

## SFA Therapeutics: White Paper of Scientific Basis for R&D Program

*“All diseases originate in the gut” Hippocrates (460-370bc)*

### **Short chain fatty acids (SCFAs): structure, concentrations in human body, production and metabolism:**

SCFAs are products of fermentation of indigestible carbohydrates (dietary fiber such as cellulose, lignin and pectin) by the gut microorganisms. These are saturated aliphatic acids consisting of one polar carboxylic acid moiety and 1-6 hydrophobic hydrocarbon chain among which acetate (C2), propionate (C3) and butyrate (C4) are the most abundant ( $\geq 95\%$ ) (Correa et al, 2016). They can be rapidly and nearly completely absorbed. Most SCFA production occurs in the colon, where they present at about 100 mmol/kg, and often at the ratio of 3 (acetate) : 1 (propionate) : 1 (butyrate). Due to difficult accessibility of the human colon, the estimates of luminal SCFAs concentrations are based on analyses in human gut contents of sudden death victims and stomal effluent of patients with transverse or sigmoid colostomy. In the autopsy samples, total SCFAs concentrations are 137-197 mmol/kg chyme in the caecum, 86-97 mmol/kg chyme in the descending colon. In transverse colostomy samples SCFAs concentrations were high (1400 mol/kg dry matter) compared to those in sigmoid colostomy samples (550 mol/kg dry matter) (Hamer et al, 2008). SCFAs are also present in the oral cavity (6-38 mM of acetate, 1-13 mM of propionate and 0-5 mM of butyrate) and female genital tract (acetate concentrations in the lower genital tract may reach 120 mM). Proportions vary depending on the fiber content of the host diet, microbiota composition, and host genotype. Human portal butyrate concentrations ranged 1.3-14.4  $\mu\text{M}$  in patients during gall-bladder surgery and 14-64  $\mu\text{M}$  in sudden death victims. Most likely, the liver subsequently extracts the majority of the remaining butyrate, resulting in even lower venous systemic serum butyrate concentrations ranging 0.5-3.3  $\mu\text{M}$ . Serum concentrations of propionate and acetate in peripheral blood range 3.8-5.4  $\mu\text{M}$  and 98-143  $\mu\text{M}$  respectively (Hamer et al, 2008). SCFAs are utilized by colonocytes (serve as a primary energy source), also transported via the portal vein to other organs (Tan et al, 2014; Jones, 2016; Verbeke et al, 2015; Correa R et al, 2016). Butyrate is quickly metabolized resulting in a half-life ( $t_{1/2}$ ) of  $\sim 6$  min. + 1.4 min. with the peak blood levels below 0.05 mM (Donohoe et al, 2012). Thus, continued administration of SCFAs most likely will be required to reach effective concentrations (Egorin et al, 1999; Vinolo et al, 2011).

Butyrate is made by *Firmicutes* (*Rosebacterium*, *Eubacterium* spp.), *Butyrivibrio* and other species using either butyryl CoA:acetate CoA-transferase or, less commonly, butyrate kinase to catalyze the final steps of the pathway (Louis et al, 2014; Bourassa et al, 2016). Butyrate can be also produced from mucin degradation by mucin-degrading butyrate-producing bacteria (e. g., *Clostridium* spp., *Eubacterium rectale*, *Rosburia intestinalis*) (Stilling et al, 2016). Butyrate and propionate may be degraded into the smaller, 2C-chain molecules by sulfate- or nitrate-reducing bacteria (such as *Acetobacterium*, *Acetogenium*, and *Clostridium* spp.) (Tan et al, 2014).

Propionate is formed mainly via the succinate pathway by *Bacteroidetes* and *Firmicutes*, also can be made by some gut bacteria in the acrylate pathway (which uses lactate) and propanediol pathway (which uses deoxyhexose sugars such as fucose and rhamnose).

Acetate is produced by acetogenic bacteria, such as *B. hydrogenotrophica* (from  $\text{H}_2$  and  $\text{CO}_2$ ) or from formate via the Wood–Ljungdahl (acetyl-CoA reduction) pathway.

Butyric acid exists in two isoforms: n-butyric and iso-butyric. **Only n-butyrate has molecular and pharmacological characteristics.**

All SCFAs are converted into acetyl-CoA by acetyl/propionyl/butyryl-CoA synthetases. Colonocytes (epithelial cells of the large intestine) derive 60–70% of their energy supply from SCFAs oxidation, butyrate in particular. Butyrate enters the colonocytes and then mitochondria (via specific transporters), and it's not clear what drives butyrate toward the mitochondria. Inside the mitochondria, butyrate undergoes beta-oxidation and is converted into acetyl-CoA. Acetyl-CoA is (1) a starting molecule for the Krebs cycle (an interesting example is that butyrate was able to rescue the diminished mitochondrial respiration in the germ free mice (Bourassa et al, 2016)), (2) a key precursor in lipid biosynthesis, (3) the source of all fatty acid carbons, (4) precursor of the neurotransmitter acetylcholine, (5) donor of the acetyl group in the histone and non-histone protein acetylation reactions (Donohoe et al, 2012; Zambell et al, 2003). Some amount of butyrate in colonocytes is diverted towards production of ketone bodies (such as beta-hydroxybutyrate, BHB), which is, in addition to serving as the alternate source of energy during starvation, involved in numerous, critical functions – inhibition of inflammation, oxidative stress, cancer growth, angiogenesis, atherosclerosis, and many more (Møller, 2020).

About 70% of the acetate is taken by the liver, where it is used as an energy source and substrate for the synthesis of cholesterol, long-chain fatty acids, glutamine/glutamate, etc. The extent to which propionate contributes to energy metabolism in humans is unknown. Concentrations of propionate in portal and hepatic venous blood suggest that ~30% of propionate is taken by the liver (where it serves as a substrate for hepatic gluconeogenesis) (Besten et al, 2013).

**SCFAs absorption and transport:** SCFAs are absorbed into the cells through (1) **simple diffusion** of undissociated form, and (2) **active transport** of SCFAs<sup>-</sup> ions **via transporters** (SCFAs dissociate, since their pKa is ~4.8, and luminal pH is 5.5–6.5). They may diffuse through the epithelium into the *lamina propria* (Besten et al, 2013).

