Molecular Interactions Between Bacteria, the Epithelium, and the Mucosal Immune System in the Intestinal Tract: Implications for Chronic Inflammation

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Abstract
In the last few years, advances in immunology, metabolomics and microbial ecology have shown that the contribution of the intestinal microbiota to the overall health status of the host has been so far underestimated. In this context, intestinal epithelial cells play a crucial role in the maintenance of intestinal homoeostasis. Indeed, at the interface between the luminal content and host tissues, the intestinal epithelium must integrate pro- and anti-inflammatory signals to regulate innate and adaptative immune responses, i.e. to control inflammation. However, under the influence of environmental factors, disturbance of the dialog between enteric bacteria and epithelial cells contributes to the development of chronic inflammation in genetically susceptible hosts. The present review covers the state of knowledge of the host response, especially in intestinal epithelial cells, to enteric bacteria, including colitogenic and probiotic bacteria. It also seeks to give an overview of potential regulatory mechanisms involved in the maintenance of intestinal homeostasis, and discusses the clinical implications for inflammatory bowel diseases.

Introduction

The mucosa and the lumen of the mammalian gastrointestinal tract harbor complex communities of bacteria. These enteric micro-organisms, often referred to as the indigenous or normal microbiota, belong to approximately 1000 species, the population size and distribution of which is variable along the gastrointestinal tract. Although the host has evolved various tolerogenic mechanisms allowing a peaceful and productive coexistence with its enteric microbiota, it remains highly responsive to enteropathogens. This discriminatory ability of the intestine toward its indigenous microbiota represents a pivotal feature of efficient tolerance and homeostatic mechanisms.

Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), are spontaneously relapsing, immunologically mediated disorders of the intestinal tract. Homeostasis (tolerance) versus chronic intestinal inflammation is determined by either a regulated or an uncontrolled response of the host to the constant antigenic drive of enteric bacteria. In the genetically susceptible host, an ineffective mucosal barrier function and the lack of appropriate mechanisms to terminate mucosal immune responses (loss of immunologic tolerance) result in continuous stimulation of the mucosal immune system, with chronic inflammation as a consequence.

Although numerous studies have detailed the cell-mediated mucosal immune response in various animal models of chronic intestinal inflammation and in human IBD, very little is known about the molecular mechanisms of bacteria-specific cross-talk at the mucosal surfaces with respect to the development of chronic intestinal inflammation in the genetically susceptible host. In the present review, after describing key players of innate and adaptative immune responses in the intestine, we focus on new insights into mechanisms underlying host-bacteria interaction in the context of intestinal inflammation.

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Defense mechanisms of the gut mucosal immune system

The gut mucosal immune system is a two-part defense system that consists of highly structured sites for the initiation of immune responses and of diffused effector cells in the lamina propria and the epithelium. Foreign antigens are encountered and taken up into gut-associated lymphoid tissue (GALT) (e.g. Peyer's patches), lymphoid nodules lining the appendix and isolated follicles in the small and large intestine. These highly organized secondary lymphoid tissues represent the inductive sites of the mucosal immune system and trigger antigen-specific effector responses. Antigen-activated B and T cell populations emigrate from the inductive sites via lymphatic drainage to mesenteric lymph nodes, circulate through the blood stream, and home to mucosal effector sites. These effector sites comprise antigen-specific T and B lymphocytes, differentiated plasma cells, macrophages, dendritic cells (DC) as well as eosinophils, basophils and mast cells. Together, the inductive and effector sites of the mucosal immune system produce mucosal and serum antibody responses, T cell-mediated immunity, local immunostimulatory or immunosuppressive mediators as well as systemic anergy (Macpherson and Harris, 2004; Mowat, 2003).

Peyer's patches, mesenteric lymph nodes, and the lamina propria

Peyer's patches are dome-like structures, which density is highest in the terminal ileum. The specialized follicle-associated epithelium contains 10–20% membranous or microfold (M) cells and separates the dome area of Peyer's patches from the enteric lumen. Although M-cells differentiate from enterocytes under the influence of B cell-derived lymphotoxin (LT)-$\alpha 1\beta 2$, they lack surface microvilli and the intestinal epithelial cell (IEC)-associated mucus layer. M-cells are adapted to uptake and transport luminal antigens, including antigens from enteropathogenic and commensal bacteria as well as food-derived antigens, to the subepithelial dome area, where processing of antigen and induction of antigen-specific immune responses

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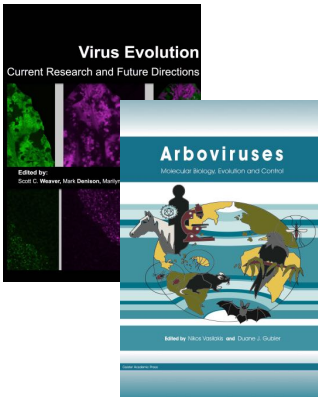

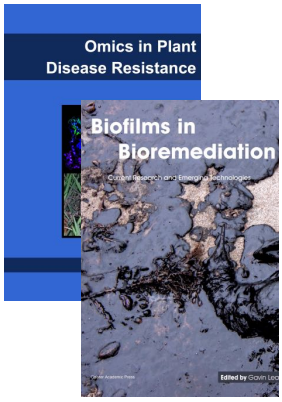

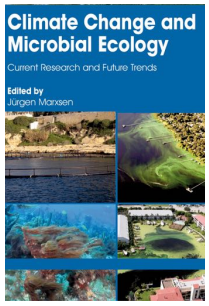

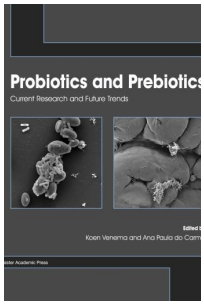

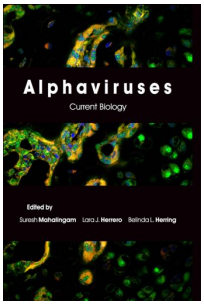

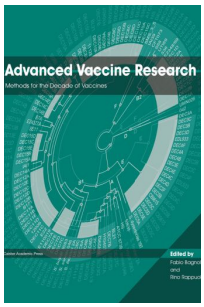

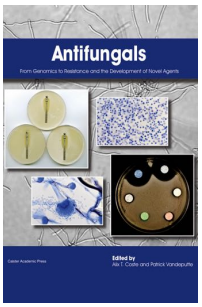

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occur. The subepithelial dome area contains follicles with germinal centers (B cell zone) and parafollicular regions enriched with T cells, macrophages and dendritic cells. The formation of germinal centers in gut-associated lymphoid follicles depends on the presence of luminal antigens, especially those of microbial origin, and infiltrating LT-α1β2-producing CD3$^-$CD4$^+$ progenitor cells. The majority of B cells in the germinal centers, where B cell immunoglobulin (Ig) class switching and affinity maturation occur, are IgA positive, suggesting that the mucosal B-cell response in the GALT is predominantly committed to protective IgA production. Local factors including transforming growth factor β (TGF-β) and interleukin 10 (IL-10) as well as cellular signals delivered by dendritic cells and CD4$^+$CD40L$^+$ T cells contribute to the isotype switch from IgM to IgA positive B cells and to the rescue of these effector cells from deletion by apoptosis (Kraehenbuhl and Neutra, 2000; Neutra *et al*., 2001).

The crossroad between peripheral and mucosal recirculation pathways are mesenteric lymph nodes (MLN). The accumulation of antigen-primed lymphocytes from Peyer's patches in MLNs requires the presence of L-selectin and α4β7 integrin. These adhesion molecules normally direct lymphocytes to enter either the peripheral circulation or the gut mucosa, respectively. LTα-deficient mice that completely lack MLNs fail to induce oral tolerance as well as specific IgA responses, demonstrating the important role of MLNs in mucosal and peripheral immune homeostasis (Brandtzaeg *et al*., 1999).

Lymphocytes that enter the gut mucosa redistribute in the lamina propria and the intestinal epithelium. CD4$^+$ T cells which remain in the lamina propria are largely unresponsive to T cell receptor (TCR)-mediated proliferative signals but contribute to the regulation of immune homeostasis through the production of membrane-bound or soluble factors, including cytokines. There are two subgroups of cytokines: Th1 cytokines, including IL-2, interferon γ (IFN-γ) and tumor necrosis factor (TNF), and Th2 cytokines, including IL-4, IL-5 and IL-10. Th1 and Th2 cytokines are associated with cell-mediated and humoral immunity, respectively. B cell blasts that enter the lamina propria mature into IgA-producing plasma cells including a primitive T-cell-independent B cell population (Macpherson *et al*., 2000). Secreted dimeric IgAs that are transported across the epithelium into the intestinal lumen by the polymeric Ig (pIg) receptor contribute to the non-inflammatory protective mucosal immune response (Fagarasan and Honjo, 2004; Williams, 2004).

