REVIEW ARTICLE

The host selects mucosal and luminal associations of coevolved gut microorganisms: a novel concept

Pieter Van den Abbeele, Tom Van de Wiele, Willy Verstraete & Sam Possemiers

Laboratory of Microbial Ecology and Technology (LabMET), Ghent University, Ghent, Belgium

Correspondence: Willy Verstraete, Laboratory of Microbial Ecology and Technology (LabMET), Ghent University, Coupure Links 653, B-9000 Ghent, Belgium. Tel.: +32 9 264 59 76; fax: +32 9 264 62 48; e-mail: willy.verstraete@ugent.be

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Abstract

Along the human gastrointestinal tract, microorganisms are confronted with multiple barriers. Besides selective physical conditions, the epithelium is regularly replaced and covered with a protective mucus layer trapping immune molecules. Recent insights into host defense strategies show that the host selects the intestinal microbiota, particularly the mucosa-associated microbial community. In this context, humans coevolved with thousands of intestinal microbial species that have adapted to provide host benefits, while avoiding pathogenic behavior that might destabilize their host interaction. While mucosal microorganisms would be crucial for immunological priming, luminal microorganisms would be important for nutrient digestion. Further, we propose that the intestinal microorganisms also coevolved with each other, leading to coherently organized, resilient microbial associations. During disturbances, functionally redundant members become more abundant and are crucial for preserving community functionality. The outside of the mucus layer, where host defense molecules are more diluted, could serve as an environment where microorganisms are protected from disturbances in the lumen and from where they can recolonize the lumen after perturbations. This might explain the remarkable temporal stability of microbial communities. Finally, commensals that become renegade or a decreased exposure to essential coevolved microorganisms may cause particular health problems such as inflammatory bowel diseases, obesity or allergies.

Introduction and central hypothesis

Introduction

The human body confronts microorganisms that enter the gastrointestinal tract with a multitude of barriers. In the mouth, saliva contains several antimicrobial factors [antimicrobial peptides (AMPs), lactoferrin, immunoglobulin A (IgA), nitrite] and exposes the microorganisms to a high flow-through environment (Xu *et al.*, 2001; Tenovuo, 2002). When leaving the mouth, microbial colonization is strongly influenced by luminal pH, retention time and secretions (Macfarlane & Macfarlane, 1997). Microorganisms are subjected to very acidic conditions and a short retention time (1–4 h) in the stomach, while they are exposed to the secretion of bile salts and pancreatic juices at the proximal part of the small intestine. Moreover, the small intestine (duodenum, jejunum and proximal ileum) has a short retention time (2–6 h) so that microbial colonization of the upper digestive tract is very restricted. The lower digestive tract, comprising the terminal ileum and the colon, is in contrast characterized by a longer retention time (48–70 h) and more favorable environmental conditions, resulting in heavy microbial colonization.

The colon harbors a highly complex microbial ecosystem at concentrations of 10^{11} microorganisms g^{-1} gut content, the highest recorded for any microbial habitat (Whitman *et al.*, 1998). As the total number of microorganisms of the human body is approximately 10 times greater than the total number of our somatic and germ cells (Luckey, 1972), it seems appropriate to view ourselves as a composite of human cells and bacteria and our genetic landscape as a 'metagenome', an amalgam of genes embedded in our genome and in the genomes of all our microbial partners. It is estimated that the collection of all microbial genomes in the gut comprises between 2 and 4 million genes, which is

70–140 times more than that of their host (Egert *et al.*, 2006). The composition of this microbial community is governed by age, diet, environment and phylogeny (Zoetendal *et al.*, 1998, 2001; Hopkins *et al.*, 2001; Ley *et al.*, 2008; Benson *et al.*, 2010) and contains all three domains of life: bacteria, archaea and eukarya (fungi, yeasts and protozoa). The microbial diversity is, however, relatively limited. From the 55 and 13 described divisions of bacteria and archaea, respectively, only a few have been identified in the gastrointestinal tract. *Firmicutes* and *Bacteroidetes* are two bacterial divisions that make up over 90% of the intestinal microbiota. The remainder consist of *Actinobacteria* (Turnbaugh *et al.*, 2009) and to a lesser extent also *Proteobacteria*, *Verrucomicrobia* and *Cyanobacteria* (Backhed *et al.*, 2005; Eckburg *et al.*, 2005; Ley *et al.*, 2006). Further, only two archaeal species have been described, with *Methanobrevibacter smithii* being more predominant than *Methanosphaera stadtmanae* (Miller & Wolin, 1985; Eckburg *et al.*, 2005). Despite this small amount of divisions, lineages terminate in broad, shallow radiations comprising hundreds of species and thousands of strains, resulting in significant interindividual variability of the microbial community composition (Zoetendal *et al.*, 1998; Backhed *et al.*, 2005; Eckburg *et al.*, 2005; Ley *et al.*, 2006; Dethlefsen *et al.*, 2008; Turnbaugh *et al.*, 2009). The hypothesis of a shared core microbiome among individuals in terms of microbial composition has been suggested (Tap *et al.*, 2009; Claesson *et al.*, 2010; Qin *et al.*, 2010). As it has been shown for thoroughly investigated probiotic bacteria that the biological outcome of different strains of a certain species can vary significantly (Rijkers *et al.*, 2010), the existence and relevance of a core microbiome at an even higher phylogenetic level remains questionable. Yet, when focusing on bacterial genes needed for digestion and not on species composition, a core microbiome in terms of functional genes was demonstrated (Turnbaugh *et al.*, 2009; Qin *et al.*, 2010).

The upper digestive tract consists of a luminal environment that is surrounded by an epithelial surface with a large surface area to avoid mass transfer limitations, thus ensuring optimal nutrient absorption. However, the requirement for optimal absorption collides with the need for host defense against food- or water-borne pathogens. In addition, a potential threat of bacterial invasion may also come from the lower digestive tract. Although the lower gut region has a much smaller surface area, the vast amount of commensals of the indigenous microbial community represents a constant challenge for the gut epithelium in terms of bacterial invasion. Therefore, the host has developed a mucosal defense barrier along the intestinal tract that protects the 20 μm thin epithelial layer that separates the gut microbiota from the internal intestinal tissues. Mucus is constantly shed off from the epithelium, which is replaced regularly (Lievin-Le Moal & Servin, 2006; Mukherjee *et al.*, 2008). Tight junctions hold the epithelial cells together so that they form a virtually impermeable barrier for molecules and ions, allowing the host to absorb specific compounds or antigens through controlled mechanisms. The mucosal barrier function also includes the innate and adaptive immune system. The importance of intestinal immunity follows from the fact that the gut-associated lymphoid tissue comprises more than 70% of the total amount of immune cells found in the human body (Gaskins, 1997).

Central hypothesis: the host selects mucosal and luminal associations of coevolved microbiota

In case of the fascinating gastrointestinal environment where the higher organism and microorganisms live in close interaction, it is not clear who is in charge: the host or the microbiota. We therefore aim to review how the host responds to the constant input of microorganisms into the gut environment and how he/she manages the indigenous microbial community. The overall structure and composition of this intestinal microbial ecosystem reflects a natural selection at both microbial and host levels, leading to a functionally stable, coevolved cooperation (O'Hara & Shanahan, 2006; Blaser & Kirschner, 2007), characterized by reciprocal adaptation and benefits. Based on the selective physical conditions along the intestinal tract (pH, temperature and hydraulics such as retention time) and the variety of specific defense mechanisms, with the innate and adaptive immunity being the most important, we hypothesize that the host selects the intestinal microbiota. This selection would particularly occur very close to the epithelium within the protective mucus layer that overlies the epithelium and contains defense molecules, resulting in a distinct mucosa-associated microbial community (MAMC) (Swidsinski *et al.*, 2002; Zoetendal *et al.*, 2002; Eckburg *et al.*, 2005; Lepage *et al.*, 2005). Recently, Laura Hooper described the gut bacteria as follows: 'symbiotic bacteria that have coevolved to submit to host strategies for sequestration on the luminal side of the epithelial barrier and for rapid elimination in the event of barrier penetration' (Hooper, 2009). We extend this hypothesis by distinguishing (1) microorganisms that are not targeted by host defense molecules upon colonization of the mucus layer, thus residing in the MAMC, and (2) commensals that are preferentially targeted by host defense molecules in the mucus layer and therefore restricted to the lumen. Microbial factors that also influence the colonization of this mucus layer are adhesion to mucus, the ability to gain nutrients from host-derived mucins and strategies to deal with the oxygen gradient along the mucus layer. Mucosal microorganisms can interact both directly and indirectly with the host epithelium and might therefore be crucial in shaping the host immune system. In contrast,

luminal microorganisms can only indirectly interact and they are, among others, important for nutrient digestion.

The host's immune system as a powerful tool to control the intestinal microbiota (Fig. 1)

The mammalian innate and adaptive immune system

The mammalian immune system consists of the evolutionary ancient, immediately responding innate immune system and the highly specific, but temporarily delayed adaptive immune system. The innate immune system detects infections and microorganisms using a limited amount of germline encoded receptors [pathogen recognition receptors (PRRs)], each recognizing conserved molecular patterns that are often essential for the life of a broad class of microorganisms [pathogen-associated molecular patterns or microorganism-associated molecular patterns (MAMPs), because pathogenic and nonpathogenic microorganisms share similar signature molecules]. As MAMPs are only produced by microorganisms, PRRs are a crucial tool for the immune system to distinguish between self and nonself (Medzhitov & Janeway, 2002) and to initiate the expression of innate immune effectors such as AMPs. These antimicrobials eliminate certain microorganisms, but as they often lack specificity, they may also cause collateral damage to the resident intestinal microbiota (Medzhitov & Janeway, 1997; Akira *et al.*, 2006). The adaptive immune system uses

