AUTOPHAGY IN AUTOIMMUNITY

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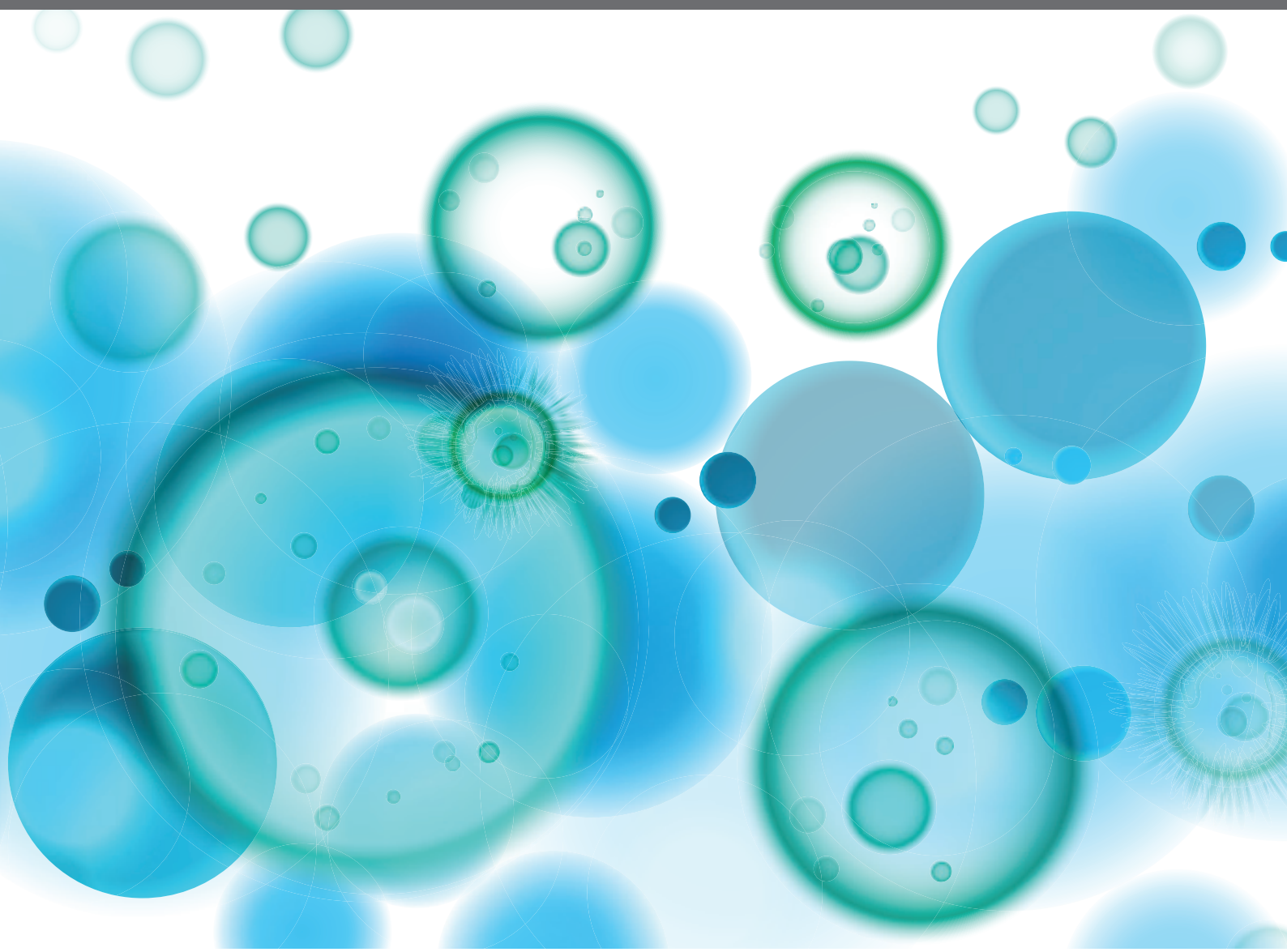

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AUTOPHAGY IN AUTOIMMUNITY

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EDITORIAL
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Editorial: Autophagy in Autoimmunity

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Editorial on the Research Topic

Autophagy in Autoimmunity

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In this Research Topic, we hosted several in-depth reviews, minireviews, and original research articles on the role of autophagy in autoimmunity.

Autophagy is an important process involved in the growth, development, physiology, and pathology of cells. It is a "double-edged sword"—exerting physiologically necessary and therapeutically useful but also potentially detrimental effects on cells and tissues. These contrasting outcomes may be accounted for by differences in the type of autophagy, the tissue or time of induction, activation mode, and stress severity. This ancient set of pathways, conserved from yeast to humans, is now emerging as a central player in nearly all aspects of immunity. Although our knowledge of the molecular mechanisms of autophagy in relation to immune-related disease has been greatly improved in recent years, additional information is needed to fully understand the role of autophagy in clinical settings and to target this set of pathways. Of importance, autophagy has been demonstrated to participate in various processes of the immune response from antigen processing to antigen presentation and therefore should be consider as an important node in the activation and expansion of autoreactive clones. Therefore, we must continue to uncover the pathogenesis and regulation of autophagy in various autoimmune diseases in order to identify new diagnostic and therapeutic approaches.

This Research Topic highlights the essential roles of autophagy in human and animal model tissues that are affected in various autoimmune diseases, including skin, lung, liver, bone, cardiovascular, and intestine systems. Arbogast and Gros discuss the mechanisms linking autophagy to lymphocyte subtype survival and the signaling pathways involved to elaborate the function of autophagy in autoimmune pathology. Reviews by Ye et al. and Hargarten and Williamson illustrate how the function of autophagy-related gene ATG5 and the epigenetic modulation of histones affect RNA transcription and half-life, miRNA expression, and directly impact immunological function that affect human autoimmune diseases. These data from targeted autophagic gene models are important and necessary to elucidate and clarify the precise functional role of autophagy in the etiology of autoimmune diseases and potentially guide future therapeutic applications. Apart from classical autophagy, Gkikas et al. showcase how mitochondrial selective autophagy (mitophagy) connects to cellular health and how its deregulation leads to impaired mitochondrial metabolism and inflammatory disorders. Sil et al. review the potential connection between the autophagic machinery and the homeostasis of skin cells (both immune and non-immune cells), in order to trace the consequences of its disruption by infections, or mechanical damage that may ultimately lead to autoimmunity. Vomero et al. elaborate how autophagy activation is involved in the pathogenesis of rheumatoid arthritis (RA) by highlighting how autophagy contributes

to the survival of synoviocytes and lymphocytes, and how autophagy is implicated in protein citrullination and osteoclastogenesis. Yin et al. address the different roles of autophagy in different autoimmune diseases and recommend future approaches to personalized therapy that consider judicious regulation of autophagy.

In mice, Amersfoort et al. explore the effects of a T cell-specific knock-out of Atg7 (Lck-Cre $Atg7^{f/f}$) on CD4+, CD8+, and NKT cells and observe a decrease in the induction of hepatic steatosis and atherosclerosis. Bendorius et al. report on experiments with the phosphopeptide P140 (Lupuzor), an inhibitor of autophagy, which shows promise for treating patients with lupus and targets chaperone-mediated autophagy (CMA) and macroautophagy rather than mitophagy. A separate effect, the inhibition of neutrophil extracellular trap (NET) release by P140 was also described. Feng et al. demonstrate that autophagy is deregulated in concanavalin A-induced autoimmune hepatitis and that the plant lignin arctigenin inhibits the IFN-γ/IL-6/Stat1 and the IL-6/Bnip3 pathways that are activated in this model of hepatic injury. Thus, arctigenin may have great therapeutic potential in immune-mediated hepatitis. Lin et al. observe that excessive mechanical ventilation with high tidal volumes triggers mitochondrial damage in lungs of rats, which activates mitophagy, results in mitochondrial membrane fracture and mtDNA release, and, ultimately promotes inflammation and injury. These interesting original research studies and expert reviews were indicate that autophagy participates in various immune related injuries. Undoubtedly, the collection of articles also supports the view that autophagy is an attractive target for developing new treatments for immune-mediated disorders.

We are happy to see the issue is welcomed in the field and the aspect of autophagy garnered significant interest from both biomarker, translational, and therapeutic standpoint.

However, at the current stage, the precise contribution of autophagy to the induction and progression of autoimmunity is still not well-understood. There have been studies that provide evidence to support a cytoprotective role of autophagy, and others that support deleterious effects of autophagy. It is plausible that the outcome is context dependent. The difference in the types of injuries and severity of injuries may produce different outcome of autophagy, in that, a certain degree of autophagic activity can maintain tissue homeostasis, whereas excessive autophagic activity results in cell death. Future investigations, for instance by using targeted autophagic gene knockout mice, and novel and systems biology assays, are still necessary.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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The Role of Mitophagy in Innate Immunity

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Mitochondria are cellular organelles essential for multiple biological processes, including energy production, metabolites biosynthesis, cell death, and immunological responses among others. Recent advances in the field of immunology research reveal the pivotal role of energy metabolism in innate immune cells fate and function. Therefore, the maintenance of mitochondrial network integrity and activity is a prerequisite for immune system homeostasis. Mitochondrial selective autophagy, known as mitophagy, surveils mitochondrial population eliminating superfluous and/or impaired organelles and mediating cellular survival and viability in response to injury/trauma and infection. Defective removal of damaged mitochondria leads to hyperactivation of inflammatory signaling pathways and subsequently to chronic systemic inflammation and development of inflammatory diseases. Here, we review the molecular mechanisms of mitophagy and highlight its critical role in the innate immune system homeostasis.

Keywords: autophagy, energy homeostasis, immunity, inflammation, metabolism, macrophages, mitochondria, mitophagy

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INTRODUCTION

The immune system is an intricate network of distinct cell types, tissues, and organs acting synergistically to protect the entire organism against the invasion of various pathogens, including bacteria, fungi, parasites, and viruses among others. The regulation of immune system is a multistep and complex process that classically includes signaling pathways initiated in the surface of immune cells and transmitted to the nucleus through a cascade of phosphorylation events. In turn, epigenetic, transcriptional, posttranscriptional, translational, and posttranslational modifications take place and influence several aspects of innate and adaptive immunity (1, 2). These molecular mechanisms are tightly coordinated and define the onset, the duration, and the magnitude of immune responses neutralizing foreign pathogenic microorganisms and resolving injury (3–6).

Recent evidence underlines the pivotal role of energy metabolism in the regulation of immunity. Mitochondria are dynamic organelles that modify their function, distribution, and structure in response to metabolic state of the cell (7). Proper mitochondrial function not only provides the required energy but also is essential for the establishment and the maintenance of immune cells phenotype and activity (8). Mitochondrial defects, characterized by cytoplasmic calcium elevation, increased reactive oxygen species (ROS) levels, and pronounced release of pro-apoptotic factors and mitochondrial DNA (mtDNA), are key stimulators of inflammatory response pathways (9). Inflammation is a cytoprotective response preserving tissue homeostasis and ensuring viability upon infection or injury (10). Innate immune cells, such as neurotrophils and macrophages, detect harmful stimuli and initiate the inflammatory signaling pathways. However, persistent and unresolved inflammation in metabolic tissues, such as adipose, liver, pancreas, and muscle, leads to the development and progression of several inflammatory pathologies, including atherosclerosis, type-2

diabetes, metabolic syndrome, and inflammatory bowel disease among others (11). The actions of macrophages in these ailments are highly appreciated (12). Inflammasomes are innate immune system receptors and sensors initiating inflammatory responses (13). Excessive mitochondrial dysfunction mediates inflammasome overstimulation in response to noxious stimuli, such as pathogens and cellular debris. In turn, caspase-1 is activated resulting in the generation of pro-inflammatory cytokines and promoting inflammatory cell death (14). Accumulating evidence interconnects impaired energy metabolism and inflammasome hyperstimulation (14–20). Therefore, repairing of mitochondrial functional deficiency or removal of damaged organelles might be beneficial against the undesired chronic systemic inflammation.

