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Endoplasmic reticulum stress in the intestinal epithelium and inflammatory bowel disease

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Abstract

The unfolded protein response as a consequence of endoplasmic reticulum (ER) stress has recently been implicated as a novel mechanism that may lead to inflammatory bowel disease (IBD). Impairment of proper ER stress resolution in highly secretory Paneth and, to a lesser extent, goblet cells within the epithelium can primarily lead to intestinal inflammation. An inability to manage ER stress may not only be a primary originator of intestinal inflammation as exemplified by genetic polymorphisms in *XBP1* that are associated with IBD but also a perpetuator of inflammation when ER stress is induced secondarily to inflammatory mediators or microbial factors. Furthermore, ER stress pathways may interact with other processes that lead to IBD, notably autophagy.

Keywords

Inflammatory bowel disease; Endoplasmic reticulum stress; Genetic susceptibility; Unfolded protein response

1. Introduction

The endoplasmic reticulum (ER) stress response allows cells to deal with endogenous stress induced by misfolded proteins via a mechanism that is termed the unfolded protein response (UPR) [1,2]. Three proximal effectors of the unfolded protein response exist in mammalian cells that sense the accumulation of misfolded proteins. These include inositol-requiring transmembrane kinase/endonuclease 1 (IRE1), pancreatic ER kinase (PERK), and activating transcription factor 6 (ATF6). Under homeostatic conditions, in the absence of significant protein misfolding, IRE1, PERK and ATF6 exist in an inactive state through an association with immunoglobulin-heavy-chain-binding protein (BIP) which is also known as glucose regulated protein 78 (GRP78). As a chaperone, BIP associates with and thus senses unfolded proteins within the lumen of the ER resulting in the conversion of IRE1, PERK and ATF6

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into an active state and consequently the ability to regulate cellular events that accommodate the cell's ability to respond to the accumulation of unfolded proteins. The cellular survival pathway that is controlled by IRE1, PERK and ATF6 is primarily mediated by downstream transcription factors. In its active state, PERK is autophosphorylated and also phosphorylates and thus inhibits eukaryotic translation-initiation factor 2α (EIF2α) resulting in the cessation of translation. The mRNA encoding activating transcription factor 4 (ATF4) is however resistant to EIF2α inhibition allowing for ATF4 production and accumulation during periods of ER stress. Similarly, during ER stress and release from BIP-mediated suppression, ATF6 is mobilized to the Golgi apparatus where its cytoplasmic tail is subject to cleavage by site-1 protease (S1P) resulting in release of a fragment of the ATF6 cytoplasmic tail (ATF6f) which is also transcriptionally active. ATF6f and ATF4 bind to endoplasmic reticulum stress element (ERSE) and unfolded protein response element (UPRE) motifs, respectively, thus directing transcriptional programs which affect protein synthesis, folding and secretion, expansion of the ER, the degradation of unfolded proteins and, ultimately, programmed cell death in the most extreme circumstance. The UPR thus allows for cellular adaptation and survival to occur in response to the accumulation of misfolded proteins within the ER.

The third arm of the UPR involves IRE1; the focus of this review. IRE1 is the most conserved among the three limbs of the UPR [3,4]. IRE1 consists of two structurally related proteins that are present in eukaryotic cells. These include the ubiquitously expressed IRE1α and IRE1β whose expression is restricted to the intestinal epithelium [5]. IRE1 is an ER resident 110 kD transmembrane protein that upon activation trans-autophosphorylates, similar to PERK, and functions as an endoribonuclease and protein kinase. The endoribonuclease function of IRE1 removes a 26 bp nucleotide stretch from cytosolic unspliced XBP1 (XBP1u) mRNA to generate a spliced version of XBP1 (XBP1s). Splicing of XBP1 leads to a frame-shift that when translated results in a modification of the carboxy-terminal portion of XBP1. Relative to XBP1u, which is highly unstable and transcriptionally inert, XBP1s functions as a potent transcription factor that binds to UPRE motifs associated with its transcriptional program [6–10]. XBP1s transactivates a core set of UPR target genes in all cell types, and a remarkably diverse set of genes in a condition-and cell type-specific manner that contribute to cellular adaptation in response to ER stress [11–13]. XBP1 is itself a transcriptional target of ATF6f showing the significant cross-talk that exists between the three arms of the UPR [2].

XBP1 is extremely important for the function (and survival) of highly secretory cells which are especially vulnerable to ER stress given their need to process and secrete high quantities of protein through the secretory pathway. These cell types include plasma cells [14,15], hepatocytes [16], pancreatic acinar cells [17] and plasmacytoid dendritic cells [18] whose homeostatic regulation requires normal XBP1 function. Moreover, it can be envisioned that these cell types are especially susceptible to environmental factors that further drive ER stress through disrupting protein quality control measures associated with the ER as would occur during hypoxia, calcium deprivation, disruption of proteasome function and other metabolic irregularities and hyperproliferative states [2]. As such, ER stress has been increasingly recognized to be both a secondary consequence of neoplasia and inflammation

[2,19,20] as well as a primary factor that is involved in inducing inflammation and potentially cancer in special tissue environments [21].