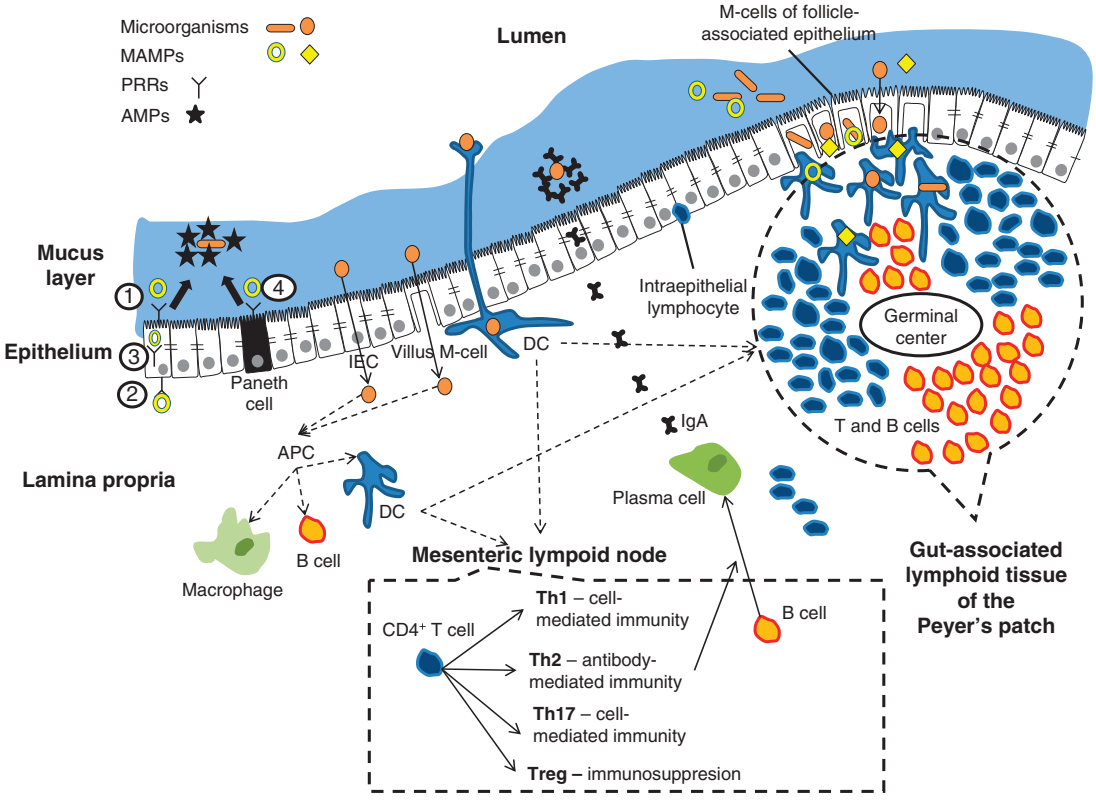

Fig. 1. The mammalian immune system consists of an innate and an adaptive arm. At the intestinal epithelial surface, there is constant signaling between microorganisms and the host. IECs and DCs extending beyond the epithelium are crucial regarding the initial recognition of microorganisms. Their PRRs of the innate immunity detect MAMPs, allowing the host to distinguish between a microbial friend or foe and to initiate the expression of AMPs. An important feature to distinguish between a microbial friend or foe is the strategic localization of PRRs and essential coreceptors: polarized (1–2), intracytosolic (3) or on specialized cells (4). Microorganisms can cross the epithelial barrier through IECs, DCs, villus M-cells and M-cells of the follicle-associated epithelium overlying the gut-associated lymphoid tissue of the Peyer's patches. After or during this transport, antigens are engulfed in APCs, being DCs, macrophages or B-cells. DCs travel from the epithelium through the lamina propria towards mesenteric lymphoid nodes, where they activate cells of the adaptive immune system. They are the only cells that can immediately bind with naive T-cells, and through cytokine release, influence their maturation to Th1, Th2, Th17 or Treg. Th2 interact with B-cells to become plasma cells that secrete IgA antibodies and coat luminal microorganisms in order to prevent them from breaching the epithelium. Alternatively, DCs can also locally activate adaptive immune cells in the gut-associated lymphoid tissue of the Peyer's patches.

Table 1. The innate immune system detects infections and microorganisms using PRRs

Localization receptor	PRR		MAMP	References
Transmembrane	TLRs (Takeda *et al*., 2003; Takeda & Akira, 2004)	TLR2/6	Diacylated lipopeptides	Takeuchi *et al*. (2001)
		TLR1/6	Triacylated lipopeptides	Takeuchi *et al*. (2002)
		TLR4	Lipopolysaccharides	Hoshino *et al*. (1999)
		TLR5	Flagellin	Hayashi *et al*. (2001)
		TLR3	Double-stranded RNA	Alexopoulou *et al*. (2001)
		TLR7 and TLR8	Single-stranded RNA	Heil *et al*. (2004)
		TLR9	CpG DNA	Hemmi *et al*. (2000)
		TLR11	Profilin and uropathogenic-derived protein	Zhang *et al*. (2004), Yarovinsky *et al*. (2008)
	Dectin-1		β-Glycans	Reid *et al*. (2009)
Cytosolic	NLRs (Fritz *et al*., 2006; Ye & Ting, 2008)	NOD/NLRC, e.g.		
		NOD1	Meso-DAP dipeptide	Chamaillard *et al*. (2003), Girardin *et al*. (2003a)
		NOD2	Muramyl dipeptide	Girardin *et al*. (2003b)
		NALP/NLRP, e.g.		
		NALP1 = NLRP1	Muramyl dipeptide, anthrax toxin	Boyden & Dietrich (2006)
		NALP3 = NLRP3	Uric acid crystals, extracellular ATP, pore-forming toxins	Mariathasan *et al*. (2006), Martinon *et al*. (2006)
		NAIP/NLRB, e.g.		
		NAIP5 = NLRB5	Flagellin	Molofsky *et al*. (2006)
	RIG-I, MDA5, DAI		Viral nucleic acids	Takaoka *et al*. (2007), Takeuchi & Akira (2008)
Secreted	Collectins		Microbial surface glycoconjugates	Rubio *et al*. (1995), Epstein *et al*. (1996), Garima & Avadhesha (2007)

One can distinguish transmembrane, cytosolic and secreted PRRs, each recognizing conserved molecular patterns that are often essential for the life of a certain broad class of microorganisms (MAMPs).

randomly generated, clonally expressed and antigen-specific receptors (B/T-cell receptors), resulting in less collateral damage of adaptive immune system effectors. In addition, these receptors provide a mechanism by which previous infections are remembered and thus protect from future infections with the same pathogen (Cooper & Alder, 2006). On the downside, adaptive immune receptors are less able to distinguish self from nonself, thus relying on the innate immune receptors to obtain information about the origin of an antigen. For example, after initial recognition by innate immune receptors, microbial antigens can be linked to small molecules such as the complement fragment C3d, so that adaptive immune cells are immediately informed about the microbial origin of this antigen (Carroll, 2004). PRRs also deliver information about the type, extent and duration of the infection, as well as about the requirement for immediate or future defense. They can turn on many cytokine genes that recruit and modify adaptive immune cells (Palm & Medzhitov, 2009). The innate and adaptive immune systems are thus complementary and render the mammalian immune system highly efficient at containing the diverse microbial communities that occupy the intestinal tract.

MAMP detection through PRRs

At the intestinal epithelial surface, constant signaling occurs between microorganisms and the host. MAMPs allow the host to distinguish between a microbial friend or foe. Bacterial MAMPs include lipopolysaccharides (Gram-negative bacteria), teichoic acids (Gram-positive bacteria), flagellins (bacterial flagellae), unmethylated CpG DNA, peptidoglycans and lipoproteins (most bacteria). An important fungal MAMP is β-glucan, whereas viruses are often detected through their nucleic acids. These MAMPs serve as ligands for PRRs, which thus specifically detect nonself microbial antigens.

Among the PRRs characterized in various cell types, one can distinguish transmembrane, cytosolic and secreted PRRs (Table 1). The best-characterized class of transmembrane PRRs are Toll-like receptors (TLRs) (Takeda *et al*., 2003; Takeda & Akira, 2004). They have an extracellular leucine-rich repeat (LRR) recognition domain and an intracellular Toll/interleukin-1 (IL-1) receptor (TIR) domain. Several types of mammalian TLRs exist, ranging from TLR1 to TLR11, each detecting a different part of the MAMP spectrum. TLRs allow the host to sense bacteria, viruses, fungi and

even protozoa (Hoshino *et al.*, 1999; Hemmi *et al.*, 2000; Alexopoulou *et al.*, 2001; Hayashi *et al.*, 2001; Takeuchi *et al.*, 2001, 2002; Heil *et al.*, 2004; Zhang *et al.*, 2004; Yarovinsky *et al.*, 2008). Although some TLRs function as homodimers, TLR2 forms heterodimers with TLR1 or TLR6, illustrating a mechanism to diversify the TLR-mediated recognition (Ozinsky *et al.*, 2000; Takeuchi *et al.*, 2001, 2002). Another well-characterized transmembrane PRR is dectin-1 that binds to β-glucans and is involved in antifungal defense (Reid *et al.*, 2009). Cytosolic PRRs include nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Fritz *et al.*, 2006; Ye & Ting, 2008) and several sensors of viral nucleic acids: retinoic-acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and DNA-dependent activator of interferon-regulatory factors (DAI) (Takaoka *et al.*, 2007; Takeuchi & Akira, 2008). NLRs are an important class of cytosolic receptors that thus respond to intracytoplasmatic MAMPs. All intracellular proteins from the NLR family contain a NOD, followed by an LRR at the C-terminus. The categorization in subfamilies is based on the domain present at the N-terminus that determines the NLR's functional properties: caspase-activation-recruitment domains (CARD) for the NOD/NLRC subfamily; pyrin for the NALP/NLRP subfamily; and BIR (baculovirus inhibitor of apoptosis protein repeat) for the NAIP/NLRB subfamily (Ting *et al.*, 2008). Like for TLRs, several types of NLRs exist, but only a few of them have been associated with specific MAMPs (Chamaillard *et al.*, 2003; Girardin *et al.*, 2003a, b; Boyden & Dietrich, 2006; Mariathasan *et al.*, 2006; Martinon *et al.*, 2006; Molofsky *et al.*, 2006). Finally, secreted PRRs such as collectins bind on the microbial surface and function as opsonins, bridging cell wall components to receptors of the complement pathway (Rubio *et al.*, 1995; Epstein *et al.*, 1996; Garima & Avadhesha, 2007).

Distinguishing between microbial friends and foes

PRRs of intestinal epithelial cells (IECs) and dendritic cells (DCs) that can extend beyond the epithelium are crucial for the initial recognition of microorganisms. There is evidence that initial recognition by these cells through their TLRs and NLRs initiates a signaling cascade, resulting in upregulated transcription of nuclear factor-κB, and subsequent proinflammatory and antimicrobial gene expression (Iwasaki & Medzhitov, 2004; Takeda & Akira, 2004; Akira *et al.*, 2006; Carneiro *et al.*, 2008). Such upregulation is necessary to react against threatening microorganisms. However, at the same time, the host has to stay unresponsive towards a vast amount of innocuous commensals. Therefore, it is of particular interest to elucidate how the host can distinguish between such microbial friends and foes through MAMP recognition with PRRs.

