

Chapter 12

Inflammatory Bowel Disease at the Intersection of Autophagy and Immunity: Insights from Human Genetics

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Abstract Studies using human genetics have identified more than 160 loci that affect the risk of developing inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC). Several of these genes have been found to play key roles in the process of autophagy, a lysosome-based degradation pathway. Although historically considered to be a relatively nonselective process of degradation of cytosolic contents, autophagy has recently been revealed to have several selective and immune-specific functions that are relevant to the maintenance of intestinal homeostasis, including xenophagy, mitophagy, antigen presentation, secretion, and inflammasome regulation. In this chapter, we review the evidence that links autophagy-related genes, their immune-specific functions, and possible mechanisms of IBD pathogenesis. We summarize the basic molecular events underlying general and selective autophagy and present evidence suggesting possible pathogenic mechanisms revealed by studies of IBD-associated risk alleles of *ATG16L1* and *IRGM*. Finally, we review chemical biology-based experimental approaches for identifying autophagy regulatory pathways that may have implications for the development of therapeutics.

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Introduction

Genome-wide association studies (GWAS) of IBD have been a major success story for genome-level studies of complex human disease. One of the particularly exciting results from these studies was the identification of autophagy as an unanticipated player in determining risk of IBD. This pathway, long considered to be a relatively nonselective process of bulk cytoplasmic degradation by the lysosome, is thought to have evolutionary roots as a mechanism to maintain metabolic homeostasis in response to decreased nutrient levels [1]. However, studies over the last 10 years have expanded the functions of autophagy to include roles in organelle clearance, antigen presentation, cell death, development, and the degradation of intracellular pathogens, among others. The role of autophagy in immune-related processes [2–4] was particularly unexpected and has served as a guide in many studies seeking to connect human genetics to mechanisms of IBD pathogenesis [5–7].

These genetic studies have identified multiple genes within the autophagy network as playing a role in CD, including *ATG16L1*, *IRGM*, *NOD2*, and *LRRK2* [8–11]. In the case of UC, the autophagy-related genes *DAP1* and *SMURF1* have also been identified as determinants of risk [12, 13]. Further studies subsequently identified polymorphisms in *ULK1*, *ATG2A*, and *GABARAPL1* as harboring significant associations with CD [14, 15]. Although autophagy has been implicated in the disease process in a range of other disorders, including neurodegenerative disease [16, 17], atherosclerosis, and cancer, to date no compelling genetic association has been found between autophagy and many immune-related diseases, including multiple sclerosis, celiac disease, rheumatoid arthritis, and psoriasis. However, targeting sequencing studies have also begun to identify coding mutations in essential autophagy genes as playing roles in disease, raising the possibility that more disease-associated autophagy-related polymorphisms have yet to be uncovered [18, 19].

In this chapter, we review studies that have employed human genetics to understand the link between autophagy and IBD. We begin by examining the idea of the intestinal mucosa as a physiological system wherein homeostasis is particularly vulnerable to disruptions in autophagy. We then present a basic overview of the molecular events involved in autophagy, followed by discussions of how autophagy may function as a selective process with critical roles in IBD-related immunity. These roles include (1) degradation of intracellular pathogens (xenophagy), (2) mitophagy, (3) antigen presentation, (4) secretion, and (5) inflammasome regulation. In the cases of xenophagy, mitophagy, and antigen presentation, we present detailed evidence of how autophagy functions in these pathways. Next, we review results from studies of two particularly well-examined CD risk single-nucleotide polymorphisms (SNPs), rs2241880 (*ATG16L1*, coding variant T300A) and rs13361189 (*IRGM*). Finally, we discuss approaches from the field of chemical biology that may be used to identify autophagy regulatory pathways with possible implications for therapeutics development.

Autophagy in the Intestinal Mucosa

As mentioned above, autophagy has been implicated in several immune-related processes that influence IBD pathogenesis, including xenophagy, mitophagy, antigen presentation, secretion and vesicular trafficking, and cytokine-based regulation of inflammasome activity. One major point of intersection between these processes is their critical importance in the intestinal mucosa. This complex environment must maintain a delicate balance of immune responses, which are continuously stimulated by metabolic stresses and antigens from food. Microbes also play a major role in this milieu, which carries the highest bacterial load in the body. This tissue, therefore, may be especially susceptible to disruption in autophagy, with consequent alteration of homeostatic processes. For example, mice with conditional knockout of *Atg7* in intestinal epithelial cells show increased severity of colitis when exposed to the infectious pathogen *Citrobacter rodentium*, while mice with deficiency in the core autophagy gene *Atg16l1* in hematopoietic cells show increased susceptibility to chemically induced models of colitis [20, 21].

Studies in human genetics have provided evidence that microbial stimulation may exist as a node of functional interaction between CD susceptibility genes, including *ATG16L1* and *NOD2*. For example, expression of the *ATG16L1* CD-associated risk allele (*ATG16L1* T300A) results in impairment of autophagy and antigen presentation, both of which are enhanced by muramyl dipeptide (MDP), the bacterial ligand for the receptor *NOD2* [22, 23]. Although no evidence has been found for an epistatic interaction between these two genes, this type of functional interaction suggests that autophagy may operate as a key point of intersection among multiple susceptibility genes.

Basic Molecular Events in Autophagy

The process of autophagy begins with an appearance of a flat membrane sheet termed the isolation membrane (also known as the phagophore assembly site in yeast), the origin of which remains unknown. The membrane elongates and expands to encompass its cargo, eventually fusing to form a double-membrane structure known as an autophagosome. The outer autophagosomal membrane then fuses with the lysosomal membrane, forming a degradative autolysosome. It should be noted that the source of the autophagosomal membrane is still a highly debated topic and the existence of multiple sources cannot be excluded. In the case of the ER, a novel Ω (omega)-shaped vesicle called the omegasome has been identified, which resides on the ER and might precede the appearance of the isolation membrane [24].