2. XBP1, Paneth cells and intestinal inflammation

It has recently been reported that conditional deletion of *Xbp1* specifically in the small and large intestinal epithelium induces spontaneous enteritis with histological features of human inflammatory bowel disease (IBD), including neutrophilic infiltration, epithelial ulcerations, and crypt abscesses [21]. Not only is this spontaneous inflammation evident in homozygotically deleted animals but also in a significant fraction of heterozygotically deleted mice. Consistent with the interdependence of the three aforementioned limbs of the UPR, *Xbp1* -deficient epithelia exhibit signs of ER stress as detected by increased transcription of *grp78* (also known as heat shock 70 kDa protein 5, *Haspa5*) and *Atf4* among other factors.

It is interesting that among the four lineages of epithelial cells (absorptive epithelial cell, goblet cell, neuroendocrine cell and Paneth cell) that are derived from the intestinal epithelial $Lrg5^+$ stem cell [22], the most highly secretory subtypes, Paneth cells and, to a lesser extent, goblet cells are the cell types most dependent upon XBP1 expression. Paneth cells, in particular, are considered to be among the most highly secretory cell type in mammals [23]. Paneth cells, which derive from $Lrg5^+$ stem cells in a pathway that is dependent upon wingless factor (Wnt) signaling mediated regulation of T cell factor (Tcf4), a transcription factor that is active in intestinal crypts [24], primarily exist in the small intestine under physiological conditions and are responsible for the secretion of unique anti-microbial peptides (α-defensins or cryptdins) [25]. $Xbp1^{-/-}$ epithelia exhibit structural alterations in which Paneth cells are absent and goblet cells are numerically reduced together with increased turn-over of intestinal epithelial cells (IECs) resulting in an increased villus:crypt ratio indicative of a regenerative response [21]. The XBP1-dependent absence of Paneth cells in $Xbp1^{-/-}$ epithelia is due to activation of programmed cell death pathways, rather than an alteration of IEC development, since conditional induction of *Xbp1* deletion using a tamoxifen-regulated Cre recombinase results in Paneth cell apoptosis in association with upregulation of CCAAT/enhancer–binding protein homologous protein (CHOP) expression. CHOP is a pro-apoptotic factor that is regulated by ATF6f and ATF4 consistent with the activation of ER stress pathways in the absence of normal XBP1 function [2].

The loss of Paneth cells in the absence of XBP1 expression is functionally important to mucosal defense. In addition to regulating the survival of Paneth cells, as noted, the function of Paneth cells is also highly dependent upon XBP1 expression. Intestinal crypts derived from $Xbp1^{-/-}$ mice are devoid of antimicrobial activity when assessed with an α-defensin sensitive strain of *Salmonella* [26,27] consistent with the phenotypic absence of Paneth cells in the $Xbp1^{-/-}$ crypts as assessed by morphology (electron microscopy and light microscopy), immunohistology (anti-cryptdin and lysozyme staining) and by molecular absence of α-defensin transcripts. Interestingly, intestinal crypts from $Xbp1^{+/-}$ mice which have no detectable phenotypic change in Paneth cells as assessed by histology also exhibit a profound alteration in antimicrobial function [21]. Moreover, $Xbp1^{-/-}$ mice exhibit a substantive defect in handling an oral challenge with a pathogen, *Listeria monocytogenes*,

that is known to be dependent upon normal Paneth cell function since *Nod2*$^{-/-}$ mice, which also possess a diminution in cryptdin expression in Paneth cells, exhibit a similar defect [28]. When challenged with *L. monocytogenes*, *Xbp1*$^{-/-}$ mice exhibit a nearly 100-fold higher quantity of *Listeria* in their faeces and 10-fold higher quantities of translocated bacteria in the liver in comparison to wild-type mice [21]. These data have several important implications. They show that a defect in antimicrobial function strictly confined to the intestinal epithelium can have dramatic effects on the quantities and potentially composition of luminal pathogenic and, potentially, commensal bacteria with important implications for understanding IBD (*vide-infra*). In a corollary fashion, overexpression of α-defensins in the epithelium has been associated with protection against a pathogenic strain of *Salmonella* [29]. In addition, consistent with the role of XBP1 in regulating ER secretory function [2], these studies reveal the important physiological function that XBP1 (and thus properly functioning ER stress pathways) have in the maintenance of normal Paneth cell secretory function and antimicrobial mucosal defense.

