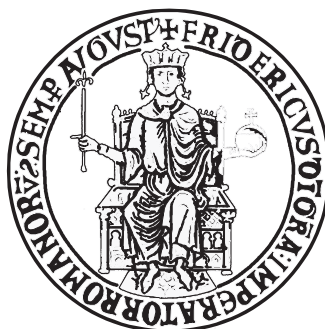


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Ph. D. thesis

**PHARMACOLOGICAL AND THERAPEUTIC EFFECTS OF
SHORT-CHAIN FATTY ACIDS IN GASTROINTESTINAL
AND EXTRA-INTESTINAL DISORDERS:
EVALUATION OF METABOLIC, HORMONAL AND
INFLAMMATORY PARAMETERS**

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ABSTRACT

The short chain fatty acid (SCFA) butyrate, a main end product of microbial fermentation of dietary fibers in human intestine, plays an important role in the maintenance of intestinal homeostasis and overall health status. The effects exerted by butyrate are multiple and involve several distinct mechanisms of action including epigenetic modifications owing to its inhibitory effects on histone deacetylases, inhibition of NF- κ B signaling, or direct agonism on the free fatty acid receptors.

At intestinal level, butyrate is the major energy source for colonocytes and acts regulating epithelial cell proliferation, defense barrier, visceral sensitivity and motility, preventing and inhibiting colonic carcinogenesis. Recent experimental evidence has suggested potential extra-intestinal therapeutic applications of butyrate, including the treatment of systemic diseases, among these not only metabolic and inflammatory disorders but also cystic fibrosis, urea cycle enzyme deficiency, X-linked adrenoleukodystrophy.

Data from literature and clinical evidence of several research groups show a wide spectrum of possibilities for potential therapeutic use of butyrate by oral administration without having serious adverse effects. Some butyrate-based products are marketed, but their spread is still very limited and greatly understaffed in view of the wide spectrum of possible indications, especially in chronic diseases, where it is possible to predict a lasting use of the compound. The main problem is the availability of butyrate formulations that could overcome the main limitations to the use of butyrate in the therapeutic field, namely its instability and poor palatability. In fact, the unpleasant taste and odour make oral administration of butyrate extremely difficult, especially in children.

On the basis of its characteristics, butyrate can be considered a “postbiotic” being a non-viable bacterial metabolic product obtained from probiotic microorganisms that have biologic activity in the host. The direct use of postbiotics, such as butyrate, may be potential alternatives to the use of live probiotic organisms or to dietary fiber intake as prebiotics exerting several and similar beneficial regulatory effects on host biological functions. Even if a growing number of studies has revealed new mechanisms and effects of butyrate with a wide range of potential clinical applications from the intestinal tract to peripheral tissues, more data are needed to elucidate the efficacy of butyrate in gastrointestinal and extra-intestinal diseases and new solutions for an easier administration.

To date, several studies have evaluated butyrate effectiveness in several animal models of colitis. In humans few studies have been performed probably due to low compliance for the oral route (for its rancid taste) or rectal enemas administration (for its cumbersome application to the patient and irritability due to acid property).

Here, about its intestinal effects, we examined the efficacy of oral butyrate and its derivative N-(1-carbamoyl-2-phenyl-ethyl) butyramide (FBA), as preventive or therapeutic treatment in a murine model of DSS-induced colitis. Both compounds are able to recover the imbalance between pro-inflammatory and anti-inflammatory mediators, altered in colitis and restore gut permeability, avoiding bacterial translocation and modulating immune cell recruitment. Butyrate anti-inflammatory effects are associated not only to the reduction in neutrophil infiltration and HDAC9 transcription in colonic mucosa, but also the restoration of PPAR- γ expression and inhibition of NF- κ B activation, protecting colonocytes from inflammation.

Alterations in the microbiota have now been implicated in the pathogenesis of some diseases, including food allergy. Intestinal microbiota influences immune system network and impairs its regulatory functions. Allergic infants exhibit an accelerated evolution of microbiota more typical of the adult one with a significantly reduced abundance of butyrate-producing species, such as Lactobacillales and Bifidobacteriales, and increased abundance of Clostridiales. Animal models of food allergy have emerged as tool for identifying mechanisms involved in the development of sensitization to normally harmless food allergens, as well as delineating the critical immune components of the effect or phase of allergic reactions to food. One of the critical advantages of using mouse models to study food allergy is that allergic sensitization or tolerance can be induced to specific allergens under controlled environmental conditions within defined genetic backgrounds, which is not possible in human subjects. This aspect of mouse models allows extensive and precise investigations into the mechanisms involved in disease etiology and responsible for loss of tolerance in patients, identifying new targets and efficacious therapies. Increasing evidence from several mouse models indicates that alterations in regulatory T (Treg) cell function and environmental factors, such as microbiota, are likely important contributors to allergic sensitization and food allergy. Here, we used a model of cow's milk allergy (CMA) induced in mice.

In this experimental model of CMA the preventive and therapeutic effects of oral sodium butyrate administration were evaluated. Butyrate was able to suppress acute skin

response to the β -lactoglobulin (BLG) allergen, one of the most important cow's milk proteins, and to reduce anaphylactic symptoms and immediate immune response. Furthermore, butyrate ability to improve gut permeability was also shown, reducing plasma levels of FITC-Dextran evaluated after oral administration. Butyrate reduced ear swelling, hypersensitivity symptoms and limited the decrease of body temperature after BLG challenge. Moreover, butyrate decreased the innate immune response, reducing IgE and IL-4 levels.

About butyrate extra-intestinal effect, our study was focused on osteoarthritis (OA), the most common form of arthritis worldwide, whose development is increased by aging, obesity and biomechanical injury. It is a cartilage degenerative disease where chondrocytes play a central role. In fact, in OA chondrocyte phenotype changes and apoptosis and extracellular matrix degradation occur. Using an *in vitro* model, we demonstrated the effect of sodium butyrate in reducing inflammatory mediators and pathways in chondrocytes activated by IL-1, and, more interestingly, we showed that its chemoattractant activity is mediated by GPR43. Butyrate, not only reduced pro-inflammatory cytokines and adipokines involved in OA, but also decreased the expression of several adhesion molecules, inhibiting inflammatory and anti-apoptotic pathways. We also show the butyrate capability to reduce MMPs production and the loss of collagen type 2, suggesting an improvement of cartilage disruption. Interestingly, butyrate anti-inflammatory effects were associated to its capability to stimulate neutrophil recruitment, increasing the expression of important chemokines (Ccl3 and Cx3cl1) and anti-inflammatory protein AnxA1, suggesting butyrate pro-resolving activity during inflammatory response. The novelty of our data is the involvement of GPR43 in the chemoattractant activity of butyrate in IL-1-stimulated chondrocytes. In fact, butyrate failed to induce the expression of Cx3cl1 and AnxA1 in GPR43-silenced cells, missing its chemoattractant effect.

The pro-resolving effect of butyrate was also analyzed in two models of wound healing induced by doxorubicin or mechanical damage. In tissue repair the immediate goal is to achieve tissue integrity, homeostasis and wound healing. Tissue injury causes the immediate onset of acute inflammation. The healing process involves three phases that overlap in time and space: inflammation, tissue formation, and tissue remodeling. Synthesis, remodeling, and deposition of structural extracellular matrix molecules and soluble mediators, are indispensable for initiating repair and progression into the healing state. In our study, we demonstrated that butyrate and FBA treatments accelerate and

promote wound resolution process. In our experimental models, we showed that butyrate and its derivative FBA reduced the repair time when used systemically (by oral administration) or topically (by intradermal injection). This protective effect appeared to be time- and concentration-dependent.

In summary, we have demonstrated the multiple protective effect of butyrate in limiting molecular events underlying the onset of several inflammatory-based pathologies, suggesting a potential clinical relevance for this compound. In particular, we have also showed the efficacy of its synthetic derivative N-(1-carbamoyl-2-phenyl-ethyl) butyramide or FBA, demonstrating that it could represent an alternative therapeutic option to sodium butyrate, sharing a comparable efficacy, but better palatability and compliance.

CHAPTER 1

1. INTRODUCTION

SCFAs are organic fatty acids with 1 to 6 carbon atoms and are the principal anions which arise from bacterial fermentation of polysaccharide, oligosaccharide, protein, peptide, and glycoprotein precursors in the colon [1]. Fermentation involves a variety of reactions and metabolic processes in the anaerobic microbial breakdown of organic matter, yielding metabolisable energy for microbial growth and maintenance and other metabolic end products for use by the host. The main end products are SCFAs together with gases (CO₂, CH₄, and H₂) and heat [2]. Various data show that fecal SCFA production is in the order of acetate > propionate > butyrate in a molar ratio of approximately 60:20:20, respectively. The ratio seems to remain fairly constant, although alterations in production and absorption may occur with dietary changes.

Carbohydrates are fermented by saccharolytic bacteria primarily in the proximal colon producing linear SCFAs, H₂, and CO₂ [3], and both the presence of carbohydrates in the colon and their fermentation can alter colonic physiology. Fermentation of proteins and amino acids by proteolytic bacteria yield branched SCFAs, H₂, CO₂, CH₄, phenols, and amines. The primary effects of SCFAs are on colonic function as a result of their uptake and metabolism by colonocytes, although SCFAs are also metabolic substrates for other cells and tissues of the host.

The production of SCFAs are determined by a number of factors, including the numbers and types of microbiota present in the colon, substrate source [4], and intestinal transit time [4]. A large microbiota population is present in the human colon at 10¹⁰ to 10¹¹ cfu/g wet weight [5], and more than 50 genera and over 400 species of bacteria have been identified in human feces [6]. Bacterial number, fermentation, and proliferation are highest in the proximal colon where substrate availability is greatest [7]. The principal site of colonic fermentation, therefore, is the cecum and proximal colon, whereas the distal colon is carbohydrate and water depleted. The total amount of SCFAs in the proximal colon is estimated to range from 70 to 140 mM [2, 4] and fall to 20 to 70 mM in the distal colon [2]. Therefore, the pH is lowest in the proximal colon and increases distally. Samples at various sites taken from patients with colonostomies indicate a decline in SCFA levels along the colon [2]. Specific species such as Bifidobacteria and Lactobacilli have been associated with improved health, resulting in the current use of probiotics, the delivery of

specific bacteria to the colon and prebiotics utility, or the administration of dietary components that promote the growth of specific bacteria with defined metabolic functions. The production of SCFAs are also determined by the substrate source where dietary intake is the most important variable. Non-digestible food components are a source of substrate for fermentation by anerobic colonic microbiota, because they are resistant to hydrolysis and digestion in the stomach and small intestine and eventually enter the colon for fermentation. Neither total SCFA nor the individual acids in the distal colon are predictive of those found proximally [8, 9]. SCFA availability in the distal colon is dynamic as water absorption and loss of digestive material alter availability of SCFAs independent of rates of production. Total SCFA and regional differences in SCFA concentration are implicated in diseases of the colon, especially in cancer and gastrointestinal disorders, where disease often occurs distally. Therefore, increased SCFA production and a greater delivery of SCFA distally, especially butyrate, may have a role in preventing these diseases.

SCFAs are produced from the fermentation of carbohydrates with the major source coming from resistant starches [10]. However, dietary fiber, unabsorbed sugars, raffinose, stachyose, polydextrose, and modified cellulose also represent significant sources of fermentable substrates in the colon [10]. It is estimated that 5% to 20% of dietary starch is not absorbed by the human small intestine [11]. Insoluble fibers (eg, lignins, cellulose, and some hemicelluloses), which are resistant to fermentation by colonic microbiota, play an important role in fecal bulking and may carry with them fermentable carbohydrate substrate, including starches and sugars [12]. Soluble fibers (eg, pectins, gums, mucilages, and some hemicelluloses) are more completely fermented by colonic bacteria and may have little effect in increasing fecal bulk. When transit and laxation are unchanged, a greater intake of fermentable carbohydrates will result in higher SCFA production because of increased substrate availability [13, 14]. Concentration and excretion of SCFAs have been shown to be greater with feeding of some non-starch polysaccharides, such as partially hydrolyzed guar gum but not others such as oat bran [14]. Therefore, there are several factors that may affect substrate fermentability that complicate their use in human studies, coupled along with a limited ability to directly measure SCFA at specific sites in the colon. Polyfructans are fermented by colonic bacteria [15] specifically the *Bifidobacterium* species [15], which have been shown to be associated with serum low density lipoprotein- cholesterol (LDL-C) reduction [16]. Furthermore, fermentation end products of polyfructans, specifically the SCFA propionate, have been shown to decrease

the acetate:propionate ratio when compared with lactulose [17] and to reduce serum cholesterol levels. Hence, there is a potential use for polyfructans (i.e, inulin and oligofructose) in combination with viscous fibers (i.e, oat bran) to lower serum cholesterol. Such combination effects on colonic bacteria may be used to achieve a range of therapeutic and preventive effects. Various sources of resistant starches [13, 14] and acarbose, the α -glycoside hydrolase inhibitor [18, 19] also raise fecal SCFAs. The SCFA increase in these studies have either been reported as higher concentrations, excretion or both, which may reflect changes in production, absorption, and intestinal transit time.

Changes in SCFA may not be observed when significant lower amount of non-digestible oligosaccharides are given, since it would be rapidly fermented and immediately absorbed in the more proximal colon [2]. Production of individual SCFAs have also been measured in various studies. Greater fecal excretion of butyrate and propionate have been observed with consumption of wheat bran compared with vegetable fiber [20]. However, feeding of partially hydrolyzed guar gum resulted in greater fecal excretion of all 3 major SCFAs, but did not change the concentration of propionate and butyrate, or decrease their relative contribution. Studies using resistant starch have been consistent in showing raised fecal butyrate [12, 14]. Starch fermentation primarily produces acetate and butyrate, whereas fermentation of pectin and xylan yields acetate alone as the main product. Recent human studies found that acute ingestion of a non-digestible monosaccharide, L-rhamnose (25 g), increased serum propionate without increasing acetate [21], and this effect did not diminish after 28 days [22].

Absorption of SCFAs in the cecum and the colon is a very efficient process with only 5% to 10% excretion in the feces [23]. Two mechanisms of absorption are proposed: (1) diffusion of protonated SCFAs and (2) anion exchange [4]. SCFAs are rapidly absorbed in the colon, and this effect is associated with enhanced sodium absorption and bicarbonate excretion [4]. Intubation studies have shown that SCFAs are taken up from the perfused human large bowel in a concentration-dependent manner [24]. At least 60% of that uptake is by simple diffusion of protonated SCFAs involving hydration of luminal CO₂, whereas the residue occurs by cellular uptake of ionized SCFAs involving transport of Na⁺ and K⁺ [25]. SCFA uptake is associated with transport of water that seems to be greater in the distal than in the proximal colon [26].

Human peripheral venous blood concentrations of SCFAs are normally low, and only acetate is present in significant amounts [2]. However, Wolever et al., [27] measured

serum propionate and butyrate, and reported values of 4.5 to 6.6 mmol/L and 2.2 to 3.9 mmol/L, respectively.

The major SCFAs: acetate, propionate, and butyrate are absorbed at comparable rates in different regions of the colon [24]. Once absorbed, SCFAs are metabolized at 3 major sites in the body: (1) cells of the ceco-colonic epithelium that use butyrate as a major substrate for maintenance-energy producing pathways; (2) liver cells that metabolize residual butyrate with propionate used for gluconeogenesis and 50% to 70% of acetate also taken up by the liver; (3) muscle cells that generate energy from the oxidation of residual acetate. Their oxidation supplies some 60% to 70% of the energy needs of isolated colonocytes [28], reduces glucose oxidation [29], and spares pyruvate [30] and glutamine [31]. In the presence of competing substrates such as glucose and glutamine, butyrate is the preferred intestinal fuel [32] suggesting that a hierarchy of oxidation exists with butyrate apparently being oxidized more in the proximal than in the distal colon [2].

The role of SCFAs has expanded to include their role as nutrients for the colonic epithelium, as modulators of colonic and intracellular pH, cell volume, and other functions associated with ion transport, and as regulators of proliferation, differentiation, and gene expression [4]. Increases in SCFAs result in decreased pH, which indirectly influences the composition of the colonic microbiota (i.e., it reduces potentially pathogenic Clostridia when pH is more acidic), decreases solubility of bile acids, increases absorption of minerals, and reduces the ammonia absorption by the protonic dissociation of ammonia and other amines (i.e., the formation of the less diffusible NH_4^+ compared with the diffusible NH_3) [33]. In **figure 1.1** the structural form of main SCFAs are shown.

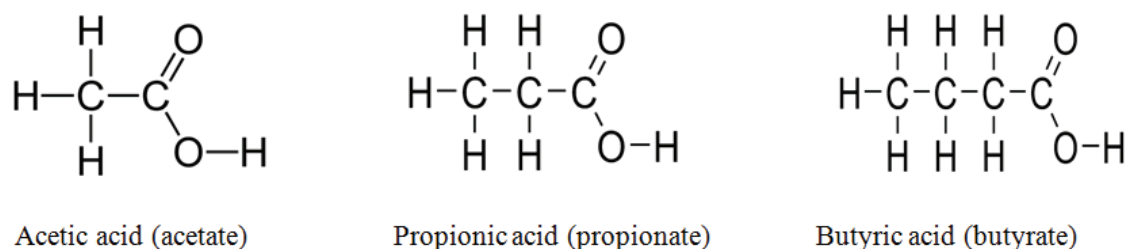


Figure 1.1: The chemical structures of main SCFAs.

Acetate, the principal SCFA in the colon, is readily absorbed and transported to the liver, and therefore is less metabolized in the colon [4]. The presence of acetyl-CoA synthetase in the cytosol of adipose and mammary glands allow the use of acetate for

lipogenesis once it enters the systemic circulation. In human studies, acetate is often used to monitor colonic events because it is the main SCFA in the blood. Acetate is the primary substrate for cholesterol synthesis. Subjects given rectal infusions of acetate and propionate showed a dose-dependent increase in serum total cholesterol and triglyceride levels, providing indirect evidence that these SCFAs are utilized for lipid synthesis [34]. However, the methodology used in this study may have resulted in non-physiologic levels of acetyl-CoA from the rapid uptake of acetate. This may have diverted SCFA to lipid synthesis rather than oxidation [35]. It is possible that substrate-dependent SCFA produced by fermentation inhibits cholesterol synthesis [36]. However, uniform agreement has not been reached on the effect of increased colonic fermentation on lipid metabolism, because the possibility exists that different substrates may produce different effects [34]. However, results from human studies have been inconsistent. One-week intakes of 2.7 g sodium propionate taken as a capsule [37] did not affect serum lipids. Only one study showed that 5.4 g of propionate given daily for 2 weeks lowered LDL-C and total cholesterol in subjects with total cholesterol > 5.5 mmol/L [18]. Studies using rectal infusions indicated that 180 mmol of propionate did not affect serum lipids or triglycerides in healthy young men and women. However, when 60 mmol of propionate was infused with 180 mmol of acetate, free fatty acids decreased by an additional 10% and acetate, when given alone, did not increase total and LDL-Cholesterol [38]. Therefore, it still seems possible that one of the determinants of the actions of propionate on serum lipids is the ratio of propionate to acetate [39, 40].

Propionate is produced via 2 main pathways: (1) fixation of CO₂ to form succinate, which is subsequently decarboxylated (the “dicarboxylic acid pathway”); (2) from lactate and acrylate (the “acrylate pathway”). Propionate is a substrate for hepatic gluconeogenesis and has been reported to inhibit cholesterol synthesis in hepatic tissue [37]. However, propionate seems to have two competing and opposite effects on gluconeogenesis. It is both a substrate for gluconeogenesis and an inhibitor of gluconeogenesis. Propionate enters in the Krebs cycle at level of succinyl CoA. The inhibiting effect of propionate on gluconeogenesis may be related to its metabolic intermediaries, methylmalonyl CoA and succinyl CoA, which are specific inhibitors of pyruvate carboxylase. Propionate enhances glycolysis, probably by depleting hepatic citrate, which is an important metabolic inhibitor of phosphofructokinase. Propionate may also influence hepatic glucose metabolism indirectly by lowering the plasma fatty acid

concentration, which is itself known to be closely related to the actual rate of gluconeogenesis [41].

The majority of our knowledge about the nutritional fate of propionate comes from studies in ruminants. However, propionate metabolism in humans is less well understood. In humans, propionate may also have systemic effects, specifically a hypolipidemic action. Observations in animals suggest that propionate inhibits cholesterol synthesis by inhibiting both 3-hydroxy-3-methylglutaryl- CoA synthase and 3-hydroxy-3-methylglutaryl-CoA reductase [42]. As previously mentioned, polyfructans are bifidogenic and decrease the acetate: propionate ratio, both of which are associated with reductions in serum lipids. The use of polyfructans (eg, Neosugar, inulin) in individuals with Type 2 diabetes mellitus (8 g/d) [43] and hyperlipidemia (18 g/d) [44] resulted in cholesterol reductions. However, no hypolipidemic effect (20 g/d) was observed in healthy subjects [17]. This inconsistency in human studies, in contrast to animal experiments, may be related to specie differences or pathological status. A number of mechanisms have been suggested to be responsible for the observed lipid lowering effect, with increased propionate production being one of the possible mechanisms of action. Increased production of propionate, through fermentation, may inhibit hepatic cholesterol synthesis [38, 39]. This has been supported in experimental studies with hyperlipidemic animals [36] but not supported in other animal studies [45].

Currently, there are limited human experimental data that have quantified the synthesis of acetate and propionate with use of prebiotics. Propionate is better absorbed in the human colon than acetate [46], and studies in ruminant mucosa show that propionate is activated to its coenzyme A derivative (a step required for its oxidation) to a greater extent than acetate. The liver extracts 90% of propionate, as opposed to 75% of acetate, during a single pass [28] and colon infusions of equal amounts of acetate and propionate suggest that the amount of colonic propionate reaching peripheral blood is only 25% of the amount of total colonic acetate.

2. BUTYRATE

Butyrate is the preferred fuel of the colonic epithelial cells, which plays a pivotal role in regulation of cell proliferation and differentiation [2]. It is the most important SCFA in colonocyte metabolism, since 70% to 90% of butyrate is metabolized by these cells [4]. Butyrate is used preferentially over propionate and acetate in a ratio of 90:30:50 [4], and is preferred over glucose or glutamine supplied by blood [47]. Butyrate oxidation has been shown to make up more than 70% of the oxygen consumed by human colonic tissue.

Sodium butyrate exerts an anti-proliferative activity on many cell types, and there are evidence from *in vivo* and *in vitro* studies, that have demonstrated preventive effects of butyrate on colon adenoma development and cancer [48]. Acetate and propionate have also been shown to induce apoptosis in colorectal tumor cell lines, but to a much lesser extent than butyrate [49]. Butyrate also stimulates immunogenicity of cancer cells [50]. Currently, the mechanisms of action of butyrate in relation to colon cancer are not clearly defined.

The drop in colonic pH caused by accumulation of SCFAs decrease the solubility of free bile acids, which may decrease the potential tumor promoter activity of secondary bile acids [51]. Furthermore, increased colonic acidification (pH below 6 to 6.5) may inhibit colonic bacterial enzyme 7 α -dehydroxylase, which degrades primary bile acids to secondary bile acids [52]. In addition, decreased colonic pH increases the availability of calcium for binding to free bile acids and fatty acids [53]. *In vitro* and *in vivo* studies have shown that butyrate is the preferred energy substrate and stimulates cell proliferation in normal colonocytes [32], yet it suppresses proliferation of colon adenocarcinoma cells.

This observed inconsistency has been termed the “butyrate paradox” [54]. This discrepancy may be explained by differences between *in vitro* and *in vivo* environments, the timing of butyrate administration in relation to the stage of cancer development, the amount of butyrate administered, the source of butyrate (i.e., different dietary fibers), and interaction with dietary fat [54].

The ratio of SCFA concentrations in the colonic lumen is about 60% acetate, 25% propionate, and 15% butyrate. As a result of increasing concentrations of acidic fermentation products, the luminal pH in the proximal colon is lower. This pH seems to boost the formation of butyrate, as mildly acidic pH values allow butyrate-producing bacteria to compete against Gram-negative carbohydrate-utilizing bacteria, such as

Bacteroides spp. [55]. The ability to produce butyrate is widely distributed among the Gram-positive anaerobic bacteria that inhabit the human colon (**Figure 2.1**). Butyrate-producing bacteria represent a functional group, rather than a coherent phylogenetic group. Numerically, two of the most important groups of butyrate producers appear to be *Faecalibacterium prausnitzii*, which belongs to the *Clostridium leptum* (or Clostridial cluster IV) cluster, and *Eubacterium rectale/Roseburia spp.*, which belongs to the *Clostridium coccoides* (or clostridial cluster XIVa) cluster of Firmicute bacteria [56]. As previously described, butyrate is the major energy source for colonocytes and is involved in the maintenance of colonic mucosal health [57]. Recently several intestinal and extra-intestinal effects of butyrate have been demonstrated [58, 59]. Butyrate has been studied for its role in nourishing the colonic mucosa and in the prevention of cancer of the colon, by promoting cell differentiation, cell-cycle arrest and apoptosis of transformed colonocytes; inhibiting the enzyme histone deacetylase and decreasing the transformation of primary to secondary bile acids as a result of colonic acidification. Therefore, a greater increase in SCFA production and potentially a greater delivery of SCFA, specifically butyrate, to the distal colon may result in a protective effect.

Butyrate can interact with GPR43 which is a G-protein-coupled receptor expressed in colonic epithelium, adipose tissue and immune cells [60]. GPR43 together with GPR109A are considered the main butyrate targets involved in suppression of colonic inflammation and carcinogenesis [61]. Moreover, butyrate modulates histone acetylation, as histone deacetylase (HDAC) inhibitor, and alters host epigenome, leading to its epigenetic mechanism [58, 59].

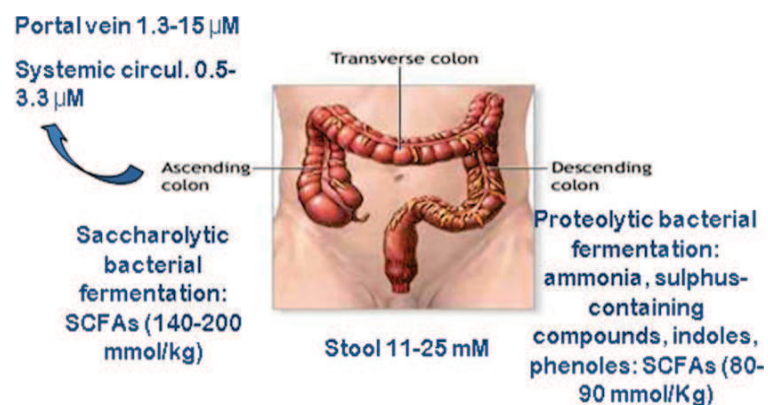


Figure 2.1. Production and absorption of SCFAs along the colon.

Butyrate derivatives

Providing butyrate can be challenging for several reasons, including short metabolic half-life, toxicity, and patient intolerance [62]. Butyrate has been provided via several routes: intravenously, rectally as enemas, and orally. There are limitations to providing butyrate intravenously (500 mg/kg body weight) in that large volumes are required, and the metabolic half-life is very short, with blood levels peaking about 6 minutes after delivery [63]. Providing higher rates of intravenous (IV) butyrate infusion is undesirable due to risk of toxicity from sodium overload. Rectal enemas (100 mmol/L) have been successful in reversing negative gastrointestinal (GI) effects in patients with inflammatory bowel disease; however, this mode of delivery lends to very poor patient compliance [63]. Tributyrin overcomes many of the problems of the parent compound. Tributyrin delivered orally in animals has a plasma half-life of 40 minutes [64]. In humans, oral delivery provided once daily for 3 weeks was without severe toxicity, and peak plasma butyrate concentrations occurred between 0.25 and 3 hours after dose and ranged from 0–0.45 mM, which is near those found to be effective *in vitro* (0.5–1 mM) [65].

Butyric acid is considered a promising bioactive food compound and has been used in clinical trials; however, its short half-life considerably restricts its therapeutic application. A butyric acid pro-drug Tributyrin (TB), is present in milk fat and honey, and shows a more favorable pharmacokinetic properties than butyric acid. Moreover its oral administration is also better tolerated. In *in vitro* and *in vivo* studies have shown that TB acts on multiple anticancer cellular and molecular targets [66]. TB induces apoptosis and cell differentiation and modulates epigenetic mechanisms. Due to its anti-carcinogenic potential and low toxicity, strategies as lipid emulsions, nanoparticles, or structured lipids containing TB are currently being developed to improve its organoleptic characteristics and bioavailability. Therefore, additional preclinical and clinical studies should be performed using TB to elucidate its molecular targets and anti-carcinogenic potential.

Moreover, another butyrate derivative is inhibitor of HDAC, sodium phenylbutyrate, an aromatic fatty acid that is converted/ oxidized *in vivo* into phenylacetate (PAA) by β -oxidation [67]. In humans the so formed PAA is eliminated by conjugation with glutamine to form phenacetylglutamine, which is excreted in the urine. This metabolic pathway is the mechanism by which phenylbutyrate acts as an ammonia scavenger in patients with urea cycle disorders (UCDs) and hyperammonemia [68]. *In vitro*, phenylbutyrate shows the ability to induce differentiation by various mechanisms [69], and the US FDA has

approved its clinical use in patients with hyperammonemia. Phenylbutyrate therapy for infants, children, and adults with UCD, which leads to nitrogen accumulation in the form of ammonia, must be undertaken daily and lifelong, and it is generally well tolerated and associated with improvements in ammonia and liver function [70]. Phenylbutyrate is relatively stable, with a 0.8–1 hour half-life in human serum. It has been shown that phenylbutyrate is able to lower very-long-chain fatty acid levels in the brain of mice with x-linked adrenoleukodystrophy, suggesting that phenylbutyrate can cross the blood-brain barrier [71].

N-(1-carbamoyl-2-phenyl-ethyl) butyramide (FBA): a new butyrate derivative

In our laboratory it was synthesized a stable amide acid of butyric acid with the amino acid phenylalanine (N-(1-carbamoyl-2-phenylethyl) butyramide, FBA), as previously described (Italian patent RM2008A000214; April 21, 2008). Its chemical structure is illustrated in **figure 2.2**. Briefly, 0.01 M of phenylalanine carboximide and 0.01 M butyryl chloride were dissolved in 50 ml of chloroform and the resulting mixture was left to react at room temperature for 24 h. The mixture, evaporated in vacuo, yields a solid white-color residue, that was washed with a 1% sodium bicarbonate solution. The aqueous bicarbonate solution was extracted twice with an equal volume of ethyl acetate to recover an additional fraction of the mixture of derivatives. To isolate the single components, the mixture was treated and processed chromatographically on a silica gel column, using dichloromethane as eluent. The compound was re-crystallised with a mixture of chloroform/n-hexane 1:1(v: v), obtaining a final yield equal to or greater than 50%. FBA is a solid, poorly hygroscopic, easily weighable form, stable to acids and capable of releasing butyric acid at small and large bowel level in a constant manner over time.

This product, referred to toxicity studies performed in our laboratory, demonstrated a toxicological profile similar to that of butyric acid, has compared to the latter, excellent physic-chemical characteristics. In fact, the product is in solid form, slightly hygroscopic, easy to be weighed, stable to acids and alkalis and it is able to release in a constant way in time butyric acid in the small intestine and colon. A particularly important aspect of FBA is that it doesn't have the unpleasant rancid odor of butyrate and is practically tasteless.

Moreover, the solubility of FBA in water is satisfactory in that it produces clear solutions up to the concentration of 0.1 M and suspensions for higher concentrations.

FBA has been studied in a model of non alcoholic fatty liver disease and the use of this derivative prevented the development of insulin resistance and decreased pro-inflammatory parameters in the liver, through the suppression of the Toll receptors and the activation of NF- κ B [72].

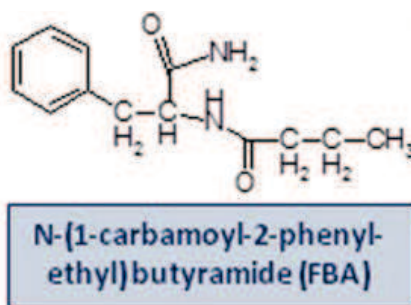


Figure 2.2. Chemical structure of N-(1-carbamoyl-2-phenylethyl) butyramide, FBA.

GPR43 and GPR41 as key receptors for SCFAs

G-protein-coupled receptors (GPCRs) are seven-transmembrane (7TM) receptors that mediate cellular responses to the majority of hormones and neurotransmitters, and are therefore attractive targets for drug discovery [73]. Free fatty acids (FFAs) have long been considered as key signalling molecules in numerous physiological and pathological processes. The identification of a family of GPCRs that bind FFAs has highlighted new potential mechanisms of action for FFAs in health and disease [74]. Among these FFA receptors, GPR43 is present in a large variety of tissues, including adipose tissue, inflammatory cells, and gastrointestinal (GI) tract and it is activated by SCFAs [75, 76]. The identification of these endogenous ligands of GPR43 has led the scientific community to propose a new appellation for GPR43, namely FFA2 or FFAR2 [74, 77]. SCFAs bind GPR43 in the following rank order of potency: propionate > acetate=butyrate > valerate > formate [75, 77]. Importantly, SCFAs also activate another receptor of the same family, GPR41, with propionate and butyrate being the most potent agonists [75, 78]. Both receptors can couple to $G\alpha_{i/0}$ resulting in inhibition of the adenylate cyclase pathway, but only GPR43 is also able to couple to $G\alpha_q$, thus leading to activation of the phospholipase C (PLC) pathway and increased intracellular calcium levels [75, 78]. GPR41 and GPR43

bind the same family of ligands (SCFAs), exhibit some overlapping expression, and partially share signalling pathways ($G\alpha_{i/0}$). Furthermore, both receptors represent potentially interesting targets for drug discovery.

