



The interplay between NLRs and autophagy in immunity and inflammation

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Since they were first described as cytosolic sensors of microbial molecules a decade ago, the Nod-like receptors (NLRs) have been shown to have many different and important roles in various aspects of immune and inflammatory responses, ranging from antimicrobial mechanisms to control of adaptive responses. In this review, we focus on the interplay between NLRs and autophagy, an evolutionarily conserved mechanism that is crucial for homeostasis and has recently been shown to be involved in the protective response against infections. Furthermore, the association between mutations of NLRs as well as proteins that form the autophagic machinery and inflammatory diseases such as Crohn's disease highlight the importance of these proteins and their interactions in the regulation of inflammation.

Keywords: NLR proteins, autophagy, inflammasomes, inflammation, infection, Crohn's disease, innate immunity

INTRODUCTION

Homeostasis in multicellular organisms is dependent on the ability to detect and adapt to a myriad of environmental variations and insults, including exposure to microbes. Early detection of microbes is a crucial step in the defense strategy. Throughout evolution, the continuous interplay between multicellular organisms and microbes has led to the selection of sensors that allow early detection and initiation of the immune response against infections. This detection is based on the recognition of “microbial-associated molecular patterns” (MAMPs), which represent a signature of microbial origin, such as lipopolysaccharide (LPS), peptidoglycan (PG), flagellin, and nucleic acids from bacteria and viruses, or of “danger-associated molecular patterns” (DAMPs), which indicate the existence of cellular damage, such as extracellular ATP and HGMB1 by “pattern recognition molecules” (PRMs). Upon activation, PRMs trigger several protective responses that include the recruitment of phagocytic cells; secretion of chemokines, cytokines, and antimicrobial peptides; and priming of dendritic cells (DCs), engaging the adaptive immune system. Several families of PRMs have been described and include “Toll-like receptors” (TLRs), “RIG-I like receptors” (RLRs), “C-lectin type like receptors” (CLR), and “Nod-like receptors” (NLRs). In addition to their role in innate and adaptive immune responses, all of them have been recently implicated in the control of autophagy, an adaptive cellular response to environmental and microbial-induced stress. In this review, we highlight the role of NLR signaling in the control of autophagy and vice versa, the

mechanisms involved and implications for inflammatory diseases such as Crohn's disease (CD) and type 2 diabetes (T2D).

NLR PROTEINS

Soon after the discovery of the transmembrane TLRs, it became evident that additional sensors were necessary for the surveillance of the cytosol. More than a decade ago, the demonstration that a mammalian homolog of plant disease-resistance (*R*) proteins called Nod1 could detect the presence of intracellular *Shigella flexneri* and activate the transcription factor nuclear factor κ B (NF- κ B) in epithelial cells *in vitro* inaugurated the studies on the role of NLRs as innate immune intracellular sensors (1). Subsequent studies have now set the number of human NLRs at approximately 20 and indicated their involvement in detecting not only microbial components but also DAMPs such as ATP, mitochondrial DNA (mtDNA) and reactive oxygen species (ROS) (2).

Due to the lack of signal peptides or transmembrane domains in their amino acid sequences, NLRs are thought to be exclusively located inside the cell. Both plant and animal NLRs are signal-transduction ATPases with numerous domains (STAND) P-loop ATPases of the AAA⁺ superfamily. The typical NLR protein contains the following domains: (a) a C-terminal leucine-rich repeat (LRR) domain, involved in sensing; (b) a central NATCH [Naip, CIITA, HET-E (plant het product involved in vegetative incompatibility)] and TP-1 (telomerase-associated protein 1 that mediates self-oligomerization and is essential for activation of NLRs); and (c) an N-terminal effector domain, responsible for

protein–protein interactions with adapter molecules and signal transduction. Based on the nature of the N-terminal domains, NLRs have been separated into the NLRC subfamily, containing a CARD domain (caspase activation and recruitment domain); the NLRP subfamily, containing a pyrin domain; and the NAIP subfamily, which includes three (BIRs) baculovirus inhibitors of the apoptosis protein repeat domain (3).

Nod1 (NLRC1) AND Nod2 (NLRC2) ARE INTRACELLULAR PEPTIDOGLYCAN SENSORS

Nod1 and Nod2 were the first NLRs identified as MAMP detectors when two concomitant studies demonstrated that Nod2 detects muramyl-dipeptide (MDP), a common motif found in Gram-negative and Gram-positive PG and a major component of adjuvants (1, 4–6). Nod1, in contrast, recognizes PG containing the minimal motif *meso*-diaminopimelic acid (DAP), an amino acid found in Gram-negative and some Gram-positive bacteria, such as *Listeria monocytogenes* and *Bacillus subtilis*. The naturally occurring PG moieties sensed by human and mouse Nod1 are GlcNAc-MurNAc-L-Ala-D-Glu-*meso*-DAP (GM-triDAP) and GlcNAc-MurNAc-L-Ala-D-Glu-*meso*-DAP-D-Ala (GM-tetraDAP), respectively. After additional studies demonstrated that PG recognition by TLR2 was due to contaminants commonly found in PG preparations, it became clear that Nod1 and Nod2 are the only known PG sensors (2, 7–9). The NLR ligands/activators are summarized in **Table 1**.

Nod1 and Nod2 have been implicated in the detection of a vast array of microbial pathogens including bacteria, parasites, and viruses. A key role for Nod1 and Nod2 in the detection of bacterial infection has been demonstrated in *Helicobacter pylori*, *Escherichia coli*, *Chlamydia* spp., *Campylobacter jejuni*, *Salmonella* spp., *Pseudomonas aeruginosa*, *S. flexneri*, and *L. monocytogenes*, *Mycobacterium tuberculosis*, and *Streptococcus pneumoniae* (**Table 1**).

More recent studies have uncovered surprising data regarding microbial recognition by Nod1 and Nod2. Nod1-deficient mice are more susceptible to infection with *Trypanosoma cruzi*, the etiologic agent of Chagas disease, apparently due to the lack of a robust nitric oxide production, suggesting that Nod1 may be involved in PG-independent microbial sensing given that *T. cruzi* does not express PG (3, 28).

Supporting a role for Nod2 in the control of infections beyond bacterial/PG detection, Shaw et al. using a *Toxoplasma gondii* infection model, described a T cell intrinsic role in Nod2-deficient mice and a consequent Th1-defective immune response. In their experiments, the authors observed lower amounts of IL-2 not only during infection with *T. gondii* but also following anti-CD28 ligation. Despite the novelty of these results, T cell activation in different models appears to be normal in Nod2-deficient mice (75, 76).

