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Innate Immunity to Intracellular pathogens: balancing microbial elimination and inflammation

Gabriel Mitchell[1] and **Ralph R. Isberg**[2]

[1]Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA 94720, USA

[2]Department of Molecular Biology and Microbiology, Tufts University School of Medicine, 150 Harrison Ave., Boston, MA 02111, USA

Summary

Recent excitement regarding immune clearance of intracellular microorganisms has focused on two systems that maintain cellular homeostasis. One system includes cellular autophagy components that mediate degradation of pathogens in membrane-bound compartments, in a process termed xenophagy. The second system is driven by interferon– regulated GTPases that promote rupture of pathogen-containing vacuoles and microbial degradation. In the case of xenophagy, pathogen sequestration and compartmentalization suppress inflammation. In contrast, interferon-driven events can lead to exposure of pathogen-associated molecular patterns to the host cytosol with consequent inflammasome activation. Paradoxically, signals and factors involved in xenophagy also mobilize interferon-regulated GTPases, which drive the inflammatory response, indicating considerable crosstalk between these pathways. How these responses are prioritized remains to be understood. In this review, we describe mechanisms of intracellular pathogen clearance that rely on the autophagy machinery and interferon-regulated GTPases, and speculate how these pathways engage each other to balance pathogen elimination with inflammation.

Introduction

The uptake of an intracellular pathogen into a mammalian host cell initiates a battle with clear downstream consequences. A traditional view of this encounter is that the pathogen and the host are in conflict, with the winner determining if health or disease will ensue. In fact, interactions between these players are much more nuanced, with several possible consequences. At the simplest level, within a single cell, the pathogen either establishes a replication site or the host prevents infection by killing the microorganism. This binary relationship rarely captures the host-pathogen relationship. For instance, establishing a beachhead in a host cell could involve forming a latent state for the microorganism as in

Correspondence: Ralph R. Isberg, Tufts University School of Medicine, 150 Harrison Ave., Boston, MA 02111, United States; ralph.isberg@tufts.edu Phone: 617-636-3993.

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Mycobacterium tuberculosis or HIV infections (Cambier et al., 2014, Churchill et al., 2016). Furthermore, blocking disease progression may result in killing of microbes, but may also involve bacteriostasis or host cell death.

In this review we discuss the several host pathways that restrict intracellular microorganisms and consequences for the outcome of disease. For instance, microbial growth can be terminated by host-derived reactive oxygen or nitrogen species, routing of a membrane-bound pathogen into a lysosomal locale or subjecting cytosolic pathogens to poorly characterized lytic defense pathways, sometimes resulting in overt inflammatory responses (Martinez et al., 2015, Haldar et al., 2015). Added to this diversity of responses is a panoply of events within the host that can either block or support microbial replication. In its most extreme expression, the disease process could involve blocking of bacterial growth in a subset of cells by innate immune pathways, but eliciting an inflammatory response that supports effective pathogen replication within the animal, as is seen with *Salmonella enterica* during intestinal growth (Winter et al., 2010).

We will focus on restriction of intracellular pathogens by the host innate immune system, concentrating on the destruction of microorganisms while they are resident in membrane-bound compartments. The mechanism of pathogen restriction modulates the nature of the global innate immune response throughout a tissue site, which can either stimulate or prevent the production of inflammatory mediators. However, there is a critical block to progress in the field, because host components that mark invading microorganisms are poor predictors of the strategy used to destroy invaders. Therefore, specific molecular components that control each of these routes of microbial destruction need to be identified in order to better predict if an inflammation will ensue.

In the following overview, we will emphasize unresolved issues that stem from the recently discovered interface between the autophagic attack against pathogens and the action of interferon-regulated GTPases in modulating levels of released inflammatory mediators. In so doing, we acknowledge that there is also cross-regulation between the host autophagy machinery and a number of other processes important to the inflammatory response, such as regulation of autophagy by pattern recognition receptors (Travassos et al., 2010), that we will not cover here. Such cross-talk may be particularly important in maintaining intestinal homeostasis, as witnessed in human allele variants in these components that are associated with inflammatory conditions such as Crohn's disease (Parkes et al., 2007). The reader is referred to one of a number of excellent reviews that exist on this topic (Salem et al., 2015, Lassen and Xavier, 2017).

Basic principles of xenophagy

The term autophagy comprises several catabolic processes that target cytoplasmic components for lysosomal degradation (Yin et al., 2016, Galluzzi et al., 2017). These various forms of autophagy utilize overlapping sets of protein complexes that are involved in a number of cellular processes, including some unrelated to autophagic degradation (Bestebroer et al., 2013). During macroautophagy, intracellular components are sequestered into double-membrane vesicles called autophagosomes and delivered to lysosome-like compartments. As observed during starvation, macroautophagy can sequester parts of the

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cytoplasm, providing building blocks for housekeeping functions. In contrast, macroautophagy can also operate in a selective manner and mediates the clearance and recycling of specific components such as protein aggregates, damaged organelles and intracellular microbes. The term xenophagy is defined as selective autophagy, during which microorganisms are sequestered into autophagosomes and digested within lysosomes (Fig. 1) (Huang and Brumell, 2014, Randow and Youle, 2014). More than one pathway can lead to xenophagic degradation, and the autophagy machinery can target either cytosolic or vacuolar pathogens. The autophagy machinery also orchestrates non-canonical pathways that play roles in cell-autonomous defense and may assist other immune processes, such as phagocytosis. These xenophagy-related processes constitute a multilayered and synergistic defense network that protects virtually every subcellular compartment during the intracellular lifecycle of microbes.

