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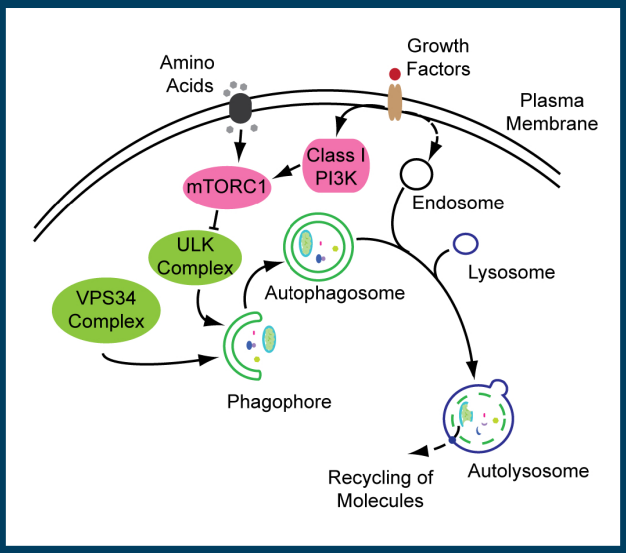

EAT THYSELF, HEAL THYSELF: AUTOPHAGY IN PATHOGEN RECOGNITION AND ANTIGEN PROCESSING

Topic Editors
Christian Muenz and Irina Caminschi

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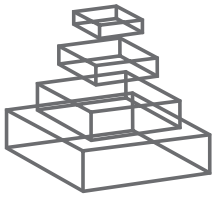

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EAT THYSELF, HEAL THYSELF: AUTOPHAGY IN PATHOGEN RECOGNITION AND ANTIGEN PROCESSING

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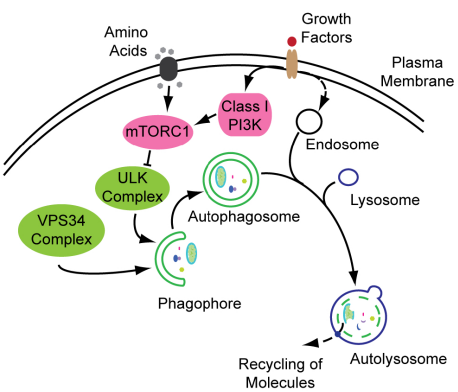

Figure reproduced from Dumit VI and Dengjel J (2012) Autophagosomal protein dynamics and influenza virus infection. *Front. Immun.* 3:43. doi: 10.3389/fimmu.2012.00043

Autophagy is a constitutive, catabolic process leading to the lysosomal degradation of cytosolic proteins and organelles. However, it is also induced under stress conditions, remodeling the eukaryotic cell by regulating energy, protein, and lipid homeostasis. It is likely that the autophagosomal/lysosomal pathway evolved primordially to recycle cell components, but further functionally developed as to become part of the immune system to defend against invading pathogens. Likewise, pathogenic, foreign agents developed strategies to fight back and even to employ the autophagy machinery to their own benefit. Hence, the regulation of autophagy has many implications on human health and disease. This eBook summarizes the molecular dynamics of autophagosome formation, maturation, and target selection. Membrane dynamics, as well as protein–protein and protein–membrane interactions are particularly addressed. In addition, it recapitulates current knowledge of the influences of influenza, measles, human immunodeficiency, hepatitis, chikungunya and herpes virus infections on the process.

Table of Contents

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EDITORIAL
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Autophagy for better or worse during infectious diseases

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Autophagy describes at least three metabolic pathways that deliver cytoplasmic constituents for lysosomal degradation (1). While micro- and chaperone-mediated autophagy engulf or translocate cytosolic material at the late endosomal or lyosomal membrane, respectively, macroautophagy can use different membrane sources, including endoplasmic reticulum, Golgi, plasma membrane, mitochondria, and outer nuclear membrane to enclose large portions of the cytoplasm in autophagosomes (2). These double membrane surrounded vesicles are generated *de novo* around macroautophagy cargo like damaged organelles, protein aggregates, and cytosolic pathogens, and more than 30 autophagy related (Atg) gene products are involved in their formation and fusion with lysosomes. The series of review articles in this *Frontiers in Immunology* e-book will high-light how regulation of macroautophagy during infections results in cytosolic restriction of pathogens, sometimes supports their replication and is connected to innate immune activation as well as adaptive immune responses to these environmental insults.

In the first set of reviews, the interactions of pathogens with macroautophagy will be discussed. Dengjel and coworkers will summarize the regulation of macroautophagy by influenza A virus and how this changes macroautophagic flux (3). This review particularly focuses on the sequential recruitment of substrates to autophagosomes and interference by influenza A virus. A second review by Biard-Piechaczyk and coauthors will then discuss the different functions that macroautophagy has during human immunodeficiency virus (HIV) infection of T cells and macrophages (4). Differences in the viral replication within these two host cells appear to determine the role that macroautophagy plays in HIV propagation in these targets. Furthermore, Faure and coworkers will high-light that there are certain nodes in the macroautophagy network that are targeted by many viruses (5). Particularly the GTPase IRGM will be discussed. Moreover, Taylor and colleague will discuss the regulation of macroautophagy by herpesviruses (6). Atg6/Beclin-1 targeting by these pathogens has resulted in fascinating insights and tools to dissect macroautophagy. Finally, this block of reviews is concluded with a text by Sasakawa and coworkers (7). They discuss the restriction of bacterial dissemination by macroautophagy and the counter responses of the bacteria aimed at escaping these immune measures. Thus, many pathogens regulate and are restricted by macroautophagy during infection.

A second set of reviews explores the role of macroautophagy in immune responses. Innate immune recognition, resulting cytokine production, antigen processing for MHC presentation, and autoimmunity will be discussed in this block. Lee and coworkers will discuss how macroautophagy regulates pathogen detection by the immune system (8). Both the turnover of cytosolic receptors of pathogen associated molecular patterns (PAMPs) and the transport of PAMPs to vesicular receptors is affected by macroautophagy. Moreover, early innate cytokine production is regulated by this pathway. Harris discusses the influence of macroautophagy on IL-1 production (9). Furthermore, Villadangos and colleague highlight the role of macroautophagy in innate and adaptive immunity, covering its role in antigen processing, as well as in T and B cell physiology (10). Expanding on some of these themes, Albert and co-worker summarize the evidence that macroautophagy contributes to exogenous antigen cross-presentation onto MHC class I molecules (11) and focus on the role of the antigen donor cell. In a second review on antigen processing via macroautophagy, the role of this pathway in MHC class II antigen processing will be discussed (12). Particularly, its contribution to both intracellular and extracellular antigen processing will be considered. Beyond antigen processing, He and colleagues will review the role for macroautophagy in lymphocyte development and function (13). This article focuses on the role of macroautophagy in T cells. Finally, Eissa and coauthor summarize how macroautophagy alterations might lead to hyperreactivity to gut commensals and autoimmunity (14). In this respect genetic predisposition to Crohn's disease, which affects essential autophagy genes, will be discussed. Thus, this second set of reviews captures the breadth of functions for macroautophagy in immunity.

Macroautophagy is, therefore, not only an essential metabolic pathway, but has also been used during the co-evolutionary struggle between pathogens and their hosts to benefit one or the other. One would predict that it may also play a role in many other infectious diseases, and consequently, could serve as a therapeutic target. However, since macroautophagy can serve the immune system or be exploited by the pathogen, its role has to be characterized for every single different pathogen in order to predict the effect its manipulation would have during infection.

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Autophagosomal protein dynamics and influenza virus infection

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Autophagy is a constitutive, catabolic process leading to the lysosomal degradation of cytosolic proteins and organelles. However, it is also induced under stress conditions, remodeling the eukaryotic cell by regulating energy, protein, and lipid homeostasis. It is likely that the autophagosomal/lysosomal pathway evolved primordially to recycle cell components, but further functionally developed as to become part of the immune system to defend against invading pathogens. Likewise, pathogenic, foreign agents developed strategies to fight back and even to employ the autophagy machinery to their own benefit. Hence, the regulation of autophagy has many implications on human health and disease. This review summarizes the molecular dynamics of autophagosome formation, maturation, and target selection. Membrane dynamics, as well as protein–protein and protein–membrane interactions are particularly addressed. In addition, it recapitulates current knowledge of the influences of influenza virus infection on the process.

Keywords: autophagy, influenza, organelle, autophagosome, protein interaction, ubiquitin

SO IT BEGINS

Autophagy comprises several diverse lysosomal degradation pathways. Chaperone-mediated autophagy involves the direct translocation of cytosolic proteins across the lysosomal membrane (Dice, 2007). During microautophagy the lysosomal membrane invaginates or protrudes to sequester and deliver portions of cytoplasm directly into lysosomes (Klionsky et al., 2007). Macroautophagy, hereafter referred to as autophagy, is a lysosomal degradation pathway mediated by specialized organelles, autophagosomes, and will be the focus of this review. Autophagosomes enclose part of the cytoplasm destined for recycling. The exact mechanism of autophagosome formation remains still unknown. However, growing evidence suggests that a subdomain of the endoplasmic reticulum (ER) is crucial for autophagosome biogenesis (Hayashi-Nishino et al., 2009, 2010; Ylä-Anttila et al., 2009). Particularly, it has been observed by electron microscopy that ER cisternae often associate with early autophagic structures (Hayashi-Nishino et al., 2010). Nevertheless, the origin of the autophagosomal membrane and how it is formed is still under debate (Chen and Klionsky, 2011). Next to the ER, mitochondria (Hailey et al., 2010), and the plasma membrane (Ravikumar et al., 2010) have been discussed as membrane sources. Almost two decades ago, complementation screening of yeast genes allowed identification of minimally 15 genes responsible for autophagosome formation (Tsukada and Ohsumi, 1993). Lately this list has grown to 33 entries of which 17 are required for all autophagy subtypes (Inoue and Klionsky, 2010). These genes are named ATG (autophagy-related) and their orthologs are essentially conserved in all eukaryotes (Noda et al., 2009). In this work, Atg refers to autophagy genes in yeast and ATG is reserved for their mammalian orthologs.

Although autophagy is a constitutive process, it can also be induced by different stress conditions, e.g., amino acid starvation or growth factor deprivation (**Figure 1A**). These treatments induce autophagy through the inhibition of the mammalian target of rapamycin (mTOR), a serine–threonine kinase central in autophagy regulation. mTOR exists as part of at least two complexes: complex 1 (mTORC1) is sensitive to nutrient abundance and is made-up of mTOR along with the subunits Raptor, mLST8 (also known as G protein beta subunit-like) and PRAS40. When activated, mTORC1 stimulates cell growth by promoting protein translation and ribosome synthesis, while it inhibits cellular degradation by autophagy (Chan, 2009). mTORC2, containing Rictor, mSin1, mLST8, and Protor next to mTOR, is discussed as acting on the cytoskeleton through other kinases, such as the serine–threonine kinase Akt and SGK1 kinase (Chan, 2009; Kim and Guan, 2011; Zoncu et al., 2011). In *Drosophila melanogaster* a third TOR complex was identified, dTTT (*Drosophila* TOR, TELO2, TTI1) which is required for dTORC1/dTORC2 activity and cell growth (Glatter et al., 2011).

It is known from yeasts, that inhibition of TORC1 by rapamycin, starvation, or other stresses induces formation of an activated Atg1 complex along with the cofactors Atg13 and Atg17, both needed for maximal Atg1 catalytic activity (Mizushima, 2010). The Atg1–Atg13–Atg17 complex has serine–threonine kinase activity and its formation leads to autophagy induction. In contrast, when TORC1 is active, it leads to Atg13 phosphorylation and subsequent destabilization of the complex and effective Atg1 inactivation (Chan, 2009). It remains unclear, however, whether TOR directly phosphorylates Atg13 (Mizushima, 2010).

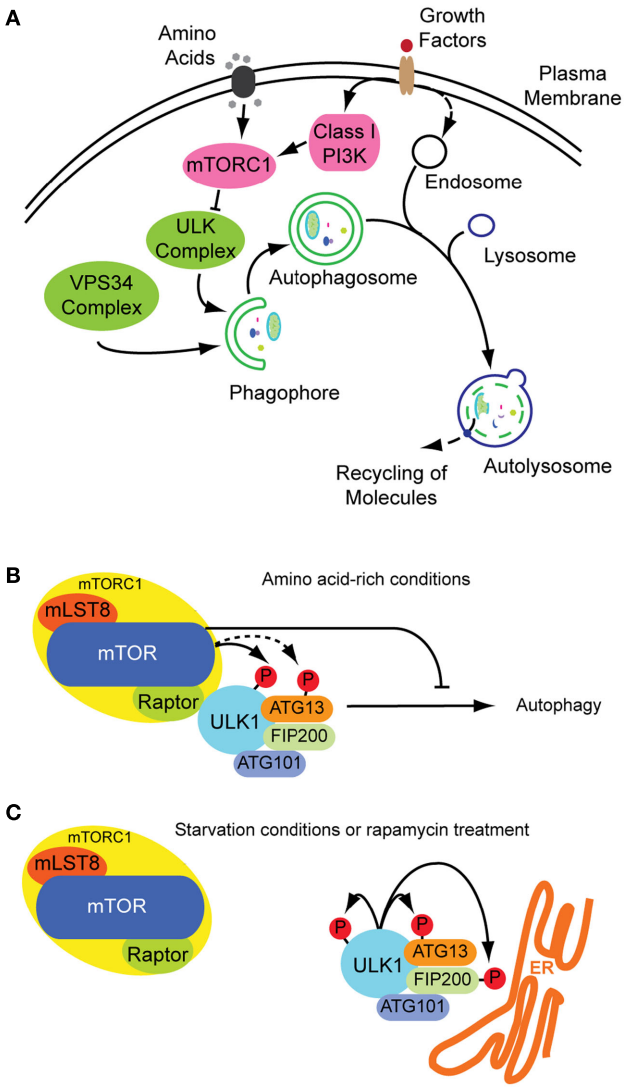

FIGURE 1 | Autophagosome formation and underlying signaling events. (A) Autophagy is a cellular stress response that can be induced *inter alia* by nutrition and growth factor deprivation. Active mTORC1 inhibits autophagosome generation, whereas active ULK- and VPS34 class III PI3K-complexes are prerequisites for autophagosome formation. The phagophore enraptures cytoplasm and matures to an autophagosome, also fusing with endosomes. Acidic hydrolases degrade the constituents in autolysosomes enabling a recycling of bio-molecules (green: stimulatory signals; pink: inhibitory signals; figure not complete). **(B)** Under growth conditions mTOR is active inhibiting the ULK-complex and by this autophagy. **(C)** Under starvation conditions mTOR is inactive and ULK active, phosphorylating itself and its binding partners and translocating to pre-autophagosomal structures.

The functional counterparts to this complex in mammals are ULKs, ATG13 and FIP200 (orthologs of Atg1, Atg13, and Atg17, respectively). ULK1 is the best characterized Atg1 homolog. The role of the other isoforms ULK2 and ULK3 in autophagy are yet less clear. Although it seems likely that ULK2 is partially redundant to ULK1 (Chan, 2009), ULK3 may not have equivalent functions (Mizushima, 2010). Under non-stress conditions, mTORC1 associates with the ULK1–ATG13–FIP200–ATG101 complex by a direct interaction between Raptor and ULK1 (Chan, 2009; Mizushima, 2010), and phosphorylates ULK1 and ATG13, inhibiting their activity (**Figure 1B**). On the other hand, when mTORC1 is inactive, it dissociates from the ULK1 complex, leading to ULK1 activation. In its active state, ULK1 undergoes autophosphorylation and phosphorylates ATG13 and FIP200 (**Figure 1C**). ULK1, ATG13, FIP200, and ATG101 accomplish their function by translocating from the cytosol to subdomains of the ER, and are thus essential for initiation of autophagosome formation (Mizushima, 2010). These proteins lead to the isolation of membrane subdomains by recruitment of a class III phosphatidylinositol-3-OH kinase (PI3K) complex to the ER. The PI3K complex includes VPS34 (also known as PIK3C3), VPS15 (PIK3R4 and p150), Beclin-1 (ATG6), ATG14, and AMBRA1 (Levine et al., 2011). At this point of autophagy induction, not only protein components have decisive functions in autophagosome formation, also the role of lipids is crucial in its regulation. In the following section, we focus on the hinge role of lipids within protein dynamics in autophagy.

THE LIPID CONNECTION

Phosphatidylinositols (PI) are negatively charged phospholipids present as minor component at the cytosolic side of eukaryotic cell membranes (Leevers et al., 1999). PI can be phosphorylated on its inositol ring to form PI-phosphate (PIP), PI-bisphosphate (PIP2), and PI-trisphosphate (PIP3; Burman and Ktistakis, 2010). PIP, PIP2, and PIP3 are collectively called phosphoinositides (Leevers et al., 1999).

In general, PI3Ks are responsible for phosphorylating the 3′OH-position of the inositol ring of PI, yielding PI3P (Burman and Ktistakis, 2010). Synthesis of PI3P is a strictly necessary requirement for all organisms undergoing autophagy (Burman and Ktistakis, 2010). The function of PI3P is to gather signaling proteins containing specific lipid-binding domains to the membrane. Particularly in autophagy, such effectors are the double FYVE-containing protein 1 (DFCP1) and WD40-repeat domain phosphoinositide-interacting (WIPI, homolog to Atg18 in yeast) family proteins (Levine et al., 2011). DFCP1, in contrast to most FYVE domain proteins that localize to endosomes, is located mainly at the ER, where PI3P is usually absent until autophagy is induced (Noda et al., 2010). Then, DFCP1 translocates to the autophagosome formation site, drawn by PI3P, to produce ER-associated Ω-like structures called omegasomes (Axe et al., 2008). The other effector of PI3P during autophagy, WIPI/Atg18, functions downstream of DFCP1 and was suggested to help the development of omegasomes into autophagosomes. WIPI2 is the major isoform among the four WIPI isoforms in most mammalian cells (Polson et al., 2010).

The FYVE domain is named after the four proteins in which it has been found: Fab1p (yeast ortholog of PIKfyve), YOTB, Vac1p (vesicle transport protein), and EEA1 (early endosome antigen 1). It is characterized by having two zinc ions and eight potential zinc coordinating cysteine residues. Additionally, several basic amino acids are localized around the cysteines. FYVE domains are part of cysteine-rich proteins, which bind PI3P in a way dependent on their metal ion coordination and interaction of their basic

amino acids with the negative charged PI3P head group (Krauss and Haucke, 2007). **Figure 2A** shows the tertiary structure of a FYVE domain and the coordination mode of the two metal ions to the eight cysteine residues. **Figure 2B** shows EEA1 and its binding mode to inositol-1,3-diphosphate, as a representative example of their interaction. It has been suggested that the binding causes conformational changes regulating protein–protein or lipid–protein interactions (Leevers et al., 1999). Most pathways regulated by PI, including autophagy, depend on their generation and likewise on their consumption. A situation where PI persist longer than the lipid signal is needed will result in loss of homeostasis. Jumpy is a PI3P phosphatase, which inhibits recruitment of WIPI to the autophagic membranes, and thus is in charge of PI3P signal termination (Vergne et al., 2009).

Independently of the conditions triggering the catabolic pathway, autophagy begins with activation of the class III PI3K Beclin-1-complex in mammals, necessary to target membranes for autophagosome generation and posterior maturation. Beclin-1, homolog of yeast Atg6, will be treated in detail below as target of various viruses to abort autophagy and to use the autophagy machinery for their own infectious purposes.

ONE WAY TICKET TO THE AUTOPHAGOSOME

Two ubiquitin-like conjugation systems (UBL), ATG12, and LC3, have been implicated in biogenesis and membrane expansion of autophagosomes (Münz, 2011a; Weidberg et al., 2011a). Modification of proteins with ubiquitin-like proteins follows a similar mechanism like modification with ubiquitin itself. For this reason, it is worthwhile to briefly summarize the general process (**Figure 3A**): Ubiquitin is a 76 amino acid-residue polypeptide, whose role is to direct proteins to the proteasome for degradation, among other regulatory functions. Ubiquitin is activated by an E1 enzyme and, subsequently, E2 enzymes pick up activated ubiquitin by transthiolation and together with E3 enzymes catalyze ubiquitination of substrates. E3 enzymes function to recognize substrates and are also capable of interacting with E2, allowing conjugation of ubiquitin to target proteins. There are two major types of E3 enzymes in eukaryotes, defined by the presence of either a HECT or a RING domain (Deshaies and Joazeiro, 2009). HECT and RING E3s catalyze ubiquitin transfer by different mechanisms (**Figure 3A**). HECT-domain containing E3s bind themselves ubiquitin from an E2 protein before transferring it to its target protein. RING E3s function as a bridge between an activated E2 and a target protein. Ubiquitination can be a repetitive process leading to the generation of polyubiquitin chains or multiple monoubiquitinations. It is also a dynamic process. Deubiquitinating enzymes are able to trim polyubiquitin chains or to remove single moieties allowing their recycling. Although eukaryotic cells have only one or few E1 enzymes, they encode more than 40 isoforms of E2 and more than 600 E3 enzymes (Grabbe et al., 2011). As it is to be expected, the number of E2 and E3 isoforms show a higher degree of complexity of the ubiquitin conjugation systems in human cells relative to yeast (Hicke et al., 2005), enabling to recognize diverse proteins in a highly specific manner (Hochstrasser, 2009).

Figure 3B shows the two UBL systems operating in autophagy. Firstly, the ATG12–ATG5 conjugation is generated by ATG7 (E1-like) and ATG10 (E2-like). ATG12–ATG5 bind to ATG16L1 and promote autophagosome formation (Fujita et al., 2008a; Weidberg et al., 2011a). It has been shown in yeast, that it is the Atg12–Atg5 complex itself that catalyzes the transfer of Atg8 from Atg3 to the substrate, phosphatidylethanolamine (PE), thus, behaving as a ubiquitin–protein ligase E3-like enzyme (Hanada et al., 2007). Secondly, LC3s, mammalian Atg8 homologs, are synthesized as precursors with an extra sequence at the C-terminus, which must be cleaved by the protease ATG4, resulting in the LC3 form I (LC3-I; Mizushima et al., 2011). LC3-I is then readily conjugated to PE (forming LC3-II). PE is a lipid found in biological membranes and lipidation of LC3/Atg8 during autophagy anchors this protein to the autophagosomal membrane. LC3/Atg8 may serve different purposes. Yeast Atg8 has been shown to be important for phagophore membrane elongation (Abeliovich et al., 2000). In addition, LC3/Atg8 functions as a membrane anchor enabling the targeting of substrates to the autophagosome (Münz, 2011a). It was also shown that it is important for membrane tethering and fusion (Nakatogawa et al., 2007; Weidberg et al., 2011b). However, these studies were performed *in vitro* and in a recent *in vivo* study it was suggested that soluble NSF attachment protein receptor (SNARE) proteins are required for membrane fusion, given that Atg8 is not able to

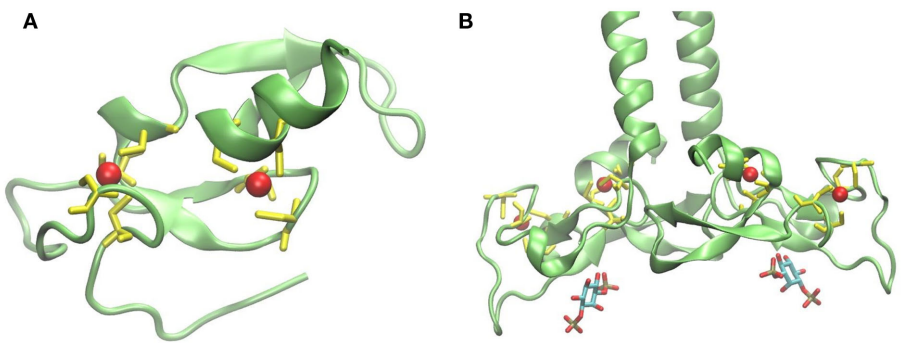

FIGURE 2 | (A) Structure of protein FYVE domain of the RUN and FYVE domain containing protein 1; Zn atoms are shown in red and coordinating cysteins in yellow (PDB code 2yw8). **(B)** Homodimer of EEA1's C-terminal FYVE domain bound to inositol-1,3-diphosphate (PDB code 1joc). The picture was prepared using the program VMD (Humphrey et al., 1996).

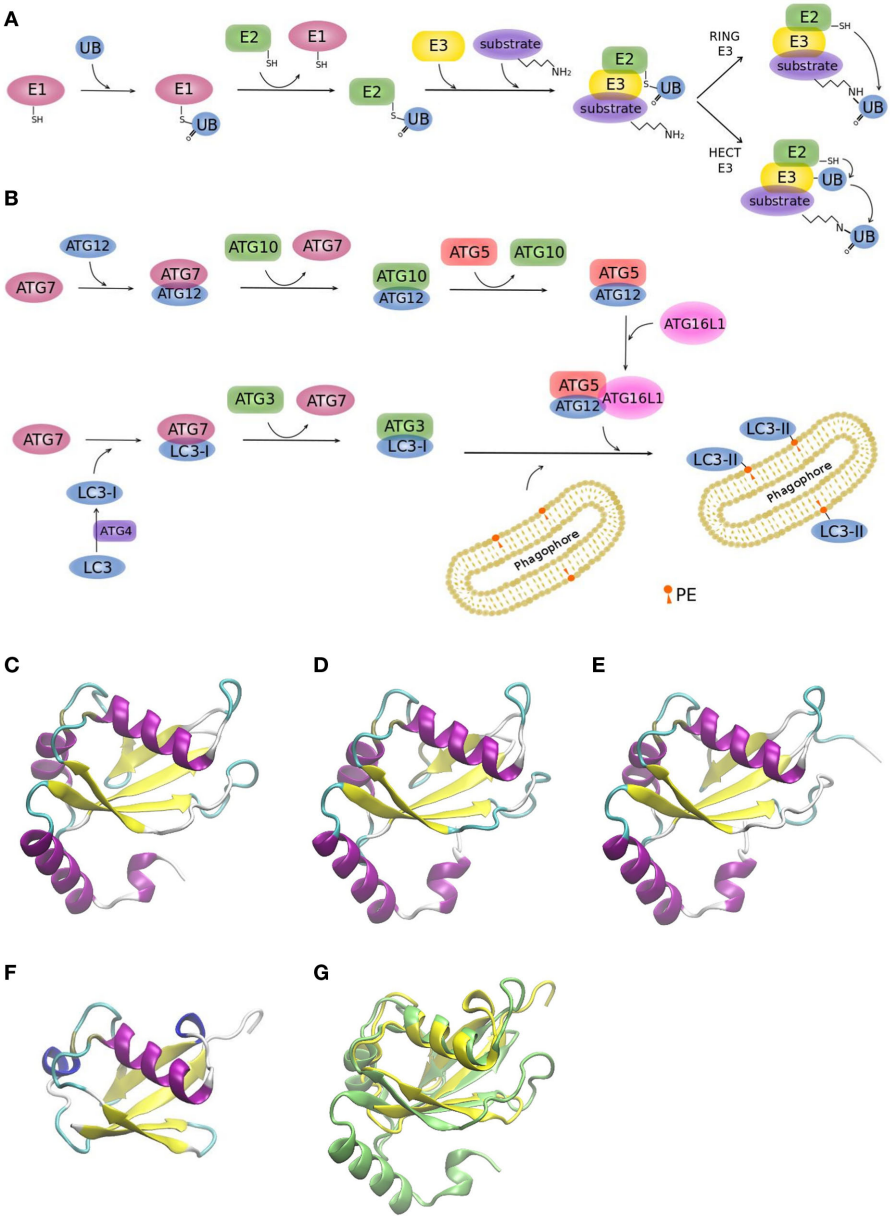

FIGURE 3 | Ubiquitin and ubiquitin-like conjugation systems. (A) The ubiquitination system. E1–E2–E3 enzymatic cascades are depicted. **(B)** The whole set of ubiquitin-like reactions taking part in autophagy involves the E1-, E2-, and E3-like enzymes ATG7, ATG3/ATG10, and ATG12–ATG5/ATG16L1, respectively. Crystallographic structure of **(C)** LC3 (PDB code 1ugm), **(D)** GABARAP (PDB code 1kjt), **(E)** GABARAPL2 (PDB code 1eo6), **(F)** ubiquitin (PDB code 1aar), and **(G)** superimposition of ubiquitin (yellow) and LC3 (green) crystallographic structures. MultiProt Server was employed for protein alignment based on their structures (Shatsky et al., 2004). The picture was prepared using the program VMD (Humphrey et al., 1996).

mediate membrane fusion under physiological PE levels (Nair et al., 2011).

Yeast has a single Atg8 protein while mammals have several paralogs: three MAP1 light chain three (LC3A, LC3B, and LC3C) and four gamma-aminobutyrate receptor associated protein (GABARAP) and GABARAP-like proteins (GABARAPL1-3), collectively referred to as LC3s. The roles of LC3s remained unclear for a long time, since the other isoforms partially compensated the loss of function of a specific LC3 form in knockout studies (Noda et al., 2009). **Figures 3C–E** displays the remarkable structural similarities of the different LC3 forms among themselves and to ubiquitin (**Figure 3F**). Superimposition of LC3 and ubiquitin structures clearly shows the high resemblance between these two proteins (**Figure 3G**).

Anchoring of autophagosomal substrates by LC3/Atg8 is accomplished by direct interaction with cargo proteins or by adaptor proteins. NIX is a mitochondrial membrane protein capable of interacting with LC3s during mitophagy, the selective removal

of mitochondria by autophagy (Novak et al., 2010). Examples of adaptor proteins are p62/sequestosome 1 (Bjorkoy et al., 2005; Pankiv et al., 2007), optineurin (Wild et al., 2011), NBR1 (Kirkin et al., 2009), or NDP52 (Thurston et al., 2009), which favor interaction between LC3/Atg8 and polyubiquitinated substrates (**Figure 4A**; Münz, 2011a). p62 and NBR1 differ in size but their structures share three domains: an N-terminal PB1 domain, a LIR motif capable of interacting with LC3 proteins, and a C-terminal UBA domain interacting with ubiquitin. Both cargo proteins, p62 and NBR1, cooperate in the targeting of ubiquitinated proteins to the autophagosome, and are both required for their degradation by autophagy (Lamark et al., 2009). The binding is mediated by the 22 amino acid-residue sequence forming the conserved LIR motif with the core W/YxxL/I, which is indispensable for LC3 recruitment into p62-positive inclusion bodies (**Figures 4B,C**; Pankiv et al., 2007; Shpilka et al., 2011). It could be shown that phosphorylation of p62's UBA domain enhances degradation of polyubiquitinated proteins (Matsumoto et al., 2011).

The next step in the maturation of an autophagosome is its closure. It is known that a defect in LC3 function leads to unclosed autophagosomes (Fujita et al., 2008b). The current model suggests that when the autophagosome double membrane is about to close forming a narrow pore, LC3 molecules, especially GABARAP proteins, meet and catalyze the mixture of lipids of the pore, forming a stalk. Disintegration of the stalk completes autophagosome closure (Noda et al., 2009). However, also here a role of SNARE proteins cannot be ruled out. The completed autophagosome is then ready to fuse with late endosomes, forming amphisomes (Gordon and Seglen, 1988), followed by fusion with lysosomes, resulting in autolysosomes, and its inner membrane and cargo get degraded by lysosomal hydrolysis (**Figure 1A**; Münz, 2011a).

Traditionally, autophagy has been described as unselective bulk degradation (Seglen et al., 1990), however, the discovery of autophagy receptors like p62 and NBR1 which also shuttle polyubiquitinated protein aggregates to autophagosomes during classical autophagy indicates the existence of target recognition and possibly directed transport. We could show by a global proteomics approach that the subcellular localization of proteins influences their degradation dynamics by autophagy (Kristensen et al., 2008). During 36 h of amino acid starvation cytosolic proteins were initially degraded, only followed at later time points by organellar proteins, such as ribosomal and mitochondrial proteins. In a follow-up study we compared the proteomic composition of autophagosomes after different autophagy-inducing stimuli (Dengjel et al., 2012). The proteomic composition of stress-induced autophagosomes during amino acid starvation differed clearly from autophagosomes induced by rapamycin treatment or by block of basal autophagy by concanamycin A, an inhibitor of the lysosomal H^+-ATPase. Comparing the proteome of autophagosomes over time also highlighted quantitative abundance differences of autophagosomal proteins. Hence, the inducing stimuli, as well as the time frame of stimulation, seem to influence the composition of autophagosomes. If (macro)autophagy is per definition unspecific, we might have to consider the possibility that only basal macroautophagy is truly unspecific and that all variants of stress-induced (macro)autophagy should instead be regarded as specific, similar to organelle-specific autophagy subtypes. Thus, we might want to discriminate, e.g., between growth factor- and amino acid-starvation induced (macro)autophagy. Another possibility might be that (macro)autophagy *per se* is not unspecific and we just did not succeed to elucidate the underlying signaling events until now. Modern high-throughput "omics" approaches in combination with systems biology allow for generation of large datasets and construction of mathematical models which may help in shedding more light on these complex biological processes (Zimmermann et al., 2010; Engelke et al., 2012).

AUTOPHAGY AND ANTIGEN PROCESSING

Even though autophagy mainly serves as protein degrading mechanism, it is likely that the same machinery has been adopted to participate in adaptive immunity (Levine et al., 2011). Thus, it could be shown that autophagy can be induced by activation of innate immune receptors in antigen presenting cells. Conveniently, fragments of proteins from infecting pathogens, which are degraded through autophagy, result in foreign peptides which can be complexed to MHC molecules and presented to T cells (for detailed review see Münz, 2011b). Classically, MHC class I molecules were regarded to present peptides from intracellular antigens to $CD8^+$ T cells. Peptides are generated by proteasomal processing and translocate to the ER via the transporter for antigenic peptides where they are loaded to nascent MHC class I complexes with the help of chaperones. In the cytosol and the ER peptides may be further processed by additional proteases before binding to MHC class I chains. Finally, MHC-I-peptide complexes transit to the

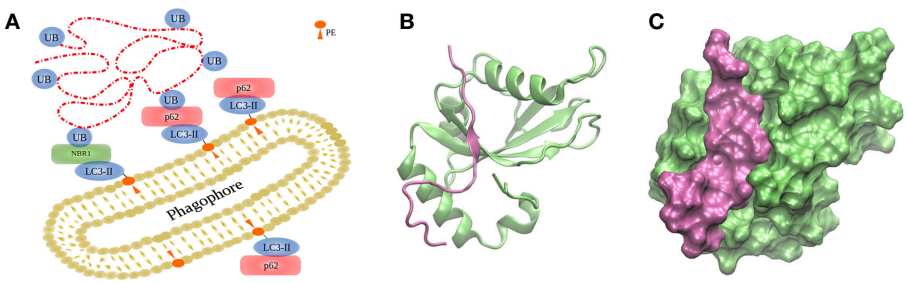

FIGURE 4 | Targeting of substrates to the autophagosome. (A) During autophagy, the autophagosome engulfs the cargo via LC3/Atg8, covalently bound to PE on both sides of the membranes by two ubiquitin-like conjugation systems depicted in **Figure 3**. **(B,C)** Interaction between human GABARAPL1 and the LIR motiv of NBR1, displayed in two different representation forms (PDB code 2l8j).

cell surface where they can be recognized by $CD8^+$ T cells. An alternative pathway, called cross-presentation, allows presentation of peptides from exogenous antigens on MHC class I molecules (Crotzer and Blum, 2010). Whereas the classical MHC class I presentation pathway seems not to be influenced by autophagy, it could be shown that autophagy modulates MHC class I presentation during late stage herpes simplex virus (HSV) infection (English et al., 2009).

Under normal conditions, MHC class II molecules present peptides on antigen presenting cells to $CD4^+$ T cells, but during infection or inflammation MHC-II expression can be induced in non-immune cells as well. MHC class II molecules were viewed to present mainly peptides from extracellular antigens. However, MHC-II peptide analyses revealed that also peptides from intracellular source proteins are presented on MHC class II molecules. MHC class II α and β chains are synthesized into the ER and the chaperone invariant chain (Ii) prevents the binding of antigenic peptides to the class II binding groove. In acidic vesicular compartments Ii is cleaved and antigenic peptides can bind to MHC class II heterodimers with the help of chaperones. In contrast to MHC-I presentation, the role of autophagy in MHC-II presentation is more clear. Thus, it could be shown by several groups that macroautophagy and chaperone-mediated autopahgy play major roles in promoting presentation of peptides derived from cytoplasmic and nuclear proteins on MHC-II (Nimmerjahn et al., 2003; Dengjel et al., 2005; Zhou et al., 2005). This is also true for the presentation of some virus derived peptides, like from Epstein Barr virus nuclear antigen 1 (Paludan et al., 2005) and from influenza virus matrix protein (Schmid et al., 2007). Interestingly, for MHC class I as well as for MHC class II presentation a dual dependency on proteasome- and autophagy-activity could be observed highlighting a crosstalk between the two degradation pathways (Dörfel et al., 2005; English et al., 2009). Along this line, we could show that the proteasome is one of the "favorite substrates" of autophagosomes and that proteasome activity is modulated by functional autophagy (Dengjel et al., 2012). However, the exact molecular mechanisms underlying autophagy–proteasome crosstalk are still not fully unveiled and more work has to be done. E.g., it is not clear if proteasomes are active inside autophagosomes and if autophagosomes may thus be regarded as scaffolds bringing together the proteasome with its substrates.

INFLUENZA VIRUS VERSUS AUTOPHAGY. WHO TAKES CONTROL?

Many pathogens compromise peptide presentation on MHC molecules by blocking the induction of autophagy or the maturation of autophagosomes. Moreover, it is known that some viruses induce autophagy but inhibit autophagosome–lysosome fusion (Deretic and Levine, 2009). Hence, several viruses inhibit autophagy at the level of autophagosome initiation by antagonizing Beclin-1. Examples of them are the α-herpervirus (HSV-1; Orvedahl et al., 2007), and γ-herpesviruses, which include human pathogens such as Epstein Barr virus, Kaposi's sarcoma associated herpesvirus (KSHV) and murine γ-HV68 (Liang et al., 2008). In contrast to DNA viruses, RNA viruses, including HIV (Kyei et al., 2009; Blanchet et al., 2010), hepatitis C (Ait-Goughoulte et al., 2008; Dreux et al., 2009), and poliovirus (Dales et al., 1965; Jackson et al., 2005), block autophagosome maturation and consequently degradation, possibly to benefit from vesicular organelles for their replication (Rossman and Lamb, 2009; Münz, 2011a). Non-maturing autophagosomes offer a propitious environment for virus replication, due to the fact that high concentrations of viral proteins can be accumulated while being unnoticed by the adaptive immune system (Rossman and Lamb, 2009). Importantly, viruses inhibiting autophagosome maturation target Beclin-1 as well, as it is also involved in the maturation process as binding partner of UVRAG.

Although it is well accepted that influenza virus infection affects autophagy, controversy still remains in many aspects of the underlying mechanisms and functional strategies employed by the virus to succeed in its infective purpose. Various independent studies suggest that an increasingly sophisticated connection exists between autophagy, apoptosis and viral replication. Moreover, interconnection between these three processes appears to be cell-line dependent, further complicating interpretation of the gathered data on the effects of influenza virus infection.

Autophagy is induced by reactive oxygen species (Huang et al., 2011) which are also produced after influenza infection (Vlahos et al., 2012) highlighting a potential point of crosstalk. Oxidizing molecules are suggested to modulate ATG4 activity leading to LC3-II accumulation (Scherz-Shouval et al., 2007). Influenza virus has been also proposed to up-regulate the expression of ATG7, ATG5, and ATG12 (Dai et al., 2012). Hence, all of these actions lead to autophagosome accumulation and may be tracked back to influenza virus infection. Significantly, it has been suggested that autophagy is involved in virus-dependent cytokine induction, which is thought to be the main cause of death of infected patients (Law et al., 2010).

A closer look at the association between influenza virus infection and autophagy raises many questions. It is well documented that influenza virus inhibits autophagy at the stage of autophagosome fusion with lysosomes, and thus leads to an accumulation of autophagosomes in human lung carcinoma-derived cells (Gannagé et al., 2009). On the other hand, it was shown that different strains of influenza virus induce functional autophagy, as detected by degradation of the autophagy receptor p62 in infected primary human blood macrophages (Law et al., 2010). A third report stated as well that influenza virus infection does induce functional autophagy in several different cell lines, with no detectable block in the pathway, as concluded from both GFP-LC3 and p62 degradation measurements (Comber et al., 2011). In an attempt to conciliate all apparently contradicting results, the authors of the latter work suggested that discrepancies may be due to the cell types or the influenza virus strains used for the experiments (Comber et al., 2011).

Another polemic aspect is the functional association between autophagy and viral replication. The purpose of compromising a key homeostatic pathway of the cell by influenza virus is still under debate. It was observed that influenza virus infection decreases cell survival by inducing apoptosis and inhibiting autophagy. The induction of apoptosis was suggested to circumvent an anti-viral immune response (Gannagé et al., 2009). However, autophagy had apparently a negligible influence on viral yields, given that loss of the degradation process did not affect virus replication. Therefore it was concluded that viral replication does not require the

autophagosome environment to take its course (Gannagé et al., 2009). Hence, it has been suggested that influenza virus seems to remain in the cytoplasm and nucleus for its replication (Rossman and Lamb, 2009). But why does influenza virus compromise autophagy? In contrast to the mentioned study, another investigation showed that inhibition of autophagy reduces replication of influenza virus (Zhou et al., 2009). On top, it was suggested that influenza virus induces autophagy only when apoptosis is first inhibited (McLean et al., 2009). This evidence was proposed to be the reason for the apparently opposing results attained before (Rossman and Lamb, 2009).

On the molecular level, there is solid evidence that binding of influenza virus M2 protein to Beclin-1 compromises autophagy at the step of lysosome fusion to autophagosomes (Gannagé et al., 2009). Beclin-1 contains a conserved BH3 domain (Oberstein et al., 2007). Such domains were first discovered in the context of apoptosis, but then could also be related to regulation of autophagy (Sinha and Levine, 2008). **Figures 5A,B** show the amphipathic BH3 helix of Beclin-1 interacting with a conserved hydrophobic groove of Bcl-XL and M11, respectively. Bcl-XL belongs to the Bcl-2 family of proteins, known to regulate apoptotic and autophagic processes in the cell (Sinha and Levine, 2008). M11 is a Bcl-2 homolog present in the human pathogen γ-herpesvirus 68, able to regulate autophagy through interaction with Beclin-1 (Sinha et al., 2008).

It could be shown that influenza virus M2 integral membrane protein is necessary and sufficient to block autophagosome–lysosome fusion (Gannagé et al., 2009). Transient expression of influenza A virus M2 protein reproduced the same phenotype as viral infection, i.e., autophagosomes accumulation due to a block in autophagosome maturation and not to an increase in autophagy. Silencing M2 expression during influenza A virus infection, or infecting cells with a M2 knockout influenza A virus, reverted the phenotype of classical infection, allowing autophagosome–lysosome fusion (Gannagé et al., 2009, 2010). M2 is a proton-selective ion channel responsible for acidification of the viral core once the virus reaches endosomes, causing dissociation of the viral particles and release of the genome into the cytoplasm (Wang et al., 2011). Surprisingly, it has been observed that autophagosome maturation is not inhibited by the M2 ion channel activity itself and that the first 60 residues of the protein are sufficient to inhibit autophagy by binding to Beclin-1 (Gannagé et al., 2009, 2010). The precise mechanism of inhibition has not been clearly determined, but it has been proposed that M2 is likely to interact with Beclin-1 through either the ectodomain (residues 1-24) or the cytoplasmic amphipatic helix (residues 46–62), but not through the transmembrane domain which should be shielded from access (Rossman and Lamb, 2009).

As shown in **Figure 5**, the secondary structures of both Bcl-XL and M11 contain mostly α-helices, as do the first 70 amino acid-residues of influenza virus M2 protein, which are needed to inhibit Beclin-1. If crystallographic structures of the complex Beclin-1–Bcl-XL and M2 are aligned, one of the transmembrane α-helices of M2 superimposes with one of the α-helices of Bcl-XL which interacts with Beclin-1 (**Figures 5C,D**). In our opinion, it cannot be excluded that this ion channel transmembrane domain of M2 binds to Beclin-1. This may happen before the M2 homotetramer complex is fully assembled in the membrane indicating a second, non-membrane-bound, function/role of this protein and possibly explaining the observation that the M2 ion channel activity seems not to be involved in its functions in autophagy regulation. To fully understand the modulations of autophagy by influenza virus and to outline the underlying molecular mechanisms more work has to be done, e.g., specifically addressing protein dynamics and protein–protein interactions under various conditions.

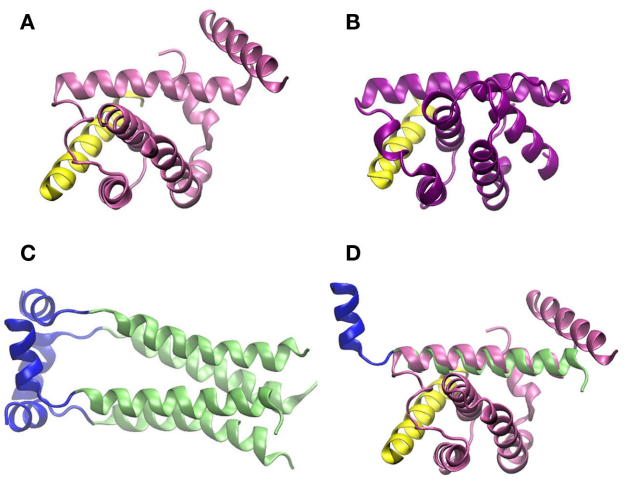

FIGURE 5 | Crystallographic structure of the BH3 helix of Beclin-1, shown in yellow, complexed to (A) Bcl-XL (PDB code 2p1l) and (B) M11 (PDB code 3bl2). (C) Crystallographic structure of M2 transmembrane protein from influenza virus (PDB code 2kih); residues 23–49 including the transmembrane domain are shown in green and the cytoplasmic helices (residues 50–60) in blue. **(D)** Superimposition of Beclin-1 complexed to Bcl-XL and influenza virus M2 protein. The picture was prepared using the program VMD (Humphrey et al., 1996), and MultiProt Server was employed for protein alignment (Shatsky et al., 2004).

CONCLUSION

Autophagy is a highly complex process and only the identification of autophagy-related genes in the genetically tractable organism yeast and the fact that the process is conserved in humans have allowed the elucidation of underlying molecular mechanisms leading to the generation of autophagosomes in mammalian cells. Although we have gained a tremendous amount of knowledge in the last decade there are still many unanswered questions, especially related to human diseases. Viruses employ autophagy for their own goods and the studying of autophagy modulation by viral infection and viral proteins will allow a deeper insight into underlying molecular mechanisms shedding more light onto this basal cell biological process. We are confident that the newly generated knowledge will not only allow the design of new anti-viral therapies but will also help in targeting autophagy in other disease settings.

