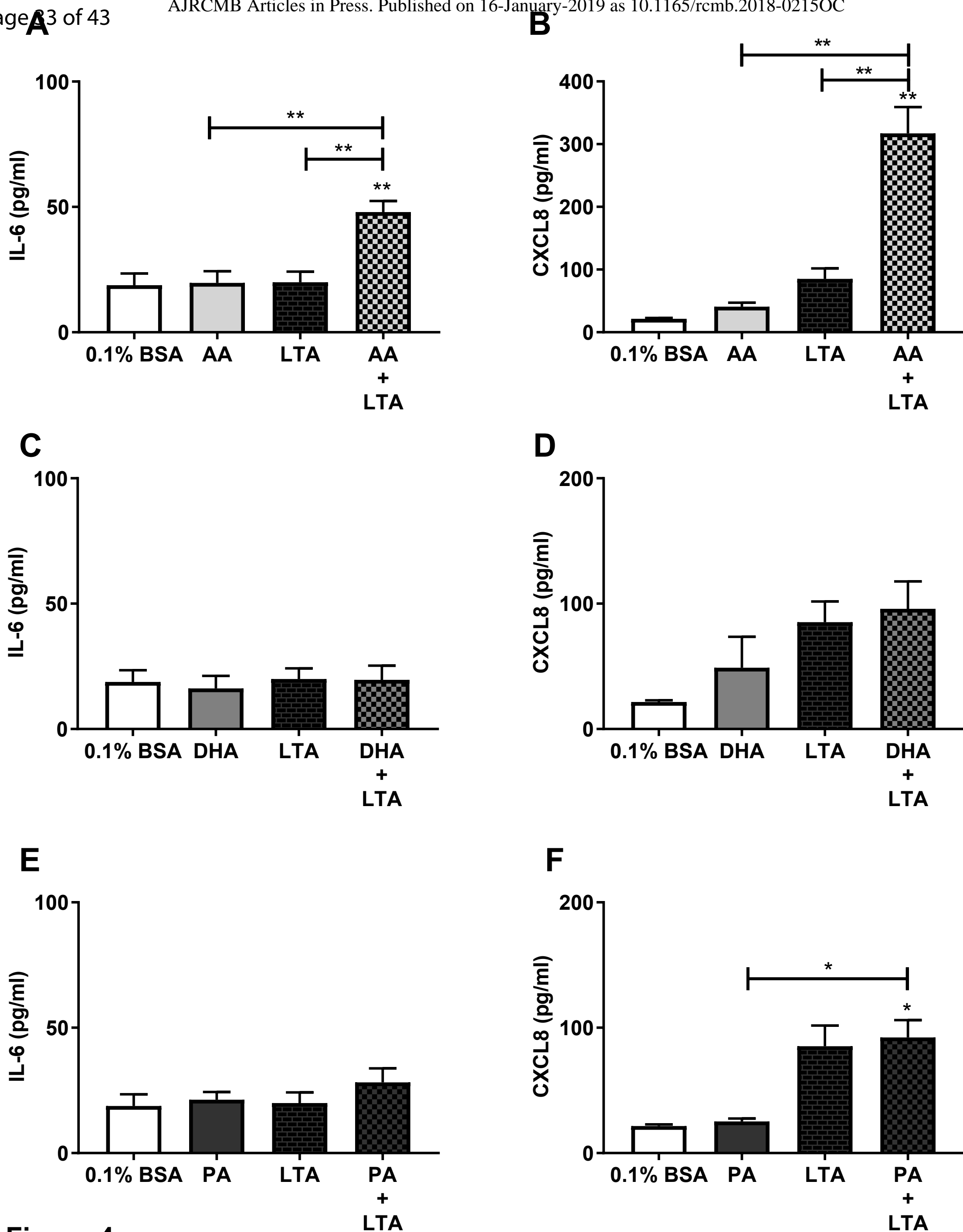

Figure 4

