Immunological Reviews

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Microbial influences on epithelial integrity and immune function as a basis for inflammatory diseases

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Acknowledgements
The authors have no conflicts of interest to declare.

This article is part of a series of reviews covering Microbial Influences on Immune Function appearing in Volume 245 of *Immunological Reviews*.

Immunological Reviews 2012
Vol. 245: 164–176

Immunological Reviews
0105-2896

Summary: Certain autoimmune diseases as well as asthma have increased in recent decades, particularly in developed countries. The hygiene hypothesis has been the prevailing model to account for this increase; however, epidemiology studies also support the contribution of diet and obesity to inflammatory diseases. Diet affects the composition of the gut microbiota, and recent studies have identified various molecules and mechanisms that connect diet, the gut microbiota, and immune responses. Herein, we discuss the effects of microbial metabolites, such as short chain fatty acids, on epithelial integrity as well as immune cell function. We propose that dysbiosis contributes to compromised epithelial integrity and disrupted immune tolerance. In addition, dietary molecules affect the function of immune cells directly, particularly through lipid G-protein coupled receptors such as GPR43.

Keywords: *microbiota, epithelium, GPR43, short-chain fatty acids*

Introduction

A new direction in immunology research has emerged recently that considers the effects of diet on the gut microbiota and immune responses. Gastrointestinal microbes have co-evolved with vertebrates and provide benefits to the host, including maintenance of epithelial integrity and regulation of immune responses. However, the composition of the gut microbiota can differ considerably between individuals, and this difference appears to relate to diet (1–3). This may be particularly relevant to human inflammatory diseases, several of which associate with western lifestyle and obesity. While several studies and reviews (including by us) have highlighted the direct effects of dietary molecules on immune cells (4, 5), a new and possibly equally important element is the gut epithelium. The gut is lined by epithelial cells, which provide an important physical barrier and defense against pathogens. The integrity of the epithelial barrier is important to reduce

contact with bacteria and their products. Thus, a straightforward model that observes high fat/low fiber diet affecting the composition of the gut microbiota and products of certain microbial communities affecting gut integrity, immune tolerance, and the development of inflammatory diseases is being developed. In this review, we explore the epidemiology of inflammatory diseases in relation to diet and defects in epithelial integrity. We then discuss the effects of bacterial products on the epithelium and immune cells.

Possible western lifestyle factors contributing to the development of inflammatory diseases

Diet as a basis for increased incidence of inflammatory diseases

There is a compelling case that diet may be responsible for at least a component of the increased incidence of inflammatory and autoimmune diseases in western countries over the past 40 years. The modern western diet has an increased overall caloric intake, as well as changes in the relative amounts of particular foodstuffs, including lower levels of fiber and higher levels of fat, compared to developing countries. The influence of diet on immune responses may be direct, for instance omega 3 fatty acids which bind to GPR120 on macrophages (5), or indirect, through effects on the composition of the microbiota, which produce metabolites such as short chain fatty acids (SCFAs), which can have profound effects on immune functions (discussed below). Dietary effects on the immune system may also occur through indirect mechanisms. For instance, fiber is fermented by certain colonic bacteria to produce SCFAs. SCFAs affect gut epithelial integrity, which may regulate exposure of the mucosal immune system to bacteria or innate signals that affect immune tolerance. Indeed, epithelial integrity is increasingly recognized in the pathogenesis of diverse inflammatory diseases from inflammatory bowel disease (IBD), to asthma, to type 1 diabetes (T1D) (6–8).

Epidemiological studies suggest that human populations that consume adequate or high amounts of dietary fiber have a decreased incidence of certain inflammatory diseases, as well as type 2 diabetes and colon cancer (9–11). The immune modulating effects of SCFAs described by us and others led us to propose diet, in particular consumption of fiber, as a contributor to the higher incidence of asthma, T1D, and other autoimmune diseases in western societies (4).

Antibiotic use and inflammatory diseases

If the composition of the gut microbiota is having a large bearing on inflammatory diseases, then the other modifying influence, in addition to diet, is antibiotic use. The precise effects of antibiotic use on microbial communities are now emerging, and evidence suggests that beneficial commensal flora never fully recover (12). In particular, the use of antibiotics in childhood might be associated with later onset asthma as well as IBD. This 'antibiotic theory' is still debated, as children wheezing are more likely to get antibiotics while they might already have signs of respiratory problems (13) and some work shows only a slight increase in incidence of asthma in people treated earlier with antibiotics. The case for an association between IBD and antibiotic use is perhaps stronger (12). If both diet and antibiotic use contribute to the incidence of inflammatory diseases, then consideration will need to be given to each in different circumstances.

Hygiene

The hygiene hypothesis (14) is probably the most popular model to account for the increase in asthma and atopic disorders in Western countries. This model supposes that excess cleanliness and diminished exposure to pathogens has led to insufficient stimuli needed for proper development of the immune system. This hypothesis arose from observations that the prevalence of asthma and allergies was lower for people who were raised on farms, or those who came from larger families or were in a lower birth order in such families. Originally, asthma and allergies incidence was also thought to relate to socioeconomic status. As we have argued previously (4), some of the observations that gave rise to hygiene hypothesis may be equally relevant to a diet hypothesis, since diet often relates to rural versus urban lifestyle as well as socioeconomic status. The main observation that challenges the hygiene hypothesis is the high incidence of asthma in communities where infections are prevalent, such as some urban poor communities in the USA who rely on low cost, high fat foods for their nutrition.

