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Commensal gut bacteria: mechanisms of immune modulation

Denise Kelly, Shaun Conway and Rustam Aminov

Gut Immunology Group, Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen, UK AB21 9SB

Mucosal immune responses to pathogenic gut bacteria and the mechanisms that govern disease progression and outcome have been researched intensely for decades. More recently, the influence of the resident non-pathogenic or 'commensal' microflora on mucosal immune function and gut health has emerged as an area of scientific and clinical importance. Major differences occur in the mucosal immune response to pathogens and commensals. In part, this functional dichotomy is explained by the presence of virulence factors in pathogenic species, which are generally absent in commensals. Additionally, immunological 'unresponsiveness' towards the resident commensal microflora is thought to permit their successful colonisation and co-existence within the host gut. However, evidence of an active dialogue between members of the commensal microflora and the host mucosal immune system is rapidly unfolding. This crosstalk is likely to affect immunological tolerance and homeostasis within the gut and to explain some of the differential host responses to commensal and pathogenic bacteria.

Human colonic microbiota, bacterial genomes and co-evolution

The human colonic microbiota comprises >500 distinct bacterial species and has an important role in human nutrition and health, by promoting nutrient supply, preventing pathogen colonization and shaping and maintaining normal mucosal immunity [1]. Evidence that host nutrition is supplemented by the metabolic capabilities of the resident microflora is derived from studies on mono-associated and conventionalized mice [2], which, unlike germ-free animals, are able to capture and store as energy, by-products released by bacterial degradation of undigested dietary substrates [2]. The genome sequences of several commensal bacterial species provide insights into how bacteria have evolved to perform their various metabolic functions within the host gut [1,3,4]. For example, large numbers of genes involved in dietary polysaccharide metabolism are encoded within the genomic sequences of two gut commensals, *Bacteroides thetaiotaomicron* and *Bifidobacterium longum* [1,5]. Yet, other metabolic genes enable these bacteria to target host glycoconjugates as alternative energy sources, in situations of limited lumenal nutrient availability, further illustrating the fine-tuning of this evolutionary adaptation [6].

With the initiation of bacterial colonization, gross changes in immune architecture occur with the expansion and phenotypic differentiation of specific cell lineages of the mucosal immune system [4,7]. The consequences of this early postnatal programming are thought to persist throughout life and to impact significantly on whole body health. An important concept, in this regard, is that commensal bacteria differ in their ability both to promote development of the gut-associated lymphoid tissues [8] and to maintain its function. Although the molecular basis for this functional distinction is currently unknown, the 'hygiene hypothesis', which links reduced exposure to important gut bacteria with the rising incidence of human allergies and autoimmune diseases, embraces this viewpoint [9]. It can thus be postulated that functionally significant bacteria of the normal commensal microflora, are required to maintain immune homeostasis in both the developing and adult gut. It might be possible that the mutualism that exists between these bacteria and the healthy host gut can be exploited for treatments of diseases, such as inflammatory bowel disease (IBD), in which inappropriate immune responses to components of the normal microflora exist.

Although the detection and assignment of a functional role for bacterial genes involved in metabolism has been relatively straightforward, the identification of genes in commensal bacteria involved in host immune modulation provides more of a challenge. Hypothetically, for example, the immunomodulatory effect of *B. longum* might involve a eukaryotic-type serine protease inhibitor (serpin) found in its genome sequence [5]. Alternatively, both the mucinase activity and the L-fucose-rich polysaccharides and glycoproteins of *B. thetaiotaomicron* might permit close contact between viable bacteria and host epithelial cells and could be important for the immunosuppressive effects of this bacterium [10,11]. Similarly, the expression of specific surface structures, such as elongation factor Tu in *Lactobacillus johnsonii* and flagellin in commensal *Salmonella*, could be important for intimate contact with the host, facilitating other effector processes involved in immune modulation [12,13]. Quorum-sensing autoinducers (AIs), which are communication molecules released by bacteria at high densities, might also modulate host responses through regulation of commensal genes involved in gut colonization and host signalling [14,15]. The recent discovery of mammalian gut hormones that mimic AI signalling molecules [16] further highlights the complexity of this crosstalk.

Corresponding author: Kelly, D. (D.Kelly@rowett.ac.uk).