Nod2 has also been implicated in the immune response to viruses. In a recent study, Sabbah et al. demonstrated that Nod2 mediated the *in vitro* production of type I IFN in cells stimulated with single stranded RNA (ssRNA) or infected with various RNA viruses. These results support the observation, made in the same study, that Nod2-deficient mice are more susceptible to respiratory syncytial virus (RSV) (29).

Finally, both Nod1 and Nod2 have been implicated in inflammatory disorders because mutations in the genes that encode these proteins were shown to be related to the establishment of genetic inflammatory diseases. The first piece of evidence of a link between mutations in *NOD2* and CD [an inflammatory bowel disease (IBD)] was provided by Hugot et al. which identified three single nucleotide polymorphisms (SNPs) in the *IBD1* locus associated with increased risk for CD (77). One of these SNPs, *Leu1007fs*, is the most common Nod2 mutation associated with the disease and encodes a protein that is no longer able to sense MDP or localize to the plasma membrane as the normal protein does upon activation (4, 78).

INFLAMMASOMES

By definition, inflammasomes are multimeric protein complexes that comprise a “sensor NLR” and function as platforms for the activation of pro-caspase-1, resulting in the processing of IL-1 β and IL-18 and their unconventional secretion (6, 79). Several inflammasomes have been described so far, and among them, the best studied are the ones that contain NLRP3 (formerly known as NALP3) or NLRC4 (formerly known as IPAF). Many NLRs, such as NLRP1, NLRP3, and NLRC4, use the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) to recruit pro-caspase-1, but this does not apply to all inflammasomes.

NLRP3 INFLAMMASOMES

NLRP3 is mostly expressed in myeloid cells and is activated by a vast array of host-derived and exogenous agonists. One common feature of NLRP3 agonists seems to be a crystalline or polymeric structure associated with danger signals or cell death. For example, monosodium urate (MSU), calcium pyrophosphate dihydrate (CPPD) (41), cholesterol crystals (42), amyloid β (80), fatty acids (47), and mtDNA (48) have all been reported to activate NLRP3. Microbial NLRP3 agonists have also been identified. NLRP3 senses bacteria, viruses, fungi, and parasites themselves or virulence factors such as pore-forming toxins. The list of pathogens detected by NLRP3 includes *Staphylococcus aureus*, *L. monocytogenes*, *Klebsiella pneumoniae*, *Neisseria gonorrhoeae*, *E. coli*, *Porphyromonas gingivalis*, *S. flexneri*, *Chlamydia* spp., the influenza A virus, *Aspergillus*, and more recently, *Leishmania* (**Table 1**).

NLRC4 AND Naip5

IPAF (also known as NLRC4) is present in the cytosol of myeloid cells, where it controls the activation of caspase-1 and IL-1 β processing in response to the presence of intracellular flagellin. NLRC4 directly binds to cytosolic flagellin, an event that promotes its oligomerization through the nucleotide-binding domain (NBD) and winged-helix domain (WHD) in the presence of adenosine diphosphate (ADP) (81). The importance of IPAF-dependent activation of caspase-1 has been highlighted in infection models *in vitro* using *Salmonella typhimurium*, *S. flexneri*, *Legionella pneumophila*, and *P. aeruginosa* (66, 70, 74). In such experiments, IPAF-deficient macrophages were impaired in their ability to activate caspase-1 and secrete IL-1 β and IL-18. Macrophages from IPAF-deficient mice infected with *S. typhimurium* have also been shown to be more resistant to cell death. Indeed, activation of NLRC4 leads to rapid cell death, a feature that differentiates IPAF

Table 1 | NLR proteins involved in autophagy and their activators.

NLR protein	Activator	Reference	
Nod1 (NLRC1)	FK156 (d-lactyl-L-Ala- γ -Glu-meso-DAP)	Uehara et al. (10) and Magalhaes et al. (11)	
	FK565 (Heptanoly)	Uehara et al. (10)	
	Meso-lanthionine, meso-DAP	Uehara et al. (12)	
	iEDAP (γ -d-Glu-meso-DAP)	Girardin et al. (159) and Chamaillard et al. (9)	
	TriDAP (l-Ala- γ -d-Glu-meso-DAP)	Girardin et al. (4)	
	TCT (GlcNAc-(anhydro) MurNAc-l-Ala- γ -d-Glu-mesoDAP-d-Ala)	Magalhaes et al. (11)	
	<i>Bacillus</i> species	Hasegawa et al. (13)	
	<i>L. pneumophila</i>	Hasegawa et al. (13)	
	<i>S. typhimurium</i>	Hasegawa et al. (13) and Le Bourhis et al. (14)	
	<i>H. pylori</i>	Viala et al. (15)	
	<i>Pseudomonas</i> species	Travassos et al. (7)	
	<i>Chlamydia</i> species	Kavathas et al. (16), Buchholz and Stephens (17), Welter-Stahl et al. (18) and Opitz (19)	
	<i>L. monocytogenes</i>	Park et al. (20), Kim et al. (21) and Opitz et al. (22)	
	<i>E. coli</i>	Kim et al. (23)	
	<i>S. flexneri</i>	Girardin et al. (24) and Carneiro et al. (25)	
	<i>C. jejuni</i>	Al-Sayeqh et al. (26) and Zilbauer et al. (27)	
	<i>T. cruzi</i>	Silva et al. (28)	
	Nod2 (NLRC2)	Muramyl dipeptide (MurNAc-l-Ala-d-isoGln)	Girardin et al. (8) and Inohara et al. (5)
		M-TriLys (MurNAc-l-Ala-d-Glu-Lys)	Girardin et al. (4)
Respiratory syncytial virus (RSV)		Sabbah et al. (29)	
<i>Bacillus</i> species		Hasegawa et al. (13)	
<i>Lactobacillus</i> species		Hasegawa et al. (13)	
<i>Corynebacterium xerosis</i>		Hasegawa et al. (13)	
<i>E. coli</i>		Hasegawa et al. (13)	
<i>Pseudomonas</i> species		Hasegawa et al. (13)	
<i>M. tuberculosis</i>		Juárez et al. (30), Ferwerda et al. (31) and Divangahi et al. (32)	
<i>S. pneumoniae</i>		Travassos et al. (33) and Liu et al. (34)	
<i>C. jejuni</i>		Al-Sayeqh et al. (26)	
<i>S. flexneri</i>		Kufer et al. (35)	
<i>S. typhimurium</i>		Keestra et al. (36) and Hisamatsu et al. (37)	
<i>L. monocytogenes</i>	Kobayashi et al. (38)		
Nalp3 (NLRP3)	Muramyl dipeptide (MurNAc-l-Ala-d-isoGln)	Martinon et al. (39)	
	Bacterial RNA	Kanneganti et al. (40)	
	Imidazoquinoline compounds	Kanneganti et al. (40)	
	MSU (monosodium urate)	Martinon et al. (41)	
	CPPD (calcium pyrophosphate dihydrate)	Martinon et al. (41)	
	Cholesterol crystals	Duewell et al. (42)	
	Silica	Hornung et al. (43), Kuroda et al. (44) and Cassel et al. (45)	
	Aluminum salts	Hornung et al. (43) and Kuroda et al. (44)	
	Amyloid-beta	Halle et al. (46)	
	Fatty acids	Wen et al. (47)	
	Mitochondrial DNA	Nakahira et al. (48) and Shimada et al. (49)	
	Aerolysin	Gurcel et al. (50)	
	Maitotoxin	Mariathasan et al. (51)	
	ATP	Mariathasan et al. (51)	
	Nigericin	Mariathasan et al. (51)	
	<i>S. aureus</i>	Craven et al. (52)	
	<i>L. monocytogenes</i>	Kim et al. (53)	
<i>P. gingivalis</i>	Huang et al. (54)		

