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# Pathogenesis of Inflammatory Bowel Diseases

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## 1. Introduction

Ulcerative colitis (UC) and Crohn's disease (CD), collectively called inflammatory bowel disease (IBD), are idiopathic, chronic, and relapsing intestinal inflammatory disorder, characterized by abdominal pain and diarrhea. UC differs dramatically from CD with the respects of disease distribution, morphology, and histopathology; for example, CD can affect any part of the gastrointestinal (GI) tract, usually discontinuously. UC is confined to the colon, it is characterized by continuous inflammation, invariably involving the rectum, and is classified according to its proximal limit (proctitis, distal, or extensive colitis). Further, unlike CD, inflammation in UC is restricted to the mucosal surface, perhaps giving weight to the emerging concept of a defective mucosal barrier in disease pathogenesis. Histologically active UC typically consists of a neutrophilic mucosal infiltrate, goblet cell depletion, "cryptitis," and prominent crypt abscesses. Acute inflammatory process in UC is associated with mucosal (particularly epithelial) cell destruction. Meantime, UC and CD share a lot of inflammatory similarities, such as epithelial barrier dysfunction, genetic susceptibility etc. IBD may result in significant morbidity and mortality, with compromised quality of life and life expectancy. While there is no cure for IBD, the last two decades have seen tremendous advances in our understanding of the pathophysiology of this intestinal inflammation. Even though the precise etiology of IBD remains elusive, it is accepted (Figure 1) that IBD arises from abnormal host-microbe interactions, including qualitative and quantitative changes in the composition of the microbiota, host genetic susceptibility, barrier function, as well as innate and adaptive immunity. In more detail, some defects occur in luminal bacterial antigen sampling by the epithelium, possibly mediated by toll-like receptors (TLRs) or nucleotide binding oligomerisation domain family (NODs), controlled by genetic factors (including NOD2 for CD etc). An over-response to the antigens then stimulates activated dendritic cells to generate Th1-type /Th17 T cells or Th2-type /NK T cells, which then generate cytokines, initiating a cascade of immunologic events resulting in tissue damage. Thus, the factors participating in what manifests as inflammation in UC and CD are part of a dynamic process in which autoantibodies are generated against mucosal antigens in a susceptible host. The autoantibodies are not primarily responsible for disease pathogenesis; rather, they mark for disease-related autoantigens, which likely cross react with bacterial antigens from the normal intestinal flora. In a genetically susceptible host, the interaction results in an exaggerated inflammatory response in which either a lack of regulatory cells or enhanced numbers of effector cells initiates disease. With time, antigenic spreading to host antigens (the autoantigens) occurs; therefore, removal of these bacteria would no longer affect disease activity (Vanderlugt et al., 1996).

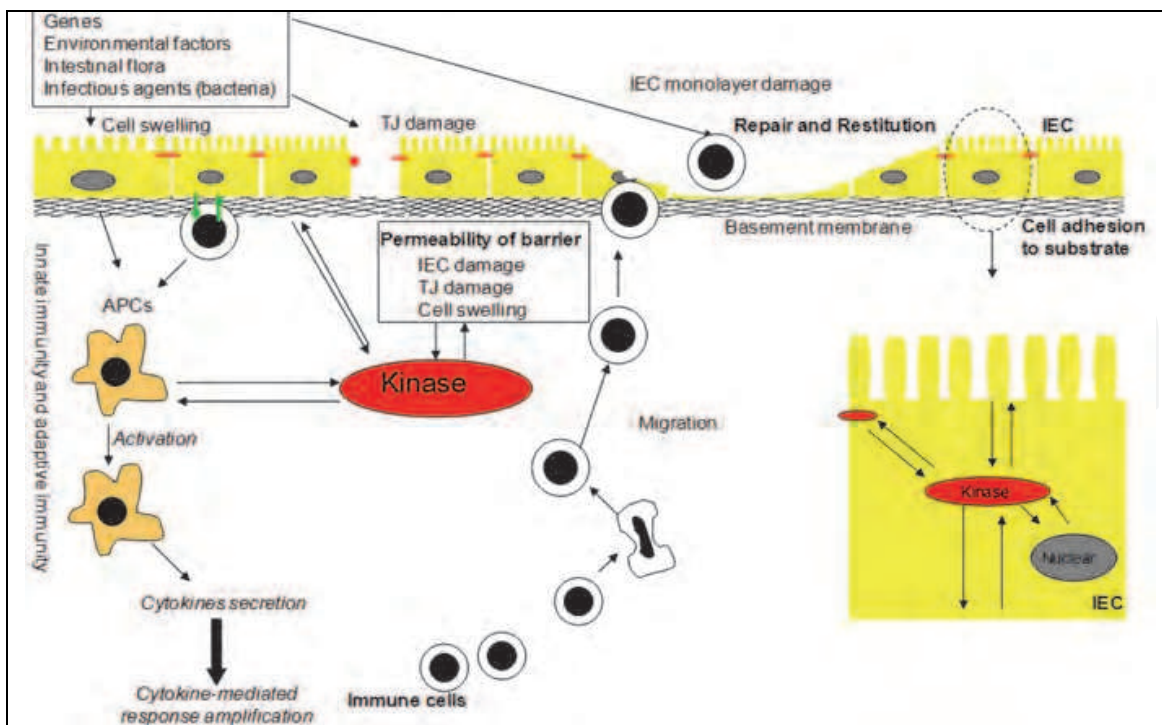


Fig. 1. Pathogenesis of IBD. Many different factors, such as genetic factors, environmental factors, and intestinal non-pathogenic or pathogenic bacteria can damage the mucus, epithelium, or the tight junction, to initiate the inappropriate regulation or deregulation of the immune response, leading to the secretion of pro-inflammatory cytokines, decrease in epithelial barrier function and initiation of the inflammation-related signaling pathways. IEC: Intestinal epithelial cell; APC: Antigen presenting cell; TJ: Tight junction. This model adapted from the model presented previously (Yan 2008)

In this chapter, we are going to focus on the involvement of diverse of factors in the pathogenesis of IBD, try to shed some light on the clues of intervention of IBD.

## 2. Genetic factor

Population-based studies provided compelling evidence that genetic susceptibility plays an essential role in the pathogenesis of IBD, evidence including an 8- to 10-fold greater risk among relatives of UC and CD and greater rates of concordance between twins in UC patients (15.4% in monozygotic vs 3.9% in dizygotic twins) and CD patients (30.3% in monozygotic vs 3.6% in dizygotic twins) (Cho & Brant, 2011). Some of genes encoding protein kinases like ERK1 (Hugot et al., 1996) and p38 $\alpha$  (Hampe et al., 1999) are located in major IBD susceptibility regions on chromosome 16 and 6. Recently, substantial advances have been achieved in defining the genetic architecture of IBD since the genome-wide association study (GWAS) analysis heralded a new era of complex disease gene discovery with notable success in CD initially and latterly also in UC. To date, near 99 published IBD susceptibility loci have been discovered and replicated, of which minimum 28 are associated with both UC and CD, although 47 are specific to UC and 24 to CD (Thompson & Lees, 2011). Generally, these genetic loci could be grouped into different categories. Importantly, most of the genes have been linked to defects in innate and adaptive immunity and epithelial barrier function. The first susceptible locus identified in IBD is the major

histocompatibility complex (MHC) class II region on chromosome 6 has been clearly demonstrated to be associated with UC (Toyoda et al., 1993).