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Bacterial community structure

Bacterial diversity and density in the gut lumen increases from the upper to the lower gastrointestinal tract, from an almost sterile content in the stomach to 10^{12} cfu g^{-1} colon and faecal samples. Recently, techniques, such as 16S rRNA gene sequencing, indicate that the majority of human faecal bacteria belong to phylogenetic clostridial clusters XIVa and IV of low G+C Gram-positive bacteria and to the *Bacteroides* class [17]. Clusters XIVa and IV are reported, along with lactobacilli and bifidobacteria, as being beneficial to health, and although bacteria belonging to clusters XIVa and IV are more abundant than lactobacilli and bifidobacteria, they have received far less attention. Current knowledge of the mucosa-associated bacterial communities in the intestine and colon is limited owing to the greater focus on characterisation of faecal diversity. Recent studies, however, indicate that the predominant mucosa-associated community is host-specific and significantly different from luminal and faecal bacterial communities [18].

Several important host factors control bacterial communities within the gut lumen and on the gut wall (Figure 1). For example, the protective role of secretory IgA, which promotes bacterial biofilm formation within the normal gut flora but also limits the expansion and translocation of undesirable pathogenic populations, has

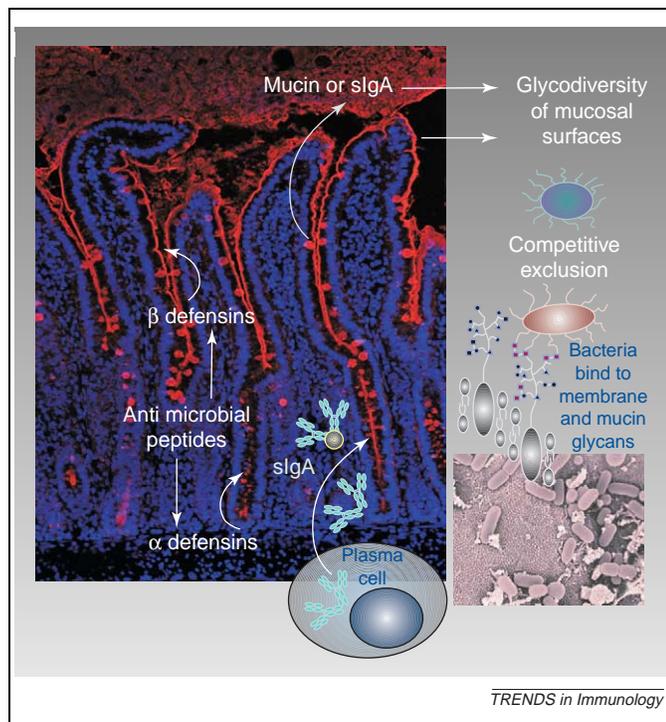


Figure 1. Host factors controlling bacterial colonisation. Histological section (left) of mouse jejunum affinity labelled with *Ulex europaeus* agglutinin-1 conjugated to rhodamine (red). This reagent shows the distribution of α -linked fucose residues on membrane and mucin glycoconjugates. Nuclei are labelled blue with DAPI (4', 6 diamidino-2-phenylindole). The contribution of mucin, sIgA, antimicrobial peptides (epithelial and Paneth cell-derived) and membrane and mucin glycans towards the anatomical distribution and density of colonizing bacterial is illustrated. The scanning electron micrograph (bottom right) shows the luminal surface of pig intestinal epithelial cells colonized by *Escherichia coli*. These bacteria express fimbrial adhesins that recognize and attach to mucin-type moieties expressed on microvillar membranes. The principal of competitive exclusion, whereby bacterial occupancy of intestinal receptor sites, specifically or sterically, occludes the attachment of competing bacteria is illustrated.

been long established [19,20]. Functioning in concert with IgA are the structurally diverse epithelial-derived antimicrobial peptides [21], which are purported to kill both pathogenic and commensal bacteria. Paneth cells, which specifically localize at the base of intestinal crypts, also secrete antimicrobial peptides, producing cryptdins or α -defensins [22]. The differential distribution of these cells along the gut, and their absence from the human colon, might contribute to the proximal to distal gradients in bacterial diversity and density. Interestingly, in relation to Paneth-cell function, *B. thetaiotaomicron*, a prevalent commensal bacterium in the human gut, induces these cells to secrete angiogenins [23]. Angiogenins represent a novel class of antimicrobial peptide that appear to selectively kill pathogenic microorganisms, inferring that *B. thetaiotaomicron* has evolved strategies that protect the 'sterility' of intestinal crypts. Although the mechanism by which *B. thetaiotaomicron* signals to Paneth cells to induce antimicrobial peptide expression is unknown, recent work has shown that Paneth-cell cryptdins can be induced by bacterial muramyl dipeptide (MDP) through nucleotide-binding oligomerization domain 2 (NOD2) activation [24].

