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MULTI-AUTHOR REVIEW

Genetically determined epithelial dysfunction and its consequences for microflora–host interactions

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Abstract The intestinal epithelium forms a highly active functional interface between the relatively sterile internal body surfaces and the enormously complex and diverse microbiota that are contained within the lumen. Genetic models that allow for manipulation of genes specifically in the intestinal epithelium have provided an avenue to understand the diverse set of pathways whereby intestinal epithelial cells (IECs) direct the immune state of the mucosa associated with homeostasis versus either productive or non-productive inflammation as occurs during enteropathogen invasion or inflammatory bowel disease (IBD), respectively. These pathways include the unfolded protein response (UPR) induced by stress in the endoplasmic reticulum (ER), autophagy, a self-cannibalistic pathway important for intracellular bacterial killing and proper Paneth cell function as well as the interrelated functions of NOD2/NF-κB signaling which also regulate autophagy induction. Multiple genes controlling these IEC pathways have been shown to be genetic risk factors for human IBD. This highlights the importance of these pathways not only for proper IEC function but also suggesting that IECs may be one of the cellular originators of organ-specific and systemic inflammation as in IBD.

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Introduction

The intestinal epithelium forms a central part of the large barrier that separates the quantitatively and qualitatively rich ecosystem of microbial life contained within the intestinal lumen from the nearly sterile environment of the host [1–3]. Until not too long ago, the epithelium was considered a rather inert physical barrier. The recent application of in vivo model systems have revealed, however, a central role of the intestinal epithelium for simultaneously regulating the composition of the microbiota and host's innate and adaptive immune response to them. In this review we will discuss pathways within intestinal epithelial cells (IECs) that are critical in directing these mutually beneficial interactions between the host and microbes, and in this context will particularly focus on those pathways that are part of the genetic underpinnings of inflammatory bowel disease (IBD) (Fig. 1).

Endoplasmic reticulum stress

The first pathway to be discussed is the response to endoplasmic reticulum (ER) stress [4, 5]. The intestinal epithelium has to cope with a substantial secretory burden. This is particularly true for goblet cells, which secrete the components that constitute the extracellular mucin layer, and Paneth cells, which secrete antimicrobial peptides [6]. Paneth cells are located at the crypt base and are

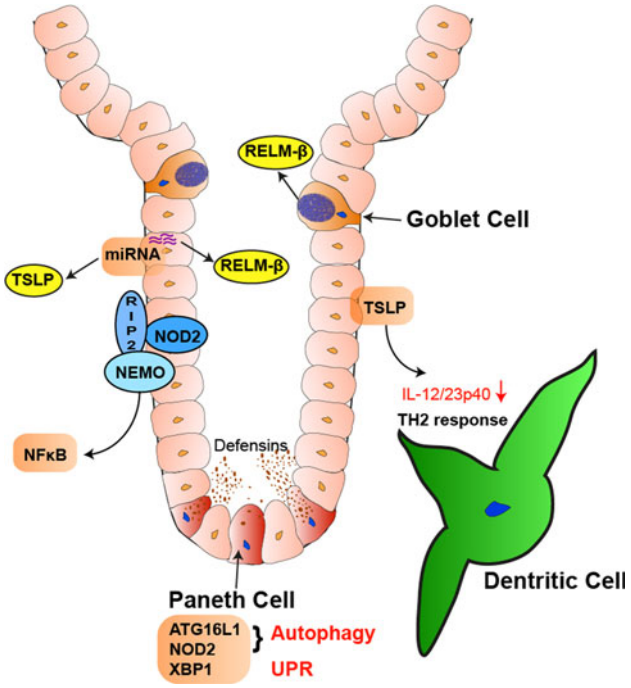

Fig. 1 Intestinal epithelial cells direct the immune state of the mucosa. IECs secrete factors like TSLP that regulate the dendritic cell function, thereby affecting the induction of mucosal T helper cell responses. NOD2 regulates NF-κB activation within IECs, among many other functions also transactivating TSLP transcription. TSLP and RELM-β expression is also regulated by specific miRNAs (miRNA-375), which involves the function of Dicer. RELM-β is secreted from goblet cells and may directly inhibit nematode mobility, constituting an important anti-nematode and anti-microbial defense mechanism. Autophagic pathways that are regulated by ATG16L1 and NOD2, as well as the unfolded protein response via XBP1, affect Paneth cell function and thereby control the release of antimicrobial peptides