Depending on the expression of αEβ7 integrin, the majority of CD8$^+$ T cells (ca. 60%) preferentially migrates to the epithelium through recognition of E-Cadherin at the basolateral membrane of enterocytes. These CD8$^+$ intraepithelial lymphocytes reside at the basal part of the epithelium lying on the basement membrane below the inter-epithelial junctions and represent an important cytotoxic effector population that can eliminate virus-infected IEC. Intraepithelial lymphocytes largely consist of both $\alpha\beta$- and $\gamma\delta$-TCR positive CD3$^+$ T cells that help to maintain appropriate immunological homeostasis and barrier function in the intestinal epithelium (Beagley and Husband, 1998).

Soluble factors from the epithelium, the lack of macrophage-derived co-stimulatory surface molecules and thiol-mediated redox regulation may contribute to the hypoproliferative status of lamina propria T cells (Christ *et al*., 1997; Haller *et al*., 2002b; Sido *et al*., 2000). The unresponsiveness of lamina propria T cells to commensal bacterial antigen can be reversed by the depletion of the anti-inflammatory mediators TGF-β and IL-10 (Khoo *et al*., 1997). The presence of regulatory T cells including TGF-β-producing CD4$^+$ Th3-cells, IL-10-producing CD4$^+$ Tr1-cells, CD4$^+$CD25$^+$ regulatory T cells, CD8$^+$ suppressor T cells and $\gamma\delta$ T cells are believed to contribute to intestinal immune homeostasis and local tolerance (Allez and Mayer, 2004). Of importance for the physiologic relevance of tolerance induction, Mayer *et al*. found that IBD patients failed to develop peripheral T cell hyporesponsiveness after oral treatment with the keyhole limpet hemocyanin (KLH) antigen, suggesting that control of mucosal immune responses is impaired in patients with UC and CD (Kraus *et al*., 2004).

The intestinal epithelium

The intestinal epithelium is a selective barrier between the luminal gut environment and underlying lamina propria immune cells. It consists of a single layer of epithelial cells that are specialized in the formation of junctional complexes and undergo a rapid and continuous renewal from pluripotent stem cells located at the base of the crypts. Epithelial cells include absorptive enterocytes (90–95%), mucus-secreting goblet cells, hormone-secreting enterochromaffin cells and Paneth cells which synthesize antimicrobial peptides and proteins. The anatomical structure of the epithelial layer is complex, with differences between the small and large intestine. In the small intestine, the formation of five to ten finger-shaped villi per crypt and the formation of microvilli on the luminal plasma membrane of differentiated enterocytes increase the absorptive epithelial surface (Falk *et al*., 1998; Gordon *et al*., 1997).

IEC are considered to be constitutive components of the mucosal immune system and to participate in innate and adaptive defense mechanisms. Indeed, IEC contribute to the initiation and regulation of the mucosal immune response to enteric bacteria by directly interacting with lamina propria dendritic cells and intraepithelial lymphocytes (Neutra *et al*., 2001; Niess *et al*., 2005). IEC constitutively express, or can be induced to express, costimulatory molecules and components of the human major histocompatibility complex (MHC) including class II, classical I and nonclassical class Ib MHC molecules. Moreover, proinflammatory stimuli (e.g. TNF and IL-1) and certain enteric pathogens (e.g. *Salmonella* spp., *Yersinia enterocolitica* and enteropathogenic *Escherichia coli*) induce the expression and secretion of a wide range of inflammatory and chemoattractive cytokines in IEC, including TNF, IL-8, MCP-1, IP-10, GROα, iNOS, COX-2 as well as the adhesion molecule ICAM-1 and defensins (Neish, 2002; Sansonetti, 2004). Together with non-immunological barrier functions such as intestinal motility, mucus secretion and cell turn-over, the regulation of IEC integrity is a key element for the mucosal defense system.

Physiological and pathological impact of host–microbiota interactions in the intestine

Savage and Dubos proposed that the enteric microbiota comprises micro-organisms natively colonizing the intestine (autochthonous populations) and transient micro-organisms (allochthonous populations) (Savage *et al*., 1968). The normal microbiota does not establish spontaneously. Instead, certain micro-organisms colonize particular regions of the gastrointestinal tract at various times after birth in a host-specific manner (Mackie *et al*., 1999; Rawls *et al*., 2006). The use of gnotobiotic animals has shown that bacteria have a profound impact on the anatomical, physiological and immunological development of the host, including effects on IEC functions and on the composition of the diffuse GALT (Cebra, 1999; Falk *et al*., 1998; Shroff *et al*., 1995).

Backhed *et al*. found that conventionalization of germfree mice led to a 60%-increase in body fat content (Backhed *et al*., 2004). This was associated with peroxisome proliferator-activated receptor (PPAR)-α-independent down-regulation of the fasting-induced adipocyte factor (Fiaf), as shown by quantitative mRNA analysis in laser-dissected ileal epithelial cells and by experiments in PPAR and Fiaf knockout mice. Also, the intestinal production of short chain fatty acids depends on bacterial enzymatic activities (Falk *et al*., 1998). Of note, butyrate oxidation contributes to up to 70% of energy intake in IEC. In addition, butyrate has been studied for its ability to inhibit the pro-inflammatory nuclear factor (NF)-κB signaling pathway (Segain *et al*., 2000). At the IEC level, the colonization of germfree mice with *Bacteroides thetaiotaomicron*, a prominent anaerobic Gram-negative commensal species in the human and mice intestine, induced the expression of a set of genes that contribute to mucosal barrier function, nutrient absorption, xenobiotic metabolism, differentiation, defense and angiogenesis. Interestingly, the gut epithelium response to colonization with *B. thetaiotaomicron* differed from the response to colonization with *E. coli*, *Bifidobacterium infantis* or a complete conventional microbiota, supporting the concept of bacteria-specific factors in the cross-talk to the host epithelium (Hooper *et al*., 2001). There is accumulating evidence that the collaboration between enteric bacteria and IEC contributes to the development of the intestinal ecosystem by modifying epithelial cell functions. For instance, the colonization of germfree mice with *B. thetaiotaomicron* induced specific host fucosylated glycoconjugate production with subsequent changes in the species ability to colonize the intestine (Hooper *et al*., 1999). In another study, the same species induced the expression of the matrix metalloprotease matrilysin, which activates antimicrobial peptides or prodefensins in the mucosal epithelium (Lopez-Boado *et al*., 2000). Recently, Cash *et al*. also found that colonization of germfree mice with an intestinal microbiota from conventionally raised mice triggered the expression of the regenerating gene RegIIIγ in Paneth cells at both mRNA and protein levels (Cash *et al*., 2006). RegIIIγ is a secreted C-type lectin with antibacterial properties, the expression of which is possibly increased in IBD (Ogawa *et al*., 2003). Conversely, reduced levels of α-defensins were found in ileal tissues from CD patients (Wehkamp *et al*., 2005).

It is also clear that the immunological responses of conventional animals differ greatly from those of germfree animals. Indeed, in germfree animals, the number and cytolytic activity of intraepithelial lymphocytes, in particular $\alpha\beta$ TCR-bearing T cells, are reduced. Moreover, germfree animals are characterized by lamina propria lymphocytes that are less abundant and less reactive to mitogens (Freter and Abrams, 1972). Microscopically, lymphoid aggregates such as Peyer's patches are small and poorly developed in the intestine of germfree animals (Rothkotter and Pabst, 1989). Early work by MacDonald and Carter showed that intestinal bacteria were required to mount a delayed-hypersensitivity (DTH) reaction in mice, suggesting that the presence of enteric bacteria influences peripheral T cell function (MacDonald and Carter, 1979). Functional proof for the importance of the indigenous microbiota in establishing a mucosal lymphocyte population was shown in severe-combined immunodeficient (SCID) mice reconstituted with mature thymus-derived T cells (Camerini *et al*., 1998). Recently, Kasper *et al*. showed that bacterial polysaccharide structures from *Bacteroides fragilis* NCTC 9343 trigger cellular and physical maturation of the mucosal immune system in the gut through mechanisms that involve polysaccharide processing and presentation by MHC class II molecules (Mazmanian *et al*., 2005).

Thus, the complex interaction between non-pathogenic bacteria, the epithelium and professional immune cells in the mucosa is a prerequisite for the development of mature immune functions and defense mechanisms in the gut. To trigger the development and maturation of the gut-associated immune system, enteric bacteria mediate pro-inflammatory processes which are tightly controlled by the host and often referred to as physiologic inflammation (Fig. 1). Yet, the intestinal microbiota is also involved in chronic inflammation (Sartor, 2006). In genetically susceptible hosts, environmental stimuli such as bacterial infections or medication may disrupt homeostatic bacteria–host interaction at the epithelium level and eventually contribute to the loss of controlled pro-inflammatory processes and to the development of chronic inflammation. A basic approach to assess the contribution of bacteria to IBD is to describe intestinal luminal and mucosal microbiota under conditions of inflammation.