Regarding macroautophagy, a lot of work has to be done to fully understand target selection. Can a cell actually allow a completely unspecific bulk degradation process to happen? Large-scale "omics" approaches should help in generating enough data to comprehensively tackle this problem on a global scale.

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Macroautophagy regulation during HIV-1 infection of CD4+ T cells and macrophages

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Autophagy is an intracellular mechanism whereby pathogens, particularly viruses, are destroyed in autolysosomes after their entry into targets cells. Therefore, to survive and replicate in host cells, viruses have developed multiple strategies to either counteract or exploit this process. The aim of this review is to outline the known relationships between HIV-1 and autophagy in CD4+ T lymphocytes and macrophages, two main HIV-1 cell targets. The differential regulation of autophagy in these two cell-types is highlighted and its potential consequences in terms of viral replication and physiopathology discussed.

Keywords: autophagy, HIV-1, CD4+ T cells, macrophages, infection

INTRODUCTION

Although progress has been made in the global fight against HIV/AIDS, more than 33 million people are still living with HIV worldwide (data from UNAIDS, 2009). HIV infection is characterized by an acute phase with very high levels of circulating viruses and a rapid decline in CD4+ T cells. Despite a strong immune response, the host cannot clear the infection and during the ensuing long, clinically latent phase, CD4+ T cells are progressively lost, eventually leading to AIDS in untreated patients. Long-lived, latent HIV reservoirs, established early during infection, prevent complete virus eradication although the anti-viral therapy effectively reduces the plasma HIV levels below detection limits (Chang and Altfeld, 2010; Mogensen et al., 2010). As a consequence, the fight against HIV is very difficult and a better understanding of the relationship between HIV and the human innate and adaptive immune systems is needed for the discovery of new drugs. Indeed, at present, drugs block the virus life cycle at different steps (entry, reverse transcription, integration, maturation) but do not act on the host response, which is responsible for the establishment of the reservoirs.

Macroautophagy (hereafter referred to as autophagy) is a physiologically controlled intracellular degradation mechanism that plays a role in both innate and adaptive immunity. Cytoplasmic pathogens can be degraded by autophagy, a process that has been termed xenophagy (Deretic and Levine, 2009). Moreover, autophagy participates in antigen processing for their presentation via the major histocompatibility complex (MHC) class I and II proteins (Schmid and Munz, 2007; English et al., 2009; Lunemann and Munz, 2009). As an evolutionary counterpoint, certain pathogens, including HIV-1, can inhibit or subvert autophagy to replicate more efficiently (Espert et al., 2007; Levine and Deretic, 2007).

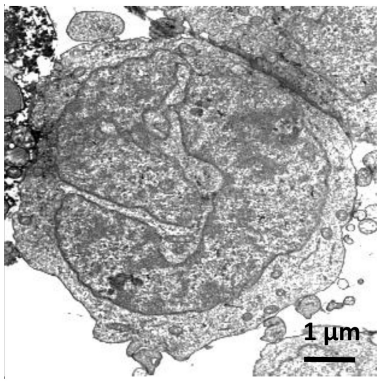
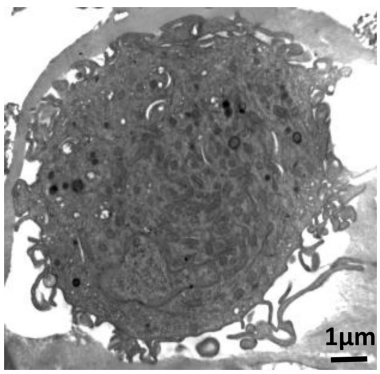
HIV-1 can infect different types of immune cells that express CD4 and a coreceptor, mainly CCR5 or CXCR4 [i.e., CD4+ T lymphocytes, macrophages, monocytes, and dendritic cells (DCs)], but replicates efficiently only in CD4+ T cells and macrophages, indicating that several aspects of the virus–host relationship are different in these target cell populations. Although both CD4+ T cells and macrophages are infected by HIV-1 and produce large amounts of virions, many differences have been identified at each step of the HIV-1 life cycle in these target cells (**Table 1**). The ultimate difference of the complex relationship between host cell and HIV-1 is the death of CD4+ T cells by apoptosis and the resistance of macrophages to HIV-1 cytopathic effects (Carter and Ehrlich, 2008). This review summarizes the most recent information on the regulation and the role of autophagy during HIV-1 infection of CD4+ T cells and macrophages and proposes that the differential regulation of autophagy in these target cells might contribute to HIV-1 physiopathology.

HIV-1 INFECTION OF CD4+ T CELLS AND MACROPHAGES

HIV-1 ENTRY IN CD4+ T CELLS AND MACROPHAGES

HIV-1 envelope (Env), composed of the glycoproteins gp120 and gp41, plays a crucial role in virus entry. In most instances, to enter a target cell, HIV-1 Env gp120, which is expressed at the surface of free virions and infected cells, must bind to CD4. The interaction between gp120 and CD4 triggers conformational changes leading to increased exposure of gp120 regions (including the V3 loop) that can bind to CCR5 or CXCR4 (Pierson and Doms, 2003). Finally, interaction of gp120 with the coreceptor induces a structural rearrangement of the transmembrane Env subunit gp41 and insertion of the fusion domain at the N-terminus of gp41 into the target cell membrane. At this stage, gp41 adopts a trimeric extended pre-hairpin intermediate

Table 1 | Major differences between CD4+T lymphocytes and macrophages during HIV-1 infection.

		HIV-1 entry	HIV-1 replication	Cell fate
CD4+T lymphocytes		Interaction of Env with HIV-1 receptors Efficient HIV-1 spread through viral synapses HIV-1 receptors-dependent endocytosis (productive infection) Syncytia observed *in vitro*	Rapid synthesis of viral DNA Viral genome integration depends on cell activation Modulation of gene expression Budding at the cell surface Susceptibility to X4 and R5 strains	**Uninfected cells** Apoptosis **Infected cells** HIV-1 spread Reservoirs
Macrophages		Interaction of Env with HIV-1 receptors and potential additional membrane components HIV-1 receptors-independent endocytosis (unproductive infection) No syncytia observed *in vitro*	Slower synthesis of viral DNA Viral genome integration is independent of cell division Modulation of gene expression Budding in endosomes and at the cell surface Greater susceptibility to R5 strains	**Uninfected cells** Resistance to apoptosis **Infected cells** HIV-1 spread Reservoirs

conformation before the formation of a stable six-helix bundle structure and virus/cell-to-cell fusion.

Infection by cell-free viruses or through contact between infected and uninfected cells leads to HIV-1 spread (Sattentau, 2008), but the efficiency of HIV-1 infection is higher when the virus is delivered through cell-to-cell contacts. DCs and macrophages, which are among the first cells encountering HIV-1, efficiently transmit HIV-1 to CD4+ T cells through virological synapses (McDonald et al., 2003; Arrighi et al., 2004; Groot et al., 2008; Felts et al., 2010). This way of spread also occurs between HIV-1-infected and uninfected CD4+ T cells (Jolly et al., 2004; Rudnicka et al., 2009). *In vitro*, cell-to-cell fusion leads to the formation of giant, multinucleated cells called syncytia.

Coreceptor (CCR5 or CXCR4) use is correlated, at least in part, with the different phases of the disease. R5 viruses, which utilize CCR5, are predominantly isolated during the early stages of HIV-1 infection. The emergence in a patient of X4 variants, which use CXCR4, is almost invariably associated with faster decline of circulating CD4+ T cells, accelerated disease progression and poor prognosis for survival (Koot et al., 1993; Richman and Bozzette, 1994). However, the presence of X4 viruses is not an obligatory prerequisite for disease progression and a significant proportion of individuals who progress to AIDS harbor exclusively R5 variants. The selective transmission of R5 viruses is not fully understood, but it may depend on the superimposition of multiple imperfect gatekeepers that restrict HIV-1 X4 transmission at different steps of the infection process (Margolis and Shattock, 2006).

Only about 15–30% of CD4+ T lymphocytes express detectable levels of CCR5 at the cell surface, whereas CXCR4 is expressed on nearly all of these T cells (Bleul et al., 1997; Grivel and Margolis, 1999). High levels of CCR5 are observed in activated/memory CD4+ T cells, which are the CD4+ T cells that can be productively infected. In addition, the first CD4+ T cells to undergo intense HIV-1 replication after infection are the resting memory CD4+ T cells present in the gut-associated lymphoid tissue (GALT), which also express CCR5 (Li et al., 2005).

Macrophages are also infected by X4 and R5 strains through its receptors, CD4, CXCR4, and CCR5. However, they are more frequently infected by HIV-1 R5 strains. This phenomenon is not fully understood, but one explanation is that R5 strains can exploit low levels of CD4 and/or CCR5 to enter macrophages (Peters et al., 2004, 2006).

Besides this route of entry, endocytosis of HIV-1 has been described, especially in macrophages. Most virions are subsequently degraded, but productive infection may nevertheless occur through this CD4-independent mechanism (Marechal et al., 2001). Entry by endocytosis has also been described in CD4+ T cells, but in such cells it requires the presence of CD4 and leads to productive infection (Pauza and Price, 1988). Recently, Miyauchi et al. (2009) have demonstrated that HIV-1 likely enters lymphoid

cells via an endocytic pathway, and that fusion between Env and its receptors occur in intracellular compartments.

Additional membrane components may support HIV-1 entry in macrophages, including syndecan, a heparan sulfate proteoglycan (Saphire et al., 2001; de Parseval et al., 2005); gp340, a cysteine-rich scavenger receptor (Wu et al., 2004); the macrophage mannose receptor (Larkin et al., 1989; Chehimi et al., 2003; Nguyen and Hildreth, 2003); elastase (Bristow et al., 2003); and α-v-integrin (Bosch et al., 2006). These constituents may facilitate virus attachment, binding, entry and/or fusion. Another potential membrane ligand of HIV-1 is annexin II, which is expressed on the membrane of macrophages, but not of T cells. Annexin II, which binds to phosphatidyl serine (PS), an anionic phospholipid captured during HIV-1 budding, contributes to the early events of macrophage HIV-1 infection (Ma et al., 2004). Other candidate host cell surface proteins that are incorporated in HIV-1 membranes and are potentially needed for HIV-1 entry might include CD28, CD44, and CD62L (Herzberg et al., 2006).

HIV-1 REPLICATION

After reaching the cytoplasm of the infected cell, HIV-1 reverse transcription takes place in the reverse transcription complex (RTC) that is constituted by virion core proteins, cellular proteins, and the RNA genome (McDonald et al., 2002; Nermut and Fassati, 2003). Double-stranded DNA is synthesized within a few hours in CD4+ T cells, whereas this process is slower in macrophages (Collin and Gordon, 1994; O'Brien et al., 1994). The RTC moves along the microtubule network to reach the microtubule organizing center (MTOC) and the nucleus. The viral DNA is then imported into the nucleus and integrated in the cell genome through a process that requires mitosis in CD4+ T cells but not in macrophages. This suggests that cellular rather than viral components of the preintegration complex (PIC), in which the viral DNA is associated with many proteins, may play a major role in viral nuclear import and integration in macrophages (Yamashita and Emerman, 2005). After integration of the viral genome, most of the assembling HIV-1 particles localize at the plasma membrane in infected CD4+ T cells, whereas in macrophages viral particles are mostly concentrated in apparently internal compartments that possess the characteristics of late endosomes/multivesicular bodies (LEs/MVBs). However, a fraction of the intracellular virus-containing compartments in macrophages could be large and complex invaginations of the plasma membrane (Deneka et al., 2007; Jouve et al., 2007; Welsch et al., 2007).

Several microarray studies also indicate that HIV-1 infection leads to changes in host gene expression, depending on the target cell-type. Specifically, modulation of genes associated with the host defense, signal transduction, cell cycle transcription, and arrest occurs predominantly in HIV-1-infected macrophages (Coberley et al., 2004; Vazquez et al., 2005). This differential modulation of gene expression contributes to the greater survival of macrophages in comparison to CD4+ T cells following HIV-1 infection (Giri et al., 2006). Transcription of the HIV-1 DNA is also a highly regulated process that exploits the specific environment of the cell host and involves multiple interplays between cell and viral factors (Rohr et al., 2003).

HIV-1 INFECTION AND APOPTOSIS OF CD4+ T CELLS

Acute HIV-1 infection leads to a dramatic decrease in the number of CD4+ T cells, which then return to normal level in the majority of patients. Afterward, the asymptomatic phase is characterized by a progressive and continuous decline in the level of circulating CD4+ T cells. In contrast, macrophages are more resistant to HIV-1 cytopathic effects and their number is stable over years, although several macrophage functions are affected by HIV-1 infection (Biggs et al., 1995; Yoo et al., 1996; Polyak et al., 1997; Kumar et al., 1999). CD4+ T lymphocyte depletion is due to continued and accelerated apoptosis, probably triggered by multifactorial mechanisms (Espert et al., 2006; Cummins and Badley, 2010). HIV-1-induced apoptosis in bystander, uninfected immune cells is likely to be a key factor in the gradual depletion of T lymphocytes observed in HIV-1-infected patients, since the degree of cell loss largely exceeds the number of infected cells. Furthermore, the vast majority of CD4+ T cells undergoing apoptosis in peripheral blood and lymph nodes of HIV-1 patients are uninfected (Krammer et al., 1994; Finkel et al., 1995; Doitsh et al., 2010).

Among the HIV-1 proteins known to induce apoptosis of CD4+ T cells, cumulative data have demonstrated a major role of Env in apoptosis of uninfected, bystander lymphocytes (Laurent-Crawford et al., 1993; Heinkelein et al., 1995; Ohnimus et al., 1997; Blanco et al., 1999, 2000; Roggero et al., 2001; Roshal et al., 2001; Ahr et al., 2004). Indeed, binding of Env to its receptors constitutes the primary interface between HIV-1 and its target cells, and both HIV-1 X4 and R5 Env can induce CD4+ T cell death (Joshi et al., 2011). Although interaction of gp120 with CD4 and the coreceptor is required for apoptosis induction, Env-mediated apoptosis of target CD4+ T cells is induced through the fusogenic function of gp41. Interestingly, hemifusion, an intermediate step in the fusion process characterized by the merger of the outer membrane leaflets of two biological membranes without the formation of a fusion pore or mixing of the inner leaflets, is sufficient to trigger Env-mediated apoptosis of bystander CD4+ T cells (Garg and Blumenthal, 2006, 2008; Garg et al., 2007).

Many fundamental questions remain unsolved, such as how host factors can influence the susceptibility to HIV-1 infection and resistance to death and what are the mechanisms leading to the establishment and maintenance of HIV-1 latency. This latter point is of major importance because the latent viral reservoirs, which are constituted mainly of resting CD4+ T cells (Chun et al., 1995, 1997; Finzi et al., 1997; Wong et al., 1997; Siliciano et al., 2003) and cells of the monocyte–macrophage lineage (Folks et al., 1988; Biswas et al., 1992, 1994; Coleman and Wu, 2009), are unaffected by highly active anti-retroviral therapies (HAART; Finzi et al., 1999) and can reinitiate systemic infection upon interruption of HAART, or following the development of resistance (Marcello, 2006; Suyama et al., 2009).

AUTOPHAGY DURING HIV-1 INFECTION OF CD4+ T CELLS AND MACROPHAGES

Autophagy is a highly regulated mechanism that involves specific genes called *Atg* (autophagy-related genes) and has an essential role in cell homeostasis. Accordingly, autophagy has

been implicated in several pathologies, including cancer, neurodegeneration, and myopathies (Marino and Lopez-Otin, 2004). Autophagy has also a specialized function in the innate immune response against intracellular pathogens through their degradation in autolysosomes (Deretic, 2006). Its implication in antigen presentation by MHC class I and II molecules extends its function to adaptive immunity as well (Nimmerjahn et al., 2003; Dengjel et al., 2005; Schmid et al., 2006). Besides its role as an intracellular host defense mechanism against viruses, autophagy can also be used by the virus for its own profit to replicate more efficiently in cells, or to control cell survival (Espert et al., 2007; Deretic and Levine, 2009; Espert and Biard-Piechaczyk, 2009; Lunemann and Munz, 2009; Lin et al., 2010; Sumpter and Levine, 2010). Data from a genome-wide RNAi screen (Brass et al., 2008) and silencing of 30 candidate cofactors (Eekels et al., 2011) indicate that HIV-1 replication in cells requires the presence of several Atg (Atg7, GABARAPL2, Atg12, and Atg16L). A very recent study underlines the role of several Atgs, in particular Atg5 and Atg16, in HIV-1 replication in CD4+ T cells (Eekels et al., 2012).

Induction of autophagy and its regulation during viral infection have different biological consequences on pathogen degradation and on the innate and adaptive immune responses, depending on both the pathogen and the host cell. The differential regulation of autophagy by HIV-1 in CD4+ T cells and macrophages (presented below) is only the tip of the iceberg, but demonstrates the importance of this process during HIV-1 infection, although many issues are not fully understood yet.

AUTOPHAGY IN UNINFECTED HIV-1 TARGET CELLS

Autophagy of uninfected CD4+ T cells, when co-cultured with lymphocytes that express Env, was described for the first time in 2006 (Espert et al., 2006). It is triggered by binding of Env to its receptors CD4 and CXCR4, but is independent of CD4 or CXCR4 signaling pathways. Autophagy can be induced in single cells (hemifusion) and syncytia (complete fusion) and depends on the fusogenic function of gp41. These results indicate that the entire process leading to HIV-1 entry into target cells through binding of Env to its receptors CD4 and CXCR4 is responsible for autophagy in CD4+ T lymphocytes (Denizot et al., 2008). As autophagy is gp41-dependent, it can be induced whatever the coreceptor used for HIV-1 entry (CCR5 or CXCR4; Espert et al., 2009). The mechanisms by which gp41 induces autophagy are unknown, but it could be a consequence of the cellular stress induced after hemifusion of the membranes. A proteomics study has already demonstrated that rapid and sustained accumulation of ROS is induced in CD4+ T cells after contact with Env-expressing cells (Molina et al., 2007), and the production of ROS is known to be directly involved in autophagy (Scherz-Shouval et al., 2007).

Importantly, Env-mediated autophagy is required to trigger CD4+ T cell apoptosis. Indeed, blockade of autophagy at different steps by drugs (3-methyladenine or Bafilomycin A1) or short interfering RNAs against *Beclin 1* and *Atg7* fully inhibits Env-mediated apoptosis. At present, nothing is known about the mechanisms that link autophagy to apoptosis, but Beclin 1 might play a key role since this Atg also binds to anti-apoptotic Bcl-2 proteins (Bcl-2 and Bcl-xL). Interaction of Beclin 1 with Bcl-2 or Bcl-xL inhibits Beclin 1-dependent autophagy, demonstrating a direct crosstalk between the core machineries regulating autophagy and apoptosis (Pattingre et al., 2005).

Interestingly, Beclin 1 is accumulated during the early steps of the Env-induced signaling cascade, and this phenomenon precedes autophagic vacuolization. Accumulation of Beclin 1 has also been reported in response to drugs that trigger cell death with autophagic features (Scarlatti et al., 2004; Shimizu et al., 2004; Furuya et al., 2005). Deciphering the molecular mechanism of Beclin 1 accumulation will be a crucial step for understanding the role of autophagy in HIV-1 pathogenicity.

Conversely, when cells that express HIV-1 R5 or X4 Env are co-cultured with cells from the monocyte/macrophage lineage, autophagy is not observed in uninfected cells, although they are susceptible to autophagy induced by different agents. Env-mediated autophagy is thus a cell-type dependent process. The state of differentiation is not responsible for their intrinsic resistance to Env-mediated autophagy because cells at different stage of differentiation (from promonocytic cells to monocyte-differentiated macrophages) are all equally resistant. Macrophages also do not undergo Env-mediated apoptosis and they are resistant to cell depletion during HIV-1 infection.

These major differences between CD4+ T cells and macrophages raise unsolved questions: what are the mechanisms that lead to Env-mediated autophagy, how autophagy is regulated in the different cell-types, and how autophagy triggers apoptosis in CD4+ T lymphocytes.

The fact that the fusogenic function of gp41 induces Env-mediated autophagy only in CD4+ T cells is surprising since fusion also occurs in uninfected macrophages co-cultured with HIV-1-infected cells. An important and still unexplained difference between these two target cells is the ability to form syncytia. This has been observed since the discovery of the disease and HIV strains were first classified based on this observation. Indeed "syncytia-inducing" viruses correspond to HIV-1 strains capable of infecting CD4+ T cells through CXCR4, while "non-syncytia-inducing" viruses are HIV-1 strains that infect macrophages through CCR5 (Goodenow and Collman, 2006). One hypothesis to explain why Env-mediated autophagy is a cell-specific mechanism is that the gp41-induced perturbations, triggered at the membrane of both macrophages and CD4+ T cells, could be different in the two cell-types or differentially regulated. Indeed, HIV-1 entry in macrophages is supported by additional specific interactions with host membrane molecules following Env binding to the receptor/coreceptor (see HIV-1 Entry in CD4+ T Cells and Macrophages). HIV-1 can also enter macrophages by endocytosis. Furthermore, recent data have demonstrated that ceramides, which are known inducers of autophagy (Pattingre et al., 2009), play an important role in the reorganization of membrane proteins (Chiantia et al., 2008) that is necessary for HIV-1 entry into cells. Another hypothesis is that gp120 binding to CD4 and to the coreceptor, two steps that precede gp41 insertion into the target membrane, transduces signals that counteract Env-mediated autophagy in a cell-type dependent manner. We also cannot exclude the involvement of secreted chemokines and/or cytokines in the regulation of gp41-induced autophagy. Further investigation is needed to elucidate this point.

AUTOPHAGY IN INFECTED HIV-1 TARGET CELLS

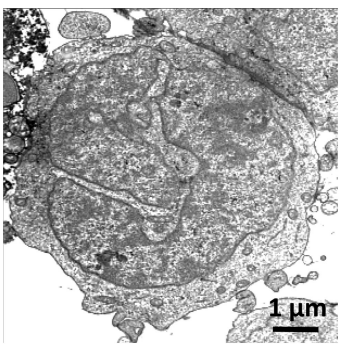
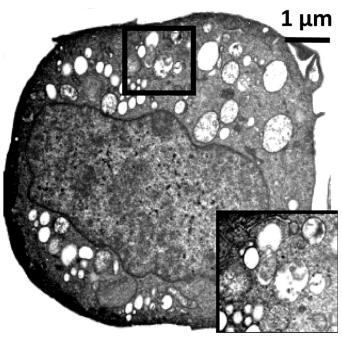
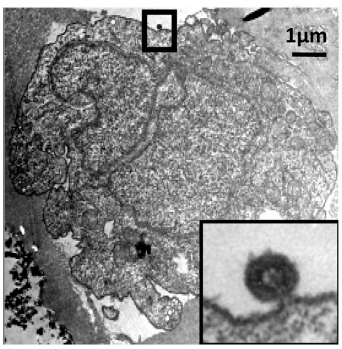
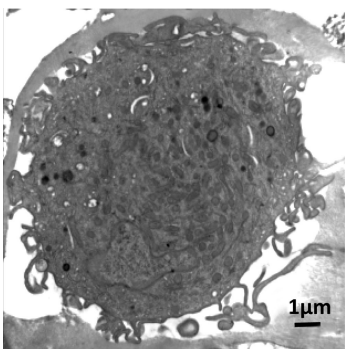
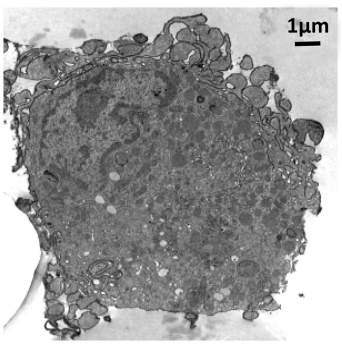
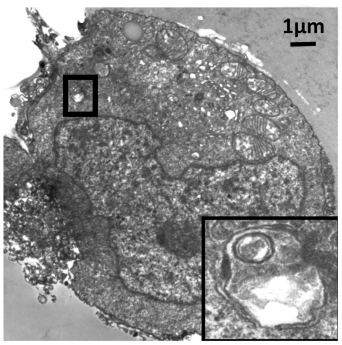
HIV-1 X4 or R5 Env expression at the cell surface (Env-transfected or HIV-1-infected cells) triggers autophagy in uninfected CD4+ T cells, leading to apoptosis. Surprisingly, when this contact leads to a productive infection, Env-mediated autophagy is inhibited in CD4+ T cells (Zhou and Spector, 2008; Espert et al., 2009). Moreover, the levels of the autophagy factor LC3-II (Zhou and Spector, 2008; Espert et al., 2009) and of Beclin 1 (Zhou and Spector, 2008) are dramatically decreased in these newly infected CD4+ T cells, suggesting that HIV-1 can actively down-regulate autophagy. Interestingly, when autophagy is triggered by different inducers in HIV-1-infected CD4+ T cells, production of HIV-1 is decreased, suggesting that autophagy can limit HIV-1 replication (unpublished data from our group).

Thus, HIV-1, as many viruses, can counteract the anti-viral function of autophagy, but the mechanism(s) by which HIV-1 can interfere with this essential cellular pathway is (are) still unknown. One or several viral proteins might block autophagy to avoid HIV-1 destruction.

Conversely, in cells from the monocyte/macrophage lineage, autophagy is induced following productive infection through contact with HIV-1 X4 or R5-infected effector cells (Espert et al., 2009). However, the observation that viruses are present only in moderately autophagic cells (Espert et al., 2009), suggests that autophagy is still controlled by HIV-1 in these cells to avoid degradation. Interestingly, early, non-degradative steps of autophagy promote HIV-1 production since blockade of this process dramatically decreases the quantity of p24 Gag (Espert et al., 2009; Kyei et al., 2009). In addition, the HIV-1 precursor Gag is found in complexes with LC3 and is present at LC3-II-enriched membranes, suggesting that autophagy could favor Gag processing and thus production of viral particles (Kyei et al., 2009). In contrast, the degradative step of autophagy has an anti-HIV-1 activity that must be controlled by the virus to prevent its degradation. Indeed, blockage of the degradative step of autophagy increases HIV-1 production (Espert et al., 2009; Kyei et al., 2009). Interestingly, the auxiliary HIV-1 protein Nef plays a major role in the inhibition of the degradative stage of autophagy by binding to Beclin 1 (Kyei et al., 2009). Nef also interacts with immunity-associated GTPase family M (IRGM), a protein known to play an autophagy-dependent anti-bacterial function (Deretic et al., 2006; McCarroll et al., 2008; Deretic, 2010a; Singh et al., 2010) and to bind to several key proteins of the autophagy process such as Atg5 and Atg10 (Pombo-Grégoire et al., 2011). Nef/IRGM interaction promotes autophagosome accumulation and improves HIV-1 replication (Pombo-Grégoire et al., 2011). In contrast, its absence is detrimental for the viral production. IRGM also triggers autophagy in cells infected by other RNA viruses, such as hepatitis C virus (HCV) and measles virus (MeV), suggesting that different RNA virus families use similar strategies, involving IRGM, to fine-tune autophagy to their own benefit.

Taken together, all these results (summarized in **Table 2**) suggest a complex, cell-type specific relationship between HIV-1 and the autophagic response and highlight the complexity of HIV-1 pathogenesis.

Table 2 | Regulation of autophagy in CD4+ T lymphocytes and macrophages during HIV-1 infection.

	Uninfected	Infected	Autophagy
CD4+ T lymphocytes	 		**Uninfected cells** Autophagy leads to apoptotic cell death contributing to AIDS **Productively infected cells** Autophagy is blocked favoring HIV-1 replication
Macrophages			 **Uninfected cells** Autophagy is not induced Macrophages are not depleted during HIV-1 pathogenesis **Productively infected cells** Autophagy is induced and blocked favoring HIV-1 replication

CONCLUSION

Although autophagy is now acknowledged to have a major role in HIV-1 infection, the available data are still quite fragmentary and more research work is needed to clarify the contribution of autophagy to viral replication, host immune responses, and viral pathogenesis. The available data indicate that autophagy is an anti-viral process and as such is inhibited by HIV-1 to avoid degradation and to promote replication. However, autophagy is also used by HIV-1 for its replication, especially in macrophages where autophagy is present all along the productive infection period.

Based on the current knowledge on the role of autophagy in different viral infections, several hypotheses can be proposed on how HIV-1 uses autophagy for its own profit in macrophages: (i) autophagy could be required to transport the RTC to the MTOC using the microtubule network. Indeed, the RTC and autophagosomes that are formed in the cytoplasm use the microtubule network to traffic toward the MTOC to reach the nucleus and to fuse with late endosomes or lysosomes, respectively (Afonso et al., 2007; Jahreiss et al., 2008; Orsi et al., 2009; Geeraert et al., 2010); (ii) autophagy could be a mechanism for membrane remodeling to support viral replication and assembly, as already described for other viruses (Miller and Krijnse-Locker, 2008). The fact that HIV-1 Gag-derived proteins interact with LC3 suggests that autophagy plays a role in promoting certain steps of HIV biogenesis (Kyei et al., 2009); (iii) autophagy could be used to specifically degrade host cell proteins that are important for the defense against HIV-1, in association with the ubiquitin–proteasome system through adapter proteins, such as p62/SQSTM1 and NBR1 (Kirkin et al., 2009; Lamark et al., 2009; Deretic, 2010b); (iv) low levels of autophagy may promote the formation of latent cell reservoirs; and (v) autophagy could process antigen determinants for their presentation by the MHC, and could hide HIV-1 from recognition by the immune system. This point is discussed in other chapters of this issue.

In addition, autophagy is a cell fate-determining process that triggers apoptosis of bystander CD4+ T cells that cannot be productively infected by HIV-1. Inhibition of autophagy in productively infected CD4+ T cells may thus both prevent HIV-1 degradation and maintain cell viability long enough to allow efficient viral replication. Autophagy has been defined as a type II programmed cell death, based on the morphology of the dying cells. However, Env-mediated autophagy is not a cell death mechanism by itself as it leads to apoptosis by a yet unknown mechanism. It is important to note that autophagy deregulation may also play a role in a variety of diseases that are related directly and/or indirectly to HIV infection, including cancer, dementia, and premature aging.

To the best of our knowledge, HIV-1 infection of CD4+ T cells and macrophages is the first example of a viral infection in which autophagy governs both viral replication and the fate of uninfected cells. The data also strongly suggest that autophagy is responsible, at least in part, for HIV-1 pathogenesis, providing new insights into therapeutic strategies for the future.

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IRGM in autophagy and viral infections

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Autophagy is a cell autonomous process allowing each individual cell to fight intracellular pathogens. Autophagy can destroy pathogens within the cytosol, and can elicit innate and adaptive immune responses against microorganisms. Nevertheless, numerous pathogens have developed molecular strategies enabling them to avoid or even exploit autophagy for their own benefit. IRGM (immunity-related GTPase family M) is a human protein recently highlighted for its contribution to autophagy upon infections. The physical association of IRGM with mitochondria and different autophagy-regulating proteins, ATG5, ATG10, SH3GLB1, and LC3, contribute to explain how IRGM could regulate autophagy. Whereas IRGM is involved in autophagy-mediated immunity against bacteria, certain viruses seem to have developed strategies to manipulate autophagy through the selective targeting of this protein. Furthermore, *irgm* variants are linked to infection-associated human pathologies such as the inflammatory Crohn's disease. Here, we discuss how IRGM might contribute to human autophagy upon viral infection, and why its targeting might be beneficial to virus replication.

Keywords: autophagy, IRGM, virus, infection, immunity, interferon

INTRODUCTION

As obligatory intracellular parasites, viruses are continually faced with the degradative mechanism of macroautophagy (thereafter referred to as autophagy; **Figure 1**). Autophagy can destroy infectious virions or virus components that are essential for replication (Levine, 2005; Richetta and Faure, 2012). Furthermore, autophagy can deliver viral genomes to TLR-containing endosomes, which sets off synthesis of antiviral type I interferon (IFN-I; Lee et al., 2007). Autophagy can also contribute to virus-derived peptide presentation on class I and class II major histocompatibility complex (MHC) molecules to trigger antiviral $CD8^+$ and $CD4^+$ T cells responses, respectively (English et al., 2009; Munz, 2009). Thus, autophagy is an intrinsic cellular antiviral process able to enhance innate responses and to link them to adaptive immunity to optimize the fight against viruses.

Numerous viruses have developed molecular strategies to counteract autophagy. Certain viruses developed properties enabling them to inhibit the autophagy flux. For instance, herpes simplex virus (HSV)-1 or the cytomegalovirus (CMV) can inhibit autophagy induction by targeting BECLIN1 (BECN1), an essential autophagy-associated protein, through interactions with viral proteins (Orvedahl et al., 2007; Chaumorcel et al., 2012). Other viruses avoid autophagy degradation by inhibiting autophagosome maturation such as human immunodeficiency virus (HIV)-1 and influenza A virus which prevent autophagosome maturation through the physical interaction of one viral protein with BECN1 (Gannage et al., 2009; Kyei et al., 2009). Finally, viruses can induce a complete productive autophagy process and exploit it as a source for metabolites or as a platform for improving their own replication (Jackson et al., 2005; Heaton and Randall, 2010). Thus, host-virus co-evolution may have led to the selection of very different mechanisms used by viruses to avoid or exploit autophagy.

The understanding of the molecular pathways linked to the proviral or antiviral functions of autophagy is still at its beginning, especially regarding the molecular interplay between viruses and autophagy proteins. The human immunity-related GTPase family M (IRGM) protein was shown to be widely targeted by RNA viruses, several among which can exploit autophagy in human cells to improve their replication (Gregoire et al., 2011, 2012). However, how IRGM regulates autophagy upon infections remains unknown. This aspect might be of great interest in several human pathologies for which *irgm* was recently found to be involved.

IRGM AND AUTOPHAGY IN INFECTIONS

Unlike its mouse ortholog, the human *irgm* gene expression is not under the control of IFN-γ (Bekpen et al., 2005). Whereas 23 immune-related genes (*irg*) genes exist in mice and play immune-related functions, the IRGM-mediated cell autonomous resistance mechanisms have been first lost in primates due to deleterious mutations. However, *irgm* gene function was restored in some primates including *Homo sapiens*, possibly due to the insertion of a retroviral element that promotes its expression (Bekpen et al., 2009). In human, five different IRGM splice isoforms that differ in their C-terminal ends could be expressed, but their individual endogenous expression has not yet been documented (Bekpen et al., 2005; Singh et al., 2010); endogenous IRGM as well as overexpressed IRGMd isoform can localize in mitochondria (Singh et al., 2010; Gregoire et al., 2011).

One of the first molecular demonstrations of a role of autophagy in immune responses against intracellular microorganisms, involved the murine ortholog of IRGM, IRGM1 (Gutierrez et al., 2004), previously described to be involved in the vacuolar trafficking of phagosomes containing *Mycobacterium tuberculosis* (MacMicking et al., 2003). *M. tuberculosis* entrapped within phagosomes can avoid its destruction by preventing

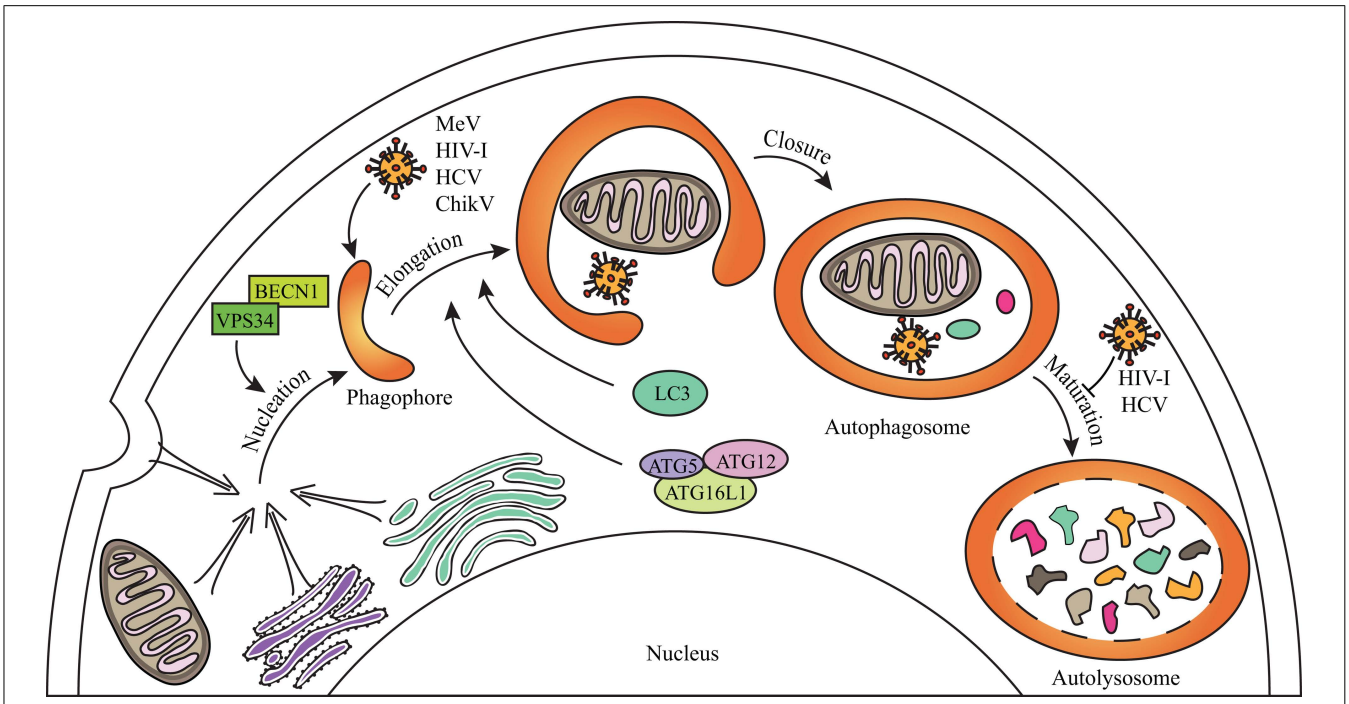

FIGURE 1 | General steps of mammalian autophagy. Autophagy engulfs portions of the cytosol through three main steps. The nucleation induces the formation of an isolation membrane which could emerge from different membrane sources (the endoplasmic reticulum, the Golgi apparatus, the plasma membrane, the mitochondria) to form a phagophore which elongates to form double-membraned autophagosome vesicles. Autophagosome can sequester cytosolic material including senescent organelles such as mitochondria, long-lived proteins or intracellular pathogens, through independent selective autophagies (for schematic simplification, all were represented within one single autophagosome). Autophagosome ultimately fuses with lysosomes during the maturation step to form autolysosomes where degradation occurs. Some of the crucial proteins involved in the different phases of autophagy are indicated. Several viruses can induce a complete autophagy flux, as MeV or ChikV, whereas others can inhibit autophagosome maturation, as HIV-1 and HCV, in order to improve their replication (see text for details).

phagosome fusion with lysosomes but the rerouting of *M. tuberculosis*-containing phagosomes to the autophagic machinery can ultimately degrade the bacteria. The treatment of murine macrophages with IFN-γ induces autophagy *via* IRGM1 and protects from *M. tuberculosis* (Gutierrez et al., 2004; Singh et al., 2006). IRGM-mediated autophagy also contributes to protection in human cells against intracellular *M. tuberculosis*, *Escherichia coli*, and *Salmonella typhimurium* (Singh et al., 2006; McCarroll et al., 2008; Lapaquette et al., 2010; Brest et al., 2011). The fact that there appears to be no role for IRGM or for its murine ortholog IRGM1 in the regulation of autophagy in absence of infection suggests a pathogen-specific function in autophagy for these proteins (Gregoire et al., 2011; Matsuzawa et al., 2012).

IRGM IN VIRUS-MEDIATED AUTOPHAGY

IRGM IS A COMMON TARGET OF RNA VIRUSES

RNA viruses genome encodes very few proteins including non-structural proteins that are often dedicated to prevent antiviral responses (Katze et al., 2008). To optimize replication, individual viral proteins could target several different host-cell proteins to counteract cellular antiviral responses. Alternatively, viral proteins could be dedicated to the efficient targeting of few host-cell proteins to counteract essential biological functions. The analysis of the interactions between 44 autophagy-associated human proteins and 83 viral proteins belonging to different RNA virus families revealed IRGM as the most targeted autophagy-associated protein by these viruses. IRGM can interact with 12 viral proteins belonging to five different viruses, Chikungunya virus (ChikV), Mumps virus (MuV), Hepatitis C virus (HCV), Measles virus (MeV), and HIV-1 (Gregoire et al., 2011). Except for MuV for whom no autophagy-related studies were yet reported, all other viruses manipulate autophagy.

IRGM AND VIRUSES EXPLOITING THE AUTOPHAGY FLUX

Measles virus infection increases the formation of *de novo* autophagosomes by inducing the autophagy flux (**Figure 1**; Joubert et al., 2009; Meiffren et al., 2010; Gregoire et al., 2011). Genetic inhibition of autophagy limits MeV viral particles production, indicating that MeV exploits autophagy to replicate. The reduced expression of cellular IRGM with specific siRNA decreased MeV replication in HeLa cells. Furthermore, the non-structural MeV-C protein can interact with IRGM, and its single overexpression induces autophagy through an IRGM-dependent pathway (Gregoire et al., 2011). Thus, in the course of MeV infection, the MeV-C/IRGM interaction might contribute to the exploitation of autophagy by MeV.

Chikungunya virus infection also induces the autophagy flux (**Figure 1**; Krejbich-Trotot et al., 2011; Joubert et al., 2012). Whereas inhibition of autophagy limits ChikV viral particles production, the experimental promotion of autophagy improved

its replication (Krejbich-Trotot et al., 2011). ChikV replication is required to induce autophagy upon infection, and, as a consequence, autophagy delays cell death, which limits ChikV-associated pathogenesis, but favors its dissemination (Joubert et al., 2012). ChikV infection induces endoplasmic reticulum and oxidative stresses that independently can trigger autophagy (Joubert et al., 2012). However, it is unknown whether ChikV proteins contribute directly to autophagy induction and/or maintenance in infected cells. Especially, IRGM was found to interact with ChikV-NS2 and E3 proteins (Gregoire et al., 2011). It will be interesting to determine whether ChikV/IRGM interaction contributes to autophagy manipulation.

IRGM AND VIRUSES INHIBITING AUTOPHAGY MATURATION

During HIV-1 infection, autophagy manipulation strategies depend on the type of infected cells. The exposition of HIV-1-ENV protein on membranes of infected cells induces autophagy in uninfected $CD4^+$ T cells leading to their apoptotic cell death (Espert et al., 2006). However, HIV-1 inhibits autophagy in infected $CD4^+$ T cells, which facilitates replication (Espert et al., 2009). In dendritic cells (DC), HIV-1 inhibits autophagy through exhaustion of the mTOR signaling pathway (Blanchet et al., 2010). However, autophagy is induced in HIV-1-infected macrophages where HIV-1–NEF protein can interact with BECN1 to inhibit the maturation of autophagosomes, what is required for an efficient replication of HIV-1 (Espert et al., 2009; Kyei et al., 2009). NEF can also interact with IRGM and the overexpression of NEF induces an IRGM-dependent accumulation of autophagosomes (Kyei et al., 2009; Gregoire et al., 2011). Thus, while NEF–BECN1 interaction could prevent autophagosome maturation, NEF–IRGM interaction could be involved in autophagy induction upon HIV-1 infection in macrophages. Through its interaction with distinct autophagy-associated proteins, a unique HIV-1 protein could finely regulate autophagy. Interestingly, a NEF deficient strain of HIV-1 does not induce autophagosome accumulation, suggesting indeed that, besides preventing autophagosome maturation, NEF is involved in the induction of autophagy by HIV-1 (Kyei et al., 2009).

Infection by HCV also induces autophagy. This induction is independent of mTOR (Su et al., 2011; Shrivastava et al., 2012), and the contribution of the unfolded protein response remains unclear (Sir et al., 2008; Mohl et al., 2012). However, autophagy is required for an optimal HCV replication since inhibition of autophagy affects HCV replication (Dreux et al., 2009; Tanida et al., 2009; Gregoire et al., 2011). Reports have shown that HCV infection could either induce a complete autophagy flux or inhibit autophagosome maturation. This discrepancy might result from the models used and/or the kinetics of infection. HCV infection was shown to prevent autophagosome maturation at an early time of infection (Sir et al., 2008; Gregoire et al., 2011; Su et al., 2011). At a later one a complete autophagy flux was reported (Ke and Chen, 2011; Mohl et al., 2012). Interestingly, a subgenomic replicon expressing the non-structural NS3-5B proteins induces autophagy (Mohl et al., 2012). Furthermore, IRGM can interact with HCV-NS3, and the reduced expression of IRGM prevents HCV-induced and HCV-NS3-induced autophagy, and limits HCV replication (Gregoire et al., 2011).

Thus, viruses that manipulate autophagy either by benefiting from the complete autophagy flux or by inhibiting the maturation step, target IRGM. Beyond its role in virus biology, how IRGM contributes to the orchestration of autophagy upon viral infection remains to be understood.

IRGM IN AUTOPHAGY INDUCTION UPON VIRAL INFECTIONS

IRGM AND AUTOPHAGY-ASSOCIATED PROTEINS

To date, only four cellular proteins were identified to interact with IRGM: ATG5, ATG10, MAP1LC3C, and SH3GLB1 (**Figure 2**). All these proteins contribute to autophagy, supporting the idea that IRGM plays an essential role in this process (Gregoire et al., 2011). ATG10, a conjugating E2-like protein, contributes to the assembly of the ATG12/ATG5 complex that binds ATG16L1 to form macromolecular ATG12/ATG5/ATG16L1 complexes essential for the elongation of the phagophore (**Figure 1**; Xie and Klionsky, 2007). MAP1LC3C is a member of the MAP1LC3 (known as LC3) sub-family and is also required for elongation of the phagophore through lipidation with phosphatidylethanolamine and anchoring within the extending phagophore (**Figure 1**; Weidberg et al., 2010). Finally, SH3GLB1 (also known as Bif-1) is a positive regulator of the nucleation process that initiates autophagosome formation, *via* its interaction with UVRAG, a protein of the BECN1/VPS34 complex. In nutrient deprived cells, SH3GLB1 colocalizes with ATG5 and LC3 to the autophagosome and potentiates the activation of the class III PI(3)-kinase VPS34 to promote autophagosome biogenesis (**Figure 2**; Takahashi et al., 2007). Thus, all the proteins known to interact with IRGM regulate one of the initial steps of autophagosome biogenesis, suggesting that IRGM might contribute to the nucleation and/or the elongation of autophagic vesicles through its interaction with one or several of these proteins. These interactions could be facilitated upon viral infection (**Figure 2**). Through the dampening of antiviral IFN-I synthesis, this targeting might be of further benefit to viruses as discussed below.

