Diet, gut microbiota and immune responses

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The fields of immunology, microbiology, nutrition and metabolism are rapidly converging. Here we expand on a diet-microbiota model as the basis for the increased incidence of asthma and autoimmunity in developed countries, and suggest one mechanism, short chain fatty acids binding to GPR43, as a link between diet, gut microbiota and the regulation of inflammatory responses.

Two important advances have emerged in recent years. First, it has been clearly demonstrated that diet has a marked effect on the composition of the gut microbiota[1, 2]. Different human populations can have vastly different intestinal microbiomes, and changes in diet lead to changes in microbiota composition. Secondly, findings from a number of laboratories show that the composition and products of gut microbiota have unexpected effects on immune and inflammatory responses[3-5]. Accordingly, diet and the effects it has on gut microbiota and immune responses are increasingly attractive explanations for the increased incidence of inflammatory diseases such as asthma and Type 1 diabetes in developed countries. New findings on gut microbiota and its immune modulating capabilities fit with persuasive epidemiological data on the connection between obesity with asthma[6] and Type 1 diabetes[7]. We suggest here that reduced intake of fibre (complex plant polysaccharides) is adversely affecting the makeup of the intestinal microbiota, leading to reduced production of immune modulating products, in particular short chain fatty acids (SCFA).

The gut microbiota can be considered as an extension of "self" and together with the genetic makeup, determines our physiology. For instance two notionally genetically identical individuals (for instance same sex inbred mice) can display widely different metabolic and inflammatory responses, depending on the makeup of their microbiota. The intestinal microbiota is at least partially derived from the mother during the birthing process, and thereafter is modified by factors such as diet, antibiotic use, host genetics and other environmental factors. Whereas microbes in the gut were often considered harmful or pathogenic, it is now clear that commensal bacteria perform many beneficial functions, such as vitamin synthesis, digestion of dietary fibre and

regulation of inflammatory responses. Microbes and vertebrates have co-evolved over the millennia, such that normal functioning of the digestive and immune systems depends on the presence of non-pathogenic "beneficial" bacteria (symbionts).

Diet and the gut microbiota

We propose that it is changes in diet and associated changes in the gut microbiota that is driving the high incidence of inflammatory diseases in developed countries (Figure 1). Similar ideas have been proposed previously[8, 9], however recent findings provide new molecular mechanisms and make a diet-microbiota hypothesis compelling. The modern western diet is characterized by food that has been processed, stored and transported, and typically contains significantly less vegetables and fibre compared to diets in developing countries, or to western diet of ~40 years ago[9]. Diet itself has a major effect on the composition of the gut microbiota[1, 10]. For instance Turnbaugh *et al.*[1] analyzed the changes in gut microbiota in mice (humanized gnotobiotic mice) after they were switched from a low-fat, plant-polysaccharide rich diet to a "western" diet high in fat and sugar, and low in plant polysaccharide. After just one day on this western diet, mice showed changes in the microbial composition, metabolic pathways, gene expression, and within 2 weeks had increased adiposity[1]. Mice on a western diet showed increases in bacteria of the Firmicutes phylum and a decrease in Bacteroidetes[1]. *ob*/*ob* mice that are genetically obese have 50% fewer Bacteroidetes, and more Firmicutes, than their lean wild type siblings. And the gut microbiota in these *ob*/*ob* mice are more effective at releasing calories from food and this trait can be transferred to germ-free recipients, resulting in greater adiposity[1, 11]. One of the important activities of the gut microbiota is to break down complex plant polysaccharides, which yields SCFAs, and levels of SCFA relate to the amount of fibre in the diet. An altered microbiome is also evident in human obesity[12] as are changes in the types of SCFA produced.

There are striking differences between the composition of the gut microbiota of children from rural Africa versus those from Europe[2]. Children from an African cohort (Berkino-Faso) had a diet very high in fibre, and their microbiota was highly enriched in Bacteroidetes, with a particular increase in bacteria known to encode genes required for hydrolysis of complex plant polysaccharides, and had markedly decreased levels of Firmicutes compared to the European cohort[2]. In fact the African

microbiome in this study contained two bacterial species (*Prevottela* and *Xylanibacter*) completely absent in western children, and which are known to contain enzymes necessary for cellulose and xylan hydrolysis. Fibre is fermented in the colon by anaerobic microbiota, in particular bacteria of the phyla Bacteroidetes (but also others as suggested above), as they have genes that encode enzymes necessary for the hydrolysis of complex plant polysaccharides[13]. In fact humans and other vertebrates rely completely on the microbiota to digest these otherwise indigestible plant polysaccharides. Fermentation of fibre results in the production of high levels of SCFA, such as acetate, propionate and butyrate. As will be discussed below, we believe that SCFA levels in the colon and blood are critically important for immune regulation. It is noteworthy, then, that SCFA production changes quickly when individuals are on different diets[14]. The European children referred to in the above study had significantly less SCFA in their faeces, compared to the African cohort[2]. Interestingly, allergies and asthma are virtually non-existent in certain rural African communities.

