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Importance of microbial colonization of the gut in early life to the development of immunity

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Abstract

The mammalian gastrointestinal tract harbors a complex microbiota consisting of between 500 and 1000 distinct microbial species. Comparative studies based on the germ-free gut have provided clear evidence that the gut microbiota is instrumental in promoting the development of both the gut and systemic immune systems. Early microbial exposure of the gut is thought to dramatically reduce the incidence of inflammatory, autoimmune and atopic diseases further fuelling the scientific viewpoint, that microbial colonization plays an important role in regulating and fine-tuning the immune system throughout life. Recent molecular diversity studies have provided additional evidence that the human gut microbiota is compositionally altered in individuals suffering from inflammatory bowel disorders, suggesting that specific bacterial species are important to maintaining immunological balance and health. New and exciting insights into how gut bacteria modulate the mammalian immune system are emerging. However, much remains to be elucidated about how commensal bacteria influence the function of cells of both the innate and adaptive immune systems in health and disease.

Keywords: Microbiota; Mucosal immune system; Probiotics; Inflammation; Tolerance

1. Introduction

The human gut microbiota, shaped by the long co-evolutionary history of symbiotic host–microbe interaction, plays an important role in maintaining human health by preventing colonization by pathogens, degrading dietary and in situ-produced compounds, producing nutrients, and maintaining the normal mucosal immunity [1–3]. Other important functions have begun to emerge over recent years suggesting that the effects of the commensal microbiota may be more profound; influencing processes as complex as lipid metabolism of the host [4], predisposition to obesity [5,6], immune development and homeostasis, inflammation, repair and angiogenesis [7–10]. The inter-relationships between the microbiota and the host are clearly important in relation to health and imbalance between these systems appears to drive a wide range of mucosal and systemic immune-mediated disorders including inflammatory bowel diseases, autoimmune and allergic conditions. In this review we discuss current data linking the human commensal microbiota to immunological development and function and highlight the potential consequences of microbe–host dialogue in early life to human health.

2. A single mucosal immune system, innate and adaptive components

The mucosal immune system relies on cells of both the innate and adaptive immune systems that function concertedly to neutralize potentially dangerous infec-

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tious agents and maintain tolerance to dietary antigens and commensal bacteria [11]. Innate immune signalling is mediated by innate or natural immune cells expressing germ-line receptors of limited repertoires that respond promptly following pattern recognition of bacterial structures (pathogen-associated molecular patterns or PAMPs) and antigens [12]. The adaptive immune system on the other hand is composed of lymphoid cells (B and T cells) bearing rearranged cell surface receptors, generated by complex gene rearrangements, that give rise to an almost unlimited diversity of recognition response elements [12]. This system has the ability to recognize and remember specific pathogens and to mount stronger attacks each time a pathogen is encountered. Importantly, both the innate and adaptive systems exhibit interdependence in driving immune maturation and functionality [11]. Thus, rather than regarding the adaptive immune system as an autonomous immunoglobulin-producing defense system that is more complex and discriminating than the evolutionary older innate system, it is more realistic to regard the innate and adaptive ‘systems’ as heterogeneous and vital elements of the same defensive network. Nowhere is the interactive nature of mucosal defense more apparent than in the mammalian gut, which has evolved highly effective mechanisms to defend against gut pathogens, exhibit tolerance to dietary and self-antigens and at the same time coexist with large populations of non-harmful or beneficial microbes. With the highest number of immune cells and the highest concentrations of foreign antigens in the body, the gut also represents the major site of immune education. There is significant evidence depicting the human gut microbiota as the single most important factor driving the processes of immune education.