Targeting of pathogens by the canonical autophagy pathway

Several studies on xenophagy describe antimicrobial processes that resemble well-studied macroautophagic pathways although xenophagy may have unique unidentified features (Fig. 1). During macroautophagy, distinct protein complexes coordinate the initiation, nucleation, elongation, closure and recycling steps of the process (Huang and Brumell, 2014, Yin et al., 2016). The proteins ULK1/2, ATG13, FIP200 and ATG101 assemble into the ULK complex to induce the formation of an isolation membrane. The kinase activity of ULK activates the Class III PI3K complex of Beclin-1, VPS34, VPS15 and ATG14 that promotes local production of phosphatidylinositol 3-phosphate (PI3P). Domains enriched in PI3P serve as docking sites for the recruitment of effector proteins such as WIPI1/2, from which the isolation membrane nucleates. The elongation and closure of the isolation membrane are then regulated by two ubiquitin-like conjugation systems, culminating in the conjugation of members of the ATG8 protein family to phosphatidylethanolamine (PE) lipids by the ATG12-ATG5-ATG16L1 complex. There are six orthologs of ATG8 in mammals divided into the LC3 and GABARAP subfamilies that act at different stages of autophagosome formation, but also serve as docking sites for autophagy adaptors that recognize cargo (Weidberg et al., 2011, Nguyen et al., 2016). Autophagosomes ultimately mature into degradative autolysosomes following a series of fusion events with endocytic compartments.

The recognition of a specific target triggers localized autophagy (Randow and Youle, 2014), requiring the activation of the protein kinase TBK1 (Thurston et al., 2016). Recognition of pathogen-associated structures is mediated by “eat-me” signals, such as ubiquitin (Ub) chains of different linkage types, which recruit autophagy adaptors that bridge cargo with the autophagy machinery for degradation (van Wijk et al., 2017, Noad et al., 2017, Randow and Youle, 2014). These autophagy adaptors, which include NBR1, NDP52, optineurin, p62 and TAX1BP1, are important for the antibacterial response and may have functions extending beyond phagophore recruitment (Verlhac et al., 2015, Randow and Youle, 2014, Tumbarello et al., 2015). Less is known about how microorganisms are targeted by Ub chains, although E3 Ub ligases targeting cytosolic *S. typhimurium* (Huett et al., 2012, Noad et al., 2017, van Wijk et al., 2017) and *M. tuberculosis*-containing vacuoles (Manzanillo et al., 2013, Franco et al., 2017) have been identified. Interestingly, the recognition of extracellular bacterial DNA by the cGAS-STING pathway, originally identified as activating

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the type I interferon response, seems to be a trigger for the ubiquitylation of *M. tuberculosis*-containing vacuoles during infection (Watson et al., 2015).

Monitoring of pathogen vacuole (PV) integrity is likely a critical step in the process of innate immune pathogen detection (Randow and Youle, 2014). While some pathogens cause damage to membranes while in transit to the cytosol, intravacuolar pathogens may also cause membrane breaches surrounding their compartment as a consequence of their replication cycle. Both processes expose β(1,4)-linked galactosides that are recognized by cytosolic galectins. Of a dozen different galectin proteins, galectins-3, -8 and -9 have been demonstrated to detect damaged microorganism-containing vacuoles (Randow and Youle, 2014). Galectin-8 directly binds the autophagy adaptor NDP52 and mediates the engulfment of *Salmonella* into autophagosomes (Thurston et al., 2012), while Ub-mediated processes may amplify this response. Accordingly, tripartite motif-containing (TRIM) E3 Ub ligases bind galectins (Chauhan et al., 2016). In addition, TRIM16 binding to galectin-3 mobilizes the core autophagy components ATG16L1, ULK1 and Beclin-1 in response to damaged endomembranes, thus triggering a localized autophagy response.

Noncanonical modification of microbial-containing vacuoles by the LC3-conjugation system

In order to initiate a localized response to a microbial threat, the host has evolved mechanisms utilizing a subset of autophagy components to recognize and mark membrane structures associated with pathogens. ATG8 family members (LC3s and GABARAPs) can be directly conjugated to PVs, bypassing some of the well characterized early steps involved in selective autophagy processes (Fig. 1) (Choi et al., 2016, Kageyama et al., 2011, Lam et al., 2013, Zhao et al., 2008). Although recruitment of the autophagy machinery may dictate the formation of an isolation membrane in close proximity to the pathogen, it is important to note that the presence of ATG8 proteins directly inserted onto the membrane compartment surrounding the pathogen represents a topologically distinct process from xenophagy. By being marked with ATG8 proteins, PVs are licensed to interact with downstream components not directly associated with xenophagy, such as those of the phagolysosomal pathway (Martinez et al., 2015) and soluble antimicrobial effectors (Choi et al., 2016, Sasai et al., 2017).

During LC3-associated phagocytosis (LAP), LC3 is directly conjugated to single-membrane vacuoles shortly after cargo internalization (Fig. 1). LAP does not rely on the formation of autophagosomes nor require the ULK complex (Martinez et al., 2015). This process was initially suggested to promote phagosome maturation, but a recent study suggests that it may play other roles, so it could interface with pathogen restriction systems in a number of fashions (Cemma et al., 2016). LAP is initiated after cell surface engagement of TLRs (Martinez et al., 2015), the immunoglobulin Fc receptor FCGR2A/FCγR2A (Cemma et al., 2016) or the CLEC7A/dectin-1 receptor (Ma et al., 2012). LC3 association with enclosed phagosomes is triggered by NOX2 NADPH oxidase complex-mediated production of reactive-oxygen species (ROS) (Huang et al., 2009). Rubicon has been proposed to act as the key molecular switch that activates LAP while it interferes with canonical autophagy. In so doing, PI3P production is stimulated, promoting the assembly of the NOX2 complex

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(Martinez et al., 2015). During LAP, Rubicon activates a Class III PI3K subcomplex containing UVRAG (UV radiation resistance-associated gene) that is devoid of ATG14L, which usually plays an essential role in canonical autophagy. In addition, diacylglycerol (DAG)-dependent signaling contributes to LAP by recruiting PKCδ (Hubber et al., 2017, Lam et al., 2013), a kinase that targets NOX2 and the kinase JNK, triggering the release of Beclin-1 from its inhibitory interaction with Bcl-2 (Wei et al., 2008). It is tempting to speculate that the activation of PKCδ is upstream to the formation of a RAB7-Rubicon-PI3K complex (Tabata et al., 2010), the production of PI3P and the recruitment of NOX2. How the NOX2-dependent production of ROS triggers LC3 lipidation is still not understood, but may be a consequence of membrane integrity disruption (Boyle and Randow, 2015).

