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Review

Commensal flora and the regulation of inflammatory and autoimmune responses

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ABSTRACT

The gut microbiota has recently been recognized for its role in immune regulation, and changes in gut microbiota may be the basis for an increased incidence of autoimmune diseases and asthma in developed countries. Beneficial microbes produce factors that are distributed systemically, and therefore can influence peripheral inflammatory responses. Such symbiosis factors are important for the control and resolution of inflammation and autoimmune diseases. Here we discuss immune regulation by recently identified symbiosis factors and how certain environmental factors favor their production and influence the composition of the gut microflora.

Microbes and vertebrates have co-evolved over millions of years, such that normal functioning of the digestive system depends on the presence of non-pathogenic "beneficial" bacteria, otherwise known as symbionts. The microbiota benefits host organisms in many ways and these benefits are not limited to metabolism and digestion: the microbiota is also essential for the development of a functional immune system and an increasing number of studies in the past years have focused on local interactions between the immune system and bacteria in the gut. Gut microbes contribute to gut-associated diseases like Crohn's disease and other inflammatory bowel diseases (reviewed in [1]). However, the gut microbiota has a much more far-reaching influence on the immune system, beyond the gastrointestinal tract. In this review we discuss recent findings on how the gut microflora regulates immune responses and how bacteria/host interactions may lead to autoimmune and allergic diseases both within and outside the gut. We will discuss recent advances in the discovery of 'symbiosis factors' that facilitate the peaceful coexistence of the microbiota and the host immune system, and also how the makeup of the gut microbiota, which is influenced by many factors including diet, antibiotic use and host genetics, affects health and disease.

1. Mechanisms of immune regulation by microbial factors

A lot of evidence for how the microbiota shapes the immune system comes from studies with germ-free (GF) mice that completely lack microbiota. Such mice exhibit profound immune defects: not only are their Peyer's Patches hypoplastic and the B and T cell compartments in the lamina propria disturbed, but also their spleens and lymph nodes have poorly developed B and T cell zones. Consequently, serum IgG and intestinal IgA levels in GF mice are reduced. Furthermore, cytokine production is greatly affected resulting in T cell responses that are skewed towards a Th2-type response (reviewed in [2]).

Normally, cells of the immune system are separated from the gut microbiota by a thin single-cell epithelial layer [3]. Hence, immune responses against the microbiota are quite rare and are elicited only when they penetrate the epithelial layer. Such responses are thought to be confined to the mucosal layer, because dendritic cells (DCs) that are primed by these bacteria stay in the Peyer's Patches or mesenteric lymph nodes, and therefore it was assumed that the systemic immune system is largely ignorant of the bacteria residing in the gut [4]. Indeed, the impact of gut bacteria on the local intestinal immune system, especially on cells of the innate immune system, like neutrophils and macrophages, is substantial. These cells recognize microbial products through pattern recognition receptors (PRRs). How these innate receptors control immune responses and how they are involved in the induction of autoimmunity were recently reviewed [5].

1.1. Shaping of T cell subsets by microbiota

Recent findings show that gut microbiota has a much greater influence on the systemic immune system than previously anticipated. Specific microbiota control T-cell differentiation in the lamina propria, where the IL-17 expressing CD4+ T cell subset (Th17 cells) is especially abundant [6,7]. Th17 cells produce many pro-

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inflammatory cytokines, like IL-17, IL-21 and IL-22 and are thought to play a pro-pathogenic role in autoimmune conditions like arthritis and experimental autoimmune encephalomyelitis (EAE) [8]. The presence of Th17 cells in the small and large intestine is dependent on gut microbiota, since their number in the LP of GF mice is drastically reduced, whereas the number of Foxp3+ regulatory T cells (Treg cells) is increased [6,7]. Both, Th17 and Treg cells require TGF-β, while differentiation of Th17 cells additionally requires IL-6 [9]. The preferential differentiation of Tregs and the absence of Th17 cells probably reflect a change in the cytokine milieu of the LP in the small intestines of GF mice. In the large intestines a $CD70^{high}CD11c^{low}$ subset of LP cells produced cytokines that promote Th17 differentiation after activation by commensal-bacteria derived ATP, which could be responsible for the preferential differentiation of Th17 cells [7].