Many of the molecular events underlying the assembly and elongation of autophagy membranes were initially identified by genetic screens in *Saccharomyces cerevisiae* [25, 26]. These screens have identified approximately 35 core autophagy proteins, about half of which have clear homologues in mammals. For the purposes

of this chapter, we divide these genes into five functional categories: (1) the ULK complex (ATG1 complex in yeast); (2) ATG9; (3) Vps34 complexes; and (4, 5) two ubiquitin-like conjugation systems anchored by ATG12 and ATG8, respectively.

The ULK complex (composed of ATG13, FIP200, and either ULK1 or ULK2) plays a central role in starvation-induced autophagy and is thought to be involved in the initiation of the autophagic cascade. The complex is recruited to sites of autophagosome formation, where it likely acts to recruit and disassemble other ATG protein complexes [27–30]. ATG9, a transmembrane protein, shuttles between autophagosomes, the trans-Golgi network, and late endosomes in a cycling process that may involve the ULK complex. ATG9 is of special interest to studies of xenophagy, since a recent report suggested that ATG9 is essential for the formation of the isolation membrane in anti-*Salmonella* autophagy in mouse embryonic fibroblasts (MEFs) [31]. In mammals, the Vps34 complex is likely required for the recruitment of several autophagy proteins to sites of autophagosome formation. This complex plays a critical role in supplying phosphatidylinositol 3-phosphate (PI3P) to the growing autophagosome, where it acts as an essential driving force in autophagosome assembly [32–36]. Furthermore, depending on the identity of its protein components, some alternative versions of the Vps34 complex may serve regulatory roles in autophagosome maturation and autophagosome-lysosome fusion [32–34, 37–39].

Two ubiquitin-like conjugation systems play important roles in autophagosome formation and maturation. The ATG12-based conjugation system is initiated by the E1-like ATG7, which activates and transfers ATG12 to the E2-like ATG10. ATG12 is then subsequently conjugated to ATG5 [40, 41]. ATG5 and ATG12 then bind with ATG16L1, forming a complex that is essential for autophagosome formation [42]. In addition to a key role in autophagosome formation, the second ubiquitin-like conjugation system may play additional roles in cargo recognition [43]. In this system, ATG8 is activated by the E1-like ATG7, is transferred to the E2-like ATG3, and is conjugated to phosphatidylethanolamine through the E3-like action of the ATG5-ATG12 complex. The conjugation of phosphatidylethanolamine to ATG8 allows subsequent incorporation of ATG8 into the inner and outer membranes of autophagosomes [44–46]. Mammals appear to have two families of ATG8 homologues, known as LC3s and GABARAPs. While all ATG8 homologues can be incorporated into the autophagosomal membrane, the incorporation of LC3s and GABARAPs might occur at different stages, with LC3s acting at the stage of biogenesis and GABARAPs playing a more important role in autophagosome maturation [47]. Given that ATG8 remains associated with autophagosomes even after their fusion with lysosomes, tracking ATG8 and its homologues allows for localization of autophagosomes. Furthermore, since the inner membranes of autophagosomes are degraded upon formation of autolysosomes, tracking ATG8 enables the measurement of autophagic flux as reflected by ATG8 degradation [44, 45].

Several layers of regulation exist in the initiation and progression through the steps of autophagic degradation. One important regulatory influence is the cytoskeleton. Autophagosome trafficking involves movement along microtubules, which facilitates fusion with lysosomes [48, 49]. Changes in lysosome positioning regulate mTORC1 signaling, since peripheral localization of lysosomes and mTORC1

increases mTORC1 activity, while perinuclear lysosomal clustering decreases activity [49]. Heat shock protein beta-1 (HSPB1), a protein that interacts with and mediates reorganization of the actin cytoskeleton, is also linked to mitophagy [50]. Disruption of HSPB1 signaling can lead to accumulation of dysfunctional mitochondria, mitochondrial fragmentation, and effects in mitochondrial respiration in MEFs [51].

Transcription also serves as a regulatory influence in the autophagic process. Autophagosome and lysosome biogenesis appear to be transcriptionally coregulated by the transcription factor EB (TFEB) [52]. Overexpression of TFEB induces autophagy, while a block of TFEB expression by RNAi decreases autophagy. Interestingly, TFEB action is implicated in basal as well as starvation-induced autophagy, and therefore TFEB might represent a transcriptional master regulator of the autophagy pathway. Furthermore, translocation of TFEB from the cytosol to the nucleus is strongly enhanced upon removal of nutrients, suggesting that TFEB function is sensitive to nutrients and growth factors. Under fed conditions, phosphorylation on serine 142 of TFEB can be mediated by the extracellular signal-regulated kinase 2 (ERK2) and might be sufficient to retain TFEB in the cytosol. A comprehensive analysis of 51 genes with known functions in the autophagy pathway sheds some light on transcriptional regulation of autophagy in the context of starvation and TFEB function. Strikingly, TFEB-dependent transcription patterns generally correlate with starvation effects, further emphasizing the role of TFEB as an autophagy regulator that can directly or indirectly sense nutritional status [52]. However, the transcription of the majority of genes is not significantly affected by starvation or TFEB expression levels [52], suggesting that transcription factors other than TFEB control the expression of most autophagy and autophagy-related genes, possibly in a nutrient-insensitive fashion.