The ATG12-ATG5-ATG16L1 complex specifies the site of ATG8 lipidation during all autophagy-related processes (Fujita et al., 2008). Although this complex can directly bind to membranes through ATG5 *in vitro*, ATG12-ATG5-ATG16L1 is not recruited to membranes without a proper inducing signal within cells (Romanov et al., 2012). The targeting of membranes by the complex is often directed by ATG16L1, through at least three different interacting partners: FIP200, the PI3P-binding protein WIPI2b (WD repeat domain, phosphoinositide interacting 2b) (Dooley et al., 2014) and Ub (Fujita et al., 2013). Notably, WIPI2b binds the membrane surrounding *Salmonella*, promoting autophagosomal engulfment of bacteria (Dooley et al., 2014). In addition, LC3 is lipidated on the *Salmonella*-containing vacuole in a Ub-dependent process that is upstream of autophagosome formation (Kageyama et al., 2011, Fujita et al., 2013). Lipidation in this fashion differs from LAP, a Ub-independent process (Lam et al., 2013, Hubber et al., 2017), and may be in response to a specific pathogenic event.

ATG8 proteins are also directly conjugated to the *Toxoplasma*- and *Chlamydia*-containing PVs in a species-specific manner through a mechanism reminiscent of LAP (Haldar et al., 2014, Choi et al., 2014). The lipidation of ATG8 proteins to these PVs does not lead unequivocally to degradation in lysosome-like compartments, but mediates the recruitment of immunity-related GTPases (Park et al., 2016, Sasai et al., 2017). This recruitment is an important link to interferon-induced clearance of intracellular pathogens as described in the following sections.

Interferon-regulated response to intracellular pathogens and regulation of inflammasome activation

Interferons (IFNs) are proteins secreted in response to infection that play a pivotal role in the immune response. IFNs are divided into three subfamilies including type I (IFN-α, IFN-β and other less characterized subtypes), type II (IFN-γ) and type III IFNs. IFN-γ, originally referred to as the macrophage-activating factor, stands out from type I and III IFNs, as the most important mediator of immunity against parasites, viruses and bacteria. IFNα/β induce an antiviral state, but, in contrast to IFN-γ, are not always protective against bacterial infections (McNab et al., 2015). Type III IFNs have been relatively recently discovered and appear to have a function similar to type I IFN, although restricted to epithelial cells (Wack et al., 2015).

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Cells induce type I IFNs as a consequence of host pattern recognition receptors binding microbial products. These receptors include TLR4, which engages lipopolysaccharide (LPS), and the RNA recognition proteins TLR7 and RIG-1-like receptors (RLRs) (Wu and Chen, 2014). In addition, cyclic dinucleotides such c-di-AMP, c-di-GMP and cGAMP drive this response, either as microbial products or generated by the host protein cGAS, an enzyme that responds to microbial double stranded DNA by synthesizing 2',3'cGAMP. Each of the cyclic dinucleotides activates the STING protein, which drives upregulation of type I IFNs (Chen et al., 2016). In addition, the release of cytokines by infected cells activates a signaling cascade that results in the secretion of IFN-γ by several types of immune cells (Schroder et al., 2004).

There are over 2000 IFN-stimulated genes, and of these, four families of GTPases are among the most abundantly expressed (Boehm et al., 1998; Pilla-Moffett et al., 2016). These include the MX viral resistance proteins (72–82 kDa), the immunity-related GTPases (IRGs; 21–47 kDa), the guanylate binding proteins (GBPs; 65–73 kDa) and the very large inducible GTPases (FLIGs/GVNs) (Pilla-Moffett et al., 2016). Bioinformatically and structurally, these subfamilies appear closest in similarity to dynamin-like GTPases, with each having an N-terminal GTPase domain linked to a C-terminal helical domain (Ghosh et al., 2004, Kim et al., 2011). That said, the biochemical behavior of these proteins does not exactly mimic dynamins or other families of GTPases, making it difficult to predict exact biochemical functions of each family member. Although the predominance of these GTPases in the IFN transcriptional response has been established for two decades, only in recent years has there been an explosion of interest in determining their functions in restricting the growth of intracellular pathogens.

For the purposes of understanding how cells respond to intravacuolar pathogens, the most important to consider are the IRGs and the GBPs, which have been linked to the disintegration of vacuole-localized microorganisms during the disease process. The IRGs consist of the GKS effectors (subdivided into the Irga, Irgb, Irgc and Irgd subgroups in the mouse) and IRGM regulatory subfamilies (Irgm1, Irgm2 and Irgm3 in the mouse; IRGM in the human). In hosts such as rodents that have both subfamilies of Irgs, the effector GKS proteins are distinguished based on the presence of a canonical catalytic GKS sequence motif in the GTPase domain, while the regulatory Irgms have the GMS substitution that defines their altered function. Although the GBPs can be grouped into those having or lacking C-terminal prenylation sites, there is no clear evidence that prenylation differentiates function among members of this subfamily.