In an effort to determine whether all gut microbiota in general or just a specific subset of bacteria were responsible for the Th17 cell development, Ivanov et al. treated mice with antibiotics selectively killing different subsets of bacteria. They identified vancomycin-sensitive bacteria as the subset able to induce Th17-cell differentiation in the LP of the small intestine [6]. Later, the same group identified segmented filamentous bacteria (SFB) to be responsible for Th17-cell induction [10]. Mice of the same strain obtained from two different suppliers varied in the number of LP Th17 cells and analysis of the microbiota revealed that SFB colonization differed between the suppliers [10]. The importance of SFB in orchestrating T-cell responses in the gut was confirmed by another group who analyzed the intestinal cytokine profile after colonization of GF mice with SFB [11]. It is interesting to note that mice engineered to express the human α-defensin gene DEFA5, which is produced by Paneth cells, lacked SFB and had reduced Th17 cell numbers in the LP, while in transgene negative littermates SFB were present. α-Defensins are antimicrobial peptides that play an important role in host defenses against enteric pathogens. Although DEFA5 is not expressed in mice and its anti-microbial activity differs from that of mouse α-defensins, the results of these studies indicate that they might be important regulators of the gut microbiota composition, counteracting colonization of potentially harmful bacteria subsets [12].

1.2. Short-chain fatty acids

While some gut bacteria residing in the intestine favor or even induce the development of inflammatory diseases (e.g. SFB), most other bacteria, especially species from the phyla Bacteroidetes have beneficial anti-inflammatory effects. They produce factors with profound anti-inflammatory capabilities and make them available systemically. By this they are able to influence immune responses throughout the whole body. Such factors include short-chain fatty acids (SCFAs) and we have recently shown how they control inflammatory responses [13]. SCFAs are produced by commensal gut bacteria, predominantly by bacteria from the phyla Bacteriodetes, by fermentation of complex plant polysaccharides (fibre) [14]. The main SCFAs produced in the colon are acetate, butyrate and propionate and their concentration in the colon is in the millimolar range [15]. Most of the SCFAs, especially proprionate and butyrate are absorbed by the colonic mucosa or liver [16], but acetate is detectable in the blood in high micromolar concentrations [17].

SCFAs have long been known for their anti-inflammatory functions [18,19], but the mechanism was unknown until the discovery that SCFAs bind the G protein-coupled receptor, GPR43. GPR43 binds the SCFAs acetate, propionate and butyrate, with that order of affinity [20]. GPR43 is expressed by innate immune cells, particularly neutrophils, eosinophils and monocytes [13,20]. SCFA–GPR43 interactions have a profound anti-inflammatory action and greatly impact on neutrophil function. Neutrophils lacking GPR43, and which are therefore unresponsive to SCFAs show a hyperactive phenotype with increased reactive oxygen species (ROS) production and higher sensitivity to chemoattractants. Consequently, mice deficient in GPR43 ($Gpr43^{-/-}$) showed exacerbated DSS-induced colitis. Interestingly, the anti-inflammatory effects of SCFA–GPR43 interactions were not limited to the colon. OVA-induced allergic airway inflammation and K/BxN serum-induced arthritis were also much more severe in $Gpr43^{-/-}$ mice. Conversely, oral administration of acetate, which has been shown to increase the acetate concentration in blood [17], had a beneficial effect on these conditions, indicating a systemic effect of SCFAs on immune cells [13]. In line with this, GF mice have very low levels of SCFAs [21], and also show exacerbated or poorly resolving responses in many inflammatory models [5,13] similar to responses by $Gpr43^{-/-}$ mice. It will be very interesting to investigate the role of SCFAs and GPR43 in the induction of autoimmune diseases that are increased in Western societies where a diet low in fibre is very common, which results in lower levels of SCFAs. The consequences of a reduced fibre intake on the development of inflammatory diseases were recently discussed in [22].

