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RESEARCH ARTICLE

Short-chain fatty acids inhibit the activation of T lymphocytes and myeloid cells and induce innate immune tolerance

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Abstract

The intestinal microbiota contributes to gut immune homeostasis, where short-chain fatty acids (SCFAs) function as the major mediators. We aimed to elucidate the immunomodulatory effects of acetate, propionate, and butyrate. With that in mind, we sought to characterise the expression of SCFA receptors and transporters as well as SCFAs' impact on the activation of different immune cells. Whereas all three SCFAs decreased tumour necrosis factor (TNF)-α production in activated T cells, only butyrate and propionate inhibited interferon (IFN)-γ, interleukin (IL)-17, IL-13, and IL-10 production. Butyrate and propionate inhibited the expression of the chemokine receptors CCR9 and CCR10 in activated T- and B-cells, respectively. Similarly, butyrate and propionate were effective inhibitors of IL-1β, IL-6, TNF-α, and IL-10 production in myeloid cells upon lipopolysaccharide and R848 stimulation. Acetate was less efficient at inhibiting cytokine production except for IFN-α. Moreover, SCFAs inhibited the production of IL-6 and TNF-α in monocytes, myeloid dendritic cells (mDC), and plasmacytoid dendritic cells (pDC), whereas acetate effects were relatively more prominent in pDCs. In monocytes and mDCs, acetate was a less efficient inhibitor, but it was equally effective in inhibiting pDCs activation. We also studied the ability of SCFAs to induce trained immunity or tolerance. Butyrate and propionate – but not acetate – prevented Toll-like receptor-mediated activation in SCFA-trained cells, as demonstrated by a reduced production of IL-6 and TNF-α. Our findings indicate that butyrate and propionate are equally efficient in inhibiting the adaptive and innate immune response and did not induce trained immunity. The findings may be explained by differential SCFA receptor and transporter expression profiles of the immune cells.

Keywords

short-chain fatty acids – lymphocytes – monocytes – receptors – tolerance

1 Introduction

Short-chain fatty acids (SCFAs) have a central role in the interplay between diet, microbiota, and host physiology. They are important players in maintaining gut homeostasis by promoting epithelial integrity and mucosal immunity, and recent evidence indicates that they are also crucial for regulating systemic immunity and preventing respiratory allergies (Corrêa-Oliveira *et al.*, 2016; Frati *et al.*, 2018; Roduit *et al.*, 2019; Yip *et al.*, 2021).

The 'Western diet' with low dietary fibre content that results in lower levels of intestinal SCFAs has been linked to many inflammatory disorders, including diabetes, certain autoimmune diseases, asthma, and allergies (Manzel *et al.*, 2014; Thorburn *et al.*, 2014). In fact, dietary fibre consumption (as the precursors for SCFAs) was found to be inversely correlated to the risk of death from cardiovascular, cancer, infectious, and respiratory disorders (Park *et al.*, 2011).

SCFAs are metabolites produced by bacteria during the fermentation of dietary fibres as well as oligo- and polysaccharides. Complex plant-derived polysaccharides and milk oligosaccharides that are not digested in the small intestine reach the large intestine, where they are metabolised by the gut microbiota. Bacterial species within the phyla *Bacteroidetes* and *Firmicutes* present in the caecum and colon act as primary or secondary fermenters, producing acetate (C2), propionate (C3), and butyrate (C4), with a colonic molar ratio of around 3:1:1, respectively (Blaak *et al.*, 2020; Cummings *et al.*, 1979, 1987). SCFAs, especially butyrate, are taken up and metabolised by the colonocytes as a preferred energy source over competing substrates such as glucose and glutamine (Roediger, 1980). In fact, most of the butyrate is metabolised locally, providing up to 60-70 percent of the energy supply for the epithelial cells in the colon (Clausen and Mortensen, 1995). The remainders pass through the intestinal epithelial cells and interact with the cells in the gut-associated lymphoid tissue (GALT) before entering the circulation. While colonocytes metabolise a small portion of propionate, most of it, along with acetate, is transported to the liver via the portal vein. In the liver, most of the propionate is metabolised by the hepatocytes. The remaining SCFAs, which mainly consist of acetate, circulate through the bloodstream and reach other organs such as the respiratory system, urinary tract, and central nervous system, where they can directly interact with cells and have a broad range of impact on other cells and tissues (reviewed in Ratajczak *et al.*, 2019; Tan *et al.*, 2014; Thorburn *et al.*, 2014).

SCFAs can exert their effects through two mechanisms: via direct effects on cellular biological processes upon internalisation and indirectly via the engagement with membrane receptors (Tan *et al.*, 2014; Van der Hee and Wells, 2021). SCFAs diffuse passively into cells; however, a more significant part is actively transported into cells via several transporter molecules located on the cell membrane among which monocarboxylate transporter 1 (MCT-1) and sodium-coupled monocarboxylate transporter 1 (SMCT-1) are most relevant for the transportation of C2-4 into the immune cells (Liu *et al.*, 2021; Van der Hee and Wells, 2021). The active transportation is essential for the SCFAs accumulation inside the cells and, subsequently, blockade of histone deacetylases (HDACs) activity, thereby directly influencing epigenetics and regulating gene expression (Singh *et al.*, 2010). Although HDACs blockade is generally considered permissive for gene transcription, inhibitory or stimulatory consequences depend on various factors, including cell type and inflammatory context (Reichert *et al.*, 2012). Another mechanism by which SCFAs modulate cells is through their interaction with G-coupled protein receptors (GPCRs) on the cell membrane (Kim *et al.*, 2013; Singh *et al.*, 2014). On the cell membrane of immune cells, three GPCRs, GPR41, GPR43, and GPR109A, have been identified as being involved and able to bind to SCFAs with varying affinities. GPR41 (free fatty acid receptor 3 – FFAR3) has the highest affinity for propionate, followed by butyrate, whilst GPR43 (free fatty acid receptor 2 – FFAR2) interacts with acetate and propionate the most (Brown *et al.*, 2003; Le Poul *et al.*, 2003). GPR109A or the Niacin receptor, exclusively binds to butyrate (Singh *et al.*, 2014; Thangaraju *et al.*, 2009). The transduced signal via these receptors also has an inhibitory effect on the enzymatic activity of HDACs (Wu *et al.*, 2012). Besides regular G-proteins, all aforementioned GPCRs engage the alternative signalling pathway mediated by β-arrestin-2 to, e.g. inhibit the NF-ϰB signalling pathway and induce anti-inflammatory effects (Chai *et al.*, 2013; Gao *et al.*, 2004).

Even though there is substantial data on SCFAs and their immunomodulatory effects, a comprehensive study covering the impact of all three SCFAs on peripheral blood mononuclear cells is missing. The current study aimed to elucidate the direct and indirect effects of exposure to individual SCFAs on innate and adaptive immune function. To this aim, human PBMCs were treated with acetate, propionate, or butyrate and were stimulated *ex vivo* with various Toll-like receptors (TLRs) ligands, including LPS and R848 to mimic bacterial and viral stimulations, respectively. We examined the effect of SCFAs on the production of cytokines by activated T- and B-cells, as well as their potential to modulate the expression of tissue homing-associated chemokine receptors, CCR9 and CCR10. These receptors were selected based on previous observations on the upregulation of CCR9 and CCR10 in activated B cells (Van Splunter *et al.*, 2018a). Additionally, we assessed cytokine production by activated innate immune cells, namely monocytes, myeloid dendritic cells (mDCs), and plasmacytoid dendritic cells (pDCs) in the presence or

absence of SCFAs. Finally, we also tested SCFAs in an *in vitro* innate immune training model. Innate immune training or trained immunity is the enhanced immune response to secondary infections by innate immune cells due to exposure to various (microbial) components. The mechanism was described initially for *Candida albicans*, which involves triggering dectin-1, which activates the Raf-1 pathway, inducing changes in the epigenome and, thereby, gene expression. Butyrate has also been described as affecting Raf-1 mediated signalling (Yu *et al.*, 2003). In addition, HDAC inhibitors, such as SCFAs, could alter the expression of genes and promote the acquisition of a trained immune phenotype by increasing the acetylation of histone proteins. With that in mind, we investigated the ability of SCFAs to epigenetically modify human monocytes' response toward training or tolerance.