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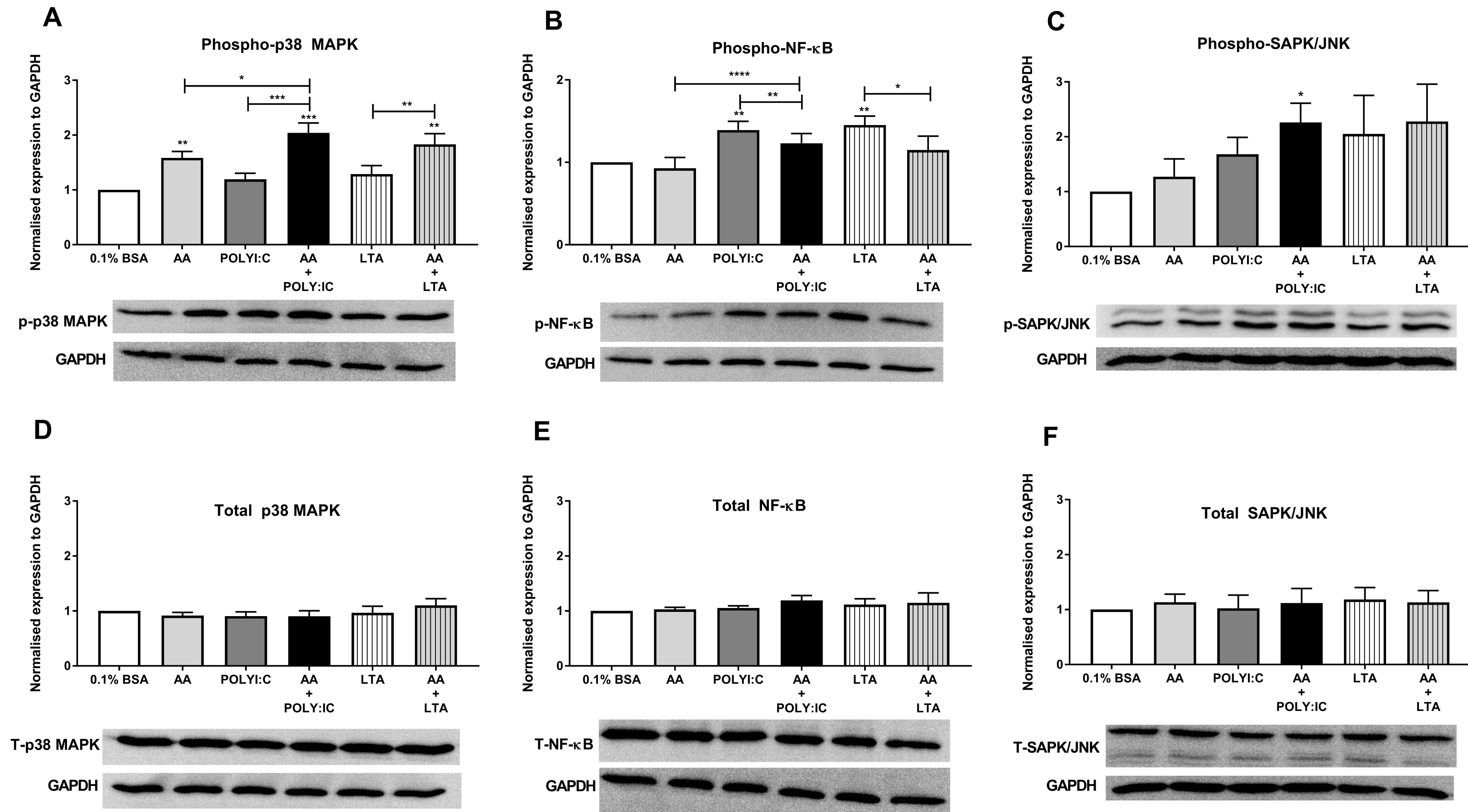

