Short chain fatty acids increase TNFα-induced inflammation in primary human lung mesenchymal cells through the activation of p38 MAP kinase.

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Abstract

Short chain fatty acids (SCFAs), produced as by-products of dietary fibre metabolism by gut bacteria, have anti-inflammatory properties and could potentially be used for the treatment of inflammatory diseases, including asthma. The direct effects of SCFAs on inflammatory responses in primary human lung mesenchymal cells have not been assessed. We investigated whether SCFAs can protect against TNFα-induced inflammation in primary human lung fibroblasts (HLFs) and airway smooth muscle (ASM) cells *in vitro*.

HLFs and ASM cells were exposed to SCFAs, acetate (C2:0), propionate (C3:0) and butyrate (C4:0) (0.01mM-25mM) with or without TNFα, and the release of pro-inflammatory cytokines, IL-6 and CXCL8, was measured using ELISA. We found that none of the SCFAs suppressed TNFα-induced cytokine release. On the contrary, challenge with supra-physiological concentrations (10mM-25mM) as might be used therapeutically of propionate or butyrate in combination with TNFα resulted in substantially greater IL-6 and CXCL8 release from HLFs and ASM cells than challenge with TNFα alone, demonstrating synergistic effects. In ASM cells challenge with acetate also enhanced TNFα-induced IL-6, but not CXCL8 release.

Synergistic upregulation of IL-6 and CXCL8 was mediated through the activation of free fatty acid receptor (FFAR)3, but not FFAR2. The signalling pathways involved were further examined using specific inhibitors and immunoblotting, and responses were found to be mediated through p38 MAP kinase signalling. This study demonstrates that pro-inflammatory, rather than anti-inflammatory effects of SCFAs are evident in lung mesenchymal cells.

Key words: Short chain fatty acids; human lung mesenchymal cells; asthma; inflammation; free fatty acid receptor 3.

Introduction

Asthma affects nearly 300 million people worldwide and is characterised by chronic airway inflammation. Anti-inflammatory treatments such as corticosteroids are commonly used to treat the disease, however around 10% of patients with severe asthma are refractory to these medications. In addition severe side effects are often observed when steroids are used at high doses, therefore new well-tolerated anti-inflammatory therapeutics are needed (43).

There is increasing evidence implicating the gut microbiota as a critical contributor to host health and immune homeostasis in inflammatory diseases including type-2 diabetes, obesity, chronic obstructive pulmonary disease and asthma (4, 5, 48). The prevailing hypothesis is that gut bacteria produce short-chain fatty acids (SCFAs) that are directly anti-inflammatory, as by-products of dietary fibre metabolism. SCFAs are fatty acids with fewer than 6 carbon (C) atoms. Important sources of dietary fibre are fruit and vegetables and the most abundant metabolites produced are acetate (C2:0), propionate (C3:0) and butyrate (C4:0). In the large intestine, SCFAs occur at concentrations ranging from 30 to 150mM. They are absorbed into the portal circulation and reach the bloodstream (0.1-5mM), where they potentially elicit anti-inflammatory effects. SCFAs can also be detected in sputum (0.1-5mM), indicating that they reach the lungs and airways (11). Possible mechanisms by which SCFAs elicit their effects are through the inhibition of histone deacetylases (HDACs) and activation of G-protein coupled receptors (GPCRs) such as GPR43 and GPR41, also known as free fatty acid receptor (FFAR)2 and FFAR3 leading to consequent effects on gene transcription. FFARs are surface receptors found on cells of the gastrointestinal tract, as well as immune cells (e.g., neutrophils and monocytes) and adipocytes (52). We recently showed that lung mesenchymal cells also express these receptors (37). FFARs differ in their affinity for SCFAs. FFAR2 has a similar affinity for acetate, propionate and butyrate, while FFAR3 has greater affinity for propionate than butyrate and low affinity for acetate (45).

Short chain fatty acids increase TNFα-induced inflammation

The potential beneficial effects of SCFAs in asthma have not been extensively studied. However, recent mouse-model studies showed that dietary fibre and propionate protect against allergic airway disease and maternal intake of dietary fibre has been associated with a reduced asthma phenotype in the offspring (42, 44). In addition, a recent human pilot study showed acute reductions in airway inflammation biomarkers, including sputum CXCL8, eNO and sputum inflammatory cell counts after consuming a high soluble fibre meal (13). However, more studies are needed to determine the potential beneficial effects of SCFAs in asthma. *In vitro* studies using colonic epithelial cells and different immune cells, including neutrophils and macrophages, show that SCFAs are anti-inflammatory, as shown by reduced chemotaxis and pro-inflammatory cytokine and reactive oxygen species release in response to inflammatory stimuli (6, 47, 52). However, the direct effects of SCFAs in human lung mesenchymal cells have not been investigated.

Tumour necrosis factor (TNF)-α is a multi-potent pro-inflammatory mediator, mainly produced by macrophages, and has been implicated in the pathology of asthma. Serum TNFα levels are increased in the airways of asthma patients and are positively correlated with the severity of the disease (19, 38). TNFα plays a critical role in the immunoregulation of asthma by contributing to bronchopulmonary inflammation and airway hyperresponsiveness. TNFα might also contribute to refractory asthma through the recruitment of neutrophils and the induction of glucocorticoid resistance (3).

We hypothesised that SCFAs could potentially be used for the treatment of asthma, specifically to reduce inflammatory responses in the lungs and airways via the activation of FFAR2 and/or 3. The aim of this study was to investigate the direct effects of SCFAs on inflammatory responses in primary human lung mesenchymal cells, *in vitro*. Since TNFα-induced cytokine release is steroid insensitive, we used this to challenge human lung fibroblasts (HLFs) and airway smooth muscle (ASM) cells and examined whether SCFAs could protect against TNFα-induced inflammation, by measuring the release of pro-inflammatory mediators.

