Rutting, Sandra; Xenaki, Dia; Lau, Edmund; Horvat, Jay; Wood, Lisa G.; Hansbro, Philip M. & Oliver, Brian G. “Dietary omega-6, but not omega-3, polyunsaturated or saturated fatty acids increase inflammation in primary lung mesenchymal cells” Published in the American Journal of Physiology: Lung Cellular and Molecular Physiology, Vol. 314, Issue 6, (2018).

Available from: http://dx.doi.org/10.1152/ajplung.00438.2017

Accessed from: http://hdl.handle.net/1959.13/1406718
Dietary omega-6, but not omega-3 polyunsaturated or saturated fatty acids, increase inflammation in primary lung mesenchymal cells

Sandra Rutting¹,², Dia Xenaki¹, Edmund Lau², Jay Horvat³ Lisa G. Wood³, Philip M. Hansbro³, Brian G. Oliver¹,⁴

¹Cellular and Molecular Biology, Woolcock Institute of Medical Research, University of Sydney, Sydney, Australia;
²Department of Respiratory Medicine, Royal Prince Alfred Hospital, University of Sydney, Sydney, Australia;
³Priority Research Centre for Healthy Lungs, Hunter Medical Research Institute and University of Newcastle, Newcastle, Newcastle, Australia;
⁴School of Life Sciences, University of Technology Sydney, Sydney, Australia.

sandra.rutting@woolcock.org.au
dia.xenaki@woolcock.org.au
edmund.lau@sydney.edu.au
jay.horvat@newcastle.edu.au
lisa.wood@newcastle.edu.au
philip.hansbro@newcastle.edu.au
brian.oliver@uts.edu.au

Corresponding author: Brian Oliver

Phone: (61) 9114-0367

Address: Woolcock Institute of medical research, level 3 cell biology

431 Glebe point road, Glebe, NSW, Australia, 2037
**Abstract**

Obesity is an important risk factor for developing severe asthma. Dietary fatty acids which are increased in sera of obese individuals and after high fat meals, activate the innate immune system and induce inflammation. This study investigated whether dietary fatty acids directly cause inflammation and/or synergise with obesity-induced cytokines in primary human pulmonary fibroblasts *in vitro*. Fibroblasts were challenged with BSA-conjugated fatty acids (ω-6 PUFAs, ω-3 PUFAs or SFAs) with or without TNFα and release of the pro-inflammatory cytokines, IL-6 and CXCL8, was measured. We found that the ω-6 PUFA arachidonic acid (AA), but not ω-3 PUFAs or SFAs up-regulates IL-6 and CXCL8 release. Combined AA and TNFα-challenge resulted in substantially greater cytokine release than either alone, demonstrating synergy. Synergistic upregulation of IL-6, but not CXCL8 was mainly mediated via COX. Inhibition of p38 MAPK reduced CXCL8 release induced by AA and TNFα alone, but not in combination. Synergistic CXCL8 release following AA and TNFα challenge, was not mediated via a single signaling pathway (MEK1, JNK, PI3K and NF-κB), nor by hyperactivation of NF-κB or p38. To investigate if these findings occur in other airway cells, effects of AA in primary human airway smooth muscle (ASM) cells and human bronchial epithelial cells were also investigated. We found pro-inflammatory effects in ASM cells, but not epithelial cells. This study suggests that diets rich in ω-6 PUFAs might promote airway inflammation via multiple pathways, including COX dependent- and independent pathways, and in an obese person may lead to more severe airway inflammation.
Introduction

Asthma and obesity are common diseases in Western countries, and the prevalence of both has increased in the last four decades. Prospective epidemiological studies show that obesity is associated with the onset of asthma, suggesting that it has a causal role (9, 26, 59). However, the underlying mechanisms are still poorly understood.

The obese asthma phenotype is characterised by increased disease severity, worse asthma-related quality of life and worse asthma control (25, 31, 56, 65, 70, 74). Obese asthmatics frequently develop an immune response that is distinct from the classical Th2 allergic response and is characterized instead by lower serum IgE levels and noneosinophilic airway inflammation (29, 74), with increased neutrophilic airway inflammation being reported specifically in obese women (72).

Obesity results from chronic metabolic excess, involving the continuous overconsumption of nutrients. Western diets contribute to obesity, being high in fat, calorie rich and nutrient poor. Consumption of high fat meals leads to increased serum triglycerides, which may affect asthma outcomes by modulating the innate immune response. Chronic exposure to high serum triglyceride levels leads to adipocyte hypertrophy and hyperplasia. These changes in adipose tissue trigger the influx of mast cells, M1 macrophages and CD8+ T-lymphocytes that secrete inflammatory cytokines such as interleukin (IL)-6 and tumour necrosis factor (TNF)-α, which contribute to the persistent but low-level systemic inflammation that is typically observed in obese individuals (48). This increased systemic inflammation in obese asthma is associated with poor asthma control and lung function (72).
Short-term exposure to fatty acids also induces an inflammatory response. A single high fat meal leads to increased circulating fatty acid levels, an increase in CXCL8 - a potent neutrophil chemoattractant - and an increase in circulating neutrophils (63, 80). We have shown that a single high fat meal augments airway inflammation in asthma, resulting in an increased proportion of sputum neutrophils and Toll like receptor (TLR) 4 mRNA expression in sputum (85). Airway neutrophilia is clinically important, as neutrophil sputum counts correlate with increased disease severity, impaired lung function and airflow obstruction in asthma in both humans and animal models (22, 46, 60). In addition, we demonstrated that fat-induced inflammation corresponds with the attenuation of airway bronchodilator recovery, which suggests that a high fat meal also affects smooth muscle function (85). Hence, the available data suggest that dietary fatty acids activate the innate immune system, induce inflammation and impair responses to treatment in the airways.

The composition of fats in diets can differentially impact inflammatory processes. ω-6 polyunsaturated fatty acids (PUFAs) and saturated fatty acids (SFAs) have predominantly been associated with pro-inflammatory effects, whereas the ω-3 PUFAs are associated with anti-inflammatory effects. ω-3 and ω-6 PUFAs also act as bio-active molecules that are metabolized by cyclooxygenase (COX) and lipoxygenase (LOX) into prostaglandins and leukotrienes respectively, that have potential anti-/pro-inflammatory actions of their own (11, 12).

Nevertheless, it remains unknown if obesity-driven local or systemic inflammation or dietary fatty acids can directly activate mesenchymal lung cells to recruit neutrophils to the lungs and therefore promote localised inflammation. We examined whether dietary fatty acids directly cause inflammation and/or synergise with obesity-induced cytokines in primary human pulmonary fibroblasts in vitro and investigated the underlying mechanisms. To investigate if these effects occur in other airway cells, effects of dietary fatty acids on inflammatory responses in primary human airway smooth muscle (ASM) cells, primary human bronchial epithelial (HBE) cells and the human bronchial epithelial cell line (BEAS-2B) were also examined.
Material and Methods

Cell culture of primary pulmonary fibroblasts, ASM cells HBE cells and BEAS-2Bs.