Figure 5

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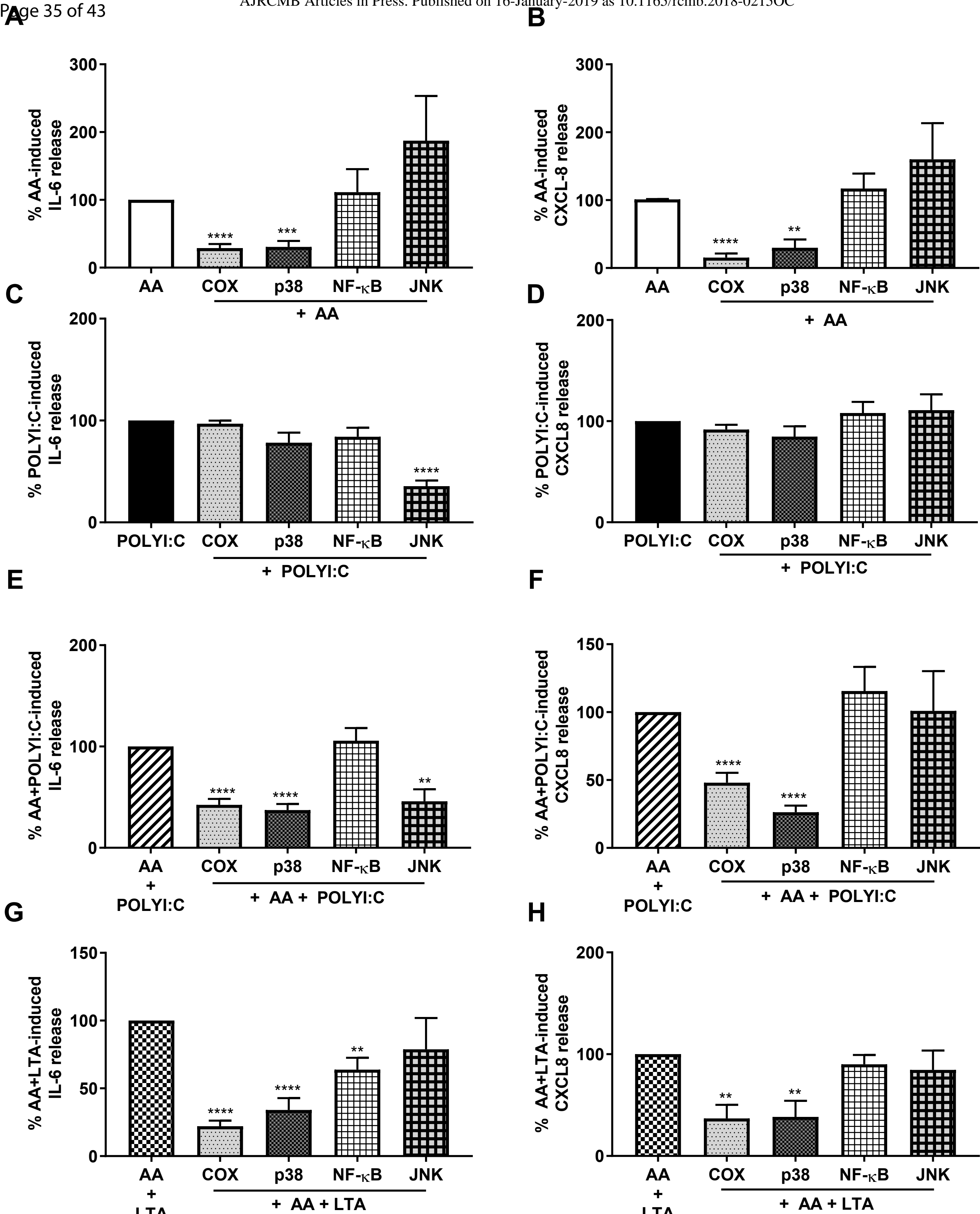