3. Describing the human gut microbiota

Historically, culture-based techniques characterizing microbial diversity dominated the field and as a result the most predominant species isolated from the human gut were considered to be Bacteroides spp., Bifidobacterium spp., Eubacterium spp., Peptostreptococcus spp., and Fusobacterium spp. [13]. These techniques, essentially based on fecal sampling and plating on specific selective media under anaerobic (up to 99.9% of colonic bacteria are obligate anaerobes) and aerobic conditions estimated that the microbiota of the human adult gut is represented by at least 400 cultivable bacterial species [14,15]. Subsequently, with the invention of molecular tools, in particular, those based on sequence analysis of PCR generated 16S rDNA libraries from luminal and mucosal samples, it has been recognized that the vast majority of human gut bacteria belong to two phyla, the Firmicutes and Bacteroidetes, with a very small number of other representatives [16–18]. Sequence analysis of 16S rDNA libraries remains the gold standard in human gut microbial diversity studies. The main attraction of this technique is that it is highly standardized and the sequence data produced can be incorporated into other studies that address comparative, genetic, developmental and environmental aspects of human gut microbiota. Other widely used techniques in human gut microbiota analysis include fluorescent in situ hybridization (FISH), which takes the advantage of the phyla- and genus-specific sequences present in a 16S rDNA molecule as well as the high copy number of 16S rRNA molecules in bacteria cell allowing detection of a single cell under a fluorescent microscope [19,20] or by flow cytometry [21]. However, by its nature this method is dependent on sequence data availability and hence fails to detect novel sequences and lacks the power of data interchange and phylogenetic reconstruction. It targets only major taxonomic groups but provides key information on the in situ location and spatial distribution of bacteria in the human gut epithelium [22].

4. Early colonization of the GIT

The mammalian fetal intestine is essentially sterile and the first exposure to maternal microbiota occurs during passage through the birth canal during the first hours of delivery. In the beginning of colonization, the microbiota of the newborn is heterogeneous [23]. In the cultivated microbiota of vaginally delivered and breast-fed infants, the majority of the fecal population is represented by bifidobacteria, with smaller numbers of Escherichia coli, bacteroides and clostridia [24]. In culture-independent real-time PCR assays, the prevalence and counts of C. difficile and E. coli are significantly lower in the gut microbiota of breast-fed infants than in that of formula-fed infants while no difference in bifidobacteria is detected between these two groups [25]. The occurrence of four species of clostridia, C. paraputrificum, C. perfringens, C. tertium, and C. difficile, in bottle-and mix-fed infants is relatively higher than in breast-fed infants [26]. These data support the notion that the composition of gut microbiota in infants can be influenced by the type of infant feeding, and other factors including mode of delivery, gestation age, infant hospitalization, and antibiotic treatment [25]. Delivery by cesarean section, for example, not only prevents the newborn from being exposed to bacteria in the birth canal but also decreases the general bacterial exposure from the mother because of routine
antibiotic prophylaxis in cesarean delivery [27]. In comparison with vaginally delivered infants, the gut of the cesarean section-delivered infant has lower numbers of bifidobacteria and Bacteroides, whereas they are more often colonized with C. difficile [25]. In general, intestinal colonization in infants delivered by cesarean section is delayed and changes in intestinal microbiota are quite persistent [28]. In later life, such infants may have an increased risk of developing atopic disease [29–32]. The present day trend, e.g., delayed colonization by “traditional” fecal bacteria, is not limited only to cesarean section-delivered newborns (although it is more profound in this group) but it is observed in vaginally delivered infants as well, possibly due to limited environmental circulation of these bacteria [33]. In their absence, skin bacteria like staphylococci and other bacteria that are usually not dominant in the gut have become the first colonizers. The consequences of these unusual gut colonization events for the development of the immune system are unknown but their potential relevance in relation to the risk of immune disorders in later life will be discussed in more detail in subsequent sections.

The second major gut microbial community succession happens at weaning and introduction to solid food. The gut microbial community at this stage become more diversified and enriched by bacteria that are common in the adult gut, in particular, Bacteroidetes and Firmicutes. For the obvious reasons, gut microbiota studies in children have been limited to fecal analyses. More detailed studies on the distribution of bacteria within the different compartments of the gastrointestinal tract have been possible only in adult volunteers and these earlier studies established that the bacterial density in the lumen is increasing from the upper to the lower gastrointestinal tract, from the almost sterile content of the stomach to the $10^5$ CFU/ml in the jejunum, to $10^6$ CFU/ml in the ileum [13,34] to $10^8$ CFU/ml in the cecum [35] and up to $10^{11}$ CFU/g wet weight in fecal samples that are considered to be representative of the colon [1,19,34]. The jejunal and ileal luminal microbiota consists of less diverse bacterial communities, mostly facultative anaerobes, such as streptococci, enterococci, lactobacilli, gamma-proteobacteria, and Bacteroides species [36]. Comparative analysis of fecal and cecum luminal contents in healthy volunteers suggested that the cecum harbors smaller proportions of the two major phyla found in feces (Firmicutes and Bacteroidetes) and is dominated by facultative anaerobes, such as enterobacteria (mainly E. coli) and enterococci [35].