A major shortcoming in studies related to IFN-induced GTPases is that there is no clear demonstration that the molecular details learned in the mouse can be applied to the human. In contrast to the impressive amount of information now available in the mouse, our knowledge of the roles of these GTPases in human cells lags, and there may be important unidentified players in the human response. Some differences in the interferon-regulated response between human and mouse can be attributed to massive contraction of the IRG family in humans (Bekpen et al., 2005). Divergent results between the two species can also be attributed to the fact that mouse studies are often performed in primary macrophages, while immortalized cell lines are commonly used to interrogate human responses. The high

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conservation of human and mouse GBPs, on the other hand, indicates that members of this protein family probably function similarly in the two species. Work on human GBPs is still rather slim compared to that of the mouse, so further work is necessary to relate function in the two species. The gap in knowledge regarding the role of GBPs in interferon-regulated restriction in humans is exemplified by the fact that GBP localization around PVs appears to be dependent on the cell type being infected by the pathogen (Johnston et al., 2016, Haldar et al., 2016). Clearly, more work needs to be performed to understand how differences in cell types control the interferon-regulated response as well as to identify evolutionarily-conserved and species-specific responses.

In the mouse, there is ample evidence that both GBPs and IRGs are involved in restricting the growth of pathogens (Fig. 2). As these family members restrict a large spectrum of pathogens, it is unlikely that there is a single mode of action for the entire family, or even that a single member plays only one role in the host innate immune response. That said, considerable evidence exists that GBPs can be recruited to the PV and promote or regulate membrane lysis of either the PV (Yamamoto et al., 2012, Meunier et al., 2014, Kravets et al., 2016) or the pathogen itself (Meunier et al., 2015, Man et al., 2015, Kravets et al., 2016). GBPs play a central role in initiating inflammatory responses to intracellular pathogens by facilitating the presentation of material from invading microorganisms to inflammasomes, which in turn activate cell death pathways executed by either caspase-1 or caspase-11 proteases. The most compelling evidence for a role in inflammasome signaling is in the mouse, in which a single deletion that eliminates five of the eleven GBPs ($Gbp^{ch3-/-}$) has profound defects in the activation of both caspase-1 and 11 (Pilla et al., 2014, Meunier et al., 2015, Meunier and Broz, 2015). It is important to point out that there is negative interplay between inflammasome-associated caspase activation and autophagy (Saitoh and Akira, 2016). As an example, during the IFN-regulated attack of *Salmonella*-containing vacuoles, inflammasome activation in mouse macrophages is increased in cells lacking ATG5 (Meunier et al., 2014). Therefore, the extent of inflammasome activation and consequent host cell death is negatively modulated by components of the autophagy machinery.

In contrast to the role of GBP proteins in promoting cascades that lead to inflammasome activation, the action of IRG proteins can lead in multiple directions. Mouse IrgM proteins participate in critical steps that allow accurate targeting of effector proteins to the PV prior to inflammasome activation. In addition, in human cells IRG-dependent restriction of pathogen replication can lead to microbial clearance via xenophagy, which potentially bypasses inflammasome activation (Chauhan et al., 2015).

There are a number of lines of evidence indicating that IRG proteins are indispensable players in IFN-regulated restriction of intracellular pathogens, including *M. tuberculosis* (MacMicking et al., 2003). One of the earliest demonstrations that this family was involved in destruction of the pathogen vacuolar membrane can be traced to work with a type II *Toxoplasma gondii* strain in mouse cells (Martens et al., 2005). The Irga6 GKS protein (also called IIGP1) localizes around the PV, forming a “membrane attack complex” and results in PV membrane disruption and eventual destruction of *T. gondii* in response to IFN-γ treatment. In contrast, IRGM members localize poorly to the PV and instead accumulate on a wide variety of organelles (Haldar et al., 2013). There is considerable evidence that this

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localization pattern is a form of self/nonself control, in which IRGM proteins prevent GKS effectors from inappropriately acting on host cell organelles. According to this “missing-self” model, the insertion of membranolytic GKS proteins happens exclusively on compartments that lack IRGMs, such as PVs (Haldar et al., 2013). Similarly, GBP proteins mislocalize in the absence of Irgm1 and Irgm3. By marking organelles, IRGM proteins prevent inappropriate targeting of self-structures by the membranolytic IFN-regulated proteins in rodent cells.

The interface of GBP proteins with the IRG system

The model proposed for self/nonself discrimination in the rodent IRG/GBP system still raises unanswered questions. First, while providing an attractive model for how GKS and GBP proteins can be blocked from targeting host membranes, there is no clear explanation for why IRGM proteins are depleted from the PV. Second, it does not provide an explanation for what pathogen-specific signatures are being recognized on the vacuole. Finally, it does not provide an explanation for how IFN-regulated restriction of intracellular pathogens occurs in humans. In humans, IRGM is the solitary IRG family member known to play a role in pathogen restriction, and there is no evidence that it plays a regulatory role in controlling a membrane attack complex. In fact, its ability to link pathogen restriction to pattern recognition, via Beclin-1 and ATG16L1, indicates that IRGM function may have diverged greatly across species (Chauhan et al., 2015).

We propose that an evolutionarily conserved function of IRG proteins is the ability to temper inflammation. For instance, *Irgm1*-deficient mice suffer from hyperinflammation (Maric-Biresev et al., 2016, Schmidt et al., 2017). In human cells, IRGM appears to play a central role in xenophagy, which we envision clears pathogens without attendant inflammasome activation (Chauhan et al., 2015). GBP proteins, in contrast, drive inflammasome activation, as the disruption of a vacuolar membrane in the absence of GBP function would be predicted to increase autophagic clearance. We hypothesize that after PVs are disrupted, GBPs intervene to present pathogen pattern molecules that cause inflammasome activation, overriding autophagic clearance of the damaged compartment and driving inflammation. The fact that GBP proteins can also recruit autophagy components, such as p62, may represent a strategy to prevent out-of-control intervention by GBP family members, allowing the inflammatory response to be dampened (Kim et al., 2011, Al-Zeer et al., 2013).