Figure 6

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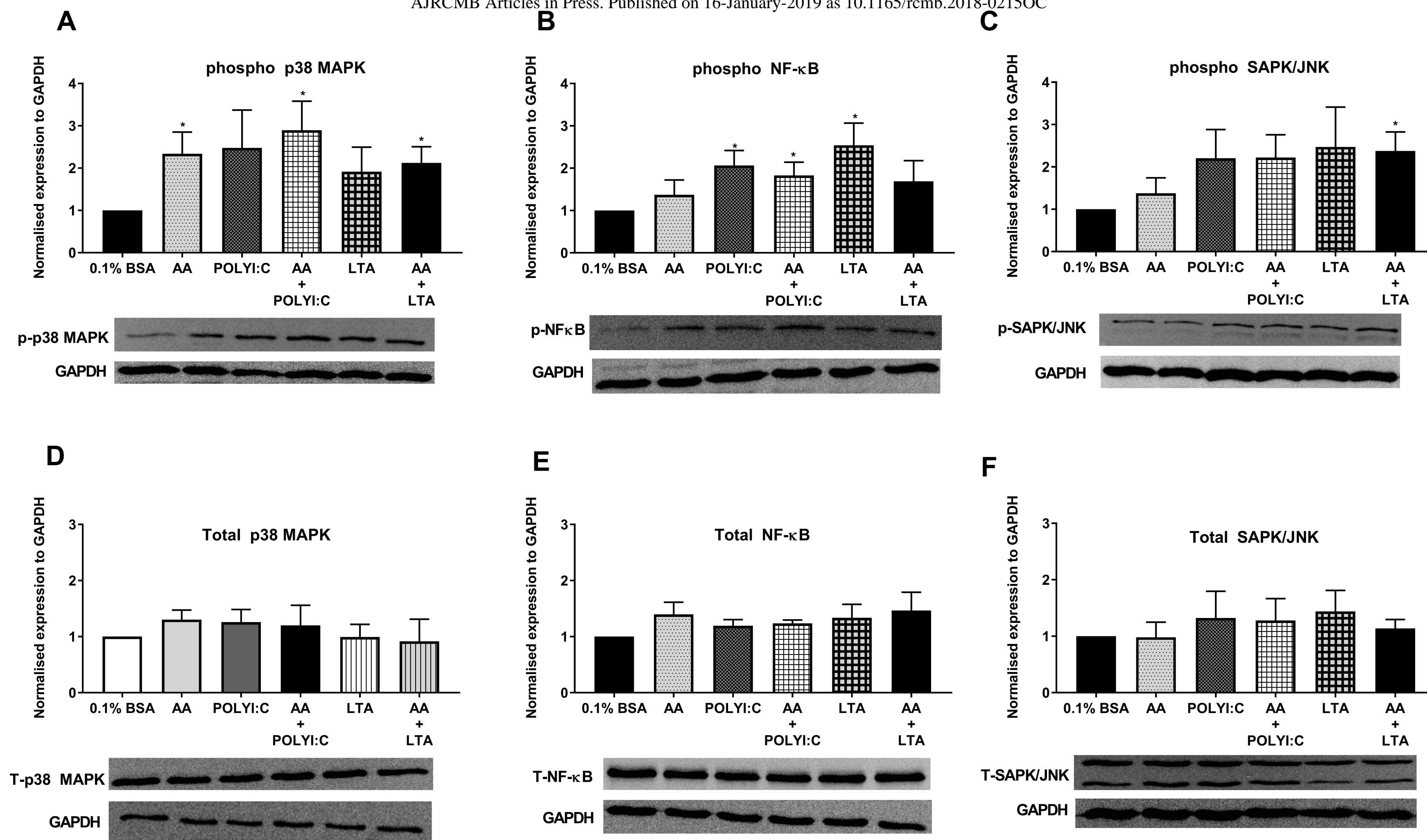

