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Lactate and short chain fatty acids produced by microbial fermentation downregulate proinflammatory responses in intestinal epithelial cells and myeloid cells

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ABSTRACT

The use of short chain fatty acids to modulate gastrointestinal inflammatory conditions such as ulcerative colitis has produced encouraging results either in animal models or also in clinical trials. Identifying the key cellular and molecular targets of this activity will contribute to establish the appropriate combinations/targeting strategies to maximize the efficacy of anti-inflammatory interventions. In the present work, we evaluated *in vitro* the interaction of lactate, acetate, propionate and butyrate on cells relevant for innate immune response of the gastrointestinal tract. All molecules tested regulate the production of proinflammatory cytokines by TLR-4 and TLR-5 activated intestinal epithelial cells in a dose response manner. Furthermore SCFAs and lactate modulate cytokine secretion of TLR-activated bone marrow derived macrophages and also TLR-dependent CD40 upregulation in bone marrow derived dendritic in a dose-dependent manner. Butyrate and propionate have been effective at concentrations of 1 to 5 mM whereas acetate and lactate produced modulatory effects at concentrations higher than 20–50 mM in different assays. Our results indicate that in concentrations similar to found in large bowel lumen, all SCFAs tested and lactate can modulate activity of relevant sentinel cell types activated by TLR signals. Modulatory activity was not inhibited by pertussis toxin treatment indicating that the effects are not related to G_i signaling. The use of these molecules in combined or separately as intervention strategy in conditions where epithelial or myeloid cells are main triggers of the inflammatory situation seems appropriate.

Abbreviations: cAMP, cyclic adenosine monophosphate; BMMs, bone marrow derived macrophages; BMDCs, bone marrow derived dendritic cells; CCL20, chemokine (C–C motif) ligand 20; dbcAMP, dibutyril cyclic adenosine monophosphate; DC, dendritic cells; FliC, flagellin; GPRs, G-protein coupled receptor; IBD, inflammatory bowel disease; IL-1β, interleukin-1β; IL-6, interleukin 6; IL-8, interleukin 8; IL-12, interleukin 12; ITF, intestinal trefoil factor; LPS, lipopolysaccharides; MFI, mean fluorescence intensity; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; *PTX*, bordetella pertussis toxin; PBMC, peripheral blood mononuclear cell; SCFAs, short chain fatty acids.

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1. Introduction

The human intestinal microbiota is a diverse community of microbes reaching up to 10^{11} bacteria/mL in the colon and serves essential functions in food digestion, immunomodulation, and establishment of a barrier effect that prevents colonization by pathogens (Tlaskalova-Hogenova et al., 2011). The human colonic microbiota produces an enormous quantity of molecules that impact on the gut homeostasis (Matsuki et al., 2013). Diet strongly influences qualitatively or quantitatively the gut microbial communities and consequently the microbial-derived molecules present in gut lumen (Vinolo et al., 2011). Dietary carbohydrates that are not digested by the host in the small bowel are fermented in the colon into short-chain fatty acids (SCFAs) including acetate, propionate and butyrate (Russell et al., 2013). Furthermore, several microbiota components produce lactate, which is the most common short chain hydroxy– fatty acid in intestinal lumen, which can be converted to other SCFAs by a subgroup of lactate-fermenting bacterial species. In turn, SCFAs have beneficial effects on the host. Particularly, SCFAs are associated with reduced risk of some diseases including the irritable bowel syndrome, inflammatory bowel disease (IBD), cardiovascular disease, and cancer (Hijova and Chmelarova, 2007; Huda-Faujan et al., 2010).

Administration of SCFAs or prebiotics that are known to enhance SCFA production was proposed as treatment in IBD (Breuer et al., 1997; Scheppach, 1996; Vernia et al., 1995). However, due to partial patient compliance or restricted indications, these treatments were not established as a standard of care. Recent studies have renewed the expectations on strategies related to intestinal SCFAs. Thus, dysbiosis in IBD patients was associated with altered butyrate fermentative pathways (Eeckhaut et al., 2013; Galecka et al., 2013; Kumari et al., 2013; Machiels et al., 2014). Interventions in animal models resulting in increased exposure of intestinal tissue to specific SCFAs have shown protective effects in intestinal mucosa (Komiyama et al., 2011; Vieira et al., 2012). In particular, the administration of probiotic bacteria with the capacity to produce butyrate has been shown to improve the symptoms in IBD models (Eeckhaut et al., 2013). These modulatory effects *in vivo* are probably due to the capacity that show different SCFA to downregulate specific inflammatory cell functions that has been partially described so far (Tedelind et al., 2007; Berndt et al., 2012; Liu et al., 2012). SCFAs have effects in different cell types mediated by different mechanisms. Various G protein-coupled receptors (GPR) such as GPR41, GPR43, and GPR109a may mediate SCFAs activities (Offermanns, 2014). Furthermore, several SCFA may mediate biological effects by inhibiting histone deacetylase (Tan et al., 2014).

An improvement in our knowledge on the capacity of the different microbial fermentation products to modulate inflammatory activation may contribute to rational design of intervention strategies in situations of altered intestinal homeostasis. In this work, the effects of lactate and various SCFAs on cells that represent the main sentinel cells of the intestine, i.e. epithelial cells, macrophages and dendritic cells were analyzed. Thus, regulation of production of pro-inflammatory responses induced by microbial stimuli or cytokine was investigated.

2. Materials and methods

2.1. Reagents

Racemic DL-Lactic (J. T. Baker, USA), acetic acid (Dorwil, Bs. As., Argentina), propionic and butyric acid (Sigma Chemical Co., USA) were used for preparation of aqueous solutions at 0.5 mol/L, neutralized to pH 7.0 ± 0.2 by the use of solution of NaOH 1 mol/L, filtered through 0.45 μm membrane filter and maintained at −20 °C until used.