characterized by large secretory granules and an elaborate smooth ER, the intracellular locale of secretory protein biogenesis [7]. Paneth cells are interspersed between intestinal epithelial stem cells and form the niche that is required for the latter [8]. Secretory protein production is under stringent control of a conserved, fundamental biological pathway, the unfolded protein response (UPR) [4, 5, 9]. Specifically, occurrence of misfolded or unfolded proteins in the ER leads to stress in this cellular compartment, which stimulates the UPR. The UPR is an adaptive response, mediated by three major proximal pathways in metazoans, IRE1/XBP1, ATF6p90/ATF6p50, and PERK/ATF4, which are focused on resolving ER stress. This is accomplished by temporarily halting protein translation, the selective transcriptional and translational induction of proteins that are important for relieving ER stress such as those involved in proper protein folding, the secretory machinery, and the protein quality control mechanisms and finally programmed cell death when ER stress is unresolved [4, 5, 9].

Genetic deletion of one of these mediators, *Xbp1*, specifically in the intestinal epithelium has profound effects on the histological architecture of the intestinal epithelium [10]. Specifically, $Xbp1^{-/-}$ mice exhibit a near total lack of Paneth cells and exhibit a $\sim$30% reduction in the number of goblet cells in the small intestine. This is due to apoptotic depletion of these highly secretory cells. Moreover, the small intestinal epithelium of $Xbp1^{-/-}$ mice exhibits a hyperproliferative phenotype [10]. As a consequence of Paneth cell depletion, $Xbp1^{-/-}$ mice have a defect in handling oral infection with a model pathogen, *Listeria monocytogenes*, as evidenced by increased *L. monocytogenes* colony counts in the feces as well as their increased translocation to the liver compared to littermate $Xbp1^{+/+}$ mice [10]. However, even minor perturbations in the UPR can have dramatic effects on Paneth cell function given the observation that isolated intestinal crypts from $Xbp1^{+/-}$ mice exhibit decreased bactericidal function against the *phoP* strain of *Salmonella typhimurium* when compared to $Xbp1^{+/+}$ mice even though Paneth cell numbers in $Xbp1^{+/-}$ mice are indistinguishable from those in $Xbp1^{+/+}$ mice [10]. Consistent with this, nearly $\sim$30% of $Xbp1^{+/-}$ mice exhibit small intestinal inflammation. Taken together, these data indicate that even incomplete deficiency of *Xbp1* is associated with functionally important impairment in the host's management of the intestinal microbiota [11].

As noted, the response of intestinal epithelium to the microbiota results in both the delivery of signals that regulate the composition of the microbes through the delivery of signals apically into the lumen and the inflammatory state of the mucosa through delivery of other signals basally into the lamina propria. Not only does hypomorphic XBP1 function affect the former but it also affects the latter responsibility as $Xbp1^{-/-}$ epithelia overreact to microbial components and soluble mediators derived from the immune system itself (e.g., TNF). This has been shown by increased activation (phosphorylation) of c-jun N-terminal kinase (JNK) in TLR5/flagellin-stimulated *Xbp1*-silenced MODE-K cells, a small intestinal epithelial cell line [10]. A similar hyperreactivity of MODE-K.*iXbp1* cells has been observed upon stimulation with TNF, a prototypical pro-inflammatory cytokine present in the intestinal mucosa [10].

As an apparent consequence of these complex alterations in intestinal epithelial biology and their interrelationship with the microbial flora, $Xbp1^{-/-}$ mice spontaneously develop small intestinal inflammation with features that are characteristic of human IBD. These include ulcerations, neutrophilic infiltration and crypt abscesses which develop in a patchy manner [10]. While $Xbp1^{-/-}$ mice exhibit a prevalence of spontaneous inflammation of $\sim$80%, $Xbp1^{+/-}$ mice also develop mild enteritis at a prevalence of $\sim$30%. Considering that mice specifically depleted of Paneth cells [12] and those with a genetic impairment in activating the Paneth cells' antimicrobial peptides ($Mmp7^{-/-}$ mice [13]) do not develop spontaneous intestinal

inflammation, it might be surmised that the development of enteritis in *Xbp1*-deficient or hypomorphic mice requires the complex interplay of a hyperreactive epithelium toward the microbiota together with predicted alterations in the structural composition of the microbiota due to Paneth cell dysfunction [10, 11].

