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Autophagy, viruses, and intestinal immunity

Elisabeth Kernbauer^{1,2} and Ken Cadwell^{1,2}

¹Kimmel Center for Biology and Medicine at the Skirball Institute, New York University School of Medicine, New York, NY 10016, USA

²Department of Microbiology, New York University School of Medicine, New York, NY 10016, USA

Abstract

Purpose of review—To highlight recent findings that identify an essential role for the cellular degradative pathway of autophagy in governing a balanced response to intestinal pathogens and commensals.

Recent findings—Following the genetic association of autophagy with inflammatory bowel disease (IBD) susceptibility, increasing evidence indicate that this pathway functions in various epithelial lineages to support the intestinal barrier. New studies are also revealing that autophagy proteins dictate the quality and magnitude of immune responses. Mouse models in particular suggest that autophagy and IBD susceptibility genes regulate inflammatory responses to viruses, a finding that coincides with an increasing appreciation that viruses have intricate interactions with the host and the microbiota beyond the obvious host-pathogen relationship.

Summary—Autophagy and other immunological or stress response pathways intersect in mucosal immunity to dictate the response to pathogenic and commensal agents. The development of novel treatment strategies as well as prognostic and diagnostic tools for gastrointestinal disorders will be greatly facilitated by a deeper understanding of these interactions at the cell type and microbe-specific manner, which includes less appreciated components of the microbiota such as eukaryotic and prokaryotic viruses.

Keywords

Inflammatory bowel disease; autophagy; ATG16L1; virome; microbiota

INTRODUCTION

The intestinal immune system is faced with the challenge of providing robust protection against transient infections while co-existing with the microbiota, the collection of microorganisms that inhabit the body's surfaces. The importance of immunity towards enteric pathogens is unfortunately too clear as diarrheal infections remain a leading cause of childhood mortality (1). In contrast, an excessive immune response is considered integral to

*To whom correspondence should be addressed: Ken Cadwell, Ph.D. New York University School of Medicine, Skirball Institute Lab 2-10, 540 First Avenue, New York, NY 10016. Telephone: (212) 263-8891. Fax: (212) 263-5711. ken.cadwell@med.nyu.edu.

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Crohn's disease (CD) and ulcerative colitis, the major forms of inflammatory bowel disease (IBD) (2). Over 150 genetic loci have been linked to IBD susceptibility, and many of these implicate pathways involved in host-microbe interactions such as autophagy (3). In this article, we will review how examination of the autophagy pathway has led to unique insight into intestinal immunity, with particular emphasis on recent findings that have altered the way we view gene-gene and gene-environment interactions and the role of virus infections in IBD.

AUTOPHAGY AND INFLAMMATORY BOWEL DISEASE SUSCEPTIBILITY

Autophagy (macroautophagy) is a conserved process by which cellular material is sequestered in a double-membrane vesicle, the autophagosome, and targeted to the lysosome for degradation and recycling (Figure 1) (4). Inhibition of mammalian target of rapamycin (mTOR) signaling by nutrient deprivation or other environmental stressors can increase the generation of autophagosomes through the coordinated action of three complexes composed of proteins encoded by autophagy-related genes (ATGs). The pre-initiation complex activates the class III PI3K complex to recruit proteins that bind phosphatidylinositol 3-phosphate (PI3P) to the nascent autophagosome membrane. This growing membrane, often referred to as the phagophore or isolation membrane, is frequently found apposed to the endoplasmic reticulum (ER), although other membrane sources and sites have been shown to contribute to elongation (5). The closure of the autophagosome is dependent on the ATG5-ATG12/ATG16L1 complex that conjugates LC3 to the lipid phosphatidylethanolamine (PE) through reactions that resemble the ubiquitination pathway. The sequestration of specific targets is mediated by the adaptor molecules p62, Nbr1, Ndp52, optineurin, and Nix that can bind both LC3 and ubiquitinated cargoes, which include protein aggregates, organelles such as damaged mitochondria, and internalized bacteria (6). Finally, together with other proteins involved in endolysosomal trafficking, the SNARE protein Syntaxin-17 incorporated in the autophagosome membrane mediates fusion with the lysosome (7).