Primary pulmonary fibroblasts were isolated from the parenchyma and ASM and HBE cells from the bronchial airways of lungs from patients undergoing lung transplantation or lung resection for thoracic malignancies. Ethical approval for the use of the lung tissue for in vitro experimentation was granted by the Human Ethics Committees of the University of Sydney and the South West Sydney Area Health Service. All volunteers provided written, informed consent. Table 1 shows the patient demographics. Isolation of pulmonary fibroblasts, ASM and HBE cells was performed, as previously described by Krimmer et al. (2013), Johnson et al. (1995) and Ge et al. (2010), respectively (27, 37, 47). Fibroblasts and ASM cells were seeded in 12-well plates at a density of $6.2 \times 10^4$ 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). HBE cells were seeded in 12-well plates at a density of $6.2 \times 10^4$ cells/mL in BEGM (Lonza) and grown to 90% confluency (3-4 days). We also used the human bronchial epithelial cell line BEAS-2B (ATCC, Manassas, VA), which was maintained in 10% FBS and 1% Antibiotic-Antimycotic in DMEM. BEAS-2Bs were seeded at a density of $1 \times 10^5$ cells/mL in 12-well plates and grown for 24 hours. All cell types, but HBE cells were quiesced for 24 hours prior to stimulation by incubation in DMEM (Gibco, Grand Island, New York, US) supplemented with 0.1% bovine serum albumin (BSA) (Sigma Aldrich, Castle Hill, NSW, Australia) and 1% Antibiotic-Antimycotic. HBE cells were quiesced for 24 hours prior to stimulation in BEBM supplemented with 0.05% insulin, 0.1% transferrin (Lonza) and 0.1% BSA (Sigma). All experiments were carried out using fibroblasts and ASM cells between passage 2 and 5, and HBE cells between passage 1 and 2.
Preparation of BSA-conjugated fatty acids

Stock solutions of 0.5M ω-3 PUFAs (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) and 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) and Pillon et al. (2012) (32, 64). These solutions were further diluted in cell culture medium to obtain final concentrations of 1, 10 and 100µM.

Treatment of cells with dietary fatty acids and TNFα

The cells were stimulated with 1, 10 and 100µM EPA, DHA, PA or AA or vehicle (EtOH/BSA/cell culture medium) and compared to untreated controls. Other cells were treated with AA (10 and 100µM) 24 hours prior to stimulation with TNFα (1ng/mL) (ThermoFisher, Scoresby, VIC, Australia). All cells were incubated at 37°C with 5% CO2 for 48 hours. Cell-free supernatants were collected and stored at -20°C until further analysis.

Treatment of cells with signaling pathway inhibitors

To investigate the cell signaling pathways activated after stimulation with AA and TNFα, specific inhibitors were used at concentrations previously shown to be effective in human airway cells (15, 27, 30, 35, 39, 79). Cells were treated with inhibitors of p38 mitogen-activated protein (MAP) kinase (SB239063, 3µM) (Tocris, Ellisville, MO, USA), MAP kinase 1 (MEK1) (PD98059, 10µM), c-Jun N-terminal kinase (JNK) (SP600125, 10µM), phosphoinositide 3-kinase (PI3K) (LY294002, 3µM) (Calbiochem, San Diego, CA), COX (indomethacin, 10µM) and NF-κB (BAY-117082, 1µM) (Sigma-Aldrich) for 1 hour before stimulation with AA (10 and 100µM) with or without TNFα (1ng/mL). Cell-free supernatants were collected after 48 hours and stored at -20°C until further analysis.
To determine whether steroids inhibit AA and TNFα-induced cytokine release, fibroblasts were treated with dexamethasone (10^{-9} M, 10^{-8} M, 10^{-7} M and 10^{-6} M) (Sigma-Aldrich) for 1 hour prior to stimulation with AA (100 µM) for 24 hours with or without TNFα (1 ng/mL) for another 24 hours or TNFα (1 ng/mL) only for 24 hours. Cell-free supernatants were collected and stored at -20°C until further analysis.

**Determination of IL-6 and CXCL8 levels**

Levels of supernatant IL-6 and CXCL8 were measured using sandwich ELISA. The amount of IL-6 release was assessed with optimized anti-IL-6 antibody pairs from BD pharmingen, BD, Franklin Lakes, NJ. A specific kit for CXCL8 was purchased from R&D Systems (Minneapolis, Minnesota, USA) and used according to the manufacturer’s instructions. The detection limit of both assays was 15.6 pg/mL.

**Western blotting**

To assess the activation of intracellular signaling molecules in fibroblasts following stimulation with AA with or without TNFα, relative levels of phosphorylated p38 MAP kinase and NF-κB from cell lysates were assessed by western blotting. Cells cultured in the presence or absence of AA (100 µM) with or without TNFα (1 ng/mL) for 30 min were lysed (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM Na$_2$EDTA, 1 mM EGTA, 20 mM Na$_4$P$_2$O$_7$, 2 mM Na$_3$VO$_4$, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% sodium deoxycholate, 1% protease inhibitor cocktail set III (Millipore, USA) and 1 mM 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 (1:1000, Cell Signaling Technology) or anti-mouse
glyceraldehyde-3-phosphate dehydrogenase (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.

**Isolation of RNA and reverse transcriptase-polymerase chain reaction**

mRNA expression of the free fatty acid receptors (FFARs) 1-4 was assessed by gel based-PCR assay. Total RNA was extracted from cultured primary pulmonary fibroblasts and BEAS-2Bs and further purified using the ISOLATE II RNA Mini Kit (Bioline, Alexandria, Australia). RNA was transcribed into cDNA using the SensiFAST™ cDNA Synthesis Kit (Bioline). Both kits were used as per the manufacturer’s instructions. PCR was performed with a Mastercycler (Eppendorf South Pacific, North Ryde, Australia) using the MyTaq™ HS Red DNA polymerase kit (Bioline). The annealing temperature and number of cycles used was optimised for each primer set. All reactions included an initial denaturation step at 95°C for 1 min followed by 26-36 cycles of denaturation (95°C for 15s) and annealing at 56.9-64.2°C for 15 sec., followed by extension at 72 °C for 10s. Primer sequences and PCR conditions are shown in table 2. PCR products were electrophoresed on 2% agarose in Tris-acetate-EDTA buffer. The bands were visualized using SYBR safe DNA gel stain (Molecular Probes, Invitrogen, Eugene, OR). Since not all 4 FFARs (FFARs 1-4) have been shown to be present in airways cells previously, we used ASM cells as a positive control for FFAR 1 and 4 and monocytes as a positive control for FFAR 2 and 3 (4, 54).
Statistical analysis was conducted using GraphPad Prism version 7 software (GraphPad Software, San Diego, CA). After testing for normal distribution and equal variance, comparisons of the data were carried out by a paired t-test or Analysis of variance (ANOVA) with repeated measures followed by a Bonferroni post-test, where appropriate. All data on bar graphs are presented as mean ± standard error of the mean (SEM). A probability ($p$) value of less than or equal to 0.05 was considered significant.
**Results**