IRGM, MITOCHONDRIA, AUTOPHAGY, AND IFN-I IN VIRAL INFECTIONS

Overexpressed GFP-fused IRGMd was suggested to be translocated to the inner membrane of mitochondria *via* its association with cardiolipin (CL; Singh et al., 2010). CL is a phospholipid abundant in the inner mitochondrial membrane that is however also found in the outer membrane and at the contact sites between the two membranes (Schlame et al., 2000; Schug and Gottlieb, 2009). Thus IRGMd might also be associated to CL linked to the outer membrane of mitochondria, and exposed to the cytosol. The four other overexpressed GFP-fused IRGM isoforms (IRGMa/b/c/e) were not found associated to mitochondria (Singh et al., 2010). However, endogenous IRGM expression, detected with an antibody with putative ability to recognize all IRGM isoforms, is found associated to mitochondria suggesting that: (i) all endogenous IRGM can be located to mitochondria, (ii) IRGMd is the most expressed isoform, or (iii) due to possibly distinct folding among the isoforms, the epitope for the antibody is only accessible on IRGMd (**Figure 2**; Singh et al., 2010; Gregoire et al., 2011). Interestingly, among the proteins interacting with IRGM at least two were found partially associated with mitochondria, SH3GLB1 and ATG5. A fraction of SH3GLB1 localizes to mitochondria where

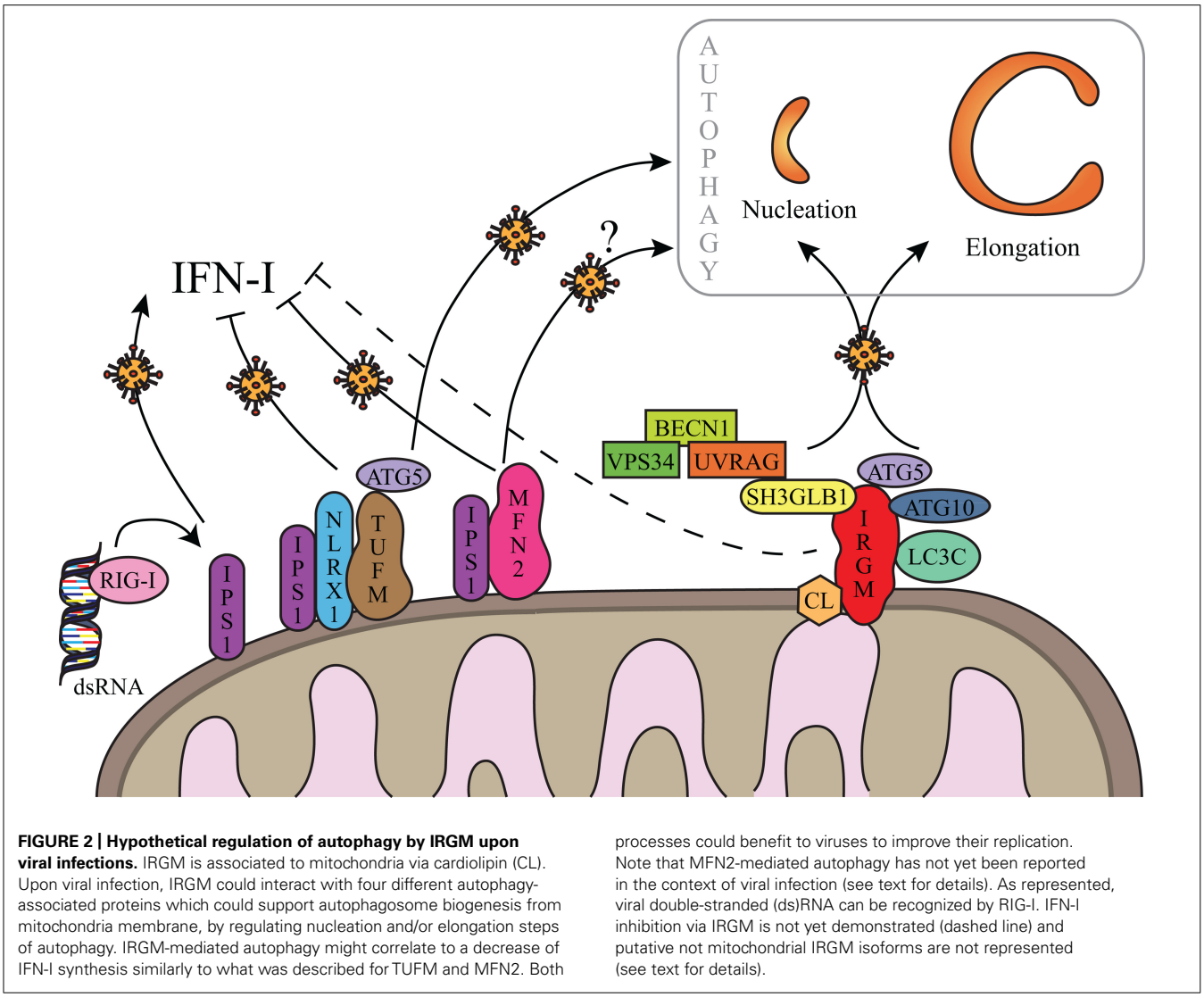

FIGURE 2 | Hypothetical regulation of autophagy by IRGM upon viral infections. IRGM is associated to mitochondria via cardiolipin (CL). Upon viral infection, IRGM could interact with four different autophagy-associated proteins which could support autophagosome biogenesis from mitochondria membrane, by regulating nucleation and/or elongation steps of autophagy. IRGM-mediated autophagy might correlate to a decrease of IFN-I synthesis similarly to what was described for TUFM and MFN2. Both processes could benefit to viruses to improve their replication. Note that MFN2-mediated autophagy has not yet been reported in the context of viral infection (see text for details). As represented, viral double-stranded (ds)RNA can be recognized by RIG-I. IFN-I inhibition via IRGM is not yet demonstrated (dashed line) and putative not mitochondrial IRGM isoforms are not represented (see text for details).

it may contribute to the regulation of morphological dynamics of the outer mitochondrial membrane (Karbowski et al., 2004), and to mitochondria-dependent apoptotic signals by interacting with the proapoptotic protein BAX (Takahashi et al., 2005). ATG5 was also shown to associate with mitochondria through its binding to IPS-1, a mitochondria-associated adaptor which relays signals from viral genome-detecting cytosolic receptors RIG-I and MDA5, in order to promote IFN-I synthesis. This interaction contributes to the down-regulation of IFN-I production during viral infection (Jounai et al., 2007). A possible hypothesis for the molecular contribution of IRGM in autophagy would be that IRGM interacts/recruits its protein partners at the mitochondria to induce autophagy upon infections especially as mitochondria is one possible source of membrane for autophagosome biogenesis (**Figures 1 and 2**; Hailey et al., 2010). Furthermore, the IRGM targeting to mitochondria could allow viruses to limit IFN-I production similarly to two other mitochondrial proteins, MFN2 and TUFM, which were shown to dampen IFN-I production while inducing autophagy (**Figure 2**). Indeed, MFN2 contributes to the supply of mitochondria membranes for the biogenesis of autophagosome (Hailey et al., 2010), and can down-regulate the production of IFN-I upon viral infection by interacting with IPS-1 (Yasukawa et al., 2009). Similarly, TUFM recruits the ATG5/ATG12 complex in order to induce autophagy, while it prevents RIG-1/IPS-1 signal transduction for IFN-I production *via* its interaction with NLRX1 (Lei et al., 2012). The antagonistic activities of TUFM and MFN2 on autophagy and IFN-I production were shown to benefit to virus replication (Yasukawa et al., 2009; Lei et al., 2012). As IRGM is associated to mitochondria and modulates autophagy induction upon virus infection, it would be important to evaluate its contribution to the ability of viruses to dampen IFN-I production; all the viruses described to date to target IRGM are known to inhibit IFN-I production. Thus, different mitochondrial proteins, including IRGM, might have dual functions upon virus infection, by inducing autophagy ultimately exploited by viruses, while restricting the innate antiviral response; the selective targeting of these proteins would offer an evident advantage for infectious viruses to

replicate within a cell. It remains however possible that cytosolic isoforms of IRGM contributes to autophagy induction upon viral infection.

IRGM AND AUTOPHAGY-MEDIATED VIRUS-DERIVED PEPTIDE MHC LOADING

It recently became clear that autophagy which is constitutively active in antigen-presenting cells (APCs), can regulate adaptive immune responses by promoting the access of antigens from intracellular pathogens to compartments that assemble peptide:MHC class II complexes for presentation to $CD4^+$ T cells (Munz, 2009). Among viruses targeting IRGM, it was observed that the response of HIV-1 gag-specific $CD4^+$ T cells to DC that process the virus was drastically reduced upon either pharmacological or genetic inhibition of autophagy, indicating a deficient capacity to process and present MHC class II-restricted HIV-1 determinants when autophagy is impaired (Blanchet et al., 2010). The negative regulation of autophagy in DCs by HIV-1 could thus help the virus evade $CD4^+$ T cell responses. As to presentation by MHC class I molecules, it is known that in mouse DCs, IRGM3/IGTP (another murine ortholog or IRGM) plays an important role in cross-presentation of phagocytosed protein antigens to conventional $CD8^+$ T cells without impacting antigen presentation to $CD4^+$ T cells (Bougneres et al., 2009).

irgm VARIANTS IN HUMAN PATHOLOGIES AND VIRAL INFECTIONS

Recent studies identified *irgm* variants as susceptibility genes for Crohn's disease (CD), tuberculosis (TB), gastric cancer and autoimmune systemic lupus erythematosus (SLE).

IRGM, CD, AND VIRAL INFECTION

Crohn's disease is a chronic inflammatory bowel disease resulting from an aberrant immune response toward the intestinal flora that leads to inflammation and tissue damages (Xavier et al., 2008). Genome-wide association studies identified polymorphisms in two autophagy-associated genes, *atg16L1* and *irgm*, that are linked to CD. CD-associated *irgm* polymorphisms, that influence or not the primary protein sequence, were both reported (Parkes et al., 2007; McCarroll et al., 2008; Moon et al., 2012). Interestingly, the gut mucosa of CD patients harbors an increased amount of the pathogenic Adherent-invasive *E. coli* (AIEC) and IRGM-dependent autophagy contributes to fight pathogenic AIEC (Lapaquette et al., 2012). Moreover, microRNA (miR)-196 binds strongly the *irgm* protective haplotype, whereas expression of the risk haplotype remains intact thus leading to overall deregulation of IRGM expression (Brest et al., 2011). miR-196 was found over-expressed in inflamed ileum and colon of patients, independently of the protective or risk *irgm* haplotype. As a result, IRGM was less expressed in individuals with the protective genotype. Furthermore, the transfection of HEK293T cells with miR-196 resulted in a decreased autophagy flux, indicating that miR-196 acts as a negative regulator of autophagy *via* IRGM upon AIEC infection. These studies suggested that the cornerstone of autophagy regulation by IRGM upon infection could be its fine tuned level of expression.

Interestingly, a viral infection-plus-susceptibility autophagy gene interaction could contribute to the onset of CD. Indeed, the hypomorphic expression of *atg16l1* develops a CD-like pathology in mice only upon infection with a viral strain of murine norovirus (Cadwell et al., 2010). This study pointed toward a genotype-specific viral trigger of a pathology very similar to CD. It would be interesting to investigate the role of viral infections in *irgm* variant expressing CD patients, for a possible contribution of virus/IRGM interactions in the onset or the development of CD.

IRGM IN TB, GASTRIC CANCER, AND SLE

As mentioned above, IRGM contributes to the control of *M. tuberculosis* in macrophages *via* autophagy. Interestingly, an *irgm* polymorphism protects from TB caused by Euro-American subgroups of *M. tuberculosis* (Intemann et al., 2009). It was proposed that when the polymorphism occurs, IRGM is more expressed resulting in enhanced autophagy and explaining a more efficient destruction of bacteria. Conversely, several different polymorphisms in the *irgm* gene have been found to result in an increased susceptibility to TB in Chinese and African American populations (Che et al., 2010; King et al., 2011). In African American populations one CD-related polymorphism was associated positively with TB suggesting a possible link between CD and an infectious etiology. *Irgm* polymorphism is possibly also a risk factor for gastric cancer (Burada et al., 2012). Although deregulation of autophagy is well established to be associated with cancer (White, 2012), a role for IRGM in these diseases has to be further determined. Similarly, a genetic-association study suggested that *irgm* variants are linked to SLE, an autoimmune disease (Zhou et al., 2011). A role of IRGM in these diseases remains to be fully depicted as well as a potential influence of viral infections on such role.

CONCLUSION

In an infected cell a virus has to counteract cell autonomous defense mechanisms while exploiting elementary cellular processes to replicate efficiently. By selectively targeting autophagy, viruses might accomplish both. As discussed here, IRGM could be a key protein for autophagy manipulation upon viral infection. The molecular organization involving IRGM in autophagy during viral infections requires further investigations. While interactions of IRGM with its protein partners were only observed in transfected cells for the time being, it would be important to visualize these interactions between endogenous proteins and during productive infections. It would also be crucial to understand why and how IRGM plays an antibacterial function, whereas it seems to act as a proviral factor. Furthermore, the role of IRGM could be cell type-specific and, as described for several autophagy-related proteins, IRGM might have non-autophagy-related functions upon infections. In regards of the link of numerous *irgm* variants with human pathologies, the comprehension of the role(s) of IRGM in autophagy-mediated immunity could be of crucial importance to fight infectious viruses and human pathologies.

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Autophagy and immunity – insights from human herpesviruses

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The herpesviruses are a family of double-stranded DNA viruses that infect a wide variety of organisms. Having co-evolved with their hosts over millennia, herpesviruses have developed a large repertoire of mechanisms to manipulate normal cellular processes for their own benefit. Consequently, studies on these viruses have made important contributions to our understanding of fundamental biological processes. Here we describe recent research on the human herpesviruses that has contributed to our understanding of, and interactions between, viruses, autophagy, and the immune system. The ability of autophagy to degrade proteins located within the nucleus, the site of herpesvirus latency and replication, is also considered.

Keywords: antigen, CD4, EBNA1, EBV, macroautophagy, nucleophagy, presentation, processing

INTRODUCTION

In mammalian cells three different autophagy pathways have been shown to deliver cytoplasmic proteins or organelles into the lumen of lysosomes for degradation. The first pathway, microautophagy, is a process in which the lysosomal membrane invaginates, capturing portions of cytoplasm. Endosomal microautophagy has also recently been described (Sahu et al., 2011). The second pathway, chaperone-mediated autophagy (CMA), involves the transport of proteins containing a specific motif into the lysosome via the concerted actions of chaperone proteins and the lysosomal membrane protein LAMP2a (Dice, 2007). The third pathway, macroautophagy (hereafter referred to simply as autophagy) is the best described of the three and can be divided into six sequential steps: initiation, nucleation, elongation, closure, maturation, and degradation. Each step is a highly regulated process involving the coordinated action of numerous autophagy-related (ATG) and other proteins. Initiation of autophagy occurs upon activation of the ULK1 and UKL2 complexes following release of their inhibition by the mTOR complex. Nucleation defines the site for the autophagic membrane to begin to form in the cytoplasm and depends on the oligomerization of Beclin-1 that acts as a platform for recruitment of multiple other autophagy-related proteins. Elongation of the autophagic membrane around a portion of cytoplasm involves the recruitment of more proteins and lipidation of the microtubule-associated protein light chain 3 (LC3) by the E2-like enzyme ATG3. Closure of the membrane completes the formation of the double membrane vacuole, called an autophagosome, containing a portion of cytoplasm within its lumen. The autophagosome then undergoes maturation by fusing with lysosomes to form an autophagolysosome. This process is again mediated by Beclin-1 as well as LC3 on the autophagosome. Following maturation, the captured cytoplasmic contents are degraded by lysosomal proteases for reuse by the cell.

Autophagy typically occurs continuously at a low level but is increased in response to cellular stresses such as nutrient deprivation in order to provide a supply of nutrients to aid cell survival. In addition to this survival function, autophagy plays other important roles that are the focus of this review.

Viruses are obligate intracellular parasites and therefore totally dependent upon a host cell for replication. This dependence has led viruses to develop ways of harnessing normal cellular processes for their own purposes. A diverse range of viruses has now been shown to manipulate autophagy. Some viruses stimulate autophagy to aid their replication. Infection with poliovirus induces the formation of membranes resembling double-membrane autophagosomes that contribute to virus production (Suhy et al., 2000; Jackson et al., 2005). Rotavirus also stimulates the formation of autophagosome-like structures with the virus replicating in close proximity to these vesicles (Berkova et al., 2006). In contrast, autophagy poses a threat for other viruses and a range of viral countermeasures have been identified (Jordan and Randall, 2011). Autophagy inhibition has been well characterized for members of the herpesvirus family. In humans, eight herpesviruses have been identified that are divided into three sub-families (alpha-, beta-, and gammaherpesvirinae). These divisions were historically based on cellular tropism but are now based on viral genome organization (**Table 1**). Viruses across all sub-families are able to manipulate autophagy, employing a range of different mechanisms. These mechanisms and the effects on both viral replication and the host antiviral immune response are described below.

HSV-1 INHIBITS AUTOPHAGY TO PREVENT VIRAL DEGRADATION

Autophagy has been shown to play important roles in many pathways of the innate immune system. These roles include modulating pathogen recognition receptors (Lee et al., 2007; Takeshita et al., 2008), controlling the production of key innate cytokines (Lee et al., 2007; Saitoh et al., 2008), and controlling phagocytosis (Sanjuan et al., 2007; Zang et al., 2012). Autophagy can directly contribute to innate immune control of viruses within infected cells, a role identified in the first study on Beclin-1

Table 1 | Classification of the eight human herpesviruses.

Formal taxonomic name	Alternative common name	Viral sub-family
Human herpesvirus 1	Herpes simplex virus 1	*Alphaherpesvirinae*
Human herpesvirus 2	Herpes simplex virus 2	*Alphaherpesvirinae*
Human herpesvirus 3	Varicella-zoster virus	*Alphaherpesvirinae*
Human herpesvirus 4	Epstein–Barr virus	*Gammaherpesvirinae*
Human herpesvirus 5	Human cytomegalovirus	*Betaherpesvirinae*
Human herpesvirus 6	HHV-6 variant A or B	*Betaherpesvirinae*
Human herpesvirus 7	HHV-7	*Betaherpesvirinae*
Human herpesvirus 8	Kaposi's sarcoma-associated herpesvirus	*Gammaherpesvirinae*

(Liang et al., 1998). Overexpression of Beclin-1 in neurons protected mice against fatal infection with the neurotropic alphavirus Sindbis (Note: Sindbis is a member of the *Togaviridae* family and not a herpesvirus; Liang et al., 1998). This protection resulted from the degradation of the Sindbis capsid protein by autophagy (Orvedahl et al., 2010). A range of intracellular bacteria and viruses are now known to be degraded by autophagy, a process termed xenophagy (Rich et al., 2003; Gutierrez et al., 2004; Nakagawa et al., 2004; Talloczy et al., 2006). The alphaherpesvirus herpes simplex virus type 1 (HSV-1) provided the first evidence of a pathogen inhibiting autophagy to escape xenophagy. Within virus-infected cells activation of cellular dsRNA-dependent protein kinase R (PKR) inhibits host and viral protein translation and viral replication. HSV-1 encodes a protein, ICP34.5, which inhibits this PKR-dependent antiviral activity (Chou et al., 1990). Talloczy et al. (2002) demonstrated that ICP34.5 also prevents PKR-dependent activation of autophagy in HSV-1-infected cells. Subsequent research revealed that ICP34.5 binds the essential autophagy gene Beclin-1 preventing the formation of autophagosomes (Orvedahl et al., 2007). Deletion of the Beclin-1 binding domain from ICP34.5 did not alter viral growth *in vitro* but could decrease viral growth and neurovirulence in a mouse model. Note that this decrease in neurovirulence could be due to a number of different factors. For example, survival of infected cells might be increased by the presence of a functional autophagy pathway. However, two observations support a contribution from xenophagy in reducing HSV-1 neurovirulence. First, HSV-1 virions can be observed in vesicles that morphologically resemble autophagosomes (Smith and de Harven, 1978; Talloczy et al., 2006). Second, biochemical studies show that inhibiting autophagy decreases the rate of degradation of HSV-1 proteins (Talloczy et al., 2006).

HERPESVIRUSES DIFFER IN THEIR ABILITY TO INHIBIT AUTOPHAGY

Other herpesviruses have also been shown to possess autophagy inhibitory mechanisms. In cells infected with the betaherpesvirus human cytomegalovirus (HCMV) autophagy is initially activated before being inhibited 24 h post infection (Chaumorcel et al., 2008; McFarlane et al., 2011). HCMV-mediated autophagy inhibition requires *de novo* viral protein synthesis and is mediated by the viral protein TRS1 (Chaumorcel et al., 2012). Interestingly, TRS1 appears to be a functional homolog of the HSV protein ICP34.5, interacting with both PKR and Beclin-1. Like ICP34.5 it is the interaction between TRS1 and Beclin-1 that inhibits autophagy.

The human gammaherpesvirus Kaposi's sarcoma-associated herpesvirus (KSHV) also inhibits autophagy by targeting Beclin-1 but uses a different mechanism to do so. The cellular Bcl-2 protein inhibits apoptosis but can also inhibit autophagy by binding Beclin-1(Pattingre et al., 2005). KSHV encodes a viral homolog of Bcl-2, called orf16, that can similarly bind Beclin-1 and inhibit autophagy (Pattingre et al., 2005). It is not currently known whether the two viral Bcl-2 homologs (BHRF1 and BALF1) encoded by the other human gammaherpesvirus Epstein–Barr virus (EBV) can similarly inhibit autophagy. However, it seems likely that one or both of these viral proteins will do so given that the viral Bcl-2 homolog encoded by another gammaherpesvirus, in this case murine MHV68, inhibits autophagy (Pattingre et al., 2005). Strong evidence for the importance of autophagy inhibition by these viral Bcl-2 homologs is provided by the MHV68 system. Mutating the Beclin binding domain of the MHV68 Bcl-2 homolog did not effect the establishment of viral latency but impaired the ability of the virus to maintain chronic infection in mice (E et al., 2009).

Interestingly, the protein interacting with carboxy terminal 1 (PICT-1), a putative human tumor suppressor protein of unknown function, has recently been shown to bind KSHV Bcl-2 (Kalt et al., 2011). Ectopic expression of PICT-1, which contains two nuclear localization sequences (NLS) and a nucleolar localization signal, caused KSHV Bcl-2 to relocalize from its normal mitochondrial localization into nucleoli thereby inhibiting its anti-apoptotic activity. The ability of PICT-1 to counteract KSHV Bcl-2-mediated autophagy inhibition was not examined in the paper. However, since Beclin-1 is almost entirely located in the cytoplasm and acts in this compartment (Liang et al., 2001), sequestration in the nucleus may well interfere with the ability of KSHV Bcl-2 to inhibit autophagy. If this proves to be the case then PICT-1 may represent the first example of a cellular countermeasure against viral inhibition of autophagy.

All of the herpesvirus autophagy inhibitory proteins described thus far act by interacting with Beclin-1. KSHV has developed an additional inhibitory mechanism, targeting a separate component of the cellular autophagy machinery. The viral FLICE like inhibitor protein (v-FLIP), a homolog of a cellular (c-FLIP), can inhibit both apoptosis and autophagy (Lee et al., 2009). v-FLIP binds Atg3 preventing it from processing LC3 thereby inhibiting autophagosome formation. Overexpression of either c-FLIP or v-FLIP inhibited cell death induced by rapamycin stimulation of autophagy (Lee et al., 2009).

In contrast to the herpesviruses described above varicella-zoster virus (VZV) is unable to inhibit autophagy. Indeed, autophagy is rapidly activated following VZV infection with many autophagosomes present in infected cells in vitro and in infected cells within skin vesicles (Takahashi et al., 2009; Carpenter et al., 2011). Unlike the other neurotropic alpha herpesviruses HSV-1 and HSV-2, VZV lacks an ICP34.5 ortholog; VZV also lacks a viral Bcl-2

homolog. The high level of autophagy present in VZV-infected cells may be the cause of the low titers of this virus obtained *in vitro*. It is interesting to note that an inability to inhibit autophagy has not proved detrimental to VZV; almost everyone is infected with the virus and in that regard it is a highly successful pathogen. Indeed, it has been postulated that VZV-induced autophagy may be beneficial to the virus, prolonging the survival of infected cells by reducing endoplasmic reticulum stress (Carpenter et al., 2011).

AUTOPHAGY AND THE ADAPTIVE IMMUNE RESPONSE TO HERPESVIRUSES

Herpesviruses have evolved an arsenal of immune evasion mechanisms in order to establish life-long latent infection of immunocompetent hosts (Horst et al., 2011). As well as playing a key role in innate anti-viral immunity, autophagy is also important in adaptive immunity and autophagy manipulation by herpesviruses affects this arm of the immune response. In agreement with the earlier work suggesting xenophagy could reduce HSV-1 neurovirulence, strains of HSV-1 incapable of inhibiting autophagy (due to deletion of the Beclin-1 binding domain of ICP34.5) caused less disease following corneal infection of mice (Leib et al., 2009). However, no disease decrease was observed for *Rag1* knockout mice that lack B and T cells suggesting that the protective effect was mediated by adaptive, rather than innate, immunity. In accordance with this result mice infected with the mutant virus had higher virus-specific CD4+ T cell responses compared to animals infected with wild-type virus. While autophagy can reduce HSV-1-mediated disease the relative contributions made by the innate and adaptive immune responses are currently unclear and may differ depending upon the model system being used.

Viral inhibition of autophagy can potentially limit adaptive immunity by acting at two key stages. First, autophagy within virus-infected cells may be important for the generation of cross-primed CD8+ T cell responses by dendritic cells (DCs). Inhibiting autophagy in influenza-infected cells reduced their ability to stimulate influenza-specific CD8+ T cells in mice (Uhl et al., 2009). Similar results have also been observed using a tumor antigen model (Li et al., 2008). Second, autophagy provides a route for antigens to enter antigen-processing pathways within pathogen-infected cells, allowing them to be processed and presented to antigen-specific T cells. Since the first landmark observation that autophagy can generate an MHC II epitope from a self antigen (Brazil et al., 1997) several pathogen-encoded antigens have been shown to be processed this way. These antigens include the bacterial protein neomycin phosphotransferase (NeoR; Nimmerjahn et al., 2003), the mycobacterial antigen Ag85B (Jagannath et al., 2009), and EBV nuclear antigen 1 (EBNA1), the first example of a viral protein processed by autophagy for recognition by CD4+ T cells (Paludan et al., 2005). Autophagy can also generate MHC class I epitopes, the first example being arising from studies on HSV-1 infection of murine macrophages (English et al., 2009). As expected, autophagy was inhibited in the HSV-1-infected cells by ICP34.5. These infected cells also presented an MHC class I restricted viral epitope generated using the classical proteasome-dependent pathway. However, at later stages of infection higher levels of the epitope were generated and an additional processing pathway was involved that was sensitive to bafilomycin A and 3-methyladenine, inhibitors of the vacuolar processing pathway and autophagy respectively. In addition to normal double membrane autophagosomes, the HSV-infected cells also contained unusual four layered membrane structures originating from the nucleus that, based on the presence of LC3, appear to be autophagosomes. These unusual nuclear-derived autophagosomes were not observed in cells treated with rapamycin to stimulate autophagy nor cells infected with recombinant HSV-1 lacking ICP34.5. They may therefore represent a cellular response to a combination of HSV-1 infection and ICP34.5-mediated blockade of conventional autophagy. A second interesting observation from this work was that presentation of the viral MHC class I epitope by cells infected with HSV-1 lacking ICP34.5 was also sensitive to bafilomycin A and therefore involved the vacuolar pathway. As these cells lacked the four layered autophagosomes this result may indicate that classical double membrane autophagosomes also contribute to the generation of MHC class I epitopes.

NUCLEAR SHELTER – ANOTHER STRATEGY TO EVADE AUTOPHAGY

Upon establishing latent infection, the herpesvirus genome persists as an episome within the nucleus. To ensure the viral genome is replicated and segregated into daughter cells the gammaherpesviruses EBV and KSHV each express a genome maintenance protein (EBNA1 and latency-associated nuclear antigen (LANA) respectively). Deletion of EBNA1 decreased EBV growth transformation 10,000-fold, clearly demonstrating the vital role of such proteins (Humme et al., 2003). Although loss of EBNA1 can be compensated by integration of the EBV genome into cellular DNA, this is a very rare event, again highlighting the importance of a correctly functioning genome maintenance protein for the gammaherpesviruses.

The dependence on the continuous presence of genome maintenance proteins to ensure life-long viral persistence represents a potential Achiles' heel for these viruses. Logically, one would therefore expect genome maintenance proteins to be well protected from immunological surveillance. This is indeed the case. EBNA1 contains a large internal glycine/alanine repeat (GAr) domain that decreases EBNA1 processing and presentation by the HLA class I processing pathway via several complementary mechanisms (Levitskaya et al., 1997; Tellam et al., 2008; Apcher et al., 2009). The KSHV genome maintenance protein LANA also contains an internal repeat domain, in this case consisting of acidic residues, that similarly reduces this protein's synthesis (Kwun et al., 2007) and degradation (Zaldumbide et al., 2007). In contrast to short-lived proteins, which are generally thought to be degraded by the proteasome, long-lived proteins are thought to be degraded by autophagy; measuring the degradation of long-lived proteins is a classical method for measuring the autophagy activity of cells (Klionsky et al., 2008). The genome maintenance proteins might therefore be potential substrates for autophagic degradation. This is indeed the case for EBNA1, which was the first viral protein identified as being degraded by autophagy (Paludan et al., 2005). Furthermore, two different EBNA1-specific CD4+ T cell clones were able to recognize EBV-transformed B lymphoblastoid cell

lines (LCLs) in an autophagy dependant manner, demonstrating that such degradation could expose EBNA1 to the immune system. Remarkably, of all the EBV latent cycle proteins EBNA1 contains the greatest number of MHC class II epitopes and most individuals possess strong CD4+ T cell immunity against this protein (Munz et al., 2000; Leen et al., 2001; Hislop et al., 2007). Taken together, these observations appear paradoxical: how can EBV persist in MHC class II positive B cells despite strong CD4+ T cell responses specific for EBNA1, the protein critical for viral persistence?

In contrast to the observations described above, other research groups using CD4+ T cell clones specific for other EBNA1 epitopes found these cells were unable to recognize LCLs (Khanna et al., 1997; Mautner et al., 2004). In our laboratory, CD4+ T cell clones specific for two epitopes could recognize LCLs expressing natural levels of the EBNA1 protein while clones specific for a third epitope could not (Leung et al., 2010). The discordant results reported by different laboratories may therefore reflect real differences in the presentation of different epitopes from this protein. Although cell surface levels were low, control experiments confirmed that the two epitopes presented by LCLs were indeed endogenously accessing the MHC II pathway. Interestingly, only one epitope was autophagy-dependent, the other being processed by another intracellular route that is currently under investigation. Different epitopes from the same source protein being processed by different routes has been observed previously for influenza hemagglutinin (Sinnathamby and Eisenlohr, 2003). When EBNA1 was over-expressed as a nuclear protein, surface levels of the two naturally presented epitopes increased slightly, but the processing routes remained the same. However, expressing EBNA1 as a cytoplasmic protein, through mutation of its NLS, resulted in all three epitopes being processed by autophagy and presented at high levels by cells. This striking result was a direct result of EBNA1's new cytoplasmic localization since addition of a heterologous NLS reversed the effect (Leung et al., 2010). While EBNA1 needs to be localized to the nucleus for its correct functioning in the virus life cycle, nuclear localization clearly brings an additional benefit, namely limiting EBNA1's endogenous processing by macroautophagy and presentation to CD4+ T cells. Potentially "nuclear shelter" (Leung and Taylor, 2010) could also help reduce CD8+ T cell presentation of EBNA1. Reducing protein turnover may decrease the need for newly synthesized replacement polypeptides that are the dominant source of MHC class 1 peptides from EBNA1 (Lee et al., 2004; Tellam et al., 2004; Voo et al., 2004; Mackay et al., 2009).

Are other nuclear-localized proteins sheltered from macroautophagy like EBNA1? Studies using non-viral proteins suggest this may be the case. The huntingtin and ataxin-1 proteins each contain a large poly-glutamine repeat that can cause these proteins to aggregate in cells. Aggregates of huntingtin or ataxin-1 were degraded by autophagy in the cytoplasm but not in the nucleus (Iwata et al., 2005). A mutation in the NLS of a tumor antigen causing it to relocalize out of the nucleus enhanced its CD4+ T cell recognition (Wang et al., 1999). Although it is not known if this epitope was processed by autophagy, the observation is consistent with the EBNA1 data. In contrast to these examples, presentation of an autophagy-dependant epitope from the NeoR protein was not reduced by the addition of a heterologous NLS (Riedel et al., 2008). The reason for this different result is unclear, but one possibility is that it might reflect different rates of nuclear import. Previous work has shown that although the addition of a minimal NLS allows nuclear import, this occurs with slow kinetics unless additional flanking sequences are included (Rihs and Peters, 1989). The import rate of the NLS-containing NeoR protein has not been measured, however EBNA1 is rapidly imported following its translation suggesting that levels of cytoplasmic EBNA1 are likely to be very low (Kitamura et al., 2006). Finally, it is important to note that other endogenous MHC class II processing pathways exist. Some of these pathways involve the proteasome (Delmas et al., 2005; Tewari et al., 2005), others CMA (Zhou et al., 2005) while others have not been mechanistically defined. Antigen localization can affect processing by one of these alternative pathways. The CMV nuclear protein IE1 contains an MHC class II epitope that is not processed by autophagy but by a proteasome-dependent mechanism. Although relocalization of IE1 into the cytoplasm did not alter the degree of presentation of this epitope, the mechanism of presentation was altered (Delmas et al., 2005).

HOW DO NUCLEAR-LOCALIZED ANTIGENS ENTER THE AUTOPHAGY PATHWAY?

The fact that some EBNA1 MHC class II epitopes are autophagy-dependent raises the interesting question of where in the cell does the nuclear-localized protein access the autophagic pathway? Can autophagy occur in the nucleus? The best-studied example of "nucleophagy" is Piecemeal Microautophagy of the Nucleus (PMN), a form of microautophagy that occurs in yeast (Roberts et al., 2003). During PMN, small pieces of the nucleus are pinched off into the yeast degradative vacuole; PMN is therefore a relatively gentle and selective process that can be tolerated by the cell. A much more dramatic example of nucleophagy has been observed in the filamentous fungus *Aspergillus oryzae*. Here, entire nuclei are degraded after being surrounded by large (1–2 μm diameter) autophagosomes (Shoji et al., 2010). While such drastic loss of genetic material would be lethal for any mononuclear cell, it can be tolerated within the multi-nucleated hyphae of this organism. Since autophagy is important for the growth of this fungus under starvation conditions, nucleophagy may be acting to recycle the constituents of redundant nuclei for use at growing hyphal tips.

Do mammalian cells undergo nucleophagy? The extent to which PMN takes place in other species is unknown, but homologs of the yeast genes essential for PMN, *Vac8*, and *Nvj1*, have not been identified in animal cells (Mijaljica et al., 2010). Granting non-specific macroautophagy access to the nucleus could result in irreversible loss of genetic material with catastrophic consequences for the cell. Indeed, to guard against such loss mammalian cells inhibit autophagy during mitosis, a time when chromosomes enter the cytoplasm following breakdown of the nuclear membrane (Eskelinen et al., 2002). However, nucleophagy has been observed in mammalian cells in select circumstances. In mouse macrophages HSV-1 infection triggers an unusual form of autophagy involving four-layer autophagosomes emerging from the nuclear envelope (English et al., 2009). It is not known whether these unusual autophagosomes can form in human cells

infected with HSV-1, or if other herpesviruses can trigger their formation.

Other examples of nucleophagy occur in the context of genetic mutations. In Bloom's syndrome, a rare cancer-predisposing disorder caused by mutation of the *BLM* gene, micronuclei bud from the nucleus into the cytoplasm (Yankiwski et al., 2000). The extent to which these cytoplasmic micronuclei are substrates of autophagy and the ability of normal cells to release micronuclei are currently not known. Finally, cells with fragile nuclei, caused by mutations in genes encoding nuclear lamina proteins, have been observed to contain perinuclear autophagosomes/autolysosomes-containing nuclear components (Park et al., 2009). Intriguingly, the authors report similar autophagosomes/autolysosomes in wild-type cells, although at a much lower frequency.

Autophagy is now known to exhibit greater selectivity for its substrates than once thought. Several autophagy adaptor proteins have now been shown to transport a range of substrates to autophagosomes for degradation. These substrates include aggregated or ubiquitinated proteins, damaged mitochondria, and intracellular bacteria (reviewed in Johansen and Lamark, 2011). Selective autophagy could be another route for nuclear proteins to enter cytoplasmic autophagosomes, one that could operate without causing genetic loss. One of the autophagy adaptor proteins, p62 (also called SQSTM1), has recently been shown to deliver capsids of Sindbis virus to autophagosomes (Orvedahl et al., 2010). With respect to the selective autophagy of nuclear proteins, p62 is of particular interest since it contains both nuclear localization and nuclear export sequences and consequently shuttles rapidly between the nucleus and cytoplasm (Pankiv et al., 2010). However, it is not yet known whether p62 is involved in the selective degradation of nuclear proteins by autophagy.

ACTIVATION OF AUTOPHAGY BY HERPESVIRUSES

Thus far we have considered autophagy as a threat to herpesviruses. However, the relationship between herpesviruses and autophagy is more complex and several examples exist of herpesviruses activating autophagy at different stages in their life-cycle. Autophagy activation can occur during lytic infection. As described earlier autophagy is rapidly activated in VZV-infected cells because the virus apparently lacks mechanisms to inhibit this pathway. However, autophagy activation has also been reported for viruses that do possess such mechanisms, suggesting that autophagy may be beneficial for viruses at particular stages of their replicative cycle. Although HSV-1 encodes the autophagy inhibitor ICP34.5 this virus has been reported to rapidly activate autophagy in human fetal foreskin fibroblasts (McFarlane et al., 2011). Autophagy induction by HSV-1 has also been reported in murine macrophages, although in this case classical macroautophagy still appeared to be inhibited by virus-encoded ICP34.5 and a morphologically distinct type of macroautophagy was observed (English et al., 2009). CMV infection also rapidly activates autophagy that is subsequently inhibited by the CMV-encoded protein TRS1 (McFarlane et al., 2011; Chaumorcel et al., 2012). KSHV also appears to activate autophagy during lytic reactivation. The KSHV protein RTA is able to activate autophagy, inhibiting this process reduced RTA-mediated lytic gene expression and KSHV replication suggesting autophagy plays a key role in the replication of KSHV during lytic cycle (Wen et al., 2010). Autophagy activation can also occur during herpesvirus latency. The EBV-encoded latent membrane protein 1 (LMP1) induces autophagy in EBV transformed B cells (Lee and Sugden, 2008). This induction does not result from LMP1's well-documented activation of cellular signaling pathways but from the presence of three of the molecule's transmembrane spanning domains. Since LMP1 is also degraded by autophagy these domains, which had previously not been assigned a functional role, may provide LMP1 with a feedback mechanism that regulates cellular levels of the protein.

CONCLUSION

During their evolution, the herpesviruses have developed a range of mechanisms to interact with the host autophagy machinery. Under some circumstances these viruses need to evade host immunity and this is achieved in several ways. One way is to target key cellular proteins required for autophagy. In this regard, several viruses have independently evolved mechanisms that target Beclin-1, reflecting the key role this protein plays in autophagy. The targeting of another autophagy protein, ATG3, by KSHV raises the possibility that a range of inhibitory mechanisms exist, waiting to be discovered. Another way to evade immunity may be through localization of genome maintenance proteins into the nucleus, thereby minimizing their access to the autophagy machinery. In this review, we have focused on macroautophagy, which is the best characterized of the three currently defined autophagy pathways. The other pathways are beginning to be elucidated and one of them, CMA, has been shown to generate an MHC class II restricted epitope from a non-viral antigen (Zhou et al., 2005). However, the contribution of these other pathways to viral immunity, and whether they too are inhibited by herpesviruses, is currently unknown. Finally, a complex balance exists between herpesviruses and autophagy within the host cell and under some circumstances herpesviruses activate autophagy, presumably for their benefit. Two recent observations – PICT-1-mediated relocalization of KSHV Bcl-2 away from its site of action, and nuclear-derived autophagosomes in mouse macrophages infected with HSV-1 – raise the possibility that cells possess mechanisms to counteract viral manipulation of autophagy. Understanding the balance between autophagy inhibition and activation within virally infected cells, and the consequences for anti-viral immunity, presents a challenge and an opportunity for future research.

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Roles of autophagy in elimination of intracellular bacterial pathogens

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As a fundamental intracellular catabolic process, autophagy is important and required for the elimination of protein aggregates and damaged cytosolic organelles during a variety of stress conditions. Autophagy is now being recognized as an essential component of innate immunity; i.e., the recognition, selective targeting, and elimination of microbes. Because of its crucial roles in the innate immune system, therapeutic targeting of bacteria by means of autophagy activation may prove a useful strategy to combat intracellular infections. However, important questions remain, including which molecules are critical in bacterial targeting by autophagy, and which mechanisms are involved in autophagic clearance of intracellular microbes. In this review, we discuss the roles of antibacterial autophagy in intracellular bacterial infections (*Mycobacteria*, *Salmonella*, *Shigella*, *Listeria*, and *Legionella*) and present recent evidence in support of molecular mechanisms driving autophagy to target bacteria and eliminate invading pathogens.

Keywords: autophagic receptors, autophagy, innate immunity, *Listeria*, *Mycobacteria*, *Salmonella*, *Shigella*, xenophagy

INTRODUCTION

Autophagy is a fundamental protein degradation pathway essential for cellular homeostasis in response to various environmental and cellular stresses. The autophagy pathway is clearly involved in multiple aspects of innate and adaptive immunity (reviewed by Deretic and Levine, 2009; Virgin and Levine, 2009; Levine et al., 2011). During infection, a specific role for autophagy has been shown in the capture and degradation of intracellular bacteria and viruses, known as "xenophagy" (Levine, 2005; Deretic, 2011). In recent years, evidence of the specific roles of autophagy in selective targeting of bacteria through autophagic adaptors has accumulated. The main autophagic adaptors or receptors include; sequestosome 1 (SQSTM1/p62), nuclear dot protein 52 kDa (NDP52), optineurin (OPTN), and neighbor of BRCA1 gene 1 (NBR1) (Kirkin et al., 2009; Thurston et al., 2009; Mostowy et al., 2011; Wild et al., 2011; von Muhlinen et al., 2012; Korac et al., 2013) (**Figure 1**). These receptors function as cargo adaptors for the connection of substrates to the autophagy-related gene 8/microtubule-associated protein 1 light chain 3 (ATG8/LC3) family of proteins (Shaid et al., 2013).

Antibacterial autophagy plays an important role in controlling bacterial replication and promoting innate immunity in host cells. Increasing evidence has revealed that intracellular bacteria in vacuoles can be targeted by autophagy activation for lysosomal fusion and degradation (Levine, 2005; Deretic, 2011). Additionally, access to the cytosol for intracellular bacteria, caused by damage to the vacuoles, enables autophagy targeting of bacteria for eventual delivery to lysosomes (Ogawa et al., 2009; Collins and Brown, 2010; Fujita and Yoshimori, 2011). Several intracellular bacteria, including *Salmonella*, *Listeria*, *Legionella*, and *Mycobacteria*, can translocate their virulent components into the host cell cytoplasm. Moreover, these intracellular bacteria often induce the formation of ubiquitinated protein aggregates, which are recognized by cargo adaptors, and are ultimately destroyed by autophagy (Ogawa et al., 2009; Collins and Brown, 2010; Fujita and Yoshimori, 2011). More recent work has revealed the structural characteristics of the conserved interactions between cargo adaptors and the ATG8/LC3 family of proteins (Shaid et al., 2013). However, LC3 is not always necessary for recruitment of the autophagic membrane structure, and mechanisms for LC3-independent targeting remain to be explored (Noda et al., 2012).

In this review, we summarize recent data describing how autophagy and cargo receptors target important human pathogens. We focus on *Mycobacteria*, *Salmonella*, *Shigella*, *Listeria*, and *Legionella*, and the autophagy-mediated elimination of these intracellular bacteria.

ANTIBACTERIAL AUTOPHAGY IN MYCOBACTERIAL INFECTION

Mycobacterium tuberculosis (Mtb) is a successful human pathogen that survives in a phagosomal environment in mononuclear phagocytes after invasion by means of inhalation (Huynh et al., 2011; Harriff et al., 2012). Phagosomal compartments containing Mtb are known to evade fusion with lysosomes, thus arresting phagosomal maturation during mycobacterial infection, while nutrient delivery continues, enabling survival and replication of the bacteria (Vergne et al., 2004; Philips, 2008). Numerous bacterial proteins and lipid effectors are known to be involved in

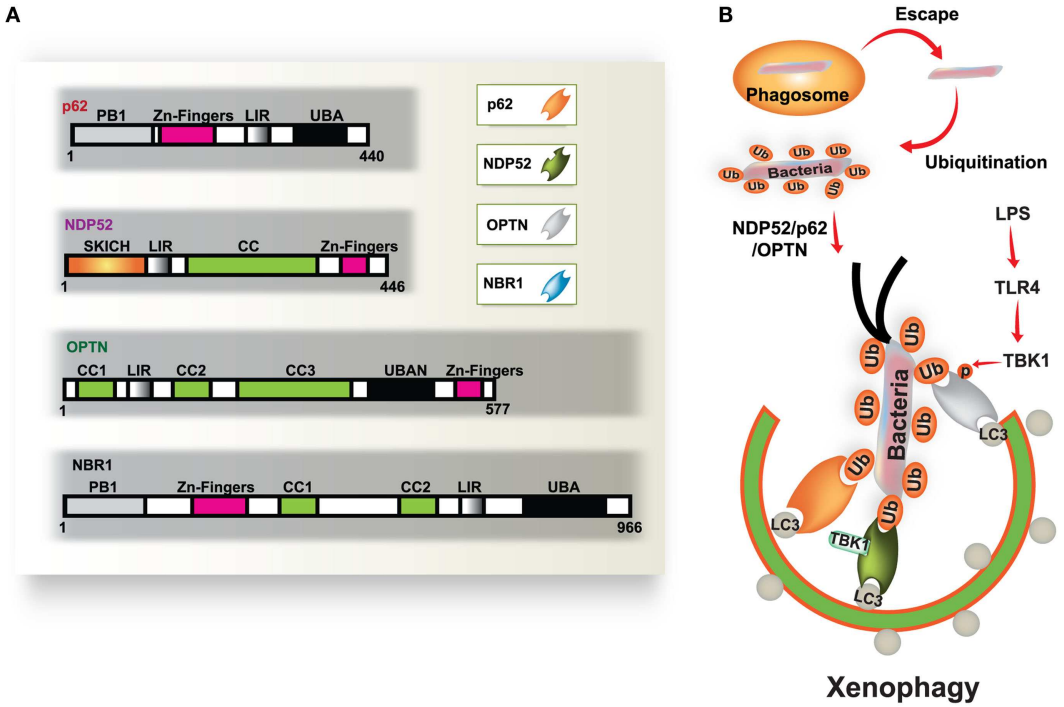

FIGURE 1 | Diverse cargo receptors are involved in the activation of selective autophagy. **(A)** Schematic model of the autophagic cargo receptors p62, NDP52, optineurin (OPTN), and NBR1. These receptors interact with both ubiquitin on substrates and LC3 on the phagophore, which results in the activation of autophagy. **(B)** Xenophagy is induced by ubiquitinated substrates derived from various bacteria. Many intracellular bacteria, such as *Salmonella*, are sequestered and replicate within *Salmonella*-containing vacuoles (SCVs), but some bacteria that escape from SCVs are recognized and ubiquitinated for recruitment to p62, NDP52, and OPTN, which results in their transportation to the phagophore. NDP52 interacts with LC3C through its CLIR domain, inducing antibacterial autophagy. TBK-1 activated by TLR4 induces phosphorylation of OPTN at Ser177, which leads to enhanced binding affinity for LC3. Moreover, TBK-1 is involved in the activation of NDP52-mediated autophagy (right). CC, coiled coil domain; PB1, Phox and Bem1p domain; UBD, ubiquitin binding domain.

delaying the fusion of Mtb phagosomes with lysosomes, and in cytokine-dependent changes in phagosomal protein composition (Philips, 2008; Steinhauser et al., 2013). Despite the ability of Mtb to interfere with phagosomal maturation, an accumulation of evidence [including immunogold electron microscopy (EM) data] which shows that Mtb, but not *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) can accumulate in the cytosol (van der Wel et al., 2007). Cytosolic translocation of Mtb depends on the 6-kDa early secretory antigenic target of Mtb (ESAT-6) Secretion System (ESX)-1 type VII secretion system, encoded in the region of difference 1 (RD1) of the Mtb genome, which has not been found in BCG or in heat-killed *Mycobacteria* (van der Wel et al., 2007) (**Figure 2A**).