Features of intestinal microbiota in IBD patients and animals models of IBD

Pathogens such as *Mycobacterium* spp. have been investigated for their possible role in the development of IBD (Sanderson *et al*., 1992). However, since given pathogens do not systematically occur in IBD patients, recent effort has been put into analyzing intestinal microbiota at taxonomic levels higher than single species. An important finding is the decrease observed in fecal bacterial diversity in IBD subjects. A lower diversity of members of the *Bacteroides fragilis* subgroup and the *Firmicutes* phylum was observed in a metagenomic library obtained from pooled feces of six CD patients in remission (Manichanh *et al*., 2006). The lower diversity of *Firmicutes* was confirmed by fluorescence *in situ* hybridization (FISH) experiments in which proportions of

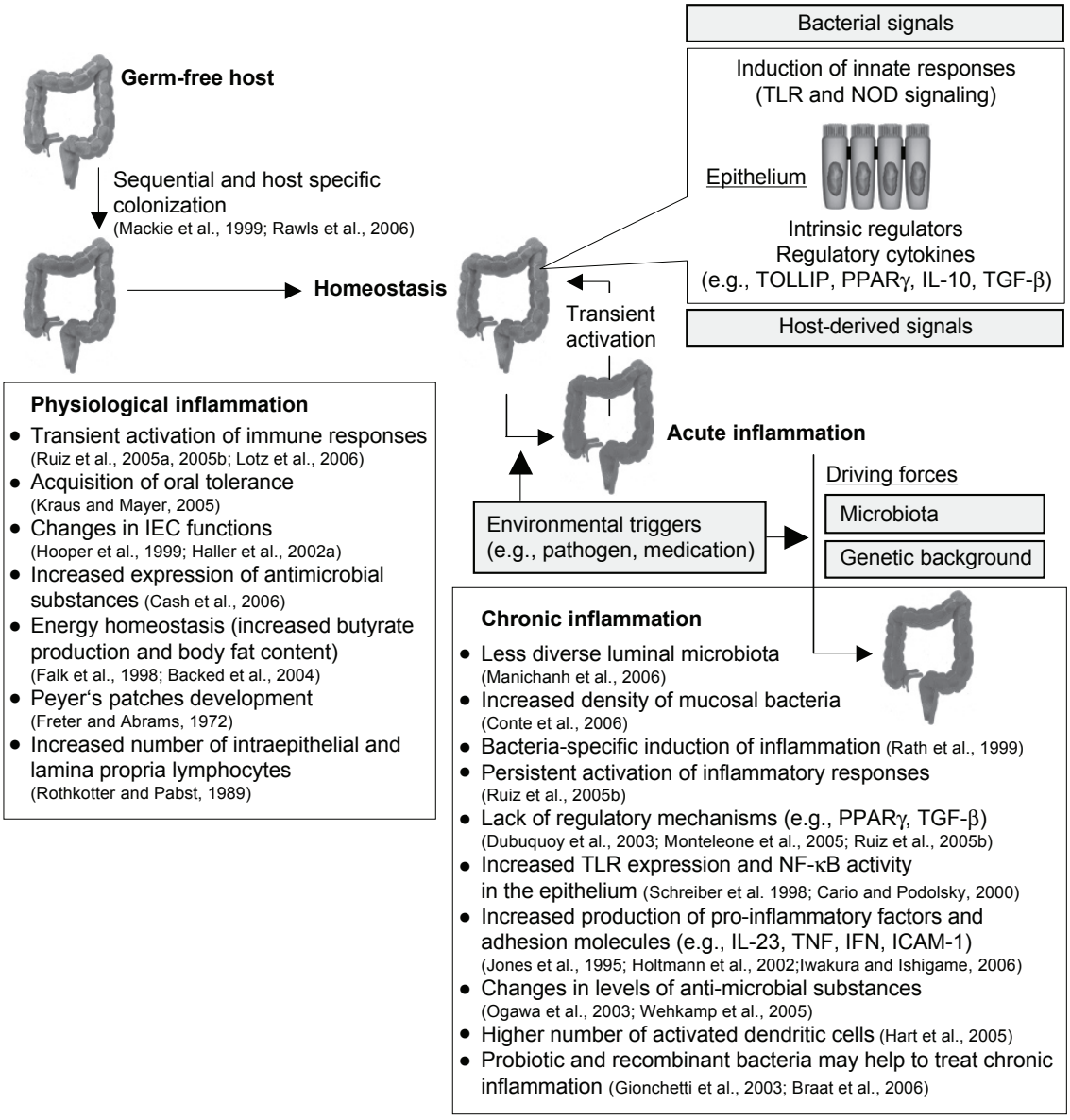

Fig. 1. Features of physiological and chronic inflammation in the intestine. Intestinal inflammation is driven by interrelated innate and adaptative immune signals. The shift from intestinal homeostasis to chronic inflammation is a long-term process controlled by environmental factors, e.g. infections, antibiotic treatments and dietary components, by enteric bacteria and by the host genetic background. The figure illustrates acknowledged trends. The list of findings and references cited is not exhaustive. Abbreviations: ICAM, intercellular adhesion molecule; IEC, intestinal epithelial cells; IFN, interferon; IL, interleukin; NF-κB, nuclear factor κB; NOD, nucleotide-binding oligomerization domain; PPAR, peroxisome proliferator-activated receptor; TGF, transforming growth factor; TLR, Toll-like receptor; TNF, tumor necrosis factor; TOLLIP, Toll-interacting protein.

the *Clostridium leptum* group dropped from ca. 25% in control subjects to <10% in CD patients. In contrast, a growing number of culture-based and molecular studies reports higher numbers of mucosa-associated bacteria, such as *Bacteroides* spp. and *Enterobacteriaceae*, in patients with IBD, including pediatric patients and children (Conte *et al*., 2006; Darfeuille-Michaud *et al*., 2004; Kleessen *et al*., 2002; Swidsinski *et al*., 2002). Nonetheless, using temporal temperature-gradient gel electrophoresis, Lepage *et al*. (Lepage *et al*., 2005) found that mucosa-associated bacterial communities in 20 CD and 11 UC patients were not dominated by a few species only, implying that inter-individual differences in species diversity are still observed in IBD patients. Altogether, it is proposed that intestinal microbiota in IBD patients are affected by global changes that go beyond the simple presence or absence of specific organisms. These changes include re-distribution of bacterial communities, e.g. appearance of more densely populated mucosa-associated bacteria. Less diverse fecal microbiota in IBD patients may be characterized by a deficit in functional redundancy, i.e. specific functions may not be any longer expressed by distinct organisms, which possibly enhances the instability of the intestinal ecosystem.

Animal models of experimental colitis such as interleukin-2- or -10-deficient (IL-$2^{-/-}$ or IL-$10^{-/-}$) mice and

HLA-B27 transgenic rats are crucial to study the influence of specific genetic backgrounds on the development of both intestinal microbiota and inflammation. Moreover, invasive techniques can be more easily used in mice than in human patients to assess mucosa-associated bacteria. However, the murine intestinal microbiota is still poorly described and few studies have analyzed the intestinal microbiota in mouse models of IBD.

In colonic mucosal samples of IL-2$^{-/-}$ mice (C57BL/6 H129/Ola background), 16S rRNA gene sequence analysis showed that *E. coli* clones accounted for 31% to 48% of total clones, whereas no such clones were found in wild type control animals (three mice in each group; 51 to 58 clones analyzed per animal) (Schuppler *et al*., 2004). Using FISH, the same authors found that up to 10% of the mucosa-associated bacteria were hybridized by an *Enterobacteriaceae*-specific oligonucleotide probe. In IL-10$^{-/-}$ C57BL/6 mice and control mice without colitis (Swiss Webster and inducible nitric oxide synthetase-deficient C57BL/6 mice), Pena *et al*. (Pena *et al*., 2004) analyzed the diversity of lactobacilli species isolated from tissue samples. The intestine of animals without colitis harbored a variety of species, including *L. reuteri*, *L. murinus*, *L. johnsonii*, *L. vaginalis*, *L. intestinalis* and *L. paracasei*. On the contrary, all strains isolated from IL-10$^{-/-}$ mice belonged to the species *L. johnsonii*. Thus, populations of lactobacilli seem to be very affected by inflammation in IL-10$^{-/-}$ mice. In parallel with the decrease in fecal species diversity observed in IBD patients, FISH counts in colonic contents of IL-10$^{-/-}$ C57BL/6 mice were significantly lower than in wild type mice (three mice in each group) (Swidsinski *et al*., 2005). However, an increase in the number of bacteria within crypts occurred under inflammation, which agrees with the idea of more densely populated mucosa-associated bacterial communities in IBD patients. Finally, the study of early state of inflammation may be crucial to understand the role of bacteria in IBD, e.g. to identify bacterial types that are becoming dominant in an environment that is losing tolerance to indigenous microbes. In this context, animal models can be used to follow changes in bacterial diversity as inflammation progresses. Using denaturing-gradient gel electrophoresis in colonic contents of five IL-10$^{-/-}$ 129 Sv/Ev mice that had been associated with specific-pathogen free (SPF) microbiota of wild type mice, Bibiloni *et al.* (Bibiloni *et al*., 2005b) noted changes in bacterial diversity as the histological score of inflammation increased after one, three and six weeks of association. Members of the *Lactobacillus acidophilus* group tended to become subdominant in IL-10$^{-/-}$ mice, while the rRNA amount related to *Bifidobacterium animalis* and the prevalence of bands corresponding to enterococci increased.