Figure 7

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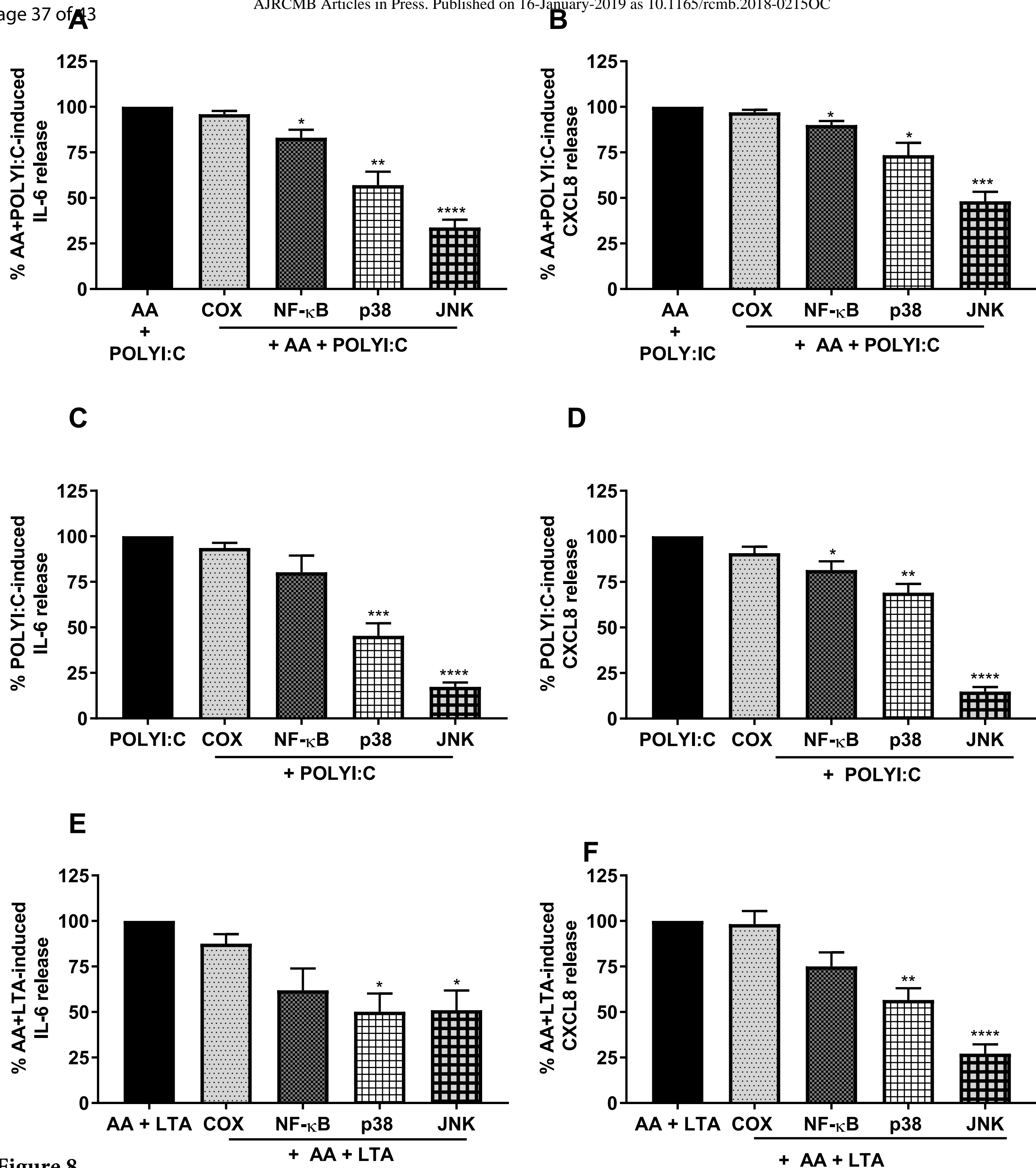

