Cell
Leading Edge
Essay

Learning Tolerance while Fighting Ignorance

Philippe J. Sansonetti[1,2,*] and Ruslan Medzhitov[3]
[1]Unité de Pathogénie Microbienne Moléculaire, Institut Pasteur, 75724 Paris Cedex 15, France
[2]Microbiologie et Maladies Infectieuses, Collège de France, 75231 Paris Cedex 05, France
[3]Howard Hughes Medical Institute and Department of Immunobiology, Yale University School of Medicine, New Haven, CT 06510, USA
*Correspondence: psanson@pasteur.fr
DOI 10.1016/j.cell.2009.07.024

Research on microbe-host interactions focuses principally on pathogens, yet our immune system must deal with the huge number of beneficial commensal bacteria in our gut. It is becoming clear that the host immune system must reach a delicate balance between destroying dangerous bacterial pathogens while preserving the beneficial gut microbiota.

We can ignore the astronomical number of microorganisms that populate our gut, skin, and other mucosal surfaces, yet our immune system cannot. In the mammalian gut, there is an increasing gradient from jejunum to ileum to rectum of resident microorganisms that peaks in the colon at a concentration of 10^{10} per gram of stool. This vast complex community comprises 1000 or more different bacterial species and has a metabolic capacity comparable to that of the liver (Frank and Pace, 2008). Most of the gut microbiota is composed of obligate anaerobic bacteria that cannot be grown in culture. The majority are harmless commensals that benefit from the nutrient-rich environment offered by the host. Some are symbionts that also provide a benefit to the host, thereby establishing a condition of mutualism. Here, we will use the term commensal in a broad sense, regardless of whether the bacteria are also symbionts.

Most of the gut microbiota belong to the Firmicutes (Gram-positive anaerobes) and Bacteroidetes (Gram-negative anaerobes), with far fewer belonging to the oxygen-adapted Proteobacteria and Actinobacteria. The resident gut microbiota can provide two major benefits to the host: release of nutrients from food and formation of a barrier against pathogens (Sansonetti, 2004). The gut microbiota exerts a right of "first occupancy" precluding other microorganisms, particularly pathogens, from invading the occupied niche. It also strengthens the gut epithelial barrier, both mechanically and immunologically. The nutritional input of the gut microbiota is indispensible for the host enabling the synthesis of certain vitamins, hydrolysis of complex plant sugars, and production of short-chain fatty acids for direct assimilation by the host.

Although colonization of the gut and other tissues by commensal bacteria is common to all metazoans, its extent increased during evolution particularly in vertebrates, due to the emergence of new organs that could be colonized (Ley et al., 2008). With this increase in colonization, metazoans became dependent on an increasingly elaborate relationship with their gut flora. The coevolution of hosts and their commensal microbiota had a particularly strong impact on the immune system, which had to develop the ability to discriminate between the continuously resident microbiota, with which it actively maintains a homeostatic balance, and the invasive pathogens, to which it must respond. Here, we will explore the elusive barrier that separates these two types of host-microbial interactions and the possible mechanisms and consequences for the failure to maintain this delicate balance.

Learning Tolerance

Tolerance of the host immune system toward gut commensals is mediated by three key players: the microbes themselves, the gut epithelium, and the immune cells that reside in lymphoid tissue in the lamina propria of the gut wall (Figure 1). Induction of immune tolerance and maintenance of the homeostatic balance reflects a series of strategies: avoidance of immune recognition by commensals, active suppression of the host response by commensals, and regulation of the immune response by the host when recognition of commensals does occur. If a group of commensals is able to avoid immune recognition, active suppression of the host response may not be necessary. Likewise, if active suppression of the immune response is established by certain commensals, regulation of the immune response by the host may not be as critical.