1.3. Peptidoglycan

Further evidence for systemic regulation of immune cells by gut microbiota came from a study conducted by Clarke et al. They found that the microbial product peptidoglycan (PTGN), a component of the bacterial cell membrane [23] influences neutrophil priming. They showed that PTGN is able to translocate across the gut mucosa and enter the circulation and bone marrow. PTGN binds to a PRR, nucleotide-binding, oligomerization domain-containing protein-1 (Nod-1). Nod1 binds specifically to meso-diaminopimelic acid-containing (mesoDAP) PTGN from Gram-negative, but not from Gram-positive bacteria, and is expressed on neutrophils. $Nod1^{-/-}$ neutrophils showed impaired killing of the pathogenic bacterial strains *Staphylococcus pneumonia* and *Staphylococcus aureus*. In line with this, neutrophils isolated from mice housed under GF conditions showed the same impairment in killing these pathogenic bacteria. Therefore, Gram-negative bacteria are able to enhance neutrophil function through PTGN to ensure a rapid and efficient immune response against pathogenic bacteria like *S. pneumonia* and *S. aureus* [24].

1.4. Polysaccharide A

Another important bacteria-derived regulator of the systemic immune system is polysaccharide A (PSA) from *Bacteroides fragilis*. This anaerobic species is very abundant in the mammalian gut [25] and expresses several different capsular polysaccharides that are able to induce T cell responses [26]. The importance of *B. fragilis* on T cell differentiation has been demonstrated by monocolonizing GF mice with *B. fragilis*, which was able to restore the reduced CD4+ T cell numbers in the spleen of GF mice. Not only were CD4+ T cell numbers restored, but also the splenic microarchitecture 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 recolonization of GF mice with *B. fragilis* lacking PSA failed to restore splenic microarchitecture and CD4+ T cell numbers. Also purified PSA alone given orally or intraperitoneally was able to induce the positive effects on T cells and splenic microarchitecture. PSA exerts its effects through CD11c+ DCs, which are able to take up orally administered PSA in the mesenteric lymph nodes (MLNs). PSA uptake increased expression of MHC II and the co-stimulatory cytokines CD80 and CD86 in DCs. Furthermore, BMDCs treated with PSA upregulated IL-12 and were able to increase IFNγ expression in T cells and their proliferation *in vivo* in an IL-12 dependent manner [27].

Microbial substances are important to establish a functional microflora/immune system collaboration and the term symbiosis factors was introduced to describe their importance for this mutually beneficial relationship [27]. Disruption of the gut microbiota, dysbiosis, could lead to dysregulation of immune responses and could ultimately result in inflammatory disease.

2. Gut microbiota and autoimmunity

2.1. Arthritis

Despite the importance of symbiosis factors produced by gut bacteria, the absence of commensal organisms, such as in GF mice, can have a positive effect on some autoimmune diseases. There are several autoimmune models that are attenuated in GF mice, which sometimes led to the wrong assumption that gut bacteria *per se* are a threat to the immune system. However, this is probably only the case when the normal gut flora becomes unbalanced and harmful bacterial species become too frequent – dysbiosis (see below). This view is supported by a study conducted by Wu et al. They investigated autoimmune arthritis in the K/BxN model under GF conditions and found that arthritis was strongly attenuated in the absence of microbiota [28]. In this model a C57BL/6 TCR transgenic mouse line (KRN) is crossed with NOD mice (K/BxN). The transgene positive F1-offspring develop an autoimmune disorder closely resembling human rheumatoid arthritis [29]. The attenuation of the disease in GF mice was caused by a reduction of splenic Th17 cells, which drive the GPI auto-antibody production in this model. Vancomycin-treatment of conventional K/BxN mice also had a beneficial effect on disease development, while the monocolonization with SFB was sufficient to restore the Th17 cell compartment, and aggravated disease in GF K/BxN mice [28]. This study clearly shows that certain harmful species of gut bacteria affect T cell populations in the periphery and thereby control development of autoimmune diseases in organs other than the gut.