Xenophagy

Autophagy has now been well described as playing a key role in the degradation of intracellular pathogens, including bacteria, in a specialized process known as xenophagy. However, the environment of the intestinal mucosa provides special challenges to this function. As this tissue is exposed to both commensal and pathogenic bacteria, the interactions between these flora and host immune responses must be tightly controlled.

Molecular Basis of Xenophagy

The molecular events surrounding the process of xenophagy follow the same basic pattern as those involved in autophagic degradation of other cellular contents, such as organelles or aggregated protein deposits. However, the mechanisms underlying

the selection of cargo for degradation by xenophagy remain largely unknown. Components of the innate immune response can interact with autophagy proteins to target bacteria for autophagic degradation. Mechanisms involved in xenophagy of other species of bacteria may share some components with anti-*Salmonella* xenophagy, but to date, detailed molecular mechanisms have been best explored for *Salmonella*.

Upon infection of epithelial cells, *Salmonella enterica* serotype Typhimurium (*S. Typhimurium*) resides in *Salmonella*-containing vacuoles (SCVs). The environment of the SCV promotes bacterial survival and replication. The SCV protective niche is established by effectors of two type III secretion systems (T3SSs) encoded by *Salmonella* pathogenicity islands 1 and 2 (SP-1 and SP-2). The secretion and activity of T3SS components is highly regulated both temporally and spatially. For example, pH sensing regulates the transition from translocon protein secretion and pore formation to effector secretion [53]. Once secreted into the host cell, the activity of effectors can be modulated. For example, the T3SS SP-1 effectors SopA, SopE, SptP, and SopB are all ubiquitinated shortly after infection by the host cell machinery [54]. The phosphoinositide phosphatase activity of SopB regulates several processes that promote internalization and survival of *S. Typhimurium*, including cytoskeletal rearrangements that promote invasion, SCV biogenesis and maturation, and pro-survival Akt activation [54]. The ability of SopB to regulate these functions is dependent upon its ubiquitination status [54, 55]. A SopB mutant that cannot be ubiquitinated has a number of defects, including a failure to relocalize from the plasma membrane to SCVs, disrupted recruitment of Rab5 to SCVs, and perturbed *S. Typhimurium* replication [54]. Thus, ubiquitination-dependent relocalization of SopB to SCVs promotes the establishment of SCVs as a niche environment for replication. This is due in part to the ability of SopB to reduce the net negative charge of PI(4,5)P₂ and phosphatidylserine lipids on SCV membranes, thereby disrupting maturation and fusion with lysosomes [55].

Although most *S. Typhimurium* resides in SCVs, a small but significant fraction is released into the cytosol from damaged SCVs. This cytosolic *S. Typhimurium* rapidly becomes ubiquitinated. Ubiquitinated bacteria may be recognized by selective cargo receptors, including p62 and NBR1, which act as bridging factors that bind to both ubiquitin and the autophagosome. p62 and NBR1 share a similar protein structure and domain composition and may show some level of redundancy, particularly in degradation of protein aggregates (aggrephagy) and mitochondria (mitophagy). However, while p62 is important for the autophagic clearance of *Salmonella*, NBR1 is not [56]. Therefore, the mechanism of xenophagy, which relies on ubiquitination events on or around intracellular bacteria, might differ from other ubiquitin-dependent selective autophagy events. Interestingly, nuclear dot protein 52 (NDP52), in addition to p62, has been implicated in the recognition of ubiquitin-coated invasive bacteria, but not in other forms of selective autophagy [57]. NDP52 contains a putative LIR motif at its N terminus, can directly bind ubiquitin through a C-terminal ubiquitin-binding zinc finger, and can form homo-oligomers via its coiled-coil domain [57–59]. p62 and NDP52 appear to act cooperatively in the recognition and killing of *S. Typhimurium*, *Listeria monocytogenes*, and *Shigella flexneri* [60, 61]. However, p62 and NDP52 are recruited

to distinct microdomains of invading *Salmonella*, suggesting that ubiquitin coats on *Salmonella* might be heterogeneous [60].

Interestingly, in macrophages but not in epithelial cells, proteasomes localize to ubiquitin-coated *Salmonella* with potential effects on efficient rupture of damaged SCVs and major histocompatibility complex (MHC) class I antigen presentation [62, 63]. Taken together, ubiquitin-dependent autophagic targeting of intracellular pathogens and by-products has implications for pathogen growth and the regulation of inflammatory responses. However, in most cases, the E3 ubiquitin ligases, the nature of ubiquitin modifications, and the substrates involved in ubiquitin-dependent xenophagy are not known and may be pathogen- as well as cell type-specific. One important exception is LRSAM1, an LRR- and RING-domain protein that was recently identified as an E3 ubiquitin ligase crucial for ubiquitin-dependent autophagy of *S. Typhimurium* [64]. Interestingly, LRSAM1 was required for ubiquitination of intracellular bacteria but dispensable for ubiquitination of aggregated proteins, confirming that LRSAM1 serves as a selective and novel antibacterial sensor that mediates target selection for the xenophagy pathway. To date, however, LRSAM1 remains the only known ubiquitin ligase associated with antibacterial autophagy, and a detailed biochemical characterization of the aforementioned factors will yield critical insights into host–pathogen interactions and may boost the development of novel pharmaceuticals aimed to combat infectious diseases.