Autophagy was initially linked to IBD by a series of genome-wide association studies (GWAS) that identified a common threonine to alanine substitution at position 300 (T300A) in *ATG16L1* that increases susceptibility to CD (8-10). The T300A substitution increases cleavage of ATG16L1 protein by caspase-3 and caspase-7 downstream of environmental stressors such as TNF- α and glucose starvation (11, 12). This destabilization of ATG16L1 leads to decreased autophagy resulting in reduced intracellular bacterial killing, Paneth cell abnormalities, and excess production of interleukin-1 β (IL-1 β). A role for autophagy in IBD has also been suggested by susceptibility mutations located near or within *innate immunity related GTPase family M protein (IRGM)*, *NDP52*, and *ULK1* (13-15). IRGM and its murine homolog *Irmg1* (Lrg-47) have been shown to increase bacterial degradation by inducing autophagy, especially during *Mycobacterium* infection (16-18). Since IRGM variants associated with CD alter binding by microRNAs rather than protein sequence, tight regulation of autophagy has been proposed to be necessary to maintain balanced immunity in the intestine (19). NDP52 has also been shown to function in antimicrobial autophagy by crosslinking LC3 with ubiquitin and galectin-8, which recognizes glycans exposed on damaged pathogen-containing vesicles (20, 21). Although the missense mutation in *NDP52*

associated with CD has been shown to inhibit responses to toll-like receptor (TLR) stimulation (14), it is unclear if and how it affects autophagy. Likewise, a CD polymorphism that tags the ULK1 locus implicates the autophagy pre-initiation complex in mucosal immune defense (15), and it will be important to further investigate the strength and nature of this association.

These genetic associations have led investigators to test whether other IBD susceptibility genes have autophagy-related functions. Among the first susceptibility factors identified for CD are mutations in the bacterial sensor *NOD2* that inhibit responses to bacterial peptidoglycan (22-24). In addition to promoting expression of cytokines and antimicrobial molecules, stimulation of *NOD2* was demonstrated to strongly induce autophagy (25-28). The IBD susceptibility genes *XBPI*, *STAT3*, *LRRK2*, *DAP*, and *PTPN22* have also been shown to regulate autophagy, mostly through their effects in upstream signaling (29-34). However, an important consideration is that association with IBD can be explained by non-autophagy functions of these genes as well. Additionally, even many bona fide autophagy genes such as *ATG16L1* participate in the non-classical form of autophagy referred to as LC3-associated phagocytosis, and also other mechanistically obscure pathways of cellular defense (35-37). Therefore, genetic association alone does not provide definitive evidence that autophagy has a primary role in IBD pathogenesis. Nevertheless, recent studies have demonstrated that autophagy proteins have a fundamental role in intestinal immunity and barrier function (Figure 1), which we review below.

AUTOPHAGY AND INTESTINAL IMMUNITY

Autophagy appears to be particularly important for maintaining secretory cell lineages. Mice with decreased *Atg16L1* levels (*Atg16L1* hypomorphs; *Atg16L1*^{HM}) display several abnormalities in small intestinal Paneth cells including structural defects in antimicrobial granules that are also observed in CD patients homozygous for the *ATG16L1*^{T300A} allele (38). Mutation of other autophagy genes or chemical inhibition of the lysosome in mice also generates Paneth cell abnormalities (38-42). In addition to Paneth cell defects, mice with cell type-specific deletion of autophagy genes in the intestinal epithelium develop goblet cell hypertrophy in the colon and a corresponding decrease in mucus-secretion (43). Interestingly, deletion of the inflammasome gene *Nlrp6*, a member of the Nod-like family of innate sensors, induces autophagy and leads to abnormal goblet cells in the colon (44). Inflammasomes are cytosolic sensors of damage that are involved in processing of caspase-1 and subsequent activation of IL-1 β and IL-18 (45). Based on this finding, the investigators suggest that *Nlrp6* functions upstream of autophagy in protection of the intestinal barrier, reminiscent of *in vitro* studies with *Nod2*.

Irgm1^{-/-} mice display Paneth cell granule and organelle defects, and deletion of the ER stress response factor *Xbp1* in the intestinal epithelium leads to Paneth cell and goblet cell depletion that is accompanied by spontaneous inflammation in ~60% of the mice (29, 46). This effect of *Xbp1* deletion implicates the ER homeostasis function of autophagy in maintenance of the secretory lineages in the intestinal epithelium. In an impressive follow-up study, the authors generate Paneth cell-specific deletions of *Xbp1* and autophagy genes to demonstrate that these stress response pathways are functioning within the Paneth cell

compartment (30). Moreover, dual deletion of *Xbp1* and *Atg16L1* (or other autophagy genes) is sufficient to induce severe enteritis. Also, several of the above autophagy-deficient mice display exacerbated intestinal inflammation upon dextran sodium sulfate (DSS) treatment (29, 46, 47). When taken together, these studies suggest that one reason autophagy genes are associated with IBD reflects an essential role for autophagy in maintaining Paneth cells and goblet cells.