Signals that allow recognition of the pathogen-containing vacuole

The initiation of IFN-regulated clearance of pathogens provides another connection to autophagy proteins. GKS-driven restriction of *T. gondii* is dependent on a noncanonical autophagy process that marks the PV with ATG8 proteins, resulting in recruitment of the GKS protein Irga6 (Fig. 1) (Zhao et al., 2008, Khaminets et al., 2010). Similar results were observed during *C. trachomatis* infection of mouse embryo fibroblasts (Haldar et al., 2014). Consistent with the model that marking of PVs is a critical step in IFN-dependent restriction in the mouse, retargeting the LC3 conjugation system to alternative target membranes results in recruitment of GKS proteins as well as GBPs following IFN-γ treatment (Park et al., 2016). This process has much in common with LAP, although the downstream consequences may be dependent on the nature of the LC3/GABARAP tagging found on the PV. For

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instance, post-translational modifications of ATG8 proteins could confer a pathogen-specific response that spares sterile compartments and those harboring nonpathogens (Wilkinson et al., 2015, Choi et al., 2016). It is also possible that specific orthologs of ATG8 are involved in the recruitment of interferon-regulated GTPases. Although still a matter of debate, a recent study strongly suggests a unique role for GABARAPs (especially Gabarapl2/Gate-16) in the IFN-γ-dependent response mediated by interferon-regulated GTPases (Sasai et al., 2017). Targeting by the IFN response allows other markers to tag the PV, possibly indicating a barcoding strategy. In mouse cells, the recruitment of GKS IRGs results in downstream ubiquitination of both *T. gondii* and *C. trachomatis* vacuoles, accompanied by attachment of p62 to the ubiquitinated sites (Haldar et al., 2015, Lee et al., 2015). The p62 adapter appears to amplify this signal by recruiting Ub E3 ligases such as TRAF6 (Haldar et al., 2015).

It is noteworthy that in mouse cells, Ub modification can instruct pathogen restriction processes in two different directions. First, Ub chains on the pathogen-containing vacuole can target the microorganism for xenophagic clearance. Second, ubiquitination can trigger the recruitment of GBPs that have membranolytic and bacteriolytic activity and mobilize microbial ligands for presentation to inflammasome receptors (Haldar et al., 2015). Ubiquitination of the vacuole, therefore, establishes a dynamic tension between two strategies for pathogen clearance, with one releasing inflammatory cytokines, and the other suppressing inflammation. Control of this process could be driven by pathogen-specific factors, such as insertion of pathogen-derived protein complexes that mark the vacuolar membrane as foreign or which expose galectin-binding β(1,4)-linked galactosides (Feeley et al., 2017).

What swings the immune response toward inflammation in response to intravacuolar pathogens?

Distinguishing whether intracellular microbes are degraded in membrane compartments or in the cytosol has a profound impact on the level of inflammation in response to pathogen attack. After internalization, intracellular pathogens have strategies to avoid phagolysosomal degradation, proliferating in either the cytosol or within a membrane-bound compartment. The establishment of a replication niche is a highly complex process involving the injection of numerous effectors into the host cytoplasm, potentially setting up booby traps for the pathogen that mark it for host recognition (Casson et al., 2013). Only the most highly adapted pathogens can grow within vacuoles without being detected. Once recognized as “non self,” PVs are marked for autophagic removal by molecular tags (e.g. Ub, p62 & galectin-3) that remarkably overlap with those that mark “aberrant-self” compartments, such as damaged organelles (Anding and Baehrecke, 2017). These tags also recruit IFN-regulated GTPases that coordinate attack of PVs, potentially inducing the inflammasome via GBP intervention.

As a model for how these pathways are coordinated, inflammasome activation could provide a fail-safe mechanism that acts as a last resort when other cell-autonomous defenses fail at clearing an infection. In this scenario, phagolysosomal processing or autophagy-related processes can sequentially act on microbes upstream of inflammasome activation, with each path having calibrated consequences on inflammation. Several lines of evidence support the

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idea that the autophagy machinery blocks inflammasome activation in the response to intracellular microbes. Autophagy-related processes reduce exposure of microbial patterns to the host cytosol by intersecting with the phagolysosomal pathway to promote LAP and bacterial clearance (Martinez et al., 2015), and by mediating the repair of damaged membranes during the infection (Kreibich et al., 2015). Further, autophagy interferes with inflammasome induction by directly digesting the pathogen (Shi et al., 2012, Meunier et al., 2014) and degrading inflammasome components, which could further serve to control inflammasome activation (Shi et al., 2012).

The IFN response may intervene in this process to drive inflammatory clearance of infection by sensitizing the host cell to inflammasome activation. Most notably, IFN induction in the mouse is tightly connected to the ability of the cell to generate and present microbial molecules to inflammasomes (Casson et al., 2013, Case et al., 2013). IFN exposure is also known to regulate the expression of thousands of genes, perhaps leading to the post-translational modification (PTM) of proteins normally involved in autophagy thereby increasing their affinity for immunity-related GTPases. For example, PTM of ATG8 proteins (Wilkinson et al., 2015) may allow the recruitment of a specific subset of guanine nucleotide exchange factors (GEFs) that could activate and recruit immunity-related GTPases on PVs. Similarly, PTM of autophagy adaptors such as p62 (Pilli et al., 2012) could increase their affinity for GBPs while decreasing their interaction with conjugated LC3.