Inheritance of microbiota from the mother

The intestinal tract is rapidly colonized by the microbiota soon after birth. Birth by cesarean is associated with a delay of gut colonization, but also a microbiota composed mainly of aerobic bacteria. Vaginal birth results in colonization with a microflora similar to vaginal and colonic microflora, i.e. anaerobic bacteria (15). Interestingly, after 6 months, cesarean deliveries were associated with significantly less *Bacteroidetes fragilis* (15), and even after 7 years of age, the differences in the microbiota were still present, with significantly higher levels of *Clostridia* in vaginally born children (16). The changes in the microbiota in cesarean born children have been associated with increased

development of atopic diseases such as asthma and rhinitis, T1D, and food allergies. Another factor may be use of formula during the neonatal period. The gastrointestinal microbiota of breastfed infants contains low populations of potentially pathogenic groups like *clostridia*, compared to that of formula-fed infants, where the microbiota is more diverse.

Obesity, the gut microbiome, and immune responses

It is now a high priority to understand how diet, antibiotics, breast feeding, etc., affect the composition of the gut microbiota, and the consequences this has on human diseases. The microbiota is a highly dynamic system with considerable changes in its composition within individuals over time (17). There is now firm evidence that diet affects the composition of the microbiota (18, 19). Nevertheless, there is disagreement in the literature as to the degree and timing diet plays in shaping the gut microbiome. The early reports indicated very rapid shifts in microbiota composition upon changes to a high fat or high fiber diet (reviewed in 18). In one recent study (19), clusters of bacterial species were identified in human subjects, which the authors referred to as 'enterotypes'. These enterotypes were not nation or continent specific. This study concluded the existence of a limited number of well-balanced host–microbial symbiotic states, each of which might respond differently to diet. They found that properties such as body mass index, age, or gender did not explain the observed enterotypes. Another important study confirmed the clustering of bacterial species into enterotypes, which could be distinguished primarily by levels of *Bacteroides*, *Prevotella*, and *Ruminococcus*. This study found that enterotypes associated strongly with long-term diets, particularly protein and animal fat (*Bacteroides*) versus carbohydrates (*Prevotella*) (20). When 10 subjects were followed in a controlled feeding study, the composition of the microbiota changed within 24 h of the diet change – high fat/low fiber or low fat/high fiber. Nevertheless, the enterotype identity remained stable, and a major conclusion from the study was that it was long-term diet that strongly associated with enterotype partitioning. It will be important to determine whether certain enterotypes associate with diet related diseases, including asthma and certain autoimmune diseases.

The role of diet and the gut microbiota in the pathogenesis of particular inflammatory diseases

IBD

While the precise etiology of IBDs such as Crohn's disease and ulcerative colitis is unknown, environmental factors play a role. The incidence of IBDs have increased in the past 40 years, and the gut microbiota may play a central role in the development of IBD (21). Studies using mice housed under germ-free conditions provide valuable insight into the importance of the intestinal microflora in IBD. Interleukin-2 (IL2)-deficient mice, for example, spontaneously develop severe intestinal inflammation very similar to human ulcerative colitis when housed under specific pathogen-free (SPF) conditions, but when housed under germ-free conditions, disease is strongly attenuated (22). Similarly, IL10-deficient mice also develop colitis under SPF-conditions, but are completely protected from disease in the absence of enteric bacteria (23). More support for the importance of the microbiota in IBD comes from the analysis of the gut bacteria composition in human patients. Frank *et al.* (24) compared the gut-wall microbiota composition of surgical samples from Crohn's disease and ulcerative colitis patients with non-IBD control subjects by sequencing the bacterial rRNA; IBD patients showed significant dysbiosis with reductions in commensal bacteria, especially of the phyla *Firmicutes* and *Bacteroidetes*. These results are in line with previous metagenomic studies performed with tissue samples from Crohn's disease and ulcerative colitis patients (25) and fecal samples from Crohn's disease patients (26), which also showed significantly altered diversity of the intestinal microbiota. However, it remains controversial whether the perturbed microbiota composition is a cause or a consequence of the inflamed gut. While it cannot be ruled out that inflammatory conditions in the gut can influence the bacterial composition and lead to dysbiosis (21), there is no doubt that dysbiosis also has profound effects on the immune system and intestinal health, be it through altered production of SCFAs that have an important effects on immune cell migration and apoptosis in the gut (27), or through changing the balance of T-regulatory cells (Tregs) and T-helper 17 (Th17) cells (28) or by altering inflammasome responses that can affect the integrity of the gut mucosa (29).