Flagellin (FliC) obtaining and purification from *Salmonella enterica* was previously described (Sierro et al., 2001). Human IL-1β was purchased from R&D Systems (Minneapolis, USA). Cyclic adenosine monophosphate (cAMP) analogue (dibutyril-cAMP, dbcAMP) was obtained from Sigma Chemical Co. Ultrapure lipopolysaccharide (LPS) from *Escherichia coli* serotype 0111:B4 was from InvivoGen (Toulouse, France). All cell cultures media, serum, and supplements were purchased from Gibco®.

2.2. Culture and stimulation of intestinal epithelial cell lines

Human colonic epithelial cell line Caco-2 were a gift from Dr. J.P. Krahenbühl. Caco-2 cells stably transfected with a luciferase reporter construction under the control of *CCL20* promoter (designated Caco-$2_{CCL20\text{-}luc}$) were previously described (Nempont et al., 2008). Cells were maintained and routinely grown as described (Iraporda et al., 2014). Caco-$2_{CCL20\text{-}luc}$ and Caco-2 cells were used after 8 days of culture at passages between 12 and 22. All experiments were performed in serum-free medium in 48 well plates.

Caco-$2_{CCL20\text{-}luc}$ cells cultured were incubated with aqueous solutions of different concentrations of lactic, acetic, propionic and butyric acid in fetal bovine serum free DMEM (1:1) for 30 min. Cells were then stimulated with FliC (1 μg/mL) or IL-1β (10 ng/mL) and incubated for 5 h at 37 °C in a 5% CO_2 – 95% air atmosphere. All experiments included a basal condition without any treatment. FliC or IL-1β were used respectively as control of 100% induction of pro-inflammatory response. Then, cells were lysed with Lysis Buffer (Promega, Madison, WI, USA). Luciferase activity was measured in a Labsystems Luminoskan TL Plus luminometer (Thermo Scientific, USA) using a Luciferase Assay System (Promega, Madison WI, USA) as previously described (Nempont et al., 2008). Luminescence was normalized to the stimulated control cells and expressed as percentage of normalized average luminescence ± standard deviation (SD) of at least three independent experiments.

For gene expression analysis, cell monolayers of Caco-2, in 24-well tissue culture plates were incubated for 30 min with 500 μL of aqueous solutions of different acids (100 mM) previously neutralized and filtered as described above, in DMEM. Then cells were stimulated with FliC (1 μg/mL) during 2 h.

2.3. Generation and stimulation of primary murine macrophages and dendritic cells

Female C57BL/6J (6–8 weeks old) mice were obtained from Janvier laboratories (St. Berthevin, France). All experiments complied with current national and institutional regulations and ethical guidelines (B59-350009–Institut Pasteur de Lille). Bone marrow (BM) from femurs and tibias were used as source of hematopoietic cell precursors as described (Van Maele et al., 2014). Briefly, BM macrophages (BMMs) were obtained upon 8 days differentiation of BM precursors (5.5×10^5/mL) in DMEM supplemented with 10% FSC, 1% penicillin–streptomycin and 30% of L929 cells-conditioned medium (mM-CSF source), initially seeded Petri culture dishes. Every 3 days, cells were supplemented with fresh medium. BM dendritic cells (BMDCs) were derived upon 9 days of differentiation by culturing 3×10^5/mL precursors in Petri culture dishes with IMDM medium supplemented with 10% FCS, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 1% penicillin–streptomycin (IMDM complete) and mGM-CSF 20 ng/mL. Every 3 days cells were supplemented with IMDM complete containing fresh mGM-CSF. Differentiation of BMDC was assessed by measurement of CD11c and I-A^b surface expression by flow cytometry. Cells were stimulated for 18 h with 100ng/mL of LPS in presence of different concentrations of SCFAs. BMDC activation was monitored by changes in surface expression

of CD40, CD80 and CD86. Moreover, the production of IL-6 and IL-12p40 was evaluated as indicator of BMM and BMDC stimulation.

2.4. Mitochondrial activity

Epithelial mitochondrial activity was evaluated to analyse treatment-induced cytotoxicity by measuring the mitochondrial-dependent reduction of colourless 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co., USA) to a coloured purple formazan (Mosmann, 1983). Caco-$2_{CCL20\text{-}luc}$ cells were seeded in 48-well plates and after reaching confluence were exposed for 5 h to the treatments described below. Subsequently, culture medium was aspirated from the wells; the cells were washed with buffer PBS, and incubated with phenol red-free DMEM medium supplemented with MTT (0.5 mg/mL) for an additional 2.5 h at 37 °C. The measure of mitochondrial dehydrogenase activity was realized as described (Iraporda et al., 2014).

2.5. Analysis of gene expression

Stimulated cells were homogenized in RA1 lysis buffer (GE Healthcare, UK) to perform the extraction of total RNA using the illustraRNAspin Mini RNA Isolation Kit (GE Healthcare, UK). Reverse transcription was performed using random primers and MMLV-Reverse transcriptase (Invitrogen, USA). Real time PCR was performed following manufacturers protocol using the iCycler thermal cycler (BioRad, USA).

Primers for genes encoding CCL20, interleukin-8 (IL-8), intestinal trefoil factor (ITF), and human β-actin and relative difference calculation were previously described (Rumbo et al., 2004).

2.6. Pertussis toxin assay

Confluent Caco-$2_{CCL20\text{-}luc}$ cells cultured in 48-well plates were incubated16 h with *Bordetella pertussis* Toxin (PTX, 100 ng/mL) in DMEM, then the culture medium was replaced and treated 30 min with aqueous solutions of lactate and SCFAs (100 mM) in DMEM for 30 min prior stimulation with FliC (1 μg/mL). Luminescence was measured after 5 h as described above. Also cells were treated 30 min with cyclic AMP analogue (dbcAMP, 1 mM) and stimulation as well as luminescence was measured as described above.