Xenophagy and IBD Risk Genes

As mentioned above, intestinal tissues are in constant interaction with both commensal and pathogenic bacteria. To prevent inappropriate responses to commensal bacteria, the cells of the intestinal mucosa must be educated to tolerate these microbes and their products [65]. Breakdown of this tolerance, with consequent inappropriate inflammation, has been proposed to play a major role in the pathogenesis of IBD. This model is supported by the finding that the IBD-associated genes *ATG16L1*, *IRGM*, and *ULK1* all play roles in the process of xenophagy. *ATG16L1*, *IRGM*, and *ULK1* each function in the degradation of *S. Typhimurium*, while *ATG16L1* and *IRGM* also play a role in antibacterial autophagy of a CD-relevant strain of *Escherichia coli* (adherent-invasive *E. coli*, or AIEC) [10, 66–69]. *ULK1* may also function in the degradation of *L. monocytogenes*. Furthermore, the function of *LRRK2* in reactive oxygen species (ROS)-driven bacterial killing, as well as its role in autophagy, raises the possibility that this protein may also be involved in xenophagy of gut bacteria [70–72]. Defects in these gene products might lead to impaired bacterial handling, promoting an environment of chronic inflammation and inappropriate host responses to commensal bacteria.

Some intracellular pathogens have also been highlighted as potential contributors to risk of CD, including mycobacteria. Genetic loci that include *IRGM*, *LRRK2*, and *NOD2* have been associated with increased susceptibility to leprosy and tuberculosis, suggesting an interesting point of interaction between autophagy genes,

CD, and mycobacteria. These studies, combined with reports that autophagy may play a key role in control of *Mycobacterium tuberculosis*, have led to the suggestion that control of mycobacteria might be altered in CD [73, 74]. Genetic studies appear to support this idea, especially in the context of granulomas, a histological hallmark of both CD and tuberculosis: polymorphisms in loci containing the autophagy genes *ATG2A* and *GABARAPL1* have been linked to granulomas in CD patients. To date, however, connections between CD and the mycobacterioses remain largely speculative, to be answered perhaps by future well-powered GWAS for tuberculosis [75].

The recent wealth of data from the human microbiome project has also highlighted how little we understand of the complex relationship between IBD and microbial stimulation. Certainly the diversity and composition of the gut microbiota are major factors influencing gut homeostasis, and particular dietary nutrients and metabolites are likely to interact with host genetics to influence host-microbiome interactions. An imbalance in the composition of the gut microbiome, termed dysbiosis, has been associated with IBD, including shifts in relative abundances of bacterial taxa, decreases in the diversity of the bacterial community, and alterations in the functional composition of the microbiome.

Mitophagy

The autophagic processing of mitochondria (mitophagy) was directly implicated in the context of IBD by a recent study that identified *SMURF1* as a susceptibility gene for UC [13]. This gene is an ubiquitin ligase that was recently identified, through an image-based genome-wide siRNA screen, as an important mediator of selective viral autophagy and mitophagy [76].

Molecular Basis of Mitophagy

In contrast to other substrates for selective autophagy, for example, surplus peroxisomes or ribosomes, mitophagy is well studied and underlying biochemical mechanisms for this process are emerging. Upon loss of mitochondrial membrane potential, PTEN-induced putative kinase 1 (PINK1) accumulates on the outer mitochondrial membrane [77, 78]. This step is accompanied by stabilization of PINK1 protein and may involve a membrane potential-dependent block of PINK1 proteolysis by the mitochondrial inner membrane protease presenilin-associated rhomboid-like protein (PARL). This protease is effective in healthy mitochondria but impaired in damaged mitochondria [77, 79]. PINK1 then recruits Parkin1 to damaged mitochondria, which results in Parkin1-dependent ubiquitination of some mitochondrial proteins such as voltage-dependent anion channel 1 (VDAC1) and mitofusins [80–83]. p62 accumulates around ubiquitin-decorated damaged mitochondria and

PB1-domain-mediated oligomerization of p62 results in mitochondrial clustering, eventually leading to autophagic degradation of mitochondria [80]. It should be noted that the mitochondrial ubiquitination event required for mitophagy may involve multiple substrates and/or may be cell type-specific, as p62 recruitment to damaged mitochondria and mitophagy is not impaired in VDAC1-deficient MEFs [78]. In addition, activating molecule in beclin 1-regulated autophagy (Ambra1), an autophagy-regulating protein initially identified in neuronal cells [84], is recruited to damaged mitochondria in a Parkin1-dependent fashion [85] and knockdown of Ambra1 expression results in impairment of mitophagy. Interestingly, Ambra1 recruitment most likely involves direct binding to Parkin1, but Ambra1 is not a substrate for Parkin1-mediated ubiquitination [85]. Thus, Parkin1-mediated mitophagy relies at least in part on effects other than ubiquitination.

Mitophagy and IBD Risk Genes

As mentioned above, the best evidence linking mitophagy to IBD comes from the identification of *SMURF1* as a gene associated with susceptibility to UC. SMURF1 is a HECT domain-containing E3 ubiquitin ligase that interacts with the selective autophagy factor p62 [76]. Smurf1^{-/-} MEFs show defects in the degradation of Sindbis and herpes simplex virus as well as impaired clearance of damaged mitochondria. Supporting this observation, Smurf1-deficient mice display an accumulation of damaged mitochondria in the heart, brain, and liver. Interestingly, Smurf1^{-/-} MEFs are competent for starvation-induced bulk autophagy, indicating that Smurf1 is a specific mediator of selective autophagy. However, a mechanistic link between *SMURF1* polymorphisms and IBD pathogenesis remains to be examined.

Antigen Presentation

Evidence for a link between IBD, antigen presentation, and autophagy originates from studies of CD-associated risk variants in NOD2 and ATG16L1. Although a role for autophagy in antigen presentation has been well described in basic studies, how this function may be altered in the context of IBD remains relatively unknown.