It is important to remember that microbes include organisms other than bacteria, such as fungi and viruses, the involvement of which in intestinal homeostasis and in the development of IBD has not yet been investigated. For instance, the intestinal tract of SPF C75BL/6 mice harbors diverse and abundant fungal populations that might colonize the mucus layer (Scupham *et al*., 2006). In humans, fungal populations have only been analyzed in a limited number of patients with pouchitis (n = 15) who received a probiotic therapy (Kuhbacher *et al*., 2006). Thus, the ecological role of fungal populations in the human intestine remains unclear. Concerning viruses, Breitbart *et al*. (Breitbart *et al*., 2003) found that viral diversity in human feces is high, and bacteriophages were detected in the mucosa of both healthy and IBD subjects (P. Lepage, unpublished).

To better understand mechanisms underlying inflammation, it is necessary to go beyond the description of intestinal bacterial communities under chronic inflammation. For instance, it is crucial to assess changes in immune signaling pathways triggered by association with specific bacterial strains or by consumption of probiotic strains.

Colitogenic effects of specific enteric bacteria on the development of chronic intestinal inflammation

In healthy individuals, the balance between tolerance to indigenous microbes and protective immune responses to enteropathogens is an intriguing immunological paradox, which is broken under conditions of chronic intestinal inflammation (Duchmann *et al*., 1995). The selective colonization of germfree rodent models of experimental colitis with non-pathogenic bacteria shows that all enteric bacteria are not equal in their ability to induce chronic inflammation, suggesting the presence of specific colitogenic enteric bacteria in the genetically susceptible host.

An early study in gnotobiotic guinea pigs showed that *Bacteroides vulgatus* TUSVM40G2–33, isolated from the cecum of carrageenan-treated pigs, played an important role in carrageenan-induced colitis (Onderdonk *et al*., 1981). The same strain triggered colitis in gnotobiotic HLA-B27 transgenic rats, whereas no pathological response to an *E. coli* strain isolated from a patient with active CD was observed (Rath *et al*., 1999). Conversely, in IL-2$^{-/-}$ mice, *B. vulgatus* mpk, isolated from SPF IL-2$^{-/-}$ mice with colitis, showed protective effects on the development of experimental colitis induced by *E. coli* mpk (serogroup H8) (Waidmann *et al*., 2003). Furthermore, *Enterococcus faecalis* and *E. coli* were identified as particularly important for the induction of colitis in gnotobiotic IL-10$^{-/-}$ mice. For instance, IL-10$^{-/-}$ 129/SvEv mice developed experimental colitis after 12–16 weeks of association with an undefined strain of *E. faecalis* (Balish and Warner, 2002). Also in IL-10$^{-/-}$ 129/SvEv mice, monoassociation with a human oral isolate of *E. faecalis* (strain OG1RF) and a murine strain of *E. coli* (randomly isolated from wild-type mice raised in SPF conditions) triggered experimental colitis with distinct kinetics and anatomic distribution, supporting the hypothesis that various colitogenic bacteria may contribute to variable disease phenotypes in IBD patients (Kim *et al*., 2005). Interestingly, different inbred rodent strains exhibit differential susceptibility to immune-mediated colitis. The impact of inheritable factors on disease expression was shown in IL-10$^{-/-}$ mice on three genetic backgrounds with different MHC alleles. Colitis developed earlier and was more severe in 129/SvEv (H-2b) and BALB/c (H-2d) strains than in the C57BL/6 (H-2b) strain (Berg *et al*., 1996). These results show that genetic factors strongly influence the susceptibility to intestinal inflammation. In wild type animals, the absence of experimental colitis and pathological immune responses to any of the bacteria

mentioned above demonstrates the apathogenic nature of indigenous microbiota and, most importantly, suggests that the normal host develops immunosuppressive mechanisms to control the constant challenge of the immune system with antigens from commensal micro-organisms.

Protective effects of bacteria on the development of chronic intestinal inflammation

Intestinal bacteria not only have the ability to induce intestinal inflammation, but also mediate beneficial activities. Probiotics are live micro-organisms, which, when administrated in adequate amount, have possible beneficial effects on the host, including the prevention of chronic intestinal inflammation. Although the specific properties of probiotic micro-organisms are not yet characterized and validated in well-designed multicenter clinical trials, several human and animal studies show therapeutic relevance.

Madsen *et al*. (Madsen *et al*., 1999) found that two-week-old SPF IL-10$^{-/-}$ 129 Sv/Ev mice displayed changes in bacterial colonization with increased colonic mucosal aerobic adherent and translocated bacteria in conjunction with reduced levels of lactobacilli. Rectal administration of a *Lactobacillus reuteri* strain isolated from 129 Sv/Ev control mice enhanced mucosal barrier function and attenuated the development of colitis at four weeks of age. Similar protective effects were demonstrated in a rat model of methotrexate-induced enterocolitis after oral administration of *Lactobacillus plantarum* DSM9843 (Mao *et al*., 1996). Treated animals were characterized by a decrease in body weight loss, in intestinal permeability and in myeloperoxidase levels. In addition, *Lactobacillus rhamnosus* GG ATCC53103 inhibited cytokine-induced apoptosis in IEC lines by activating the Akt/protein kinase B signaling pathway (Yan and Polk, 2002), supporting the concept that probiotic bacteria may help to maintain the barrier function of the intestinal epithelium. A mechanistic role for loss in barrier function in the pathogenesis of mucosal inflammation is shown in N-Cadherin-dominant-negative mice (Hermiston and Gordon, 1995) and mice with disruption of the multidrug resistance gene 1a (mdr1a$^{-/-}$) (Panwala *et al*., 1998). In the latter model, chronic inflammation is most probably due to defective IEC rather than defective lymphocyte function, since irradiated mutant mice that were reconstituted with bone marrow cells from wild-type donors developed disease, whereas normal mice reconstituted with bone marrow cells from mdr$^{-/-}$ donors did not. An additional molecular mechanism of probiotic activity was shown at the level of Epidermal Growth Factor (EGF) receptor signaling. *Lactobacillus acidophilus* ATCC 4356 and *Streptococcus thermophilus* ATCC 19258 restored EGF receptor phosphorylation (activation) in IEC cultures after infection by the enteroinvasive strain *E. coli* O29:NM (Resta-Lenert and Barrett, 2003). These effects were associated with increased expression of tight junction proteins and with improved barrier function in the model epithelium.

Concerning protective activities of lactic acid bacteria in human IBD, Gionchetti *et al*. showed that the VSL#3 mixture of 8 different lactic acid bacteria including lactobacilli, bifidobacteria and streptococci inhibited relapse of chronic pouchitis, with inhibition of mucosal TNF and up-regulation of IL-10 (Gionchetti *et al*., 2003; Ulisse *et al*., 2001). In UC patients too, the VSL#3 probiotic mixture has been associated with remission of the disease (Bibiloni *et al*., 2005a). Beneficial effects of VSL#3 were also shown in SPF IL-10$^{-/-}$ mice (Madsen *et al*., 2001). VSL#3 treatment resulted in the normalization of physiologic colonic function, barrier integrity and histopathology, in conjunction with a reduction of mucosal TNF and IFNγ secretion. In trinitrobenzene sulfonic acid (TNBS)-treated mice, VSL#3 ameliorated Th1-mediated colitis by inducing IL-10 and IL-10-dependent TGF-β-bearing regulatory T cells (Di Giacinto *et al*., 2005). In IEC, VSL#3 induced cytoprotective heat shock protein expression and blocked proteasome function followed by the inhibition of NF-κB (Petrof *et al*., 2004). Additional mechanistic evidence for the protective effects of VSL#3 was shown at the level of Toll-like receptor (TLR) 9 signaling, using CpG sequences from bacterial DNA for the treatment of dextran sodium sulfate (DSS)-induced experimental colitis in TLR2$^{-/-}$, TLR4$^{-/-}$ and TLR9$^{-/-}$ mice (Rachmilewitz *et al*., 2002).