As reviewed elsewhere (48, 49), autophagy also has a general function in promoting innate and adaptive immunity including bacterial degradation, delivery of products generated by these internalized pathogens to the endo-lysosome for activation of endosomal TLRs and antigen presentation (50-54), and mediating survival and differentiation of B and T lymphocytes (55-62). Several recent studies demonstrate that the ability of autophagy to limit the survival of intracellular pathogens is important in the intestine. Upon oral inoculation with *Salmonella enterica* typhimurium, mice in which *Atg5* or *Atg16L1* is deleted in the intestinal epithelium display an increase in bacterial burden within enterocytes and dissemination to extra-intestinal tissue (63, 64). Mice expressing *Atg16L1*^{T300A} (*Atg16L1*^{T300A} knock-in mice) also display an increase in bacterial burden leading to an increased inflammatory cytokine response (11, 12). Therefore, it is possible that decreased immunity due to autophagy inhibition can lead to an unresolved infection in the intestine, which may initiate or sustain inflammation.

Other models also need to be considered since autophagy can benefit viruses and bacteria including *Salmonella* by providing membranes or other factors that support replication (65-69). Additionally, autophagy genes have potent immuno-suppressive functions. As originally described in cells derived from *Atg16L1*^{-/-} mice, the autophagy pathway suppresses inflammasome activity, likely by removal of internal damage signals such as reactive oxygen species (ROS) produced by damaged mitochondria (70, 71). Although the mechanism remains the focus of ongoing studies, autophagy appears to have a similar role in suppressing type I IFN (IFN-I) production downstream of viral infection (72-75). Thus, a role for autophagy in suppressing immunity is another potential mechanism by which this pathway contributes to IBD.

INTESTINAL VIRUSES AND AUTOPHAGY

Viral infections have long been suspected to have a role in IBD (76, 77). Experimental support was lacking until the Paneth cell abnormalities in *Atg16L1*^{HM} mice were found to be dependent on persistent infection by a murine norovirus (MNV), a positive-strand RNA virus related to noroviruses that cause acute gastroenteritis in humans (47). In addition to Paneth cell abnormalities, MNV-infected *Atg16L1*^{HM} mice develop inflammatory pathologies in response to DSS that resemble pathologies observed in CD patients (47). An important aspect of these findings is that neither *Atg16L1* mutation nor MNV infection alone generated these abnormalities, thereby supporting a multi-hit disease model in which genetic and environmental factors act in concert to create disease pathologies.

The mechanism by which MNV induces intestinal abnormalities in *Atg16L1*^{HM} mice involves an excessive inflammatory response rather than uncontrolled viral replication (47).

In another context, the Atg5-Atg12/Atg16L1 complex was shown to be necessary for the ability of IFN- γ to restrict MNV replication in macrophages through a process that does not require lysosomal degradation (37). In contrast, other enteric viruses have been shown to subvert the autophagy pathway. Echovirus induces autophagy to enter cultured polarized intestinal epithelial cells (78), and rotavirus uses the autophagy pathway for virion assembly (79, 80). When several studies with Poliovirus and coxsackievirus B are taken together, a model emerges in which many of these viruses utilize the autophagy machinery to generate membranous platforms for replication and egress (81-85). *NOD2* and *IRGM1* also mediate virus-host interactions (86-88), and it will be interesting to examine the function of these and other IBD genes in additional models of intestinal viral infections.

Viral gastroenteritis can precede a diagnosis of IBD (89, 90), and *Retroviridae* and certain phage species are overrepresented in patients affected by CD (91). Reactivation of herpesviruses, potentially in response to ongoing inflammation, could exacerbate IBD (92). Thus, a viral trigger can conceivably lead to IBD onset or recurrence. However, as demonstrated by studies with MNV and Atg16L1^{HM} mice, a potential viral trigger may not act in isolation. Two additional studies demonstrate that MNV requires other factors (*Helicobacter* infection or IL-10 deficiency) to exacerbate intestinal disease in mice (93, 94). Also, the observation that neither intentional nor natural infection by persistent strains of MNV causes obvious disease in wild-type control mice suggests that this particular virus is a conditional pathogen (pathobiont). This relationship between MNV and the host could even be classified as commensalism since infection is typically innocuous, much like colonization by a given commensal bacterial species.

Although it is unclear whether a functionally analogous virus exists in humans, deep sequencing efforts have revealed the presence of a complex intestinal microbiota that includes phages, eukaryotic viruses, and fungi in addition to bacteria that are the traditional focus of attention (95). Phages and fungi can have beneficial functions for the mammalian host (96, 97). Also, the presence of intestinal helminths has a striking inverse correlation with the incidence of IBD and other inflammatory disorders, and is currently under scrutiny as therapeutic agents (98). Given the widespread existence of viruses, the contribution of our 'virome' to health and disease is likely underappreciated (99).