Methods

Cell culture

HLFs were isolated from the parenchyma and ASM cells from the bronchial airways of lungs from patients undergoing lung transplantation or lung resection for thoracic malignancies, as previously described (15, 23). Ethical approval for all experiments was provided by The University of Sydney Human Ethics Committee and the Sydney South West Area Health Service, and written informed consent was obtained. *Table 1* shows the patient demographics. HLFs and ASM cells were seeded in 12-well or 6-well plates at a density of 6.2 x 10^4 cells/mL in DMEM medium containing 5% fatal bovine serum (FBS) and 1% Antibiotic-Antimycotic (Gibco, Grand Island, New York, US) and grown to sub confluence (3 days). HLFs and ASM 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.
We also used the human monocyte cell line THP-1 (ATCC, Manassas, VA). THP-1 cells were maintained in RPMI 1640 medium (Gibco), supplemented with 10% FBS, 1% antibiotic-antimycotic and 1% HEPES (Gibco). THP-1 cells were seeded at a density of 1 x 10^6 cells/mL in 12-well plates and treatments were added. All experiments were carried out using HLFs and ASM cells between passage 2 and 5, and THP-1 cells between passage 3 and 6.

Treatment of cells with SCFAs and FFAR agonists

Cells were unstimulated (control) or stimulated with propionate (0.5mM-25mM), butyrate (0.01mM-10mM), acetate (0.5mM-25mM) (Sigma Aldrich, Castle Hill, NSW, Australia), FFAR2 agonist 4-CMTB (10μM) (Sigma), FFAR3agonist AR420626 (10μM) (Sigma), FFAR3 antagonist β-hydroxybutyrate (BOH) (100mM) (Sigma) or vehicle (0.1% DMSO) for 24h or 96h, with or without TNFα (1ng/mL) (ThermoFisher, Scoresby, VIC, Australia) or LPS (1μg/mL) (Sigma) for another 12 or 24h. The total incubation time was 36, 48 or 120h. All cells were incubated at 37°C with 5% CO_2.

Inhibition of signaling pathways

HLFs were treated with inhibitors of p38 mitogen-activated protein kinase (MAPK) (SB239063, 3μM) (Tocris, Ellisville, MO, USA), MAP kinase 1 (MEK1) (PD98059, 10μM), c-Jun N-terminal kinase (JNK) (SP600125, 10μM), (Calbiochem, San Diego, CA, USA), COX (indomethacin, 10μM) and NF-κB (BAY-117082, 1μM) (Sigma-Aldrich) for 1 hour before stimulation with propionate (25mM) with or without TNFα (1ng/mL).

ELISA

Levels of IL-6 and CXCL8 in supernatants were measured using commercial antibody kits according to the manufacturer's instructions (R&D Systems, Minnesota, USA). The detection limit of both assays was 15.6pg/ml.

Quantitative PCR

Total RNA was extracted using the ISOLATE II RNA Mini Kit and transcribed into cDNA using the SensiFAST™ cDNA Synthesis Kit (Bioline, Alexandria, Australia). qPCR was performed using the StepOne Plus detection system and data were analysed with StepOne software (Applied Biosystems, Melbourne, Australia). Assays were carried out in triplicate using a reaction mixture containing the Bioline SensiFAST Probe Hi-ROX Master Mix, primer for IL-6 or CXCL8 and for ubiquitously expressed ribosomal RNA (18S rRNA) as a housekeeping gene. Relative expression was normalised to 18S rRNA expression and quantification performed using the 2ΔΔCT method.

Western blotting

To assess the activation of intracellular signaling molecules in HLFs following stimulation with propionate with or without TNFα, relative levels of phosphorylated p38 MAPK, JNK, ERK, Akt and NF-κB from cell lysates were assessed by western blotting. Cells cultured in the presence or absence of propionate (25mM) with or without TNFα (1ng/mL) for 30 min were lysed (20mM Tris, pH 7.4, 150mM NaCl, 1mM Na2EDTA, 1mM EGTA, 20mM NA4P2P7, 2mM Na3VO4, 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 MAPK (Thr180/Tyr182) (No. 9211) , rabbit anti-p38 MAPK (No. 9212), rabbit anti-phospho SAPK/JNK (Thr183/Tyr185) (No. 9251), rabbit anti-SAPK/JNK (No. 9252), rabbit anti-phospho ERK (Thr202/Tyr204) (No. 9101), rabbit anti-ERK (No. 9102), rabbit anti-phospho AKT (Thr308) (244F9) (No. 4056), rabbit anti-AKT (No. 9272), rabbit anti-phospho NF-κB p65 (Ser536) (93H1) (No. 3033), rabbit anti-NF-κB p65 (D14E12) XP (No. 8242) (all 1:1000, Cell Signaling Technology) or anti-mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (MAP374) (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.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism version 7 software (San Diego, CA, USA). Comparisons of data were carried out using 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.

Results

Stimulation with propionate or butyrate and TNFα increases cytokine release from fibroblasts. To assess whether SCFAs inhibit the inflammatory response to TNFα in human lung mesenchymal cells, HLFs (*n* = 10-24) were challenged with propionate, butyrate or acetate prior to stimulation with TNFα, and IL-6 and CXCL8 release was measured. None of the SCFAs supressed TNFα-induced cytokine release. Challenge with propionate (25mM), butyrate (10mM) and acetate (25mM) alone did not induce cytokine release from HLFs (*Figure 1A-F*). However, challenge with the combination of propionate (10mM and 25mM) and TNFα (1ng/ml) resulted in substantially greater IL-6 ($p<0.05$*)* and CXCL8 ($p<0.001$) release than challenge with TNFα alone (*Figure 1A and 1B)*. The effect of the combination of propionate and TNFα on IL-6 and CXCL8 release was greater than the sum of the individual effects of propionate and TNFα, demonstrating a synergistic effect. Challenge with butyrate (10mM) and TNFα also resulted in greater IL-6 ($p<0.001$) and CXCL8 ($p<0.0001$) release, than TNFα alone (*Figure 1C and 1D*). There was no interaction between acetate and TNFα (*Figure 1E and 1F*).