Another manifestation of *Xbp1* deficiency in the epithelium which links directly to inflammation, as opposed to antimicrobial function, is the massive overactivation of IRE1 that is observed in the absence, or even partial deletion, of XBP1 expression. This has been shown by an examination of XBP1 mRNA splicing status in *Xbp*1-deficient mice [21]. In the animal model that was originally established, the conditionally targeted *Xbp1* allele was designed to delete exon 2 which introduced a premature stop codon, with the splicing site in exon 4 remaining intact [30]. As such, the splicing status of XBP1 mRNA can be monitored in wild-type and mutant (i.e. deleted) alleles alike in the engineered mice. XBP1 is the only known substrate of IRE1 and thus mRNA splicing of XBP1 is a highly accurate measure of IRE1 activation [10,31]. Deletion of even one *Xbp1* allele results in almost complete XBP1 splicing in the small intestinal epithelium, and deletion of both alleles increases the level of XBP1 splicing even further in the intestinal epithelium of both the small and large intestine [21]. The mechanism by which loss of XBP1 expression results in overactivation of IRE1 remains to be elucidated. However, consistent with IRE1 activation, the loss of XBP1 results in activation of both c-Jun-N-terminal kinase (JNK), which typically regulates an inflammatory response via the AP-1 transcription factor, and NFκB (A Kaser and RS Blumberg, unpublished observation). Under conditions of ER stress, IRE1α is known to interact with the adaptor protein tumor necrosis factor (TNF)-receptor-associated factor (TRAF2) leading to JNK phosphorylation via the activation of apoptosis signal-regulating kinase 1 (ASK1) [32]. Consistent with increased IRE1 activity, phosphorylated JNK can be detected in the intestinal epithelium of *Xbp1*$^{-/-}$, but not *Xbp1*$^{+/+}$, mice [21]. This *in vivo* phosphorylation of JNK is directly due to the absence of normal XBP1 function rather than inflammation *per se*, and thus a cause rather than a consequence of inflammation, since it is observed in intestinal epithelial cell lines only when XBP1 expression is silenced and in the context of an inflammatory signal [21]. The range of inflammatory signals that induce enhanced JNK phosphorylation in IECs in the absence of normal XBP1 function remains to be established but clearly includes those derived from cytokines (TNFα) and Toll like receptors (e.g. flagellin). Both of these factors are interesting with respect to intestinal inflammation and IBD [33]. Under conditions of ER stress, the TNF receptor has been observed to retrotranslocate into the ER in association with IRE1α [34] such that when

epithelial cells which are deficient in XBP1 expression are stimulated with TNFα, a major pathogenic cytokine in IBD [35], JNK phosphorylation is significantly enhanced. In a similar manner, JNK phosphorylation is increased in XBP1-deficient epithelial cells when stimulated with flagellin, a dominant pathogenic antigen that binds to Toll like receptor (TLR) 5 and is associated with IBD [36]. In the context of exposure to inflammatory cytokines (TNFα) and pathogenic bacterial antigens (flagellin), XBP1-deficient intestinal epithelial cells secrete increased quantities of chemokines such as CXCL1 in a JNK-dependent pathway [21].

These studies show that XBP1, and thus ER stress pathways, define both the antimicrobial activity of the epithelium and the degree of sensitivity that the epithelium exhibits to signals that are delivered by bacteria (and cytokines). In this manner, the epithelium is capable of primarily organizing the composition of and its responsiveness to its local microbial and inflammatory environment which, if reaching the proper pitch, can evolve into spontaneous inflammation. The cell autonomous instruction of inflammation that emanates directly from the epithelium, which these studies teach, builds upon a significant number of recent observations which show that the epithelium can primarily determine the composition of T cell responses within the lamina propria [37]. For example, IECs, through their secretion of thymic stromal lympopoietin protein (TSLP), can regulate dendritic cell production of interleukin-12p40 or B cell activating factor (BAFF) which determines T helper 1 responses [38] and B cell production of IgA and IgG [39], respectively. In the case of XBP1-deficiency within the intestinal epithelium this results in the secretion of inflammatory mediators, the range of which have yet to be defined but likely include TNFα and chemokines, that result in spontaneous inflammation of the small intestine and increased susceptibility to colitis in the context of dextran sodium sulphate (DSS) administration [21]. Consistent with this, *Ire1β*$^{-/-}$ mice are also more susceptible to DSS colitis compared to wild-type controls [5]. IRE1β is an intestinal epithelial cell specific isoform of IRE1 which emphasizes the importance of IRE1/XBP1 to the intestinal epithelium [5,21]. As such, it might be predicted that in the absence of IRE1β, IRE1α activity and thus the activation of JNK in response to bacterial and inflammatory signals is significantly augmented.

3. XBP1 as a genetic risk factor for Crohn's disease and ulcerative colitis

XBP1 function within the epithelium thus converges on both the regulation of the intestinal microbiota and responsiveness of the mucosal immune system to components of the intestinal microbes and as such determines the ability of the epithelium to primarily orchestrate intestinal inflammation in a cell autonomous manner. Given the central role that the relationship between the commensal microbiota and host immune responsiveness to the microbiota has for the pathogenesis of human IBD [33,35], it is interesting that polymorphisms have been identified as risk factors for both Crohn's disease (CD) and ulcerative colitis (UC) [21]. Three independent whole genome analyses previously reported linkage between IBD (both CD and UC) and chromosome 22 in close proximity to the *XBP1* locus [40–42]. Selecting tagging single nucleotide polymorphisms (SNP) from the HapMap database, the relevant portions of chromosome 22 surrounding the *XBP1* locus were recently examined in three cohorts and indeed identified and replicated an association with IBD, both CD and UC [21]. Given that several lines of evidence indicated that the *XBP1* locus was