In this review, we focus on the role of mitophagy in innate immune system. We first describe the molecular pathways that govern mitophagy as well as its complex interplay with microbe selective autophagy, known as xenophagy. Furthermore, we discuss the essential role of energy metabolism and mitophagy in macrophage homeostasis and inflammasome stimulation. Better understanding of mitochondrial degradation mechanisms is a key requirement for the development of novel therapeutic interventions to tackle numerous pathologies in humans, including inflammatory diseases.

MOLECULAR MECHANISMS OF MITOCHONDRIAL TURNOVER

Cellular homeostasis is often undermined by misfolded and aggregated proteins, damaged organelles, and invading microbes, among others. As a consequence, cells have developed sophisticated quality control mechanisms that remove superfluous and/or damaged cytoplasmic components. Autophagy serves as such a clearance mechanism that is highly responsive to the nature of the stimulus. Based on the response, three different types of autophagy have been described including microautophagy, chaperon-mediated autophagy, and macroautophagy (21, 22). For the purpose of this review, the prominent type of macroautophagy (hereby referred to as autophagy) will be described. General autophagy machinery comprises autophagosome formation and maturation *via* irreversible steps of double-membrane vesicle nucleation and elongation. Mature double-membrane autophagosomes followed by induction of autophagic adaptor proteins can recognize, sequester, and enclose cellular cargo. Ultimately, fusion of the mature autophagosome with the lysosome mediates cargo degradation and recycling of intracellular material (23).

In the immune system, proper mitochondrial function is a prerequisite for inflammatory responses and host defense (24). Accumulation of damaged mitochondria results in excessive ROS production, elevated cytoplasmic calcium levels, and mtDNA release to the cytosol, which in turn triggers inflammasome activation (25–27). Aberrant inflammatory responses have been associated with the development of several autoimmune diseases. Therefore, targeting damaged mitochondria for degradation could be a promising therapeutic strategy against progressive inflammatory pathologies. The removal of damaged mitochondria required the activation of a selective autophagic process, known as mitophagy. Although the crosstalk between mitophagy mechanisms and host defense has been established only recently, a growing body of evidence supports the importance of their coordination.

Following, recent evidence regarding the intricate role of mitophagy in inflammatory responses will be discussed in detail. The involvement of receptors and adaptors molecules is essential for mitophagy initiation and progression. Up to date, several mitochondrial proteins, located either in the outer (OMM) or the inner mitochondrial membrane (IMM), have been characterized as mitophagy receptors. Malfunctioning mitochondria are recognized by a microtubule-associated protein light chain 3 (LC3) in either ubiquitin-dependent or -independent manner (**Figure 1**). In turn, mitophagy receptors, which harbor an LC3-interacting region (LIR) motif, associate directly with LC3 and promote autophagosome formation (28).

The PTEN-Induced Kinase 1 (PINK1)/Parkin Pathway

Mutations in the PINK1 and the E3-ubiquitin ligase (Parkin) were primary associated with Parkinson's disease. Both PINK1 and Parkin are needed for proper mitochondrial function, although their role in mitochondrial turnover was appreciated only recently (29). Under physiological conditions, the transport of PINK1 preprotein into the IMM is followed by sequential proteolytic cleavage by the mitochondrial processing peptidase and presenilin-associated rhomboid-like protease (30–32). The remaining fragment of 52 kDa, which harbors the kinase domain of PINK1, is exposed to the cytosol until its final degradation by the proteasome. Under challenged conditions and loss of mitochondrial integrity, PINK1 fails to translocate to the IMM, and its proteolytic cleavage is blocked. Consequently, active PINK1 accumulates on the OMM though its interaction with the translocons of the outer mitochondrial membrane complex (TOM complex) (33). Then, PINK1 recruits Parkin through a circuit of modifications including phosphorylation of both Parkin and ubiquitin (34–38). Damaged mitochondria are tagged with active Parkin, which, in turn, mediates the polyubiqutination of several OMM proteins, including mitofusin 1 and 2 (MFN1/2), voltage-dependent-anion-selective channel 1, and mitochondrial import receptor subunit TOM20 homolog (TOMM20) among others (**Figure 1**) (39). In certain cases, Parkin-mediated polyubiquitination triggers the proteosomal degradation, as it has been documented for MFN1 and MFN2 (40, 41). As a consequence, mitochondrial fusion is prevented isolating damaged organelles form the healthy mitochondrial network. Thus, mitofusins degradation generates smaller mitochondria that can easily be sequestered by autophagosomal membranes.

The Role of Adaptor Proteins in Mitochondrial Selective Autophagy

Following, Parkin-mediated ubiqutination of mitochondrial substrates, several adaptors proteins have been described to bind ubiquitin chains on the OMM promoting LC3 recruitment (42). Similar to the canonical autophagy mechanism, LC3 recognizes

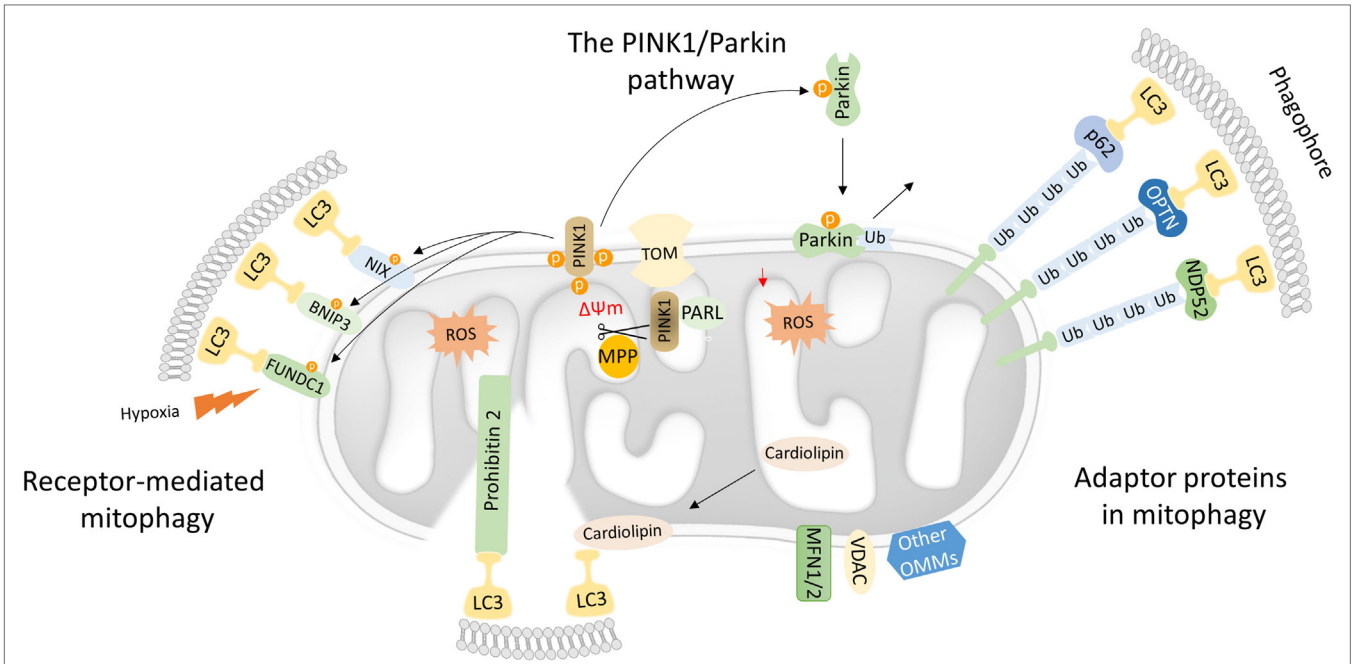

FIGURE 1 | Mechanistic insights into mitophagy process. Dysfunctional mitochondria redirect PTEN-induced kinase 1 (PINK1) to the OMM while its proteolytic cleavage through mitochondrial processing peptidase (MPP) and presenilin-associated rhomboid-like (PARL) proteases is blocked. Concomitantly, PINK1 recruits Parkin through a series of modifications, such as phosphorylation of both Parkin and ubiquitin. In turn, Parkin triggers the polyubiquitination of various OMM proteins including voltage-dependent-anion-selective channel 1 (VDAC1) and MFN1/2. Polyubiquitinated proteins are recognized by several adaptor molecules, including p62, optineurin (OPTN), and NDP52, promoting their recognition by light chain 3 (LC3) and autophogosomal formation. Receptor-mediated mitophagy relies on various OMM proteins such as BNIP3, NIX, and FUN14 domain-containing protein 1 (FUNDC1). In addition, PHB2 and cardiolipin serve as inner mitochondrial membrane receptors in response to mitochondrial damage. Subsequently, PHB2 and cardiolipin are exposed to the cytosol mediating LC3 recruitment *via* their LIR motifs.

and interacts with the adaptor molecules through LIR motifs initiating autophagosomal formation. Numerous autophagy adaptors have been identified so far, including p62/sequestosome-1 (SQSTM1), optineurin (OPTN), next to BRCA1 gene 1 (NBR1), nuclear domain 10 protein 52 (NDP52), and TAX1 binding protein 1 (TAX1BP1) (**Figure 1**) (43).

While the autophagy adaptor p62/SQSTM1 binds ubiquitin chains on depolarized mitochondria and is essential for mitochondrial clustering in a Parkin-dependent manner, the exact role of p62/SQSTM1 in mitophagy has not been verified yet (44–46). Despite the similar kinetics of NDP52, TAX1BP1, and OPTN to dysfunctional mitochondria, cells lacking these adaptors fail to induce mitophagy (47–49). Particularly, loss of OPTN results in most prominent inhibition of mitophagy. Studies in mammalian cells demonstrate that PINK1-mediated recruitment of OPTN and NDP52 autophagy adaptors albeit Parkin was dispensable for mitophagy induction (45). Recent findings suggest that both NDP52 and OPTN are phopshorylated by the Tank-binding kinase 1 (TBK1) and, thereby, enhancing their binding affinity (48, 50–52). Interestingly, TBK1 is activated and phosphorylates OPTN in response to mitochondrial damage. Then, OPTN is recruited on the OMM-promoting mitochondrial elimination (49).

Receptor-Mediated Mitophagy

Mitophagy receptors are commonly found on the outer and IMM. Certain OMM receptors of mitophagy have been identified, including BCL2 interacting protein 3 (BNIP3), Nip3-like protein X (NIX), and the FUN14 domain-containing protein 1 (FUNDC1) among others (43). Surprisingly, cardiolipin and prohibitin 2 (PHB2), which are located in IMM, have been also shown to serve as receptor proteins upon stress conditions (53–55). Mitophagy receptors contain LIR motifs that indicate their direct interaction with LC3 to promote the engulfment of defective mitochondria (**Figure 1**).