There have been few attempts to cultivate the bacteria from gut mucosal surfaces and most of the microbial diversity work has been conducted using the 16S rDNA library approach. A 16S rDNA survey of the human esophageal surfaces revealed the presence of 95 phylotypes belonging to six major phyla, Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and uncultivated TM7 [37]. Unlike the SSU rRNA libraries from other intestinal locations, the vast majority of human esophageal bacterial sequences (82.1%) was classified as belonging to culture-defined bacterial species and shared a significant similarity with the species found in the oral cavity. Although the stomach content is considered to be almost sterile due to the harsh acidic environment populated with proteolytic enzymes, the use of the 16S library approach demonstrated the presence of at least 128 phylotypes belonging to the Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, and Fusobacteria phyla [38]. However, since 67% of these phylotypes correspond to sequences from the human oral cavity, the majority of sequences retrieved from the gastric mucosal specimens may belong to transient bacteria rather than to the bona fide indigenous microbiota. Comparative sequence analysis of 16S rDNA libraries from the mucosal biopsies of the jejunum, distal ileum, ascending colon and rectum revealed that the jejunum harbors the least microbial diversity and is dominated by sequences closely related to Streptococcus (67%), while the populations from distal ileum, ascending colon and rectum are dominated by sequences affiliated with Bacteroidetes (27–49%) and Firmicutes (27–47%) [39].

5. Genomic and metagenomic approaches to functionality of the human microbiota

The major drawback of descriptive microbial diversity studies is their limited value in discerning the functional role of the majority of bacteria in the human gut. To address this several new approaches have been proposed. The human gut microbiome initiative, which aims to generate draft genome sequences of hundreds of gut bacteria as well as new metagenomic analyses have been proposed as routes of analysing the functional diversity of the human gut microbiota [40,41]. Studies utilizing these technologies have concentrated on the metabolic aspects of host–microbe interactions [40,42] and somewhat predictably, have identified that the microbiome is significantly enriched in genes encoding for the metabolism of host substrates including glycans and amino acids, and the biosynthesis of vitamins and isoprenoids. Genome/metagenomic analyses, which specifically addresses the functionality of the human microbiota in relation to immune development and homeostasis is currently unavailable. As progress
continues such approaches will address the intriguing question of which structures and components of commensal bacteria drive the developmental and regulatory effects on the host immune system. Recently, the role of bacterial carbohydrate structures in promoting T cell activity has been demonstrated [43]. In this regard, the presence of several hundred genes with eukaryotic signatures within prokaryotic genomes may not be coincidental; perhaps these genes encode proteins that facilitate important processes of host–microbe immune regulation.

6. Microbial colonization and development of immunity

Extensive intercellular signalling between lymphoid cell types is a key feature of a highly regulated innate and adaptive immune system. These interactions occur within germinal centres (GCs) and secondary lymphoid follicles (LFs) associated with the structurally complex gut-associated lymphoid tissues (GALT). There is increasing awareness that the GALT plays a role in shaping the repertoire of the gut microbiota and vice versa. In young mammals GC and LF regions of the GALT are incompletely developed. The developmental process is dependent on microbial and other exogenous stimuli [44]. As successive populations of bacteria colonise the gut lumen they contribute significantly to both nutrient processing and education of the immune system [45]. Maternal microbial antigen experience can influence T cell and myeloid cell recruitment and activation in the neonate [46]. A single bacterial polysaccharide (PSA) from a commensal bacterium, Bacteroides fragilis, is capable of directing the cellular and physical maturation of the developing immune system [43,47]. Other non-cultivated bacteria have been shown to direct immune development [48]. Although the precise mechanisms are unknown, accumulating evidence demonstrates that the commensal gut microbiota can influence epithelial and stromal cell biology, the function of dendritic cells (DCs), T and B cells suggesting that at the level of the mucosal immune system their effects are likely to be profound.