(Continued)

Table 1 | Continued

NLR protein	Activator	Reference
	<i>Chlamydia</i> species	Abdul-Sater et al. (55) and He et al. (56)
	Influenza A virus	Thomas et al. (57) and Allen et al. (58)
	<i>Aspergillus</i>	Saïd-Sadier et al. (59)
	<i>Leishmania</i>	Lima-Junior et al. (60)
	ROS	Zhou et al. (61)
IAPAF (NLRC4)	Cytosolic flagellin	Franchi et al. (62)
	<i>L. pneumophila</i>	Case et al. (63), Vinzing et al. (64) and Coers et al. (65)
	<i>S. typhimurium</i>	Mariathasan et al. (66), Broz et al. (67) and Miao et al. (68)
	<i>S. flexneri</i>	Suzuki et al. (69)
	<i>P. aeruginosa</i>	Cohen and Prince (70), Sutterwala et al. (71) and Franchi et al. (72)
Naip5	Cytosolic flagellin (in cooperation with IAPAF)	Zamboni et al. (73)
	<i>L. pneumophila</i>	Lightfield et al. (74) and Zamboni et al. (73)

from the other NLRs (66). Another pathogen whose detection induces cell death through IAPAF is *L. pneumophila*. However, in this case, another NLR protein, Naip5 (also known as Birc1e), is required. Both Naip5 and IAPAF have been reported to physically interact, but the role of Naip5 in caspase-1 activation remains to be fully elucidated, as *A/J* mice (mice with a mutation that results in a non-functional Naip5) are able to secrete IL-1 β following infection with *S. typhimurium*, *P. aeruginosa*, and *L. monocytogenes* (66, 71, 73) (Table 1).

Shigella flexneri also triggers IAPAF-dependent activation of caspase-1 and secretion of IL-1 β . These data are intriguing considering that *S. flexneri* is a non-flagellated bacterium, suggesting that other factors are able to activate IAPAF (69). Indeed, more recent studies revealed that *P. aeruginosa* strains lacking flagellin are still able to induce secretion of IL-1 β through NLRC4 (71) (Table 1).

Although the importance of IAPAF in cytosolic flagella sensing is broadly recognized, it has been demonstrated that flagellin-dependent responses may occur in the absence of IAPAF. Recently, a new pathway was reported in which macrophage stimulation with flagellin leads to cell death in a cathepsin B and D-dependent manner even in IAPAF-deficient cells. It has yet to be determined whether a new flagellin sensor is involved in such events (82).

NLRX1

In contrast to the huge amount of data regarding other NLR proteins, little is known about the biological function of NLRX1. This protein is highly conserved among species and has sequence homology with Nod3. Unlike other NLRCs, NLRX1 has no CARD in its N-terminal portion but does have a putative mitochondrial-targeting sequence (83). Indeed, what we know about NLRX1 is derived from its mitochondrial localization, even though its precise localization inside this organelle is still a matter of debate. Studies from two independent groups report conflicting results; while Arnoult et al. claimed that NLRX1 is located in the mitochondrial matrix, Moore et al. proposed that the protein localizes to the outer mitochondrial membrane (84, 85). There are also discrepancies concerning the attributed function of NLRX1. Initial results from Tattoli et al. reported that NLRX1 amplifies NF- κ B and JNK through the production of ROS. Opposing results from Moore et al. suggest that NLRX1

functions as a brake on innate immune pathways by inhibiting mitochondrial antiviral signaling (MAVS)-dependent NF- κ B and IFN- β production upon poly I:C stimulation *in vitro* (85, 86). Further studies are required to clarify of the function of NLRX1.

NLRP4

Very little is known about NLRP4, a 113-kDa protein also known as Nalp4 or PYPAF4. This protein is expressed in tissues as diverse as testis, oocytes, spleen, placenta, thymus, kidney, and lung. NLRP4 has been recently reported as a negative regulator of type I IFN signaling by targeting tank binding kinase-1 (TBK-1) for degradation as well as of TNF- α and IL-1 β by inhibiting NF- κ B activation by interacting with IKK α (87, 88).

AUTOPHAGY

The term autophagy (meaning “self-eating”) was first introduced at the CIBA Foundation Symposium on Lysosomes in 1963 by cell biologist Christian de Duve, who also discovered lysosomes in 1955 (Nobel Prize in Physiology in 1974) and coined several other terms currently used today, such as “endocytosis” and “exocytosis.” Autophagy was first characterized by the presence of single- or double-membrane vesicles harboring cytoplasmic content in different stages of degradation – the autophagosomes. At that time, de Duve and others considered autophagy to be a non-selective degradation pathway. However, under specific circumstances, autophagy is highly specific and plays essential roles in maintaining homeostasis. For a complete historical perspective on autophagy and its importance in different pathologies, please refer Ref. (89–91).

Autophagy is a highly conserved cellular homeostatic process in which long-lived proteins, damaged organelles, or parts of the cytosol are delivered to lysosomes for degradation and recycling of functional blocks for anabolic reactions, especially during nutrient shortages. Indeed, for years, autophagy was considered a mere response to nutritional stress given that initial observations demonstrated that glucagon or amino acid deprivation triggered the formation of autophagosomes while exogenous amino acids supplementation inhibited autophagy and protein breakdown (92).