As virulent Mtb strains can resist and inhibit autophagosome formation and its fusion with lysosomes (Deretic et al., 2006; Vergne et al., 2006; Deretic, 2008), divergent exogenous stimuli have been proposed to induce antibacterial autophagy targeting Mtb to inhibit its intracellular replication through enhancement of Mtb phagosomal maturation (summarized in **Figure 2B**). Autophagy activation via nutrient starvation, interferon (IFN)-γ, Toll-like receptor (TLR) stimuli, or by vitamin D treatment, has promoted phagosomal acidification and inhibited the survival of intracellular Mtb (reviewed by Deretic et al., 2006; Basu et al., 2012). In IFN-γ-induced mycobacterial xenophagy, LRG-47 (Irgm1; LPS-stimulated RAW 264.7 macrophage protein 47), a downstream effector of IFN-γ, plays an essential role in induction of autophagy and generation of autolysosomal organelles to inhibit intracellular mycobacterial replication (Singh et al., 2006). A recent study showed that bactericidal antibiotics activated the antibacterial autophagy process and contributed to successful antimicrobial responses during treatment for Mtb infection (Kim et al., 2012). This strongly implies that autophagy activation can overcome the Mtb-induced phagosomal maturation blocking process, and that it enhances host defense against Mtb. Several important questions remain to be answered, such as how to destroy Mtb in lysosomal compartments. Previous findings indicate that induction of autophagy in Mtb-infected macrophages promotes the delivery of ubiquitin conjugates to the lysosome, showing that at least one mechanism involving the generation of ubiquitin-derived peptides can enhance the bactericidal capacity of the lysosomal fraction (Alonso et al., 2007; Purdy and Russell, 2007).

The mechanisms by which Mtb phagosomes recruit autophagic machinery are also not fully understood. Recent studies have revealed that extracellular Mtb-DNA released from Mtb can be recognized by the stimulator of IFN genes (STING)-dependent

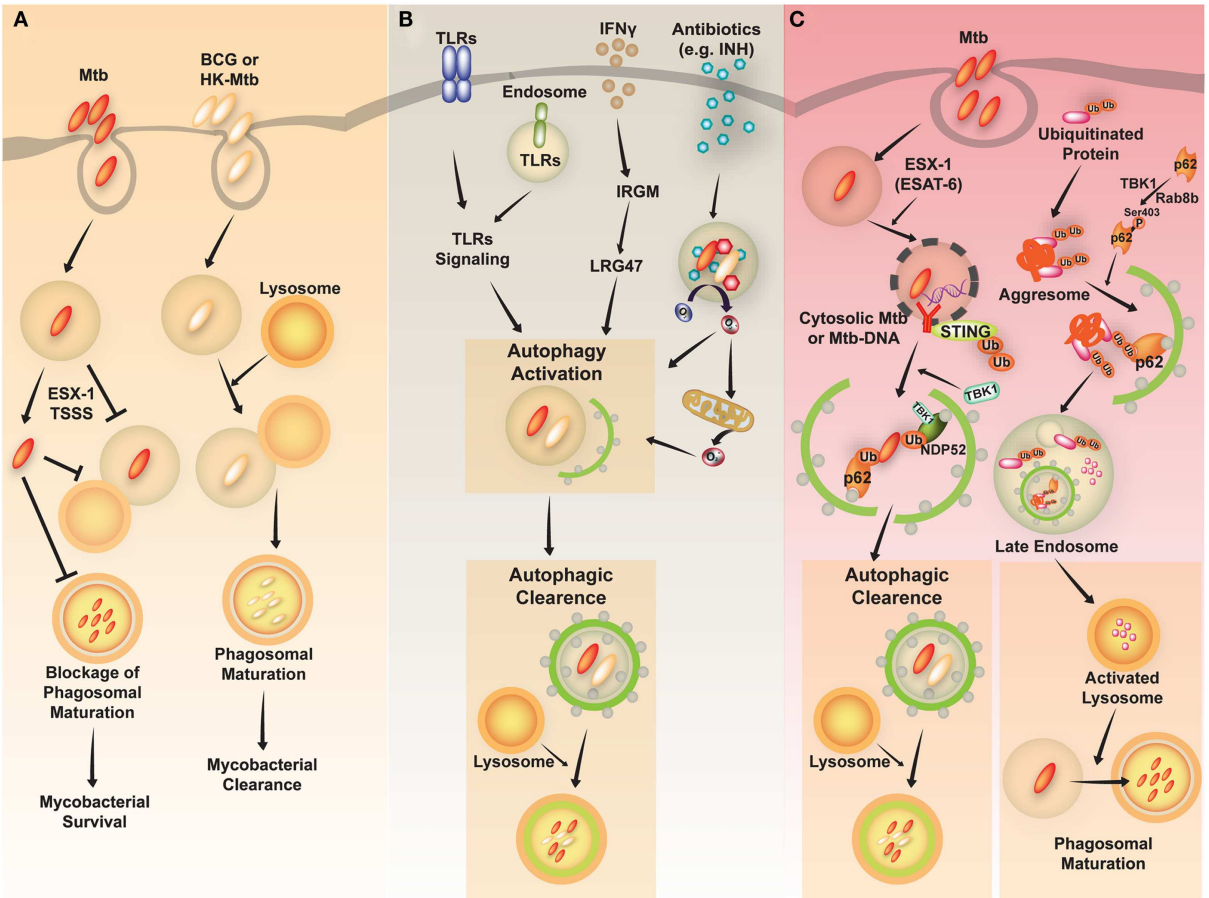

FIGURE 2 | A schematic diagram of *Mycobacterium tuberculosis* (Mtb) infection and clearance via the autophagic pathway. **(A)** Mtb, but not *M. bovis* BCG, has diverse strategies for evading host immune system. Phagosomes containing Mtb do not fuse with lysosomes and mature into the phagolysosomes by preventing phagolysosome biogenesis. The restricted fusion of phagosomes with lysosome is attributable to limited entrance of lysosomal hydrolases to Mtb, preventing acidification of phagosomes. Mtb, but not *M. bovis* BCG or heat-killed (HK)-*Mycobacteria*, can evade to the cytosol depending on the ESX-1 Type VII secretion system. **(B)** Diverse stimuli including toll-like receptors (TLRs), interferon (IFN)-γ, and antimycobacterial antibiotics induce activation of the autophagic pathway to eliminate Mtb. The activation of endosomal and plasma membrane TLRs is linked to the induction of xenophagy of phagocytosed Mtb. IFN-γ induces autophagy through a downstream effector, Irgm1, in human macrophages, which then results in autophagic clearance of Mtb. Antimycobacterial antibiotics activate autophagy, which depends on cellular and mitochondrial reactive oxygen species. **(C)** The activation of autophagy plays a critical role in the clearance of intracellular *Mycobacteria* through diverse signaling pathways. *First*, ubiquitinated proteins are internalized and delivered via vesicles to the late endosome. Cargo receptors, such as p62, recognize ubiquitinated proteins and bind to LC3, contributing to autophagy activation. The autophagic vacuoles which contain ubiquitinated proteins traffic to the late endosome. This process promotes activation of lysosomes and fusion of Mtb-containing phagosomes with the lysosomes. *Second*, cytosolic recognition of Mtb-DNA via the STING-dependent pathway promotes ubiquitination of Mtb, and delivery of bacteria to autophagosomes through the cargo receptors p62 and NDP52. Finally, various stimuli induce autophagic clearance.

cytosolic pathway, marked with ubiquitin, and delivered to the autophagic machinery through the selective autophagic receptors p62 and NDP52 (Watson et al., 2012) (**Figure 2C**, left). Importantly, the Mtb ESX-1 secretion system is critical for cytosolic sensing of bacterial DNA, and activation of the ubiquitin-mediated selective autophagy pathway in natural Mtb infection (Watson et al., 2012). Moreover, cytosolic sensing of Mtb-DNA is mediated through the STING/TANK-binding kinase 1 (TBK-1)/IFN regulatory factor 3 (IRF3) axis, and results in IFN-β secretion. Note that IRF3$^{-/-}$ mice are protected from long-term Mtb infection, indicating that cytosolic sensing of Mtb-DNA and type I IFN signaling may contribute to the pathogenesis of tuberculosis (Manzanillo et al., 2012). Moreover, another study hinted at novel roles for Rab8b, a member of the Rab family member of membrane trafficking regulators, and TBK-1, with regard to autophagic elimination of *Mycobacteria* in macrophages (Pilli et al., 2012) (**Figure 2C**, right). TBK-1 phosphorylates the autophagic receptor p62, thus playing an important role in linking the innate immune response to cargo recruitment into autophagosomes (Pilli et al., 2012).

Other recent studies have shown that virulent Mtb inhibits autophagosome maturation in dendritic cells, and that this is dependent on the ESX-1 system (Romagnoli et al., 2012). The recombinant BCG and Mtb H37Ra strains with genetic complementation, using either the ESX-1 region from Mtb

(BCG::ESX-1) or the PhoP gene (Mtb H37Ra::PhoP), a regulator of ESAT-6 secretion, restored their inhibitory activities against autophagy (Romagnoli et al., 2012). Classic autophagy activation by rapamycin treatment led to an increased interleukin (IL)-12 production and T helper cell (Th)1-oriented response in dendritic cells infected with Mtb (Romagnoli et al., 2012). These data partly correlated with previous findings in which mammalian target of rapamycin (mTOR) signaling negatively regulated the synthesis of IL-12 and IL-23 in human monocyte-derived macrophages infected with Mtb (Yang et al., 2006). These conflicting results are most likely due to the use of different cell types from different species (e.g., mouse or human), and variations of Mtb strains (e.g., Erdman strain, BCG, or others). Therefore, we must understand how antibacterial autophagy is activated in different cells and through which mechanisms. This information will help to identify and develop new therapies against Mtb infection.

ANTIBACTERIAL AUTOPHAGY IN *SALMONELLA* INFECTION

Salmonella enterica serovar *typhimurium* (*S. typhimurium*) is a facultative intracellular pathogen with a bimodal life style inside host cells. The pathogen usually resides in a membrane-bound, *Salmonella*-containing vacuole (SCV). In this compartment, *S. typhimurium* can replicate and deliver a variety of effectors through type III secretion systems (TTSSs), allowing bacteria to enter the cytosol. SCVs can also develop into long tubular structures, also known as spacious vacuole-associated tubules, sorting nexin 3 (SNX3) tubules, and *Salmonella*-induced filaments (SIFs) (Bakowski et al., 2008; Schroeder et al., 2011). Some bacteria within damaged SCVs escape into the cytosol and can be detected by the autophagy process, which depends on the *Salmonella* pathogenicity island 1 (SPI-1) TTSS (Birmingham et al., 2006).

S. typhimurium that enter the cytosol are initially coated with polyubiquitinated proteins, and are then detected by the cargo adaptor, NDP52 (Thurston et al., 2009) (**Figure 3A**). In addition, *S. typhimurium* activates TLR4 signaling pathways, leading to phosphorylation of TBK-1. Through molecular interaction with adaptor proteins Nap1 and Sintbad, TBK-1, an important signaling molecule for regulation of TIR domain-containing adapter-inducing IFN-β (TRIF)-dependent IRF3 signaling (Yuk and Jo, 2011), is recruited to NDP52, and it phosphorylates OPTN on Ser177, another autophagic receptor (Thurston et al., 2009; Wild et al., 2011). Phosphorylated OPTN has an enhanced ability to interact with the autophagic LC3 protein, driving bacteria toward the autophagic machinery and elimination by xenophagy activation (Thurston et al., 2009; Wild et al., 2011). A more recent study revealed that NDP52 selectively and preferentially interacts with LC3 isoform C (LC3C) through its non-canonical LC3C-interacting region (CLIR) domain structure. Notably, this interaction between LC3C and NDP52 is involved in the recruitment of all ATG8 family members to cytosolic bacteria and successful elimination of *S. typhimurium* (von Muhlinen et al., 2012).

Another cargo adaptor, p62/SQSTM1, is recruited by polyubiquitin-decorated *S. typhimurium* for the xenophagic control of bacteria (Zheng et al., 2009). NBR1 is a cargo adaptor that has a similar domain structure containing an N-terminal PB1 domain, a LIR motif (interacting with LC3 proteins), and a C-terminal UBA domain which interacts with ubiquitin (Kirkin et al., 2009; Lamark et al., 2009). It is known to interact with p62 to form oligomers, it is recruited to polyubiquitinated cargos and degraded by autophagy processes (Kirkin et al., 2009; Lamark et al., 2009). However, it is not known whether NBR1 is involved in *Salmonella* infection. Determining whether it plays a role in the autophagic clearance of intracellular bacteria and whether it can co-operate with other cargo receptors including p62 and NDP52 would be of interest.

In *Salmonella* infection, bacteria initiate an early state of intracellular amino acid deprivation, which is induced by host membrane damage, suggesting that xenophagy is activated by a metabolic switch induced by amino acid starvation (Tattoli et al., 2012). In addition, diacylglycerol (DAG)-induced and ubiquitin-independent autophagy has been reported in host defense against *Salmonella*. DAG, a lipid second messenger generated by phospholipase D, is associated with autophagy-targeted *Salmonella* and is required for antibacterial autophagy through protein kinase Cδ signaling (Shahnazari et al., 2010). Recent studies have also revealed a novel role of cytosolic lectin Galectin 8 (LGALS8) in detecting bacterial invasion through binding to host glycans during invasion by *Salmonella* and *Shigella*. LGALS8 recruits NDP52 (CALCOCO2) to activate antibacterial autophagy (Thurston et al., 2012).

ANTIBACTERIAL AUTOPHAGY IN *SHIGELLA* INFECTION

Shigella is an invasive bacterium that exploits a harmful niche enabling it to replicate inside host cells. During a *Shigella* infection, the bacterium uses an array of pathogenic strategies including; induction of macrophage cell death, a massive inflammatory response, which results in subsequent infection, multiplication within epithelial cells, disruption of the vacuolar membrane surrounding the bacteria, and movement through promotion of actin polymerization (Ashida et al., 2011).

Shigella can manipulate the autophagy pathway through escape from and induction of the host autophagic system. *Shigella* can escape autophagy by secreting IcsB through a TTSS (**Figure 3B**, left), whereas VirG (a protein for intracellular actin-based motility) induces autophagy via interaction with the autophagy protein ATG5 (Ogawa et al., 2005). Additionally, Shiga toxins induce autophagy in THP-1 cells and human macrophages, and enhance cell death of renal epithelial cells through an autophagy-dependent mechanism. Especially in toxin-sensitive cells especially, those toxins are translocated to the endoplasmic reticulum (ER) and activate calpains and caspase-8 and -3, resulting in the cleavage of the autophagy-related genes ATG5 and Beclin-1 (Lee et al., 2011).

Upon invasion of epithelial cells by *Shigella* the vacuolar membrane fragments ruptured by the bacteria are targeted to the autophagy pathway by recruiting ubiquitin, TNF receptor associated factor 6 (TRAF6), p62, and LC3 (Dupont et al., 2009). Interestingly, guanosine triphosphatase (GTP)-binding protein septin assemblies are recruited to intracytosolic *Shigella*, which they entrap in cage-like structures (Mostowy et al., 2010). Moreover, the cargo adaptors p62 and NDP52 direct *Shigella* to an autophagy pathway that is dependent upon septin and actin (Mostowy et al., 2011). During infection, host-derived pro-inflammatory cytokine TNF-α enhances septin caging and p62-mediated autophagic activity, thereby limiting *Shigella* survival

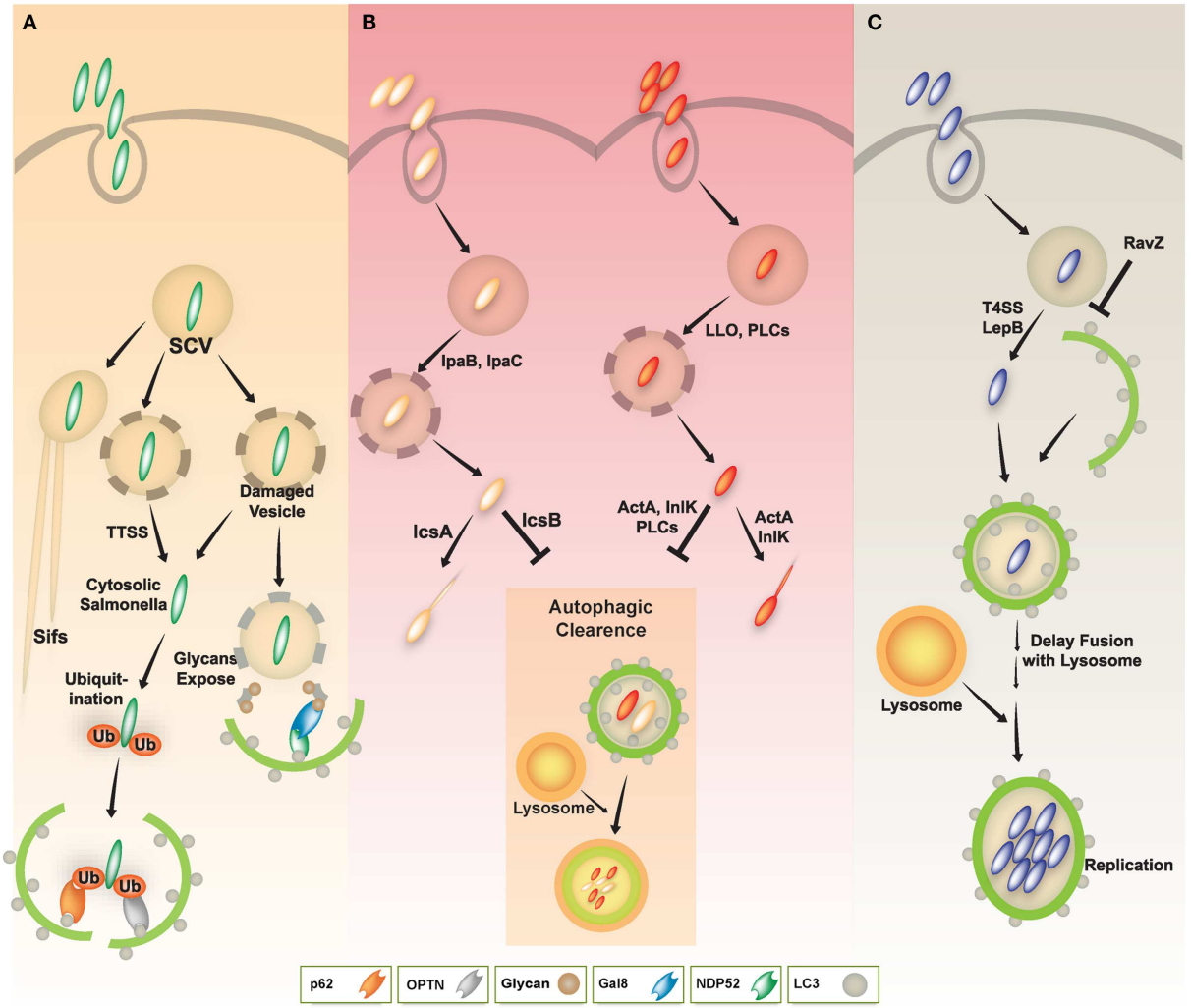

FIGURE 3 | A schematic diagram of diverse intracellular pathogen infections and clearance via autophagy pathway. **(A)** In *Salmonella* infection, the majority of *S. typhimurium* resides in *Salmonella*-containing vacuoles (SCVs) and allow establishment of a niche permissive for growth, which then form *Salmonella*-induced filaments (SIFs). Some *Salmonella* enter the host cytosol via type III secretion system (TTSS). The cytosolic *Salmonella* via TTSS-dependent damage to the SCV was targeted by the autophagy system through ubiquitin-dependent or -independent pathways. The cytosolic *Salmonella* can become coated with ubiquitin and then be recognized by the cytosolic cargo receptors such as NDP52, OPTN, or p62, and bind to ATG8/LC3, delivering the bacteria into autophagosomes for autophagic clearance. Otherwise, NDP52 binds to Galectin 8, a cytosolic lectin that detects host glycans on vesicles damaged by *Salmonella* during the process of entering the host cell. The NDP52-Galectin-8 interaction delivers bacteria for autophagic degradation. Galectin-8 can detect a wide variety of vesicle-damaging pathogens in addition to *Salmonella*, e.g., *Shigella* and *Listeria*. **(B)** In *Shigella* and *Listeria* infection, the bacteria can escape from vacuoles to the host cytosol via their bacterial products (e.g., IpaB, IpaC, LLO, or PLCs). The cytosolic *Shigella* and *Listeria* have actin-based motility, contributing to their escape from autophagy. Essential bacterial products (e.g., IcsA, IcsB, ActA, or InlK) are involved in actin-based motility and inhibition/evasion of autophagy. **(C)** In *Legionella* infection, *L. pneumophila* also escapes from vacuoles to the host cytosol via LepB through Type IV secretion system. Cytosolic *L. pneumophila* is recognized by its autophagic machinery; however, *Legionella* delays fusion of the autophagosome with lysosomes until it develops into an acid-resistant form. The acid-resistant *Legionella* can replicate in the acidic autophagolysosome. *Legionella* also interferes with autophagy by using its bacterial effector protein RavZ.

and cell-to-cell spread (Mostowy et al., 2010, 2011). A highly conserved Tectonin domain-containing protein, Tecpr1, plays a major role in antibacterial autophagy, targeting *Shigella* through interaction with ATG5 (Ogawa et al., 2011a). Tecpr1-deficient mouse embryonic fibroblasts (MEFs) have a defect in selective autophagy, which is manifested by accumulation of depolarized mitochondria and miss-folded protein aggregates, and an increased replication of *Shigella* (Ogawa et al., 2011a). Importantly since Tecpr1 offers the fusion of autophagosomes and lysosomes by interacting with ATG12-ATG5 and PtdIns3P (Chen et al., 2012), Tecpr1 may play an important role in triggering autophagy in general (Behrends et al., 2010; Ogawa et al., 2011a). *Shigella flexneri* VirA, which harbors TBC-like dual-finger motifs that exhibit GTPase-activating protein (GAP) activity, is known to direct host Rab1 to inhibit IL-8, and counteract autophagy-mediated host defense in infected cells (Dong et al., 2012). Collectively, these studies indicate that the host

defense system and the bacterial tactics against the host autophagic machinery, as well as the immune response may determine the outcome of *Shigella* infection.

ANTIBACTERIAL AUTOPHAGY IN *LISTERIA* INFECTION

Listeria monocytogenes (L. monocytogenes) is a facultative Gram-positive bacteria and an intracellular pathogen that causes listeriosis. Listeriosis commonly affects pregnant women and people with suppressed immune systems, e.g., those with cancer or HIV (Vazquez-Boland et al., 2001). Intestinal epithelial cells are the primary targets of *L. monocytogenes*. After primary infection of the epithelium, the bacterium translocates to phagocytic cells, such as dendritic cells and macrophages, through M cell-dependent or M cell-independent pathways (Barbuddhe and Chakraborty, 2009; Ogawa et al., 2011b). After internalization by the host cell, *L. monocytogenes* escapes from the phagosome to the cytosol by secreting listeriolysin O (LLO), which is a pore-forming hemolysin (Tweten, 2005; Schnupf and Portnoy, 2007; Birmingham et al., 2008). *L. monocytogenes* in the host cytosol expresses the bacterial protein ActA, which engages the host cell actin machinery, to assist bacterial motility and eventually cell-to-cell spread (Moors et al., 1999; Lambrechts et al., 2008). By spreading from cell-to-cell, *L. monocytogenes* disseminates and expands into other cells or tissues.

L. monocytogenes has been reported to induce autophagic responses. During the early phase of (∼2 h of post) *Listeria* infection, autophagy plays a crucial role in the host immune defense in mice (Birmingham et al., 2007; Py et al., 2007). *L. monocytogenes* replicates more efficiently in ATG5-deficient MEFs, compared to wild-type (WT) MEFs, suggesting an essential role for autophagy in inhibition of bacterial growth inside the cells (Birmingham et al., 2007; Py et al., 2007). It has also been reported that *L. monocytogenes* induces autophagy activation in *Drosophila* hemocytes (Yano et al., 2008). Moreover, Zhao et al. (2008) revealed that the autophagy protein ATG5 in phagocytic cells, such as macrophages and neutrophils, is essential for *in vivo* immunity to *Listeria* infection (Zhao et al., 2008).

Several possible mechanisms exist by which *L. monocytogenes* triggers the autophagy pathway; one possibility involves the bacterial components, and another is recognition of bacterial invasive process via cytosolic receptors. LLO, a major virulence factor of *L. monocytogenes*, was reported to be a key component of *L. monocytogenes*-induced autophagy (Birmingham et al., 2007; Py et al., 2007). *L. monocytogenes* lacking LLO failed to induce autophagy, cleavage from LC3 I to LC3 II, and co-localization with LC3. Similarly, LLO-mediated membrane remnants of phagosomal rupture were found to be sufficient to activate autophagy (Meyer-Morse et al., 2010). First, LLO-containing liposomes were shown to be recruited to autophagosomes even in the absence of infection (Meyer-Morse et al., 2010). Second, cytosolic receptors, such as peptidoglycan recognition protein (PGRP)-LE or nucleotide-binding oligomerization domain-containing (NOD) 1, play a role in the positive regulation of autophagy during *Listeria* infection (Yano et al., 2008; Travassos et al., 2010). In *Drosophila*, sensing of peptidoglycan by PGRP-LE is required for the induction of autophagy, which can inhibit intracellular growth of *L. monocytogenes* and induce host survival after *Listeria* infection (Yano et al., 2008). In murine and human cells, both NOD1 and ATG16L are recruited to the membranes of vesicles containing *L. monocytogenes*. Notably, the levels of autophagosome-containing *L. monocytogenes* in NOD1 deficient MEFs were significantly lower, compared with those in NOD1 WT MEFs (Travassos et al., 2010).

As autophagy is essential for inhibiting the intracellular growth of *L. monocytogenes* (Birmingham et al., 2007; Py et al., 2007; Zhao et al., 2008), *L. monocytogenes* has evolved diverse evasion strategies against the host autophagy machinery (Birmingham et al., 2007; Py et al., 2007; Yoshikawa et al., 2009; Dortet et al., 2011; Ogawa et al., 2011b) (**Figure 3B**, right). *L. monocytogenes* has several bacterial components that negatively regulate host autophagy activation. Phospholipases C (PLCs) from *L. monocytogenes*, such as PI−PLC (encoded by PlcA) and PC−PLC (encoded by PlcB), act synergistically with LLO to lyse phagosomal vesicles to promote invasion into the host cytosol. PLCs, however, inhibit host autophagy induced by *L. monocytogenes* (Birmingham et al., 2007; Py et al., 2007). Additionally, ActA, a *L. monocytogenes* surface protein, is involved in intra- and inter-cellular motility enabling escape from autophagy (Dortet et al., 2011; Ogawa et al., 2011b). The ability of the ActA protein to induce recruitment of the Arp2/3 complex and Ena/VASP, contributes to the bacterial ability to evade host autophagic recognition (Yoshikawa et al., 2009). Thus, *L. monocytogenes* lacking ActA is not able be recruited to the Arp2/3 complex and Ena/VASP, it instead becomes ubiquitinated, bind to p62 and LC3, and finally undergoes autophagic clearance (Yoshikawa et al., 2009). Another *L. monocytogenes* surface protein, InlK, acts similarly to ActA (Dortet et al., 2011). Moreover, *L. monocytogenes* lacking ActA showed increased expression of InlK, enabling comparable intracellular survival, similar to WT bacteria. Thus, InlK has a redundant function in *L. monocytogenes* lacking ActA, by replacing ActA and enabling the bacteria to escape autophagic clearance (Dortet et al., 2011). Collectively, these studies indicate that *L. monocytogenes* has dual autophagy regulation mechanisms. While autophagy activation via LLO is as an important defense mechanism against infection, *Listeria* has evolved several evasion mechanisms involving various virulence factors, such as PLCs, ActA, and InlK.

ANTIBACTERIAL AUTOPHAGY IN *LEGIONELLA* INFECTION

Legionella pneumophila (L. pneumophila), although usually found in freshwater protozoa and amebae, is an accidental infectious pathogen that can replicate in alveolar macrophages in the human lung, and especially in immune compromised patients (Dubuisson and Swanson, 2006; Joshi and Swanson, 2011). *L. pneumophila* resides within vacuoles that have features typical of autophagolysosomes, containing the autophagy-related protein ATG8/LC3, lysosomal-associated membrane protein 1 (LAMP1), and the lysosomal acid hydrolase cathepsin D (Dubuisson and Swanson, 2006; Joshi and Swanson, 2011). Notably, the biogenesis of *L. pneumophila*-harboring vacuoles is similar to the formation of autophagosomes. For example, the ER is one source of these two vacuoles, as are the *L. pneumophila* vacuole and the autophagosomal membrane (Joshi and Swanson, 2011). Moreover, this pathogen continuously replicates within acidic lysosomal vacuoles in macrophages, and inhibits immediate delivery to the lysosomes, thus persisting in immature autophagosomal vacuoles (Amer and Swanson, 2005; Joshi and Swanson, 2011).

Subsequent secretion of Type IV effectors, including LepB, causes delayed maturation of autophagosomes, and may provide sufficient time for inducing acid resistance and other traits within the autophagolysosomes (Joshi and Swanson, 2011) (**Figure 3C**).

Several host defense mechanisms, including apoptosis, autophagy, and inflammasome-associated cell death, are thought to form part of the host defense against *L. pneumophila* infection (Swanson and Molofsky, 2005; Banga et al., 2007). *L. pneumophila*-mediated inflammasome activation and pyroptotic cell death is likely to be linked to the autophagy pathway through a mechanism involving the cytoplasmic translocation of flagellin, and its detection via Naip5, a NOD-like receptor (NLR) adaptor protein of the inflammasome (Dubuisson and Swanson, 2006). *In vitro* studies, including treatment of A/J mouse peritoneal macrophages with 2-deoxy-D-glucose, support the role of autophagy in inhibiting the intracellular replication of *L. pneumophila* (Matsuda et al., 2009). *In vivo* studies using the ATG9 mutant *Dictyostelium discoideum* show a critical defect in phagocytosis and clearance of *L. pneumophila*, as well as in growth and development, indicating an important role for autophagy in protection during *L. pneumophila* infection (Tung et al., 2010). Recent studies have revealed a mechanism by which the *L. pneumophila* effector protein RavZ inhibits autophagy by functioning as a deconjugating enzyme that targets ATG8/LC3 proteins attached to phosphatidylethanolamine on autophagosome membranes (Choy et al., 2012) (**Figure 3C**). Although *Legionella* RavZ can inhibit autophagy by irreversibly inactivating ATG8/LC3 proteins during infection (Choy et al., 2012), whether RavZ-mediated inhibition of autophagy could affect any phenotype of host cells remains to be determined.

CONCLUDING REMARKS

To conclude, the data to date indicate that xenophagy functions selectively target intracellular bacteria through autophagic receptors including SQSTM1/p62, NDP52, OPTN, and NBR1. Cytosolic access of intracellular bacteria or their components, from bacterial vacuoles, initiates the formation and ubiquitination of protein aggregates. During mycobacterial infection, cytosolic sensing of extracellular Mtb-DNA activates ubiquitin-mediated selective autophagy that targets Mtb in an ESX-1 system-dependent manner. However, the cytosolic sensing of Mtb and IRF3-dependent type I IFN signaling are likely to be associated with the pathogenesis of tuberculosis, because IRF deficiency leads to a more protective phenotype against long-term Mtb infection in mice. Whatever the autophagic stimuli, the induction of autophagy by IFN-γ, vitamin D, and TLR ligands is of paramount importance for the elimination of intracellular Mtb in macrophages. In *Salmonella* infection, cytosolic bacteria from damaged SCVs are coated with ubiquitin and recruited to the cargo receptor p62, which interacts with the autophagic machinery. In *Shigella* infections, several cargo receptors, including p62, NDP52, and Tecpr1, contribute to antibacterial autophagy targeting of *Shigella*. *L. monocytogenes* exhibits a dual regulatory function in autophagic regulation through its bacterial components or by modulating host-originated proteins as follows: (1) positive regulation via the virulence factor LLO and host cytosolic receptors, NOD1 or PGRP-LE; (2) negative regulation through *Listeria*-derived components, such as PlcA, PlcB, ActA, and InlK. Curiously, *Legionella* can persist and replicate in immature autophagosomal vacuoles. The *Legionella* effector RavZ was found to subvert host autophagy through delipidation and inactivation of ATG8/LC3. Regardless of the intracellular bacterial strain, host autophagic clearance systems and bacterial manipulation of the host autophagic machinery may determine the outcome of intracellular bacterial infection. Further studies are needed to elucidate the role of bacterial effectors in manipulating host autophagy and to clarify the pathogenesis of intracellular bacterial infections. We believe that this will facilitate the development of innovative treatments for such bacterial infections.

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Modulation of pathogen recognition by autophagy

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Autophagy is an ancient biological process for maintaining cellular homeostasis by degradation of long-lived cytosolic proteins and organelles. Recent studies demonstrated that autophagy is availed by immune cells to regulate innate immunity. On the one hand, cells exert direct effector function by degrading intracellular pathogens; on the other hand, autophagy modulates pathogen recognition and downstream signaling for innate immune responses. Pathogen recognition via pattern recognition receptors induces autophagy. The function of phagocytic cells is enhanced by recruitment of autophagy-related proteins. Moreover, autophagy acts as a delivery system for viral replication complexes to migrate to the endosomal compartments where virus sensing occurs. In another case, key molecules of the autophagic pathway have been found to negatively regulate immune signaling, thus preventing aberrant activation of cytokine production and consequent immune responses. In this review, we focus on the recent advances in the role of autophagy in pathogen recognition and modulation of innate immune responses.

Keywords: autophagy, Toll-like receptors, RIG-I-like receptors, NOD-like receptors

INTRODUCTION

Eukaryotic cells contain two major protein degradation systems for maintaining cellular homeostasis: the ubiquitin-proteasome system, responsible for degradation of soluble short-lived proteins (Kloetzel, 2001), and autophagy, a conserved system that degrades long-lived proteins and organelles (Klionsky and Emr, 2000). Autophagy was originally described as a starvation-induced response that provides nutrients by degrading long-lived proteins and recycling intracellular organelles (Kuma et al., 2004; Komatsu et al., 2005). There are three types of autophagy: microautophagy, chaperone-mediated autophagy, and macroautophagy (**Figure 1A**; Mizushima and Klionsky, 2007). Macroautophagy is the main route of degradation. It involves the formation of a double-membrane vesicle called autophagosome, which is formed by elongation of a cup-shaped membrane, followed by wrapping of the cellular constituents and fusion with lysosomes for degradation (Mizushima et al., 1998). The molecular mechanism underlying autophagy has been thoroughly covered in excellent reviews elsewhere (Klionsky and Emr, 2000; Ohsumi, 2001; Mizushima et al., 2002). Here, we briefly describe the process relevant to innate pathogen recognition by macroautophagy. Autophagy-related gene (Atg) 6 (Beclin-1) and type III phosphatidylinositol 3-kinase (PI3K) are required for the initiation of the isolation membrane. The elongation and termination of the autophagosome are regulated by at least two ubiquitin-like systems: the microtubule-associated protein 1 light chain 3 (LC3; mammalian homolog of the yeast autophagic protein Atg8) and the Atg12 conjugation pathways. The C-terminal amino acids of LC3 are cleaved by Atg4, and this C-terminal residue then gets transferred to phosphatidylethanolamine (PE) in the newly formed isolation membrane by the E1- and E2-like enzymes Atg7 and Atg3, respectively. Although LC3 gets recycled from the outer autophagosomal membrane by deconjugation from its phospholipids, it remains attached to the inner autophagosomal membrane, and this portion is degraded along with the inner autophagosomal membrane in lysosomes and late endosomes after fusion with these vesicles (Ohsumi, 2001). Autophagosome-associated LC3 (LC3-II) and free cytosolic LC3 (LC3-I) can be distinguished by their apparent molecular weight, and autophagosomes can be visualized by using green fluorescent protein (GFP)-conjugated LC3 molecules. In the other ubiquitin-like system, Atg12 gets coupled through its C-terminal glycine residue to a lysine residue of Atg5 by the E1- and E2-like enzymes Atg7 and Atg10, respectively. The Atg12–Atg5 complex associates with Atg16 and then binds to the outer surface of the isolation membrane. Upon completion of the autophagosome, the Atg5–Atg12–Atg16 complex dissociates from the outer autophagosomal membrane and only LC3 remains associated with the completed autophagosome. Autophagosomes then fuse with late endosomes and lysosomes for degradation of their contents and membrane (**Figure 1B**; Levine and Deretic, 2007).

Besides maintaining cellular homeostasis, autophagy plays important roles in multiple biological processes including development, aging, and degeneration (Levine and Klionsky, 2004). Not surprisingly, aberrant regulation of autophagy induces many diseases such as cancer, neurodegenerative diseases, and myopathies (Shintani and Klionsky, 2004; Levine and Kroemer, 2008). Recently, autophagy was found to be involved in immunity. It can act as a direct effector by eliminating invading pathogens (Gutierrez et al., 2004; Nakagawa et al., 2004; Deretic, 2005; Ogawa et al., 2005), regulating innate pathogen recognition (Sanjuan et al., 2007; Xu et al., 2007; Delgado et al., 2008), contributing to antigen presentation via major histocompatibility complex (MHC) class II molecules (Dengjel et al., 2005; Paludan et al., 2005; Schmid and Munz, 2007; Schmid et al., 2007), and controlling B- and T-cell development (Li et al., 2006; Pua and He,

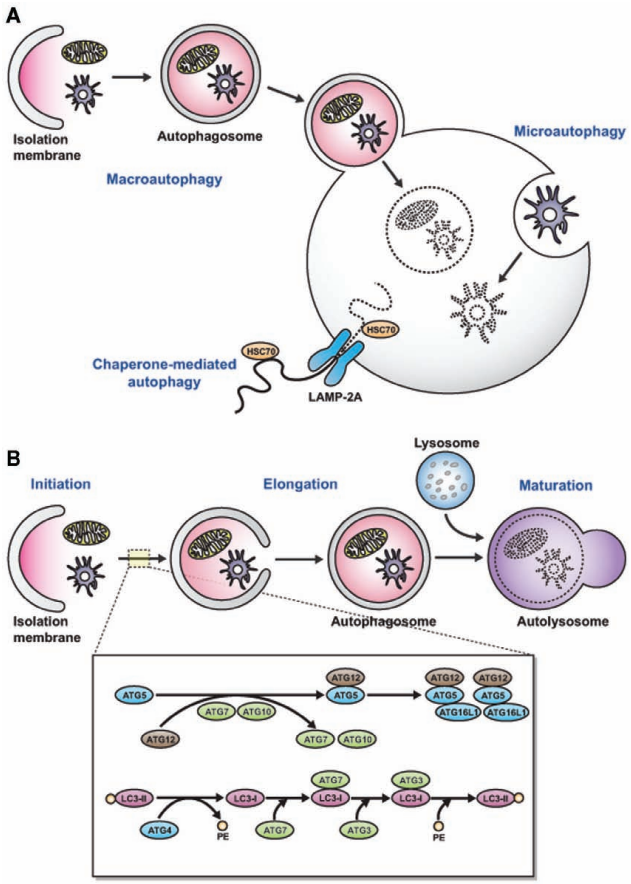

FIGURE 1 | Three types of autophagy and cellular and molecular events in an autophagic pathway. (A) There are three types of autophagy. In macroautophagy, which is the main route of cellular degradation, an autophagosome (a double-layered membrane vesicle) is formed, which fuses with lysosomes to degrade the cellular constituents or pathogens. Microautophagy is characterized by the removal of cellular constituents via the budding of an autophagic body at the lysosomal membrane. In chaperone-mediated autophagy, a signaling motif that contains molecules is transported with the chaperone HSC70 via the LAMP-2A protein into lysosomes. **(B)** The autophagic pathway consists of three distinct stages. For process initiation, nucleation of the autophagic vesicle is needed. Atg6 (Beclin-1) and type III PI3-kinase are required for the initiation of isolation-membrane formation. Elongation and closure of the autophagosome membrane are regulated by two ubiquitin-like conjugation systems: LC3 and Atg12 conjugation systems. The autophagosome fuses with lysosomes and subsequently matures into an autolysosome to degrade the materials present inside the cell.

2007; Pua et al., 2007; Miller et al., 2008). In this review, we focus on the role of autophagy in innate pathogen recognition and its regulation.

AUTOPHAGY IN PATHOGEN RECOGNITION

PAMPs STIMULATING CERTAIN TLRs INDUCE AUTOPHAGY INDUCTION

The innate immune system recognizes only a limited number of microbial molecular structures, so-called pathogen-associated molecular patterns (PAMPs; Iwasaki and Medzhitov, 2004; Akira et al., 2006), which are conserved within the same class of microbes but differ across classes (e.g., viruses, Gram-negative bacteria, Gram-positive bacteria, and fungi). Pattern recognition receptors (PRRs) bind to these conserved structures and initiate downstream signaling pathways. PRRs are located in various sites such as plasma membranes, endosomal vesicles, and cytoplasm, thereby enabling the recognition of various types of microbes in any of these locations. Toll-like receptors (TLRs) are the most well-known PRRs in innate pathogen recognition. TLRs can be divided into two groups based on their locations: cell surface TLRs, TLRs 1, 2, 4, 5, and 6, that mainly recognize bacterial components, and endosomal TLRs, TLRs 3, 7, 8, and 9, that generally recognize viral nucleic acids. All these receptors contain leucine-rich repeats (LRRs) in their extracellular domain for ligand binding, and a cytoplasmic Toll/IL-1 receptor (TIR) homology domain, which is essential for signaling. After recognition of PAMPs, TLRs initiate common or distinct signaling pathways via different kinds of adaptor molecules. All TLRs except TLR3 activate the transcription factors, nuclear factor (NF)-κB and activator protein-1 (AP-1), via MyD88, leading to the production of proinflammatory cytokines. TLR3 and TLR4 activate the transcription factor interferon regulatory factor 3 (IRF3) via TIR domain-containing, adapter-inducing interferon-β (TRIF), leading to the production of type I IFNs (Lee and Kim, 2007; Delgado and Deretic, 2009).

Recently, some studies have shown that activation of TLR can lead to induction of autophagy (Xu et al., 2007; Delgado et al., 2008; Shi and Kehrl, 2008). After stimulation with lipopolysaccharide (LPS), TLR4 can induce autophagy in primary human macrophages and in the murine macrophage cell line RAW264.7 (Xu et al., 2007). It was shown that LPS stimulation induces redistribution of LC3 protein from a diffuse to a punctate pattern and increases the levels of the lipidated form of LC3 (LC3-II), both of which are reliable markers of autophagy induction. Interestingly, this LPS-induced autophagy occurs via a TRIF-dependent, MyD88-independent TLR signaling pathway, for which receptor-interacting protein 1 (RIP1) and p38 mitogen-activated protein kinase (MAPK) are required (**Figure 2**). Moreover, Xu et al. (2007) also reported that LPS-induced autophagy results in mycobacterial colocalization with the autophagosomes, suggesting that autophagy could enhance the elimination of mycobacteria. In another study, mycobacteria elimination has been demonstrated using starvation and rapamycin for autophagy induction(Gutierrez et al., 2004).

A study reporting the effect of TLR agonists on autophagy induction in RAW264.7 macrophages (Delgado et al., 2008) showed that ligands of TLR3, TLR4, and TLR7 could induce autophagy, and those of TLR7 generated the most potent effects (**Figure 2**). Two different ligands of mouse TLR7, single-stranded RNA (ssRNA) and imiquimod, induced formation of autophagosomes characterized by LC3 puncta formation in murine primary macrophages (Delgado et al., 2008). Induction of autophagy via TLR7 signaling was dependent on MyD88 and required Beclin-1. Moreover, autophagy activation by TLR7 agonists induced killing of intracellular mycobacteria, even though mycobacteria are normally not associated with TLR7 signaling. This ability of pathogen elimination was diminished by siRNA knockdown of Beclin 1, thus depending on autophagy.