To avoid immune recognition, commensal microorganisms must fail to express key virulence factors. One issue that needs more study is the degree of diversity in pathogen-associated molecular patterns (PAMPs) in commensals compared to pathogens. PAMPs, such as lipopolysaccharide (LPS) and peptidoglycans, are the structural elements of prokaryotes to which immune cells respond. For example, lipid A, the endotoxin moiety of LPS in Gram-negative bacteria, is pentacylated in the Bacteroidetes species that dominate the gut microbiota. This modification makes the lipid A of Bacteroidetes a poor agonist for Toll-like receptor 4 (TLR4), the receptor expressed by immune cells that is activated by LPS (Coats et al., 2007). Conversely, the lipid A of commensal Proteobacteria is hexacylated, making it strongly endotoxic and more likely to elicit an immune response (Munford and Varley, 2006). The effect of Proteobacteria lipid A as a potent agonist for TLR4 is diluted out in the gut because of the predominance of Bacteroidetes species. However, during inflammation associated with inflammatory bowel disease or intestinal infection, there is a decrease in Bacteroidetes and Firmicutes species and an increase in Proteobacteria, which are more resistant to inflammatory medi-

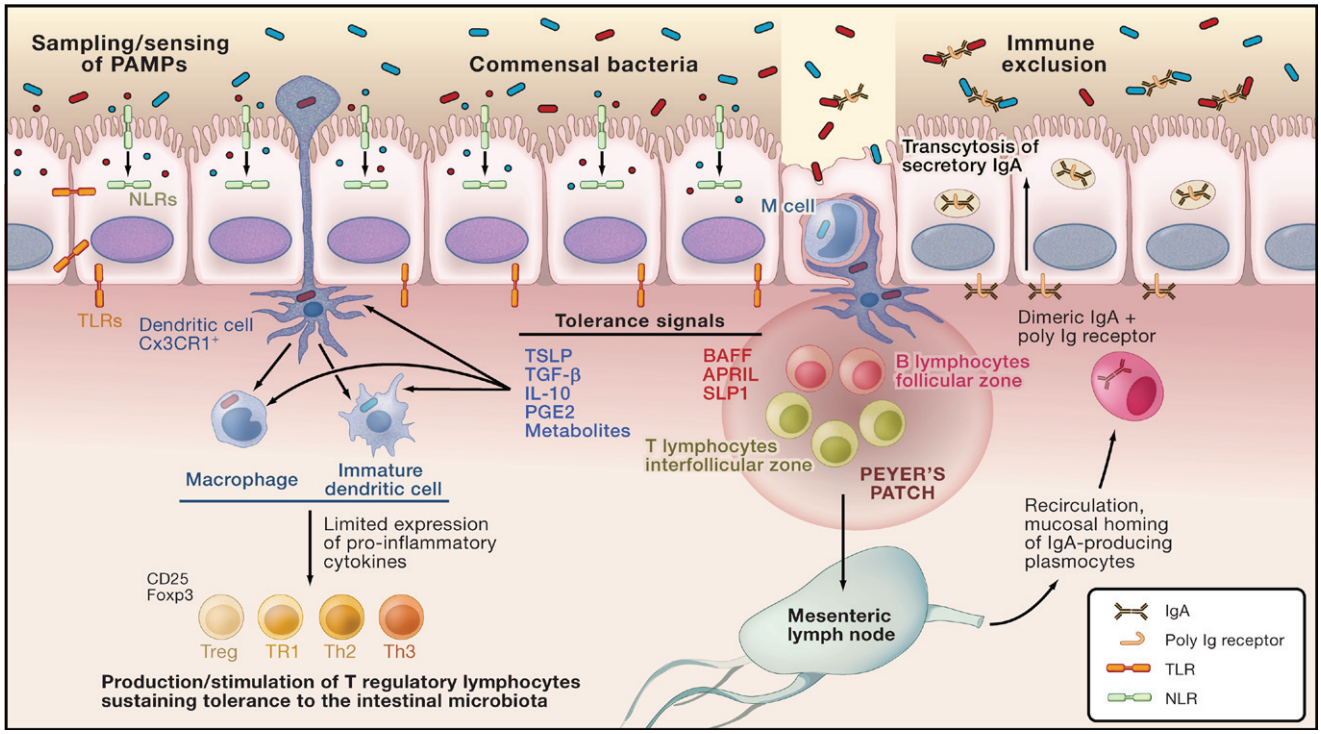

Figure 1. The Interface between the Gut Epithelium and Its Resident Microbiota

The gut epithelium is on the frontline of interactions with the resident microbiota and is a major effector of immune tolerance. It induces tolerance by sensing the number of bacteria and their distance from the epithelial surface using a combination of receptors such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs) that sample pathogen-associated molecular patterns (PAMPs). The gut epithelium then integrates this information and strategically responds to bacterial pathogens with inflammatory signals or to harmless commensals with immune tolerance signals. Molecules such as thymic stromal lymphopoietin (TSLP), transforming growth factor-β (TGF-β), interleukin-10 (IL-10), and prostaglandin E2 (PGE2) are essential mediators released by intestinal epithelial cells that regulate the production of proinflammatory cytokines. In this way, macrophages and dendritic cells, which are resident in the gut lamina propria are maintained in an anergic noninflammatory state resulting in the predominance of T regulatory lymphocytes (Tregs). Dendritic cells can transcytose between epithelial cells, capturing lumenal bacteria or sensing PAMPs, and also may participate in the induction of immune tolerance. In response to the presence of commensal bacteria, the intestinal epithelium also produces mediators such as B cell activation factor (BAFF), proliferation-inducing ligand (APRIL), and SUN-like protein (SLP1). These factors stimulate the maturation and proliferation of B lymphocytes that have been primed by the commensal bacteria sampled through M cells in the lymphoid tissue of the intestinal tract and mesenteric lymph nodes. The resulting mature B cells (plasmocytes) express a repertoire of IgA antibodies against commensal antigens. The plasmocytes enter the circulation and home to the intestinal mucosa where they produce secretory dimeric IgA antibodies that move into the gut lumen via the poly Ig receptor expressed by epithelial tissue. IgA participates in the homeostatic process by removing commensals from the epithelial surface.

ators (Lupp et al., 2007). This may result in an exacerbation of intestinal inflammation. Thus, homeostasis is highly dependent on maintaining the balance between strictly anaerobic and aero-tolerant microbes and their relative expression of immunostimulatory PAMPs.

Not Just Ignorant Bystanders