**Major SCFA transporters** are [H<sup>+</sup>]-coupled **MCT-1** (or Slc16a1) and [Na<sup>+</sup>]-coupled **SMCT-1** (or SLC5A8). Besten et al (2013) and Goncalves et al (2016) provide good reviews for both receptors. SCFAs which are not consumed by the colonocytes are transported across the basolateral membrane via (most likely) different MCT transporters. The transporters for the uptake of SCFAs from the blood into the tissues are not well studied. The organic anion transporters (OAT2 and OAT7) were found to transport propionate and butyrate, respectively, across the sinusoidal membrane of hepatocytes. OAT2 was also found in the kidney (Natarajan et al, 2014). Butyrate did induce apoptosis in colon cancer cell lines if SMCT-1 was expressed in these cells and this process correlated with HDAC inhibition (Thangaraju et al, 2009; Tan et al, 2014; Gupta et al, 2006). Both SMCT-1 (known as tumor suppressors) and MCT-1 are downregulated in colon cancer (Natarajan et al, 2014; Lambert et al, 2002).

### **SCFAs act as**

**(1) ligands for the corresponding G-protein coupled receptors (GPCRs)** and regulate downstream pathways,

**(2) HDAC inhibitors (HDACi):** key mechanisms involve activation of GPCR (which reduces expression of HDAC-encoding genes) and inhibition of the HDAC enzyme by binding to and blocking substrate

access to its active site (Abouzeid L et al, 2007); butyrate, in particular, is the most potent HDACi among all known natural compounds).

**(3) modifiers of the activity of some transcriptional factors:** butyrate binds to the butyrate-responsive elements in the selected gene promoter regions, which may contribute to the pleiotropic effects of butyrate (Tabuchi et al, 2006; Bugaut et al, 1993; Stilling et al, 2016).

**G Protein Coupled Receptors (GPCRs)** are activated by conformational changes caused by interaction with diverse ligands. GPCRs trigger downstream signaling such as RAS, PKA, Src, PI3K (Lappano et al, 2011; Dranse et al, 2013), contribute to tissue remodeling and repair, inflammation, angiogenesis, cell growth, etc. (Katritch et al, 2016; Dorsam et al, 2007). Receptors **GPR43 (or FFAR2**, coupled with  $G_{i/o}$  and  $G_q$  membrane-associated G-proteins) and **GPR41 (or FFAR3**, coupled with the  $G_{i/o}$  only) are activated by acetate, butyrate, and propionate. GPR43 and GPR41 share ~33% amino acid identity, and they differ in affinity for SCFAs, tissue distribution, and physiological roles. Activity relationship for GPR43 is acetate = propionate > butyrate, and for GPR41 is butyrate = propionate > acetate (Besten et al, 2013). The  $EC_{50}$  values of the endogenous ligands range ~0.01-1.0mM.  $EC_{50}$  of propionate for GPR41 is 12 $\mu$ M but for GPR43 is 300 $\mu$ M ( $EC_{50}$  is concentration necessary for half-maximal activation of the receptor) (Natarajan et al, 2014). **GPR109a (or HCAR2) is activated by butyrate only** (at  $EC_{50}$  of ~1.5 mM) (Thangaraju et al, 2009) and is expressed on e. g., hepatocytes, gut/retinal epithelium, vascular endothelium, immune cells (dermal dendritic cells, macrophages, and neutrophils), etc. (Tan et al, 2014). See also reviews by Besten et al, 2013, Goncalves et al, 2016 and Natarajan et al, 2014 for details about SCFA transporters and receptors.

Below are some examples highlighting the importance of **SCFA-activated GPCR pathways in health and disease:**

1) SCFAs-FFAR are involved in **regulation of lipid and glucose metabolism**, thus controlling **metabolic homeostasis**. In particular, SCFAs regulate the balance between fatty acid synthesis and oxidation. Fatty acid oxidation is activated by SCFAs in multiple tissues, while *de novo* synthesis is inhibited, resulting in a net reduction of the concentrations of free fatty acids in plasma and in a decrease of fat storage in white adipose tissue (thus, decrease in body weight). *In vitro* and *in vivo* experiments showed that SCFAs increase leptin (adipocytokine) expression via FFAR2 and FFAR3-dependent pathways. Kimura et al (2013) demonstrated that GPR43-deficient mice are obese on a normal diet, whereas mice overexpressing GPR43 (specifically in adipose tissue) remain lean even when fed a high-fat diet. Same authors also observed reduction of glucose and fatty acid uptake in the white adipose tissue associated with suppressed AKT phosphorylation upon acetate administration. Interestingly, oral administration of acetate and propionate reduced glycemia in diabetic hyperglycemic KK-A(y) mice. Via activation of the FFAR2 and FFAR3 receptors, SCFAs may also affect plasma glucose levels by increasing the gut hormones PYY and GLP-1 (PYY and GLP-1 inhibition resulted in the return of appetite and increased food intake). FFAR2 and FFAR3 knockout mice had reduced colonic PYY expression and glucose tolerance. Type of microbiota is of key importance. The complexity of the interactions between gut microbiota, SCFA concentrations, and host energy metabolism in humans are not so conclusive due to the lack of human data and because not all results obtained in rodents can be directly translated to humans.

2) In the context of **cancer**, GPR43 expression is lost in human colon cancer cell lines and significantly downregulated in colorectal adenocarcinomas. Molecular events associated with the activity of GPR43

(stimulated by propionate) included downregulation of PCNA (plays an essential role in nucleic acid metabolism), cyclin D3, CDK1 and CDK2, and stimulation of p21 in p53-independent manner, thus negatively regulating cell cycle progression (increase in the G0/G1 phase arrest) (Tang et al, 2011). GPR109A expression is silenced in human colon cancer and in a mouse model of intestinal/colon cancer (Chong, 2014; Tan et al, 2014) via methylation of *CPR109A* DNA (Thangaraju et al, 2009). However, re-expression of GPR109a in human colon cancer cells and its activation by butyrate has been associated with increased apoptosis (via downregulation of Bcl-2, Bcl-xL, cyclin D1, NF-κB and upregulation of the death receptor pathway) (Sivaprakasam et al, 2016). Singh et al (2014) concluded that expression of GPR109a in immune cells and colonic tissue is necessary for protection against colitis and colon carcinogenesis. SCFAs receptors may act as regulators of carcinogenesis in extraintestinal organs. Forced expression of GPR109A induced apoptosis and cell cycle arrest in breast cancer cell lines (mammary epithelium cells express GPR109A, whereas human breast tumor tissue does not).