Figure 8

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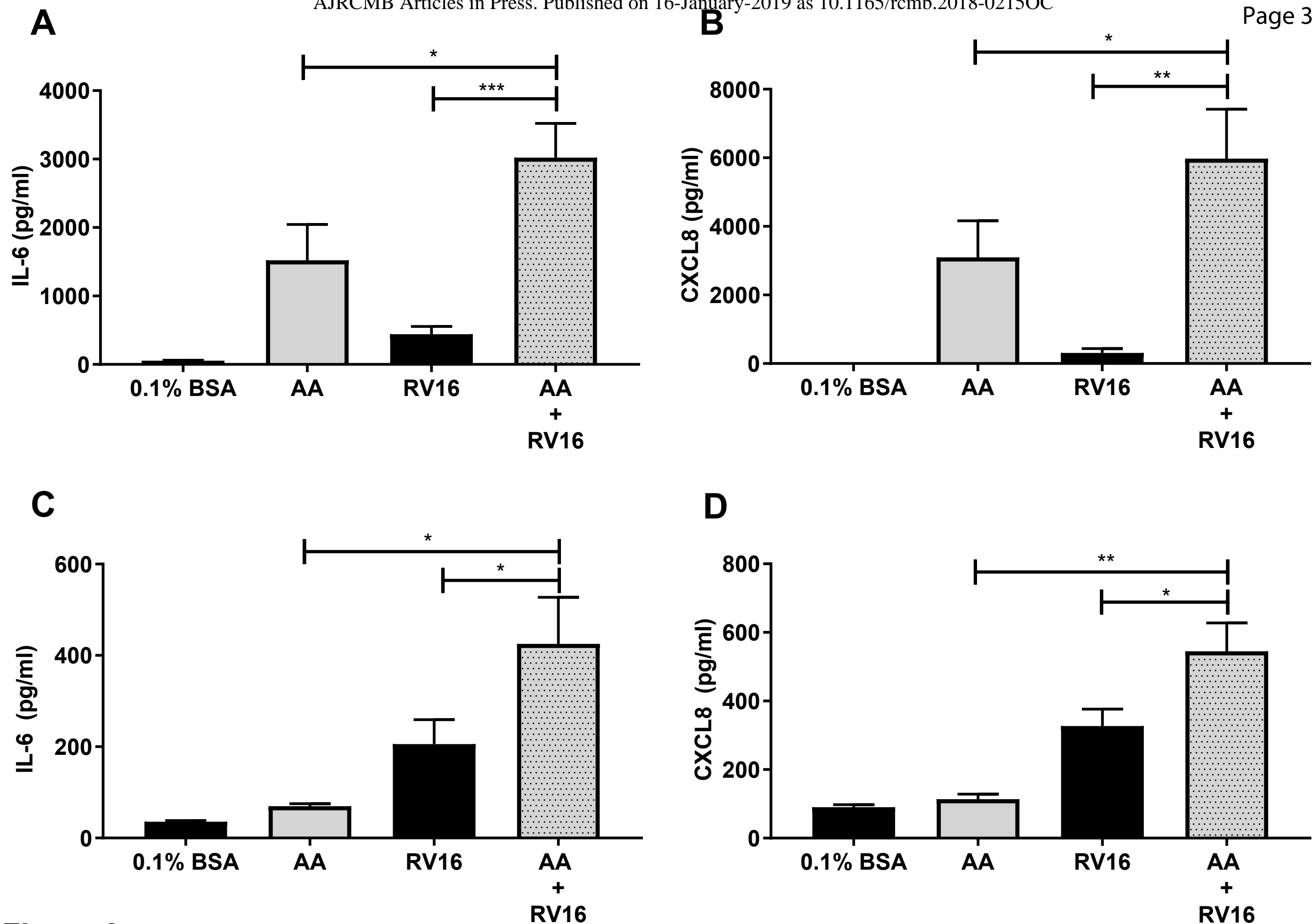

Figure 9

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ONLINE SUPPLEMENT

Dietary fatty acids amplify inflammatory responses to infection through p38 MAP kinase signaling

Sandra Rutting, Razia Zakarya, Jack Bozier, Dia Xenaki, Jay C. Horvat, Lisa G. Wood, Philip M. Hansbro, Brian G. Oliver

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Supplementary Methods

Cell culture HPFs and BEAS-2Bs

HPFs were seeded in 12-well plates at a density of 6.2 x 10^4 cells/mL in DMEM containing 5% fetal bovine serum (FBS) and 1% Antibiotic-Antimycotic (Gibco, Grand Island, New York, US) and grown to sub confluence (3 days). The human bronchial epithelial cell line BEAS-2B was maintained in 10% FBS and 1% Antibiotic-Antimycotic in DMEM. BEAS-2Bs were seeded at a density of 1 x 10^5cells/mL in 12-well plates and grown for 24 hours. Both cell types were quiesced for 24 hours prior to stimulation, by incubation in DMEM supplemented with 0.1% bovine serum albumin (BSA) (Sigma Aldrich, Castle Hill, NSW, Australia) and 1% Antibiotic-Antimycotic. All experiments were carried out using fibroblasts between passage 2 and 6.