In this context it is notable that *Nod2*$^{-/-}$ mice (see below) also exhibit impaired handling of oral *L. monocytogenes* infection as demonstrated by increased translocation to the liver and spleen [14]. *Nod2*$^{-/-}$ mice express decreased levels of specific alpha-defensins, and correspondingly, patients with Crohn's disease (CD) harboring the risk-conferring *NOD2*^{3020insC} variant also exhibit decreased expression of human alpha-defensins (HD4, HD5) in their Paneth cells [15]. Remarkably, colonization of specific pathogen-free *Nod2*$^{-/-}$ mice with *Helicobacter hepaticus*, a pathobiont, leads to the development of granulomatous ileocolitis [16]. In that model, the inflammatory infiltrate appears skewed to a Th1 phenotype. Notably, this *H. hepaticus*-induced enteritis phenotype in *Nod2*$^{-/-}$ mice could be rescued by transgenic overexpression of human alpha-defensin 5 (HD5) in the intestinal epithelium [16]. Further supportive evidence that this phenotype relates to NOD2 function in the epithelium came from studies in bone marrow chimeric mice, in which transfer of wild-type bone marrow into irradiated *Nod2*$^{-/-}$ mice did not rescue the phenotype [16].

The similarity of the Paneth cell phenotype in *Xbp1*$^{-/-}$ and *Nod2*$^{-/-}$ mice is interesting as both corresponding human genetic loci have been linked to IBD. *NOD2* is the pre-eminent genetic risk factor of CD, with three main risk-conferring variants and multiple rare variants associated with CD [17–21]. In contrast, the *XBP1* locus has been associated with both forms of IBD, CD and ulcerative colitis (UC), and several rare variants have been described in IBD and functionally characterized as hypomorphic inducers of the UPR [10]. While *Xbp1*-deficient mice spontaneously develop intestinal inflammation as discussed above [10], *Nod2*$^{-/-}$ mice do not exhibit any spontaneous histological abnormalities [14], but rather respond in an abnormal manner to certain types of microorganisms as noted above [16]. Although, the mechanism(s) of this dysfunctional responsive in the context of NOD2 deficiency is unknown, the recent discovery that NOD2 plays an important role in autophagy induction may represent one important converging pathway [22–24].

Autophagy

The recognition that autophagy is an important pathophysiologic pathway that is involved in IBD is originally based on the discovery of a risk-conferring *ATG16L1* coding variant in one of the first genome-wide association studies (GWAS) [25]. *ATG16L1* and further IBD risk genes that functionally map to autophagy have been found in several additional GWAS [26, 27]. Autophagy (or macroautophagy) is an ancient auto-cannibalistic process that allows cells to survive periods of starvation by degrading cellular organelles or macro-protein structures thereby releasing quintessential cellular nutrients such as amino acids [28, 29]. This pathway plays an important role in many processes (e.g., development, cancer, neurodegeneration), and has also been co-opted by the innate immune system in the process of degrading intracellular pathogens (xenophagy). Autophagy functions by engulfment of intracellular organelles or ingested microbes via double-membraned vesicles which fuse with lysosomes (so-called autophagolysosomes) which leads to their contents' degradation [29]. Gene-trap-targeted mice expressing a hypomorphic variant of *Atg16l1* (*Atg16l1*^{HM}) exhibit a remarkable alteration that affects the exocytosis pathway of Paneth cells with inappropriate cytoplasmic location of typical Paneth cell granule content [30]. A similar structural alteration has been reported for Paneth cells in patients homozygous for the CD risk-conferring *ATG16L1*^{T300A} variant [30]. Further support of impaired autophagy within Paneth cells as the fundamental basis for this phenotype has come from mice with a conditional deletion of *Atg5* specifically in IECs [30]. In addition to these morphological changes, *Atg16l1*^{HM} Paneth cells exhibit profound transcriptional alterations that include increased production of inflammatory mediators such as adipokines, and others [30]. Despite these changes in Paneth cell morphology and function, *Atg16l1*^{HM} mice orally infected with *L. monocytogenes* do not exhibit increased translocation of this model bacterium to their inner organs or mesenteric lymph nodes. The consequences of hypomorphic Atg16l1 function on fecal *L. monocytogenes* colony counts has not been evaluated, and hence it is currently unknown whether hypomorphic Atg16l1 might affect the intraluminal control of microbial challenges or—by inference—influence the structural composition of the intraluminal and the epithelially attached microbial flora [30].