Host genetics affecting microbial composition

The innate immune system is another factor that probably determines the composition of the intestinal microbiota. This was probably best illustrated in a study by Chervonsky and colleagues, who showed that NOD mice deficient in the innate signaling molecule, MyD88, were protected from development of Type 1 diabetes[3]. Surprisingly, protection was lost when *MyD88*$^{-/-}$ NOD mice were housed under germ-free conditions, i.e. when they were devoid of gut microbiota. Absence of MyD88 in NOD mice led to an over-representation of the bacterial phyla Bacteroidetes[3] and this microbiota was somehow actively suppressing development of diabetes, presumably through production of an immuno-modulatory product. TLR5 is another innate component important for determining the makeup of the microbiota. *TLR5*$^{-/-}$ mice display hyperphagia (overeating) and hallmark features of metabolic syndrome, including insulin resistance and increased adiposity[15]. Transfer of *TLR5*$^{-/-}$ microbiota to wild type mice conferred many aspects of the *TLR5*$^{-/-}$ phenotype including hyperphagia and obesity[15]. Loss of TLR5 resulted in alterations in the gut microbiota, which somehow fed through to hyperphagia and other aspects of metabolic syndrome[15]. From these two studies it is reasonable to suggest that any element that affects innate immunity, for instance immune subversion by pathogens, or

polymorphisms in innate immunity genes, might affect the makeup of the gut microbiota.

Inflammatory disease-associated gut microbiota

There is now mounting evidence that the microbiota is altered in people with allergy or asthma[16, 17] (reviewed in ref[8]). One of the first studies to demonstrate this examined the intestinal microflora from 76 infants at high risk of atopic diseases at 3 weeks and 3 months of age. Micriobiota composition differed significantly between infants in whom atopy was and was not developing, and these microbiota differences preceded the development of atopy[18]. Daily consumption of fermented foods may be important for maintaining levels of *Lactobacilli*, and may reduce the prevalence of allergic disease[8]. Communities where consumption of fermented foods is high, and with no antibiotic use, there are low levels of allergy and asthma. Indeed a similar notion dates back to the early 1900's, when Elie Metchnikoff reported on a population of Bulgarians who had an extremely long life expectancy, which he attributed to their consumption of yoghurt, and the requirement of "good" microbiota to maintain harmony (homeostasis).

A sophisticated analysis of microbiotas associated with human inflammatory diseases is now only just commencing. However differences in gut microbiota have been observed in patients with rheumatoid arthritis (RA)[19, 20], particularly with erosive RA[19], and changes included decreased *Bifidobacteria* and *Bacteroides*[20]. In the KxB/N mouse model of inflammatory arthritis, removing anaerobic bacteria (using antibiotics) exacerbates disease[21], presumably through a mechanism relating to a changed microbiota. In inflammatory bowel disease patients the gut microbiota is often changed, compared to healthy individuals. Again these changes are seen in the "beneficial" anaerobic microbes such as Bacteroidetes and a subgroup of Firmicutes[22]. It is therefore becoming clear that certain gut microbiota are required for regulation of immune responses, and that perturbations in the microbiota could result in a lack of immune regulation, outgrowth of more pathogenic microbes, and promotion of inflammation, particularly in individuals that are genetically susceptible. A summary of all the factors that potentially affect the composition of the intestinal microbiota are depicted in Figure 1.

Mechanisms whereby gut microbiota influence immune and inflammatory responses

It has only recently been recognized that the gut microbiota can influence immune function beyond the gut. We found that mice deficient in a single G-protein coupled receptor, GPR43, had profoundly altered inflammatory responses. The only known ligands of GPR43 are SCFA, particularly acetate and propionate, which are primarily a product of gut microbial metabolism of fibre. *Gpr43*$^{-/-}$ mice have increased and poorly resolving inflammation in KxB/N serum-induced arthritis model, OVA/alum-induced allergic airway inflammation, as well as colitis models, and *Gpr43*$^{-/-}$ neutrophils display an intrinsic hyper-reactive phenotype[4]. GPR43 is expressed mainly on innate/inflammatory immune cells such as neutrophils, eosinophils and activated macrophages.