Intestinal and colonic mucins provide additional layers of protection that influence both bacterial colonization and the host response [25]. Commensal bacteria specifically modulate mucin synthesis and structure and affect other goblet cell proteins, such as the resistin-like molecules (RELM β) [26]. Other than the obvious physical and chemical constraints imposed by mucin, bacterial colonization is also controlled by novel antimicrobial activities associated with specific mucin glycans [27]. Because their natural diversity is extensive, other intestinal and colonic glycan structures might exert similar antimicrobial effects. Glycans, similar to those of mucin, are also found associated with gut membrane glycoconjugates. These structures act as receptor sites, capable of promoting bacterial attachment and adherence to the gut wall. Although these structures have been defined mainly for gut pathogens, they might represent docking sites for commensals [12,28]. Such glycan-mediated adherence might also provide a molecular basis for competitive exclusion and explain some of the genotypic differences in intestinal and colonic wall bacteria.

Recognition of commensal bacteria by intestinal epithelial cells

Bacteria that penetrate mucins and resist host antimicrobial peptides potentially attain close proximity to epithelial cells that line the intestine. In this scenario, Toll-like receptors (TLRs) and nucleotide-binding oligomerisation domain isoforms (NODs) have a crucial role in their recognition by the mucosal immune system. TLRs, by recognizing components of microbes, trigger both innate and adaptive immune responses that eliminate pathogens and shape the intestinal microflora. Included among the innate immune responses are, the synthesis of antimicrobial peptides, proinflammatory cytokines and chemokines [29], as well as the secondary anti-inflammatory responses required for the resolution of inflammation [30]. TLR signalling also impacts on subsequent T-cell

responses through activation of dendritic cells (DCs) that overcome the suppressive effects of T regulatory (Treg) cells [31]. The NOD proteins, which localize to the cell cytosol, are also capable of triggering both innate and acquired immune responses, following cellular uptake and recognition of muramyl dipeptide and meso-diaminopimelic acid motifs, components of virtually all Gram-positive and Gram-negative bacteria [24,32]. Although the role of TLRs and NODs in pathogen recognition by immune cells has been well studied, their function in relation to the complex commensal flora, which in the healthy gut does not evoke inflammatory immune responses, is relatively unexplored.

The absence of TLRs from the apical surfaces of epithelial cells, either owing to their intrinsic polarization to basolateral surfaces or as a result of ligand-induced downregulation, might contribute to the hyporesponsive tone of the gut towards its diverse commensal microflora. Consistent with this notion, TLR3, 7, 8 and 9 appear to localize within intracellular compartments of positively expressing cells [33], whereas TLR5 localizes to basolateral membrane surfaces [34]. TLR2, similar to TLR4, is either absent or attenuated in intestinal epithelial cells [35–37]. Hence, the ability of epithelial cells to mount rapid innate immune responses to luminal pathogens has hitherto been explained by rapid translocation and/or internalization of pathogen-specific ligands and migration to cognate TLRs [37–40]. Recent data, however, confirming the presence of functional TLR2 and TLR5 receptor populations on the apical surfaces of intestinal epithelial cells conflicts with this explanation [41,42]. Irrespective of their precise location in intestinal epithelial cells, TLRs and NODs are likely to be crucial to bacterial host communication. In support of this, TLR signalling mediated by commensal bacteria is essential for intestinal barrier function and repair [43]. In this study, MyD88 (TLR signalling adaptor molecule)-, TLR2- and TLR4-deficient mice, mouse strains markedly impaired in their ability to respond to the commensal microflora, exhibit severe mortality following dextran sulphate sodium-induced intestinal injury. This outcome contrasts with wild-type mice, which survives with low morbidity. Analyses of colonic tissues reveal elevated expression of cytoprotective factors, including heat-shock proteins, in wild-type mice compared to TLR-defective mice [43]. Barrier-promoting function might be an inherent feature of TLR-mediated signalling [41], illustrating how these receptor systems might regulate many diverse cellular processes.