Stimulation with propionate and TNFα increases IL-6 and CXCL8 mRNA expression in fibroblasts. Next, we assessed whether propionate increases TNFα-induced IL-6 and CXCL8 mRNA expression using qPCR. Challenge with the combination of propionate (25mM) and TNFα (1ng/ml) resulted in substantially greater mRNA expression of IL-6 ($n = 8$, $p<0.05$) and CXCL8 ($n = 8$, $p<0.05$) *(Figure 2)* than challenge with TNFα alone at both time points (12h and 24h). The effect of the combination of propionate and TNFα on IL-6 and CXCL8 mRNA expression was greater than the sum of the individual effects of propionate and TNFα, again demonstrating synergistic effects.

SCFAs enhance TNFα-induced IL-6 and CXCL8 release through FFAR3 signalling.

To investigate whether these pro-inflammatory effects are mediated through activation of FFAR2 and/or FFAR3, HLFs were challenged with specific agonists for FFAR2 (4-CMTB) or FFAR3 (AR420626), prior to stimulation with TNFα. Challenge with the combination of AR420626 (10μM), but not 4-CMTB, and TNFα resulted in greater IL-6 (n = 14, p<0.05) and CXCL8 (n = 14, p<0.001) release than TNFα alone (*Figure 3A-3D*), suggesting the activation of FFAR3, but not FFAR2 to be the signalling mechanism for SCFAs. To further confirm the involvement of FFAR3 signalling, HLFs were incubated with FFAR3 antagonist BOH (100mM) for 60 minutes prior to challenge with the combination of propionate (10mM) and TNFα. Blocking of FFAR3 signalling with BOH suppressed propionate and TNFα-induced IL-6 (n = 8, p<0.05) and CXCL8 release (n = 8, p<0.01) (*Figure 3E and 3F*) **Stimulation with propionate and TNFα leads to hyperactivation p38 MAPK.** To investigate the mechanisms underlying the effects of combined propionate and TNFα-induced IL-6 and CXCL8 release, we used protein immunoblotting to investigate the activation of signalling pathways. We focussed on five known major signalling pathways (NF-κB, p38 MAPK, AKT, ERK and SAPK/JNK), all of which have been shown to stimulate IL-6 and/or CXCL8 production (22, 26, 36, 41). Phosphorylation of NF-κB was increased 30 minutes after stimulation with TNFα alone (n = 10, p<0.01), but was not increased by concomitant treatment with propionate (p<0.01) (*Figure 4A*). p38 MAPK phosphorylation was increased upon challenge with propionate alone (n = 10, p<0.05), TNFα alone (p<0.01) and the combination of propionate and TNFα (n = 10, p<0.01) (*Figure 4C*). The combination of propionate and TNFα led to greater phosphorylation of p38 MAPK, than TNFα alone (p<0.05), showing hyperactivation of this pathway. Phosphorylation of AKT did not increase with any of the treatments (*Figure 4E*) and phosphorylation of ERK was increased upon challenge with TNFα alone (n = 10, p<0.01), but not in combination with propionate (*Figure 4G*). Finally, phosphorylation of JNK was increased upon challenge with TNFα alone (n = 10, p<0.05) and the combination of propionate and TNFα (p<0.01) (*Figure 4I*). Total NF-κB, p38 MAPK, AKT, ERK and SAPK/JNK did not change with any treatment *(Figure 4B, D, F, H, J).*

Inhibition of p38 MAPK suppresses and propionate and TNFα-induced cytokine release. To further investigate and confirm the mechanisms underlying the effects of propionate and TNFα-induced IL-6 and CXCL8 release, specific inhibitors were used to block COX, p38 MAPK, JNK, NF-κB or MEK activation, at concentrations previously shown to be effective in human airway cells (7, 10, 12, 14, 17, 49). Inhibition of COX, JNK or MEK did not suppress cytokine release induced by propionate in combination with TNFα or TNFα alone. However, inhibition of p38 MAPK with SB239063 suppressed IL-6 (n = 10, p<0.05) and CXCL8 (n = 10, p<0.05) release induced by TNFα alone *(Figure 5A and 5B*) and by the combination of propionate and TNFα (n = 11, p<0.05 for IL-6 and p<0.01 for CXCL8) *(Figure 5C and 5D).* Inhibition of NF-κB suppressed IL-6 (p<0.05), but not CXCL8 release, induced by propionate in combination with TNFα. This suggests p38 MAPK to be the main pathway. However, the only partial (30-60%) inhibition of propionate and TNFα-induced cytokine release achieved by blocking the p38 MAPK signaling pathway, indicates that other pathways are also involved.**Chronic exposure of SCFAs also enhances TNFα-induced cytokine release from fibroblasts**

To explore whether chronic exposure to SCFAs has similar effects as acute exposure, HLFs (n = 7*)* were challenged with propionate (25mM), butyrate (10mM) or acetate (25mM) for 96h before TNFα was added for another 24h. Challenge with propionate or butyrate, but not acetate led to substantially greater IL-6 (p<0.01) and CXCL8 (p<0.001), than challenge with TNFα alone (*Figure 6*). These results demonstrate that chronic or acute exposures of SCFAs have similar effects on TNFα-induced IL-6 and CXCL8 release.

Stimulation with acetate, propionate or butyrate and TNFα increases cytokine release from ASM cells. To explore whether other lung mesenchymal cells respond in a similar way to HLFs, we

repeated selected experiments in primary human ASM cells (*n* = 8-20). The combination of propionate (10mM and 25mM) and TNFα resulted in substantially greater IL-6 ($p<0.01$) and CXCL8 release ($p<0.01$), than challenge with TNFα alone (*Figure 7A and 7B*). Challenge with butyrate (10mM) and TNFα also resulted in greater IL-6 (10mM) ($p<0.05$) and CXCL8 release ($p<0.01$), than TNFα challenge alone (*Figure 6C and 6D*). The combination of acetate (10mM and 25mM) and TNFα had no effect on IL-6, but resulted in greater CXCL8 ($p<0.01$) release from ASM cells (*Figure 7E and 7F*). Thus, challenge of ASM cells shows similar effects as in the HLFs.