Molecular Basis of Autophagy in Antigen Presentation

MHC molecules on the cell surface present peptide antigens to T cells. MHC class I molecules interact with the T cell receptor (TCR) of CD8⁺ cytotoxic T cells, while MHC class II molecules interact with the TCR of CD4⁺ helper T cells. MHC class I

antigens are derived from proteasomal degradation of cytosolic proteins. In contrast, MHC class II antigens are delivered to the lysosome before transfer to the MHC class II loading compartment and transport to the cell surface. Delivery of these antigens to the lysosome can occur either via endocytosis of protein antigens from the extracellular space or by autophagy of cytoplasmic material [86–88]. Autophagy also plays an important role in the presentation of pathogen antigens by dendritic cells (DCs). Mice lacking Atg5 specifically in DCs show impaired CD4⁺ T cell priming following infection with herpes simplex virus, an impairment that is due to a decreased ability of Atg5-deficient DCs to process and present antigens for presentation on MHC class II molecules [89]. Furthermore, these cells show delayed fusion of phagosomes to lysosomes. Autophagic degradation may also combine with vesicular trafficking to facilitate presentation of citrullinated self-peptides by DCs to CD4⁺ T cells. This finding is particularly relevant to IBD, since immune responses against citrullinated self-proteins are associated with autoimmunity [90, 91].

Antigen Presentation and IBD Risk Genes

Interestingly, DCs from patients with CD-associated risk variants of *ATG16L1* or *NOD2* show defects in autophagy induction, bacterial trafficking, and MHC class II antigen presentation to CD4⁺ T cells [23]. Furthermore, autophagy was recently reported to destabilize the immune synapse between DCs and T cells; in this report, decreased expression of *ATG16L1* or *IRGM* resulted in hyperstable interactions between DCs and T cells as well as increased activation of T cells, suggesting a mechanism by which adaptive immunity might be increased in patients with CD who carry *ATG16L1* risk alleles [92].

Vesicular Trafficking and Secretion

The suggestion that the secretory pathway might be modulated by autophagy in the context of CD arose from the GWAS-based identification of *ATG16L1* as a common CD-associated risk gene. Patients carrying the CD-associated variant of this gene (*ATG16L1* T300A), as well as *Atg16l1* hypomorphic (*Atg16l1*^{HM}) mice, were found to show abnormalities in specialized epithelial cells called Paneth cells. These cells play a central role in innate immunity and are an important source of antimicrobial peptides in the small intestine, serving to prevent microbial invasion and control the composition of the gut microflora [93]. *ATG16L1* T300A patients and *Atg16l1*^{HM} mice exhibit decreased numbers of granules and diffuse staining for lysozyme in their Paneth cells, raising the possibility that autophagy plays a key role in secretion in these cells [94, 95]. Supporting this hypothesis, mice lacking *Atg5* or *Atg7* in

intestinal epithelial cells also show Paneth cell defects [96]. Interestingly, the Paneth cell phenotype in *Atg16l1^{HM}* mice can be influenced by exposure to a pathogen, since the cellular defects can be eliminated by maintaining mice in a virus-free environment [94]. Paneth cell defects have also been observed in mice lacking other CD-associated genes, including *Nod2* and *Xbp1* [97, 98]. More recently, a role was reported for Paneth cells in sensing nutrient availability, demonstrating that caloric restriction reduces Paneth cell-specific signaling by mTORC1, a regulator of autophagy [99]. Taken together, these reports suggest that Paneth cell homeostasis and function are intimately linked to autophagy. However, whether autophagy directly regulates vesicular trafficking and secretion remains unknown, since the molecular mechanisms by which ATG16L1 might regulate these cellular functions are poorly understood.

Inflammasome Hyperactivation and Cytokine Secretion

Inflammasomes are molecular scaffolds that activate caspase 1 and maturation of the proinflammatory cytokines interleukin (IL)-1 β and IL-18. Several types of inflammasomes have been described, which are activated by a number of endogenous and exogenous signals.

Recent studies have shown that autophagy defects can lead to inflammasome hyperactivation. For example, stimulation of *Atg16l1^{-/-}* fetal macrophages with lipopolysaccharide (LPS) induces elevated levels of activate caspase 1 and enhanced secretion of IL-1 β and IL-18 [21]. Mice with specific deletion of *Atg16l1* in hematopoietic cells also show higher serum levels of IL-1 β and IL-18 in response to dextran sulfate sodium (DSS), accompanied by increased inflammation and mortality.

The underlying mechanism linking autophagy to regulation of the inflammasome remains unknown. One possibility is that defects specifically in mitophagy result in increased levels of ROS, which are known to cause hyperactivation of inflammatory activity [100–102]. Supporting this idea, *Atg16l1^{-/-}* macrophages treated with a ROS scavenger did not show the increased IL-1 β secretion described above [21].

Samples from patients have also shown ATG16L1 coding variant-dependent alterations in IL-1 β secretion. In these studies, stimulation of peripheral blood mononuclear cells with MDP, but not LPS, resulted in a relative increase of IL-1 β secretion from ATG16L1 T300A-expressing cells. Although no difference was found in levels of activated caspase 1, protein levels of pro-IL-1 β and mRNA levels of IL-1 β were increased in the context of the ATG16L1 risk allele [103].

The autophagy adapter p62 may also provide a connection between IL-1 β signaling and ATG16L1 [104]. In a recent study, we showed that levels of p62 are normally regulated by ATG16L1-based activation of the ubiquitin ligase Cullin-3, which promotes proteasomal degradation of p62. Loss of ATG16L1, however, can result in decreased levels of Cullin-3 and decreased degradation of p62. As p62 can act as a scaffold in the IL-1 β signaling pathway, elevated levels of p62 may result in amplified IL-1 β signaling [104].