NIX contains a mitochondrial BH3 domain and interacts with LC3. NIX has been shown to mediate mitochondrial turnover during reticulocytes' maturation (56). Specifically, NIX-mediated mitophagy relies on a specific motif within NIX cytoplasmic region, which acts as a signaling amplifier to launch additional mitophagic proteins (57). Under low oxygen levels, NIX transcriptional activity is regulated by hypoxia-inducible factor 1 (HIF1), while posttranslational phosphorylation at Ser81 drives mitochondrial clearance in ischemic stroke (58, 59). In addition, NIX phosphorylation at Ser34 and Ser35 residues surrounding LIR motif increases its binding affinity to LC3 (60). A recent study has also documented an alternative role of NIX-mediated mitochondrial quality control in human fibroblasts lacking PINK1 and Parkin (61, 62). This non-canonical regulation of mitophagy by NIX can give rise to novel therapeutic approaches for removal of malfunctioning mitochondria in Parkinson's disease.

BNIP3 was also characterized as a BH3 protein on the OMM initially involved in cell death process (63). Despite its

role in cell death, a potent role of BNIP3 in mitophagy has been reported. Specifically, its N-terminal LIR motif serves as a signaling platform for LC3-mediated mitochondrial sequestration through autophagosomes. Notably, sufficient LC3 binding is accompanied by BNIP3 phosphorylation at Ser17 and Ser24, proximal to the LIR motif (64–66). Surprisingly, BNIP3-deficient mammalian cells showed induction of PINK1 proteolysis and subsequently failed to promote mitophagy (67). Upon hypoxia, HIF1 triggers BNIP3 expression levels, which, in turn, inhibits cleavage of PINK1 proteolysis and promotes mitophagy (67). Depletion of DCT-1 the *Caenorhabditis elegans* homolog of both BNIP3 and NIX results in mitophagy inhibition suggesting a conserved role of autophagy receptors among species (68).

In mammalian cells, hypoxia promotes the binding of mitophagy receptor FUNDC1 to LC3 (69). Under normal oxygen levels, LC3 binding is perturbed due to phosphorylation of FUNDC1 at Tyr18 and Ser13 by Src and casein kinase II, respectively (70). In response to hypoxic conditions, phosphoglycerate mutase family member 5 phosphatase is activated and dephosphorylates FUNDC1 enabling its functional association with LC3 autophagosomal protein (71). Recently, it has been reported that FUNDC1 is a substrate of the serine/threonine-protein kinase unc-51-like kinase 1 (ULK1) (72). ULK1 translocates to damaged mitochondria and phosphorylates FUNDC1 at Ser17 triggering mitophagy in response to stress conditions (72). However, several homeostatic mechanisms have been evolved to regulate and fine-tune mitophagy during hypoxia (23, 73). FUNDC1-mediated mitophagy is block due to activation of receptor-interacting serine/threonine-protein kinase 3 followed by phosphorylation of FUNDC1 upon reperfusion injury (73). These results highlight the interplay between mitophagy and necroptosis to maintain cellular homeostasis during hypoxic conditions. Furthermore, mitochondrial E3-ubiquitin protein ligase 5 ubiquitinates FUNDC1 mediating its proteasomal degradation in response to hypoxia (23, 74).

Although the aforementioned receptors are located on the OMM, the possibility that an IMM protein could serve as a mitophagy receptor is not excluded. Toward this direction, PHB2 was recently characterized as an IMM mitophagy receptor (55). Particularly, it has been showed that Parkin-dependent loss of mitochondrial integrity and permeabilization of the OMM enhance the interaction between LC3 and PHB2, thereby promoting mitophagy. In addition, PHB2-mediated mitophagy is involved in selective clearance of paternal mitochondria in *C. elegans* embryos (55).

Similar to PHB2, cardiolipin belongs to the group of IMM mitophagy receptors. Biosynthesis of cardiolipin occurs in the IMM, where it is primary located. In response to mitochondrial stress, cardiolipin migrates to the OMM setting up a signaling platform for mitophagy and apoptosis initiation. Furthermore, migration of cardiolipin on the OMM is essential for its direct binding of with LC3 and mitophagy stimulation (53). A recent study in yeast showed that both the mitogen-activated protein kinase and the protein kinase C (PKC) are involved in cardiolipin-mediated mitophagy. Interestingly, activation of PKC was sufficient to reverse mitophagy defects phenotypes in cardiolipin-depleted cells (54). Taken together, detail mechanistic insights relative to the activation and function of IMM mitophagy receptors will provide novel therapeutics targets in numerous mitochondrial disorders.

THE INTERPLAY BETWEEN MITOPHAGY AND XENOPHAGY

The bacterial origin of mitochondria is a result of an endosymbiotic event that happened billions of years ago. Although the evolutionary changes, mitochondria retained several vestiges of their prokaryotic ancestors. First, mitochondria are semi-autonomous organelles that could expand or shrink their population through fission/fusion events independently of cell division (75). Mitochondria contain their own circular genome that displays evident bacterial characteristics such as decreased methylation events, lack of histones, polycistronic, and intron-less genetic loci (76). Furthermore, mitochondrial inner membrane is composed of cardiolipin, a specific phospholipid that exist uniquely in prokaryotic membranes (77). In addition, mitochondrial protein translation begins with *N*-formylmethionine, which is a derivative of methionine and a common feature of bacterial and organellar protein synthesis (78). Given the ancestral similarities between mitochondria and bacteria, it is worthwhile to investigate the common molecular mechanisms that regulate mitophagy and microbe selective autophagy, known as xenophagy.

The function of innate immunity is driven by the recognition of endogenous and exogenous signals by innate immune system receptors, such as toll-like receptors (TLRs), formyl peptide receptors, nucleotide oligomerization domain-like receptors (NLRs), retinoic acid-inducible gene 1 (RIG-1)-like receptors (RLRs), C-type lectin receptors (CLRs), and inflammasomes. Both pathogen-associated molecular pattern (PAMP) molecules, which are microbial derived stimulators (e.g., microbial nucleic acids, lipoproteins, and carbohydrates), and damage-associated molecular pattern (DAMPs) molecules, which are released by the cells of the host in response to injury or necrotic cell death (e.g., mtDNA, cardiolipin, ATP, and formyl peptides), are recognized by the immunity receptors mediating, in turn, inflammatory signaling pathways (10, 11).

Similar to mitophagy induction, PAMPs promote the recruitment of autophagic machinery through a series of ubiquitination events and the stimulation of several receptor and adaptor molecules in response to pathogen invasion. Following *Mycobacterium tuberculosis* infection in macrophages, bacterial DNA is recognized by cGMP-AMP synthase/stimulator of IFN genes mediating type 1 interferon generation and xenophagy initiation (51). Galectin-8 is a cytosolic PAMPs receptor that binds *Salmonella typhimurium* and prevents its proliferation. Interestingly, galectin-8 recruits NDP52 adaptor protein in a ubiquitin-dependent manner and promotes xenophagy (79). Moreover, several autophagy adaptor proteins, including p62, OPTN, and NBR1 with a well-established role in mitophagy, bind ubiquitinated bacteria and mediate autophagosome formation (**Figure 2**) (50, 80, 81). Thus, ubiquitination events have an important role in the recognition and the elimination of pathogenic microorganisms

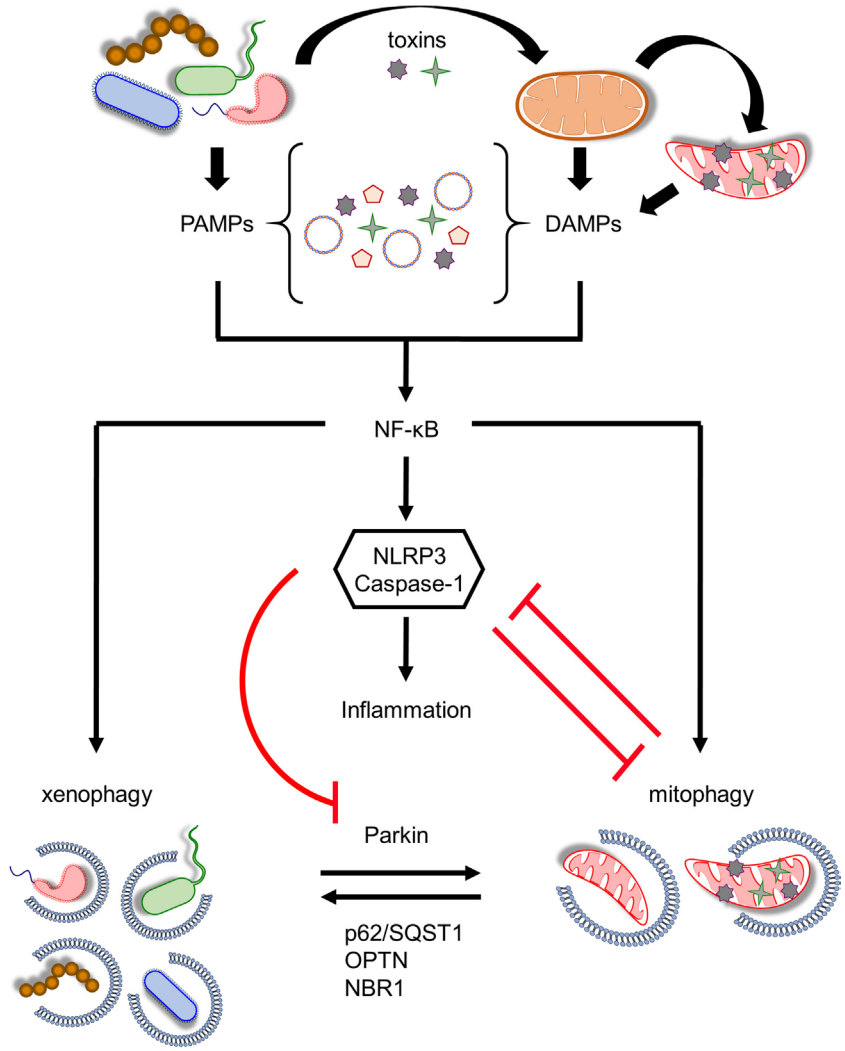

FIGURE 2 | Coordination between mitophagy and xenophagy during infection. Pathogen-derived toxins and secreted proteins impair mitochondrial homeostasis leading to mitochondrial DNA and formyl peptides release and excessive generation of mitochondrial ROS. Consequently, pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) trigger nuclear factor-κB (NF-κB) transcription factor promoting immune responses through NLR family, pyrin domain-containing 3 (NLRP3) inflammasome activation and pro-inflammatory cytokines production. In parallel, NF-κB establishes a self-limiting program to prevent persistent and uncontrolled inflammation by augmenting mitophagy and pathogen removal *via* xenophagy. NLRP3 stimulation amplifies mitochondrial defects by inhibiting mitophagy through the direct caspase-1-mediated proteolytic cleavage of Parkin. Mitochondrial and bacterial autophagic processes share several common regulatory factors, including Parkin, p62/SQST1, optineurin (OPTN), and NBR1 among others, highlighting their tight communication. This intricate interplay between energy metabolism and innate immune responses upholds cellular and tissue homeostasis and survival during pathogen invasion.

bearing a strong resemblance to mitophagy (82). Strikingly, the E3-ubiquitin ligase Parkin has been shown to mediate xenophagy (83). *M. tuberculosis* ubiquitination is abolished in murine and human Parkin-depleted macrophages resulting in defective bacterial elimination (83). Furthermore, Parkin-deficient nematodes, flies, and mice are more vulnerable to multiple pathogenic bacteria, such as *M. tuberculosis*, *Mycobacterium marinum*, *S. typhimurium*, *Listeria monocytogenes*, and *Pseudomonas aeruginosa* (83, 84). Congruently, several polymorphisms in the *PARK2* genetic locus are correlated with enhanced susceptibility to *Mycobacterium leprae* and *S. typhimurium* in humans, highlighting the evolutionary conserved function of Parkin in innate immunity (85–87).