**Human primary pulmonary fibroblasts express messenger RNA of all 4 FFARs.**

First, we examined whether pulmonary fibroblasts express receptors that can directly be activated by (dietary) fatty acids by assessing if the FFAR 1, 2, 3 and 4 were expressed in human pulmonary fibroblasts. We detected mRNA encoding the FFAR 1, 2, 3 and 4 in cultured fibroblasts \( (n = 7) \) \( \text{(figure 1)} \). However, the level of expression of these receptors varied from patient to patient.

**ω-6 PUFA AA induces IL-6 and CXCL8 release.**

To assess the inflammatory response of pulmonary fibroblasts to the different dietary fatty acids, fibroblasts were treated with 1, 10 and 100µM of EPA, DHA, PA or AA for 48 hours and IL-6 and CXCL8 release in supernatant was measured. Challenge with ω-3 PUFAs (EPA and DHA) or SFA (PA) did not cause IL-6 and CXCL8 release from pulmonary fibroblasts. However, we found that the ω-6 PUFA AA challenge resulted in a dose-dependent increase of IL-6 \( (n = 11, p<0.05) \) and CXCL8 release \( (n = 11, p<0.01) \) \( \text{(figure 2)} \).

**Combined AA and TNFα treatment results in greater cytokine release, than either alone.**

TNFα is a pro-inflammatory cytokine, known to be elevated in sera of obese individuals \( (41, 71) \) and is further elevated in obese asthma patients \( (13) \). To replicate the events in an obese person, we challenged the fibroblasts with AA \( (10 \text{ and } 100\mu M) \) in combination with TNFα \( (1\text{ng/mL}) \). Stimulation with the combination of AA and TNFα resulted in substantially greater IL-6 \( (n = 10, p<0.0001) \) and CXCL8 \( (n = 10, p<0.0001) \) release, than AA alone \( \text{(figure 3)} \). The effect of the combination of AA with TNFα on IL-6 and CXCL8 release was greater than the sum of the individual effects of AA and TNFα, demonstrating a synergistic effect.
Inhibition of COX results in suppressed AA- and combined AA and TNFα-induced IL-6 release.

AA can act as a bio-active molecule, and is the precursor that is metabolized by COX to produce eicosanoids, including the prostaglandins (11, 12). Prostaglandins are known to play a key role in the generation of inflammatory responses. To investigate whether COX-mediated prostaglandins contribute to AA-induced IL-6 and CXCL8 release, we pre-treated pulmonary fibroblasts with a non-selective COX-inhibitor, indomethacin. We found that indomethacin (10⁻⁵M) treatment inhibited IL-6 release, induced by AA alone (n = 7, p<0.0001) and in combination with TNFα (n = 13, p<0.0001).

Indomethacin pre-treatment also attenuated CXCL8-release induced by AA combined with TNFα (n = 13, p<0.05), but had no effect on CXCL8 release induced by AA alone (figure 4). The lack of inhibition of AA-induced CXCL8 release and the limited inhibition (29%) of AA and TNFα induced-CXCL8 release, suggest the involvement of COX-independent pathways.

Inhibition of p38 MAP kinase suppresses AA-induced cytokine release, but not combined AA and TNFα-induced CXCL8 release. To further investigate the mechanisms underlying the effects of AA- and combined AA and TNFα-induced IL-6 and CXCL8 release, specific inhibitors were used to block some common protein kinases that are known to be downstream of TNFα or prostaglandin receptors. Inhibition of MEK1, JNK, PI3K and NF-κB did not inhibit IL-6 and CXCL8 release upon stimulation with AA (100µM), TNFα (1ng/ml) or the combination of AA with TNFα (table 3).

However, inhibition of p38 MAP kinase with SB239063 attenuated IL-6 and CXCL8 release induced by AA and TNFα alone (n = 7-8, p<0.05). Inhibition of P38 MAPK also suppressed IL-6 release induced by the combination of AA and TNFα (n = 13, p<0.01), but not CXCL8 release (figure 5). The partial (30%) inhibition of TNFα-induced cytokine release and the lack of inhibition of AA and TNFα-induced CXCL8 release, achieved by blocking the p38 MAP kinase signaling pathway, suggest involvement of other pathways.
TNFα- with or without AA activates p38 MAPK and NF-κB signaling to a similar extent.

The use of single pathway inhibitors did not elucidate all underlying signaling pathways used upon stimulation with AA and TNFα and suggests either the involvement of multiple pathways or hyperactivation of single pathways. Activation of two main signaling pathways, p38 and NF-κB, were investigated using western blotting. AA alone had no effect on the phosphorylation of p38 MAP kinase and NF-κB. However, p38 MAP kinase phosphorylation was increased 30 minutes after stimulation with TNFα alone, as well as TNFα in combination with AA (5.9- and 6.0-fold increase, respectively, n = 9, p<0.05). NF-κB phosphorylation was also increased after stimulation with TNFα alone and in combination with AA (6.6- and 7.8-fold increase, respectively, n = 9, p<0.05). There was no difference in phosphorylated p38 MAP kinase or NF-κB expression between TNFα alone and combined TNFα and AA stimulated cells (figure 6). Total p38 MAP kinase and NF-κB did not change with any treatment.

AA also induces IL-6 and CXCL8 release in ASM cells, but not epithelial cells.