2.7. cAMP determination

Caco-$2_{CCL20\text{-}luc}$ cells in 24-well tissue culture plates incubated 30 min with 500 μL of aqueous solutions of lactate and SCFAs (100 mM) in DMEM and then lysed with 500 μL distilled water. The cell debris were heated 5 min 90 °C for enzyme inactivation, centrifuged 5 min 5000 × *g*. The cAMP in the supernatant was measured by radioimmuno-assay as previously described (Del Punta et al., 1996). Concentration of cAMP was expressed in relation to that found in cells without treatment. Results were obtained from the average relative concentration of cAMP of at least three independent experiments.

2.8. Flow cytometry

After incubation with anti-CD16/CD32 Fc block antibodies (2.4G2), cells were stained during 30 min at 4 °C with specific antibodies: anti-mCD11c- PeCy7(N418), anti-mMHCII-eFluor 450(M5/114.5.2), anti-mCD40APC(1C10), anti-mCD80-PE(16-10A1) and anti-mCD86-AlexaFluor 700(GL-1)(Becton Dickinson, BioLegend and eBioscience). Sample acquisition was done on a BD FACSCanto™ II Flow Cytometer and data analyzed using FlowJo software (version 7.6, TreeStar, Inc.,USA). Cells $CD11c^+$ $MHCII^+$ were gated for analysis of co-stimulatory molecules surface expression.

2.9. Determination of cytokine production

IL-6 and IL-12p40 content in cell free supernatants collected after 18 h of stimulation of BMDC and BMM with medium alone as negative control, SFCA or LPS alone or combination of LPS and SCFAs at different doses were measured using ELISA kits (eBioscience, USA) following manufacturer's instructions.

2.10. Statistical analysis

The Student *t*-test was used for comparison between two groups, the differences were considered statistically significant for $p < 0.05$. All analyses were performed using the program Graph Pad Prism version 5.00 for Windows. For multiple comparisons ANOVA test was used. If heterogeneity of variance was detected by Bartlett test, this was reduced by logarithmic transformation of the data before analysis. After that, data were then subjected to one-way ANOVA followed by Dunnet test for multiple-range comparisons. A value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Short chain fatty acids and lactate downregulate innate activation of myeloid cells

Bone marrow-derived macrophages (BMM) were used to evaluate whether lactate and SCFAs regulate macrophage pro-inflammatory response (Fig. 1). In these experiments BMMs were incubated with lipopolysaccharide (LPS) and various concentrations of monocarboxylates including lactate, acetate, propionate or butyrate. Cells exposed with media, the monocarboxylates or LPS alone were used as controls. After 18 h, secretion of pro-inflammatory cytokines IL-6 and IL-12p40 was evaluated as surrogate indicator of activation. While basal levels of cytokines were observed upon propionate treatment, LPS strongly elicited the secretion of both cytokines. Furthermore, co-incubation with propionate inhibited LPS-induced production of IL6 and IL12p40 in a dose dependent manner (Fig. 1A). Similar experiments were performed using different doses of butyrate, acetate and lactate. In all cases, the treatment of BMM by monocarboxylates was devoid of any stimulatory or inhibitory effect when compared to mock conditions (Fig. 1B and C). In contrast, increasing doses of propionate, butyrate and acetate reduced significantly the production of IL-6 and IL-12p40 of LPS-treated BMM. Butyrate showed the most prominent effect, since the same% of reduction of cytokines secretion was maintained with a concentration of butyrate ten times lower than propionate or acetate, which modulate IL-12p40 production at 10 mM and 50 mM, respectively ($p < 0.01$–$p < 0.05$). Lactate also downregulated BMM activation since the secretion of IL-12p40 induced by LPS was significantly reduced with lactate concentrations ≥20 mM. Interestingly, the production of IL-6 was not altered by lactate (Fig. 1C).

The ability of lactate and SCFAs to regulate dendritic cells' response to LPS was next investigated by measuring expression of co-stimulatory functions using flow cytometry and production of cytokines by ELISA. Similarly to BMM, BMDC activation promoted by LPS was also inhibited by propionate. Thus, whereas LPS significantly upregulated surface expression of the co-stimulatory molecule CD40, propionate was able to reduce the expression in a dose dependent manner ($p < 0.01$), without affecting basal levels of expression. Furthermore, LPS-induced secretion of IL6 and IL12p40 was also modulated by increasing doses of propionate (Fig. 2A).