THE MACHINERY OF AUTOPHAGOSOME BIOGENESIS

So far, three types of autophagy have been described, chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy (hereafter, autophagy) (93). The hallmark of autophagy is the generation of autophagosomes. This process occurs in a stepwise manner controlled by over 30 *Atg* genes that were initially identified in yeast species. Interestingly, most of these genes have mammalian orthologs or paralogs with high structural and functional similarities. Briefly, the process starts with the formation of a cup-shaped membrane or phagophore. Once formed, the membrane elongates and selectively and/or non-selectively enwraps the cargo (i.e., the cytosolic target), eventually sealing, completing the formation of the autophagosome. The outer membrane of the autophagosome fuses with a lysosome membrane, forming the autolysosome, where all the degradation steps of the autophagic response take place (Figure 1). The source of the autophagosomal membrane is still a matter of debate. Various studies have proposed the plasma membrane, the endoplasmic reticulum or the outer mitochondrial membrane as the source (92).

THE CORE AUTOPHAGY PATHWAY

At the molecular level, the number of proteins implicated in the control of autophagy is still expanding and linking autophagy with several other pathways. Here, we focus on the core of the autophagic pathway and its links to NLR signaling. For autophagosome formation, the following two ubiquitin-like (UBL) systems are required: (i) in the Atg12 conjugation system, Atg5 and Atg12 form complexes through the covalent binding of Atg12 to the C-terminal glycine of ATG5 in UBL reactions involving Atg7

and Atg10. Atg16L1, a scaffold protein, is then conjugated to the Atg5-Atg12 complex by binding to the N-terminus of Atg5. The Atg5-Atg12-Atg16L1 complex multimerizes, forming large 800 kDa complexes that are found in the cytosol and in the forming membrane. It has been shown that the Atg16L1 complex acts as a E3-like enzyme, targeting microtubule-associated protein 1 light chain 3 (LC3) to its membrane site of lipid conjugation. (ii) The Atg8 conjugation system is crucial for inducing modifications in LC3. Under normal conditions, LC3 has a diffuse cytosolic distribution pattern. LC3 is cleaved at its C-terminus by Atg4, a cysteine protease, and undergoes UBL modifications by the E1-like enzyme, Atg7, and the E2-like enzyme, Atg3, to form LC3-I (94). During the induction of autophagy, the C-terminal carboxyl group of LC3-I is eventually conjugated with phosphatidylethanolamine to form LC3-II, which is found exclusively on the autophagosomal membrane. For this reason, LC3-II is widely used as an autophagy marker (95).

The *Atg* proteins, the autophagic machinery, seem to require other proteins to form autophagosomes. Similar to the Atg1-Atg13-Atg17 complex found in yeast, the Unc-51-like kinase (ULK1), focal adhesion kinase family integrating protein (FIP200), and Atg13 proteins were described to colocalize at the nascent isolation membrane after induction of autophagy in mammalian cells (96,97). Another protein that was shown to participate in autophagy is Atg9. Studies in mammals have demonstrated that Atg9 is essential for autophagosome formation as it associates with the trans-Golgi, endosomes, LC3, and Rab GTPases (Rab7 and Rab9) and redistributes following the induction of autophagy (92). In Figure 1, we summarize the main proteins and all the steps that are part of the formation of autophagosomes.

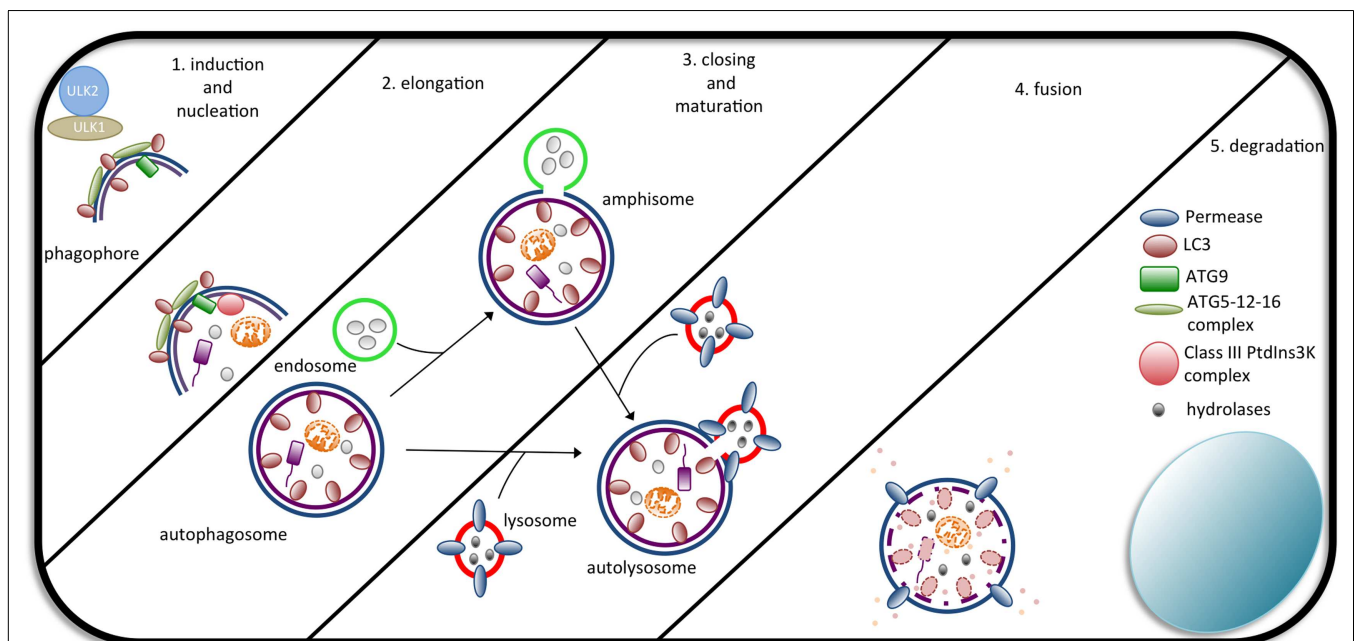


FIGURE 1 | The steps of autophagosome formation. Macroautophagy begins with the formation of a cup-shaped structure called a phagophore as a consequence of the activity of the ULK1/2 complex. In the sequence, the ATG5-ATG12-ATG16L1 complex, Class III PtdIns3K, ATG9, and LC3 assist in the formation of the autophagosome. Autophagosomes

completely wrap their cargo and fuse with endosomes (forming an amphisome, which will later fuse with lysosomes) or directly with lysosomes, forming an autolysosome. Upon fusion, the cargo is degraded by lysosomal hydrolases and exported back to the cytosol to be used by the cell.