To explore whether other structural lung cells respond the same as pulmonary fibroblasts, we repeated selected experiments in ASM cells, BEAS-2BS and HBE cells. Challenge with AA (100µM) increased IL-6 (n = 7, p<0.05) and CXCL8 release (n = 7, p<0.01) from ASM cells (figure 7E and 7F). In ASM cells, AA in combination with TNFα resulted in substantially greater IL-6 (n = 8, p<0.001) and CXCL8 (n = 8, p<0.0001) release, than AA alone (figure 8A and 8B). The effect of the combination of AA with TNFα was greater than the sum of the individual effects of AA and TNFα, showing similar effects as in fibroblasts. We also confirmed that COX-mediated prostaglandins and the p38 pathway contribute to AA and TNFα-induced IL-6 and CXCL8 release in ASM cells. Pre-treatment with indomethacin (10⁻⁵M) (Figure 8C and 8D) or SB239063 (3µM) (figure 8E and 8F) suppressed IL-6 and CXCL8 release upon stimulation with AA and TNFα (n = 7-8). Indomethacin was more effective in inhibiting IL-6 release, compared to CXCL8-release, showing similar effects as in the pulmonary fibroblasts.
BEAS-2Bs, however, did not respond to any of the dietary fatty acids (ω-3-PUFA, ω-6 PUFA or SFAs) (figure 7A and 7B, only the response to AA is shown). HBE cells also did not respond to AA (figure 7C and 7D). To assess whether differences in the expression of FFARs could explain the lack of effect of AA in epithelial cells, expression of FFAR1-4 in BEAS-2Bs was investigated. We detected mRNA encoding the FFAR-1, 2, 3 and 4 in cultured BEAS-2Bs (n = 5) (figure 9).

Steroid treatment attenuates AA and TNFα induced cytokine release.

Steroids have beneficial effects in many inflammatory diseases by exerting their anti-inflammatory effects through inhibiting multiple signal transduction pathways (33). To assess whether steroids could inhibit AA and TNFα-induced IL-6 and CXCL8 release, fibroblasts were pre-treated with dexamethasone (10⁻⁹M-10⁻⁶M). Dexamethasone pre-treatment attenuated IL-6 and CXCL8 release induced by AA and TNFα alone and the combination of AA and TNFα (figure 10). Dexamethasone at the highest concentration of 10⁻⁶M reduced AA-induced IL-6 (n = 9, p<0.05) and CXCL8 release (n = 9, p<0.01) both by 95% (figure 10A). IL-6 release induced by TNFα (n = 8 p<0.01) and the combination of AA and TNFα (n = 7, p<0.001) was inhibited by 82% and 84%, respectively. TNFα, and combined AA and TNFα-induced CXCL8 release, however was inhibited by only 39% (n = 8, p<0.05) and 54 % (n = 7, p<0.01), respectively (figure 10B and 10C).
This study is the first to investigate whether dietary fatty acids directly cause inflammation and/or synergise with obesity-induced cytokines in human pulmonary fibroblasts. Asthma is characterised by chronic airway inflammation, declining airway function and structural changes within the airway wall also referred to as ‘airway remodeling’. The impact of obesity on airway and lung parenchyma remodeling has been investigated in mouse models and some human studies. Saraiva et al. (2011) showed that diet-induced obesity enhances lung remodeling and results in higher airway responsiveness in a mouse model of chronic allergic asthma (68). A positive correlation between body mass index and airway remodeling in asthma has also been shown in human studies (14, 36).

The role of pulmonary fibroblasts in obese asthma has not been investigated and is an area that requires further investigation. Pulmonary fibroblasts are phenotypically altered in asthma (58), and play a crucial role in tissue homeostasis, the production of pro-inflammatory cytokines and extracellular matrix (ECM) proteins and, therefore, contribute to airway inflammation and subsequent remodeling in asthma (8, 38, 45, 84). Fibroblasts are located at the interface of the lumen and the blood supply and are 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.

Our data demonstrates that the ω-6 PUFA AA up-regulates IL-6 and CXCL8 release from primary human pulmonary fibroblasts in a dose-dependent manner, suggesting that exposure to ω-6 PUFAs promotes airway inflammation. The ω-3 PUFAs and SFAs did not induce inflammatory responses in fibroblasts. Other In vitro studies of inflammatory responses to dietary fatty acids have shown that SFAs including PA, but not the ω-3 PUFA DHA or the ω-6 PUFA linoleic acid, initiate innate immune responses through mechanisms such as TLR activation, particularly TLR2 and 4, in adipocytes and macrophages (1, 34, 49, 57, 69). These results have been replicated in in vivo human studies, where within 4 hours of consumption of a high fat meal, innate immune responses are stimulated with increased TLR2, -4 and NF-κB activity in mononuclear and polymorphonuclear cells (28, 62). The non-
responsiveness to SFAs in this study may be explained by the lack of functional TLR4 signaling in pulmonary fibroblasts. CD14 acts as a co-receptor for TLR4, and has been shown to be essential for LPS-induced TLR4 signaling and (10) lung mesenchymal cells do not express CD14 (2, 86). Studies that examined the effects of ω-6 PUFAs on inflammatory processes have conflicting data. A human study found that supplementation of healthy men with AA significantly increased the number of circulating neutrophils and in vitro production of LPS-induced leukotriene B4 by leukocytes. However, 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 (42, 44). In our study a clear pro-inflammatory effect of AA was observed. Other in vitro studies examining the ω-3 PUFAs, DHA and EPA, have predominantly shown anti-inflammatory effects, including inhibition of LPS-induced production of TNFα, IL-6 and CXCL8 in macrophages (83) and endothelial cells (16). Other studies investigating the effects of ω-3 PUFA supplementation on immune responses have reported inhibitory effects on in vitro cytokine production of IL-6 and TNFα by peripheral blood mononuclear cells (24, 51). However, not all studies have shown anti-inflammatory responses in leukocytes (43, 87).

A possible mechanism by which fatty acids could affect inflammatory processes is through direct activation of FFARs. FFAR 1 and 4 have shown to be activated by dietary fatty acids and are expressed in various tissues and influence important metabolic functions. Activation of FFAR1 by the selective agonist GW9508 in neutrophils leads to superoxide production, whereas FFAR4 activation by DHA has been shown to have anti-inflammatory effects in macrophages (53). Recently, Mizuta et al. (2015) (54) identified expression of FFAR 1 and 4 in primary ASM cells and freshly native human tracheal airway smooth muscle. We are the first to detect mRNA encoding FFAR 1-4 in cultured fibroblasts, thereby identifying these receptors as a possible pathway for fat-induced inflammation in pulmonary fibroblasts. The level of expression of these receptors varied between patients and could contribute to variability in responses between different patients. However, since only AA and not the other dietary fatty acids had biological activity, and AA-induced cytokines were largely COX
dependent, we do not consider that the FFAR system was activated by the dietary fatty acids used in this study.