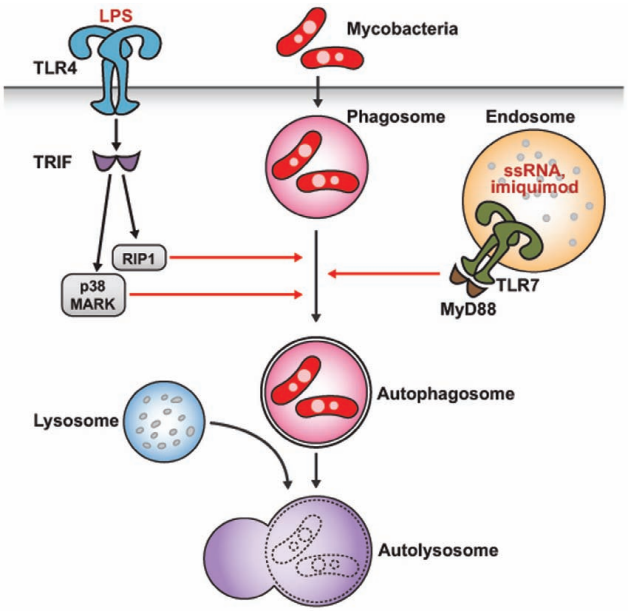

FIGURE 2 | Autophagy induction by TLR stimulation. Activation of TLR can induce autophagy for pathogen elimination. Stimulation of TLR4 with LPS in a TRIF-dependent, MyD88-independent signaling pathway, and stimulation of endosomal TLR7 with two different TLR7 ligands via the MyD88-dependent pathway induces autophagosome formation and eliminates *Mycobacteria bacilli,* even though mycobacteria are normally not associated with TLR7 signaling. Atg proteins such as Atg5, Beclin-1, and PI3K are required for the formation of an autophagosome.

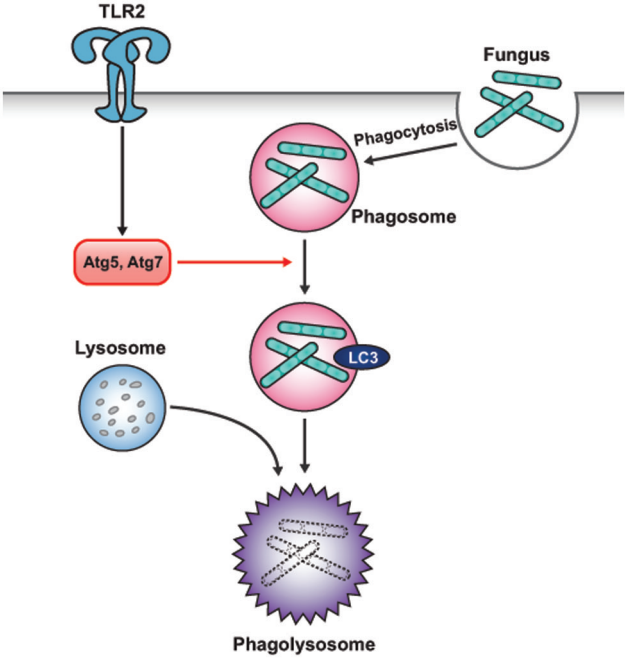

FIGURE 3 | Interplay between autophagy and phagocytosis. TLR signaling can enhance the maturation of phagosomes with the autophagic machinery. LC3 recruitment to phagosomal membranes promotes the fusion of phagosome with lysosome for maturation. However, LC3 recruitment to phagosomal membranes is not associated with the characteristic double-membrane structure of the autophagosomes. The Atg5 and Atg7 proteins are involved in the development of this structure.

INTERPLAY BETWEEN AUTOPHAGY AND PHAGOCYTOSIS

Unlike TLR4- and TLR7-mediated induction of autophagy, which enhances autophagosome fusion with the pathogen-containing phagosomes and promotes elimination of intracellular pathogens, certain TLR signaling pathways enhance the maturation of phagosomes by the autophagic machinery (Sanjuan et al., 2007). Upon phagocytosis of zymosan (a component of the fungal cell wall), phagosomes rapidly recruit LC3 and fuse with lysosomes for maturation (**Figure 3**). Pam_3CSK_4 coating of latex beads also induces the rapid recruitment of LC3 to phagosomes in RAW264.7 macrophages. This zymosan-mediated translocation of LC3 to phagosomes was found to be independent of MyD88 but dependent on Atg5 and Atg7. Live *Saccharomyces cerevisiae* engulfed by macrophages survived more frequently in Atg7 knockout macrophages than in wild-type macrophages. Interestingly, LC3 recruitment to phagosomal membranes was not associated with the double-membrane structures characteristic of autophagosomes. Instead, it was associated with phagosome fusion with lysosomes, which enhanced killing of the engulfed pathogens. Collectively, this study has shown a new way of utilizing the autophagic machinery to promote conventional functions of phagocytes after TLR activation, in the absence of autophagosome formation.

NOD-LIKE RECEPTOR SIGNALING AND AUTOPHAGY

To the best of our knowledge, there are only two reports on the NOD-like receptor (NLR) family in autophagy (Cooney et al., 2010; Travassos et al., 2010). NLRs recognize bacterial cell wall components (specifically, peptidoglycans) in the eukaryotic cell's cytosol. NLRs are composed of three distinct domains: a C-terminal LRR, a NACHT domain, and an N-terminal effector domain, which mediate ligand sensing, activation of the NLRs, and initiation of downstream signaling, respectively. When NLRs recognize bacterial peptidoglycans, they initiate signaling transduction by recruiting the protein kinases, which, in turn, activate NF-κB and AP-1 leading to production of cytokines and other molecules involved in innate immunity (Lee and Kim, 2007).

Recent studies have shown that activation of NOD2 by muramyl dipeptide (MDP) induces autophagosome formation, which in turn enhances bacterial clearance (**Figure 4**; Kuma et al., 2004; Travassos et al., 2010). In human DCs, NOD2 stimulated with MDP induces autophagosome formation, which promotes MHC class II-associated antigen presentation. Atg5, Atg7, Atg16L1, and receptor-interacting serine-threonine kinase-2 (RIPK2), the latter being one of the downstream regulators of the NOD2 signaling pathway, are required for autophagosome formation and antigen presentation by MDP (Cooney et al., 2010). Another study also showed that stimulation of NOD1 and NOD2 by bacterial peptidoglycans activates the autophagy pathway in mouse embryonic fibroblasts (MEFs; Travassos et al., 2010). Upon bacterial invasion, NOD2 recruits Atg16L1 to the bacterial entry sites, facilitating bacterial trafficking to the autophagosomes. This, in turn, induces the fusion of the autophagosomes with the

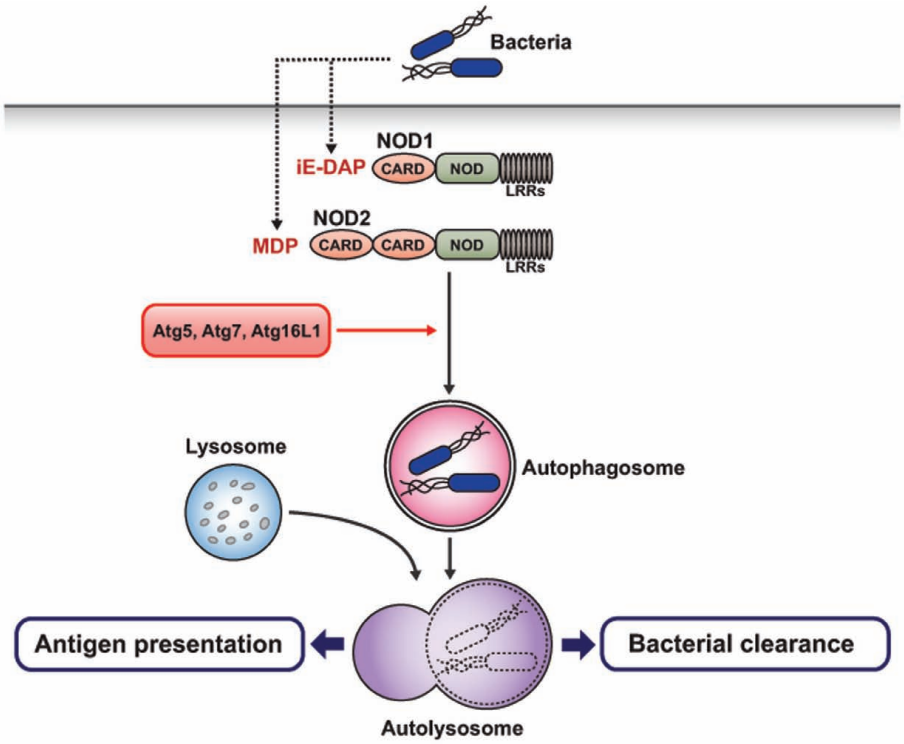

FIGURE 4 | NOD-like receptor-mediated autophagy induction. NOD-like receptors recognize bacterial peptidoglycan in the cytosol. C-terminal leucine-rich repeats (LRRs) of NOD1 and NOD2 detect γ-d-glutamyl-*meso*-diaminopimelic acid (iE-DAP) and muramyl dipeptide(MDP), respectively. NOD1 and NOD2 can induce the autophagosome formation after stimulation with bacterial peptidoglycan, which promotes bacterial clearance and MHC class II-associated antigen presentation. This process depends on Atg5, Atg7, and Atg16L1.

lysosomes to form the autophagolysosomes and promotes antigen presentation via MHC class II molecules. In MEFs, this process does not require the adaptor RIP2 and the transcription factor NF-κB. Interestingly, NOD2 mutation and single-nucleotide polymorphism in the *Atg16L1* gene have been known to be associated with the development of Crohn's disease (Cho and Weaver, 2007; Hampe et al., 2007; Rioux et al., 2007). DCs isolated from patients with Crohn's disease and risk alleles for *NOD2* or *Atg16L1* showed impaired function in autophagy induction and antigen presentation (Cooney et al., 2010). Collectively, these reports revealed a close relationship between two of the most important Crohn's disease-associated susceptibility genes. Furthermore, they functionally link bacterial sensing by NOD proteins to the autophagy pathway.

ROLE OF AUTOPHAGY IN VIRAL SENSING

In addition to the aforementioned direct effector function, autophagy also works in TLR activation by delivering cytosolic PAMPs to endosomal TLRs (Lee et al., 2007). In plasmacytoid dendritic cells (pDCs), viral RNA or DNA recognized by TLR7 or TLR9, respectively, induces type I IFN and proinflammatory cytokine production. These TLRs are located in the endosomal compartment and sense the viral nucleic acids endocytosed by the host cells (Barton, 2007). In addition to NF-κB and MAPK activation, MyD88, an adaptor molecule for these TLRs, activates IRF7, leading to type I IFN production.

In the case of vesicular stomatitis virus (VSV), the replicating virus rather than the viral genome is required to initiate TLR7 signaling and produce IFN-α in pDCs. However, how these cytosolic replication intermediates gain access to the endosomal compartment where TLR7 resides is not completely known. A recent study demonstrated that cytosolic PAMP is delivered by autophagy to the lysosomes for TLR7 recognition (**Figure 5**; Lee et al., 2007). Thus, Atg5-deficient pDCs fail to sense VSV via TLR7, and are unable to secrete IFN-α and IL-12p40. Consequently, mice lacking Atg5 fail to defend themselves from systemic VSV infection in vivo. Moreover, IFN-α production is impaired in Atg5-deficient pDCs in response to herpes simplex virus-1 (HSV-1), which is recognized by TLR9, while IL-12 response remained intact in these cells. Thus, autophagy plays a critical role in the induction of innate immune responses by delivering viral replication intermediates from the cytosol to the endosome for recognition after ssRNA virus infection (Lee and Iwasaki, 2008; Tal and Iwasaki, 2009; Yordy and Iwasaki, 2011).

NEGATIVE REGULATION OF INNATE IMMUNE RESPONSES VIA AUTOPHAGY

The autophagic machinery plays key roles other than activating PRR signaling. In contrast to viral recognition in pDCs, which is mediated by endosomal TLRs, most of the other cell types utilize cytosolic sensors such as those encoded by retinoic acid-inducible gene I (*RIG-I*) and melanoma differentiation associated

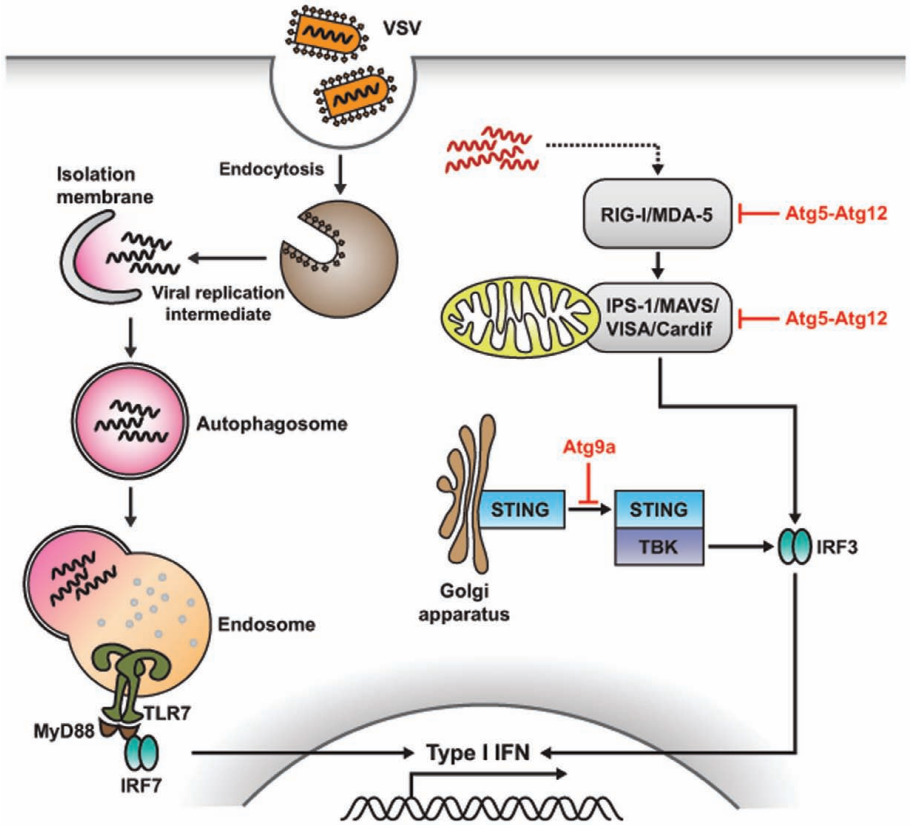

FIGURE 5 | Role of autophagy in antiviral immune response. Autophagy and Atg proteins are required for viral sensing and regulation of antiviral immune responses. In pDCs, autophagy mediates the recognition of viral infection by delivering the viral replication intermediates in the cytosol to lysosomes, where TLR recognition occurs, which, in turn, enhances type I IFN production. However, in non-pDCs, such as mouse embryonic fibroblasts (MEFs), autophagy negatively regulates type I IFN production in response to viral infection. Atg5-Atg12 conjugates block RLR signaling by direct CARD-mediated association with RIG-I and IPS-1, resulting in the suppression of type I IFN production. In the case of dsDNA recognition, STING, a multispanning membrane protein, is translocated from the ER to the Golgi apparatus and assembled with TBK1, which phosphorylates the transcription factor IRF3, resulting in the production of type I IFN. During this process, Atg9a, an essential component of autophagy, colocalizes with STING in the Golgi apparatus, where it controls the assembly of STING.

gene 5 (*MDA-5*) to detect the virus invasion (Yoneyama et al., 2004, 2005; Foy et al., 2005). RIG-I and MDA-5, both of which are RIG-I-like receptors (RLRs), contain a DExD/H box RNA helicase domain for ligand recognition and two caspase-recruiting domains (CARDs) for initiating downstream signaling. When these CARD-containing RNA helicases recognize double-stranded RNA (dsRNA), which is synthesized during active viral replication in the cytosol, signals through IFN-β promoter stimulator-1 [IPS-1; also known as mitochondrial antiviral signaling (MAVS), virus-induced signaling adaptor (VISA), or Cardif] activate the transcription factors IRF-3 and NF-κB, and subsequently lead to the production of type I IFN. IPS-1 is an essential adaptor molecule consisting of an N-terminal CARD domain (through which it associates with RIG-I and MDA-5) and a C-terminal domain (for mitochondrial localization; Lee and Kim, 2007).

A recent study revealed that Atg5–Atg12 conjugates, essential components of the autophagic process, regulate innate viral recognition by RIG-I and MDA-5 in MEFs (**Figure 5**; Jounai et al., 2007). Atg5- and Atg7-deficient MEFs, lacking Atg5–Atg12 conjugates, overproduce type I IFN in response to VSV. Conversely, biochemical analysis indicated that Atg5–Agt12 conjugates block RLR signaling by direct CARD-mediated association with RIG-I and IPS-1; additionally, they suppress type I IFN production. Thus, autophagy-related proteins involved in RLR-mediated viral sensing repress type I IFN response, acting as negative regulators of antiviral responses.

Similarly, in another report, Atg5-deficient cells showed overproduction of type I IFN through enhanced RLR signaling pathway (Tal et al., 2009). That study also showed that dysfunctional mitochondria and mitochondria-associated IPS-1 were accumulated in the absence of autophagy. In Atg5-deficient cells, reactive oxygen species associated with dysfunctional mitochondria may potentiate RLR signaling, which can be blocked by antioxidant treatment. Thus, autophagy plays important roles in the balanced regulation of innate antiviral response by acting as a scavenger of dysfunctional mitochondria.

Another recent study showed that *Atg16L1*, a candidate gene for Crohn's disease, is involved in endotoxin-induced inflammasome activation in mice (**Figure 6A**; Saitoh et al., 2008). Atg16L1 is an essential component of the autophagosome. It forms a

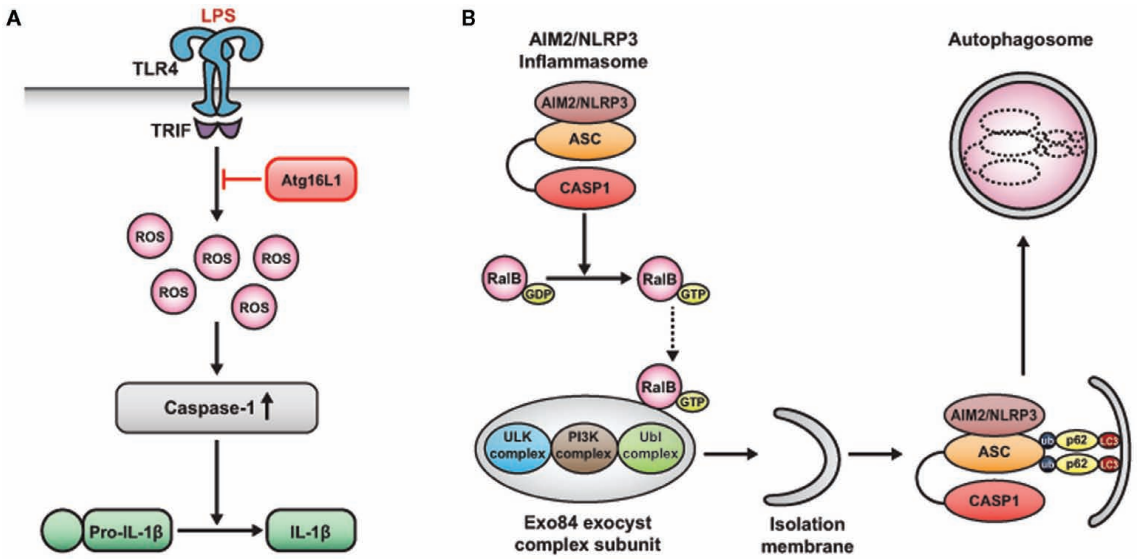

FIGURE 6 | Suppression of inflammasome activity by autophagy. (**A**) Atg16L1, a candidate gene for Crohn's disease, regulates endotoxin-induced inflammasome activation. Atg16L1-deficient macrophages exhibit enhanced IL-1β and IL-18 production in response to LPS. This process is mediated by excessive ROS, which activates caspase-1, leading to the processing of IL-1β. (**B**) Autophagy limits IL-1β production by eliminating active inflammasomes. Induction of AIM2 or NLRP3 inflammasomes triggers the nucleotide exchange of RalB, which promotes autophagosome formation by the binding activated RalB to the exocyst complex subunit Exo84.This binding induces the assembly of active kinase complexes on the exocyst, which are required for the formation and maturation of isolation membranes into autophagosomes. Activation of inflammasomes leads to the ubiquitination of ASC, and ubiquitinated inflammasomes are engulfed by autophagosomes via the autophagic adaptor protein, p62.

complex with Atg5–Atg12 conjugates and induces LC3-PE conjugation (Fujita et al., 2008). Atg16L1-deficient macrophages exhibit enhanced IL-1β and IL-18 production in response to LPS. This process is mediated by TRIF-dependent activation of caspase-1, which, in turn, activates IL-1β production. Moreover, mice lacking Atg16L1 in hematopoietic cells are highly susceptible to dextran sulfate sodium-induced acute colitis, which is alleviated by the injection of anti-IL-1β and IL-18 antibodies (Saitoh et al., 2008). Hence, Atg16L1 plays an important role in negatively regulating endotoxin-induced inflammatory immune responses.

Very recently, an interesting study showed the relationship between autophagy and inflammasome activity. This study suggested that autophagy induced by inflammatory signals targets ubiquitinated inflammasomes for destruction, thereby limiting IL-1β production (**Figure 6B**; Shi et al., 2012). The activation of AIM2 or NLRP3 inflammasomes in macrophages triggers nucleotide exchange on RalB, thereby effecting autophagosome assembly (Bodemann et al., 2011; Shi et al., 2012). Autophagic adaptors such as p62 (sequestosome 1) and neighbor of BRCA1 gene (NBR1), which have ubiquitin-associated (UBA) domains and LC3-interacting regions (LIR), recognize ubiquitinated molecules and facilitate their elimination by autophagy (Johansen and Lamark, 2011; Deretic, 2012). Various kinds of intracellular pathogens are recognized by different kinds of autophagic adaptors and thereby eliminated by autophagy (Dupont et al., 2009; Zheng et al., 2009; Orvedahl et al., 2010; Mostowy et al., 2011). Similarly, in the present study, assembled inflammasomes were ubiquitinated, and these ubiquitinated complexes were recruited by the autophagic adaptor p62, which assisted in their entry into the autophagy pathway. Thus, autophagy limits inflammasome activity by eliminating active inflammasomes, and this tempers inflammation.

The exact mechanisms underlying recognition of dsDNA derived from bacteria or DNA viruses are still unclear, and so are the subsequent immune responses. Nonetheless, it becomes apparent that stimulation with dsDNA induces the production of type I IFNs and other inflammatory cytokines (Stetson and Medzhitov, 2006; Charrel-Dennis et al., 2008). Recent studies indicated that translocation and assembly of stimulator of IFN genes (STING) and TANK-binding kinase 1 (TBK1) are required for the induction of type I IFN responses (Ishikawa and Barber, 2008; Jin et al., 2008; Zhong et al., 2008; Sun et al., 2009). STING is a multispanning membrane protein, which is translocated from the endoplasmic reticulum to the Golgi apparatus after sensing dsDNA, followed by assembly with TBK1, which phosphorylates the transcription factor IRF3, leading to the production of type I IFNs. During this process, Atg9a colocalizes with STING in the Golgi apparatus, where it controls the assembly of STING (Saitoh et al., 2009). In Atg9a-deficient MEFs, but not in Atg7- and Atg16L1-deficient MEFs, the translocation of STING from the Golgi apparatus to the cytoplasmic punctate structures, and its assembly with TBK1, are greatly enhanced. This, in turn, induces aberrant activation of type I IFN responses (Saitoh and Akira, 2010). Overall, these findings underline a role for Atg9a in the regulation of innate immune responses.

CONCLUSIONS

Recent studies have demonstrated that autophagy acts as an important regulator of immune responses. In addition to elimination of intracellular pathogens by its original function of

degradation (xenophagy), autophagy can be involved in innate pathogen recognition and modulate the downstream signaling pathway. Autophagy promotes the clearance of cytosolic pathogens via autophagosome formation, which is elicited by TLR sensing. Autophagic machinery is utilized to enhance phagosome–lysosome fusion and efficiently eliminate extracellular phagocytosed pathogens. Moreover, autophagy can promote adaptive immune responses such as MHC class II-restricted antigen presentation after bacterial sensing via NLRs. In case of viral recognition, autophagy delivers the cytosolic replication intermediates to the lysosomes, where recognition by the endosomal TLRs occurs, thus enhancing the production of type I IFN essential for antiviral response. However, autophagy does not play a role in enhancing pathogen elimination alone. It can also negatively regulate the signaling pathway mediating pathogen recognition and elimination. In case of viral recognition by cytosolic viral sensors such as RLRs, autophagy represses the signaling downstream of the innate immune response, such as that mediating the production of type I IFN. Autophagy-related proteins, including Atg16L1, are also involved in the regulation of endotoxin-induced inflammasome activation, which has been associated with Crohn's disease. Recent advances in the study of autophagy have largely helped understanding the mode of function of the autophagic machinery in PRR-mediated innate pathogen recognition and its regulation. Considering the complexity of autophagy function in immunity, it is still unclear whether activation or suppression of autophagy could have therapeutic benefits in the treatment of infectious diseases or inflammatory disorders such as Crohn's disease. A better understanding of the modulation of the immune system by autophagy is essential to unveil new therapeutic avenues in the future.

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Autophagy and IL-1 family cytokines

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Autophagy is an important intracellular homeostatic mechanism for the targeting of cytosolic constituents, including organelles, for lysosomal degradation. Autophagy plays roles in numerous physiological processes, including immune cell responses to endogenous and exogenous pathogenic stimuli. Moreover, autophagy has a potentially pivotal role to play in the regulation of inflammatory responses. In particular, autophagy regulates endogenous inflammasome activators, as well as inflammasome components and pro-IL-1β. As a result, autophagy acts a key modulator of IL-1β and IL-18, as well as IL-1α, release. This review focuses specifically on the role autophagy plays in regulating the production, processing, and secretion of IL-1 and IL-18 and the consequences of this important function.

Keywords: autophagosome, autophagy, cytokines, inflammasome, inflammation, interleukin, IL-1, IL-18

INTRODUCTION

Autophagy exists in three forms. Microautophagy describes the direct engulfment of small volumes of cytosol by lysosomes (Ahlberg et al., 1982). Alternatively, in chaperone-mediated autophagy specific proteins are recognized by a cytosolic chaperone and targeted to the lysosome (Dice, 1990). This review will focus on macroautophagy (hereafter referred to as *autophagy*), a highly conserved homeostatic mechanism for the lysosomal degradation and recycling of cytosolic components, including macromolecules and organelles (Shintani and Klionsky, 2004). Macroautophagy is characterized by the formation of an isolation membrane, or phagophore, which elongates around its target and fuses with itself to form a double-membraned autophagosome. This can then fuse with lysosomes to form an autolysosome, leading to degradation of the luminal contents.

Autophagy acts as an important survival mechanism, sequestering and degrading damaged/toxic cytosolic constituents, such as dysfunctional mitochondria or peroxisomes. In addition, autophagy regulates energy and nutrient homeostasis and plays an essential role in development (Yang and Klionsky, 2010). Autophagy can be induced by numerous different stimuli, including environmental and cellular stresses, such as nutrient deprivation/amino acid starvation, growth factor withdrawal, and endoplasmic reticulum (ER) stress (Lum et al., 2005; Ogata et al., 2006; Yorimitsu et al., 2006).

Autophagy can also regulate a number of important immune responses, including clearance of intracellular bacteria (Deretic, 2010), antigen presentation (Munz, 2010), and the regulation of cytokine production and secretion (Harris, 2011). In addition, autophagy is important for immune cell homeostasis; deficiencies in the autophagy pathway cause defects in ER and leave T cells more prone to cell death (Jia and He, 2011; Jia et al., 2011) and is required for immunoglobulin production by plasma cells (Pengo et al., 2013). Moreover, autophagy in thymic epithelial cells facilitates the presentation of endogenous self-antigens and is thus important for central $CD4^+$ T cell tolerance (Aichinger et al., 2013).

Importantly, autophagy is induced by numerous immune stimuli, including exogenous pathogen-associated molecular (PAMPs), such as LPS (Xu et al., 2007), as well as endogenous damage-associated molecular (DAMPs), including HMGB1 (Tang et al., 2010a,b). Moreover, cytokines can regulate autophagy; IFN-γ, TNF-α, IL-1α, and IL-1β all induce autophagy in macrophages (Gutierrez et al., 2004; Harris and Keane, 2010; Shi and Kehrl, 2010), while IL-4, IL-13, and IL-10 have all been shown to inhibit autophagy in macrophages (Harris et al., 2007, 2009; Van Grol et al., 2010; Ní Cheallaigh et al., 2011; Park et al., 2011).

AUTOPHAGY REGULATES IL-1α, IL-1β, AND IL-18 SECRETION

Interleukin 1 family cytokines include IL-1α, IL-1β, IL-18, IL-33, IL-36, IL-37, and IL-38 and orchestrate a wide range of immune and physiological roles. In particular, IL-1α, IL-1β, which signal through the same receptor (IL-1R1), have strong pro-inflammatory effects, largely through the induction of cyclooxygenase type 2 (COX-2), type 2 phospholipase A, and inducible nitric oxide synthase (iNOS) (Dinarello, 2002) and are responsible for the recruitment of myeloid cells, including neutrophils, to sites of inflammation (Rider et al., 2011). IL-18 is similarly pro-inflammatory and both IL-1β and IL-18 are tightly regulated; they are produced as inactive pro-forms that are cleaved by caspase-1 to form the mature, bioactive, cytokines. Caspase-1 is itself activated by an inflammasome, a large multimeric structure that includes an intracellular sensor, such as the NOD-like receptor (NLR) NLRP3 or the DNA sensor, absent in melanoma 2 (AIM2) (Davis et al., 2011). IL-1α, while active in its pro-form, has recently been shown to be more potent as a granzyme B-cleaved truncated peptide (Afonina et al., 2011). Recently, studies have suggested that IL-1β can drive the release of both IL-1α and IL-23 (Harris et al., 2008; Fettelschoss et al., 2011), further highlighting the importance of this cytokine in regulating inflammatory responses.

Studies have demonstrated that autophagy can regulate the transcription, processing, and secretion of IL-1β, as well as the secretion of IL-1α and IL-18 (**Figure 1**; **Table 1**). This occurs through at least two distinct mechanisms. Firstly, in macrophages and dendritic cells, inhibition of autophagy, either pharmacologically with 3-methyladenine (3-MA) or through siRNA deletion of autophagy genes, leads to increased release of IL-1β, IL-1α, and IL-18 in response to TLR3 or TLR4 agonists (Saitoh et al., 2008; Harris et al., 2011; Nakahira et al., 2011; Zhou et al., 2011a). Typically, the release of IL-1β is a two stage process. First, transcription of pro-IL-1 is induced by inflammatory stimuli (such as LPS). This is followed by activation of inflammasome assembly by a second stimulus, such as reactive oxygen species (ROS), ATP, particulates (e.g., silica, alum), protein aggregates, or lysosomal disruption. Thus, the inhibition of autophagy results in the accumulation of a second, endogenous, inflammasome-activating stimulus. The second mechanism is more direct; autophagosomes can sequester and degrade inflammasome components, including the adaptor molecule apoptosis-associated speck-like protein containing a CARD (ASC), AIM2, and NLRP3 (Shi et al., 2012), as well as pro-IL-1β(Harris et al., 2011). Studies have not yet addressed whether autophagy might regulate other IL-1 family cytokines, including IL-33, IL-36, and IL-37, but this would be of considerable interest given role in mediating the production and release of IL-1 and IL-18, as well as other cytokines (Saitoh et al., 2008; Crisan et al., 2011; Harris et al., 2011; Nakahira et al., 2011).

A ROLE FOR MITOCHONDRIA

Two studies have addressed the role of mitochondria in driving the release of IL-1β by autophagy-compromised cells. Zhou et al. (2011a) demonstrated that inhibiting the sequestration of mitochondria by autophagosomes (mitophagy) with 3-MA leads to the accumulation of damaged, ROS-producing mitochondria, which in turn activates the NLRP3 inflammasome, leading to the processing and release of IL-1β. Similarly, Nakahira et al. (2011) established that depletion of the autophagy proteins beclin 1 or LC3B in macrophages leads to the activation of caspase-1 and the release of IL-1β and IL-18 by promoting the accumulation of dysfunctional mitochondria. Moreover, secretion of both cytokines was dependent on mitochondrial DNA (mtDNA), which translocated to the cytosol, a process dependent on ROS and NLRP3. These data would suggest that mitochondrial dysfunction represents an endogenous stimulus for inflammasome activation. Studies have demonstrated that enhanced IL-1β release by autophagy-deficient mouse cells is dependent on

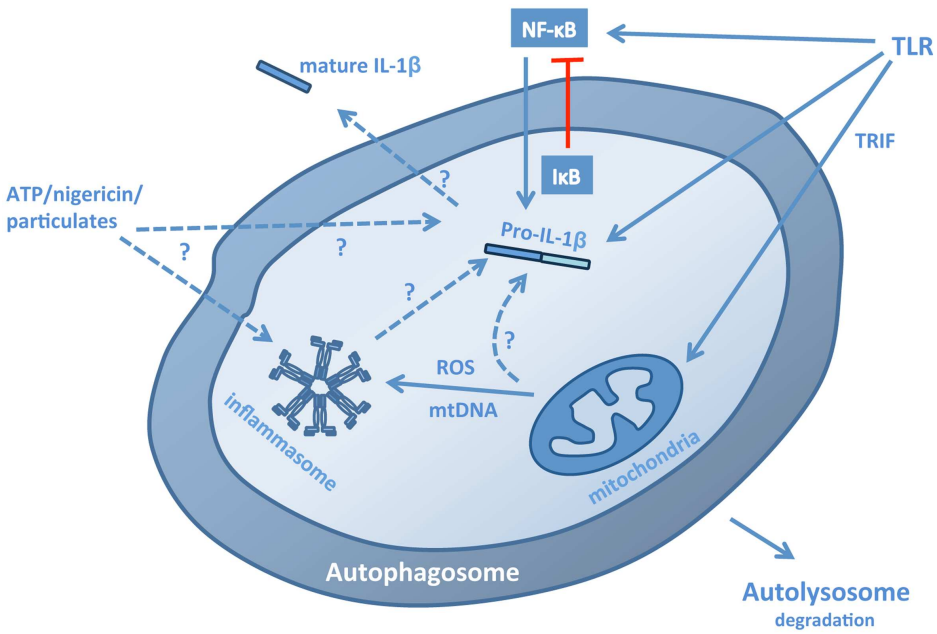

FIGURE 1 | Pathways for the regulation of IL-1β by autophagy. Autophagy regulates IL-1β production, processing, and secretion through a number of mechanisms. In the absence of autophagy, stimulation of macrophages or dendritic cells with TLR3 or TLR4 ligands leads to a TRIF-dependent, mitochondrial ROS/DNA-dependent increase in the production, processing, and secretion of IL-1β, suggesting that autophagy normally limits the presence of these stimuli in the cytosol. These stimuli induce inflammasome assembly, but may also increase transcription of pro-IL-1β. Conversely, induction of autophagy in cells stimulated with TLR ligands leads to the sequestration and lysosomal degradation of pro-IL-1β, thus limiting the availability of the cytokine for subsequent processing and secretion. In addition, the inflammasome components ASC, NLRP3, and AIM2 can also be sequestered into autophagosomes. The effects of autophagy on transcription of pro-IL-1β are complex: autophagy down-regulates p62, which may be required for NF-κB activation, but also down-regulates IκB, promoting NF-κB nuclear translocation. Induction of autophagy in the presence of inflammasome-activating stimuli, such as ATP, nigericin and particulates, and crystals, can lead to increased secretion of IL-1β suggesting that autophagosomes may act as part of an exocytic pathway and possibly also a platform for inflammasome assembly, although it is not yet clear whether fully assembled inflammasomes are sequestered.

Table 1 | Interactions between autophagy and IL-1 family cytokines.

Interaction	Notes	Reference
IL-1α and IL-1β induce autophagy	Induction of autophagy by IL-1 family cytokines suggests a potential negative feedback loop for the control of inflammation by autophagy, as well as a possible anti-microbial response mediated by inflammatory cytokines	Shi and Kehrl (2010)
Inhibition of autophagy increases IL-1α, IL-1β, and IL-18 secretion	Inhibition of (or deficiency in) autophagy leads to increased secretion of IL-1 family cytokines in response to TLR ligands and *Mycobacterium tuberculosis*. This is dependent on TRIF (in mice), reactive oxygen species, and mitochondrial DNA. These results suggest that autophagy normally regulates endogenous factors that would otherwise induce inflammasome assembly, caspase-1 activation, and subsequent processing and secretion of IL-1β and IL-18. In humans, regulation of IL-1β by autophagy occurs at the transcriptional level	Saitoh et al. (2008), Crisan et al. (2011), Harris et al. (2011), Kleinnijenhuis et al. (2011), Nakahira et al. (2011), Zhou et al. (2011a)
Induction of autophagy reduces IL-1β secretion *in vitro* and *in vivo*	Induction of autophagy with drugs (e.g., rapamycin) or starvation reduces secretion of IL-1β by macrophages and dendritic cells in response to LPS with alum or ATP or in response to *Mycobacterium tuberculosis*. Induction of autophagy also decreases intracellular levels of pro-IL-1β. In a mouse model of LPS-induced sepsis, rapamycin decreases serum levels of IL-1β.	Crisan et al. (2011), Harris et al. (2011), Shi et al. (2012)
Autophagosomes sequester and degrade IL-1β and inflammasome components	In macrophages stimulated with TLR ligands, autophagosomes sequester IL-1β. The inflammasome component ASC is ubiquitinated in response to inflammasome activation and delivered to autophagosomes. NLRP3 and AIM2 are also sequestered by autophagosomes	Harris et al. (2011), Shi et al. (2012)
Activation of the NLRP3 and AIM2 inflammasomes induces autophagosome formation	Induction of the AIM2 inflammasome by transfection of macrophages with poly(dA:dT) or the NLRP3 inflammasome with uric acid crystals or nigericin leads to an increase in autophagosome formation	Shi et al. (2012)
Autophagy can act as secretory pathway for the release of IL-1β	Induction of inflammasome activation in parallel with autophagy induction can lead to an increase in IL-1β secretion. This novel secretory pathway is dependent on inflammasome assembly, Atg5, Rab8a, and GRASP55 (GORASP2; a mammalian Golgi reassembly stacking protein paralog)	Dupont et al. (2011)

TIR-domain-containing adapter-inducing interferon-β (TRIF), an adaptor molecule involved in TLR3 and TLR4 signaling (Saitoh et al., 2008; Harris et al., 2011). However, the role of TRIF in this response is not yet known. In addition, autophagy-deficient NLRP3$^{-/-}$ dendritic cells are still able to secrete IL-1β in response to LPS (albeit at much lower levels than wild type control cells) (Harris et al., 2011), suggesting that other inflammasomes may be activated by mitochondrial instability, at least in mice.

Inhibition of autophagy with 3-MA has also been shown to limit IL-1β transcription in humans through a process independent of caspase-1 activation (Crisan et al., 2011; Kleinnijenhuis et al., 2011). While the mechanism underlying these observations is not yet clear, Lee et al. (2012) have demonstrated that autophagy down-regulates p62, which is important for IL-1β signaling and activation of NF-κB, which leads to increased IL-1β production. However, this is potentially complicated by a number of studies that have demonstrated that autophagy is required for NF-κB activation, as autophagosomes target ubiquitinated IκB for degradation, allowing increased nuclear transcription of NF-κB (Meng and Cai, 2011; Criollo et al., 2012; Jia et al., 2012). Interestingly, in mice, inhibition of autophagy, either with 3-MA or by siRNA targeting of beclin 1, had no effect of IL-1β transcription (Peral de Castro et al., 2012), suggesting important differences between mice and humans in the mechanism through which autophagy regulates IL-1β.

AUTOPHAGY AND THE INFLAMMASOME

While inhibition of autophagy leads to increased release of IL-1β and IL-18 (as well as IL-1α) in response to TLR3 or TLR4 ligands, induction of autophagy with the mTOR inhibitor rapamycin can inhibit IL-1β release in response to LPS with alum or ATP (Harris et al., 2011). Moreover, activation of IL-1β transcription with TLR agonists, in the absence of an inflammasome-inducing signal, leads to sequestration and degradation of pro-IL-1β by autophagosomes. This process is independent of TRIF (Harris et al., 2011) and would suggest that autophagy acts a self-regulatory mechanism for the control of inappropriate and potentially deleterious inflammatory responses. More recently, Shi et al. (2012) have demonstrated that activation of the NLRP3 and AIM2 inflammasomes induces autophagy in macrophages. Inhibition of autophagy with 3-MA increased inflammasome activation, while induction of autophagy with rapamycin or amino acid starvation limited it. Moreover, inflammasome components, including

AIM2, NLRP3, and ASC partially co-localized with GFP-LC3 (an autophagosomal marker) and LAMP-1 (a lysosomal marker) (Shi et al., 2012), suggesting that inflammasomes are degraded within autophagosomes. Interestingly, caspase-1 does not co-localize with GFP-LC3 (Harris et al., 2011), suggesting that sequestration of inflammasome components by autophagosomes is a highly specific process. Again, these data suggest that autophagy represents a regulatory mechanism for the control of inflammatory responses in macrophages.

A recent report has demonstrated that caspase-11 can contribute to NLRP3-dependent IL-1β secretion in a TRIF-dependent manner in response to Gram-negative bacteria. In this response, TRIF activates caspase-11 via type I IFN signaling, which in turn interacts with the NLRP3 inflammasome to regulate caspase-1 activation (Rathinam et al., 2012). It is not yet clear whether autophagy intersects with this TRIF-dependent pathway to regulate caspase-11-dependent IL-1β secretion.

However, the role of autophagy in the regulation of inflammasome activation may not be quite so straightforward. A recent study has demonstrated that induction of autophagy with mTOR inhibitors or by amino acid starvation can lead to increased IL-1β secretion in response to inflammasome-activating treatments, including LPS with nigericin, alum, or silica crystals (Dupont et al., 2011). This effect was partially dependent on Atg5 and at least one of the two mammalian Golgi reassembly stacking protein paralogs, GRASP55 and Rab8a. In these experiments, autophagy was induced at the same time that the inflammasome-activating stimulus was added. Thus, the role of autophagy in regulating IL-1β secretion may depend on timing and context; in the absence of an inflammasome-activating signal, autophagy may act to remove pro-IL-1β and inflammasome components from the cell, while in the presence of such a signal, autophagy may act as a secretory pathway for IL-1β release.

IL-1α AND IL-1β INDUCE AUTOPHAGY

Numerous cytokines are known to regulate autophagy in macrophages, including IFN-γ (Gutierrez et al., 2004), TNF-α (Harris and Keane, 2010), IL-10 (Van Grol et al., 2010; Park et al., 2011), IL-4, and IL-13 (Harris et al., 2007). Amongst those that have been shown to activate autophagosome formation are IL-1α and IL-1β (Shi and Kehrl, 2010; Peral de Castro et al., 2012). Moreover, other cytokines associated with inflammatory responses, including IL-23, have been shown to drive autophagy (Peral de Castro et al., 2012). Thus autophagy may represent an important mechanism in a negative feedback loop to control the secretion of inflammatory cytokines.

FURTHER CONSEQUENCES OF AUTOPHAGIC REGULATION OF IL-1: EFFECTS ON IL-23 AND IL-17

The regulation of IL-1β release by macrophages and DC subsequently affects IL-23 secretion by the same cells; inhibition of autophagy with 3-MA or by depletion of beclin 1, leads to an increase in IL-23 secretion, driven directly by IL-1β, while induction of autophagy with mTOR inhibitors reduces IL-23 secretion (Peral de Castro et al., 2012). Together, IL-1 (α or β, or IL-18) and IL-23 potently induce the secretion of IL-17 by Th17 cells and innate γδ T cells (Sutton et al., 2009; Mills et al., 2013). Thus, supernatants from LPS-stimulated autophagy-deficient dendritic cells and macrophages, high in IL-1α, IL-1β, IL-18, and IL-23, have been shown to stimulate the secretion of IL-17 by T cells, predominantly γδ T cells (Peral de Castro et al., 2012). This is also relevant *in vivo*, as mice lacking the autophagy protein Atg5 in myeloid cells secrete higher levels of IL-1α, IL-12p70, CXCL1, and IL-17 in response to infection with *Mycobacterium tuberculosis* (Castillo et al., 2012).

AUTOPHAGY AND INFLAMMATION *IN VIVO*

In humans, single nucleotide polymorphisms (SNPs) in the autophagy-related protein 16-like 1 (*atg16l1*) gene have been linked with increased susceptibility to Crohn's disease (Hampe et al., 2007; Rioux et al., 2007), while Mice lacking Atg16L1 in hematopoietic cells are more susceptible to dextran sulfate sodium (DSS)-induced colitis (Saitoh et al., 2008). Similarly, polymorphisms in the genes encoding other autophagy-related proteins, including Atg2a, Atg4a, Atg4d, Immunity-related GTPase M (IRGM), and ULK-1, have also been associated with Crohn's disease (Craddock et al., 2010; Henckaerts et al., 2011; Brinar et al., 2012). IRGM is a known modulator of autophagy in human macrophages (Singh et al., 2006), while the mouse ortholog, Irgm1 (formerly LRG-47), modulates IFN-γ-induced autophagy (Gutierrez et al., 2004). Moreover, polymorphisms in IRGM have been linked to the multifactorial autoimmune disease systemic lupus erythematosus (SLE) (Zhou et al., 2011b), as have polymorphisms in Atg5 and Atg7 (Harley et al., 2008; Gateva et al., 2009; Han et al., 2009; Zhou et al., 2011b). In mice with a conditional deletion of Atg7 in the intestinal epithelium, LPS induces higher levels of IL-1β mRNA, compared to wild type controls (Fujishima et al., 2011), while LC3B$^{-/-}$ mice produce higher levels of IL-1β and IL-18 in response to LPS- or cecal ligation and puncture (CLP)-induced sepsis (Nakahira et al., 2011). Moreover, autophagy has a role to play in obesity-related inflammation in mice and humans. Expression of LC3 is higher in the subcutaneous adipose tissues of obese mice and humans, compared to lean controls and correlated with systemic insulin resistance and adipose tissue inflammation (Jansen et al., 2012). In addition, inhibition of autophagy with 3-MA increased the expression of IL-1β, IL-6, and IL-8 mRNA in human adipose tissue explants and IL-1β, IL-6, and keratinocyte-derived chemoattractant (KC) mRNA in mouse explants and this effect was greater in samples from obese individuals/animals (Jansen et al., 2012).

CONCLUSION

Autophagy is a highly conserved and ubiquitous process that has many roles to play in cellular homeostasis. Amongst these is the regulation of inflammatory responses to both pathogenic and endogenous stimuli. In particular, autophagy modulates the transcription, processing, and secretion of IL-1β, acting as an important negative feedback mechanism for the control of inflammatory responses, both *in vitro* and *in vivo*. As such, autophagy may represent a potent target for novel anti-inflammatory therapeutics.