Interestingly, it has been proposed that autophagy may occur even in the absence of ATG proteins in both insects and mammals. However, the conditions that trigger ATG-independent autophagy and whether this type of autophagy is particular to some cell types remain to be determined (98, 99).

CONTROL OF AUTOPHAGY

The vast number of pathways that connect to autophagy gives rise to an intricate network that makes our understanding of autophagy regulation far from complete. A major breakthrough in understanding autophagy regulation was made when the target of rapamycin (TOR) in yeast and mammals was discovered, implicating the involvement of phosphatidylinositol kinase-related kinases in the process (100–102). Both pathways are linked by the key serine/threonine kinase Akt and are known to participate in several cellular responses such as proliferation and metabolic adaptation (92). Activators of phosphatidylinositol-3 kinase (PI3K) range from cytokines to TLR ligands. After receptor activation, phosphatidylinositol-4,5-bisphosphate is phosphorylated by class I PI3K, activating Akt. mTOR complexes are effectors downstream of Akt and integrate a myriad of cellular signals, especially those related to protein synthesis and translation (103–106). Under optimal nutrient conditions, autophagy is negatively regulated by Akt and mTOR (107). This negative regulation of autophagy by mTOR has been recently shown to require inhibition of ULK kinase-complex activity through its phosphorylation (108). At least in yeast, ULK1 seems to regulate autophagy not only by inhibiting mTOR but also by interacting with Atg8 (109). It remains to be tested whether these findings also apply in mammals. Another mechanism involved in the control of mTOR activation is the recruitment of TNF receptor-associated factor 6 (TRAF6) to the lysosome by p62, where it drives the polyubiquitination of mTOR during optimal nutrient conditions (110).

Upon the induction of autophagy, vacuolar protein sorting 34 (Vps34), a class III phosphatidylinositol-3-phosphate (PtdIns3P) kinase (PI3K) enzyme, specifically phosphorylates phosphatidylinositol and is implicated in trafficking, nutrient sensing, and autophagy (111, 112). In yeast, the role of PtdIns3P seems to go beyond autophagosomal membrane elongation. It has been proposed that the levels of PtdIns3P in the phagophore assembly site (PAS) regulate autophagosome turnover due to the accumulation of ATG proteins in the membrane (113). Other important players in autophagosome formation are Vps15, beclin-1, ultraviolet radiation resistance associated gene (UVRAG), and ambra1, which together form a multiprotein complex with Vps34 that is necessary for the initial steps in autophagosome formation. For a complete view of the autophagy pathway, see the review by Boya et al. (114).

AUTOPHAGY AND IMMUNITY TO INFECTIONS

The contamination of the cytosolic compartment with invasive pathogens is a major step in the activation of innate immune defenses. In this regard, autophagy has emerged in recent years as another component of the innate immune system's arsenal. Several microbial agents are recognized by the autophagic machinery, and their fates can vary from destruction to the creation of a replicative niche. It has been shown that the autophagic machinery can selectively segregate microbes in the cytosol. The mechanisms by which such specificity is achieved are not completely understood

but seem to involve both microbial and host factors. Similar to MAMPs, which themselves were shown to induce autophagosome formation, toxins secreted by pathogens also induce autophagy (115). In a landmark paper, Nakagawa et al. demonstrated that group A *Streptococcus* (GAS) lacking streptolysin O (SLO) do not escape from phagocytic vacuoles, and thus, do not activate autophagic sequestration (116).

Gram-negative bacteria are recognized by the autophagic machinery as well. For example, *S. flexneri* uses a type 3 secretion system (T3SS) to deliver effector proteins directly into the host cell. Ogawa et al. showed that, in epithelial cells, the wild-type (WT) *S. flexneri* strain is capable of evading autophagic sequestration. This is dependent on the T3SS effector IcsB, as the mutant strain lacking IcsB is trapped by autophagy. The role of IcsB seems to be to camouflage the bacterial target molecule (VirG) from the autophagy machinery (117, 118). Interestingly, these observations seem to vary depending on cell type, given that in bone marrow-derived macrophages, no difference in bacteria sequestration was observed between the WT and IcsB mutant strains (69). The autophagic machinery apparently relies on redundant strategies to fight *Shigella*; different mechanisms have been described for the induction of autophagy by this pathogen. In one mechanism, the phagocytic vacuolar membrane remnants, rather than bacterium itself, trigger autophagy in response to bacterial invasion (119).

Avoidance of autophagic destruction has also been reported for other bacteria. The *Burkholderia pseudomallei* T3SS effector, BopA, which shares some homology with IcsB, contributes to bacterial evasion from autophagosome targeting (120). The Gram-positive bacterium *L. monocytogenes* is able to invade host cells, where it finds a replicative niche within the cytosol following autophagosome escape. These events are dependent on the expression of listeriolysin O (LLO), ActA, and phospholipase C (121).

However, autophagy can serve as a back-up control mechanism for bacteria that are able to escape from other defense mechanisms. For example, after invading the host cell, *S. typhimurium* resides within vacuoles called *Salmonella*-containing vacuoles (SCV). Following SCV damage mediated by its T3SS, *S. typhimurium* gains access to the cytosol where autophagy is immediately activated to confine the bacteria and restrict the infection (122). In the case of *M. tuberculosis*, which is known to subvert host cell phagosomal maturation and survive within macrophages, autophagy induction via rapamycin or IFN- γ circumvents the phagosomal maturation blockade, leading to *M. tuberculosis* elimination (123).

Viral pathogens interact with the autophagic pathway as well. Herpes simplex virus (HSV) ICP34.5 interacts with beclin-1 and blocks autophagosome formation (124). In vesicular stomatitis virus (VSV)-infected DCs, autophagy is essential for the delivery of viral ligands to endosomes to induce type I IFN production (125).

NLR-MEDIATED AUTOPHAGY AND INFECTION

As mentioned before, the first line of host defense against infection relies on various families of PRMs. As it became evident that autophagy is also an innate immune effector mechanism, considerable efforts were made to understand the role of PMRs in the autophagic response to pathogens.

The first study linking MAMP sensing and autophagy induction was the work by Xu et al. who showed a role for TLRs in

autophagy. TLRs are transmembrane proteins that recruit myeloid differentiation primary response protein 88 (MyD88) and Toll-IL-1 receptor (TIR) domain-containing adapter-inducing IFN (Trif) adapter proteins through their TIR domain to initiate downstream signaling. It was demonstrated that LPS induces autophagy in a TLR4-p38-RIP1-Trif-dependent manner (126). Later, a report from Delgado et al. showed that TLR7 could elicit similar responses upon stimulation of macrophages with ssRNA, and this was also dependent on the recruitment of MyD88 (127). The balance between Beclin-1 and Bcl-2 is a major checkpoint in the pathway for autophagy induction. Shi et al. (128) proposed that MyD88 and Trif both target Beclin-1, resulting in decreased binding to Bcl-2 and subsequent autophagy activation upon TLR stimulation. MyD88 and interferon-regulatory factors (IRFs) 5 and 7 are also recruited by mTOR to control cytokine production (128, 129).