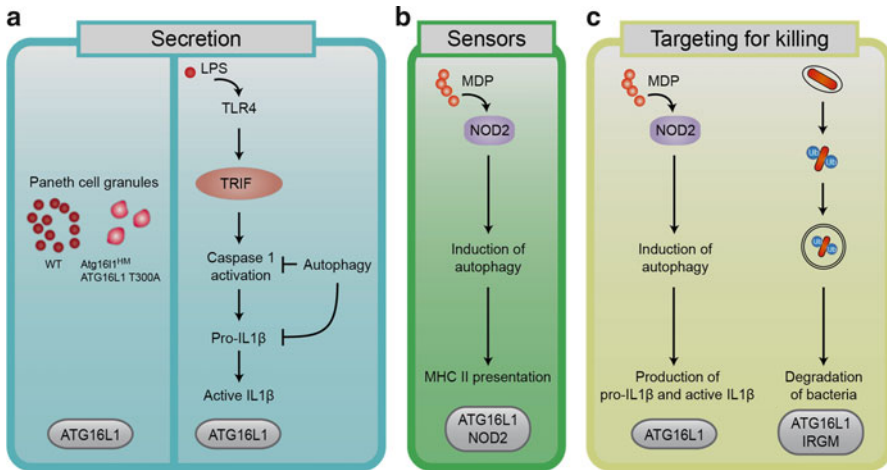


Fig. 12.1 Immune-related autophagy functions. (a) Paneth cells from CD patients expressing the CD-associated *ATG16L1* T300A allele, as well as mice expressing a hypomorphic allele of *Atg16l1* (*Atg16l1^{HM}*), show altered granule morphology, suggesting a role for autophagy in secretion of antimicrobial peptides. Another role for autophagy in secretion is suggested by observations in macrophages, in which secretion of active IL-1 β (as well as active IL-18, not shown) upon LPS stimulation is regulated by autophagy at two levels. In this pathway, loss of *ATG16L1* expression is associated with increased cytokine secretion. (b) Autophagy can also act as a sensor for bacterial products. In dendritic cells, activation of NOD2 by MDP induces autophagy that leads to MHC class II antigen presentation. This pathway appears to be altered in the context of CD-associated SNPs in *ATG16L1* and *NOD2*. (c) Autophagy plays a role in targeting bacteria and bacterial products for degradation/killing. In one pathway, observed in primary blood mononuclear cells, activation of NOD2 by MDP induces autophagy that leads to production of pro-IL-1 β and secretion of active IL-1 β . The CD-associated variant *ATG16L1* T300A is associated with increased amounts of active IL-1 β upon MDP stimulation. Autophagy can also directly degrade bacteria. In epithelial cells, bacteria become ubiquitinated and targeted for autophagic degradation in a process that requires the CD-associated genes *ATG16L1* and *IRGM*

ATG16L1 T300A

One of the best-studied CD-associated risk alleles is the T300A coding polymorphism in *ATG16L1*. *Atg16l1*^{-/-} mice are not viable [21], but an alternative hypomorphic model (*Atg16l1^{HM}*) has yielded important clues to how the T300A coding polymorphism may result in altered autophagy and increased inflammation [94, 95]. The site of the T300A polymorphism is near a WD-repeat domain, which is present in mammalian *ATG16L1* but absent in yeast *Atg16*. This domain is not required for autophagy, consistent with the finding that the T300A polymorphism does not affect classical autophagy [42, 67].

As a core autophagy protein, *ATG16L1* has been shown to play multiple roles in IBD-relevant processes, including xenophagy, antigen presentation, IL-1 β production, and secretion (see Fig. 12.1). Interestingly, the CD-associated risk allele

appears to have different effects on these functions depending on the cell type examined [42, 67]. It is perhaps not surprising, then, that T300A has also been associated with defects in antigen presentation increases in IL-1 β production and abnormalities in Paneth cell secretion in patient samples [23, 95, 103]. In addition, antibacterial autophagy of *Salmonella* is affected by the presence of the ATG16L1 CD risk allele [67], and patients with the T300A allele show increased susceptibility to infection by *Helicobacter pylori* [105]. These findings illustrate that disease genes are likely to have specific functions in specific cell types, and researchers must consider the cell type- and stimulation-specific contexts used to examine disease-associated phenotypes.

IRGM rs13361189

IRGM (immunity-related GTPase family M) is a human gene recently identified as playing an important role in antibacterial and antiviral autophagy. To date, xenophagy of pathogens including *S. Typhimurium*, *M. tuberculosis*, and CD-associated AIEC has been reported to be dependent on IRGM [66, 68, 69, 106].

A synonymous SNP within the human *IRGM* locus (rs13361189) is associated with CD, and a 20 kb deletion polymorphism within the 5' untranslated region of *IRGM* is in perfect linkage disequilibrium with this SNP [68, 107]. However, both rs13361189 and the deletion polymorphism have also been reported to be in perfect linkage disequilibrium with a synonymous exonic SNP (rs10065172). This SNP is associated with decreased binding of the microRNA miR-196 to the 3' UTR of *IRGM*, an event that downregulates expression of IRGM [69]. This finding is consistent with the observation that expression of miR-196 is increased in epithelial cells of the inflamed ileum and colon of CD patients compared with controls [69]. This result may be relevant to autophagic targeting of pathogenic bacteria, since knockdown of IRGM or overexpression of miR-196 affects the autophagic targeting of AIEC. Furthermore, an additional *IRGM* polymorphism (–261TT) is associated with an increase of IRGM expression and protection against *M. tuberculosis* [74].