GPR43 and GI tract functions

Since GPR43 is largely expressed throughout the gut, several authors have suggested that some effects of SCFAs could be GPR43-dependent. In 2006, Karaki et al. demonstrated that GPR43 was expressed in rat distal ileum and colon. Interestingly, peptide YY (PYY)-containing enteroendocrine L cells were immunoreactive for GPR43, whereas 5-hydroxytryptamine (5-HT) immunoreactive mast cells coexpressed GPR43 [79]. PYY is a satietogenic peptide that inhibits upper GI motility and SCFAs have been shown to induce its release in the blood [80]. Therefore, SCFAs might stimulate L cells to release PYY via GPR43 activation, thus slowing intestinal transit. GPR43 expression in enteroendocrine L cells was observed likewise in the human colon [81]. SCFAs also exert physiological effects on colonic motility and secretion via 5-HT release [82] and Karaki et al. proposed this might be attributable to the activation of GPR43 on 5-HT-containing mast cells [79]. The presence of GPR43 throughout the rat gut, with the lowest mRNA levels observed in the esophagus and stomach and the highest levels detected in the colon, was also confirmed [83].

Glucagon-like peptide 1 (GLP-1) is another gut hormone released by enteroendocrine L cells that is involved in the control of intestinal function and glucose metabolism [84]. SCFA infusion was shown to induce plasma GLP-1 release in animals and humans [85]. Interestingly, co-localization of GPR43 and GLP-1 in enteroendocrine L cells was demonstrated in both rat and human colon and terminal ileum [86]. In rodents, supplementation with fermentable carbohydrates increased GLP-1 production and the density of GPR43/GLP-1-positive enteroendocrine L cells in the proximal colon [86]. Therefore, a higher colonic production of SCFAs following dietary fiber fermentation increase GLP-1 secretion, *via* GPR43 activation. This hypothesis has been recently confirmed by Tolhurst et al. [87]. The SCFA-triggered secretion of GLP-1 was almost completely abolished in primary colonic cultures from GPR43 KO mice but was also reduced, to a lesser extent, in mice lacking GPR41. GPR43-deficient mice had significantly reduced colonic GLP-1 protein content. Moreover, basal and glucose-

stimulated levels of active GLP-1 were reduced in both GPR43 and GPR41 KO mice. These effects were associated with impaired glucose tolerance. Even if mice lacking GPR43 also exhibited decreased colonic expression of GPR41, a dominant role for GPR43 in SCFA-induced L cell activation was suggested based on the prevailing involvement of $G_{\alpha q}$ coupled pathways in this process (**figure 2.3**) [87]. These results reveal again the difficulty to generate GPR43 KO mice without affecting GPR41, thus introducing uncertainties about their interpretation. Studies conducted in germ-free (GF) mice highlighted a potential link between gut microbiota and the expression of FFA receptors. The conventionalization (colonization by normal mouse microbiota) of GF mice increased adiposity and decreased the expression of GPR41 and GPR43 in the distal small intestine [88]. However, another study reported that GF mice exhibited decreased intestinal expression of GPR43, GPR41, PYY, and GLP-1 as compared with conventional mice. It is worth noting that the expression of GPR43 and GPR41 was differentially affected in GF mice, with a 10% and 70% decrease, respectively. This was associated with lower levels of circulating PYY [89]. Overall, these results suggest that gut microbiota can influence the intestinal expression of SCFA receptors and the secretion of gut peptides, but further studies are needed to elucidate the underlying mechanisms.

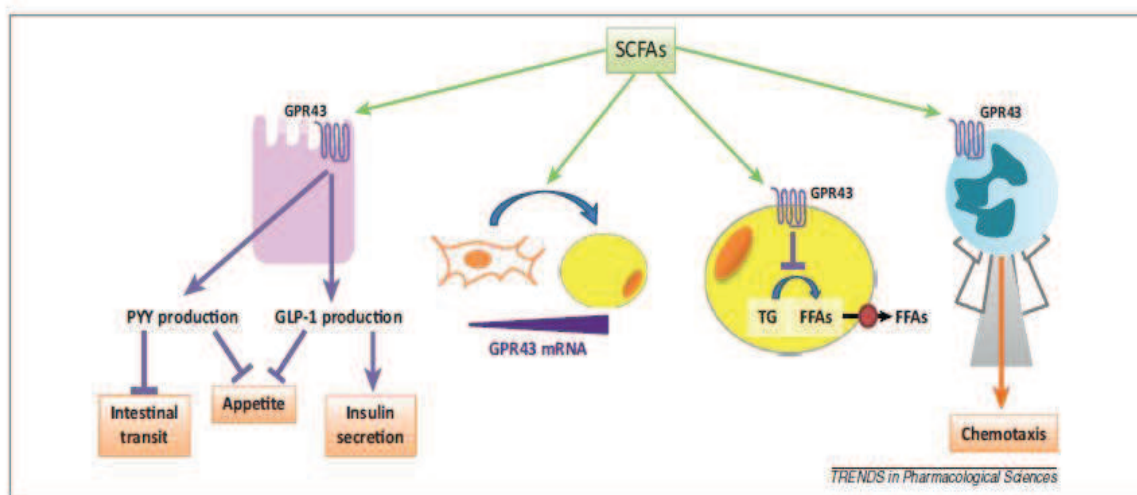


Figure 2.3. Location and physiological functions of G-protein-coupled receptor 43 (GPR43). Studies in rodents have highlighted that SCFAs bind to GPR43 to exert several physiological actions. GPR43 activation on intestinal enteroendocrine cells (in pink) induces the production of PYY and GLP-1. PYY inhibits intestinal transit and appetite, whereas GLP-1 is anorexigenic and stimulates insulin secretion. In mice, GPR43 expression increases during adipogenesis and SCFAs stimulate adipocyte differentiation (fibroblast in orange). Through their binding to GPR43, SCFAs also inhibit lipolysis in mature adipocytes (in yellow). Finally, SCFAs induce chemotaxis of neutrophils (in blue) through GPR43 activation. Abbreviations: TG, triglycerides; FFAs, free fatty acids. *Image from : Bindels, Dewulf, Delzenne. GPR43/FFA2: physiopathological relevance and therapeutic prospects. Trends Pharmacol Sci. 2013 Apr;34(4):226-32. doi: 10.1016/j.tips.2013.02.002. Epub 2013 Mar 13.*

GPR43 and inflammation

SCFAs have long been known to modulate the production of pro- and anti-inflammatory mediators [90]. For instance, production of prostaglandin E2 is reduced by SCFAs. This effect can be inhibited by pertussis toxin, suggesting the involvement of a G-protein-mediated signalling [91]. The formal proof of GPR43 involvement in the management of inflammation was simultaneously provided by two research teams. They both established the contribution of GPR43 to the recruitment of immune cells [6,48], and this observation was further confirmed by others [92]. However, these studies showed divergent findings on the potential impact of GPR43 in inflammatory diseases. Maslowski et al., [76] demonstrated that stimulation of GPR43 by acetate allowed resolution of colitis-related inflammatory response. GPR43 KO mice showed exacerbated or unresolved inflammation in cases of acute and chronic colitis, arthritis, and asthma. This could be related to increased immune cell recruitment [76]. By contrast, Sina et al. reported that, in an acute colitis model, GPR43 KO mice showed an increased mortality compared with control mice, despite reduced immune cell recruitment, decreased colonic inflammation, and attenuated colonic tissue damage. The increased mortality was attributed to septic complication. In a chronic colitis model, GPR43 deficiency led to reduced colonic inflammation, without any sign of sepsis and any lethality. The authors pointed out the bipotential pathophysiological role of immune cells at the intestinal level, being a protective factor against acute bacterial transmigration, but having a detrimental role in chronic inflammatory responses [93]. Clearly, GPR43 is involved in the SCFA-induced neutrophil chemotaxis in mice (**figure 2.3**) [76, 92]. However, demonstrating GPR43 contribution to human neutrophil chemotaxis remains to be accomplished. Interestingly, GLPG0974, an orally available small GPR43 inhibitor from Galapagos with undisclosed structure, has been claimed to reduce neutrophil migration. GLPG0974 is currently being tested in a second Phase I study and results are expected in early 2013 (ClinicalTrials.gov; identifier: NCT01721980). The global role of GPR43 in inflammatory conditions needs to be clarified before ruling on the therapeutic potential of GPR43 in this context. The dualistic action of SCFAs, being anti-inflammatory while recruiting neutrophils, might be one of the keys to full understanding of how SCFAs and GPR43 manage inflammation. Importantly, a comparison of several studies highlights that the biological and molecular responses to SCFAs differ in the several type of immune cell [92]. This might be consistent with the various temporal roles of these cells in an inflammatory response [91]. Therefore,

the different molecular pathways downstream of GPR43 remain to be elucidated. Finally, Inulin-type fructans (ITF) prebiotic feeding can control inflammation in rodent models of colitis, obesity, diabetes, and leukemia [94, 95]. It is possible to postulate that prebiotics, through their fermentation into SCFAs, might exert some of their anti-inflammatory effects in a GPR43-dependent manner, this hypothesis need to be confirmed.

3. EFFECTS OF BUTYRATE AT INTESTINAL LEVEL

Effects on inflammatory process and oxidative stress

Butyrate plays a role as an anti-inflammatory agent, primarily via inhibition of NF- κ B activation in human colonic epithelial cells [96], which may result from the inhibition of histone deacetylase (HDAC). NF- κ B regulates many cellular genes involved in early immune inflammatory responses, including IL-1 β , TNF- α , IL-2, IL-6, IL-8, IL-12, iNOS, COX-2, intercellular adhesion molecule-1 (ICAM-1), vascular cellular adhesion molecule-1 (VCAM-1), T cell receptor- α (TCR- α), and MHC class II molecules [97]. The activity of NF- κ B is frequently dysregulated in colon cancer [98] and in IBDs, such as ulcerative colitis (UC) and Crohn's disease (CD) [99]. In CD patients, butyrate decreases pro-inflammatory cytokine expression via inhibition of NF- κ B activation and I κ B α degradation [99]. The upregulation of PPAR- γ and the inhibition of IFN- γ signaling, are another two of butyrate's anti-inflammatory effects [100]. Butyrate can act on immune cells through GPR41 (or FFA3) and GPR43 (or FFA2), which are both expressed on immune cells, including polymorphonuclear cells, suggesting that butyrate might be involved in the activation of leucocytes [101]. The possible immune-modulatory functions of SCFAs are highlighted by a recent study on GPR43^{-/-} mice. These mice exhibit aggravated inflammation, related to increased production of inflammatory mediators and increased immune cell recruitment [93]. Most clinical studies analyzing the effects of butyrate on inflammatory status focused on UC patients. Hallert et al. [102] instructed 22 patients with quiescent UC to add 60 g oat bran (corresponding to 20 g dietary fiber) to their daily diet. Four weeks of this treatment resulted in a significant increase of fecal butyrate concentration and in a significant improvement of abdominal symptoms. In a double blind, placebo-controlled multicenter trial, Vernia et al., [103] treated 51 patients with active distal UC with rectal enemas containing either 5-aminosalicylic acid (5-ASA) or 5-ASA plus sodium butyrate (80 mmol/L, twice a day). The combined treatment with topical 5-ASA plus sodium butyrate significantly improved the disease activity score more than 5-ASA alone. These and other intervention studies [104] suggested that the luminal administration of butyrate or stimulation of luminal butyrate production by the ingestion of dietary fiber results in an amelioration of the inflammation and symptoms in UC patients.

Numerous studies have reported that butyrate metabolism is impaired in intestinal inflamed mucosa of patients with IBD. Recent data show that butyrate deficiency results from the reduction of butyrate uptake by the inflamed mucosa through downregulation of monocarboxylate transporter isoform 1 (MCT1). The concomitant induction of the glucose transporter GLUT1 suggests that inflammation could induce a metabolic switch from butyrate to glucose oxidation. Butyrate transport deficiency is expected to have clinical consequences. Particularly, the reduction of the intracellular availability of butyrate in colonocytes may decrease its protective effects toward cancer in IBD patients [105]. Limited evidence from pre-clinical studies shows that oxidative stress in the colonic mucosa can be modulated by butyrate. Oxidative stress is involved in both inflammation [106] and the process of initiation and progression of carcinogenesis [107]. During oxidative stress there is an imbalance between the generation of reactive oxygen species (ROS) and the antioxidant defense mechanisms, leading to a cascade of reactions in which lipids, proteins, and/or DNA may get damaged. In healthy humans, it has been demonstrated that locally administered butyrate in physiological concentrations increased the antioxidant GSH and possibly decreased ROS production, as indicated by a decreased uric acid production [108]. As the human colon is continuously exposed to a variety of toxic stimuli, enhanced butyrate production in the colon could result in an enhanced resistance against toxic stimuli, thus improving the barrier function. This might be relevant for the treatment of gastrointestinal disorders, such as post-infectious irritable bowel syndrome (IBS), microscopic colitis, IBDs, and diversion colitis.

Butyrate and intestinal epithelial permeability

Intestinal epithelial permeability has been widely studied as an important parameter of the intestinal defense barrier. Under normal conditions, the epithelium provides a highly selective barrier that prevents the passage of toxic and pro-inflammatory molecules from the external milieu into the submucosa and systemic circulation. Macromolecules pass the epithelial barrier mainly *via* the paracellular route for which tight junctions are the rate-limiting structures [109]. Increased permeability, indicating impaired epithelial barrier function, is thought to be involved in the pathophysiology of several gastrointestinal inflammatory diseases, but can either be a cause or a consequence of inflammation [110]. Several studies have assessed the effects of butyrate on intestinal permeability *in vitro* as

well as *ex vivo*. At low concentrations, butyrate (up to 2 mM) induces a concentration-dependent reversible decrease in permeability in a Caco-2 and HT-29 cell lines [111]. This decrease in permeability may be related to the butyrate associated increased expression of tight junction proteins observed in different cultured cell lines, but this effect was shown to be cell type dependent [112]. At higher concentrations (8 mM), however, butyrate increased the permeability in a Caco-2 cell line [111]. An *ex vivo* study, using adult rat distal colon mucosa mounted in an Ussing chamber, demonstrated that acute exposure to butyrate at a concentration of 10 mM, but not 1 or 5 mM increased paracellular permeability in rat colon [113]. This has also been demonstrated in rats fed a diet-containing fermentable FOS. The rapid bacterial fermentation of FOS led to accumulation of high concentrations of SCFAs that increased intestinal permeability and was associated with increased translocation of *Salmonella* [114]. However, in humans, daily FOS supplementation of 20 g did not increase intestinal permeability [115]. It can be concluded that the effect of butyrate on intestinal permeability depends on its concentration and on the model system or species used. The effects of butyrate at different concentrations remain to be evaluated in *in vivo* studies.

Effects on transepithelial ion transport

Potentially, SCFAs are absorbed by each intestinal segment, as demonstrated in animal models and human volunteers. The colonocytes absorb butyrate and other SCFAs through different mechanisms of apical membrane SCFA uptake, including non-ionic diffusion, SCFA/HCO₃⁻ exchange, and active transport by SCFA transporters. The transport protein involved are MCT1, which is coupled to a transmembrane H⁺-gradient, and SLC5A8, which is Na⁺-coupled co-transporter [116]. The absorption of these fatty acids has a significant impact on the absorption of NaCl and, generally, on the electrolyte balance [117]. In particular, butyrate is able to exert a powerful proabsorptive stimulus on intestinal NaCl transport and an anti-secretory effect towards Cl⁻ secretion. The powerful regulatory pro-absorptive/anti-secretory effects induced by butyrate on the trans-epithelial ion transport occurs through several mechanisms: (1) stimulation of NaCl absorption by the action of two coupled transport systems on the intestinal brush border: Cl⁻/HCO₃⁻ and Na⁺/H⁺ and Cl⁻/butyrate and Na⁺/H⁺; and (2) inhibition of Cl⁻ secretion by blocking the activity of the co-transporter Na⁺-K⁺-2Cl⁻ (NKCC1) on the enterocyte basolateral

membrane. *In vitro* studies have shown that butyrate has an inhibitory effect on Cl⁻ secretion induced by prostaglandin E₂, cholera toxin, and phosphocholine. This effect is due to reduced production of intracellular cAMP secondary to the expression and regulation of adenylate cyclase [116]. Comparison studies showed that the pro-absorptive and anti-secretory effects of butyrate are significantly higher than those of all other SCFAs [117]. Clinical studies in children with acute diarrhea caused by *V. cholerae* showed a reduction in stool volume and a more rapid recovery in patients who received oral rehydration therapy in addition to resistant starch, a precursor of butyrate, in the diet [118]. These results were confirmed in other forms of infectious diarrhea in children and in animal models studies [55]. Moreover, butyrate therapy is beneficial in patients affected by Congenital Chloride Diarrhea (CLD) [56]. This rare genetic disease is caused by mutations in the gene encoding the solute-linked carrier family 26-member A3 (SLC26A3) protein, which acts as a plasma membrane anion exchanger for Cl⁻ and HCO₃⁻ [119]. The mechanism underlying this therapeutic effect could be related, at least in part, to stimulation of the Cl⁻/butyrate exchanger activity [56]. It is also possible that butyrate could reduce mistrafficking or misfolding of the SLC26A3 protein, as demonstrated for other molecules involved in transepithelial ion transport [120]. Alternatively, butyrate may enhance gene expression: the SLC26A3 gene contains a 290-bp region between residues -398 and -688 that is crucial for high-level transcriptional activation induced by butyrate. This may explain the variable response of patients affected by CLD to butyrate [57]. In fact, depending on the patient's genotype, mutations in the above-mentioned regulatory regions of the SLC26A3 gene could affect the gene transcription rate. It is also conceivable that other channels could be involved in the therapeutic effect of butyrate in CLD.

Effects on visceral perception and intestinal motility

Little is known about the environmental and nutritional regulation of the enteric nervous system (ENS), which controls gastrointestinal motility. Butyrate regulates colonic mucosa homeostasis and can modulate neuronal excitability. Soret et al., [121] investigated the effects of butyrate on the ENS and colonic motility, and showed, *in vivo* and *in vitro*, that butyrate significantly increased the proportion of choline acetyltransferase (ChAT), but not neuronal nitric oxide synthase (nNOS) immunoreactive myenteric neurons. Butyrate increases the cholinergic-mediated colonic circular muscle contractile response *ex*

in vivo. The authors suggest that butyrate might be used, along with nutritional approaches, to treat various gastrointestinal motility disorders associated with inhibition of colonic transit. A recent study by Vanhoutvin et al., [122] shows that intraluminal administration of a physiologically relevant dose (50 to 100 mmol/L-) of butyrate into the distal colon increases compliance and decreases pain, urge, and discomfort measured with a rectal barostat procedure in healthy subjects. This study suggests a potential beneficial effect of butyrate in disorders that are associated with visceral hypersensitivity, such as IBS and infantile colics, and provides a basis for future trials with dietary modulation resulting in intracolonic butyrate production in both healthy and IBS subjects. The decrease in visceral perception induced by butyrate treatment could be due to an increased 5-HT release, as previously suggested by others [123]. Another possible mechanism by which butyrate could affect visceral perception is the previous reported inhibition of histone deacetylase. In fact, Chen et al., [124] showed that these inhibitors induce microglial apoptosis and attenuate inflammation-induced neurotoxicity in rats, which may affect visceral perception. Butyrate has been reported to induce enhancement of colonic motility via the release of 5-HT [125]. In functional studies, butyrate and propionate induced phasic and tonic contractions in rat colonic circular muscle. The dose-dependent contractile effect occurred only when SCFAs were applied on the mucosal side and disappeared in mucosal free preparations, suggesting the presence of sensory mechanisms near the epithelium [82].

The effects of butyrate at intestinal levels are resumed in schematic **figure 3.1**.

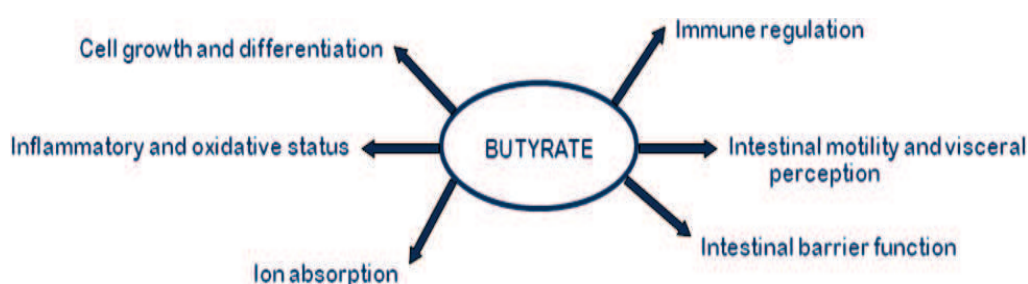


Figure 3.1. Effects of butyrate at intestinal level.

Effects on non-specific intestinal defense mechanisms

Besides the effects of butyrate on carcinogenesis, inflammation and oxidative stress, butyrate has been shown to affect several components of the colonic defense barrier

leading to enhanced protection against luminal antigens. One important component of this barrier is the mucous layer covering the epithelial lining consisting of mainly mucin glycoproteins and trefoil factors (ITF or TFF3). Mucin glycoproteins are classified into neutral and acidic subtypes and the latter category further includes sulfomucins and sialomucins. Sulphated mucins are generally considered to be more resistant to bacterial degradation [126]. Several epithelial mucin (MUC) genes have been identified in humans, of which MUC2 is predominantly expressed in the human colon [127]. Alterations in goblet cell function, composition and thickness of the intestinal mucous layer have been found in several intestinal disorders. For example, a reduced mucous thickness and a decreased MUC2 production have been reported in UC patients [128]. In *in vitro* studies, butyrate increased the MUC2 gene expression in specific cell lines [129, 130]. In addition, 0.1–1 mM butyrate administered to human colonic biopsy specimens *ex vivo* stimulated mucin synthesis [131]. Luminal butyrate administration of 5 mM, but not 100 mM, increased mucous secretion in an isolated perfused rat colon [132]. In another rat study, caecal and faecal SCFA concentrations were found to correlate with mucous thickness. In humans, effects of butyrate on mucous synthesis, thickness of the mucous layer and MUC expression *in vivo* have not been reported. The effects of a number of fermentable dietary fibers on the mucous layer have been studied with variable results. For example, resistant starch increased the number of acidic mucins, but did not affect the number of goblet cells in rats [133]. In contrast, FOS increased the number of goblet cells in piglets [134]. In a human intervention study with patients with an ileo-anal pouch, inulin supplementation did not alter MUC2 expression or the ratio between sulfomucins and sialomucins [135]. Trefoil factors are mucin-associated peptides that contribute to the viscoelastic properties of the mucous layer. TFFs are thought to reduce the recruitment of inflammatory cells and to be involved in the maintenance and repair of the intestinal mucosa, although the exact mechanism for this effect is not yet known [136]. Intestinal trefoil factor is almost exclusively secreted by the intestinal goblet cells [137]. In a rat TNBS model of colitis, TFF3 expression was decreased during active disease, and intracolonic administration of butyrate increased TFF3 expression [138]. However, butyrate inhibited the expression of TFF3 in colon cancer cell lines and in colonic tissue of newborn rats [139]. Other components of the colonic defense barrier that are involved in the maintenance of the colonic barrier, which may be influenced by butyrate are transglutaminase, antimicrobial peptides and heat shock proteins (HSPs). The enzyme transglutaminase is actively

involved in intestinal mucosal healing and correlates with the severity of inflammation in UC [140]. In a rat model of colitis, butyrate restored the colonic transglutaminase levels [141]. Antimicrobial peptides such as cathelicidin (LL-37) and defensins, protect the gastrointestinal mucosa against the invasion and adherence of bacteria and thereby prevent infection [142]. Several *in vitro* studies have shown that butyrate up-regulates the expression of LL-37 in different colon epithelial cell lines as well as in freshly isolated colorectal epithelial cells [143]. HSPs confer protection against inflammation by suppressing the production of inflammatory modulators [144, 145]. Butyrate induced the expression of HSP70 and HSP25 in Caco-2 cells [145] and in rats [133, 144]. However, in DSS induced colitis butyrate inhibited HSP70 expression in rats. This effect was related to protection against the decrease in cell viability, increase in mucosal permeability and neutrophil infiltration in DSS colitis. It was concluded that the induction of heat shock response had a protective effect before an injury, whereas activation of heat shock response leads to cytotoxic effects after a pro-inflammatory stimulus [146]. In addition, there is evidence from *in vitro* studies with human colon cancer cell lines that butyrate is involved in repair after mucosal damage through an increase in the rate of cell migration. Efficient repair of superficial injuries and mucosal ulcers is important in maintaining and re-establishing the epithelial barrier [147]. In conclusion, there are several lines of evidence suggesting that butyrate reinforces the colonic defense barrier by affecting several components of this barrier, such as the promotion of epithelial migration and the induction of mucins, TFF, transglutaminase activity, antimicrobial peptides and HSPs. However, most of these effects still have to be confirmed in the human studies.

Effects on cell growth and differentiation

Several epidemiological studies have supported the role of dietary fiber in the protection against colorectal cancer [148, 149]. Different mechanisms have been proposed for fiber's cancer preventive properties: reduction in transit time of the feces in the gut, which reduces exposure of the mucosa to luminal carcinogens; absorption of bile acids, biogenic amines, bacterial toxins, and production of butyrate. Most of the anti-carcinogenic effects of butyrate were observed *in vitro* carcinoma cell lines. In these models, addition of butyrate led to inhibition of proliferation, induction of apoptosis, or differentiation of tumor cells [150]. Butyrate's anti-carcinogenic effects are in contrast with the effects of

this compound in normal enterocytes. In fact, it has been shown that butyrate stimulates the physiological pattern of proliferation in the basal crypt in the colon, whereas it reduces the number and the size of aberrant crypt focus, which are the earliest detectable neoplastic lesions in the colon [151]. These contradictory patterns of butyrate represents the so called “butyrate paradox” [150]. An important mechanism by which butyrate causes biological effects in colon carcinoma cells is the hyperacetylation of histones by inhibiting HDAC. An imbalance of histone acetylation can lead to transcriptional dysregulation and silencing of genes that are involved in the control of cell cycle progression, differentiation, apoptosis and cancer development [152, 153]. In particular, in human colon cancer cell lines butyrate, acting as HDAC inhibitor, increases the p21 (WAF1) gene expression by selectively regulating the degree of acetylation of the gene-associated histones, and induces G1 cell cycle arrest [154]. A novel contributory mechanism to the chemopreventive effect of butyrate is the downregulation of the key apoptotic and angiogenesis regulator Neuropilin-1 (NRP-1), which has been shown to promote tumor cell migration and survival in colon cancer in response to vascular endothelial growth factor (VEGF) binding [155]. Several reports have shown that the apoptosis triggered by butyrate *in vitro* is associated with dysregulation of Bcl2 family proteins, especially up-regulation of BAK and downregulation of Bcl-xL [156], rather than cellular damage. A study by Thangaraju et al suggests a novel mode of action of butyrate in the colon involving GPR109A, a G-protein-coupled receptor for nicotinate [157], which recognises butyrate with low affinity. This receptor is expressed in the normal colon on the lumenfacing apical membrane of colonic epithelial cells, but is silenced in colon cancer *via* DNA methylation. Thangaraju et al., [157] showed that inhibition of DNA methylation in colon cancer cells induces GPR109A expression and that activation of the receptor causes tumor cell-specific apoptosis. Butyrate is an inhibitor of HDAC, but apoptosis induced by activation of GPR109A with its ligands in colon cancer cells does not involve inhibition of histone deacetylation. The primary changes in this apoptotic process include downregulation of Bcl-2, Bcl-xL, and cyclin D1 and upregulation of death receptor pathway. Moreover, a recent study suggested that the protective role of dietary fiber, and its breakdown product butyrate, against colorectal cancer could be determined by a modulation of canonical Wnt signaling, a pathway constitutively activated in the majority of colorectal cancers [158]. Butyrate is recognized for its potential to act on secondary chemoprevention, by slowing growth and activating apoptosis in colon cancer cells , but it can also act on primary

chemoprevention. The mechanism proposed is the transcriptional up-regulation of detoxifying enzymes, such as glutathione-S-transferases (GSTs). This modulation of genes may protect cells from genotoxic carcinogens, such as H₂O₂ and HNE [152].

4. EXTRA-INTESTINAL EFFECTS OF BUTYRATE

Hemoglobinopathies

Clinical trials in patients with sickle cell disease and β -thalassemia confirmed the ability of butyrate to increase fetal hemoglobin (HbF) production [159, 160]. Butyrate is an inducer of HbF through an epigenetic regulation of fetal globin gene expression via HDAC inhibition, resulting in global histone hyperacetylation, including nucleosomes at the γ -globin promoters [161]. Other experiments have shown that butyrate can cause a rapid increase in the association of γ -globin mRNA with ribosomes [162]. It has been demonstrated that activation of p38 mitogen activated protein kinases and cyclic nucleotide signaling pathways is associated with butyrate induction of HbF [163]. Taken together, these studies suggest that global histone hyperacetylation induced by HDAC inhibition is not the unique mechanism underlying butyrate stimulation of HbF.

Genetic metabolic diseases

Sodium phenylbutyrate 4 (4-PBA) was approved by the Food and Drug Administration (FDA) for use in patients with urea cycle enzyme deficiency, in which it acts as a scavenger of ammonia. Indeed, 4-PBA is oxidized to phenylacetate, which binds to glutamine and determines the urinary excretion. In patients with ornithine transcarbamylase deficiency, the use of 4-PBA allows for better metabolic control and increased intake of natural protein in the diet [164]. The possible use of butyrate in the treatment of X-linked Adrenoleukodystrophy (X-ALD), a disorder of peroxisomes characterized by altered metabolism and accumulation of very long chain fatty acids, has also been studied. Sodium phenylbutyrate 4 induces an increase in β -oxidation of very long chain fatty acids and peroxisome proliferation, both in vitro on fibroblasts from patients with X-ALD than in vivo in X-ALD knockout mice [165].

Hypercholesterolemia

Under normal lipidemic conditions, the liver is the most important site of cholesterol biosynthesis, followed by the intestine. Biosynthesis in the liver and intestine account for

about 15% and 10%, respectively, of the total amount of cholesterol biosynthesis each day [166]. In hypercholesterolemia, when cholesterol biosynthesis is suppressed in liver by fasting, the intestine becomes the major site of cholesterol biosynthesis, and its contribution can increase up to 50%. Importantly, recent evidence shows that the global effect of butyrate is to downregulate the expression of nine key genes involved in intestinal cholesterol biosynthesis, potentially inhibiting this pathway [167].

Obesity and insulin resistance

Dietary supplementation with butyrate can prevent and treat diet-induced obesity and insulin resistance in mouse models. After a 5-wk treatment with butyrate, obese mice lost 10.2% of their body weight. Consistent with the change in body weight, fat content was reduced by 10%. Furthermore, fasting glucose was reduced by 30%, insulin resistance was reduced by 50%, and intraperitoneal insulin tolerance was improved significantly by butyrate. The mechanism of butyrate action is related to promotion of energy expenditure and induction of mitochondrial function. Stimulation of peroxisome proliferator-activated receptor (PPAR) coactivator (PGC-1 α) activity has been suggested as the molecular mechanism of butyrate. Activation of AMP-activated protein kinase (AMPK) and inhibition of histone deacetylases may contribute to the PGC-1 α regulation. These data suggest that butyrate may have potential application in the prevention and treatment of metabolic syndrome in humans [168].

Butyrate and satiety

It has been hypothesized that SCFAs produced in the large intestine also can influence upper gut motility and satiety [169]. Endocrine L-cells present in large concentrations in the colonic mucosa secrete peptides such as GLP-1, peptide YY and oxyntomodulin, which are involved in appetite regulation and satiety [170]. In several animal studies using fermentable carbohydrates such as inulin [170], lactitol [171] and FOS [172], an increased satiety, decreased weight gain and increased endogenous production of GLP-1 and/or PYY were reported. In humans, FOS increased satiety [173] and increased plasma GLP-1 concentrations [174]. However, lactitol did not affect plasma concentrations of this gut peptide [171]. The increased satiety is possibly promoted through the production of SCFAs. This is supported by a number of studies. Butyrate

increased the expression of PYY and pro-glucagon *in vitro* in rat epithelial cells [175] and increased PYY release, but not that of GLP-1, in the isolated colon of rats [176] and rabbits [177]. In addition, colonic SCFA infusion in rats stimulated PYY release [178]. However, colonic infusion with SCFAs in humans did not increase plasma levels of either PYY or GLP-1 [179]. Activation of the SCFA receptor GPR43 expressed in endocrine L-cells may play a role in this effect on satiety [81]. There is increasing evidence that the effect of fermentable dietary fiber on satiety is mediated through the colonic production of SCFAs. However, most evidence originates from rat studies, while again human evidence remains limited. In **figure 4.1** the main intestinal SCFA receptors and transporters are shown.