Beneficial effects of probiotic bacteria on chronic intestinal inflammation are not limited to lactic acid bacteria. For example, *E. coli* Nissle 1917 maintained remission in UC patients, showing protective effects equal to those of mesalamine treatment (Rembacken *et al*., 1999). In mice, it was found that the positive effect of *E. coli* Nissle 1917 on DSS-induced colitis was TLR2- and TLR4-dependent (Grabig *et al*., 2006). Interestingly, *E. coli* Nissle 1917 triggered the induction of β-defensins in IEC cultures through the induction of the NF-κB signaling cascade, which may contribute to enhance innate defense mechanisms in the epithelium (Wehkamp *et al*., 2004). However, *E. coli* Nissle 1917 also induced the NF-κB-dependent proinflammatory chemokine MCP-1 in the epithelial cell line Caco-2 (Ukena *et al*., 2005). Bacteria may also exert beneficial effects thanks to the production of metabolites. In this context, oral administration of the butyrate-producing species *Butyrivibrio fibrosolvens* and *Clostridium butyricum* had beneficial effects on DSS-induced colitis in rodents (Araki *et al*., 2004; Ohkawara *et al*., 2006). Also, microorganisms other than bacteria, such as the fungus *Saccharomyces boulardii*, may prevent the development of IBD (Chen *et al*., 2006; Guslandi *et al*., 2003). In mice, the inhibition of intestinal inflammation by *S. boulardii* was at least partly due to the accumulation of IFN-γ-producing T cells in mesenteric lymph nodes (Dalmasso *et al*., 2006). Finally, genetically engineered bacteria, including *Lactococcus lactis* strains that secrete cytoprotective murine trefoil factors or the immunosuppressive cytokine IL-10, have already shown the potential of future immunotherapy approaches for treatment of IBD (Steidler *et al*., 2000; Vandenbroucke *et al*., 2004). A recent phase I trial showed that the use of IL-10-producing *L. lactis* for mucosal delivery of IL-10 is a feasible strategy in human beings (Braat *et al*., 2006).

Despite these findings, the specific molecular mechanisms underlying colitogenic- and probiotic-bacteria-induced host responses in the intestine are virtually unknown. The inflammatory processes observed in the intestine are the consequence of dysregulated

activation of numerous mucosal cells such as IEC, mesenchymal cells, macrophages, neutrophils, dendritic cells and lymphocytes. Although the contribution of these various cells to the initiation and regulation of IBD is difficult to determine, IEC are likely the primary target of the various luminal constituents present in the intestine.

Mechanisms of bacteria–epithelial cell cross-talk under normal conditions and under chronic intestinal inflammation

Over recent years, it has become evident that IEC are important players in bacteria-induced intestinal host responses (Haller and Jobin, 2004). Various luminal agents, including cell wall bacterial products, adherent and invasive bacteria and cytokines, stimulate IEC to release proinflammatory products (chemokines, cytokines and adhesion molecules). The production and accumulation of these proinflammatory molecules in the vicinity of the mucosa has a dual effect on the inflammatory process. It leads to the activation of adjacent lamina propria mononuclear cells (macrophages, dendritic cells and mast cells) and contributes to the recruitment of peripheral mononuclear and polymorphonuclear cells. Although bacterial distribution and population levels vary throughout the intestine, bacteria and bacterial products are certainly, among others, the most relevant IEC stimuli, given their high content in the intestinal lumen.

Even if bacteria can trigger host responses in many different manners, the cornerstone of innate signaling is initiated by a set of well conserved receptors named Toll-like receptor (TLR) located in the extracellular membrane and by a family of cytosolic nucleotide-binding oligomerization domain (NOD)-like receptors. The combined actions of both sets of receptors play a pivotal role in the detection of various microbial molecular signatures and in the transmission of various signaling cascades that lead to the induction of a complex innate gene program (Fig. 2).

From pattern recognition receptors to the NF-κB transcriptional system

To date, over 10 different TLR and more than 20 NOD-like proteins have been identified, but only a handful have been assigned a specific ligand. For example, lipopolysaccharides (LPS) are recognized by the pattern recognition receptor (PRR) TLR4, whereas Gram-positive bacterial products (e.g. lipoteichoic acid and peptidoglycan), bacterial flagellin and unmethylated CpG DNA are recognized by TLR2, TLR5 and TLR9, respectively (Beutler, 2000; Hayashi *et al*., 2001; Hemmi *et al*., 2000). The primary role of these PRR is the immunosurveillance of the host and, as such, their expression pattern in the lung, in the gastrointestinal tract and within hematopoietic-derived cells correlates with their function (Zarember and Godowski, 2002). TLR activate down-stream target effector systems, including the mitogen-activated protein kinase (MAPK), extracellular activated kinase (ERK), p38 and c-jun NH2-terminal kinase (JNK) pathways and the IκB/NF-κB transcriptional system (Zhang and Ghosh, 2001).

The NF-κB transcription factor plays a key role in the induction of numerous cytokines, chemokines and adhesion molecules, all of which are involved in various inflammatory disorders, including IBD (Pahl, 1999a; Schmid and Adler, 2000). The molecular structure and organization of the cytoplasmic portion of TLR is similar to IL-1R and is named the Toll/IL-1R (TIR) domain (Daun and Fenton, 2000). The extracellular portion of TLR is characterized by a leucine-rich repeat (LRR) domain located at the C-terminal portion of the protein. It seems that the TIR domain of TLR promotes the homophilic interaction with the TIR of the cytoplasmic myeloid differentiation protein 88 (MyD88) (Burns *et al*., 1998), followed by the recruitment of the IL-1-receptor-associated kinase (IRAK-1, 2, 4 or M) (Cao *et al*., 1996; Croston *et al*., 1995; Muzio *et al*., 1997; Suzuki *et al*., 2002; Wesche *et al*., 1999). Recently, a novel TIR domain-containing protein was identified and named TIRAP (TIR-domain containing adapter protein) (Horng *et al*., 2001) or Mal (MyD88-adapter-like) (Fitzgerald *et al*., 2001). This protein binds the TIR of TLR4, but not TLR9, and transduces the signal to NF-κB, independently of MyD88. After assembly of the TLR-induced membrane proximal signaling complex, IRAK is phosphorylated, dissociates from the complex, and recruits both the TNF receptor-associated factor-6 (TRAF-6) (Cao *et al*., 1996; Daun and Fenton, 2000) and the transforming growth factor-β activated kinase 1 (TAK1) (Ninomiya-Tsuji *et al*., 1999). The signal coming from the TRAF6/TAK1 is transmitted to the NF-κB-inducing kinase (NIK), which in turn associates and activates the IκB kinase (IKK) complex (Malinin *et al*., 1997; Stancovski and Baltimore, 1997). This complex is controlled by the structural regulatory protein IKKγ, also known as NF-κB essential modifier (NEMO). IKKγ directs the activation of the catalytic IKKα and IKKβ subunits (Delhase *et al*., 1999), which subsequently phosphorylates IκBα at serine residue 32 and 36 (DiDonato *et al*., 1996). This is followed by the activation of a complex enzymatic system (E1, E2, E3) that add multiple ubiquitin proteins at lysine residues 21 and 22 of phosphor-IκBα. The enzyme responsible for ubiquitin conjugation of phosphorylated IκBα is the $E3RS^{I\kappa B}$ (E3 receptor subunit of IκB) (Delhase *et al*., 2000; Yaron *et al*., 1998). Ubiquitinated IκBα is selectively and rapidly degraded via the non-lysosomal, ATP-dependent 26S proteolytic complex composed of a 700 kDa proteasome. Destruction of IκBα liberates NF-κB and allows its nuclear transmigration. In the nucleus, NF-κB binds to κB-promoter elements and induces gene transcription.

Studies in human IBD have reported increased TLR expression and NF-κB activity in lamina propria macrophages and in the intestinal epithelium under chronic intestinal inflammation (Andresen *et al*., 2005; Cario and Podolsky, 2000; Hausmann *et al*., 2002; Schreiber *et al*., 1998). In TNBS-treated mice, local administration of anti-sense NF-κB RelA oligonucleotides abrogated clinical and histological signs of experimental colitis (Neurath *et al*., 1996b). This also suggests a role for sustained NF-κB activity in the pathogenesis of chronic inflammation. On the other hand, but equally important, the inhibition of NF-κB activity with pharmacological inhibitors during the resolution phase of carrageenan-induced acute inflammation had adverse effects on the host (Lawrence *et al*., 2001). This suggests dual functions of activated