7. Microbial colonization, gut barrier and maintenance of immune tolerance

Several lines of evidence from various animal and disease models support the view that there is intensive communication between the host and gut bacteria. The net outcome of this communication, in immunological terms, is dictated largely by the nature of the bacterium. In healthy individuals commensal non-harmful bacteria induce immunological tolerance, contrasting with the response generated by pathogenic bacteria, which is characterized by strong pro-inflammatory reactions that trigger immune activation. The ability of the gut to respond differentially to gut bacteria implies that complex mechanisms of microbial surveillance, immune recognition/exclusion and regulation operate to ensure appropriate immune responses are mounted. It is likely that components of this complex network are present at birth and others are educated as a result of microbial exposure and sampling. Immune tolerance of the commensal microflora is, in part, attributable to the ‘barrier’ and ‘gatekeeper’ characteristics of the innate and adaptive intestinal IgA system [49]. Intestinal mucus, tight junctions and anti-microbial peptides [50,51] also contribute to this barrier function. Many commensal bacteria that preferentially colonize the gut mucus layer can also access epithelial cell membrane glycans [52] and at certain periods in life, for example the suckling period, preferentially utilize host mucosal substrates as energy sources [53]. As a consequence gut bacteria gain closer contact with the host. The fact that immune hyporesponsiveness is maintained in this scenario suggests that, in addition to exclusion, other microbe/host factors are involved. For example, commensals typically lack virulence factors such as specific adhesions and invasins that enable pathogens to penetrate mucus and possibly invade the epithelial layer. Commensal or symbiotic bacteria also exert direct immunosuppressive effects that could be responsible for the unique tolerance of the gastrointestinal mucosa [8,54,55].

8. The IgA barrier—milk and B1 cells in young mammals

The presence of microbial-reactive IgA in the lumen strongly augments the physical barrier function of the developing and adult gut and plays a key role in shaping the immune response to microbial colonization. The intestinal IgA shields the commensal flora from both the innate and the systemic immune system [49]. This function is likely to be very critical in early life when mechanisms of immune regulation are not fully operational. In the absence of IgA, gut commensal bacteria overgrow, have inappropriate access to mucosal epithelia and trigger abnormal systemic immune responses [56]. Maternal milk is an important primary source of IgA (and IgM) in sucking animals and infants, which transfers the ability from mother to neonate to exclude non-pathogenic luminal bacteria. This exclusion is an apparently primitive process that does not require diver-
sification of the primary natural antibody repertoire [57]. Other maternal antibodies, particularly IgG are delivered in milk or retrotransported via neonatal Fc receptor (FcRn) from neonatal serum into the intestinal lumen and give protection against pathogenic species [57]. In addition to passively derived IgA, neonates generate their own natural poly-reactive IgA repertoire of limited diversity but sufficient for the exclusion function [57]. In the immature gut, this synthesis of intestinal IgA occurs in the absence of GC formation. Peritoneal B1 cells generate large amounts of low affinity gut IgA independently of T cell help [58]. The production of these intestinal IgAs requires the presence of commensal flora [59] and hence it is possible that activation of TLRs on B1 cells by PAMPs in the gut flora enhances the production of IgA [60]. Thus, a significant fraction of B1 cells sense commensal gut bacteria and are constantly induced to migrate out of the peritoneal cavity [45]; through persistent activation of these cells “natural antibody” production is enhanced. These antibodies probably play an important role in the early defense against bacteria and viruses, and are thus likely to protect against infections in infancy [60]. An alternative pathway for the production of intestinal IgA has been proposed in which IgM + B cells in the lamina propria switch to production of the IgA isotype, again without the need for T cell help [58].