2.2. Experimental autoimmune encephalomyelitis

Another recent study showed that SFB can also promote EAE, a mouse model for the CNS autoimmune disease multiple sclerosis, where immune cells attack the myelin sheath of neurons [30]. In addition to Th1 cells, pro-inflammatory Th17 cells are important drivers of the disease. EAE can be induced by a subcutaneous injection of CNS antigens, like myelin oligodendrocyte glycoprotein (MOG) [31]. Similar to the arthritis in K/BxN mice, EAE was strongly attenuated in GF mice. To investigate whether CD4+ T cells from GF mice were incapable of inducing EAE, Lee et al. harvested CD4+ T cells from MOG/CFA-immunized GF and SPF mice and restimulated them in vitro with MOG-peptide before injecting them into *Rag1*$^{-/-}$ mice. Both CD4+ T cells from GF and SPF mice were able to induce disease in the recipient mice, although disease in mice receiving CD4+ T cells from GF mice was slightly attenuated demonstrating that CD4+ T cells from GF mice were not unresponsive *per se*. Comparison of the pro-inflammatory cytokines IFNγ and IL-17A produced by T cells harvested from GF and SPF mice immunized with CFA and MOG revealed a reduced production of these cytokines in GF mice, along with increased numbers of Treg cells. Co-culture experiments with DCs and MOG-specific T cells showed that DCs from GF mice were unable to induce efficient IL-17A and IFNγ production by T cells. In accordance with previous studies, after monocolonization of GF mice with SFB, IL-17A and IFNγ production was restored and disease severity strongly increased, confirming the great immunomodulatory potential of this bacterial subset [30]. This immunomodulatory effect seemed to be mediated through DCs, which indirectly resulted in altered T cell function.

2.3. Type-1 diabetes

While the above-mentioned experiments convincingly show that some bacteria species can induce autoimmunity – a harmful effect on the immune system is probably the exception. In other autoimmune conditions, the lack of microbiota increases disease severity. The development of Type-1 diabetes (T1D) for example does not require the presence of microbiota, since GF animals readily develop disease [32]. A recent study showed that gut microbiota could protect from T1D. Diabetes-prone NOD mice deficient in the toll-like receptor (TLR) adapter molecule Myd88 were protected from development of T1D when kept under specific pathogen free (SPF) conditions [33]. Myd88 is required for signaling through all known TLRs except TLR3 [34], and these results apparently confirmed the importance of TLRs in the development of T1D. However, analysis of Myd88-deficient diabetogenic T cells revealed that their proliferation was only reduced in the pancreatic lymph node, but not in spleen or mesenteric lymph nodes, which indicated that there was not a systemic suppression of diabetogenic T cells. Interestingly, the protection from development of T1D in the absence of TLR signaling required the presence of gut microbiota, since Myd88-deficient NOD mice readily developed T1D under GF conditions. These results indicate that TLR signaling controls components of the gut microbiota that protect against T1D. To test if Myd88-deficiency altered the composition of gut microbiota, 16s RNA sequencing of caecal contents of *Myd88*$^{+/-}$ NOD and *Myd88*$^{-/-}$ NOD littermates were analyzed. The results were very intriguing and showed that *Myd88*$^{-/-}$ NOD mice had a significantly lower ratio of Firmicutes to Bacteroidetes. This increase in Bacteroidetes could explain the protection of *Myd88*$^{-/-}$ NOD mice under SPF conditions suggesting an anti-inflammatory effect of these commensals [33]. Interestingly, Bacteroidetes are the main producers of SCFAs from dietary fibre [14]. SCFAs could potentially be the anti-inflammatory factor protecting against disease in the Myd88-deficient NOD mice.

Diet can have a significant impact on the incidence of T1D. New insights on how anti-diabetogenic diets influence T1D came from a study conducted by Alam et al. They reported that young NOD mice suffer from mild colitis as evidenced by villous hyperplasia. This hyperplasia was not observed when mice were fed the anti-diabetogenic ProSobee diet, which is based on a soy protein isolate [35,36]. Mice on this diet had a reduced diabetes incidence and also significant changes in their bacterial fatty acid profiles indicating that the composition of the gut bacteria was affected by the diet change. ProSobee diet also affected the cytokine profile in the colon and resulted in reduced levels of IL-17, IL-23 and IL-10. Although this study did not convincingly show that these alterations were responsible for the reduced diabetes incidence in ProSobee-diet fed mice, it showed clearly that diet can have a profound immunomodulatory effect presumably by altering the composition of the gut microflora [37].

3. Disease-associated gut microflora

Changes in the gut microbiota composition is becoming an increasingly popular theory for the increased incidence of inflammatory diseases in Western society. As outlined below, many environmental, as well as genetic factors, affect host colonization. It is becoming clear that the gut microbiota can influence both gut and peripheral immune development and responses, and as such a few studies have demonstrated clear differences in gut microbiota colonization between healthy and diseased individuals. This section summarizes findings from studies reporting differences in

the gut microbiota of individuals with asthma, rheumatoid arthritis and colitis.