Gene *NOD2* is the breakthrough discovery whose mutations are associated with CD lying either within or near the C-terminal, leucine-rich repeat domain, which is required for microbial sensing. *NOD2* is expressed by many leukocytes, including antigen presenting cells, macrophages, and lymphocytes, as well as ileal Paneth cells, fibroblasts, and epithelial cells. Activation of *NOD2* by microbial ligands activates the transcription factor nuclear factor  $\kappa$ B (NF- $\kappa$ B) and mitogen activated protein kinase (MAPK) signaling, and functions as a positive regulator of immune defense (Hugot et al., 2001; Ogura et al., 2001). The *NOD2* ligand muramyl dipeptide (MDP) is ubiquitous, indicating that broad classes of bacteria are capable of activating *NOD2*. However, the N-glycosylated form of muramyl dipeptide found in mycobacteria and actinomycetes more potently activates *NOD2* compared to the N-acetylated form, found more frequently in gram-positive and gram-negative bacteria (Coulombe et al., 2009). Autophagy 16-like 1 (*ATG16L1*) has been strongly associated with CD and encodes a protein component of the autophagy complex (Levine & Deretic, 2007). *ATG16L1* is broadly expressed, including in small intestinal Paneth cells (Cadwell et al., 2008) where it mediates exocytosis of secretory granules that contain antimicrobial peptides. IL-10 gene SNPs were found to be involved in the UC by GWAS analysis (Franke et al., 2008), and IL-10<sup>-/-</sup> mouse is one of the oldest and most widely used animal models of UC, in which spontaneous colitis develops in specific pathogen-free conditions (Kuhn et al., 1993). IL10 is expressed by many different cells of the adaptive and innate immune system including Th1, 2, and 17 cells, subsets of regulatory T cells, dendritic cells, macrophages, mast cells, and natural killer cells (Mosser et al., 2008). It has pleiotropic effects on T and B cells, and importantly limits the release of proinflammatory cytokines like TNF- $\alpha$  and IL-12. In the IL-10<sup>-/-</sup> mouse model, defective counter regulatory anticytokine responses result in inflammation affecting intestinal mucosa which is characterized by enlarged and branched crypts, reduced number of goblet cells, degeneration of superficial epithelial cells, and increased expression of MHC class II molecules in mouse colon. But these IL-10<sup>-/-</sup> mice require gut microbia to develop inflammation, giving rise to an attractive theory that IL10 could be involved in restricting the mucosal immune response to enteric flora (Louis et al., 2009; Sellon et al., 1998). Interestingly, this IL-10<sup>-/-</sup> model of UC have, in another hand, elegantly shown a protective role of IL10: transfer of IL10 producing regulatory T cells to immunodeficient mice prevents or cures colitis (Uhlir et al., 2006). Further, IL10 has been shown to exert a protective effect on carcinogenesis in mice (Erdman et al., 2003). The anti-inflammatory response of IL-10 is mediated through IL10 receptor (IL10R) and subsequent activation of signal transducer and activator of transcription 3 (STAT3). IL10R is a heterotetrameric molecule; while IL10R1 is specific to IL10R, IL10R2 is found as a subunit of receptors to other cytokines, notably IL22, IL26, and IFN $\gamma$ . Extracellular matrix gene 1 (ECM1) (Festen et al., 2010), E-cadherin gene (CDH1), Hepatocyte nuclear factor 4 alpha gene (HNF4a), and laminin B1 (Barrett et al., 2009) are another four genes implicated in mucosal barrier function, conferring risk of UC; ECM1 interacts with the basement membrane, inhibits matrix metalloproteinase 9 (MMP9), and strongly activate NF $\kappa$ B (Chan et al., 2007; Matsuda et al., 2003). The Wnt/beta-catenin signal transduction pathway has been shown to influence ECM1 expression (Kenny et al., 2005). E-cadherin is the first genetic correlation between colorectal cancer and UC, Chimeric mice with impaired E-cadherin function due to expression of dominant-negative N-cadherin developed colitis despite possessing an intact immune system (Hermiston & Gordon 1995a, 1995b). Notably, all of



these 4 genes are regulated or related to protein kinases, for example, HNF4 $\alpha$ -DNA binding activity is dependent on its phosphorylation by protein kinase A (PKA) (Viollet et al., 1997), while its transcription activity was dependent on AMP-activated protein kinase (AMPK) (Hong et al., 2003).

Similarly, by GWAS analysis, the strongest association in CD was found in interleukin-12 receptor (IL23R) (Duerr et al., 2006)—as well as the previously identified NOD2 gene. Knockout of or antibodies to IL23 prevent the development of intestinal inflammation in such models (McGovern & Powrie, 2007). It is now evident that much of the function previously ascribed to IL12 appears to relate to IL23, both of these cytokines sharing a p40 subunit in their heterodimeric structures. The IL23/IL12 pathway has become the subject of intensive study in the field of immunology as it plays a key role in determining differentiation of naive T cells into effector Th1 cells (driven by IL12) or Th17 cells (driven by IL23). Some specific bacterial components such as peptidoglycan can differentially induce antigen presenting cells to produce IL23 rather than IL12, leading to distinct patterns of inflammatory response (Begum et al., 2004). Th17 cells are particularly interesting for their role in organ-specific inflammation—raising the hope that therapeutic disruption of the IL23 pathway will control such inflammation without impairing systemic immunity.

### 3. Microbiota and immune responses

The human GI tract contains as many as  $10^{14}$  individual bacteria, comprising over 500 different species. These commensal bacteria serve as a primary barrier between the intestinal epithelial cells and the external environment, which is critical to the healthy host, as it modulates intestinal development, maintains a healthy intestinal pH, promotes immune homeostasis, and enhances metabolism of drugs, hormones and carcinogens. Evidence from immunologic, microbiologic, and genetic studies implicates abnormal host-microbial interactions in the pathogenesis of UC. But the mechanisms underlying the involvement of microbiota are elusive, and the effects of microbiota are due to their interaction with other factors, such as immunologic factors, genetic factor or epithelial junction proteins. The postulated mechanisms (Packey & Sartor, 2008) are as followed with little modification: (A) Pathogenic bacteria or abnormal microbial composition. A traditional pathogen or functional alterations in commensal bacteria, including enhanced epithelial adherence, invasion, and resistance to killing by phagocytes or acquisition of virulence factors, can result in increased stimulation of innate and adaptive immune responses. Luminal bacterial concentrations are increased in IBD, microbial diversity is diminished, particularly in patients with active disease. The involvement of pathogen mycobacterium avium subspecies paratuberculosis (MAP) in the pathogenesis of IBD is still controversial. Commensal bacteria that undergo functional alterations might contribute to the pathogenesis of IBD. *Escherichia coli* are commensal aerobic Gram-negative bacteria that play an important role in maintaining normal intestinal homeostasis. Modifications of luminal bacteria concentrations, including *E. coli*, have been observed in Crohn's disease patients (Frank et al., 2007). Reduced numbers of *Bacteroides fragilis* might also contribute to inflammation because this prominent human symbiont has protective effects: it protects mice from colitis induction by *Helicobacter hepaticus*, a murine commensal bacterium with pathogenic properties (Mazmanian et al., 2005). *Faecalibacterium prausnitzii* has anti-inflammatory properties; its numbers are reduced in patients with CD and associated with risk of postresection recurrence of ileal CD (Sokol et al., 2008). There is a decreased ratio of protective commensal bacterial species compared to aggressive species in patients with IBD.

Decreased concentrations of bacteria that produce butyrate and other short-chain fatty acids (SCFA) may compromise epithelial barrier integrity. (B) Defective host containment of commensal bacteria. Increased mucosal permeability can result in overwhelming exposure of bacterial to TLR ligands and antigens that activate pathogenic innate and T cell immune responses. (C) Defective host immunoregulation. Inflammation might arise from lack of tolerance to antigens present in autologous microflora; cells derived from inflamed intestinal tissues of patients with IBD are activated by exposure to sonicated samples of autologous or heterologous GI microflora, whereas cells from normal individuals respond only to sonicates of heterologous microflora (Duchmann et al., 1997). Antigen-presenting cells and epithelial cells overproduce cytokines due to ineffective down regulation, which results in TH1 and TH17 differentiation and inflammation. Dysfunction of regulatory T cells (Treg) leads to decreased secretion of IL-10 and TGF- $\beta$ , and loss of immunological tolerance to microbial antigens (an overly aggressive T cell response.).