SCFA are also beneficial in other ways. Butyrate is the major energy source for colonocytes, and is thus associated with epithelial maintenance. SCFA can also bind other GPCRs including GPR41 (but with differing affinity and SCFA preference), and SCFA, particularly butyrate, inhibit histone deacetylases and inhibit Nf□B activation[23-25]. Germ-free mice devoid of microbiota have very low levels of SCFA[26], and also show exacerbated or poorly resolving responses in many inflammatory models[4, 27] similar to responses by *Gpr43*$^{-/-}$ mice. The many different aspects of SCFA actions on immune and epithelial cells are illustrated in Figure 2.

SCFA/GPR43 is probably just one of several pathways by which the microbiota regulates inflammatory responses in the gut and elsewhere. Bacteroidetes also utilize fibre for glycan synthesis[13]. The commensal bacterium *Bacteroides fragilis* produces a particular glycan, polysaccharide A (PSA), which has strong anti-inflammatory effects. Colonization of germ-free mice by *B. fragilis* or treatment with purified PSA protects mice against the induction of experimental inflammatory bowel disease[28]. PSA increases local production of IL-10 by induction of Tregs[29]. Peptidoglycan (PTGN) is another example of a bacterial product that can modulate peripheral immune function. PTGN derived from the gut microbiota enters the blood and primes the innate immune system, promoting killing of certain bacterial pathogens[5]. Depletion of the microbiota in mice markedly lowered systemic PTGN concentrations, and this led to less killing of *S. pneumoniae* and *S. aureus* by

neutrophils[5]. PTGN signals via the pattern recognition receptor nucleotide-binding, oligomerization domain–containing protein-1 (Nod1), which recognizes meso-diaminopimelic acid (mesoDAP)-containing PTGN found predominantly in Gram-negative bacteria. All these mechanisms support the idea that certain beneficial bacteria have developed molecules that induce protective intestinal immune responses, but which also affect or regulate systemic immune responses.

It is likely that the gut microbiota influences the adaptive and innate immune systems in completely different ways. The microbiota is well recognized for its role in the proper development of the immune system. For instance germ-free mice have poorly developed lymphoid tissues, and show perturbations in development of T and B cell subsets, and in some cases GF mice do not develop disease present in conventional mice. This probably relates to an inability in mounting adaptive immune responses due to defects in the adaptive immune system in the absence of microbiota, rather than to lack of microbes *per se* (see Chervonsky[27] for more on this). On the other hand innate components in germ-free mice show hyperactivity, for example germ-free macrophages display higher basal levels of lysozymal enzymes compared to conventional mice[30]. The presence of segmented filamentous bacteria (SFB) are important for the development of IL-17 producing T cells (Th17 cells), and these bacteria are necessary for the development of autoimmunity in the T cell transgenic K/BxN model of arthritis[21]. However this model has an easily distinguishable initiation stage (T and B cell dependent) and an effector stage dependent on mast cells, complement activation and neutrophils. The effector stage is exacerbated in germ-free mice, as well as in *Gpr43*$^{-/-}$ mice[4]. This is because inflammatory cells selectively express receptors (such as GPR43) that regulate immune cell functions.

Does diet affect inflammatory diseases?

If diet affects the composition of the microbiota, and the microbiota regulates immune and inflammatory responses, then diet should have easily quantifiable effects on immune responses. Although the studies on this topic are highly promising, most of the evidence to date has been indirect, or derives from studies with limited numbers of trial subjects. The affect of diet on asthma and allergies has been reviewed recently[9, 31]. One of the noted changes in western diet has been the decreased consumption of dietary fibre (complex plant polysaccharides). Human populations that consume

adequate or high amounts of dietary fibre have a decreased incidence of inflammatory diseases, such as colitis, type 2 diabetes and colon cancer[32-34]. In one study, 1861 children were followed from birth to see if nutrient intake by their mothers during pregnancy correlated with development of asthma at 5 years. There were clear differences in dietary intake in mothers of children who later developed childhood wheeze and asthma[35]. Perhaps one of the strongest associations have been the numerous epidemiological studies that have linked obesity with the development and severity of asthma, in both children and adults[6]. A direct assessment on intake of dietary fibre and inflammation has mostly been studied in inflammatory bowel diseases, with encouraging results[34]. In addition, some trials have reported positive effects of SCFA in patients with inflammatory bowel conditions, in fact patients often have severely reduced levels of SCFA[34]. Several studies have demonstrated benefits of a vegan diet (which is high in fibre) on reducing arthritis[36], and although indirect, could suggest that a high fibre diet might benefit patients with RA.