Commensals are often trapped in the thick mucus layer loaded with antimicrobials and commensal specific IgA as an instrument to further exclude them from internal tissues (Brandtzaeg & Pabst, 2004; Macpherson & Uhr, 2004; Hooper, 2009). Whereas commensal bacteria tend to retain to the luminal side of the epithelium, pathogenic bacteria possess strategies to cross the epithelial border. Based on this different behavior, PRRs and essential coreceptors are strategically localized in order to distinguish between both microbial groups (Fig. 1). Firstly, PRRs can be expressed on the apical and/or the basolateral side of IECs (= polarized expression). An example of such refined PRR localization is TLR5, which is solely expressed on the basolateral side (Gewirtz *et al.*, 2001). This allows the host to detect infection mediated by flagellated and enteropathogenic bacteria, only when these microorganisms have breached the epithelial border. Moreover, epithelial cells express low levels of TLR2, TLR4 and its aiding molecules MD-2 and CD14 (cluster of differentiation 14) on their apical side (Cario & Podolsky, 2000). As a result, the host avoids the unnecessary detection of commensal bacteria, numerously present on the apical side and accounting for a large amount of lipopolysaccharides in the gut lumen, which could otherwise result in hyper-responsiveness. A second aspect of strategic localization of PRRs involves the cytosolic detection of MAMPs based on the fact that pathogenic bacteria possess different mechanisms by which they can internalize their MAMPs in epithelial cells. In contrast to the ignorance of luminal lipopolysaccharides due to the absence of apical TLR4/MD-2/CD14, epithelial cells do express NOD1 in order to detect this MAMP in the cytosol, because cytosolic lipopolysaccharides is potentially derived from more threatening bacteria (Girardin *et al.*, 2001). A third feature is the appearance of PRRs on specialized cells occurring in well-defined parts of the intestinal tract. The epithelium of the small intestine and proximal colon harbors for example Paneth cells that produce AMPs such as defensins, lysozymes, regenerating islet-derived protein III (reg III) or polylactide microspheres that kill food- or water-borne pathogens, regulate the microbial community composition and maintain sterile conditions during cell production in the crypts (Ogura *et al.*, 2003; Ayabe *et al.*, 2004; Mukherjee *et al.*, 2008). In contrast to enterocytes and colonocytes, Paneth cells possess much more apical PRRs because of these specific functions.

Recent insights into the nature of the NALP/NLRP3 inflammasome reveal that there is more than strategic localization of PRRs in order to label potentially dangerous bacteria. NALP3 seems to serve as an activator of the inflammasome in response to danger signals. NALP3 inflammasome activation requires a first signal (such as lipopolysaccharides detection through transmembrane TLRs) to initiate activation and induce IL-1β production,

but also a second signal that leads to caspase-1 activation and further processing and secretion of the proinflammatory cytokine IL-1β. For this second signal, NALP3 does not just sense cytosolic MAMPs, but rather senses danger-associated molecular patterns such as pore-forming exotoxins, the type III secretion system used to inject virulence effectors in host cells (Kaparakis *et al.*, 2007; Ye & Ting, 2008), as well as danger signals released by damaged cells or tissues (uric acid crystal, elevated ATP) (Mariathasan *et al.*, 2006; Martinon *et al.*, 2006). Mutations in NALP3 result in human diseases such as familial cold autoinflammatory syndrome and Muckle–Wells syndrome (Ting *et al.*, 2006).

Another indication that PRRs could be involved in distinguishing between commensal and pathogenic bacteria follows from the correlation between Crohn's disease and NOD2, another member of the NLR family. Crohn's disease is characterized by hyper-responsiveness towards nonself, innocuous antigens and thus by a lack of properly distinguishing commensal from pathogenic bacteria. Interestingly, mutations of the intracytosolic PRR NOD2 on locations important for bacterial recognition (LRRs) strongly increase the vulnerability to this disease. This indicates the need for PRRs to inform the host about the nature of antigens (Ogura *et al.*, 2003; Cho, 2008). In addition, NOD1 potentiates several antimicrobial Th responses [T-helper cell type 1 (Th1), Th2 and Th17] (Fritz *et al.*, 2007), enhancing the possible role of NOD receptors to distinguish between innocuous and noninnocuous antigens.

Microbial transport through the epithelium and further processing by antigen-presenting cells (APCs) and immune cells

Besides initial sensing of luminal antigens through PRRs of IECs and DCs that extend beyond the epithelium, antigens can also cross the epithelial barrier, after or during which they are engulfed in APCs (B lymphocytes, macrophages and DCs). APCs engulf, process and present antigens in class I or class II major histocompatibility complexes (MHC class I or II) to adaptive immune cells in order to elicit proper immune responses (Fig. 1).

Bacteria can cross the epithelium through several pathways (Didierlaurent *et al.*, 2002). The epithelial cells that account for the largest contact surface with microorganisms are villus enterocytes and colonocytes. These epithelial cells can sample antigens through bulk uptake and receptor-mediated endocytosis. A second pathway to cross the epithelial barrier is through M-cell delivery (Neutra, 1998). M-cells are epithelial cells that efficiently deliver samples of foreign material by transepithelial transport from the lumen to organized lymphoid tissues within the mucosa. They are located in the follicle-associated epithelium overlying the gut-associated lymphoid tissue. Recently, villus M-cells have also been characterized (Niedergang & Kweon, 2005). A third mechanism is transport through DCs, which were shown to have the capacity to migrate between epithelial cells, while opening tight junctions and capturing antigens via their dendrites (Rescigno *et al.*, 2001). Upon sampling from the lumen or the mucus layer, DCs travel from the epithelium through the lamina propria towards mesenteric lymphoid nodes and the gut-associated lymphoid tissue (inductive sites), where they activate cells of the adaptive immune system. DCs are the only cells that can immediately bind with naive T-cells and influence their maturation [to Th1, Th2, Th17 or regulatory T-cells (Treg)] through cytokine release (Kapsenberg, 2003). Moreover, DCs provide antigens to B-cells (Qi *et al.*, 2006). In contrast to the local trafficking of sampled commensal microorganisms, DCs carrying pathogen antigens travel throughout the body and elicit systemic immune responses (Macpherson & Uhr, 2004).

When APCs encounter an exogenous antigen, they engulf it by endocytosis, process its proteins or polysaccharides with lysosomes and display the resulting short fragments at the surface within an MHC class II. These surface molecules can be recognized by $CD4^+$ T-cells (cluster of differentiation 4^+ T-cells) using their T-cell receptor in combination with CD4 (binds to the CD4 receptor of APC) (Busch *et al.*, 2000). When antigens are, however, produced inside the cell (e.g. viral proteins), degraded fragments are displayed in MHC class I, where they can be recognized by T-cell receptors in combination with its CD8 of $CD8^+$ T-cells (Pamer & Cresswell, 1998). Although $CD4^+$ T-cell epitopes are normally of exogenous origin and $CD8^+$ T-cell epitopes of endogenous origin, deviations may exist (Rammensee *et al.*, 1999). Lipids are also degraded and lipid fragments are presented to T-cells by cell surface molecules designated CD1 (Porcelli & Modlin, 1999). After these first steps of antigen presentation by APCs and initial binding of a T-cell with an APC, the APC needs to send a second and third signal in order for a T-cell to become active. Therefore, the APC can express a molecule called CD80/86 on its surface that binds to CD28 on the T-cell. The importance of initial PRR–MAMP sensing follows from the fact that CD80/86 is only expressed on the APC after the APC has detected a MAMP through PRR signaling (Dabbagh *et al.*, 2002). Finally, the third signal involves the secretion of cytokines by APCs, which directs the differentiation of T-cells into an effector T-cell subtype (Gutcher & Becher, 2007).

Different T-cell subtypes

As mentioned above, a first important subdivision between T-cells is that between $CD8^+$ and $CD4^+$ T-cells. $CD8^+$ T-cells are mostly cytotoxic and secrete molecules that destroy

the cell to which they have bound. $CD4^+$ T-cells can in turn be divided into several subsets, which each have distinct functions: different Th-cells (Th1, Th2 and Th17) and Treg. The first type of Th-cells (Th1) require T-bet as a transcription factor for differentiation (Szabo *et al.*, 2000; Glimcher, 2007) and Th1 bind with their T-cell receptors to DCs or macrophages, releasing lymphokines to attract other cells. This results in cell-mediated immunity and inflammation. In contrast, the key regulator of Th2 differentiation is GATA-3 (Zheng & Flavell, 1997), and Th2 interact with B-cells, resulting in antibody-mediated immunity. B-cells initially bind to soluble antigens with B-cell receptors. Being an APC, B-cells endocytose and digest these antigens in order to display them at their surface, where Th2 cells bind to these fragments with their T-cell receptors, followed by the secretion of lymphokines. This secretion by Th2-cells stimulates the development of B-cells into plasma cells that produce the soluble form of the B-cell receptors, i.e. specific antibodies against the antigen (Macpherson & Uhr, 2004). The Th1/Th2 balance is very important because this determines how the immune system will react against the antigens encountered. When the balance goes towards Th1, the killing efficiency of macrophages and proliferation to $CD8^+$ T-cells will be maximized. In contrast, when the balance goes towards Th2, a rather humoral, antibody-mediated immune response is induced that marks suspicious antigens rather than killing them. It has been postulated that too much Th1 may cause autoimmunity, while too much Th2 may cause asthma (Romagnani, 1992; Barnes, 2001). In addition to Th1 and Th2, Th17 are present in the lining of the gastrointestinal tract and they require RORγt as a transcription factor (Ivanov *et al.*, 2006). Th17 bind to DCs, secrete defensins and recruit scavenging cells to eliminate invaders through cell-mediated immunity and inflammation. They may play a major role in inflammation diseases (Ivanov *et al.*, 2008). Finally, Treg can be divided into Treg type 1 (Roncarolo & Levings, 2000) and $CD4^+CD25^+$ Tregs (Shevach, 2002). Importantly, Tregs are T-cells that have an immunosuppressive function and are crucial in the induction of peripheral tolerance to self and foreign antigens.