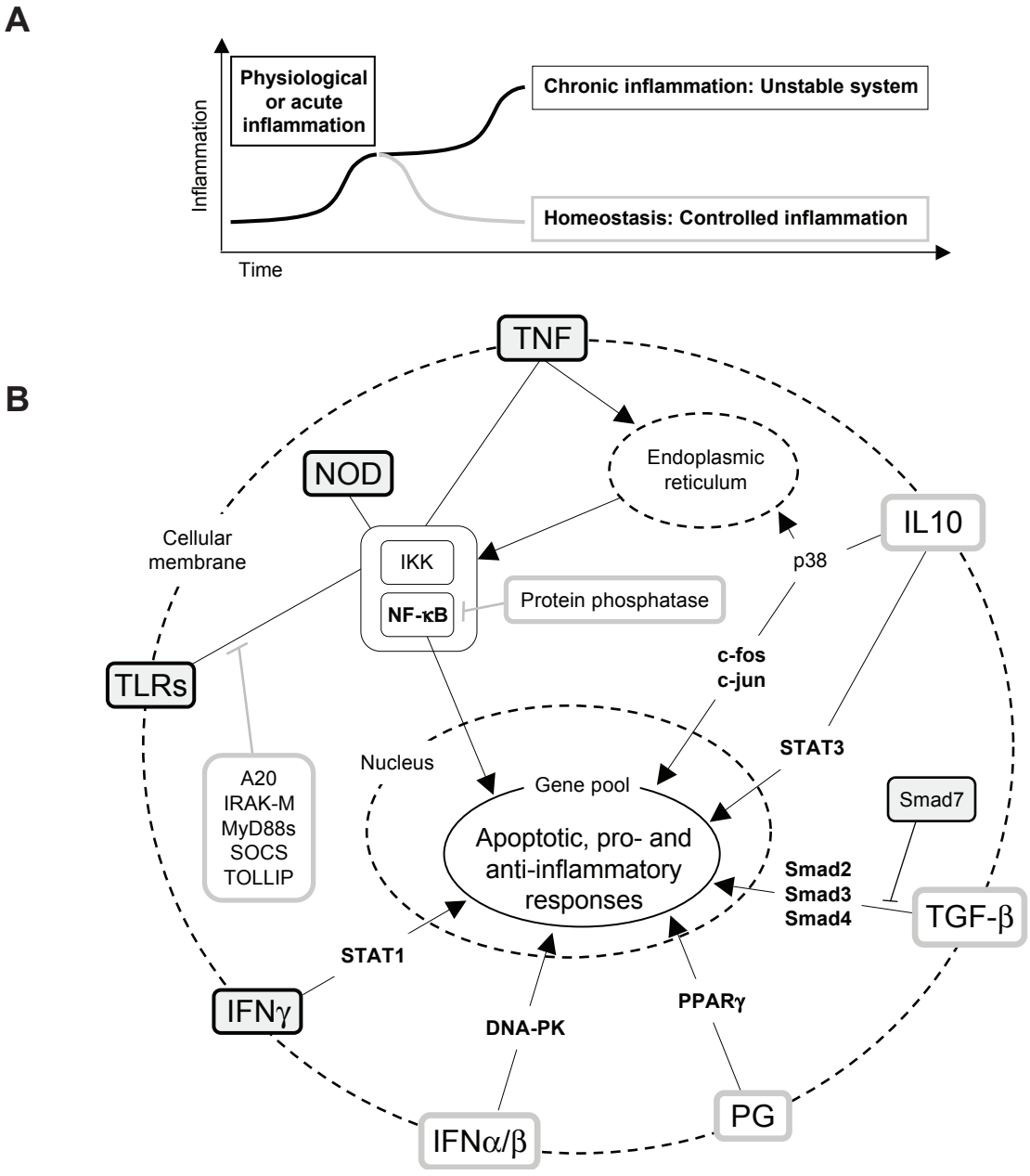

Fig. 2. (A) Time course of intestinal inflammation. After the initial activation of inflammatory responses, intestinal homeostasis is characterized by the equilibrium between defense and regulatory mechanisms. Conversely, pro-inflammatory signals rule the immune response under chronic inflammation. (B) Simplified molecular network of pro- and anti-inflammatory pathways in IEC. Intestinal inflammation is controlled by complex and interacting signaling pathways, whose precise functioning and regulation is still not understood. Grey boxes with a black frame and boxes with a grey frame show defense and regulatory effectors, respectively. The molecules depicted are: A20, cytoplasmic zinc-finger protein also referred to as TNFAIP3; DNA-PK, DNA-dependent protein kinase; IFN, interferon; IL, interleukin; IRAK, IL-1-receptor-associated kinase; IKK, IκB kinase; MyD88s, spliced variant of myeloid differentiation protein 88; NF-κB, nuclear factor κB; NOD, nucleotide-binding oligomerization domain; p38, member of the mitogen-activated protein kinase family; PG, prostaglandins; PPAR, peroxisome proliferator-activated receptor; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; TGF, transforming growth factor; TNF, tumor necrosis factor; TOLLIP, Toll-interacting protein.

NF-κB including protective and detrimental mechanisms during the course of inflammation. Accordingly, the selective ablation of NF-κB activity in IKKβ-deficient IEC sensitized mice to acute ischemia-reperfusion-induced enterocyte apoptosis and was associated with the loss of mucosal integrity (Chen *et al*., 2003). This local intestinal tissue injury is likely due to the failure of IKK to activate an NF-κB-dependent protective gene program that protects IEC against the deleterious effects of intestinal ischemia-reperfusion. These results support the hypothesis that the acute and transient activation of NF-κB may be protective for the host, while sustained and uncontrolled NF-κB signaling in the intestinal epithelium may contribute to the immunopathology of experimental colitis.

Physiological relevance of non-pathogenic-bacteria-induced signaling in IEC

We have shown that *B. vulgatus* induces RelA phosphorylation, NF-κB transcriptional activation and pro-inflammatory gene expression in primary and IEC lines via the TLR4 signaling cascade (Haller *et al*., 2003; Haller *et al*., 2002a). Immunostained intestinal sections of *B. vulgatus* monoassociated rats showed that the induction of RelA phosphorylation was restricted to the epithelium, with no induction in underlying lamina propria immune cells. This implies a compartimentalized activation of NF-κB in the gut mucosa. In addition, Hornef *et al*. showed that LPS from *E. coli* K12 D31m4 were internalized by murine IEC to stimulate the IκB/NF-κB system via intracellular located TLR-4 (Hornef *et al*., 2002), supporting the concept that non-pathogenic Gram-negative bacteria can activate pro-inflammatory signaling processes in the gut epithelium. Also, non-virulent *Salmonella* strains inhibit NF-κB activity by preventing IκB ubiquitination, possibly through inhibition of $E3RS^{IκB}$ (Neish *et al*., 2000). This suggests that some intestinal bacteria have evolved sophisticated mechanisms to down-regulate the host innate immune response by targeting regulatory elements of the NF-κB pathway.

Most important for the pathological relevance of bacteria-epithelial cell signaling, an oral isolate of *E. faecalis* induced transient TLR2-mediated RelA phosphorylation and NF-κB-dependent gene expression in native IEC from wild type mice, but led to persistent activation of the TLR/NF-κB pathway in IL-$10^{-/-}$ mice. After one week of colonization, bacteria-mediated activation of the epithelium preceded any histological evidence of colitis in IL-$10^{-/-}$ mice. However, after 14 weeks, persistently active TLR/NF-κB signaling in IEC of IL-$10^{-/-}$ mice was associated with the development of intestinal inflammation (Ruiz *et al*., 2005b).

Interestingly, *Bifidobacterium lactis* BB12 targeted the TLR2 signaling cascade in primary and IEC lines, showing that probiotic bacteria may also initially trigger innate host responses in the gut epithelium. Similar to the colonization of wild type rodents with *B. vulgatus* and *E. faecalis*, colonization of Fisher F344 rats with *B. lactis* BB12 induced transient NF-κB activation and pro-inflammatory gene expression in the native epithelium (Ruiz *et al*., 2005a). We also found that the colonization of reconstituted lactobacilli free (RLF) mice with *Lactobacillus reuteri* 100–32 trigged a transient activation of a NF-κB-dependent pro-inflammatory gene program (M. Hoffmann *et al*., unpublished). It seems paradoxical that colitogenic and probiotic bacteria signal through the same pattern recognition receptor system to initially trigger pro-inflammatory signaling cascades in IEC, but these results clearly support the hypothesis that the normal host develops hard-wired mechanisms to inhibit persistent activation of IEC. The recognition of non-pathogenic enteric bacteria by the TLR system may even be required for intestinal homeostasis and maintenance of intestinal barrier function. For instance, TLR2-mediated protein kinase C (PKC) signaling enhanced IEC integrity through the induction of the tight junction protein ZO-1 (Cario *et al*., 2004). Additional data on transient activation of pro-inflammatory signals in IEC was recently published by Lotz *et al*. (Lotz *et al*., 2006). The authors showed that primary IEC from SPF C57BL/6 mice acquired postnatal endotoxin tolerance in response to TLR-induced signals. IEC not only became tolerant to LPS but also to pro-inflammatory host-derived signals such as IL-1β and TNF.

Recently, Rakoff-Nahoum *et al*. (Rakoff-Nahoum *et al*., 2006) showed that the development of experimental colitis in IL-$10^{-/-}$ mice was abrogated in the absence of TLR/MyD88-derived signals using IL-$10^{-/-}$ × MyD$88^{-/-}$ mice. The lack of the TLR/MyD88 innate signaling pathway prevented the development of colitis at the level of T cell-mediated adaptive immune responses. In contrast, TLR/MyD88 deficient mice showed increased histopathology in the DSS-induced model of colitis, suggesting protective effects of the TLR/MyD88 signaling cascade at the epithelial cell level (Rakoff-Nahoum *et al*., 2004). This agrees with the fact that TLR4 mutant C3H/HeJ mice are more sensitive to DSS-induced colitis than wild-type mice (Mahler *et al*., 1998; Sundberg *et al*., 1994). In addition, the prevention of allergic responses to food antigens by enteric bacteria has been associated with TLR4-mediated signals (Bashir *et al*., 2004), supporting the hypothesis that the loss of pattern recognition receptor signaling may prevent the host to mount an appropriate innate response leading to dysregulated adaptive immune responses (Schnare *et al*., 2001). Further evidence for protective TLR-mediated effects on experimental colitis was recently shown by Katakura *et al*. (Katakura *et al*., 2005). The authors demonstrated that the induction of TLR9 signaling resulted in the activation of interferon regulated factors (IRF1 and 8) and triggered protective type I IFN (IFN-α/β) production through MyD88- and DNA-dependent protein kinase (DNA-PK)-dependent mechanisms.