PTM of galectin also has the potential to modulate the interaction of PVs with Tripartite motif-containing (TRIM) proteins. TRIM proteins interact with autophagy regulatory proteins (e.g. Beclin-1, ULK1) that can recruit membranes to the vicinity of PVs (Kimura et al., 2016), and this could be modulated by PTM of galectin. Several TRIMs are upregulated by IFNs (Ozato et al., 2008) which could promote the conjugation of ATG8 proteins on PVs. The topology and the nature of the membrane on which ATG8 proteins are lipidated is likely to be an important determinant of the downstream outcome, especially considering that human IRGM is likely to have tropism for autophagosomes (Chauhan et al., 2015) and may not directly bind to PVs. Another strategy that could modulate these events is the Ub-like modifier ISG15, which controls the function of key immune response players through ISGylation. Interestingly, ISGylation of Beclin-1 negatively regulates canonical autophagy in response to type I IFN exposure (Xu et al., 2015). One hypothesis is that ISGylation of Beclin-1 inhibits Class III PI3K complexes involved in autophagy, but still allows the formation of PI3P on the PV through a mechanism resembling LAP (Martinez et al., 2015). This would allow immunity-related GTPases to be recruited to the PV while simultaneously blocking xenophagy.

As summarized by Fig. 3, the host recognizes and decorates PVs with Ub proteins, members of the ATG8 (LC3/GABARAP) family, and autophagy adaptors such as p62. These markers are well-characterized coordinators of autophagic removal of organelles and microbes. It is now appreciated that they also have a broader function in innate immunity, allowing the recruitment of IFN-regulated GTPases, which participate in pathogen dissolution and drive inflammasome induction. Therefore, seemingly identical markers of pathogen attack can lead to multiple pathways for pathogen clearance. Future research should aim to identify microbial and host factors that push microbial clearance toward pathways that alter the

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dynamic tension between inhibition and stimulation of downstream inflammatory responses. We suspect that several host checkpoints will be identified that allow host cells to keep inflammation in check and remain intact as they eliminate microbes through xenophagy. In the absence of these checkpoints the trigger will be pulled, driving an inflammatory response with potentially extreme consequences for the cell.

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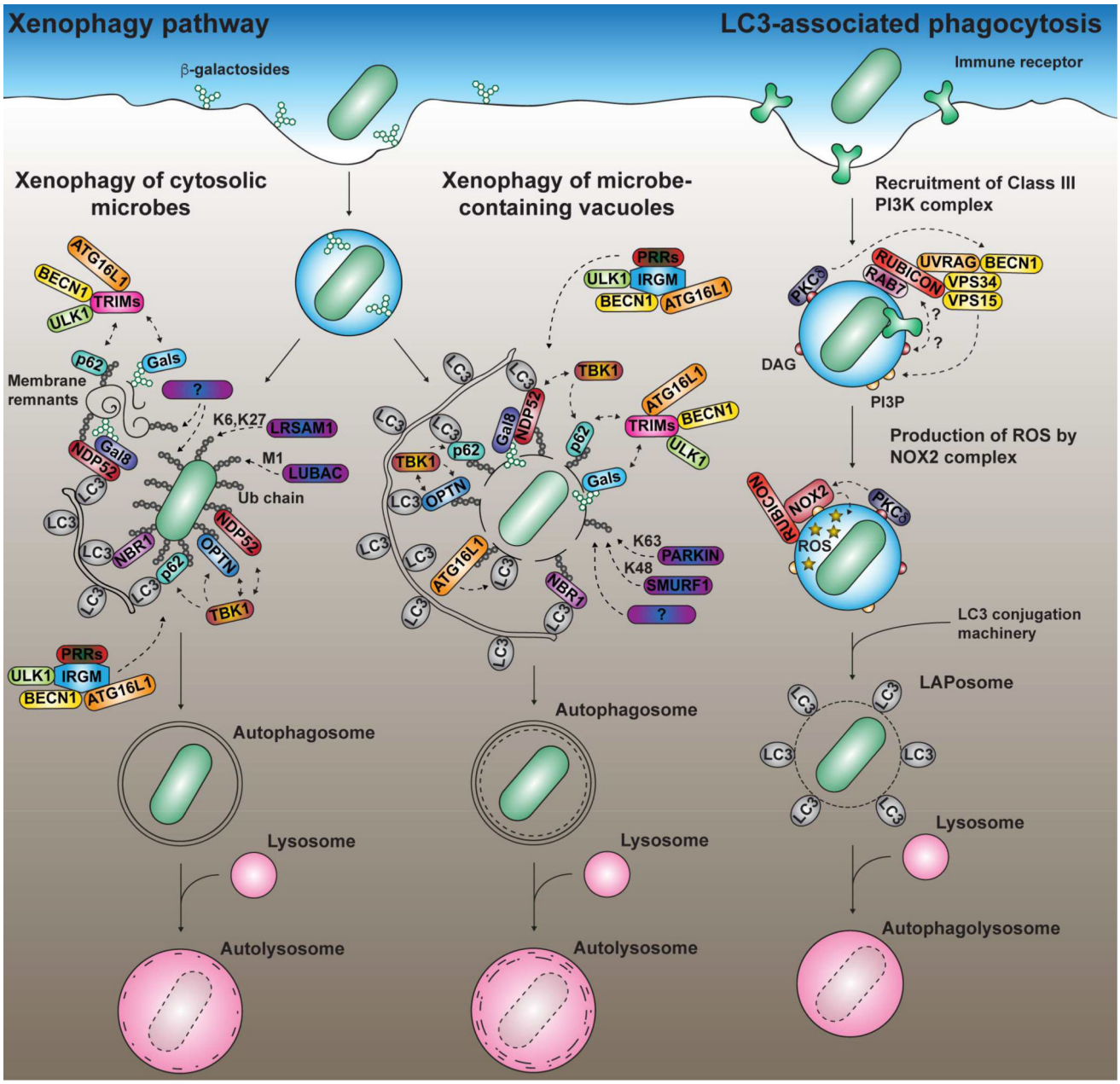