Normal health status: partitioning between mucosal and luminal microorganisms (Fig. 2)

Factors that affect microbial colonization of the mucus layer

Previous studies have shown the layered composition of the protective mucus overlying the epithelium along the entire length of the intestinal tract (Atuma *et al.*, 2001). While the inner mucus layer is very dense, firmly attached and hardly colonized, the outer mucus layer is less dense, loosely attached and more strongly colonized. Both layers have a similar composition, with Muc2 being the main constituent, which suggests that the loose layer is generated from the firm one. The concentration of several compounds can, however, vary between both the layers. This has been shown for the Muc2 concentration, which is approximately four times higher in the inner layer compared with the outer one (Johansson *et al.*, 2008).

It is shown that host defense molecules, produced at the epithelial surface, are trapped in the inner and outer mucus layer (Meyer-Hoffert *et al.*, 2008). Similar to that for Muc2, AMPs and IgA could reach a maximal concentration in the inner mucus layer, while being substantially diluted in the outer one. Interestingly, mucosal microorganisms are also confronted with an oxygen gradient as oxygen is continuously released from the blood towards the mucus layer, where partial pressures have been measured at ~30 mm Hg (Kirk, 1949; Bornside *et al.*, 1976). Because aerobic conditions seem to increase the killing efficiency of several AMPs (Nuding *et al.*, 2009), the killing efficiency of AMPs would be higher in the inner mucus layer. Recently, AMPs produced by Paneth cells have indeed been shown to serve as a tool for the host in selecting bacteria that approach the epithelium (Vaishnava *et al.*, 2008). Bacterial signals were sensed directly by Paneth cells through TLR activation, resulting in the expression of multiple antimicrobial factors (reg III γ, reg III β, CRP-ductin and RELMβ). Whereas the amount of luminal microorganisms was not affected by this AMP production, the amount of microorganisms in mesenteric lymphoid nodes and the spleen was strongly decreased for AMP-producing mice, showing that the expression of these AMPs is essential in controlling intestinal barrier penetration by both commensal and pathogenic bacteria (Vaishnava *et al.*, 2008).

On the other hand, microbial characteristics also influence the composition of the MAMC. Specific mechanisms for microbial adhesion to the mucus layer have been described and include extracellular mucus-binding (mub) proteins (Roos & Jonsson, 2002), proteins for mannose-specific binding (Pretzer *et al.*, 2005) and mucus-binding pili (Alander *et al.*, 1999; Kankainen *et al.*, 2009). Another important factor is the ability to gain nutrients from the host-derived mucins (Derrien *et al.*, 2004; Martens *et al.*, 2008; Ruas-Madiedo *et al.*, 2008; Harrington *et al.*, 2009). Survival in the presence of the oxygen gradient along the mucus layer further selects for specific mucosal microorganisms. Moreover, some abundant, generally nonpathogenic gut colonizers, such as the *Bacteroidetes*, have evolved resistance to several host AMPs (Nuding *et al.*, 2009). Therefore, these microbial characteristics, together with the concentration gradient of host defense molecules along the mucus layer, shape the mucosal microbiota, resulting in a

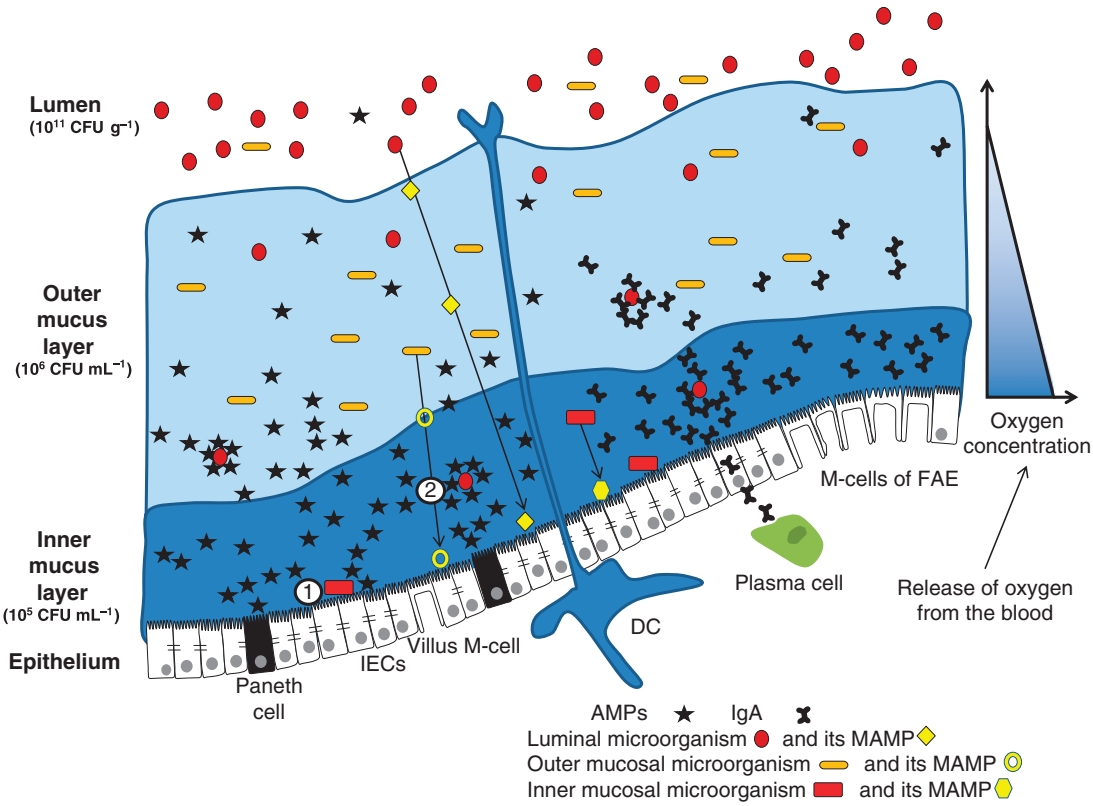

Fig. 2. *Hypothesis*. The harsh physical conditions along the intestinal tract together with the variety of specific defense mechanisms of innate and adaptive immunity allow the host to select its commensal microbiota. Mucus is secreted and forms a double protective layer: a very dense, firmly attached quite sterile inner mucus layer and a less dense, loosely attached, more strongly colonized outer mucus layer. The concentration of oxygen and secretion products can vary among both layers as shown for Muc2, which is approximately four times higher concentrated in the inner layer. It is assumed that secreted host defense molecules such as AMPs and antibodies such as IgA are trapped in the mucus layer and reach a maximal concentration in the inner part, while being substantially lower in the outer one. Along with distinct microbial characteristics such as specific adhesion mechanisms, mucin-degrading capacities, oxygen tolerance and AMP resistance, the selective pressure from the host side generates a unique mucosa-associated microbiota. Recent studies have shown that the microbial composition of the inner mucus layer differs from that in the outer layer and also from the one in the lumen. Several mucosal microorganisms are especially important as they fine-tune the immune system. A direct interaction would take place when these mucosal microorganisms reach the epithelium and make cell contact (1), while indirect interaction through the diffusion of MAMPs is also possible (2). Microorganisms that are targeted by host defense in the mucus layer are restricted to reside in the lumen, which is less controlled by the host. They make no direct contact with the epithelium, although their MAMPs might reach the epithelium after diffusion. The very outside of the mucus layer, where host defense molecules are quite diluted, would comprise an overlap between mucosal and luminal microbiota. Therefore, it might contain a backup of most microorganisms in a protected microenvironment, potentially serving as an inoculum to restore previously existing intestinal microbiota after disturbances (e.g. disruption during antibiotic treatment).

unique microbial association (MAMC) that closely interacts with the host at the host–microbial interface.

Unique composition of MAMC

While the composition of the human fecal microbiota has been revealed in considerable detail (Tap *et al.*, 2009; Claesson *et al.*, 2010; Qin *et al.*, 2010), sampling restrictions limit the current knowledge about the MAMC. An important issue is that goblet cells empty stored mucus after death, destroying the separation between the inner and the outer mucus layer. By analyzing the mucus layer as a whole, it was revealed that the dominant mucosal microorganisms are uniformly distributed along the intestinal tract and, importantly, that they differ substantially from fecal microorganisms (Swidsinski *et al.*, 2002; Zoetendal *et al.*, 2002; Eckburg *et al.*, 2005; Lepage *et al.*, 2005).

Johansson *et al.* (2008, 2010) reported previously that the inner mucus layer would be devoid of bacteria. They used FISH, but without lysozyme treatment, impairing the detection of Gram-positive bacteria. Recently, fascinating results were generated by separately sampling the inner and the outer mucus layer from living mice (Schreiber, 2010). It was shown that the inner mucus layer is colonized with approximately 5 log CFU anaerobes mL^{-1} mucus, while the outer mucus layer contains 10 times more bacteria. Interestingly,

terminal restriction fragment length polymorphism analysis revealed that the inner and outer mucus layer not only differ in numbers, but that they also have a completely different microbial composition. This shows that the microbiota of the inner mucus layer is very different from that in the outer, loosely adherent mucus layer and obviously also from the one in the luminal content and feces.

Mucosal microorganisms and immune system regulation

The normal mucosal microbiota protects the host tissues from invading microorganisms by locally producing organic acids that lower the pH (Bomba *et al.*, 1996), excreting natural antimicrobial compounds (Collado *et al.*, 2005) or competing with pathogens for nutrients (Hooper *et al.*, 1999) and adhesion sites (Bernet *et al.*, 1994; Hooper *et al.*, 1999). Because mucosal microorganisms reside very close to the epithelium compared with microorganisms that are targeted by host defense and restricted to the lumen, they also have a stronger potential to interact with the epithelium and modulate the immune system, directly (through cell contact) or indirectly (through diffusion of MAMPs or metabolites). In this way, mucosal microorganisms can balance host defense mechanisms so that foreign antigens are properly dealt with: commensals are tolerated and pathogens are cleared.