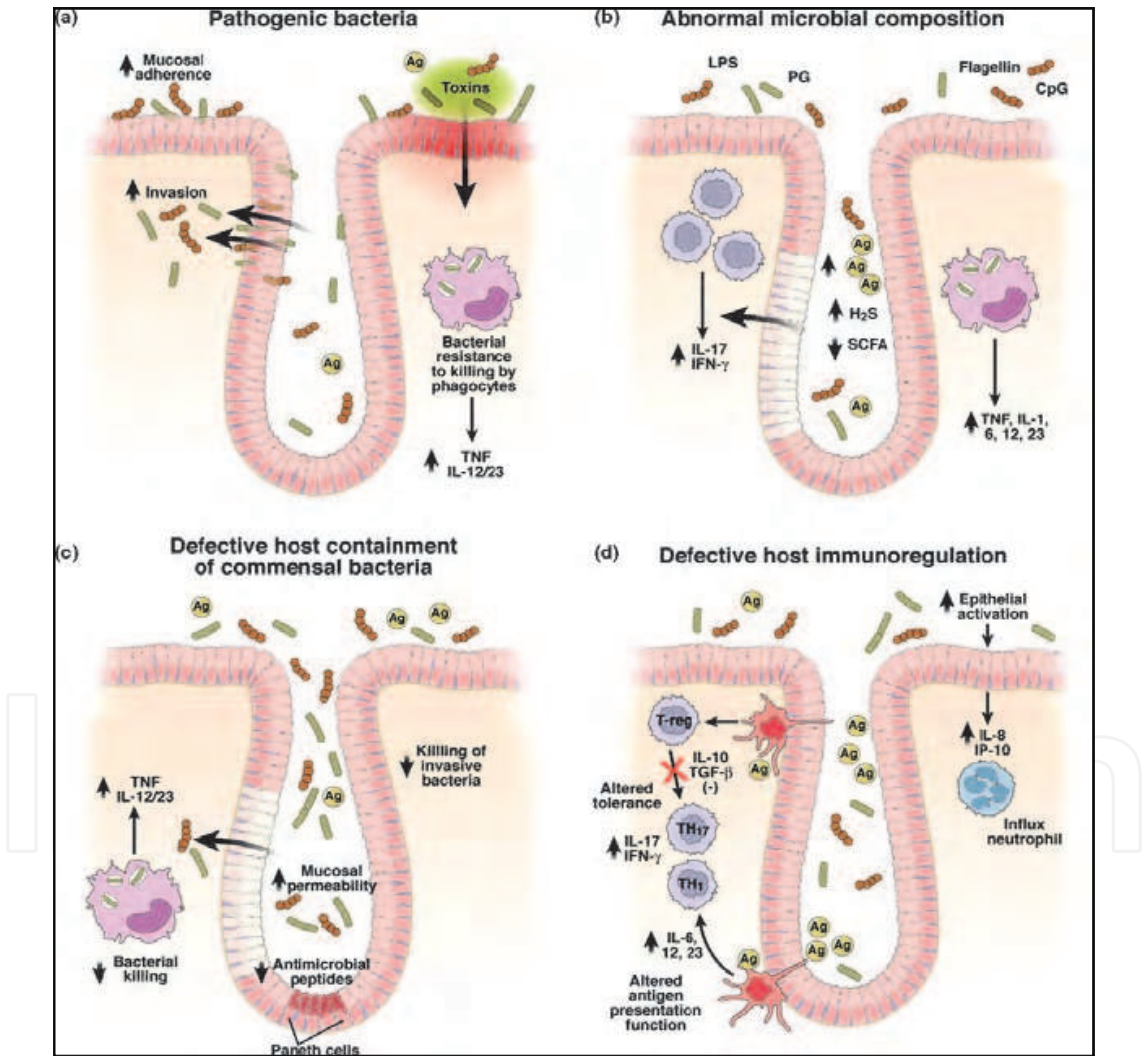


Fig. 2. Proposed mechanisms by which bacteria and fungi induce chronic immune-mediated inflammation and injury of the intestines. This model adapted from the model presented in the work by Dr Sartor (Packey & Sartor 2008) (a) Pathogenic bacteria. (b) Abnormal microbial composition. (c) Defective host containment of commensal bacteria. (d) Defective host immunoregulation.

The bowel is the largest immunological organ of the body, with continuous interaction between the mucosal immune system and the intestinal flora. IBD is commonly regarded as the consequences of an enhanced inflammatory response or the lack of a down regulatory response to bacteria abnormality (Sartor et al., 2008; Xavier et al., 2007). The dysregulated immune response involving the innate (for example, TLR, DC, etc) and the adaptive immune system (e.g. effector T-cells, regulatory T-cells, eosinophils, neutrophils, etc) may follow or precede the macroscopic lesions. Th-1 and Th17 immune responses play a role in the pathogenesis of Crohn's disease (Sartor, 2008; Strober et al., 2007). The Th1 cytokine profile, which includes IFN- $\gamma$  and IL-12 p40, is dominant in patients with Crohn's disease. Traditional Th1 responses are mediated by IFN- $\gamma$ , the production of which is stimulated by IL-12, produced by antigen-presenting cells (APCs). Most experimental colitis models also have a dominant Th1 response, although in several models Th1 responses can change into Th2 (type 2 T-helper lymphocyte) responses as the inflammatory process matures (Spencer et al., 2002; Bamias et al., 2005). How we think about Th1 responses has been influenced by the discovery of an additional Th17 pathway. IL-17 mediates Th17 responses (Kolls & Linden, 2004). The production of this cytokine is stimulated by the production of IL-6, TGF $\beta$  and IL-23 by innate immune cells and APCs, especially dendritic cells. Bacterial colonization stimulates IL-23 expression by ileal dendritic cells (Becker et al., 2003). The levels of both IL-23 and IL-17 are increased in Crohn's disease tissues and most forms of experimental colitis (Fujino et al., 2003; Schmidt et al., 2005; Yen et al., 2006). Of pathogenic importance, the IL-12-IFN- $\gamma$  and IL-23-IL-17 pathways seem to be mutually exclusive, since IFN- $\gamma$  suppresses IL-17, and vice versa (Kolls et al., 2004). The immunopathogenesis of UC has been a more difficult disease to ascertain, neither IFN- $\gamma$  (a major Th1 cytokine) nor IL-4 (the major Th2 cytokines) was increased (Fuss et al., 2008). In fact, IL-4 production was found to be decreased in cells extracted from UC tissue and only the fact that an additional Th2 cytokine IL-5 secretion by these cells was somewhat increased hinted that the disease may have a Th2 character. A further, enhanced level of IL-13 was noticed in lamina propria from UC specimens, whereas those from Crohn's disease specimens were producing IFN- $\gamma$  (Fuss et al., 1996). Fuss (Fuss et al., 2004) found that antigen-presenting cells bearing a CD1d construct (and thus expressing CD1d on its surface, which presents lipid rather than protein antigens to T cells.) could only induce lamina propria mononuclear cells from UC patients but not that of Crohn's disease to produce IL-13. Thereby, the cytokine secretion profile seen in UC was produced from a non-classical CD1 dependent NK T cell whereas the cytokines produced in Crohn's disease were from that of an activated classical Th1 CD4 + T cell. In addition, Lamina propria cells enriched for NK T cells from the patients could be shown to be cytotoxic for epithelial cells and such cytotoxicity was further enhanced by IL-13. Antigens in the mucosal microflora activate NK T cells because of barrier dysfunction that, in turn, cause cytolysis of epithelial cells and the characteristic ulcerations associated with the disease. As suggested, enhancement of cytolytic activity was observed *in vitro* in the presence of IL-13. Further, IL-13 was shown to have direct effects on activation of cytokine, transcription. These studies demonstrated that TGF- $\beta$  transcription was dependent upon IL-13. In short, UC is associated with an atypical Th-2 response mediated by a distinct subset of NK T cells that produce IL-13 and are cytotoxic for epithelial cells (Fuss et al., 2008). Further, UC is characterized by the presence of various types of autoantibodies which confirm a key role of defect of host/bacterial interface to this disease. Approximately 70% of patients were diagnosed in the traditional manner with ulcerative colitis express pANCA (Saxon et al., 1990). The site of production of pANCA has been localized to the



gastrointestinal mucosa (Targan et al., 1995). pANCA-primed B cells have been demonstrated in the mesenteric nodes and were not found in detectable amounts in the periphery (Targan et al., 1995). These findings represent further confirmation that the pANCA-reactive antigen(s) also originates in the mucosa. Despite the fact that pANCA can be found in the peripheral circulation, the mucosal origin is the evidence that the antigen(s) to which pANCA reacts is mucosa specific and thus is more closely related to mucosal immune responses and mucosal inflammation. This finding corroborates that disease results from a defect in the host's reaction to bacteria. The antigens to pANCA have been localized to the nucleus of neutrophils by the use of electron microscopy (Billing et al., 1995).