Maintenance of immune homeostasis toward commensal microorganisms is not mediated exclusively by tolerance. Increasing evidence indicates that some commensal bacteria have the capacity to actively suppress inflammation, particularly by targeting NF-κB, a key mediator of the inflammatory response. Some bacteria, such as nonpathogenic *Salmonella*, block the ubiquitination of IκBα catalyzed by the E3-SCF (βTrCP) ubiquitin ligase by neddylating cullin-1. This prevents degradation of IκBα, which keeps NF-κB in its inactive state and prevents transcription of NF-κB target genes including those encoding proinflammatory factors (Collier-Hyams et al., 2005). The bacterium *Bacteroides thetaiotaomicron* targets the NF-κB subunit RelA, enhancing its export from the nucleus through a mechanism that is independent of the nuclear export receptor Crm-1 but dependent on formation of a complex with PPARγ. This prevents transcription of the proinflammatory genes activated by NF-κB (Kelly et al., 2003). Meanwhile, *Lactobacillus casei* induces transcriptional downregulation of Roc-1 (a subunit of E3-SCF) and of several components of the proteasome complex, thereby protecting IκB from degradation (Tien et al., 2006). The challenge now is to identify the effectors of commensal microorganisms that mediate the blockade of NF-κB activity. One clue comes from the study of a mouse model of colitis (Mazmanian et al., 2008). This study shows that the exopolysaccharide of a gut bacterial species *Bacteroides fragilis* exerts a strong protective anti-inflammatory action against the induction of colitis by the pathogen *Helicobacter hepaticus*. It induces the protective effect by regulating production of the cytokine interleukin-10 (IL-10) by immune cells of the gut. A systematic analysis of the characteristics of a limited number of gut microorganisms is

needed to obtain a "commensal signature," as has been done for gut pathogens. Particularly important questions to ask are, what are the effector molecules that enable commensals to occupy a niche and stay there, and what are the factors and mechanisms that alter the host immune response?

Gut Epithelium on the Frontline

The intestinal epithelium is a critical barrier to invasion by pathogenic microbes. It is on the frontline of sensing microbes in the gut and participates in the innate immune response as a bona fide component of the host immune system. The gut epithelium had to evolve under the double constraints of sensing microbes and adjusting the immune response to the perceived degree of threat. The result has been either constitutive "physiological inflammation" that balances the constant presence of the resident gut microbiota or induced "pathological inflammation," the degree of which depends on the number and virulence of the invading pathogens. Physiological inflammation can be viewed as a basal sensing-responding loop that maintains a dynamic yet fragile homeostatic balance. The epithelium secretes factors such as mucins that establish a protective film of variable thickness and density, with the layer closest to the epithelial surface forming a dense lattice in which secreted antimicrobial molecules remain embedded (Liévin-Le Moal and Servin, 2006). In general, commensal bacteria seem to grow at a respectable distance from this zone establishing a sort of "bacterial no man's land." This notion has been nicely confirmed with the demonstration that the densely packed inner mucin layer that is immediately adjacent to the epithelial surface is devoid of bacteria in the mouse colon (Johansson et al., 2008). At the lumenal edge of the peripheral zone of the mucus layer, bacteria seem to establish complex communities often referred to as biofilms (Swidsinski et al., 2005).