3.1. Asthma and allergic diseases

Similar to GF mice, the immune systems of newborns have a Th2-type skewed immune system – this is probably important for the survival of the fetus during pregnancy to prevent an inflammatory response from the mother against the fetus. So it is important in early life to be exposed to immune challenges that can change this Th2 orientation. The prevailing theory to explain asthma and allergy etiology is that reduced exposure to pathogens and parasites is causing an inability to switch the immune system from Th2-based to Th1-based responses – the hygiene hypothesis. Another idea could be that microbial colonization of the gut is a key event in helping prime the host immune system, enabling this switch away from Th2 dominated responses. Specific alterations in the gut microbiota have been observed in children with allergy or asthma [38]. Compared to nonallergic children, allergic children from Sweden and Estonia had reductions in anaerobes *Lactobacilli*, *Bacteroides* and *Bifodobacterium* and an increase in aerobes, particularly *S. aureus* and *Clostridia* [38,39]. In a separate prospective study of children at high risk of developing allergy (family history of atopic disease), children who developed allergy by twelve months of age (29% of the cohort) were found to have had differences in their gut microbiota at three weeks of age compared to those individuals that did not develop allergy. Differences observed were increased levels of *Clostridia* and reduced levels of *Bifidobacteria* in those that developed allergy [40]. Therefore, changes in the gut microbiota present before detection of allergy could have a profound effect on the developing immune system and thereby affect susceptibility to disease [38,39] in addition to genetic susceptibility [40].

3.2. Rheumatoid arthritis

Similarly, differences in gut microbiota have been observed in patients with rheumatoid arthritis (RA) [41,42]. In one study, patients with early RA, who had symptoms for $\leq$6 months and were not on anti-rheumatic drugs or glucocorticoid medication, were compared to patients with fibromyalgia. RA patients had decreased *Bifidobacteria* and bacteria of the *Bacteroides* and *Eubacterium* groups compared to fibromyalgia patients [42]. Another study comparing early RA patients with healthy controls demonstrated a significant difference between early RA patients and healthy controls, with the largest differences seen in those patients with erosive RA [41]. Differences in the gut microbiota were mainly attributable to changes in the anaerobic bacteria population in RA patients [41].

3.3. Colitis

In colitis there is a more complex link between the microbiota and disease. While in many cases the gut microbiota is required for inflammation in the colon, this is not true in all cases [13]. It has long been viewed that the commensal microbiota is somehow involved in the development of colitis, however since we all have a microbiota, there must be additional factors that lead to disease. Disease may result from 'dysbiosis', where more pathogenic microbes are present. Particular beneficial microbes are probably required to maintain homeostasis within the gut. Abnormal gut colonization has been observed in subsets of Crohn's disease and ulcerative colitis patients. Again these changes are seen in the "beneficial" anaerobic microbes such as Bacteroidetes and a subgroup of Firmicutes [43]. Other studies have identified *Escherichia coli* in the ileal mucosa that are adherent and invasive (adherent-invasive *E. coli* – AIEC) in a proportion of Crohn's disease patients, and not in healthy controls [44,45], which would support the idea that pathogenic microbes are involved in colitis pathogenesis.

3.4. A disease-associated microbiome

The above studies in mice and humans indicate that certain inflammatory diseases are associated with an altered microbiome. Whether these changes contribute to disease pathogenesis, or whether it is just a by-product of disease, remains to be determined, but considering mounting evidence from studies in mice showing that gut microbiota can influence peripheral immune responses it does appear very likely that an altered microbiome influences the progression of disease. A prospective study of children with a high risk of developing asthma suggested that changes in the microbiota occur before disease development. This study also demonstrated that genetically susceptible individuals showing 'normal' colonisation did not develop disease, suggesting that the combination of genetic susceptibility and an altered microbiome are required for disease development [40]. It is therefore becoming clear that certain species of gut commensals 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.