2 Material and methods

Study samples and PBMC isolation

The experiments were performed using buffy coats from the Sanquin blood bank in Nijmegen, the Netherlands, or freshly collected blood from several individuals after obtaining written consent. The blood collection centre at the university collects blood for experimental purposes under the supervision of the Medical Ethics Committee (METC). Blood samples from 5-8 individuals were used to stimulate innate and adaptive immune cells for PBMC work, whereas eleven buffy coats were used for the innate immune training model. The PBMCs were isolated by density gradient centrifugation using Ficoll Paque Plus (GE Healthcare, 17-1440-02, Chicago, IL, USA). The Leucosep tubes (Greiner Bio-One, #227290, Monroe, NC, USA) were filled with 15 ml of Ficoll and briefly centrifuged to move the Ficoll below the porous barrier. Blood samples or the buffy coats were diluted 1:1 with warm phosphate-buffered saline (PBS) (Gibco, #20012027, Cincinnati, OH, USA) and then pipetted on top of the porous barrier. The tubes were then centrifuged, and the buffy layer above the porous barrier was poured into new 50 ml falcon tubes (Corning, #352070, Corning, NY, USA). The tubes were topped up with warm PBS and centrifuged to wash away the Ficoll and diluted plasma residuals. The cells were washed three times in total, resuspending the pellet before every wash. After washing and discarding the PBS, the cell pellets were resuspended in RPMI 1640 Glutamax (Gibco, #61870010) enriched with 10% FCS (Gibco, #10270106) and 1% penicillin/streptomycin (Gibco, #15140122). Cells were counted using the flow cytometer CytoFLEX LX (Beckman Coulter, #C11186, Indianapolis, IN, USA), and appropriate dilutions for downstream processes were prepared.

SCFA receptors and transporters expression on immune cells

The expression of SCFA receptors (GPR41, GPR43, GPR109A) and transporters (MCT-1 and SMCT-1) on the cell membrane of innate and adaptive immune cells were studied. PBMC from three donors (7.5×10^5/well) were stained with antibodies for immune cell phenotyping to discriminate between T- and B-lymphocytes, monocytes, mDCs, and pDCs. The expression of GPR41 (Invivogen, #PA5-25146, San Diego, CA, USA), GPR43 (R&D system, #FAB10082R, Minneapolis, MN, USA), and GPR109A (R&D system, #FAB2760T, Minneapolis, MN, USA) were analysed by flow cytometry. Similarly, antibodies against MCT-1 (R&D system, #FAB8275T) and SMCT-1 (R&D system, #FAB8398R) were used to screen the expression of SCFAs transporters on the aforementioned immune cells. The full antibody panel is summarised in Supplementary Table S1 and S2 and Supplementary Figure S1 describes the gating strategy.

Adaptive immune cell activation

SCFAs were evaluated for their potential to modulate the expression of tissue homing-associated chemokine receptors CCR9 and CCR10 on T and B lymphocytes, as well as their cytokine induction profile. 5×10^5/well PBMCs were cultured on 48-well flat-bottom cell culture plates (Sigma-Aldrich, Costar 3548, St. Louis, MO, USA) containing 15 millimolar (mM) of either sodium acetate (Sigma-Aldrich, #S2889), sodium propionate (Sigma-Aldrich, #P1880), sodium butyrate (Sigma-Aldrich, #3034100), or RPMI 1640 as a negative control. For B cell activation, CpG (1.5 μg/ml) (Invitrogen, #tlrl-2216, San Diego, CA, USA) was used, and a combination of soluble anti-CD3 (α-CD3) (eBioscience, #14-0038-82, Cincinnati, OH, USA)(1 μg/ml) and anti-CD28 (α-CD28)(BD, #555725, Franklin lakes, NJ, USA)(1 μg/ml) was applied to activate T cells. The cell stimulation was done in the presence or absence of retinoic acid (RA, 1 μM) (Merck, #R2625, St. Louis, MO, USA) for its known ability to induce cell homing markers. After 48 hours of incubation, the plates were centrifuged, and 100 μl/well of the supernatants were transferred to a NUNC plate and stored at −20 °C for cytometric bead array (CBA) and cytokine measurement. Individual cytokine Flex-sets were used to measure the level of IFN-γ (#558269), IL-13 (#558450), IL-17 (#562151), TNF-α (#558273), and IL-10

(#558274) (BD, Franklin Lakes, NJ, USA). IFN-γ, IL-13, IL-17, and IL-10 were measured as signature cytokines of Th1, Th2, Th17, and regulatory T cells (Tregs), respectively.

Then the cells were harvested and transferred to a 96-wells NUNC plate (ThermoFisher, #267245, Cincinnati, OH, USA) on ice for fluorescence-activated cell sorting (FACS) staining. Extracellular staining with α-CD4 (Biolegend, #300506, San Diego, CA, USA) and α-CD19 (Biolegend, #302234) for selecting T- and B-lymphocytes was performed, and the expression of homing markers was quantified by staining the cells with α-CCR9 (Biolegend, #358904, San Diego, CA, USA) and α-CCR10 (Biolegend, #341506). In summary, the PBMCs were washed with cold FACS buffer (PBS supplemented with bovine serum albumin (0.5% v/v), ethylenediaminetetraacetic acid (EDTA, 2.5 mM), and sodium azide (NaN_3, 10% v/v)), and the supernatant was discarded after centrifugation. Subsequently, the cells were incubated with the antibody mixture (Supplementary Table S3) diluted in FACS buffer at 4 °C in the dark for 20 min. Following that, the cells were washed twice with cold FACS buffer and resuspended in 200 µl of FACS buffer before being used for the measurement.

The samples were measured on CytoFLEX LX, and the generated flow cytometry data were analysed with FlowJo (FlowJo LLC, v10). The analysis was carried out to determine the percentage of T- and B-cells expressing CCR9 and CCR10 (Supplementary Figure S2). The experiment included two replicates, and the analysis employed the arithmetic mean of the replicates.

Innate immune cell activation

SCFAs were investigated for their capacity to modulate the innate immune response by assessing cytokine levels in the PBMC culture supernatant and intracellularly in monocytes, mDCs, and pDCs. PBMCs were seeded (5×10^5/well) in flat-bottom 48-well culture plates (Sigma-Aldrich, Costar 3548, St. Louis, MO, USA) where acetate, propionate, or butyrate with a final concentration of 15 mM was added. The innate cells were stimulated with TLR4 ligand (LPS, 0.2 µg/ml) (Sigma-Aldrich, #L2880) or TLR7/8 ligand (R848, 1 µg/ml) (Sigma-Aldrich, #SML0196). Brefeldin A (0.1 µg/ml) (Invitrogen, #00-4506-51) was added to the wells to keep the produced cytokines inside the cell. The plates were incubated at 37 °C with 5% CO_2 for 3 h. After incubation, the cells were harvested, transferred to a 96-wells NUNC plate, and kept on ice for FACS antibody staining. Extracellular and intracellular FACS labelling of the markers was used for phenotyping the cells and determining IL-6 and TNF-α production. In summary, the cells were washed with cold FACS buffer and then incubated with an antibody mixture against extracellular markers (Supplementary Table S4). The plates were then incubated at 4 °C in dark conditions for 20 min. Following live/dead staining with fixable viability dye 520 (FVD520) (Invitrogen, #65-0867-14), the cells were fixed, and the cell membrane was permeabilised according to the manufacturer's protocol using the IC fix/perm kit (Invitrogen, #88-8824-00). Then, antibodies against IL-6 (Biolegend, #501107) and TNF-α (Biolegend, #502916) were added to quantify the percentage of cells that were producing these cytokines. The samples were measured on CytoFLEX LX, and the generated flow cytometry data were analysed with FlowJo (FlowJo LLC, v10). The innate immune cells were discriminated, and the percentage of IL-6 and TNF-α producing cells) was quantified (Supplementary Figure S3).

A second plate with the same conditions and stimuli but without BFA was incubated at 37 °C for 24 h to measure cytokine levels in the culture supernatant. The culture supernatant was collected after 24 h and was stored at –20 °C until CBA analysis. Individual cytokines Flex-sets were used for quantifying IL-1β (#558279), IL-6 (#558276), TNF-α (#558273), IFN-α (#560379), and IL-10 (#558274) (BD, Franklin lakes, NJ, USA). Each condition was replicated twice, and the arithmetic mean of the replicates was used for analysis.

Monocyte isolation and training

The training or tolerogenic properties of SCFAs on monocytes was investigated in an *in vitro* innate immune training model (Domínguez-Andrés *et al.*, 2021; Hellinga *et al.*, 2020; Van Splunter *et al.*, 2018b). The EasySep Human Monocyte Isolation Kit (Stemcell technologies, #19359, Vancouver, BC, Canada) was used in accordance with the manufacturer's protocol to isolate $CD14^+$ monocytes from donors' fresh PBMCs. In summary, the PBMCs were resuspended at a concentration of 5×10^7 cells/ml in a Monocyte Isolation Buffer (PBS containing 2% FBS and 1 mM EDTA without Ca^{2+} or Mg^{2+}). Subsequently, 50 µl/ml of the isolation cocktail and then the platelet removal cocktail were added, and the cells were incubated at room temperature for 5 min. Next, magnetic particles (50 µl/ml) were added, and the tubes were put in a magnet following 5 min of incubation. The negative fraction was collected and washed with the monocytes isolation buffer before being resuspended in the enriched RPMI 1640 (+1% gentamicin and 1% pyruvate). The cells were counted and checked for $CD14^+$ monocyte purity by staining with antibod-

ies against CD14 (Biolegend, #301830) and CD3 (BD, #555334). All donors had a >80% $CD14^+$ monocyte purity with <3% $CD3^+$ T cell contamination.