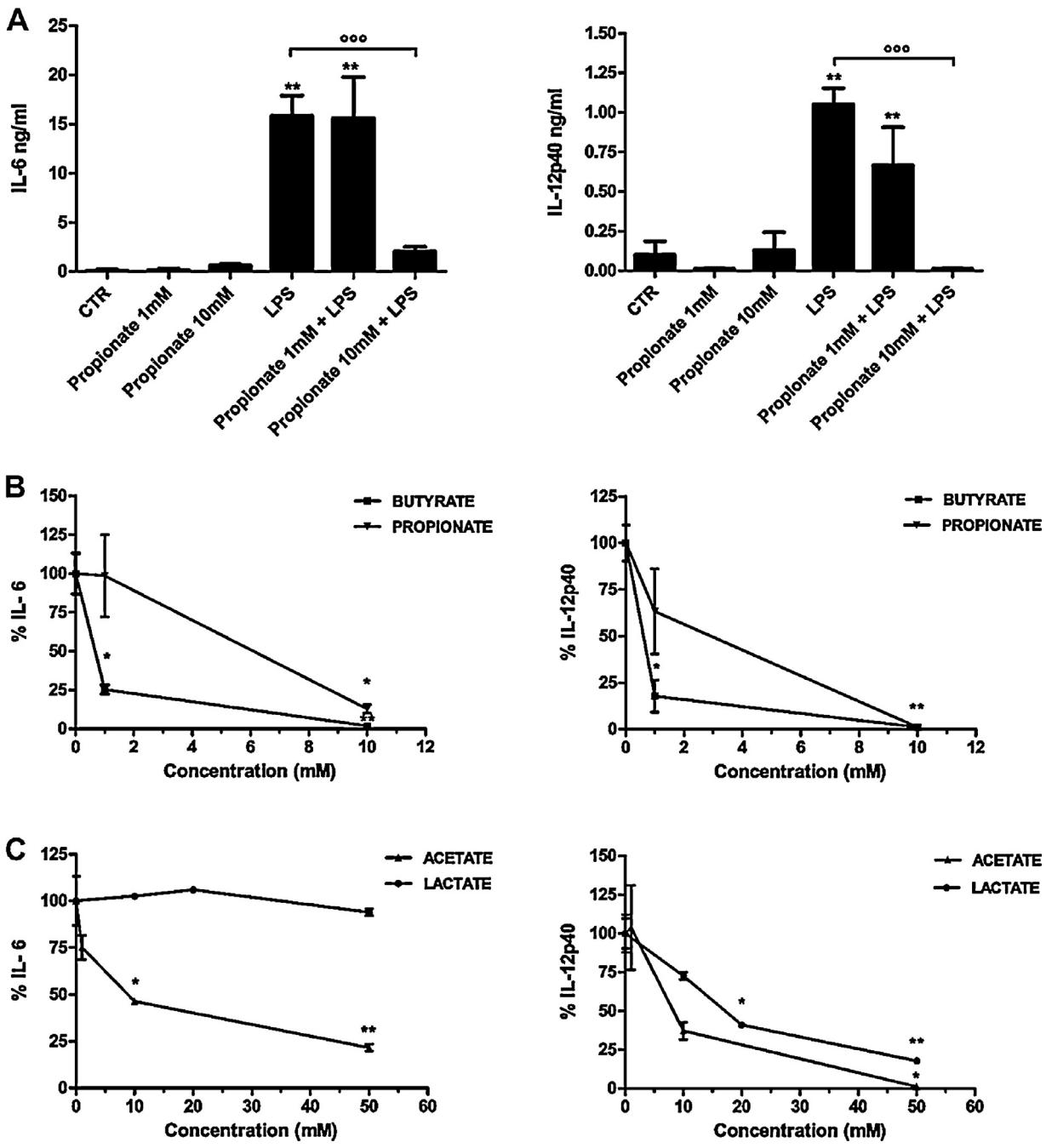

Fig. 1. Lactate and SCFA downregulate cytokine secretion of LPS-activated macrophages.
Bone marrow-derived macrophages (BMM) were stimulated for 16 h with LPS 100 ng/mL in presence of different concentration of organic acids. Production of IL-6 and IL-12p40 was evaluated by ELISA in the supernatants. A- Effect of propionate used at different concentrations on cytokine production. ** Indicates $p < 0.01$ when compared to untreated control; ◦◦◦ indicates $p < 0.005$ when compared to LPS treated condition. B- Using similar design as shown in A, effect of propionate and butyrate were evaluated in the 0–10 mM range. Results are expressed as percentage of cytokine produced, using LPS treated condition as reference of 100% of stimulation. * Indicates $p < 0.05$; ** indicates $p < 0.01$. C- Effect of lactate and acetate were evaluated in the 0–50 mM range. Results are expressed as percentage of cytokine produced, using LPS treated condition as reference of 100% of stimulation. * indicates $p < 0.05$; ** indicates $p < 0.01$. Similar results were observed in three independent experiments.

A similar approach was followed to study the effects of the other SCFA and lactate. The regulatory activity was evident at concentrations higher than 1 mM for butyrate and propionate whereas acetate and lactate required concentrations of 10 mM and 50 mM respectively (Fig. 2B and C). Cytokine secretion induced by LPS on BMDC was also modulated by SCFA but only in the case of propionate and butyrate. While propionate was the only SCFA decreasing secretion of both IL-6 and IL-12p40 butyrate affected IL-6 generation. Both metabolites showed effect at doses higher than 1 mM ($p < 0.01$) (Fig. 2B). Contrarily to the effect observed on BMDM, nor lactate nor acetate modulated cytokine secretion in BMDC at doses employed in this study (Fig. 2C). Similarly to BMDM, any of the metabolites assayed induced changes by themselves in cytokines secreted by BMDC giving values of negative control treatment (not shown).

Although several particular differences were observed related to the parameters analyzed, lactate and all the SCFA tested showed a dose–response capacity to modulate aspects of myeloid cells proinflammatory activation. Butyrate and propionate showed the highest modulatory activity.

3.2. Downregulation of the proinflammatory response of intestinal epithelial cells by SCFAs and lactate

To define whether the main sentinel cells of the gut, i.e. intestinal epithelial cells are impacted by SCFA, Caco-$2_{CCL20\text{-}luc}$ cells which, in response to stimulation with FliC as well as IL-1β manifest a high luciferase activity were used as model system. Pre-incubation of epithelial cells for 30 min with 50 mM of lactate, acetate, propionate or butyrate produced a significant reduction of luciferase response induced with Il-1β (Fig. 3A). The same inhibitory dose-dependent effect was evidenced when intestinal epithelial cells were stimulated with FliC (Fig. 3B). Propionate and butyrate reduced 50% of luciferase expression at concentration of 1 and 5 mM. Meanwhile lactate and acetate produced similar effects at concentrations above 10 mM. Importantly, treatments with lactate and SCFAs did not induce any cytotoxicity as measured by, the mitochondrial activity of treated cells (not shown).