An immune-calming mechanism was described recently for *Lactobacillus acidophilus* NCFM. This bacterium can bind with a surface protein (SlpA) to host receptors of DCs [DC-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN)], stimulating anti-inflammatory IL-10 production. Moreover, DCs mature rather to Th2 that mark suspicious antigens instead of Th1 that eliminate these suspicious antigens. *Lactobacillus acidophilus* NCFM thus ensures that there is less inflammation and a more bacteria-friendly environment (Konstantinov *et al.*, 2008). It is also demonstrated that *Lactobacillus rhamnosus* GG and *L. acidophilus* strain LB protect the epithelial tight junctions when they are loosening due to external stress (Montalto *et al.*, 2004; Seth *et al.*, 2008). Loosening tight junctions might lead to the infiltration of MAMPs across the epithelium, disrupting the MAMP detection that is based on the strategic localization of PRRs. For example, the vast amount of commensal lipopolysaccharides is normally ignored due to the lack of apical TLR4 on epithelial cells. However, weaker tight junctions resulting in the infiltration of lipopolysaccharides lead to basolateral detection of this MAMP, ultimately alarming the host (Cani *et al.*, 2007, 2009). Further, it was shown that a mix of *Lactobacillus reuteri* strains is able to reach the epithelium and prevent inflammation and translocation in DSS-treated mice. It was proposed that this might be due to a strengthening of tight junctions or an increased expression of membrane-bound mucins (MUC3) (Schreiber, 2010).

Recently, segmented filamentous bacteria (SFB) were also identified as crucial microorganisms in shaping host immune responses in rodents (Gaboriau-Routhiau *et al.*, 2009; Ivanov *et al.*, 2009). SFB were shown to simultaneously stimulate several immune responses that complete and balance each other. The introduction of SFB induced a proinflammatory gene response and stimulated the production of Th17 cells in mice that were otherwise Th17 deficient. This SFB-induced immune response protected mice from infection with *Citrobacter rodentium*. A striking feature of SFB is their capacity to adhere, especially in the ileum (Ivanov *et al.*, 2009). Even if a direct extrapolation of the importance of SFB in rodents to humans is questionable, these results demonstrate that a single member of the mucosal microbiota can play a major role in setting up the intestinal mucosal defense.

Another example of a putative immune regulator at the host–microbiota interface is *Faecalibacterium prausnitzii*. A study with monozygotic twins, discordant for Crohn's disease (one is sick, one is healthy), revealed that *F. prausnitzii* was consistently and abundantly present in the MAMC of healthy individuals as opposed to ileal Crohn's disease patients (Willing *et al.*, 2009), a result confirmed by others (Margarita *et al.*, 2006; Vasquez *et al.*, 2007). Moreover, lower proportions of this bacterium in the MAMC were correlated with a higher risk of postoperative recurrence of ileal Crohn's disease (Sokol *et al.*, 2008). Both *in vivo* and *in vitro* studies revealed the anti-inflammatory effects of *F. prausnitzii* (Sokol *et al.*, 2008). As this bacterium is a well-known butyrate producer (Duncan *et al.*, 2002), the beneficial effects may be attributed to this metabolite (Scheppach & Weiler, 2004). Especially when present in the MAMC, *F. prausnitzii* would be able to produce butyrate very close to the epithelium, which is of distinct benefit to the host.

Recently, it was demonstrated that polysaccharide A (PSA) of *Bacteroides fragilis* could serve as an archetypal molecule that mediates the maturation of the intestinal immune system. Germ-free animals have lower amounts of $CD4^+$ T-cells, suggesting that bacteria are involved in the development of these cells. Moreover, germ-free animals develop an immune system skewed towards Th2, suggesting that Th2 and thus antibody-mediated immunity may be a default pathway. PSA of *B. fragilis* was shown to be sufficient for the development of the Th1 cell-mediated arm of the immune system. PSA is taken up by APCs, which process and present PSA to T-cells, thus directing the maturation of T-cells to Th1 (Mazmanian *et al.*, 2005). Further, it was shown in a model of experimental colitis that *B. fragilis* could only protect animals from disease when *B. fragilis* was able to produce PSA. PSA-induced $CD4^+$ T-cells to express IL-10, which suppresses inflammation and offers protection from inflammatory bowel disease (IBD) (Mazmanian *et al.*,

2008). Microorganisms such as *B. fragilis* might thus play an important role in fine tuning the Th1/Th2 balance.

Another immune-calming mechanism is the induction of intestinal alkaline phosphatase. Lipopolysaccharides of certain indigenous Gram-negative bacteria is necessary and sufficient to upregulate intestinal alkaline phosphatase activity, which is probably important in detoxifying the endotoxin component of lipopolysaccharides by dephosphorylation. This prevents excessive intestinal neutrophil infiltration and inhibits inflammatory responses to the microbiota (Bates *et al.*, 2007).

Besides calming down the immune system, specific species can also induce strong antimicrobial responses if necessary. *Bacteroides thetaiotaomicron* can, for example, induce AMPs of the innate immune system to create an environment buffered against inflammatory perturbations by fortifying the gut epithelium and promoting the establishment of nonpathogenic microbiota (Stappenbeck *et al.*, 2002; Hooper *et al.*, 2003). In addition, it has been shown that as yet identified commensals upregulate AMP expression when pathogenic *Salmonella typhimurium* enters the MAMC (Vaishnava *et al.*, 2008). Interestingly, Vaishnava *et al.*, (2008) found that pathogenic bacteria themselves did not trigger enhanced AMP expression, but that commensal bacteria signaled the host that pathogens were present through an MyD88-dependent pathway in the host epithelium, resulting in upregulated AMP expression and less *S. typhimurium* penetration through the epithelium.

Luminal microorganisms and nutrient digestion

Compared with mucosal microorganisms, microorganisms restricted to the lumen can only interact indirectly with the host through the diffusion of their MAMPs and metabolites over a relatively long distance. As a result of the high luminal substrate availability, luminal microorganisms are less subjected to mass transfer limitations for microbial growth. They possess a large metabolic degradation capacity to convert substrates that would otherwise be indigestible and leave the host as such (Hooper *et al.*, 2002; Flint *et al.*, 2008). Compared with the microbial community (Flint *et al.*, 2008), the host proteome has a limited repertoire of glycoside hydrolases needed to digest complex polysaccharides. For example, the single species *B. thetaiotaomicron* possesses 208 paralogs of two outer-membrane proteins that bind and import starch (Martens *et al.*, 2009), 256 predicted glycoside hydrolases and 16 polysaccharide lyases, while the human genome only contains 97 glycoside hydrolases (http://www.cazy.org). Such members of the microbial community thus process complex dietary polysaccharides to short-chain fatty acids (SCFAs). Acetate, propionate and butyrate confer both energy and health-promoting effects to the host (Macfarlane & Macfarlane, 2003). Other important bacterial products are essential vitamins, amino acids and several bioactive compounds.

Recently, approaches have been developed to correlate the gut microbiota and the host metabolic phenotype in order to define key microorganisms that influence host metabolism and hence host health. It was shown that (fecal) *F. prausnitzii* is associated with the modulation of at least eight urinary metabolites of diverse structures (glycolate, glycine, 2-hydroxyisobutyrate, lactate, dimethylamine, 3,5-hydroxybenzoate, taurine and 3-aminoisobutyrate), indicating that this single member is already able to influence numerous host pathways. The microbial community as a whole thus has a major impact on the metabolic phenotype of its host (Li *et al.*, 2008). This metabolic phenotype is very important with regard to human health as investigations across and within four human populations (United Kingdom, United States, China and Japan) revealed that metabolic phenotypes are clustered according to geography, but interestingly also according to BMI, hearth stroke rate and diabetes, with almost every discriminatory metabolite being of microbial origin (Holmes *et al.*, 2008). Other studies have shown that the gut microbiota modulates absorption, storage and energy harvest from the diet at the systems level and thus impacts metabolite concentrations in tissues of all organs, resulting in a tremendous impact on host health (Martin *et al.*, 2007).

To sense and monitor these luminal commensals and to know what they are doing in terms of metabolite production, the host utilizes dendrites that extend in the chyme (Niess *et al.*, 2005) and several metabolite sensors (Le Poul *et al.*, 2003). Among these sensors, two G protein-coupled receptors [G protein-coupled receptor 41 (Gpr41) and Gpr43] have been demonstrated to bind with SCFA. They are expressed in intestinal enteroendocrine cells of the distal small intestine, colon and adipocytes. After binding with SCFAs, feedback mechanisms are initiated that impact leptin expression, a polypeptide hormone with pleiotropic effects on appetite and energy metabolism. This was shown in a study with Gpr41 $-/-$ and $+/+$ mice, cocolonized with *B. thetaiotaomicron* and *M. smithii*. Whereas SCFAs were produced in all mice, increased adiposity and *de novo* lipid production was only observed in $+/+$ mice. Also in conventional mice, Gpr41 $-/-$ animals were not able to sense and subsequently use the SCFAs. Such receptors thus transduce information about key microbial activities that impact host physiology (Samuel *et al.*, 2008).

Coevolution in the gastrointestinal tract

Coevolution between the host and the intestinal microorganisms

Coevolution between the host and the intestinal microorganisms is defined as a reciprocal adaptation. For example,

genetic changes that increase the production of a microbial metabolite may trigger the selection of changes in the host genome that promote uptake rather than the synthesis of that metabolite (Zaneveld *et al.*, 2008). The metabolic function of the luminal intestinal microorganisms is so beneficial that it is assumed to be the main evolutionary driving force to include microorganisms in the gastrointestinal tract and to acquire carefully designed defense mechanisms (McFall-Ngai, 2007; Mukherjee *et al.*, 2008). A host that allows every single microorganism in his gut would indeed risk severe microbial infections. On the other hand, if a host is too restrictive towards microbiota, he/she will not be able to benefit from their advantages and risk autoimmunity. Therefore, coevolution probably led to a steady state of a competitive host with a well-functioning mucosal barrier, fine-tuned by specific mucosal microorganisms so that the host is very vigilant to exclude potentially dangerous microorganisms, although still tolerant enough to grasp benefits from the luminal nutrient fermentation. Blaser introduced the concept in which coevolved host–microorganism systems such as humans and the intestinal microbiota have developed cross-signaling that allows homeostasis to conform to evolutionary stable strategies (ESS) (Blaser & Kirschner, 2007). This hypothesis is based on a game theory: an ESS is a subset of the Nash equilibrium, which is a strategy profile in a game with ≥ 2 players in which none of them can win by changing strategy unilaterally (Nash, 1951). Players who do change strategy unilaterally are defined as cheaters and penalties have evolved to lower their fitness. When the abundance of a cheater reaches a threshold, this may trigger new innate or adaptive immune responses (Blaser & Kirschner, 2007).