4. Factors affecting the composition of gut microbiota

A wide range of environmental factors affect the composition of the gut microbiota. It is now clear that the environment plays a major role in the development of inflammatory diseases, for instance the incidence of asthma differs between Western and developing countries. Given that the gut microbiota is important for regulating inflammatory responses, and that they are dramatically affected by the environment, it seems apparent that one of the major ways the environment is affecting our susceptibility to disease is through altering the gut microbiota. This section will discuss environmental and genetic influences that affect the gut microbiota.

4.1. Diet

Diet directly affects gut microbial composition due to bacteria having different preferences for energy sources. Complex plant polysaccharides are the substrate source for beneficial microbes and promote their growth over other microbes. In fact, the move into a herbivorous niche was enabled by the symbiotic relationship with gut microbes: digestion of complex plant polysaccharides can not be achieved without the enzymatic capacity of the gut commensals [14,46,47]. It has been suggested that differences in the Westernized diet could be driving the rapid increase in asthma [48]. Modifying the diet can very rapidly change the microbiota composition. Turnbaugh et al. demonstrated that switching from a low fat, plant polysaccharide rich diet to a high fat, high sugar "Western" diet could alter the microbiome within one day [49]. These experiments were conducted in mice stably colonised with a human microbiome. Changes in the microbiome after switching to the Western diet included a general reduction in Bacteroidetes phyla and increases in Firmicutes taxa, Clostridia, Bacilli and Erysipelotrichi [49]. Modifications of the microbiota composition resulted in changes in gene expression and metabolic pathways utilised by the microbiota [49], and within 2 weeks mice on the Western diet had greater adiposity, which could be transferred to GF recipients following fecal transplantation.

Vast differences in the gut microbiota have also been observed between human populations. The microbiota of children from rural Africa and from Europe was analyzed and compared [50]. Children

from the African cohort (Burkino-Faso) have a predominantly vegetarian diet high in fibre, starch and plant polysaccharides and low in animal protein and fat, whereas the diet of children from the urban European cohort was high in fat, sugar and starch and low in fibre. There were also differences in breast-feeding between the groups. Children from the African cohort were breastfed for two years, along with a mixed diet, whereas children from the European cohort were breastfed for an average of one year. The microbiota of the African cohort was highly enriched in Bacteroidetes, with a particular increase in bacteria known to encode genes required for hydrolysis of complex plant polysaccharides, including two bacterial species (*Prevottela* and *Xylanibacter*) that were completely absent in the Western cohort [50]. This was reflected by an increase in SCFA production in African children [50]. Given the strong immunomodulatory function of SCFAs, their increase in the African population could be one explanation for the reduced occurrence of allergies and autoimmune diseases in this population.

4.2. Hygiene

The hygiene hypothesis [51] is currently the prevailing explanation for the increase in asthma and atopic disorders in western countries. It suggests that excessive "cleanliness" in the environment has led to a decline in the number of infectious stimuli required for the proper development of the immune system, affecting the switch from Th2-predominant immunity following birth, to Th-1 predominant responses. But hygiene could also relate to altered exposure to commensals that might be important for immune educating events. See also information below on antibiotic use and maternal transfer.

4.3. Antibiotic use

The development of antibiotics has revolutionised Western medicine and has dramatically reduced infectious diseases and associated morbidity. However, use of antibiotics may be linked to the increase in allergic diseases in Western countries [52–57]. Antibiotics can alter the ecology of the gut microflora, reducing beneficial microorganisms and colonization resistance causing an increased risk of infection, or outgrowth, by pathogenic microorganisms [56,58]. In infants, antibiotics reduced numbers of *Bifidobacteria* and *Bacteroides* [59]. Furthermore, antibiotic use is associated with higher risk of developing allergy [52,55]. In one study this was only the case in individuals who had a genetic predisposition (one parent with allergy) [55]. Antibiotic use can enable yeasts, such as *Candida albicans* to flourish. Over growth of *Candida* was demonstrated to drive the development of allergic airway responses to mould spores [57]. *Candida* has been shown to produce prostaglandins [60,61], which could provide a mechanism for how yeast could drive immune responses. All of these studies indicate the potential for antibiotics to induce dysbiosis.