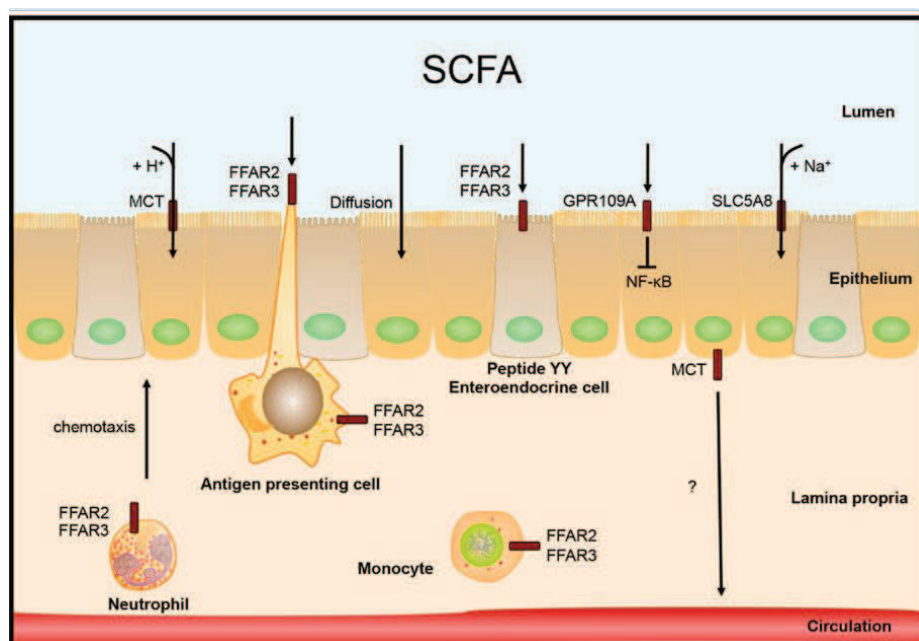


Fig. 4.1. Intestinal SCFA receptors and transporters. SCFAs are taken up by the epithelial cells by diffusion, H⁺ coupled transport by monocarboxylate transporters (MCT) or by Na⁺ coupled transport by SLC5A8. Other receptors that are activated by SCFA are localized on colonocytes, peptide YY expressing enteroendocrine cells, or different immune cells. Receptor FFAR2 is involved in neutrophil chemotaxis toward sources of SCFA. *Image from: Schilderink, Verseijden and de Jonge. Dietary inhibitors of histone deacetylases in intestinal immunity and homeostasis. Front. Immunol., 01 August 2013 doi: 10.3389/fimmu.2013.00226.*

5. ADVERSE EFFECTS OF BUTYRATE

In contrast to the wide range of positive effects of butyrate on the intestinal mucosa, a small number of studies have also shown some adverse effects. Two studies on rat

revealed that rectal administration of butyrate (8–1000 mM), dose dependently increased colonic visceral sensitivity [180]. However, these effects have not yet been reported in humans. In faeces of weaning children, low butyrate concentrations have been measured [181]. It has been hypothesized that overproduction or accumulation of SCFAs may be toxic to the intestinal mucosa of premature infants and might play a role in the pathogenesis of neonatal necrotizing enterocolitis. It has been demonstrated that the severity of mucosal injury to butyrate, measured in newborn rats, was dose dependent and also depended on the maturation of the intestine [139, 182]. It remains to be established whether luminal butyrate in premature infants can increase towards levels that are toxic for the intestinal mucosa [182]. In addition, as mentioned before, increased permeability and Salmonella translocation has been found after FOS supplementation in a study with rats, which may be the result of SCFA accumulation [114]. However, this was not confirmed in the humans [115].

Moreover, despite the potential benefits of SCFA as a therapeutic treatment, the review of Lei et al. [183] describes that SCFA, in particular propionic acid and butyric acid, are implicated in non genetically related Autism Spectrum Disorder (ASD), a conditions with multiple aetologies, in children, through the elevated levels of these SCFA produced by gut microbiome or from food as propionic acid is a popular food preservative [184]. The unscoring mechanism has yet been elucidated. Epidemiological investigations in children exposed to VPA treatment in uterus have reported a significant risk associated with ASD and other neurodevelopmental disorders [185]. Indeed, VPA could induce ASD-like behaviour in rodents when exposed in uterus [186, 187]. Thus, careful consideration must be taken to decide whether or not SCFA would be suitable as therapeutics, especially in pregnant women or women who are planning to conceive.

CHAPTER 2

6. GASTROINTESTINAL DISORDERS

6.1 ULCERATIVE COLITIS

Ulcerative colitis (UC) was first described in the mid-1800s, whereas Crohn's disease (CD) was first reported later, in 1932, as "regional ileitis." Because Crohn's disease can involve the colon and shares clinical manifestations with ulcerative colitis, these entities have often been conflated and diagnosed as inflammatory bowel disease (IBD), although they are clearly distinct pathophysiological entities. Ulcerative colitis is the most common form of inflammatory bowel disease worldwide. In contrast to Crohn's disease, ulcerative colitis is a disease of the mucosa that is less prone to complications and can be cured by means of colectomy, and in many patients, its course is mild [188]. The literature on the pathogenesis and treatment of so-called IBD has tended to focus on Crohn's disease [189, 190], and few articles expressly discuss ulcerative colitis [191]. Ulcerative colitis and Crohn's disease are disorders of modern society, and their frequency in developed countries has been increasing since the mid-20th century. When IBD is identified in a new population, ulcerative colitis invariably precedes Crohn's disease and has a higher incidence. Among children, however, ulcerative colitis is less prevalent than Crohn's disease [192]. The highest incidence and prevalence of IBD are seen in the populations of Northern Europe and North America and the lowest in continental Asia, where ulcerative colitis is by far the most common form of inflammatory bowel disease [193]. A westernized environment and lifestyle is linked to the appearance of IBD, which is associated with smoking, diets high in fat and sugar, medication use, stress, and high socioeconomic status [194]. IBD has also been associated with appendectomy [194]. Of these factors, only cigarette smoking and appendectomy are reproducibly linked to ulcerative colitis.

Bloody diarrhea with or without mucus is the hallmark of ulcerative colitis. The onset is typically gradual, often followed by periods of spontaneous remission and subsequent relapses. Active disease is manifested as mucosal inflammation commencing in the rectum (proctitis) and in some cases spreading to the rest of the colon (**figure 6.1.1A**). Although proctitis is frequently associated with faecal urgency and the passage of fresh blood, constipation may paradoxically occur.

Proctosigmoiditis, left-sided colitis, extensive colitis, or pancolitis (**figure 6.1.1B**) may lead to diarrhea, frequent evacuations of blood and mucus, urgency or tenesmus, abdominal pain, fever, malaise, and weight loss, depending on the extent and severity of the disease. The prognosis for patients with ulcerative colitis is generally good during the first decade after diagnosis, with a low rate of colectomy; over time, remission occurs in most patients [188]. Assessment of the clinical activity of ulcerative colitis helps the clinician choose, diagnostic tests and make therapeutic decisions.

A



B



Figure 6.1.1. Gross morphological appearance of typical UC specimens. A. Severe inflammation of the rectum, sigmoid, splenic flexure and part of the transverse colon, where it stops abruptly and transitions to normal mucosa; B. Severe pancolitis; Pictures taken from: Danese, *Fiocchi Ulcerative colitis. N Engl J Med.* 2011 Nov 3;365(18):1713-25. Doi 10.1056/NEJMra1102942.Review.

In ulcerative colitis, inflammation is characteristically restricted to the mucosal layer, with infiltrates varying in density and composition during active disease or stages of remission. Infiltrates consist primarily of lymphocytes, plasma cells, and granulocytes; the last are being particularly prominent during acute flare-ups and accumulate in crypt abscesses [195]. Other typical features include goblet- cell depletion, distorted crypt architecture, diminished crypt density, and ulcerations. However, epithelioid granulomas, which are typical of Crohn's disease, are not present. There are no exact criteria for the diagnosis of ulcerative colitis, but in most cases, the presence of two or three of the aforementioned histological features will suffice [196]. The severity of inflammation on histological examination and the severity of disease on endoscopic examination may not coincide; for instance, histological findings (**figure 6.1.2**) may indicate severe disease even in a patient with endoscopically quiescent disease.

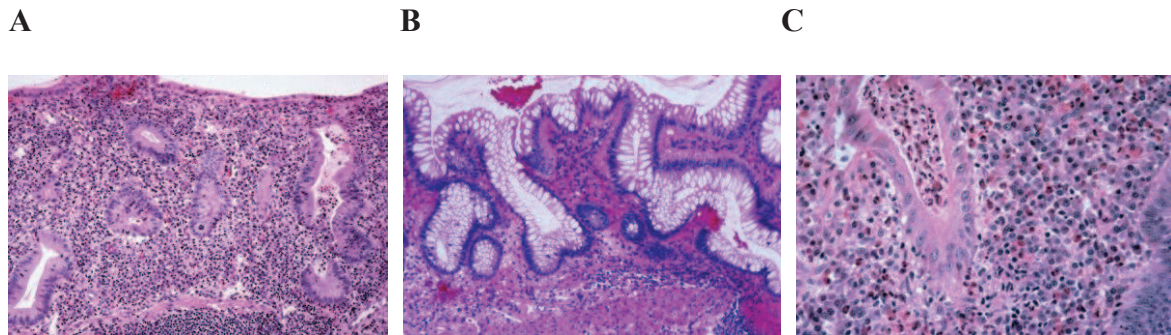


Figure 6.1.2. Histologic appearance of typical UC mucosal tissue. A. Chronic active colitis with dense and diffuse inflammatory infiltrates; B. Chronic quiescent colitis with crypt distortion; C. Neutrophil accumulation in a crypt (crypt abscess) in severe colitis with massive leukocyte infiltration of the *lamina propria*. Pictures taken from: Danese, *Fiocchi Ulcerative colitis. N Engl J Med. 2011 Nov 3;365(18):1713-25. Doi 10.1056/NEJMr1102942.Review.*

The gut immune system is generally tolerant of this microbial load, and a breakdown in tolerance is postulated to be central to the pathogenesis of IBD [197]. Although loss of tolerance to gut microbiota is demonstrable in animal models of IBD, there are none evidence for this finding in patients with ulcerative colitis. It has also been postulated that alterations in the composition of the gut microbiota, defects in mucosal immunity, or the two factors combined could lead to ulcerative colitis; however, supportive evidences are sparse. A key issue is the characterization of the gut microbiota in the normal intestine and in the intestine in patients with IBD. This issue awaits answers from the Human Microbiome Project, which aims to define the composition of the intestinal microbiota in conditions of health and disease [198]. There is a consensus that the density of microbiota is greater in patients with ulcerative colitis or Crohn's disease than in healthy control subjects, but whether there are reproducible, disease-specific alterations is unclear [199]. The fact that antibiotic therapy has no clinical effect on ulcerative colitis argues against an important role of bacteria in this disease, whereas antibiotics do provide some benefit in luminal Crohn's disease. Although serum antibacterial antibodies are present in patients with ulcerative colitis, they are much more common and are found in higher titers in patients with Crohn's disease. Furthermore, the range of antibodies against bacterial antigens is broader in Crohn's disease, whereas the only ulcerative colitis-associated antibody is perinuclear antineutrophil cytoplasmic antibody (pANCA), which recognizes nuclear antigens that may cross-react with bacterial antigens [200].

Intestinal homeostasis requires a controlled innate immune response to the microbiota, which is recognized by toll-like receptors and NOD receptors on epithelial and immune cells [201]. This recognition process contributes to tolerance, but when the

process is dysregulated, inflammation ensues. At present, there is no clear evidence of specific, innate immune defects in ulcerative colitis; an increased expression of TLR2 and TLR4 by colonocytes [202] is probably secondary to inflammation. In contrast, in Crohn's disease, abnormalities of innate immunity are linked to variants of the NOD2, ATG16L1, and IRGM genes, the products of which normally mediate microbial recognition [203, 204]. The production of proinflammatory cytokines, such as IL-1 β , IL-6, TNF- α , and tumor necrosis factor-like ligand 1 (TL1A), is universally increased in patients with IBD. Abnormalities in humoral and cellular adaptive immunity occur in ulcerative colitis. Elevated IgM, IgA, and IgG levels are common in inflammatory bowel disease, but there is a disproportionate increase in IgG1 antibodies in ulcerative colitis [205]. Abnormalities of adaptive immunity that differentiate ulcerative colitis from Crohn's disease are defined by mucosal CD4⁺ T cells, which were initially divided into two lineages: Th1 and type 2 helper T cells (Th2). UC represents an atypical Th2 response, as indicated by the presence of non classical natural killer T cells in the colon that secrete abundant IL-13, which mediates epithelial cell cytotoxicity, apoptosis, and epithelial barrier dysfunction [206]. IL-5-producing Th2-polarized T cells are also present in ulcerative colitis. However, additional helper-cell lineages have recently been delineated, including Th17 cells that produce the pro-inflammatory cytokine IL-17, the levels of which are increased in the mucosa of patients with IBD [207].

Because inflammation in ulcerative colitis typically does not extend into the small intestine and occurs in proximity to the epithelium, colonocytes are implicated in the pathogenesis of this disease. It has been proposed that the epithelium is diffusely abnormal, irrespective of inflammation [208]. Other reported abnormalities in ulcerative colitis include an epithelial-barrier defect and impaired expression of PPAR- γ , a nuclear receptor that regulates inflammatory genes [209]. In both ulcerative colitis and Crohn's disease, epithelial cells have a decreased ability to activate suppressor CD8⁺ T cells, but this abnormality is probably secondary to other immune events [210]. Variants of the XPB1 gene, the product of which is a component of the stress response of the endoplasmic reticulum in epithelial cells, have been linked to IBD, reinforcing the idea that colonocytes are involved in its pathogenesis [211].

Butyrate and ulcerative colitis

Host-microbial homeostasis requires appropriate immune regulation within the gut mucosa, preventing uncontrolled immune responses against the beneficial commensal microbiota, which could potentially lead to inflammatory bowel diseases (IBDs), such as UC [212]. Butyrate, the main SCFA, is an organic acid produced by intestinal microbial fermentation of undigested dietary carbohydrates, specifically resistant starches and dietary fibers, but also in a minor part by endogenous proteins. It is absorbed by the colonic cell and extensively metabolized, constituting the main source of energy [58]. The rate and amount of SCFAs production depends on the species and levels of microbiota present in the colon, the substrate source and gut transit time [58].

At intestinal level butyrate exhibits several effects, for example, on transepithelial ion transport, on cell growth and differentiation, on inflammatory and oxidative status, on non-specific intestinal defense mechanisms and finally on visceral perception and intestinal motility [58].

The importance of butyrate supplementation has been demonstrated by the impaired butyrate metabolism in intestinal inflamed mucosa of patients affected by IBD [213]. In fact, recent data show that butyrate deficiency results from the reduction of butyrate uptake by the inflamed mucosa due to down regulation of the monocarboxylate transporter (MCT)-1 expressed on the apical membrane of intestinal epithelium [214]. Particularly, the reduction of the intracellular availability of butyrate in colonic cells may decrease its protective effects toward cancer in IBD patients [214].

The concomitant induction of the glucose transporter GLUT1 suggests that inflammation could induce a metabolic switch from butyrate to glucose oxidation. Butyrate transport deficiency is expected to have clinical consequences. Particularly, the reduction of the intracellular availability of butyrate in colonocytes may decrease its protective effects against cancer in IBD patients [105]. In spite of these several preclinical and clinical studies analyzing the efficacy of SCFAs mixture or butyrate alone in various models of UC, often the results are contradictory.

To date, several studies have evaluated butyrate effectiveness in several animal model of UC [215, 216]. In humans few studies have been performed probably due to low compliance with the oral route (for its rancid taste) or rectal enemas administration (for its cumbersome application to the patient and irritability due to acid property). Moreover,

rectal administration of butyrate or mixture of SCFAs did not show beneficial effects or displayed only trends towards clinical amelioration [103, 217]. The discrepancy in human studies using enemas may be due to differences in treatment, duration, use of butyrate alone or mixture of SCFAs enemas, and use of several concentrations and volumes of these mixtures. Conversely, other studies reported that fermentable dietary fiber supplementation, which resulted in increased fecal butyrate levels, was effective in maintaining remission in UC, revealing a significant improvement in clinical and inflammatory aspects [218].

Some butyrate-based products are marketed even if their spread is still very limited and greatly understaffed in view of the wide spectrum of possible indications, especially in chronic diseases where it is possible to predict their lasting use. The unpleasant taste and odor make extremely difficult the oral administration of butyrate reducing the compliance.

6.2 COW'S MILK ALLERGY

Food Allergy

Food allergy affects more than 1% to 2% but less than 10% of the population'' and that it remains unclear whether the prevalence is increasing, as confirmed by a comprehensive review of the literature.¹¹ In particular, the Centers for Disease Control reported that the prevalence of food allergy increased from 3.4% to 5.1% between 1997 and 2011 in the United States [219].

Cow's milk (2.2%), peanut (1.8%), and tree nuts (1.7%) were the most common allergens in children, and shellfish (1.9%), fruits (1.6%), and vegetables (1.3%) were the most common allergens in adults. Taking a different perspective using food allergen-specific serum IgE (sIgE) results obtained in the National Health and Nutrition Examination Survey (NHANES) in the United States (2005-2006), Liu et al. [220] estimated clinical allergy to cow's milk, egg, and peanut at 1.8% each in children age 1 to 5 years. The 2 most recent NHANES performed from 2007-2010 with 20,686 US participants included queries on self-reported food allergies. Overall, 8.96% reported food allergy, with 6.53% among children [221].

Cow's milk allergy (CMA) is the most common food allergy of infancy and early childhood with an estimated prevalence of 2-3% worldwide [222]. Berni Canani et al. [223, 224] have demonstrated that dietary management with an extensively hydrolyzed casein formula (EHCF) containing the probiotic *Lactobacillus rhamnosus GG* (LGG) results in a higher rate of tolerance acquisition in infants with CMA. The mechanistic basis for this effect is not known.

Although pediatric patients with CMA may be allergic to various milk protein, casein, β -lactoglobulin (BLG) and α -lactalbumin are considered the cow's milk protein with the greatest antigenic potential [225].

Depending on the nature of the immune response, the pathophysiology of CMA involves IgE-mediated (immediate reactions) and non-IgE-mediated (delayed reactions, mediated by T cells) hypersensitivity reactions (**figure 6.2.1**). IgE antibodies are produced by plasma cells that are derived from lymphocytes B. IgE production is stimulated by a particular subpopulation of helper T cells, TH2 lymphocytes: the differentiation of T cells in this particular subpopulation is stimulated by the interaction with specific antigens,

including the ones on the surface of parasites and helminths and allergens. When T helper cells evolve into TH2 cells, they begin to produce cytokines such as IL-4 and IL-5, which stimulate isotype switching of B cells into antibody-secreting IgE cells. These cells are involved in adaptive or cell-mediated immunity [226]. Interleukin-4 (IL-4), in turn, stimulates the mast cells to release histamine and prostaglandins, triggering the inflammatory process. Clinical manifestations of CMA include skin reactions (eg, urticaria, angioedema, contact dermatitis), respiratory reactions (eg, dyspnea, rhinitis, cough, laryngeal edema, and asthma with severe respiratory distress), and gastrointestinal reactions (such as diarrhea and vomiting, bloody stools, constipation, severe irritability of the colon). IgE-mediated allergic reactions occur within a few minutes up to an hour after exposure to allergens (usually within 10-20 minutes) with a risk of anaphylaxis, while non-IgE-mediated reactions occur within one hour up to several days after ingestion of an allergen [227].

In general, healthy individuals with mature immune system develop tolerance to the food proteins more ingested and commensal bacteria [228]. Patients can show food allergy when the specific oral tolerance to the food eaten is compromised or not properly developed.

Alterations in the microbiota have now been implicated in the pathogenesis of food allergy [229]. Intestinal microbiota influences the network of the immune system and impairs regulatory functions and T_H2 skewing. In human studies, germ-free (GF) conditions are almost impossible, limiting the types of analysis that can be performed, while a role for commensal microbiota in promoting oral tolerance has been clearly defined by using gnotobiotic mice, in which reconstitution of GF mice with well-characterized communities of microbiota or defined bacteria has been performed. Il4raF709 mice carrying a gain of function mutation in IL-4 receptor α -chain, which are susceptible to allergic sensitization and anaphylaxis [230, 231], exhibit an altered gut microbiota signature from that seen in control mice. GF mice reconstituted with these microbiota exhibit allergic sensitization and anaphylaxis. Transfer of antigen-specific Treg cells to Il4raF709 mice is capable of both restoring the normal microbiota and suppressing the allergic responses [231]. A recent study demonstrated a successful reconstitution of mice with human microbiota that resulted in an increase in Treg cell numbers and amelioration of allergic diarrhea [232].

Intriguingly, mice co-housed with or progeny of reconstituted mice with human microbiota also exhibited increased Treg cell numbers [232]. These findings suggest that susceptibility to or protection against food allergy might be a transmissible trait. These murine approaches are powerful tools for dissecting the interaction between the microbiota and disease pathogenesis, opening potential investigations into a myriad of human microbiota that are beneficial or harmful in the treatment and management of allergic conditions.

The implication of butyrate in cow's milk allergy

Allergic infants exhibit an accelerated ecological succession to a community structure more typical of the adult microbiota with a significantly reduced abundance of Lactobacillales and Bifidobacteriales and increased abundance of Clostridiales. This increase in Clostridia is maintained in CMA infants after treatment with extensive hydrolyzed casein formula (EHCF) associated to *Lactobacillus Rhamnosus GG* (LGG) or rice hydrolyzed formula (RHF). When examined at the genus level, treatment with EHCF+LGG, but not with RHF, is associated with a significant enrichment of the butyrate-producing *Clostridia Faecalibacterium* and *Roseburia*. CMA infants treated with EHCF+LGG had correspondingly significantly higher levels of butyrate detectable in their feces than infants treated with RHF, which correlated with an accelerated acquisition of tolerance. Moreover, the murine model showed promising protective effects of induction of oral tolerance on food allergies [233].

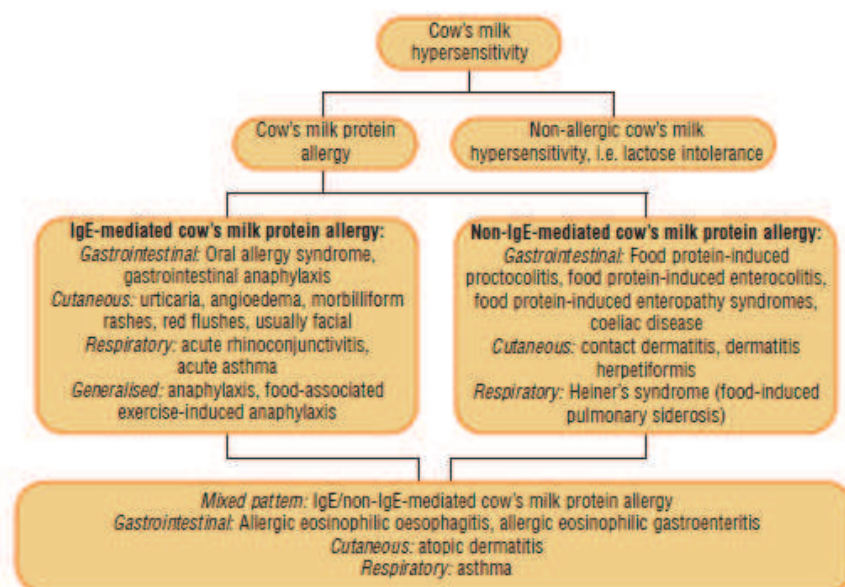


Figure 6.2.1. Difference between two types of cow's milk hypersensitivity. Image taken from Venter, Journal of Family Health 2010.

7. EXTRA-INTESTINAL DISORDERS

7.1 OSTEOARTHRITIS AND CHONDROCYTES

Osteoarthritis (OA), the most prevalent form of arthritis, affects up to 15% of the adult population and is principally characterized by degradation of the articular cartilage of the joint, associated with subchondral bone lesions. Chronic, low-grade inflammation contributes to symptoms and disease progression. Networks of diverse innate inflammatory danger signals, including chemokines, cytokines and alarmins are activated in OA. Besides inflammatory mediators, biomechanical injury and oxidative stress compromise the viability of chondrocytes, leading to hypertrophic differentiation and pro-catabolic responses with further extracellular matrix (ECM) degradation. Better understanding the inflammatory pathophysiology should help identifying different OA subtypes in the population and should lead to the development of new therapeutic options. OA is one of the most prevalent diseases of the elderly and is a top cause of disability. There are few treatment options for OA patients and most of them aim at reducing pain and controlling inflammation to improve function. Non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroid injections are largely used since many years but the current treatment strategies have no impact on the progressive degeneration of joint tissues [234, 235]. Recent studies suggest that disease-modifying treatments are possible.

Similar to the approach that has been successful for rheumatoid arthritis (RA), biotherapies targeting inflammatory mediators such as TNF- α , IL1 or IL6 have been tested. Although these strategies led to a majority of disappointing results [236-238], some biotherapies are still under evaluation. In a recent study using adalimumab (a humanized monoclonal antibody targeting TNF α) induces statistically significant less erosive evolution on the radiological image hand of OA patients with clinical joint swelling [239]. The current data indeed suggest that co-inhibition of several pro-inflammatory cytokines may be more efficient in OA [240]. In this context, mesenchymal stromal/stem cell (MSC)-based therapy seems attractive because this innovative therapeutic strategy could provide an enlarged anti-inflammatory potential. MSCs are immunosuppressive cells, which can decrease inflammation through the release of anti-inflammatory factors (including IL1RA) and decrease monocyte activation [241].

The role of inflammation in OA

Although OA has generally been proposed as a degenerative disease, recent work suggested that low-grade inflammatory processes could promote disease symptoms and accelerate disease progression [242]. Some of the cartilage matrix catabolic products probably activate macrophages and other innate immune cells to release inflammatory cytokines, which in turn promote cartilage damage progression by altering chondrocyte function [243]. The interplay between the immune system and cartilage is not well understood but evidence of regulation of acute-phase response signaling pathway, the complement pathway, and the coagulation pathway in the joint fluid of OA patients has been reported, suggesting a contribution of inflammation to joint damage [244]. However a correlation with the expression level of PGE₂ in the synovial fluid has not been demonstrated. Synovial membranes from patients with OA demonstrate low grade synovitis compared to RA but with high expression of cytokines. OA synovial tissue shows an increase in immune cell infiltrates associated with pro-inflammatory cytokine expression, including TNF α , IL1 β , IL6, IL8 and IL22. Moreover, activation of the innate immune system contributes to the persistence of OA synovial low-grade inflammation. Damage to cells and cartilage ECM resulting from repeated microtrauma and senescence generates damage-associated molecular patterns (DAMPs) that activate the innate immune system through the TLR pathway [245]. DAMPs include fragments generated from ECM degradation such as proteoglycans, intracellular proteins such as heat-shock proteins or DNA. By inducing the release of alarmins (high mobility group box protein 1 S100A8 and S100A9) by monocytes, they contribute to the inflammatory cascade. The inflammatory process activates the release of enzymes by chondrocytes and monocytes resulting in enhanced catabolic process. These enzymes include proteins of A Disintegrin And Metalloproteinase with Thrombospondin motifs (ADAMTS) family and matrix metalloproteinases (MMP)1, 3, 13, which are directly responsible of ECM remodeling. It has also been shown that the joint synovial fluid from OA patients contains a small number of MSCs but their role in OA pathogenesis or cartilage regeneration has yet to be established [246]. OA is therefore an inflammatory musculoskeletal disease involving both innate and adaptive immune response as shown by high levels of pro-inflammatory cytokines and downstream target factors. All these factors are showed in **figure 7.1.1**.

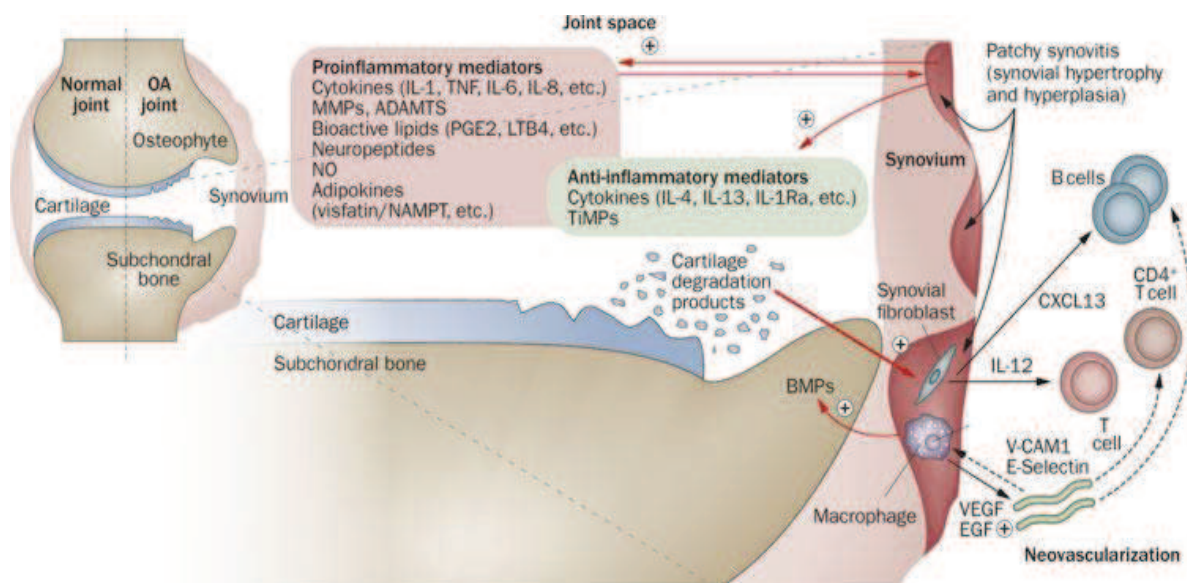


Figure 7.1.1. Osteoarthritis and inflammation. Image taken from *The role of synovitis in pathophysiology and clinical symptoms of osteoarthritis*, Sellam & Berenbaum, *Nature Reviews Rheumatology* 2010, 06, 625-635, doi:10.1038/nrrheum.2010.159.

Inflammatory biomarkers in osteoarthritis

Inflammatory OA is a debilitating and highly prevalent disease, but is often sub-clinical. There is an increasing body of evidence that inflammatory and destructive responses of the synovium play a major role in OA [247]. Moreover, the role of inflammation in the illness of OA has been recognized through the association of joint effusion with joint pain [248]. It is still unclear to what extent inflammation is an initiator vs an outcome of the joint destructive process [249]. Of particular interest is the emerging evidence that the degree to which the immune and wound healing responses can be activated in part controls the predisposition of an individual to chronic diseases, among them OA. Despite the global burden of OA, diagnostic tests and treatments for the molecular or early subclinical stages are still not available for clinical use. It would of great value to be able to readily identify subclinical and/or sub-acute inflammation, particularly in such a way as to be appropriate for a clinical setting. Daghestani et al. [250] reviewed some important types of biomarkers associated with OA in human studies that point to a role of inflammation in OA.

Among these, cytokines are small proteins released from a wide array of cells, including immune cells (macrophages, B lymphocytes, T lymphocytes, mast cells, natural killer cells, epithelial cells, endothelial cells, dendritic cells, fibroblasts, and stromal cells)

[251]. Various cells utilize cytokines, chemokines (chemotactic cytokines), and adipokines (cytokines released by adipose tissue) as part of the inflammatory response to regulate cell signaling and interactions within the cell itself and between other cells [252, 253]. Cytokines can have either pro-inflammatory or anti-inflammatory properties and often work concordantly to maintain cell homeostasis [254]. It has been shown that the concentrations of key SF cytokines (IL-1a, IL-18 and TNF- α) were associated with the level of OA severity; baseline IL-18 also predicted OA progression [255]. Serum levels of IL-6 and TNF- α have also been associated with the prevalence of joint space narrowing and prediction of knee cartilage loss [256]. Serum TNF- α levels have effectively been used to monitor the efficacy of various OA treatments in rabbits [257]. However, in a multi-center, randomized, double blind, placebo-controlled trial, injection of anti-TNF- α agents did not show any improvement in patients with unmanageable hand OA [237]. Soluble TNF- α receptor levels were decreased in the synovial fluid and plasma of patients with OA in comparison with healthy controls, with synovial fluid levels being negatively correlated with joint symptoms [258] evaluated by the Western Ontario and McMaster University Osteoarthritis Index (WOMAC) questionnaire. In a small pilot study, Vangness et al. [259] showed that higher levels of SF IL-2, IL-5, and monocyte chemoattractant protein (MCP)-1 were associated with greater degrees of OA severity. Synovial fluid levels of IL-6, IL-13, and macrophage inflammatory protein (MIP)-1 β were elevated in patients with endstage knee OA while granulocyte colony-stimulating factor (G-CSF) levels were lower when compared to SF levels of healthy controls [260].

Chemokines are a class of small protein cytokines that act as chemoattractants to guide cells to migrate to a specific location, and in the context of inflammation, toward the site of injury or pathogenic invasion [251]. Interferon gamma inducible protein 10, also known as CXCL-10, is a chemokine that has been shown to be inversely associated with radiographic knee OA in both plasma and synovial fluid. Serum fractalkine, or CX3CL1, has been shown to be significantly elevated in patients with knee OA in comparison to the sera from healthy individuals [261]. Synovial CXCL12, but not serum CXCL12, was associated with radiographic severity of OA [262]. A recent study has demonstrated the feasibility of using serum and synovial MCP-1, also known as chemokine ligand 2 (CCL2), as a biomarker for self-reported pain and physical disability in patients with knee OA [263]. To further support the role of macrophages in OA related inflammation, MCP-1

has been shown to increase the recruitment of macrophages into adipose tissue [264] and atherosclerotic lesions [265].

A number of adipose tissue-secreted hormones mediate inflammatory effects [266] and cartilage catabolism in OA [267, 268]. Plasma adipokines adiponectin, which is responsible for modulating metabolic processes, and leptin, a regulator of fat storage, have been shown to be positively associated with joint symptom burden, while the glucose modulating adipokine, adiponectin, was lower in patients with hip and knee OA [269]. Adiponectin is also known as complement factor D and it is responsible for activating the alternative pathway of the complement system [270]. Serum adiponectin levels have also been associated with radiographic hand OA progression after 6-year follow-up [271]. Leptin is believed to play an important role in the pathogenesis of OA via its stimulation of anabolic functions of chondrocytes in cartilage [272]. Serum leptin levels were also associated with prevalent and incident knee OA in the Study of Women's Health Across the Nation (SWAN) cohort [273].