Despite increasing evidence showing a role for TLRs in the induction of autophagy, it remains unclear how the autophagic machinery is directed to trap an entire microorganism during infection, especially considering that they are transmembrane proteins. Several bacteria, such as *Salmonella*, *Mycobacterium*, and *Listeria*, grow within host cells and by doing so can avoid antibody and cellular dependent defenses. Intracellular PRMs, such as the NLR family, are known for their essential role as cytosolic sentinels that can trigger robust cytokine production and inflammation. However, little was known regarding how these sensors contribute to the elimination of intracellular invaders. The first evidence implicating NLRs in autophagy-dependent control of an intracellular infection came from studies using *Drosophila* as a model. Yano et al. (130) reported that, upon infection of hemocytes with *L. monocytogenes*, *Drosophila* PGRP-LE detects diaminopimelic (DAP)-containing PG to trigger autophagy directed against the bacterium. Consistent with these observations, PGRP-LE null mutants were more susceptible to infection (130). The recognition of intracellular PG and subsequent induction of autophagy seem to be conserved features of the innate immune system. In 2010, two independent studies reported Nod1- and Nod2-dependent autophagy upon PG detection. We showed that Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane during bacterial entry into the host cell (Figure 1). Interestingly, the most common mutation in Nod2 associated with CD, Nod21007fs, results in a protein that fails to recruit ATG16L1 to initiate the formation of autophagosomes, although they still interact in the cytosol. In another study, Cooney et al. (131) found that Nod2 induces autophagy in human DCs, increasing bacterial killing, and antigen presentation. DCs expressing CD-associated variants displayed lower autophagy and antigen presentation levels upon MDP stimulation. Interestingly, while we demonstrated that the adaptor protein Rip2 and NF- κ B activation is dispensable for autophagy induction because Rip2-deficient fibroblasts displayed similar numbers of *S. flexneri* targeted to autophagosomes, Cooney et al. found that Rip2-deficient DCs had reduced levels of autophagy. The difference in the cell types used could account for such differences (131, 132).

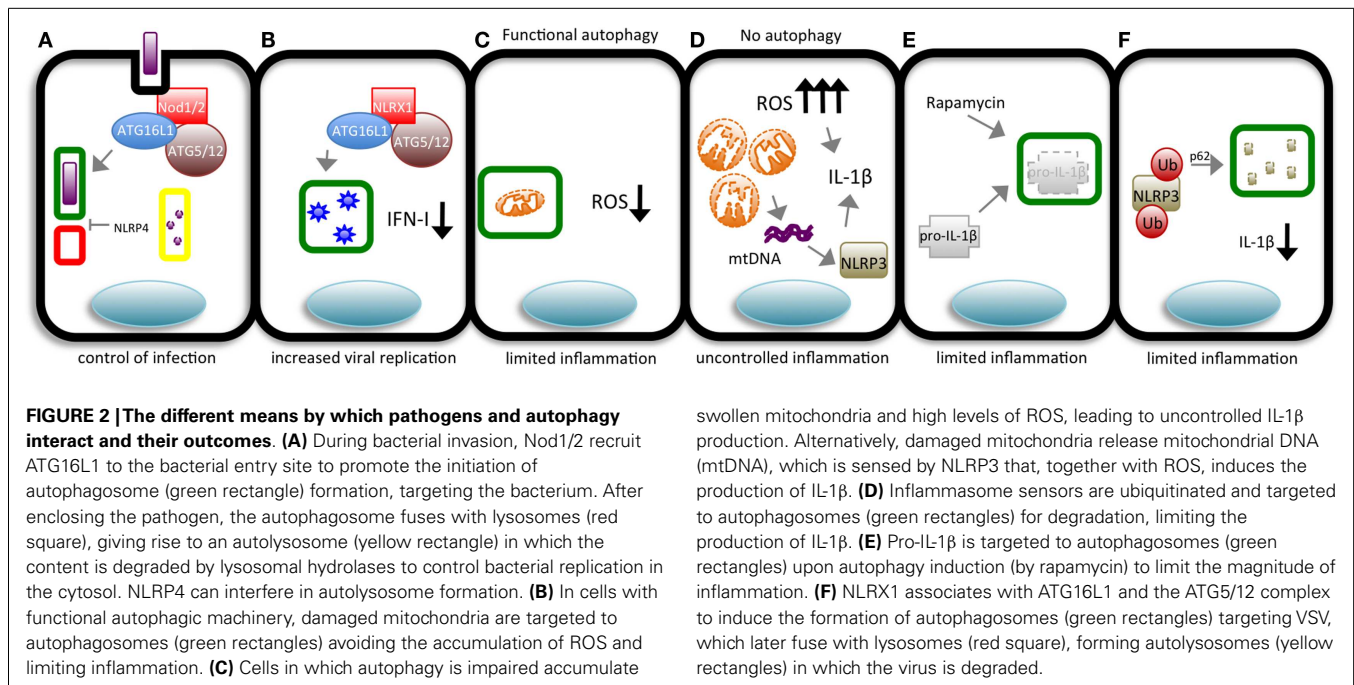
These studies gained additional relevance as a recent link between polymorphisms in ATG16L1 and CD was uncovered. CD and ulcerative colitis (UC) are common presentations of idiopathic IBD. It is estimated that their prevalence in Caucasian individuals reaches 100–150 per 100,000 (133). IBD is the

outcome of combined genetic and non-genetic risk factors, and recently genome wide association studies (GWAS) have identified a non-synonymous single nucleotide polymorphism in ATG16L1 (T300A) as one of the most important genetic risk factors for CD. Studies in which the implications of this polymorphism were analyzed show that MDP-induced but not canonical autophagy is impaired in cells of individuals carrying the T300A variant (131, 132). The effect of this polymorphism on the restriction of bacterial growth varies depending on the cellular and bacterial models used. Epithelial cells expressing the T300A variant show decreased bacteria-targeted autophagy during infection with *S. typhimurium* (134). Monocytes from patients with CD and carrying the T300A allele infected with *M. avium paratuberculosis* display no difference in bacterial growth in comparison to patients with the normal allele (135). The impact of this variant on cytokine release has also been evaluated, and again, contradictory findings were observed. Plantinga et al. (136) reported that upon Nod2 stimulation with MDP (but not with TLR ligands), PBMCs from healthy volunteers carrying the T300A variant secreted increased amounts of IL-1 β (136). In contrast, in another study, the same group demonstrated that PBMCs with the variant allele do not produce more IL-1 β in comparison to the normal allele upon infection with *M. tuberculosis* (137).