The *Atg16l1*^{HM} model has also served as an excellent means to obtain fundamental insights into gene-environment interactions in general and in IBD in particular [31]. Specifically, the morphological and functional phenotype of Paneth cells as described above is dependent on the presence of a persistent infection with a specific murine norovirus strain (MNV CR6) [31]; norovirus infection is commonly present in typical specific pathogen-free (SPF) animal facilities. *Atg16l1*^{HM} mice rederived into an MNV-free enhanced barrier facility exhibit perfectly normal Paneth cells. Only infection with the persistent MNV CR6 strain, but not the transiently infective MNV CW3 strain, re-introduces Paneth cell abnormalities to *Atg16l1*^{HM} mice

[31]. These 'abnormalities' also extend to the profound alterations in the inflammatory transcriptional profile known to be associated with *Atg16l1*^{HM} mice [31]. Paneth cell abnormalities have not been detected in wild-type mice infected with MNV CR6. Of note is the fact that MNV is not found in Paneth cells within infected mice, but is present in mononuclear cells of the lamina propria [31]. This suggests that Paneth cells in *Atg16l1*^{HM} mice may 'inappropriately' respond to a host-derived signal induced in a non-Paneth cell compartment through the presence and/or replication of the virus. This has obvious implications for the differential responsiveness towards MNV strains CR6 and CW3. Despite these MNV-CR6-dependent abnormalities in Paneth cells, neither MNV-free nor MNV-infected *Atg16l1*^{HM} mice develop spontaneous intestinal inflammation [30, 31]. However, MNV CR6-infected *Atg16l1*^{HM} mice (but not non-infected) mice exhibit increased severity towards dextran sodium sulphate (DSS)-induced colitis [31]. This is extremely interesting because Paneth cells are not normally present in the colon except when Paneth cell metaplasia occurs during inflammation. Moreover, when exposed to DSS, MNV CR6 infected *Atg16l1*^{HM} mice exhibit ileal pathology (not usually seen in DSS colitis) with villus blunting similar to what may be observed in human CD and celiac disease [31]. Moreover, antibody-mediated neutralization of IFN-γ or TNF, as well as reduction of microbial communities via broad-spectrum antibiotics results in amelioration of DSS colitis in MNV CR6-infected *Atg16l1*^{HM} mice [31]. In summary, these studies reveal a complex interaction between host genetics, viral infection, and the microbiota in determining susceptibility to intestinal inflammation.

NOD2, the gene that confers the highest degree of genetic risk to the development of CD, has recently been discovered to regulate autophagy [22–24]. Specifically, it has been shown that activation of NOD2 via its specific molecular ligand MDP or bacteria stimulates the formation of autophagosomes through recruitment of NOD2 to the bacterial entry site [22, 23]. Remarkably, NOD2 has been shown to recruit ATG16L1 to the isolation membrane in epithelial cells [23]. While this process appears to be independent of RIP2 and NF-κB signaling in epithelial cells [23], another study performed in dendritic cells has reported NF-κB-dependency for NOD2-initiated autophagosome formation [22]. The absence of NOD2 function (either genetically or by gene silencing) results in a marked impairment of bacterial clearance in these model systems [22–24]. Importantly, CD-associated *NOD2* and *ATG16L1* variants exhibit a similar profound impairment in NOD2-dependent induction of autophagy [22–24]. In addition to these autophagy-related effects on innate immune functions, one of these studies also predicted that impaired NOD2 function may cause a substantial reduction in adaptive immunity, in that it decreased MHC class II antigen presentation by DC in an elegant model system using recombinant *S. enterica* expressing tetanus toxin [22].