Propionate suppresses LPS-induced CXCL8 release from THP-1 monocytes. Our findings show that SCFAs have pro-inflammatory and not anti-inflammatory effects on lung mesenchymal cells. This contradicts our hypothesis, as well as published literature demonstrating that SCFAs are generally anti-inflammatory including in white blood cells such as monocytes (33). To confirm and replicate these findings in our study, THP-1 cells were challenged with acetate, propionate or butyrate prior to stimulation with LPS, and CXCL8 release was measured. Propionate (25mM), but not acetate or butyrate suppressed LPS-induced CXCL8 release from THP-1 cells (n = 7, $p<0.001$) (*Figure 8A-C*). None of the SCFAs increased LPS-induced cytokine release, demonstrating that the pro-inflammatory effects of SCFAs that we have found are cell specific.

Discussion

This study is the first to investigate whether SCFAs directly suppress innate immune responses in primary human lung mesenchymal cells. We found that the SCFAs propionate, butyrate or acetate

did not suppress TNFα-induced cytokine release from HLFs. Furthermore, challenge with high concentrations (10mM and 25mM) of propionate in combination with TNFα led to greater IL-6 and CXCL8 release than TNFα alone. The effect of the combination of propionate and TNFα on cytokine release was substantially greater than the sum of the individual effects of propionate or TNFα alone which indicates that the effects are synergistic. Butyrate, but not acetate also increased TNFα-induced cytokine release, although the effect on IL-6 release was less profound compared to propionate. These effects were observed with acute (24h) and chronic exposure (96h) of SCFAs. Several studies have demonstrated that SCFAs have therapeutic potential in protecting against allergic airways disease in animal models (42, 44), and asthma in human studies (13), potentially through their anti-inflammatory properties. SCFAs have been shown to inhibit the production of pro-inflammatory mediators such as TNFα in LPS-stimulated immune cells, including neutrophils, monocytes and macrophages (30, 34, 51). Inhibitory effects have also been observed in human intestinal cell lines, with reduced LPS-induced CXCL8 release, associated with the inhibition of HDAC activity (1). However, not all studies have reported anti-inflammatory effects. SCFAs have also been shown to increase pro-inflammatory cytokine production in toll like receptor (TLR)-stimulated polymorphonuclear cells and epithelial cells *in vitro* (28, 32) and well as in a mouse-model study (20). In addition, orally administered SCFAs have been shown to induce inflammation in the renal system in mice (35). Moreover, there is evidence for SCFA enhancement of neutrophil chemotaxis in mouse-model studies (50). In bronchial epithelial cells, depending on the concentration of SCFAs, either inhibitory or stimulatory effects on pro-inflammatory cytokine production are observed (11). Thus, observations of the effects of SCFAs on inflammatory processes in immune cells and structural cells are divergent. They can be pro- or anti-inflammatory depending on the cell type that is studied and on the conditions, type and concentration of SCFA and type of co-stimulation. The concentrations of SCFAs used in this study were chosen based on concentrations found in the colonic lumen (30-150mM), the airways (0.1-5mM) and from previous studies, and based on individual concentrations of SCFAs with acetate being the most prevalent followed by propionate and butyrate, respectively

(11, 55). To investigate the use of SCFAs as a therapeutic strategy, we also used concentrations that are higher than physiological concentrations, as typically occurs when exogenous cytokines, prostaglandins or other mediators are used as a therapeutics. We examined the release of IL-6 and CXCL8 from mesenchymal cells, as these are pro-inflammatory mediators and are important in the pathogenesis of asthma (2). IL-6 is a marker of systemic inflammation and its levels are increased in the serum and BAL fluid of asthma patients. Increased IL-6 levels have also been associated with asthma exacerbations, disease severity and poor lung function (25). CXCL8 is a potent neutrophil chemoattractant, and its levels are increased in sputum in severe asthma patients and during virus-induced asthma exacerbations (2).

This is the first study to investigate the direct effects of SCFAs specifically in primary HLFs. HLFs are one of the main structural cells in the airway wall and play an important role in inflammation and the production of potent pro-inflammatory mediators, including IL-6 and CXCL8, and provide a good representation of airway mesenchymal cells (16, 18). In addition, fibroblasts are located at the interface of the airway lumen and the blood supply and are directly exposed to constituents of tissue fluids (plasma), including SCFAs which are present in millimolar concentrations. Hence, these cells are likely to be key cells in driving inflammatory responses to serum derived factors in asthma and consequently our study primarily focussed on pulmonary fibroblasts.

A possible mechanism by which SCFAs elicit biological responses is the activation of FFAR2 and/or FFAR3. These two GPCRs share around 40% peptide sequence, but differ in their tissue distribution, physiological roles and affinity for SCFAs. FFAR2 has a similar affinity for acetate, propionate and butyrate, whereas FFAR3 has a greater affinity for propionate than butyrate and the lowest affinity for acetate. Acetate mainly activates FFAR2, propionate mainly activates FFAR3, and butyrate equally activates FFAR2 and FFAR3 (24, 45). Despite growing interest in these receptors, many questions regarding their function and effect on inflammatory responses remain unanswered. Studies using FFAR2 (GPR43) and/or FFAR3 (GPR41) deficient (-/-) mice show inconsistent results; Maslowski and colleagues showed that FFAR2 was necessary for the resolution of a number of

inflammatory responses in models of colitis and asthma using FFAR2-/- and germ-free mice, however, not all studies confirm these findings (29). Sina *et al*. showed that FFAR2-/- mice had reduced polymorphonuclear leucocyte infiltration that was associated with less tissue damage in a mouse-model of colitis (39). These results suggest a potential pro-inflammatory role of FFAR2 in colitis. Trompette and colleagues, however, showed that a high fibre diet led to a reduction in inflammatory markers, including eosinophil infiltration and goblet cell hyperplasia in a mouse-model of allergic asthma compared to a low fibre diet (44). This finding was also observed in FFAR2-/- but not FFAR3-/- animals, suggesting that activation of FFAR3 is protective. In addition, *in vitro* studies using bronchial epithelial cells from cystic fibrosis patients found increased release of the pro-inflammatory mediator CXCL8 upon stimulation with SCFAs that was reduced by siRNA knockdown of FFAR3 (31). In this study, we investigated whether activation of FFAR2 and/or FFAR3 is responsible for the observed pro-inflammatory effect of propionate using specific synthetic agonists for these receptors. We used 4-CMTB, which is a selective allosteric ligand for FFAR2 (40), and AR420626, which is a selective agonist of FFAR3 that does not activate FFAR2 at concentrations up to 100μM (9). Interestingly, we found that AR420626, but not 4-CMTB in combination with TNFα, resulted in greater IL-6 and CXCL8 release, than challenge with TNFα alone. These results suggest that activation of FFAR3, but not FFAR2 enhances the pro-inflammatory effects of TNFα in HLFs. This could also explain the lack of pro-inflammatory effect of acetate in HLFs, as acetate primarily acts on FFAR2. We further confirmed these findings using the FFAR3 antagonist BOH. Several studies have shown BOH to inhibit FFAR3 signalling *in vitro* (21, 27, 54). We found that BOH pre-treatment suppressed propionate and TNFα-induced IL-6 and CXCL8 release, providing further evidence for FFAR3 to be the main signalling pathway.