Overall, in coincidence with was observed for myeloid cells, butyrate and propionate show higher capacity than lactate and acetate to modulate inflammatory activation of intestinal epithelial cells.

3.3. Activity of SCFAs and lactate on epithelial cells operates upstream of transcription and is independent of G_i protein

The effect of monocarboxylates on the transcription of pro-inflammatory genes in epithelial cells was next studied using real-time quantitative PCR (Fig. 4). As expected, the transcription of the genes coding for the chemokines CCL20 and IL-8 was significantly upregulated >100-fold when stimulated by flagellin. Standalone incubation with lactate and SCFAs pre-treatments did not affect transcription with regards to mock cells. Exposures to lactate, acetate, propionate or butyrate abrogated the flagellin-mediated induction of pro-inflammatory gene expression (Fig. 4A and B). Remarkably, all treatments had no effect on the transcription of the enterocyte -specific genes TFF3 coding for intestinal trefoil factor 3 (Fig. 4C), thus indicating a specific inhibition of proinflammatory pathways by monocarboxylic short chain organic acids.

In order to determine the contribution of inhibitory G-protein in the effects triggered by SCFAs exposure, Caco-$2_{CCL20\text{-}luc}$ cells were pretreated with *Bordetella pertussis* toxin. Whatever the conditions, the toxin was not able to modulate the SCFA-dependent