complex and potentially harbored multiple rare SNPs, deep sequencing of the *XBP1* gene in more than 1000 IBD patients and controls was undertaken and identified 51 novel SNPs in the coding region and the 4.2 kb promoter region of *XBP1*. Among these were 39 rare SNPs occurring only once in either/or the CD, UC, or control cohorts [21]. Notably, rare SNPs were found three-times more frequently in the IBD subjects in comparison to the control subjects. Among the novel SNPs were 5 rare non-synonymous (ns) coding SNPs whose actual frequencies were assessed in the largest cohort by Taqman genotyping. Among those 5 SNPs, 4 were only detected in the IBD subjects, but not controls, while the fifth occurred at comparable frequencies in IBD subjects and controls [21].A nominal association with *XBP1* was found in arecent UC GWAS (personal communication, A. Franke and S. Schreiber), but not published, as *XBP1* was not among the main hits of that GWAS [43]. The *XBP1* association was not published in any of the recent GWAS, which is not unexpected since the stringent statistical corrections applied may have resulted in a type II error with true associations being missed [44]. Moreover, loci featuring rare variants (as is the case at the *XBP1* locus) may be missed by GWAS, which test for common variants, and this may in general account for a substantial part of 'missed heritability' in GWAS as recently discussed [45].

Two of the four "IBD-only" *XBP1* nsSNPs (M139I, A162P) as well as the "non-selective" nsSNP (P15L) that occurred in IBD patients and controls alike were engineered into *XBP1u* and *XBP1s*. These mutants were tested for their capacity to transactivate a UPRE promoter luciferase reporter construct in an intestinal epithelial cell line both under baseline and ER stress conditions as induced by tunicamycin. While the "IBD-only" mutants exhibited decreased transactivation capacity, the "non-selective" mutant was indistinguishable from the wild-type *XBP1* construct [21]. In a second approach, retroviral constructs were generated from these mutants and transduced into $Xbp1^{-/-}$ mouse embryonic fibroblasts (MEF), and expression of aprototypical XBP1-regulated gene, ERdj4, assessed. Notably, the "IBD-only" mutants exhibited decreased ERdj4 mRNA induction compared to wild-type constructs [21]. These studies reveal the presence of rare nsSNPs that are associated with IBD (both CD and UC) which exhibit hypomorphic function. Given that IRE1 is dramatically overactivated by even a biologically minor reduction of XBP1 expression of 50% as revealed by an examination of $Xbp1^{+/-}$ epithelia, it can be predicted that even minor decreases in XBP1 function due to mutant *XBP1* alleles might have significant consequences for individuals that carry risk-associated alleles [21]. These functional studies on *Xbp1* in mice *in vivo* as well as on IBD-associated *XBP1* mutants *in vitro* point towards the existence of a very delicate balance between the manner in which ER stress is handled and mucosal homeostasis [21]. Moreover, they predict that other genetically determined pathways will be identified that confer risk for the development of IBD either through their direct involvement in ER stress pathways and/or by regulating pathways that secondarily intersect with the UPR and thus the ability of cells to accommodate ER stress. With regards to the former is the identification of *ORMDL3*, an ER resident protein of unknown function, as a risk factor for CD [46]. As will be discussed below, the biologic pathway of autophagy, which is associated with CD, interacts significantly with ER stress responses [47].

4. ER stress and goblet cells

Goblet cells are also among the most highly secretory cells within the intestinal epithelium which provide the majority of mucus glycoproteins to the lumen and a variety of soluble mediators involved in host defense such as RELMβ [48] and trefoil factors [49]. The secretion of mucins, and other soluble mediators, not surprisingly creates a significant secretory burden on this cell type. Consistent with this, conditional deletion of *Xbp1* in intestinal epithelium leads to an approximately 30% reduction in number of goblet cells in the small intestine presumably through the induction of programmed cell death [21]. Interestingly, alterations in goblet cells are not observed in the colon of $Xbp1^{-/-}$ mice. Recently, Heazlewood et al. developed two strains of mice (termed *Winnie* and *Eeyore*) by *N*-ethyl-*N*-nitrosourea mutagenesis which develop spontaneous colitis and linked the strains to two distinct noncom-plementing missense SNPs in the *Muc2* gene [50]. *Muc2* encodes a mucin glycoprotein which is the major macromolecular component of intestinal mucus, secreted by goblet cells. Each MUC2 subunit protein is over 5000 amino acids in length and over 70% carbohydrate by weight. These subunits dimerize in the ER, which allows for glycosylation in the Golgi whereupon MUC2 undergoes further homo-oligomerization to form complex multimers that are primarily responsible for the viscous properties of mucus [51]. The penetrance of the phenotype of *Winnie* and *Eeyore* is complete in both strains, with mice developing chronic diarrhea and mild spontaneous distal intestinal inflammation by week 6 with a phenotype that resembles human ulcerative colitis [50]. Among the mice, 25% and 40% in the two strains, respectively, developed severe disease. Mutant mice exhibited aberrant MUC2 biosynthesis, less stored mucin in goblet cells, and a diminished mucus barrier [50]. This pathology was accompanied by accumulation of the MUC2 precursor and ultrastructural evidence of ER stress in goblet cells. In addition to showing evidence of mucin misfolding, the *Winnie* and *Eeyore* strains of mice also exhibit biochemical evidence of ER stress as shown by increased expression of grp78 and XBP1 mRNA splicing [50]. Consistent with observations in $Xbp1^{-/-}$ mice, the intestinal epithelium of *Winnie* and *Eeyore* mice also display an increased rate of apoptosis and hyperproliferation [50]. Colonic explants from *Winnie* and *Eeyore* mice exhibit increased secretion of pro-inflammatory cytokines IL-1β, TNF-α, and IFNγ compared to wild-type mice.