A flat-bottom 96-wells cell culture plate (Sigma-Aldrich, Costar 3596) was filled with isolated monocytes (1×10^5 monocytes/well) and was incubated at 37 °C for 2 h to allow the cells to settle down and adhere to the plate. After incubation, the cells were stimulated with either medium as a negative control, 100 μg/ml whole glucan particles (WGP) (Invivogen, #tlrl-wgp) as a positive control, 5 mM of SCFAs (acetate, propionate, or butyrate), 0.1 μg/ml LPS, or 10 μg/ml R848 and were incubated at 37 °C for 24 h. The plate was washed twice with warm PBS the next day, and fresh RPMI medium enriched with 10% human pooled serum (Sigma-Aldrich, #H3667) was added. The medium was refreshed on day 4. During the resting phase, the monocytes were differentiated into macrophages which were stimulated on day 7 with TLR-ligands LPS (0.1 μg/ml), R848 (10 μg/ml), or Pam3CSK4 (10 μg/ml) (Invivogen, #tlrl-pms). The plate was incubated with stimuli at 37 °C for 24 h. On day 8, the supernatant was collected and stored at −20 °C until CBA analysis. The CBA was performed using Flex-sets for measuring IL-6 (BD, #558276) and TNF-α (BD, #558273).

Statistical analysis

GraphPad Prism (GraphPad Software, v9, San Diego, CA, USA) was used for statistical analysis and for preparing the charts. One-way ANOVA with Dunnett's post-test was used for comparing the conditions to the no SCFAs group within each stimulation. The innate immune training data was normalised and expressed as fold changes compared to the negative control (RPMI medium). Friedman test was used for pair-wise comparisons. The differences were considered significant when the *P*-value was <0.05 (*), <0.01 (**), <0.001 (***), or <0.0001 (****), as indicated in the graphs. The summary of mean, standard deviation, number of donors and the statistical analysis applied for every experimental condition are presented as Supplementary Figures S5-S10.

3 Results

Differential expression of SCFA receptors and transporters

The relative expression of SCFA receptors and transporters was evaluated on different immune cells from PBMCs. When individual cell types were examined, GPR41 was expressed higher than other receptors, with monocytes and B cells showing relatively the highest expression levels (Figure 1A). GPR109A was abundant in monocytes, expressed at low levels on mDC and B cells but not detectable on T cells or pDC. The expression levels of GPR43 in mDC, pDC, and B cells were considerably higher than the expression of GPR109A. On monocytes, GPR43 expression was much lower compared to GPR109A and GPR41 expression. T cells did not express detectable levels of GPR43 or GPR109A and only expressed GPR41.

For the SCFA transporters, MCT-1 was only detected in T cells (Figure 1B). SMCT-1, on the other hand, is extensively expressed on monocytes, mDCs, pDCs, T-, and B lymphocytes as a key SCFAs membrane transporter. The highest levels of SMCT-1 expression were found in monocytes, DCs (both mDCs and pDCs), and B cells. In fact, SMCT-1 expression in B lymphocytes was more than 6-fold higher than in T cells.

Overall, the expression patterns of SCFA receptors and transporters varied substantially across the cell types studied. Monocytes showed the highest expression of GPR41 and GPR109A, low levels of GPR43, and considerable SMCT-1 with no detectable MCT-1 expression. mDCs express GPR41 and GPR43 much higher than GPR109A. While SMCT-1 was abundantly present on mDCs, MCT-1 was entirely absent. No GPR109A was detected on pDCs, while GPR41 and GPR43 were equally expressed. pDCs highly expressed SMCT-1 with marginal MCT-1 expression. Although GPR41 is present in both T and B cells, the levels were considerably higher in B cells. Besides, B cells also express GPR43 and GPR109A, while both receptors were absent in T cells. The MCT-1 molecule was detectable in T cells, and they also expressed SMCT-1, while only SMCT-1 was present in B cells.

Effects of SCFAs on cytokine release from adaptive immune cells

The production of cytokines was measured in the culture supernatant of PBMCs. Cells were incubated with SCFAs and with soluble α-CD3/α-CD28 antibodies (T-cell activation) or CpG (for B-cell activation) for 48 h, and cytokines were measured in the supernatants. Stimulation of T cells with α-CD3/α-CD28 resulted in the production of TNF-α, IFN-γ, IL-17, IL-13, and IL-10, reflecting the determination of Th1, Th17, Th2, and Treg, respectively (Figure 2). All three SCFAs, with different potency, had an inhibitory effect on TNF-α production in activated T cells (p_v C2 = 0.032, C3 = 0.002, C4 = 0.003) (Figure 2A). Similarly, butyrate and propionate, but not acetate, significantly decreased IL-17

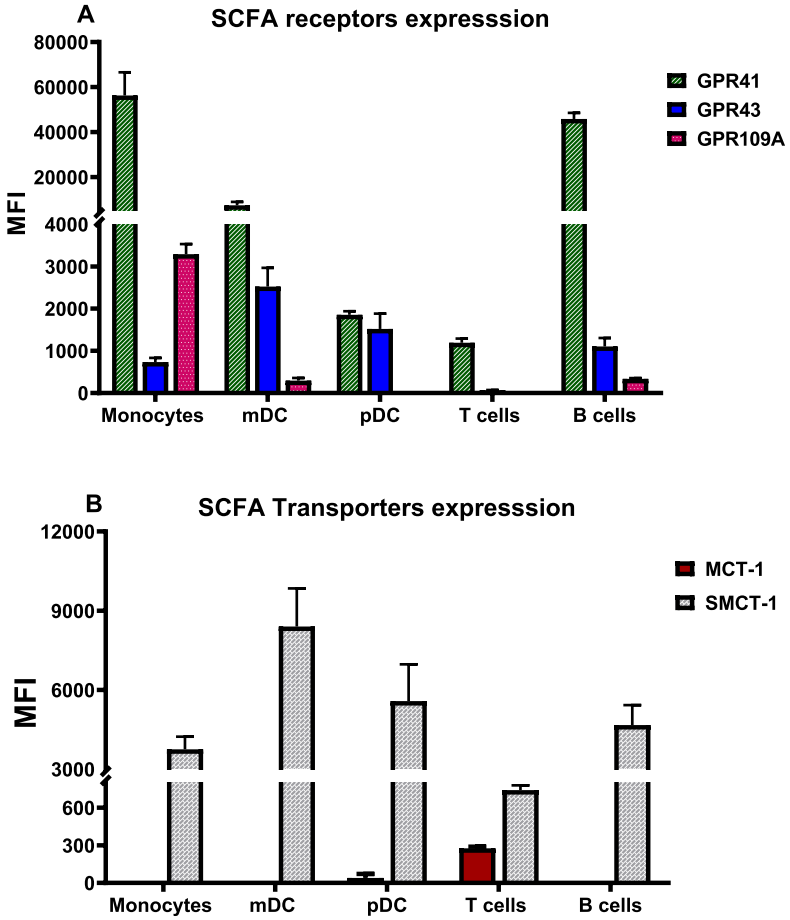

FIGURE 1 Differential expression pattern of short chain fatty acid (SCFA) receptors and transporters on PBMCs. The relative expression of SCFA receptors, GPR41, GPR43, and GPR109A (A) and SCFAs membrane transporters, MCT-1, and SMCT-1 (B) on monocytes, mDCs, pDCs, T- and B-lymphocytes. Data were generated from 3 independent donors.

(p_v C3 = 0.041, C4:0.040), IL-13 (p_v C3 = 0.016, C4 = 0.015), and IFN-γ (p_v C3 = 0.0255, C4 = 0.025) levels after T cell stimulation with soluble α-CD3/α-CD28 (Figure 2B-D). Stimulation with α-CD3/α-CD28 antibodies also resulted in the production of the anti-inflammatory cytokine IL-10. Production of IL-10 was blocked in the cells treated with butyrate (p_v = 0.028) and propionate (p_v = 0.029) but not by acetate (Figure 2E). Stimulation of B cells with CpG did not induce substantial production of cytokines, with the exception of IL-10, which was inhibited by butyrate (p_v = 0.0168), propionate (p_v = 0.016), and to a lesser extent by acetate (p_v = 0.118) (Figure 2A-E).

The effects of SCFAs on the expression of homing receptors on B and T cells

In order to study the ability of SCFAs to modulate the expression of the tissue homing-associated chemokine receptors CCR9 and CCR10, PBMCs were co-incubated with individual SCFAs and T- and B-cell stimuli for 48 h in the presence or absence of retinoic acid (RA). RA is known for its ability to upregulate CCR9 expression and homing to the small intestine (Takeuchi *et al.*, 2010). CCR9 expression was only induced after activation of the T cells with α-CD3/α-CD28 in the presence of RA, and it was inhibited by butyrate (p_v = 0.003) and propionate (p_v = 0.005) (Figure 3A). The average of T cells expressing CCR9 declined from 20% in the no SCFAs group to 5% in the group of cells treated with butyrate and propionate. In contrast, stimulation of B cells by CpG increased (however trivial) CCR10 expression, which was not affected by the addition of RA (Figure 3B). This expression was inhibited by butyrate (p_v = 0.004) and propionate (p_v = 0.001) but not by acetate. None of the stimuli or SCFA resulted in the expression of CCR9 on B cells or CCR10 on T cells (data not shown).