We also demonstrated that propionate increases TNFα-induced IL-6 and CXCL8 mRNA expression, indicating that the transcription of these cytokines is enhanced. To further understand the mechanisms involved, signaling pathways were investigated using protein immunoblotting. We focussed on five main signalling pathways, NF-κB, p38 MAPK, AKT, ERK and SAPK/JNK, all of which

have been shown to stimulate IL-6 and/or CXCL8 production (22, 26, 36, 41). We demonstrated that in HLFs, TNFα alone activates NF-κB, p38 MAPK, ERK and JNK, but not AKT signalling. TNFα is known to stimulate multiple signal transduction pathways, including JNK, p38 and NF-κB, resulting in IL-6 and CXCL8 release in other cell types (8, 53). More importantly, we found that hyperactivation of p38 MAPK is the underlying mechanism for the pro-inflammatory effects of propionate as challenge with this SCFA alone led to an increase in phosphorylation of p38 MAPK, and the combination of propionate and TNFα resulted in greater p38 MAPK phosphorylation than TNFα alone. We further investigated and confirmed the mechanisms involved in propionate and TNFα-induced IL-6 and CXCL8 release using specific signaling inhibitors. SB239063 is a potent and selective inhibitor of p38 MAPK and displays specific and high-affinity binding (IC50 = 44nM) (46). It suppressed IL-6 and CXLC8 release induced by TNFα alone and by the combination of propionate and TNFα. Inhibition of NF-κB partially suppressed IL-6, but not CXCL8 release induced by propionate and TNFα. These results confirm that p38 MAPK signalling is the main signal transduction pathway responsible for propionate and TNFα-induced cytokine release.

To explore whether other structural lung cells respond in the same way as pulmonary fibroblasts, we repeated selected experiments in primary ASM cells. In ASM cells, propionate and butyrate in combination with TNFα also resulted in synergistic cytokine release, but the effect of butyrate was less profound compared to propionate. These results show that SCFAs have similar effects in ASM cells and HLFs. Interestingly, acetate also enhanced TNFα-induced CXCL8, but not IL-6 release from ASM cells, indicating that this SCFA has pro-inflammatory effects in ASM cells, but not HLFs. These results show different cells respond differently in some way to SCFAs, but the consistent observation is that propionate and butyrate are the most potent SCFAs in enhancing pro-inflammatory effects in primary lung mesenchymal cells. This is interesting, as based on previous findings from others, we expected SCFAs to be anti-inflammatory and potentially beneficial in reducing inflammation in asthma, but found opposite results in lung mesenchymal cells.

We next used a monocyte cell line (THP-1) and investigated whether SCFAs suppressed LPS-induced

CXCL8 release, and found an inhibitory effect of propionate. These results confirm SCFAs to have both anti-inflammatory and pro-inflammatory effects, depending on the stimulus and cell type studied. Although the studies in this manuscript utilized primary human mesenchymal cells, an important limitation of this study is that all studies were done *in vitro.* In future studies effects of SCFAs on inflammatory markers will be investigated using an *in vivo* model. In summary, this study demonstrates that exposure of primary HLFs and ASM cells to supra-physiological concentrations of SCFAs synergistically enhances TNFα-induced inflammatory responses, as measured by IL-6 and CXCL8 release, through activation of FFAR3 and p38 MAPK signalling. Contrary to our hypothesis, this study demonstrates that pro-inflammatory, rather than anti-inflammatory effects of SCFAs are evident in lung mesenchymal cells.

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Disclosures

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

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Author contributions

S.R, D.X, P.M.H and B.G.O conceived and planned the experiments. S.R. and D.X carried out the experiments. S.R, D.X, 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.

Table 1. Summary of patient demographics

Table 1. Patient demographics (*n* = 58)