Serum leptin measured at baseline has been associated with increased levels of bone formation biomarkers (osteocalcin and procollagen type I N-terminal propeptide (PINP)) after 2-year follow-up [274]. In addition, baseline soluble leptin receptor was associated with lower levels of procollagen type II N-terminal propeptide (PIIANP) and decreased cartilage volume [274]. Visfatin (also known as nicotinamide phosphoribosyltransferase (Nampt)), which is an adipokine and enzyme involved in the metabolism of nicotinate and nicotinamide, has recently been suggested to be a therapeutic target for OA given its role in chondrocyte and osteoblast activation [275]. As an inflammatory biomarker, levels of synovial visfatin were significantly greater in patients with knee OA compared to control subjects [276]. In addition, synovial visfatin was positively correlated with the cartilage degradation markers collagen II and aggrecan 1 and 2 and was significantly elevated in more severe knee OA grades (Kellgren Lawrence 4 vs 3) [276]. In middle-aged women, lower levels of serum estradiol, which is a hormone that has anti-inflammatory properties [277], and the urinary estrogen metabolite 2-hydroxyestrone were associated with both the prevalence and incidence of knee OA [278].

The role of chondrocyte in osteoarthritis

OA is a progressive degenerative disease characterized by gradual loss of articular cartilage. Since the OA lesion is often localized to weight-bearing cartilage or to sites of trauma, repetitive mechanical injury has been proposed as the critical signal for the initiation and progression of OA. It is now generally accepted that the chondrocyte is the target of these abnormal biomechanical factors, and that biochemical and genetic factors also contribute to alterations in the normal functional activities of these cells.

Even in the absence of classic inflammation, which is characterized by infiltration of neutrophils and macrophages into joint tissues, elevated levels of inflammatory cytokines have been measured in OA synovial fluid. Although the OA cartilage lesion is present at sites remote from the synovium, the fibroblast- and macrophage-like synovial cells, as well as the chondrocyte itself, are potential sources of cytokines that could induce chondrocytes to synthesize and secrete cartilage degrading proteases, cytokines, and other inflammatory mediators [279]. These synovium- and chondrocyte-derived products represent potential targets for the development of therapeutic agents, such as proteinase inhibitors, cytokine antagonists, and cytokine receptor blocking antibodies, which could be used to prevent or retard the progression of the OA articular lesion.

Biochemical and genetic factors, as well as mechanical stress, contribute to the OA lesion in cartilage by disrupting chondrocyte–matrix associations and altering metabolic responses in the chondrocyte [280]. In early OA, the chondrocyte exhibits a transient proliferative response (clonal growth), increased synthesis of cartilage matrix as an early attempt at repair, and increased synthesis of catabolic cytokines and matrix degrading enzymes. Catabolic cytokines may also be generated by the fibroblast- and macrophage-like cells in the synovium in response to breakdown products from the damaged cartilage. Local loss of proteoglycans and cleavage of type II collagen occur initially at the cartilage surface, resulting in an increase in water content and loss of tensile strength in the cartilage matrix.

The involvement of chondrocyte-derived MMPs in the degradation of cartilage collagens and proteoglycans in OA is well established. The development of antibodies that detect specific cleavage epitopes has permitted the analysis of degradation products of type II collagen and aggrecan in synovial fluid and cartilage of OA patients [281-283]. MMPs have been localized in regions of cartilage degradation [284], and they are elevated in synovial fluid and cartilage from OA patients [282, 285]. The levels of the tissue inhibitor

of metalloproteinases 1 (TIMP-1) are also increased in OA synovial fluid and correlate with the MMP levels [282, 285], possibly reflecting an endogenous adaptive response to the increased levels of active proteinase activities. However, the progressive degradation of the cartilage matrix that occurs in OA indicates that there is a local imbalance in the proteinase/inhibitor content [286].

Relationship among inflammation, obesity, the gut microbiota, and metabolic osteoarthritis

Emerging evidence suggests that intrinsic inflammatory mediators secreted by body fat, or adipose tissue, including cytokines, adipokines, and advanced glycation end products, may be sufficient to lead to onset and progression of OA [287]. It appears that these obesity-associated, intrinsic inflammatory factors define a metabolic subtype of OA [288, 289]. Characterizing the factors that comprise this unhealthy metabolic phenotype is critical to understanding the influence of obesity on OA. Furthermore, establishing the “indirect” role of the microbiota and the gut is required to fully understand the initiators and drivers of metabolic OA.

Experimental high fat diets (45-60% of energy derived from lard-based fat rich in saturated fatty acids) have been associated with an increase in OA independent of gains in body weight [290, 291]. However, these diets do not accurately represent the typical human Western-type diet, as the high percentage of fat (>50% kcal from fat) would be considered extreme. Rather, the obesity epidemic in North America appears driven by processed foods high in fat and simple carbohydrates, which is better modeled by a high fat/high sucrose (HFS) diet [292].

When fed a HFS diet, Sprague Dawley rats, as well as Wistar rats and C57BL/6C mice, exhibit obesity prone (top ~50% of weight gainers) and obesity resistant (bottom ~50% of weight gainers) phenotypes, where obesity resistant animals grow normally and the prone become obese [293]. This feature allows for experimental evaluation where in animals are all exposed to the same obesogenic diet but develop disparate body weight [293]. Moreover, the effect of HFS diet-induced obesity on OA in prone and resistant animals was experimentally evaluated [294]. Although the extent to which inflammatory mediators contribute to metabolic OA remains unknown, several cytokines and adipokines detected in serum and synovial fluid of obese animals have been implicated [291, 295, 296]. In

particular, leptin is elevated in serum and synovial fluid of obese individuals and animals with OA [272]. However, the detailed molecular origins of such low-grade systemic inflammation remain unknown [297]. Systemically, visceral adipocytes, macrophages, and adipose tissue mast cells have been suggested to contribute to this chronic inflammatory state [298]. In knee joints, the infrapatellar fat pad and synovial fluid are thought to contribute to the local inflammatory environment [268, 299]. Recently, a link between low-grade inflammation and changes in the composition and activity of the microbes that reside in the gastrointestinal tract, collectively termed the gut microbiota, has been established. Notably, high fat diets enhance translocation of the bacterial membrane component lipopolysaccharide into the bloodstream, initiating obesity and insulin resistance [300]. New evidence suggests that the gut microbiota, through activating innate immune responses that lead to systemic inflammation, represent a possible mechanistic link to metabolically induced OA [301].

Butyrate, as histone deacetylase inhibitor and its role in OA

Acetylation and deacetylation of nucleosomal histones play an important role in the regulation of gene expression [302]. The histone acetylation status is controlled by the opposing actions of two classes of enzymes: histone acetyl transferases (HATs) and histone deacetylases (HDACs). Acetylation of histones loosens nucleosomal structures, thereby promoting gene transcription. In contrast, deacetylation of histones stabilizes nucleosomal structures and represses gene transcription [302]. However, emerging evidence indicates that gene regulation by acetylation/ deacetylation is more dynamic and complex, and that HATs can act as repressors and HDAC as activators of transcription. Indeed, global analysis of gene expression has shown that inhibition of HDAC activity results both in induction and repression of gene expression [303, 304].

In recent years, significant interest has emerged in the inhibition of HDAC activity as a possible anti-cancer treatment. HDAC inhibitors induce growth arrest, differentiation and apoptosis of cancer cells in vitro and reduce the growth of experimental tumors in vivo [305]. Presently, several HDAC inhibitors are in clinical trials for the treatment of solid and hematological tumors [306, 307]. In addition to their anti-cancer effects, recent studies have demonstrated that HDAC inhibitors modulate inflammatory responses. For instance, HDAC inhibitors reduce the production of IL-1, tumor necrosis factor- α (TNF- α), and

interferon- γ (IFN- γ) in lipopolysaccharide (LPS)-stimulated human peripheral blood mononuclear cells [308, 309]. Likewise, HDAC inhibitors prevent LPS-induced production of TNF- α , IL-6 and reactive oxygen species in neuroglia cultures, and primary microglia [124, 310, 311]. HDAC inhibitors have also been reported to suppress IL-12 production in dendritic cells and macrophages [312]. Moreover, it has been partially demonstrated whether HDAC inhibitors regulate inflammatory responses in articular chondrocytes [313].

7.2 WOUND HEALING

Skin ulcers are one of the most common diseases in the worldwide healthcare sector. The ulcer is clinically well defined as chronic wound, one of the most common ailments, which seriously affects the life quality of the patient and creates a huge financial burden for the health system. Ulcers are caused by chronic injury from a variety of events such as trauma, exposure to heat, cold, corrosive material, or radiation, and blood circulation problems. It is estimated that in the socially evolved countries the appearance of chronic skin ulcers interests a minimum of 1.5% up to a maximum of 3% of the population, with an index of hospitalizations of 0.4%, and of the days hospitalization of approximately 1% [314]. In Italy it is estimated that ulcers affect approximately two million of people, thus assuming the contours of a social disease. Skin ulcers are characterized by loss of skin, superficial and deep dermis and subcutaneous tissue, they tend to recur and difficultly to heal spontaneously. The ulcers represent a significant risk factor for hospitalization, amputation, sepsis and mortality. From the point of view of the patient the therapy of the lesions is often painful and uncomfortable [315].

It is well known that the wound healing involves a series of processes that require the interaction of cytokines and growth factors, produced by different highly specialized cells. Many factors can prevent the healing of an ulcer and determine the development of an ulcer lesion with associated and high morbidity. Among these may be poor nutrition, metabolic disorders such as diabetes. During normal wound healing, it is possible to identify four overlapping phases: inflammation, granulation tissue formation, epithelialization and remodeling. When these phases are delayed for more than a couple of weeks, there is a slow healing, as happens for example in diabetic foot ulcers [316] or, more generally necrotic ulcer [317].

Inflammation in Wound Repair

Wound healing is a highly dynamic process and involves complex interactions of extracellular matrix molecules, soluble mediators, various resident cells, and infiltrating leukocyte subtypes. The immediate goal in repair is to achieve tissue integrity and homeostasis [318]. To achieve this goal, the healing process involves three phases that overlap in time and space: inflammation, tissue formation, and tissue remodeling (**figure 7.2.1**) [319]. During the inflammatory phase, platelet aggregation is followed by infiltration of leukocytes into the wound site. In tissue formation, epithelialization and newly formed granulation tissue, consisting of endothelial cells, macrophages and fibroblasts, begin to cover and fill the wound area to restore tissue integrity. Synthesis, remodeling, and deposition of structural extracellular matrix molecules, are indispensable for initiating repair and progression into the healing state.

Cellular responses to injury involve direct cell–cell and cell–matrix interactions, as well as the indirect crosstalk between different cell populations by soluble mediators. Indeed, complex interactions between the epidermal and dermal compartment are essential.

During the past decade numerous factors have been identified that are engaged in a complex reciprocal dialogue between epidermal and dermal cells to facilitate wound repair [320]. The sensitive balance between stimulating and inhibitory mediators during diverse stages of repair is crucial to achieving tissue homeostasis following injury. The inflammatory response is regarded as the first of a number of overlapping processes that constitute wound healing. In skin repair, the infiltrating leukocytes are the principal cellular components of the inflammatory response. They are not only effector cells combating invading pathogens but are also involved in tissue degradation and tissue formation. As such, an excessive or reduced influx or activation of infiltrating leukocytes into the damaged tissue may have profound effects on downstream cell migration, proliferation, differentiation, and ultimately the quality of the healing response. Continuing progress in understanding the essential and complex role of the inflammatory response in wound repair will provide strategies to modulate diseases with pathologic tissue remodeling, such as healing disorders, various chronic inflammatory disease states, and cancer. Tissue injury causes the immediate onset of acute inflammation. It has long been considered that the inflammatory response is instrumental to supplying growth factor and

cytokine signals that orchestrate the cell and tissue movements necessary for repair [321], as shown in **figure 7.2.2**. In various experimental animal models and human skin wounds, it has been demonstrated that the inflammatory response during normal healing is characterized by spatially and temporally changing patterns of various leukocyte subsets [318, 322]. The well-defined chronology of these events is essential for optimal repair.

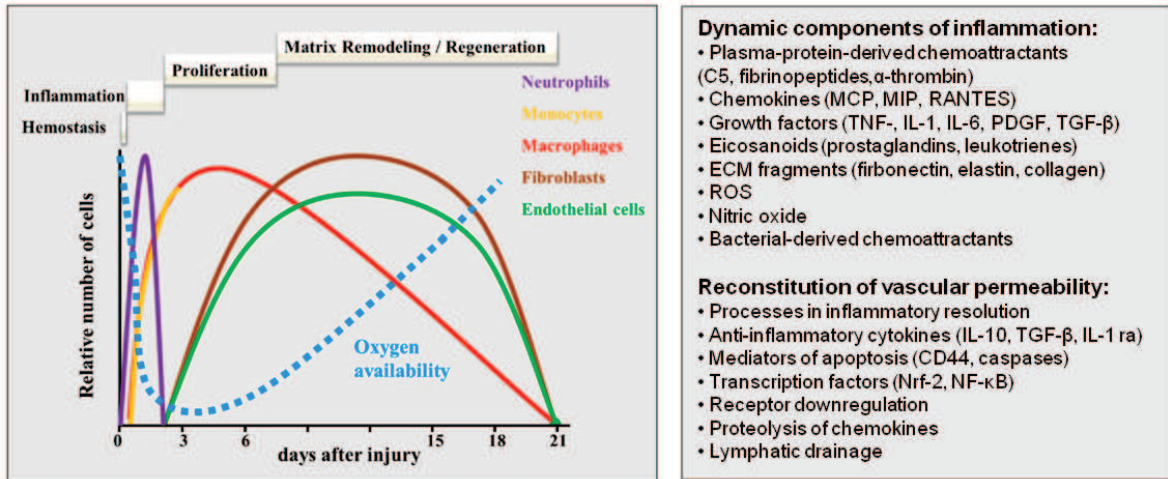


Figure 7.2.1. Mediators and mechanisms of inflammation and inflammatory resolution in repair. Tissue injury causes the immediate onset of acute inflammation mediated by chemoattractants derived from plasma proteins, resident and recruited hematopoietic cells, extracellular matrix and bacteria. Progression to complete wound healing is accompanied by resolution of the inflammatory response, which is essential for successful repair. Resolution of inflammation is directed by downregulation of proinflammatory mediators and the reconstitution of normal microvascular permeability, which contributes to the cessation of local chemoattractants, synthesis of anti-inflammatory mediators, apoptosis, and lymphatic drainage. An excessive or prolonged inflammatory response results in increased tissue injury and poor healing. Successful wound repair requires the coordinate expression of both inflammation and resolution of inflammation. (Image taken from *Journal Of Investigative Dermatology*, Eming et al., 2007)

Successful repair after tissue injury requires resolution of the inflammatory response. However, whereas the knowledge about mechanisms and molecules inducing and perpetuating the inflammatory response is constantly increasing, mechanisms, that limit and down regulate this activity are less appreciated. Such mechanisms might include: downregulation of chemokine expression by anti-inflammatory cytokines such as IL-10 [323] or TGF- β 1 [324], or upregulation of anti-inflammatory molecules like IL-1 receptor antagonist or soluble TNF receptor; resolution of the inflammatory response mediated by the cell surface receptor for hyaluronan CD44 [325]; apoptosis [326], receptor unresponsiveness or downregulation by high concentrations of ligands (**figure 7.2.3**). Interestingly, recent in vitro data suggested that MMPs can downregulate inflammation via cleavage of chemokines, which then act as antagonists [327].

Cell type	Functions	Mediators
PMN	Phagocytosis of infectious agents	ROS, cationic peptides, eicosanoids, proteases (elastase, cathepsinG, PR-3, and uPA)
	Mφ activation through phagocytosis	
	Amplify inflammatory response	TNF-α, IL-1β, IL-6
	Stimulate repair response	VEGF, IL-8
Mφ	Phagocytosis of PMN and fragments of tissue degradation	
	Amplify inflammatory response	TNF-α, IL-1β, and IL-6
	Anti-inflammatory function	IL-10, TGF-β1
	Stimulate repair response: angiogenesis, fibroplasia	PDGF, VEGF, bFGF, TGF-α, and TGF-β
	Fibrolysis	t-PA, uPA, u-PAR, and PAI-1/2
MC	Control vascular permeability	Histamine
	Control influx of PMN	Chymase, tryptase
	Regulate tissue remodeling	
T cell; Th1/Th2	Regulate tissue remodeling	CD40 ligand; IL-2, TNF-α/IL-4, -5, -10
γδT cells	Keratinocyte proliferation, differentiation, hyaluronan synthesis in Kc	FGF-7, FGF-10, and IGF-1

bFGF, basic fibroblast growth factor; FGF, fibroblast growth factor; MC, mast cell; PDGF, platelet-derived growth factor; PMN, polymorphonuclear leukocyte; ROS, reactive oxygen species; TGF, transforming growth factor; TNF, tumor necrosis factor; uPA, urokinase-type plasminogen activator; VEGF, vascular endothelial growth factor.

Figure 7.2.2. Inflammatory cells, their functions and mediators released in tissue repair. (Image from *Journal Of Investigative Dermatology*, Eming et al., 2007)

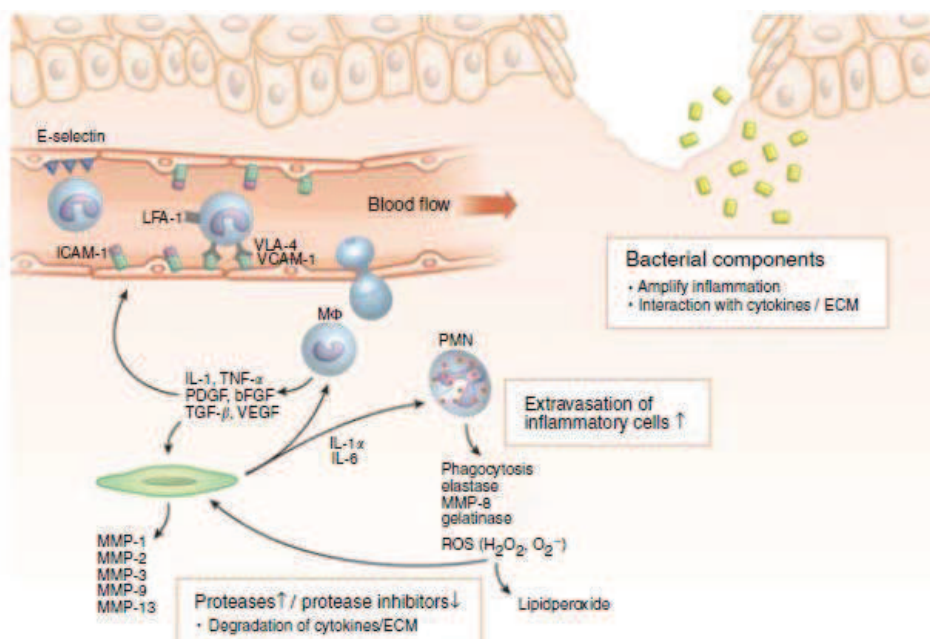


Figure 7.2.3. Model of multifactorial molecular and cellular mechanisms deleterious in tissue repair. Chronic wounds fail to progress through the normal pattern of wound repair, but instead remain in a state of chronic inflammation predominantly characterized by abundant PMN and MF infiltration. Persisting inflammatory cells play a major role in the generation of proinflammatory cytokines (IL-1, TNF-α, and IL-6) and a protease rich and pro-oxidant hostile microenvironment. Increased proteolytic activity (neutrophil elastase, MMP-8, and gelatinase) leads to degradation of growth factors and structural proteins of the extracellular matrix crucial for repair. Increased ROS (H₂O₂, O₂⁻) can lead to direct damage of cells or extracellular matrix molecules, or contribute to increased expression of MMPs (MMP-1, -2, -3, -9, and 13). Bacterial components (extracellular adherence protein (Eap), formyl methionyl peptides, N-acetylmuramyl-L-alanyl-D-isoglutamine) may contribute to impaired repair mechanisms of the host by interference with cell-matrix interactions or promoting the inflammatory response. (Image taken from *Journal Of Investigative Dermatology*, Eming et al., 2007)

However, the relevance of these mechanisms for cutaneous tissue repair has to be further investigated. In recent studies, Nrf-2, a target of the keratinocyte growth factor-1 was identified as novel transcription factor regulating the inflammatory response during repair. The wound healing response in Nrf-2 knockout mice was characterized by a prolonged inflammatory response following wound closure, which was most likely mediated by prolonged expression of IL-1 β and TNF- α [328].

The role of butyrate on Wound Healing

SCFAs (propionate, butyrate and valerate) derive from the bacterial fermentation of oligo-polysaccharides and a small part of proteins, peptides and glycoproteins by the normal intestinal saprophytic flora (especially at the level of the colon). A derivative of butyrate, butyryl-glycerol, seems to have angiogenic activity [329], and the topical application of short chain fatty acids facilitates the healing of intestinal ulcers [330, 331].

The mechanisms of action of butyric acid, which contribute to the wound healing, may include the ability of butyrate to stimulate the platelet-derived growth factor (PDGF), which has an important function in wound healing. It was also demonstrated that butyrate is able to block the production or signaling of TNF- α and of the factor TGF β in a variety of systems [99, 319, 332]. Butyrate may also down-regulate MMPs, and it can modify the gene expression of MMPs [333].

The butyrate, besides being a source of energy to epithelial cells, may have potential anticancer and anti-inflammatory properties, may also have effects on the intestinal barrier and play a role in the sense of satiety and oxidative stress [72].

Some factors related to the physic-chemical properties of butyric acid invalidated the assumption of this substance. It is also important to remember that butyric acid has a strong smell of rancid cheese, it is liquid at room temperature and in time undergoes degradation phenomena that alter the stability.

EXPERIMENTAL SECTION

CHAPTER 3

8. MATERIAL AND METHODS

8.1 *IN VIVO* MODELS

8.1.1 DEXTRAN SODIUM SULPHATE-INDUCED COLITIS IN MICE

DSS-induced colitis and animal treatments

Experimental colitis was induced in ten weeks old BALB/c male mice (25 ± 2 g) (Harlan-Corezzano, Italy) by 2.5% DSS (wt/vol) (MP Biomedicals, Irvine, CA) in sterile drinking water *ad libitum* for five days, followed by drinking water without DSS for twelve days. Mice were randomly divided into six groups (n=10 each group) as following: 1. a control animals (CON), 2. DSS treated mice (DSS), 3. DSS mice treated with sodium butyrate as preventive therapy (PREV Butyrate), 4. DSS mice treated with N-(1-carbamoyl-2-phenylethyl) butyramide (FBA) as preventive therapy (PREV FBA) 5. DSS mice treated with sodium butyrate as curative therapy (CUR Butyrate). 6. DSS mice treated with FBA as curative therapy (CUR FBA). We have recently obtained a high palatable synthetic butyrate derivative, N-(1-carbamoyl-2-phenylethyl) butyramide (FBA; Italian patent RM2008A000214; April 21, 2008). FBA is present in a solid, poorly hygroscopic, easily weighable form, stable to acids and alkalis and capable of releasing butyric acid at small and large bowel level in a constant manner over time. This product has demonstrated a toxicological profile comparable to that of butyrate; it shows physicochemical characteristics distinctly more suitable for extensive clinical use than those of butyrate. A particular aspect of FBA is that it does not present the unpleasant odour of butyrate and is practically tasteless, thus making possible to overcome the main limitation to the use of butyrate in the therapeutic field, namely its very poor palatability. Moreover, the solubility of FBA in water is satisfactory in that it produces clear solutions up to the concentration of 0.1 M and suspensions for higher concentrations. The oral

treatment with Butyrate (20 mg/kg/die) or FBA (42.5 mg/kg/die), started 7 days before DSS challenge (PREV) or two days after (CUR) DSS challenge and continued for all experimental period (20 days). DSS group without pharmacological treatments received H₂O+Tween20 0.01% as both drug's vehicle. Colitis was assessed by the daily monitoring of body weight, stool consistency and fecal blood. All procedures involving animals were carried out in accordance with the Institutional Guidelines and complied with the Italian D.L. no.116 of January 27, 1992 of Ministero della Salute and associated guidelines of the European Communities Council Directive of November 24, 1986 (86/609/ECC). Prior to sample collection, animals, kept overnight fasted, were euthanized by an isoflurane anesthesia, followed by cervical dislocation to minimize pain. All efforts were made to minimize animal suffering. Blood samples from animals were collected by cardiac puncture and serum obtained. At 20th days mice were killed and full intestine and colons measured. Colon tissue was excised and immediately frozen. Segments of colon were assessed histologically by hematoxylin and eosin staining. In **figure 8.1.1a** the scheme of experimental protocol and animal treatments are summarized.

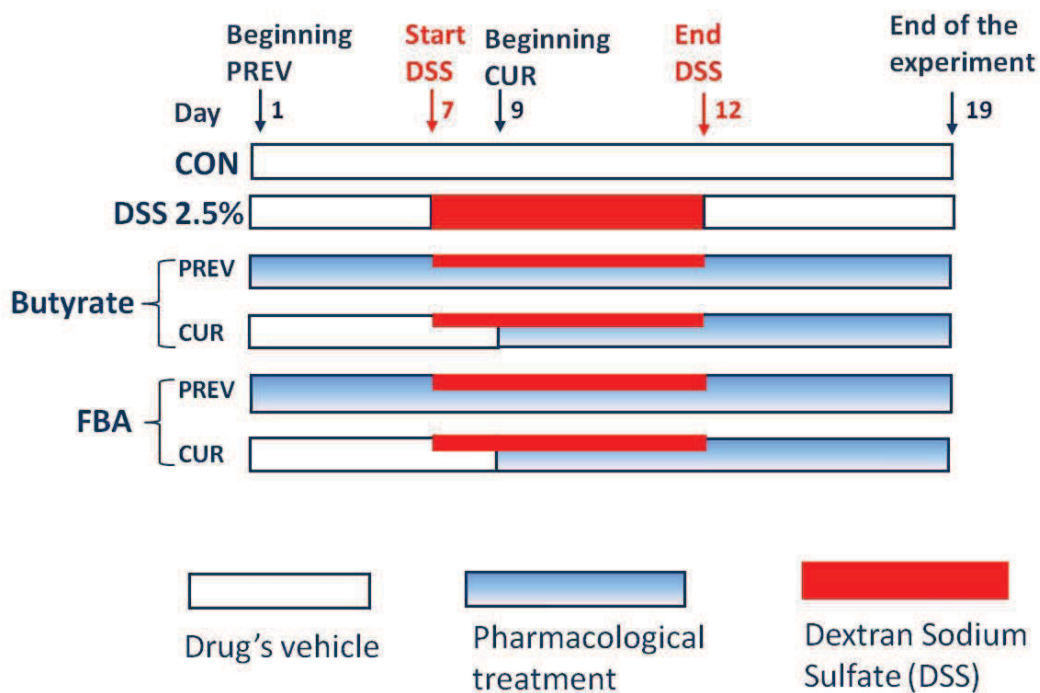


Figure 8.1.1a. Experimental protocol used in this study

Evaluation of experimental colitis

In all animals, weight, presence of blood and stool consistency were determined daily as previously described [334]. Disease activity index (DAI) was determined by combining scores of a) weight loss b) stool consistency and c) bleeding (divided by 3). Each score was determined as follows, change in weight (0:<1%, 1: 1–5%, 2: 5–10%, 4:>15%), stool blood (0: negative, 2: positive) or gross bleeding (4), and stool consistency (0: normal, 2: loose stools, 4: diarrhea) as previously described [335]. Body weight loss was calculated as the percent difference between the original body weight and the actual body weight on any particular day. Typically in DSS colitis animals will lose 10–15% body weight over the course of 10 days. The appearance of diarrhea is defined as mucus/fecal material adherent to anal fur. The presence or absence of diarrhea was scored as either 1 or 0, respectively, and the cumulative score for diarrhea was calculated by adding the score for each day and dividing by the number of days of exposure. Rectal bleeding was defined as diarrhea containing visible blood/mucus or gross rectal bleeding and scored as described for diarrhea.

Measurement of MPO activity

Proximal colonic tissues were homogenized twice for 30s at 4°C in 0.5% hexadecyltrimethylammonium bromide (HTAB) in 50 mM sodium phosphate, pH 6.0, according to Bradley et al., [336]. The homogenates were clarified by centrifugation at 13000xg for 15 min, at 4°C, and were assessed for MPO activity in 3 ml 50 mM sodium phosphate, pH 6.0, containing 16 mM aqueous guaiacol and 5.9 mM H₂O₂, as previously described [337]. The increase in absorbance was measured for 2 min at 470 nm using The iMark microplate absorbance reader (Bio-Rad). Protein concentrations were determined using the Bio-Rad protein assay. MPO activities were expressed in U.MPO/mg protein with 1 U hydrolyzing 1 μmol H₂O₂/min.

Real-time semi-quantitative PCR

Total RNA isolated from colon was extracted using TRIzol Reagent (Invitrogen Biotechnologies), according to the manufacturer's instructions. cDNA was synthesized using a reverse transcription kit (Maxima First Strand cDNA Synthesized Kit, Fermentas,

Ontario, Canada) from 2 µg total RNA. PCRs were performed with Bio-Rad CFX96 Connect Real-time PCR System instrument and software (Bio-Rad Laboratories). The primer sequences for GPR43, IL-10, IL-6, TNF- α , Occludin and ZO-1 were purchased by Eurofins MWG Operon (Huntsville, AL, USA) and are reported in **Table 1**. For Annexin A1 (AnxA1), Ccl2, CD14, CD68, Fpr1, Fpr2, GAPDH, Ly-6G and NOS2 we used QuantiTect[®] Primer Assays for SYBR Green by Qiagen. The PCR conditions were 15 min at 95°C followed by 40 cycles of three-step PCR denaturation at 94°C for 15 s, annealing at 55 or 60°C for 30 s and extension at 72°C for 30 s. Each sample contained 40-100 ng cDNA in 2X Power SYBRGreen PCR Master Mix (Applied Biosystem) and 200 nmol/l of each primer in a final volume of 25 µl. The relative amount of each studied mRNA was normalized to GAPDH as housekeeping gene, and the data were analyzed according to the $2^{-\Delta\Delta CT}$ method.

Table 1. Real-Time PCR Primer Sequence

Target gene	Forward primer (5'→3')	Reverse primer (3'→5')	Accession Number
GPR43	TTCTTACTGGGCTCCCTGCC	TACCAGCGGAAGTTGGATGC	NM_146187
HDAC9	GCGGTCCAGGTAAAAACAGAA	GCCACCTCAAACACTCGCTT	NM_001271386.1
IL-10	GGTTGCCAAGCCTTATCGGA	ACCTGCTCCACTGCCTTGCT	NM_010548.2
IL-6	ACAAGTGGGAGGCTTAATTACACAT	TTGCCATTGCACAACCTTTTTC	NM_031168.1
MCT-1	GAGCGCGCGAAGCTGCATTTGCT	TGCTCCCAGGCCCGCTTTACA	NM_009196.3
PPAR-γ	CTGCTCAAGTATGGTGTCCATGA	ATGAGGACTCCATCTTTATTCA	NM_001127330.1
Occludin	ATGTCCGGCCGATGCTCTCTC	CTTTGGCTGCTCTTGGGTCTGTAT	NM_008756.2
TNF-α	CATCTTCTCAAACTCGAGTGACAA	TGGGAGTAGATAAGGTACAGCCC	NM_012675.3
ZO-1	ACCCGAAACTGATGCTGTGGATAGA	AAATGGCCGGGCAGAAGCTTGTGTA	NM_001163574.1

Immunofluorescence analysis of GPR43

Colonic and liver tissue samples for immunofluorescence were embedded in O.C.T. (Pelco Cryo-Z-T, Ted Pella inc, Redding, California), and cryosectioned (10 µm thick). Tissue sections were then fixed in 4% paraformaldehyde for 10 min at room temperature (RT) and washed in TBS with 0.05% TX-100. For immunofluorescence detection of Ly-6G, sections were blocked with 10% FCS in TBS for 30 minutes at RT and then incubated with a monoclonal antibody anti Ly-6G-FITC (BD Biosciences) overnight at 4°C. To

examine co-localization of AnxA1 and GPR43 with Ly-6G, rabbit monoclonal anti-AnxA1 antibody [338] and goat polyclonal anti-GPR43 antibody (Santa Cruz Biotechnology, Inc.) were incubated alongside anti Ly-6G-FITC antibody overnight at 4°C. Sections were washed in 1% FCS plus 0.025% TX-100 and incubated with Alexa-Fluor[®] 546 goat anti-rabbit IgG (for AnxA1) and with Alexa-Fluor[®] 594 donkey anti-goat IgG (for GPR43) 1 h at RT. After incubation with secondary antibody, sections were washed in TBS, and then incubated with DAPI to visualize nuclei. Slides were mounted in mounting medium (Vectashield; Vector Laboratories, Burlingame, California, USA). Fluorescence was visualized on a Olympus BX51 fluorescence microscope (Olympus) equipped with a DS-QiMc monochromatic camera (Nikon) and X-Cite[®] Series 120Q Xenon lamp. NIS-Elements BR3.1 software (Nikon) was used for all analyses. Merge images were performed with ImageJ software. Two negative controls were used: slides incubated with or without primary antibody. Images were recorded at identical gain settings, performed in duplicate in non-serial distant sections, and analyzed in a double-blind manner by two different investigators. Four image fields were taken of each section.

Serum adiponectin detection

Whole blood taken through cardiac puncture was centrifuged after 24h at 1500xg at 4°C for 15 min. So serum obtained was stored at -80°C and then used for adiponectin levels detection by Enzyme Linked Immuno Sorbent Assay (ELISA) (Quantikine[®] Immunoassay, RD & SYSTEMS, Minneapolis, MN) following manufacturer's instructions.

Statistical analysis

Data are presented as mean \pm SEM. Statistical analysis was performed by ANOVA test for multiple comparisons followed by Bonferroni's test, using Graph-Pad Prism (Graph-Pad software Inc., San Diego, CA). Statistical significance was set at $P < 0.05$.