Effects of SCFAs on cytokines release from innate immune cells

To address the effects of the SCFA on stimulated innate immune cells (monocytes, mDC, pDC), PBMCs were

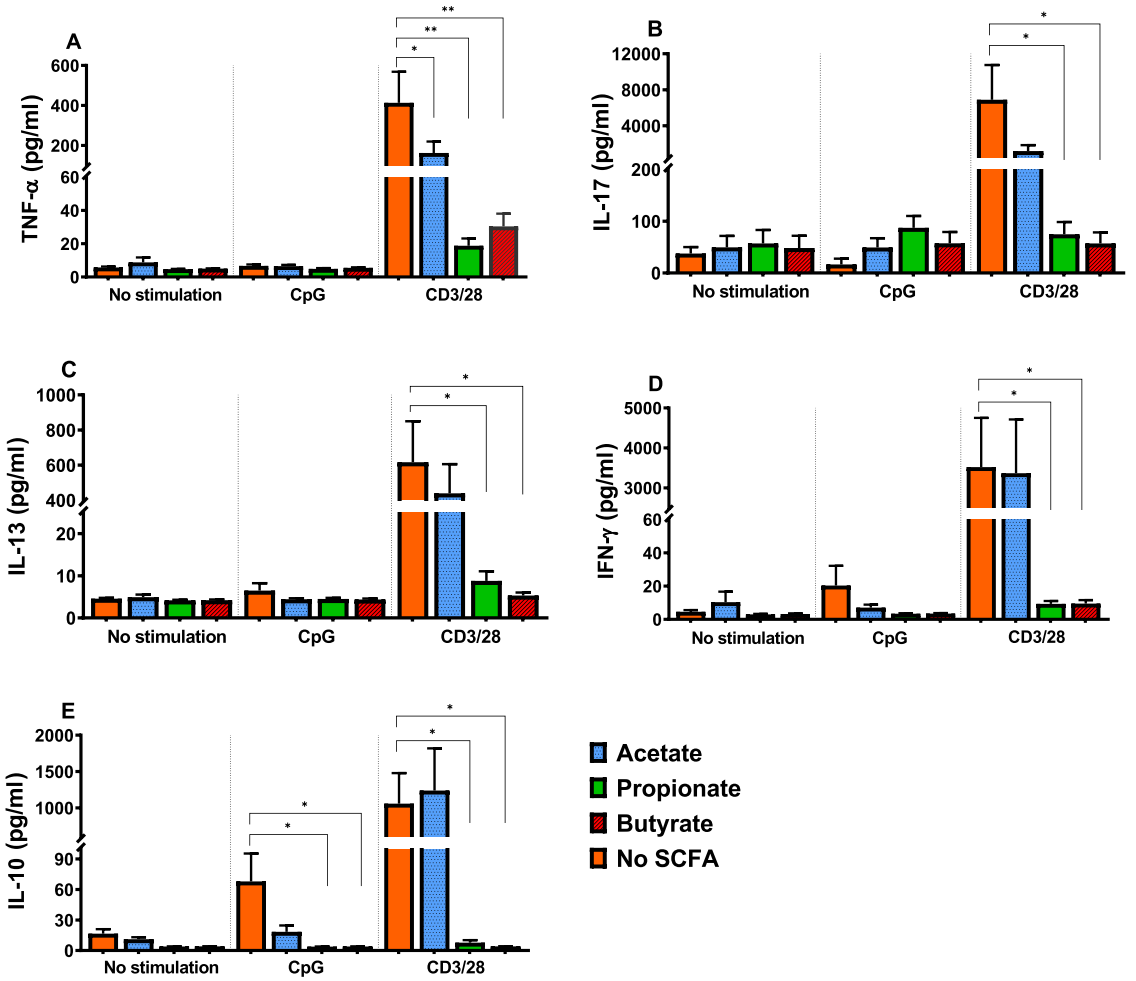

FIGURE 2 The effects of short chain fatty acids (SCFAs) on the production of cytokines from adaptive cells. The levels of tumour necrosis factor (TNF)-α (A), interleukin (IL)-17 (B), IL-13 (C), interferon (INF)-γ (D), and IL-10 (E) in the 48 h culture supernatant of the PBMCs incubated for 48 h with acetate, propionate, butyrate, or RPMI and stimulated with CpG or the combination of α-CD3/α-CD28 antibodies. The significance of differences is shown as $P < 0.05$ (*) or $P < 0.01$ (**). Data were generated from 5 independent donors.

incubated with TLR4 (LPS) and TLR7/8 (R848) agonists in the presence or absence of individual SCFAs for 24 h. The production of IL-1β, TNF-α, IL-6, IL-10, and IFN-α was quantified in the culture supernatant, and the results were compared to the group treated with no SCFAs. Upon LPS and R848 stimulation, IL-1β, TNF-α, IL-6, and IL-10 production was induced, but IFN-α production was only induced after stimulation with R848 (Figure 4A-E). Following R848 stimulation, all three SCFAs were competent in decreasing IL-1β production in innate immune cells (p_v C2 = 0.001, C3 < 0.001, C4 < 0.001); however, the differences between conditions were not significant following LPS stimulation (Figure 4A). Butyrate and propionate both inhibited the production of IL-6 following stimulation with LPS (p_v C3 < 0.001, C4 < 0.001) and R848 (p_v C3 < 0.001, C4 = 0.004), while acetate did not have an effect (Figure 4B). TNF-α production in the innate cells was significantly reduced by all three SCFAs upon stimulation with R848 (p_v C2 < 0.001, C3 < 0.001, C4 < 0.001), whereas in the LPS stimulated group, this decrease was only observed in cells treated with butyrate (p_v = 0.013) and propionate (p_v = 0.037), but not acetate (p_v = 0.055) (Figure 4C). As mentioned before, LPS did not induce the production of IFN-α in any of the groups, but on the contrary, stimulation with R848 resulted in IFN-α production, which was blocked by acetate (p_v = 0.21) and butyrate (p_v = 0.15), but not propionate (p_v = 073) (Figure 4D). Finally, all three SCFAs significantly decreased IL-10 levels in LPS-stimulated cells (p_v C2 = 0.039, C3 < 0.001, C4 < 0.001), while only butyrate inhibited this production in R848 treated cells (p_v = 0.005). Nevertheless, treatment with acetate marginally increased the production of IL-10 in the R848-stimulated cells (Figure 4E). In summary, under described conditions the addition of SCFA reduced cytokines released from stimulated cells, butyrate was the most potent inhibitor, followed by propionate. Except for a few stimulation

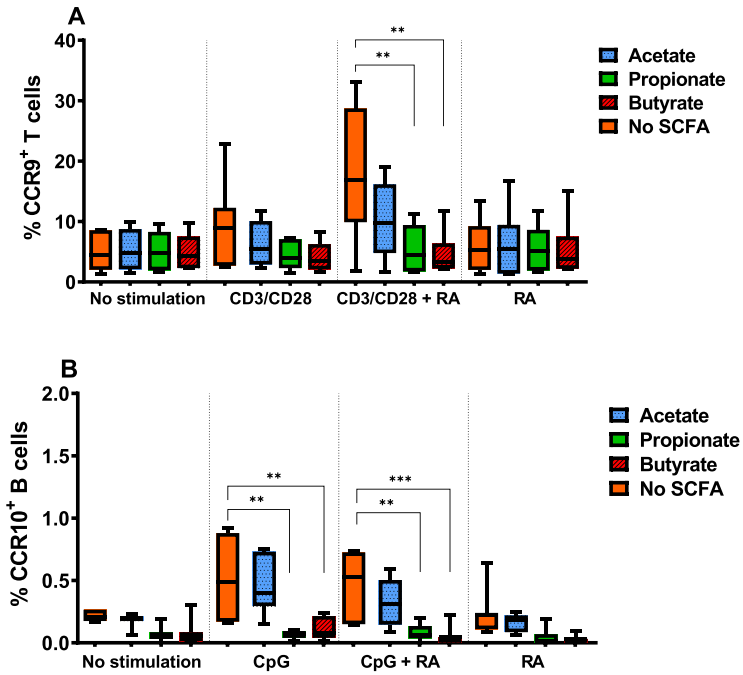

FIGURE 3 Expression of CCR9 and CCR10 in the presence of short chain fatty acids (SCFAs). Expression of CCR9 in T cells cultured for 48 h with acetate, propionate, butyrate, or RPMI and stimulated with α-CD3/α-CD28 in the presence of retinoic acid (RA) (A). Expression levels of CCR10 in B cells incubated for 48 h with acetate, propionate, butyrate, or RPMI and activated by CpG in the presence or absence of RA (B). The significance of differences is shown as $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***). Data were generated from 7 independent donors.

conditions, the cytokine production in the acetate-treated group was comparable to those without any SCFAs.