These studies show that an inability to properly fold and secrete functional mucin glycoproteins in goblet cells can lead to both, the consequences of mucin deficiency and/or the consequences of ER stress. As a highly secretory cell type, the goblet cell may be prone to an ER stress-induced inflammatory phenotype and ultimately programmed cell death. In a similar manner, it has recently been observed that ER stress is readily demonstrable in HLA-B27 transgenic rats [52]. This rat model of colitis is also dependent upon the presence of commensal microbiota [53], and occurs as a consequence of transgenic overexpression of the human HLA-B27 protein in rats [54]. HLA-B27 is a notoriously unstable protein predicting that its overexpression in rat cells may burden the secretory machinery and thus induce an ER stress response and consequently inflammation [52,55]. Regarding the effects of mucin deficiency, $Muc2^{-/-}$ mice have been observed to develop spontaneous colitis [56] as well as intestinal tumors [57]. On the other hand, selective depletion of goblet cells via diphtheria toxin expressed under the intestinal trefoil factor (ITF) promoter which reduces

goblet cell numbers by approximately 60% renders mice more resistant to DSS colitis [58]. Similarly, mice with a goblet cell lineage defect due to *Math1* or *Gfi* deficiency do not develop intestinal inflammation, albeit such mice die fairly early in life due to other complications [59,60]. It might be conjectured that in the context of total goblet cell loss as occurs in *Math1*$^{-/-}$, *Gfl*$^{-/-}$ and ITF-diphtheria toxin transgenic mice there is not only a loss of mucin production but also other proinflammatory mediators from goblet cells such as RELMβ which activates TNFα production by macrophages [48,56,57,59–63]. Goblet cells in *Muc2*$^{-/-}$ mice would be expected to continue to produce such factors since goblet cells are present and remain secretory including compensatory metaplastic expression of the gastric mucin, MUC6 [57]. Moreover, the absence of MUC2, a significant contributor to mucus viscosity, is likely to be a major causative factor in the development of colitis in MUC2-deficient mice by allowing for significant contact between the luminal microbiota and the intestinal epithelium. Thus, the colitis that is observed in the *Winnie* and *Eyore* strains of mice can be envisioned to be due to two interrelated processes: the inflammatory effects of selective mucin deficiency which allows for increased contact of the epithelium with luminal microbiota leading to increased secretion of inflammatory mediators from the epithelium (including goblet cells that remain intact) and the potentially inflammatory effects of enhanced ER stress due to the accumulation of unfolded mucin proteins. It can further be predicted that, similar to *Xbp1*$^{-/-}$ mice, these two pathways are synergistically pathogenic as epithelial cells which are subjected to ER stress will be more sensitive to signals delivered by microbial antigens through Toll-like receptors. Although the cellular mechanisms that define these responses are yet to be defined in *Winnie* and *Eeyore* mice, they likely include increased JNK-mediated signaling which is driven by the distorted architecture and potentially composition of the luminal microbiota that is anticipated to exist in the absence of normal mucin secretion [51].

Although genetic polymorphisms have not yet been defined in *MUC2*, it is interesting that polymorphisms of the *MUC19* gene have been identified in Crohn's disease making it possible that variants may be identified which are phenotypically convergent with that observed in *Winnie* and *Eeyore* mice [46]. The expression pattern of MUC19 in humans (and mice) is poorly understood, however, and has only been studied in mucus cells of various glandular (non-intestinal) tissues [64].

5. ER stress and intestinal inflammation in humans

Increased XBP1 splicing, consistent with ER stress is observed in the colon and small intestine of subjects with both Crohn's disease and ulcerative colitis [21]. Heazlewood et al. have also reported evidence for MUC2 precursor accumulation with staining present throughout the cytoplasm in IECs from ulcerative colitis patients together with ultrastructural changes in goblet cells that are suggestive of either inappropriate granule formation or premature dissolution of stored granules prior to secretion [50]. Furthermore, these changes are accompanied by increased grp78 expression in IECs in support of the presence of ER stress. Given that *MUC2* polymorphisms have not been identified in human IBD, it is likely that the identification of ER stress in ulcerative colitis as shown by Heazlewood and colleagues [50] represents either the secondary effects of inflammation since stress inducing factors such as hypoxia and hypersecretion are characteristic of these

disorders [33,65] and/or that they are representative of primary (genetic)-based abnormalities in ER stress pathways such as that associated with *XBP1*. Consistent with the notion that intestinal inflammation can secondarily induce ER stress in IBD is the observation by Shkoda and colleagues that grp78 expression is increased to a similar degree in epithelial cells from patients with CD, UC as well non-IBD inflammatory controls (sigmoid diverticulitis) compared to uninflamed controls [66]. Taken together, these studies show that ER stress in the epithelium can be due to a primary (genetic) predisposition and/or secondary to inflammation *per se.* Moreover, it can be predicted that individuals who are unable to cope with ER stress due to genetic susceptibility will not only be predisposed to the primary development of inflammation emanating directly from the epithelium but will also be especially sensitive to the effects of inflammation. Thus ER stress can both promote and perpetuate inflammation.