Autophagy Interaction Network

Despite the approaches described in the studies above, in which individual autophagy genes are examined in relative isolation, it is important to note that a complex network of autophagy-related proteins exists in the cell and that cellular functions captured within this network likely extend beyond the strict bounds of lysosomal degradation.

As mentioned above, autophagy is implicated in many cellular pathways, including adaptations to changes in environmental factors such as nutrient

availability. Many autophagy and autophagy-related proteins have been characterized, and distinct autophagic complexes with functions in autophagosome assembly and maturation have been identified and analyzed in great detail. The current picture suggests a stepwise process, but the precise regulation of autophagy in changing environmental settings—including fluctuations in growth factor supply, cytokine signaling, and the presence of pathogens—likely involves complex cross talk between pathway components. Behrends and colleagues recently used an approach which might be described as “pathway proteomics” to investigate the network organization of the human autophagy system [108]. In doing so, they provided a basis for understanding the autophagy network on a protein level. In brief, interaction partners for 32 epitope-tagged stably expressed proteins, representing core autophagy as well as autophagy-related processes, were identified using mass spectrometry following immunoprecipitation of the respective tagged protein. A subset of proteins interacting with primary baits were used as baits in secondary screens, leading to the identification of 751 high-confidence candidate interactions between a total of 65 baits and an additional 409 proteins. A high coverage of known interactions, a high level of validation (reciprocal immunoprecipitations and *in vitro* assays), and validation of autophagy functions of network components via RNAi allow for the designation of these results as a true autophagy protein interaction network. Interestingly, different autophagy subnetworks such as the ULK complex, Vps34 complex, ATG8 conjugation system, and PI3P signaling members were revealed to have an underappreciated high level of interconnectivity. Novel interaction partners for the mammalian ATG8 homologues were also identified. These interactors may be shared among all ATG8 homologues, specific for LC3 or GABARAP subfamily members or specific for individual ATG8 homologues. However, differential cellular abundance of distinct ATG8 homologues could in part account for different efficiencies with respect to the co-immunoprecipitation of ATG8 interaction partners [108]. Nevertheless, differences in LC3-interacting region (LIR) motif composition among different ATG8 homologues can affect binding to p62, NBR1 [109], and possibly control differential requirements of LC3 and GABARAP subfamily members in early or late phases of autophagosome biogenesis [47]. In addition, it will be interesting to see if some of the novel ATG8 interactors might function as receptors for certain types of selective autophagy. Good candidates might be recovered by a comparison of ATG8 interactors identified by these proteomics studies and a list of proteins predicted to combine LIR and ubiquitin-binding motifs (Kay Hofmann, unpublished data in [110]).

Interestingly, pharmacological induction of autophagy led to alterations of protein–protein interactions within some, but not all, subnetworks [108]. It will be interesting to see if such clusters might be targets of microRNAs, which generally modify the translation of multiple genes at the same time. In addition, it is possible that different subnetworks are altered by different stimuli, possibly in a cell type-specific fashion, which could ultimately help in the design of disease-specific autophagy-enhancing or autophagy-inhibiting drugs.

Chemical Modulators of Autophagy

As highlighted throughout this chapter, many unanswered questions remain regarding the mechanistic basis of multiple autophagy functions. We believe that the field of chemical biology is poised to help answer these questions, providing the tools to dissect the regulatory nodes that exist at the intersections of autophagy and immunity. Furthermore, studies in this field may help to identify druggable targets within the autophagy pathway and its regulatory influences.

Small molecules have been proven to be useful tools in autophagy research, demonstrating success by increasing our knowledge of autophagy regulation while simultaneously identifying potential therapeutic compounds. Small-molecule screens are particularly important in identifying probes for dissecting complex biological processes such as autophagy, as they allow the dissection of multiple steps in a pathway, provide temporal control over target function, and are often reversible. These screens are also particularly powerful in enabling researchers to identify molecules that parallel gene activity. Furthermore, by pursuing the mechanism of action (MOA) of active compounds, novel regulatory pathways can be identified.

Target identification and MOA determination is a rate-limiting step to small-molecule discovery in phenotype-based screens and relies on the integration of multiple complementary approaches. One such approach is candidate based, which can be used to determine whether novel small molecules target known MOAs. Several unbiased approaches to determine small-molecule MOAs can also be used, including (1) combining quantitative proteomics (SILAC) with affinity enrichment to identify proteins that interact with the small molecule and yield the observed phenotype, (2) compound profiling and connectivity analysis using gene expression signatures, and (3) genetic complementation of small-molecule effects by RNAi or overexpression screening to identify genes that function in the same pathway as the small molecule [111, 112].

Several forward chemical screens have been used to identify compounds with relevance to autophagy. These cell-based phenotypic screens employed libraries of FDA-approved drugs and known bioactive compounds to identify chemical modulators of autophagy [113–116]. Various readouts were used to measure autophagic activity, including GFP-LC3 puncta formation, the clearance of mutant huntingtin and A53T α (alpha)-synuclein aggregates, and degradation of luciferase-fused LC3. Autophagy-related small molecules discovered in these screens can be broadly classified into two groups: (1) mTOR-dependent molecules, which consist of compounds that induce autophagy, and (2) mTOR-independent molecules, which can be either inducers or suppressors of autophagy. The first group includes inhibitors of class I PI3K/Akt/mTOR signaling, such as allosteric and ATP-competitive mTOR kinase, Akt, PI3K, and dual mTOR/PI3K inhibitors. The second (mTOR-independent) group includes inhibitors of cAMP-Epac-PLC- ϵ (epsilon)-IP3 and Ca^{2+} -calpain-Gs- α (alpha) signaling [117].