The effects of SCFAs on IL-6 and TNF-α production in monocytes, mDCs, and pDCs

To measure the effects of the SCFA on stimulated monocytes, mDC, and pDC, PBMCs were incubated with or without the individual SCFAs and were stimulated with the TLR4 ligand LPS or the TLR7/8 ligand R848 for 3 h. This assay was executed to characterise the effects of SCFAs on individual myeloid innate immune cells within PBMC. BFA was added to the culture to block cytokine excretion. Following stimulation, the cells were labelled with the fluorochrome-conjugated monoclonal antibodies to identify monocytes, mDC, and pDC; they were permeabilised, and the intracellular presence of IL-6 and TNF-α was detected by flow cytometry. Both LPS and R848 stimulation induced the production of IL-6 in more than 85% of monocytes. The IL-6 production was blocked in the group of cells treated with butyrate ($p_v < 0.001$) and propionate ($p_v < 0.001$) but not acetate ($p_v = 0.582$) (Figure 5A). In fact, the percentage of IL-6 producing monocytes dropped to less than 40% in the butyrate and less than 45% in the propionate group, where acetate did not exert clear effects (Figure 5A). On the contrary, exposure to all three SCFAs significantly decreased the TNF-α production in monocytes induced by R848 (p_v C2 = 0.001, C3 < 0.001, C4 < 0.001) (Figure 5B). R848 but not LPS stimulation induced TNF-α production in more than 80% of monocytes while acetate, propionate, and butyrate decreased this percentage to around 60, 30, and 35%, respectively (Figure 5B). When mDCs were examined, it was noted that acetate, butyrate, and propionate potently inhibited the IL-6 production induced by both LPS (p_v C2 = 0.007, C3 < 0.001, C4 < 0.001) and R848 (p_v C2 = 0.007, C3 < 0.001, C4 < 0.001) (Figure 5C). The percentage of IL-6 producing mDCs declined from more than 50% to around 25% in acetate-treated cells and around 10% after butyrate and propionate treatment (Figure 5C). From the same mDCs, stimulation with LPS and R848 resulted in the production of TNF-α in more than 40 and 70% of cells, respectively. However, TNF-α positive cells were significantly decreased by butyrate to 25% (p_v = 0.036) in the LPS-stimulated cells and less than 40% by butyrate (p_v = 0.015) and propionate (p_v = 0.008) in the cells activated by R848 (Figure 5D). R848 but not LPS, induced the production of IL-6 and TNF-α in pDCs. However, IL-6 production was blocked efficiently by all three SCFAs (p_v C2 = 0.012, C3 = 0.004, C4 < 0.002) (Figure 5E). The percentage of IL-6 producing pDCs decreased more than 4-fold from above 8% to less than 2% (Figure 5E). Moreover, more than 50% of

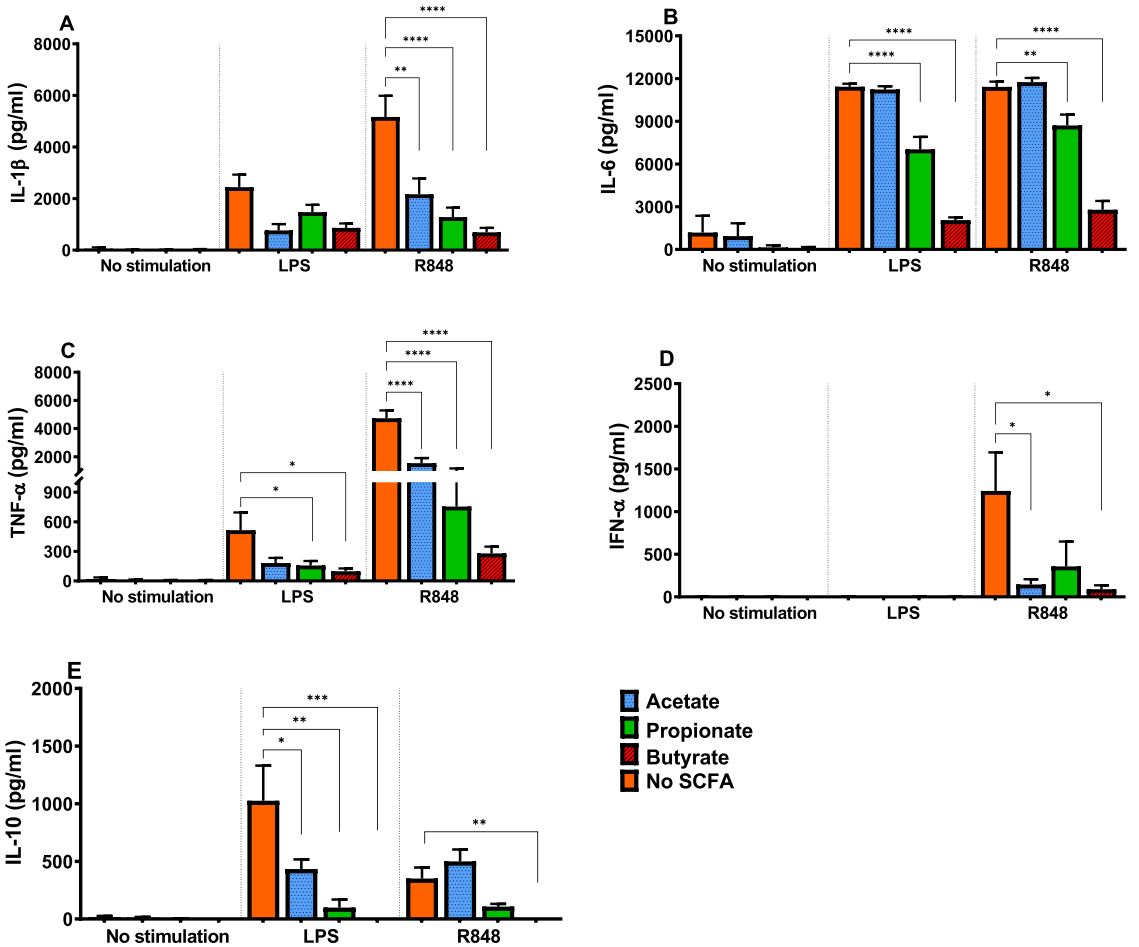

FIGURE 4 The effects of short chain fatty acids (SCFAs) on the production of cytokines in innate immune cells. The cytokine levels of interleukin (IL)-1β (A), IL-6 (B), tumour necrosis factor (TNF)-α (C), interferon (IFN)-α (D), and IL-10 (E) were measured in the 24 h culture supernatant of PBMCs incubated with acetate, propionate, butyrate, or RPMI and activated with lipoproteins (LPS) or R848. The significance of differences is shown as $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), or $P < 0.0001$ (****). Data were generated from 7 independent donors.

pDCs responded to R848, but not LPS stimulation, and produced TNF-α. Nevertheless, the cytokine production was decreased by butyrate ($p_v = 0.042$) and propionate ($p_v = 0.024$), and the levels dropped to 25% of cells. It is worth mentioning that acetate also lowered the percentage of TNF-α producing cells to less than 30%, but the difference was not quite significant ($p_v = 0.068$) (Figure 5F).

Innate immune training by SCFAs

Using an *in vitro* trained immunity model (Domínguez-Andrés *et al.*, 2021), we investigated the ability of SCFAs to modulate the responsiveness of human monocytes to secondary stimulation. The monocytes were incubated/trained with individual SCFAs, WGP, or TLR ligands (LPS and R848) as tolerogenic controls for 24 h. Thereafter, the monocytes were rested for 6 days and were stimulated with the TLR ligands PAM3CSK4 (TLR2/TLR1), LPS (TLR4), or R848 (TLR7/8). The monocytes trained with butyrate and propionate showed a decreased production of IL-6 and TNF-α upon stimulation with LPS, R848, and Pam3CSK4 compared to the negative control (RPMI) (Figure 6A-F). Training with acetate did not significantly lower IL-6 and TNF-α levels in any of the stimulations.

Training with the tolerogenic controls strongly inhibited IL-6 and TNF-α production in all cases, in a similar manner to butyrate and propionate (Figure 6A-F), except IL-6 production in response to LPS stimulation. This could be explained by the high variability of responses in this group of participants (Figure 6C). In our hands, training with WGP induced slightly higher IL-6 and TNF-α production than the RPMI control, but the differences did not reach statistical significance (Figure 6A-F). Table 1 provides the *P*-value of the trained immunity analyses.