6. Interleukin-10 and the UPR

Interleukin-10 (IL-10) is an important regulatory [67] and barrier protective [68] cytokine. In the absence of IL-10 as observed in *Il10*$^{-/-}$ mice, one of the first genetic models of intestinal inflammation, colitis and small intestinal enteritis may be observed [69]. *Il10*$^{-/-}$ mice develop colitis histologically resembling human UC when held under specific pathogen free conditions, but not under germ-free conditions [69], underscoring the importance of the commensal microbiota for the perpetuation of intestinal inflammation in this as well as most other animal models [70]. The role of IL-10 in mucosal inflammation that is associated with IBD has gained increased attention in view of a recent genome wide association study involving UC by Franke and colleagues that identified polymorphisms in the *IL10* gene as an important genetic susceptibility factor for the development of this disease [43].

Proteomic analysis of primary intestinal epithelial cells obtained from germ-free *Il10*$^{-/-}$ mice monocolonized with *Enterococcus faecalis* has revealed the upregulation of grp78 expression [66]. In a small intestinal epithelial cell model system using MODE-K cells, it was shown that IL-10, but not TGF-β, triggers decreased grp78 mRNA and increased degradation of grp78 protein in an IL-10 receptor reconstituted small intestinal epithelial cell line. In this model, it has been suggested that IL-10 inhibits recruitment of ATF6 to the grp78 promoter. Although grp78 is classically considered to be an ER-resident chaperone which promotes the UPR and provides cellular protection by enhancing the folding of proteins [71], these authors interestingly propose a role for grp78 within the cytosol in the context of inflammation. In this model, in the absence of IL-10, but the presence of TNFa, they propose that grp78 is increased, translocates into the cytosol and promotes RelA phosphorylation by facilitating its association with the IK-kinase (IKK) complex. [66].

It is interesting in this regard that IKK is able to physically associate with IRE1 α through the adapter protein TRAF2 [34]. Consistent with this, ER stress-induced NFκB activation is impaired in IRE1α silenced cells and *Ire1α*$^{-/-}$ mouse embryonic fibroblasts. Moreover, gene expression analysis has revealed that ER stress-induced expression of TNF-α is IRE1α and NFκB dependent and that blockade of TNFR1 signaling significantly inhibits ER stress-induced cell death [34]. This suggests that apoptosis in the context of ER stress is due to

both expression of CHOP and TNFR-signaling. Further studies have also suggested that ER stress impairs TNF-α-induced activation of NFκB and JNK and converts TNF-α from a weak to a powerful inducer of apoptosis [34]. Together with earlier work by Urano et al. which reported JNK phosphorylation secondary to ER stress that is dependent on adaptor protein TRAF2 [32], these studies provide further evidence in support of a direct relationship between ER stress and inflammation and vice versa. Returning now to the work of Shkoda et al. [66], it might be predicted that IL-10 inhibits a yet to be confirmed pro-inflammatory role for grp78 within the cytosol. How this reconciles with a role of grp78 as an ER-resident chaperone, the effects of IL-10 on other components of the ER stress pathway and the cell biology of grp78 translocation into the cytosol remains to be established.

7. Intestinal microbiota and ER stress pathways in the epithelium

The identification of markers indicative of increased ER stress in the tissues and epithelia of the vast majority of human subjects with IBD analyzed to date and evidence that ER stress pathways can in and of them selves initiate and/or promote inflammation [21,50,66], it is logical to propose that inflammation-induced ER stress is likely to be a perpetuator of intestinal inflammation. As a corollary, it might be surmised that environmental factors which promote (or enable) the UPR might make cells able to cope with ER stress and thus tend to deter pro-inflammatory pathways at a cellular level. One of the key drivers of intestinal inflammation associated with IBD is that associated with the commensal microbiota [33,35,72]. It is therefore reasonable to consider the manner in which microbes intersect with ER stress pathways. Microbes can be envisioned to directly initiate ER stress, and thus inflammation, but also cause ER stress indirectly through their ability to induce inflammation and/or to prevent inflammation through enabling UPR pathways as already alluded to above.

Regarding the possibility that products derived from microbes may inhibit an UPR with all of its attendant consequences, a screen for inhibitors of XBP1 activation has recently identified a 21-membered macrocyclic lactam termed “trierixin” derived from *Streptomyces* sp. as an XBP1 inhibitor [73,74]. Trierixin was able to inhibit thapsigargin-induced XBP1-mediated activation of a XBP1-luciferase reporter in Hela cells as well as endogenous XBP1 splicing in untransfected HeLa cells [74]. In the process of trierixin isolation, two structurally related compounds, mycotrienin II and trienomycin A, were identified as inhibitors of ER stress-induced XBP1 activation [74]. While such compounds would be predicted to function in a manner comparable to the phenotype observed with XBP1-deficiency [21], this remains to be established.