Donor #	**Cell type**	**Diagnosis**	**Age**	**Gender** (F/M)	**Surgery** (T/R/B)	**Smoking history** (Current/ex/non) (pack years)	**Medication before surgery**	**LTOT** (yes/no)	**Experiments**
1.	HLF	sarcoidosis/ pulmonary fibrosis	50	M	T	N/A	budesonide/formoterol, terbutaline	N/A	qPCR, inhibitors, FFAR agonists
2.	HLF	NSCLC	62	M	R	Non-smoker	travoprost	No	qPCR, inhibitors, FFAR agonists
3.	HLF	tumour	70	F	R	Ex-smoker (45 pack years)	tiotropium, budesonide/formoterol, ezetimibe, rosuvastatin, felodipine, sertraline, indapamide.	No	qPCR, inhibitors, FFAR agonists
4.	HLF	COPD	55	M	T	Ex-smoker (30 pack years)	tiotropium, fluticasone/salmeterol, salbutamol, prednisolone	No	qPCR, inhibitors, FFAR agonists
5.	HLF	COPD	52	F	T	Ex-smoker (50 pack years)	venlafaxine, prednisolone, fluticasone/salmeterol, tiotropium	No	qPCR, SCFAs + TNFα, inhibitors, FFAR agonists, WB
6.	HLF	adenocarcinoma	64	F	R	Ex-smoker	levothyroxine, telmisartan, furosemide, spironolactone, rosuvastatin, warfarin	No	qPCR, SCFAs + TNFα, inhibitors, FFAR agonists, WB
7.	HLF	sarcoidosis	46	M	T	Ex-smoker (<2 pack years)	methotrexate, folinic acid, budesonide/formoterol, amoxicillin/clavulanic acid, omeprazole	No	qPCR, SCFAs + TNFα, inhibitors, FFAR agonists, WB
8.	HLF	emphysema	54	M	T	Ex-smoker (60 pack years)	N/A	Yes	qPCR, SCFAs + TNFα, inhibitors, FFAR agonists, WB
9.	HLF	IPF	58	F	T	N/A	salbutamol, warfarin, pravastatin, tralokinumab, fenofibrate, celecoxib, levothyroxine, mometasone	No	SCFAs + TNFα
10.	HLF	COPD	56	F	T	Ex-smoker (120 pack years)	fluticasone/formoterol, tiotropium, pantoprazole, terbutaline	Yes	SCFAs + TNFα
11.	HLF	Emphysema	59	M	T	Current (35 pack years)	fluticasone/formoterol, prednisolone, salbutamol, tiotropium, meloxicam, doxycycline, ipratropium, glycopyrronium bromide, tapentadol, oxycodone, rabeprazole, pregabalin	No	SCFAs + TNFα
12.	HLF	pulmonary hypertension	36	M	T	Non-smoker	dobutamine, bumetanide, empagliflozin, entecavir, folic acid, gabapentin	No	SCFAs + TNFα
13.	HLF	emphysema	62	F	T	Ex-smoker (40 pack years)	terbutaline, ciclesonide, tiotropium, formoterol, salbutamol, ipratropium, irbesartan, rosuvastatin, prednisolone, azithromycin, pantoprazole	Yes	SCFAs + TNFα, FFAR agonists, WB
14.	HLF	IPF	57	M	T	Ex-smoker (40 pack years)	sildenafil, bumetanide, fluticasone/formoterol, salbutamol	Yes	SCFAs + TNFα, FFAR agonists, WB
15.	HLF	IPF	62	M	T	Ex-smoker (10 pack years)	N/A	N/A	SCFAs + TNFα, FFAR agonists, WB
16.	HLF	NSCLC	72	F	R	Ex-smoker (>20 pack years)	telmisartan, propionate/salmeterol, furosemide, ranitidine.	No	SCFAs + TNFα, FFAR agonists, WB
17.	HLF	adenocarcinoma	57	F	R	N/A	N/A	No	SCFAs + TNFα, FFAR agonists, WB

18.	HLF	IPF	63	M	T	Ex-smoker (>40 pack years)	prednisone, pantoprazole, lorazepam, escitalopram, morphine	Yes	SCFAs + TNFα, FFAR agonists, WB
19.	HLF	IPF	52	M	T	Ex-smoker (15 pack years)	clonazepam, esomeprazole, clotrimazole, hydrocortisone, irbesartan, nintedanib, paracetamol, rosuvastatin, temazepam, trimethoprim/ sulfamethoxazole,	Yes	SCFAs + TNFα , inhibitors, WB
20.	HLF	sarcoidosis/ pulmonary hypertension	57	M	T	Non-smoker	prednisolone, sildenafil, warfarin, ambrisentan	Yes	SCFAs + TNFα , inhibitors, WB
21.	HLF	IPF	63	F	T	Ex-smoker (15 pack years)	gabapentin, lorazepam, pantoprazole, prednisolone, sildenafil, trimethoprim/sulfamethoxazole	Yes	SCFAs, inhibitors, WB
22.	HLF	IPF	55	M	T	Ex-smoker (10 pack years)	pantoprazole, nintedanib, olmesartan, fluticasone /vilanterol	Yes	SCFAs + TNFα
23.	HLF	IPF	59	M	T	Ex-smoker (26 pack years)	prednisolone, omeprazole, budesonide/formoterol, glycopyrronium bromide, perindopril	Yes	SCFAs + TNFα
24.	HLF	rejection/IPF	61	M	T	N/A	cyclosporin, prednisolone, trimethoprim/ sulfamethoxazole, azithromycin, mycophenolate mofetil, posaconazole, ezetimibe, pravastatin, irbesartan, metformin, pantoprazole	No	SCFAs + TNFα
25.	HLF	IPF	65	M	T	Ex-smoker (35 pack years)	omeprazole, sildenafil, budesonide/formoterol, nizatidine, ergocalciferol	Yes	SCFAs + TNFα
26.	HLF	pulmonary hypertension	62	F	T	Non-smoker	prednisolone, sildenafil, furosemide, pantoprazole	Yes	SCFAs + TNFα
27.	HLF	ILD	40	M	T	Ex-smoker (5 years)	trimethoprim/sulfamethoxazole, prednisolone, pantoprazole, azathioprine, mycophenolic acid,	No	SCFAs + TNFα
28	HLF	COPD	69	F	T	Ex-smoker (100 pack years)	tiotropium, budesonide/formoterol, atorvastatin, furosemide, baclofen, glucosamine, ciclesonide, rabeprazole, terbutaline, perindopril/amlodipine	No	Chronic exposure of SCFAs, FFAR3 antagonist
29.	HLF	Interstitial pneumonitis	59	M	T	Non-smoker	trimethoprim/sulfamethoxazole, prednisolone, metformin, atorvastatin, escitalopram	Yes	Chronic exposure of SCFAs, FFAR3 antagonist
30.	HLF	IPF	64	M	T	Ex-smoker (70 pack years)	furosemide, atorvastatin, thyroxine, aspirin, sildenafil, bisoprolol, pantoprazole, umeclidinium bromide/vilanterol, olmesartan medoxomil	Yes	Chronic exposure of SCFAs, FFAR3 antagonist
31.	HLF	IPF	54	M	T	Ex-smoker (>30 pack years)	azathioprine, prednisolone, rosuvastatin, trimethoprim, pregabalin, warfarin	No	Chronic exposure of SCFAs, FFAR3 antagonist
32.	HLF	IPF	63	M	T	Ex-smoker (2 pack years)	prednisolone, pirfernidone, n-acetylcysteine	Yes	Chronic exposure of SCFAs, FFAR3 antagonist
33.	HLF	Adenocarcinoma	57	F	R	N/A	N/A	N/A	Chronic exposure of SCFAs, FFAR3 antagonist
34.	HLF	Squamous Cell Carcinoma	62	F	R	Ex-smoker (60 pack years)	Unknown	No	Chronic exposure of SCFAs, FFAR3 antagonist
35.	HLF	Adenocarcinoma	75	F	R	Ex-smoker (>20 pack years)	rosuvastatin, aspirin, clopidogrel	No	Chronic exposure of SCFAs, FFAR3 antagonist
36.	HLF	Extrinsic allergic alveolites	69	M	T	Ex-smoker (23 pack years)	prednisolone, olmesartan, trimethoprim/ sulfamethoxazole, aspirin, atorvastatin, temazepam, venlafaxine	No	SCFAs + TNFα
37.	ASM	emphysema	44	F	T	Ex-smoker (15 pack years)	prednisolone, salbutamol, salmeterol/fluticasone, tiotropium,	N/A	SCFAs + TNFα