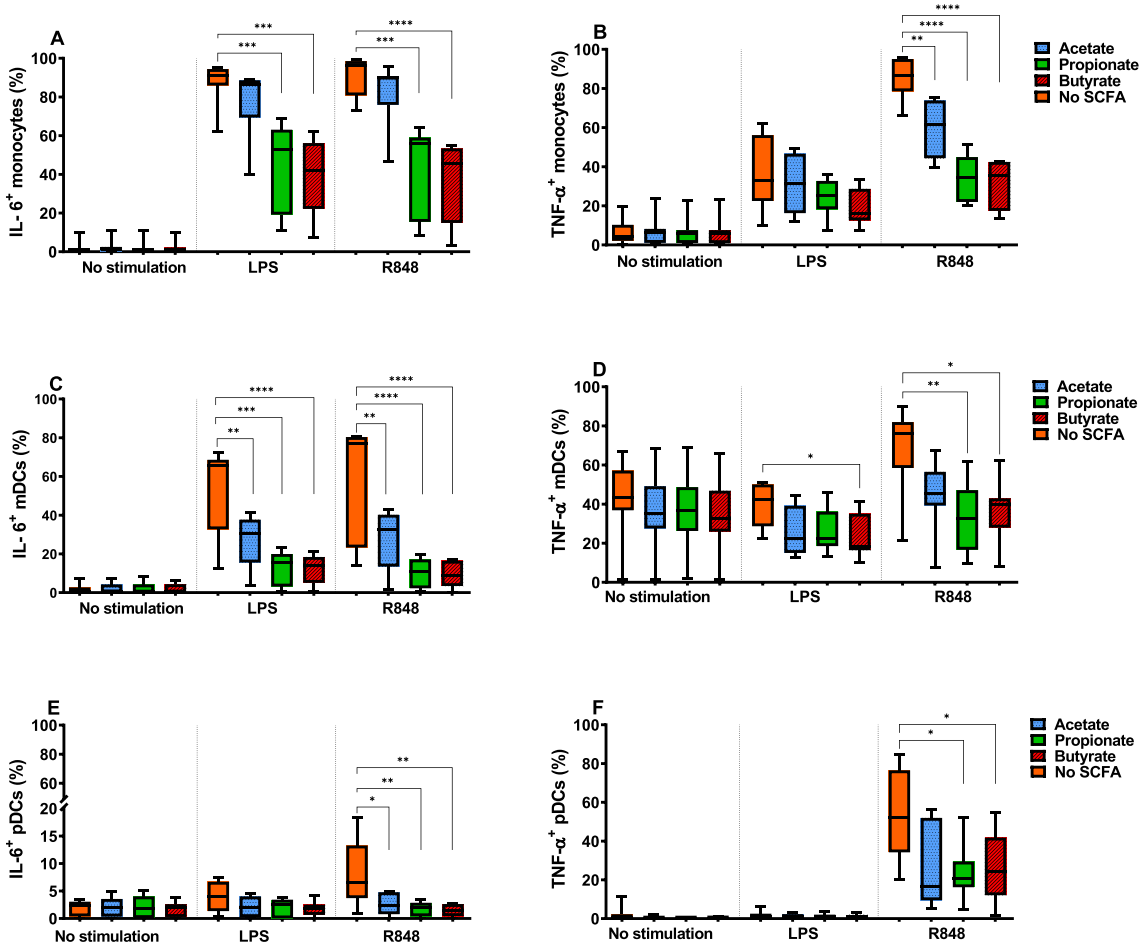

FIGURE 5 Intracellular production of IL-6 and TNF-α in innate immune cells. After 3 h of PBMCs incubation with acetate, propionate, butyrate, or RPMI, the percentage of cells producing IL-6 and TNF-α upon stimulation with LPS or R848 was quantified. The IL-6 (A) or TNF-α (B) producing monocytes, IL-6 (C) or TNF-α (D) producing mDCs, and IL-6 (E) or TNF-α (F) producing pDCs are shown in the figure. The significance of differences is shown as $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), or $P < 0.0001$ (****). Data were generated from 8 independent donors.

4 Discussion

In this study, we studied the effects of individual SCFAs on the activation of innate and adaptive immune cells, as well as the ability to induce immune training on monocytes. Here, we show that butyrate and propionate were the most potent tested SCFAs at inhibiting the activation of monocytes, mDC, B- and T-cells. Noteworthy, activation of pDC was inhibited by all three SCFAs, including acetate, which could be explained by the differential expression profiles of SCFA receptors on these cells. In addition, although GPRs have some downstream signalling pathways in common with Dectin-1, the receptor through which β-glucans induce innate immune training, SCFAs did not induce innate immune training.

SCFAs are well-known for their ability to maintain local and systemic homeostasis and to prevent excessive inflammatory responses to commensal bacteria and dietary components (Corrêa-Oliveira *et al.*, 2016; Van der Hee and Wells, 2021). However, to our knowledge, no studies to date have addressed the effects of all three most common SCFAs on innate as well as adaptive immune cell types in a single study, as most studies focus on single SCFA or single cell type. This approach enabled us to not only investigate the overall impacts of SCFAs but also to compare the effects of SCFA on individual cell types linked to the expression of their receptors.

Acetate, propionate, and butyrate contain 2, 3, and 4 carbon atoms, respectively, produced by colonic bacteria via the glycolytic pathway or pentose phosphate pathway (Tan *et al.*, 2014). While colonocytes consume most of the butyrate as their energy source, propionate and acetate enter the portal vein and are taken to the liver. Hepatocytes in the liver metabolise propionate; therefore, acetate is the SCFA with the highest concentrations in the peripheral blood (Cummings *et al.*,

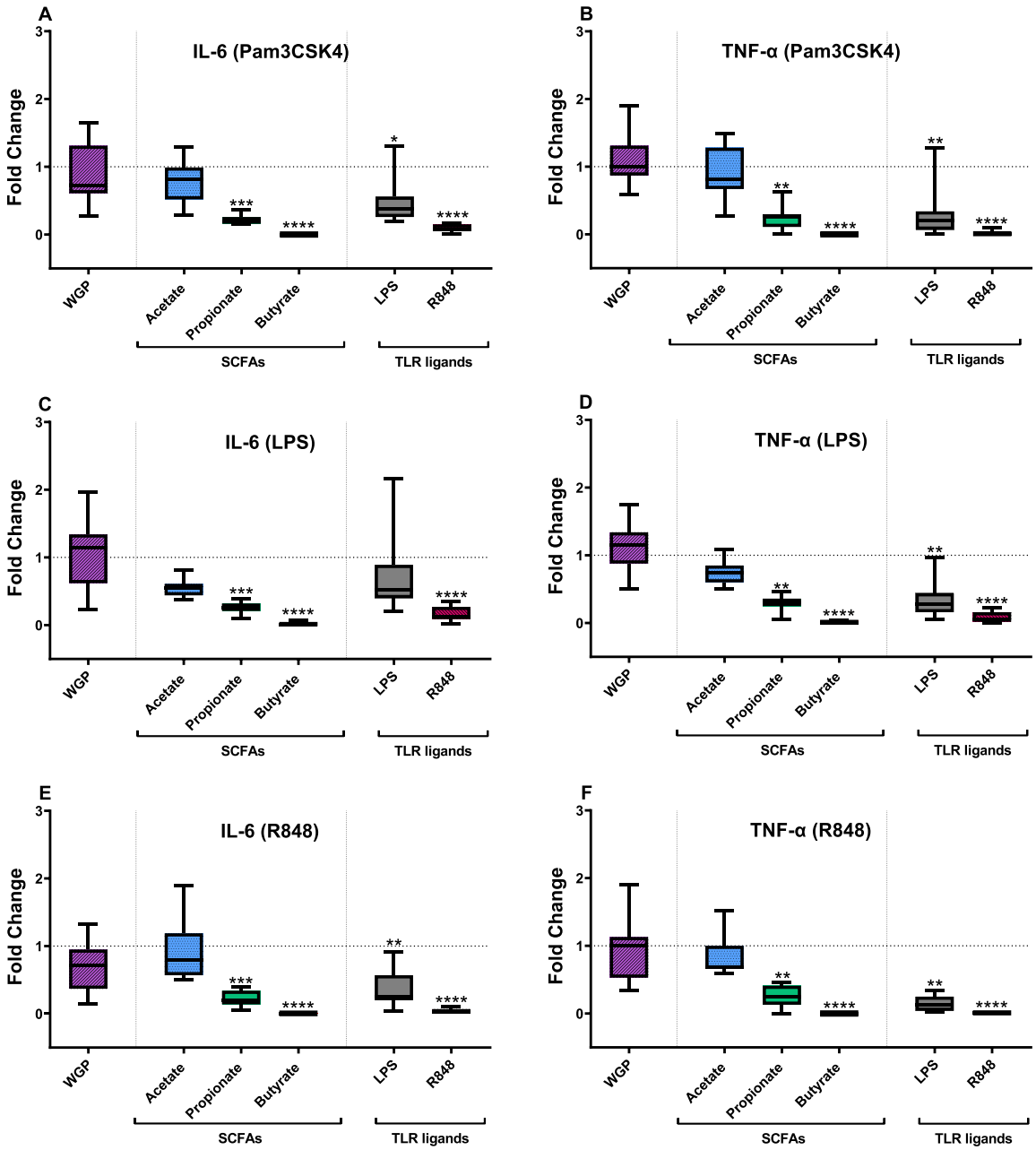

FIGURE 6 The effects of short chain fatty acids (SCFAs) training on monocytes responsiveness. Monocytes were treated for 24 h with WGP, different SCFAs, and tolerogenic TLR ligands (LPS or R848) and then rested for 6 days before being stimulated with TLR ligands. The production of IL-6 in monocytes stimulated with Pam3CSK4 (A), LPS (C), or R848 (E) as well as TNF-α upon stimulation with Pam3CSK4 (B), LPS (D), or R848 (F) are expressed as fold changes compared to untrained cell (RPMI) which are expressed as 1. The average (Range) of cytokines in the RPMI controls were 2734 (632-5103 pg/ml), 328 (85-873 pg/ml), 5174 (2199-10,957 pg/ml), 1371 (825-2377 pg/ml), 9001 (3037-30,641 pg/ml), and 1941 (914-4929 pg/ml) for conditions A to F, respectively. The boxes represent 50% of the data, and the line is the median value where upper and lower whiskers present upper and lower 25% of the data, respectively. The significance of differences is shown as $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), or $P < 0.0001$ (****). Data were generated from 11 independent donors.