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Autophagy and mechanisms of effective immunity

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Macroautophagy (autophagy) is a cellular pathway facilitating several critical functions. First, autophagy is a major pathway of degradation. It enables elimination of microbes that have invaded intracellular compartments. In addition, it promotes degradation of damaged cellular content, thereby acting to limit inflammatory signals. Second, autophagy is a major trafficking pathway, shuttling content between the cytosol and the lysosomal compartment. Given these two key roles, autophagy can have significant and sometimes unexpected consequences on mechanisms that initiate robust immunity. Here, we will discuss the impact of autophagy on pathways of innate and adaptive immune responses including microbe elimination, inflammatory cytokine production, antigen processing and T and B lymphocyte immunity.

Keywords: autophagy, innate immunity, adaptive immunity, antigen presentation, T lymphocytes

Macroautophagy (referred herein as autophagy) is a process that involves the formation of an autophagosome, a double membrane vesicle, which is trafficked to lysosomes where autophagosomal contents are degraded. Autophagy is considered both a major pathway of degradation, providing an alternative to the proteasome, and a significant trafficking pathway between the cytosol and lysosomal compartments. Activating autophagy mostly occurs via serine/threonine kinase, mammalian target of rapamycin (mTOR) that impedes autophagy by binding and inactivating the UNC-51-like kinase (ULK)1/2 kinase. Signaling via the ULK1/2 kinase complex is critical for recruiting autophagy-related gene (ATG) proteins to the site of autophagosome biogenesis (Jung et al., 2010; Weidberg et al., 2011). One ATG of particular importance is Atg8 (microtubule-associated light chain 3, LC3 in mammals), a widely used marker of autophagosomes. LC3 acts in autophagosome expansion and closure (Nakatogawa et al., 2007), in addition to selectively recruiting autophagosomal cargo (Noda et al., 2008). Prior to association with the autophagosome, LC3 is conjugated to phosphatidylethanolamine by a series of reactions that involves Atg7, Atg3 and an Atg5–Atg12–Atg16 complex (Tanida et al., 2004). Other critical contributors to autophagy are phosphatidylinositol kinase Vsp34 and Beclin 1, that promote autophagosomal membrane and fusion events (Liang et al., 2008; Matsunaga et al., 2009; Zhong et al., 2009). Here, we will discuss examples of the contribution of the autophagy pathway to innate (**Figure 1**) and adaptive (**Figure 2**) effector mechanisms that are critical for robust immunity.

AUTOPHAGY AND INNATE EFFECTOR MECHANISMS

Autophagy is an innate effector mechanism that eliminates intracellular pathogens. Autophagy is induced downstream of signaling by "danger" receptors including Toll like receptors (TLR; Xu et al., 2007; Delgado et al., 2008), retinoic acid inducible gene I-like receptors (RLR; Yano et al., 2008; Cooney et al., 2010; Travassos et al., 2010), and alarmins (Tang et al., 2010). "Xenophagy" describes the process by which intracellular microbes are selectively degraded by autophagy (Levine, 2005). Xenophagy eliminates bacteria that access the cytosol including *Streptococcus pyogenes* (Nakagawa et al., 2004) and *Mycobacterium tuberculosis* (Gutierrez et al., 2004). Vacuolar bacteria are also targeted. Following RAW cell infection with *M. tuberculosis* var. *bovis* bacillus Calmette–Guerin (BCG), autophagy facilitates the fusion of mycobacteria containing vacuoles with late endosomes/lysosomes to promote mycobacteria degradation (Singh et al., 2006). *Salmonella*-containing vacuoles are targeted by LC3 (Birmingham et al., 2006) and can be engulfed by autophagosomes (Kageyama et al., 2011). Interestingly, recruitment of autophagy machinery to damaged bacterial vacuoles involves the exposure of host sugar residues that attract receptor galectin 8 (Thurston et al., 2012). Xenophagy also facilitates removal of viral pathogens. One example is the degradation of the Sindbis virus capsid by the autophagy pathway (Orvedahl et al., 2010). Autophagy can also impair viral replication by trafficking cytosolic viral replication products to the endolysosomal compartment where they are detected by TLR and a type I interferon (IFN) response is triggered. This is the case for vesicular stomatitis virus (VSV) infection of plasmacytoid dendritic cells (pDC; Lee et al., 2007). Given its role in anti-viral defense, many viruses encode proteins to disable autophagy. Gammaherpes virus 68 (Ku et al., 2008), influenza A virus (Gannage et al., 2009), herpes simplex virus-1 (HSV-1; Orvedahl et al., 2007), human cytomegalovirus (Chaumorcel et al., 2012), and human immunodeficiency virus (HIV; Kyei et al., 2009) are examples of viruses that encode proteins to block autophagy initiation by interfering with beclin 1 activity. Other interesting examples of viral interference with the autophagy pathway include Kaposi's sarcoma-associated herpesvirus encoded Fadd-like interleukin-1 beta-converting enzyme (FLICE)-like inhibitor protein that blocks interaction between LC3 and Atg4 (Lee et al., 2009) and HIV nef that prevents autophagosomal maturation (Kyei et al., 2009). Like viruses, bacteria also subvert the autophagy pathway. *L. monocytogenes*, via the expression of ActA, recruits host proteins to its surface to disguise itself from autophagic

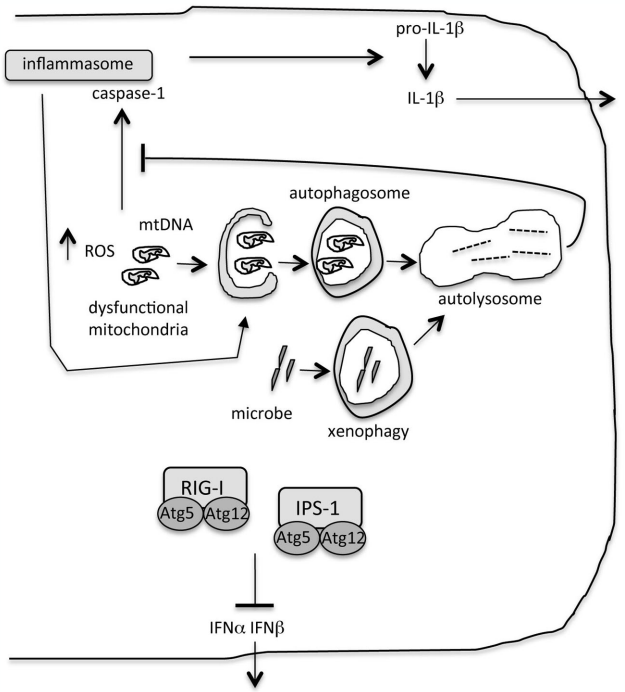

FIGURE 1 | Role of autophagy during innate immunity. "Xenophagy" eliminates intracellular microbes. Autophagy limits inflammatory cytokine production by ensuring removal of dysfunctional organelles, including mitochondria. Accumulation of damaged mitochondria results in increased reactive oxygen species (ROS) and the escape of mitochondrial DNA into the cytosol. These are triggers of the inflammasome and interleukin-1β (IL-1β) production. Inflammasomes are targeted for degradation by autophagy. Proteins in the autophagy machinery bind to and inhibit signaling of key proteins in the type I interferon pathway, retinoic acid inducible gene-I (RIG-I) and the signaling molecule IFNβ promoter stimulator-1 (IPS-1).

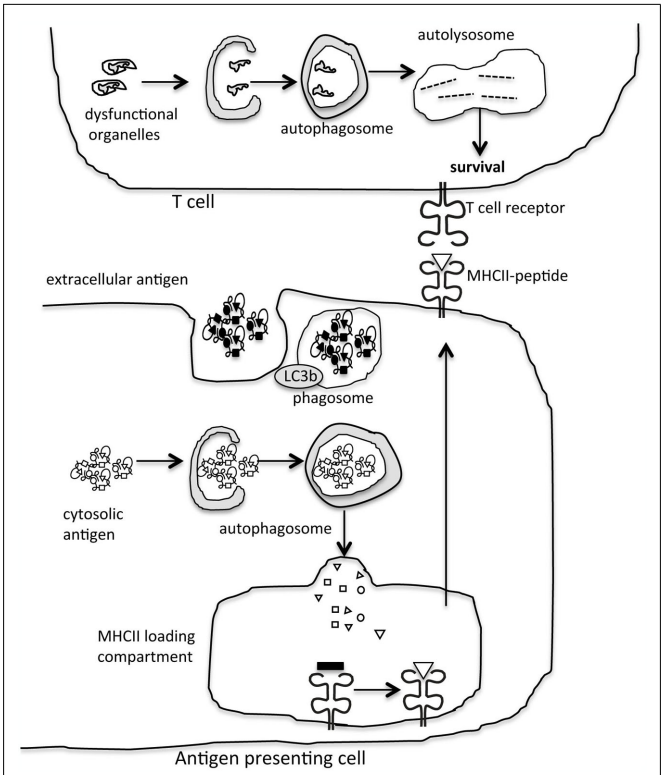

FIGURE 2 | Autophagy modulation of adaptive immunity. Autophagy is a significant trafficking pathway for the delivery of cytosolic antigen to the MHCII loading compartment in antigen presenting cells. In addition, proteins in the autophagy machinery contribute to phagocytosis of extracellular antigen. In lymphocytes, the role of autophagy in eliminating damaged organelles promotes cell survival.

recognition (Yoshikawa et al., 2009). Similarly, *Shigella* hides from the autophagy machinery by expressing IcsB, that inhibits Atg5 binding to the *Shigella* surface (Ogawa et al., 2005). In summary, autophagy is a major mode of innate immune defense against intracellular pathogens.

Autophagy participates in the control of pro-inflammatory cytokine cascades. Many examples illustrate a shutdown in autophagy results in excessive inflammatory cytokine production. This is particularly well described for interleukin (IL)-1β and IL-18, where the absence of autophagy exacerbates their secretion by murine macrophages when stimulated with pro-inflammatory compounds. Specifically, this has been described for *beclin1*$^{+/-}$ (Nakahira et al., 2011), *LC3b*$^{-/-}$ (Nakahira et al., 2011), *Atg16L1*$^{-/-}$ (Saitoh et al., 2008), and *Atg7*$^{-/-}$ (Saitoh et al., 2008) macrophages or following expression of inactive Atg4B or due to treatment with the chemical autophagy inhibitor 3-methyladenine (3-MA; Saitoh et al., 2008; Harris et al., 2011). Enhanced secretion of IL-1β also occurs when human peripheral blood mononuclear cells are stimulated in the presence of an impaired autophagy pathway due to treatment with 3-MA (Crisan et al., 2011). Conversely, stimulation of murine DC with rapamycin suppresses IL-1β production in response to inflammatory stimuli (Harris et al., 2011). Rapamycin administration *in vivo*, inhibits the detected increase in IL-1β but not IL-12, in the serum 4 h following lipopolysaccharide (LPS) treatment (Harris et al., 2011). Not all stimuli provoke excessive IL-1β secretion in the absence of autophagy. Stimuli that do include LPS, or treatment of LPS-primed macrophages with adenosine 5'-triphosphate (ATP) or monosodium urate (MSU). *Salmonella typhimurium* or muramyl dipeptide, a nucleotide-binding oligomerization domain-containing protein 2 (NOD2) ligand, do not provoke such a response (Saitoh et al., 2008). Ligands that engage TLR4 and to some extent those recognized by TLR3, TLR7, and TLR9, but not TLR2 or TLR5, elicit enhanced IL-1β in autophagy-deficient macrophages (Saitoh et al., 2008). In murine macrophages, excessive cytokine production following autophagy shutdown requires TIR-domain containing adapter inducing IFNβ (TRIF), an adapter molecule for TLR signaling (Saitoh et al., 2008), while in human cells the response is dependent on p38 mitogen activated protein kinase (MAPK) phosphorylation (Crisan et al., 2011). Excessive IL-1β secretion in the absence of autophagy is not associated with alterations in NFκB activation (Saitoh et al., 2008). Importantly, exaggerated inflammatory cytokine production in the absence of autophagy translates to the development of severe disease *in vivo*. *Atg16L1*-deficient mice display enhanced inflammatory infiltrates in the distal colon, elevated IL-1β, and IL-18 serum levels and reduced survival in a dextran

sulfate sodium induced model of experimental colitis (Saitoh et al., 2008). *LC3b*-deficient mice exhibit enhanced susceptibility to LPS-induced lethality (Nakahira et al., 2011), while both *LC3b*-deficient and *beclin1*$^{+/-}$ mice have increased IL-1β and IL-18 serum levels in response to cecal ligation and puncture-induced sepsis (Nakahira et al., 2011). In addition to IL-1β/IL-18, the type I IFN pathway is also susceptible to modulation by autophagy. Again the absence of a functional autophagy pathway promotes amplified secretion of type I IFN in response to a given stimulus. VSV infection of *Atg5*-deficient murine embryonic fibroblasts (MEF) elicits increased production of IFNα, IFNβ, IL-6, and IP-10 and as such dramatically attenuates viral replication rendering cells resistant to infection (Jounai et al., 2007; Lee et al., 2010). Another example is *Atg9a*-deficient MEF that exhibit enhanced IFNβ production in response to stimulation with inflammatory double stranded DNA (Saitoh et al., 2009). Therefore, autophagy has the potential to exert a critical influence on the regulation of pro-inflammatory cytokine production and downstream effector mechanisms of innate immunity. The ability of autophagy to limit inflammatory cytokine production renders it a target pathway for pathogens to activate as a strategy to facilitate replication. Hepatitis C virus (HCV) is an example of a pathogen that is proposed to do this. If HCV infection occurs in the absence of a functional autophagy pathway, HCV viral replication is severely attenuated due to the corresponding activation of the type I IFN pathway. Therefore, HCV infection elicits active autophagy in the infected cell, limiting type I IFN production and enabling successful viral replication (Ke and Chen, 2011). Triggering autophagy is therefore a putative mode of immune escape.

How does autophagy act to regulate inflammatory cytokine activity? Secretion of IL-1β/IL-18 is a tightly regulated process. Pro-inflammatory stimuli induce the transcription of inactive precursors pro-IL-1β or pro-IL-18. Cleavage of the inactive proteins into their active secreted forms requires the assembly of an inflammasome, a large protein complex that, under inflammatory conditions, processes pro-caspase-1 to caspase-1. Active caspase-1 then cleaves pro-IL-1β and pro-IL-18 liberating the active cytokines for secretion by the cell. In mouse cells, the levels of precursor IL-1β are not altered following LPS stimulation in the absence of autophagy (Saitoh et al., 2008), rather there is an increase in the cleaved form of caspase-1 (Saitoh et al., 2008; Nakahira et al., 2011). In contrast, in human cells, transcription of IL-1β is elevated (Crisan et al., 2011). Autophagy exerts its influence on IL-1β and IL-18 secretion by several mechanisms. A major role for autophagy in inflammatory cytokine secretion is considered to be via its contribution to maintaining a healthy intracellular environment. Namely, autophagy has a pivotal role in the clearance and elimination of damaged and dysfunctional organelles. Studies have demonstrated autophagic degradation of depolarized mitochondria (Sandoval et al., 2008), expanded endoplasmic reticulum (Bernales et al., 2006), mature ribosomes (Kraft et al., 2008; Kundu et al., 2008), and excess peroxisomes (Dunn et al., 2005). The role of autophagy in the maintenance of mitochondrial integrity is a process known as "mitophagy." In the absence of functional mitophagy, cells accumulate damaged mitochondria that produce high levels of reactive oxygen species (ROS), a side product of cellular respiration (Zhou et al., 2011). The accumulation of damaged mitochondria is further exacerbated following stimulation with inflammatory compounds as observed for LC3b or beclin 1-deficient macrophages treated with LPS plus ATP (Nakahira et al., 2011). Without the autophagy pathway to remove these dysfunctional organelles, ROS levels increase (Saitoh et al., 2008), a known trigger for the nucleotide binding domain, leucine rich-containing family, pyrin domain containing 3 (NLRP3) inflammasome (Zhou et al., 2011). Damaged mitochondria also display increased membrane permeability with mitochondrial DNA (mtDNA) then being able to translocate to the cytosol. mtDNA is detected in higher abundance in the cytosol of LC3b and beclin 1-deficient macrophages, most notably following stimulation with LPS plus ATP (Nakahira et al., 2011). As such, autophagy, by acting to maintain turnover of damaged organelles and preserving mitochondrial integrity, indirectly restricts excessive inflammatory cytokine production by removing endogenous triggers of the inflammasome.

The second mechanism by which autophagy controls inflammatory cytokine activity is through its direct interaction with proteins involved in inflammatory cascades. In this case, proteins are targeted by autophagosomes or the autophagy machinery. Interestingly, inflammasomes are engulfed and degraded by autophagy. Following inflammasome induction, components of the absent in melanoma 2 (AIM2) inflammasome associate with p62 and beclin1 and exhibit K63-linked ubiquitination (Shi et al., 2012). Other examples of the direct targeting of inflammatory proteins by autophagy include the co-localization of the autophagosomal protein LC3 and IL-1β in punctate autophagosomal structures following stimulation of bone marrow-derived macrophages with a variety of TLR ligands including LPS, Pam3Cys, poly (I:C), R837, or CpG (Harris et al., 2011). The authors suggest that autophagy limits IL-1β secretion by facilitating its degradation in the autolysosome. A direct role for autophagy in modulating components of the type I IFN response is illustrated by several examples where the autophagy machinery targets proteins involved in this signaling cascade. Atg5–Atg12 directly interacts with the cytosolic viral RNA sensor RIG-I and the signaling molecule IFNβ promoter stimulator-1 (IPS-1). Interaction with Atg5–Atg12 blocks caspase recruitment domain (CARD)-mediated signaling and the downstream production of type I IFN (Jounai et al., 2007). Another interesting example is stimulator of interferon genes (STING), a molecule critical for type I IFN signaling. Atg9a is critical for the localization of STING to cytoplasmic punctae following treatment of cells with dsDNA (Saitoh et al., 2009). Mislocalization of STING to the endoplasmic reticulum results in exacerbated IFN responses (Saitoh et al., 2009), although the specific details of why this is the case remain to be elucidated.

Not all inflammatory cytokine production is enhanced in the absence of functional autophagy. In contrast to IL-1β, IL-18 and type I IFN, mouse tumor necrosis factor (TNF)α and IL-16 (Harris et al., 2011), and human TNFα (Crisan et al., 2011) production is inhibited when autophagy is suppressed. This is the case for macrophages following stimulation with LPS or Pam3Cys in the presence of 3-MA. Another example is the reduced production of IL-8 by intestinal epithelial cells with silenced *Atg7* (Li et al., 2011b). The mechanism of why these cytokine pathways require

autophagy may involve autophagic inhibition of p38 phosphorylation (Crisan et al., 2011). Therefore the role of autophagy in modulating cytokine output is complex with its contribution varying for individual cytokines.

Another mechanism by which autophagy modulates inflammation is by restricting the expression of extracellular inflammatory signals. During programmed cell death, autophagy enables the generation of a cellular corpse that is efficiently phagocytized and less likely to elicit detrimental inflammatory outcomes. This is elegantly illustrated in a model of embryonic cavitation, where apoptotic embryonic stem cells lacking *Atg5* or *beclin1* fail to exhibit critical engulfment signals (Qu et al., 2007). These include failing to expose phosphatidylserine (PS) at the outer membrane and lack of secretion of the chemoattractant lysophosphatidylcholine. As a consequence, autophagy-deficient cellular corpses are not engulfed for clearance by immune cells. The failure to eliminate autophagy-deficient apoptotic corpses is implicated in the neonatal death of *Atg5*-deficient mice. Excess apoptotic corpses are detected in the lungs and retina, together with the presence of an abnormal inflammatory infiltrate in these tissues. Interestingly, $Atg5^{-/-}$ (Uhl et al., 2009), $beclin1^{+/-}$ (Li et al., 2008), or $Atg12^{-/-}$ (Li et al., 2008) cells loaded with antigen are less efficient at facilitating DC antigen presentation to $CD8^+$ T cells (cross-presentation). This is postulated to be due to their poor engulfment. The requirement for autophagy in the display of engulfment signals is thought to be due to the pathway's role in maintaining cellular bioenergetics. Restoration of ATP levels in autophagy-deficient cells by treatment with methylpyruvate restores corpse clearance (Qu et al., 2007). Induction of autophagy in dying cells also stimulates the release of immune modulator high mobility group B1 (HMGB1; Thorburn et al., 2009) that promotes immunogenicity and removal of apoptotic corpses (Scaffidi et al., 2002).

AUTOPHAGY AND ADAPTIVE IMMUNITY

Proteolysis provides the peptide epitopes displayed by major histocompatibility complex (MHC) molecules to elicit T cell responses. All nucleated cells utilize the proteasome, a multiproteolytic complex located in the cytosol that generates ligands for MHCI presentation. In MHCII expressing cells, lysosomal proteases generate ligands for these molecules. Autophagy participates in generating the pool of peptides displayed by MHCII molecules due to its role in trafficking cytosolic proteins to the lysosomes, the same compartment where biosynthesis and loading of MHCII molecules occurs. Indeed, the autophagosomal protein LC3 co-localizes with MHCII loading compartments in DC (Schmid et al., 2007). Fusion of influenza matrix protein M1 (Schmid et al., 2007) or the hemagglutinin (HA) site 1 epitope (Comber et al., 2011) to LC3 enhances their presentation by MHCII to $CD4^+$ T cells as a result of the intersection of autophagosomes and MHCII loading compartments. Autophagy contribution to MHCII antigen presentation is further supported by detailed analysis of the steady state MHCII repertoire where up to a third of peptides displayed by MHCII are shown to be derived from cytoplasmic or nuclear sources (Chicz et al., 1993; Dongre et al., 2001) and would potentially rely on autophagy trafficking to access the MHCII loading compartment. Induction of autophagy increases the relative display of these peptides compared to those derived from membrane proteins (Dengjel et al., 2005). Peptides derived from LC3b and gamma-aminobutyric acid receptor-associated protein (GABARAP), components of the autophagy machinery are eluted from MHCII molecules (Dengjel et al., 2005). There is now a growing list of antigens that are documented as being trafficked by autophagy for MHCII display. These include self proteins together with virus and bacterial-derived antigens (Munz, 2009). The role of autophagy in presenting self antigen by MHCII, means that autophagy contributes to $CD4^+$ T cell selection in the thymus. Studies in which $Atg5^{-/-}$ thymi were grafted into normal recipients elegantly illustrate that autophagy trafficking in thymic epithelial cells participates in the display of self antigen by MHCII (Nedjic et al., 2008). Transplant of *Atg5*-deficient thymi into MHCII-restricted TCR transgenic hosts results in reduced positive selection of HA and SEP-specific $CD4^+$ TCR T cells. Selection of AND, DO11.1, or DEP-specific $CD4^+$ TCR transgenic T cells, on the other hand, proceeds efficiently in the absence of autophagy. Autophagy is therefore considered to facilitate the thymic MHCII presentation of some self proteins but not others. In doing so, autophagy contributes to the complex composition of the repertoire of MHCII epitopes that are displayed by the thymic epithelium. The role for autophagy in thymic selection is further evidenced by an increase in $IE\alpha_{52-68}$–IA^b complexes at the surface of cortical thymic epithelial cells that lack *Atg5* (Nedjic et al., 2008). In this case, it is postulated that the removal of autophagy dependent endogenous epitopes due to the absence of *Atg5*, reduces competition and increases access of the IEα peptide to MHCII. In addition to autophagy shaping the $CD4^+$ T cell repertoire that emerges from the thymus, it also participates in effective $CD4^+$ T cell immunity during infection. Autophagy dependent-MHCII presentation of cytosolic model antigens has been described for complement C5 (Brazil et al., 1997), tumor antigen mucin 1 (Dorfel et al., 2005) and neomycin phosphotransferase (Nimmerjahn et al., 2003) where presentation is inhibited following treatment with 3-MA. Nuclear antigen 1 of Epstein-Barr virus (EBNA-1) is a prominent example of a viral-derived antigen that requires autophagy for presentation by MHCII (Paludan et al., 2005). Treatment with 3-MA or silencing of *Atg12* inhibits EBNA-1 specific $CD4^+$ T cells from recognizing a lymphoblastoid cell line (Paludan et al., 2005). Antigen presenting cell types that utilize autophagy to deliver antigen for MHCII presentation include B cells (Brazil et al., 1997), macrophages (Brazil et al., 1997), and DC (Schmid et al., 2007; Jagannath et al., 2009; Lee et al., 2010). Autophagy-mediated delivery of antigen for MHCII presentation to $CD4^+$ T cells means that triggering autophagy represents a promising immunotherapeutic strategy to promote robust $CD4^+$ T cell responses. Indeed, immunization with rapamycin-treated DC, enhances MHCII presentation and $CD4^+$ T cell immunity to the anti-tuberculosis BCG vaccine antigen (Jagannath et al., 2009). This is an exciting outcome, with major implications for improving vaccine efficacy in multiple settings.

Whether autophagy exhibits selectivity in the substrates targeted is currently not well understood. This is important if the autophagy pathway is to be manipulated for delivery of antigen to MHCII. While basal autophagy initiated under starvation conditions is considered to be a non-selective process, autophagy

initiated under steady state conditions in immune cells is considered to be selective. A general observation is that long lived proteins are considered to be turned over by lysosomal proteolysis and hence are autophagy substrates, while the proteasome degrades short lived polypeptides (Henell et al., 1987). Aggregates of long lived poly-ubiquitinated proteins are known substrates of autophagy (Mizushima and Klionsky, 2007). Of interest, protein aggregates form in activated DC (Lelouard et al., 2002) and in macrophages following TLR4 stimulation (Fujita et al., 2011). Several "autophagy receptors" have been described that act to recruit ubiquitinated proteins to autophagosomes via an LC3-interacting motif (LIR). Known autophagy receptors identified to date include p62/sequestosome1 (Komatsu et al., 2007), neighbour of Brca1 (NBR1) (Kirkin et al., 2009), nuclear dot protein 52kDa (NDP52) (Thurston et al., 2009), and optineurin (Wild et al., 2011) with all of these proteins possessing binding domains for both ubiquitin (UBD) and LC3 (LIR). The interplay of these receptors in targeting autophagy substrates is beginning to begin explored. For example, p62, optineurin, and NDP52 all target ubiquitinated *Salmonella enterica* for destruction in lysosomes (Wild et al., 2011). While optineurin and NDP52 colocalize, p62 binds to a separate subdomain of the ubiquitinated bacteria. This implies selective recognition of ubiquitin chains by specific autophagy receptors. How upstream events regulate the activity of autophagy receptors is mostly unknown. Interestingly, optineurin is subject to phosphorylation by tank binding kinase (TBK)1, providing the molecular trigger to promote autophagic clearance of cytosolic bacteria (Wild et al., 2011). Identification of a growing number of autophagy receptors is exciting with the detail of the specific E3 ubiquitin ligases involved, the selective recognition of ubiquitin chains and their specific regulation by phosphorylation all remaining to be further studied.

Somewhat unexpectedly, in addition to assisting the display of cytosolic antigen by MHCII molecules, autophagy also contributes to the presentation of extracellular antigen and phagocytosis. Individual components of the autophagy machinery are recruited to phagosomes in the presence of TLR signaling. During a process termed "LC3-associated phagocytosis (LAP)" (Sanjuan and Green, 2008), LC3 is recruited to latex-bead phagosomes in macrophages, when beads are coupled with the TLR agonists LPS or Pam3Cys or in response to phagosomes containing killed yeast particles (zymosan; Sanjuan et al., 2007). LAP also contributes to the phagocytosis of apoptotic, necrotic, or receptor interacting kinase-3 (RIPK3)-necrotic cells with this response being triggered by the T cell immunoglobulin and mucin domain containing molecule (TIM)4 receptor (Martinez et al., 2011). Entosis, the engulfment of live cells, also involves localization of LC3 to the entotic vacuole (Florey et al., 2011). The recruitment of LC3 to phagosomes requires other components of the autophagy machinery given that it does not take place in the absence of *Atg5* (Sanjuan et al., 2007; Martinez et al., 2011), *Atg7* (Sanjuan et al., 2007; Martinez et al., 2011), or *beclin-1* (Martinez et al., 2011). The role of autophagy in extracellular phagocytosis and MHCII antigen presentation is evident given that *Atg5*-deficient DC show impaired MHCII presentation of soluble and cell-associated antigen and elicit impaired $CD4^+$ T cell responses in response to antigens derived from herpes simplex virus-2 (HSV-2; Lee et al., 2010). Infection of *Atg5*-deficient mice with HSV-2 results in a failure of *Atg5*-deficient mice to mount protective $CD4^+$ T cell immunity and enhanced disease progression occurs (Lee et al., 2010). Autophagy also contributes to exogenous processing and MHCII presentation of HIV-1-derived protein (Blanchet et al., 2010). The autophagy machinery is considered to contribute to phagocytosis by facilitating phagosome to lysosome fusion and the delivery of lysosomal proteases to phagosomes, required for effective antigen degradation. Extracts of phagosomes isolated from *Atg5*-deficient DC displayed impaired cathepsin (Cat) S, CatB/L activity when assayed with fluorogenic substrates (Lee et al., 2010).

A contribution of autophagy to MHCI antigen presentation is less obvious, given that the major proteolytic source of peptide generation for MHCI is the proteasome and not lysosomal proteases. Indeed, there are several reports discarding a role for autophagy in MHCI presentation of antigen derived from extracellular sources (cross-presentation; Blanchet et al., 2010; Lee et al., 2010). Its role in MHCI presentation of cytosolic antigen is less well studied. One report demonstrates the requirement for autophagy in macrophage MHCI presentation of glycoprotein B (gB), a HSV-1-derived protein (English et al., 2009). In this case, treatment with 3-MA or silencing of *Atg5* reduced presentation of gB by macrophages late after infection. HSV-1 gB is proposed to be trafficked from the nuclear envelope to the autophagosome from where its escapes for degradation by the proteasome and MHCI display. Whether this applies only to HSV-1 infection, macrophages, or is relevant to other antigens or cell types, remains to be elucidated. Another example is the cross-presentation of antigen loaded αAl_2O_3 nanoparticles (Li et al., 2011a). MHCI presentation of αAl_2O_3 nanoparticles loaded with OVA is inhibited following 3-MA treatment or silencing of *Atg12* or *beclin1*. The mechanism is unknown, however internalized nanoparticles are shown to access autophagosomes that are considered to be immunogenic (Li et al., 2008). Autophagy may also impact MHCI presentation by degrading MHCI molecules themselves (Li et al., 2010).

An important contribution of autophagy to adaptive immunity is its cell intrinsic role in lymphocyte survival. An active autophagy pathway operates in T cells, with autophagosomes detected, particularly following activation via the T cell receptor (TCR) (Espert et al., 2006a; Li et al., 2006; Pua et al., 2007). Analysis of T cell compartments in *Atg5*-deficient mice illustrates a role for autophagy in mature T cell survival (Pua et al., 2007). While thymus cellularity is reduced in the absence of *Atg5*, thymocyte development is unperturbed. In the periphery, reduced numbers of $CD4^+$ and $CD8^+$ T cells are present in the spleen and lymph node. The $CD8^+$ T cell compartment is more affected, with a large proportion of *Atg5*-deficient $CD8^+$ T cells staining with Annexin V, indicative of cells undergoing early apoptosis. Both $CD4^+$ and $CD8^+$ T cells that survive in the absence of *Atg5* display proliferative defects following TCR stimulation, with fewer cells undergoing division, and fewer divisions completed in response to anti-CD3 compared with wildtype T cells. The proliferative defect is not rescued by anti-CD28 or IL-2 and the cells display normal levels of TCR and upregulate T cell activation markers CD69 and CD25. A similar phenotype is observed in $Atg7^{fl/fl}$Lck-Cre mice where the specific deletion of *Atg7* in the T cell lineage results in mostly normal thymocyte development, however $CD4^+$ and $CD8^+$ T cell numbers

are greatly reduced in the peripheral lymphoid compartment. Both subsets of peripheral T cells display evidence of apoptosis; with a large proportion staining with annexin V and exhibiting caspase 9 activity. Impaired T cell survival in the absence of autophagy is likely to arise from its pivotal role in maintaining intracellular organelle integrity. *Atg7*-deficient T cells possess increased numbers of mitochondria and increased ROS levels (Pua et al., 2009). For the B cell compartment, the absence of *Atg5* impairs B cell development in the bone marrow, with reduced survival of pre-B cells and consequently reduced numbers of mature B-1a cells in the periphery (Miller et al., 2008). In some settings autophagy facilitates lymphocyte death, rather than survival. Examples include autophagy-mediated T cell death following growth factor withdrawal (Li et al., 2006) and following binding of the HIV-1 envelope protein to C-X-C chemokine receptor 4 (Espert et al., 2006b). Therefore, autophagy serves as a critical pathway in lymphocytes that facilitates the intricate balance between survival and death.

AUTOPHAGY AND DISEASE

The critical impact of autophagy on effector functions of innate and adaptive immunity outlined herein, is highlighted by the association of autophagy gene mutations with increased incidence of inflammatory disease, susceptibility to microbial infection and autoimmune disease. A prominent example is Atg16L1, a susceptibility gene for Crohn's disease (Massey and Parkes, 2007), where the small intestine exhibits chronic inflammation triggered by a breakdown in the clearance of commensal bacteria. Atg16L1-deficient Paneth cells possess elevated pro-inflammatory cytokine transcripts, while *Atg16L1*-deficient mice develop exacerbated intestinal inflammation following environmental triggers, similar to that observed in human patients (Cadwell et al., 2008). Other examples include cystic fibrosis, a chronic inflammatory disease of the pulmonary airways, where autophagy-mediated maintenance of lung epithelial cell homeostasis is important to limit lung inflammation and disease (Luciani et al., 2010). Autophagy is also postulated to contribute to inflammation-associated responses underlying metabolic diseases, such as obesity (Yang et al., 2010). In infectious disease settings, mouse models illustrate a contribution of autophagy to a growing number of microbial pathogens. IRGM, a human immunity-related GTPase that plays a critical role in regulating autophagy-mediated clearance of mycobacteria, is a genetic risk factor for tuberculosis (Intemann et al., 2009). Finally, a link between single nucleotide polymorphisms in the Atg5 gene and susceptibility to systemic lupus erythematosus, a debilitating autoimmune disease has been reported (Gateva et al., 2009). The emergence of often surprising roles for autophagy in diseases of the immune system means that a wide range of immune-related diseases may now be amenable to targeting by autophagy modulating drugs. As such studies of autophagy in innate and adaptive pathways provide excellent insights into this complex pathway, uncovering new roles for autophagy in disease.

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Antigen cross-priming of cell-associated proteins is enhanced by macroautophagy within the antigen donor cell

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Phagocytosis of dying cells constitutes an important mechanism of antigen capture for the cross-priming of $CD8^+$ T cells. This process has been shown to be critical for achieving tumor and viral immunity. While most studies have focused on the mechanisms inherent in the dendritic cell that account for exogenous antigen accessing MHC I, several recent reports have highlighted the important contribution made by the antigen donor cell. Specifically, the cell stress and cell death pathways that precede antigen transfer are now known to impact cross-presentation and cross-priming. Herein, we review the current literature regarding a role for macroautophagy within the antigen donor cell. Further examination of this point of immune regulation is warranted and may contribute to a better understanding of how to optimize immunotherapy for treatment of cancer and chronic infectious disease.

Keywords: autophagy, cross-priming, antigen presenting cells, dendritic cells, cell death, tumor immunity, viral immunity

INTRODUCTION

Significant evidence for an indirect pathway for the loading of MHC class I molecules has emerged over the last 35 years (Bevan, 1976; Rock et al., 1990). The most compelling data comes from *in vivo* experiments in mouse models demonstrating that viral, tumor, and histocompatibility antigens can be transferred from MHC-mismatched donor cells to host bone marrow derived conventional dendritic cells (cDCs), and elicit antigen-specific CTL responses that are restricted to self MHC molecules (Falo et al., 1995; Reis e Sousa and Germain, 1995; Sigal et al., 1999; Mellman and Steinman, 2001; Boon et al., 2006; Petersen et al., 2010). Bevan (1976) originally coined this phenomenon "cross-priming," as antigen is "crossing the MHC barrier" that had initially been invoked in the generation of MHC class I peptide epitopes. As it is now understood that the activation of naïve T cells is a property restricted to cDCs, these *in vivo* observations offered a solution to the question of how $CD8^+$ T cells are activated for the targeting of cells which express antigen that is not directly expressed by cDCs. Examples of such antigen include tumor-restricted proteins and viruses which do not infect professional antigen presenting cells (APCs; e.g., human papillomavirus; Fausch et al., 2003). While these observations indicate that the immune system possesses a natural mechanism by which exogenous antigens may access MHC I molecules of APCs, there remains much to be discovered regarding the mechanisms of antigen transfer.

Our *in vitro* studies and the *in vivo* work of others demonstrated that immature cDCs are capable of capturing antigen derived from internalized dying cells and cross-presenting donor antigen on MHC I molecules for engagement of $CD8^+$ T cells (Albert et al., 1998, 2001; Kurts et al., 2010; Pang and Neefjes, 2010; Flinsenberg et al., 2011). cDC trafficking of tissue-restricted antigen derived from internalized dying cells has been demonstrated for models of gut-, skin-, and pancreas-restricted protein antigen (Huang et al., 2000; Belz et al., 2002; Scheinecker et al., 2002; Turley et al., 2003). In the latter model system, the use of transgenic mice expressing inhibitors of apoptosis in beta cells and the *in vivo* injection of biochemical modulators of death pathways have confirmed the critical role for cell death in both antigen transfer and T cell activation (Hugues et al., 2002; Turley et al., 2003; Giodini and Albert, 2010; Locher et al., 2010; Flinsenberg et al., 2011).

Over the last decade, there has been an explosion of information regarding cell stress and cell death. These death pathways may synergize and/or compete, each vying to deliver the fatal blow. Importantly, the mechanisms of cell stress and cell death are now recognized as critical determinants of the subsequent immune response – impacting trafficking of the APC, altering the antigenic repertoire that is transferred upon phagocytosis and influencing the cDC activation state (Albert, 2004). While most studies have focused on apoptotic cell death vs. necrotic cell death, there is increasing awareness that macroautophagy, within the antigen donor cell, influences the outcome of cross-presentation. Herein we focus on the ability of DCs to capture and cross-present cell-associated antigen, reviewing in detail the recent evidence for macroautophagy in the donor cell as an important mechanism for facilitating antigen delivery to cDCs.

MACROAUTOPHAGY AND ANTIGEN PRESENTATION

Macroautophagy (referred to herein as autophagy) has been defined as an "auto-digestive" process that promotes the delivery of intracytosolic components to lysosomal or vacuolar compartments for terminal degradation and recycling (Deretic and Levine, 2009; **Figure 1**). Autophagy has distinct roles in different cellular contexts and occurs at a basal level in all nucleated cells. Constitutive autophagy is important for the turnover of unfolded proteins

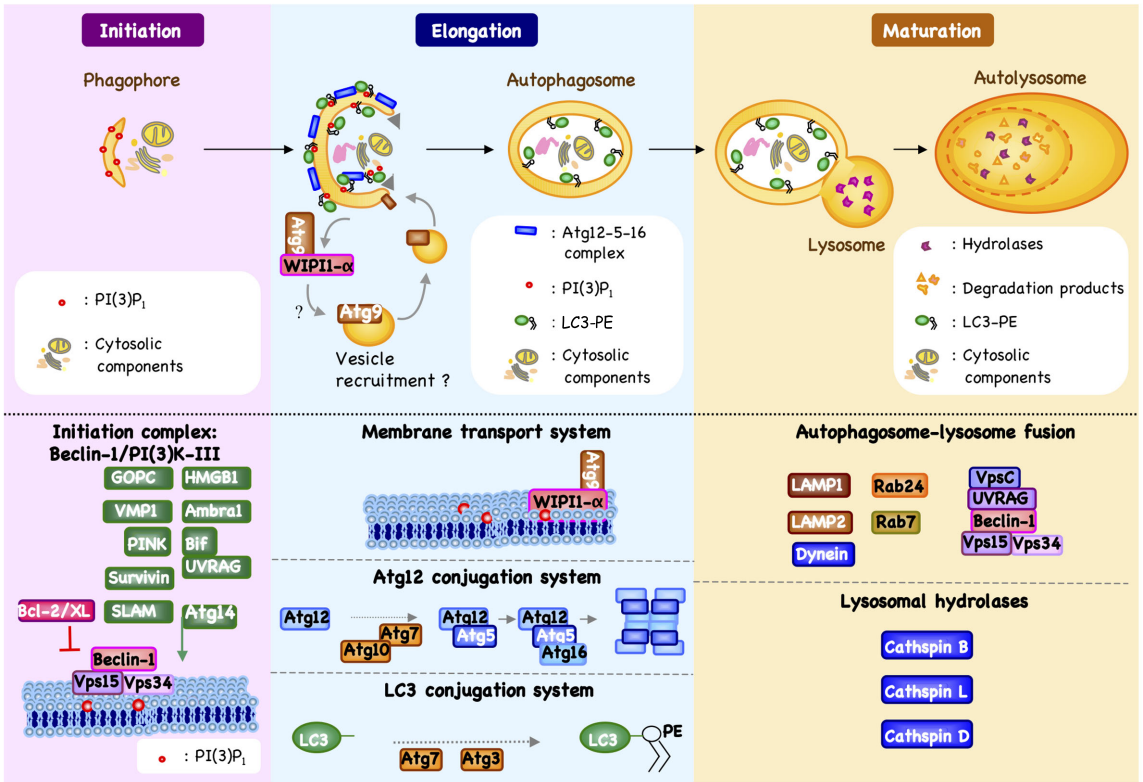

FIGURE 1 | Machinery of autophagy. Beclin-1/PI3K-III complex activation, which is regulated by different mechanisms, results in the formation of phosphatidylinositol 3-phosphate [PI(3)P1] and the induction of an autophagic vesicle, which is characterized by a double-membrane, and termed an autophagosome (Crotzer and Blum, 2010; Mehrpour et al., 2010; Kang et al., 2011). Two ubiquitin-like systems have been shown essential for autophagosome formation. In the first, autophagy-related gene-12 (Atg12) is conjugated to Atg5, together forming a complex with Atg16L1, which decorate the outer membrane of the isolation membrane. Microtubule-associated protein 1 light chain-3 (LC3, also known as Atg8) constitutes the second ubiquitin-like system and conjugates phosphatidylethanolamine (PE) at the outer and inner autophagosomal membrane. Unlike the Atg12/Atg5/Atg16L1 complex that is recycled by the protease Atg4, the LC3-PE (referred to as LC3-II) remains associated with the inner membrane of autophagosome (Mehrpour et al., 2010). The incorporation of phospholipid into the autophagosome membrane is essential for its elongation, and regulates the membrane transport system. Autophagosome maturation is characterized by the formation of an autolysosome, the product of fusion with the lysosome.

or damaged organelles and maintains cellular homeostasis (Kroemer et al., 2010). For example, autophagy is important for reducing oxidative stress by selectively targeting damaged mitochondria (Wang and Klionsky, 2011; Mai et al., 2012). Similarly, endoplasmic reticulum (ER), peroxisomes, ribosomes, protein aggregates, and even intracellular pathogens may be eliminated *via* autophagy (Yu et al., 2008; Joubert et al., 2009; Komatsu and Ichimura, 2010; Mizushima and Komatsu, 2011). Autophagy is also considered as part of the host response to cellular stress, including nutrient deprivation or adenosine triphosphate (ATP) depletion. Under such conditions, autophagy protects cells by supplying recycled nutrients to support essential cellular process until restoration of homeostasis. Autophagy induced by cellular stress is generally considered to be a non-selective, bulk degradative process.

More recently, autophagy has been recognized as an important modulator of host immunity, with a particular role for the processing of antigen for presentation by MHC and the initiation of adaptive immune responses (Dengjel et al., 2005; Deretic and Levine, 2009; Crotzer and Blum, 2010). Specifically, autophagy has been described to participate in the translocation of endogenous protein into the MHC class II loading compartment, which facilitates MHC class II presentation and $CD4^+$ T cells activation (Dengjel et al., 2005). As one key example, it has been demonstrated that the constitutive degradation of cellular components through autophagy provides a critical source of self antigen in the thymus for education of $CD4^+$ T cell precursors (Nedjic et al., 2008). In addition to $CD4^+$ T cell activation, there is now evidence to suggest a role for autophagy – or at least autophagic genes – in the generation of MHC I/peptide complexes, within the APC. Based on early studies using silencing of *Atg12* or *Atg5*, or pharmacological inhibitors, it was argued that autophagy does not have a major impact on MHC class I presentation of multiple endogenous or exogenous antigens (Nimmerjahn et al., 2003; Paludan et al., 2005; Lee et al., 2010). Moreover, conjugation of a viral epitope to LC3 did not alter MHC class I presentation of this epitope (Schmid et al., 2007). Recent data however, has challenged these conclusions, at least in the context of selected model systems. Interestingly, inhibition of autophagy in IFN-γ-treated

B16 mouse melanoma cells diminished MHC class I protein surface expression and tumor cell cytolysis by $CD8^+$ T lymphocytes (Li et al., 2010). MHC class I presentation by these tumors was proteasome dependent, suggesting a possible connection between autophagy and the conventional pathway for MHC class I presentation. In another study, the group of Desjardins has highlighted an unconventional autophagy pathway that modulates MHC class I presentation in macrophages infected by Herpes simplex virus (HSV; English et al., 2009). Early after infection (6–8 h), viral capsid antigen presentation in infected macrophages occurs *via* the conventional MHC class I pathway, but during the late stages of infection (8–12 h), viral capsid presentation is dependent on acidic organelles as well as *Atg5* expression. This study also reported that HSV-infected cells contain specific and unconventional $LC3^+$ vesicles that are closely associated with the nuclear envelope. This led the authors to suggest that virus infection induced a novel form of autophagy, which may contribute to the escape of antigen into the cytosol.

CONNECTING AUTOPHAGY AND CROSS-PRIMING

While basal or induced autophagy in APCs does not seem to be strongly implicated in their ability to cross-present exogenous antigen, some early evidence suggested a role for autophagy within the antigen donor cell. In 2002, a study from Vile and colleagues suggested for the first time that autophagic vacuoles within the donor cell could be correlated with efficient cross-priming of associated-antigens (Bateman et al., 2002). Viral fusogenic membrane glycoprotein (FMGs) had been known to kill solid tumor cells after the formation of large multinucleated syncytia. The authors observed that FMG-mediated killing of syncytia was not dependent on a classical apoptosis pathway. Instead, death of syncytia was associated with nuclear fusion and premature chromosome condensation as well as severe ATP depletion (Bateman et al., 2002). Interestingly, cytoplasmic vacuoles were demonstrated to be acidic, suggesting that the death of syncytia was correlated with autophagic activity. In the same study, the authors demonstrated that FMG-mediated death was accompanied by release of vesicles reminiscent of exosomes, which they called syncitiosomes. Importantly, dying syncytia produced significantly more syncitiosomes than normal cells or cells killed by pro-apoptotic or pro-necrotic signals, including irradiation, freeze thaw, or osmotic shock (Bateman et al., 2002). These syncitiosomes served as an efficient source of antigen for cDCs, out-performing classical exosomes, or dead cell corpses. Lacking from this study, however, was the mechanistic association of Atg proteins with death of syncytia and effective cross-priming.