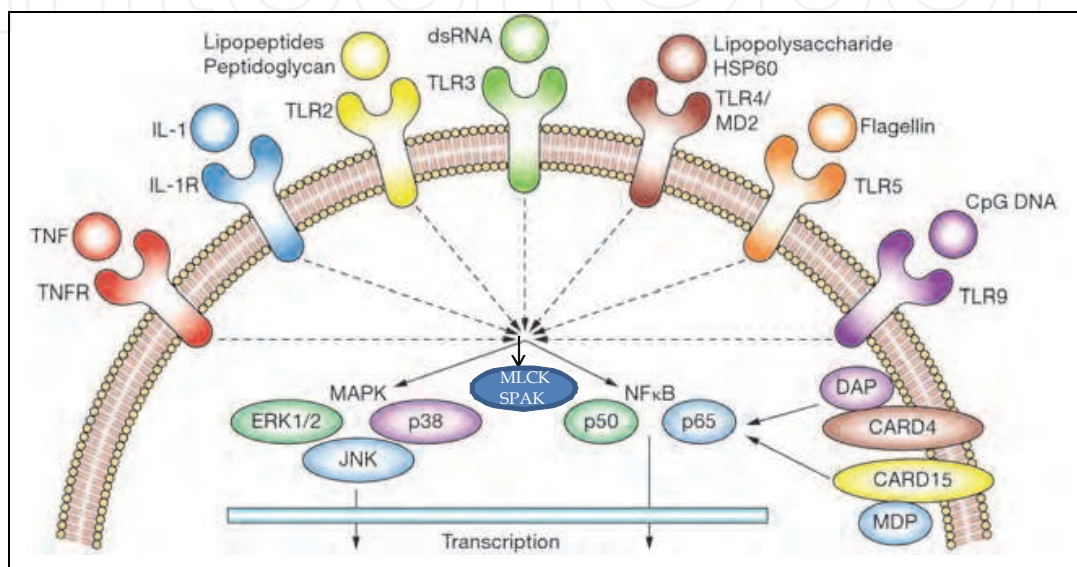


Fig. 3. Binding of microbial adjuvants to extracellular and intracellular pattern-recognition receptors and initiate their function by activating protein kinases. Toll-like receptors on the cell membrane selectively bind to various bacterial, viral or fungal components. This ligation activates conserved signaling pathways that activate NF- $\kappa$ B and mitogen-activated protein kinases. These transcription factors stimulate the expression of a number of proinflammatory and antiinflammatory genes. This model adapted and modified from the model presented previously.

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The intestinal mucosa must rapidly recognize detrimental pathogenic threats to the lumen to initiate controlled immune responses but maintain hyporesponsiveness to omnipresent harmless commensals. Pattern recognition receptors (PRRs) may play an essential role in allowing innate immune cells to discriminate between "self" and microbial "non-self" based on the recognition of broadly conserved molecular patterns. Toll-like receptors (TLRs), a class of transmembrane PRRs, play a key role in microbial recognition, induction of antimicrobial genes, and the control of adaptive immune responses. Polymorphisms in TLRs have been linked to Crohn's disease (Franchimont et al., 2004; Torok et al., 2004), and immunofluorescence studies reveal that epithelial TLR expression is markedly upregulated in IBD (Cario et al., 2000). TLR4, for example, is induced by proinflammatory cytokines and is highly expressed in IECs, resident macrophages and dendritic cells in active IBD (Cario, 2000; Hausmann et al., 2002). The functional variant Asp299Gly of TLR4 is associated with IBD and increased susceptibility to Gram-negative infections (Franchimont et al., 2004). Disrupted



TLR4 signalling could engender an inappropriate innate and adaptive immune response necessary to eradicate pathogens, which would result in severe inflammation. Polymorphisms of TLRs 1, 2 and 6 are associated with more extensive disease localization in IBD (Pierik et al., 2006). UC patients have an association between a TLR7 variant and the prevalence of pANCA antibodies, which crossreact with enteric bacterial antigens (Vermeire et al., 2004; Seibold et al., 1998). Blockade of bacterial signalling through NF $\kappa$ B in IECs potentiates chemically induced colitis in TLR4 and TLR9-deficient mice (Fukata et al., 2006; Lee et al., 2006). Individual TLRs differentially activate distinct signaling events via diverse cofactors and adaptors. To date, at least five different adaptor proteins have been identified in humans: MyD88, Mal/TIRAP, TRIF/TICAM-1, TRAM/Tirp/TICAM-2, and SARM (O'Neill et al., 2003). The first identified so-called "classical" pathway (Cario, 2005) involves recruitment of the adaptor molecule MyD88, activation of the serine/threonine kinases of the interleukin 1 receptor associated kinase (IRAK) family, subsequently leading to degradation of inhibitor  $\kappa$ B (I $\kappa$ B) and translocation of nuclear factor  $\kappa$ B (NF $\kappa$ B) to the nucleus, then result in activation of specific transcription factors, including NF $\kappa$ B, AP-1, Elk-1, CREB, STATs, and the subsequent transcriptional activation of genes encoding pro- and anti-inflammatory cytokines and chemokines as well as induction of costimulatory molecules. All of these various downstream effects are critically involved in the control of pathogen elimination, commensal homeostasis, and linkage to the adaptive immunity. Signaling through different TLRs can result in considerable qualitative differences in TH dependent immune responses by differential modulation of MAPKs and the transcription factor c-FOS (Agrawal et al., 2003). So TLR signalling protects intestinal epithelial barrier and maintains tolerance, but aberrant TLR signalling may stimulate diverse inflammatory responses leading to UC. TLR comprise a family of (so far) 11 type-I transmembrane receptors. Different pathogen associated molecular patterns selectively activate different TLRs: (Lipoteptoteins) TLR1, 2 and 6; (dsRNA) TLR3; (LPS) TLR4; (Flagellin) TLR5; (ssRNA) TLR7 and 8; (CpG DNA) TLR9. These signals all converge on a single pathway via myeloid differentiation primary response protein MyD88, which activates NF $\kappa$ B. the NF $\kappa$ B pathway was thought to have predominantly pro inflammatory activities and NF $\kappa$ B is activated in the tissues of UC patients and its inhibition can attenuate experimental colitis (Neurath et al., 1996). In intestine, tolerance is an essential mucosal defence mechanism maintaining hyporesponsiveness to harmless luminal commensals and their products. Several molecular immune mechanisms that ensure tolerance via TLRs in intestinal epithelial cells (IEC) have recently been described, for example, low expression of TLRs at resting conditions in IEC can maintain hyporesponsiveness to microbiota; high expression levels of the downstream signaling suppressor Tollip which inhibits IRAK activation (Otte et al., 2004), ligand induced activation of peroxisome proliferator activated receptor  $\gamma$  (PPAR $\gamma$ ) which uncouples NF $\kappa$ B dependent target genes in a negative feedback loop (Dubuquoy et al., 2003. Kelly et al., 2004), and external regulators which may suppress TLR mediated signalling pathways. Commensal bacteria may assist the host in maintaining mucosal homeostasis by suppressing inflammatory responses and inhibiting specific intracellular signal transduction pathways (Neish et al., 2000), uncoupling NF $\kappa$ B dependent target genes in a negative feedback loop (Dubuquoy et al., 2003) which may lead to attenuation of colonic inflammation (Kelly et al., 2004).