FIGURE 1. Targeting of microbes by xenophagy and LC3-associated phagocytosis

Disruption of a pathogen-containing vacuole (PVs) can lead to xenophagy. Cytosolic microbes (LEFT) and microbe-associated membranes (MIDDLE) are targeted by ubiquitin (Ub) ligases such as PARKIN, SMURF1, LRSAM1 and LUBAC (and other unknown Ub ligases as indicated by question marks) and decorated by a ubiquitin (Ub) coat of different chain linkages (M1, K6, K27, K48 and K63). These Ub chains recruit autophagy adaptors such as NBR1, NDP52, p62 and optineurin (OPTN), which bind LC3 on autophagosomal membranes. TBK1 can be recruited and phosphorylates p62 and OPTN, increasing affinity for Ub. The ATG12-ATG5-ATG16L1 complex binds ubiquitylated membranes and transfers LC3/GABARAP proteins onto PVs. Breaches in pathogen-containing vacuoles expose β-

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galactosides, which recruit galectins (Gals), and downstream partners. TRIM proteins bind galectins and p62, and interact with ATG16L1, Beclin-1 (BECN1) and ULK1. In humans, these autophagy regulatory proteins may also be recruited in complex with IRGM and pathogen-recognition receptors (PRRs) such as NOD2. RIGHT: TLRs, Fc receptor and CLEC7A/dectin-1 trigger LC3-associated phagocytosis (LAP). Rubicon associates with a Class III PI3K subcomplex, driving production of phosphatidylinositol 3-phosphate (PI3P). Rubicon and PI3P promotes the production of reactive oxygen species (ROS) generated by the NOX2 NADPH oxidase. Crosstalk between TLR signaling, Rubicon, the Class III PI3K subcomplex and NOX2 is likely to involve production of diacylglycerol (DAG), as well as recruitment of PKCδ and RAB7 (indicated by question marks). ROS production triggers conjugation of LC3 through an unknown mechanism. These three pathways ultimately delivered microbes to lysosome-like compartments. LC3: ATG8 orthologs (LC3/GABARAP).

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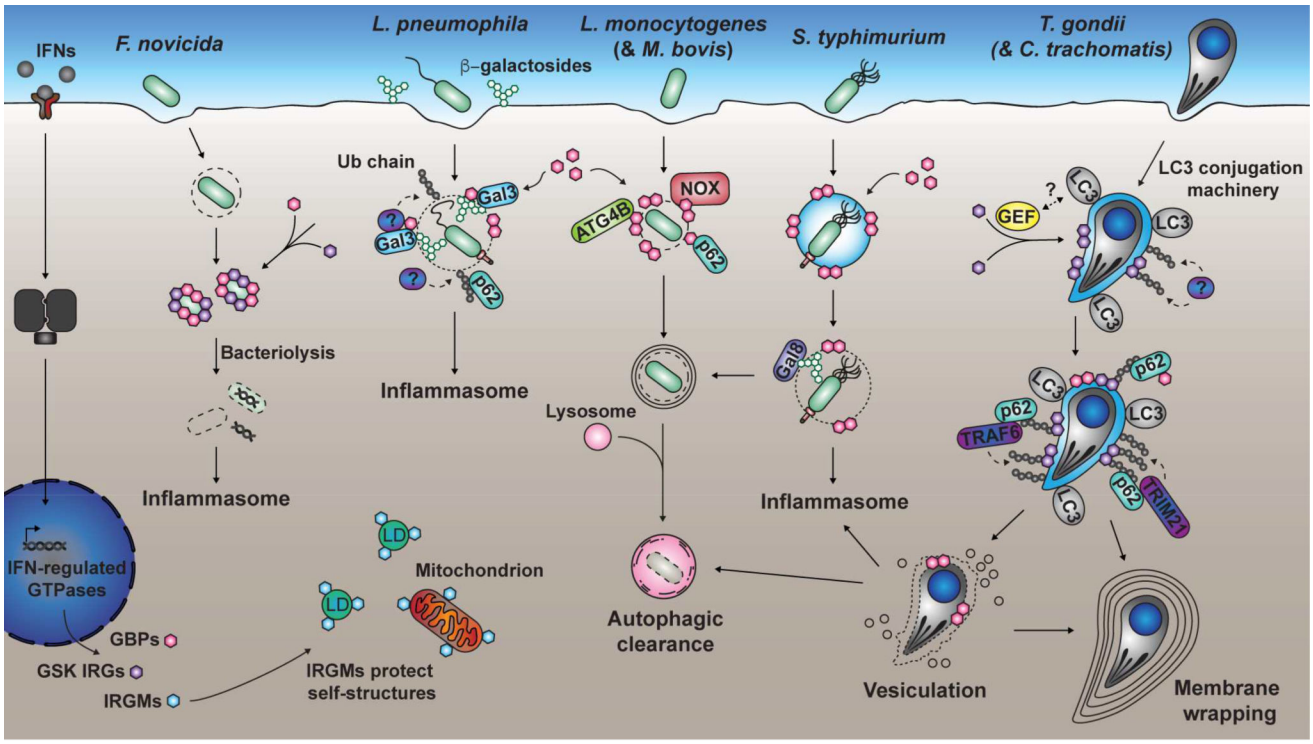

FIGURE 2. IFN-regulated response to intracellular pathogens

Binding of IFNs to receptors leads to upregulation of GSK IRGs, GBPs and IRGMs. IRGMs have regulatory functions and protect "self" structures from effector activity of GSK IRGs and GBPs. GSK IRGs and GBPs can lyse cytosolic *F. novicida* bacteria, releasing DNA and activating an inflammasome. GBPs bind to *L. pneumophila* PVs decorated by galectin-3 (here Gal-3), promoting the formation of a Ub coat, the recruitment of p62 and inflammasome induction. During *L. monocytogenes* and *M. bovis* infection, GBPs recruit ATG4B, NADPH oxidase components and p62, and were suggested to trigger xenophagy. GBPs disrupt *S. typhimurium* PVs, driving both xenophagic clearance and inflammasome induction. IRGs are recruited to *T. gondii* and *C. trachomatis* PVs downstream of LC3/GABARAP proteins and trigger formation of a Ub-associated compartment. Ub, in turn, recruits p62, which binds TRAF6, TRIM21, and GBPs. The exposed pathogens can be directly digested in the cytosol via IFN-regulated GTPases with a potential for inflammasome activation, clearance by xenophagy or wrapping in layers of membranes. A speculative interaction between an unknown guanine nucleotide exchange factor (GEF) and LC3/GABARAP proteins is indicated by a question mark. Gal8: galectin-8; LC3: ATG8 orthologs (LC3/GABARAPs); LDs: Lipid droplets.