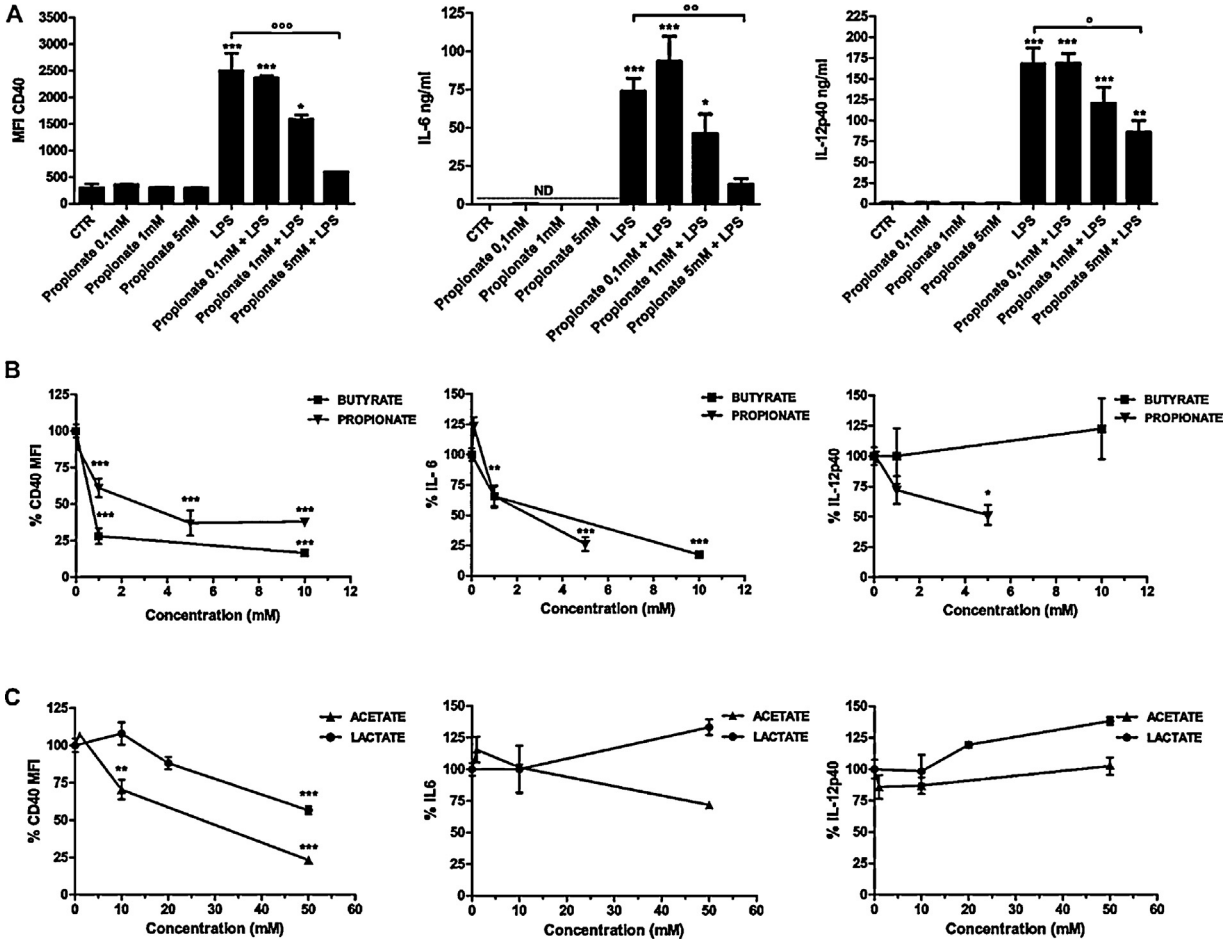

Fig. 2. Lactate and SCFA modulate cell surface markers and cytokine secretion of LPS-activated dendritic cells.
Bone marrow-derived dendritic cells (BMDC) were obtained after 9 days of culture *in vitro* and stimulated with LPS 100 nM in presence of different concentration of organic acids. Surface expression of CD40 was assessed by flow cytometry and generation of IL6 and IL12p40 was evaluated by ELISA in the supernatants upon 16 h post-stimulation. A- Effect of propionate used at different concentrations on mean fluorescence intensity (MFI) of detection of CD40 or cytokine production. *** Indicates $p < 0.005$, ** indicates $p < 0.01$ and * indicates $p < 0.05$ when compared to untreated control; °°° indicates $p < 0.005$, °° indicates $p < 0.01$ and ° indicates $p < 0.05$ when compared to LPS treated condition. B- Using similar design as shown in A, effect of propionate and butyrate were evaluated in the 0–10 mM range. Results are expressed as percentage of cytokine produced (or percentage of MFI when applicable), using LPS treated condition as reference of 100% of stimulation. * Indicates $p < 0.05$; ** indicates $p < 0.01$ and *** indicates $p < 0.005$. C- Effect of lactate and acetate were evaluated in the 0–50 mM range. Results are expressed as percentage of cytokine produced (or percentage of MFI when applicable), using LPS treated condition as reference of 100% of stimulation. * Indicates $p < 0.05$; ** indicates $p < 0.01$ and *** indicates $p < 0.005$. Similar results were observed in three independent experiments.

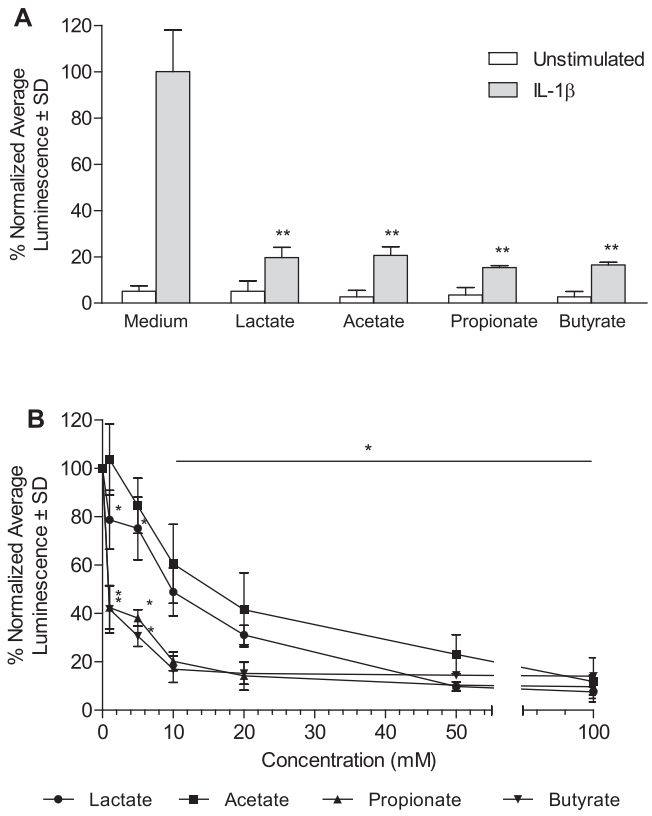

Fig. 3. Pretreatment with lactate and SCFAs downregulates the luciferase activity induced by stimulation with human IL-1β and FliC in intestinal-epithelial cells.
A- Percentage normalized average luminescence of control Caco-$2_{CCL20\text{-}luc}$ cells and preincubated 30 min with 50 mM lactate, acetate, propionate or butyrate unstimulated (□) or stimulated with IL-1β (■). Statistically significative differences with control cells stimulated with IL-1β obtained by Student t test are indicated as $^{**}p$-value < 0.01.
B- Percentage normalized average luminescence of Caco-$2_{CCL20\text{-}luc}$ cells preincubated 30 min with different concentrations (mM) of lactate (●), acetate (■), propionate (▲) or butyrate (▼). Statistically significative differences with control cells stimulated with FliC obtained by ANOVA followed by Dunnet test are indicated as * for p-value < 0.05.