The hygiene hypothesis re-visited

The hygiene hypothesis[37] is currently the prevailing explanation for the increase in asthma and atopic disorders in western countries. It suggests that excess "cleanliness" in the environment has led to a decline in the number of infectious stimuli needed for the proper development of the immune system. Prevalence of asthma and allergies are lower for individuals raised on a farm, belonging to larger families or in a lower birth order in such families, and socioeconomic status. Some of the observations that gave rise to this hypothesis may be equally relevant to a "diet hypothesis" or "fibre hypothesis". For instance children who live on farms probably have different diets than children from urban environments. An interesting case is that of Japan, where there is a high level of hygiene and urbanization, but a much lower level of asthma than in Australia or the USA. Diet in Japan and other Asian countries typically contains high amounts of rice, beans and fermented or pickled foods, which yield high levels of SCFA. Another observation that would argue against the hygiene hypothesis is that the urban poor in the USA have higher levels of infectious diseases, such as TB, yet have high levels of asthma, and this appears to correlate better with diet and obesity in these populations.

Manipulation of microbiota for control of inflammatory diseases

If the microbiota does have a substantial bearing on immune responses, as the preliminary reports discussed above suggest, then this opens up new avenues for therapy. Probiotics have been tested in a number of clinical trials, with some notable successes[8], although few large-scale trials with human inflammatory diseases have been undertaken. It may be that clinical trials with probiotics will also need to incorporate considerations on diet, because a probiotic requires fibre for it's metabolism, and it is likely that SCFA is one of the mechanisms by which probiotics can be beneficial. Moreover individuals may vary significantly in their capacity to support expansion of newly introduced microbes in their gastrointestinal tract. In some clinical trials with probiotics, it was not clear whether oral administration allowed for survival during their transit through the stomach. It may be necessary also for constant dietary support of these microbes[8] or selecting for microbes that are better at surviving passage through the stomach and colonizing the colon. Preliminary trials with fecal transplantation (the process of transferring a fecal sample from a healthy person to treat another) for *C. difficile* colitis appear very promising and could be used to treat severe cases of dysbiosis[38].

Future directions

The recent flurry of papers on diet and its effects on gut microbiota, together with the new findings on the regulation of immune responses by microbiota, opens up an entirely new approach to the understanding and treatment of human inflammatory diseases. Likely suspects in this equation are SCFAs but there may be numerous molecules produced by gut microbes that affect immune responses. Rather than developing new anti-inflammatory drugs, it might be more cost effective to devote more effort to new approaches, such as monitoring of our intestinal microbiota and manipulation if required using probiotics and/or prebiotics. The opportunity exists to develop new probiotics based on emerging knowledge on the mechanisms by which the microbiota modulates inflammatory responses. Another possibility is to develop probiotics from microbiota derived from human communities where allergies and asthma are virtually non-existent. It will also be important to determine how antibiotics change the composition of the gut microbiota and how relevant this is to inflammatory diseases. Finally, if diet is a major contributing factor to the prevalence of allergies, asthma and even autoimmune diseases, should we consciously alter our intake of fibre or other foodstuffs? Carefully controlled trials are needed to establish

whether diet is directly affecting inflammatory diseases, and if so, at what point in human development does it operate, and through what cellular and molecular mechanisms. Increasing knowledge emerging from the human microbiome project, and increasing capabilities for metagenome sequencing, will allow a systematic analysis of the intestinal microbiota in human inflammatory diseases.

Conclusion

Diet plays a major role in determining the types of microbes that colonize the gut. Moreover the microbiota has a much bigger influence on inflammatory processes than previously appreciated. It follows that changes in the gut microbiota brought on by western diet (particularly lack of fibre) represent promising new ideas to explain the increased incidence of inflammatory disease in western countries. Manipulation of the microbiota might be a promising new approach for the prevention or treatment of inflammatory diseases.