An additional clue linked autophagy to the removal of apoptotic corpses in mouse embryoid bodies (EBs) and during chick retinal development (Qu et al., 2007; Mellen et al., 2008). In both studies, autophagy is required for dying cells to have sufficient energy to generate the engulfment signals necessary for the clearance of corpses by phagocytes. Indeed, EBs derived from cells lacking autophagy genes (e.g., *atg5* or *beclin-1*), failed to express "eat-me" signal such as phosphatidylserine (PS) exposure, and secreted lower levels of "come-get-me" signals, such as lysophosphatidylcholine (LPC; Qu et al., 2007). These defects were associated with low levels of cellular ATP and could be reversed by treatment with the metabolic substrate, methyl pyruvate. Similar results were observed after treatment of retinas with 3-methyladenine (3-MA), a pharmacological inhibitor of autophagy (Mellen et al., 2008). Extrapolation of these studies might suggest another mechanism by which autophagy regulates cross-presentation. Indeed, the role of autophagy in metabolism may be generally relevant for phagocyte function, and aspects of innate and adaptive immune responses.

AUTOPHAGY WITHIN TUMOR CELLS FAVORS CROSS-PRIMING OF TUMOR-ASSOCIATED ANTIGEN-SPECIFIC $CD8^+$ T CELLS

Seminal studies from Hu and colleagues illustrated a direct role for autophagy in the cross-priming of associated-antigen (Li et al., 2009). In their initial studies, HEK 293T cells expressing ovalbumin (V-TfR-GFP-OVA) or melanoma cells that endogenously express the gp100 tumor antigen were used as antigen donor cells (ADC). The authors showed that autophagy in ADC regulates the efficiency of cross-presentation both *in vitro* and *in vivo* (Li et al., 2009). Inhibition of autophagy with 3-MA or siRNA knockdown of the essential autophagic genes *beclin-1* and *Atg12*, demonstrated that early steps of autophagy – including initiation and elongation of the double-membrane structure, sequestration of cytosolic antigens, and formation of autophagosomes – were required for efficient antigen cross-presentation. Providing additional support, drug, or stress-induced autophagy (i.e., treatment with rapamycin or starvation) resulted in enhanced cross-priming of OVA or gp100-specific $CD8^+$ T cells. In these studies, it is important to note that late steps of autophagy, including late lysosomal fusion and degradation, seemed to have little impact on antigen cross-presentation (Li et al., 2009). Perhaps most interestingly, autophagosomes were isolated, purified, and exposed to DCs, demonstrating efficient delivery of antigen and cross-presentation (Li et al., 2009). Future studies will be required to confirm that contaminating microsomes or secreted exosomes did not contribute to antigen transfer in these experiments.

More recently, the same group observed that isolated autophagosomes from dying tumor cells expressed not only long-lived protein – well know to be sequestered in autophagosomes – but also short-lived proteins (SLiPs), including defective ribosomal initiation products (DRiPs; Yewdell et al., 1996; Li et al., 2011). Immunization with these autophagosomes – named "Dribbles" by the authors to refer to DRiPs-containing blebs – were effective in initiating tumor immunity and inducing the regression of 3LL Lewis lung tumors as well as delaying growth of B16F10 melanoma. Strikingly, DRibbles were more potent than GM-CSF gene modified tumor cells. Importantly, DRibble-derived antigen processing by cDCs was mediated by classical components of the MHC I processing machinery, including TAP1 and proteasome, and it did not involve the lysosomal pathway of the cDC (Li et al., 2011). These observations suggested that DRibble-derived antigen must be released into the cytosol after phagocytosis to be processed by the classical MHC class I pathway. Although mechanisms by which DRibbles could favor antigen cross-presentation remain to be confirmed, these studies were the first to identify capture of autophagosome-associated antigen as a mechanism for achieving tumor immunity.

VIRUS-MEDIATED ABORTIVE AUTOPHAGY ENHANCES ANTIGEN CROSS-PRIMING

Studies from our own laboratory have also identified an important role for autophagy within the ADC, serving to enhance antigen cross-priming of $CD8^+$ T cells (Uhl et al., 2009). The experimental system employed permitted direct comparison between two forms of programmed cell death (PCD): (i) the classical caspase-dependent apoptosis, which it occur in wild-type (WT) mouse embryonic fibroblast (MEFs); and (ii) caspase-independent cell death, which occurs with increased features of autophagy, and achieved experimentally through the use of $Bax^{-/-}/Bak^{-/-}$ MEFs (Shimizu et al., 2004). Both cell types were infected with influenza A virus as a source of antigen, followed by UV irradiation – serving to both inhibit viral replication and induce genotoxic stress – and injected *in vivo* as a source of antigen for studying the efficiency of cross-priming (Uhl et al., 2009). Interestingly, mice immunized with cells undergoing enhanced autophagy showed a significantly higher $CD8^+$ T cell response specific for both $HA_{518–526}$ (in Balb/c hosts) and $NP_{366–374}$ (in C57BL/6 hosts). Strikingly, silencing of the essential autophagic gene *Atg5* in both WT and $Bax^{-/-}/Bak^{-/-}$ MEFs inhibited antigen cross-priming. Careful evaluation of influenza infected WT MEFs indicated that viral infection induced accumulation of autophagosomes, now known to be a result of influenza M2-inhibition of autophagosome/lysosome fusion (Gannage et al., 2009). Notably, influenza infection is capable of inducing both autophagy and apoptosis; different from other triggers of cell stress and cell death, these two processes can be found simultaneously within the same cell (de la Calle et al., 2011). We argue that the finding of double positive cells – co-labeled with anti-caspase 3 antibodies and harboring LC3 punctae – is a result of abortive autophagy. This is supported by studies using chloroquine (de la Calle et al., 2011), which interestingly, has also been shown to enhance antigen cross-priming (Accapezzato et al., 2005), and is currently being tested in combination with rapamycin as a means of inducing *in situ* tumor immunity (Amaravadi et al., 2011). Additional work is required in order to establish abortive autophagosomes as the critical source of antigen for facilitating efficient transfer from donor cells to cDCs.

AUTOPHAGY MAY FAVOR RELEASE OF "IMMUNOGENIC" PROTEINS

The first established link between autophagy genes and pro-inflammatory responses was established in plasmacytoid DCs (pDCs), with the demonstration that *Atg5* is involved in autophagy-mediated delivery of TLR7 agonists from the cytosol of infected cells into the lumen of the endosome, thus accounting for induction of type I interferon (IFN; Lee et al., 2007). Somewhat paradoxically, several studies have shown that the absent or hypomorphic expression of autophagic genes in certain cell types can result in enhanced production of type I interferon or other cytokines; including pro-inflammatory molecules such as IL1β and IL-18, as well as adipocytokines, such as leptin and adiponectin (Jounai et al., 2007; Cadwell et al., 2008; Saitoh et al., 2008; Tal et al., 2009). Thus, autophagy machinery could have a dual function in regulating cytokine production, acting not only to stimulate antiviral type I IFN responses in pDCs, but may also limit excess innate immune activation in other cell type, including fibroblasts. Alternatively, autophagy genes may differentially regulate distinct PRRs: enhancing TLR engagement through the delivery of ligands into the endosome; while inhibiting cytosolic sensors through direct or indirect mechanisms.

Defining how autophagy alters the inflammatory milieu is an important issue, as type I IFNs has been shown to regulate antigen cross-priming (Jounai et al., 2007; Uhl et al., 2009; Wei et al., 2010). Using the same model as previously described, our group has demonstrated that mice immunized with $Bax^{-/-}/Bak^{-/-}$ dying cells – undergoing high level of autophagy – induced in cDCs a significantly higher type I IFN production in cDCs as compared to mice immunized with WT MEFs (Uhl et al., 2009). Supporting a role for type I IFN, it was shown that immunization of mice deficient for the IFNα/β receptor (IFNAR) resulted in a dramatically reduced cross-priming response (Uhl et al., 2009). The mechanism by which autophagy within dying cells favors the production of type I IFN by APCs remains to be defined; one possibility is that viral nucleic acids present in the autophagic corpse serves to engage sensors within the phagocytic cDC (Schulz et al., 2005).

In addition to pro-inflammatory cytokines, other "immunogenic proteins" may be released during autophagic processes, which in turn favor cross-priming. For example, the high-mobility group box 1 protein (HMGB1) provides an interesting connection between autophagy and cross-priming. HMGB1 is a highly conserved chromatin-binding protein that facilitates DNA bending and promotes transcription (Maruyama, 2011). In addition to its intra-nuclear role, HMGB1 also functions as an extracellular signaling molecule and can interact with at least three different surface receptors that are expressed on APC, namely the receptor for advanced glycosylation (RAGE), TLR2, and TLR4 (Nogueira-Machado et al., 2011). The binding of HMGB1 to TLR4 may not be a direct interaction as recent data indicates that HMGB1 chaperones LPS (Yang et al., 2012), as well as other PRR ligands (Yanai et al., 2009). Indeed, the role of HMGB1 as a co-factor for PAMPs may be central to its role in stimulating the processing and presentation of derived antigens. Importantly, secretion of HMGB1 by dying tumor cells has been shown to inhibit fusion of the APC's phagosome with lysosomes, thereby preventing rapid degradation of tumor antigens and enabling processing and presentation onto MHC I (Apetoh et al., 2007). Furthermore, Scaffidi et al. (2002) showed that HMGB1 participates in the recruitment of phagocytes. Indeed, $HMGB1^{-/-}$ cells have a greatly reduced ability to promote inflammation, which indicates that the release of HMGB1 can signal the demise of a cell to its neighbors. While initial studies indicated that HMGB1 is released (passively) from necrotic cells and that during apoptosis HMGB1 becomes hypoacetylated and remains bound to the chromatin of the dying cell (Sims et al., 2010), the biology now seems a bit more complex. Recent work from Ferguson and colleagues indicate that the redox state, in addition to the acetylation state, may impact the bioactivity of HMGB1 (Kazama et al., 2008). ROS mediated oxidation of HMGB1 inhibited its pro-inflammatory potential. The authors went on to demonstrate that oxidation was caspase-dependant, acting via the cleavage of mitochondrial components of the electron transport system (Kazama et al., 2008). Based on the ability of autophagic processes to limit accumulation of ROS, it is interesting

to consider that this may contribute to the pro-inflammatory effects of autophagy within dying ADC. This hypothesis is supported by evidence that HMGB1 released from autophagic tumor cells is immune stimulatory (Thorburn et al., 2009). Inhibition of autophagy resulted in HMGB1 retention, and in the induction of caspase-mediated cell death. The mechanism by which autophagy regulates secretion of HMGB1 remain unknown, but seems to be occurring in a manner similar to other leaderless cytokines. Indeed, exogenous HMGB1 can modulate the future of tumor cells and reduced HMGB1 induced pro-survival autophagy *via* the activation of RAGE receptor and beclin-1 whereas oxidized HMGB1 favored activation of caspase-9 and -3 that lead to apoptotic cell death (Tang et al., 2010). Thus, autophagy could favor the release of reduced HMGB1 by limiting cytoplasmic ROS level; in turn reduced HMGB1 may increase autophagy in neighboring cells as well as activate the recruitment of immune cells, stimulate cytokines secretion, and facilitate antigen cross-priming.

Other immunogenic signals may also be regulated by autophagy, including exposure of PS and secretion of LPC (Qu et al., 2007; Mellen et al., 2008). Recently, the groups of Zitvogel and Kroemer established a link between autophagy and ATP release during chemotherapy treatment (Michaud et al., 2011). This study showed that autophagy is dispensable for chemotherapy-induced cell death but required for its immunogenicity both *in vitro* and *in vivo*. In response to chemotherapy, autophagy-competent, but not *Atg5* or *Atg7*-shRNA transfected tumors, were capable of recruiting cDCs and T cells into the tumor micro-environment, via ATP mediated chemoattraction. Although this work did not formally show T cell cross-priming, it helped establish a new concept related to autophagy regulation of immunity. Further investigation

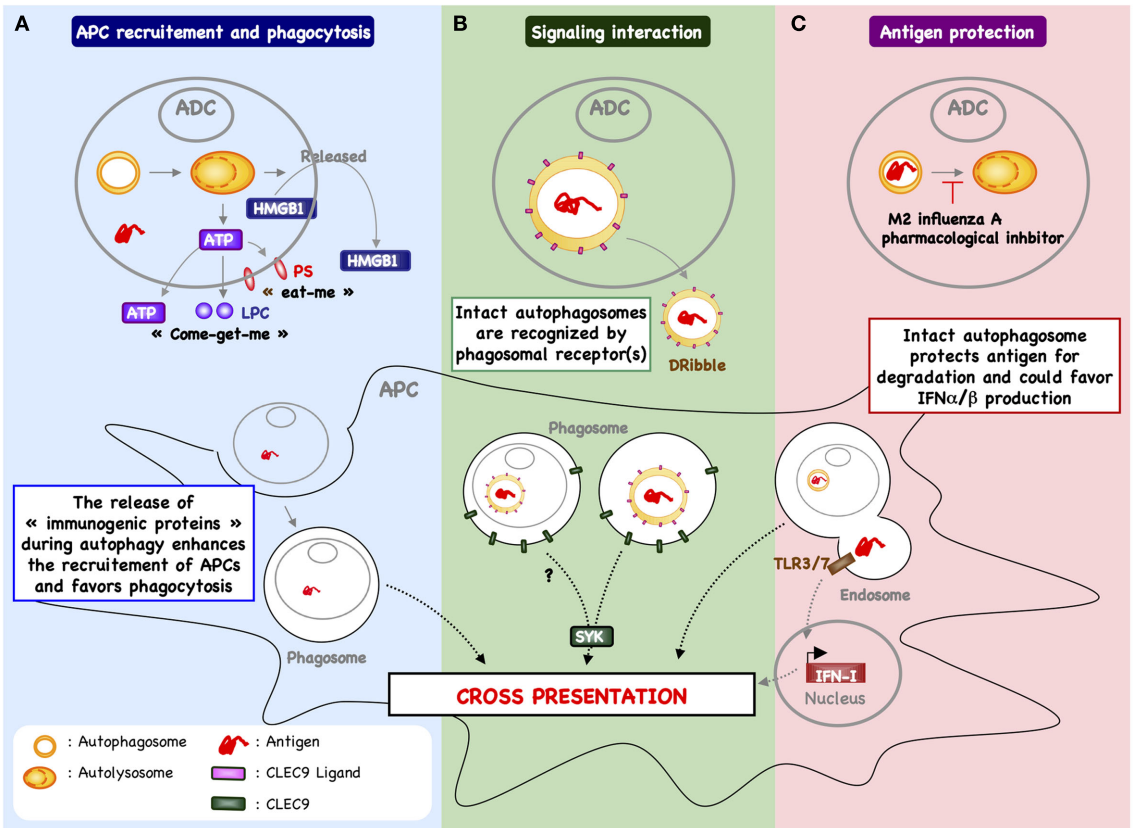

FIGURE 2 | Autophagy within antigen donor cells and cross-priming. **(A)** The process of autophagy within stressed or dying cells has been demonstrated to enhance recruitment of antigen presenting cells (APCs) and phagocytosis. For example, in response to chemotherapy, autophagy within cancer cells favors the release of ATP and enhances the recruitment of cDC and T cells into the tumor micro-environment (Michaud et al., 2011). In addition, the release of ATP triggers the exposure of "eat-me" signal (e.g., PS) and the release of "come-get-me" signal (e.g., LPC), an essential feature of efficient capture of dying cells by APC (Qu et al., 2007; Mellen et al., 2008). Autophagy can also be involved in the release of other "immunogenic proteins" known to support the cross-priming (e.g., HMGB1). The mechanism by which autophagy regulates the release of HMGB1 remain to be determined. **(B)** Free autophagosome containing antigen – passively released following cell death or actively secreted – may be recognized directly by APC, inducing a signal transduction pathway that leads to increased cross-presentation. One example includes DRibbles-derived antigen cross-priming that is partially dependent of CLEC9A (Li et al., 2011). These data suggest that autophagosome express the ligand of CLEC9A, which is recognized by APC to enhance cross-priming. **(C)** Blocking autophagic flux in antigen donor cells – using pharmacological inhibitor or during influenza A infection – favors antigen cross-priming (Uhl et al., 2009). In this context, it is possible that inhibition of lysosomal fusion serves to protect epitope within the autophagosome of dying cells and facilitates delivery of intact or partially processed antigen to APC. In addition, the capture of intact autophagosome could enhance cytokine production (e.g., type I interferon) and cDC activation.

regarding other secreted and/or exposed proteins would help further clarify the mechanisms by which autophagy within ADC favors antigen cross-priming.

AUTOPHAGIC FLUX OR AUTOPHAGOSOMES?

One critical unknown concerns the cell biology of autophagy within ADC as it relates to protection and delivery of antigen to APCs. Some evidence suggests that autophagosomes, but not necessarily autophagic flux, are required for efficient cross-priming. This is supported by the importance of early steps of autophagy – including initiation and elongation of the double-membrane structure, sequestration of cytosolic antigens, and formation of autophagosomes – for achieving efficient antigen cross-presentation of tumor cells (Li et al., 2009, 2011). Additionally, our own studies indicate that blocking autophagic flux within dying cells enhanced the cross-priming of viral antigens (Giodini and Albert, unpublished data). Accumulation of autophagosomes may also account for the seminal observations of Barnaba and colleagues, who shown that chloroquine enhance cross-priming (Accapezzato et al., 2005). In this context, it is possible that inhibition of lysosomal fusion serves to protect epitope within the autophagosome of dying cells and facilitates delivery of intact or partially processed antigen to APC.

One final consideration is the capture of free autophagic vesicles – passively released following cell death or actively secreted via a mechanism referred to as exophagy (Abrahamsen and Stenmark, 2010) – could be recognized by APCs as a source of antigen or immunogenic signals to enhance cross-priming. It has indeed been demonstrated *in vitro* that tumor cell-derived autophagosomes can be isolated from culture media; and may engage the C-type lectin receptor, CLEC9A (Li et al., 2011). These data may extend the previous role for CLEC9A as a receptor for necrotic cells and a mediator of efficient SYK-dependant cross-priming (Sancho et al., 2009).

CONCLUSION

In addition to cell death pathways, increasing evidence indicates an important role for cell stress, within ADC, as a regulator of adaptive immune responses. Autophagic processes have been shown to enhance the delivery of tumor and viral antigen to cDCs. Moreover, autophagy triggers release of immunostimulatory proteins (e.g., HMGB1) and bioactive molecules (e.g., LPC, ATP), which together favor recruitment and activation of the APC (**Figure 2**). Once within the phagosome, autophagic cells may serve as stimulators of PRRs, further enhancing the phagocyte's cross-priming potential. Another interesting line of investigation will be the autophagosome itself, which may serve to protect antigen from degradation and facilitate delivery APCs. A detailed evaluation of how autophagy favors cross-priming may help launch new immunotherapy strategies for treating cancer or chronic disease. Moreover, it may provide a mechanistic understanding of currently used chemotherapies (e.g., cyclophosphamide or methotrexate) or experimental compounds (e.g., rapamycin given in combination with hydroxychloroquine).

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Antigen processing for MHC class II presentation via autophagy

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T cells recognize proteolytic fragments of antigens that are presented to them on major histocompatibility complex (MHC) molecules. MHC class I molecules present primarily products of proteasomal proteolysis to $CD8^+$ T cells, while MHC class II molecules display mainly degradation products of lysosomes for stimulation of $CD4^+$ T cells. Macroautophagy delivers intracellular proteins to lysosomal degradation, and contributes in this fashion to the pool of MHC class II displayed peptides. Both self- and pathogen-derived MHC class II ligands are generated by this pathway. In addition, however, recent evidence points also to regulation of extracellular antigen processing by macroautophagy. In this review, I will discuss these two aspects of antigen processing for MHC class II presentation via macroautophagy, namely its influence on intracellular and extracellular antigen presentation to $CD4^+$ T cells.

Keywords: autophagosome, $CD4^+$ T cell, amphisome, MHC class II containing compartment, lysosome

INTRODUCTION

T cells recognize antigenic fragments presented to them by major histocompatibility complex (MHC) molecules. $CD4^+$ T helper cells, which orchestrate adaptive humoral and cell-mediated immune responses, are stimulated by MHC class II molecules. These are present in the steady-state on antigen presenting cells (APCs), like B cells, macrophages and dendritic cells, leukocytes that are thought to initiate immune responses, but they can be up-regulated on most human cells upon inflammation or activation (Neefjes et al., 2011). Antigenic fragments and self-peptides are loaded onto MHC class II molecules in late endosomes or MHC class II containing compartments (MIICs). Most likely proteins that are transported to MIICs bind to MHC class II and are then trimmed by lysosomal proteolysis to yield peptides of at least nine amino acids length, but often 15-mers or longer, which then stabilize MHC class II for export from MIICs to the cell surface for T cell stimulation (Trombetta and Mellman, 2005). In addition to proteases, the oxidoreductase GILT (gamma-IFN-inducible lysosomal thiol reductase) participates in the unfolding of antigens by reducing disulfide bonds (Maric et al., 2001). MHC class II molecules reach MIICs with the help of the invariant chain (Ii), which associates with them in the endoplasmic reticulum, where it prevents premature peptide loading onto MHC class II and guides MHC class II transport to MIICs via its cytoplasmic tail. Ii is degraded in MIIC by lysosomal hydrolysis, and its last remnant the CLIP peptide (class II associated invariant chain peptide) is then released from the MHC class II peptide binding groove under the influence of the HLA-DM chaperone, which is negatively regulated by HLA-DO in some cell types. Some self-protein ligands of MHC class II might reach the MIICs via a similar route as MHC class II molecules themselves. Indeed, membrane proteins including MHC class I and II constitute around 40% of natural MHC class II ligands (Dengjel et al., 2005). Classically, endocytosis delivers non-self antigens to MIICs, but might also account for some surface receptor delivery for MHC class II loading. However, in B cell lines only around 10% of natural MHC class II ligands are derived from bona fide extracellular proteins. In addition, a substantial amount of MHC class II ligands originates from cytosolic and nuclear antigens (Dengjel et al., 2005). This fraction makes up 20–30% of natural MHC class II ligands. In this review I will discuss how macroautophagy, a pathway that engulfs cytoplasmic constituents with a double-membrane surrounded autophagosome and delivers them to lysosomes for degradation (**Figure 1**), might contribute not only to the transport of cytosolic and nuclear antigens to MIICs, but also how it might facilitate the delivery of endocytosed cargo to this compartment.

ENDOGENOUS PROCESSING FOR MHC CLASS II PRESENTATION OF SELF- AND FOREIGN ANTIGENS VIA AUTOPHAGY

SELF-ANTIGEN PROCESSING BY AUTOPHAGY FOR MHC CLASS II PRESENTATION ON LEUKOCYTES AND IN THE THYMUS

Indeed, when macroautophagy was induced in B cell lines by starvation, cytosolic, and nuclear antigen presentation was increased by 50%, while membrane bound antigen presentation remained largely unaffected (Dengjel et al., 2005). For individual ligands the increase even exceeded 130%. This increase in intracellular antigen presentation correlated with macroautophagy induction as determined by autophagic vacuole content. Among the cytosolic MHC class II ligands also two components of the macroautophagic machinery could be found, namely LC3 and GABARAP (Dengjel et al., 2005; Suri et al., 2008). Both of them are mammalian homologs of the autophagy related gene (atg) product Atg8, which are coupled to the autophagosome membrane and involved in the extension of the double-membrane around the cargo and

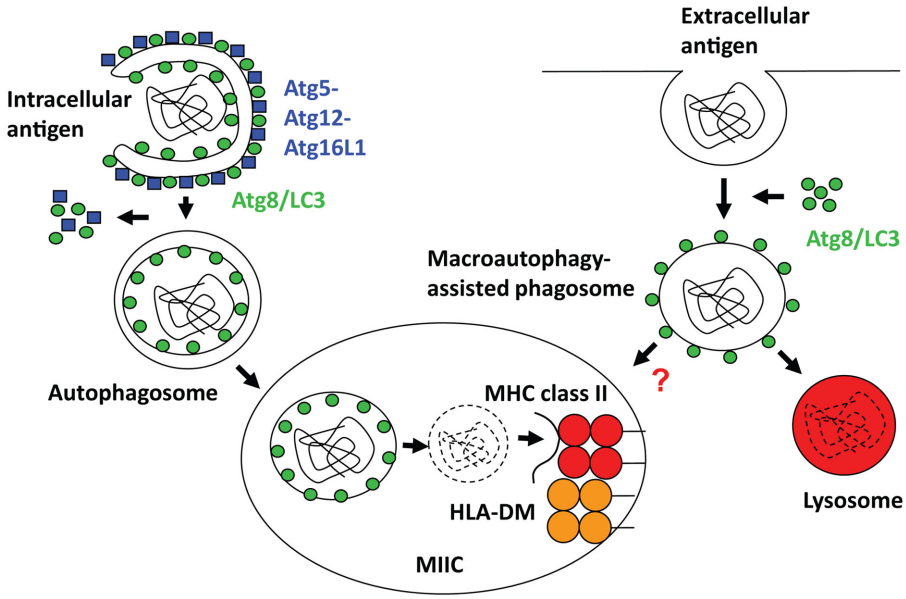

FIGURE 1 | Regulation of intra- and extracellular antigen processing for MHC class II presentation by macroautophagy. Left side: Autophagosomes, which recruit their cargo through binding to Atg8/LC3, fuse with MHC class II containing compartments (MIICs), in which limited lysosomal hydrolysis breaks down these antigens for MHC class II loading with the assistance of HLA-DM (H2-M in mice). Right side: phagosomes, especially those carrying Toll-like receptor (TLR) ligands or apoptotic cells, get decorated with Atg8/LC3, which enhances fusion with lysosomes, and might also increase fusion with MIICs for antigen loading onto MHC class II molecules.

closure of the final autophagosomes, as well as substrate recruitment into the autophagosome (Weidberg et al., 2010, 2011a,b). Interestingly, Atg8 homologs are also the only essential macroautophagy proteins that remain with the completed autophagosome on its inner membrane and get degraded with the autophagosome cargo by lysosomal proteolysis. Therefore, their turn-over can be monitored to analyze macroautophagy. These findings suggest that autophagosome cargo, including LC3 and GABARAP proteins gain access to MHC class II presentation and, therefore, autophagosomes might frequently fuse with MIICs. Indeed, such fusion events were observed in epithelial cells that expressed MHC class II upon IFN-γ treatment and also B cell lines and dendritic cells, as classical APCs (Schmid et al., 2007). The fusion vesicles contained hallmarks of MIICs with expression of HLA-DM and the lysosome associated membrane protein (LAMP) 2. Furthermore they resemble multivesicular bodies (MVBs) with both MHC class II and LC3 molecules primarily on the intravesicular membranes. Such autophagosome fusion delivers also melanosomes and tumor differentiation antigens to MIICs in melanoma cells (van den Boorn et al., 2011). Thus, autophagosomes fuse quite frequently with MIICs in APCs and some of their cargo is loaded onto MHC class II. This self-protein presentation on MHC class II molecules after macroautophagy seems to be especially important during thymic $CD4^+$ T cell education. Indeed, also in thymic epithelial cells (TECs), which constitutively express MHC class II molecules, autophagosomes fuse with MIICs (Kasai et al., 2009). This fusion seems to deliver self-proteins for both positive $CD4^+$ T cell selection in cortical TECs and negative selection in medullary TECs (Nedjic et al., 2008). Only some, but not other $CD4^+$ T cell specificities were selected by cortical TECs deficient in the essential autophagy protein Atg5, which is involved in Atg8/LC3 coupling to the autophagosome membrane. In contrast, $CD8^+$ T cells were correctly educated through Atg5 deficient thymic cortex. In addition, defective negative selection through Atg5 deficient thymii has been suggested to cause autoimmune inflammation in the intestines and other organs. This suggests that a substantial portion of self-protein derived ligands originates from macroautophagy cargo and that these are required to positively select some T cell specificities through the thymus and to induce central tolerance in this organ.

ENDOGENOUS ANTIGEN PROCESSING OF VIRAL AND BACTERIAL ANTIGENS VIA MACROAUTOPHAGY

In addition to self-proteins, some viral and bacterial antigens are presented on MHC class II molecules after macroautophagy. The first viral example for this pathway was the nuclear antigen 1 (EBNA1) of the human tumor virus Epstein Barr virus (EBV; Paludan et al., 2005). This viral protein, which limits both its translation and its proteasomal degradation via a glycine–alanine (GA) repeat domain, is turned over by lysosomal degradation after macroautophagy. The engulfment by autophagosomes leads to MHC class II presentation on EBV transformed B cells to EBNA1 specific $CD4^+$ T cell clones, a T cell specificity that is consistently found in healthy EBV carriers (Münz et al., 2000). Its nuclear localization, however, limits antigen processing via macroautophagy, and EBNA1 that lacks its nuclear localization domain is more efficiently presented to $CD4^+$ T cells (Leung et al., 2010). More efficient processing of cytosolic EBNA1 via macroautophagy leads to $CD4^+$ T cell recognition of more EBNA1 derived epitopes on EBV transformed B cells.

In addition to EBNA1, processing of bacterial antigens has been reported to require macroautophagy. The bacterial transposon-derived neomycin phosphotransferase II (NeoR) is presented to $CD4^+$ T cells after macroautophagic processing for MHC class II presentation (Nimmerjahn et al., 2003; Comber et al., 2011). Interestingly and in contrast to EBNA1, forced nuclear localization of this after transfection cytosolic protein resulted in similar or even slightly enhanced presentation on MHC class II molecules (Riedel et al., 2008). This presentation was still dependent on macroautophagy and lysosomal degradation. While NeoR was in these studies introduced into the cytosol by transfection, some bacteria inject antigens via secretion systems into the same cellular compartment. Among these is the mycobacterial Ag85B antigen, whose MHC class II presentation by DCs is enhanced upon macroautophagy stimulation (Jagannath et al., 2009). DCs with macroautophagically increased Ag85B presentation elicit then more efficiently protective immune responses after vaccination by adoptive transfer. Thus, macroautophagy might enhance antigen presentation during mycobacterial infection. Furthermore, *Yersinia* outer proteins YopE and H block MHC class II loading via endocytosis. In the absence of this extracellular antigen processing, YopE fusion proteins get endogenously processed for MHC class II presentation (Russmann et al., 2010). This antigen processing is sensitive to lysosomal and macroautophagy inhibition. Thus bacterial cytosolic, and maybe even nuclear antigens are degraded via macroautophagy and this leads to MHC class II presentation to $CD4^+$ T cells.

SUBSTRATE RECRUITMENT FOR ENDOGENOUS MHC CLASS II ANTIGEN PROCESSING VIA AUTOPHAGY

The above discussed evidence suggests that cytosolic and nuclear antigens get processed for MHC class II processing via macroautophagy. But how are these substrates recruited to autophagosomes? In higher eukaryotes two pathways of substrate recruitment to macroautophagy have been described. Both rely on anchoring of cytoplasmic constituents to Atg8/LC3, presumably on the inner autophagosomal membrane and deliver cell organelles like mitochondria as well as protein aggregates to autophagosomes. One pathway uses integral organelle proteins, like NIX for mitochondria (Schweers et al., 2007; Sandoval et al., 2008), to recruit them to forming autophagosomes or autophagosomal membranes to their cargo. The second mechanism relies on protein adaptors that link polyubiquitinylated substrates to Atg8/LC3 via ubiquitin-binding domains (UBA or UBZ) and LC3 interacting domains (LIRs). The four identified members of this class of proteins are p62/sequestosome 1, NBR1, NDP52, and optineurin (Bjorkoy et al., 2005; Pankiv et al., 2007; Kirkin et al., 2009; Thurston et al., 2009; Wild et al., 2011). They recruit protein aggregates, mitochondria, and bacterial pathogens, like *Salmonella*, to autophagosomes. Because they anchor these substrates to the inner autophagosomal membrane, they end up in the completed autophagosomes and are degraded with their content. Thus, they can be used to monitor autophagosome turn-over or macroautophagic flux. While none of these adaptor proteins has so far been directly linked to antigen processing for MHC class II presentation, covalent coupling of antigens to the N-terminus of Atg8/LC3 enhances their presentation on MHC class II molecules to $CD4^+$ T cells by epithelial cells, B cells, and dendritic cells up to 20-fold. Such increase has been observed for the influenza A virus antigens matrix protein 1 (MP1; Schmid et al., 2007) and hemagglutinin (HA; Comber et al., 2011), as well as the tumor antigen NY-ESO-1 (unpublished data). Delivery of these fusion constructs to MIICs was dependent on the macroautophagy machinery, namely Atg7 and 12, and mutating the C-terminal glycine residue of Atg8/LC3, which is used to couple this protein to the autophagosomal membrane, abolishes enhanced MHC class II presentation of these fusion constructs (Schmid and Münz, 2007; Comber et al., 2011). These data argue that Atg8/LC3 can recruit antigens for MHC class II presentation to autophagosomes, which then frequently fuse with MIICs. To which extent the UBA/LIR anchor proteins and organelle specific LIR containing proteins contribute to self- and foreign-protein recruitment for MHC class II presentation remains to be determined.

EXTRACELLULAR ANTIGEN PROCESSING FOR MHC CLASS II PRESENTATION WITH THE HELP OF AUTOPHAGY

CHARACTERISTICS OF PROCESSING VESICLES FOR MHC CLASS II LOADING

Major histocompatibility complex class II containing compartments are usually characterized as late endosomal compartments with intravesicular membranes that morphologically appear either as MVBs, multilamellar (MLBs), or electron dense bodies (EDBs; Stern et al., 2006; Neefjes et al., 2011). Rarely, they have a clear lysosomal appearance, which suggests that their hydrolytic potential is controlled, and does not readily degrade antigens and MHC class II molecules all the way to amino acids. This notion is also supported by recent studies on limited acidification of endosomes in dendritic cells by alkalinization via for example reactive oxygen species (ROS) production by phagosomal NADPH oxidase 2 (NOX2; Savina et al., 2006). Elevated pH causes less efficient hydrolysis by for example lysosomal cathepsins. Along these lines MHC class II antigen presentation of macroautophagy substrates can be enhanced by slightly neutralizing the endolysosomal compartment with pharmacological reagents in macrophages (Brazil et al., 1997). In support of these cell biological studies, dendritic cells, and macrophages handle antigen also differently *in vivo*. While macrophages, which have a decreased ability to initiate immune responses, degrade injected antigen rapidly within 1 day, dendritic cells, superior in activation of adaptive immune compartments, retain injected antigens even 2 days after injection (Delamarre et al., 2005). Accordingly, antigen formulations, which are highly sensitive to lysosomal degradation are less well presented to $CD4^+$ T cells and induce weaker immune responses than antigens with some resistance to hydrolysis (Delamarre et al., 2006). These data suggest that extracellular antigen is more efficiently presented on MHC class II molecules, when it is degraded less efficiently, and that rapid fusion with lysosomes would rather be detrimental for $CD4^+$ T cell stimulation. This has to be kept in mind when we now discuss what the macroautophagic machinery contributes to phagocytosis and phagosome maturation.

ALTERATIONS OF PHAGOSOMES THROUGH THE MACROAUTOPHAGY MACHINERY

Fusion of autophagosomes and endosomes is a frequent process in higher eukaryotic cells and the resulting vesicles were termed amphisomes (Berg et al., 1998). While Rab7 seems to be involved in direct fusion of autophagosomes with lysosomes, endosome, and MVB fusion is mediated by Rab11 (Gutierrez et al., 2004b; Fader et al., 2008). Amphisomes are then also delivered to lysosomes. Thus, autophagosomes might modify phagosomes via delivering additional cargo to amphisomes. Along these lines macroautophagy has been described to transport source proteins of microbial peptides to phagosomes (Alonso et al., 2007; Ponpuak et al., 2010). These were found to be derived from ubiquitin itself or proteins with ubiquitin-like domains, that were imported into autophagosomes by some of the anchor proteins described above. In part due to this cargo delivery autophagosome fusion with phagosomes renders the resulting amphisomes more degradative for endocytosed bacterial pathogens (Gutierrez et al., 2004a; Birmingham et al., 2006). ROS production by NOX2 and diacylglycerol (DAG) formation at the phagosomal membrane was proposed to enhance autophagosome fusion with bacteria containing phagosomes (Huang et al., 2009; Shahnazari et al., 2010). Thus, amphisome formation can enhance bactericidal activity of phagosomes.

In addition, macroautophagy has been described to accelerate phagosome fusion with lysosomes (Sanjuan et al., 2007; Florey et al., 2011). However, this pathway might not require autophagosome fusion with lysosomes, but coupling of Atg8/LC3 to the phagosomal membrane (**Figure 1**). Accordingly, Atg8/LC3 co-localization to phagosomes and enhanced fusion of phagosomes with lysosomes is dependent on the core machinery of Atg8/LC3 lipidation, for example Atg5 and Atg7, but not on factors that are required for starvation induced macroautophagy, like mTOR and Atg1/ULK1. Toll-like receptor (TLR) agonist carrying particles and apoptotic cells have been identified in Atg8/LC3 positive phagosomes. How Atg8/LC3 on the outside of the phagosome, however, mediates enhanced fusion with lysosomes remains unclear. It is tempting to speculate that Atg8/LC3, which is involved in autophagosome membrane extension by possibly mediating fusion with additional membranes (Nakatogawa et al., 2007; Weidberg et al., 2011a), could also promote other vesicular fusion events directly, like phagosome fusion with lysosomes. Irrespective of the mechanism, the macroautophagy machinery seems to modify phagocytosis by delivering precursors of bactericidal peptides to phagosomes and accelerating their fusion with lysosomes.

ENHANCED EXTRACELLULAR ANTIGEN PROCESSING VIA MACROAUTOPHAGY

Although rapid degradation of phagosomal content rather destroys antigens than leads to their presentation and phagosomes have been described to mature faster with the help of the macroautophagy machinery, it was found that mice with macroautophagy deficiency in their dendritic cell compartment were less able to prime $CD4^+$ T cell responses after herpes simplex virus (HSV) infection (Lee et al., 2010). Similarly, HSV lacking its ICP34.5 antigen, which blocks autophagosome formation (Orvedahl et al., 2007), stimulates stronger $CD4^+$ T cell responses than wild-type HSV infection (Leib et al., 2009). Even so these data could still suggest intracellular HSV antigen processing by macroautophagy and cytosolic ovalbumin was also less efficiently presented on MHC class II molecules by Atg5 deficient dendritic cells, in addition, extracellular ovalbumin was less efficiently presented to $CD4^+$ T cells by macroautophagy deficient dendritic cells (Lee et al., 2010). This was correlated with less efficient recruitment of lysosomal hydrolases, particularly cathepsins, to phagosomes in the absence of macroautophagy. However, to which extend this more rapid phagosome maturation with the help of the macroautophagy machinery contributes to extracellular antigen presentation on MHC class II molecules remains unknown, and influenza antigen processing for MHC class II presentation to $CD4^+$ T cells was unaffected by macroautophagy inhibition (Comber et al., 2011). Therefore, $CD4^+$ T cell responses only to some, but not other pathogens might be dependent on macroautophagy in dendritic cells for their induction, and the mechanism of macroautophagic assistance for extracellular antigen processing onto MHC class II molecules remains poorly defined.

CONCLUSION AND OUTLOOK

Macroautophagy delivers cytoplasmic constituents for lysosomal degradation. This machinery is also used to visualize intracellular antigens to $CD4^+$ T cells by processing them for MHC class II presentation. However, in the process of studying dependency of antigen processing on macroautophagy an additional pathway was revealed, by which macroautophagy influences extracellular antigen processing for MHC class II presentation to $CD4^+$ T cells. How coupling of Atg8/LC3 to the phagosomal membrane, however, modifies phagosome maturation, potentially accelerating fusion with lysosomes remains unknown. Furthermore, the role of this accelerated phagosome maturation for MHC class II presentation remains to be determined. A better understanding of these molecular processes during antigen presentation should allow us to design antigens that are then more efficiently processed for $CD4^+$ T cell stimulation and choose adjuvants, like TLR agonists, that utilize macroautophagy to optimize antigen presentation.

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Macroautophagy in T lymphocyte development and function

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Macroautophagy (referred to as autophagy) is a fundamental intracellular process characterized by the sequestration of cytoplasmic compartments through double-membrane vesicles, termed autophagosomes. Recent studies have established important roles of autophagy in regulating T lymphocyte development and function. Resting T lymphocytes have basal levels of autophagy that is upregulated by T cell receptor stimulation. Several specific knockout or transgenic models have been developed during the past few years, and it has been revealed that autophagy plays an essential role in regulating thymocyte selection, peripheral T cell survival, and proliferation. The regulation of T cell development and function by autophagy is mediated through its role in regulating self-antigen presentation, intracellular organelle homeostasis, and energy production. Here we will review the current findings concerning how autophagy regulates T cell function, as well as compare different models in studying autophagy in T lymphocytes.

Keywords: autophagy, apoptosis, T lymphocyte, thymocyte selection, organelle homeostasis

INTRODUCTION

Macroautophagy is a cellular process characterized by the sequestration of cytoplasmic compartments through double-membrane vesicles, termed autophagosomes. Macroautophagy (therefore referred as autophagy) starts with the generation of double-membrane bound structures referred as isolation membranes or phagophores. The phagophore then retains intracellular components to form autophagosomes. Consequentially, autophagosomes fuse with lysosomes, in which the inside content is digested (Klionsky and Emr, 2000). Autophagosome biogenesis requires a group of evolutionarily conserved genes, referred to as autophagy-related genes (*atg*s; Longatti and Tooze, 2009). The initiation of the phagophore requires the class III phosphatidylinositol 3-kinase (PtdINs3K) complex, which further recruits two ubiquitin-like conjugation systems: The Atg12–Atg5 conjugation and LC3 processing (Ohsumi and Mizushima, 2004; He and Klionsky, 2009; **Figure 1**). Autophagy has been shown to be important for providing nutrients during starvation, clearing long-lived proteins as well as unwanted organelles, and fighting intracellular infections in multiple types of mammalian cells (Mortimore and Poso, 1987; Levine et al., 2011). The primary role of autophagy in certain cells depends on the cell type and environment. To study autophagy in primary T lymphocytes, several specific knockout or transgenic models have been developed during the past few years, and it has been revealed that autophagy plays an essential role in T cell homeostasis and function. The phenotypes of different *atg*-deficient T cells showed a great deal of similarity, with certain differences, which can be explained as a function of deletion timing or efficiency, accumulating effects, or the molecular functions outside of autophagy. Here we will review the current findings concerning how autophagy regulates T cell function, as well as compare different models in studying autophagy in T lymphocytes.

AUTOPHAGY INDUCTION IN T LYMPHOCYTES

Although relatively low, autophagy can be detected in all subsets of thymocytes and freshly isolated naïve T lymphocytes (Li et al., 2006; Pua et al., 2007; Stephenson et al., 2009). These results were fairly surprising, since T cells contain limited cytoplasm. The existence of autophagy in T lymphocytes suggests a regulatory role of intracellular program. Aging T lymphocytes, on the other hand, showed an accumulation of autophagic vacuoles. In a study of long-term *in vitro* human lymphocyte culture, the percentage of cells with autophagosomes increased during culture, which was associated with the increase in lysosomal mass and accumulation of lipofusion events (Gerland et al., 2004).

The T cell receptor (TCR) mediates activation signal upon interaction with the antigenic peptide presented by the major histocompatibility complex (MHC) on antigen presenting cells. TCR activation is a strong trigger for autophagy in T lymphocytes (Pua et al., 2007, 2009; Hubbard et al., 2010; Jia and He, 2011; Jia et al., 2011; Kovacs et al., 2012). $CD4^+$ T cells upregulate Beclin-1 (Atg6) and LC3 (Atg8) upon TCR stimulation (Arsov et al., 2008). Ultrastructural studies revealed an increase in the number and a decrease in the size of autophagosomes after TCR stimulation. Interestingly, while mitochondria are frequently contained in the autophagosomes of resting T lymphocytes, the autophagic cargo switches to almost exclusively cytosolic material in activated T lymphocytes (Hubbard et al., 2010). Mitochondria undergo morphological changes during autophagy induction to escape autophagic degradation and maintain energy production in mouse embryonic fibroblasts (MEFs; Gomes et al., 2011). It is important to determine whether T lymphocytes apply similar strategies or utilize unique pathways to regulate the mitochondrial content.

T cell receptor-induced autophagy requires the key autophagy machinery, as deleting Atg5, Atg7, and Atg3 can abolish autophagosomal induction (Pua et al., 2007, 2009; Hubbard et al., 2010;

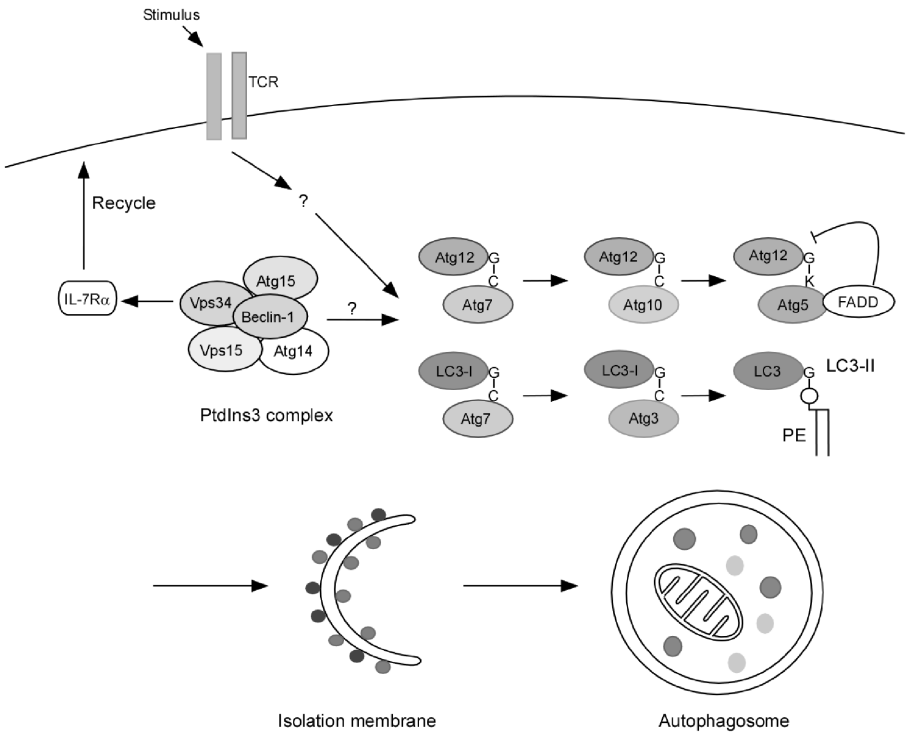

FIGURE 1 | Autophagy in T lymphocytes. Published results indicate that TCR engagement is a strong inducer of autophagy in primary T cells. Although autophagy is induced by the PtdIns3k complex in many types of cells, the role of this complex in autophagy induction in T lymphocytes may not be the same as in other cells. Vps34 is dispensable for autophagy induction in T cells while it promotes T cell survival by regulating IL-7Rα surface expression. The role of Beclin-1 in T cell autophagy induction remains to be further defined. However, the two autophagosome processing pathways: Atg5–Atg12 conjugation and LC3 processing are essential for autophagy induction as deleting Atg3, Atg5, and Atg7 all results in impaired autophagy in T cells. Furthermore, FADD, the death adaptor protein in the extrinsic death pathway, may play a key role in mediating a crosstalk between the apoptosis and autophagy pathways.