8.1.2 FOOD ALLERGY MODEL

Experimental design

Female specific pathogen free C3H/HeO_uJ mice were purchased from Charles River Laboratories Italia (Calco, Lecco, Italy), maintained on cow's milk protein free standard mouse chow and housed in the animal facility at the Department of Pharmacy, University of Naples "Federico II". All experiments were performed in accordance with the Institutional Animal Care and Use Committee of the University of Naples "Federico II". Mice were treated by daily gavage with 20 mg/kg/day of sodium butyrate (Sigma-Aldrich, Steinheim, Germany) beginning at two weeks prior to sensitization and continuing throughout the sensitization protocol. Sensitization to the cow's milk protein β -lactoglobulin was performed according to a previously established protocol, with slight modifications [226]. Briefly, 5 week old mice received 20 mg of β -lactoglobulin (BLG, Sigma-Aldrich) in 0.2 ml of PBS mixed with 10 μ g cholera toxin (CT) (Sigma-Aldrich) intragastrically on day 0, 7, 14, 21, and 28; control mice received CT in PBS alone. The experimental design is described in **figure 8.1.2a**.

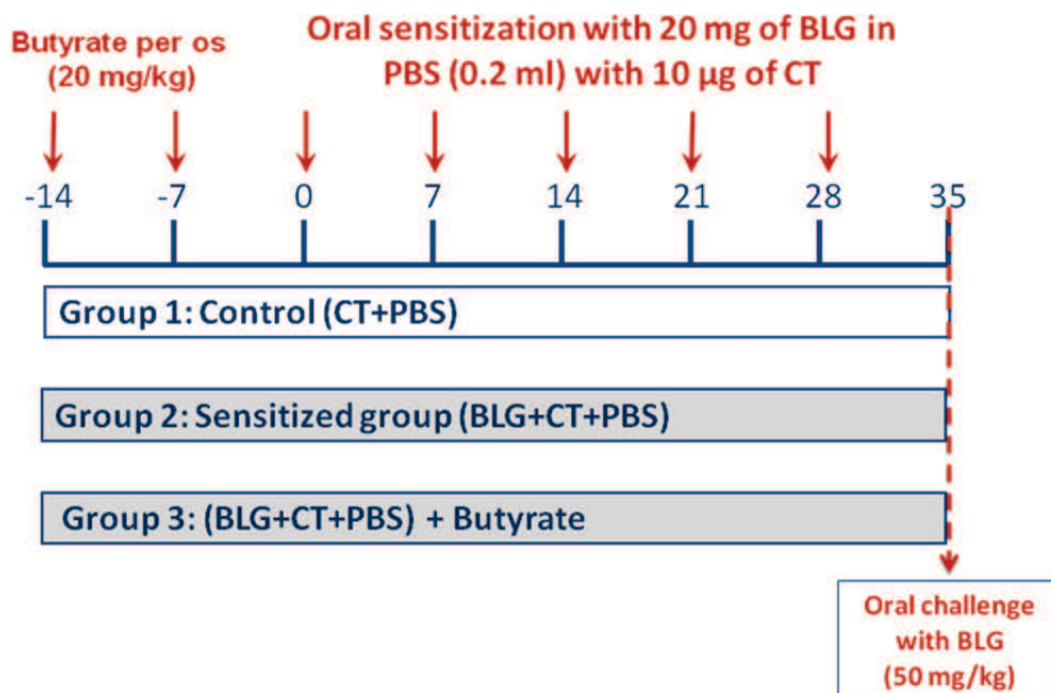


Figure 8.1.2a. Experimental protocol of sensitization with BLG and of butyrate treatment

Acute skin response and hypersensitivity symptoms

One week after the last sensitization, the acute allergic skin response was determined at 1 h after intra-dermal challenge with 10 µg BLG in the ear pinnae [339]. Ear thickness was measured in duplicate using a digital micrometer (Mitutoyo Italiana Srl, Milan, Italy). Allergen-specific net ear swelling was calculated by correcting the allergen-induced increase in ear thickness with the baseline value obtained before intra-dermal challenge. The ear swelling is expressed as delta micrometers. On the day after acute allergic skin response evaluation the same mice were challenged intragastrically with 0.2 ml of BLG (50 mg). One hour following the oral BLG challenge, hypersensitivity symptoms were scored by a person blind to the study, using a scoring system previously described [339]. The scores were as follows: 0 = no symptom; 1 = scratching and rubbing around the nose and head; 2 = reduced activity; 3 = activity after prodding and puffiness around the eyes and mouth; 4 = no activity after prodding, labored respiration, and cyanosis around the mouth and the tail; and 5 = death. Within 2h later, blood samples were collected, centrifuged for 15 min at 20,000g and stored at -70°C for measurement of BLG specific IgE by ELISA assay.

Measurement of BLG-specific IgE

Serum IgE anti-β-lactoglobulin (BLG) levels were measured by ELISA as previously described [340]. Briefly, 96-well plates were coated with 1 µg/ml of BLG in 0.05 M sodium carbonate-bicarbonate coating buffer. After overnight incubation at 4°C, the plates were washed 3 times with 50 mM Tris, 0.14 M NaCl, 0.05% Tween 20 and blocked with 50 mM Tris, 0.14 M NaCl, containing 1% bovine serum albumin for 1 hour. The plates were washed repeatedly prior to addition of serum samples and incubation for 2 hours at 37°C. Plates were then washed four times and 100 µl of biotin-labeled rat anti-mouse IgE antibody (BD, Pharmingen) was added for 1 hour at 37°C. The plates were washed again prior to incubation with streptavidin-horseradish peroxidase (Dako, Denmark) for 1 h. The plates were developed by the addition of 3, 3'-5, 5'-tetramethylbenzidine (TMB) (Sigma) for 30 min at 25°C in the dark, stopped with 1 M H₂SO₄, and read on a micro-plate reader (Bio-RAD Model 680) at 450 nm. Results are expressed as optical density (OD).

Measurement of gut permeability in vivo

An additional group of mice was sensitized with BLG plus CT for 5 weeks as described above. After oral challenge with 50 mg of BLG the mice were fasted for 6 h and then gavaged with 4,000 kDa FITC-labeled dextran diluted in water (TdB Consultancy AB, Uppsala, Sweden) (500 mg/kg, 125 mg/ml). After 2 h, blood (500 µl) was collected from intracardiac puncture and centrifuged (3,000 rpm for 15 min at RT), and FITC dextran concentration in plasma was determined by spectrophotometer (excitation wave length 485 nm; emission wave length 535 nm; HTS-7000 Plus-plate-reader; Perkin Elmer, Wellesley, Massachusetts, USA), as previously described [95].

Measurement of IL-4 from spleen lysates

Spleen lysates were prepared as previously described [341]. Briefly, individual spleens were placed in Eppendorf tubes containing 0.5 ml of lysate buffer. The spleen cells were lysed and homogenized by sonication for 30 s on ice. Supernatants were collected after centrifugation at 17,500 g for 10 min at 4°C and stored at -20°C. Interleukin-4 (IL-4) from spleen lysates was analyzed by commercially available ELISA kits following the manufacturer's protocol (R&D systems, Space Import-Export srl, Milan, Italy).

Statistical analysis

Two-group comparisons were made by t test. One-way ANOVA with Bonferroni post-test was used for comparisons between three or more groups. Fisher's exact test was used for categorical variables.

8.1.3 WOUND HEALING MODELS

Skin Ulceration induced by doxorubicin

Housing of animals and anesthesia were performed following the guidelines established by the Institutional Animal Welfare committee with the European guide for care and use of laboratory animals. Standardized skin ulceration was performed by intradermal doxorubicin (Doxorubicin Teva 0.2%) injection on the shaved dorsum of male Sprague-Dawley rats, as previously described in mice [342]. In brief, animals were anesthetized intraperitoneally with uretan (5 g/50 ml and 1,2 ml/100 g w/w of animals). The backs of the mice were shaved with a hair clipper and depilated with Veet depilatory cream (Reckitt Benckiser, Massy, France). Two days after depilation, mice received 500 µl of a 2 mg/ml doxorubicin solution by intradermal injection on the depilated area. The maximum of skin ulcer area was reached 11 days after doxorubicin injection. That day (day 1) was the first day of treatment with sodium butyrate (20 mg/Kg) and its derivative FBA (42.2 mg/Kg). Ulcers were photographed every 2 days (days 1, 3, 5, 8, 10, 12, 14, 17, 19, and 21) and cleaned until their complete closure. The lesion size was measured three times by using ImageJ software (<http://imagej.nih.gov/ij/>) for each ulcer, and the mean was calculated. Biological samples for molecular analysis were taken from sacrificed animals on days 1, 3, 5, 10, 17, 19, and 30 after the first application of the ointment, whereas biochemical analysis was done on days 1, 5, and 10. In **figure 8.1.3a** the scheme of experimental protocol and animal treatments are summarized.

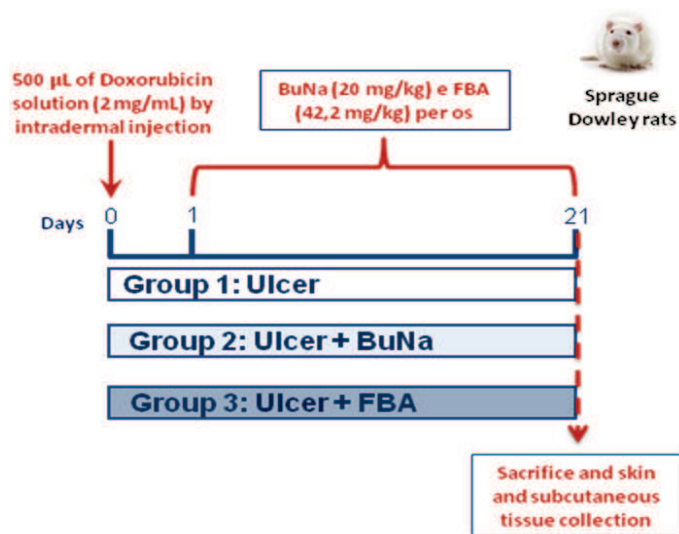


Figure 8.1.3a Experimental protocol of wound healing induced by doxorubicin.

Wound healing induced by mechanical damage

Six week old male C57BL/6J (Charles River, Calco, Italy) mice were anesthetized with 2–3% isoflurane, shaved, and a full thickness middorsal wound (0.5-cm² surface, circle shaped) was created by excising the skin and the underlying panniculus carnosus with bioptic instrument. The wounds were allowed to dry and form a scab. The subcutaneous treatment (10 µl/ mouse) with vehicle (80% of sterile saline solution/10% of PEG 400/10% of TWEEN 80), sodium butyrate (1-10 µg) or FBA (1-10 µg) was performed daily just after the induction of wound until complete healing. Wound closure was measured after 3, 7 and 14 days in a double blinded fashion on control and treated mice. To measure the wound area for each mouse was taken a picture of wound and analyzed using ImageJ software (<http://imagej.nih.gov/ij/>). In **figure 8.1.3b** the scheme of experimental protocol and animal treatments are summarized.

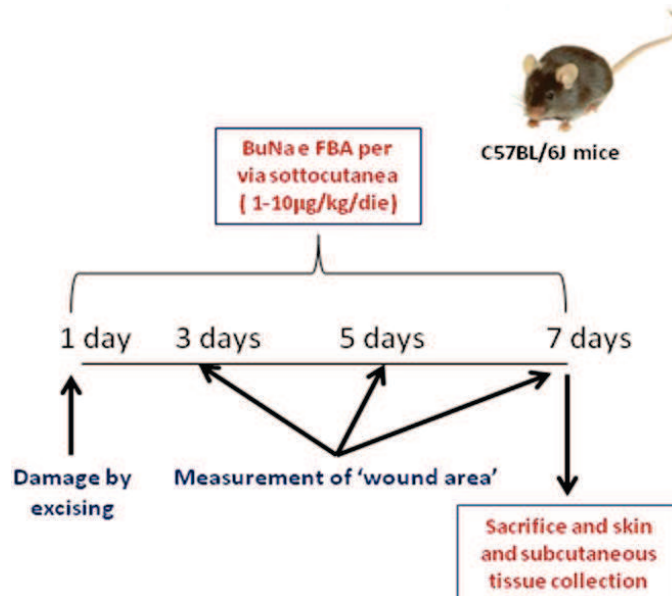


Figure 8.1.3b Experimental protocol of wound healing induced by excising.

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed by ANOVA test for multiple comparisons followed by Bonferroni's test, using Graph-Pad Prism (Graph-Pad software Inc., San Diego, CA). Statistical significance was set at P<0.05.

8.2 *IN VITRO* MODEL

8.2.1 INDUCTION OF INFLAMMATION IN MURINE CHONDROCYTES

Cell culture

ATDC5 cells were a kind gift from Dr. Agamemnon E. Grigoriadis (King's College, London Guy's Hospital, London, UK). Cells were grown in a standard medium of Dulbecco's modified Eagle's medium (DMEM)/F-12 containing 5% fetal bovine serum (FBS), 10 µg/ml human transferrin, 3 X 10⁻⁸M sodium selenite, and antibiotics (50 units/ml penicillin and 50 µg/ml streptomycin) at 37°C under 5% CO₂. The medium was replaced every two days. Fetal bovine serum (FBS), tissue culture media, media supplements were purchased from Sigma (St. Louis, MO).

MTT assay

Cell metabolic activity was examined using a colorimetric assay based on the MTT labeling reagent. Cells were seeded in 96-well plates (8,000 cells/well). Assays were performed according to the instructions and protocol provided by the manufacturer (Sigma–Aldrich). Spectrophotometrical absorbance was measured using a microtiter ELISA reader at 550 nm (Multiskan EX, Termo Labsystem, Barcelona, Spain).

Cell treatments and nitrite assay

ATDC5 cells were seeded at a density of 10⁶ cells/well into 6-well dishes. After 12 hours of starvation in serum-free DMEM/Ham's F-12 medium, cells were stimulated for 24 hours with mouse interleukin (IL)-1 (0.5 ng/ml) (Sigma Aldrich) and pre-treated or not for 1 h with 10-50-100-250 µM of sodium butyrate (Sigma Aldrich) under an atmosphere of 5% CO₂ for 24 h. Twenty-four hours was the time selected for optimal observation according to our time-course experiments.

Nitrite accumulation was measured in the culture medium by Griess reaction. Briefly, 100 μ l of cell culture medium was mixed with 100 μ l of Griess reagent (equal volumes of 1% [weight/volume] sulfanilamide in 5% [volume/volume] phosphoric acid and 0.1% [w/v] naphthylethylenediamine HCl), incubated at room temperature for 10 minutes, and then the absorbance at 550 nm was measured in a microplate reader (Titertek Multiscan; Labsystems, Helsinki, Finland). Fresh culture medium was used as blank in all the experiments. The amount of nitrite in the samples (in μ M) was calculated from a sodium nitrite standard curve freshly prepared in culture medium.

RNA isolation and reverse transcriptase–polymerase chain reaction (RT-PCR)

RNA was extracted using a NucleoSpin kit, according to the instructions and protocol provided by the manufacturer. For relative quantification, we performed an RT reaction with a First Strand Kit from SABiosciences. Next, real-time PCR was performed with a SABiosciences Master Mix and specific RT² qPCR primers for: mouse *Nos2* (122 bp, NM_010927), *Ptgs2* (135 bp, NM_011198.3), *Interleukin-6* (178 bp, NM_031168.1), *Vcam-1* (146 bp, NM_011693.3), *ICAM-1* (122 bp, NM_000201.2), *lipocalin-2* (81 bp, NM_008491.1), *nesfatin-1* (NUCB2, from SOLARIS, Thermo Scientific), *MMP13* (88 bp, NM_008607), *Col2a1* (138 bp, NM_031163.3), *Ccl3* (112 bp, NM_011337.2), *Cx3cl1* (113 bp, NM_009142.3), *Annexin-a1* (70 bp, NM_010730.2), *Ffar2* (156 bp, NM_146187.4). These gene expressions were normalized with mouse *Gapdh* (140 bp, NM_008084.2). Results of comparative real-time PCR were analyzed with MxPro version 4 software (Stratagene, La Jolla, CA).

Western blot analysis

To determine iNOS, VCAM-1 and lipocalin-2 protein expression, ATDC5 cells were incubated with IL-1 for 24 hours. Moreover, to evaluate p65 translocation and I κ B- α degradation, cells were pre-treated with sodium butyrate (250 μ M) for 12 hours and then challenged with IL-1 α (0.5 ng/mL) for 15 or 30 min. Afterwards, cells were subjected to a differential lysis to obtain the nuclear and cytosolic fractions as previously described [343]. In other set of experiments, to assess the phosphorylation of extracellular signal-regulated

kinase (ERK1/2), p38, Akt and AMPK α , cells were pre-treated with sodium butyrate (250 μ M) for 12 hours and then challenged with IL-1 α (0.5 ng/mL) for 30 min. In both experiments, after the stimulation, cells were washed twice with ice cold PBS, harvested, and resuspended in lysis buffer for protein extraction (10 mM Tris/HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 0.5% Triton X-100, 1mM PMSF, protease inhibitor cocktail). Cell lysates were obtained by centrifugation at 14.000xg for 20 min at 4°C. Protein concentrations were estimated by the Bio-Rad protein assay using bovine serum albumin as standard. Lysates from control or stimulated cells were collected and separated by SDS/PAGE on a 10% polyacrylamide gel. Proteins were subsequently transferred to a polyvinylidene difluoride transfer membrane (Immobilon-P transfer membrane, Millipore, MA) using a transfer semidry blot cell (BioRad Laboratories). Blots were incubated with the appropriate antibodies: anti-iNOS and anti-VCAM-1 (Cell Signaling Technology, Denver, Massachusetts, USA), lipocalin-2 (R&D Systems, Inc., Minneapolis, MN, USA), anti-phospho ERK1/2 and anti-ERK1/2 (Millipore, MA, USA), anti-phospho-p38 and anti-p38 (Millipore, MA, USA)), anti-NF κ B p65 (Santa Cruz, CA,USA), anti-I κ B- α , anti-phospho-Akt and anti-Akt, anti-phospho-AMPK- α and anti-AMPK- α (Cell Signaling Technology, Denver, Massachusetts, USA). Immunoblots have been visualized with Immobilon Western Detection kit (Millipore, MA) using horseradish peroxidase labelled secondary antibody. To confirm equal loading in each sample, the membranes were stripped in stripping buffer (100 mM β -mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) and re-blotted with anti-GAPDH (Sigma, MO, USA) or anti-lamin- β 1 antibody (GeneTex, CA, USA). The images were captured and analyzed with an EC3 imaging system (UVP). Densitometric analyses were performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

siRNA-mediated gene silencing of GPR43 receptor

In order to silence GPR43 gene, we used the siRNA that targets GPR43 receptor (Integrated DNA Technologies, USA) and the siRNA negative control, that does not target any known sequence. Transfection with 10nM of siRNA duplex was performed using the cationic lipid siLentFect (BioRad, CA, USA) according to the manufacturer's

recommendations. Cells were transfected for 48 h and then they were stimulated with 0.5 ng/ml of IL1 in presence or not of 250 μ M of sodium butyrate for 24 h.

Statistical analysis

Data are reported as mean \pm standard error mean (S.E.M.) values of independent experiments, which were done at least three times, each time with three or more independent observations. Statistical analysis was performed by analysis of variance test, and multiple comparisons were made by Bonferroni's test or, when appropriate, with Dunnet's test. Statistical significance was set at $P < 0.05$.

CHAPTER 4

9. RESULTS

9.1 N-(1-CARBAMOYL-2-PHENYL-ETHYL) BUTYRAMIDE, A NEW SYNTHETIC BUTYRATE DERIVATIVE, REDUCES INTESTINAL INFLAMMATION IN DEXTRAN SODIUM SULPHATE-INDUCED COLITIS.

Effect of Sodium butyrate and FBA on weight change and disease activity index in DSS mice

As shown in **figure 9.1.1 A** both pharmacological treatments (preventive and curative protocol) were able to preserve weight loss induced by DSS challenge (*P<0.05; **P<0.01 vs. CON). As reported in AUC graph, curative treatment appeared more effective than preventive one. Assessment of disease activity index (DAI) after 7 days from 12th day showed a strong increase of colitis gravity in DSS-challenged mice (***P<0.001 vs. CON). Instead, butyrate and FBA (PREV and CUR) significantly prevented the development of colitis manifestations (**figure 9.1.1 B**) (### P<0.001 Vs. DSS).

Butyrate and FBA improve tissue histopathology and prevent colon shortening in DSS- induced colitis

Control colon sections showed the intact epithelium, well defined crypt length, no edema, no neutrophil infiltration in mucosa and submucosa, and no ulcers or erosions. In contrast, colon tissue from DSS treated mice showed clear and severe inflammatory lesions extensively throughout the mucosa (**figure 9.1.2 A**). Ulcers, shortening and loss of crypts were seen focally at the beginning progressing to more extensive areas of mucosal involvement and finally the whole colon. Infiltration of immune cells including neutrophils and lymphocytes were seen in the lamina propria in DSS mice. In spite of DSS treatment, butyrate and FBA were able to protect colonic mucosa structure and to reduce immune cellular recruitment.

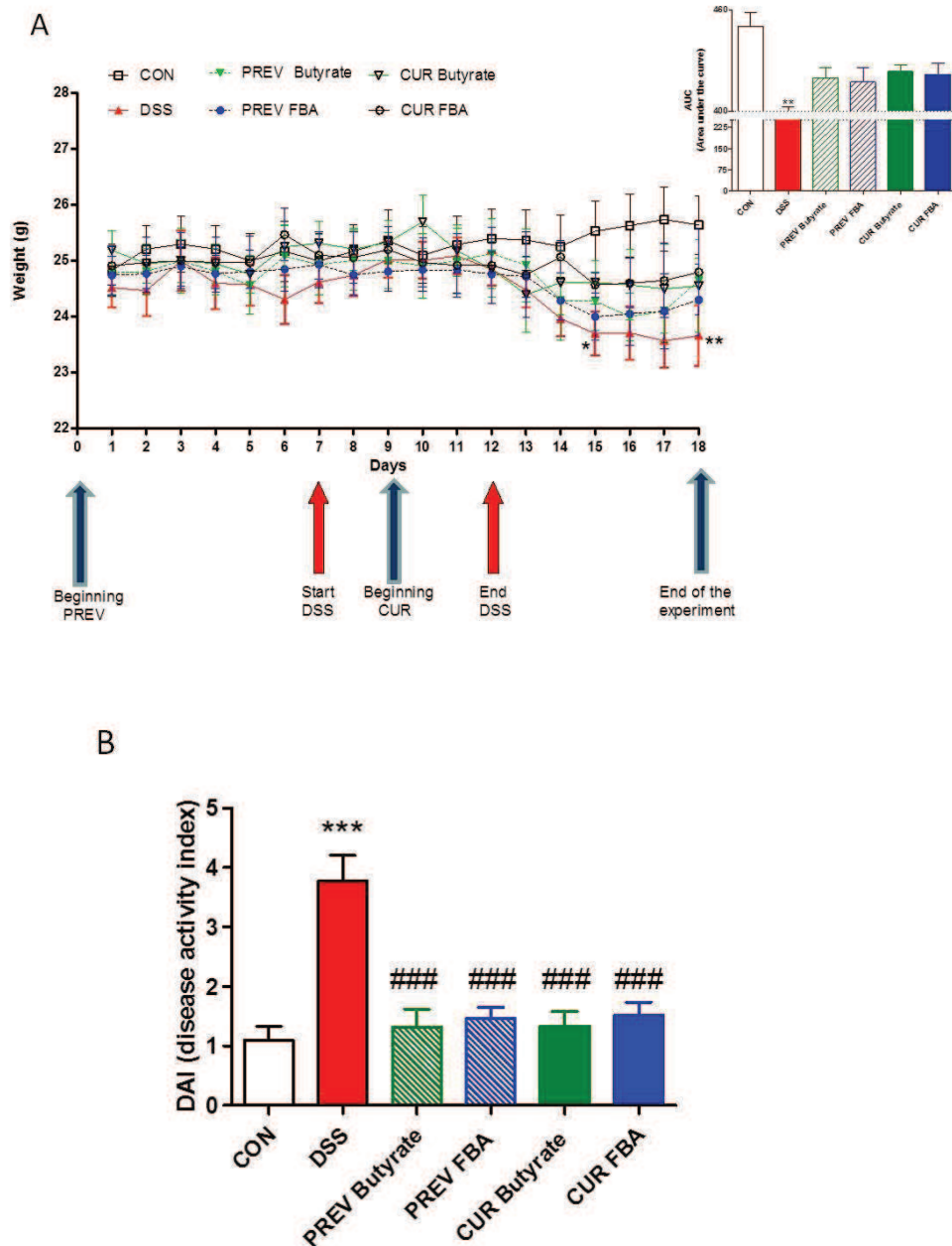


Figure 9.1.1. Effects of Butyrate and FBA on induction and recovery from DSS induced colitis. WT mice received 2.5% DSS for 5 days followed by treatment and then returned to normal drinking water for an additional 7. Mice were sacrificed on day 12. (A) Evolution of body weight and (B) DAI values on day 12. Data are mean \pm SD from eight mice/group.

In addition both preventive and curative treatments ameliorated mucosa integrity and crypt structure improving epithelial surface. Beneficial effects of all therapeutics schemes were shown macroscopically after colon excision. In fact, as depicted in **figure 9.1.2 B**, butyrate and FBA preserved colon from inflammation and bleeding induced by DSS. Furthermore, both therapeutic protocols, in particular curative ones, with butyrate and FBA

reduced colon shortening shown in DSS-challenged mice (**figure 9.1.2 C**) (* $P < 0.05$ vs CON; # $P < 0.05$ and ## $P < 0.01$ vs. DSS).

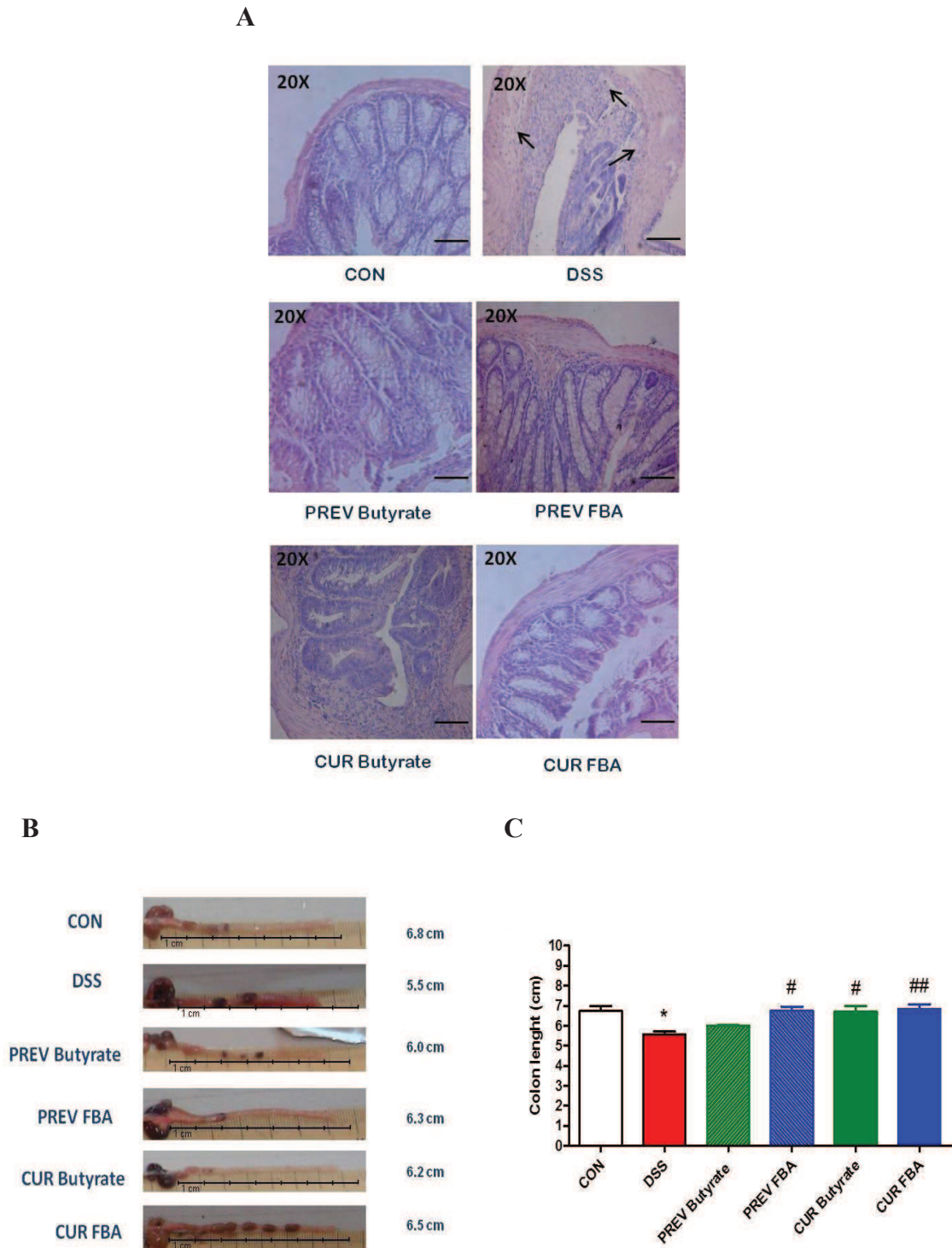


Fig. 9.1.2. Therapy with Butyrate and FBA ameliorates DSS colitis. (A) Representative histology of the distal DSS colon (H&E staining; original magnification 200X, bar 0.2 mm). Black arrows indicates infiltrated cells in the submucosa. (B) Colon images and (C) assessment of total colon length after DSS treatment. Data are mean \pm SD from eight mice/group. Histological images are representative of 5 slides for each group.

Anti-inflammatory effect of butyrate and FBA in colon and serum

Colonic inflammation was determined by TNF- α , IL-6, COX-2 and IFN γ mRNA (figure 9.1.3 A-B-C-D). All these cytokines were significantly up-regulated in DSS-challenged mice, conversely mRNA of anti-inflammatory mediators such as IL-10, adiponectin, PPAR γ were reduced (figure 9.1.4 A-B-C-D) (*P<0.05; **P<0.01 vs. CON). Protective effect of butyrate and FBA (PREV and CUR) was shown not only in reducing pro-inflammatory mediators but also in recovering anti-inflammatory factors in colon tissue and serum (#P<0.05; ##P<0.01; ###P<0.001 vs. DSS).

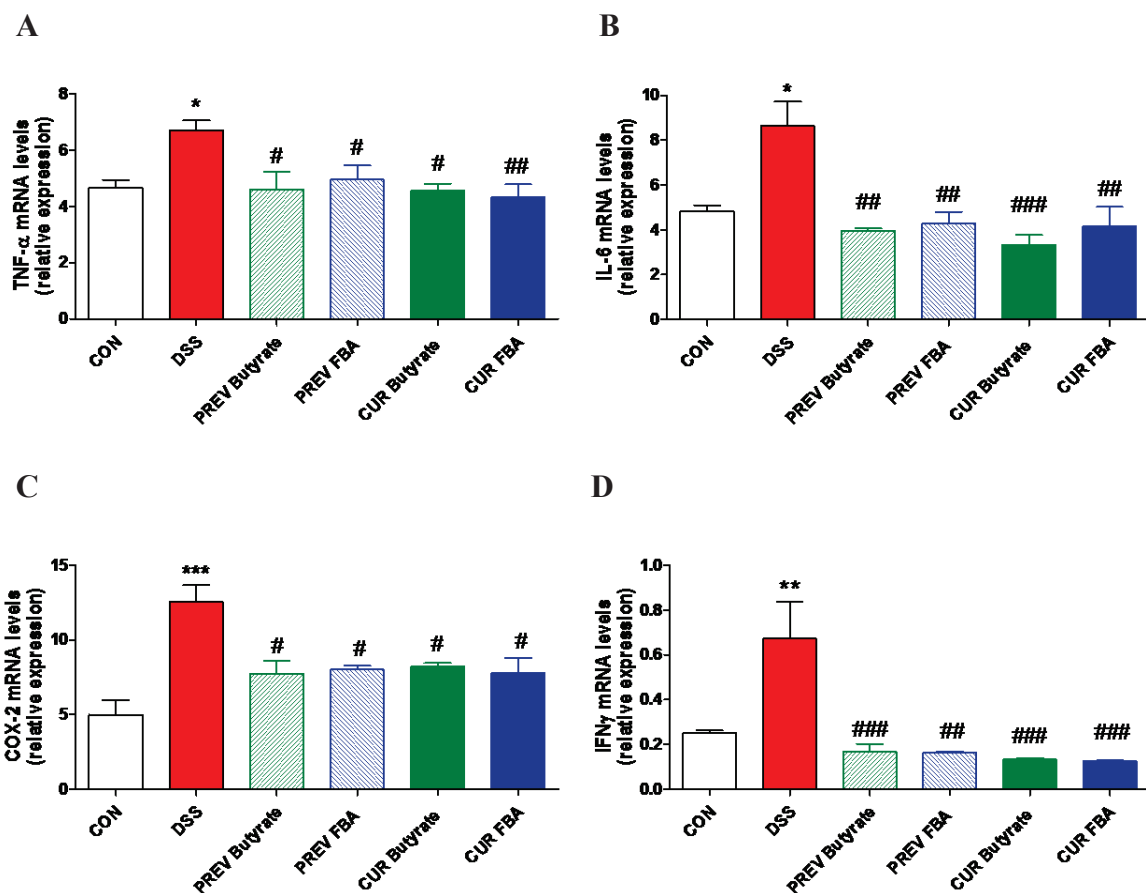


Figure 9.1.3. Butyrate and FBA inhibit secretion of pro-inflammatory mediators. mRNA transcriptional levels of (A) TNF- α , (B) IL-6, (C) COX-2 and (D) IFN γ in colon tissue. Data are presented as means \pm standard error of 5 animals for each group.

Moreover, DSS was able to induce significantly HDAC9 mRNA transcript (figure 6.1.4 D). Instead, butyrate and FBA, when used as curative treatments, down-regulated HDAC9 mRNA levels (#P<0.05; ###P<0.001 vs. DSS).