Epithelial cell responses to flagellated commensal bacteria

Commensal bacteria appear to activate TLR signalling pathways [43]. It is a strong possibility that this signalling capability also extends to TLRs expressed on gut epithelial cells. In the context of large populations of commensal bacteria that express high densities of TLR-specific ligands, strong activation of TLRs could be potentially damaging. For example, many of the dominant commensal groups, particularly those belonging to cluster XIVa of low-G+C *Clostridium* subphylum of Gram-positive

bacteria, are flagellated. Hence, the predominance of flagellated *Roseburia* in the human gut is several orders of magnitude higher than that encountered in a typical infection by flagellated pathogens. The question therefore arises as to how the gut accommodates such high levels of flagellin protein in the absence of an inflammatory response because monomeric flagellin, through its activation of TLR5 and NF- κ B, is a potent proinflammatory ligand [44]. Although flagellae with low intrinsic activity, in terms of TLR5 activation, have been documented [45], for many gut bacteria, the regions of flagellin involved in receptor recognition and activation are highly conserved and hence likely to be biologically active within the gut environment [46]. One possibility is that proinflammatory responses induced by commensal bacteria are rapidly attenuated either by host systems or by gut bacteria, in ways analogous to the immune evasion strategies used by pathogenic bacteria [47]. The ability of certain commensal bacteria to attenuate NF- κ B has been recently demonstrated [10,48], however, whether this contributes significantly to the overall immune tolerance to the large populations of commensal bacteria, including those flagellated species, is currently unknown.

Host systems that attenuate TLR signalling and inflammation

The magnitude and duration of TLR signalling is carefully regulated in the healthy gut. TLR2, for example, induces both proinflammatory and anti-inflammatory cytokine synthesis [30]. Other host mechanisms that modulate TLR-mediated responses include, interleukin-10 (IL-10) [49], Trefoil [50], transforming growth factor- β (TGF- β) or SMAD7 (intracellular signalling molecule of the TGF- β receptor cascade) [51,52], peroxisome proliferators-activated receptor γ (PPAR γ) and PPAR γ ligands [10,53,54], the deubiquitinating enzyme A20 [55,56], single Ig IL-1R-related protein (SIGIRR) [57], anti-inflammatory TLR9 signalling [58], cytosolic NOD2 [59] and ST2 (secreted soluble molecule of the Toll-IL-1 receptor family) [60]. Genetic defects in these regulatory systems can predispose to inflammatory diseases. Specifically, mutations in the *NOD2* or caspase activation recruitment domain 15 (*CARD15*) gene have been linked to an enhanced susceptibility to Crohn's disease. Although wildtype *NOD2* is an activator of NF- κ B, intact *NOD2* signalling appears to inhibit TLR2-driven activation of NF- κ B, particularly the NF- κ B c-Rel subunit [59]. However, it is worth noting that this does not appear to be a universal phenomenon [24]. Irrespective of the precise mechanism, *NOD2* deficiency in mice and the Crohn's disease-like *Card15* mutation, are associated with aggressive Th1 responses that can promote tissue damage and inflammatory disease [59]. These Th1 responses might be directed towards common bacterial antigens produced by gut commensal bacteria. Supporting this mechanism, many strains of colitic mice have elevated Th1 responses directed towards commensal bacterial flagellins [61]. Similarly, in patients with Crohn's disease, serum IgG levels against flagellins are elevated [61]. Local immune responses directed against bacterial flagellin might exacerbate the disruption to the natural balance of

bacterial groups associated with inflammatory bowel diseases [43,62].

Commensal bacteria effector mechanisms and attenuation of TLR signalling

In addition to the host mechanisms that control inflammation, recent evidence supports a role of the indigenous commensal microflora in maintaining immune homeostasis within the gut. [10,11,13]. For example, the ability of the gut to tolerate large numbers of potentially proinflammatory flagellated commensal bacteria might be explained, in part, by the anti-inflammatory effects of commensal anaerobes, such as *B. thetaiotaomicron* [10], which restrict the signalling induced by both flagellin protein and flagellated pathogens [10]. Importantly, the biological action of *B. thetaiotaomicron* is downstream of

TLR receptor signalling and NF- κ B activation [10], whereas other bacterial commensals block the activation of NF- κ B by inhibiting I κ B- α ubiquitination [13]. The anti-inflammatory activity of *B. thetaiotaomicron* operates at the level of the nucleus, promoting the nuclear export of transcriptionally active RelA (Figure 2), with the upstream TLR-mediated signalling unaffected. The physiological significance of the distinct modes of action of anti-inflammatory gut bacteria is not fully appreciated but perhaps has relevance for other cellular processes that share components of the NF- κ B signalling pathway, such as cellular integrity, survival and repair [41,43].