That dysbiosis contributes significantly to IBD rather than being a secondary effect of IBD is supported by a recent study that investigated the correlation of antibiotic use in childhood and the development of IBD. In this retrospective study, the antibiotic use in the first year of life of 36 IBD patients was compared with 360 controls. Children diagnosed with IBD were almost three times more likely to have been prescribed antibiotics in their first year of life than children without IBD (30). A prospective study conducted with 117 children in Denmark also identified a strong correlation between antibiotic use in childhood and IBD (31).

IBD is more prevalent in countries with a western lifestyle (32). The two most likely culprits are antibiotic use and/or western diet, both of which may alter the makeup of the intestinal microbiota and lead to dysbiosis. However, such observational studies are yet to establish conclusively that antibiotic use may favor dysbiosis in later life and consequently an increased chance of developing IBD. Hence, more detailed prospective studies that correlate additional parameters like the diversity of the bacterial composition, production of SCFA and other microbial factors, and immunological data on cell subsets and cytokine production with antibiotic use and/or a specific diet are required.

T1D

Similar to asthma, T1D incidence has increased in western countries. Studies in the non-obese diabetic (NOD) mouse model show that microbial factors strongly affect the development of T1D (8, 33, 34). Likewise, interactions between the gut microbiota and the intestinal immune system have been implicated in the development of T1D (35–37). Previous work with germ-free NOD mice has shown that germ-free conditions significantly exacerbate the development of diabetes and suggests that the prevalence of certain bacterial strains may be more relevant to the outcome of the disease (38). For instance, innate immune mechanisms are important in determining the composition of the microbiota. Thus, absence of Cd1d (also known as Cd1d1), Nod2, or Myd88 qualitatively and quantitatively alters the microbiota compared with wild-type littermates (34, 39, 40). Intriguingly, Myd88-deficient mice only develop diabetes under germ-free conditions. Absence of MyD88 in NOD mice led to an over-representation of the bacterial phyla *Bacteroidetes* (34), and this altered microbiota somehow actively suppressed the development of diabetes, presumably through production of a systemically distributed immuno-regulatory product. Candidates that may be involved include SCFAs (4) but also other bacterial products (see below).

The absence of beneficial gut microbiota significantly reduced relative and total numbers of Treg cells in the mesenteric lymph nodes (MLNs) (41, 42). In addition, segmented filamentous bacteria, which colonize the small intestine, efficiently induce ileal IL-17 (42). An increase in IL-17 in the colon of mice under germ-free conditions induces the expansion and/or survival of Th17 cells (43). It has been suggested that IL17 is upregulated in the colon and MLN in young SPF NOD mice (44). Intriguingly, peritoneal B cells, which are regulated by microbial factors and actively participate in the defense against invasion by intestinal microbiota, are abnormally activated in NOD mice under SPF conditions (45).

Rheumatoid arthritis

The gut microbiota may play a role in the pathogenesis of rheumatoid arthritis (RA). Similar to mouse models of IBD, the absence of gut microflora attenuated disease in mouse models of RA, as was the case in a study using the K/B×N model of RA, where C57BL/6 mice expressing a T-cell receptor specific for glucose-6-phosphate isomerase (GPI) are crossed with NOD mice (46). During the effector stage, autoantibodies aggregate in the joints of K/B×N mice, resulting in activation of the complement cascade and recruitment of inflammatory cells such as mast cells, neutrophils, and macrophages (47, 48). Under SPF conditions, the F1 offspring of these mice develop RA similar to the human disease, while the disease was strongly attenuated in the absence of microbiota. The attenuation under germ-free conditions was associated with a reduction of Th17 cells in the spleen, while mono-colonization with SFB increased Th17 cell numbers and exacerbated disease. However, results by our group indicate that certain gut bacteria have a beneficial function in RA. Mice deficient in the SCFA receptor Gpr43 show aggravated disease in the serum transfer model, which is independent of T and B cells but dependent on mast cells, macrophages, and neutrophils. We have shown that SCFAs have an anti-inflammatory effect, particularly on cells expressing the SCFA receptor Gpr43 such as neutrophils and macrophages (27) (see below and *Fig.* 1).

Evidence for the contribution of gut bacteria to the development of RA also comes from human studies. Eerola *et al.* (49) investigated the composition of bacterial cellular fatty acids (CFA) in fecal samples by gas–liquid chromatography and identified significant differences in the CFA spectrum of RA patients compared to non-RA controls. The observed differences in the CFA composition indicate that the diversity of the intestinal bacteria was strongly affected in RA patients. A recent study compared the composition of the gut microbiota in patients with early RA to patients with fibromyalgia (a non-inflammatory musculoskeletal disease). Using fluorescent probes that were able to specifically distinguish 16S rRNA from eight different bacterial species, a significant difference between RA patients and fibromyalgia patients was found. RA patients exhibited a reduction in *Bifidobacteria* as well as *Bacteroides* and *Eubacterium* (50). Now that there are much better methods for identifying the makeup of the gut microbiome of patient groups through metagenome sequencing, a more thorough analysis of the gut microbiota in human RA patients should soon emerge.