3) SCFA–GPR43 interactions profoundly affect **inflammatory responses**. For example, in a chronic model of colitis, *Gpr43*<sup>-/-</sup> mice showed greater morbidity and marked reduction in the ability to regain weight compared to wildtype littermates. *Gpr43*<sup>-/-</sup> mice also showed more severe inflammation in an acute allergic airway inflammation model, increased numbers of cells in the broncho-alveolar lavage fluid, and greater levels of inflammatory cells in the lung tissue (Maslowski et al, 2009). The activation of GPR109A by butyrate prevented colitis through increased expression of anti-inflammatory effector molecules by monocytes and induced differentiation of Treg and IL-10-producing T cells. GPR109a deficiency enhanced susceptibility to colitis (Rooks et al, 2016; Singh et al, 2014). Treatment with β-hydroxybutyrate induced anti-inflammatory effects in both *in vitro* and *in vivo* models of Parkinson's disease (PD) through GPR109a activation and down regulation of NF-κB. Thus, CPR109a could be a good target for therapeutics in PD (Bourassa et al, 2016).

SCFAs reach out other organs (such as the brain and lungs), where they directly or indirectly act on local or resident antigen-presenting cells to decrease inflammatory responses (in case of brain and lungs, associated with neuroinflammation and allergic airway disease, respectively). Tecfidera (dimethyl fumarate, DMF) is an FDA-approved drug for the treatment of relapsing multiple sclerosis (MS). DMF is metabolized into monomethyl fumarate (MMF), which is GPR109A agonist. Butyrate is also GPR109A agonist – can butyrate act as DMF?

GPR43 expression is necessary for SCFA-induced neutrophil chemotaxis (which involved intracellular Ca<sup>2+</sup> mobilization) and for the expansion of Treg cells (Vinolo et al, 2011; Rooks et al, 2016).

### **Immunomodulatory function of SCFAs, role in inflammation:**

SCFAs influence the anti/pro-inflammatory balance, and such immunomodulation is important for homeostasis. SCFAs regulate innate immune responses (by tightly controlling activation and chemotaxis of immune cells to the inflammatory sites) and adaptive immune responses (by direct or indirect modulation of T cell differentiation and proliferation) (Tan, 2014; Rooks et al, 2016).

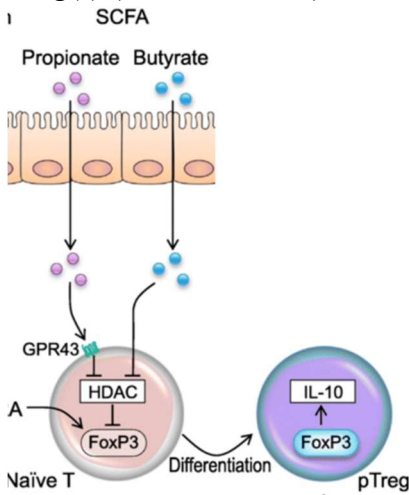
## The principle mechanisms through which SCFAs exert anti-inflammatory effects include:

### - Suppression of TNF- $\alpha$ and NF- $\kappa$ B

Butyrate may inhibit *TNF- $\alpha$*  gene expression via facilitating its mRNA degradation (*cis*-acts on the ARE locus in the 3'-UTR known to be involved in the regulation of mRNA degradation) (Fukae et al, 2005). There are numerous studies explaining mechanisms whereby butyrate suppresses NF- $\kappa$ B (Vinolo et al, 2011, Inan et al, 2000; Lührs et al, 2001, Yin et al 2001, Usami et al, 2008, Segain, 2000). Examples of such mechanisms include inhibition of I $\kappa$ B $\alpha$  degradation (which is NF- $\kappa$ B inhibitor) and upregulation PPAR $\gamma$  (nuclear hormone receptor capable of inhibition the NF- $\kappa$ B-dependent transcriptional activation) (Canani et al, 2012).

### - Stimulation of differentiation of naïve T-cells into Treg cells (Treg)

Treg are FoxP3<sup>+</sup> T<sub>CD4</sub> cells that maintain self-tolerance by suppressing activation/expansion of effector lymphocytes and thus playing a critical role in preventing autoimmunity. HDAC inhibition by propionate and butyrate is one of the downstream mechanisms that modify Foxp3<sup>+</sup> Treg(s) frequency and function (Kinoshita et al. 2014). **Butyrate** exerts epigenetic control over the Foxp3 locus by promoting histone acetylation at the promoter and at the enhancer Conserved Non-coding Sequence 1 (CNS1), thereby enhancing gene expression along with effector function (Furusawa et al, 2013; Arpaia et al. 2013; Skelly et al, 2019). **Propionate** signals through GPR43 to induce IL-10-producing FOXP3<sup>+</sup> Treg(s) (Smith et al 2013). Park et al (2015) showed that SCFAs affect T cell differentiation into Treg(s) also via regulation of the mTOR pathway: inhibition of mTOR during T cell activation is known to promote the generation of Treg(s) (Sun et al, 2018).



One of the characteristics of Treg(s) is expression of anti-inflammatory IL-10 at the steady state. It was shown that, induced by butyrate and propionate, Treg(s) efficiently promoted production of IL-10 (Kinoshita et al. 2014) and blocked the IL-6/STAT3/IL17 signaling. Park et al (2015) showed that SCFAs affect T-cell differentiation into either effector Th1/Th17 or anti-inflammatory Treg cells also via enhancement of mTOR-S6 kinase activity (i. e., increased acetylation of S6 kinase and phosphorylation of mTOR target ribosomal S6 protein, rS6, regulating the mTOR pathway). Inhibition of mTOR during T cell activation is known to promote the generation of Treg(s).