TABLE 1 The *P*-values of the analyses observed after the incubation of cells with various components compared to no short chain fatty acids (SCFAs)[1]

	Stimulation	WGP	Acetate	Propionate	Butyrate	LPS	R848
IL-6	Pam3	0.277	0.277	<0.001	<0.001	0.015	<0.001
	LPS	0.96	0.757	<0.001	<0.001	0.167	<0.001
	R848	0.167	0.429	<0.001	<0.001	0.007	<0.001
TNF-α	Pam3	0.843	0.621	0.002	<0.001	0.005	<0.001
	LPS	0.999	0.138	0.002	<0.001	0.003	<0.001
	R848	0.729	0.587	0.004	<0.001	0.001	<0.001

1 The differences were considered significant when $P < 0.05$.

1987). We speculate that even though SCFA concentrations measured in the circulation are relatively low, their concentration might be closer to the luminal SCFA concentrations in the intestinal mucosa where they first encounter immune cells. Interestingly, in some studies, no obvious effects were observed when using low concentrations of acetate and propionate (Iraporda *et al.*, 2015; Maslowski *et al.*, 2009; Usami *et al.*, 2008). In this regard, acetate, propionate, and butyrate were shown to decrease LPS-stimulated TNF-α release from neutrophils at a concentration as high as 30 mM (Tedelind *et al.*, 2007). After 3 or 24 h of incubation with SCFAs, the viability of T- and B-cells, as well as monocytes, was still > 80%. However, incubation of monocytes with 5 mM of butyrate for 24 h resulted in a reduction to 60% of viable cells (Supplementary Figure S4). The applied concentrations of SCFA used in the experiments were based on the toxicity profile of individual SCFAs as well as their levels in batch culture supernatants (Perdijk *et al.*, 2019). It should be noted that SCFAs exert concentration-dependent responses and increasing or decreasing the concentrations might lead to somewhat different outcomes. As the levels of the SCFA tested may approach luminal levels *in vivo*, considerably lower concentrations reach the circulation, and the results discussed here may not reflect *in vivo* effects of microbiota-derived SCFA at distant sites in the body.

SCFAs can exert their effects by directly inhibiting the enzymatic effects of HDACs upon cell entry or indirectly by signalling via GPR receptors. Butyrate and propionate inhibited HDAC activity in effector and regulatory T cells independently of GPR41 and GPR43 (Park *et al.*, 2015). Furthermore, butyrate and propionate have been shown to limit the development of dendritic cells (DCs) in mice. This effect has been observed due to HDACs inhibition following SMCT-1-mediated cell entry (Singh *et al.*, 2010). On the other hand, a growing body of research indicates that the direct involvement of GPR proteins is crucial for the SCFAs to exert their immunomodulatory effects. Colonic inflammation was suppressed by activating GPR109A (Singh *et al.*, 2014), whereas the signal transduced via GPR41 and GPR43 promoted an inflammatory response in mouse intestinal epithelial cells (Kim *et al.*, 2013). It is unclear which mechanism is more prominent or whether both pathways are necessary for SCFAs to induce their effects.

Incubation with SCFAs inhibited the production of cytokines in activated T cells *ex vivo*. TNF-α, IL-13, IL-17, and IFN-γ levels were significantly lower in cells treated with butyrate and propionate compared to the absence of SCFAs (Figure 2). Butyrate was previously found to block HDAC activity in murine $CD4^+$ T cells, resulting in cell anergy (Fontenelle and Gilbert, 2012), while propionate was demonstrated to suppress T helper 2 (Th2) response and decrease IL-4, IL-5, and IL-17 levels in allergic murine lungs (Trompette *et al.*, 2014). Similarly, in another study, butyrate decreased IFN-γ and IL-2 levels, thereby inhibiting cell proliferation in rat mesenteric lymph node lymphocytes (Cavaglieri *et al.*, 2003). Regulating the inflammatory cytokines produced by various T cell subsets, executed effectively by SCFAs, is critical for preventing excessive inflammatory or allergy responses at mucosal surfaces. The decreased T-cell activity observed following SCFA administration is consistent with the differential expression of relevant transporter molecules. Although GPRs are not highly expressed in the T lymphocytes, these cells express MCT-1 and particularly SMCT-1, which was found to be essential for intracellular accumulation of butyrate and propionate and direct HDAC activity inhibition (Singh *et al.*, 2010). Additionally, an indirect effect via decreased DC capacity for naive T cell stimulation may partially account for the observations (Liu *et al.*, 2012; Millard *et al.*, 2002).

Previously, SCFAs were shown to prevent allergic inflammation in animal models (Trompette *et al.*, 2014;

Yip *et al.*, 2021). Therefore, we were interested to know whether individual SCFAs might influence lymphocyte homing to distant tissues. Our findings indicate that SCFAs cannot stimulate homing to the respiratory or intestinal tract but rather inhibit the activation-induced expression of homing receptors. Both butyrate and propionate prevented the upregulation of the CCR9 expression in activated T cells. Likewise, the same two SCFAs suppressed the expression of CCR10 in activated B lymphocytes, albeit the overall expression was relatively low. CCR9 interaction with CCL25 directs intestinal intraepithelial lymphocytes and DCs to the intestine (Pathak and Lal, 2020; Sumida, 2020), and CCR10 is required for tissue-specific migration to various mucosal tissues, including the airways (Xiong *et al.*, 2012). The absence of GPR43 may thus explain why acetate does not affect T cell activity, whereas at the same time, the high availability of GPR41 and SMCT-1 may justify the observations for CCR10 expression in B lymphocytes. Although specific effects of SCFA on intestinal and respiratory tract homing have not been described so far, an inhibitory effect of SCFAs on immune cell trafficking has been demonstrated previously. Butyrate downregulated intercellular adhesion molecule 1 (ICAM-1) on monocytes (Bohmig *et al.*, 1997), butyrate and propionate significantly decreased the expression of chemokine ligands CCL5, CXCL9, CXCL10, and CXCL11 in DCs (Nastasi *et al.*, 2015), and propionate showed the ability to inhibit vascular cell adhesion molecule 1 (VCAM-1) and ICAM-1 expression (Zapolska-Downar and Naruszewicz, 2009). These findings suggest that SCFAs exert tight control over immune cell migration by retaining them in draining lymph nodes where they can exhibit their effects. Additional research could be conducted to determine these applications for treating inflammatory diseases.

Butyrate, and to a lesser extent, propionate, demonstrated a suppressive effect on inflammatory cytokines production from innate immune cells, while acetate was shown to be more selective in its impact. Proinflammatory IL-1β, IL-6, TNF-α, and IFN-α levels were elevated in PBMCs stimulated with TLR agonists (Figure 4). However, butyrate, followed by propionate, significantly reduced the production of all these cytokines, whereas acetate did so primarily for IFN-α, the cytokine typically produced by pDC upon viral(like) stimulation. Further experiments on the intracellular synthesis of IL-6 and TNF-α in innate immune cells confirmed and extended the results described for cytokine production in the culture supernatant. Monocytes and mDCs produced IL-6 and TNF-α in response to both LPS and R848, but pDCs only responded to R848 stimulation. Human pDCs do not possess TLR4; therefore, they do not react to LPS activation but respond to pathogen-derived nucleic acids due to the presence of TLR7 and TLR9 (Hornung *et al.*, 2002; Kaisho, 2012; Reizis, 2019). Butyrate and propionate were able to inhibit IL-6 and TNF-α production in monocytes and mDCs in a similar fashion. Moreover, acetate also had significant effects by decreasing TNF-α in monocytes and IL-6 in mDCs, but this influence was less pronounced than those of butyrate and propionate. Subsequently, we tried to link these findings to the expression of SCFA receptors on different immune cells. Monocytes were shown to express high levels of GPR109A and GPR41 but not GPR43. As a result, significant effects from butyrate and propionate are expected, while acetate has essentially little effect. On the other hand, acetate showed inhibitory effects on mDCs and reduced the percentage of IL-6 producing cells by half, in accordance with the higher expression of GPR43 as the primary receptor of acetate on mDCs. The effect of acetate on pDC, though, was comparable to the effects of both butyrate and propionate, with a three- to four-fold reduction of cytokine-producing cells. GPR41 and GPR43 are equally present on pDCs mediating the inhibitory effects of propionate and acetate, respectively, whereas the high availability of SMCT-1 on pDCs may explain the butyrate effect in a comparable manner to T cells.