38.	ASM	COPD	52	F	T	Ex-smoker (50 pack years)	venlafaxine, prednisolone, fluticasone/salmeterol, tiotropium	No	SCFAs + TNFα
39.	ASM	COPD	56	F	T	Ex-smoker	symbicort, tiotropium, terbutaline		SCFAs + TNFα
40.	ASM	Emphysema	59	M	T	Current (35 pack year)	fluticasone/formoterol, prednisolone, salbutamol, tiotropium, meloxicam, doxycycline, ipratropium, glycopyrronium bromide, tapentadol, oxycodone, rabeprazole, pregabalin	No	SCFAs + TNFα
41.	ASM	emphysema	62	F	T	Ex-smoker (40 pack years)	terbutaline, ciclesonide, tiotropium, formoterol, salbutamol, ipratropium, irbesartan, rosuvastatin, prednisolone, azithromycin, pantoprazole	Yes	SCFAs + TNFα
42.	ASM	COPD	65	M	T	ex-smoker (40 pack years)	salmeterol/fluticasone, tiotropium, pantoprazole, risedronic acid	No	SCFAs + TNFα
43.	ASM	IPF	57	M	T	Ex-smoker (40 pack years)	sildenafil, bumetanide, fluticasone/formoterol, salbutamol	Yes	SCFAs + TNFα
44.	ASM	IPF	62	M	T	Ex-smoker (10 pack years)	N/A	N/A	SCFAs + TNFα
45.	ASM	malignant neoplasm	75	M	R	Ex-smoker (>20 pack years)	simvastatin, allopurinol, metformin, Amlodipine, bimatoprost/timolol, perindopril, prochlorperazine maleate	No	SCFAs + TNFα
46.	ASM	healthy donor	65	M	T	N/A	N/A	No	SCFAs + TNFα
47.	ASM	pulmonary hypertension	30	F	T	N/A	sildenafil, furosemide, epoprostenol, macitentan	N/A	SCFAs + TNFα
48.	ASM	IPF	58	F	T	N/A	N/A	N/A	SCFAs + TNFα
49.	ASM	pulmonary hypertension	36	M	T	Non-smoker	dobutamine, bumetanide, empagliflozin, entecavir, folic acid, gabapentin	No	SCFAs + TNFα
50.	ASM	emphysema	54	M	T	Ex-smoker (60 pack years)	N/A	Yes	SCFAs + TNFα
51.	ASM	Asthma	51	M	B	N/A	N/A	No	SCFAs + TNFα
52.	ASM	IPF	63	F	T	Ex-smoker (15 pack years)	gabapentin, lorazepam, pantoprazole, prednisolone, sildenafil, trimethoprim/ sulfamethoxazole	Yes	SCFAs + TNFα
53	ASM	IPF	62	M	T	Ex-smoker (10 pack years)	N/A	N/A	SCFAs + TNFα
54.	ASM	Sarcoidosis	57	M	T	Non-smoker	prednisolone, sildenafil, warfarin, ambrisentan	Yes	SCFAs + TNFα
55.	ASM	IPF	65	M	T	Ex-smoker (35 pack years)	omeprazole, sildenafil, budesonide/formoterol, nizatidine	Yes	SCFAs + TNFα
56.	ASM	Emphysema	59	M	T	Current (40 pack years)	salbutamol, tiotropium, mirtazapine, ciclesonide		SCFAs + TNFα
57.	ASM	rejection/IPF	61	M	T	N/A	cyclosporin, prednisolone, trimethoprim/ sulfamethoxazole, azithromycin, mycophenolate mofetil, posaconazole, ezetimibe, pravastatin, irbesartan, metformin, pantoprazole	No	SCFAs + TNFα
58.	ASM	ILD	40	M	T	Ex-smoker (5 pack years)	trimethoprim/sulfamethoxazole, prednisolone, pantoprazole, azathioprine, mycophenolic acid, vitamin D, calcium	No	SCFAs + TNFα

HLF: human pulmonary fibroblast, ASM: airway smooth muscle, COPD: chronic obstructive pulmonary disease, NSCLC: non-small cell lung carcinoma, IPF: idiopathic pulmonary fibrosis, ILD: Interstitial

Lung Diseases, F: Female, M: Male, T: transplantation, R: resection, B: biopsy, SCFA: short chain fatty acid, FFAR: free fatty acid receptor, WB: western blotting. LTOT: long term oxygen therapy. N/A: data not available.

Figure legends

Figure 1. Synergistic increase in cytokine release with combined propionate or butyrate and TNFα challenge, than either alone in human pulmonary fibroblasts. Primary human lung fibroblasts (*n* = 10-24 patients) were unstimulated (control) or challenged with short-chain fatty acids (SCFAs) propionate (Pr) (0.5mM, 10mM, 25mM) (A, B), butyrate (Bu) (0.01mM, 0.5mM, 10mM) (C, D) or acetate (Ac) (0.5mM, 10mM, 25mM) (E, F) in 0.1% BSA-DMEM for 24h with or without 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. All data are represented as mean ± standard error of the mean. All challenges are compared to control and challenges with SCFAs and TNFα are compared to with TNFα alone, using a one-way ANOVA and a Bonferroni post-test. Significance is represented as **** (p<0.0001), *** (p<0.001), ** (p<0.01) or * (p<0.05).