NODs comprise at present more than 20 different members with C terminal ligand recognition (LRR) domain, central nucleotide binding domain (NBD), and N terminal caspase recruitment domains (CARDs). Recent research has mostly focused on two cytosolic receptors of this family, NOD1 and NOD2, which both play a major role in intestinal regulation of

proinflammatory signalling through NF $\kappa$ B in response to distinct bacterial ligands. NOD2 is constitutively or inducibly expressed in all kinds of cells throughout the gastrointestinal tract. MDP has been identified as (so far) the sole ligand of NOD2 (Inohara et al., 2003; Girardin et al., 2003). NOD2 has been found to exert antibacterial activity in intestinal epithelial cells limiting survival of enteric bacteria after invasion. Bacterial clearance of *Salmonella typhimurium* is strongly accelerated in IEC expressing a functional NOD2 protein, whereas L1007fsinsC mutant expressing IEC are virtually unable to clear the pathogen *in vitro* (Hisamatsu et al., 2003). NOD2 (Chin et al., 2002) knockout mice, exhibit a profoundly decreased ability to clear intracellular *Listeria monocytogenes*, inducing persistent immune activation by combined loss of antibacterial activity, dysregulation of cytokine production, and imbalance of T cell activation. Emerging studies have started to reveal the molecular mechanisms by which NOD2 influences innate immune responses in the intestinal mucosa. It seems that different NOD2 mutations may span a spectrum of diverse phenotypes, ranging from complete “loss of function” to maximal “gain of function”. NOD2 mutations within NBD lead to constitutive ligand independent NF $\kappa$ B activation, causing a chronic systemic inflammatory disorder known as “Blau syndrome” (Inohara et al., 2003a). Conversely, it has been suggested that CD associated NOD2 mutants which are predominantly found in the microbial ligand dependent LRR domain rather reflect “loss of function” phenotypes. Several *in vitro* transfection studies showed that human CD associated NOD2 mutants significantly abolish NF $\kappa$ B activation in response to MDP (Inohara et al., 2003b; Girardin et al., 2003; Chamaillard, 2003). However, paradoxically, macrophages within the intestinal lamina propria of CD patients overproduce NF $\kappa$ B targets, including exaggerated production of proinflammatory cytokines, such as TNF- $\alpha$  and IL-1 $\beta$  (Podolsky, 2002). Accordingly, a recent *in vivo* study now demonstrates that MDP stimulated macrophages isolated from mice generated with a murine NOD22932iC variant, homologous to the human NOD23020insC (=L1007fsinsC) variant, exhibit enhanced NF $\kappa$ B activation, increased apoptosis, and elevated IL-1 $\beta$  secretion (Maeda et al., 2005), possibly implying an important mechanism of how dysfunctional NOD2 may trigger intestinal inflammation in some types of CD. Thus this murine NOD2 frame-shift mutation in the LRR region may imbalance functions of both terminal parts of the whole protein: bacterial dysrecognition through the impaired LRR domain, ligand independent NF $\kappa$ B activation, as well as uncontrolled apoptosis and subsequent induction of IL-1 $\beta$  processing and release through the hyperactive CARD domains. The NOD2 gene product is most abundant in ileal Paneth cells (Lala et al., 2003; Ogura et al., 2003) which express a diverse population of microbicidal defensins restricting colonization or invasion of small intestinal epithelium by bacteria (Ouellette et al., 1994). Stimulation with MDP elicits cryptidin secretion from Paneth cells (Ayabe et al., 2000). In addition, NF- $\kappa$ B is normally grouped into one of the pro-inflammatory mediators, a protective role for epithelial NF- $\kappa$ B signaling by either bacteria, IL-1, or TNF stimulation of TLRs, or cytokine receptors is demonstrated by conditional ablation of NEMO (I $\kappa$ B kinase) in intestinal epithelial cells causing spontaneous severe colitis (Nenci et al. 2007). Blockade of epithelial NF- $\kappa$ B signaling led to increased bacterial translocation across the injured epithelium, similar to TLR4-deficient mice treated with DSS (Fukata et al., 2006).

#### 4. Barrier dysfunction

Generally, intestinal barrier function consists of different level of defense lines, the mucus layer, commensal microbiota, epithelial cells themselves, the junction between lateral

epithelial cells, innate and adaptive immune systems and enteric nerve system. Any stresses which interfere with any level of this defense lines could potentially lead to intestinal barrier dysfunction and result in intestinal inflammation.

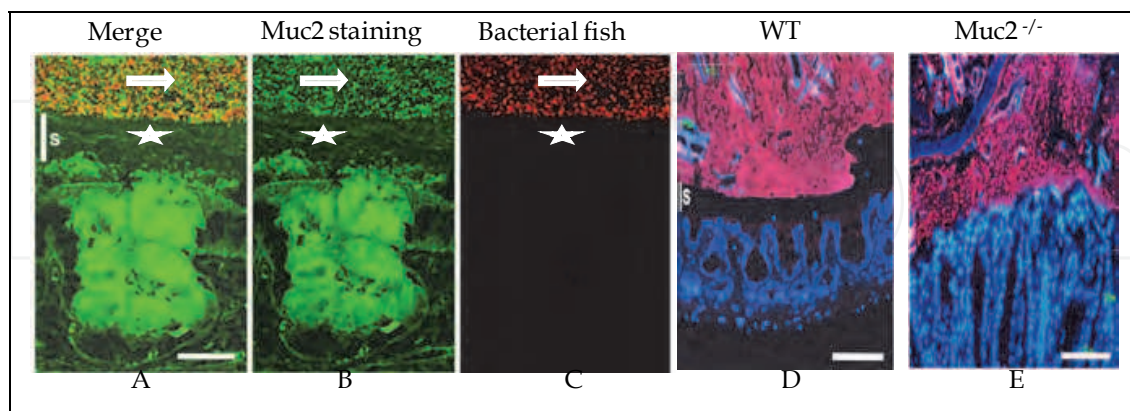


Fig. 4. Merged figure (A) of Muc2immunostaining (green, B) and FISH analysis using the general bacterial probe EUB338-Alexa Fluor 555 (red, C) of distal colon, it was shown muc2-positive goblet cells and the outer mucus layer (Arrow) and inner mucus layer (Star) on the epithelium. The inner layer (Star) is devoid of bacteria, which can only be detected in the outer mucus layer. The inner mucus generates a spatial separation between the cells and the microflora. (Scale bar: 20 $\mu$ m.). (D) FISH using the EUB338-Alexa Fluor 555 probe staining bacteria and DAPI DNA staining in colon show a clear separation of the bacterial DNA and epithelial surface in WT mice, but not in Muc2<sup>-/-</sup> mice. This separation corresponds to the inner mucus layer (s). (Scale bar: 100 $\mu$ m.). These models adapted from the models presented previously (Johansson 2008).

Epithelial cells form a continuous, polarized monolayer that is linked together by a series of dynamic junctional complexes. Except function as a physical barrier, epithelial cells maintain a mucosal defense system through the expression of a wide range of PRRs, such as TLRs and NODs. These PRRs form the backbone of the innate immune system through the rapid response and recognition of the unique and conserved microbial components, (Medzhitov & Janeway. 2002; Akira et al., 2006). Tight junctions are composed of transmembrane proteins (claudins, occludins, and junctional adhesion molecule [JAM]), peripheral membrane or scaffolding proteins (zonula occludens [ZO]), and intracellular regulatory molecules that include kinases and actin. An anatomically and immunologically compromised intestinal epithelial barrier allows direct contact of the intestinal mucosa with the luminal bacteria and plays a crucial role in the development and maintenance of IBD by initiating chronic inflammatory responses, although it is unclear whether this is a primary pathogenic process or secondary to inflammation. Since the contribution of genetic factors, microbiota and immune responses to the pathogenesis to IBD, we high light the involvement of mucus layer, tight junction itself in the pathogenesis of IBD.