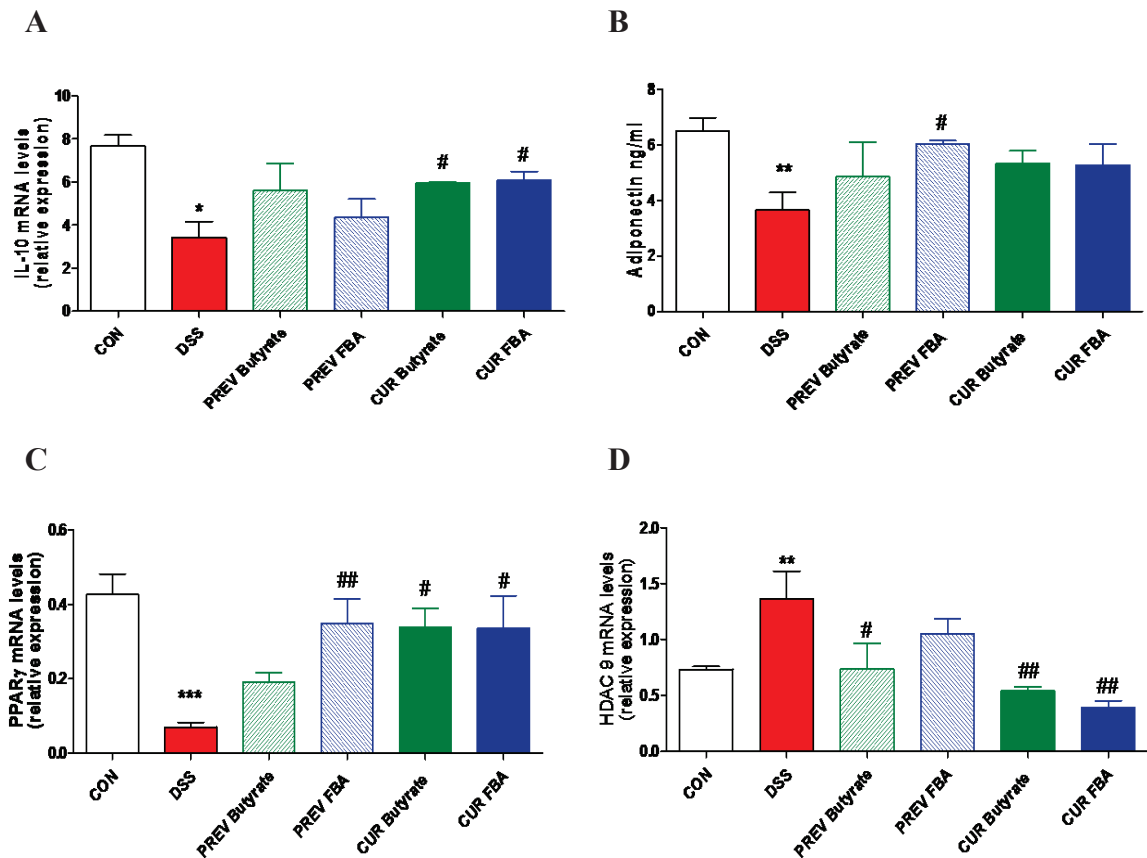


Figure 9.1.4. Butyrate and FBA restore anti-inflammatory factors and inhibit HDAC9. mRNA transcriptional levels of (A) IL-10, (B) adiponectin, (C) PPAR γ and (D) HDAC9 in colon tissue. Data are presented as means \pm standard error of 5 animals for each group.

Protective effect of Butyrate and FBA in restoring intestinal barrier integrity

IBDs are characterized by inappropriate and ongoing activation of the mucosal immune system driven by penetration of normal luminal flora due to tight-junction defects of the intestinal epithelial barrier. In our experiment, we demonstrate that butyrate and its derivative FBA were able to significantly restore the intestinal barrier integrity compromised by DSS, inducing the increase of mRNA levels of occluding and zonulin (ZO)-1, two of most important tight-junction of the intestinal tract(**figure 9.1.5 A-B**) (* $P < 0.05$ and *** $P < 0.001$ vs CON; # $P < 0.05$ vs DSS).

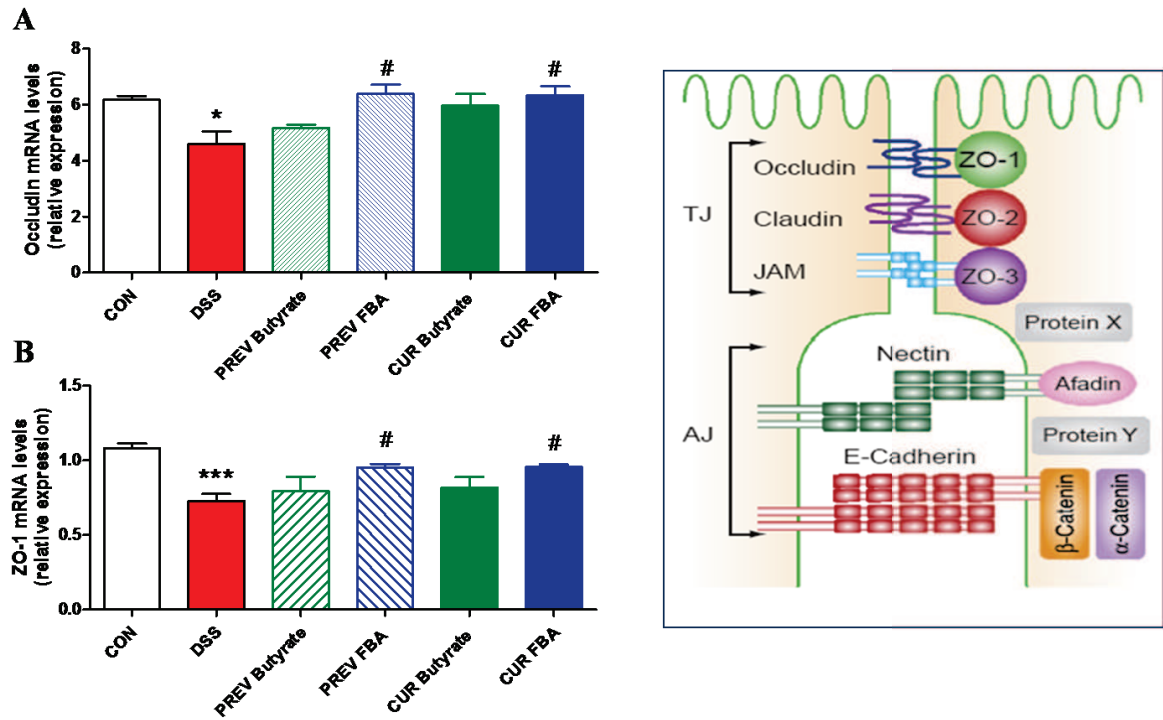


Figure 9.1.5. Butyrate and FBA impair the gut barrier integrity. Real-Time PCR of (A) occludin and (B) zonulin (ZO)-1 in colon tissue. Preventive treatment with FBA and curative treatment with butyrate increased mRNA expression of occludin and ZO-1 in colon, altered by DSS. Data are mean \pm SD from eight mice/group.

Butyrate and FBA increase GPR43 expression and distribution along intestinal epithelium and induce MCT1 transporter in colonic mucosa

As depicted in **figure 9.1.6 A-B** butyrate and FBA, in particular curative protocol, were able to increase the transcriptional levels of its main receptor GPR43 and its transporter MCT1 compared to control and DSS-untreated mice, suggesting their important implication in butyrate anti-inflammatory activity (**P<0.01 vs. CON and #P<0.05 vs. DSS). This effect was confirmed by immunofluorescence staining for GPR43. this receptor was particularly expressed along intestinal epithelium, and only in DSS-alone group was co-localized with PMNs positive cells (data not shown).

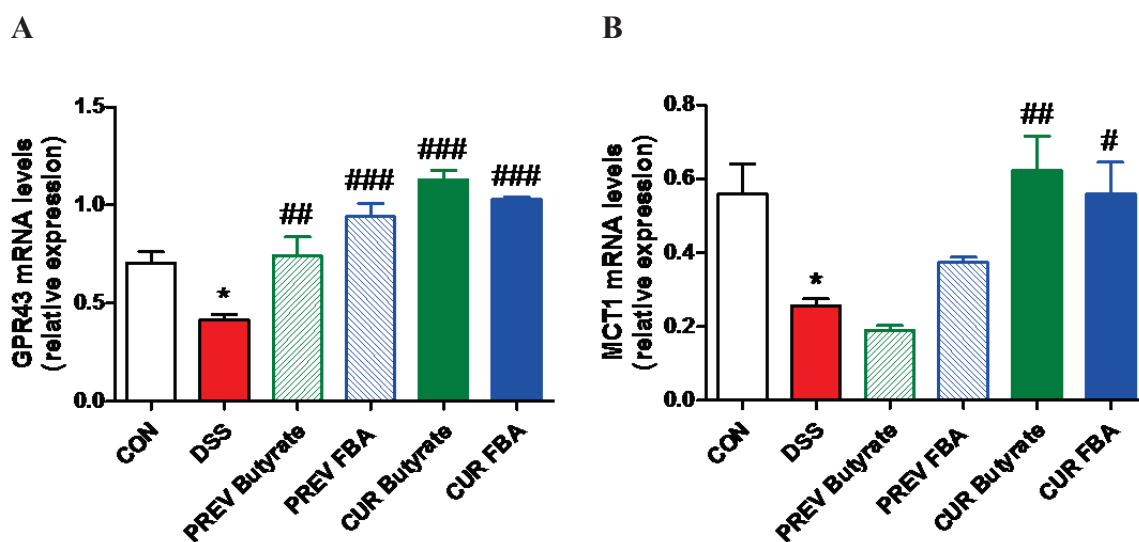


Figure 9.1.6. Effect of Butyrate and FBA on GPR43 and MCT1 expression in colon. Real-Time PCR of (A) GPR43 and (B) MCT1 in colon tissue. Real-Time data are presented as means \pm standard error of 5 animals for each group.

Effects of butyrate-based compounds on mucosal infiltration by monocytes / macrophages

Butyrate and FBA reduced monocytes infiltration in colonic mucosa by impairment of chemokine expression and lowering macrophages markers. In particular, either pharmacological treatments, both preventive and curative protocol, preserved NOS2, CD14 CD68, Ccl2 enhancement induced by DSS challenge (**figure 9.1.7 A-D**) (*P<0.05; **P<0.01 vs. CON; #P<0.05; ##P<0.01 vs. DSS).

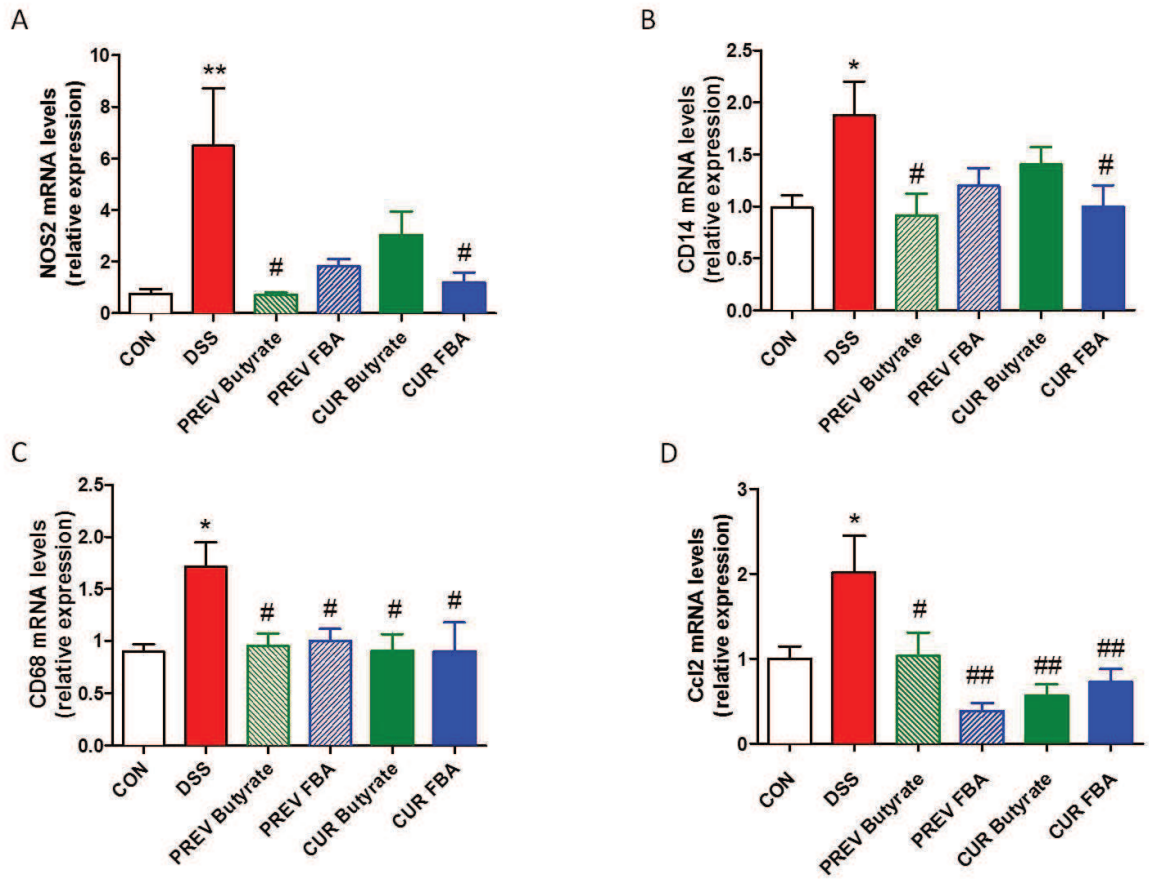


Figure 9.1.7. DSS treatment induces up-regulation of monocytes / macrophages markers in colon tissue. Relative amount of (A) NOS2, (B) CD14, (C) CD68 and (D) Ccl2 mRNA was significantly increased in DSS-challenged mice treated with vehicle alone. Both Butyrate and FBA were able to prevent infiltration of immune cells reducing chemoattractant release in colonic mucosa. Data are presented as means \pm standard error of 5 animals for each group.

9.2 THERAPEUTIC EFFECT OF SODIUM BUTYRATE IN A MODEL OF COW'S MILK ALLERGY (CMA) INDUCED IN MICE

Butyrate treatment reduce acute skin response

As showed in **figure 9.2.1** shows the sensitization with BLG led in mice to an exacerbated local ear swelling response upon intradermal BLG challenge when compared to controls. Daily administration of butyrate significantly reduced the local ear swelling response by sensitization (** $P < 0.001$ vs CON; ## $P < 0.01$ vs BLG).

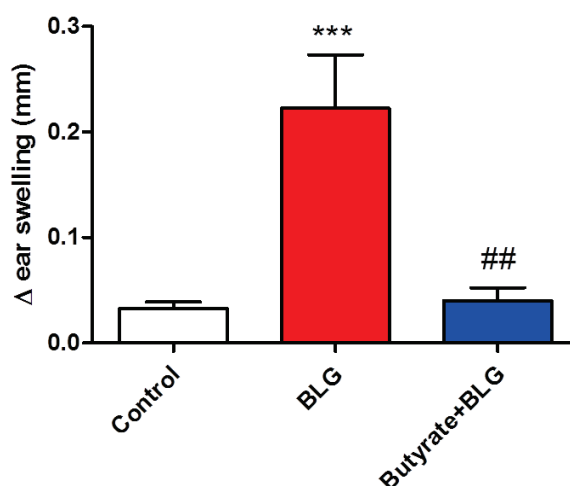


Figure 9.2.1. Acute allergic ear swelling response. sensitization with BLG led to an exacerbated local ear swelling response. butyrate significantly alleviated the local response. Data are presented as means \pm standard error of 5 animals for each group.

Effect of butyrate oral administration after intragastric challenge with BLG

BLG sensitization also led to a systemic anaphylactic response to intragastric challenge with BLG, as assessed by anaphylactic symptom scores (**figure 9.2.2 A**) and body temperature measurement (**figure 9.2.2 B**). The oral administration of butyrate was able to reduce symptom scores and to prevent a decreasing of body temperature after the oral challenge with BLG (* $P < 0.05$, *** $P < 0.001$ vs CON; # $P < 0.05$ vs BLG).

Finally, we demonstrated that BLG sensitization increased gut permeability, measured through the detection of DX-4000 FITC in plasma, compared to control group. Butyrate was able to remarkably improve the intestinal barrier integrity (**figure 9.2.4**) (*P<0.05 vs CON and vs BLG).

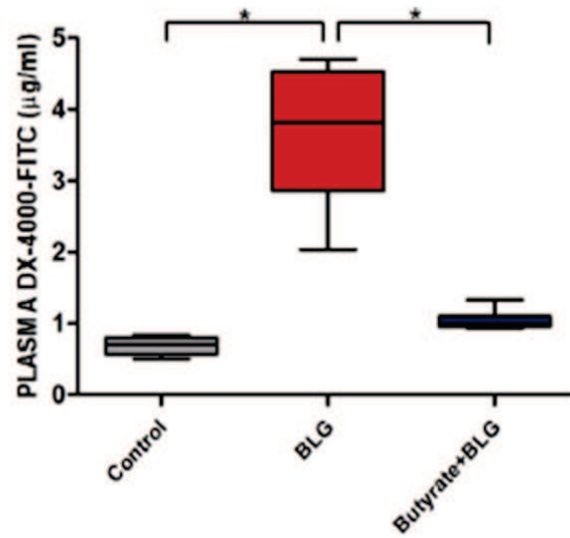


Figure 9.2.4. Intestinal permeability to plasma DX-4000 FITC. BLG sensitization increases gut permeability. Butyrate improves the intestinal barrier integrity.

9.3 ANTI-INFLAMMATORY EFFECT OF SODIUM BUTYRATE AND ITS CHEMOATTRACTANT ACTIVITY MEDIATED BY GPR43 RECEPTOR IN MURINE CHONDROCYTE CELL LINE

Butyrate decreases IL-1-induced NO production but not cell viability

ATDC5 chondrogenic cells were stimulated with 0,5 ng/ml IL-1 in the absence or presence of increasing concentrations of sodium butyrate (10-250 μ M) and the production of NO was evaluated using Griess reagent. The treatment with butyrate marked decreased IL-1-induced nitrite amount in a concentration-dependent manner (**figure 9.3.1 B**) (** $P < 0,001$ vs CON; # $P < 0,05$; ## $P < 0,01$ vs IL-1). The observed inhibition was not due to the reduction of cell viability as confirmed by the methyl thiazolyl tetrazolium (MTT) assay (**figure 9.3.1 A**).

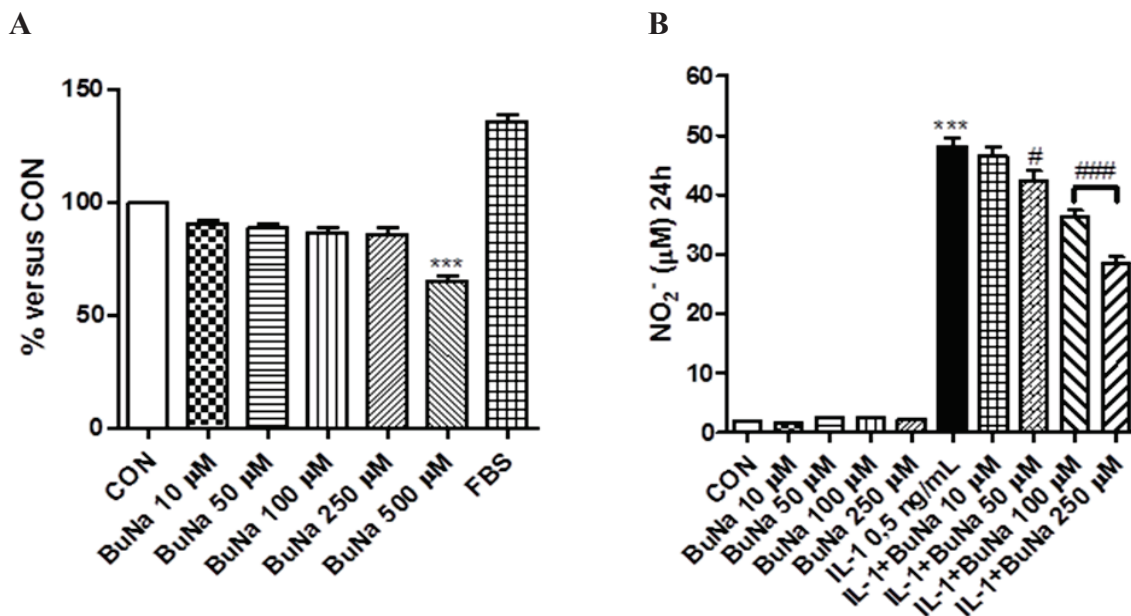


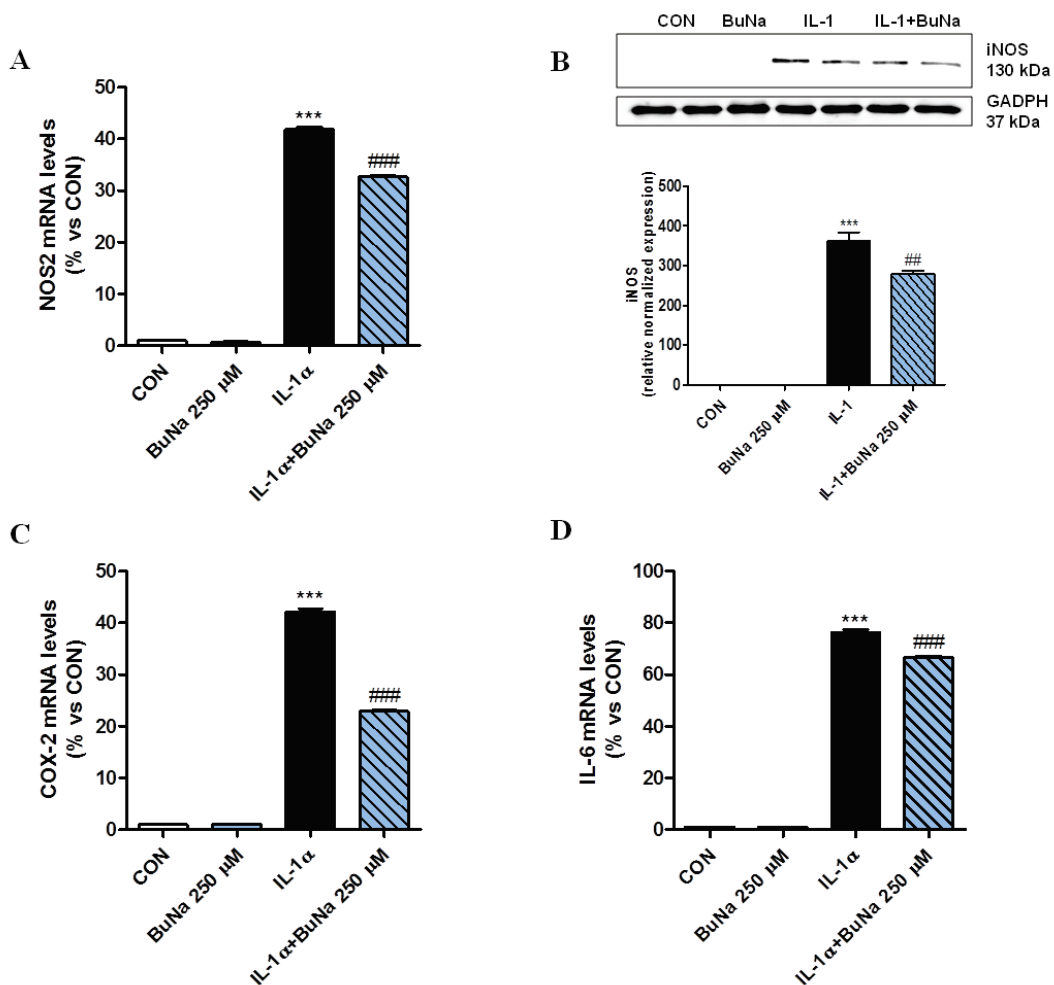
Figure 9.3.1. Cell viability assay and nitrite accumulation. The treatment with butyrate marked decreased IL-1-induced nitrite amount (B) in a dose-dependent manner but not cell viability (A). Data are presented as means \pm standard error of 3 different experiments.

Butyrate reduces pro-inflammatory mediators and adipokines in chondrocytes stimulated by IL-1

Consistently with the reduction of NO accumulation, we evaluated the effect of butyrate treatment on mRNA expression of *NOS-2*, *Ptgs2* and *Interleukin-6*, analyzed by

real time PCR. The relative expression level of each gene mRNA was plotted as fold changes *versus* untreated control cells. GAPDH gene expression was used for normalization. Under basal conditions, *Nos2*, *Cox2* and *Il-6* levels were undetectable, while IL-1 stimulation induced a significant increase of all gene mRNA levels. Butyrate at the dose of 250 μ M was able to suppressed markedly this induction, suggesting that the anti-inflammatory effect of butyrate was at transcriptional level (**figure 9.3.2 A-C-D**) (** $P < 0,001$ vs CON; ### $P < 0,001$ vs IL-1). Next, we analyzed the iNOS protein by Western blotting and butyrate significantly reduced this expression, induced by IL-1 challenge (**figure 9.3.2 B**) (** $P < 0,001$ vs CON; ## $P < 0,01$ vs IL-1).

We also examined lipocalin-2 (LCN2) and nesfatin-1 (or NUCB2) production in response to IL-1 stimulation showing a significant increase of both genes mRNA expression compared with control. In contrast, the treatment with butyrate markedly decreased LCN2 and NUCB2 (**figure 9.3.2 E-F**) (** $P < 0,001$ vs CON; ### $P < 0,001$ vs IL-1).



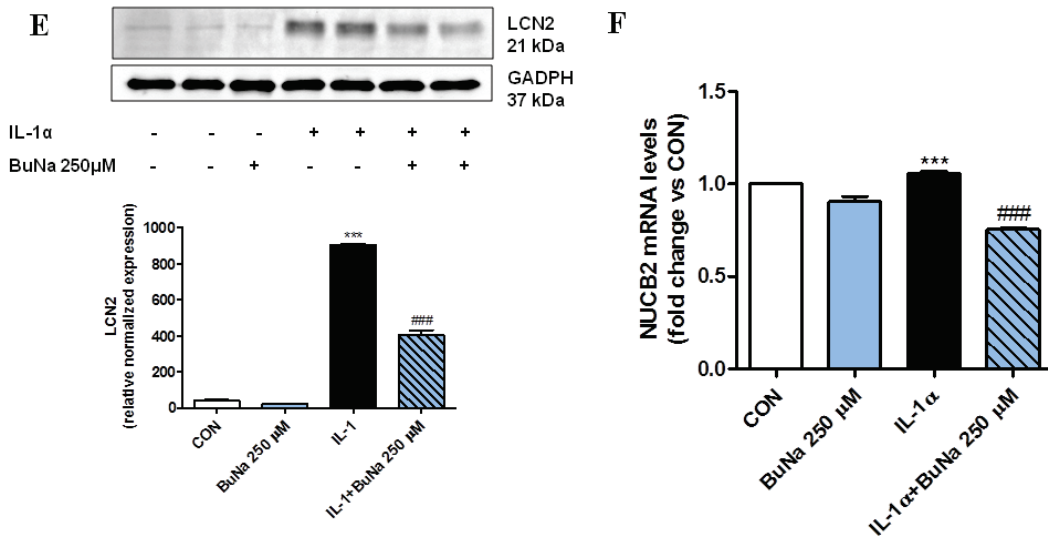


Figure 9.3.2. Expression of pro-inflammatory cytokines and adipokines in murine chondrocytes. IL-1 stimulation induced a significant increase of *Nos2* (A-B), *Ptsg2* (C) and *Il-6* (D) expression. Butyrate at the dose of 250 μ M was able to suppressed markedly this induction. Butyrate also reduces the expression of pro-inflammatory adipokines lipocalin-2 (E) and NUCB2 (F). Western blot and Real Time data are presented as means \pm standard error of 3 different experiments.

Butyrate reduces adhesion molecules expression

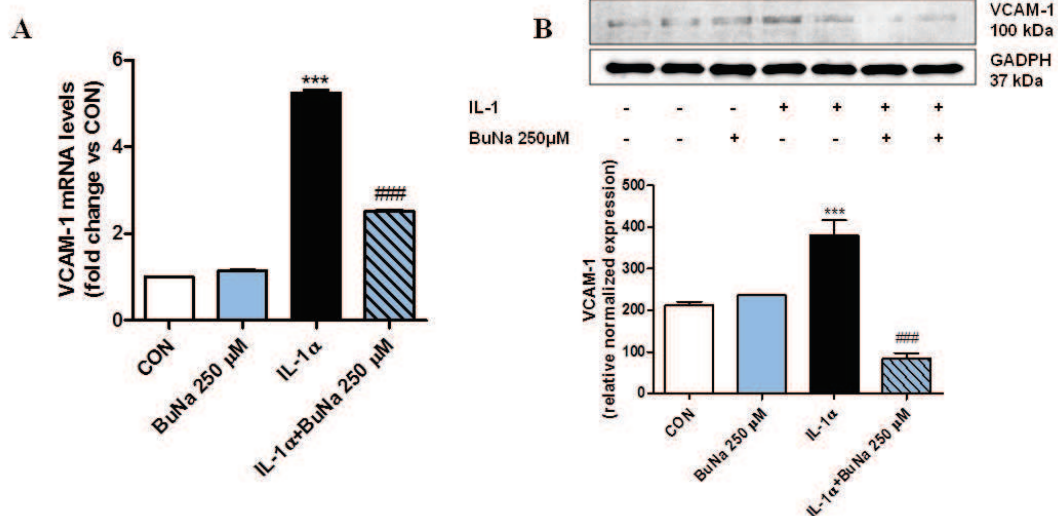
The vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) proteins mediate the adhesion of lymphocytes, monocytes, eosinophils, and basophils to vascular endothelium and may play a role in the development of atherosclerosis and rheumatoid arthritis. Here, we showed that, upon IL-1 stimulation, the mRNA levels of these molecules were deeply increased (**figure 9.3.3 A-B-C**). (***) $P < 0,001$ vs CON; (###) $P < 0,001$ vs IL-1). Similarly, the protein expression of VCAM-1 was markedly increased. Butyrate treatment, 1 h before IL-1 stimulation until 24 h, was able to significantly reduce these levels.

Effect of butyrate on pro-inflammatory and anti-apoptotic pathways in ATDC5 cells

In response to inflammatory stimulation the p65 subunit of Nfk-B is activated and translocates in the nucleus and in the same time I κ B- α is degraded in cytoplasm. In our experiments on ATDC5 cells, after IL-1 stimulation for 15-30 minutes, the activation of p65 subunit and consequently I κ B- α degradation were induced and evaluated by Western blotting in nucleus and cytoplasmic lysates, respectively. Butyrate significantly reduced

NFκB p65 expression in nucleus and consistently increased IκB-α in cytoplasm (**figure 9.3.4 A-B**). Moreover, it is well known that mitogen-activated protein (MAP) kinase pathway is involved in the inflammatory response. Here, we showed that butyrate significantly decreased the phosphorylation of p44/42 MAPK (or 44/42 ERK) and of p38 subunit, significantly activated by IL-1, suggesting that its anti-inflammatory effect involved MAP-kinase cascade signaling (**figure 9.3.4 C-D**).

AMPK is currently known to act as a key regulator of metabolic homeostasis. In fact, several biosynthetic enzymes for fatty acid or glycogen are recognized as targets of AMPK. It was well known that IL-1 induces phosphorylation of AMPK-α, which regulates AMPK activities [344]. Also in chondrocytes the phosphorylation of AMPK- α was significantly increased by IL-1 stimulation compared with no-treated cells, while sodium butyrate markedly reduced this phosphorylation, inducing AMPK-mediated anti-inflammatory activity (**figure 9.3.4 E**). At the same time, butyrate acted on anti-apoptotic phosphatidylinositol 3-Kinase/Akt pathway, that is activated by inflammatory stimulation [345]. Consistently, we demonstrated that IL-1 induced an increase of Akt phosphorylation, which was remarkably reduced by butyrate treatment, as shown by Western blot analysis (**figure 9.3.4 F**), suggesting its involvement also in regulating the phosphatidylinositol 3-kinase/Akt pathway in inflammation (*P<0,05 vs CON, ***P<0,001 vs CON; ### P<0,001 vs IL-1).