In agreement with our findings, the reduction of LPS-induced TNF-α by SCFAs was previously demonstrated (Tedelind *et al.*, 2007; Vinolo *et al.*, 2011). Similarly, butyrate and propionate-treated cells produced less IL-6 and IL-12, whereas acetate treatment did not have the same effects on monocyte-derived DCs (Nastasi *et al.*, 2015). Butyrate also showed the ability to suppress NF-κB activity and hence decrease IL-6 and TNF-α levels in mouse macrophages (Ohira *et al.*, 2013). The cytokine suppressing effects were found to be related to SCFAs' ability to modulate NF-κB activity (Segain *et al.*, 2000). Upon NF-κB activation, depending on the stimuli and immunological context, a wide range of proinflammatory cytokines, such as IL-6 and TNF-α are produced. Nevertheless, both butyrate and propionate were shown to effectively decrease NF-κB activity in PBMCs in a manner comparable to that of trichostatin A (TSA) (Usami *et al.*, 2008) Albeit, it is worth mentioning that NF-κB inhibition is dose and time-dependent.

Here, one remarkable result was found for pDCs. As the primary producers of the antiviral cytokine IFN-α, these cells generated detectable quantities of the cytokine after stimulation of unseparated PBMCs with the TLR7/8 ligand (R848) despite their lower prevalence

in blood compared to monocytes (less than 0.5% compared to about 10% of PBMCs). pDCs do not express GPR109A and have a low level of GPR41 expression; however, GPR43 with a higher affinity for acetate is abundantly present on their cell membrane. Notably, IFN-α is the only cytokine that was reduced by acetate as effectively as butyrate and propionate, which could be explained by higher GPR43 expression on pDCs.

Previously, low-concentration butyrate and propionate exposure was shown to increase the production of IL-10 in PBMCs and T cells, respectively (Säemann *et al.*, 2000; Smith *et al.*, 2013). However, in our study, we observed a reduction in the synthesis of this cytokine when SCFAs were applied. Differences in the SCFA concentrations could explain the contradictory results applied. It was previously demonstrated that adding sodium butyrate to human regulatory T cells (Tregs), the primary producers of IL-10, inhibited their proliferation *in vitro* while enhancing their inhibitory effect on other T cells (Akimova *et al.*, 2010). The inhibition of IL-10 production reported in the current study is most likely due to decreased proliferative activity of Tregs exposed to higher concentrations of butyrate and propionate.

Apart from the dissimilar potential of SCFAs, the diversity of immunomodulatory effects could have been reflected by the differential expression of receptor and transporter molecules on immune cells. Here we showed that the expression of GPR43, with the highest affinity for acetate followed by propionate, is relatively low in monocytes and lymphocytes while it is present on both mDCs and pDCs. As a result, one should anticipate that acetate administration will have little or no effect on monocytes and lymphocyte-associated cytokines but will substantially impact mDCs and pDCs. This is entirely consistent with our findings in the current study. GPR41, on the other hand, is abundantly expressed on monocytes, B cells, and mDCs examined. GPR41 is the primary receptor for propionate followed by butyrate, the same two SCFAs with the most significant effect on the aforementioned cell types. Furthermore, GPR109A, which exclusively interacts with butyrate, is not highly expressed in the immune cells (except monocytes), but in return, SMCT-1 availability may make up for that, as described earlier. Taken together, the lower effects of acetate on lymphocytes and monocytes may be attributable in part to the low level of GPR43 expression on these cells, while the higher impact on mDCs and especially pDCs may confirm this notion.

Considering SCFAs' documented ability to activate extracellular signal-regulated kinases (ERK)1/2 (Seljeset and Siehler, 2012) as well as inhibiting HDAC activity (hence alter gene expression), we hypothesised that these metabolites might prime monocytes towards trained immunity or tolerance. Modifying epigenome via HDAC modulation and metabolic rewiring was shown to be the underlying mechanism for both trained immunity and tolerance (Divangahi *et al.*, 2021; Quintin *et al.*, 2014). As a result, we investigated SCFAs in an established *in vitro* trained immunity/tolerance induction model. Our findings indicate that butyrate and propionate modulate monocytes' responsiveness to TLR ligands upon secondary stimulation, confirming their higher potency for HDAC inhibition than acetate. It should be mentioned that for butyrate at 5 mM – but not for propionate or acetate – reduced viability of the monocytes was noted after 24 h incubation, which may partly explain these findings. Butyrate and propionate, but not acetate, lowered monocytes' response to Pam3CSK4, LPS, and R848 stimulation a week after training. In line with our findings, oral supplementation with butyrate decreased the trained immunity induced by ox-LDL and β-glucan *in vivo* (Cleophas *et al.*, 2019). The findings can be explained by the differential expression pattern of SCFA receptors on monocytes similar to what was described for direct SCFA stimulation. Monocytes with high GPR109A and GPR41 expression responded similarly to butyrate and propionate treatment but not to acetate, most likely due to the low availability of GPR43. In general, HDAC inhibition is regarded as a permissive factor for gene expression, which may result in increased gene expression. However, depending on the chromatin status and promoter, HDAC inhibition may also suppress gene expression (Reichert *et al.*, 2012). Our observation on decreased IL-6 and TNF-α production in monocytes is in accordance with this statement. As previously stated, trained immunity and tolerance are two sides of the same coin, sharing epigenetic and metabolic alterations as the underlying mechanism (Divangahi *et al.*, 2021). In this study, however, we demonstrate the inhibitory effects of SCFAs on IL-6 and TNF-α production after secondary stimulation in a similar manner to responses of established tolerogenic controls in this training model. To confirm the tolerogenic properties of SCFAs, further in-depth research on altered epigenetic markers, signalling cascades, and cell metabolism are necessary.

Here we provide extensive evidence for the immunomodulatory effects of individual SCFA on human PBMCs. Both butyrate and propionate inhibited the production of lymphocyte cytokines in activated T cells and prevented the upregulation of the tissue homing mark-

ers expression in activated T- and B-cells, thus reducing their potential to migrate to distant tissues. Whereas butyrate and propionate strongly inhibited the activation of monocytes and mDC, acetate administration had a selective effect on the TLR-mediated activation of pDC. Finally, butyrate and propionate inhibited the IL-6 and TNF-α secretion in a trained immunity/tolerance setting, whereas acetate did not affect these responses. These dissimilar SCFAs findings could be due to the differential expression of relevant receptors and transporters on different immune cells. Acetate is found in higher quantities in circulation and can reach the most remote organs. Furthermore, pDCs revealed the most pronounced response to acetate. Thus, it would be intriguing to investigate the effects of acetate on antiviral response regulation in pDCs in organs such as the respiratory tract in order to discover new therapeutic or prophylactic applications for this important SCFA.

Supplementary material

Supplementary material is available online at: https://doi.org/10.6084/m9.figshare.23579658

Table S1. Flow cytometry antibody panel for phenotyping immune cells and assessing the differential expression of SCFAs receptors.

Table S2. Flow cytometry antibody panel for phenotyping immune cells and assessing the differential expression of SCFAs transporters.

Table S3. Flow cytometry antibody panel for quantifying CCR9 and CCR10 in $CD3^+$ T cells and $CD19^+$ B cells.

Table S4. Flow cytometry antibody panel for staining monocytes, mDCs, and pDCs and quantifying IL-6 and TNF-α levels.

Table S5. The summary of mean, SD, and number of donors analysed for the effects of SCFAs on cytokine release from adaptive immune cells.

Table S6. The summary of mean, SD, and number of donors analysed for the effects of SCFAs on the expression of homing receptors on T cells.

Table S7. The summary of mean, SD, and number of donors analysed for the effects of SCFAs on the expression of homing receptors on B cells.

Table S8. The summary of mean, SD, and number of donors analysed for the effects of SCFAs on cytokines release from innate immune cells.

Table S9. The summary of mean, SD, and number of donors analysed for the effects of SCFAs on IL-6 and TNF-α production in monocytes, mDCs, and pDCs.

Table S10. The summary of mean, SD, and number of donors analysed for the effects of SCFAs on trained immunity induction.

Figure S1. Gating strategy for identifying $CD3^+$ T cells, $CD19^+$ B cells, $CD14^+$ monocytes, $CD11c^+$ mDCs, and $CD123^+$ pDCs from PBMCs. The expression of SCFA receptors and transporters was determined for each cell type.

Figure S2. The gating strategy for identifying CD3+ T cells and CD19+ B cells in PBMCs and quantifying CCR9 and CCR10 expression within each cell type.

Figure S3. Gating strategy for discrimination of $CD14^+$ monocytes, $Cd11c^+$ mDCs, and $CD123^+$ pDCs in PBMCs. The percentage of IL-6 and TNF-α producing cells was quantified within each population.

Figure S4. The % of viable T- and B-cells, and monocytes after 3 or 24 h incubation with different concentrations (45, 15, 5, 1.5, 0.5, and 0 mM) of acetate, butyrate, and propionate.

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