Legionella pneumophila, a Gram-negative pathogen of amoebae, is also able to replicate within alveolar macrophages and cause the pneumonia known as Legionnaire's disease. Mouse macrophages, in contrast to human cells, restrict *L. pneumophila* replication through the activation of Naip5 and NLRC4 by cytosolic flagellin and activation of caspase-1 resulting in pyroptosis. In a recent study, Byrne et al. demonstrated that flagellin recognition by Naip5 and NLRC4 increases autophagosome turnover (138).

NLRX1 has been shown to enhance autophagy. A recent study demonstrated that NLRX1 enhances autophagy through interaction with the Tu translation elongation factor (TUFM) which, in turn, interacts with the Atg5-Atg12 complex. It is not clear, however, how this NLRX1-TUFM-Atg5-Atg12 interaction leads to increased autophagy. Still, NLRX1 plays an important role as a pro-autophagic factor during vesicular stomatitis (VSV) infection. Lei et al. (139) showed decreased viral replication in NLRX1-deficient fibroblasts, suggesting that autophagy is important for VSV replication, although a previous work demonstrated that VSV succumbs to autophagy in a *Drosophila* model (139, 140). Of note is the fact that the role of NLRX1 in autophagy varies among studies using the different NLRX1 knockout mice available. While Soares et al. and Rebsamen et al. found that the MAVS pathway is fully functional in their NLRX1-deficient mice, Allen et al. reported an enhancement in this signaling pathway using a different NLRX1 knockout mice [the same used by Lei et al. (139, 141–143)]. It remains to be determined whether the role of NLRX1 in autophagy is specific to the knockout animals used by Lei et al. or if it is a general feature of all NLRX1-deficient mice (Figure 2B).

Finally, in contrast to what was described above, NLRs can also act as negative regulators of autophagy through mechanisms that are not yet completely elucidated. Suzuki et al. reported that NLRC4- and caspase-1-deficient macrophages display increased targeting of *S. flexneri* to autophagosomes (69). Similar results were found in a study analyzing the impact of NLRC4 and NLRP4



on autophagy, where it was demonstrated that epithelial cells with both proteins silenced displayed enhanced autophagy. A partial explanation for these observations is that NLRP4 is part of the Beclin-1 and C-VPS (a complex consisting of VPS11, VPS16, VPS18, and Rab7 that controls membrane tethering and fusion of vacuolar membranes) complexes, which are essential for the biogenesis and maturation of autophagosomes, respectively (144). The elucidation of the precise mechanisms by which NLRP4 exerts its negative effects on autophagy requires further studies (Figure 2A).

AUTOPHAGY-DEPENDENT CONTROL OF NLR-DEPENDENT INFLAMMATION

Another function of autophagy seems to be the control of the magnitude of inflammatory responses (145). In the last few years, several groups have reported that autophagy blockade by pharmacological or genetic means leads to increased production of cytokines by different mechanisms. Two independent studies have shown that ATG5-deficient fibroblasts produce significantly more IFN-I and IL-6 than WT cells during viral infection. The mechanisms likely involve the interaction between ATG12-ATG5 complexes with IPS-1, RIG-I, and MDA-5 and accumulation of damaged mitochondria in the autophagy-deficient cells, as discussed below (146, 147).

AUTOPHAGY AND INFLAMMASOMES

A ROLE FOR ROS

As discussed above, the cells deficient in ATG genes produce increased levels of pro-inflammatory cytokines (146, 147). Tal et al. (147) demonstrated a vital role of autophagy in the removal of damaged mitochondria (mitophagy), and thus, in cell homeostasis. Cells with defects in autophagy, such as Atg5-deficient cells, accumulate damaged mitochondria, and consequently present increased levels of ROS. This in turn results in the enhancement

of the levels of IFN α and IL-6 production upon infection with VSV. In addition, the authors show that by using the antioxidants *N*-acetyl-*L*-cysteine (NAC) and propyl-gallate (PG) during VSV infection, they were able to revert the increase in cytokine production. The removal of damaged mitochondria can be regulated by adapter proteins that participate in NLR-activating pathways. For example, besides its well-known and crucial role linking Nod1 and Nod2 sensing to NF- κ B activation, Rip2 has also been implicated in mitophagy. In a recent study, Lupfer et al. showed that Rip2 regulates mitophagy through ULK1 to keep ROS at basal levels. Genetic deletion of Rip2 leads to the accumulation of ROS and significantly higher levels of IL-18 and IFN- γ upon influenza infection (148) (Figure 2C).

Among all the cytokines that have been studied, IL-1 β seems to be the cytokine whose production is most dramatically affected by autophagy. Processing and activation of pro-IL-1 β into its active form depends on the assembly of inflammasomes. Increased ROS levels seem to be a prerequisite for inflammasome activation. The manipulation of autophagy by pharmacological or genetic means has a profound impact on IL-1 β production and secretion during infection or LPS treatment. Indeed, the use of the autophagy inhibitor 3-methyladenine (3-MA), deletion of LC3B or ATG16L1, silencing of Beclin-1 or dominant negative forms of the cysteine protease ATG4B all lead to remarkably higher amounts of IL-1 β (48, 149–151). The first evidence to show that autophagy can modulate IL-1 β production surfaced in 2008 with the work of Saitoh et al. These authors reported that macrophages from ATG16L1-deficient mice produced much higher levels of IL-1 β after LPS exposure and that this was not due to defects in the generation of pro-IL-1 β and pro-caspase-1. These authors proposed a model in which TRIF and loss of K⁺ and ROS are required for the activation of inflammasomes, and subsequently, IL-1 β processing (150). However, it remains to be shown that these observations were not due to defective mitophagy. Further studies not only