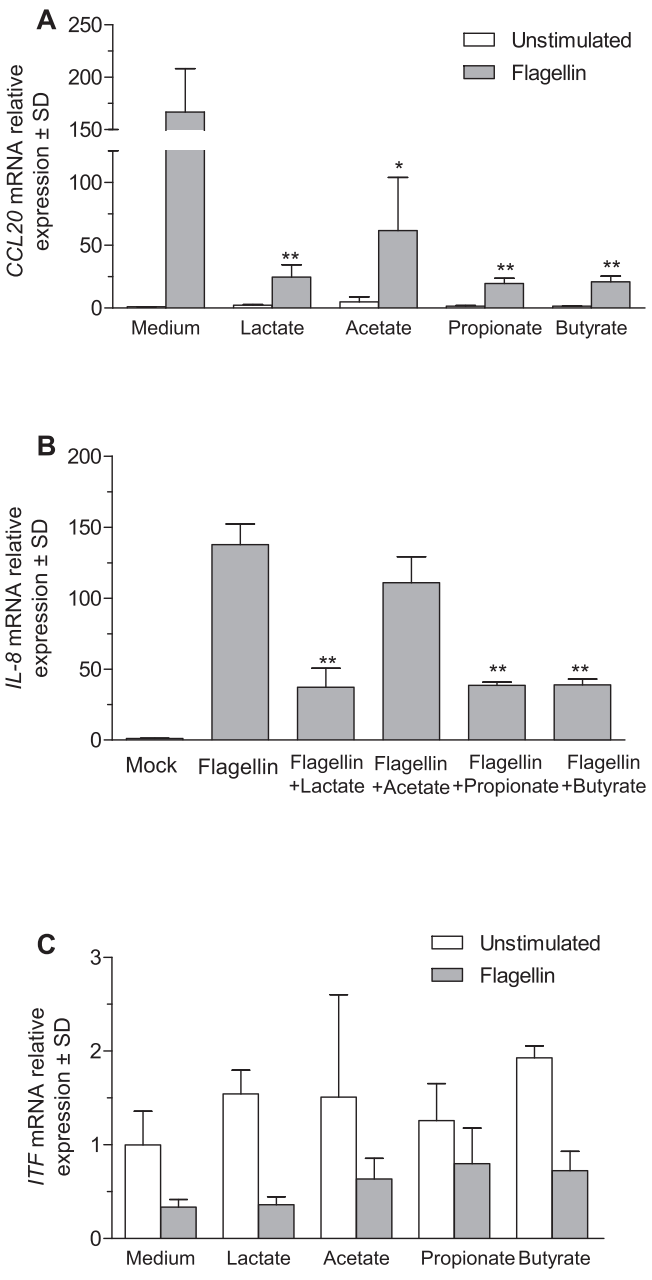

Fig. 4. Lactate and SCFAs modulates the expression of pro-inflammatory cytokines without affecting normal enterocyte functions.
mRNA relative expression of pro-inflammatory chemokines/cytokines genes: A- CCL20, B- IL-8 and C- ITF of Caco-2 cells in basal condition (mock), pre-incubated with lactate and SCFAs solutions 100 mM pH 7, non-treated and stimulated with flagellin. Statistically significative differences with control cells stimulated with flagellin obtained by Student t test are indicated as * for p-value < 0.05, $^{**}p$-value < 0.01.

inhibition of expression of luciferase activity (Fig. 5A). Besides, flagellin-specific activation of Caco-$2_{CCL20\text{-}luc}$ cells in presence of dbcAMP, an analogue of cAMP, did not produce any changes in luciferase activity, thereby indicating that cAMP signalling pathway does not mediate the modulatory activity (not shown). Nevertheless, slight changes in cellular cAMP levels were observed when intestinal epithelial cells were treated with acetate ($p = 0.048$) or butyrate ($p = 0.010$), whereas lactate and propionate did not produce significant modifications (Fig. 5B). Altogether, these results suggested that lactate and SCFAs do not regulate pro-inflammatory signalling using G-inhibitory subunit of GPRs.

4. Discussion

This study demonstrates that four monocarboxylic short chain organic acids: lactate, acetate, propionate and butyrate are potent inhibitors of TLR-mediated pro-inflammatory responses in epithelial and myeloid cells. In a molar ratio, the relative effects of butyrate and propionate tend to be stronger than effects elicited by lactate and acetate.

Butyrate is a well-characterized SCFA that was shown to downregulate innate responses in various biological systems. Using PBMC stimulation with LPS, Kovarik et al. (Kovarik et al., 2011) showed modulatory effects on cytokine secretion. Modulatory effects of butyrate 1 mM on surface marker modulation on either human or mouse dendritic cells were also reported (Berndt et al., 2012; Liu et al., 2012). This work evidenced changes in CD40 expression levels upon BMDC activation, whereas changes in CD80 and CD86 were not detected (Figs. 1 and 2). Modulation on IL-6 secretion was observed at lower doses than modulation on IL-12 secretion for most organic acids tested. Differences in cytokine modulation in DCs are also reported in literature, since several works indicate different extent of modulation of IL-6, TNF-α and IL-12 (Kovarik et al., 2013; Saemann et al., 2002; Wang et al., 2008).This may be due to differences in the DC derivation and activation protocols used.

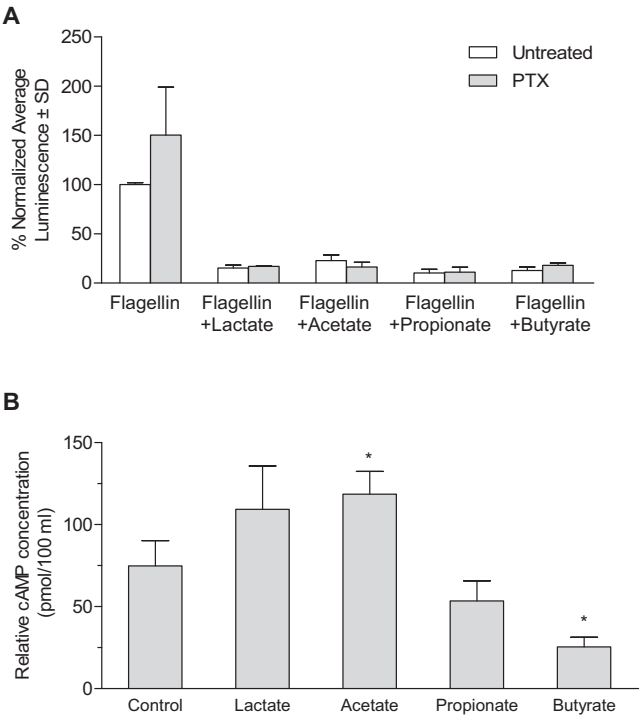

Fig. 5. Lactate and SCFAs inhibition of luciferase activity in intestinal epithelial cells is independent of heterotrimeric G-inhibitory protein.
A- Percentage normalized average luminescence of control Caco-$2_{CCL20\text{-}luc}$ cells untreated (□) and treated with PTX 16 h (■), incubated 30 min with 50 mM lactate, acetate, propionate or butyrate and stimulated with flagellin.
B- Relative concentration of cAMP (pg/100 μl of lysate) found in lysates of intestinal-epithelial cells incubated 30 min with 50 mM of lactate, acetate, propionate or butyrate. Results are expressed as relative to concentration found in controls cells without treatment. Statistically significative differences with control cells obtained by Student *t*- test are indicated as * for *p*-value < 0.05.