The question then arises: How does bacterial infection stimulate the E3 ligase activity of Parkin? During mitochondrial removal, PINK1 is stabilized on the OMM mediating Parkin translocation and activation (74). Recently, PINK1 has emerged as a critical regulator of innate immunity, as it has been shown that loss of PINK1 enhances inflammation by attenuating the levels of pro- and anti-inflammatory cytokines leading subsequently to cell death (88). Moreover, PINK1-depleted nematodes are sensitive to *P. aeruginosa* infection (84). However, the role of PINK1 in xenophagy needs further to be elucidated. An alternative candidate of Parkin activation could be the serine/threonine kinase TBK1. Interestingly, TBK1 has an essential role in mitophagy regulation, as it has been found to phosphorylate

several autophagy adaptor proteins including p62, OPTN, and NDP52, enhancing cargo recognition and autophagosomal engulfment (48, 50–52). Notably, TBK1 is required for efficient identification and removal of *M. tuberculosis* and *S. typhimurium* in mammals (50, 51). Hence, TBK1 kinase could mirror PINK1 activity during infection; however, further investigation of the functional association between TBK1 and Parkin is needed.

MITOCHONDRIA: A SIGNALING HUB OF INNATE IMMUNE SYSTEM STIMULATION

Considering the structural similarities between mitochondria and bacteria, an intricate question follows: Could mitochondria be misrecognized by the innate immunity as "invaders" promoting lethal inflammatory responses? Severe physical injury and/or trauma could lead to tissue disruption and cellular damage mediating the release of mitochondrial DAMPs molecules, such as formyl peptides and mtDNA, into the host bloodstream (89). Then, the immune system is alarmed resulting in the development of systemic inflammatory response syndrome (SIRS), which is characterized by fever, increased heart rate, low blood pressure, shortness of breath, multiple organ failure, and increased lethality rates. Although, the characteristic features of SIRS resemble sepsis, an inflammatory response to severe infection, pathogenic microorganisms are need not to be present. Thus, mitochondria lie in the heart of innate immunity initiating uncontrolled immune response upon noxious stimuli.

Several studies have shown the immunogenic capabilities of defective mitochondria (90). Impaired mitochondrial metabolism results in increased mitochondrial ROS (mtROS) levels and defective ion homeostasis. Compelling evidence has been accumulated suggesting that the cytoplasmic levels of mtDNA and mtROS signaling are critical factors in innate immunity *via* inflammasome activation (16–18). The NLR family, pyrin domain-containing 3 (NLRP3) inflammasome is one of the well-studied inflammasomes protecting the cell against pathogens invasion (13). However, dysregulation of NLRP3 activity leads to chronic inflammation and the development of several pathologies, such as neurodegeneration, metabolic disorders, and sepsis (91).

Infections impair mitochondrial homeostasis mediating mtDNA release, excessive mtROS production, and subsequently inflammasome stimulation (**Figure 2**). NLRP3 is triggered in response to mitochondrial damage promoting caspase-1 activation. Consequently, caspase-1 generates mature interleukin (IL)-1β and IL-18 promoting inflammatory cell death (14, 92). To this direction, there is evidence suggesting that LC3B-, ATG5-, ATG16L1-, and Beclin1-deficient macrophages display accrual of defective mitochondria, increased cytosolic levels of mtDNA, and mtROS in response to noxious stimuli, resulting in NLRP3 activation and IL-1β secretion (15, 17, 18). In addition to mtDNA and mtROS, NLRP3 is also activated by cardiolipin. Mitochondrial membrane depolarization triggers the translocation of cardiolipin from the IMM to OMM promoting its direct association with NLRP3 (93). NLRP3–cardiolipin interaction is pivotal for inflammasome stimulation indicating that mitochondria act as a central signaling platform for innate immune responses. Experimental evidence highlights the existence of a positive feedback loop between inflammasome and mitochondria, since caspase-1 amplifies mitochondrial dysfunction by impairing mitochondrial membrane potential, increasing mitochondrial membrane permeabilization, and promoting mitochondrial network fragmentation to enhance inflammatory responses (19). Notably, mitophagy is also inhibited upon inflammasome stimulation, since it is reported that Parkin is cleaved by caspase-1 preventing the degradation of damaged organelles (19, 94). Concurrently, accumulation of defective mitochondria results in enhanced mtROS production and hyperstimulation of NLRP3 (**Figure 2**).

Nuclear factor-κB (NF-κB) is the master coordinator of inflammatory signaling acting downstream of immune receptors (95, 96). In addition to the production of multiple inflammatory chemokines and cytokines, NF-κB also regulates inflammasome activation (97). The TLR9 innate immune receptor recognizes mtDNA, which is released from necrotic cells, resulting in NF-κB nuclearization and the induction of several pro-inflammatory cytokines, such as tumor necrosis factor α (TNFα) and IL-6 (98). A recent study uncovered a self-regulatory and anti-inflammatory pathway, whereby NF-κB restricts NLRP3 function through p62-dependent mitophagy (20). NF-κB enhances the expression of p62 adaptor molecule mediating the removal of damaged mitochondria. Moreover, p62-, Parkin-, and ATG7-depleted macrophages display pronounced NLRP3 activity, since they accumulate defective organelles releasing inflammasome-activating signals in response to harmful stimuli (20). Therefore, NF-κB establishes a self-limiting program to inhibit unresolved inflammation, whereby mitophagy has a central role preventing tissue damage through the maintenance of mitochondrial metabolism (**Figure 2**).

Mitochondrial antiviral signaling protein (MAVS) is an RLR immune receptor that is localized on the OMM (99). Elevated mtROS levels trigger oligomerization of MAVS, which subsequently activate NF-κB to regulate host defense and inflammation (100). Interestingly, MAVS recruits NLRP3 on the OMM during viral infection (**Figure 3**). Thereby, inflammasome assembly and activity is enhanced due to the close proximity of NLRP3 with the sites of mtROS generation (101, 102). MAVS signaling is negatively regulated by ubiquitination events mediated by the ubiquitin E3 ligases SMURF1, Gp78, and Mul1 (103–105). Notably, SMURF1, Gp78, and MUL1 are also involved in the regulation of mitochondrial removal indicating the immunosuppressive role of mitophagy in response to noxious stimuli (**Figure 3**) (106–108). Indeed, a very recent study revealed that anti-inflammatory cytokine IL-10 promotes mitophagy to restrain inflammasome activity and the uncontrolled inflammatory responses upon lipopolysaccharide (LPS) treatment (109).

Altogether, these results demonstrate the pivotal role of mitochondria in the innate immune signaling pathways and underline mitophagy as a key regulatory mechanism limiting excessive inflammation and preserving tissue homeostasis. Although the delineation of mitophagy–innate immunity interplay represents a milestone in the field of immunometabolism, several mechanistic questions still remain elusive, including how mitophagy and

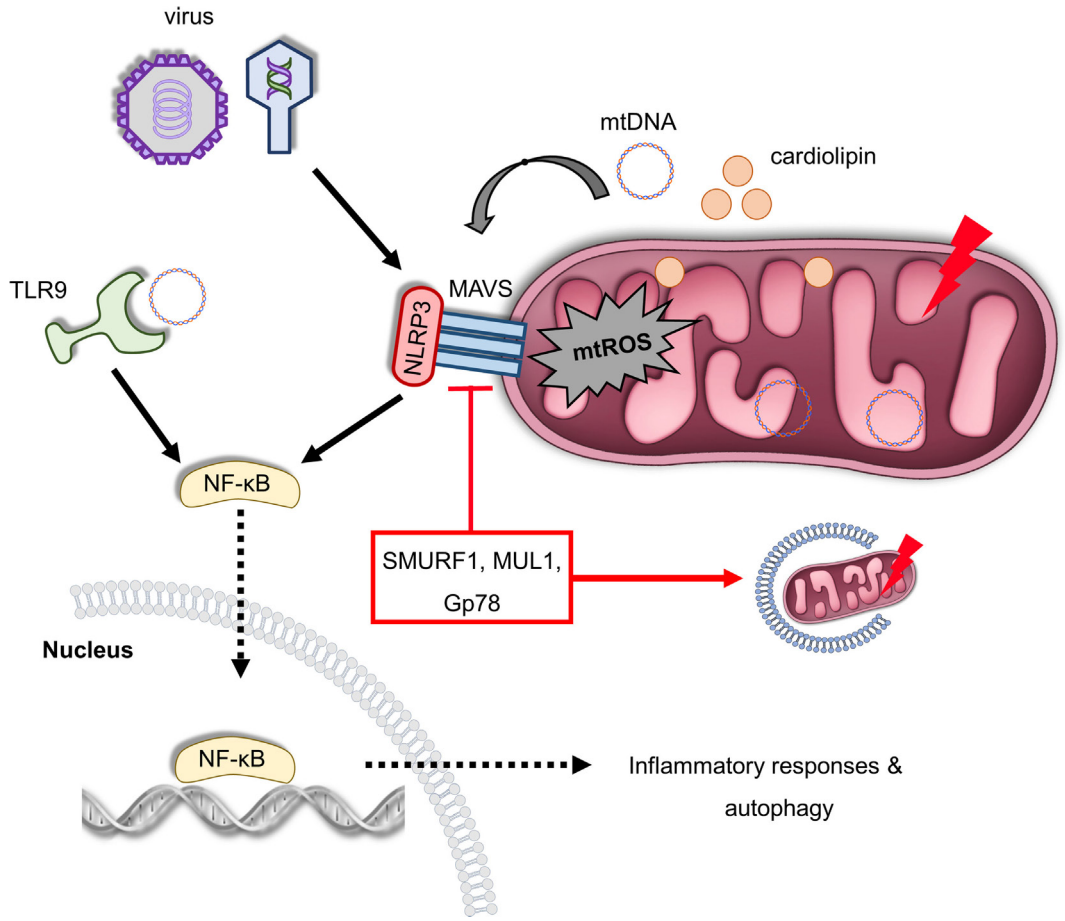

FIGURE 3 | Mitochondria act as a signaling platform of innate immunity. Viral infection triggers mitochondrial antiviral signaling protein (MAVS) oligomerization in OMM, which promotes the expression of several inflammatory and immune genes *via* nuclear factor-κB (NF-κB) activation. Furthermore, MAVS can be activated by excessive mitochondrial ROS (mtROS) production recruiting NLRP3 on the OMM in response to infection and mitochondrial damage. In turn, NLRP3 assembly and activity is enhanced due to elevated reactive oxygen species levels. In addition, depolarized mitochondria release cardiolipin and mitochondrial DNA (mtDNA) in the cytosol-stimulating NLRP3, which generates pro-inflammatory cytokines and mediates inflammation. mtDNA can be also sensed by toll-like receptor (TLR) 9 immune receptors promoting activation of both NLRP3 and NF-κB. The immunosuppressive role of the ubiquitin E3 ligases SUMRF1, MUL1, and Gp78 is characterized both by the negative regulation of MAVS signaling and mitophagy stimulation. Thus, multiple innate immune signaling pathways, such as MAVS, NLRP3 inflammasome, and TLR9, depend on mitochondrial function to regulate immune responses.

inflammasome activity are coordinated in response to infection, physical injury, and/or trauma and which already known mitophagy factors are involved during mitochondrial removal in immune cells.