Western blotting

To investigate the cell signalling pathways activated after challenge with the combinations of AA and POLYI:C or LTA, relative levels of phosphorylated p38 mitogen-activated protein (MAP) kinase, NF-κB and stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) from cell lysates were assessed by western blotting. Cells cultured in the presence or absence of AA (100μM) with or without POLYI:C (10μg/ml) or LTA (10μg/ml) for 30 min were lysed (20mM Tris, pH 7.4, 150mM NaCl, 1mM Na_2EDTA, 1mM EGTA, 20mM $NA_4P_2P_7$, 2mM Na_3VO_4, 1% Triton X- 100, 10% glycerol, 0.1% SDS, 0.5% sodium deoxycholate, 1% protease inhibitor cocktail set III (Millipore, USA) and 1mM phenylmethylsulfonyl fluoride (PMSF) (Amresco, Solon, OH, USA)). Cell lysates were separated by SDS/polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels and transferred to polyvinylidene difluoride (PVDF) membranes using a Trans-Blot Turbo transfer system (Bio-Rad). The membranes were incubated with rabbit anti-phospho p38 MAP kinase, rabbit anti-p38 MAP kinase, rabbit anti-phospho NF-κB p65, rabbit anti-NF-κB p65, rabbit anti-phospho SAPK/JNK, rabbit anti-SAPK/JNK (1:1000, Cell Signaling Technology) or mouse anti-glyceraldehyde-3-phosphate dehydrogenase

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(GAPDH) (1:5000, Merck Millipore, USA) overnight at 4°C. After washing with Tris-buffered saline-containing Tween 20 (0.05%), bound antibody was visualized using horseradish peroxidase-conjugated goat anti-rabbit IgG or horseradish peroxidase-conjugated anti-mouse IgG antibody (Dako, USA) and enhanced chemiluminescence, and imaged (Image Station 4000MM; Kodak Digital Science, New Haven, CT). GAPDH served as the control.

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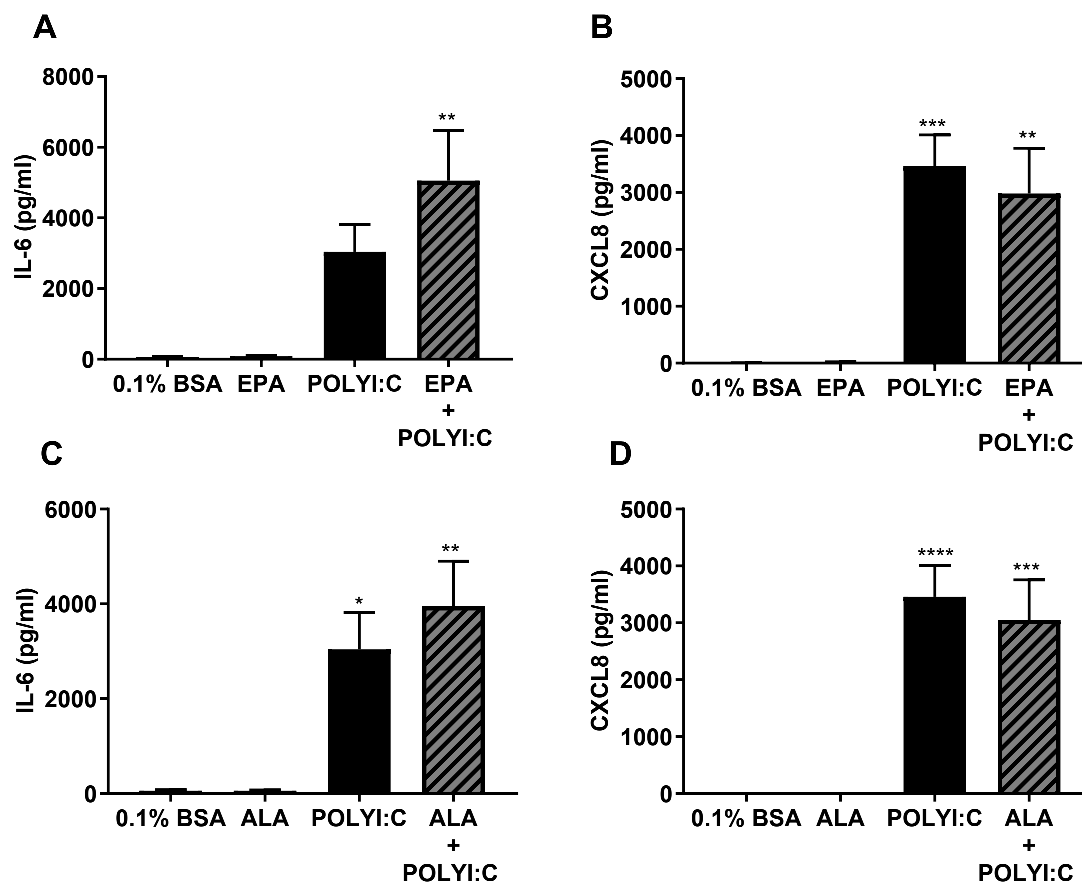
Supplementary Figure 1



ω-3 PUFAs do not suppress POLYI:C-induced IL-6 and CXCL8 release.