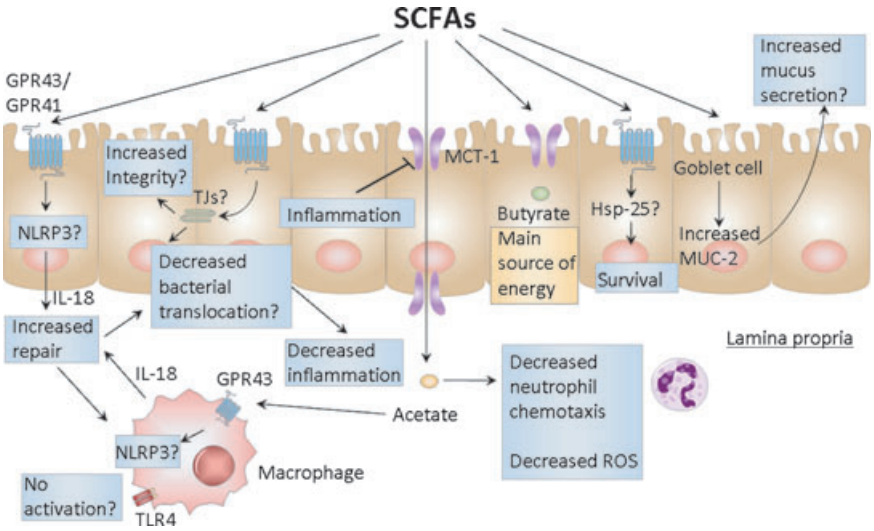

***Fig. 1.* Potential effects of SCFAs on gut epithelial biology and immune cells.** SCFAs may exert their effects through multiple mechanisms including those illustrated above. SCFA signaling via GPR43/GPR41 on epithelial or immune cells may activate the NLRP3 inflammasome, promote IL-18, and lead to epithelial integrity. SCFAs may promote epithelial integrity by preserving tight junctions (TJs). MCT-1 may regulate the transport of SCFAs across the epithelial barrier to access immune cells, such as macrophages and neutrophils and in turn, regulate chemotaxis and function. SCFAs may upregulate Hsp-25 to promote cell survival. SCFAs may promote maintenance of the mucus layer.

Asthma

Asthma has been the inflammatory disease that has increased most dramatically over the past 40 years, and has been associated with western lifestyle. The effect of diet on asthma and allergies has been reviewed recently (51), and there is now mounting evidence that the gut microbiota is altered in people with allergy or asthma (reviewed in 52). Numerous epidemiological studies that have linked obesity with the development and severity of asthma, in both children and adults (53). In one study, 1861 children were monitored from birth to assess nutrient intake by their mothers during pregnancy and correlated this with the development of asthma in the children later in childhood. The mothers of children who later developed childhood wheeze and asthma had a distinctly different dietary intake (54).

Asthma is a heterogeneous disease of unknown origin usually characterized as an adverse Th2 response. However, the majority of novel genes identified in asthma are located in lower airway epithelium rather than the Th2 inflammatory cascade (55). In a genome wide association study on asthma, single-nucleotide polymorphisms (SNPs) of epithelium related genes have been identified (56), specifically SNPs flanking IL-33 and IL-18R1, which may modify the inflammatory response to epithelial damage.

In animal studies, conditional transgenic mice that had reduced airway epithelial expression of thyroid transcription factor 1 (Ttf-1) and forkhead box A2 (Foxa2) caused an increase in goblet cell metaplasia and Th2 responses (57, 58). Hence, epithelial-related genes may play a previously unappreciated role in the development of asthma.

In human studies, asthmatics have defects in tight junctions (TJs) (59). Indeed, epithelial cells brushed from the airways of asthmatics and cultured as monolayers show defects in TJ function. This may be representative of a defect in repair, since addition of epidermal growth factors improves dysfunction (60). Other defects closely associated with the epithelium include defects in antioxidant defenses (61) and innate immunity (60). Defects in innate immunity in asthmatic epithelium are evident in viral infections, where asthmatics have impaired interferon responses (62). Furthermore, the critical role of epithelium in asthma is highlighted as corticosteroids, which target the inflammatory response, given over prolonged periods have little effect on the natural history or remodeling of the airways (63). These defects in epithelium help to explain why asthma is characterized by both structural and immunological features, both of which are essential components of disease. This also provides rational for the different subtypes of asthma. While most of the above studies have concentrated on airway epithelium, it will also be important to assess gut epithelial integrity in asthma patients.

Bacterial products that affect immune cell functions

There are two broad mechanisms how dietary molecules or products of gut microbes might facilitate immune regulation.

Dietary molecules or factors produced by microbes may act directly on immune cells and affect immune cell survival, activation, or cell recruitment, by binding surface expressed receptors on immune cells. GPR120 and GPR43 are excellent examples. Another attractive proposition is that dietary molecules or bacterial metabolic products regulate epithelial integrity, which may affect barrier function and exposure of the immune system to gastrointestinal antigens as well as factors such as TLR agonists. Poor epithelial barrier function may therefore allow inappropriate costimulation through PAMPs and compromise normal tolerance mechanisms. This topic is discussed below, particularly in relation to SCFAs, and readers are directed to a recent comprehensive review describing the factors affecting epithelial integrity (64).