MITOPHAGY AND MACROPHAGE HOMEOSTASIS

Macrophages are indispensable phagocytic cells coordinating both pro-inflammatory and anti-inflammatory responses as wells as tissue homeostasis and repair during infection (**Figure 4**) (110). Halted macrophages can be stimulated and adapted to the host and pathogen nature while their activity undergoes dynamic changes (111). Following pathogen invasion and recognition through the innate immune receptors, such as TLRs and CLRs, immune cells produce inflammatory cytokines (112–114). Particularly, secretion of interferon γ (IFNγ) by helper T cells 1, triggers macrophages pro-inflammatory polarization, known as classically activated or M1 macrophages (115, 116). Similar to IFNγ production, LPS signaling also stimulates M1 macrophages through TLRs (117). The tumoricidal and microbicidal properties of M1 macrophages are highlighted by their ability to produce and release several pro-inflammatory cytokines, such as IL-1β and TNFα, and cellular byproducts including ROS and nitric oxide (NO) (118–120). On the contrary, polarization of macrophages toward an anti-inflammatory phenotype is known as alternatively activated or M2 macrophages. In response to specific stimulus, a large spectrum of mediators has been reported to activate M2 macrophages (121). Stimulation of M2 macrophages requires in part, secretion of IL-4 and IL-13 cytokines through Th2 cells (122,123). Particularly, IL-4- and IL-13-induced M2 macrophages are important for wound healing, while IL-10-induced M2 macrophages regulating host immunity and tissue homeostasis

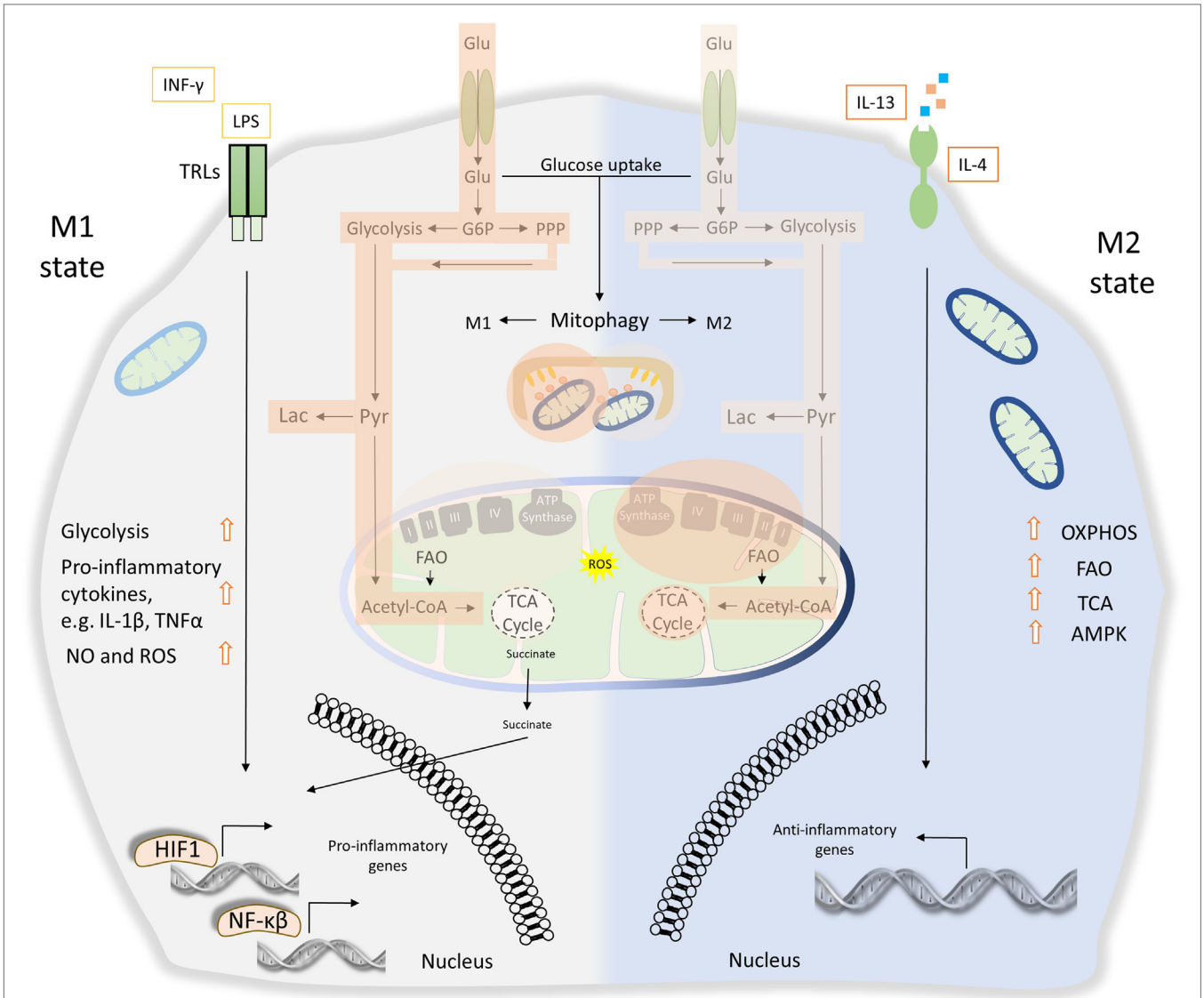

FIGURE 4 | The role of energy homeostasis and mitophagy in metabolic signature of M1 and M2 polarization. M1 macrophages are characterized by secretion of pro-inflammatory cytokines, such as interleukin (IL)-1β and tumor necrosis factor α (TNFα). Thus, M1 macrophages display high tumoricidal and microbicidal properties. M1 macrophages rely heavily on glycolysis accompanied with increased glucose uptake and pentose phosphate pathway (PPP). Metabolic byproducts, such as reactive oxygen species (ROS) and nitric oxide (NO), are abundantly produced. During M1 macrophage polarization, succinate is released to the cytosol and promotes stabilization of hypoxia-inducible factor 1 (HIF1), which in turn drives the transcription of several genes involved in glycolysis. Translocation of both HIF1 and nuclear factor-κB (NF-κB) to the nucleus mediates the expression of numerous pro-inflammatory genes. On the other hand, anti-inflammatory function of M2 macrophages relies more on mitochondrial oxidative phosphorylation (OXPHOS), tricarboxylic cycle (TCA) fluxes, and fatty acid oxidation (FAO). AMP-activated protein kinase (AMPK) is activated and triggers FAO, which fuels OXPHOS. Mitophagy act as a key factor of M1/M2 differentiation. During M1 polarization, mitochondria clearance through mitophagy stimulation favors metabolic reprogramming to glycolysis. Activity of glycolysis, OXPHOS, TCA, and FAO is labeled with bright orange frames (less active state) and dark orange frames (more active state).

(124). In addition, the secretion of immune complexes together with agonist of TLRs triggers M2 macrophages exerting an immunoresponsive function (125). Non-activated macrophages could be differentiated to M2 phenotype by the transforming growth factor-β activity (124, 126). Given the distinct modes of action, further classification of M2 macrophages has been proposed (57, 115, 127).

Apparently, numerous pathogen-derived molecules and biochemical signals orchestrate macrophage activation from pro-inflammatory M1 toward to anti-inflammatory M2 phenotype, including their intermediate responses (110). As a consequence of these extreme heterogeneities, M1 and M2 macrophages undergo broad transcriptional and metabolic alternations, beyond their energy demands (8). Metabolic signature of both M1 and M2 macrophages activation imposes a tight coordination between glycolysis, pentose phosphate pathway (PPP), fatty acid oxidation (FAO), mitochondrial oxidative phosphorylation (OXPHOS), and tricarboxylic cycle (TCA) fluxes (128).

It has been shown that M1 macrophages activity is mostly affected by glycolysis and PPP while mitochondrial OXPHOS and TCA capacities are decreased (129, 130). Specifically, LPS-activated M1 macrophages promote the release of succinate dehydrogenase to the cytosol resulting in the stabilization of HIF1, which in turn regulates the expression of several pro-inflammatory genes such as IL-1β (**Figure 4**). Absence of HIF1 or inhibition of glycosis in bone marrow-derived macrophages (BMDMs) failed to induce LPS-mediated IL-1β expression (131). Metabolic rewiring from OXPHOS toward glycolysis followed by IL-1β induction requires the stimulation of pyruvate kinase M2 in LPS-activated M1 macrophages (132). On the other hand, M2 macrophages activity relies more on FAO, mitochondrial OXPHOS, and TCA, but less in glycolysis and PPP fluxes (133). Particularly, the stimulation of M2 macrophage requires AMP-activated protein kinase activation as well as induction of FAO to fuel OXPHOS (**Figure 4**) (134). Notably, byproducts of mitochondrial metabolism, such as mtROS, have also been involved in innate immune responses and macrophages activity. Production of mtROS has been shown to mediate inflammatory cytokine secretion (135). Accordingly, several studies suggest that augmented mtROS levels are required for the bactericidal activity of macrophages (119, 120).

Only recently, the metabolic signature underpinning macrophage activation has been associated with mitochondrial clearance through mitophagy. A latter report suggests that NIX-mediated mitophagy regulates metabolic shift during macrophage differentiation (136). Mitophagy is triggered during M1 macrophage polarization in response to LPS/IFNγ treatment favoring metabolic rewiring to glycolysis. Interestingly, NIX-depleted M1 macrophages present decreased levels of glycolytic enzymes and pro-inflammatory cytokines, indicating metabolic defects during their differentiation process (136). IFN-stimulated gene 15 (ISG15) has also been shown to regulate both cellular metabolism and mitophagy of BMDMs in response to vaccinia virus infection (137). In addition to impaired mitochondrial function, ISG15-deficient macrophages present reduced Parkin protein levels and inhibition of mitophagy upon IFNγ stimulation. Moreover, loss of ISG15 leads to defective macrophages polarization and subsequently to enhanced virus susceptibility (137).

As reported, IL-10-depleted murine BMDMs favors glucose uptake and glycolysis while inhibits OXPHOS in response to LPS treatment (109). IL-10-deficient macrophages display accumulation of damaged mitochondria due to inhibition of mitophagy. Interestingly, IL-10 regulates mitochondrial homeostasis through the inhibition of mTOR signaling (109). A recent study in mouse macrophages has also demonstrated that high glucose supplementation results in mitophagy defects and promotes M1 macrophages activation (138). Therefore, mitophagy regulation is indispensable for the proper determination of M1/M2 macrophage phenotypes (**Figure 4**). Recently, the essential role of fine-tuned mitochondrial metabolism and mitophagy was underlined in a mouse model of sepsis (139). Bone marrow-derived mesenchymal stem cells (BMSCs) promote survival and performance of various organs during septic shock. It is shown that the beneficial effects of BMSCs were mediated by mitophagy induction in cocultured BMDMs resulting in decreased mtROS levels and inflammasome restriction during cecal ligation and puncture-induced sepsis (139). Although the beneficial effect of mitophagy induction during pronounced inflammatory conditions, mitophagy hyperstimulation could also be detrimental for cellular physiology. Runaway mitophagy mediates mitochondrial content elimination conferring resistance to apoptosis in alveolar macrophages and subsequently leads to the development and progression of idiopathic pulmonary fibrosis (IPF) (140).