NOD2 and nuclear factor κB signaling

The exact mechanism whereby CD risk-conferring *NOD2* variants contribute to the induction of intestinal inflammation in CD is still unresolved [1]. In addition to the role of NOD2 in regulating Paneth cell defensins and autophagy, other recent studies suggest that NOD2 regulation of TLR signaling and NF-κB pathways are also potentially important in converting IBD-associated, NOD2 genotypes into phenotypic IBD. After stimulation with its natural ligand MDP, NOD2 is recruited to a RIP2 (receptor interacting serine/threonine kinase 2)/BID (BH3 interacting domain death agonist)/NEMO (NF-κB essential modifier) complex, which results in ubiquitination of NEMO via the E3 ubiquitin ligase TRAF6 (TNF receptor-associated factor 6) [32–35]. The upstream kinases of the NF-κB pathway, IKK1 and IKK2, are then phosphorylated via recruitment of TAK1 to NEMO [36]. CD-associated variants of NOD2 appear to be hypomorphic inducers of this activation cascade, at least after acute NOD2 stimulation [17], whereas prolonged stimulation might result in NOD2 acting as a negative regulator of NF-κB activation in the context of parallel TLR2 stimulation [37, 38]. Considering this close interaction between NOD2 and NF-κB signaling, the critical role of NF-κB signaling in directing IEC function deserves further commentary.

Substantial insight into the fundamental role of the intestinal epithelium in regulating the mucosal immune system and its interaction with the microbial content has come from studies investigating the role of NF-κB signaling in the intestinal epithelium [39, 40]. NF-κB is activated via kinases (IKK1 and IKK2) that phosphorylate inhibitor of kappa B kinase α (IκBα) resulting in its ubiquitination and proteasomal degradation, thereby releasing NF-κB transcription factors for their nuclear translocation, DNA binding and transactivation of their target genes [36]. IKK1 and IKK2 form a complex with NEMO, which lacks kinase activity [36]. Deletion of *Nemo* in the intestinal epithelium results in severe colitis, a phenotype that is also recapitulated by deletion of both upstream kinases, *Ikk1* and *Ikk2*, but not either alone [40]. *Nemo* deficiency in the epithelium results in epithelial apoptosis and impaired expression of antimicrobial peptides which results in the translocation of bacteria into the mucosa [40]. Colitis induction in this model is dependent upon microbial signals, as germ-line *Myd88* deletion is protective. Moreover, TNF signaling through its type 1 receptor is critical as well, since *Nemo*$^{IEC-/-}$.*Tnfr1*$^{-/-}$ mice are also protected from colitis [40].

In contrast to overt colitis occurring in the absence of epithelial NEMO, *Ikk2* deletion does not lead to any form of spontaneous intestinal inflammation in mice held under SPF conditions [39]. However, IKK2 has an important role in directing the induction of mucosal immunity as revealed through an infestation model with the gut-dwelling nematode *Trichuris muris* [39]. Specifically, mice with an intestinal epithelium-specific deletion of *Ikk2* cannot clear the nematode via an inability to mount a protective Th2 immune response characterized by increased IL-13 expression in particular [39]. Instead, these mice develop severe colitis characterized by a Th1 and Th17 immune response driven by IL-12/23p40 secreted by mucosal dendritic cells [39]. TSLP, whose promoter harbors two NF-κB-binding sites, had earlier been suggested by in vitro studies as an IEC-derived factor that may instruct mucosal dendritic cells towards a 'tolerogenic' phenotype [41]. Indeed, intestinal epithelium deficient in *Ikk2* has been shown to exhibit impaired TSLP expression upon *T. muris* infestation [39]. Impairment of this TSLP-IL-13 pathway also results in decreased expression and release of RELM-β (resistin-like molecule beta) [39], a goblet cell derived peptide that has been shown to directly affect nematode motility and hence constitutes an important anti-nematode and anti-microbial defense mechanism [42–44].