Results from such chemical screens have been carried forward into cell and animal models of autophagy-related diseases. For example, (SMER) compounds were originally identified in a screen for enhancers of rapamycin-induced growth defects

in *S. cerevisiae* [115]. SMERs 10, 18, or 28 were found to be active in mammalian cell models of Huntington's and Parkinson's disease, promoting autophagic clearance of mutant huntingtin and A53T α (alpha)-synuclein aggregates [115]. In vivo, SMERs protect against neurodegeneration in a *Drosophila* model of Huntington's disease [115].

Like the SMERs, the pan-nitric oxide synthase (NOS) inhibitor N ω (omega)-nitro-L-arginine methyl ester hydrochloride (L-NAME) can block neurodegeneration in the same *Drosophila* Huntington's disease model and, similar to rapamycin, can clear mutant huntingtin aggregates in a zebrafish Huntington's disease model, suggesting that NOS inhibitors have therapeutic potential [118]. L-NAME triggers functional autophagy in a variety of cell types as measured by LC3 processing, the accumulation of RFP-LC3 puncta, and the ATG5-dependent clearance of mutant huntingtin aggregates [118]. While NOS inhibitors such as L-NAME decrease NO, complementary experiments to increase NO levels using NO donors and NO synthase overexpression revealed an inhibitory role for NO in autophagy [118]. The MOA of NO was found to involve two different mechanisms of inhibiting autophagy. First, S-nitrosylation of JNK1 impairs phosphorylation of BCL2, leading to increased BCL2-bcl-2 interaction. Second, S-nitrosylation of IKK β promotes the TSC2-dependent activation of mTORC1 [118].

The role of HMGB1 in regulating mitophagy following rotenone-induced disruption of oxidative phosphorylation is a further example that highlights the utility of small-molecule–gene interactions in identifying key autophagy pathways [51]. Mitochondrial stress mediated by rotenone, a small molecule that inhibits complex I in the electron transport chain, led to mitophagy that was facilitated by HMGB1 and its transcriptional target HSPB1. HMGB1 and HSPB1 were found to control mitochondrial homeostasis, as knockdown of either HMGB1 or HSPB1 resulted in perturbed morphology, glycolysis, ATP production, and mitochondrial fragmentation [51, 119]. Furthermore, rotenone-induced mitophagy was disrupted by cytochalasin D, suggesting a role for the actin cytoskeleton in HMGB1-HSPB1-mediated mitophagy [51].

To enhance the therapeutic utility of small molecules, the relationships between compound structure and biological activity (structure-activity relationships or SARs) can be studied to determine which chemical groups present in the compound are important for activity and how modifications of the structure can improve selectivity and potency. In one example, Chen et al. synthesized a novel set of diphenyl-butylpiperidines that demonstrated tenfold improved potency over lead compounds fluspirilene and penfluridol as measured by LC3-GFP puncta [120].

In addition to autophagy activators, autophagy inhibitors can also be useful as probes in determining whether a given state is dependent on classical autophagy or whether a compound truly induces autophagic flux. PI3K inhibitors 3-MA and wortmannin and the vacuolar H⁺ ATPase inhibitor bafilomycin A1 are typically used for these purposes. Autophagy inhibitors can also have therapeutic potential. Chou and colleagues identified DBE-Q, a reversible ATP-competitive inhibitor of the p97 ATPase [121]. Inhibition of p97 following DBE-Q treatments triggers diverse phenotypes, including a blockage of autophagosome maturation, activation of caspase 3 and 7, and inhibition of cancer cell proliferation, suggesting a potential therapeutic utility for p97 inhibitors in cancer [121].

Unlike the PI3K class I inhibitors discussed above, the discovery of potent and selective inhibitors of class III VPS34 has proven difficult. Class III VPS34 contains a smaller, more rigid ATP binding pocket compared to class I p110 γ , possibly adding to the difficulty in finding inhibitors [122]. 3-MA, a widely used inhibitor of VPS34, is typically used at millimolar concentrations, thus likely triggering many off-target effects that complicate results. To gain insight into developing VPS34 selective inhibitors, the structures of VPS34 in complexes with 3-MA, PIK-90, PIK-93, and PI103 were solved [122]. By employing structure-based design, Miller and colleagues were able to synthesize PT210, an analog of PIK-93, with tenfold more selectivity for VPS34 than for class I p110 γ [122]. Improved VPS34 inhibitors will be useful to probe the role of VPS34 in autophagy with more selectivity and without confounding effects of class I p110 γ inhibition.

The success of small-molecule screens in identifying and dissecting autophagy regulatory pathways is notable for its repercussions for therapeutics. The identification of new druggable targets may lead, after extensive optimization, to potential therapeutics in human diseases with autophagy-dependent and/or autophagy-modulatory features. Experimental results such as those obtained using SMERs and NOS inhibitors suggest that small molecules may also be employed to target anti-bacterial autophagy at multiple stages.

Concluding Remarks

Recent research has provided significant insight into autophagic functions in immunity, including xenophagy, mitophagy, antigen presentation, vesicular trafficking and secretion, and cytokine activity. However, substantial questions remain regarding the mechanistic basis of such functions as well as how individual disease risk alleles modulate these functions. In particular, studies of the microbiome may be critically important in understanding the relationship between xenophagy, immunity, and IBD. We anticipate that microbiome-wide studies (MWAS), in combination with detailed insights into the function of autophagy in health and disease, will help inform the development of novel biological and chemical entities for the treatment of patients suffering from these inflammatory disorders.

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