Studies evaluating proinflammatory cytokines in obese asthma have shown that serum levels of IL-6 and TNFα are significantly higher in obese patients compared to lean patients (13, 41, 71). Thus, to mimic events in an obese person, we investigated the response to AA in combination with TNFα. This is clinically important, as pro-inflammatory mediators negatively correlate with lung function and asthma control and might have synergistic effects on the airways (72). We found that stimulation with the combination of AA and TNFα results in greater IL-6 and CXCL8 release than either AA or TNFα alone. Interestingly, the effect of the combination on cytokine release was substantially greater than the sum of the individual effects of AA and TNFα, which indicates that the effects are synergistic. These results suggest that exposure to AA in an obese environment may lead to more severe airway inflammation. We used TNFα rather than IL-6 in our experiments, as we have previously shown the IL-6 receptor to be absent in mesenchymal cells (3).

To understand the underlying mechanisms involved in AA and TNFα-induced IL-6 and CXCL8 production, signaling pathways were investigated. AA can act as a bio-active molecule and is the precursor of eicosanoids, including prostaglandins, through metabolism by COX. Prostaglandins are known to play a key role in the generation of inflammatory responses (66). Prostaglandin E2 (PGE₂) has been shown to affect the functions of different immune and lung cells, including the induction of pro-inflammatory cytokine release, such as IL-6 from airway epithelial cells (40, 73). To investigate whether COX-mediated prostaglandins contribute to AA-induced IL-6 and CXCL8 release, we pre-treated the pulmonary fibroblasts with indomethacin, which inhibits both COX-1 and COX-2. Our study demonstrates that indomethacin inhibits IL-6 release, induced by AA alone and in combination with TNFα, but had no effect on AA-induced CXCL8 release and only little effect on AA combined with TNFα-induced CXCL8. Our data suggest that (synergistic) IL-6 production is dependent on prostaglandin synthesis, whereas CXCL8 release involves mainly prostaglandin independent mechanisms. We believe that these effects are mediated through COX-2, rather than COX-1.
are several reasons for this. COX-1 is responsible for constitutive prostaglandin production under basal conditions, whereas COX-2 is up-regulated during inflammation and is responsible for PGE2 biosynthesis at sites of inflammation. A number of proinflammatory cytokines are capable of inducing COX-2 in lung epithelial and mesenchymal cells (21, 52). TNFα has been shown to induce COX-2 expression in ASM-cells (67), but not all studies confirm this finding (61). These differences in TNFα responses could be due to the different time points assessed and instability of the COX-2 protein (67). TNFα has consistently been shown to enhance the ability of other pro-inflammatory cytokines such as IL-1β to induce COX-2 expression and PGE2 release in ASM-cells (7, 55, 81).

Similarly, in human pulmonary fibroblasts transcripts for COX-2, but not COX-1, have shown to be induced by TNFα (19). We did not further investigate the role of COX-1 versus COX-2 in this study.

The biological effects of PGE2 depend on activation of the PGE2 receptor (EP) subtypes (1-4). All belong to the family of G protein-coupled receptors. PGE2 has been linked to IL-6 synthesis in human epithelial cells (73). This is accomplished through EP2 and EP4 stimulation, leading to cAMP formation, which activates cAMP response element-binding protein (CREB). In turn, CREB is a transcription factor that promotes IL-6 production (18). PGE2 has also been linked to IL-6 synthesis though cAMP-independent mechanisms acting on EP4, resulting in p38 MAP kinase and protein kinase C (PKC) activation (23). Thus, the influence of PGE2 and cAMP on IL-6 production appears to be cell specific, and in the current study, involvement of EP receptors and prostaglandin signaling specifically in pulmonary fibroblasts was not further investigated.

Release of CXCL8 however, was only partially inhibited by indomethacin. We have not explored the reason for this. The most likely explanation is that when COX is inhibited with indomethacin, the production of prostaglandins is shunted into leukotrienes (20, 50, 76).

We further investigated the underlying mechanisms involved in AA and TNFα-induced IL-6 and CXCL8 release by identifying activated signaling pathways using specific signaling inhibitors. The p38 MAP kinase inhibitor SB239063, attenuated IL-6 and CXLC8 release induced by AA and TNFα alone, and IL-6 release induced by the combination of AA and TNFα, but not CXCL8 release. SB239063 is a
potent and selective inhibitor of p38 MAP kinase and displays specific and high-affinity binding to p38 MAP kinase (IC50 = 44nM) (77). These data suggest that AA and TNFα activate the p38 pathway, leading to both IL-6 and CXCL8 release, but that other pathways are also involved AA and TNFα-induced IL-6 and CXCL8 release. Inhibition of other pathways, including MEK1, JNK, PI3K and NF-κB had no effect on IL-6 or CXCL8 release. TNFα is known to stimulate multiple pathways, including JNK, p38 and NF-κB (17, 82), resulting in IL-6 and CXCL8 release in other cell types (17, 82). The lack of inhibition of AA and TNFα-induced CXCL8 release using single inhibitors can be explained by the absence of a dominant single pathway; instead multiple pathways are involved. This is suggested by the limited (30%) inhibition of TNFα-induced CXCL8 release achieved by blocking the p38 MAP kinase signaling pathway and the lack of effect on AA and TNFα-induced CXCL8 release. We were unable to simultaneously inhibit multiple pathways by combining pathway inhibitors, as it led to (visually observed) cell death in our experiments. Pathway inhibitors are a useful means for indicating the involvement of a specific pathway in a given response, but the measurement of protein phosphorylation, translocation and binding to gene promoter regions is needed to confirm direct involvement. Thus, the activation of pathways by stimulation with AA and TNFα necessitated the use of western blotting. We focused on p38 MAP kinase and NF-κB, as these pathways have been shown to be mainly involved in prostaglandin and TNFα-signaling (23, 82). We found significant p38 MAP kinase- and NF-κB phosphorylation after challenge with TNFα alone as well as with AA in combination with TNFα. There was no difference in phosphorylated p38 or NF-κB or expression between TNFα only and combined AA and TNFα treatments, showing that increased activation of p38 or NF-κB cannot explain the synergy between TNFα and AA. Presumably, multiple pathways are responsible for synergistic AA and TNFα-induced CXCL8 release.

To explore whether AA induces inflammatory responses in other structural lung cells, we also examined the effects of dietary fatty acids in ASM cells, BEAS-2Bs and HBE cells. In ASM cells, AA also up-regulated IL-6 and CXCL8 release, and in combination with TNFα resulted in synergistic cytokine release. We also confirmed the involvement of COX-mediated prostaglandins and the p38-pathway
leading to AA and TNFα-induced IL-6 and CXCL8 release in ASM cells. Interestingly, BEAS-2Bs did not respond to any of the dietary fatty acids, including AA. Initially, we used BEAS-2Bs as they have shown to be a good representative for human epithelial cells including in our own studies (75). Because the lack of induction of cytokine release by AA was surprising, we then confirmed the response in primary airway epithelial cells, and found that AA also did not induce IL-6 and CXCL8 release in HBE cells. We do not know the reason why epithelial cells do not have a pro-inflammatory response to AA, but in our previous studies we have shown that PGE2 production from epithelial cells is lower than that from mesenchymal cells, and furthermore with the same stimulus mesenchymal, but not epithelial derived PGE2 is insufficient to desensitise the β2 adrenoceptor (78).