4.4. Maternal transfer

Infants are born sterile, and in the hours and days following birth the gut becomes colonised with microbes, derived mostly from the mother. This is thought to be an important time for immune education; therefore disruption of this process could have consequences for immune function. Method of delivery, hospital hygiene and breast-feeding all contribute to colonization of a newborn [59,62–65]. Differences in colonization were observed between vaginally and caesarean section-born infants, with *Bifidobacteria* and *Bacteroides* dominating in vaginally born infants, while infants born by caesarean section were more often colonised with *Candida difficile* [59,62]. Also, premature infants were more often associated with, and had higher counts of, *C. difficile* [59]. Formula feeding was associated with colonisation by *C. difficile*, *E. coli*, *Bacteroides* and *lactobacilli* [59]. Differences in microbial products were also found between breastfed and formula fed infants, with breastfed infants having higher levels of acetic acid (acetate) [66]. Differences in frequency of *bifidobacterium* colonisation were observed between different wards, suggesting that the birthing environment has a major impact on the colonisation of infants [64,65]. These differences are thought to have a major effect on susceptibility to allergic diseases (reviewed in [57]).

4.5. Host genetics

Genetics of the host may itself affect microbial colonization. *Tlr5*$^{-/-}$ mice were shown to have an altered microbiome compared to WT mice. *Tlr5*$^{-/-}$ mice display hyperphagia (overeating) and hallmark features of metabolic syndrome, including insulin resistance and increased adiposity [67]. While hyperphagia could itself cause a change in microbiota, transfer of *Tlr5*$^{-/-}$ microbiota to wild type mice conferred many aspects of the *Tlr5*$^{-/-}$ phenotype including hyperphagia and obesity [67] suggesting that the changes in microbiota did precede hyperphagia and induce this phenotype. From this and other studies performed with *Myd88*$^{-/-}$ NOD mice described earlier, it is reasonable to suggest that any element that affects innate immunity, such as infections or polymorphisms in innate immunity genes, might affect the makeup of the gut microbiota.

Genetically obese mice (*ob/ob* mice, leptin gene deficient) also have an altered microbiota. *ob/ob* mice have an over-representation of bacteria from the phyla Firmicutes and an under-representation of Bacteroidetes [68]. Gut microbiota transferred from *ob/ob* mice to WT GF mice could induce obesity, due to the ability of the obesity-associated microbes to extract more energy from the diet. Similar changes in the gut microbiota were also found in a cohort of obese human subjects [69]. Relative abundance of Bacteroidetes could be restored to the same levels as lean subjects after weight loss [69]. This suggests a positive feedback mechanism in obesity, where obesity can induce changes in the gut microbiota (through diet and genetics), which results in a microbiome more capable of extracting energy from the diet, thereby helping perpetuate obesity.

4.6. Stress

Stress is known to have effects on the immune and metabolic systems, and it also appears that stress can alter the gut microbiota. One study demonstrated that exposure to stress during pregnancy in monkeys caused altered microbial colonization in the stressed monkeys' offspring, with decreases in *Bifidobacteria* and *Lactobaccili* [70]. Other studies have demonstrated that stress reduces *Lactobacilli* and increases growth and epithelial adherence of *E. coli* and *Pseudomonas*. Early life stress in rats was shown to alter the brain-gut axis, with increases in plasma corticosterone, increased peripheral immune response to LPS and change in gut function and microbiota [71]. Also gut microbiota can upregulate virulence factors in response to host stress [reviewed elsewhere 72]. One mechanism for stress affecting gut microbiota could be through changes in bowel function. Stress can cause increased bowel movement and would therefore affect substrate availability for microbes. Given the intricate links between the central nervous system and immunity, this adds further interest to the field of neuroimmunology.

4.7. Pathogens

Commensal organisms provide protection against pathogens through competition for nutrients and space, this is known as colonisation resistance. However pathogens sometimes outsmart commensals. Different pathogens may have different survival techniques. *Salmonella enterica* induces intestinal inflammation in order