Interestingly, in mice, butyrate facilitated extrathymic generation of Treg cells and was dependent on the CNS1 (conserved non-coding sequence 1, essential for extrathymic but dispensable for thymic Treg

differentiation). CNS1, 2 and 3 sequences define the size, composition and stability of the Treg cell population. CNS1 contains sites for binding with NFAT and SMAD (key member of the canonical TGF- $\beta$  signaling). CNS2 has telomerase activating protein Est-1 binding site, while CNS3 is responsive to c-Rel of NF- $\kappa$ B (Maruyama et al, 2011). *De novo* Treg generation in the periphery was also potentiated by propionate. The capacity for each SCFA to induce Treg cells positively correlated with the level of HDAC inhibition (with butyrate being most potent) and led to increased histone H3 acetylation within genetic loci required for Treg cell induction (Arpaia et al, 2013).

#### **- Inhibition of IFN- $\gamma$**

IFN- $\gamma$  acts via STAT1 signaling (Krause et al, 2006). Butyrate inhibited activation of STAT1 through blocking its Tyr/Ser phosphorylation, nuclear translocation, and DNA binding (Klampfer et al, 2003).

#### **- Inhibition of IL-17**

IL-17 is capable of activating the NF- $\kappa$ B and MAPK/ERK pathways (Hata et al, 2002). IL-17 promotes inflammation by inducing the expression of chemo-attractants that are found in psoriatic lesions. It was shown that butyrate administration suppressed IL-17 levels in plasma and colonic mucosa (in rats) and inhibition of IL-17 release by monocytes (in humans) (Zhang et al, 2016). Microarray analysis of the colonic epithelial cells exposed to butyrate showed down-regulation of IL-17 (Daly et al, 2006).

#### **- Inhibition of IL-23**

In human monocytes and in a rat model of colitis (induced by 2,4,6-trinitrobenzene sulfonic acid), the IL-23 levels were decreased following butyrate treatment (Zhang et al, 2016). Also, it was shown that activation of GPR109a resulted in suppression of IL-23 production in dendritic cells and reduced colonic inflammation (Bhatt et al, 2018). Butyrate is a strong ligand for GPR109a, thus may suppress IL-23 via activation of GPR109a. Another, not direct mechanism of IL-23 suppression by butyrate could be explained by inhibition of IFN- $\gamma$ : since IFN- $\gamma$  upregulates IL-23 (Shaikh et al, 2010), inhibition of IFN- $\gamma$  may result in the decreased levels of IL-23.

#### **- Inhibition of VEGF**

Butyrate was found to block angiogenesis *in vitro* and *in vivo*, and reduce expression of pro-angiogenic factors HIF-1 $\alpha$  and VEGF (Canani et al, 2012). For example, butyrate inhibited transcription factor Sp1 binding to DNA and reduced expression of VEGF gene (Prusanna et al, 2008).

The crosstalk between SCFA and immune cells is shown in **Fig. 1**.

#### In macrophages butyrate

(a) significantly inhibited TNF $\alpha$  and nitric oxide (NO) production and prevented NF- $\kappa$ B activation by stabilizing I $\kappa$ B $\alpha$ /I $\kappa$ B $\beta$  and DNA binding (Qiao et al. 2014; Park et al. 2007);

(b) enhanced the anti-inflammatory IL-10 production;

(c) suppressed migration of macrophages (as a result of HDAC inhibition and, consequently, suppression of Src, an enzyme that phosphorylates focal adhesion kinase (FAK) important in macrophage locomotion) (Arpaia et al. 2013);

(d) reduced production of macrophage chemoattractant MCP-1 (Singh et al. 2014);

(e) stimulated production of prostaglandin E2 (PGE2) (which is anti-inflammatory due to ability to attenuate production of IL-1 $\beta$  and TNF- $\alpha$  and enhance accumulation of Treg cells) (Vinolo et al. 2011a; Kalinski, 2012);



(f) reduced the phagocytic activity (due to inhibitory action on cell differentiation and maturation) (Millard et al. 2002). Murine bone marrow-derived macrophages treated with butyrate showed a dose-dependent inhibition of proliferation (Corrêa-Oliveira et al, 2016). “Butyrate imprints a non-inflammatory antimicrobial programme in gut macrophages, which is consistent with the concept that butyrate-producing commensal bacteria shape host–microbial crosstalk to promote a stable relationship” and homeostasis (Flemming, 2019).

#### In monocytes, butyrate

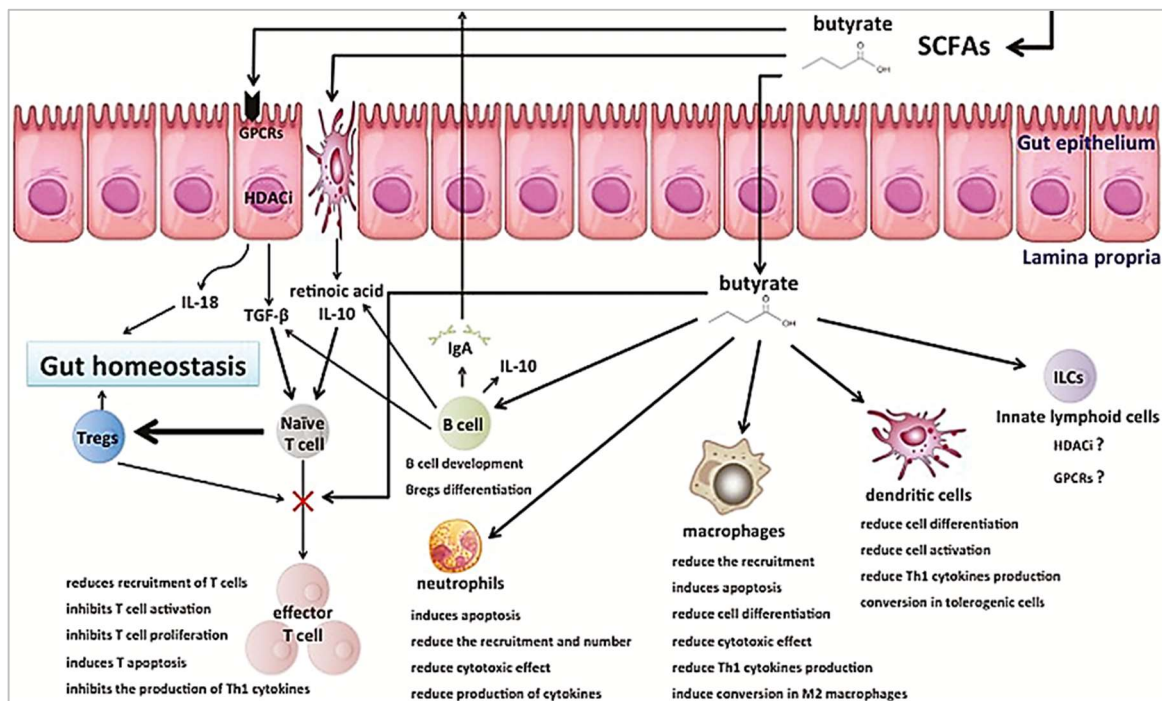
- (a) elicited anti-inflammatory effects via inhibition of pro-inflammatory IL-12 and upregulation of anti-inflammatory IL-10, also suppressed production of  $\text{TNF}\alpha$ , IL-1 $\beta$ , and NO (Park et al. 2007);
- (b) blocked expression of IFN- $\gamma$ -inducible CXCL-10 (chemoattractant for monocytes);
- (c) inhibited HMGP-1 (nuclear protein which activates downstream NF- $\kappa$ B signaling) (Tan et al. 2014).
- (d) upregulated an important lipid mediator of immune responses, PGE2 (Vinolo et al. 2011a).