All cell types have their own functions and different cell types can respond differently to the same stimulus. ASM cells and pulmonary fibroblasts are mesenchymal lung cells and share the same embryonic origin, which might explain why these cells respond similarly to AA (5, 45). To further investigate why epithelial cells do not respond to AA, we assessed expression of FFARs in BEAS-2Bs. We found that BEAS-2Bs express all FFARs further supporting our hypothesis that FFARs are not involved in AA-induced IL-6 and CXCL8 release.

Due to the complexity of the pathways involved in AA and TNFα signalling, inhibition of any single signalling pathway is unlikely to be effective as a potential therapeutic agent in dietary fat and obesity-related inflammation. Corticosteroids are the most effective anti-inflammatory therapy for many chronic inflammatory diseases and influence multiple signal transduction pathways, and therefore are an alternative (6). We found that pre-treatment with dexamethasone at the highest concentration of $10^{-6}$M results in inhibition of AA and TNFα-induced cytokine release, with 84% inhibition of IL-6 release versus only 54% for CXCL8 release, demonstrating steroid insensitivity. This seems to be driven by TNFα rather than AA, as dexamethasone almost fully attenuated AA-induced CXCL8-release, and only 39% of TNFα-induced CXCL8 release. These results suggest that steroids do not completely attenuate dietary fat and obesity-related inflammation.
In summary, this study demonstrates that in primary mesenchymal lung cells, but not epithelial cells, dietary fatty acids can directly cause inflammation and synergise with the obesity-induced cytokine, TNFα. Exposure to ω-6, but not ω-3 PUFA or SFAs led to IL-6 and CXCL8 release via multiple pathways, including COX dependent- and independent pathways. This suggests that diets rich in ω-6 PUFAs might promote airway inflammation, and in an obese person may lead to more severe airway inflammation.

**Grants**

P.M.H. is supported by a fellowship from the National Health and Medical Research Council (NHMRC) of Australia, a Brawn Fellowship from the Faculty of Health and Medicine and funding from and the Rainbow Foundation. The authors thank F. Thomson and M. Thomson for their continued support. B.G.O is supported by a fellowship from the National Health and Medical Research Council (NHMRC) of Australia (APP1110368). L.G.W is supported by a fellowship from the National Health and Medical Research Council (NHMRC) of Australia (APP1081355).

**Disclosures**

No conflicts of interest, financial or otherwise, are declared by the authors.

**Acknowledgements**

We would like to acknowledge the collaborative effort of the cardiopulmonary transplant team and the pathologists at St Vincent’s Hospital (Sydney, Australia), and the thoracic physicians and pathologists at the Royal Prince Alfred Hospital (Sydney) and Strathfield Private Hospital (Strathfield, Australia).
References


51. Meydani SN, Endres S, Woods MM, Goldin BR, Soo C, Morril-Labrode A, Dinarello CA, and Gorbach SL. Oral (n-3) fatty acid supplementation suppresses cytokine production and lymphocyte...


68. **Saraiva SA, Silva AL, Xisto DG, Abreu SC, Silva JD, Silva PL, Teixeira TP, Parra ER, Carvalho AL, Annoni R, Mauad T, Capelozzi VL, Silva PM, Martins MA, and Rocco PR.** Impact of obesity on


Table 1. Summary of patient demographics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>All patients (n = 62)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age, y (SD)</td>
<td>57.3 (11.8)</td>
</tr>
<tr>
<td>Sex n (F/M)</td>
<td>28/34</td>
</tr>
<tr>
<td>Surgery n (R/Tx)</td>
<td>16/46</td>
</tr>
<tr>
<td>Diagnosis n (%)</td>
<td></td>
</tr>
<tr>
<td>idiopathic pulmonary fibrosis</td>
<td>14 (22.6)</td>
</tr>
<tr>
<td>COPD and/or emphysema</td>
<td>24 (38.7)</td>
</tr>
<tr>
<td>Eisenmyer’s syndrome</td>
<td>2 (3.2)</td>
</tr>
<tr>
<td>thoracic malignancies</td>
<td>11 (17.7)</td>
</tr>
<tr>
<td>other</td>
<td>11 (17.7)</td>
</tr>
</tbody>
</table>