Distance does not mean ignorance, however. TLRs are expressed by gut epithelial cells enabling them to detect bacteria, although receptors such as TLR4 are present at low levels and may be devoid of coactivating molecules (Abreu et al., 2005). In addition, tolerance of the gut epithelium to major immune activators, such as bacterial LPS, is established very early in life (Lotz et al., 2006). And recent evidence in the zebrafish indicates that alkaline phosphatase in the epithelial brush border actively detoxifies bacterial endotoxins in the gut lumen (Bates et al., 2007). From a coevolutionary perspective, these observations suggest that LPS may be a major effector driving the mechanisms leading to immune tolerance to the gut microbiota.

The gut epithelium also actively monitors the proximity and density of the resident microbiota by sampling bacterial molecules via apical channels such as hPepT1 (Vavricka et al., 2004). Bacterial cell wall components, such as muramyl dipeptide, are transported into cells via these channels and may induce maintenance of a minimal level of "physiological inflammation" by activating NOD (nucleotide-oligomerization domain) proteins (pattern recognition receptors of the innate immune system) in the cytoplasm of gut epithelial and immune cells. In parallel, OCTN2, a cation transporter on the apical surface of epithelial cells, is able to sample quorum-sensing molecules produced by bacterial communities, leading to activation of stress signaling pathways mediated by MAPK, Akt kinase, and heat-shock proteins that maintain the integrity of the epithelial barrier (Fujiya et al., 2007). Similarly, the quorum-sensing regulator C12 selectively impairs the activation of NF-κB in mammalian cells (Kravchenko et al., 2008). TLRs also play a role in this complex choreography: they are sequestered in the intestinal crypts that act as sanctuaries for stem cells and so must be protected. TLRs also mediate essential signals for proliferation of epithelial stem cells in these critical zones (Rakoff-Nahoum et al., 2004). Thus, the intestinal epithelium provides a highly suitable and adaptable frontline for microbiota to interact with the host and is much more than just a static defensive barrier.

Vertebrate hosts have had to develop both innate and adaptive tolerance toward commensal bacteria. Commensals are a source not only of PAMPs but also of foreign antigens that potentially can be recognized by the adaptive immune system. Immune cells in the lamina propria are actively maintained in a state of tolerance to the gut microbiota by epithelial cells. For example, intestinal epithelial cells produce factors such as thymic stromal lymphopoietin that ensure dendritic cells and macrophages in the gut do not induce an inflammatory response (Rimoldi et al., 2005). In addition, IKKβ produced by gut epithelial cells maintains this homeostatic balance by promoting mucosal immunity and controlling intestinal inflammation (Zaph et al., 2007). Similarly, epithelial IKKγ/NEMO is critical for maintaining epithelial integrity and immune homeostasis (Nenci et al., 2007). The resident microbiota, by producing TLR-mediated signals, stimulates expression of inhibitory factors that maintain tolerance. The local production of IgA antibodies directed against microbial antigens is also part of the dynamic epithelial barrier that keeps the resident flora in check and allows downregulation of proinflammatory signals (Peterson et al., 2007). Finally, T regulatory lymphocytes (Tregs) in the gut subepithelium produce IL-10 and TGF-β, factors that are major contributors to inducing tolerance of the innate and adaptive immune systems to the resident gut microbiota (Izcue et al., 2006).

Failures of Tolerance

On the host side, any broken link in the chain that maintains physiological inflammation can cause loss of the gut mucosa's ability to keep the resident microbiota in check, leading to a switch from physiological to pathological inflammation. This may be a major cause of a type of inflammatory bowel disease called Crohn's disease. In Crohn's disease, mutations in the gene encoding NOD2, a key element of the homeostasis network, lead to decreased production of antimicrobial β-defensins by gut epithelial cells (Nuding et al., 2007). This enables commensals to make intimate contact with the epithelial surface (Swidsinski et al., 2002) resulting in a destructive mucosal inflammation.