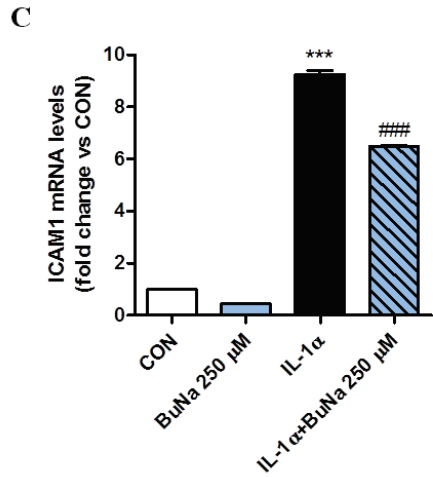
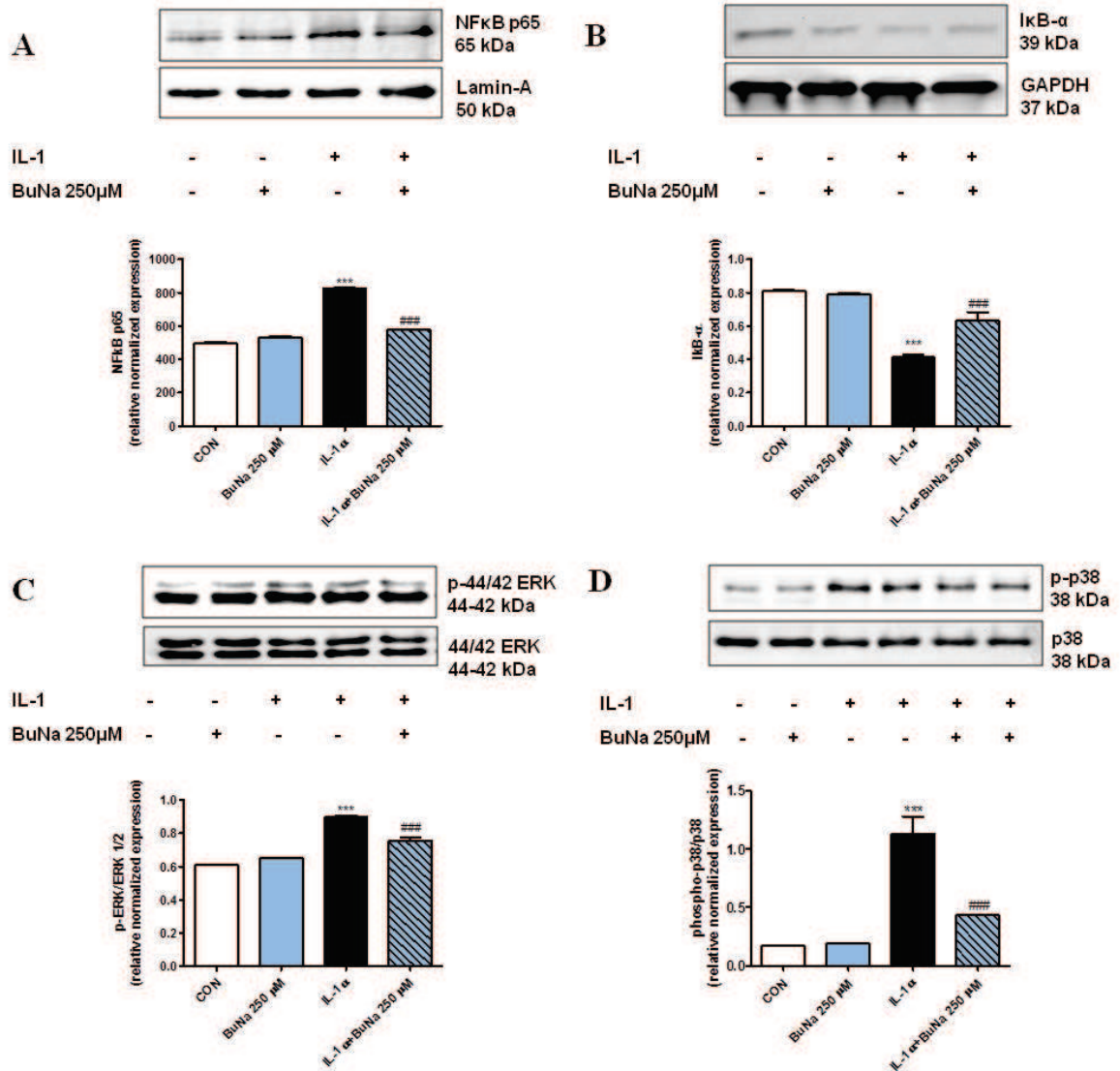


Figure 9.3.3. Expression of VCAM-1 and ICAM-1 after IL-1 stimulation. Upon IL-1 stimulation, the mRNA levels of these molecules (A and C) and, similarly, the protein expression of VCAM-1 (B) were greatly increased. Butyrate significantly reduced these levels. Real-Time and Western blot data are presented as means \pm standard error of 3 different experiments.



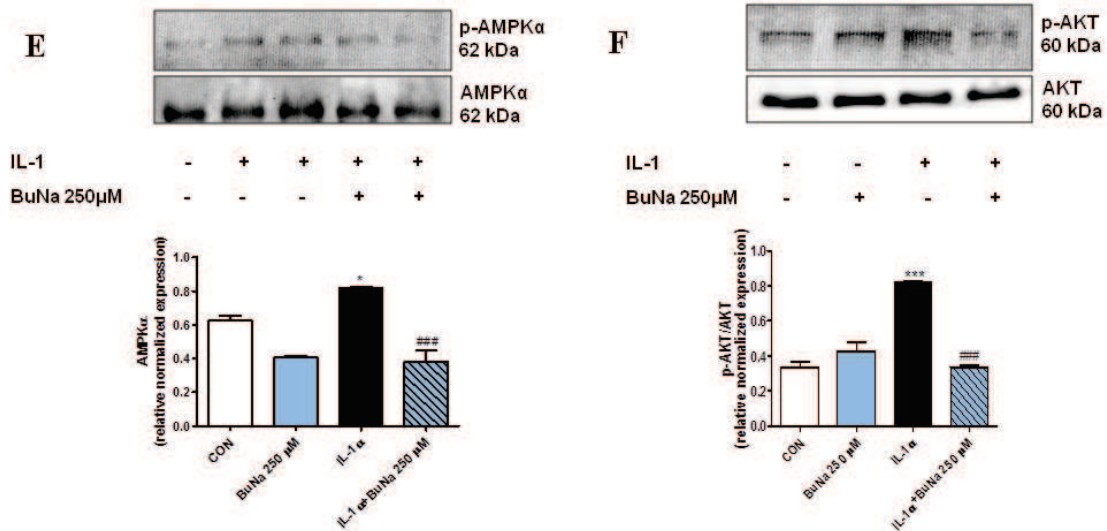


Figure 9.3.4. Effect of butyrate on pro-inflammatory pathways. After IL-1 stimulation for 15-30 minutes, the translocation of p65 subunit and IκB-α degradation were induced. Butyrate significantly reduced p65 expression in nucleus and increased IκB-α in cytoplasm and significantly decreased the phosphorylation of p44/42 MAPK (or 44/42 ERK) (A) and of p38 subunit (B), activated by IL-1. Also butyrate was able to reduce the phosphorylation of AMPKα (E) and of Akt (F) significantly induced by IL-1 stimulation. Western blot data are presented as means ± standard error of 3 different experiments.

Butyrate suppresses MMP-13 production and increases collagen type II levels after inflammatory stimulation

Cartilage destruction in OA is thought to be mediated mainly by MMPs family, which are responsible for cartilage collagen breakdown. For this reason, we studied the effect of butyrate on MMP-13 expression induced by IL-1. Cytokines stimulation produced a marked increase of MMP-13 expression and consistently a reduction of collagen type II levels. Butyrate (250 μM) resulted in suppressing MMP13 mRNA expression and increasing collagen type II (figure 9.3.5 A-B) (***P<0,001 vs CON; ### P<0,001 vs IL-1).

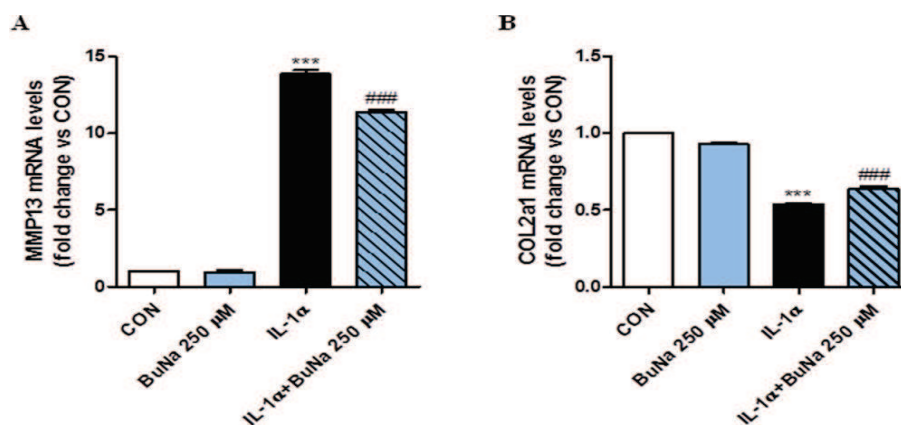


Figure 9.3.5. MMP13 and collagen type II mRNA levels. Cartilage destruction in the osteoarthritis is mediated mainly by the matrix MMPs. Sodium butyrate (250 μM) resulted in suppressing MMP13 mRNA expression (A) and in increasing collagen type II (B) levels. Real-Time and Western blot data are presented as means ± standard error of 3 different experiments.

Butyrate induces chemokines recruitment after several inflammatory challenges

In chondrogenic cells, IL-1 stimulation induced mRNA of important chemokines involved in inflammatory process and in neutrophils and leucocytes recruitment, such as *Ccl3* and *Cx3cl1*, and it induced the activation of annexin-1 (*AnxA1*), also known as lipocortin, a protein that inhibits various inflammatory events, including chemotaxis (**figure 9.3.6 A-C-F**). Interestingly, butyrate showed a chemoattractant effect in ATDC5 cells, increasing significantly mRNA expression of these genes. Behind anti-inflammatory effect of butyrate, this activity supports its capability to modulate immune response as pro-resolving factor in inflammation, due to the chemokine increase and resolvin induction (**figure 9.3.6 A-C-F**). (**P<0,001 vs CON; ###P<0,001 vs IL-1).

To demonstrate that the chemoattractant effect of butyrate was independent by the type of inflammatory challenge, we treated ATDC5 cells with other two inflammatory stimuli such as LPS (1 ng/mL) and TNF- α . The cells were pre-treated for 1 hour with sodium butyrate (250 μ M) and for 24 hours with challenges; then the expression of *Ccl3* and *Cx3cl1* was evaluated. These parameters were markedly increased by both stimulations and butyrate confirmed its chemoattractant effect, suggesting a general cross-section mechanism as pro-resolving factor (**figure 9.3.6 B-D-E**) (**P<0,001 vs CON; ### P<0,001 vs IL-1).

Anti-inflammatory effect of butyrate in mature chondrocytes after differentiation

Anti inflammatory effect of butyrate was confirmed in mature chondrocytes, after IL-1 stimulation (**figure 9.3.7**). After 14 days of ATDC5 cell differentiation, butyrate treatment of mature chondrocytes significantly reduced inflammatory enzymes and adipokines, also limiting the loss of collagen type 2 and inducing chemokine recruitment and resolving factor *AnxA1* (**figure 9.3.7**) (**P<0,001 vs CON; ### P<0,001 vs IL-1).

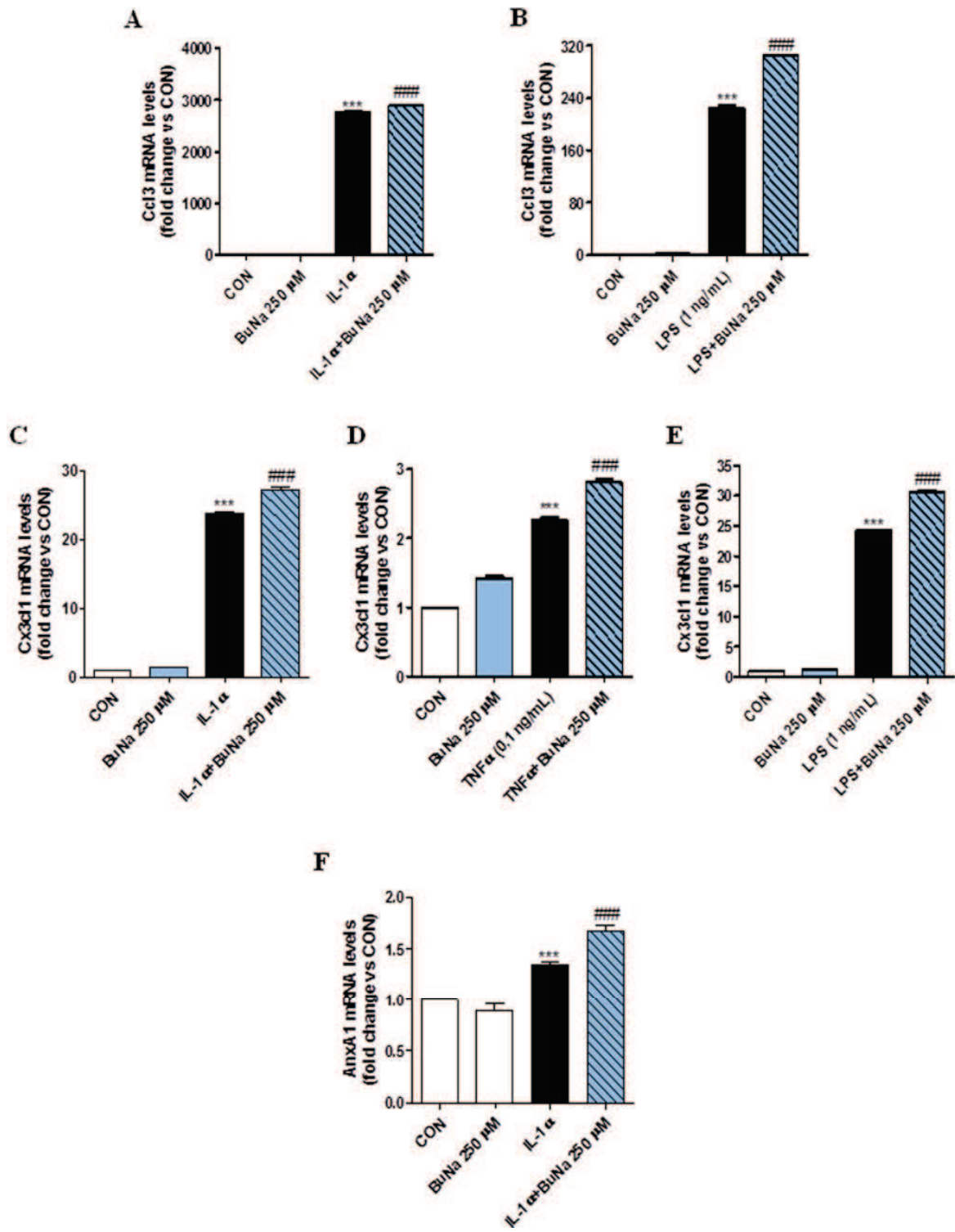


Figure 9.3.6. Effect of butyrate on immune response to inflammatory process induced by IL-1. IL-1 stimulation induced neutrophils and leucocytes recruitment, increasing Ccl3 (A) and Cx3cl1 (C) levels and induced the activation of annexin-1 (F). Butyrate showed a chemoattractant effect, increasing significantly mRNA expression of these genes. After both LPS and TNF- α stimulation (B-D-E), butyrate showed its chemoattractant effect, suggesting a possible general mechanism as pro-resolving factor. Real-Time data are presented as means \pm standard error of 3 different experiments.

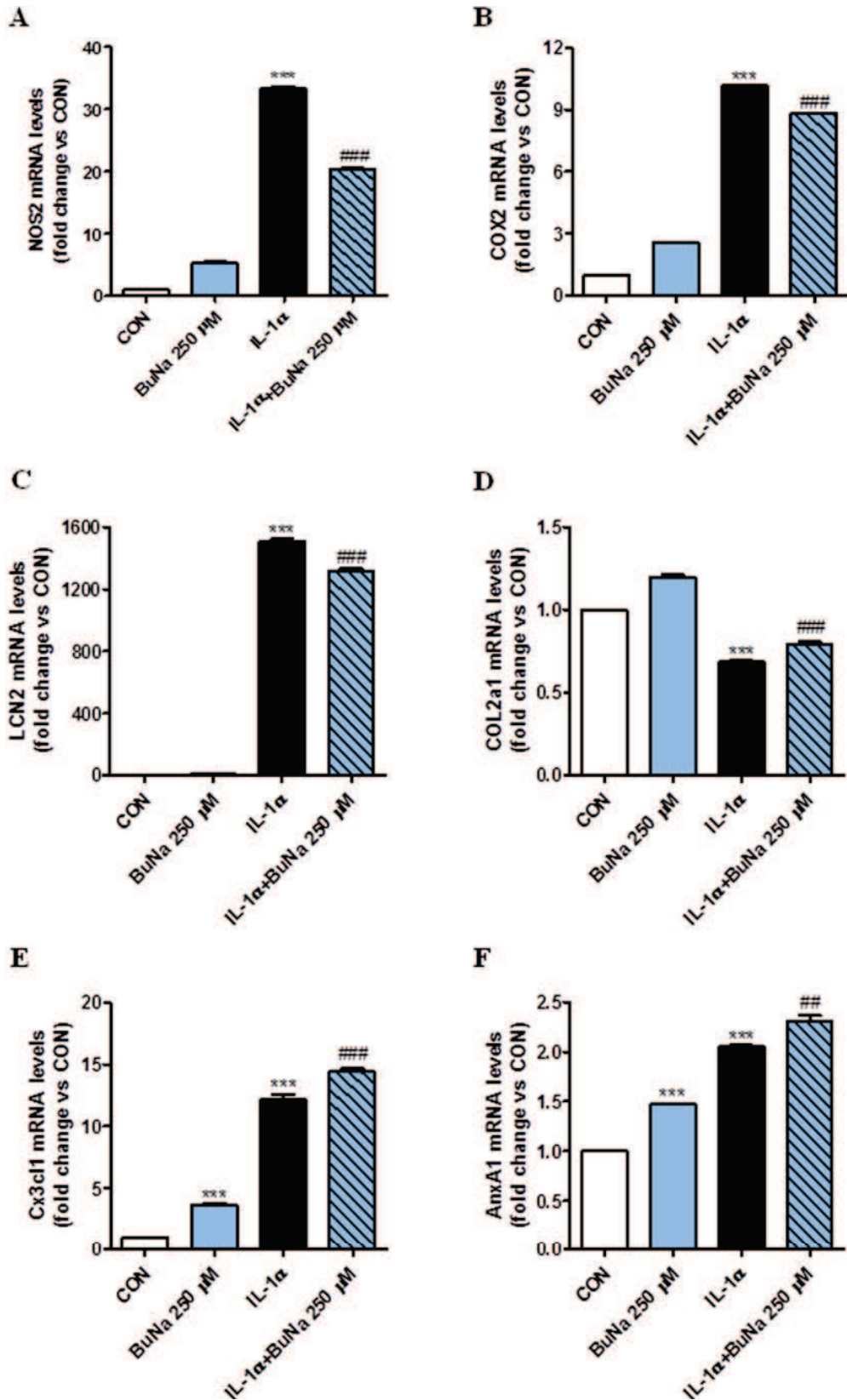


Figure 9.3.7. Effect of butyrate on inflammatory response in mature chondrocytes. After 14 days of cell differentiation, butyrate significantly reduced inflammatory cytokines (A-B) and adipokines (C), and limited the loss of collagen type 2 (D) induced by IL-1, confirming its anti-inflammatory activity during the differentiation. Butyrate also was able to act as pro-resolving factor, increasing Cx3cl1 (E) and AnxA1 (F) levels. Real-Time data are presented as means \pm standard error of 3 different experiments.

GPR43 receptor mediates chemoattractant effect of butyrate in ATDC5 cell line

To clarify a possible mechanism of butyrate chemoattractant effect we hypothesized that it was modulate by GPR43, the main butyrate receptor. To this purpose we silenced GPR43 gene in ATDC5 cells for 48 hours and then mRNA was extracted and chemokine expression was evaluated. The chemoattractant effect of butyrate after IL-1 stimulation was significantly inhibited and reverted by GPR43 silencing, leading to a significant reduction of Cx3cl1 and AnxA1 transcriptional levels, as shown in **figure 9.3.8 A-B**. This result suggests the direct implication of this receptor in modulating butyrate chemoattractant activity (**P<0,001 vs CON; ###P<0,001 vs IL-1).

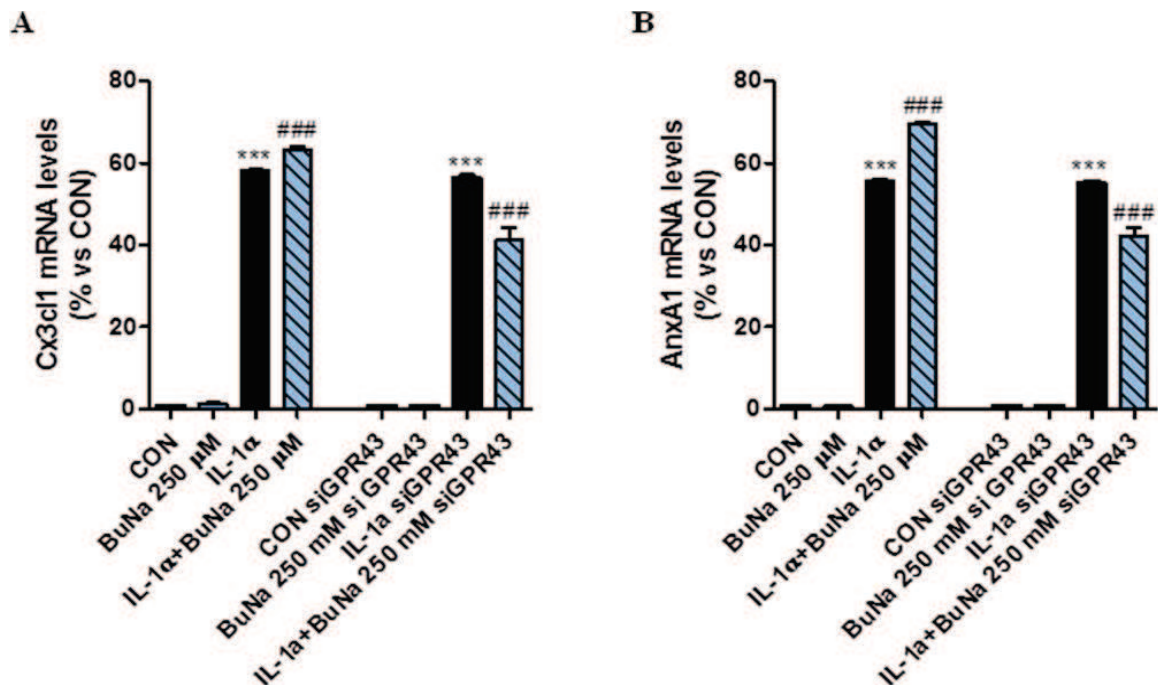


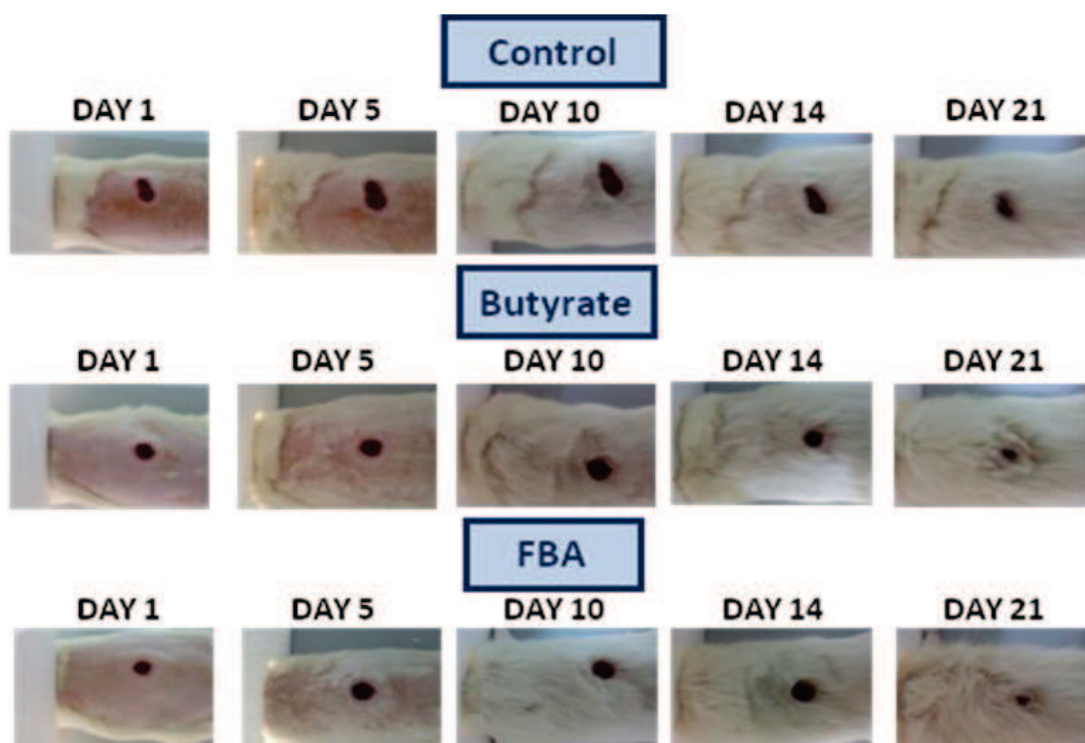
Figure 9.3.8. Chemoattractant activity of butyrate mediated by its receptor GPR43. siRNA-mediated gene silencing of GPR43 receptor for 48 hours in ATDC5 cells. The chemoattractant effect of butyrate on Cx3cl1 (A) and AnxA1 (B) was inhibited by Gpr43 silencing. Real-Time data are presented as means ± standard error of 3 different experiments.

9.4 THERAPEUTIC EFFECT OF BUTYRATE AND ITS DERIVATIVE FBA IN ANIMAL MODELS OF WOUND HEALING INDUCED BY DOXORUBICIN OR MECHANICAL DAMAGE

Effect of butyrate and FBA on doxorubicin-induced wound healing

As previously described [342], standardized skin ulceration was performed by intradermal injection of 2 mg/ml doxorubicin solution on the shaved dorsum of male Sprague-Dawley rats. The maximum of skin ulcer area was reached 11 days after drug injection. The lesion size was measured at day 1, 5, 10, 14 and 21 by using ImageJ software. We showed that the oral treatment with sodium butyrate (20 mg/Kg) and its derivative FBA (42.2 mg/Kg) remarkably reduced the skin ulcer area compared with control group. Pictures and wound area graphic confirmed butyrate effect (figure 9.4.1 A-B) (* $P < 0,05$, ** $P < 0,01$ and *** $P < 0,001$ vs CON).

A



B

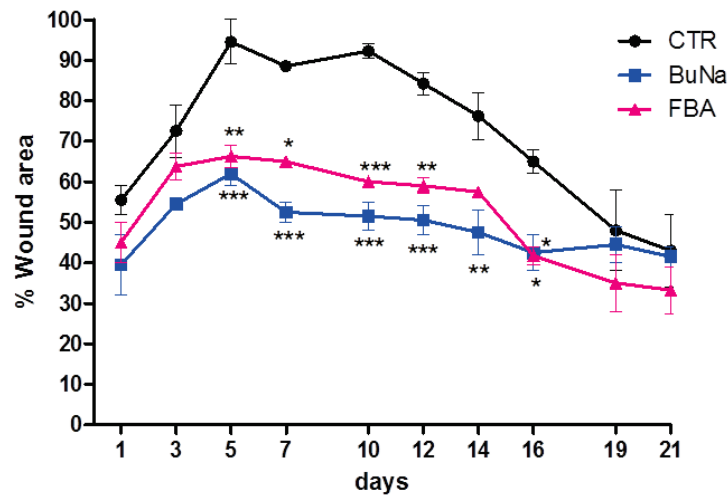


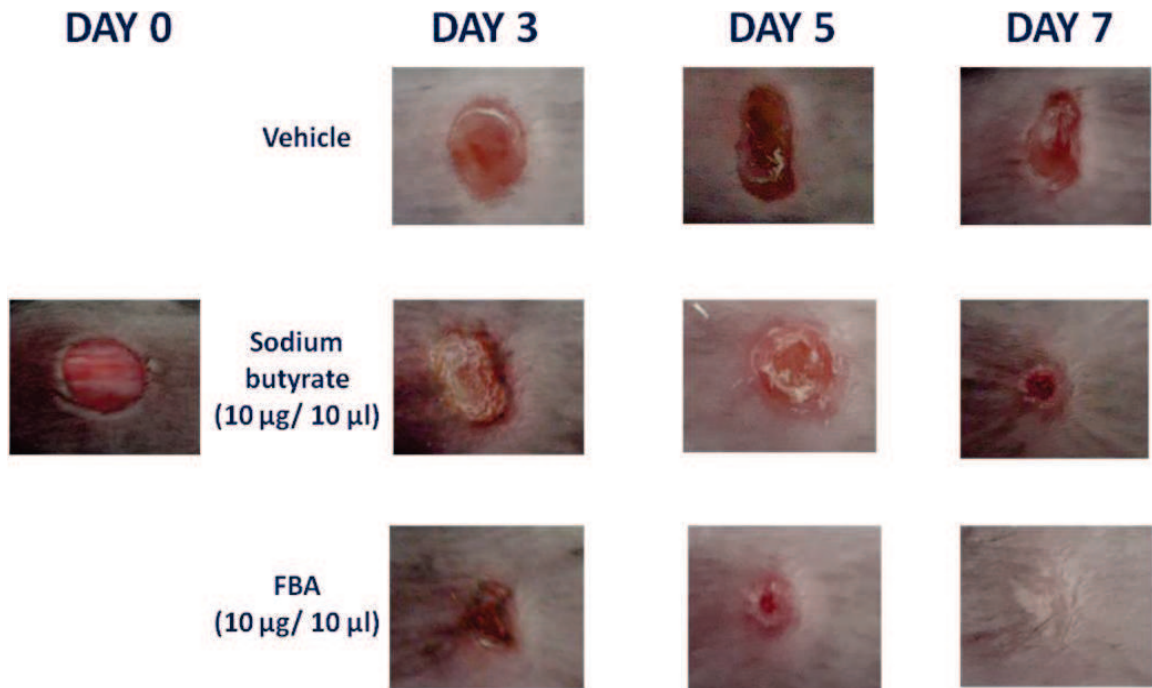
Figure 9.4.1. Determination of wound resolution time. The maximum of skin ulcer area was reached 11 days after doxorubicin injection. The lesion size was measured at day 1, 5, 10, 14 and 21 by using ImageJ software. The oral treatment with sodium butyrate and FBA remarkably reduced the skin ulcer area, as shown by pictures (A) and wound area graphic (B).

Sodium butyrate and FBA promote the excising-induced wound healing

We confirmed the beneficial effect of butyrate in another model of wound healing induced by mechanical damage. For this reason we created a full thickness middorsal wound by excising the skin and the underlying panniculus carnosus with bioptic instrument. Wound closure was measured after 3, 7 and 14 days and to evaluate the change of the wound area for each mouse, we took a picture of wound and analyzed using ImageJ software. We showed that the subcutaneous treatment with sodium butyrate (1-10 μ g), and more with its derivative FBA (1-10 μ g), was able to significantly accelerate the wound healing compared with control mice, as shown by pictures and wound area analysis in **figure 9.4.2 A-B** (* $P < 0,05$, ** $P < 0,01$ and *** $P < 0,001$ vs CON).

Further study will be needed to determine the mechanism by which butyrate could act independently by route of administration.

A



B

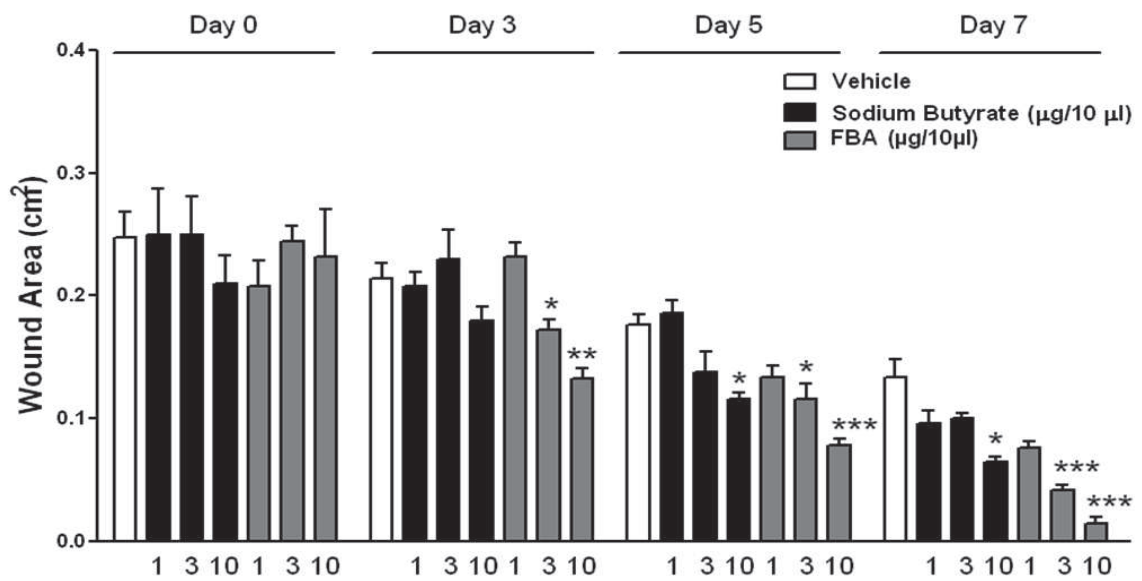


Figure 9.4.2. Evaluation of wound healing during the time after excising. Wound closure was measured after 3, 7 and 14 days, using ImageJ software. The subcutaneous treatment with sodium butyrate and FBA significantly accelerated the wound resolution, as shown by pictures(A) and wound area analysis (B).

10. DISCUSSION

10.1 N-(1-CARBAMOYL-2-PHENYL-ETHYL) BUTYRAMIDE, A NEW SYNTHETIC BUTYRATE DERIVATIVE, REDUCES INTESTINAL INFLAMMATION IN DEXTRAN SODIUM SULPHATE-INDUCED COLITIS.

Despite the wide spectrum of possible indications, the major limits of butyrate in clinical practice are its unpleasant taste and odour, when orally administered, or discomfort, by rectal preparations.

In this study we demonstrated that Butyrate and its derived amide FBA are able to protect from colitis injury induced by DSS administration in mice. In particular, they play their effects restoring epithelial barrier integrity and reducing colon inflammation.

The efficacy of derivative FBA, compared to butyrate, was evaluated in an animal model of colitis induced by DSS.