Model system for host–microbiota interaction: *Vibrio fischeri–Euprymna scolopes*

The mutualistic symbiosis between *V. fischeri* and the squid *E. scolopes* provides a model system for the examination of mechanisms by which bacteria–host communication and ESS development occur (McFall-Ngai & Ruby, 1991). The squid is bacteria-free at hatching, rapidly acquires *V. fischeri* and promotes its growth in a special symbiotic light organ. One *V. fischeri* gene, encoding the two-compartment sensor kinase RscS, is particularly important for host specificity. RscS activates the production of exopolysaccharides that mediate bacterial aggregation during initial infection (Mandel *et al.*, 2009). Reciprocal benefits are the driving force for the ESS: in exchange for nutrients and a safe niche, *V. fischeri* provides bioluminescence, which is used by *E. scolopes* to camouflage itself from predators (eliminate the shadow of the moon). Interestingly, *V. fischeri* mutants defective in light production are unable to persist at wild-type levels and, moreover, they are outcompeted by the wild-type *V. fischeri* (Visick *et al.*, 2000). Even though closely related *E. scolopes* can become infected by wild-type *V. fischeri* strains isolated from closely related squid species, the non-native strains are outcompeted when the animals are exposed to their native strain (Nishiguchi *et al.*, 1998). The squid thus selects specific coevolved microorganisms.

Vertebrates need their coevolved intestinal microbiota

Compared with the squid, vertebrate models such as mice and zebrafish are more closely mimicking the physiology of the human host. Studies with these vertebrates provide an insight into the need for coevolved microbial communities. Both mice and zebrafish possess conserved responses to their coevolved microbiota. Upon conventionalization, mice and zebrafish reveal similarities in transcriptional responses to microbiota, with changed expression of genes involved in cell proliferation, nutrient utilization and immune function (Rawls *et al.*, 2006). Microbial colonization induces, in both cases, changes in gut epithelial homeostasis (change in cell proliferation and the relative number of secretory cells) and maturation of the gut epithelium (shifts in surface-expressed glycans and increase of gut-associated immune cells) (Cheesman & Guillemin, 2007). Moreover, inoculation of mice and zebrafish with the complex coevolved microbiota of one another leads to parallels in gene expression regarding nutrient metabolism, showing that the vertebrate gut is flexible in handling microbiota. However, less induction of cell proliferation and innate immune genes in the new hosts illustrates that vertebrates such as humans may have coevolved with a certain microbial consortium that is necessary for specific functions such as cell proliferation and immune instruction.

In order to elucidate such host-specific lineages within the intestinal microbiota, Ley *et al.* (2008) performed whole microbiome 16S rRNA gene surveys of 60 mammalian hosts. While host phylogeny and microbial community composition were clearly correlated, the existence of a coevolution was obscured by effects of host-related diet and environmental conditions. Moreover, 16S rRNA gene approaches offer insufficient genetic resolution to fully grasp the diversity and evolutionary history of the gut microbiota (Koeppel *et al.*, 2008). Therefore, Oh *et al.* (2010) investigated the genomic content of 165 *L. reuteri* strains, isolated from different vertebrate hosts (human, mouse, rat, pig, chicken and turkey), as the shared ancestry of the genomic content of these *L. reuteri* strains offers much higher-resolution comparisons. Oh and colleagues demonstrated highly diverged, host-specific subpopulations of *L. reuteri*. Similar to that for the squid, this coevolution also increased the persistence of the coevolved strain because when a mix of *L. reuteri* strains was supplied to mice, only coevolved strains

of mice and rats were able to maintain at high levels (Oh *et al.*, 2010). A similar clustering according to the host genotype was obtained for several *Lactobacillus johnsonii* strains (Danin-Poleg *et al.*, 2010). Interestingly, *L. johnsonii* specifically colonizes C57BL mice as opposed to BALB/C mice. By making reciprocal crosses between both mouse lines, it was concluded that the presence of *L. johnsonii* was determined by host genotype (Buhnik *et al.*, 2010). Another example that highlights the phylogenetic link between the host and the intestinal microorganisms is the occurrence of methanogens in vertebrates, which obeys 'Dollo's rule': if methanogens are lost in the course of evolution, they do not appear in any of the descendants of the common ancestor that lost them (Hackstein & Stumm, 1994; Hackstein & van Alen, 1996). Recently, host genetic control of the microbiota in mice was demonstrated as several quantitative trait loci were shown to control the presence of specific microbial species, groups of related taxa or even distantly related organisms (Benson *et al.*, 2010). These observations of *L. reuteri*, *L. johnsonii* and methanogens confirm that at least some gut microorganisms have diversified into host-adapted (including human-adapted) lineages by a long-term evolutionary process.

Coevolution within the intestinal microbiota as such

We hypothesize that besides between the host and the intestinal microorganisms, coevolution is also prominent within the intestinal microbial community as such. Most microorganisms are, due to the limitations of their genomes, indeed bound to be organized in coevolved associations and behave as a multicellular organism (Shapiro, 1998). We hypothesize that when microorganisms cooperate for a long time in a certain spatial and functional configuration, they will coevolve so that a driving force is developed, in order to maintain the composition and function of the microbial association. Functional redundancy would apply, meaning that subdominant microorganisms can perform similar functions. These subdominant microorganisms may be crucial for preserving community functionality during disturbances. Yet, after such a disturbance, they are repressed by more efficient coevolved microorganisms, resulting in a resilient microbial community. This might explain the remarkable temporal stability of the intestinal microbiota (Zoetendal *et al.*, 1998; Claesson *et al.*, 2010).

Interestingly, the intestinal microbial community reveals the existence of resilient associations, occupying specific niches within the complex community. The microbial bioactivation of lignans, a type of phytoestrogens, is a first example of such a resilient intestinal microbial consortium. Bioactivation involves the conversion of lignans to enterolignans (enterodiol and enterolactone), which would be more potent in decreasing colon and breast cancer risk and that may even influence prostate cancer risk (Adlercreutz, 2007). Bacteria are shown to be crucial in catalyzing four sequential reactions needed to convert lignans in enterolignans: *O*-deglycosylation, *O*-demethylation, dehydrogenation and dehydroxylation. Moreover, depending on the type of lignan, bacteria also assist in additional reduction steps. Clavel *et al.* (2006) provided an overview of the bacteria that catalyze these different reaction steps. *Bacteroides* and *Clostridium* species are involved in *O*-deglycosylation, *Ruminococcus productus* in *O*-demethylation, *Eggerthella lenta* in dehydroxylation and *Lactonifactor longoviformis* in dehydrogenation. These microbial partners are phylogenetically and functionally distantly related, but interact very closely and are complementary to one another.

The fermentation of indigestible polysaccharides by colonic microorganisms to SCFAs such as acetate, propionate and butyrate (Macfarlane & Macfarlane, 2003; Louis *et al.*, 2007) is a second example of such resilient microbial associations. The microorganisms involved are also phylogenetically distantly related and form a cross-feeding web to ferment these substrates. First of all, a very diverse spectrum of polysaccharides is entering the colon: plant cell storage glycans (e.g. starch and fructans), plant cell wall glycans (e.g. cellulose, hemicelluloses and pectin), salivary and colonic mucin *O*- and *N*-linked glycans and glycosaminoglycans produced along the intestinal surface. While early surveys revealed that the initial degradation of such complex polysaccharides is mediated by versatile microorganisms mostly belonging to the *Bacteroidetes* (Salyers *et al.*, 1977a), and to a lesser extent also to the *Actinobacteria*, *Proteobacteria* and *Firmicutes* (Salyers *et al.*, 1977b), these studies also revealed that the degradation of specific substrates was often species and even strain dependent, which indicates that numerous microorganisms are needed to digest this wide range of polysaccharides. Mechanistic research with *B. thetaiotaomicron* revealed the existence of a cell envelope-associated multiprotein system, called the starch utilization system (Sus), whose functioning was reviewed recently by Martens *et al.* (2009). This and many derivate systems are highly represented in the genome of *Bacteroidetes* members and each degrade particular glycans, confirming that this phylum is crucial in polysaccharide fermentation. Interestingly, by combining less versatile microorganisms that depend on one another to produce certain SCFAs, complex cell–cell communication and cross-feeding between specific gut microorganisms has been demonstrated (Fig. 3) (Duncan *et al.*, 2004; Belenguer *et al.*, 2006; Falony *et al.*, 2006; Kovatcheva-Datchary *et al.*, 2009). While bifidobacteria and lactobacilli are often potent acetate and/or lactate producers, they are unable to produce propionate or

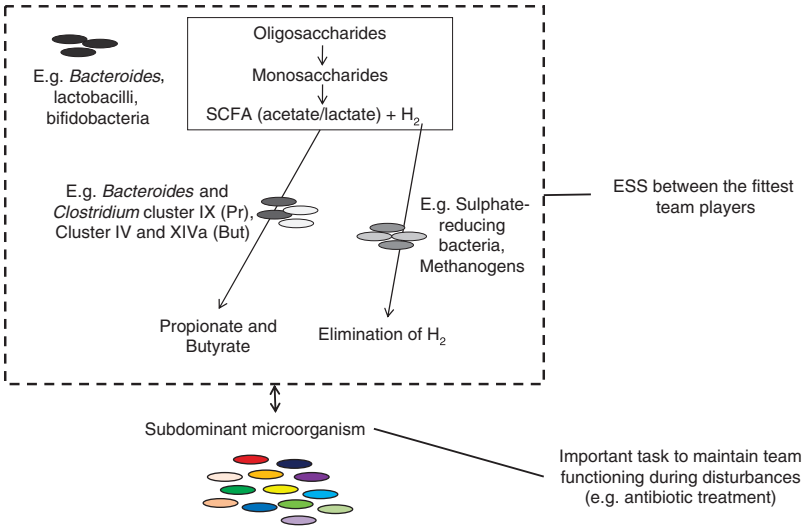

Fig. 3. *Hypothesis*. When bacteria cooperate for a long time in a certain spatial and functional configuration, they coevolve so that a collective recognition is developed that is a driving factor to restore the association after perturbations. In other words, an ESS has evolved within the microbial community. The intestinal microbial community, which as a whole performs specific tasks, may be defined as a resilient partnership. An example of such a specific task is the degradation of polysaccharides through a complex food chain of several phylogenetically distantly related microorganisms. The initial degradation is often mediated by versatile microorganisms mostly belonging to the *Bacteroides*. Members of the *Clostridium* cluster IX group and *Bacteroides* are able to generate propionate, while microorganisms of the *Clostridium* cluster IV and cluster XIVa are among the dominant butyrate producers. The assistance of other microorganisms is needed to prevent the accumulation of the generated waste products such as H_2, which is eliminated by nitrate- and sulfate-reducing bacteria, methanogenic or acetogenic microorganisms. Microorganisms that cooperate for a long time in such syntrophic partnerships may evolve and become very complementary. Because of a severe disturbance such as an antibiotic treatment, these associations can be perturbed. Functional redundancy, however, applies to the community, meaning that subdominant microorganisms can perform similar functions in order to maintain the community functioning. However, after the perturbation, there is a driving force to restore the initial microbial community.