Beyond the effects of butyrate on DC activation, we extended this analysis to other SCFAs and lactate, which are less studied. We could observe that propionate elicits comparable modulatory effects to butyrate on the TLR4-mediated activation of BMDCs (Fig. 2). Although there is evidence that propionate may influence DC biology (Schulz et al., 2014; Trompette et al., 2014), direct effects of this SCFA on DC activation were not described so far.

Studies on the effect of these metabolites on other cell types are also rare. Cavaglieri et al. (Cavaglieri et al., 2003) have shown that acetate and propionate have weaker effects than butyrate on lymphocyte activation; the capacity to modulate endothelial cell proinflammatory activation has also been described (Zapolska-Downar and Naruszewicz, 2009). There are also few reports comparing the effects of different SCFAs on intestinal epithelial cells proinflammatory activation (Tedelind et al., 2007) that have shown similar results than reported here (Fig. 3) indicating higher modulatory capacity of propionate than acetate on epithelial cell activation, supporting its possible role as modulatory agent in proinflammatory conditions.

We have recently reported the modulatory activity of lactate on epithelial cell proinflammatory activation (Iraporda et al., 2014), being the first report indicating this capacity. We extend here this observation on cells of myeloid origin. Our results are coincident with previous evidence that lactate may modulate macrophage and monocyte activation at doses ranging 10–20 mM depending on acidity (Dietl et al., 2010; Goetze et al., 2011; Hoque et al., 2014; Yabu et al., 2011); however, information on the influence of lactate on DC activation is very scarce.

Dose response studies performed here showed that lactate and SCFAs can modulate proinflammatory activation of relevant sentinel cell types at concentrations that are expected to find along specific parts of the gastrointestinal tract. Most SCFAs are locally produced in large intestine by specific bacterial species. Butyrate formation occurs in certain Firmicutes bacteria, acetate is produced by most anaerobes. Producers of succinate and propionate largely belong to the phylum Bacteroidetes, but also include some Firmicutes. Lactate can be formed by many bacterial groups, as well as being also a metabolic product of the host cells. However, high levels of lactate similarly to used here are normally found in intestinal microenvironment. Luminal lactate is generally converted into acetate, propionate or butyrate by a subset of lactate-utilizing species (Flint et al., 2012). The net outcome of all of these complex cross-feeding interactions for a typical healthy microbiota is that, in faecal samples, acetate is the dominant SCFAs detected (typically 40–70 mM) followed by propionate and butyrate (each ranging between 10 and 30 mM) (Cummings, 1998). Moreover, SCFAs concentration in the colonic lumen is in the range of 70–130 mM or up to 200 mM (Maslowski and Mackay, 2011; Sengupta et al., 2006), with molar ratios of acetate:propionate:butyrate 60:20:20, depending on the diet (Tedelind et al., 2007; Bergman, 1990). Approximately 80–90% of SCFAs are absorbed from the colonic lumen and partly metabolized by colonic epithelial cells, a proportion also enters the portal and peripheral circulation and the rest are excreted in faeces (Meijer et al., 2010). This indicates that physiological concentrations of SCFAs in different compartments may be in a range compatible with modulation of epithelial or myeloid cell activation. This also opens the possibility of using diverse strategies to stimulate local production of specific fermentation products with the desired modulatory profile, as shown by different studies that use prebiotic to modulate microbial metabolic profile (Delzenne et al., 2011; O'Flaherty et al., 2010).

There are different mechanisms that may explain the modulatory capacity of lactate and SCFA on epithelial and myeloid cells described here. GPR are essential mediators of SCFA modulation on immune cells. It has been shown that GPR43 mediates the protective effect of acetate on IBD mouse models (Maslowski et al., 2009; Masui et al., 2013). GPR109a contributes to butyrate modulatory activity on epithelial and dendritic cells (Chang et al., 2014; Thangaraju et al., 2009) and GPR41 and propionate in modulating DC biology (Trompette et al., 2014); however, since there is some promiscuity in ligand-receptor interaction, it is difficult to dissect the exact contribution of each ligand-receptor pair. GPR81, which form a GPR subfamily with GPR109a and GPR109b, has been associated to hydroxy-carboxylic acid ligands which are intermediates of energy metabolism such as lactate (Ahmed et al., 2009) and administration of lactate reduced inflammation and organ injury in mice with immune hepatitis in a GPR81 dependent fashion (Hoque et al., 2014). Different reports indicate that GPR dependent anti-inflammatory effects of SCFAs and lactate on macrophages are independent on G_i proteins and dependent on β-arrestin2 mediated signaling (Hoque et al., 2014; Liu et al., 2014). Our results showing that modulatory activity of SCFAs and lactate are not inhibited by pertussis toxin treatment and differently affect cAMP intracellular levels (Fig. 5) are consistent with these observations and indicate that modulatory effects are not related to G_i signaling.

Beyond the signaling capacity through GPRs, SCFAs and lactate may also modulate histone deacetylase activity and several modulatory effects on macrophages and epithelial cells were also associated with this capacity (Chang et al., 2014; Latham et al., 2012; Schilderink et al., 2013). Furthermore, high concentrations of lactate in extracellular milieu have also effects on modulation of cell metabolism that can contribute to modulation of proinflammatory mediator production (Dietl et al., 2010), we cannot exclude that some of the effects observed here are related to these latter mechanisms.

5. Conclusion

This study established a dose-dependent capacity of butyrate, propionate, acetate and lactate to modulate pro-inflammatory activation of epithelial and myeloid cells. In any conditions, butyrate and propionate were shown to be effective at lower concentrations than acetate and lactate. These molecules in combination or separately may be used as intervention strategy in gut inflammatory diseases where epithelial and myeloid cells are the main triggers of inflammation, thereby restoring gastrointestinal tract homeostasis.

Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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