9. IgA barrier—T cell-dependent

In the maturing gut, high affinity neutralising IgA is generated in a T cell-dependent fashion within the organised follicular structures of the GALT. The major inductive sites in the small intestine are LFs, either solitary or clustered as in Peyer’s patches (PP). These follicles are covered by a specialised follicle associated epithelium (FAE) containing M cells (microfold cells) that take up intact macromolecules, particles and pathogens, and deliver them by transepithelial transport to underlying subepithelial dendritic cells (DCs). The latter antigen-presenting cells are attracted to the subepithelial region by chemokines that are released by the FAE [61–63]. Adherence of antigens and microorganisms to M cells greatly increases the efficiency of uptake in PP [64]. It has recently been shown that innate TLR signalling regulates this gatekeeping function of the FAE, promoting antigen capture by DCs in organised lymphoid tissue [65]. Within the subepithelial dome (SED) DCs and other resident antigen-presenting cells present antigen to helper T lymphocytes that in turn secrete cytokines that stimulate B cell proliferation, class switch recombination, somatic hypermutation and production of IgA. After leaving lymphoid follicles and passing through the systemic circulation, IgA + lymphocytes migrate back to the lamina propria where they differentiate into plasma cells capable of secreting large amounts of antibody. Upon reintroduction of the antigen, plasma cells secrete specific IgA that is transported back to the intestinal lumen. In addition to its role in exclusion, luminal immunoglobulins, specifically IgG by forming complexes with its receptor (FcRn), can also function in bacterial antigen retrieval from the gut lumen and initiate adaptive immune responses in regional lymphoid structures [66]. FcRn may thus integrate luminal antigen encounters with systemic immune compartments and as such provide essential tuning of the immune system, host defense and other immunoregulatory functions at the mucosal surfaces [66]. As FcRns are constitutively expressed throughout life, their immunomodulatory functions in relation to the commensal microbiota are probably active in both neonates and adults.

10. Commensal bacteria, signalling through extra- and intra-cellular recognition receptors

Although exclusion and immune suppression are key features of immune tolerance to commensal bacteria, the expansion and function of the immune system is built upon recognition and sampling. These later events are critical for immune education. A wide range of TLRs [67] and non-TLRs [68] for PAMP recognition have been characterised on gut epithelial cells, associated DCs, monocytes and macrophages [69]; lineages involved in microbial-immune surveillance. Yet there is a paradox, the commensal microflora express many of the same ‘molecular patterns’ encountered on pathogenic bacteria; somehow commensals induce tolerance whereas pathogens, stimulate proinflammatory processes and active immunity [70]. Careful regulation of TLR signalling is therefore critical as inappropriate activation of TLRs can trigger both inflammatory and autoimmune diseases [71–73]. In this context, the surveillance role of TLRs localized to epithelial cells is particularly intriguing given their close anatomical proximity to the gut microbiota. Several mechanisms have been postulated to explain immune hyporesponsiveness to colonizing microbes. Firstly, the subcellular location of TLRs on epithelial cells may provide some explanation for the differential responsiveness to commensal and pathogenic bacteria. For example, TLR5 signalling in response to flagellate commensals is thought to be negligible due to the basolateral distribution of these receptors, which only become activated when encountered by invasive pathogens [74]. In the case
of TLR4, immunological tolerance to LPS is achieved through the developmental down-regulation of interleukin-1 receptor-associated kinase (IRAk), which is essential for TLR4 signalling and responsiveness to LPS [75]. Many other host proteins modulate the downstream signalling activity of TLRs (reviewed by [55]); some of these may be activated/deactivated by commensal bacteria. At odds with the notion that tolerance to the microbiota is maintained by exclusion and/or active down-regulation of TLR signalling, is the recent evidence that activation of TLR signalling by commensal bacteria appears to maintain intestinal epithelial homeostasis and confers protection against gut injury [7]. Conceptually this is very important and suggests that commensal bacteria are both recognized by, and can activate, TLR receptors and downstream signalling pathways. Clearly, such responses require regulation; IL2 and IL10 cytokines appear to prevent the deleterious consequences of commensal-induced immune signalling while preserving its beneficial physiological effects [76]. Additional work is needed to dissect out how both protective (anti-inflammatory) and immune-activating (pro-inflammatory) effects of TLR signalling are regulated in response to commensal microbiota and to define the factors that account for the diversification of these signalling pathways [77].