Gut microbiota and short chain fatty acids

One of the main functions of the gut microbiota is to break down complex polysaccharides into SCFAs, mostly acetate, butyrate, and propionate. SCFAs are an energy source for colonocytes but also affect immune cell functions. This fermentation process is specific to the anaerobic bacteria, mostly of the phyla *Bacteroidetes* and *Clostridium*, and occurs in the cecum and colon (65). Levels of SCFAs produced in the colon vary according to diet. In a study of children in Burkina Fasso, high consumption of dietary fiber, as well as presence of bacterial species efficient at digestion of fiber, correlated with high production of SCFAs (1). These three SCFAs are found at the highest concentrations in the colon, approximately 50 mM, but are also found in peripheral tissues, particularly acetate at approximately 50 μM (65). Concentrations of acetate in the blood can be much higher (∼low mM) particularly in individuals on a high fiber diet. SCFAs promote health benefits for inflammatory diseases, infection, and cancer. For instance, clinical recovery from cholera in malnourished children associates with increased levels of SCFAs (66). Moreover, patients with IBD show decreased levels of acetate, butyrate, and propionate (67).

Gpr43

Acetate and other SCFAs act on cells through two possible pathways: they can enter cells and inhibit histone deacetylases and affect physiologic cellular processes, such as gene transcription. SCFAs also bind the G protein-coupled receptors GPR43 and GPR41 (*Fig. 1*). Mice deficient in Gpr43 showed profoundly altered inflammatory responses (27). The only known ligands of GPR43 are SCFA, particularly acetate and propionate. GPR43-deficient mice (also known as *Ffar2*$^{-/-}$) showed exacerbated and poorly resolving inflammation in the K/B×N serum-induced arthritis model, a model of allergic airway inflammation, as well as in colitis models (27). Neutrophils lacking GPR43 are hyperactive and are more responsive to chemoattractants such as C5a and inflammatory chemokines (27). However, not all inflammatory conditions are exacerbated in GPR43-deficient mice, including uric acid crystal induced gout. This result is explained by a compromised activation of NLRP3 inflammasome in macrophages lacking GPR43 (unpublished observations).

Microbial effects on epithelial integrity

The gut epithelial barrier is not only composed of epithelial cells but also antimicrobial products and a mucus layer. The cellular component consists of four types of cells: the enterocytes, the goblet cells, the Paneth cells, and the enterochromaffin cells (68). These cells are apically closely attached by tight junctions limiting the paracellular transport of luminal contents such as microbial products. The major components of these tight junctions include zonulin and occludin (69), which are commonly used as markers of gut epithelial integrity, and are downregulated in DSS-induced colitis (70), and also obesity (71). Certain assays measure either *in vivo* or *in vitro* the leakiness of these junctions. In humans, non-invasive methods measuring gut epithelial leakiness are used to diagnose food allergies (72). In animal models, presence of FITC-Dextran in plasma after gavage is commonly used to gauge gut integrity. Finally, *in vitro*, a decrease of the transepithelial resistance and an increase of the transepithelial passage of defined products such as beads through confluent epithelial cell lines is a marker of increased epithelial permeability.

Antimicrobial peptides released by Paneth cells play a dual role in maintaining microbiota homeostasis and in the stimulation of the immune system. Particularly, proteins belonging to the defensin family play a critical role in maintaining homeostasis. Active β-defensins result from the cleavage of precursors. Mice lacking the enzyme involved in this maturation, like the Matrilysin-deficient mice, have reduced capacity to clear enteric pathogens (73). α-defensins have been shown to actively regulate the gut microbial ecology with lack of α-defensins associated with an increase ratio of *Firmicutes*:*Bacteroidetes*. Complete loss of *segmented filamentous bacteria* in the small intestine, and thus fewer Th17 T cells, were observed in mice expressing human α-defensins (74, 75). Finally, patients with Crohn's disease have decreased antimicrobial effects in the *lamina propria* leading to impaired epithelial barrier function with increased bacterial translocation through the epithelium (76).

Another major component of the gut epithelial barrier is the mucus. The goblet cells secrete the protein Mucin-2, belonging to the mucin family, which with a complex glycocalyx network ensures the physical separation between the epithelium and the microbiota. The mucus is made of two layers, the outer layer, which is loose and 'populated with bacteria', and the inner layer, which is dense and devoid of bacteria (77). The mucus is critical to maintain an efficient epithelial barrier. In DSS colitis, the thickness of the mucus layer decreases while inflammation develops (78) and mice lacking Mucin-2 are more susceptible to develop disease (79). The transmembrane Mucin-13 has a potent anti-apoptotic effect on epithelial cells, highlighting the key role of mucus in the epithelial barrier integrity (80).

A compromised epithelial barrier may also allow passage of whole bacteria as well as their products. High fat diet-fed mice show higher translocation of commensal bacteria to the blood and adipose tissue compared to standard chow-fed mice (81). This translocation involves the Nod1 receptor, as Nod1 knockout mice had a blunted bacterial translocation, whereas translocation was strongly increased in Myd88-deficient and leptin-deficient *ob/ob* mice. This translocation was reversed by the use of probiotic bacteria that increased epithelial integrity.