In neutrophils, SCFAs altered their ability to regulate production of  $\text{TNF}\alpha$ , IL-17, ROS, chemoattractants CSCL1 and CSCL8, and phagocytosis (Corrêa-Oliveira et al. 2016).

In polymorphonuclear cells, SCFAs altered cytoplasmic pH, calcium concentration, phagocytosis, cytoskeletal actin distribution, motility, and chemotaxis (Nilsson et al. 2003; Vinolo et al. 2011).

In dendritic cells, butyrate and propionate promoted overexpression of IL-10 and retinoic acid, and decreased production of pro-inflammatory IL-6, IL-12B and T-cell chemoattractants (CXCL9, -10, -11 and -19) (Nastasi et al. 2015; Singh et al. 2014). Microarray analysis showed that butyrate and TSA (HDACi) induced similar, if not identical, gene-expression changes in dendritic cells (Arpaia et al. 2014).

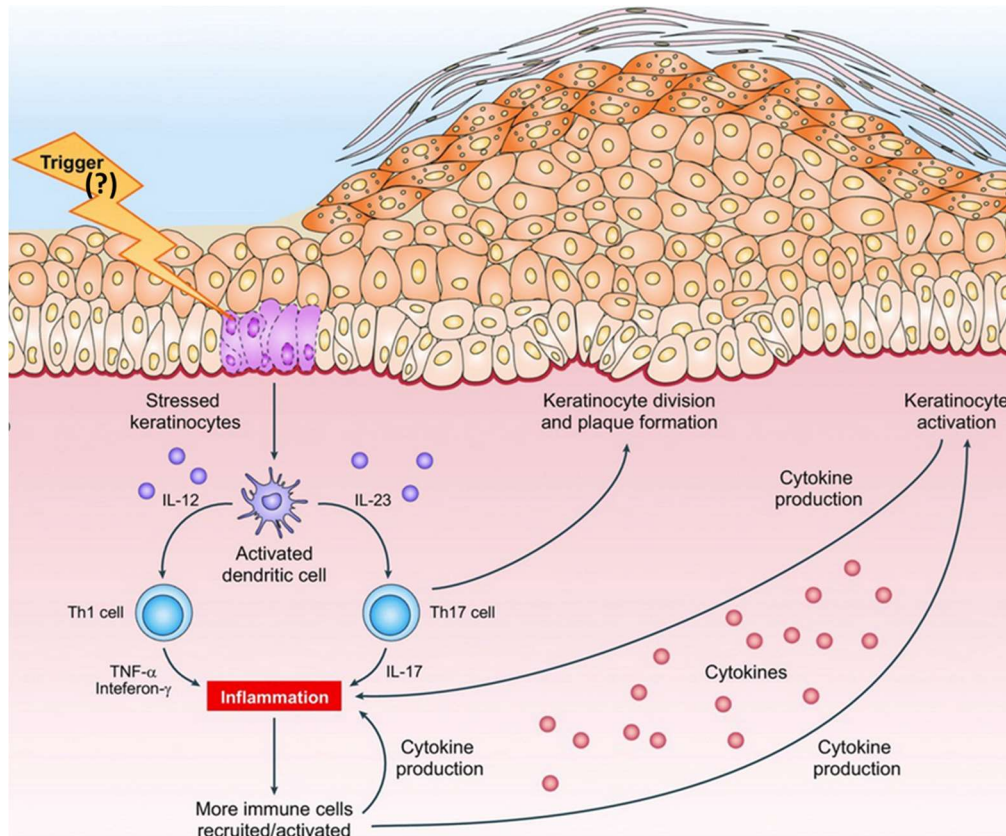
In B cells, SCFAs changed the expression of some genes involved in the B cell differentiation, and IgA and IgG production (Kim et al. 2016).



**Fig. 1.** The crosstalk between microbiota and cells of the immune system: the central role of butyrate in down-regulation of pro-inflammatory effectors and functions. Image is modified from Gonçalves et al, 2018.