A further point worth mention is the emerging evidence of the link between inflammation and colorectal cancer (CRC). Bacterial mechanisms that modulate cellular processes of ubiquitination and proteosomal-mediated

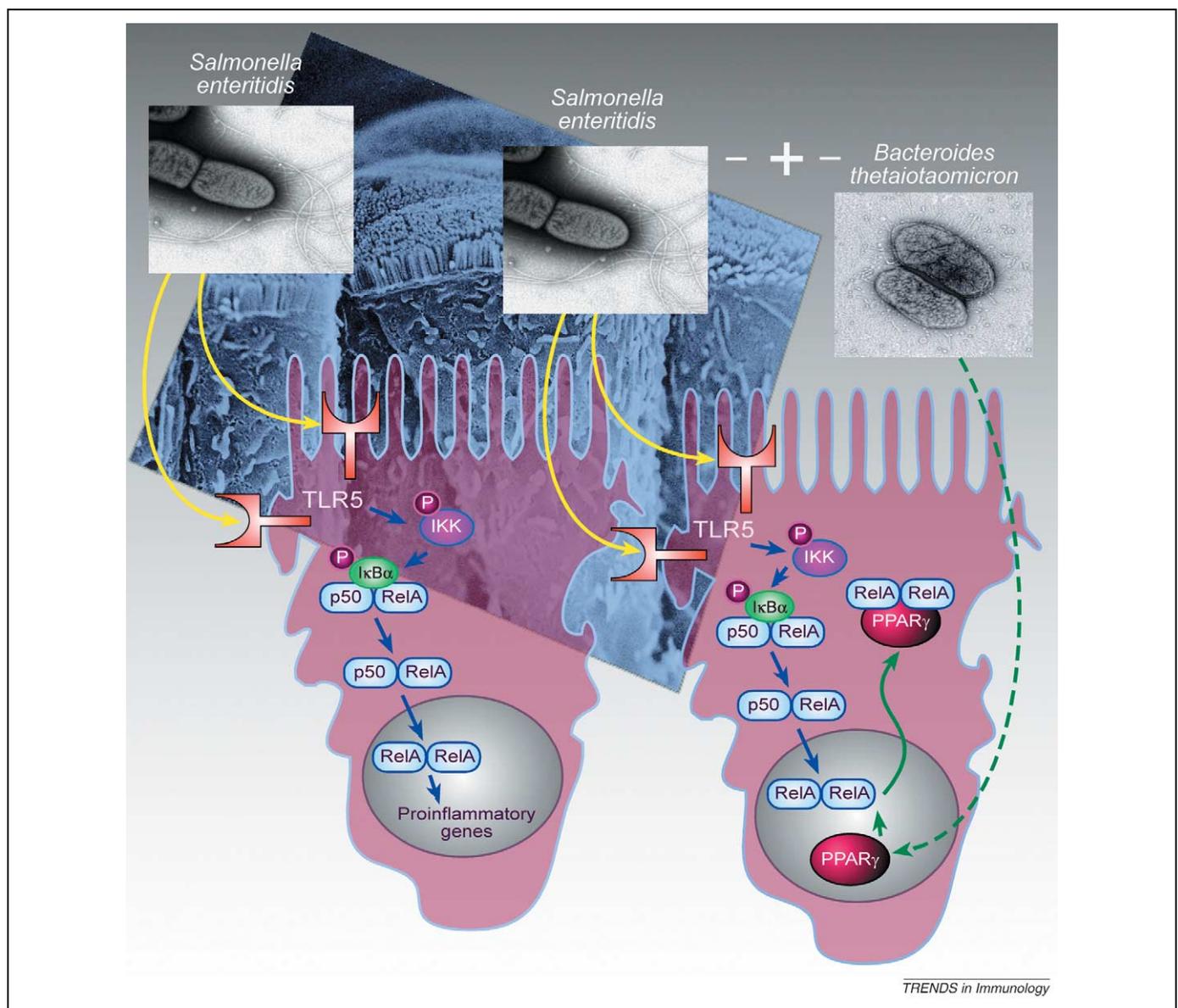


Figure 2. Anti-inflammatory *Bacteroides*. Following contact with intestinal epithelial cells (IECs), enteroinvasive *Salmonella enteritidis* triggers I κ B kinase (IKK) activation, I κ B α degradation and the nuclear translocation of the NF- κ B RelA subunit. *Salmonella* flagellin, through its activation of epithelial TLR5 receptors, is crucial for both NF- κ B activation and NF- κ B-mediated proinflammatory gene transcription. Co-incubation of IECs with a human commensal bacterium, *B. thetaiotaomicron*, attenuates proinflammatory gene expression induced by flagellated *S. enteritidis*. *B. thetaiotaomicron* derives its anti-inflammatory effects by promoting the nuclear export of transcriptionally active NF- κ B RelA. This effect is dependent on the nuclear receptor, PPAR γ , which physically associates with, and exports, RelA from the nucleus, thereby terminating the proinflammatory response.

degradation are likely to influence other important events, such as β -catenin degradation, stability and localization [63]. This might alter the expression of genes, such as *c-myc*, implicated in oncogenesis and CRC. Hence, the mechanisms by which bacteria regulate the processes of inflammation might have long-term consequences on gut health and perhaps helps draw a distinction between beneficial commensal bacteria and those that might cause long-term harm.

Intriguing questions arise in relation to the nature of bacterial effector systems that permit host immune modulation by commensals. In pathogenic bacteria, several mechanisms that interfere with the processes of immune recognition and activation have been documented [47,64–66], which essentially result in a self-limiting infection, ensuring both host and bacterial survival. Many of these mechanisms rely on the transfer of structurally diverse bacterial effector molecules, either directly into the cytosol of eukaryotic target cells or into the extracellular milieu, which then subvert host signalling [47,65,67]. Such transfer proceeds through type III secretion systems or a functional flagellar export system [68,69]. The findings from recent comparative genomic analysis, bioinformatic