butyrate. Other microorganisms are shown to convert acetate, lactate and/or partially degraded carbohydrates to propionate and butyrate. Using distinct pathways, *Bacteroides* species (the succinate pathway or randomizing pathway) and members of the *Clostridium* cluster IX group (acrylate pathway) are able to generate propionate (Walker *et al*., 2005), while microorganisms of the *Clostridium* cluster IV (related to *F. prausnitzii*) and cluster XIVa (related to *Eubacterium rectale* and *Roseburia intestinalis*) are among the dominant butyrate producers (Pryde *et al*., 2002; Louis & Flint, 2009). As these fermentation pathways form waste products, the assistance of other microorganisms is needed to prevent the accumulation of these waste products. The excess H_2 is, for example, eliminated by nitrate- and sulfate-reducing bacteria, but also by methanogenic and acetogenic microorganisms in order to prevent H_2 accumulation and the resulting fermentation inhibition (Macfarlane & Macfarlane, 2003). These insights into the complex microbial food chain of polysaccharide fermentation reveal the existence of intimate cooperation between specific microbial groups within the vast intestinal microbial community.

The intestinal microbiota may thus be defined as a resilient association. We believe that once such coevolved microbial associations are established, they will continue to dominate their joint niche as the different members cooperate so well and are probably spatially and functionally structured. A recent in-depth study with pyrosequencing indeed shows the close relationship between members of the intestinal microbial community (Dethlefsen *et al*., 2008). An antibiotic treatment with ciprofloxacin influenced one third of the bacterial taxa and the largest part of the disturbances was attributed to the impact on cross-feeding between different species. The drastic change in the composition did not influence the intestinal function as assessed subjectively by the human participants, which supports the hypothesis of functional redundancy. Finally, 4 weeks post-treatment, the microbial communities returned to their initial composition, indicating that previously subdominant microorganisms occupied a niche during the perturbation, but were replaced afterwards. Other studies where intestinal microbial communities were subjected to severe perturbations such as an antibiotic treatment (De La Cochetiere *et al*., 2005) or chemotherapy (van Vliet *et al*., 2009) confirmed that several species are no longer detected in feces during the treatment while most of them reappear after treatment. This shows that within a timeframe of a few weeks, the intestinal

microbial community recovers to a similar composition as before the disturbance, a composition specific for the individual subject.

What factors cause and maintain the ESS within the microbial community?

It will be very interesting to unravel the factors that are responsible for the temporal stability of the intestinal microbiota (Zoetendal *et al.*, 2002; Claesson *et al.*, 2010). These factors probably include selective pressures such as the diet, which can force the community in a certain direction. It has been shown that by continuously eating a polysaccharide-rich diet, humans coevolved with a specific microbiota. Recently, the drastically changed diet resulted in an altered intestinal microbial composition, which deviates from our coevolved community (De Filippo *et al.*, 2010). Another important environmental factor is early life colonization. Because of functional redundancy among microbial species, several microorganisms can potentially occupy a specific niche within the microbial community. The first microorganisms that arrive in the intestine have the first possibility to fill these niches and establish an ESS with other microorganisms, thus resulting in a stable microbial community. It has been shown that early life colonization can have a life-long impact on the intestinal microbial community composition (Mulder *et al.*, 2009). Moreover, each host imposes specific conditions on the microbial community regarding pH, temperature, secretions and hydraulic parameters. These parameters can differ substantially among vertebrates (Karasov & Martinez del Rio, 2007), but also among individuals within a certain vertebrate group. Further, these parameters may change during aging of the host. Other important factors influencing the ESS within a microbial community can be sought in typical microbial characteristics such as metabolic (growth rate, substrate affinity) or signaling (quorum sensing) parameters.

Finally, the MAMC of the outer mucus layer might serves as a backup community. As the outside of the mucus layer is characterized by lower concentrations of host defense molecules, it could comprise an overlap between microorganisms of the inner mucus layer and the lumen. It has been shown that antibiotics are up to 1000 times less efficient in combating biofilm-forming microorganisms compared with planktonic ones (Stewart & Costerton, 2001), meaning that the outer mucus layer might be an environment that is buffered and protected from factors that disturb the luminal microorganisms, and might have the important function to retain a backup of coevolved microorganisms. After perturbations, this community could serve as an inoculum to redevelop the previously existing communities.

Disease status: when commensal bacteria become renegade

Introduction

Numerous diseases are correlated with often pathogenic intestinal microbiota and it is of great interest to unravel this interconnection (Lederberg, 2000; Frank *et al.*, 2007; Stecher & Hardt, 2008). It is often questioned whether changes in the microbial community are the cause or rather the consequence of health problems. According to our hypothesis, a healthy host is able to control its microbiota. With several examples, we show that commensal bacteria can become renegade, meaning that they persistently maintain particular health problems. The term renegade refers to specific microorganisms with altered behavior (renegade microorganisms), but can also refer to a microbial community that is unbalanced as a whole (renegade microbial community).

Helicobacter pylori

Blaser *et al.* (2008) extensively reviewed how *H. pylori* can affect human physiology. During the first part of a human life, this bacterium confers benefits to the host by instructing Treg cells, preventing diarrheal illnesses, gastroesophageal reflux disease, asthma, energy imbalances and esophageal adenocarcinoma. However, after many years of reproduction and transmission, *H. pylori* becomes a renegade opportunistic pathogen and may cause gastric ulceration and gastric adenocarcinoma. *Helicobacter pylori* could therefore be seen as an example of how the host becomes betrayed by one of the indigenous commensals.

IBD

It is debated whether IBD is caused by deficient host immunity, by harmful intestinal bacteria or by environmental factors (Sartor, 2006). Recently, Garrett *et al.* (2007) used T-bet-deficient mice to show that, besides immune deficiencies, commensal microbiota can cause colitis. As mentioned earlier, T-bet is a key transcription factor for Th1 differentiation (Szabo *et al.*, 2000; Glimcher, 2007). T-bet-deficient mice with no T- or B-cells (TRUC mice) develop a disease that remarkably resembles human ulcerative colitis. Interestingly, transfer of the microbiota of these sick mice to healthy wild-type mice caused similar disease patterns in the latter. Investigation of the causative microbiota showed that they do not belong to known groups of disease-causing agents, but that they are members of the commensal microbiota. The microbial community as a whole was unbalanced and assumed to cause colitis, even in genetically intact (T-bet sufficient), healthy hosts (Garrett *et al.*, 2007). More detailed characterization revealed that *Klebsiella*

pneumoniae and *Proteus mirabilis* correlated with colitis in TRUC mice, they were involved in maternal transmission of the colitis and interestingly they also required the endogenous microbiota for maximal inflammation (Garrett *et al.*, 2010). Mechanistic research revealed that in DCs, T-bet binds to the promoter region of the tumor necrosis factor-α (TNF-α) gene and suppresses the production of this inflammatory cytokine (Fig. 4). In the absence of T-bet, there is no such suppression resulting in excessive TNF-α production and injuring the colonic epithelial lining. This new colonic environment transforms the commensal microbiota into an unbalanced, renegade community that inflames the host and causes disease even in genetically intact, healthy hosts (Garrett *et al.*, 2007).

SFB were recently identified as a group of beneficial commensals residing in the mucus layer, as mentioned in the previous chapter (Gaboriau-Routhiau *et al.*, 2009; Ivanov *et al.*, 2009). Interestingly, Gaboriau-Routhiau and colleagues stated that these SFB might, however, become pathogenic ('renegade') in genetically predisposed hosts depending on the local bacterial environment and participate in IBDs such as Crohn's disease. SFB stimulate the production of Th17 cells that produce cytokines that can be highly protective in the case of infection, but at the same time, in the wrong context or in the wrong amount, they can lead to disease. SFB may thus skew the balance of the immune system towards the development of inflammatory, autoimmune disease, psoriasis and even arthritis.

Additionally, disturbance of the interaction with mucosal microorganisms could lead to or maintain disease states such as IBD. It is for example observed in active IBD patients that the mucus layer becomes thinner and more discontinuous (Strugala *et al.*, 2008). It is suggested that in such cases, the host is less able to exclude bacteria from the mucus layer that are normally only present in the lumen (Swidsinski *et al.*, 2002). In contrast, there will be increased interaction