## SCFAs in the context of a disease treatment

**PSORIASIS:** Psoriasis is a chronic inflammatory skin disease (**Fig. 2**) characterized by T cell-mediated hyperproliferation and incomplete differentiation of keratinocytes which, under the normal conditions, are continuously produced by stem cells of the basal layer, or *stratum basal*, and completely differentiate during 14 days. Psoriasis is considered as an autoimmune disease, but the antigen(s) that triggers autoimmunity remain elusive. Mechanisms regulating skin barrier integrity and immune responses in the epidermis are important for the maintenance of skin immune homeostasis, and disruption of this homeostasis (by infections, trauma, medications, stress, and genetic predisposition) triggers the pathogenesis of psoriasis. Such factors activate keratinocytes which in turn activate resident skin macrophages and dendritic cells to release cytokines (IL-12 to activate TH1 and IL-23 to activate TH17) that play role in sustained inflammation (TNF- $\alpha$ , INF- $\gamma$  and IL-17 recruit /activate more immune cells). Since neutrophils have a short life span (about 3 days), their persistent presence in the epidermis suggests that they are constantly recruited. Epidermal infiltration of T<sub>CD8</sub> (predominantly) and T<sub>CD4</sub> cells is a striking feature of chronic psoriasis lesions, indicating that these cells are responding to specific antigens (Fallen et al, 2013). IFN- $\gamma$  inhibits apoptosis of keratinocytes by stimulating expression of the anti-apoptotic protein Bcl-x, also promotes IL-23 production. Regarding IL-12, Säemann et al (2000) demonstrated that butyrate strongly inhibited IL-12 at the mRNA level

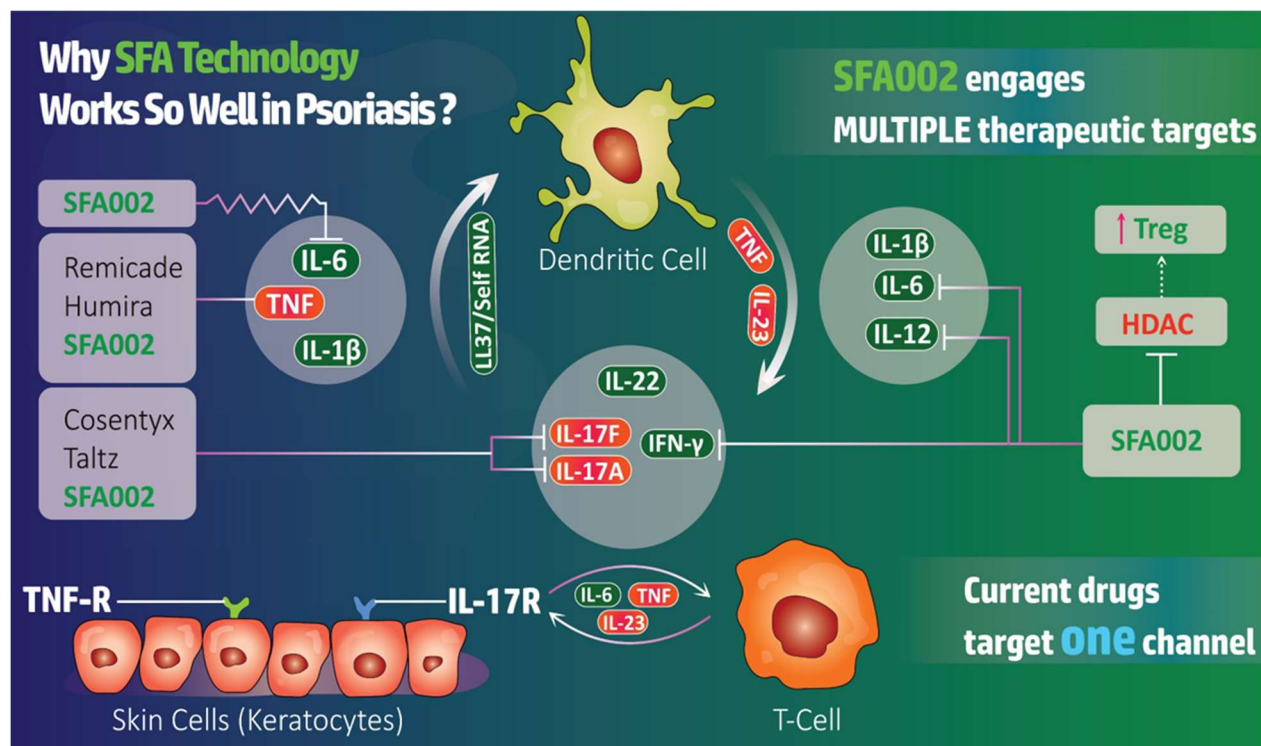




**Fig. 2.** The pathogenesis of psoriasis outlining key pro-inflammatory cytokines involved in the disease onset and development. Image from Young et al, 2017.

Psoriasis has substantial negative effects on patient quality of life.

**Key molecules involved in the pathogenesis of psoriasis**  $\text{TNF-}\alpha$ , IL-23, IL-12, IL-17  $\text{INF-}\gamma$  are altered by SCFAs (butyrate mainly). Mechanisms are explained above (pp 5-6). **Importantly, SFA-002 (1) simultaneously acts on the multiple therapeutic pathways (unlike the other drugs on the market which target a single molecule, Fig. 3), and (2) provides an immunomodulatory effect - brings back to the normal the up-regulated levels of pro-inflammatory cytokines.**



**Fig. 3.** Key targets of SFA-002.

### Vitamin D3 (Vit D3)

Another ingredient in SFA-002 is Vit D3. Vit D3 (or cholecalciferol) is naturally synthesized in the skin (primarily in the *stratum basale*) and metabolized to the biologically active hormonal form 1,25-dihydroxyvitamin D (1,25(OH) $_2$ D) which binds to the vit D3 Receptor (VDR) found in nearly all tissues. VDR is a ligand-activated transcription factor that controls gene expression via binding directly to the vit D Response Elements (VDREs) within DNA and recruiting a variety of co-regulatory complexes (Pike et al, 2012; Bikle, 2014). Vit D3 has diverse immunomodulatory, antioxidant, and anti-fibrotic actions, and may be involved in the pathways that ameliorate immune-mediated tissue injury (Czaja et al, 2019). Differentiation of skin keratinocytes, suppression of T cell proliferation, induction of Treg(s), and regulation of dendritic cell maturation/migration are among the vit D3 associated immunomodulatory functions (Adorini, 2005; Bikle, 2014), **Table 1**.