Besides signalling through extracellular receptors, some bacterial signals are recognized by intracellular receptors, which also have leucine-rich repeat regions involved in recognition of bacterial ligands [78]. Two families, NOD-like receptors and RIG-like helicases have been described (reviewed by [68]) and these receptors survey the cytoplasm for the presence of intracellular invaders/PAMPs. Again, although these receptors are believed to have evolved to recognize the specific patterns of pathogenic bacteria, structurally, there are no differences, between muramyl dipeptide molecules (a NOD2 ligand) found commonly on the surfaces of commensal and pathogenic bacteria. Since mutations in the gene encoding NOD2, which interfere with muramyl dipeptide recognition, were recently shown to be associated with the chronic inflammatory disease, Crohn’s disease, NODs may have important roles in detection of commensal bacteria and in controlling bacterial aggression that leads to inflammatory diseases [79]. The mechanisms by which NOD2 and indeed other intracellular recognition receptors regulate immune responses to microbes are not currently understood [68]. So although knowledge of both extracellular and intracellular receptors involved in pathogen recognition is increasing at an extraordinary rate, their exact role in immune surveillance of the normal benign gut microbiota and in tolerance induction is not yet fully explained [68].

11. DCs and mesenteric lymph nodes in microbe/host interaction

The development of the GALT sees an increasingly vital sentinel role for cellular components of the innate system including DCs, macrophages and epithelial cells that together monitor the microbial environment and coordinate immune responses to danger signals [80]. A subpopulation of DCs (CX3CR1+) populate the entire lamina propria as well as the dome regions of the PP [81]. These DCs are responsible for continuous antigen acquisition from the intestinal lumen and transport of this antigen for presentation from the lamina propria to the intestinal mesenteric lymph nodes (MLNs) that drain the gut submucosa. Whether these DCs are involved in tolerance induction remains to be determined but by employing such routes, DCs transport sampled commensal bacteria or their constituents to MLNs, potentially inducing T cell proliferation and production of T cell-dependent IgA by B cells [82, 83]. Significantly, the DCs carrying the commensal load are restricted to the mucosal immune compartment by the MLNs, which ensures that immune responses to commensal bacteria are induced locally without potentially damaging systemic immune responses [82]. Where previously PP were considered critical for the induction of tolerance, MLN are now recognized to be key sites for induction of oral tolerance to food antigens [84] and probably commensal microbes [82]. Although there may be similarities in relation to the tolerance mechanisms operating in response to food antigens and commensal bacteria, in the case of microbes other factors come into play. For example, TLR-dependent and TLR-independent signalling generated in response to microbes would potentially influence DC maturation and hence T and B cell function [60]. In the case of pathogens, induction of TLRs on DCs renders them immunogenic [85]. Hence, how tolerance to commensals is achieved when such bacteria trigger TLR signalling events on DCs is an intriguing question that requires explanation [45, 60]. In this regard differential engagement of different types and subtypes of TLRs and non-TLRs may be important [56]. Furthermore, differences in downstream signalling events may also be relevant. For example, MyD88-independent TLR4 signalling in response to LPS may be important for oral tolerance induction [56] whereas the MyD88-dependent pathway activated by commensal bacteria appears to be important for cytoprotection [7]. Irrespective of precise
mechanisms, complex regulation at the level of DC/T cell interaction must play a key role in maintaining immune hyporesponsiveness and tolerance to commensal bacteria.

12. Tregs and mucosal tolerance

In the steady state, prior to acute infection and inflammation, DCs are in an immature state and not fully differentiated to carry out their known roles as inducers of immunity. However, these immature cells are not inactive. They continuously circulate through tissues and into lymphoid organs, capturing self-antigens as well as innocuous environmental and microbial proteins. Recent experiments have provided direct evidence of antigen-loaded immature DCs in vivo that silence T cells either by deleting them or by expanding regulatory T cells [86]. Furthermore, although many commensal bacteria may be shielded from immune cells by the epithelial, mucin and IgA barriers, there is good evidence that the exclusion is incomplete and that local immune responses may be activated in response to their presence. Finely tuned and possibly tissue-specific interactions between effector and regulatory T cell responses serve to diminish inflammatory responses to microbial and dietary antigens [87,88]. Production of anti-inflammatory cytokines such as IL-10 and TGF-β by regulatory T cells (Tregs) is often dominant during immune responses at mucosal surfaces, and is an important adaptive mechanism to prevent the development of chronic inflammation [87,89]. The importance of these regulatory cytokines to the maintenance of mucosal tolerance is demonstrated in IL-10 and TGF-β deficient mice that develop chronic inflammation in response to their commensal microbiota [10]. Loss of these cytokines generally correlates with diminished Treg function and expansion of effector CD4+ T cells of either Th1 or Th2 phenotype. There is an emerging consensus that bacterial antigens are crucial for the generation and/or expansion of Treg cells in healthy individuals [56,90,91]. Although adoptive transfer of CD4+ CD45RBhi T cells from germ-free mice, which contain cells of regulatory phenotype can inhibit colitis induced by CD4+ CD45RBlo T cells [92] suggesting that bacterial antigen exposure is not required for the generation of Tregs, other evidence suggests that mucosal Treg subsets involved in maintaining tolerance require microbial antigen exposure [90]. This subject has been reviewed very comprehensively [56,88] and the prevailing opinion is that the commensal microbiota, through mechanisms involving TLR signalling, promotes the development of a fully functional Treg population [56,93]. Recent research [94] demonstrates that peripheral antigen displayed by lymph node stroma promotes T cell tolerance to intestinal self. The potential role of intestinal or lymph node stromal cells in tolerance to commensals should be investigated.