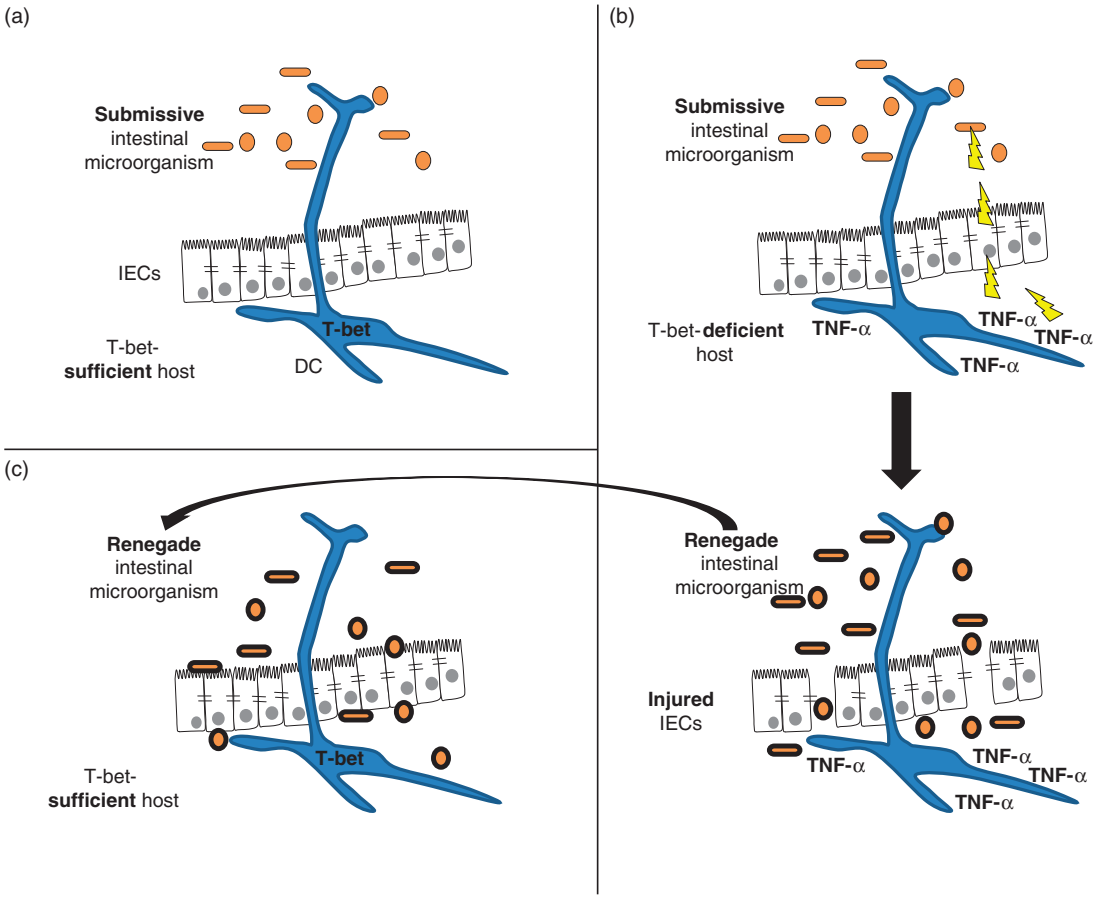

Fig. 4. (a) The transcription factor T-bet, expressed in DCs that sample bacteria, suppresses the production of the inflammatory cytokine TNF-α by binding to the TNF-α promoter region. (b) However, in T-bet-deficient mice with no T- or B- cells (TRUC mice), no such repression occurs and DCs respond to bacteria by releasing excessive amounts of TNF-α. As a result, the IECs are injured and the submissive intestinal bacteria are transformed (into 'renegade bacteria') and maintain inflammation of the epithelium. (c) Moreover, when these renegade bacteria are transferred to a T-bet-sufficient, healthy host, they persistently cause inflammation (based on Garrett *et al.*, 2007).

with microorganisms that were previously restricted to the lumen. This is additional evidence that the MAMC may be considered as a selection of specific microorganisms that are crucial for the host to maintain a normal health status.

Renegade microorganisms can cause obesity

Besides IBD, obesity has been recognized as an important growing health problem in our society (Mokdad *et al.*, 2001). Obesity has been found to coincide with decreased gut barrier function and elevated lipopolysaccharides levels in the blood (Cani *et al.*, 2007). This endotoxemic effect was decreased by improving the gut barrier function through the modulation of the gut microbiota with inulin (Cani *et al.*, 2009). Several other studies also link obesity to the intestinal microbiota, more specifically to a microbiota that consists of more *Firmicutes* and less *Bacteroidetes* (Ley *et al.*, 2006; Turnbaugh *et al.*, 2006, 2008). However, the final relevance of this change is unclear, as other research groups observed that obesity coincided with less *Firmicutes* and more *Bacteroidetes* (Duncan *et al.*, 2007, 2008; Schwiertz *et al.*, 2010). Interestingly, Turnbaugh *et al.* (2006) demonstrated an increased body weight and fat adiposity in germ-free mice after receiving microbiota from genetically obese mice compared with microbiota of genetic lean mice. However, as the experiments used abnormally lean germ-free mice as a baseline to report increased adiposity upon inoculation with microbiota from different donors, neither group of transplant mice exhibited obese adiposity levels. Transplanting an obese microbiota into a germ-free mouse indeed only increases its adiposity level back to a normal level. Finally, the only available time-course data (Ley *et al.*, 2006) suggest that diet is the main driving force for adiposity and probably also effects on the microbiota structure.

Health problems of the 20th and 21st century

Human health problems such as inflammatory diseases and allergies have been increasing since the 20th century. The hygiene hypothesis was a first attempt to explain this trend. It states that diminished exposure to parasites and pathogens early in life might be responsible for increased allergic and autoimmune disorders in later years (Strachan, 1989; von Hertzen, 2000). This decreased exposure is caused by improved vaccines, the use of antibiotics and cleaner water. However, the concept of childhood infections is now considered too narrow. Most of the human childhood viruses were only picked up from animals during husbandry (10 000 years ago) so that endemicity is very recent. Also, nonviral childhood infections are sporadic, therefore making it very unlikely that they have the role of delivering essential genes (Armelagos & Harper, 2005). It is now believed that the environmental changes mentioned together with replacement of breast milk by formula milk, the modern western diet, cesarian sections and demographic developments (e.g. trend towards smaller families) have also had an impact on the composition of our normal commensal microbiota (Bjorksten *et al.*, 1999; Blaser, 2006; Dethlefsen *et al.*, 2006; De Filippo *et al.*, 2010). As mentioned previously, specific commensals are shown to fine-tune the immune system and act as peacekeepers that prevent inflammatory disease including *Lactobacillus* spp., SFB, *F. prausnitzii*, *B. fragilis* and *B. thetaiotaomicron*. Disappearance or decreased appearance of childhood infections and normal mucosal commensal microorganisms can cause health problems, often explained by Th cell maturation to Th1/Th2 (Biedermann & Rocken, 1999; Infante-Duarte & Kamradt, 1999; von Hertzen, 2000) or Th17 (Ivanov *et al.*, 2008). In addition to these commensals, other 'old friends' have been identified. They include pseudocommensals, harmless organisms associated with mud, untreated water and fermenting vegetable matter such as environmental saprophytic mycobacteria and lactobacilli that were always present in food and water throughout mammalian evolution. A second group of 'old friends' is formed by helminthes, parasites that need to be tolerated by the host because any effort to eliminate them is likely to make the situation worse. These 'old friends' influence the maturation of DCs, leading to Treg rather than Th1 or Th2 effector cells, resulting in Treg cells for the 'old friends,' but also self-antigens, gut content antigens and allergens. This, respectively, results in bystander suppression of other responses, downregulated autoimmunity, IBDs and allergies (Rook, 2007, 2009). Overall, throughout mammalian evolution, we were continuously exposed to a specific coevolved microbial community. Changes in human ecology resulted in changes in the microorganisms that populate our bodies. This different microbial spectrum interacts differently with the innate immune receptors (PRRs) and elicits different programs of differentiation of the adaptive immune system, therefore inducing a different immune development, ultimately affecting human physiology and health. The steep increase of inflammatory diseases could be caused not only by less sporadic childhood infections but also due to the fact that humans do not become exposed to essential coevolved microorganisms.

Other studies illustrate that changes of the coevolved commensal microbial community can lead to health problems. The influence of changes in composition community on vulnerability to *Salmonella* infections was investigated by administering antibiotics at a low dose to mice. This did not significantly decrease the total bacterial amount, but markedly altered the community composition. This perturbation before infection was sufficient to increase the ability of *S. typhimurium* to colonize the murine intestinal tract, further perturb the intestinal microbiota and induce intestinal pathology (Sekirov *et al.*, 2008). Moreover, changing the

microbial community with prebiotic fructo-oligosaccharides (FOS) has been shown to stimulate pathogenic infection and translocation in rats (Ten Bruggencate *et al.*, 2003). Recent studies at the National Food Institute of the Technical University of Denmark illustrate that changing the intestinal microbial communities of mice and guinea pigs with prebiotics such as FOS, xylo-oligosaccharides (XOS), galacto-oligosaccharides (GOS), inulin, pectin and β-glucan results in a very case-dependent outcome with regard to protection against infections (*Salmonella* or *Listeria*) (Ebersbach *et al.*, 2009; Petersen *et al.*, 2009). Interventions with antibiotics, but also probiotics and prebiotics thus change the coevolved microbial community of humans. We must be aware that microbial members can be influenced directly, but also indirectly through the complex intestinal microbial food chain. By forcing the host–microorganism interaction in a certain direction through such interventions, an artificial system could be created, potentially leading to a disruption of the ESS between the host and the intestinal microbiota in the long term.

Concluding remarks

Throughout evolution, the host coevolved with its intestinal microbiota. An intriguing interaction came to existence, characterized by reciprocal adaptation and benefits. The microbiota adapted to harvest nutrients from the diet of their host while also inducing proper development of the host's immune system. Thus, through evolution, several commensal bacteria have become essential for us in order to deal with the complex microbial communities that we face. In this attained steady state, the host is carefully controlling its intestinal microorganisms through various defense mechanisms in order to exclude potentially dangerous microorganisms, although still being tolerant enough to grasp their benefits.

The central hypothesis of this review is that the host selects its microbiota, particularly those occurring very close to its epithelium within the MAMC. It will be necessary to gain more information about the unique composition and expression profile of these mucosal microorganisms, as they have a huge potential biological outcome and could be referred to as an immune-regulating core microbiome. Additionally, microorganisms that are targeted by host defense when entering the mucus are restricted to the lumen, where they perform important metabolic pathways while not directly interacting with the host epithelium. These two distinct communities could overlap in the outer mucus layer, where host defense molecules have a lower impact. The outer mucus layer might thus contain a backup of intestinal microorganisms, serving as an inoculum to restore the initial microbial associations after perturbations, thus ensuring a stable community. We also introduced the concept of coevolution within the intestinal microbial communities themselves. This could additionally explain why microbial communities return to their initial composition, even after severe disturbances. Such community behavior could be of importance for microbial applications extending way beyond intestinal microbiology.

Recent insights into the nature of host–microorganism interactions could have a huge potential in medical healthcare applications to deal with IBDs. Such diseases are often characterized by hyper-responsiveness towards nonself, innocuous antigens, meaning that the host is not able to properly distinguish between innocuous and threatening microbiota. This can be caused by mutations in the host genome, but also due to the composition of the intestinal microbial community itself. During the 20th century, human ecology changed drastically, resulting in profound changes in our coevolved intestinal microbial communities. Moreover, microorganisms can become renegade and cause inflammation.

In conclusion, grasping the true nature of the mechanisms that steer the host–microorganism interactions is of fundamental importance to understand how the gut microbiota may contribute to the increasing incidence of diseases such as allergies, asthma, IBD and obesity. Unraveling these interactions may lead to a gastrointestinal resource management that supports our coevolved intestinal microbiota, with a special emphasis on mucosal microorganisms.

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