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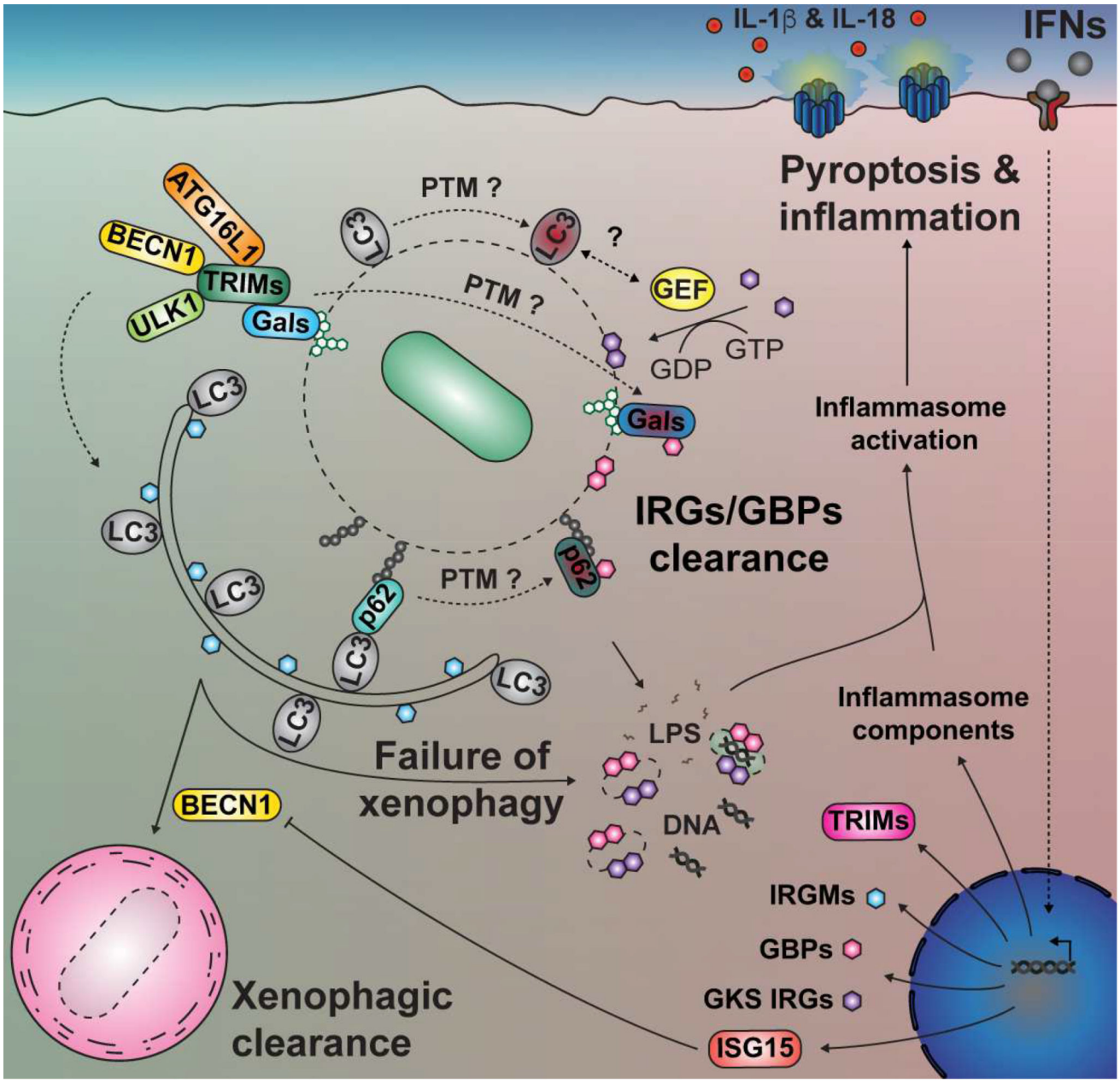

FIGURE 3. Factors that predispose inflammasome induction

Pathogen-containing vacuoles (PVs) are marked by Ub, galectins (Gals), autophagy adaptors (e.g. p62) and LC3/GABARAP proteins. These markers can trigger xenophagy, but also recruitment of IFN-regulated GTPases, activating inflammasomes. The IFN response sensitizes cells to the action of IRGs/GBPs and inflammasome induction by transcriptional up-regulation. Post-translational modifications (PTMs) of galectins, p62, LC3/GABARAP proteins and autophagy regulatory proteins have the potential to change their interacting partners and may participate (as indicated by question marks) in checkpoint mechanisms that distinguish a xenophagic pathway to pathways that induce cell death and inflammation. A speculative interaction between an unknown guanine nucleotide exchange factor (GEF)

and LC3/GABARAP proteins is also indicated by a question mark. In addition, particular orthologs of ATG8 (e.g. Gabarapl2/Gate-16) may be more specifically involved in recruiting interferon-regulated GTPases to PVs (not illustrated here). GEF: Guanine nucleotide exchange factor; BECN1: Beclin-1; LC3: ATG8 orthologs (LC3/GABARAPs).

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