Jia and He, 2011). However, the class III PI3K, Vps34, seems to be dispensable for autophagy induction in mature T lymphocytes (McLeod et al., 2011). JNK1/JNK2 is required for TCR-induced autophagy in $CD4^+$ T cells (Li et al., 2006). Beclin-1, a component of PtdINs3K complex, was shown to be crucial for autophagy initiation (Yue et al., 2003). The level of autophagy in Beclin-1 deficient primary T lymphocytes remains to be measured (Kovacs et al., 2012). Nevertheless, overexpression of Beclin-1 in T lymphocytes by a BAC transgene did not change the basal level of autophagy in multiple organs including thymus and spleen (Arsov et al., 2008), suggesting that additional autophagy initiators may be required in T lymphocytes for autophagy induction.

T cell receptor-induced autophagy is compromised in aged $CD4^+$ T lymphocytes (Mattoo et al., 2009). The mechanism by which autophagy induction is defective during aging is unclear. Some evidence suggested that the Rel family member, p65, might be involved, as the nuclear translocation of p65 upon TCR signaling is impaired in aged $CD4^+$ T cells (Mattoo et al., 2009), and p65 has been shown to be essential for autophagy induction by upregulating the transcription of Beclin-1 in multiple cell lines (Copetti et al., 2009). It is also unknown whether defective autophagy induction leads to other defects in aged T lymphocytes, such as increased mitochondrial damage, reduction in glycolysis, or enhanced apoptosis upon primary TCR stimulation. It would be appealing to investigate whether modulating autophagic levels can restore the function of aged T lymphocytes.

AUTOPHAGY IN T LYMPHOCYTE HOMEOSTASIS: DIFFERENT GENETIC MODELS, SIMILAR DEFECTS

Several tissue-specific knockout models have been developed during the past few years to study the role of autophagy in T lymphocytes. The deficiency in autophagy-related genes leads to a blockage in autophagic flux, as well as impaired T cell homeostasis. In $Atg5^{-/-}$ fetal liver chimeric mice, the thymocytes undergo full maturation, but the thymic cellularity is reduced by half. The peripheral T lymphocyte number is dramatically decreased, which may be the result of both the loss of thymocytes and increased cell death rate (Pua et al., 2007). The $Atg5^{f/f}$Lck-Cre^+ mice generated later showed an almost identical phenotype to the $Atg5^{-/-}$ chimera (Stephenson et al., 2009). The question was whether the loss of autophagy was the real reason for the enhanced cell death, since Atg5 interacts with Fas-associated death domain (FADD) protein and the anti-apoptotic protein Bcl-x_L (Pyo et al., 2005; Yousefi et al., 2006). Atg5 may regulate apoptosis through the extrinsic or intrinsic cell death pathways (Zhang et al., 2005).

To address whether Atg5 regulates T cell homeostasis through autophagy, several other tissue-specific knockout models have been analyzed since. Autophagosome formation requires two interrelated protein conjugation systems: LC3 processing and

Atg5–Atg12 conjugation. During LC3 processing, Atg3, along with Atg7, catalyzes the cleavage and conjugation of LC3-I to generate the membrane bound LC3-phosphatidylethanolamine (LC3-II/LC3-PE; Levine et al., 2011). Tissue-specific knockout of Atg3 using an Lck-Cre system [deletion occurs during double negative (DN) stage in thymus] revealed an almost identical phenotype as the Atg5$^{-/-}$ chimera, characterized by normal frequencies of DN, double positive (DP), and single positive (SP) thymocytes in the thymus, but a reduced cellularity in both the thymus and peripheral T cell pools (Jia and He, 2011). On the other hand, the thymocyte cellularity in Atg7$^{f/f}$Lck-Cre^{+} mice was barely decreased (Stephenson et al., 2009), with only a minor reduction in SP cells. Despite the difference inside the thymus, T lymphocytes in all these models (Atg5$^{-/-}$ chimera, Atg5$^{f/f}$Lck-Cre^{+}, Atg7$^{f/f}$Lck-Cre^{+}, Atg3$^{f/f}$Lck-Cre^{+}, and BECN$^{f/f}$CD4-Cre^{+}) showed decreased T cell numbers in the periphery and an enhanced apoptosis (Pua et al., 2007, 2009; Stephenson et al., 2009; Jia and He, 2011; Kovacs et al., 2012).

Ortiz's group utilized a Rag-1$^{-/-}$ (recombination activating gene-1 null) blastocyte complementation to study the role of Beclin-1 in hematopoietic cells. Beclin-1-deficient (BECN$^{-/-}$) blastocytes were transferred to a Rag-1$^{-/-}$ host. The data showed that Beclin-1 is required for the maintenance of early thymocyte progenitors. Controversially, survival and TCR-induced proliferation remained normal in BECN$^{-/-}$ T lymphocytes developed in this system. Moreover, autophagy can be detected in those cells, though decreased compared to controls (Arsov et al., 2011). These results indicate that a Beclin-1 independent autophagy-inducing pathway may occur in T lymphocytes. The mechanism of autophagy induction in the absence of Beclin-1 needs to be determined.

Vps34, a key component in PtdINs3K complex, regulates the assembly of phagophore. Surprisingly, Vps34 deficient T cells showed no change in LC3 puncta formation after TCR stimulation or starvation. Moreover, autophagosomes could be detected in naïve Vps34$^{-/-}$ T cells by transmission electronic microscopy (McLeod et al., 2011). These results suggest that Vps34 is not essential for autophagy induction in T lymphocytes. The differences in findings between Vps34 and Beclin-1 knockout T cells can be explained by the accessory molecules associated with the complex. Beclin-1 also has affinity for Bcl-2, and is an interaction partner with Bcl-2 during conditions of stress (Liang et al., 1998), inhibiting the ability of Bcl-2 to repress pro-apoptotic Bax/Bak (Wei et al., 2008). Additionally, other functions of Beclin-1 include recruitment of proteins that bend and flex membranes, including UVRAG and Bif1, to provide the physical machinery for autophagosomal elongation (Takahashi et al., 2007, 2009). Finally, Vps34 can function as a scaffold for the autophagic process, but the lipid kinase function of this molecule can potentially be compensated for by phosphatidylinositol metabolism. This has been recently confirmed by data in MEFs showing that the class Ia PI3K, p110β, is a positive regulator of autophagy (Dou et al., 2010).

AUTOPHAGY IN THYMOCYTE SELECTION

Autophagy plays an important role in the generation and selection of thymocytes which have little autoreactivity. Interestingly, this autophagy occurs in thymic epithelial cells (TECs), which although are not hematopoietic in nature, are the major selecting cells in thymic education and tolerance induction. As thymocytes mature, they undergo a functional rearrangement of VDJ gene segments in various TCR loci (either αβ or γδ TCR subunits) to express a surface TCR receptor (Schatz and Ji, 2011). These maturing thymocytes depend on receiving TCR-mediated survival cues. During this process, DP thymocytes of the αβ lineage are selected by cortical thymic epithelial cells (cTECs) by a process termed "positive selection" (Takahama et al., 2010) and migrate inward to be selected by medullary TECs by "negative selection." Through this process, TCR clones are generated that have low affinities for self-peptides presented in the context of a MHC. One of the mechanisms that TECs use for this process is the expression of *autoimmune regulator* (AIRE), which regulates the expression of a host of tissue-specific proteins against which developing thymocytes can be selected (Heino et al., 1999). Another possibility is that thymocytes develop in the presence of antigens derived from peripheral cells that are transported back to the thymus by dendritic cells. However, from studies performed by Nedjic et al. (2008a), it is clear that autophagy is a major mechanism by which self-antigens are processed and presented for selection of developing thymocytes.

Thymic epithelial cells have extraordinarily high levels of constitutive autophagy (Nedjic et al., 2008a). Using Atg5-deficient TECs engrafted into nude mice, Nedjic observed normal thymocyte numbers. However, these cells had undergone a disrupted process of positive and negative selection, despite the normal expression of AIRE, suggesting a role of macroautophagy in the selection. Moreover, when T cells selected by Atg5-deficient TECs were transferred, they recapitulated a wasting disease with colitis and lung infiltration highly reminiscent of typical autoimmune disorders (Nedjic et al., 2008a). These results are consistent with that autophagy is essential for the expression of self peptide–MHC complexes on TECs to foster normal T cell development. When the abnormally low phagocytic activity of TECs is accounted for, the prominence of autophagy in generating peptides for MHC display is enhanced even further (Nedjic et al., 2008b). It would be of great interest to investigate the opposite effect, namely would increasing autophagic activity in TECs promote T cell production?

Conversely, a study showed that autophagic activity in thymocytes (including TECs) was greatly reduced in aged mice, correlating with a reduced pool of mature T cell production in these mice (Uddin et al., 2012). It would be of great interest to determine whether increased levels of autophagy could be stimulated in TECs of aged mice to increase the pool of mature T lymphocytes and maintain clonal diversity into old age.

AUTOPHAGY IN T CELL FUNCTION

AUTOPHAGY AND INTRACELLULAR ORGANELLE HOMEOSTASIS

Autophagy selectively degrading mitochondria, termed as mitophagy (Kraft et al., 2009), plays a role in controlling mitochondria content during T cell development (Pua et al., 2009; Stephenson et al., 2009). Transcriptional profiling of Atg5-deficient T lymphocytes revealed a remarkable enrichment in mitochondrion-associated genes (Stephenson et al., 2009). The mitochondrial contents in T lymphocytes are gradually decreased during the

transition from thymocytes to peripheral T lymphocytes. The level of mitochondria was shown to be the highest in DP thymocytes, followed by SP thymocytes, and mature T lymphocytes contained the lowest volume (Pua et al., 2009). This contraction of mitochondria requires autophagy, as least partially, as mitochondrial content was higher in Atg5$^{-/-}$, Atg7$^{-/-}$, and Atg3$^{-/-}$ than in wild-type T lymphocytes (Pua et al., 2009; Stephenson et al., 2009; Jia et al., 2011). On the other hand, it was reported that cells from BECN$^{f/f}$ CD4-Cre^{+} mice showed no change in mitochondrial content (Kovacs et al., 2012). One possible explanation is that genomic deletion only starts in DP thymocytes in this model, and persistent level of Beclin-1 protein in cells is enough to manage the autophagic degradation of mitochondria. Still, one cannot rule out the possibility that mitochondrial content regulation and even autophagy are achieved independent of Beclin-1 in T lymphocytes. Current data indicates that autophagy protects T lymphocytes migrating to the periphery by eliminating excessive mitochondria. Mitochondria are assumed to be the major reservoir of toxic reactive oxygen species (ROS; Hildeman et al., 2003). The oxidative tension is much higher in the blood than the thymus (Braun et al., 2001; Sitkovsky and Lukashev, 2005). Therefore, maintaining the same level of mitochondria in peripheral T lymphocytes as in thymocytes may lead to intolerable levels of ROS for T lymphocytes.

Endoplasmic reticulum (ER) levels are as well regulated by autophagy. Selective autophagy toward ER is termed as reticulophagy (or ER-phagy; Kraft et al., 2009). Different from mitochondria, ER volume is downregulated in the transition of DN to DP thymocytes, and maintained to a similar level afterward. An increase in ER volume was observed in autophagy-deficient T lymphocytes (Jia et al., 2011). The accumulation of ER, as well as mitochondria, requires a long period of time to be established (up to 18 days *in vitro*) once autophagy is abolished by induced-deletion in mature T lymphocytes. Interestingly, lymphocytes did not show elevated cell death until 24 days after inducible deletion of Atg3. These data suggest that the elevated cell death in autophagy-deficient T lymphocytes is possibly the outcome of cellular organelles' abnormality.

AUTOPHAGY IN T CELL ACTIVATION

Different groups have identified that autophagy-related genes are required for T cell proliferation upon TCR stimulation. T lymphocytes lacking Atg5, Atg7, Atg3, or Beclin-1 all showed impaired proliferation (Pua et al., 2007, 2009; Stephenson et al., 2009; Hubbard et al., 2010; Jia et al., 2011; Kovacs et al., 2012). TCR stimulation induced cell death was also observed in Beclin-1-deficient lymphocytes (Kovacs et al., 2012). The survival defect may contribute to impaired proliferation in Beclin-1-deficient T lymphocytes. Atg7$^{-/-}$ T cells displayed impaired calcium influx upon TCR stimulation, as well as increased calcium efflux from ER to cytosol. The excessive ER in Atg7-deficient T cells may lead to abnormal redistribution of calcium into the ER upon TCR stimulation (Jia et al., 2011). Interestingly, other TCR signaling components such as PLCγ-1(the upstream signal of calcium efflux), p38, ERK, and NF-κB activation all remain unchanged in Atg7-deficient T lymphocytes.

Autophagy is involved in the metabolism of activated T lymphocytes. Upon receipt of a TCR signal, T lymphocytes undergo a metabolic switch and produce more ATP to ensure sufficient energy for protein synthesis, cytokine secretion, and cellular division (Fox et al., 2005). Without autophagy, the ATP production in activated T lymphocytes is reduced to resting levels. The lack of ATP, at least partially, contributes to impaired transcription and production of IFN-γ and IL-2 in those cells, as methyl pyruvate, a cell-permeable intermediate of glucose, incompletely rescues both ATP generation and cytokine production (Hubbard et al., 2010). However, another study showed increased levels of IL-2 secretion in autophagy-deficient T lymphocytes after TCR activation (Jia et al., 2011). This controversy may be the consequence of the autocrine nature of IL-2: IL-2 is consistently produced and utilized by activated T lymphocytes (Cantrell et al., 1988). Therefore, the IL-2 detected in culture medium at a single time point may or may not reflect the capacity of IL-2 production. To settle the debate, careful study in the kinetics of the transcription and secretion of cytokines is needed.

Autophagy is differentially regulated in each T helper subset. T lymphocytes cultured in Th2 polarizing condition *in vitro* contain more autophagosomes than T lymphocytes induced by Th1 polarizing conditions (Li et al., 2006). Th17 cells are relatively more (Wu et al., 2011) resistant to cell death without Beclin-1, compared to Th0, Th1, and Th2 (Kovacs et al., 2012). Th17 cells, along with Th1 cells, mediate the pathogenesis of experimental autoimmune encephalomyelitis (EAE) (El-behi et al., 2010). Though Th17 cells manage to survive when autophagy is blocked, mice with Beclin-1-deficient T lymphocytes still showed resistance to EAE development. The number of MOG specific Th1 and Th17 cells were reduced in the periphery and were undetectable in the central nerve system (CNS) in Beclin-1 knockout mice (Kovacs et al., 2012). The protective role of Beclin-1-deficient T lymphocytes in EAE induction may be the result of defective antigen-induced proliferation.

AUTOPHAGY IN HIV INFECTION

Autophagy was reported to be involved in the progressive decline in the number of CD4^{+} T lymphocytes during HIV infection. HIV infection induces cell death in both infected and uninfected "bystander" CD4^{+} T cells (Laurent-Crawford et al., 1993). The repertoire of HIV-1 envelope glycoproteins (Env) is composed of gp41 and gp120 and expressed on infected human lymphocytes. Via CXCR4 and CCR5, Env triggers autophagy in uninfected "bystander" CD4^{+} T cells, which eventually leads to apoptosis (Espert et al., 2006, 2009). The gp41 fusion to target membranes is required for Env-mediated autophagy (Denizot et al., 2008). Interestingly, autophagy is repressed in HIV-infected CD4^{+} T cells (Zhou and Spector, 2008). The effect of killing "bystander" T cells through surface protein Env seems to be T cell specific, as Env expressed on macrophages with CXCR4 and CCR5 fails to induce autophagy in uninfected CD4^{+} T cells in a coculture experiment (Espert et al., 2009). From the perspective of virus, down-regulation of autophagy in host cells may be beneficial as autophagy attacks virus through xenophagic degradation and facilitates antigen presentation (Kim et al., 2010). At the same time, provoking excessive autophagy in uninfected surrounding

lymphocytes mediates cell death, further weakening the host defense by reducing potential adaptive immunity against the virus.

AUTOPHAGY IN T CELL DEATH

As mentioned above, the loss of autophagy-related genes generally leads to increased cell death (Pua et al., 2007, 2009; Stephenson et al., 2009; Jia et al., 2011). The crosstalk between autophagy and apoptosis has been observed in various eukaryotic cells (Thorburn, 2008), but how autophagy regulates apoptosis in T lymphocytes remains unclear. Atg7$^{-/-}$ T lymphocytes upregulate the expression of Bcl-2 (Pua et al., 2009). Beclin-1-deficient T lymphocytes demonstrate highly increased protein levels, but not mRNA levels of pro-apoptotic pro-caspase-8, pro-caspase-3, and Bim as well as a moderate increase in Bcl-2. Interestingly, Caspase-8 is detected in p62/ubiquitin-containing aggregates in Beclin-1$^{-/-}$ T cells (Kovacs et al., 2012). Although p62 may target caspase-8 for autophagic degradation, pro-caspase-8 level is comparable between wild-type and p62-deficient T cells. Further studies are required to identify whether Beclin-1 controls pro-apoptotic protein levels through Bcl-2 binding, autophagy induction, or other pathways.

Although it is clearly established that autophagy is required for T cell survival, excessive autophagy seems to be destructive for T lymphocytes. Autophagy promotes, rather than protects, growth factor withdrawal-induced cell death in a Th2 cell line (Li et al., 2006). In long-term human CD8^{+} T cell cultures, cells with higher numbers of autophagosomes died out first. These results might imply that autophagy renders lymphocytes more vulnerable to apoptosis under certain conditions (Gerland et al., 2004). However, it is still unclear whether the high number of autophagosomes reflects an increase in flux or a blockage of autophagosome–lysosome fusion. Whether those cells undergo enhanced autophagy remains to be verified. Other data supporting the pro-death role of autophagy involves Irgm 1 (Interferon-γ inducible, immunity-related GTPase$^{-/-}$). When exposed to IFN-γ, Irgm 1$^{-/-}$ T cells showed greatly enhanced death in an autophagy-dependent manner (Feng et al., 2008). Consistent with this, Ginsenoside Re suppresses autophagy by inhibiting Irgm 1 and IFN-γ production in human T lymphocytes, which leads to enhanced proliferation and reduced cell death (Son et al., 2010).

Blocking caspase-8 activity was shown to induce autophagy and non-apoptotic death (Yu et al., 2004; Li et al., 2006; Wu et al., 2011). In T lymphocytes, loss of capase-8 activity may lead to necroptosis upon TCR stimulation. Necroptosis is a programmed necrotic cell death, regulated by Rip-1/Rip-3 kinases and independent of the apoptotic pathway (Galluzzi and Kroemer, 2008). It is still controversial whether autophagy is involved in TCR-induced necroptosis in T lymphocytes. Caspase-8 activation requires the adaptor protein FADD (Zhang et al., 1998). FADD*dd* (dominant negative) T lymphocytes showed hyperactive autophagy and RIP-1-dependent cell death (**Figure 1**). Inhibiting autophagy by either 3-MA administration or silencing of Atg7, rescued those cells from cell death (Bell et al., 2008). However, studies in FADD$^{-/-}$ and caspase-8$^{-/-}$ T cells suggested that enhanced cell death cannot be rescued by 3-MA treatment or deletion of Atg7 (Osborn et al., 2010; Ch'en et al., 2011). It is imprudent to draw any conclusions from the current results, as thoroughly abolishing autophagy may create additional stress, thus killing instead of protecting T lymphocytes. Careful analyses of cell death pathways in caspase-8-deficient T cells, especially with modest inhibition of autophagy, will provide us valuable information to understand the relationship between autophagy, apoptosis, and necroptosis.

CONCLUSION

Autophagy, a fundamental cellular process, is required for the homeostasis and function of T lymphocytes. Deletion of different autophagy-related genes results in similar phenotypes in T lymphocytes, indicating that the defects are caused by a loss of autophagy instead of other functions of those proteins. T lymphocytes utilize autophagy to maintain intracellular organelle homeostasis, intact TCR signaling, and metabolic switch upon TCR activation. It is worthy to notice that autophagy plays a dual role in T lymphocytes: autophagy induction is critical for cell survival and T cell response, while too many autophagic vesicles can create intolerable stress. Future studies can focus on how to modulate the levels of autophagy to promote/inhibit T cell responses in various disease models.

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Autophagy and autoimmunity crosstalks

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Autophagy, initially viewed as a conserved bulk-degradation mechanism, has emerged as a central player in a multitude of immune functions. Autophagy is important in host defense against intracellular and extracellular pathogens, metabolic syndromes, immune cell homeostasis, antigen processing and presentation, and maintenance of tolerance. The observation that the above processes are implicated in triggering or exacerbating autoimmunity raises the possibility that autophagy is involved in mediating autoimmune processes, either directly or as a consequence of innate or adaptive functions mediated by the pathway. Genome-wide association studies have shown association between single nucleotide polymorphisms (SNPs) in autophagy related gene 5 (*Atg5*), and *Atg16l1* with susceptibility to systemic lupus erythematosus (SLE) and Crohn's disease, respectively. Enhanced expression of *Atg5* was also reported in blood of mice with experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (MS), and in T cells isolated from blood or brain tissues from patients with active relapse of MS. This review explores the roles of autophagy pathway in the innate and adaptive immune systems on regulating or mediating the onset, progression, or exacerbation of autoimmune processes.

Keywords: autophagy, autophagosome, autoimmunity, encephalomyelitis, autoimmune, experimental, lupus erythematosus, systemic

The autophagy pathway, an evolutionary conserved mechanism, starts with the development of an isolation membrane within the cell that engulfs damaged organelles, misfolded proteins or pathogens, and eventually develops into an autophagosome. The autophagosomes, in turn, fuse with lysosomes to form the autophagolysosomes where the actual degradation of the substrates takes place (Levine et al., 2011). For the individual cell, the autophagy pathway is important not only to get rid of foreign or unwanted materials but also for efficient energy recycling during periods of stress. For the whole organism, the immune and physiological consequences of aberration of the autophagy pathway are much more profound. The immune system, responsible for surveillance and communication between different organs and cells types, is one such system in which the role of autophagy and the consequences of defects in autophagy go far beyond the degradative role of the pathway (Deretic, 2012a). **Figure 1** shows potential roles of the autophagy pathway in the adaptive and innate immune systems that might modulate the onset and outcome of an autoimmune disease.

AUTOPHAGY, THE ADAPTIVE IMMUNE SYSTEM AND AUTOIMMUNITY

Autophagy plays important roles in both innate and adaptive immunity. Because there have been several excellent reviews on this topic (Munz, 2009; Sumpter and Levine, 2010; Kuballa et al., 2012; Randow and Munz, 2012), we will only discuss brief aspects of these roles as they might pertain to autoimmunity. Autophagy is essential for survival and homeostasis of lymphocytes and there exist at least two broad stages where autophagy might affect the adaptive immune cells. As the development of lymphocyte is a complex process involving inputs from other cells, both lymphocyte-intrinsic and extrinsic defects in autophagy might affect development and/or maturation of lymphocytes.

AUTOPHAGY IN LYMPHOCYTE DEVELOPMENT

T cell development in the thymus undergoes positive and negative selections, processes in which extrinsic inputs from thymic epithelial cells (TECs) play a major role in shaping the T cell repertoire. TECs show high levels of constitutive autophagy essential for proper display of MHC-antigen complex on their surface (Mizushima et al., 2004; Kasai et al., 2009), thereby facilitating appropriate T cell selection. Mice with *Atg5* deficiency in TECs showed severely impaired central tolerance and autoimmune organ destruction, suggesting that autophagy-mediated display of MHC-antigen complex on surface of TECs is essential for proper T cell development (Nedjic et al., 2008). Autophagy deficiency in the TECs impaired both positive and negative selection mechanisms resulting into autoimmunity and it was proposed that autophagy-dependent display in the peripheral tissue needed to be counterbalanced by a similar tolerogenic mechanism in the thymus in order to prevent such autoimmune processes (Nedjic et al., 2008). Further, a recent report demonstrated the requirement of autophagy in TECs for loading endogenous antigens onto MHC-II and that this process was essential for negative selections of CD4 T cells (Aichinger et al., 2013). Because both DCs and TECs might be important in differentiation of regulatory T cells (Tregs) (Wirnsberger et al., 2009; Hinterberger et al., 2010), this report suggested that autophagy might be important in differentiation of Tregs (Aichinger et al., 2013). As Tregs are among the major players controlling autoimmunity (La Cava, 2009), this might

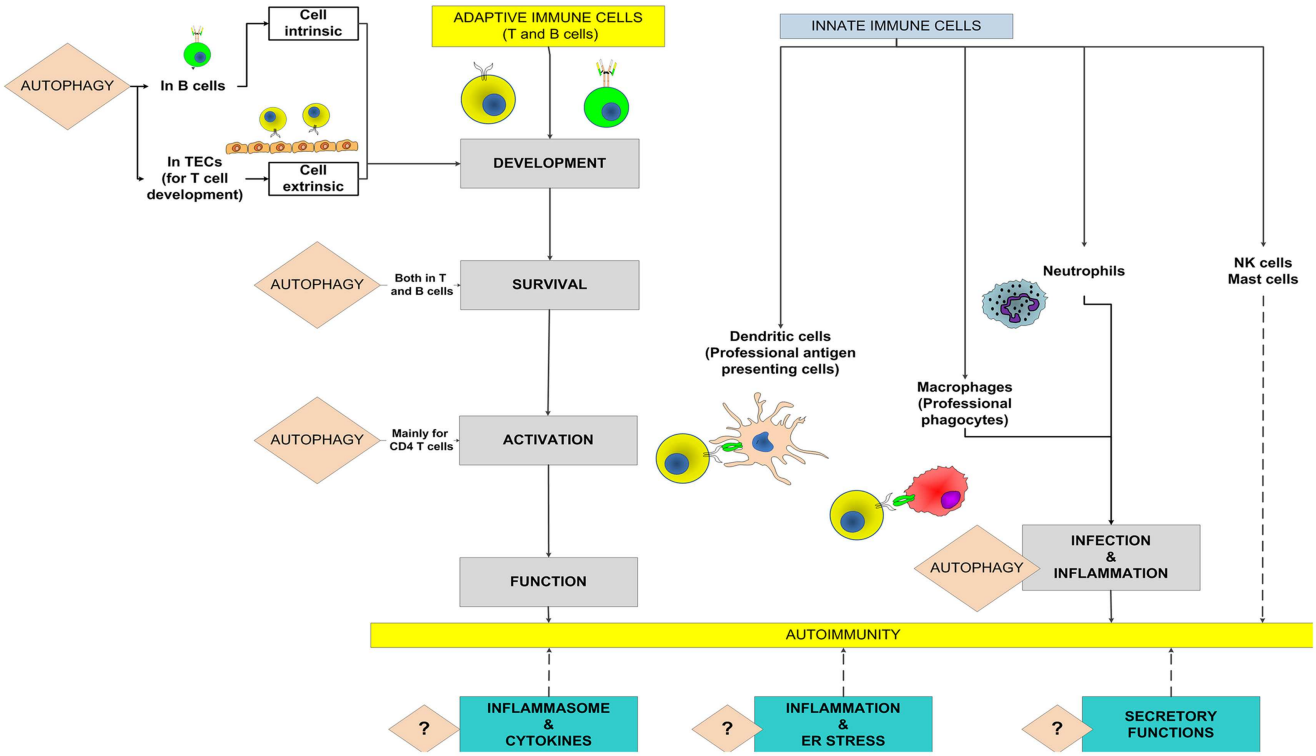

FIGURE 1 | Potential roles of autophagy in the adaptive and innate immune systems to mediate autoimmunity. The known roles of autophagy in the contributing processes are highlighted and the broken lines show potential contributions toward autoimmunity. A question mark denotes the possibility that the autophagy pathway might modulate autoimmune diseases through these processes. TECs, thymic epithelial cells.

be another potential link between autophagy and autoimmune diseases.

Fetal liver chimera and conditional knock-out studies have shown that T cell development remained normal in mice lacking *Atg5* in T cells but peripheral T cell compartment showed reduction in numbers, particularly in CD8 T cells (Pua et al., 2007). These results were attributed to the pro-survival role of autophagy in mature T cells. Studies showed considerable interaction between the autophagy and apoptotic pathways (Maiuri et al., 2007). *Atg3, 5, or 7*-deficient mature T cells showed defective Endoplasmic reticulum (ER) homeostasis and mitochondrial clearance and, consequently, an elevated levels of ROS, which might serve as one of the potential links between the autophagy and apoptotic pathways (Pua et al., 2007; Jia and He, 2011; Jia et al., 2011). However, increased levels of mitochondria were observed in *Atg7−/−* but not in *Atg5−/−* thymocytes at the single positive stage (Pua et al., 2009). A possible explanation could be different stages or extent of involvement of these proteins in mitochondrial clearance. These findings potentially brings another layer of complexity into focus, namely autophagy-independent effect of various *Atg*.

In contrast to T cells, autophagy in B cells plays a very important role in development and the requirement of *Atg5* has been found to be highly stage-specific, with a defective pro- to pre-B cell transition in B cell-specific *Atg5−/−* knock-out mice. In these mice, the levels of pre and immature B cells, along with peritoneal B1 cells were reduced to a great extent. This finding was also attributed to a role of autophagy in maintaining B cell survival (Miller et al., 2008).

AUTOPHAGY IN LYMPHOCYTE FUNCTIONS

Autophagy induction in response to starvation and TCR stimulation has been observed in mouse T cells (Pua et al., 2007) and in cultured human T cells during aging (Gerland et al., 2004) and in HIV infection (Espert et al., 2006). *Atg5*-deficient T cells showed reduced proliferation upon both TCR and PMA-ionomycin stimulation (Pua et al., 2007). This finding highlights potentially different roles of autophagy in naïve versus activated T cells. Most studies involving autophagy in T cells focused on roles of autophagy in cell survival and found autophagy to be a pro-survival mechanism (Pua et al., 2007). However, some studies have also suggested that autophagy might be required for T cell death (Espert et al., 2006; Bell et al., 2008). Uninfected lymphocytes undergo autophagy-mediated cell death upon engagement of the receptor CXCR4 by HIV envelop glycoprotein (Espert et al., 2006). Another study has also shown that autophagy could be an important cell death machinery in T cells lacking caspase-8 or Fas-associated death domain (FADD) activity, thereby raising the possibility that interaction between autophagy and apoptosis might be context dependent (Bell et al., 2008). It is possible that, in activated T cells, autophagy plays different roles compared to naïve cells and might be involved in activation-induced cell death following T cell proliferation in immune response.

Recent findings suggest that autophagy might affect overall T cell functions under different conditions of polarization and activation. Rapamycin, an mTOR inhibitor and inducer of autophagy, has been found to promote T cell memory when administered in low doses, although it is not clear if this effect is mediated by autophagy (Araki et al., 2009). Moreover, low dose rapamycin exacerbated autoimmune experimental uveitis, and this action of rapamycin was thought to be mediated by autophagy (Zhang et al., 2012). Interestingly, expression of *Atg5* has been shown to correlate with severity of experimental autoimmune encephalitis (EAE), a mouse model of multiple sclerosis (MS), and to be increased in T cells of MS patients during relapses (Alirezaei et al., 2009), which can worsen by prolonged autoreactive T cell survival. EAE is considered predominantly a CD4 mediated disease and further studies are required to dissect how autophagy in T cells influences the onset or progression of autoimmune diseases in animal models and if these roles of autophagy are also dependent on cell survival. These studies can develop *in vivo* models in which the roles of autophagy in CD4 or CD8 cells could be studied independent to its pro- or anti-survival functions, particularly in the context of an infection or autoimmune disease.

In mature B cells, BCR signal can lead to B cell activation or apoptosis, depending on the context. Autophagy is involved in both processes, with BCR-activation-mediated cell death being associated with extensive autophagosome formation (Watanabe et al., 2008). B cells are capable of antigen processing following BCR ligation and autophagy might be involved in such process (Watanabe et al., 2008). BCR signaling recruits TLR-9 to autophagosome for further interaction with its ligand (Chaturvedi et al., 2008). Systemic lupus erythematosus (SLE) is perhaps the most studied autoimmune disease with respect to the roles of autophagy in autoimmune processes (Pierdominici et al., 2012). There are a number of potential mechanisms by which autophagy might influence the pathogenesis of SLE, modulating both the adaptive and innate immune system. As B cells represent a major player in SLE, in which they act by both antibody-dependent and antibody-independent mechanisms, autophagy-mediated B cell modulation might directly influence the pathophysiology of SLE.

AUTOPHAGY, THE INNATE IMMUNE SYSTEM AND AUTOIMMUNITY

Autoimmunity results from uncontrolled action of the adaptive immune cells, however, activation of the adaptive system depends on the innate immune cells and the innate immune system is perhaps the most extensively studied component with respect to the role of autophagy in shaping the organization and functions of the system (Deretic, 2012b).

The innate immune functions can be broadly categorized into four overlapping stages, migration, recognition and phagocytosis, antigen processing and presentation, and cytokine secretion. Autophagy plays particularly important roles in the last three stages, thereby not only shaping the innate immune response but also influencing the activation of the adaptive immune compartment.

LEVEL ONE: PHAGOCYTOSIS, AUTOPHAGY, AND AUTOIMMUNITY

The role of autophagy in innate immunity is best characterized with respect to pathogen elimination. Both pathogen recognition and intracellular killing can be controlled by autophagy. The autophagy pathway interacts extensively with a number of pattern recognition receptors (PRR) and PRR activation in a wide variety of cases has been shown to induce autophagy (Tang et al., 2012). However, recent evidences suggest that this process might also extend beyond pathogen control.

Phagocytosis can be viewed as a coordinated interaction between two different kinds of players, a predator that engulfs the materials to be cleared, macrophages being the professional phagocytes in the body, and prey to be engulfed such as a pathogen, foreign materials, or dead cells. Autophagy has been found to be an essential process for dead cell clearance. Apoptotic cells release lysophosphatidylcholine (LPC) as a "come-get-me" signal for phagocytes and upregulate phosphatidylserine (PS) as an "eat me" signal on their surface. Autophagy genes are essential for efficient release of LPC and in absence of autophagy, apoptotic cells fail to express PS properly on their surface (Qu et al., 2007).

On the other hand, proteins involved in autophagy pathway, such as LC3-II (Microtubule-associated protein 1 light chain 3 alpha), Beclin 1, and VPS34, are recruited to phagosomes following phagocytosis of particles containing TLR ligands by macrophages (Sanjuan et al., 2007). LC3-associated phagocytosis, a process distinct from classical autophagy, has also been found to be necessary to carry out efficient dead cell clearance (Martinez et al., 2011) and defects in expression of MARCO (macrophage receptor with collagenous structure), a receptor involved in dead cell clearance, has been shown to result into reduced dead cell clearance and SLE in mice (Rogers et al., 2009). Thus, an absence of autophagy or autophagic proteins might result into defective clearance of apoptotic cells. As defects in apoptotic cell clearance have been linked to a number of autoimmune diseases, such as SLE, it is possible that autophagy might modulate the susceptibility to autoimmunity.

It should also be noted that autophagy induction in macrophages has been shown to affect phagocytosis of pathogens, though the reports are conflicting, indicating both increase and decrease in phagocytosis following induction of autophagy (Martinet et al., 2009; Lima et al., 2011). A number of autoimmune diseases are precipitated or exacerbated following infection (Kivity et al., 2009). It would be important to determine if changes in apoptotic cell clearance occur following infection-induced modulation in autophagy, which in turn could modulate the induction or exacerbation of autoimmune processes.

LEVEL TWO: ANTIGEN PRESENTATION, AUTOPHAGY, AND AUTOIMMUNITY

The classic definition of antigen presentation is that extracellular antigens are presented in the context of class II MHC following endocytosis and phagolysosomal degradation (Gannage and Munz, 2010). Recent evidence suggests that this process depends on the autophagy pathway. Characterization of the MHC-II ligands, called ligandome, in a human B lymphoblastoid cell line showed that peptides from intracellular sources are presented on

MHC-II and starvation-induced autophagy enhanced this process (Dengjel et al., 2005). Further, autophagosomes colocalize with MHC-II loading compartments in two important antigen presenting cells (APCs) that shape up the entire adaptive immune repertoire. These cells are TECs, that shape up the T cell repertoire, and the dendritic cells (DC) that act as the professional APCs (Schmid and Munz, 2007; Schmid et al., 2007). Mice with DCs lacking *Atg5* succumbed to HSV-2 infection and showed defective CD4 T cell priming. These DCs showed defective antigen presentation resulting from a profound defect in processing and delivery of antigens containing TLR ligands to MHC-II compartment and delayed phagolysosomal fusion and degradation of the antigens (Lee et al., 2010). Autophagy induction in bone-marrow DCs also enhanced presentation of mycobacterial antigen and mice immunized with rapamycin-treated DCs showed stronger T cell response upon challenge with *Mtb* (Jagannath et al., 2009). Autophagy in APCs is involved in presentation of citrullinated peptide, a hallmark of rheumatoid arthritis, in context of class II MHC (Ireland and Unanue, 2011). It also has been suggested that autophagy may be involved in class I antigen presentation to CD8 T cells, particularly in context of viral infection (English et al., 2009; Uhl et al., 2009).

Dendritic cell-mediated antigen presentation in the context of MHC-II is perhaps an area where the role of autophagy could directly influence autoimmune diseases. Activation of the adaptive immune cells, the major players in most autoimmune diseases, depends primarily on DC-mediated antigen presentation. Genome-wide association studies have identified *Atg5* as one of the susceptibility loci in SLE (Harley et al., 2008; Gateva et al., 2009; Han et al., 2009; Zhou et al., 2011a), though the functional significance of this finding is yet to be established. A number of possibilities have been raised ranging from increased survival of pathogenic T cells to defects in apoptotic cell clearance and several autophagy modulators are currently in clinical trials for SLE (Pierdominici et al., 2012). It is interesting to note that the *PRDM1-ATG5* intergenic region has also been associated with susceptibility to rheumatoid arthritis (Raychaudhuri et al., 2009) and a common role of the autophagy pathway in different autoimmune diseases has been proposed (Zhou et al., 2011a). Another autophagy gene associated with autoimmunity is *Atg16l1*, being implicated in Crohn's disease (CD) (Parkes et al., 2007). DCs with patients of CD, harboring particular *Atg16l1* risk variant, showed defects in autophagy induction and in presentation and priming of pathogen-specific CD4 T cells (Cooney et al., 2010). Interaction between the gut microflora and the mucosal immune system plays a pivotal role in CD (Manichanh et al., 2012) and autophagy in mucosal immune cells might also influence the pathophysiology and outcome of CD. Indeed, a recent report showed that an intact autophagy pathway restricted intracellular replication of adherent-invasive *Escherichia coli*, implicated in the pathogenesis of CD; without affecting the replication of other commensal or pathogenic strains of *E. coli* involved in gastroenteritis (Lapaquette et al., 2010).

It would be informative to determine the phenotype of mice with autophagy deficiency in DCs, in autoimmune disease models of MS, a predominantly CD4 T cell mediated disease, or rheumatoid arthritis.

LEVEL THREE: CYTOKINES, ER STRESS, AUTOPHAGY, AND AUTOIMMUNITY

The third important link between autophagy and autoimmunity could be through modulating cytokine secretion, particularly in the context of inflammasome activation. Autophagy plays a negative role with respect to inflammasome activation and autophagy deficiency leads to increased production of IL-1β and IL-18 (Nakahira et al., 2011; Zhou et al., 2011b). Diseases resulting from increased activation of the immune system comprise two different categories: autoinflammatory diseases, characterized by inflammation mediated predominantly by innate immune cells, including macrophages and neutrophils, and autoimmune diseases in which the adaptive immune cells target the self-antigens (McGonagle et al., 2009). The inflammasome-mediated effects belong to the former category and the role of inflammasomes in these diseases has been reviewed (Shaw et al., 2011). However, given the extensive effects of IL-1β on adaptive immune cells, autophagy might also affect the outcome of autoimmune diseases by modulating IL-1β production. As a whole, IL-1β and IL-18 enhance the functional responses of B and T cells including IL-2 receptor expression and lifespan, antibody production by B cells, and T_H1 and T_H17 polarization effects (Ben-Sasson et al., 2009; Chung et al., 2009). Thus IL-1β and IL-18 might well serve as a bridge between autophagy in innate cells and the adaptive immune response. In this context, IL-1β receptor blockade had beneficial effects in rheumatoid arthritis and has been suggested as a therapy for autoinflammatory diseases (Goldbach-Mansky, 2009). Conflicting reports exist regarding the role of inflammasome activation in EAE, with one study showing roles of NLRP3 inflammasome in EAE progression (Gris et al., 2010), whereas another study found no such role but reported an inflammasome-independent role of ASC (Shaw et al., 2010). The gut microbiota have important roles in shaping the immune system as a whole and particularly in models of MS (Ivanov et al., 2009; Ochoa-Reparaz et al., 2009; Berer et al., 2011). Given the extensive interaction between autophagy and different microbes, it would be informative to determine how autophagy and gut microflora interact to influence autoimmune diseases.

Recent evidence showed that autophagy played an important role in pancreatic beta cell functions and might modulate glucose homeostasis as a whole (Ebato et al., 2008; Jung et al., 2008). ER stress has an important role in the pathogenesis of diabetes and autophagy plays a role in this process as well (Quan et al., 2012). Since ER stress is involved in insulin resistance (Ozcan et al., 2006), autophagy might also be involved in insulin resistance by modulating ER stress response.

LEVEL FOUR: SECRETION, AUTOPHAGY, AND AUTOIMMUNITY

Secretion from cells can proceed through two broad pathways: a well-characterized canonical pathway in which proteins with a signal peptide go through ER and Golgi. However, secretion of proteins without a signal peptide proceeds through an ER-Golgi independent pathway. Interestingly, it was proposed that secretion of such proteins might, in part, be mediated by autophagy (Giuliani et al., 2011). Autophagy-mediated secretion of acyl coenzyme A (CoA) binding protein (ACBP), a cytosolic protein

without a signal peptide, was reported in yeasts (Duran et al., 2010; Manjithaya et al., 2010) and recent reports also suggested that autophagy is involved in a number of secretory processes in immune and non-immune cells. Autophagy modulates secretory processes in the context of osteoclastic bone formation (DeSelm et al., 2011), from mast cells (Ushio et al., 2011), intestinal Paneth cells (Cadwell et al., 2008), presynaptic neurotransmission (Hernandez et al., 2012), and secretion of IL-1β (Dupont et al., 2011). Though the relationship between autophagy and IL-1β secretion is complicated owing to the fact that autophagy inhibits inflammasome activation (Nakahira et al., 2011), a recent report showed that baseline autophagy inhibits IL-1β secretion whereas induced autophagy increases secretion of IL-1β (Dupont et al., 2011).

Elevated levels of type I interferon, interferon-alpha (IFN-α) being the prototypic one, is the hallmark of SLE and clinical trials are going on with monoclonal antibodies against IFN-α in SLE (Lichtman et al., 2012). Interestingly, autophagy is also involved in type I IFN secretion. Autophagy is required in plasmacytoid dendritic cells (pDCs), a major source of IFN-α, for sensing ssRNA virus and secretion of IFN-α (Lee et al., 2007). Similarly mTOR inhibition has also been shown to reduce IFN-α secretion by pDCs in response to TLR-9 ligands (Cao et al., 2008), though whether this is mediated by autophagy remains to be elucidated. However, in contrast to pDCs which use Toll-like receptor 7 (TLR7) for sensing ssRNA viruses, most other cell types in the body use cytosolic RNA sensors such as RIG-I and MDA-5, belonging to the RLR family, for this purpose. *Atg5* deficiency in MEF has been shown to increase IFN-α secretion in context of viral infections by suppressing RLR signaling (Jounai et al., 2007; Tal et al., 2009). This finding represents a non-canonical role of *Atg5* (Takeshita et al., 2008; Tal et al., 2009). Non-canonical autophagy was also shown to mediate IFN-α secretion in response to DNA-immune complex (Henault et al., 2012). Thus, modulation of IFN-α secretion by autophagy pathway might play a role in SLE. In a recent study, analysis of SLE metabolome in serum samples of SLE patients showed increased oxidative stress (Wu et al., 2012). Autophagy deficiency is generally associated with increased oxidative stress secondary to accumulation of damaged mitochondria (Zhou et al., 2011b). On the other hand, autophagy inhibition leads to accumulation of p62 (Mizushima and Komatsu, 2011) which, in turn, activates Nrf2 (nuclear factor erythroid 2-related factor 2) (Komatsu et al., 2007). Nrf2 works as a major player in the oxidative stress response pathway (Kaspar et al., 2009). The effect of modulation of autophagy on oxidative stress of SLE warrants further studies.

It would be interesting to test how autophagy-mediated secretory functions influence autoimmune processes. Though considered to be mediated by adaptive immune cells, autoimmune processes, as in MS, are influenced by innate immune cells (Gandhi et al., 2010). The role of autophagy in secretion might have added significance in cells such as NK cells and neutrophils, which function mainly through secretion and degranulation. Another important area for future exploration would be the role of autophagy in myeloid-derived suppressor cells that suppress T cell function.

CONCLUSION

Given the above potential implications of autophagy in autoimmunity, it is rather surprising that there are only few *in vivo* reports on the functional correlation between autophagy and autoimmune diseases. Non-specific autophagy-lysosomal inhibitors, such as chloroquine, have long been used in clinics to treat SLE and rheumatoid arthritis (He et al., 2011). It is essential to understand the complex interplay between autophagy and autoimmunity in order to develop effective and more specific therapeutic strategies. Autophagy might play different roles in an autoimmune disease depending on the cell types involved and thus the ultimate results of pharmacological modulation might depend on the downstream effector involved. Given the paucity of *in vivo* data, it will be important to determine how the findings from animal models translate to human conditions, as pathophysiology of autoimmune diseases vary considerably between humans and lower animals.

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