Although the purpose of TLR signaling pathways is to alert and protect the host against pathogenic micro-organisms, continuous activation of these pathways, either due to the lack of negative immunoregulatory mechanisms or to persistent stimulation, may lead to chronic inflammation.

Negative regulators of TLR signal transduction and bacteria-mediated IEC activation

The common concept in innate immunity is that Pathogen Associated Molecular Patterns (PAMP) bind to TLR and induce a host response. However, Commensal Associated Molecular Patterns (CAMP) have also the ability to trigger an innate host response through these receptors, without inducing histopathology in the normal gut mucosa, suggesting that sophisticated mechanisms tightly regulate proinflammatory signaling in the intestine and help maintain homeostasis (Cario *et al*., 2002; Haller *et al*., 2003).

Intrinsic negative regulators of TLR signaling

Intrinsic regulatory mechanisms may operate in a negative feedback loop fashion or may be induced by independent signaling cascades that interact with the TLR cascade. As mentioned above, an intricate network of kinases, adapter proteins and scaffolding proteins assures the transmission of TLR signals to various effector signaling cassettes, including the NF-κB signaling machinery. Among the various signaling proteins involved in the regulation of TLR-mediated gene expression, four specific proteins are

critical regulators of innate immune responses: IRAK-M, Toll-interacting protein (TOLLIP), A20 (also referred to as TNF-induced protein 3) and the peroxisome proliferator-activated receptor (PPAR)γ (Fig. 2).

IRAK-M appears to prevent TLR signaling by blocking IRAK1 and IRAK-4 binding to TRAF-6, thereby cutting off signaling to downstream effector targets (Kobayashi *et al*., 2002). IRAK-M$^{-/-}$ mice displayed increased inflammatory responses to bacterial infection, enhanced Peyer's patch number and size in the small intestine and showed higher susceptibility to the toxic effect of LPS. Moreover, after LPS stimulation, macrophages isolated from IRAK-M$^{-/-}$ mice secreted higher amount of the proinflammatory cytokines IL-12, p40, IL-6 and TNF than macrophages isolated from wild-type mice. Since IRAK-M expression is strongly induced by LPS exposure, this regulatory protein may be at the forefront of LPS tolerance. This feedback mechanism may be important to downmodulate LPS response and to prevent constant bacterial stimulation.

The second intrinsic negative regulator of TLR signal transduction is the Toll-interacting protein (TOLLIP). Because interaction between TLR4/MyD88/IRAK is critical to transduce LPS signaling to downstream effector proteins, disruption in this complex impairs signal transduction. Interestingly, TOLLIP was shown to associate with TLR4 and TLR2 and suppress LPS-induced IRAK phosphorylation and activity, thus impairing transcriptional activity of NF-κB and activator protein (AP)-1. Noteworthy, TLR hyporesponsiveness to bacterial ligands in colonic epithelial cell lines was associated with the induction of TOLLIP, suggesting that TOLLIP modulates innate signaling in the intestine (Melmed *et al*., 2003; Otte *et al*., 2004). Whether TOLLIP is essential for the control of intestinal homeostasis remains to be demonstrated using a more definitive approach such as transgenic or gene deleted mice.

PPARγ is a member of the steroid receptor superfamily with various cellular functions including differentiation, apoptosis, lipid metabolism and anti-inflammatory responses (Auwerx, 2002; Blanquart *et al*., 2003). Although PPARγ is expressed in multiple tissues, the highest levels are found in adipose tissue and colonic epithelium (Fajas *et al*., 1997). Ligand-specific activation of the PPARγ transcription factor has been shown to inhibit pro-inflammatory gene expression and experimental colitis (Jiang *et al*., 1998; Ricote *et al*., 1998; Straus *et al*., 2000; Su *et al*., 1999). For example, studies with mice heterozygous for a deficiency of PPARγ (PPARγ$^{/+}$) were significantly more susceptible to the development of experimental colitis than wild type mice (Nakajima *et al*., 2001; Saubermann *et al*., 2002). Accordingly, PPARγ expression in colonic epithelium was substantially reduced in patients with UC (Dubuquoy *et al*., 2003) and in DSS-treated mice (Katayama *et al*., 2003). Interestingly, *B. thetaiotaomicron* triggered PPARγ-mediated nuclear export of transcriptionally active RelA and abolished *Salmonella enteriditis*-induced inflammatory effects in IEC (Kelly *et al*., 2004). Of note, the PPARγ-specific ligand 15-deoxy-$^{\Delta 12,14}$-prostaglandin J_2 triggered protein phosphatase (PP2A) activity in IEC which induced dephosphorylation of *B. vulgatus*-induced phospho-RelA and, as a consequence, inhibited NF-κB-dependent gene expression (Ruiz *et al*., 2004). Although monoassociation of germfree Fisher F344 rats induced PPARγ nuclear expression in native epithelium, the anti-inflammatory effect of 15-deoxy-$^{\Delta 12,14}$-prostaglandin J_2 in IEC was independent on the presence of PPARγ. Nevertheless, SPF TLR4$^{-/-}$ mice failed to trigger PPARγ expression in native IEC, suggesting that the TLR signaling cascade directly modulates nuclear hormone receptor expression (Dubuquoy *et al*., 2003). These results provide compelling evidence that the induction of PPARγ expression in the intestinal epithelium is closely regulated by the presence of the enteric microbiota, and thus may play an important role in the regulation of mucosal inflammation.

The zinc finger protein A20 is a cytokine and LPS inducible molecule involved in the negative regulation of NF-κB activity and TLR signaling (Beyaert *et al*., 2000). Interestingly, the colonization of Fisher F344 rats with *B. lactis* BB12, but not *B. vulgatus*, triggered A20 mRNA expression in primary and IEC lines (Ruiz *et al*., 2005a). While the physiological relevance of bacteria-induced A20 expression in IEC remains to be defined, A20 may negatively regulate IKK activity by binding to TRAF/RIP signaling proteins (Beyaert *et al*., 2000; Zhang and Ghosh, 2000). The preponderant role of A20 in controlling LPS signaling is highlighted by a study in A20 gene deficient mice, which display multiple spontaneous inflammatory disorders including colitis (Dumitru *et al*., 2000). Moreover, LPS-induced NF-κB activity and TNF secretion is strongly enhanced in macrophages isolated from A20$^{-/-}$ mice compared with macrophages from wild-type mice (Boone *et al*., 2003). Interestingly, A20$^{-/-}$ × RAG$^{-/-}$ (recombination activating gene deficient) mice still developed colitis, suggesting that A20 controls innate immunity and LPS responsiveness. The generation of gnobiotic A20$^{-/-}$ mice will help to address the role of the enteric microbiota in triggering intestinal inflammation in this animal model.

Cytokine-mediated regulation of TLR signaling: TGF-β and IL-10

Since the intestinal epithelium is renewed every three to five days, the biological information for immunosuppressive effects in the colonized host should be imprinted in the gene program of pluripotent epithelial stem cells or mediated by the recruited professional immune cells in the lamina propria. In addition to the aforementioned TLR-related mechanisms underlying hypo-responsiveness of the intestinal epithelium towards enteric bacteria, host-derived immune signals are critical in maintaining epithelial cell homeostasis. In this context, IL-10 and TGF-β signaling cascades are of high relevance to IBD.

Powrie *et al*. (Powrie *et al*., 1994) showed the importance of the immunosuppressive mediators TGF-β and IL-10 using SCID and RAG$^{-/-}$ mice. The adoptive transfer of CD4+ CD45RB^{high} T cells from congenic donor mice into T and B cell deficient SCID or RAG$^{-/-}$ mice triggered experimental colitis. The development of chronic inflammation was associated with the production of high amounts of the pro-inflammatory Th1 mediator IFN-γ. In contrast, the adoptive transfer of CD4+ CD45RB^{low} T cells revealed protective mechanisms, depending on the presence of the immunosuppressive mediators TGF-β and IL-10 (Hara *et al*., 2001; Maloy *et al*., 2003).

In accordance with these observations, IL-10$^{-/-}$ mice develop immune-mediated colitis in a specific pathogen free (SPF) environment but remain healthy under germ-free conditions. Thus, in the absence of host-derived immune regulators, bacterial antigens drive inflammatory processes. In addition, the protective mechanisms of IL-10 in TNBS-induced experimental colitis were indirectly mediated through its inductive effect on TGF-β secretion in lamina propria T cells, suggesting an interrelated role of these protective cytokines (Fuss *et al*., 2002; Neurath *et al*., 1996a). Moreover, TGF-β1 deficient mice spontaneously develop colitis (Kulkarni *et al*., 1995) and the over-expression of TGF-β1 in lamina propria immune cells inhibited Th1-mediated experimental TNBS-induced colitis (Kitani *et al*., 2000).