Figure 2. Increased IL-6 and CXCL8 mRNA expression upon challenge with propionate and TNFα in human pulmonary fibroblasts. Primary human lung fibroblasts (*n* = 8 patients) were unstimulated (control) or challenged with propionate (Pr) (25mM) in 0.1% BSA-DMEM for 24h with or without TNFα (1ng/mL) for another 12h (A, C) or 24h (B, D). Total RNA was extracted and IL-6 (A, B) and CXCL8 (C, D) mRNA was measured using qPCR. All data are represented as mean ± standard error of the mean. Challenges with Pr and TNFα are compared to challenge with TNFα alone, using a one-way ANOVA with a Bonferroni post-test. Significance is represented as * (p<0.05).

Figure 3. SCFAs enhance TNFα-induced IL-6 and CXCL8 release via FFAR3 signalling. Primary human lung fibroblasts (*n* = 14 patients) were unstimulated (control) or challenged with free fatty acid receptor (FFAR)2 agonist 4-CMTB (10μM) (A, B) or FFAR3 agonist AR420626 (10μM) (C, D) in 0.1% BSA-DMEM for 24h with or without TNFα (1ng/mL) for another 24h. Other cells (*n* = 8) were pre-treated with FFAR3 antagonist β-hydroxybutyrate (BOH) (100mM) for 60 minutes, prior to challenge with propionate (Pr) 10mM for 24 hours and TNFα (1ng/ml) for another 24h (E, F). 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, challenges with FFAR agonist and TNFα are compared to challenge with TNFα alone, and challenges with FFAR3 antagonist (BOH) are compared with their respective control in the absence of the FFAR3 antagonist using a one-way ANOVA and a Bonferroni post-test. Significance is represented as *** (p<0.001), ** (p<0.01) or * (p<0.05).

Figure 4. Hyperactivation of p38 MAPK upon stimulation with propionate and TNFα. Primary human lung fibroblasts (n = 6-10 patients) were unstimulated (control) or challenged with propionate (Pr) (25mM), TNFα (1ng/ml) or Pr (25mM) in combination with TNFα (1ng/mL) for 30 minutes. Whole cell lysates were collected and levels of phosphorylated NF-κB p65 (A), p38 mitogen-activated protein (MAP) kinase (C), protein kinase B (Akt) (E), extracellular signal-regulated kinases (ERK) 1 and 2 (G) or Stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) (I). Total NF-κB p65 (B), p38 MAP kinase (D), Akt (F) ERK1 and 2 (H) and SAPK/JNK (J) were also assessed. Densitometry was performed and 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 fisher's LSD test. Significance is represented as *** (p<0.001), ** (p<0.01) or * (p<0.05). Representative western blots are shown under each graph.**Figure 5. Inhibition of p38 MAPK supresses combined propionate and TNFα-induced cytokine release in human pulmonary fibroblasts.** Primary human lung fibroblasts *(n* = 10-11 patients) were treated with or without the cyclooxygenase (COX) inhibitor indomethacin (10μM), p38 mitogen-activated protein (MAP) kinase signaling inhibitor SB239063 (3μM), mitogen-activated protein (MAP) kinase 1 (MEK1) inhibitor PD98059 (10μM), the c-Jun N-terminal kinase (JNK) inhibitor SP600125 (10μM) or the NF-κB inhibitor BAY-117082 (1μM) for 60 minutes before challenge with TNFα (1ng/ml) (A, B) or propionate (Pr) (25mM) in combination with TNFα (1ng/ml) (C, D). Cell free supernatants were collected after 48h 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 treatments with inhibitor

are compared to their respective control in the absence of the inhibitor using a one-way ANOVA and a Bonferroni post-test. Significance is represented as ** (p<0.01) or * (p<0.05).

Figure 6. Chronic exposure to propionate or butyrate enhances TNFα-induced cytokine release in human pulmonary fibroblasts. Primary human lung fibroblasts (n = 7 patients) were unstimulated (control) or challenged with short-chain fatty acids (SCFAs) propionate (Pr) (25mM (A, B), butyrate (Bu) (10mM) (C, D) or acetate (Ac) (25mM) (E, F) in 0.1% BSA-DMEM for 96h with or without 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. All data are represented as mean ± standard error of the mean. All challenges are compared to control and challenges with SCFAs and TNFα are compared to with TNFα alone, using a one-way ANOVA and a Bonferroni post-test. Significance is represented as *** (p<0.001) ** (p<0.01) or * (p<0.05).

Figure 7. Greater cytokine release with combined acetate, propionate or butyrate and TNFα challenge, than each alone in airway smooth muscle cells. Primary human airway smooth muscle cells (n = 8-20 patients) were unstimulated (control) or challenged with short-chain fatty acids propionate (Pr) (0.5mM, 10mM, 25mM) (A, B), butyrate (Bu) (0.01mM, 0.5mM, 10mM) (C, D) or acetate (Ac) (0.5mM, 10mM, 25mM) (E, F) in 0.1% BSA-DMEM for 24h with or without 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. All data are represented as mean ± standard error of the mean. All challenges are compared to control and challenges with TNFα are compared to their respective challenge without TNFα, using a one-way ANOVA and a Bonferroni post-test. Significance is represented as **** (p<0.0001), *** (p<0.001), ** (p<0.01) or * (p<0.05).

Figure 8. Propionate suppresses LPS-induced CXCL8 release in THP-1 cells. THP-1 cells (n = 7 replicates) were unstimulated (control) or challenged with short-chain fatty acids propionate (Pr)

(0.5mM, 25mM) (A), butyrate (Bu) (0.01mM, 0.5mM) (B) or acetate (Ac) (0.05mM, 25mM) in 10% FBS-RMPI for 24h with LPS (1ng/mL) for another 24h. Cell free supernatants were collected and CXCL8 release was measured using ELISA. All data are represented as mean ± standard error of the mean. Challenges with LPS are compared to their respective challenge without LPS, using a one-way ANOVA and a Bonferroni post-test. Significance is represented as **** (p<0.001).

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