data mining and genome-wide functional screening, have revealed the presence of type III secretion systems in commensal bacteria [68]. Similarly, type IV secretion systems in commensal bacteria might also be important [70]. By using both type III and/or type IV secretion systems, commensal bacteria, following intimate contact with the host, might be able to deliver bacterial effector molecules to intestinal cells, which modify the signalling outcome. Such bacterial effector molecules already defined for pathogenic bacteria include AvrA, YopJ and leucine-rich proteins [64–66]. Alternatively, it might be possible that surface structures act through host-cell receptors, to promote anti-inflammatory responses and immune modulation [11,12]. Clearly, the more highly evolved and successful such evasion strategies are in negating host immune surveillance mechanisms, the greater the opportunity for bacterial survival within microniches close to mucosal surfaces.

Commensal bacteria and adaptive immunity

The normal physiological response to commensal flora is one of immunological tolerance. Shifts away from this are thought to underlie pathological conditions, such as IBD and food allergies. The 'hygiene hypothesis', which in many ways has pushed the science of gut microbiology and immunology to the fore, postulates that reduced exposure to gut bacteria and childhood infections, alters the mechanisms and signals that determine T-cell differentiation and the susceptibility to immunological tolerance [9,71]. For example, a bias away from Th1, towards Th2 hyper-responsiveness in the lung is thought to account for the rising incidence in allergic disease, as a result of reduced exposure to Th1 respiratory pathogens; by contrast, the loss of Th2-promoting helminth infections in the gut accounts for the increase in Th1-dominant immunity and related gut diseases. More recent evidence derived from TLR4-mutant mice, which fail to respond to Gram-negative bacteria but which are capable of

mounting Th1 responses, has further established the importance of commensal bacteria in preventing excessive Th2 allergen-specific IgE responses [72]. Although bacteria are clearly important, both gut-related food allergies (Th2) and inflammatory bowel diseases (Th1) are increasing within the human population, suggesting that a bias in individual effector T-cell responses does not provide a full explanation.