Of importance to understand the biological function of TGF-β at the epithelial cell level, the molecular blockade of TGF-β signaling in tissue specific transgenic mice that express a dominant-negative TGF-β receptors in the intestinal epithelium triggered colitis under conventional conditions (Hahm *et al*., 2001). TGF-β1 mediates its biological effect through activation of various signaling cascades including the Smad and MAPK pathways (Shi and Massague, 2003). The lack of TGF-β-activated Smad signaling in lamina propria T cells of IBD patients due to over-expression of the specific inhibitor Smad7 was associated with disease progression (Boirivant *et al*., 2006; Monteleone *et al*., 2005). Thus, although immunosuppressive mediators may be present in diseased tissues, the intracellular blockade of these protective signals may lead to development of chronic intestinal inflammation.

TGF-β inhibits pro-inflammatory NF-κB signal transduction pathways in IEC

In *B. vulgatus*-monoassociated wild type Fisher F344 rats and in *E. faecalis*-monoassociated wild type SvEv129 mice, we showed that nuclear RelA phosphorylation was followed by the induction of Smad2 phosphorylation in IEC isolated at early stages of bacterial colonization (Haller *et al*., 2003; Haller *et al*., 2002a; Ruiz *et al*., 2005b). Thus, under normal conditions, the presence of NF-κB and TGF-β1 signals in the intestinal epithelium follows bacterial colonization. Interestingly, TGF-β-activated Smad signaling induced rapid TLR2 degradation and blocked CBP/p300-mediated histone phosphorylation in IEC, leading to the inhibition of pro-inflammatory gene expression. Additional evidence for proteasome-mediated degradation of TLRs as a strategy of the host to control pattern recognition receptor signaling was recently shown by Chuang *et al*. (Chuang and Ulevitch, 2004). The authors demonstrated that the intrinsic RING finger protein TRIAD3 enhanced ubiquitination and proteolytic degradation of TLR4 and TLR9 but not TLR2 due to its E3 ubiquitin-protein ligase activity. Hence, various negative feed-back regulators of the TLR signaling cascade may have distinct effects depending on the TLR subsets. Importantly, TGF-β1-induced Smad2 signaling was absent in IEC isolated from *E. faecalis*-monoassociated IL-10$^{-/-}$ mice (Ruiz *et al*., 2005b). This implies that, in the absence of the activated TGF-β/Smad cascade in the intestinal epithelium, bacteria-mediated TLR signaling may lead to the development of chronic intestinal inflammation. In conclusion, we propose that host-derived feed-back mechanisms control epithelial cell responses towards enteric bacteria under normal conditions, but the lack of these protective immune signals is associated with the loss of epithelial cell homeostasis and with the chronic activation of pro-inflammatory immune mechanisms (Haller, 2006).

IL-10 inhibits endoplasmic reticulum stress responses: implications for chronic intestinal inflammation

Adverse environmental and metabolic conditions trigger cellular stress responses, including endoplasmic reticulum (ER)-specific mechanisms, to ensure the transit of correctly folded proteins to the extracellular space, plasma membrane and exo- and endocytic compartments. Various biochemical and physiologic stimuli can induce ER stress, such as changes in calcium homeostasis or redox status, elevated protein synthesis and expression of unfolded or misfolded proteins, glucose deprivation and altered protein glycosylation, cholesterol depletion and microbial infections (Zhao and Ackerman, 2006). Distinct signal transduction pathways, including the unfolded protein response (UPR), the ER-overload response (EOR) and the sterol regulatory element binding protein pathway, direct specific ER stress signals towards the nucleus (Pahl, 1999b). In parallel, ER-associated degradation processes reduce the accumulation of mis- or unfolded proteins through the initiation of proteasomal degradation. Likely upon failure of these adaptation mechanisms, the excessive and prolonged ER stress response results in cell death through mitochondria-dependent and -independent apoptotic mechanisms (Wu and Kaufman, 2006; Zhang and Kaufman, 2006). The glucose-regulated protein (grp)-78 (also referred to as the immunoglobulin heavy chain-binding protein BiP) was first identified as a prototypic ER stress marker and master regulator of the UPR (Zhang and Kaufman, 2006). The accumulation of mis- or unfolded proteins in the ER triggers grp-78 liberation from ER trans-membrane proteins including the transcription factor (ATF)-6, the bifunctional serine/threonine protein kinase/endoribonuclease (IRE-1/Ern1p) and the PKR-like ER-associated kinase (PERK). Interestingly, ER stress responses have been linked to the activation of NF-κB pathways through mechanisms that involve IRE-1 signaling, the induction of the TNF receptor-associated factor (TRAF) 2, Ca2+ signaling and the production of reactive oxygen species (ROS) (Hu *et al*., 2006; Pahl, 1999b). So far, ER stress responses have been associated with the development of chronic pathologies such as type I and type II diabetes, cancer and neurodegenerative diseases (Zhao and Ackerman, 2006). However, little is known about the role of ER stress responses in IBD.

We performed proteomic analysis in *E. faecalis*-monoassociated IL-10$^{-/-}$ mice and showed that the expression of grp-78 was increased in primary IEC under conditions of chronic inflammation (Shkoda *et al*., 2007). IEC from patients with CD and UC were also characterized by increased grp-78 protein levels in inflamed but not in control tissues. Interestingly, grp-78 modulated cytoplasmic TNF signal transduction through recruitment

of grp-78 into the IKK complex (Fig. 2). Consistently, small interfering (si) RNA-mediated knock-down of grp-78 prevented TNF-induced NF-κB RelA phosphorylation, supporting the hypothesis that the association of grp-78 with the IKK/NF-κB signalsome facilitates the activation of the TNF pro-inflammatory cascade. Since TNF triggers ROS-dependent ER stress (Xue *et al*., 2005) independent of grp-78 re-synthesis (Pahl and Baeuerle, 1995), the appearance of grp-78 in the IKK complex may reflect TNF-induced ER stress and redistribution of grp-78 from the ER lumen into the cytoplasmic space. These findings agree with a limited number of studies showing that ER stress inducers trigger the redistribution of grp-78 from the ER lumen. Grp-78 may either migrate to the cytoplasm (Hendershot *et al*., 1995) or act as a transmembrane protein (Rao *et al*., 2002).

IL-10 signals through JAK1/STAT3 and p38 MAPK-dependent pathways to trigger anti-inflammatory mechanisms mediated by suppressors of cytokine signaling (SOCS) or heme oxygenase (HO)-1 (Alexander and Hilton, 2004; Lee and Chau, 2002; Moore *et al*., 2001). Although IL-10 signaling in IEC is still unclear, we found that IL-10-receptor-reconstituted IEC cultures regained IL-10-mediated p38 phosphorylation, suggesting a direct protective role of IL-10-mediated p38 signaling at the epithelial cell level. In addition, we showed that the activation of the p38 MAPK signaling cascade is present in primary IEC from *E. faecalis*-monoassociated wild type but not from IL-10$^{-/-}$ mice. Together with the findings that IL-10-mediated p38 signaling blocked ER stress responses in the intestinal epithelium through mechanisms that inhibit nuclear recruitment of ATF-6 to the grp-78 promoter, we suggest that IL-10 may directly confer protective mechanisms to the intestinal epithelium by regulating ER stress response mechanisms (Fig. 2). Considering our previous findings that protective TGF-β-mediated Smad signaling was present at the early but not at the late phase of bacterial colonization (Ruiz *et al*., 2005b), we propose that TGF-β and IL-10 may both contribute to the maintenance of epithelial cell homeostasis but differ in the timing and molecular mechanisms of their effects. The presence of sustained ER stress response mechanisms in the intestinal epithelium may contribute to the development of epithelial cell dysfunctions and chronic intestinal inflammation. An attractive hypothesis is that transient induction of NF-κB activity in epithelial cells triggers biologically active IL-10-mediated TGF-β responses in the lamina propria or the epithelium, suggesting that IL-10 and TGF-β1 have interrelated roles in maintaining epithelial cell homeostasis to commensal enteric bacteria.

Conclusion

The intestinal microbiota is a key stimulant of mucosal immune responses. Various bacteria present in the intestine of a normal host activate innate immune responses and trigger physiological inflammation. However, a failure to terminate these responses may lead to persistent inflammation and to chronic inflammation in a susceptible host (Fig. 1). The aforementioned data show that bacteria-mediated signaling is controlled by a complex network of regulatory cascades that assure a proper activation and most importantly a sequential inactivation of immune responses (Fig. 2). Numerous studies in different experimental models of colitis have shown that enteric bacteria are not all equal in their contribution to the development of IBD. The challenge is to build a comprehensive overview of host-specific mechanisms underlying both colitogenic activities of otherwise non-pathogenic bacteria and protective activities of endogenous or probiotic bacteria. The understanding of signal transduction mechanisms in the intestinal epithelium will likely help to develop new strategies to terminate the immunopathology of chronic intestinal inflammation. New technologies such as proteomics (expression, functional and structural) will contribute to the discovery of novel molecules involved in the interaction of bacteria with the host under normal conditions and under chronic inflammation.

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