#### 4.1 Mucus layer

As mentioned in previous part of this chapter, the digestive tract is home to 10<sup>14</sup> bacteria and bacteria genome is as many 10 times as human genome, which has evolved to ensure homeostasis. How to manage this enormous bacterial load without overt immune responses from the adaptive and innate systems is not well understood. When the equilibrium is

altered, as in CD and UC, inflammatory responses are initiated against the commensal bacteria. An important component, often neglected due to lack of understanding, is the mucus layer that overlies the entire intestinal epithelium as a protective gel-like layer (Johansson et al., 2008). This thick and hyperviscous mucus layer secreted by goblet cells overlies the entire intestinal epithelium as a protective gel-like layer that can extend up to as much as 150  $\mu\text{m}$  thick in mouse colon (and 800  $\mu\text{m}$  thick in rat colon). There exist two different kinds of mucus layer—outer layer and inner layer. The majority of microorganisms in the lumen can be found in the outer mucus layer, there is an inner, protected, and unstirred layer that is directly adjacent to the epithelial surface and is relatively sterile. The sterility of this layer contributes to the retention of a high concentration of antimicrobial proteins (such as cathelicidins, defensins, and cryptidins) produced by various intestinal epithelial lineages, including enterocytes and Paneth cells. The inner firmly attached mucus layer forms a specialized physical barrier that excludes the resident bacteria from a direct contact with the underlying epithelium. This organization of the colon mucus, as based on the properties of the Muc2 mucin, should be ideal for excluding bacteria from contacting the epithelial cells and thus also the immune system. Alterations or the absence of these protective layers, as in the Muc2<sup>-/-</sup> mouse colon, allow bacteria to have a direct contact with epithelial cells, to penetrate lower into the crypts and also translocate into epithelial cells. That such a close contact between bacteria and epithelia can trigger an inflammatory response (Johansson et al., 2008; Shen et al., 2009). The surface mucus layer also impacts mucosal permeability, as demonstrated by spontaneous colitis in Muc-2- deficient mice (Bergstrom et al., 2010), and increased dextran sulphate sodium-induced colitis in intestinal trefoil factor-deficient mice (Mashimo et al., 1996) and in human UC, particularly in mucus composition and concentration in phospholipids (Braun et al., 2009). Aberrant mucin assembly causes endoplasmic reticulum stress and spontaneous inflammation that resembles UC in mice (Kaser et al., 2008; Heazlewood et al., 2008); defects in the mucus layer could also influence the pattern of microbial colonization and the maintenance of microbial community structure and function. The importance of the Muc2 mucin in organizing the colon mucus protection is further strengthened by the report that two mouse strains with diarrhea and colon inflammation were shown to have two separate spontaneous mutations in the Muc2 mucin (Heazlewood et al. 2008). Importantly, the production of mucin is regulated by protein kinases, for example, resistin and resistin-like molecule (RELM) beta upregulated mucin expression which dependent on the kinase activities of protein kinase C (PKC), tyrosine kinases, and extracellular-regulated protein kinase (Krimi et al., 2008); Cathelicidin stimulates colonic mucus synthesis by up-regulating MUC1 and MUC2 expression through a mitogen-activated protein kinase pathway (Tai et al., 2008).

#### 4.2 Epithelial cell and its tight junction

The intestinal defect was first reported in studies showing that the intestinal mucosa of patients with CD had a decreased ability to exclude large molecules (Hollander et al., 1986). The cellular components of the intestinal barrier consist of the complete array of columnar epithelial cell types (enterocyte, paneth cells, enteroendocrine cells, and goblet cells) present within the intestine. These cells are polarized with an apical membrane and a basolateral membrane, and apical membrane composition is distinct from the basolateral membrane, for example, the nutrient transporters are located on the apical membrane; they use Na<sup>+</sup> ions cotransport to provide the energy and directionality of transport. In contrast, the Na<sup>+</sup>K<sup>+</sup>-ATPase, which establishes the Na<sup>+</sup> electrochemical gradient, is present on basolateral, but



not apical membranes. In addition, the lipid composition of the membrane differs; the apical membrane is enriched in sphingolipids and cholesterol relative to the basolateral membrane. One result of this cellular polarization is that the apical membranes of intestinal epithelial cells are generally impermeable to hydrophilic solutes in the absence of specific transporters. Thus, the presence of epithelial cells, particularly the apical membranes, contributes significantly to the mucosal barrier (Shen et al., 2009). Among the most important structures of the intestinal barrier are the epithelial tight junctions (TJs) that connect adjacent enterocytes together to determine paracellular permeability. The tight junction is composed of multiple proteins including transmembrane proteins such as occludin, tricellulin, claudins and junctional adhesion molecule (JAM). The intracellular portions of these transmembrane proteins interact with cytoplasmic peripheral membrane proteins, including zona occludens (ZO)-1,-2,-3 and cingulin (Mitic & Anderson. 1998). These tight junction and cytoplasmic proteins then interact with F-actin and myosin II, thereby anchoring the tight junction complex to the cytoskeleton. Once thought to be static, the association of these proteins with the tight junction is highly dynamic (Shen et al., 2009) and may play a role in epithelial barrier regulation. Occludin was the first tight junction-associated integral membrane protein identified (Furuse et al., 1993). Although occludin knockout mice exhibit intact intestinal epithelial tight junctions and display no observable barrier defect (Schulzke et al., 2005, Saitou et al., 2000). But *in vitro* studies demonstrate crucial roles in tight junction assembly and maintenance (Yu et al., 2005; Suzuki et al., 2009; Elias et al., 2009). This suggests that further analysis of occludin knockout mice under stressed condition may reveal *in vivo* functions of occludin and provide new insight into mechanisms of tight regulation (Turner, 2006). Given the phylogenetic and structural similarities between occludin and tricellulin (Ikenouchi et al., 2005), it may be that the tricellulin accounts for normal intestinal barrier function in occludin knockout mice. This hypothesis could also be applied to inflammatory bowel disease, where intestinal epithelial occludin expression is reduced (Heller et al., 2005). The fact that occludin knockout mice exhibit intact intestinal epithelial barrier function led to the search for additional tight junctional components and ultimately to the discovery of the claudins (Furuse et al., 1998). The claudins are a large family of proteins that also interact with partners on neighboring cells to affect junctional adhesions via extracellular loops. At least 24 different claudin proteins are present in mammals (Van Itallie et al., 2003, 2004, 2006), and these proteins are the primary component of tight junction strands (Furuse et al., 2006). Claudins are expressed in a tissue-specific manner, studies on human intestine confirm the expression of claudins-1, -2, -3, -4, -5, -7, and -8 in the colon, expression of claudins-1, -2, -3, and -4 in the duodenum, and expression of claudins-2 and -4 in the jejunum (Burgel et al., 2002; Escaffit et al., 2005, Szakal et al., 2010; Wang et al., 2010; Zeissing et al., 2007).