The defective function of mucosal DCs and Treg cells, which are pivotal in directing and controlling T-cell responses, might prove to be of greater importance in the pathology associated with immune-based disorders, irrespective of whether they are Th1- or Th2-mediated. Colonization by gut commensal bacteria facilitates the development of oral tolerance [71,73] and it is feasible that such bacteria influence tolerance-inducing mucosal DCs and/or the induction of Treg cells (Figure 3). Consistent with this, lamina propria and Peyer's patch DCs appear to be able to sample luminal bacteria [74]. Mucosal DC subsets can contribute to Th1, Th2 and Treg cells and, under certain conditions, induce Th1- and Th2-mediated inflammation and pathology. In particular, the CD11c⁺ CD11b⁺ CD8 α ⁻ DC subset preferentially polarizes antigen-specific T cells towards Th2 cytokines and IL-10, promoting T cell-dependent IgA production [75,76]. Conversely, the presence of CD11c⁺ CD11b⁻ CD8 α ⁻ DCs in terminal ileum Peyer's patch, which under steady-state conditions also contain bacteria but constitutively express IL-12p40, contribute to Th1-mediated responses [77] and, if unchecked, could give rise to Th1 inflammation, characteristic of Crohn's disease [78]. Yet another DC population, prevalent in mucosal tissues, and thought to be of importance in maintaining tolerance to harmless dietary antigens and commensal bacteria, is the CD8⁺ plasmacytoid DCs, which induce IL-10-producing Treg cells [76]. In the healthy gut, excessive Th1 responses to the commensal flora are prevented by the controlling influence of Treg cells [79,80]. Factors that promote the appearance of both tolerance-inducing DCs and Treg cells include probiotic bacteria, which are potent inducers of IL-10-producing DCs and also inhibit the generation of Th1 cells [81]. The contribution of the commensal flora is likely to be of paramount importance, as implicated by the hygiene hypothesis. Sampling of IgA–bacteria complexes through FcR α receptors [82] and potentially attenuation of TLR-specific signalling and NF- κ B-mediated responses by commensal bacteria might also be important for tolerance induction [10].

Although the commensal flora is subject to mucosal immune sampling and local tolerance responses, the systemic immune system is thought to remain largely ignorant of these bacteria [74]. Contrary to this viewpoint, colitogenic Th1 cells are present in peripheral lymphoid tissues in normal mice [83] and small populations of circulating intestinal, β 7 integrin (CCR9)-positive memory (CD45RA⁻) T cells that produce the effector cytokine IL-10, have been detected in peripheral blood [84,85]. These data suggest that peripheral tolerance, as well as local tolerance to the commensal flora, is in fact achieved by active suppression mediated by Treg cells [79,84–86]. Their importance is further supported by the phenotypic