In other studies, Paton et al. have identified a new family of potent AB_5 cytotoxins produced by Shiga toxigenic *Escherichia coli* [75]. AB_5 cytotoxins also include toxins derived from *Shigella* sp., *Vibrio cholera* and *Bordetella pertussis*. This class of toxins are comprised of a single 35 kD A subunit and a pentamer of 13kD B subunits. Both the A and B proteins are encoded by two closely linked and cotranscribed genes (*subA* and *subB*) [75]. Intraperitoneal injection of the purified AB_5 subtilase (subAB) cytotoxin is fatal for mice due to the induction of extensive microvascular thrombosis resulting in necrosisin the brain, kidneys,

and liver [75]. The extreme cytotoxicity of this toxin for eukaryotic cells is due to the serine protease activity of the A subunit which specifically disrupts grp78 function and activates the ER stress pathways [76]. This is due to the fact that subAB cleaves grp78 and, assuch, activates all three effector pathways of the UPR; PERK, ATF6 and IRE1 leading to XBP1 splicing [77]. Mutagenesis of grp78 leading to a single amino acid substitution at the cleavage site prevents subAB-induced cleavage of grp78 *in vitro* [76]. Notably, oral challenge of mice with an *E. coli* expressing subAB confers toxigenicity to the *E. coli* with mice appearing ill and lethargic together with steady loss of body weight [75]. Although the mechanism of the toxicity remains unexplored, it is likely that a profound activation of the UPR response within the epithelia is occurring in mice infected with *E. coli* expressing subAB similar to that which has been observed in $Xbp1^{-/-}$ mice [21].

It is also noteworthy that the B pentamers of AB_5 cytotoxins recognize specific oligosaccharide moieties displayed by host cell glycolipids such that differences in target glycolipids have a major impact on host susceptibility and tissue tropism [78]. Paton et al. identified GM2 among a panel of glycolipids as being able to bind and neutralize subAB and characterized a strain of *E. coli* as an expressor of the particular GM2 glycolipid to which subAB binds [75]. This raises the possibility that certain *E. coli* strains may express receptor mimics that could provide ‘probiotic’ function by neutralizing subAB activity. It should be noted that the subtilases are a family of serine proteases which are found in a wide variety of microorganisms, but to date, no other members have been shown to exhibit cytotoxic activity [75]. Bioinformatics have identified a *Bacillus anthracis* sequence as the closest homologue of subA, while *Yersinia pestis* and *Salmonella typhi* contain homologues of subB, albeit none of the strains encode the required second subunit of the toxin within the immediate genetic vicinity of the first which is required for a pathogenic cyototoxin [75]. These studies show that the microbiota can express factors capable of eliciting an UPR and that complex interactions are likely to exist among the microbiota themselves and the host that will determine the set-point of an ER stress response within the epithelia and other mucosa-associated cell types.

8. ER stress mechanisms interact with autophagy

Genome wide association studies (GWAS) have recently identified autophagy as an important mechanism that is involved in intestinal inflammation associated with CD through the identification of genetic polymorphisms within the *ATG16L1* [79,80], *IRGM* [81–83] and, potentially, *LRRK2* genes [46,79]. Macroautophagy represents a lysosomal pathway that is involved in the turnover of cellular macromolecules and organelles which is involved in cellular homeostasis and defense with implications for biological processes as diverse as the maintenance of immunologic tolerance and host defense against pathogens [47,84,85]. The first step of autophagy is the envelopment of cytosol and/or organelles in the isolating membrane, which wraps around the cargo forming an autophagosome, a vesicle surrounded by a double-membrane as opposed to the single membrane associated with a phagosome [84]. The autophagosome then undergoes a progressive maturation by fusion with endolysosomal vesicles creating an autolysosome whereupon the cargo is then degraded [47,84].

The *ATG16L1* association was first discovered in a GWAS of approximately 20,000 nsSNPs and haplotype and regression analysis identified a single coding nsSNP (*rs2241880*, T300A) that carried virtually all of the disease-associated risk exerted by the *ATG16L1* locus [79]. Based mostly upon sequence homology with yeast *ATG16*, it was predicted that *ATG16L1* might be an autophagy gene [86]. Together with the *IL23R* and *NOD2* loci, *ATG16L1* exhibits the strongest association with CD of all the currently known risk-associated loci [46,81]. Three recent studies have yielded insights into the potential mechanisms by which ATG16L1 may be associated with CD [87–89]. All three studies have confirmed the prediction that ATG16L1 is indeed a 'bona fide' autophagy protein. Studying mice with hypomorphic *Atg16l1* alleles, Cadwell et al. reported ultrastructural alterations of Paneth cell granules together with altered expression of inflammatory genes in Paneth cells [87]. Saitoh et al. reported that ATG16L1 regulates endotoxin-induced inflammosome activation and that chimeric mice lacking ATG16L1 expression in hematopoietic cells exhibit increased IL-1β- and IL-18-mediated susceptibility to DSS colitis [88]. In a third report, Kuballa et al. transfected human epithelial cells with the disease-associated T300A variant of ATG16L1 and described impairment in the capture of internalized *Salmonella* within autophagosomes [89]. The second documented autophagy gene that is associated with CD is *IRGM*, which belongs to the p47 immunity related GTPase family [81–83]. Its mouse homolog, LRG-47 (encoded by *Irgm1*), controls intracellular pathogens through autophagy, and *Irgm1*$^{-/-}$ mice exhibit a marked increase in the susceptibility to *Toxoplasma gondii* and *L. monocytogenes* infection [90]. Consistent with this, IRGM induces autophagy and thereby controls intracellular *Mycobacterium tuberculosis* in human macrophages [91].