13. Childhood infections and vaccination

Vaccination is the most effective means of preventing infectious diseases and is largely responsible for the eradication of diseases such as polio-myelitis, smallpox and diphtheria that, in early periods of history, caused high mortality amongst children. Whilst disease control in this way is absolutely essential much of the ongoing work in vaccine development aims to derive ‘good vaccines’ with improved biological efficacy [60]. Commonly used vaccines promote antibody responses but have little impact on the cellular immune response [60] and hence their use in the developing immune system may not be optimal or provide appropriate ‘education’. Advances in our understanding of how PAMPs, derived from both commensal and pathogenic microbes, interact with the innate immune system to regulate the adaptive arm, may help resolve this limitation. The concept of using multiple TLR ligands to induce immune synergy and diversity is emerging in vaccine development field [60]. By promoting a ‘healthy gut microbiota’ in early life through appropriate microbial colonization (environmental exposure), nutrition and possibly through the use of new probiotics, mucosal immunity and natural resistance to infections will be enhanced.

14. Hygiene hypothesis-link between gut microbiota and disease

Microorganisms colonising the human gastrointestinal tract are now recognized to contribute significantly to improved nutrient status and health of the host. These outcomes are achieved mainly through improved nutrient availability and nutrient capture in the human colon but also through enhanced immune functionality. Such positive effects on the immune system are though to confer protection against many human diseases including inflammatory bowel disease (IBD), atopic diseases and other organ-specific autoimmune diseases. This correlation, which is embraced by the hygiene hypothesis, is supported by epidemiological and clinical data, suggesting that the decreased incidence of infections observed over the last three decades, particularly in westernized societies, may predispose to diseases such as type I diabetes mellitus, autoimmune thyroiditis, atopic and inflammatory diseases including Crohn’s disease, systemic lupus erythematosus, rheuma-
toid arthritis and psoriasis. However, before elaborating on the evidence favouring the hygiene hypothesis and the potential mechanisms of microbial influence [72] two main points should be noted. Firstly, multiple gene linkage studies have identified several genetic loci linked to these immune-mediated disorders including cytotoxic T lymphocyte-antigen 4 (CTLA4), caspase recruitment domain (CARD15), DLG5, SLC22A4/A5, programmed cell death 1 (PDCD1), RUNX1, SLC9A3R1/NAT9, PADI4, ADAM33, DPP10, PHF11 and GPRA) [95] indicating a strong interplay between genetic predisposition and environmental factors in the etiology of allergic and autoimmune diseases. Secondly, and in conflict with the concepts presented by the hygiene hypothesis, some viral and parasitic infections, which may or may not contain cross-reactive epitopes mimicking self-antigens, but importantly induce TLR activation may or may not contain cross-reactive epitopes mimicking self-antigens, but importantly induce TLR activation.