Even if dietary fiber intake, leading to SCFA production, has shown benefits in IBD [218, 346, 347], other data had demonstrated an inverse association between intake of fruits and vegetables and risk of IBD [348] and more recently dietary intake and risk of developing IBD have been reviewed [349, 350]. Very recently the rational identification of diet-derived postbiotics in restoring intestinal microbiota composition and function has been reviewed [351]. For all these reasons the use postbiotics, such as butyrate, may be a potential alternative to the use of live probiotic organisms or dietary fiber intake as prebiotics. In fact, the beneficial effects of sodium butyrate in different models of DSS-induced colitis in mice have been already reported after oral or topical administration [215, 216].

In our experiment, DSS mice showed a reduction of body weight after five days of DSS administration, while both butyrate-based treatments (PREV and CUR) reduced this loss in DSS-challenged mice. Our results on mice weight change were comparable to those shown in other DSS induced models of colitis [352, 353]. The clinical and histological changes were determined by phenotypic and pathologic changes, such as diarrhea, rectal bleeding, body weight loss and colon shortening, which were the common phenomena seen in DSS-induced experimental colitis. Diarrhea is due to the increased permeability of epithelium or hyper-osmolarity in lumen led by DSS [354]. Weight loss and colon

shortening, as indicators for the severity of intestinal inflammation, correlate with the pathologic and histological changes and are consistent markers for colitis.

Here, we tested oral FBA efficacy in DSS colitis characterized by a significant loss of body weight, associated to diarrhea, rectal bleeding and colon shortening. These phenotypic and pathologic changes were counteracted by FBA, and similarly by sodium butyrate, especially when used as therapeutic protocol.

In this experimental model, establishment of inflammation and tissue injury in DSS-challenged mice was confirmed by the increase in colon tissue of nitric oxide synthase-2 (NOS2), TNF- α , IL-6 and IFN γ and by the reduction in colonic mRNA levels of IL-10 and PPAR γ and in serum adiponectin. IL-10 plays an important role in preventing colitis pathology. In fact, IL-10 knockout mice are prone to develop spontaneous colitis [355]. Anti-inflammatory effect of preventive and curative treatments with butyrate or FBA was also evident in reducing pro-inflammatory mediators and in restoring anti-inflammatory ones. In spite of its clinical beneficial effects for patients suffering from IBD [356], there is a lack of understanding of how SCFAs exert their anti-inflammatory effects. Recent studies of Chang et al. [357] demonstrate that the short chain fatty acid n-butyrate, which is secreted in high amounts by commensal bacteria as Clostridiales species, can modulate the function of intestinal macrophages, the most abundant immune cell type in the *lamina propria*. In fact, treatment of macrophages with n-butyrate led to the downregulation of LPS-induced pro-inflammatory mediators, including nitric oxide, IL-6, and IL-12, but did not affect levels of TNF- α or MCP-1. The authors attributed these effects to inhibition of HDAC activity in macrophages, as it is known that SCFAs inhibit HDAC activity in many cell types [358].

Butyrate regulates epigenetically gene expression by inhibiting HDAC, specifically class IIA and I [359], and its anti-inflammatory effects are related to this mechanism in many cell types [357]. In particular class IIA HDAC has been reported to suppress regulatory T cells (Tregs) expansion [358] and the inhibition of HDAC9 increases Treg function, reducing colitis in mice [360]. Through HDAC inhibition, butyrate could avoid NF- κ B activation in human colonic epithelial cells (CECs) [99]. NF- κ B regulates many cellular genes involved in early immune inflammatory response frequently dysregulated in IBDs [361]. Here, FBA reproduced the same effect of butyrate, limiting the *Hdac9* transcript up-regulation induced by DSS challenge. Accordingly, FBA and butyrate,

especially when used as therapeutic treatment, inhibit NF- κ B activation and promote histone H3 acetylation.

NF- κ B regulates many cellular genes involved in early immune inflammatory responses, including IL-1 β , TNF- α , IL-2, IL-6, IL-8, IL-12, NOS2, COX-2, ICAM-1, VCAM-1, TCR- α , and MHC class II molecules [97]. The activity of NF- κ B is frequently dysregulated in colon cancer [98] and in IBDs, such as ulcerative colitis and Crohn's disease [99, 100]. So, we can hypothesize that one of the possible mechanisms of action underlining its anti-inflammatory effects is linked to butyrate and FBA ability in preventing or reducing NF- κ B activation by HDAC9 inhibition.

These findings not only link butyrate to commensal microbe-mediated induction of functional T-reg cells in the colonic mucosa, but also provide molecular insight into the therapeutic application of butyrate, indicating how a metabolite produced by colonic microbial fermentation mediates host-microbial crosstalk for the establishment of gut immune homeostasis [362]. Numerous studies have reported that butyrate metabolism is impaired in intestinal inflamed mucosa of patients with IBD. Recent data show that butyrate deficiency derives from the reduction of butyrate uptake by the inflamed mucosa through downregulation of MCT1 [105]. Butyrate transport deficiency is expected to have clinical consequences. Particularly, the reduction of the intracellular availability of butyrate in colonocytes may decrease its protective effects on cancer in IBD patients [214]. So, in our model we observed a strong reduction of this transporter in colonic mucosa of DSS mice, confirming an impairment of butyrate utilization, and oxidative stress, in colonocytes. Both butyrate and FBA, only when applied alongside a curative protocol, prevented this down-regulation restoring MCT1 transcription to physiological levels. Aside from inhibition of NF- κ B activation, butyrate may exert an anti-inflammatory activity through the up-regulation of PPAR- γ [361, 363]. This nuclear receptor is a ligand-activated transcription factor highly expressed in colonic epithelial cells, whose activation induces anti-inflammatory effects [364]. Our hypothesis is that butyrate up-regulates PPAR- γ in epithelial cells, where this acts in a negative feedback loop, uncoupling NF κ B-dependent target genes, involved in the inflammatory response [365]. In fact, butyrate and FBA restored PPAR- γ mRNA levels in colonic mucosa, reducing cytokines release and inflammatory status and improving intestinal homeostasis.

IBDs are also thought to result from inappropriate and ongoing activation of the mucosal immune system driven by penetration of normal luminal flora due to tight-

junction defects of the intestinal epithelial barrier. Our results showed a protective effect of butyrate and FBA in restoring transcriptional levels of two major tight junctions as occludin and ZO-1. These data demonstrate that butyrate has a noticeable effects on colonic epithelial integrity and physiology. The altered TJ structure in ulcerative colitis results in impaired barrier function, which may lead to increased uptake of luminal antigens and/or adjuvants that overwhelm the net suppressive tone of the mucosal immune system [366]. SCFAs modulate key epithelial cell functions that help to maintain intestinal epithelial barrier integrity against injury [367]; The most important effect of gut permeability integrity is the reduction of bacterial translocation and maintenance of mucosal immunity homeostasis. In fact DAMPs release and PAMPs penetration determine a strong recruitment of immune cells in infection site and subsequent inflammation establishment. FBA showed an higher effect than that of butyrate in restoring transcriptional levels of *Ocln* and *Tjp1*.

In this study, we also show that butyrate and FBA up-regulated GPR43 mRNA levels in colonic mucosa. Moreover, we confirmed the involvement of this receptor in neutrophil recruitment. Indeed, GPR43 was co-localized with neutrophil cells only in DSS-challenged mice (data not shown). These data suggest that in inflammatory conditions GPR43 can regulate neutrophil chemotaxis (**figure 10.1.1**), while butyrate and FBA treatments, improving intestinal barrier integrity and reducing immune cells recruitment, are able to increase GPR43 expression only on intestinal enteroendocrine L cells . Taken together these actions, both butyrate-based compounds displayed anti-inflammatory properties.

Butyrate and FBA reduced transcriptional levels of *Ccl2* gene, which encodes for MCP-1, in colon tissue and thus could impair monocyte recruitment as confirmed by reduced CD68 and CD14 mRNAs, both markers of macrophages. Besides, both butyrate-based compounds, as demonstrated in another experiment, down-regulated *AnxA1*, *Fpr1* and *Fpr2* mRNA levels, reducing PMN infiltration (data not shown).

Conclusions

In conclusions these data show that butyrate and FBA are able to attenuate colitis and colon injury, reducing the inflammatory response *via* gut integrity improvement, and suppression of neutrophil recruitment in colon tissue. Both compounds restore PPAR- γ

levels and block NF- κ B activation, leading to colonocyte protection from inflammation. In addition, gut integrity is improved, avoiding bacterial translocation and limiting immune cell recruitment. These anti-inflammatory effects are visible as reduction in neutrophil infiltration and HDAC9 transcription in colonic mucosa and as modulation of butyrate transporter and receptor. These events improve intestinal immune homeostasis and tolerance *vs.* commensal bacteria. The efficacy of butyrate in limiting early molecular events responsible of the inflammatory process linked to the intestinal damage suggests its potential clinical utility as a preventive or therapeutic strategy for UC.

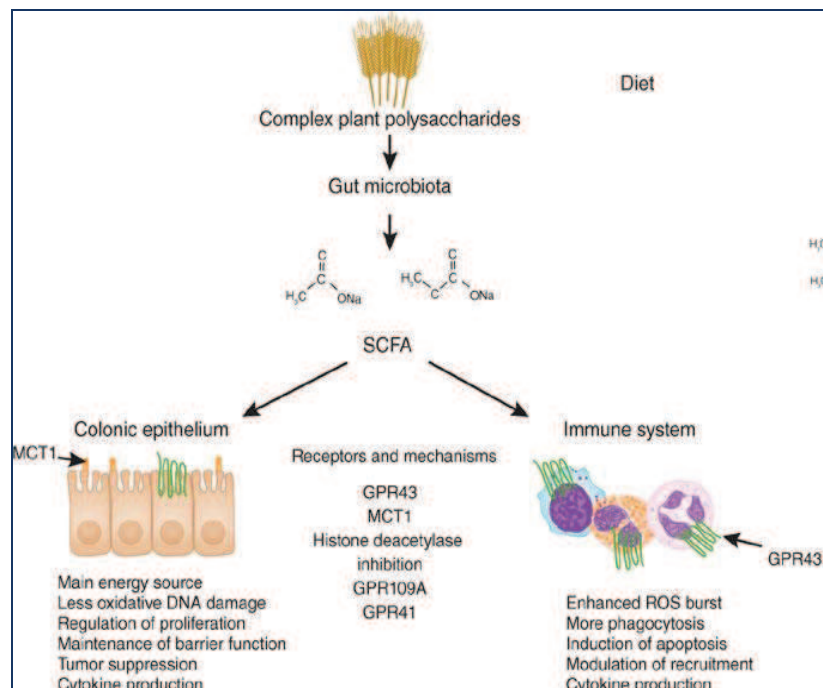


Figure 10.1.1. GPR43 involvement in inflammation and immune system modulation.

10.2 THERAPEUTIC EFFECT OF SODIUM BUTYRATE IN A MODEL OF COW'S MILK ALLERGY (CMA) INDUCED IN MICE

In the experimental model of CMA, we show the therapeutic effect of oral sodium butyrate administration in suppressing acute skin response to the allergen BLG and in reducing anaphylactic symptoms and immediate immune response. Moreover, butyrate is able to restore gut integrity, compromised by BLG sensitization.

The Centers for Disease Control reported that the prevalence of food allergy increased from 3.4% to 5.1% between 1997 and 2011 in the United States [219]. To understand which factors might be driving this increase, attention has turned to the trillions of bacteria that populate human gastrointestinal tract, known collectively as gut microbiota [368]. Recent environmental interventions, including widespread antibiotic use, consumption of a high fat/low fiber Western diet, elimination of previously common enteropathogens (including *H. pylori* and helminthic parasites), increased vaccination/reduced exposure to infectious disease, and Caesarean birth/formula feeding have perturbed mutually beneficial interactions established with commensal microbiota over millions of years of co-evolution. In genetically susceptible individuals, this dysbiosis can predispose to both allergic and chronic inflammatory diseases [369]. The National Institute of Allergy and Infectious Diseases also defined food allergy as “an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food” and food intolerance as no immune reactions that include metabolic, toxic, pharmacologic, and undefined mechanisms [370].

Animal models of food allergy have emerged as tool for identifying mechanisms involved in the development of sensitization to normally harmless food allergens, as well as delineating the critical immune components of the effect or phase of allergic reactions to food [371]. However, animal models might play a role in understanding human unclear diseases.

Interestingly, allergy is a field in which the transcriptional analysis approach has shown remarkable consistency between murine and human species. For example, a recent study using a murine model of atopic dermatitis (AD) included comparisons with data from affected human skin and showed a high degree of homology in the gene expression profile [372]. Also the choice of a specific animal strain is important. Using genetically modified mice, the authors definitively showed key roles for T cells and mast cells in

disease pathogenesis. Similarly, in a murine model of severe asthma, Yu et al [373] performed transcriptional comparison analysis between the murine lung and human lung biopsy specimens. Their data showed a highly significant association in gene expression patterns that was lost in mast cell-deficient mice, but restored if mast cells were reconstituted by adoptive transfer. There is no doubt that such validation approaches will be an important aspect of mechanistic studies moving forward.

The review by Oyoshi et al. [371] aimed to outline the role that animal models might play in understanding the potential mechanisms of pathogenesis in food allergy, as well as to highlight how animal models might contribute to the development of future therapies. There are generally 3 main types of approach in modeling human disease: homologous (in which the underlying cause, symptoms, and treatments are shared), isomorphic (in which the symptoms and treatments are shared), and predictive (in which symptoms might be different but treatments show efficacy). Within the allergy field, most models are isomorphic.

In the field of food allergy, there is not enough information regarding the nature of food allergens and the mechanisms responsible for loss or lack of tolerance in patients. However, it is important to note that food allergens to mice elicits oral tolerance, as it occurs in most human subjects. Instead, mucosal adjuvants, such as cholera toxin (CT) [374] or staphylococcal enterotoxin B (SEB) [375, 376] or genetically manipulated mouse strains susceptible to enteral sensitization [230] have been used. In our study, we used CT as allergy adjuvant in all sensitizations with BLG (20 mg/ml) ad day 1, 7, 14, 21 and 28, and at day 35 with BLG oral challenge (50 mg/ml).

One of the critical advantage of using mouse models to study food allergy is that allergic sensitization or tolerance can be induced to specific allergens under controlled environmental conditions within defined genetic backgrounds, which is not possible in human subjects. This aspect of mouse models allows extensive and precise investigations into the mechanisms involved in disease etiology or therapy, such as identification of possible triggers, as well as pathways involved in food allergy. Normally, ingestion of food results in oral tolerance in mice, as in most human subjects.

Increasing evidence from mouse models indicates that alterations in regulatory T (Treg) cell function and environmental factors, such as microbiota, are likely important contributors to allergic sensitization and food allergy, although the immune mechanisms responsible for breakdown in oral tolerance are not fully understood. Enteral sensitization

to food allergens can also be elicited in the absence of CT or SEB in mice genetically manipulated to enhance IL-4 responses. For instance, *Il4raF709* mice, in which IL-4 signaling is enhanced because of disruption of the inhibitory signaling motif in the IL-4 receptor α -chain, exhibit sensitization to food proteins, mast cell expansion, anaphylactic responses after food challenge, and a food allergy-specific gut microbiota. [230, 231, 377]. These findings implicate strong IL-4 signals, such as those that might be encountered in the T_H2 milieu of atopic patients, in subverting Treg cell responses to oral antigens and fostering the development of food-specific IgE, intestinal mast cell expansion, and susceptibility to anaphylaxis. Consistently, in our experiment we showed that the sensitization with the allergen BLG increased IgE levels in serum and IL-4 content in spleen lysates.

Moreover, increased intestinal permeability has been suggested as a potential cause of food allergy, possibly through increased exposure to the intact protein. In our experiments, we demonstrated that the sensitization with BLG affected gut barrier integrity, while the treatment with butyrate was able to limit the increase of intestinal permeability and, consequently, to improve gut integrity.

Moreover, alterations in the microbiota have been implicated in the pathogenesis of atopic dermatitis (AD), asthma, and food allergy [229]. Intestinal microbiota influences the network of the immune system and results in impaired regulatory functions and T_H2 skewing. While germ-free (GF) conditions are almost impossible in human studies, limiting the types of analysis that can be performed, a role for commensal microbiota in promoting oral tolerance has been clearly defined by using gnotobiotic mice, in which reconstitution of GF mice with well-characterized communities of microbiota or defined bacteria has been performed. Numbers of CD4⁺ Foxp3⁺ Treg cells are reduced in antibiotic-treated mice or GF mice [378, 379], which exhibit a predisposition toward allergic sensitization [379, 380].

Administration of defined commensal microbiota, such as *Clostridia* species and *Bacteroides fragilis*, or SCFAs to GF mice induced Treg cells [358, 378, 381, 382] and reduced allergic sensitization [378], supporting the notion that intestinal commensal microbiota promotes Treg cells and limits allergic responses to foods.

To this purpose, we have clearly demonstrated that the oral administration of sodium butyrate before and during sensitizations with BLG was able to significantly reduce the acute skin response to this allergen and to markedly decrease the innate immune response,

leading to the suppression of allergic responses to BLG, one of the most important cow's milk proteins.

Conclusions

In conclusion, in our experimental model we demonstrate that the oral administration of sodium butyrate before and during sensitization with the allergen BLG is able to reduce acute allergenic response, decreasing of ear swelling and hypersensitivity symptoms and limiting the decrease of body temperature. Butyrate treatment also significantly decreases the innate immune response, reducing IgE and IL-4 levels, measured in plasma and spleen lysates respectively. Finally, butyrate is able to restore intestinal barrier integrity altered by BLG. Efficacy of sodium butyrate in limiting acute and immune allergenic response can suggest its clinical application as a therapeutic strategy for the treatment of cow's milk protein allergy, especially in children.

10.3 IN VITRO ANTI-INFLAMMATORY EFFECT OF SODIUM BUTYRATE AND ITS CHEMOATTRACTANT ACTIVITY MEDIATED BY GPR43 RECEPTOR IN CHONDROCYTE

In this *in vitro* study, we demonstrated the anti-inflammatory effect of sodium butyrate in reducing inflammatory mediators and pathways in chondrocytes activated by IL-1, and, more interestingly, that butyrate chemoattractant activity is mediated by its receptor GPR43.

It is well known that OA is the most common form of arthritis worldwide, whose development is increased by aging, obesity and biomechanical injury [383]. The central characteristic of OA is a progressive degeneration of cartilage, which leads to permanent functional joint failure and disability, but it also involves the whole synovial joint organ [383].

OA is related to degenerative disease in which chondrocytes play a central role, because in this pathology chondrocyte phenotype changes and apoptosis and extracellular matrix degradation occur [279, 384].

Here, we have demonstrated that after 24 hours of IL-1 (0,5 ng/ml) stimulation the nitrite production is increased. Our experiments are in agreement with other studies showing that all severe perturbations previously described in cartilage homeostasis result to be mediated by nitric oxide, an important mediator induced by several pro-inflammatory cytokines, including IL-1 which plays a central role in the pathophysiology of OA [385]. Butyrate treatment is able to limit nitrite accumulation, suggesting its involvement in maintaining and regulating cartilage homeostasis.

Moreover, some evidences clarify that the role of inflammation is pivotal in OA [247], and recognized through the association of joint effusion with joint pain [248] and significant production of several pro-inflammatory mediators [279, 386, 387]. Indeed, we showed that IL-1 stimulation markedly increased the expression of iNOS, COX-2 and IL-6. According to our findings, some studies showed that OA severity and progression depend on the concentration of key SF cytokines, such as IL-1, IL-18 and TNF- α [250, 255]. In particular, IL-1 β induces a cascade of inflammatory and catabolic events in chondrocytes, including the synthesis of prostaglandin E2 (PGE2) and NO [313]. At the same time, also adipokine dysregulation is a clear component of metabolic triggered inflammation that appears to play a major role in OA and RA [388, 389]. In particular,

lipocalin-2 was found in human and murine chondrocytes; and highly expressed in hypertrophic chondrocytes [390, 391]. It is modulated by several factors, including IL-1 β [390-393] and increased in different rheumatic diseases, especially OA [394] and inflamed cartilage results highly positive for this adipokine. Similarly, Li et al. [395] showed that nesfatin-1 (or NUCB2) increased bone mineral density of ovariectomized rats, suggesting a pro-osteogenic activity of this adipokine, that would represent a valuable treatment of bone metabolic diseases, such as osteoporosis. For this reason and for its strong similarities in terms of metabolic actions with other members of adipokine superfamily, NUCB2 was found to be involved in rheumatic disorders, such as OA and associated inflammation [396].

Consistently with all these data, we investigated the expression of LCN2 and nesfatin-1/NUCB2 in ATDC5 cell line after IL-1 stimulation and simultaneous treatment with butyrate for 24 hours and we demonstrated that their expression is highly induced by IL-1. Interestingly, butyrate is able to reduce the levels of both pro-inflammatory adipokines.

It is well known that the recruitment of circulating leukocytes to sites of inflammation is facilitated by adhesion molecules [397]. For example, stronger attachment of leukocytes to endothelium is mediated by ICAM-1 and VCAM-1 [397] and these cell adhesion molecules are commonly more detected in human atherosclerotic lesions than in healthy arterial tissue [398, 399] and their expression is regulated by pro-inflammatory cytokines [397, 400]. In our conditions, IL-1 stimulation increased expression of VCAM-1 and ICAM-1, that was reduced by sodium butyrate.

In articular chondrocytes, IL-1 β is able to trigger several signaling cascades, as that of MAP-kinases [401], which may activate consequentially the DNA binding activity of NF- κ B and AP-1. It has been showed that high NF- κ B and AP-1 binding activity has been found in the synovium of patients with RA and OA, pointing out their involvement in disease pathogenesis [401]. Consistently with these findings, in our study we demonstrated that IL-1 stimulation causes a marked increase of NF- κ B p65 migration in the nucleus. Butyrate not only significantly reduced NF- κ B activation, also limiting I κ B- α degradation. In addition, we clearly demonstrated butyrate-induced inhibition of MAP-kinase pathway, altered by inflammatory stimulus. Other studies have demonstrated that many pro-inflammatory genes transcription is mediated by NF- κ B [402, 403] and sequential

activation of important cytoplasmic protein kinases, such as MAPKs (ERK1/2 and p38, in particular) [404, 405].

Moreover, it was clearly showed that IL-1 activated anti-apoptotic phosphatidylinositol 3,4,5-trisphosphate (PI3) -kinase/Akt pathway and induced the anti-apoptotic effects of Akt [345]. Certain inhibitors of PI 3-kinase blocked ERK activation, suggesting a regulatory role for the PI 3-kinase pathway in the MAPK cascade [406, 407]. In agreement with these data, we demonstrated that IL-1 stimulation increases the phosphorylation of Akt, while butyrate blocks this activation, leading to a remarkable decrease of Akt anti-apoptotic effect, paralleled by the reduction of ERK1/2 phosphorylation.

Furthermore, IL-1 β and hydrogen peroxide led to an activation of the redox dependent transcription of NF- κ B and AP-1 in bovine chondrocytes [408, 409], triggering a remarkable decrease in type II collagen transcription, associated with a significant increase in MMP synthesis. Moreover, Terkeltaub et al. [410] showed that IL-1 β and TNF α induce expression of MMP-3 and MMP-13. Consistently with these evidences, in our study we demonstrated that IL-1 stimulation in ATDC5 chondrogenic cells induced a remarkable increase of MMP13 levels and in the same time it reduced type II collagen compared with untreated cells. Butyrate reduces MMP13 expression and increases type II collagen, underlying its important role in limiting extracellular matrix disruption and consequentially in reducing the loss of collagen, important structural protein in healthy cartilage. Interestingly, all these butyrate activities were confirmed in mature chondrocytes derived by ATDC5 differentiation.

We also showed that inflammatory response by IL-1 promote the significant increase of chemokines, class of small protein cytokines that act as chemoattractants to guide cells to migrate to a specific location, and in the context of inflammation, toward the site of injury or pathogenic invasion [251]. According to our findings, it has been clearly demonstrated that among these chemokines, serum fractalkine, or Cx3cl1, was significantly elevated in patients with knee OA in comparison to healthy individuals sera, while both serum and SF concentrations of the chemokine were associated with knee OA scores [261]. Furthermore, these chemokines, together with other pro-inflammatory mediators, are released in the synovial fluids of OA patients and contribute to the disruption of the balance between anabolism and catabolism [247, 411, 412].

In our study we showed a marked increase in IL-1-induced Ccl3 (or MIP1- α), Cx3cl1 and AnxA1, known mediator of some glucocorticoid effects through the inhibition of PLA2 activity [413] and related to cyclooxygenase and inducible nitric oxide synthase expression [414]. Although its multiple functions [415], the main role ascribed to AnxA1 is an anti-inflammatory activity. We hypothesized that AnxA1 increased expression is a compensatory mechanism, leading to limit inflammation. Interestingly, butyrate treatment increased the expression of Ccl3, Cx3cl1 and AnxA1 after stimulation with IL-1, suggesting its pro-resolving activity in inflammatory response.

Some recent studies, which demonstrated the association and the strong positive connections between several members of HDACs inhibitors, such as butyrate, and the anti-inflammatory pro-resolving molecule AnxA1 [416, 417]. However, the underlying mechanism of this chemoattractant effect of butyrate in chondrocyte was not still well investigated.

The novelty of our data is the involvement of GPR43 in chemoattractant activity of butyrate in chondrocytes. Previously, the role of GPR43 in SCFA-induced neutrophil chemotaxis was determined in mice [76, 92], supporting the relevant role of this SCFA receptor on immune cells during inflammation [76, 93]. In particular, GPR43 seems to be the unique functional receptor for SCFAs on neutrophils [76]. In according with these data, we demonstrated the direct involvement of GPR43 on butyrate-induced chemotaxis in chondrocytes after IL-1 stimulation. In fact, GPR43 silencing in IL-1-stimulated chondrocytes made butyrate unable to induce the expression of Cx3cl1 and AnxA1, missing its chemoattractant effect.

Conclusions

In conclusion, we demonstrated the anti-inflammatory effects of sodium butyrate in ATDC5 chondrogenic cell line not only in reducing pro-inflammatory cytokines and adipokines, but also in decreasing the expression of important adhesion molecules and the activation of inflammatory or anti-apoptotic pathways. We also show that butyrate reduces MMPs production and the loss of collagen type 2, suggesting its capability to preserve cartilage integrity. Interestingly, butyrate anti-inflammatory effects were associated to the stimulation of neutrophil recruitment, increasing the expression of important chemokines

(Ccl3 and Cx3cl1) and the anti-inflammatory and resolving protein AnxA1. The efficacy of sodium butyrate in reducing inflammation in chondrocyte, closed-related with its chemoattractant effect, can suggest its clinical application as pro-resolving factor of cartilage inflammation for the treatment of OA and its related complications.

10.4 THERAPEUTIC EFFECT OF BUTYRATE AND ITS DERIVATIVE FBA IN ANIMAL MODELS OF WOUND HEALING INDUCED BY DOXORUBICIN OR MECHANICAL DAMAGE

Preliminary conclusions

In the gut, butyrate enhances cell proliferation, differentiation and maturation, and reduces apoptosis of normal enterocytes and colonocytes, through its influence on gene expression and protein synthesis [59]. A clear role for these cells has been identified in maintaining homeostasis and in repairing tissue damage.

Wound healing is a highly dynamic process involving complex interactions of extracellular matrix molecules, soluble mediators, various resident cells, and infiltrating leukocyte subtypes. The healing process involves three phases that overlap in time and space: inflammation, tissue formation, and tissue remodeling. Synthesis, remodeling, and deposition of structural extracellular matrix molecules, are indispensable for initiating repair and progression into the healing state. Tissue injury causes the immediate onset of acute inflammation. It has long been considered that the inflammatory response is necessary in supplying growth factor and cytokine signals that orchestrate cell and tissue movements necessary for repair. Successful repair after tissue injury requires resolution of the inflammatory response [319].

However, whereas the knowledge about mechanisms and molecules inducing and ongoing the inflammatory response is increasing, molecules and underlined mechanisms that limit and down regulate this activity are less known. Such mechanisms might include: downregulation of chemokine expression by anti-inflammatory cytokines (i.e. IL-10, TGF- β 1), or up-regulation of anti-inflammatory molecules; or resolution of the inflammatory response mediated by the surface cells. Dysregulation of critical parameters of these interactions results in chronic inflammatory disease states that impair the quality of healing. However, the relevance of these mechanisms for cutaneous tissue repair has to be further investigated.

In our study using two models of ulcers by doxorubicin or mechanical damage, we demonstrated that butyrate and its derivative FBA are able to improve wound healing in

rats and mice. These preliminary data demonstrate that both treatments, through oral or intradermic administration, accelerate and promote wound resolution process.

In both experimental models, butyrate and FBA were able to accelerate and promote wound healing, inducing an early tissue repair. In particular, butyrate was able to act after local or systemic administration. Indeed, both intradermal and oral administration of the examined products reduced the time necessary to obtain wound resolution and tissue repair. The effect of butyrate and its derivative appears to be time- and concentration-dependent. Further studies shall be required to determine butyrate mechanism of action to confirm its role as pro-resolving factor.

The analysis of treated-skin lysates will allow the understanding of the mechanisms involved in butyrate-induced wound healing improvement. In particular, we will analyze whether butyrate would be able to modulate the expression of pivotal mediators involved in the first inflammatory phase, in tissue proliferation and matrix remodeling/regeneration. This study would strengthen butyrate capability in regulating the balance between inflammation and repair.

GENERAL CONCLUSIONS

Short-chain fatty acids are important end-products of probiotic fermentation, therefore they are considered “postbiotics”. Among the SCFAs produced in the human intestine, butyrate has been widely studied and has been shown to play an important role in the maintenance of colonic health. Increased butyrate production in the large intestine seems to be responsible for at least some of the protective effects of fermentable dietary fibre. The effects of butyrate are diverse and complex, and involve several distinct mechanisms that go beyond the classical connotation as an energy source for the intestinal epithelial cells. Frequently, butyrate mechanisms are ascribed to its effect on gene expression, as histone deacetylase inhibitor, and suppression of NF- κ B activation. However, butyrate exerts many other activities, such as the inhibition of colonic carcinogenesis, inflammation and oxidative stress, the improvement of the colonic defence barrier function and the promotion of satiety by different mechanisms, acting not only at intestinal but also at extra-intestinal level.

Here, about its intestinal effects, we examined the efficacy of butyrate and its derivative FBA in a murine model of DSS-induced colitis. Both compounds were able to restore gut integrity, avoiding bacterial translocation and modulating immune cell recruitment. Butyrate anti-inflammatory effects are associated not only to the reduction in neutrophil infiltration and HDAC9 transcription in colonic mucosa, but also to the recovery of the unbalance between pro-inflammatory and anti-inflammatory mediators, involved during colitis. In particular, we have demonstrated that the butyrate-releasing compound, FBA, restore PPAR- γ levels and block NF- κ B activation, protecting colonocytes from inflammation. These events improve intestinal immune homeostasis and tolerance *vs.* commensal bacteria.

Efficacy of butyrate to limit early molecular events underlying inflammatory process linked to intestinal damage, suggests its potential clinical utility as a preventive and therapeutic strategy for colitis. Indeed, FBA may represent an important therapeutic alternative to butyrate, favoring a better compliance and a greater effectiveness, since it lacks the characteristic odor of rancid cheese, its main negative characteristic associated to its instability.

However, the precise innate immune mechanisms of butyrate on beneficial host-microbial interactions are not yet fully understood. Peng et al. [418] demonstrated that butyrate enhances the intestinal barrier by facilitating tight junction assembly *via* activation of AMPK in Caco-2 cell monolayer. Furthermore, butyrate ability to improve gut integrity was also shown in a model of cow's milk allergy (CMA) induced by BLG sensitization. In this model, mice were treated by daily gavage with 20 mg/kg of sodium butyrate, beginning two weeks prior to sensitization and continuing throughout the sensitization protocol. In this study we have displayed that oral administration of sodium butyrate ameliorated gut barrier integrity.

BLG sensitization induces acute allergenic response, with the increase of ear swelling and hypersensitivity symptoms and the decrease in body temperature. Butyrate significantly decreases the innate immune response, reducing IgE and IL-4 levels, measured in plasma and spleen lysates, respectively. Efficacy of sodium butyrate to limit acute and immune allergenic response can suggest its possible clinical application for the treatment of cow's milk protein allergy, especially in children.

From June 2015 to February 2016, my study was focused on the anti-inflammatory effects of sodium butyrate in chondrocytes, and its chemoattractant activity, that was demonstrated for the first time mediated by GPR43. These experiments were performed in the laboratory of Dr. Oreste Gualillo (IDIS Institute), located in the Clinical Hospital in Santiago de Compostela, NEIRID Lab, thank to the COINOR fellowship (Programma STAR-Linea 2 "Mobilità Giovani Ricercatori").

We used IL-1 stimulated-ATDC5 cells, as *in vitro* model of OA. Butyrate not only reduces pro-inflammatory cytokines and adipokines involved in OA, but also decreases the expression of important adhesion molecules, inhibiting inflammatory and anti-apoptotic pathways. We also showed the butyrate capability to reduce MMPs production and the loss of collagen type 2, suggesting an improvement of cartilage disruption. Interestingly, butyrate anti-inflammatory effects were associated to its capability to stimulate neutrophil recruitment, increasing the expression of several chemokines (Ccl3 and Cx3cl1) and the anti-inflammatory protein AnxA1. This last effect was directly mediated by butyrate receptor GPR43.

The pro-resolving effect of butyrate was also analyzed in two models of wound healing induced by doxorubicin or mechanical damage. Here, butyrate and FBA were able to reduce the repair time, when used systemically (by oral administration) or topically (by

intradermal injection). The protective effect of butyrate and its derivative on wound healing and tissue repair appeared to be time- and concentration-dependent. Further studies will be needed to determine the resolving pathway that are induced by butyrate.

Since FBA does not have the characteristic odor of rancid cheese, this derivative may represent a viable therapeutic alternative to butyrate, favoring a better compliance and a greater effectiveness.

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