The molecular anatomy of transport through tight junction is not yet clear, at least two routes allow transport across the tight junction, and the relative contributions of different paracellular transport are regulated independently (Fihn et al., 2000; Van Itallie, 2008; Watson et al., 2005). One route, the size-dependent pathway, allows paracellular transport of large solutes, including limited flux of proteins and bacterial lipopolysaccharides (Van Itallie 2008; Watson et al., 2005). Although at what size particles are excluded from the leak pathway has not been precisely defined, it is clear that materials as large as whole bacteria cannot pass. Flux across the leak pathway may be increased by cytokines and protein kinases, including IFN $\gamma$ , TNF (Watson et al., 2005; Wang et al., 2005; Clayburgh et al., 2006), MAPKs, myosin II light chain kinase (MLCK) (Turner 2006) and SPAK (Yan et al., 2011). A second pathway is charge-dependent pathway, characterized by small pores that are

defined by tight junction-associated pore-forming claudin proteins (Amasheh et al., 2002; Colegio et al., 2003; Simon et al., 1999). These pores have a radius that excludes molecules larger than 4 Å (Van Itallie 2008; Watson et al., 2005). Thus, tight junctions show both size selectivity and charge selectivity, and these properties may be regulated individually or jointly by physiological or pathophysiological stimuli. It needs to be pointed out that barrier dysfunction may be caused by increased paracellular permeability, but mainly by epithelial damage, including erosion, and ulceration (Zeissig et al., 2004; Schulzke et al., 2006). In addition, in epithelial cells, the site of claudin protein polymerization to form strands depends on ZO family protein expression (Furuse & Tsukita, 2006), and cells lacking ZO-1 and ZO-2 fail to form tight junctions at all.

Generally, TJ proteins can be subdivided into “tightening” TJ proteins that strengthen epithelial barrier properties (such as occluding and claudin-1 and -4 etc) and “leaky” TJ proteins (like claudin-2) that selectively mediate paracellular permeability. Dysfunctional intestinal barrier is a feature of gut inflammation in humans and has been implicated as a pathogenic factor in IBD for the last 30 years. The factors responsible for barrier dysfunction in UC are similar to those in CD, including an increase in epithelial antigen transcytosis and a change in TJ structure with a reduction in TJ strand count and in the depth of the TJ main meshwork; although, in contrast to CD, strand breaks are not as frequent as in UC (Schmitz et al., 1999; Schurmann et al., 1999). Again, the downregulation of occludin and downregulation of several “tightening” TJ proteins like claudin-1 and -4, together with an upregulation of the pore-forming TJ protein claudin-2 contribute to the barrier defect observed in UC (Heller et al., 2005; Oshima et al., 2008). These disruptions of tight junction proteins could lead to a breakdown in the protective barrier and can be used as a portal of entry by the luminal bacteria. This breach in intestinal barrier can result in inflammatory infiltrate and enhanced production of cytokines and other mediators (such as neutrophil) that can further contribute to the altered barrier function.

Mucosal permeability is influenced by several factors. The surface mucus layer also impacts mucosal permeability, as demonstrated by spontaneous colitis in Muc-2- deficient mice (Van der Sluis et al., 2006), and increased dextran sulphate sodium-induced colitis in intestinal trefoil factor-deficient mice (Mashimo et al., 1996). Luminal microbiota can also compromise the intestinal barrier function (Packey & Sartor, 2008). The third is the integrity of the epithelial cell layer and the basement membrane. Molecularly this can be compromised by downregulating tight junction components Claudins 5 and 6, upregulating pore-forming Claudin 2 (Zeissig et al., 2007), which can be accomplished by TNF and IL-13, or increasing epithelial apoptosis, which has been achieved in mice by blocking nuclear factor kappa-B (NFκB) signalling. Genetic factors are involved in the loss of intestinal barrier function (Cho & Brant, 2011). Dysregulated innate and adaptive immune system can lead to the enhanced epithelial permeability (Fuss, 2008). Finally, autonomic nerve system function affects epithelial permeability, as demonstrated by mice that develop fulminant jejunoileitis following ablation of enteric glial cells (Bush et al., 1998).

The increased uptake of antigens and macromolecules from the intestinal lumen mediated through this epithelial barrier dysfunction can further exacerbate the inflammatory process, ending up in a vicious circle. In this manner, barrier dysfunction is a perpetuating principle during gastrointestinal inflammation. Since epithelial TJs are important in the maintenance of barrier function, regulatory changes in their function that are commonly found during intestinal inflammation can have severe consequences. For example, the resulting passive loss of solutes into the intestinal lumen and the subsequent osmotically driven water flow

results in “leak flux diarrhea”, one of the main consequences of UC. The tight junction is, therefore, the rate-limiting step in transepithelial transport and the principal determinant of mucosal permeability. But it has to be pointed out that barrier dysfunction itself is not sufficient to cause intestinal diseases, such as in MLCK (Turner 2006) and SPAK (Yan et al., 2011) transgenic mouse models, these two different transgenic mice revealed increased transepithelial permeability, but neither of them demonstrated any UC characterization, for example, these mice develop normal, no significant weight loss, histologically normal crypts were found, no abscesses was noticed.

Recent molecular advances as well as studies of cellular physiology in model epithelia have instead revealed that both the permeability and selectivity of tight junctions can be modulated dynamically by a variety of signals (Mitic et al., 2000). Much of the progress in this field has rested on a significantly enhanced understanding of the proteins that make up the junction itself, as well as those components of the junction on its cytoplasmic face that link the junctional region both to the cellular cytoskeleton and to signal transduction modules (González-Mariscal et al., 2003).

## **5. Protein kinase and pathogenesis of IBD**

### **5.1 mitogen activated protein kinases (MAPK)**

Interestingly, protein kinases are associated with all different level of aspects, demonstrated promising potential as intervention targets against UC. Intracellular signaling cascades are the main route of communication between the plasma membrane and regulatory targets in various intracellular compartments. The evolutionarily conserved mitogen activated protein kinases (MAPK) signaling pathway plays an important role in transducing signals from diverse extra-cellular stimuli (including growth factors, cytokines and environmental stresses) to the nucleus in order to affect a wide range of cellular processes, such as proliferation, differentiation, development, stress responses and apoptosis. MAPK (Coskun et al., 2011) signaling cascades, which comprise up to seven levels of protein kinases, are sequentially activated by phosphorylation and also involved in intestinal inflammation. These families can be divided into two groups: the classical MAPKs, consisting of ERK1/2, p38, JNK and ERK5, and the atypical MAPKs, consisting of ERK3, ERK4, ERK7 and NLK (Coulombe & Meloche, 2007). The signalling pathways which the members of these families influence can be independent of each other or overlapping. The classical pathway leading to activation of ERK1/2 is through the upstream activation of the Raf MAPKKKs, which activate sequentially the MAPKKs, MEK1/2, which can specifically bind and phosphorylate ERK1/2. At this stage, and depending upon the signal being propagated, the ERK1/2 proteins commonly then phosphorylate the downstream MAPK activated proteins (MAPKAP) 1/2. However, other proinflammatory proteins such as cytosolic phospholipase A<sub>2</sub> can be activated, as well as several transcription factors including Ets-1, Elk and c-myc. These transcription factors aid the inflammatory process by inducing other related cellular processes such as cell migration and proliferation. Interestingly, a role for ERK1/2, using an ERK1/2 inhibitor, was found in cells of the immune system and colonocytes in the development and progression of IBD, through its mediation in the signalling pathways induced by various cytokines, for example IL-21, and IL-1 (Caruso et al., 2007; Kwon et al., 2007). Indeed, several studies, cell line cultures and isolated crypts from human biopsies, have shown that it is not only over-expressed in IBD tissue (both colonocytes and cells in the underlying lamina propria), but that its phosphorylation state and therefore activation state is increased significantly during the active stages of IBD (Waetzig et