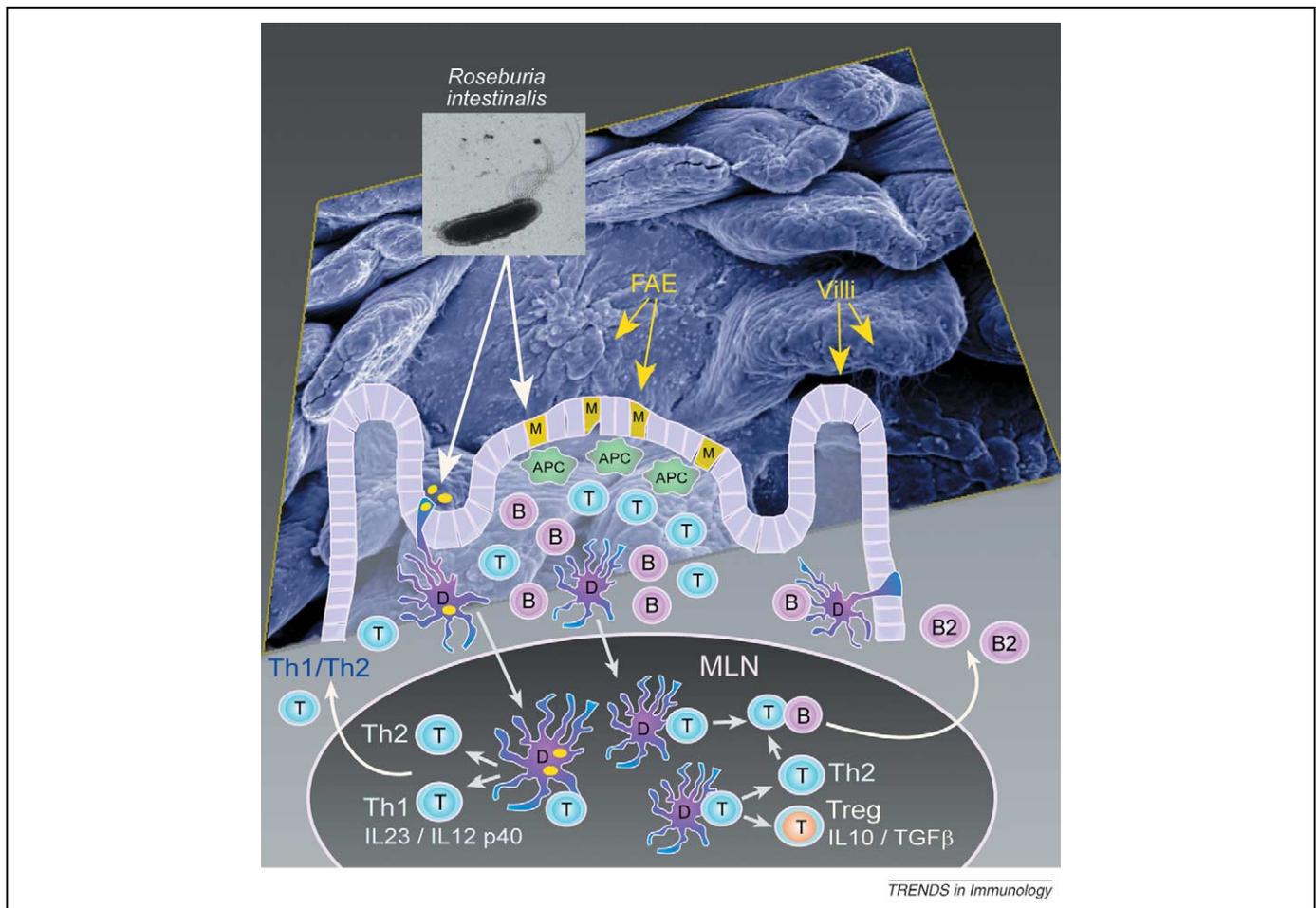


Figure 3. Commensal bacteria and interactions with mucosal DCs and T cells. Mucosal DCs can sample commensal bacteria directly from luminal contents or following translocation across M cells. Bacterial loaded DCs traffic to MLN, where they interact with T and B cells to induce T cell differentiation and IgA secretion. Specific DC subsets are capable of inducing Th1, Th2 and Tregs. In the healthy gut, Th1 and Th2 responses are carefully regulated by Tregs, expressing IL-10 and TGF- β . Abbreviations: APC, antigen-presenting cell; B, B cell; FAE, follicle-associated epithelium; M cell, microfold cell; MLN, mesenteric lymph node; T, T cell.

alterations observed in these cell populations in IBD patients [85]. The specific contribution of individual species of commensal bacteria towards mucosal tolerance and the differentiation of Treg cells is difficult to dissect but further study might unravel their importance in this immune function.

Human disease and commensal bacteria as therapeutics

Many clinically relevant diseases have been linked with dysfunctional immune responses directed against the commensal microflora. Such aberrant immune responses, in conjunction with genetic predisposition, contribute to the pathogenesis of IBD [87]. Furthermore, the hygiene hypothesis, which attempts to explain the rising incidence of atopic, autoimmune and inflammatory diseases among the human population, postulates that the innate immune response to commensal bacteria influences the adaptive response to both food and environmental antigens [88]. Hence, an imbalance between aggressive and protective bacterial species, or loss of gut bacteria that promote tolerance and Treg-cell polarization, could lead to excessive Th1 or Th2 responses, thus promoting inflammatory or autoimmune diseases or allergic diseases, respectively.

Targeting the immune system of the human gut with live bacterial probiotics, bacteria with health-promoting properties, could provide benefit for the treatment of both acute and chronic intestinal diseases, including IBD. The use of probiotic products, such as VSL#3, *Escherichia coli* Nissle 1917 and *Lactobacillus* GG, for the treatment of Crohn's disease, ulcerative colitis and pouchitis, is attracting increasing interest, although much more clinical data are necessary before these are accepted therapeutics [88]. Pure products derived from commensal bacteria might be more applicable in the clinical setting. Progress in identifying such bacterial effector molecules is likely to be rapid with the available commensal genomes and metabolomes [3,4]. Delivery of such effector molecules to appropriate target cells might alleviate inflammation. The possibility of targeting intracellular signalling pathways, including NF- κ B and/or mitogen-activated protein kinase (MAPK) cascades, at distinct points in the cascade, from the cytosol to nucleus, might overcome global immunosuppressive effects associated with current therapies. With the established link between inflammation and colorectal cancer, therapies based on alleviating inflammation will undoubtedly have a major impact on the prevention and treatment of many intestinal diseases.

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