Taken together, mitochondrial homeostasis and mitophagy are crucial for the determination of macrophages functional behavior. However, the mechanistic details that orchestrate macrophage intracellular metabolism remain still elusive. A better understanding of the interconnection between mitophagy and macrophages fate and function in response to injury and/or infection could lead to unpreceded understanding of several immune disorders.

MITOCHONDRIAL FUNCTION AND MITOPHAGY IN SEPTIC SHOCK

Infection triggers the activation of immune system promoting the production and release of several cytokines and chemokines into the host circulation. In turn, inflammatory responses are initiated mediating the signal throughout the body of the organism to confer protection against pathogens. However, persistent systemic inflammatory conditions, such as sepsis, impairs cellular metabolism leading to generalized shock, compromised function of multiple organs and eventually to death. Sepsis or septicemia is a life-threatening condition and a leading cause of morbidity and mortality worldwide (141–143). The pronounced mitochondrial defects, which are described in septic conditions, and the significant role of mitochondria in innate immune signaling indicate their involvement in the development and progression of sepsis (144).

Peripheral mononuclear blood cells, isolated form septic patients, present hyperactivated mitogen-activated protein (MAP) kinase kinase 3 (MKK3) (145). Notably, MKK3 stimulates p38 MAP kinase signaling to promote septic shock (146, 147). MKK3-depleted macrophages display improved energy metabolism, which is characterized by reduced mtROS production, larger and elongated mitochondria, elevated membrane potential and ATP generation during LPS challenge (148, 149). These results suggest that MKK3 alters mitochondrial function to further enhance inflammatory responses. Indeed, MKK3 depletion restricts NF-κB nuclearization and inflammasome stimulation conferring resistance to septic injury (147, 148). Recently, MKK3 has revealed as an essential factor of mitochondrial homeostasis, since MKK3 deficiency influences the modulation of several proteins, including sirtuin 1, PINK1, and Parkin among others, to promote both the induction of mitophagy and mitochondrial biogenesis (145).

Further supporting the immunosuppressive role of mitophagy in sepsis, a recent study showed that senstrin 2 (SESN2) restrains NLRP3 activity by promoting the elimination of damaged mitochondria in macrophages (150). Interestingly, SESN2 mediates the association between p62 and the ubiquitin chains on the

OMM, thereby, promoting the perinuclear localization of dysfunctional organelles. In turn, SESN2 initiates autophagosomal formation and mitochondrial turnover by increasing the levels of the autophagy initiator protein ULK1 (150). It has been reported that NO prevents NLRP3 activation and protects against LPS-induced septic shock (151). Notably, NO, generated by nitric oxide synthase 2, upregulates SESN2 protein levels contributing to inflammasome suppression during LPS-induced sepsis (150). In addition, basal levels of NO could also promote mitochondrial translocation of Parkin mediating PINK1-independent mitophagy (152). On the other hand, under nitrosative stress conditions, the excessive NO generation induces *S*-nitrosylation of PINK1 inhibiting its kinase activity and preventing Parkin mitochondrial recruitment (153). Thus, further investigation is needed to delineate the role of NO activity in the regulation of mitophagy and energy metabolism in immune cells.

CONCLUDING REMARKS

Mitophagy holds an essential role in the regulation of inflammatory responses. Several molecular mechanisms are coordinated to mediate mitophagy, preserving cellular and organismal survival in response to intracellular and environmental stimuli (68). Mitophagy deregulation leads to impaired mitochondrial metabolism and eventually to systemic unresolved inflammation and tissue collapse. While basal changes in mitochondrial number and function induce mitophagy, under severe mitochondrial damage excessive mitophagy leads to programmed cell death (154). Therefore, mitophagy impacts organismal health and disease in a context- and dose-dependent fashion (66, 140, 155, 156). To this direction, shortage of mitochondrial population due to induction or persistent mitophagy has also been reported (140, 157, 158). It is becoming evidence that mitophagy defects as well as excessive mitophagy events represent common features of several pathologies. In particular, runaway mitophagy lowers mitochondrial population in alveolar macrophages conferring resistance to apoptosis, which in turn, leads to IPF progression (140). As noted, growth of tumor cells upon hypoxia requires stimulation of glycolysis and lactate production accompanied by increased mitophagy (159, 160). Within this scope, mitochondrial localization of valosin-containing protein drives hyperactivation of mitophagy and leads to neurodegeneration in Huntington's disease (161). Overall, the degree to which mitophagy contributes to these pathologies has not been elucidated yet. Moreover, it remains to be clarified whether pharmacological stimulation or inhibition of mitophagy represents a potent therapeutic strategy for several pathologies including immune disorders, cancer, and neurodegeneration among others. Although great progress has been already made in the field of mitophagy-inducing drugs, better understanding of the molecular mechanisms would ensure the identification of novel targets for maximum therapeutic efficiency.

Several synthetic and/or natural compounds have been shown to induce mitophagy maintaining cellular and organismal homeostasis (162). p62/SQST1-mediated mitophagy inducer (PMI) is a chemical compound that promotes mitochondrial removal through nuclear factor E2-related factor 2 (Nrf2) stimulation (163). Nrf2 is the master regulator of cellular homeostasis orchestrating the gene expression of multiple cytoprotective proteins, including antioxidant, anti-inflammatory, and detoxification enzymes among others, to enhance survival and viability during stress (164). Moreover, Nrf2 activity is pivotal for proper mitochondrial function and metabolism, since it regulates the expression levels of several mitochondrial related genes (165). PMI prevents the proteasomal degradation of Nrf2 by disrupting its association with Kelch-like ECH-associated protein 1 (163). In turn, Nrf2 is stabilized and enhances p62/SQST1 expression promoting PINK1/Parkin-independent mitophagy upon PMI supplementation (163). Notably, deregulation of Nrf2 function results in the development of autoimmune diseases and increased susceptibility to pathogens, since Nrf2 is implicated in several innate immune responses (166–169). Therefore, PMI administration could be beneficial against the aforementioned pathological conditions.

Natural occurring compounds able to induce mitochondrial turnover have attracted much attention in recent years. As such, spermidine and urolithin A have been shown to induce mitochondrial elimination promoting longevity and stress resistance in many organisms, including mice, flies, and nematodes (170–172). In addition to mitophagy, both spermidine and urolithin A present anti-inflammatory properties modulating mitochondrial metabolism and subsequently NF-κB activity (173–178).

The balanced interplay between the inflammatory and the immunosuppressive signaling pathways highlights that sustaining mitochondrial network integrity and energy metabolism safeguard tissue and organismal homeostasis in response to constant exposure to immunogenic signals. Hence, examination of already FDA-approved drugs and several pharmacological screenings are taking place to characterize novel molecules that can be used to enhance immune system homeostasis through the regulation of mitophagy. Although, experimental evidence underlines the cytoprotective effects of mitophagy modulators on animal disease models, the therapeutic potential and the levels of cytotoxicity on humans remain to be determined. Thus, interventional clinical studies need to be organized to monitor and validate the therapeutic capacity of mitophagy-inducing agents against immune system diseases.

AUTHOR CONTRIBUTIONS

IG and KP wrote the manuscript. NT organized and edited the manuscript.

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REVIEW
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Lymphocyte Autophagy in Homeostasis, Activation, and Inflammatory Diseases

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Autophagy is a catabolic mechanism, allowing the degradation of cytoplasmic content *via* lysosomal activity. Several forms of autophagy are described in mammals. Macroautophagy leads to integration of cytoplasmic portions into vesicles named autophagosomes that ultimately fuse with lysosomes. Chaperone-mediated autophagy is in contrast the direct translocation of protein in lysosomes. Macroautophagy is central to lymphocyte homeostasis. Although its role is controversial in lymphocyte development and in naive cell survival, it seems particularly involved in the maintenance of certain lymphocyte subtypes. Its importance in memory B and T cells biology has recently emerged. Moreover, some effector cells like plasma cells rely on autophagy for survival. Autophagy is central to glucose and lipid metabolism, and to the maintenance of organelles like mitochondria and endoplasmic reticulum. In addition macroautophagy, or individual components of its machinery, are also actors in antigen presentation by B cells, a crucial step to receive help from T cells, this crosstalk favoring their final differentiation into memory or plasma cells. Autophagy is deregulated in several autoimmune or autoinflammatory diseases like systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, and Crohn's disease. Some treatments used in these pathologies impact autophagic activity, even if the causal link between autophagy regulation and the efficiency of the treatments has not yet been clearly established. In this review, we will first discuss the mechanisms linking autophagy to lymphocyte subtype survival and the signaling pathways involved. Finally, potential impacts of autophagy modulation in lymphocytes on the course of these diseases will be approached.

Keywords: autophagy, mitophagy, metabolism, unfolded protein response, autoimmunity, lymphocytes

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INTRODUCTION

Autophagy is a catabolic process related to lysosomal activity. Several forms of autophagy coexist in vertebrate cells. They have been historically separated into three major mechanisms: macroautophagy, chaperone-mediated autophagy (CMA), and microautophagy. But in fact, as mentioned later, in each form, several mechanism subtypes have been described, with different physiological roles and particular molecular effectors. Microautophagy, consists in the direct invagination of lysosomal membrane, to engulf cytoplasmic content for degradation. The physiological relevance of this autophagy pathway is not studied in lymphocytes. We will thus focus on macroautophagy and CMA in this review.

Macroautophagy occurs through the formation of double-membrane vesicles named autophagosomes, which emerge from structures named called phagophores. It has been described that autophagosome formation can occur at the contact site between endoplasmic reticulum (ER) and mitochondria, in structures named omegasomes. Some studies also propose the plasma membrane as a source for autophagy initiation in certain contexts such as primary cilium formation (1). Macroautophagy execution is under the control of autophagy-related (*ATG*) gene products, conserved from yeast to vertebrates and extensively reviewed elsewhere (2). CMA is a selective form of autophagy allowing the direct translocation of protein substrates from the cytosol into the lysosomal lumen. Although cross-regulations with macroautophagy have been described, CMA does not rely on ATG activity but on the expression of the chaperone HSPA8/Hsc70 and lysosomal-associated membrane protein 2 isoform a (LAMP2a).