Modulation of epithelial permeability by SCFAs

Bacteria in the gut contribute to epithelial integrity. As described above, one of the important elements in the gut mucosa is the mucus that physically protects against invasion of both pathogenic and non-pathogenic bacteria. In germ-free mice, the mucus layer is thinner than in conventional mice, but normalizes when exposed to bacterial products such as LPS or peptidoglycan (78). Interestingly, acetate and butyrate stimulate the release of mucin (82), although whether this requires Gpr43 remains unclear (*Fig. 1*). *In vitro*, addition of butyrate to goblet cell lines HT29-Cl.16E increased the expression of MUC-2 23-fold (83), further suggesting that SCFAs might be critical bacterial products for maintenance of the mucus layer.

Gut epithelial cells express the same receptors to detect MAMPs (microbial associated molecular patterns) as the innate immune cells uses to sense pathogenic bacteria and clear infections. These receptors include TLRs, the nucleotide-binding oligomerization domain protein-like receptors (NLRs), and the C-types lectin receptors (46). Interestingly, instead of triggering an inflammatory reaction, the interaction of the commensal microbiota with the epithelium is necessary to ensure a functional epithelial barrier and to prevent diseases such as IBD (84). A level of sub-stimulation of these receptors contributes to epithelial barrier function. Thus, germ-free mice lacking TLR stimulation are more susceptible to DSS colitis, as are mice that have received a broad-spectrum antibiotic treatment to deplete their colonic microbiota (84). Conversely, injection of LPS alleviates the severity of colitis in mice depleted of their microbiota and is associated with upregulated cytoprotective heat shock protein-72 (Hsp-72) and Hsp-25 in epithelial cells (84), which serve a defense mechanism under stress conditions. New therapeutics aim to target them to treat gut inflammatory diseases (85). It was shown in rats that supplementation of the diet with non-fermentable fiber suppressed the expression of Hsp-72 and Hsp-25, and that addition of butyrate to gut mucosal cells *in vitro* upregulated their expression (86). Butyrate is not the only SCFA mediating this effect, as propionate also induced the upregulation of Hsp-25 on the cell line IEC-18 (87) (*Fig. 1*). To our knowledge, the role of GPR41 and GPR43 or histone deacetylases are yet to be investigated. However, this result suggests that the composition of the microbiota and the type of diet consumed, leading (or not) to higher SCFAs production, might be important to maintain a functional epithelial barrier.

Some studies have revealed dysbiosis in patients with IBD, including a reduced gut microbial diversity and a decrease in *Clostridia* groups IV and XIVa, which are the main butyrate-producing bacteria (6, 24, 88). This result probably accounts for the decreased SCFAs in these patients. Butyrate is a major source of energy for colonocytes (89) and is transported from the lumen to the cytoplasm via the transporter monocarboxylate transporter 1 (MCT-1) (90) (*Fig. 1*). Inflammation that develops in the *lamina propria*, in both rodent models of DSS-induced colitis or in human IBD, downregulates the expression of MCT-1, blocking the entrance of butyrate into the cells (91). Butyrate oxidation is thus reduced in patients with IBD, which might directly account for the increase in reactive oxygen species in epithelial cells (91). MCT-1 is not specific for the transport of butyrate but also participates in the transepithelial transport of propionate and acetate and other short chain carboxylic acids. Since acetate is a potent anti-inflammatory mediator (27), the reduction of MCT-1 expression in IBD might impede acetate transfer to gut mucosa and other sites, and affect immune cell recruitment and survival.

Another major effect of the SCFAs is their ability to directly enhance epithelial integrity *in vitro* and *in vivo* (*Fig. 1*). Addition of acetate, propionate, and butyrate enhances transepithelial resistance *in vitro* on cell lines and *ex vivo* on rat cecum, with the strongest effect mediated by acetate. Interestingly, addition of

a G-protein inhibitor strongly attenuated the effects of acetate suggesting that acetate signals via a G-protein-coupled receptor (GPCR) (92). As mentioned earlier, GPR43 is the GPCR with the highest affinity for acetate, although GPR41 or an unknown receptor may also play a role. Acetate has also been shown to increase epithelial integrity *in vivo*, as reconstitution of germ-free mice with *Bifidobacteria Longum*, a high producer of acetate, protected from a lethal *E. Coli* infection by increasing epithelial survival and integrity (93).

SCFAs also inhibit histone deacetylase (HDAC) activity. HDACs are a conserved family of chromatin-modifying enzymes that repress transcription by deacetylating nucleosomal histones. SCFAs (particularly butyrate) induce hyperacetylation of histones by inhibiting histone deacetylation, and thereby modulate gene transcription. SCFAs have well documented anti-inflammatory effects and at least part of this is through NF-κB inhibition and related suppression of inflammatory cytokines (94). To what degree acetate and other SCFAs mediate their disease modifying effects through GPR43, GPR41, or through inhibition of HDAC activity, are topics for future investigation.