Defects in the adaptive arm of immune tolerance to the resident microbiota also may lead to inflammation, and experimental models confirm this (Hoffmann et al., 2003). Immunological analyses of gut tissue from Crohn's disease patients indicate a strong bias in favor of T helper

(Th) 1 and Th17 cells in the gut lamina propria, which are lymphocytes of the adaptive immune system that induce an inflammatory response (Arseneau et al., 2007). Genetic analyses of patients with inflammatory bowel disease will undoubtedly continue to provide key information regarding the essential molecules that regulate the homeostatic balance between the innate and adaptive immune systems of the gut and its resident microbiota.

Discriminating Commensals from Pathogens

Pathogens produce an arsenal of effectors that enable them to achieve close proximity and colonization of the epithelial surface followed by subversion and destabilization of the epithelial barrier. These effectors include adhesins, invasins, proteases (mucinases), toxins, as well as PAMPs such as hexacylated lipid A. Some pathogens are able to dampen expression or destroy epithelial defenses such as mucus. Invasive microorganisms, such as *Shigella*, induce decreased production of antimicrobial molecules such as β-defensins and cathelicidin, thereby achieving efficient colonization of epithelial surfaces by escaping the bactericidal effects of these molecules (Spérandio et al., 2008). Together, these pathogenic properties result in a massive delivery of PAMPs and other signaling molecules to the epithelial and subepithelial surveillance network of TLRs and Nod-like receptors (NLRs) expressed by gut epithelial cells and immune cells (Figure 1).

The host immune system thus appears to have evolved under an antagonistic selective pressure enabling it to tolerate the beneficial gut microbiota on the one hand and to combat and eradicate pathogens on the other. The virulence of pathogens may be specifically sensed by the host resulting in an immune response, but what is sensed may be the specific and stereotypic effects of virulence on host tissues. An example of this includes disruption of the extracellular matrix and basement membranes of epithelial cells by bacterial proteases. Another example is disruption of membrane integrity by the pore-forming exotoxins of Gram-positive pathogens or by the type III secretion systems of Gram-negative pathogens, which is sensed by the NALP3 inflammasome of macrophages and other immune cells (Pétrilli et al., 2007). It is not simply tissue damage per se, but rather the very specific alterations in tissue homeostasis associated with pathogen virulence that signal the immune system to mount a protective response.

Conclusion

Commensal bacteria are highly heterogeneous, and what counts is the combination of all of their individual properties. It is interesting to consider a "social" view of the ecology of this vast community. Genomic sequencing of individual species, together with metagenomics that provides information on the genomes of most of the gut microbiota (the majority of which cannot be cultured), no doubt will confirm that some commensal microorganisms are potentially more harmful than others. This may be particularly true for Proteobacteria species including probiotic strains like the Nissle 1917 strain that carries virulence genes (Grozdanov et al., 2004). These bacteria may be essential for the development of at least some signals that induce immune tolerance, such as those inducing tolerance to LPS, as long as their density is kept in check. Thus, the concept of "good" and "bad" commensals may not withstand a sociological view of the intestinal flora. Moreover, maintaining the balance among the different bacterial species that comprise the microbiota depends on the characteristics of the bacteria themselves and on certain environmental cues, particularly nutrition, which introduces another degree of complexity. Inflammation of the gut epithelium also contributes to loss of homeostatic balance and expansion of potentially more aggressive microorganisms such as the Proteobacteria. This process may explain in part the chronic inflammation of inflammatory bowel disease. Likewise, innate immune signaling through the TLR/MyD88 pathway affects the composition of the intestinal microbiota, and this in turn affects the development of autoimmune diseases such as type 1 diabetes (Wen et al., 2008). The notion of "good" and "bad" commensals thus may be a reflection of the balance between representative commensal species and the particular characteristics of the gut milieu. We still have a lot to learn about how homeostasis is maintained between the gut and its microbiota. Deciphering the mechanisms of this homeostasis and its possible rupture will yield essential information about how development, tissue repair, and physiology can be affected by our microbial environment. At stake is our understanding and treatment of major inflammatory diseases such as inflammatory bowel disease as well as colon cancer and other tumors. Other epithelial surfaces, such as those of the oral cavity and skin, will benefit from this global concept according to which only fighting ignorance guarantees the virtue of tolerance.

ACKNOWLEDGMENTS

P.J.S. is a foreign scholar of the Howard Hughes Medical Institute. The authors thank members of their respective groups for invaluable discussions.

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