THE MACROAUTOPHAGY MACHINERY

Two main activating pathways control the phagophore formation (**Figure 1**). The first one is the mammalian target of rapamycin (mTOR)-ATG1/unc-51 like autophagy activating kinase (ULK1) axis, which regulation is tightly linked to the metabolic state of the cell. Indeed, macroautophagy was first described as a response to nutrient depravations. In these conditions, for example, amino acid starvation, mTOR pathway is inhibited which leads to ULK1 kinase activation and autophagy initiation. Alternatively, the adenosine monophosphate-activated protein kinase (AMPK) can be activated under nutrient stress, leading both to ULK1 activation and mTOR inhibition, ultimately inducing autophagy. The second, and major, pathway involves the generation of phosphatidylinsositol-3-phosphate (PInst3p) by Beclin-1-BECN1/Vps34 complex, exerting a class III phosphatidylinositol 3 kinase (PIK3CIII) activity. ULK1 complex can directly phosphorylate

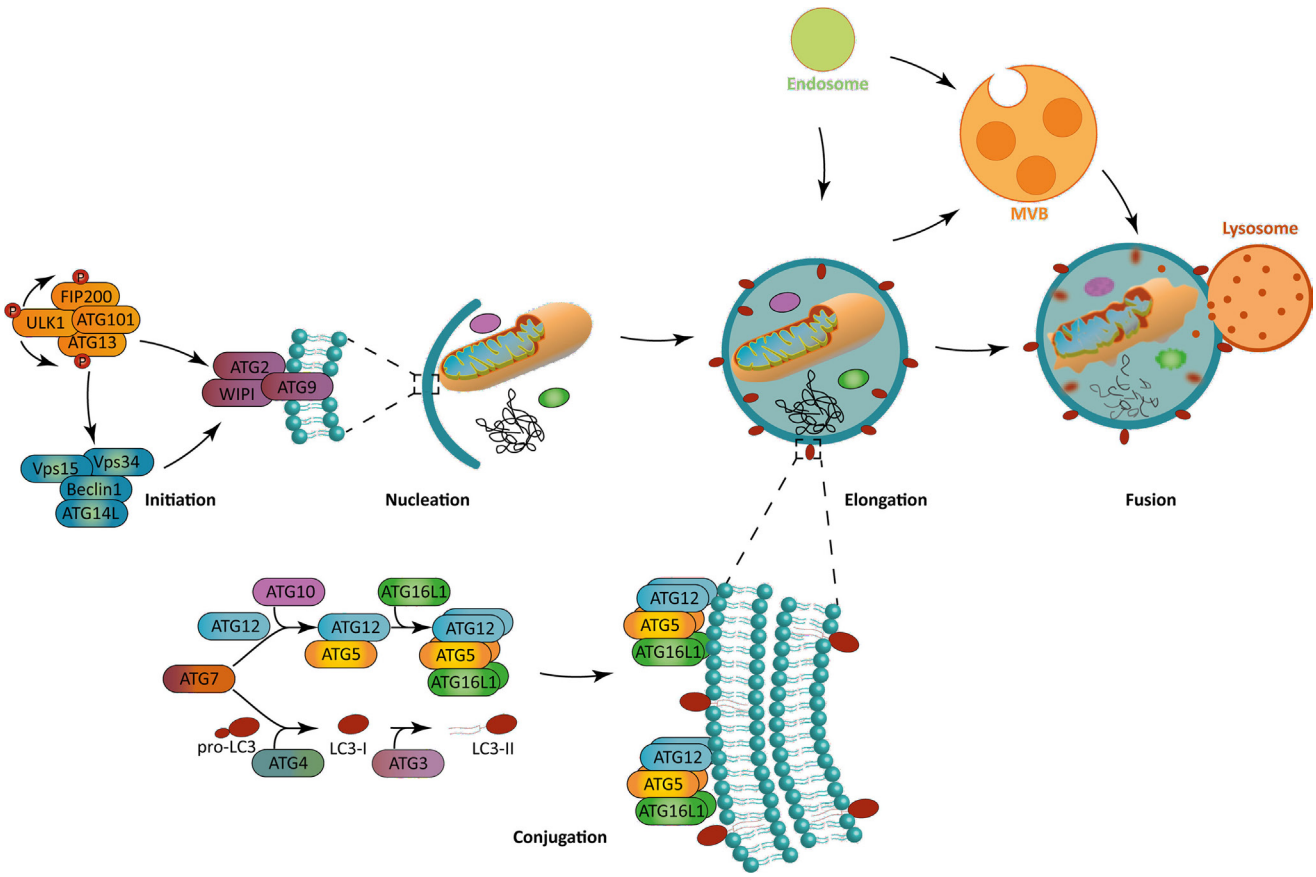

FIGURE 1 | The macroautophagy process. (Left) Autophagy initiation is mediated in a context-dependent manner by ULK1 complex, Beclin-1 complex, or both. These complexes allow the recruitment to the phagophore assembly site of the further effectors ATG8, WIPI, and ATG2 during the nucleation step. (Bottom) The ATG12-ATG5/ATG16L1 complex allows the incorporation of LC3-II in the phagophore, which is crucial for its elongation. Both ATG12-ATG5/ATG16L1 complex and LC3-II are formed by the combined action of two ubiquitin-like systems. While the first one mediates ATG5 complex formation, the second one is responsible for the pro-LC3 cleavage to form LC3-I and a further addition of a phosphatidylethanolamine residue on it to form LC3-II. (Right) Macroautophagy allows the engulfment of cytoplasmic portions. The fusion with lysosomes leads to the degradation of autophagosomal content. Alternatively, autophagosomes can fuse with endocytic vesicles or multivesicular bodies, prior to fusion with lysosomes. The blue lipids layers represent the phagophore membrane. Abbreviations: ATGs, autophagy-related genes; FIP200, FAK-family interacting protein of 200 kDa; LC3, short for MAP1LC3 microtubule-associated protein 1 light chain 3; ULK1, Unc-51 like autophagy activating kinase 1; Vps15/34, vacuole protein sorting 15/34; WIPI, WD-repeat *protein* interacting with phosphoinositides.

and activate Beclin 1/Vps34 complex after its recruitment to the initiation site, but also indirectly trigger it *via* AMBRA1 phosphorylation. Depending on the context, only ULK1, Beclin 1/Vps34 pathway, or both are necessary for autophagy initiation. Non-canonical forms of autophagy have indeed been described, needing only parts of core ATGs for initiation or for further steps (3). The formation of the phagophore can give rise to the autophagosome at the elongation phase. During this step, the ATG7 and ATG10 ubiquitin-ligase-like (E1 and E2-like, respectively) allow the covalent conjugation between ATG5 and ATG12, which can then recruit ATG16L1. PInst3P generated by Beclin1/Vps34 complex activity allows the recruitment of molecules like members of the WD-repeat protein interacting with phosphoinositides (WIPI) family that indicate the site of elongation by recruiting ATG12-ATG5/ATG16L1 complex. The latter leads to the conjugation of microtubule-associated protein light chain 3 (MAP1LC3), often abbreviated LC3, with a phosphatidylethanolamine (PE) that can be integrated into the autophagosomal membrane. This lipidated form is then named LC3-II, in opposition to LC3-I referring to the soluble cytosolic form. Other members of LC3 family, such as GAPARAP (gamma-aminobutyric acid A receptor) proteins can also associate with autophagosome membranes. Before lipidation, LC3 is processed by ATG4 to expose a glycine at the C-terminal domain. The E1-like ligase ATG7 activates LC3 C-terminal glycine residue forming with it a thioester bond. The E2-like ligase ATG3 then replaces ATG7 allowing the action of ATG5-ATG12/ATG16L1 as a putative E3-like enzyme, transferring PE to LC3. ATG5-ATG12/ATG16L1 complex is present on the autophagosomal membrane until vesicle closure, whereas LC3-II remains associated during the whole autophagic process. The closed autophagic vesicle is then addressed to lysosomes during the maturation phase. The low pH and the activity of degradative enzymes lead to the digestion of the autophagosome content in a so-called autolysosome. Macroautophagy was first thought to be largely non-specific, regarding the nature of the cytoplasmic content targeted for degradation. It is now clear that several forms of macroautophagy coexist, selecting organelles, protein aggregates, microorganisms, for degradation (4). This selectivity is ensured by cargo-specific adapter proteins that contain LC3 interacting regions (LIR), which can dock to LC3 expressed on autophagosomes, ultimately leading them to degradation.

AUTOPHAGY, GLUCOSE, AND LIPID METABOLISM

Although the role of autophagy during amino acid starvation has been extensively studied, it appears that autophagy is also modulated by glucose availability and involved in lipid metabolism. Indeed, mTOR complex 1 (mTORC1) is not only activated during amino acid starvation but also under limited glucose availability, independently of AMPK activity (5). It has been described that inhibition of hexokinase II (HK2), enzyme essential for glycolysis, by 2-deoxyglucose leads to inhibition of autophagy. In cardiomyocytes, HK2 specifically induces autophagy in the absence of glucose, protecting cells from death. HK2 can directly bind mTORC1 complex, inhibiting its activity, and thus inducing autophagy. In the presence of glucose-6-phosphate generated by HK2 activity under glucose availability, mTOR binding to HK2 is abolished and autophagy is inhibited.

Autophagy also plays a role in free fatty acid (FFA) mobilization. Lipid droplets (LDs) are organelles present in every eukaryotic cell. In specialized tissues, like adipose tissue and liver, LDs are needed for energy storage. During lipolysis, a multi-protein complex mainly assures FFA mobilization from LDs (6). However, in cells expressing few lipolysis enzymes, during starvation or increased energy demand, macroautophagy is required to execute LD degradation and a quick increase in FFA availability (7, 8). This selective form of macroautophagy was named lipophagy, and while it consists in a ubiquitous pathway, yet it remains poorly characterized. However, as for other forms of selective autophagic degradation, several receptors, such as huntingtin and p62, might be recruited to facilitate LD degradation (9, 10). Increase in FFA availability is crucial to generate succinate, the primary substrate for mitochondrial β-oxidation, but also for gluconeogenesis thus creating fuel for glycolysis and so increasing adenosine tri-phosphate (ATP) level (11). However, increasing FFA availability might induce cellular toxicity. Consequently, after their mobilization to sustain the energetic demand, some ATGs of the core machinery might induce LD *de novo* formation (12). LD generation is also needed to limit an accumulation of potential toxic lipids such as triacylglycerols, diacylglycerols, and ceramids. LDs are then created to sequestrate those overabundant lipids maintaining cellular homeostasis (13). Therefore, macroautophagy and its machinery play a role in lipid homeostasis by lowering their toxicity potential, as well as in their metabolism by insuring FFA efficient mobilization.

SELECTIVE FORMS OF AUTOPHAGY

Basal macroautophagic activity plays a major role in cell homeostasis. It allows the degradation of malfunctioning organelles, especially mitochondria, part of the ER, lysosomes, and peroxisomes (14). A specialized form of macroautophagy, aggrephagy, can also target ubiquitin-tagged aggregates, which cannot be processed by the proteasome, after ubiquitin linking with p62/SQSTM1 (sequestosome 1) (15). Housekeeping functions of macroautophagy linked to organelle and protein homeostasis are particularly required in cell populations that rely on self-renewal to maintain tissue integrity. For example, long-lived cells like neurons are highly dependent on aggrephagy and mitophagy for proper survival (16). As mentioned above, nutrient stress can induce macroautophagy, leading to the degradation of macromolecules, supporting energy production, and providing new building blocks for synthesis of new molecules.