Thymic stromal lymphopoietin

As implied, TSLP is an excellent example of the factors which the epithelium secretes in response to microbes that controls subsequent immune responses (recently reviewed in [45]). TSLP is an IL-7-like cytokine expressed in IECs which acts through a heterodimeric receptor composed of the TSLP-R (that is related to the common-γ-chain) and the IL-7R α-chain [45]. The receptor is relatively broadly expressed in myeloid and lymphoid cells, with particularly high expression in myeloid DC [45]. Its ligand TSLP is constitutively expressed by intestinal epithelial cells [46], in particular in the colon, and appears to be a tonic signal emanating from the epithelium for the generation and maintenance of 'tolerogenic' or 'non-inflammatory' tissue-resident DCs in the gut [41, 45]. TSLP-stimulated DCs secrete less IL-12/23p40 and are deviated to drive a Th2 immune response [41]. The effects of TSLP on basophils further support helminth- and allergen-induced Th2 responses, albeit the exact pathway whereby TSLP exerts these effects on basophils has not yet been revealed [45]. Genetic deficiency of *Tslpr* or antibody-mediated neutralization of TSLP recapitulates the aforementioned pathology in the *T. muris* model which is characterized by an impaired ability to elicit a protective Th2 immune response while inducing severe intestinal inflammation characterized by overexpression of IFN-γ and IL-17A [46]. Similar to results in the $Ikk2^{\Delta IEC}$ model infected with *Trichuris,* neutralization of IFN-γ restores the necessary levels of Th2 induction, suggesting that in the absence of pro-inflammatory Th1 cytokines, Th2 protective immunity can develop despite the absence of TSLP/TSLPR signaling [46]. In addition to pathological inflammation in *Trichuris* infected mice, defective TSLP/TSLPR signaling also leads to increased severity of DSS-induced colitis, again characterized by increased IFN-γ and IL-12/23p40 expression [46]. Of note in this context is that colonic epithelial cells of CD patients exhibit lower TSLP expression compared to healthy control subjects [41], and in an in vitro model system, such colonic IEC supernatants from CD patients are not able to induce a 'non-inflammatory' DC phenotype.

Epithelial regulation by miRNAs

A final example of microbial regulation of the immune system through interactions with the epithelium can be found in the specific miRNAs that appear to be critically important in the epithelium [47]. Genetic deletion of *Dicer* in the intestinal epithelium, a gene encoding a protein that is required for the biogenesis of miRNAs, results in an impaired ability to clear *T. muris* infestation together with intestinal inflammation that is characterized by a Th1 infiltrate [47]; an immunopathologic response that is similar to the response observed in $Ikk2^{\Delta IEC}$ mice [39]. $Dicer^{\Delta IEC}$ mice exhibit a defect in colonic goblet cell differentiation together with decreased expression of RELM-β and TSLP [47]. Several lines of evidence indicate that absence of a specific miRNA, miRNA-375, accounts for the phenotype associated with epithelial *Dicer* deficiency [47]. miRNA-375 does not only control RELM-β and TSLP expression, thereby affecting downstream Th2/IL-13 induction, but is induced by IL-13 itself via a phosphoinositide-3-kinase-dependent pathway [47]. These data suggest that miRNA-375 and Dicer within IECs control a pathway that promotes Th2 differentiation and appears critical for mounting an effective mucosal immune response towards nematodes.

Conclusions

Recent genetic studies in humans coupled together with functional studies in murine model systems have shown the central importance of IECs as organizers of the mucosal immune response by bi-directionally relaying signals from and to the microbiota and thereby contributing profoundly to determining the balance between homeostasis and overt inflammation in the intestine. The human relevance of

these pathways is highlighted by the multiple genetic loci that have been linked to IBD and which functionally map to the intestinal epithelium and especially Paneth and goblet cells. Our understanding of the structural and functional composition of the intestinal microbiota is still in its very infancy, and we are only beginning to discover how specific constituents of the microbiota interact with IECs to affect mucosal immunity (e.g., SFB and Th17 cells [48, 49]; murine norovirus and autophagy [31] and polysaccharide antigen A and IL-10 production [50–52]). Studies of such relationships at the molecular level will undoubtedly provide many more eureka moments.

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