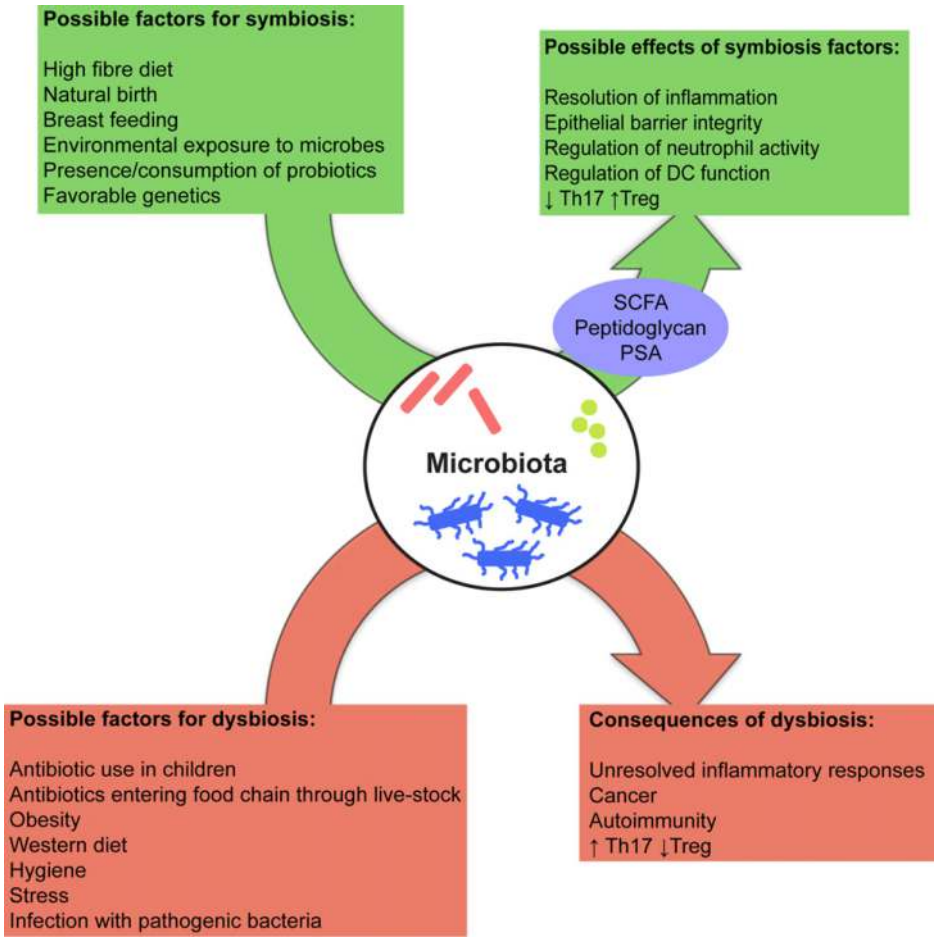

Fig. 1. Possible factors controlling the gut microflora. Environmental factors have a great influence on the gut microbiota. Some factors, such as a high-fibre diet, promote the colonization with beneficial bacteria that produce symbiosis factors like SCFA, PSA and peptidoglycan, which have a favourable regulatory function on immune responses. Other factors, such as antibiotic use might lead to dysbiosis and an increase of harmful bacteria and potentially a reduced production of symbiosis factors. This can result in inflammatory diseases, autoimmunity and cancer.

to benefit its growth by reducing competition with the healthy microbiota [73]. Inflammation itself could be damaging to the microbiota, up-regulation of, or increased number of immune cells expressing, innate immune molecules, such as MyD88 may exert some pressure on the microbiota (as mentioned above), and provide an environment more suitable for a pathogen such as *S. enterica*.

5. Concluding remarks

We are only beginning to understand how environmental factors affect the composition of the gut microflora, and the consequences of this for immune responses (summarized in Fig. 1). We still know very little about how genetic factors, diet and other environmental factors affect immune function and future research will have to focus more on how the composition of the gut microbiota is affected in different experimental settings. Technical advances, like high-throughput 16s RNA sequencing will greatly facilitate this task. It will be of great importance to identify conditions that lead to overgrowth of harmful bacteria that result in diminished production of beneficial microbial products, such as PSA or SCFAs and to find ways how to counteract colonisation of the gastrointestinal tract by harmful bacteria. The increase in allergies and autoimmune diseases in Western countries strongly suggests that something associated with a Western lifestyle, possibly diet and/or antibiotic use, might promote a gut flora that is reduced in beneficial bacteria. Therefore, understanding of how we can manipulate gut bacteria to alleviate such conditions might represent a new frontier for immunology research and therapy.

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