The inflammasome and epithelial integrity

Another mechanism whereby microbiota may preserve epithelial integrity is via the activation of the inflammasome. The inflammasomes are cytoplasmic multi-protein complexes that sense microbial products, and are composed of NLRs, adapter proteins, and procaspase-1, triggering IL-1β and IL-18 maturation (95). Genetic studies have shown the importance of NLRs in IBD, as mutation of Nod2 is associated with increased susceptibility to Crohn's disease (96). Activation of the inflammasome is well described in innate immune cells, but non-immune cells such as gut epithelial cells also show functional inflammasome activation. In one recent study (97), various components of the NLRP3 inflammasome such as NLRP3, Pycard, and Caspase 1 were all necessary to protect against DSS colitis. This study found that release of IL-18 by epithelial cells following NLRP3 inflammasome activation was critical for epithelial repair. Interestingly, we found that GPR43-deficient mice do not develop gout when injected with uric acid crystals into their joints (A. Viera, unpublished data). Gout-associated uric acid crystals activate the NLRP3 inflammasome (98), and we found that the activation of the inflammasome was impaired in GPR43-deficient macrophages. GPR43-deficient mice are more susceptible to DSS-induced colitis (27). Thus, it is possible that SCFAs, binding to GPR43, might participate in inflammasome activation in mucosal tissues and promote epithelial integrity (*Fig. 1*). The decrease of SCFAs in patients with IBD might also exacerbate epithelial damage, with lower SCFA levels leading to sub-optimal inflammasome activation in either immune cells or epithelial cells.

While immune cells express the NLRP3 inflammasome, epithelial cells in the gut express the NLRP6 inflammasome. Lack of NLRP6 exacerbated the development of colitis in DSS-treated mice through alterations to the gut microbial composition (29). This was dependent on IL-18, although whether this was related to epithelial integrity was not examined. The transfer of the NLRP6 knockout mice microbiota to wildtype mice, achieved simply through co-housing the mice, also transferred the increased disease severity (29). We do not know yet whether SCFAs or GPR43 influence NLRP6 activation in epithelial cells.

Other bacterial products that directly modulate immune cell functions

Numerous other microbial products may act similarly to SCFAs and affect either immune cell functions, or epithelial integrity. Microbiota-derived peptidoglycan (PTGN) can modulate peripheral immune function. PTGN derived from the gut microbiota enters the blood and bone marrow and primes the innate immune system, promoting killing of certain bacterial pathogens (99). Depletion of the microbiota in mice markedly lowers systemic PTGN concentrations, leading to less killing of *Streptococcus pneumoniae* and *Staphylococcus aureus* by neutrophils (99). PTGN signals via the pattern recognition receptor Nod1 (nucleotide-binding, oligomerization domain-containing protein-1), which recognizes meso-diaminopimelic acid-containing PTGN found predominantly in the cell wall of Gram-negative bacteria.

Another important bacteria-derived regulator of the systemic immune system is polysaccharide A (PSA) from *Bacteroides fragilis*. This anaerobic species expresses several different capsular polysaccharides capable of inducing T-cell responses (100). The importance of *B. fragilis* on T-cell differentiation has been demonstrated by mono-colonizing germ-free mice with *B. fragilis*, which restored $CD4^+$ T-cell numbers in the spleen of GF mice. Not only were $CD4^+$ T cell numbers restored, but also the splenic micro-architecture returned to normal and the increased IL-4 cytokine production that causes a Th2-bias in GF mice was corrected. These effects were dependent on the expression of the zwitterionic capsular PSA, since re-colonization of germ-free mice using *B. fragilis* lacking PSA failed to restore splenic micro-architecture and $CD4^+$ T-

cell numbers. Also, purified PSA given orally or intraperitoneally was able to induce the positive effects on T cells and splenic micro-architecture. These effects on T cells were mediated through $CD11c^+$ DCs, which are able to take up orally administered PSA and migrate into the mesenteric lymph nodes (MLNs). PSA activates DCs, as shown by increased expression of MHC class II and the costimulatory cytokines CD80 and CD86. Furthermore, bone marrow-derived DCs treated with PSA upregulate IL-12 and increase IFNγ expression in T cells and their proliferation *in vivo*, in an IL-12-dependent manner (101). PSA also directly promotes regulatory T-cell development by signaling through TLR2 in T cells (102).

Bacteria from the phylum *Clostridium* play a critical role in T-cell development. Indeed, reconstitution of germ-free mice with a *Clostridium* cocktail promoted the development of intestinal Treg cells (103). Moreover, reconstitution of germ-free mice with SFB belonging to the *Clostridia* species not only promoted the development of Th17 responses (42, 104) but also Th1 and Th2 cells in the intestine (104). The SFBs are among the rare bacteria that tightly adhere to the epithelium and have been shown to promote the release of serum amyloid A in the terminal ileum, which promotes the development of Th17 cells by acting on DCs (42).