Importantly, vit D3 modulates the expression of important keratins K1 and K10 in the *stratum spinosum* (known to be suppressed in the psoriatic skin), and normalizes the distribution of integrins (Soleymani et

al, 2015). Vit D3 stimulates synthesis of glucosyl-ceramides required for the barrier integrity and permeability in the *stratum corneum*. Vit D3 deficiency reduces the levels of involucrin and loricrin (proteins of the skin cornified envelop) and keratohyalin granules, resulting in hyperproliferation of the basal layer. The anti-proliferative action of vit D3 in keratinocytes is mediated by the decreased expression of c-Myc and cyclin D and by the increased expression of the cell cycle inhibitors p21<sup>cip</sup> and p27<sup>kip</sup> (Umar et al, 2018).

Vitamin D actions on skin biology and psoriasis pathogenesis
Regulation of keratinocytes proliferation, differentiation and apoptosis
Regulation of cutaneous immune system (inhibition of T cell proliferation, Tregs induction)
Down-regulation of pro-inflammatory cytokines
Stimulation of antimicrobial peptides expression
Regulation of barrier integrity and permeability

**Table 1.** Vit D action in psoriasis (Barrea et al, 2017).

The vit D3 deficiency is observed in the patients with psoriasis (Barrea et al. 2017; Kechichian et al. 2018), and the main goal of the phototherapy is to increase vit D3 production in the skin. Low serum levels of vit D3 contribute to co-morbidities associated with psoriasis (metabolic syndrome, cardiovascular risk, etc.) (Hambly et al. 2017). The therapy with vit D3 (oral and topical, singly or in combination with topical corticosteroids) is often prescribed as the first-line treatment.

**RHEUMATOID ARTHRITIS (RA):** RA is a chronic inflammatory disease characterized by cartilage destruction and extracellular matrix degradation in multiple joints. The pathogenesis of RA involves TNF $\alpha$  activation followed by the induction of other pro-inflammatory cytokines (IL-1 $\beta$ , IL-6, and IL-8) and some matrix metalloproteinases involved in cartilage and bone destruction. The pathogenesis of RA involves activation of pro-inflammatory cytokines (TNF $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8) and some matrix metalloproteinases resulting in cartilage and bone destruction. Butyrate suppressed both TNF $\alpha$  protein and mRNA production in macrophage-like synoviocytes isolated from patients with RA, in human peripheral monocytes, and in murine RAW264.7 macrophages (Fukae et al, 2005). Using the model of rat adjuvant arthritis, treatment with butyrate resulted in downregulation of TNF $\alpha$  and decrease in joint swelling, synovial mononuclear cell infiltration, and pannus formation. There was also a remarkable absence of cartilage or bone destruction (Chung et al, 2003).

**INFLAMMATORY BOWEL DISEASE (IBD):** IBD is a chronic inflammation of the digestive tract and has two forms: ulcerative colitis (affects the inner lining of the colon) and Crohn's disease (affects all layers of the bowel walls and occurs anywhere between the mouth and the anus). Administration of 4g of butyrate/day for 8 weeks (via an enteric-coated tablet) induced clinical improvement and remission in 53% of Crohn's disease patients (as result of downregulation of the mucosal levels of NF- $\kappa$ B and IL-1 $\beta$ ) (Vernia, et. al, 2000). Mice treated with butyrate had reduced neutrophil infiltration and inflammation in colonic mucosa (Tan et al, 2014). In a mouse model of colitis, butyrate administration increased the levels of Treg cells and improved disease symptoms (Furasawa et al, 2013). A recent work confirmed that inflammatory

state in Crohn's disease is associated with a reduction of multiple butyrate-producing bacteria (Takahashi et al, 2016).

**LUPUS:** Lupus is a systemic autoimmune disease with multi-organ inflammation and is characterized by production of pathogenic autoantibodies directed against nucleic acids and their binding proteins, reflecting a global loss of self-tolerance. B cells have been implicated in the pathogenesis of lupus because of their ability to produce autoantibodies (as result of self-antigen presentation to autoreactive T lymphocytes). In a mouse model of lupus, treatment with butyrate led to the suppression of high-affinity autoantibody generation, thus reduction of disease severity (Richards et al, 2016). Study using butyrate treatment in lupus-prone mice showed it could ameliorate kidney damage by increasing F/B ratio and microbial diversity (Anshory, et al 2023).

**OBESITY AND DIABETES:** Obesity is caused by a long-term imbalance between energy intake and expenditure which in turn influences multiple metabolic pathways. Butyrate and propionate are anti-obesogenic because they increase insulin sensitivity and leptin gene expression, reduce cholesterol synthesis (Chakraborti, 2015) and inhibit gut hormone secretion (Lin et al, 2012). Canfora et al (2015) provide an excellent review about the link between the gut microbiota (and their metabolites including SCFAs) and obesity, as well as potential role of SCFAs in the prevention of obesity and associated disturbances in glucose metabolism and insulin resistance. It's not surprising that subjects with Type 2 Diabetes have dysbiosis, particularly significantly lower numbers of butyrate-producing bacteria (Tilg et al, 2014). In a comparative study with metformin using a rat model of Type 2 diabetes, butyrate reduced insulin-resistance, fat accumulation and dyslipidemia resulting in the improved glucose homeostasis (Khan et al, 2016). Acetate and propionate have also been shown to reduce plasma concentrations of cholesterol in rodents and humans. For example, propionate lowered cholesterol synthesis rate by decreasing the enzyme activity of hepatic HMGCS and HMGCR (which are enzymes of cholesterol synthesis pathway) in vitro and in vivo. Acetate supplementation decreased hypercholesterolemia in humans (Besten et al, 2013).

**NASH:** Nonalcoholic Steatohepatitis (NASH) is the advanced form of Nonalcoholic Fatty Liver Disease (NAFLD) characterized by accumulation of fat, inflammation, hepatocellular injury and different degree of fibrosis which may progress to cirrhosis and/or hepatocellular carcinoma (HCC). NASH is the result of interplay of metabolic, inflammatory and fibrogenic processes. The metabolic root (which is studied better) includes insulin resistance, adipose tissue dysfunction, lipid flux in the liver, de novo lipogenesis and imbalance between energy intake and energy expenditure (Ratziu et al, 2022). All these pathways are affected by butyrate. In particular, butyrate (1) potentiates PPAR signaling (PPAR regulates the expression of genes involved in fatty acid beta-oxidation, glucose metabolism, inflammation responses, and is a major regulator of energy homeostasis) (Nepelska et al, 2017), (2) suppresses TNF- $\alpha$ , CCL2/CCR2 and CCL5/CCR5 (key cytokines involved in adipocyte-related inflammation, hepatic stellate cell activation, initiating a fibrogenic response) (Gart et al, 2021), (3) improves insulin sensitivity, (4) promotes expression of GLP-1R (GLP-1 regulates glucose homeostasis, gastric motility and food intake) (Chen et al, 2020), and (5) inhibits fibrosis development (as demonstrated by decreased hepatic collagen content) (Gart et al, 2021).