Human primary pulmonary fibroblasts (*n* = 5-9) (patients) were unstimulated or challenged with ω-3 polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA) (A, B) or α-linolenic acid (ALA) (C, D) in 0.1% BSA-DMEM (100μM) for 4h with or without the viral mimic polyinosinic:polycytidylic acid (POLYI:C) (10μg/ml) for another 24h. Cell free supernatants were collected and IL-6 (A, C) and CXCL8 (B, D) release was measured using ELISA. All data are represented as mean ± standard error of the mean. All challenges are compared to control and challenges with POLYI:C are compared to challenge with POLYI:C alone, using a one-way ANOVA with a Bonferroni post-test. Significance is represented as * (p<0.05), ** (p<0.01), *** (p<0.001) or **** (p<0.0001).

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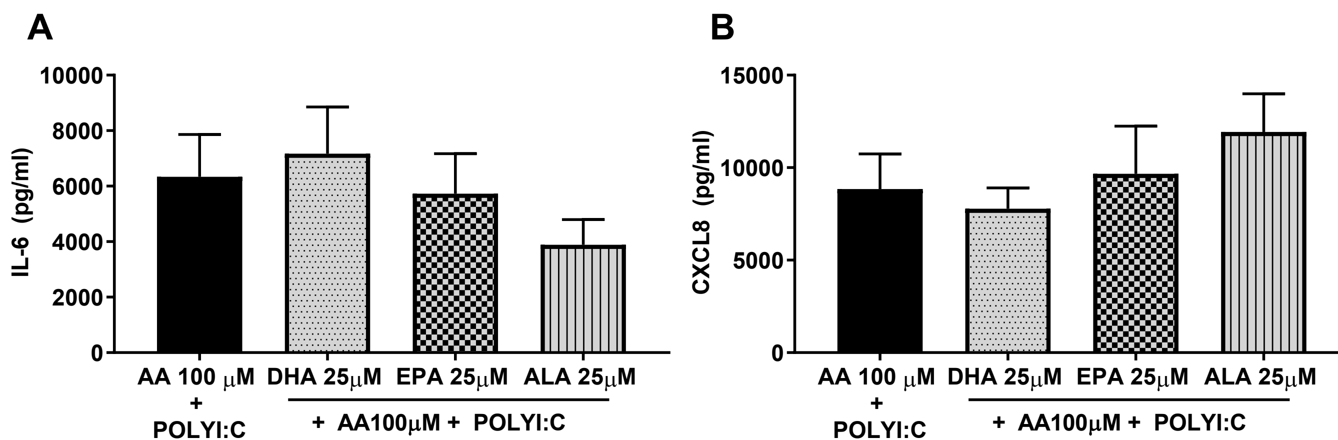
Supplementary Figure 2



ω-3 PUFAs do not suppress combined arachidonic acid and POLYI:C-induced IL-6 or CXCL8 release. Human primary pulmonary fibroblasts (*n* = 9) (patients) were challenged with ω-6 polyunsaturated fatty acid (PUFA) arachidonic (AA) (100µM) with or without ω-3 PUFAs docosahexaenoic acid (DHA) (25µM), eicosapentaenoic acid (EPA) (25µM) or α-linolenic acid (ALA) (25µM) in 0.1% BSA-DMEM for 4 hours prior to challenge with the viral mimic polyinosinic:polycytidylic acid (POLYI:C) (10µg/ml) for another 24h. Cell free supernatants were collected and IL-6 (A) and CXCL8 (B) release was measured using ELISA. All data are represented as mean ± standard error of the mean. All challenges with ω-3 PUFA are compared to their respective challenge without ω-3 PUFA, using a one-way ANOVA with a Bonferroni post-test. There were no significant differences.

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