Therapeutic possibilities for inflammatory diseases

Recent advances in our understanding of the role of the gut microbiota in inflammatory diseases may give rise to entirely new approaches to treating or preventing inflammatory diseases. This may simply arise through testing of new prebiotics or probiotics. The concept of transferable beneficial bacteria is now scientifically credible and is also accepted by many consumers who purchase probiotics or foodstuffs with live bacteria such as *Bifidobacterial* strains. Indeed, Elie Metchnikoff proposed more than a hundred years ago that bacteria in the gut could play a role in maintaining homeostasis. Results of human clinical trials with probiotics have been mixed, although there have been some notable successes (105). Disappointing results may relate to failure of probiotic species to colonize the colon or to be supported by the appropriate diet. This topic is likely to be investigated intensely in the coming years.

Prebiotics and probiotics

Currently, the most commonly used probiotics include *Lactobacilli* and *Bifidobacteria*. The sequencing of the human microbiome and the identity of factors from probiotic bacteria that confer health benefits should lead to the development of a new generation of probiotics. In one important study, a Japanese group used genomics and metabolomics approaches to identify the factor produced by a probiotic strain of *Bifidobacteria longum* that provided protection against infection with a pathogenic strain of *E. coli*. They identified acetate (a SCFA) as the single factor produced by a strain of *B. longum* (protective strain) that provided protection against *E. coli* infection. Protective, but not non-protective strains of *Bifidobacteria*, expressed an ABC transporter that enabled metabolism of fructose and production of acetate (93). Acetate acted on gut epithelial cells and affected their integrity and barrier function, which prevented passage of shiga toxin. An inference from this study was that acetate production could be one of the principle features of probiotic bacteria that provide immune benefits and protection against certain pathogens. This study did not address how acetate acted on epithelium or immune cells, and how it mediated its effects.

Commensal bacteria use several mechanisms to promote intestinal homeostasis, and presumably probiotic bacteria show these same or even exaggerated features. In particular, probiotics and their effector molecules influence gut barrier function, including modulation of mucus production, reduction of bacterial adhesion, enhancement of tight junctions, enhancement of cell survival, induction of defensins, and stimulation of IgA production. Commenal bacteria also stimulate TLRs to promote gut homeostasis, and maintain epithelial barrier function (84, 106–110). Thus, TLRs serve both a pro-inflammatory role designed for protection against pathogenic bacteria as well as a role in maintenance of epithelial barrier function.

The probiotic VSL#3 promoted epithelial integrity in a murine model of colitis, by preventing apoptosis and maintaining tight junction protein expression (110). In a human clinical trial in patients with multiple organ dysfunction, most patients that received live, but not sonicated, VSL#3 over the study period showed decreased intestinal permeability and an enhancement of immune activity (111). Another study found that diversity in gut microbial flora was reduced by VSL#3 and TNBS-induced chronic colitis was significantly reduced in rats fed with this probiotic compared to controls (112). VSL#3 may alter the composition of intestinal microbiota to protect against disease (112).

In a mouse model carrying a humanized microbiome, administration of the probiotic *Lactobacillus paracasei* or *Lactobacillus rhamnosus* modified the microbiome and resulted in altered hepatic lipid metabolism coupled with lowered plasma lipoprotein levels and stimulated glycolysis. The probiotic treatments also altered amino acid metabolism, methylamines and SCFAs (113). Similarly, Yan *et al.* (114) found that this bacterium secretes a soluble protein (p40), which prevents death

of intestinal epithelial cells through activation of the epidermal growth factor receptor signaling pathway. In three separate murine models of intestinal inflammation, administration of recombinant p40 reduced disease severity (114).

Prebiotics are non-digestible foodstuffs that stimulate the growth and/or activity of bacteria, conferring benefits upon the host. Dietary fiber is a common prebiotic. Prebiotics can modulate the composition of the gut microbiota as well as serve as a substrate for the production of metabolites, such as SCFAs. Prebiotic supplementation increased fecal secretory IgA and postnatal immune development in infants (115, 116). The recognition that diet may affect immune and inflammatory responses should lead to new clinical trials with prebiotics. For a summary of some of the clinical trial results obtained in human IBD using different probiotics or prebiotics, see ref (117).

Future directions

Diet and other factors that affect the composition of the gut microbiota represent new possibilities for investigating the basis of many inflammatory diseases and developing new therapeutic approaches. This will require cross-disciplinary approaches that bring together expertise in microbiology, metabolism, and immunology and inflammatory diseases. The use of probiotics and prebiotics for the treatment of human diseases holds promise, but trials with these agents need to be rigorous. Consideration must be given to use of diet together with probiotics, and careful monitoring to ensure that a probiotic has actually colonized the gastrointestinal tract. The mechanisms that underlie the stimulation of epithelial integrity, as well as the expansion of Treg versus Th17 cells are important topics for investigation. It will also be important to determine how the immune system shapes the composition of the microbiota and how to stably manipulate the gut microbiota for health benefits. Colonization of germ-free mice with defined bacterial strains or human gut enterotypes (118, 119) should be a powerful way to address several questions about the relationships between diet, the gut microbiota, and effects on epithelial biology and immune and inflammatory responses. The hope is that diet or probiotics may be an effective and economical means to improve human health in both the developing and the developed world.

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