Numerous ideas have been proposed regarding the hygiene hypothesis including the nature of protective infectious agents, their mechanisms of action and the timing of their involvement with regard to the natural history of immune diseases [72]. Antigenic competition is the first hypothesis, which attempts to explain the inverse relationship between natural infection rate and the occurrence of immune-mediated diseases. Simply put, immune responses against pathogens compete with and hence reduce the autoimmune and the allergic immune response. Infectious agents may also intervene through components that are not recognized as antigens but which bind to specific receptors on cells of the innate immune system, in particular TLRs, and via such signals promote immune homeostasis [7,98]. Another hypothesis proposes immune regulation as the prevailing mechanism, preventing immune dysfunction and suggesting that infectious agents stimulate a large variety of regulatory cells (Th2, CD25+, Tr1, NKT) whose biological functions generate bystander suppression.

15. Hygiene hypothesis and the normal commensal microbiota

The hygiene hypothesis may be extended to encompass the protective effects of benign, non-infectious gut microbes. The basis of this assumption is that altered microbial diversity and functional activities of the luminal microbiota may be linked to the etiology of immune-mediated diseases. In children with allergic diseases, the fecal microbiota composition is markedly different from the norm. For example, the fecal microbiota of children with eczema is characterized by lower number of bifidobacteria, higher numbers of lactic acid bacteria (particularly enterococci) and is frequently associated with E. coli [25,99,100]. Babies who develop allergy are less often colonized by bifidobacteria but more often with Staphylococcus aureus and have higher counts of clostridia [101]. Feces of allergic infants have higher levels of the rarely detected i-caproic acid, which is associated with the presence of Clostridium difficile [102]. Adult patients with atopic dermatitis also harbor lower numbers of bifidobacteria in the gut than healthy subjects [103].

The normal microbial diversity in IBD, such as Crohn’s disease, is diminished on mucosal surfaces [104] and in fecal material, especially the Firmicutes [105]. This loss of diversity is compensated by expansion of bacteria that are unusual in the gut [106]. Such microbial diversity shifts may be due to elevated immune reactivity against dominant bacterial structures including flagellins [107]. Another hallmark of IBD is a much closer association of intestinal bacteria with epithelial surfaces and increased incidence of bacterial invasion [22,108]. Biofilms in IBD patients consist mainly of Bacteroides fragilis [109], which is not surprising in the light of close association of this bacterium with mucosal surfaces and highly developed mechanisms of evading the immune surveillance. The mechanisms by which commensal/symbiotic bacteria contribute to immune development, immune homeostasis and regulation, are now being actively researched and debated. As a result of this scientific evidence [47,82] the hygiene hypothesis is gaining credibility.

16. Probiotic approaches to restore microbial balance and treat human diseases

Due to the potential beneficial effects of commensal bacteria on immune function, there is now considerable interest in using probiotics in clinical medicine for the treatment of a wide range of disorders including necrotizing enterocolitis in pre-term infants, atopic eczema, inflammatory bowel diseases and cancer (reviewed by [110,111]). Some clinical trials suggest that probiotics exert either prophylactic or therapeutic benefit [112,113] whereas others have shown that they have no effect [114]. For many probiotic preparations little is known about their precise mode of action and their selection and application for treatment of human disease has not been wholly evidence-based. Recently, some progress...
has been reported on mode of action of the probiotic, *Saccharomyces boulardii*, which inhibits IBD by trapping Th1 cells in the MLN thereby limiting their infiltration into the inflamed colon [115]. Similarly robust scientific analyses of other probiotic preparations may open other clinical avenues. The use of probiotics in infant nutrition can only be justified once we have derived a detailed understanding of their action, in the longer term.

17. Summary

The functionally immature immune status of a newborn must be properly educated to deal with the challenges of autonomous life such as the development immune competence against pathogens, while ensuring that tolerance to common food antigens and commensal microbiota is maintained. Appropriate antigen exposure of the infant gut at the right time is crucial for the healthy development of gut-associated and systemic immune responses. This includes colonization of the gut by bacteria from the mother’s birth canal and skin, provision of immunological factors with breast milk, and importantly overall exposure to bacteria in the environment. Patterns of microbial colonization in human infants have undergone significant changes in the last 60+ years; this has been attributed to higher numbers of caesarean and premature deliveries, introduction of formulae, essentially sterile food, and generally cleaner living environments. It may not be a simple coincidence that during the same period we have witnessed an increase in allergic diseases in children and inflammatory disorders in adults. There is clearly a correlation between the gut microbiota and education of the immune system and continued analysis of this interplay is essential to advance our understanding of gut health and disease.

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