Of note is the fact that several of the genetic risk factors for IBD exhibit a phenotypic convergence on the Paneth cell [92]. *Nod2*$^{-/-}$, *Atg16l1*-hypomorphic, as well as *Xbp1*$^{-/-}$ mice all show some form of alteration related to Paneth cell structure and/or function that also link to the induction of inflammation [92]. Interestingly, human subjects harboring the *ATG16L1* risk allele and similarly *NOD2* risk alleles have been suggested to exhibit comparable morphological and transcriptional changes of Paneth cells, suggesting that these studies in mice may extend to humans [87,93]. These observations suggest that these three genetically mediated pathways either affect Paneth cells independently or that the pathways which they affect are interrelated. In support of the latter possibility, evidence is accumulating that ER stress may be a potent trigger of autophagy. It was initially noted in the yeast *Saccharomyces cerevisiae* [47,94] that autophagy may counterbalance the expansion of the ER during the UPR [95]. In mammalian cells, induction of autophagy during ER stress has been linked to PERK/eIF2α [96,97], while Ogata et al. have presented evidence to suggest that IRE1 rather than PERK might constitute the link between ER stress and autophagy [98]. Using mouse embryonic fibroblasts (MEFs) deficient for IRE1α or ATF6 and embryonic stem cells deficient for PERK, these latter authors have shown that accumulation of LC3 (microtubule-associated protein 1 light chain 3)-positive autophagic vesicles triggered by tunicamycin or thapsigargin fully depends on IRE1, but not PERK or ATF6 [98]. Thapsigargin-induced accumulation of LC3-positive vesicles is also completely inhibited in MEFs deficient in TRAF2 expression which shows that the link from IRE1 to autophagy is mediated byJNK [32]. Consistent with this, pharmacological inhibition of JNK effectively inhibits the translocation of LC3 in this model system, suggesting that the IRE1-

TRAF2-JNK pathway may be essential for the induction of autophagy in MEFs challenged with ER stressors [47,98].

As described above, IRE1 overactivation is an important feature of XBP1-insufficiency [21]. Active IRE1 can bind to TRAF2 which participates in the activation of JNK [32] through apoptosis signal-regulating kinase 1 (ASK1) [99]. It is predicted that this may allow cells to adapt to ER stress by initiating autophagy [98]. This might be particularly important for situations in which XBP1 function is hypomorphic as modeled in *Xbp1*$^{+/-}$ mice and in human subjects harboring IBD-associated hypomorphic variants of *XBP1* [21]. In contrast to XBP1-deficient (*Xbp1*$^{-/-}$) mice which develop enteritis and lack Paneth cells due to the induction of programmed cell death, *Xbp1*$^{+/-}$ mice develop spontaneous enteritis in the context of dysfunctional, but intact, Paneth cells. In this setting, it is anticipated that increased IRE1 (and UPR pathway) function is associated with JNK activation but not to a degree that apoptosis occurs but rather to a level that allows for adaptive mechanisms, such as autophagy, to develop in order to cope with ER stress [21]. The Bcl2-family molecules Bax and Bak, which may form a complex with active IRE1, might also represent another means by which active IRE1 could affect apoptosis *versus* autophagy decisions in cells with a stressed ER [85,100].

9. Conclusion

The UPR is a phylogenetically conserved mechanism involving three proximal effectors (IRE1, PERK and ATF6) that allows cells to cope with the ER stress associated with the burden of secreting high levels of protein. Through an examination of XBP1 function within the intestinal epithelium, a highly secretory cell type, it is now clear that a proper ER stress response is necessary to prevent organ specific inflammation. Deletion of *Xbp1* specifically within the intestinal epithelium results in Paneth cell, and to a lesser extent, goblet cell dysfunction which leads to spontaneous intestinal inflammation in a pathway that involves IRE1 overactivation of JNK and downstream inflammatory pathways. Since the *XBP1* locus contains polymorphisms that function as genetic susceptibility factors for both forms of IBD (CD and UC), it is now evident that ER stress represents a new pathway from which intestinal, and perhaps other forms of organ-specific inflammation, can emerge. Moreover, these studies confirm the importance of the intestinal epithelium as a central cell type that is involved in the initiation of IBD which is highly significant given the strategic location of the epithelium in between the two major factors that determine intestinal inflammation, the microbiota on the one side and the immune system on the other. As such, the intestinal epithelium functions as both a sensor and determining factor of the composition of the luminal microbiota and the tone of the local inflammatory milieu. In this context, the ability of the epithelium to manage the level of ER stress that accompanies these obligations is a major determinant of the luminal microbial content and inflammatory tone and whether homeostasis *versus* inflammation will occur. It is now clear that a broad range of factors (both microbial, cellular and immune-derived) can affect the UPR as recently revealed by an examination of bacterial toxins (e.g. subAB), mucins (e.g. MUC2), autophagic proteins (e.g. ATG16L1) and cytokines (e.g. TNFα and IL-10) and their ability to primarily cause or exacerbate homeostatic ER stress which is of special importance to highly secretory cells (e.g. Paneth and goblet cells). Overall, recent evidence shows that ER stress has a unique

role in the epithelium and can be both a primary cause as well as a consequence of intestinal inflammation. The genetically determined ability to mount an appropriate ER stress response will therefore likely be a major determinant of both the initiation and perpetuation of mucosal inflammation as it pertains to IBD.

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