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References

1. Turnbaugh, P.J. *et al. Sci Transl Med* **1** (2009).
2. De Filippo, C. *et al. Proceedings of the National Academy of Sciences of the United States of America*.
3. Wen, L. *et al. Nature* **455**, 1109-1113 (2008).
4. Maslowski, K.M. *et al. Nature* **461**, 1282-1286 (2009).
5. Clarke, T.B. *et al. Nat Med* **16**, 228-231.
6. Sin, D.D. & Sutherland, E.R. *Thorax* **63**, 1018-1023 (2008).
7. Patterson, C.C. *et al. Lancet* **373**, 2027-2033 (2009).
8. Noverr, M.C. & Huffnagle, G.B. *Trends Microbiol* **12**, 562-568 (2004).
9. Devereux, G. *Nat Rev Immunol* **6**, 869-874 (2006).
10. Ley, R.E. *et al. Nat Rev Microbiol* **6**, 776-788 (2008).
11. Turnbaugh, P.J. *et al. Nature* **444**, 1027-1031 (2006).
12. Ley, R.E. *et al. Nature* **444**, 1022-1023 (2006).
13. Comstock, L.E. *Cell Host Microbe* **5**, 522-526 (2009).
14. Duncan, S.H. *et al. Appl Environ Microbiol* **73**, 1073-1078 (2007).
15. Vijay-Kumar, M. *et al. Science*.
16. Bjorksten, B. *et al. Clin Exp Allergy* **29**, 342-346 (1999).
17. Bjorksten, B. *et al. J Allergy Clin Immunol* **108**, 516-520 (2001).
18. Kalliomaki, M. *et al. J Allergy Clin Immunol* **107**, 129-134 (2001).
19. Eerola, E. *et al. Br J Rheumatol* **33**, 1030-1038 (1994).

20. Vaahtovuo, J. *et al. J Rheumatol* **35**, 1500-1505 (2008).
21. Wu, H.J. *et al. Immunity* **32**, 815-827.
22. Frank, D.N. *et al. Proceedings of the National Academy of Sciences of the United States of America* **104**, 13780-13785 (2007).
23. Le Poul, E. *et al. J Biol Chem* **278**, 25481-25489 (2003).
24. Segain, J.P. *et al. Gut* **47**, 397-403 (2000).
25. Grunstein, M. *Nature* **389**, 349-352 (1997).
26. Hoverstad, T. & Midtvedt, T. *J Nutr* **116**, 1772-1776 (1986).
27. Chervonsky, A.V. *Nat Immunol* **11**, 28-35.
28. Mazmanian, S.K. *et al. Nature* **453**, 620-625 (2008).
29. Round, J.L. & Mazmanian, S.K. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 12204-12209.
30. Morland, B. & Midtvedt, T. *Infect Immun* **44**, 750-752 (1984).
31. McKeever, T.M. & Britton, J. *Am J Respir Crit Care Med* **170**, 725-729 (2004).
32. Venn, B.J. & Mann, J.I. *Eur J Clin Nutr* **58**, 1443-1461 (2004).
33. Lim, C.C. *et al. Molecular nutrition & food research* **49**, 609-619 (2005).
34. Galvez, J. *et al. Molecular nutrition & food research* **49**, 601-608 (2005).
35. Devereux, G. *et al. Am J Respir Crit Care Med* **174**, 499-507 (2006).
36. Muller, H. *et al. Scand J Rheumatol* **30**, 1-10 (2001).
37. Strachan, D.P. *Thorax* **55 Suppl 1**, S2-10 (2000).
38. Khoruts, A. *et al. Journal of clinical gastroenterology* **44**, 354-360.

FIGURE LEGENDS

Figure 1: Diet, microbial composition and regulation of the immune system.
Diet, and other environmental and host factors, has a major effect on gut microbial composition. Our model would suggest that balanced microbial composition results in symbiosis, which provides regulation of immune and inflammatory responses through anti-inflammatory/immune modulatory products such as short chain fatty acids (SCFA), polysaccharide A (PSA) and peptidoglycan (PTGN), helping maintain homeostasis. Dysbiosis would lead to dysregulation of the immune system, through lack of beneficial microbial products and perhaps an increase in virulence factors, which could leave the host susceptible to inflammation. Dysbiosis could occur through consumption of a "western diet", as well as through changes induced by factors such as host genetics, maternal transfer and antibiotic use.

Figure 2: Effects of SCFA on colonic epithelium and the immune system.
Short chin fatty acids (SCFA) produced by the gut microbiota as a by-product of fermentation of dietary fibre have many beneficial effects on the host. **Colonic epithelium:** Butyrate is the major energy source of colonic epithelial cells, and is transported into cells via monocarboxylate transporter 1 (MCT1). SCFA are therefore important for maintaining epithelial barrier function, regulation of proliferation, and tumor suppression. SCFA also reduce oxidative DNA damage and regulate cytokine production. Actions of SCFA in epithelial cells are mostly through their role as an energy source and also through inhibition of histone deacetylase's. Actions could also operate through GPCRs, GPR41, GPR43 and GPR109A. **Immune system:** SCFA have several anti-inflammatory effects but are also important for stimulating immune function, and their role therefore appears to be important for regulating timely immune responses and in resolution of inflammation. Acetate enhances reactive oxygen species (ROS) production and phagocytosis but also induce apoptosis. Many anti-inflammatory effects are mediated through GPR43, but could also possibly act through inhibition of histone deacetylase's and GPR41.