SPECIFICITY OF GENE-MICROBE INTERACTIONS IN IBD

Recent observations in Atg16L1 and Nod2 mutant mouse models suggest that interactions between genetic and environmental susceptibility factors are specific. The same Atg16L1^{HM} mice that are susceptible to virus-mediated intestinal pathologies are highly resistant to bladder and intestinal disease induced by uropathogenic *Escherichia coli* and *Citrobacter rodentium* respectively (100, 101). In the example of *C. rodentium*, a model pathogen related to enteropathogenic *E. coli*, Atg16L1 deficiency increases the innate immune response leading to a 500-1000 reduction in bacterial burden and near absolute protection from diarrheal disease. This unexpected protective effect of Atg16L1 mutation could be related to the role of autophagy in suppressing excess cytokine production since the Atg16L1^{HM} mice display an enhanced IFN-I gene expression signature in the intestine (101). In striking contrast, Nod2^{-/-} mice are highly susceptible to *C. rodentium* due to

impaired recruitment of monocytes to the site of infection (102). Also, instead of an adverse response to MNV infection, *Nod2*^{-/-} mice display intestinal epithelial defects including impaired mucus production by goblet cells and susceptibility to piroxicam-mediated enteritis in a manner dependent on the commensal species *Bacteroides vulgatus* (103). Side-by-side comparisons of *Atg16L1* and *Nod2* mutant mice demonstrate that these two IBD susceptibility genes have opposing functions in their responses to these various microbial agents (101, 103).

CONCLUSION

The close proximity of bacteria, eukaryotic and prokaryotic viruses, fungi, and parasites in the gastrointestinal tract suggest that “trans-kingdom interactions” are common place and intricate mechanisms that allow coexistence are in place (104). A prime example is that the mouse helminth *Trichuris muris* requires the presence of the intestinal bacteria for egg hatching (105), and helminth colonization in humans is associated with increased diversity in the bacterial composition of the gastrointestinal tract (106). Bacteriophages can also shape the intestinal bacterial community through horizontal transfer of virulence factors and selective killing of individual species (97, 107-109), but what about interactions between eukaryotic viruses and bacteria? The pathogenic effect of MNV in *Atg16L1*^{HM} mice is dependent on commensal bacteria, and poliovirus, reovirus, and mouse mammary tumor virus (MMTV) exploit the presence of intestinal bacteria for viral propagation (110-112). Host factors likely influence these interactions, as demonstrated by the expansion of the enteric virome in immuno-compromised primates infected with SIV (113). Also, a recent finding demonstrating that herpesviruses reactivate in response to T helper 2 cytokines induced by helminth infection reinforces the need for co-infection models to address these questions (114). Given the central role autophagy plays in intestinal immunity, future studies will likely reveal new ways in which this pathway and specific IBD susceptibility genes influence interactions with and between various components of the intestinal microbiota.

Based on the findings described in this article, another important research direction is to understand the relationship between IBD susceptibility genes. It is tempting to link various genes by demonstrating overlapping function within the same pathway (e.g. *NOD2* and *ATG16L1* in autophagy), but it is also possible that individual genes or groups of genes have distinct contributions to IBD pathogenesis. It is also likely that mutations will combine with environmental factors to evoke specific pathologies, or in some instances act synergistically with another mutation, as in the example of dual inhibition of *Atg16L1* and *Xbp1* (30). Such functional characterization of genes and pathways identified in population genetic studies will continue to yield insight into intestinal immunity and tolerance. Deep mechanistic studies will surely lead to novel therapeutic targets for improving treatment of enteric infections, IBD, and other gastrointestinal disorders.

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KEY POINTS

- Functional characterization of IBD genes implicates a role for the autophagy pathway in intestinal immunity.
- Autophagy proteins support the intestinal barrier through cell type and context-specific functions.
- The virome is potentially an unappreciated contributor to intestinal health and disease host physiology.
- *In vivo* studies support specific gene-microbe and gene-gene interactions in IBD pathogenesis.

Figure 1. Autophagy in intestinal immunity.

A. Autophagy can be activated in response to various signals including nutrient sensing, ER stress, cytokines, and activation of innate immune mediators such as NOD2 and IRGM. Downstream of these signals, the ULK1 pre-initiation complex, the Class III PI3K complex, and the ATG5-ATG12/ATG16L1 complex act in concert to mediate the generation, elongation, and closure of the phagophore, which becomes the autophagosome. Adaptors such as NDP52 and p62 facilitate the inclusion of cargo (e.g. damaged organelles, protein aggregates, viral proteins, and bacteria) by crosslinking LC3 on the phagophore with

ubiquitin (Ub) attached to the cargo. The completed autophagosome fuses with an endo-lysosome leading to the formation of an autophagolysosome in which the cargo is degraded and recycled. Genetic associations with IBD are in bold.

B. Autophagy maintains homeostasis in the intestine by supporting Paneth cell and goblet cell function, degrading internalized pathogens (xenophagy), regulating cytokine production, supporting B and T cell viability and differentiation, and delivering microbial products to the endo-lysosome for TLR recognition and antigen presentation. Autophagy inhibition disrupts these intestinal immune processes, which alters resistance to enteric infection and may contribute to IBD.