confirmed but expanded the evidence for the requirement of functional autophagy for the maintenance of basal levels of ROS, and subsequently, for the control of inflammasome activation. In an elegant study, Nakahira et al. dissected the role of autophagy in the activation of the NLRP3 inflammasome. In a series of experiments in which autophagy was inhibited either by deletion of LC3B or by heterozygous deletion of Beclin-1, a robust increase in IL-1 β processing and release was observed following stimulation with LPS plus ATP. According to the authors, these stimuli led to swollen mitochondria and the release of its DNA (mtDNA). In ρ^0 J774A.1 macrophages, the release of mtDNA into the cytosol is blocked, and IL-1 β secretion is impaired. Similar results were obtained when cells were treated with DNase I. Interestingly, the production of ROS and consequent activation of the inflammasome were dependent on the presence of cytosolic mtDNA. Altogether, the results from this study delineate a model in which LPS plus ATP induce mitochondrial damage, ROS production, and NLRP3-dependent release of mtDNA into the cytosol, resulting in the activation of caspase-1 and release of IL-1 β (48). The findings reported by Nakahira et al. were partially contradicted by a more recent study that suggests that ATP actually induces the release of oxidized mtDNA, which in turn binds to NLRP3 to induce IL-1 β production. Oxidized mtDNA was not detected in the cytosol of NLRP3-deficient macrophages. According to the authors, this could be explained by the fact that unbound oxidized mtDNA is rapidly degraded, but this later observation still lacks experimental confirmation (49) (**Figure 2D**). It is important to note that the idea of ROS as an activator of NLRP3 inflammasomes was challenged by a study demonstrating that ROS is key for priming of NLRP3 but not for its activation. One way or another, it is clear that ROS is necessary for IL-1 β production (152).

The source of ROS required for inflammasome activation is also a matter of debate. Initial reports suggested that nicotinamide dinucleotide phosphate (NADPH) oxidases were the main source for inflammasome activation (153). The NADPH complex comprises the membrane-bound gp91^{phox} and p22^{phox} glycoproteins and the cytosolic components p47^{phox} and p67^{phox}. Patients with chronic granulomatous disease (CGD) can have mutations in any of the NADPH oxidases and as a result present defective phagocytes because their cells have impaired capacity to generate superoxide anion and its metabolites, hydrogen peroxide, the hydroxyl anion, and hypohalous acid (154). Meissner et al. reported that in monocytes from CGD patients with mutations in gp91^{phox}, p22^{phox}, and p47^{phox}, stimulation with LPS plus ATP led to the activation of caspase-1 and secretion of mature IL-1 β (155). Furthermore, monocytes from CGD patients presented elevated IL-1 β levels in comparison to monocytes from healthy controls (154). These findings challenged the notion that NADPH oxidases are the source of ROS necessary to induce inflammasome activation. This question was apparently clarified by the work of Zhou et al. which demonstrated that ROS generated by dysfunctional mitochondria [mtROS, achieved by either by treating cells with the complex I inhibitor, rotenone, or silencing of the voltage-dependent anion channel (VDAC)] activates the Nlrp3 inflammasome. The requirement of mtROS for activation of the inflammasome seems to be specific for Nlrp3 because VDAC1 silencing did not influence activation of NLR4 of AIM2 inflammasomes. Once again, the

crucial role of autophagy in clearing damaged mitochondria was demonstrated in the work of Zhou et al. in which 3-MA treatment or beclin-1 or Atg5 silencing resulted in ROS accumulation and inflammasome activation (61).

As already mentioned, mutations in NLR genes are associated with increased risk for inflammatory diseases. Auto-activation of the NLRP3 inflammasome has been linked to several autosomal dominant cryopyrinopathies or cryopyrin-associated periodic fever syndromes (CAPS), such as familial cold-induced autoinflammatory syndrome (FCAS), Muckle–Wells syndrome (MWS), and neonatal onset multisystem inflammatory disorder or chronic infantile neurologic cutaneous and articular syndrome (NOMID/CINCA) (156). These syndromes, despite their different names, represent a continuum of disease severity where FCAS is the mildest and NOMID/CINCA the most severe. These cryopyrinopathies are associated with periodic fever, rashes, arthralgia, and conjunctivitis, and the aberrant production of IL-1 β is the most prominent feature related to all these manifestations (6).

The increasing incidence of T2D has become a global health burden. T2D has been associated with low-grade inflammation that leads to insulin resistance. In this context, IL-1 β is one of the main cytokines implicated in T2D, mediating the destruction of beta cells and resulting in insulin resistance in cells that were initially sensitive to the hormone. A high-fat diet (HFD) is one of the factors associated with T2D. Indeed, T2D patients display augmented levels of free fatty acids in the serum. In a recent study, Wen et al. demonstrated in a bone marrow-derived model that palmitate, an abundant saturated fatty acid in the plasma, inhibits AMP-activated protein kinase (AMPK), leading to defective autophagy, and thus, ROS accumulation. These events contribute to the elevation of IL-1 β production and impairment of insulin signaling *in vitro* (47).

A recent report showed that autophagy is involved not only in IL-1 β production but also in its secretion. Macrophages from Atg5 conditional knockout mice secreted significantly more IL-1 β during the induction of autophagy triggered by starvation. These observations need further confirmation (157).

A ROLE FOR UBIQUITIN

As in many aspects of its biology, autophagy has ambiguous roles in regulating inflammasome activation and acts both as a positive and negative regulator depending on the experimental model.

In a recent study, Harris et al. provided experimental evidence that autophagy controls inflammasomes by targeting its components to autophagosomes. These authors showed that pro-IL-1 β is delivered to autophagosomes after TLR stimulation. Upon autophagy induction by rapamycin treatment, pro-IL-1 β is degraded, limiting the amount available for the processing and secretion of IL-1 β (149) (**Figure 2E**).

One mechanism underlying the targeting of inflammasome proteins to autophagosomes seems to be ubiquitination. In a recent study by Shi et al. it was demonstrated that inflammasomes containing ASC are directed to autophagosomes during NLRP3 or AIM2 activation in primary macrophages. They also provided evidence that beclin-1 and p62 are involved in targeting ASC to autophagosomes after it is K63 ubiquitination. The results presented suggest that by using its separate UBA

and LIR domains, p62/SQTM1 bridges ASC K623 ubiquitination, and autophagy-dependent degradation (151). In addition to ASC, NLRP3 is also ubiquitinated in a mtDNA-, ROS-, and ATP-dependent manner, but its delivery into autophagosomes has not yet been demonstrated (158) (Figure 2F).

CONCLUDING REMARKS

The NLR and autophagy fields are two exciting research areas in biology with many unanswered questions related to the precise mechanisms that coordinate the “talk” between NLR proteins and autophagy. It will be interesting to discover in more detail whether autophagy modulation can be used to control NLR-dependent immune pathways to improve therapeutic strategies for inflammatory and infectious diseases. In light of the recent findings that connect autophagy and inflammasome regulation, it remains to be determined whether alterations in autophagy could explain, at least in part, the dysregulated production of IL-1 β in NLRP3-associated cryopyrinopathies. We believe that in the near future, some of the findings discussed in the present review have the potential to be translated into new therapeutic strategies that can be applied in daily medical practice.

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