F: Female, M: Male, R: resection, Tx: Transplant, COPD: chronic obstructive pulmonary disease
<table>
<thead>
<tr>
<th>Target</th>
<th>Primer sequence</th>
<th>Amplicon Size (bp)</th>
<th>Annealing temperature (°C)</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>FFAR1</td>
<td>FP: 5’ ATGGACCTGCCCCCGCAGCTC 3’ RP: 5’ CTTCTGGGAACCTTGCCCCCTTG 3’</td>
<td>900</td>
<td>64.2</td>
<td>36</td>
</tr>
<tr>
<td>FFAR2</td>
<td>FP: 5’ GCCCTGGTGCTCTTCTCTCATC 3’ RP: 5’ AGGTGGGACACGTTGTAAGG 3’</td>
<td>178</td>
<td>56.9</td>
<td>36</td>
</tr>
<tr>
<td>FFAR3</td>
<td>FP: 5’ CACCATCATCTCACCACGCCC 3’ RP: 5’ CCTCTTCCATGATGCTTTTGTGATCTGA 3’</td>
<td>141</td>
<td>56.9</td>
<td>36</td>
</tr>
<tr>
<td>FFAR4</td>
<td>FP: 5’ TGGAGATGCAATTGTGTTGGAGA 3’ RP: 5’ AGCCTCAAGGGGATGAGTA 3’</td>
<td>130</td>
<td>60.8</td>
<td>36</td>
</tr>
<tr>
<td>GAPDH</td>
<td>FP: 5’ CCACCATGGGAAATTCATGGA 3’ RP: 5’ TCTAGACGGCAGGTCCACC 3’</td>
<td>598</td>
<td>60.0</td>
<td>26</td>
</tr>
</tbody>
</table>
Table 3. Effect of MEK1, NF-κB, PI3K and JNK inhibition on AA, TNFα and combined AA and TNFα-induced IL-6 and CXCL8 release.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Inhibitor</th>
<th>Cytokine measured</th>
<th>Treatments</th>
<th>AA 100µM pg/ml</th>
<th>p-value</th>
<th>TNFα pg/ml</th>
<th>p-value</th>
<th>AA 100µM + TNFα pg/ml</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No pathway</td>
<td>No inhibition</td>
<td>IL-6</td>
<td></td>
<td>8317 ± 3485</td>
<td>-</td>
<td>7481 ± 961</td>
<td>-</td>
<td>35492 ± 5696</td>
<td>-</td>
</tr>
<tr>
<td>MEK1</td>
<td>PD98059</td>
<td>IL-6</td>
<td></td>
<td>8551 ± 3210</td>
<td>&gt;0.05</td>
<td>7019 ± 950</td>
<td>&gt;0.05</td>
<td>32993 ± 7853</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>NF-κB</td>
<td>BAY-117082</td>
<td>IL-6</td>
<td></td>
<td>8724 ± 3789</td>
<td>&gt;0.05</td>
<td>6549 ± 1517</td>
<td>&gt;0.05</td>
<td>29688 ± 5388</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>PI3K</td>
<td>LY294002</td>
<td>IL-6</td>
<td></td>
<td>10614 ± 3624</td>
<td>&gt;0.05</td>
<td>7814 ± 1492</td>
<td>&gt;0.05</td>
<td>44485 ± 6373</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>JNK</td>
<td>SP600125</td>
<td>IL-6</td>
<td></td>
<td>8426 ± 2688</td>
<td>&gt;0.05</td>
<td>7396 ± 1400</td>
<td>&gt;0.05</td>
<td>31425 ± 7041</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>No pathway</td>
<td>No inhibition</td>
<td>CXCL8</td>
<td></td>
<td>15509 ± 4128</td>
<td>-</td>
<td>14583 ± 1746</td>
<td>-</td>
<td>43774 ± 11235</td>
<td>-</td>
</tr>
<tr>
<td>MEK1</td>
<td>PD98059</td>
<td>CXCL8</td>
<td></td>
<td>17049 ±4423</td>
<td>&gt;0.05</td>
<td>14024 ± 2500</td>
<td>&gt;0.05</td>
<td>39688 ± 13097</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>NF-κB</td>
<td>BAY-117082</td>
<td>CXCL8</td>
<td></td>
<td>16984 ± 4847</td>
<td>&gt;0.05</td>
<td>18033 ± 4276</td>
<td>&gt;0.05</td>
<td>33531 ± 10782</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>PI3K</td>
<td>LY294002</td>
<td>CXCL8</td>
<td></td>
<td>19059 ± 4306</td>
<td>&gt;0.05</td>
<td>18006 ± 1969</td>
<td>*</td>
<td>65275 ± 8715</td>
<td>*</td>
</tr>
<tr>
<td>JNK</td>
<td>SP600125</td>
<td>CXCL8</td>
<td></td>
<td>15344 ± 3202</td>
<td>&gt;0.05</td>
<td>20089 ± 5147</td>
<td>&lt;0.05</td>
<td>40596 ± 9482</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

All values are mean ± SEM; n = 7-8. All treatments with inhibitor are compared to their respective treatment in the absence of inhibitor, using a paired t-test. Significance is represented as * (p<0.05).
Figure legends

Figure 1. Pulmonary fibroblasts express free fatty acids receptors 1-4.

Total RNA was extracted from cultured human pulmonary fibroblasts and mRNA expression of the free fatty acid receptors (FFARs) \((n = 7)\) (patients) 1 (A), 2 (B), 3 (C) and 4 (D) was assessed by gel-based PCR. GAPDH (housekeeping gene) served as the control (E). Lane 1 is negative control (water); lane 2 till 8 is total RNA from cultured pulmonary fibroblast from 7 different patients; lane 9 is the positive control, either total RNA from cultured smooth muscle cells (A, D) or monocytes (B, C).

Figure 2. \(\omega-6\) PUFA arachidonic acid induces IL-6 and CXCL8 release from primary pulmonary fibroblasts.

Human primary pulmonary fibroblasts were unstimulated (control) or challenged with \(\omega-3\) polyunsaturated fatty acids (PUFAs): eicosapentaenoic acid (EPA) \((n = 10)\) (patients) (A, E) and docosahexaenoic acid (DHA), \((n = 9)\) (patients) (B, F) saturated fatty acid (SFA): palmitic acid (PA), \((n = 8)\) (patients) (C, G) and \(\omega-6\) PUFA: arachidonic acid (AA), \((n = 11)\) (patients) (D, H) in 0.1% BSA-DMEM (1, 10, 100μM) for 48h. Cell free supernatants were collected and IL-6 (A-D) and CXCL8 (E-H) release was measured using ELISA. All data are represented as mean ± standard error of the mean. All concentrations are compared to their respective control values and other concentrations using a one-way ANOVA with a Bonferroni post-test. Significance is represented as * \((p<0.05)\) and ** \((p<0.01)\).

Figure 3. Greater cytokine release with combined arachidonic acid and TNF\(\alpha\) treatments, than either alone.

Human pulmonary fibroblasts \((n = 10)\) (patients) were unstimulated (control) or challenged with arachidonic acid (AA) (10, 100μM) for 24h with or without TNF\(\alpha\) (1ng/ml) for another 24h. Cell free supernatants were collected and IL-6 (A) and CXCL8 (B) release was measured using ELISA. Striped bars represent combination treatment with AA and TNF\(\alpha\). Black bars represent treatment with AA alone. All data are represented as mean ± standard error of the mean. Challenges with TNF\(\alpha\) are compared to their respective challenge without TNF\(\alpha\), using a two-way ANOVA with a Bonferroni post-test. Significance is represented as * \((p<0.05)\), ** \((p<0.01)\) and **** \((p<0.0001)\).

Figure 4. Indomethacin inhibits arachidonic (AA)- and combined AA and TNF\(\alpha\)-induced IL-6 release.

Human pulmonary fibroblasts \((n = 7-13)\) (patients) were unstimulated (control) or treated with or without indomethacin \((10^{-5}M)\) for 60 minutes prior to challenge with arachidonic acid AA (100μM) for 24h with or without TNF\(\alpha\) (1ng/ml) for another 24h. Cell free supernatants were collected and IL-6 (A-B) and CXCL8 (C-D) release was measured using ELISA. Blocked bars indicate pre-treatment with indomethacin \((10^{-5}M)\), black bar represent no pre-treatment. All data are represented as mean ± standard error of the mean. All treatments with indomethacin are compared to their respective controls in the absence of indomethacin, using a two-way ANOVA with a Bonferroni post-test. Significance is represented as * \((p<0.05)\) and **** \((p<0.0001)\).

Figure 5. Inhibition of p38 MAP kinase suppressed arachidonic (AA)-induced cytokine release, but not combined AA and TNF\(\alpha\)-induced CXCL8 release.