**NEUROLOGICAL DISEASES:** Butyrate (a) protected neurons from cell death in the models of Parkinson's disease (PD) and in cisplatin-induced hearing loss; (b) reduced the infarct size in models of ischemic stroke (limiting the damage to the brain and improving behavioral outcome); (c) had a strong effect on improving learning and memory in cases of traumatic brain injury or toxicity-induced dementia. The anti-inflammatory effects of butyrate treatment were shown in both *in vitro* and *in vivo* models of PD (Bourassa et al, 2016). Colonization of germ-free mice with butyrate-producing bacteria or butyrate administration orally (PO) restored Blood Brain Barrier (BBB) permeability to healthy levels (Braniste et al, 2014). The gut microbiota of the patients with relapsing-remitting Multiple Sclerosis (MS) showed depletion of a large subset of butyrate producers when compared with healthy people, thus suggesting a possible link between altered gut microbiota and the pathogenesis of MS (Miyake et al, 2015). The use of butyrate as an experimental drug in the models for neurological disorders (ranging from depression to neurodegenerative diseases and cognitive impairment) was reviewed by Stilling et al (2016). Authors also suggested that butyrate could play a role in psychiatric disorders (e. g., depression, which has a pro-inflammatory phenotype).

## **EYE DISEASES**

***Uveitis:*** Uveitis is a disease of different parts of the uvea and is caused by autoimmune disorders or infections. PO administration of SCFAs (butyrate and propionate) attenuated uveitis severity through Treg cell induction, alteration of lymphocyte migration into the eye, and restoration of intestinal homeostasis (Nakamura et al. 2017). The therapeutic effect of butyrate on autoimmune uveitis (via regulation of Th17/Treg cell balance) was confirmed by independent *in vivo* studies (Chen et al, 2017). Interestingly, Na-butyrate and hydroxybutyric acid eye drops decreased inflammation in animals subjected to alkali burns of cornea and were shown to be safe for *in vivo* use (Bian et al, 2017).

***Dry Eye Syndrome:*** Dry eye occurs when the quantity and/or quality of tears fail to keep the surface of the eye sufficiently lubricated. Nakamura et al. (2003) treated rats (which have abnormal tear dynamics and superficial punctate keratopathy similar to that in humans) with eye drops containing hydroxybutyrate. Significant reductions in blink frequency, Schirmer score, and tear clearance were observed (Nakamura et al 2003).

**VASCULITIS:** Vasculitis is a rare autoimmune disease which involves inflammation of the blood vessels, and can affect people of all ages. Vasculitis is often linked to certain blood cancers. Different types of vasculitis are classified according to the size and location of the blood vessels that are affected. One important pathway in the pathogenesis of vasculitis involves GPR-109a receptor activation of which by butyrate down-regulates NF- $\kappa$ B, thus associated inflammation. (Chai et al, 2013).

## **CANCER:**

SCFAs target hallmarks of cancer. What is remarkable about SCFAs is their selective toxicity to cancer cells, while showing little or no toxicity to normal cells which is based on differences in the way cancer and normal cells metabolize SCFAs. The excellent review by Feitelson et al (2023) explains the pleiotropic properties of SCFAs in the context of carcinogenesis. In particular, SCFAs:

- Reduced angiogenesis *in vitro* and *in vivo* by repressing expression of pro-angiogenic factors HIF-1 $\alpha$  and VEGF in human epithelial colorectal adenocarcinoma cells (Canani et al. 2012; Zgouras et al, 2003).

- Arrested cell cycle (at the G0/G1 stage) by activation of p21, down-regulation of cyclins D1 and E1, and triggered apoptosis in multiple cancer cells (colon, breast, liver, etc.) (Jiang et al, 2012). Lallemand et al (1996) showed the direct inhibition of *cyclin D1* gene transcription (in mouse fibroblasts and human epidermoid carcinoma cells) caused by butyrate binding to 11 bp butyrate-response consensus element in the gene promoter region.

- Triggered apoptosis by downregulation of Ras (McBrearty et al, 2021; Jung et al, 2005) and Akt (Chen et al 2006) signaling pathways as well as up-regulation of proapoptotic p53 (de Conti et al. 2013) and p21 (Chopin et al, 2004).

- Stimulated production of GST involved in detoxification of the compounds that can cause oxidative damage and mutations (Ebert et al, 2001).

- Inhibited HDACs (Benjamin et al, 2001). Butyrate inhibited growth of the cancer colon cells but not normal colonocytes and even increased proliferation of the latter, so-called “butyrate paradox” (Bugaut et al, 1993, Vanhoutvin et al. 2009) which may be explained by the Warburg effect (Donohue et al, 2012). Similar effects were observed in the experiments with HCC cells lines (Jiang et al, 2012).

- Down-regulated NF- $\kappa$ B signaling in several cell types (butyrate>propionate>acetate) (Tan et al. 2014). Butyrate prevented degradation of I $\kappa$ B $\alpha$  via suppressing cellular proteasome activity, up-regulated the level of inhibitory I $\kappa$ B protein and thus attenuated NF- $\kappa$ B nuclear translocation (Yin et al. 2001; Lührs et al. 2001).

Important conclusion is homeostasis, which contributes importantly to the definition of "health," is maintained, in part by the production of SCFAs, which, without overt toxicity, can either delay or prevent the development of various diseases. This demonstrates the scientific feasibility of using SCFA-based technology to treat various, serious unmet medical conditions, with a high therapeutic index, even with a potential for durable responses.

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