al., 2002; Dahan et al., 2008). Study also found that Erk activation is involved in claudin-4 protein expression and claudin-4 is involved in the maintenance of the intestinal epithelial cell barrier function (Pinton et al., 2010) as a “tightening” junction protein. Activation of p38/MAPK and Akt signal transduction pathways in the epithelial cells have also been implicated as key mediators of these protective effects (Resta-Lenert & Barrett. 2006). For example, *Lactobacillus* GG (LGG) prevents cytokine-induced apoptosis in both human and mouse intestinal epithelial cells through activating antiapoptotic Akt in a phosphatidylinositol-3 $\kappa$ -kinase (PI3K)-dependent manner and inhibiting proapoptotic p38/MAPK activation (Yan & Polk. 2002). The p38 family is composed of four members:  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ . Expression of the isoforms varies between tissues. Different ligands, via their respective receptors, are able to activate one or several of p38 targets TAK1, ASK1, MLK3, MEKK1-4 and TAO1-3 (Thalhamer et al., 2008). Several studies using the p38 inhibitor, SB203580, have indicated that p38 phosphorylation is increased significantly in IBD tissue (Waetzig et al., 2002; Dahan et al., 2008). This finding is substantiated further by an *in vitro* study, indicating that inhibition of p38 using the natural IL-1 receptor antagonist, in a colonocyte cell line, leads to reduced IL-6 and -8 production, and an *in vivo* study using a murine model of IBD, where inhibition of p38 reduced significantly cytokine mRNA and NF $\kappa$ B activation (Garat et al., 2003; Hollenbach et al., 2004). However, Heat-killed *L. brevis* SBC8803 induced Hsps, phosphorylated p38 MAPK, regulated the expression of tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin (IL)-1 $\beta$  and IL-12, and improved the barrier function of intestinal epithelia under oxidant stress (Ueno et al., 2011).

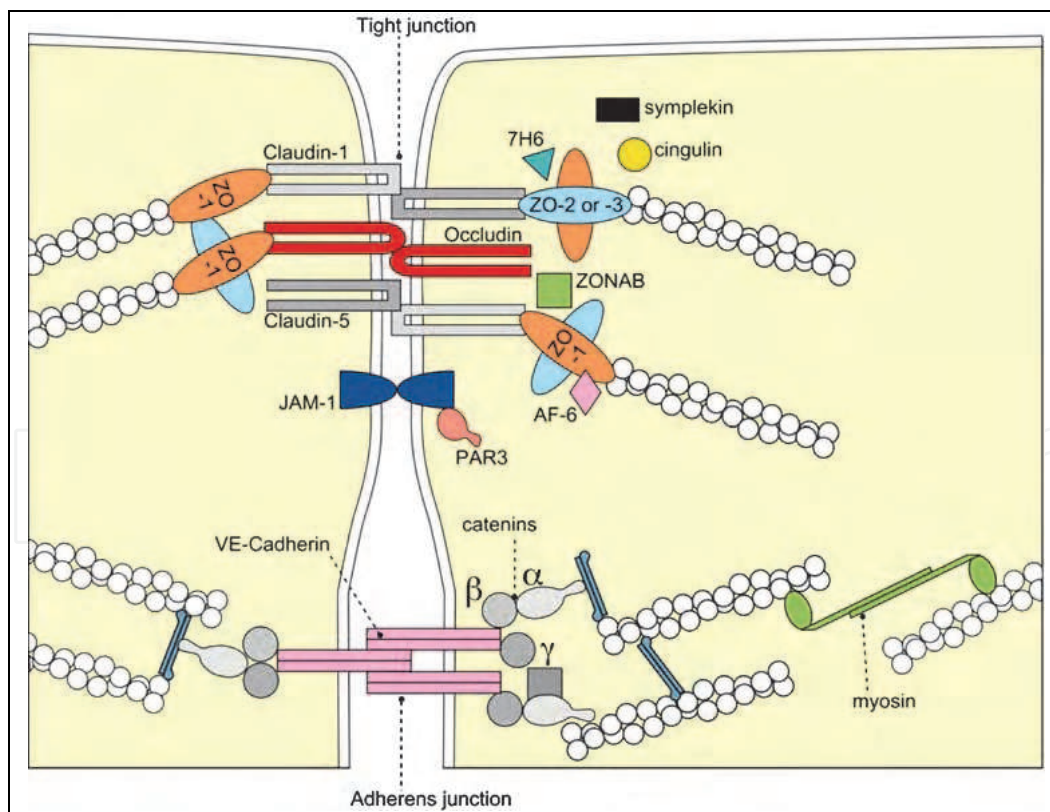


Fig. 5. Molecular composition of tight junctions. This model adapted from the model presented previously:

<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2413111/?tool=pubmed>.



There are three JNK isoforms, JNK1, 2 and 3, of which there are 10 splice forms in total. Studies using a specific inhibitor against JNK1/2 in induced IBD in rodent models or with isolated colonic tissue found that proinflammatory cytokine production was reduced in conjunction with reduced inflammatory cell infiltration. Similarly, increased phosphorylation of JNK1/2 was seen in inflamed tissue from IBD patients (Dahan et al., 2008; Assi K et al., 2006; Mitsuyama et al., 2008). RDP58 (Loftberg et al., 2002) is a peptide consisting of 9 D-amino acids blocking p38 and JNK, further attenuate UC.

## **5.2 Serine and threonine kinase**

### **5.2.1 Ste20 related proline/alanine rich kinase (SPAK)**

SPAK is defined as a ste20-like proline-/alanine rich kinase that contains an N-terminal series of proline and alanine repeats (PAPA box) followed by a kinase domain, a nuclear localization signal, a consensus caspase cleavage motif, and a C-terminal regulatory region (Johnston et al., 2000). Colonic SPAK exists as a unique isoform that lacks the PAPA box and F- $\alpha$  helix loop in the N-terminus (Yan et al., 2007). The diversity of domains present in SPAK protein might be associated with a variety of biological roles. For example, SPAK has been shown to play roles in cell differentiation, cell transformation and proliferation, and regulation of chloride transport (Piechotta et al., 2002; Gagnon et al., 2006). More importantly, a linkage has been established between SPAK and inflammation, SPAK, as an upstream kinase to Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>-</sup> co-transporter 1 (NKCC1), can phosphorylate Thr203, Thr207, and Thr212 amino acids on NKCC1, which play an important role in inflammation (Topper et al., 1997). Furthermore, we have demonstrated that SPAK can activate p38 pathway (Yan et al., 2007) that is well known involving inflammation. SPAK caused an increase in intestinal permeability, and SPAK transgenic (TG) mice were more susceptible to experimental colitis. Additionally, increased cytokine production and bacterial translocation were associated with the increased colitis susceptibility (Yan Y et al., 2011).

### **5.2.2 Myosin II light chain kinase (MLCK)**

MLCK is a specific Serine and threonine kinase which can phosphorylate MLC. It has been found that MLCK activity is required for TNF-induced acute diarrhea. Further, TNF treatment resulted in increased myosin light chain kinase expression (Wang et al., 2005), as a result of transcriptional activation (Graham et al. 2006) *in vitro* and *in vivo*. Constitutive MLCK activation accelerates onset and increases severity of experimental UC. MLCK inhibition, either pharmacologically or by genetic knockout, prevented both intestinal epithelial MLC phosphorylation and barrier dysfunction. More remarkably, MLCK inhibition also restored net water absorption, and therefore corrected the TNF-dependent diarrhea (Clayburgh et al., 2006).

## **6. Conclusions**

Different aspects of factors are implicated in the pathogenesis of a variety of human intestinal inflammatory disorders including IBD, continuing progress in the understanding of the involvement of these factors in intestinal barrier dysfunction, further in IBD pathogenesis offers hope for a new generation of therapeutic strategies targeted at the modulation of transcription factor activity.

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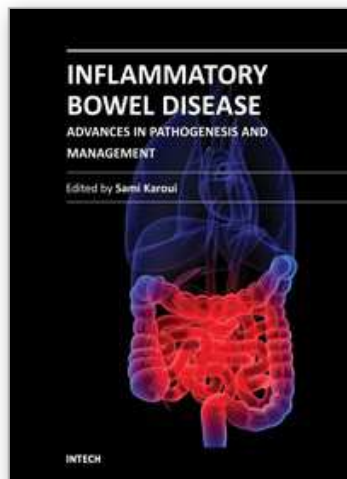
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## **Inflammatory Bowel Disease - Advances in Pathogenesis and Management**

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