Primary human pulmonary fibroblasts \((n = 7-13)\) (patients) were treated with or without the p38 mitogen-activated protein (MAP) kinase signaling inhibitor SB239063 (3μM) for 60 minutes before challenge with arachidonic acid (AA) (100μM) (A, D), TNF\(\alpha\) (1ng/ml) (B, E) or AA (100μM) in combination with TNF\(\alpha\) (1ng/ml) (C, F). Cell free supernatants were collected after 48h and IL-6 (A-C) and CXCL8 (D-F) release was measured using ELISA. All data are represented as mean ± standard error of the mean. All treatments with the p38 MAP kinase inhibitor are compared to their respective control in the absence of the inhibitor using a paired t-test. Significance is represented as * \((p<0.05)\) and ** \((p<0.01)\).
Figure 6. TNFα alone activates p38 MAP kinase and NF-κB signaling to similar extent as arachidonic acid combined with TNFα. Primary human pulmonary fibroblasts (n = 9) (patients) were unstimulated (control) or challenged with arachidonic acid (AA) (100μM), TNFα (1ng/ml) or AA (100μM) in combination with TNFα (1ng/ml) for 30 minutes, before whole cell lysates were collected and p38 mitogen-activated protein (MAP) kinase (A) or NF-κB p65 (B) phosphorylation was assessed by western blotting. Total p38 MAP kinase (C) or NF-κB p65 (D) 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. Data was analysed using a one-way ANOVA with a Bonferroni post-test. Significance is represented as * (p<0.05). Representative western blots of phosphorylated and total p38 MAPK and NF-κB p65 are shown under each graph.

Figure 7. Arachidonic acid induces IL-6 and CXCL8 release from primary airway smooth muscle cells, but not bronchial epithelial cells. Bronchial epithelial cells (BEAS2Bs) (n = 7) (replicates) (A, B) and human primary airway smooth muscle (ASM) cells (n = 7) (patients) (E, F) were unstimulated (control) or challenged with arachidonic acid (AA) in 0.1% BSA-DMEM (1, 10, 100μM) for 48h. Primary human bronchial epithelial (HBE) cells (n = 7) (patients) (C, D) were unstimulated (control) or challenged with AA (10, 100μM) or TNFα (1ng/ml) (positive control) in BMEM supplemented with 0.05% insulin, 0.1% transferrin and 0.1% BSA. 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 concentrations are compared to their respective control value using a one-way ANOVA with a Bonferroni post-test. Significance is represented as * (p<0.05) and ** (p<0.01).

Figure 8. Greater cytokine release with combined arachidonic acid and TNFα-treatments from airway smooth muscle cells that is suppressed by inhibition of COX and p38. Human airway smooth muscle (ASM) cells (n = 7-8) (patients) were unstimulated (control) or challenged with arachidonic acid (AA) (10, 100μM) for 24h with or without TNFα (1ng/ml) for another 24h (A, B). Some cells were treated with or without the COX-inhibitor indomethacin (10^{-5}M) (C, D) or the p38 mitogen-activated protein (MAP) kinase signaling inhibitor SB239063 (3μM)(E, F) for 60 minutes prior to challenge with AA (100μM) for 24h with TNFα (1ng/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. Striped bars represent combination treatment of AA with TNFα, blocked bars indicate pre-treatment with indomethacin (10^{-5}M) and dotted bars pre-treatment with SB239063. All data are represented as mean ± standard error of the mean. Challenges with TNFα, indomethacin or SB239063 are compared to their respective challenge without TNFα, indomethacin or SB239063, using a two-way ANOVA with a Bonferroni post-test. Significance is represented as * (p<0.05), ** (p<0.01), *** (p<0.001) and **** (p<0.0001).

Figure 9. Human bronchial epithelial cell line (BEAS-2B) expresses free fatty acids receptors 1-4. Total RNA was extracted from cultured BEAS-2Bs and mRNA expression of the free fatty acid receptors (FFARs) (n = 5) (replicates) 1 (A), 2 (B), 3 (C) and 4 (D) was assessed by gel-based PCR. GAPDH (housekeeping gene) served as the control (E). Lane 1 is negative control (water); lane 2 till 6 is total RNA from cultured BEAS-2Bs (5 replicates); lane 7 is total RNA from cultured fibroblasts (positive control).
**Figure 10. Dexamethasone suppresses combined AA and TNFα-induced IL-6 and to lesser extent CXCL8 release.** Primary human pulmonary fibroblasts (n = 7-9) (patients) were treated with or without dexamethasone (10^{-9}, 10^{-8}, 10^{-7} or 10^{-6} M) for 60 minutes, before challenge with arachidonic acid (AA) (100μM) (A), TNFα (1ng/ml) (B) or AA (100μM) in combination with TNFα (1ng/mL) (C). Cell free supernatants were collected after 48h and IL-6 (dotted line) and CXCL8 (solid line) release was measured using ELISA. Data is represented as % inhibition of AA-, TNFα- or combined AA and TNFα-induced IL-6 and CXCL8 release. All concentrations are compared to their challenge in the absence of dexamethasone using a one-way ANOVA with a Bonferroni post-test. Significance is represented as *(p<0.05), ** (p<0.01) and *** (p<0.001).
A. FFAR1

B. FFAR2

C. FFAR3

D. FFAR4

E. GAPDH

Legend
Lane
1 = negative control
2-8 = cultured fibroblasts (n = 7) (patients)
9 = cultured smooth muscle cells or monocytes (positive control)
IL-6 (pg/ml) vs. Control 1 μM 0 μM 100 μM

ω-3 PUFA (EPA)

ω-3 PUFA (DHA)

SFA (PA)

ω-6 PUFA (AA)
A

- AA only
- + TNFα (1ng/mg)

Control AA10μM AA100μM

IL-6 (pg/ml)

B

Control AA10μM AA100μM

CXCL8 (pg/ml)
Figure A: Comparison of IL-6 and CXCL8 levels in control and AA 100μM treated groups with and without indomethacin pre-treatment.

Figure B: Comparison of IL-6 and CXCL8 levels in control, TNFα, AA100μM + TNFα treated groups.

Figure C: Same as Figure A but focusing on CXCL8 levels.

Figure D: Same as Figure B but focusing on CXCL8 levels.
A. FFAR1

-900bp

B. FFAR2

-178bp

C. FFAR3

-141bp

D. FFAR4

-130bp

E. GAPDH

-598bp

Legend

Lane
1 = negative control
2-6 = cultured BEAS-2Bs (n = 5) (replicates)
7 = cultured fibroblasts (positive control)
% inhibition AA-induced IL-6 and CXCL8 release

% inhibition TNF-α-induced IL-6 and CXCL8 release

% inhibition AA+TNF-α-induced IL-6 and CXCL8 release