Mitophagy

In eukaryotic organisms, mitochondria form a sophisticate and dynamic network, with a central role in energetic production mainly through oxidative phosphorylation (OXPHOS), in relation with glycolysis and/or fatty acid oxidation (FAO). During OXPHOS, electrons are transported in the inner chain of the mitochondria to generate an H^+ gradient, crucial for the final step of ATP generation. Electron leakage or damaged transport

chain might lead to the formation of reactive oxygen species (ROS), which are neutralized by ROS scavenging enzymes. Eventually some ROS might not be neutralized and act on cellular homeostasis. At low levels, ROS have been demonstrated to contribute to cell proliferation and survival. However, at higher levels, their actions as proteins/lipids oxidizers and DNA damages inducers, contribute to tumorigenesis and/or apoptosis. Since accumulation of defective mitochondria directly impairs energetic production and cellular homeostasis, cells might try to repair them *via* fusion/fission mechanisms or degrade them through a selective autophagic pathway named mitophagy (17, 18).

In mammalian cells, mitophagy relies on both ubiquitin-dependent and -independent mechanisms (**Figure 2**). The first one, which is also the predominant one, is mediated by the PTEN-induced putative kinase 1 (PINK1)/Parkin-mediated pathway (19). PINK1 is imported into the inner membrane of healthy mitochondria in a membrane potential-dependent manner, *via* translocases (translocase of the outer inner and translocase of the outer membrane). In that case, PINK1 is continuously processed by matrix processing peptidases and rhomboid protease presenilin-associated rhomboid like (PARL). This processed form is sensitive to protease-mediated degradation in the cytosol. In case of damaged mitochondria and compromised membrane potential, PINK1 is not imported in the inner membrane but accumulates at the outer membrane, where it is not accessible to PARL-induced degradation. PINK1 is then autophosphorylated and exerts its kinase activity, which allows Parkin recruitment. Parkin belongs to the family of ubiquitin ligases. Parkin recruitment to damaged mitochondria is mediated by PINK1-induced phosphorylation of ubiquitinylated proteins located at the outer membrane of mitochondria. Recruitment of Parkin by phosphorylated ubiquitin leads to the triggering of its ubiquitin ligase activity. Mitochondrial outer membrane proteins then get highly ubiquitinylated and become targets for degradation *via* specific receptors. At least five identified receptors, such as the previously mentioned NBR1 and p62, but also optineurin, TAX binding protein 1, and nuclear domain 10 protein NDP52, recognize these ubiquitin chains. Each of these proteins harbors an LIR motif, suggesting that they can interact with LC3 to recruit the autophagosome membrane, and sequestrate mitochondria for lysosomal degradation. It has also recently been shown that components of the initiation complex could be recruited at the mitochondrial membrane in a PINK1-dependent manner (20).

The first ubiquitin-independent mechanism identified relies on Nip3-like protein X (NIX) or BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3) (18). These molecules play a role in the elimination of mitochondria during erythrocyte development, and in other cells under hypoxia. NIX is localized at the outer membrane of mitochondria and is upregulated during erythrocyte maturation. NIX contains an LIR domain that could allow its interaction with LC3, although other parts of the proteins have been involved. Both NIX and BNIP3 are upregulated during hypoxia and can interact with LC3. Altogether, these pathways direct damaged mitochondria to autophagosomes. NIX and BNIP3 recruitment and activation are regulated by various phosphorylations, although the kinases involved remain to be identified. Bcl2-L13, FUN14 domain containing 1, and FK506-binding protein 8 can also interact with LC3, inducing mitophagy. Interestingly, some mitochondrial lipids might also act as degradation signals by directly interacting with members of the LC3 family (21).

Autophagy and Proteostasis

Autophagy plays important roles in the equilibrium between protein synthesis and degradation. First, autophagy is induced upon ER stress as a part of the unfolded protein response (UPR, **Figure 3**) (22). The activation of the protein kinase RNA-like endoplasmic reticulum kinase pathway is known to induce the expression of several ATGs and the macroautophagic process. The autophagic machinery can sequestrate portions of the ER, after recognition of several cargo receptors, namely reticulophagy regulator 1, SEC62, and reticulon 3 (RTN3). These molecules contain LIR domains, allowing linkage to LC3 and sequestration into autophagosomes. This so-called ER-phagy contributes to limit stress and trigger of apoptosis.

Macroautophagy can also selectively degrade proteins outside the ER. Large aggregates are cleared by macroautophagy *via* p62 recruitment, targeting them to autophagosomes. More generally, several reports show a selective degradation of proteins involved in signaling pathways, modulating cell activation and survival.

Chaperone-Mediated Autophagy

Another well-characterized form of selective autophagy, CMA, consists in the direct translocation of cytosolic content in the lysosome lumen (23). The constitutive chaperone HSPA8/Hsc70, localized at the cytosolic side of lysosomal membrane, can recruit proteins bearing a particular peptide motif [glutamine at one extremity of the motif, one or two positive residues (lysine or arginine), one or two hydrophobic residue, and one negatively charged residue]. This motif is named and KFERQ-like as a reference for the first motif identified in ribonuclease A, targeted to lysosomal degradation. Approximately 40% of proteins in mammals bear such a motif. HSC70 binding to the substrate is modulated by cochaperones like HSC70 interacting protein, heat shock protein 40, and HSC70-HSP90 organizing protein. The HSC70-substrate complex associates with LAMP2a leading to its oligomerization. LAMP2a oligomers are then stabilized by the interaction with HSP90 localized at the luminal side of lysosomes. The substrate is then unfolded and translocates into lysosomes for degradation, with the contribution of luminal HSC70. This selective form of autophagy can be induced during stresses, like nutrient starvation, oxidative stress, DNA damage, and hypoxia (24). Several signaling pathways have also been involved in CMA induction like calcium/nuclear factor of activated T-cells (NFAT) signaling, retinoic acid receptor-α signaling. CMA can also be modulated by mTORC2 complex activity. CMA is involved in protein quality control, generation of amino acids upon starvation, regulation of the cell cycle, and glucose and lipid metabolism (25). It can finally regulate transcription by degrading transcription factors, and signaling intermediates, tuning cell survival and activation.

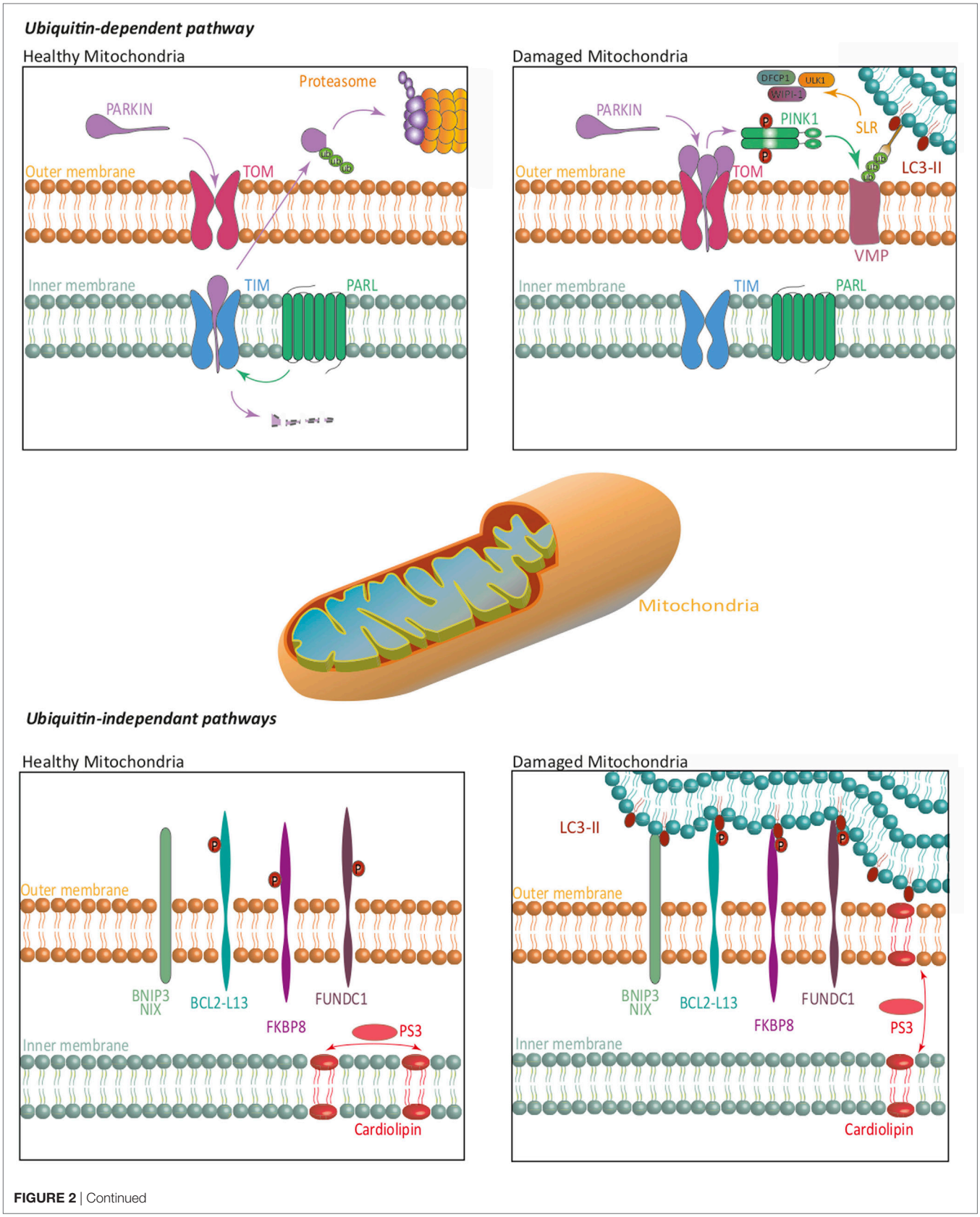

FIGURE 2 | Continued

FIGURE 2 | Mitophagy. The ubiquitin-dependent PINK1/Parkin pathway, and ubiquitin-independent pathways regulate mitophagy. On healthy mitochondria, Parkin is constitutively imported and retained on the inner mitochondrial membrane where it is cleaved by PARL, then re-exported for a proteasome-mediated degradation. On damaged mitochondria, Parkin is retained in the outer mitochondrial membrane, where it activates PINK1. Together, they ubiquitinate mitochondrial substrates, leading to their selective recognition by the autophagic machinery. Independently of Parkin, PINK1 induces, *via* NDP52 and optineurin, the recruitment of ULK1, DFCP1, and WIPI-1 to focal spots proximal to mitochondria, to promote mitophagy. In ubiquitin-independent pathways, several receptors are repressed by specific phosphorylations, in healthy mitochondria. Phosphorylations on their LC3-interacting region, lead them to act as degradation signal, and thus recruit the autophagic machinery to insure mitophagy. Some lipids translocation, such as cardiolipin, act in a similar way. The blue lipids layers represent the phagophore membrane. Abbreviations: BCL2-L13, BCL2-like 13; BNIP3, BCL2/adenovirus E1B 19 kDa interacting protein 3; DFCP1, double FYVE-containing protein 1; FKBP8, FK506-binding protein 8; FUNDC1, FUN14 domain containing 1; NIX, Nip3-like *protein* X; PARL, presenilin-associated rhomboid like; PINK1, PTEN-induced putative kinase 1; SLR, sequestosome-like receptor; TIM, translocase of the outer inner; TOM, translocase of the outer membrane; ULK1, Unc-51 like autophagy activating kinase 1; VMP, variety of mitochondrial proteins; WIPI, WD-repeat *protein* interacting with phosphoinositides.

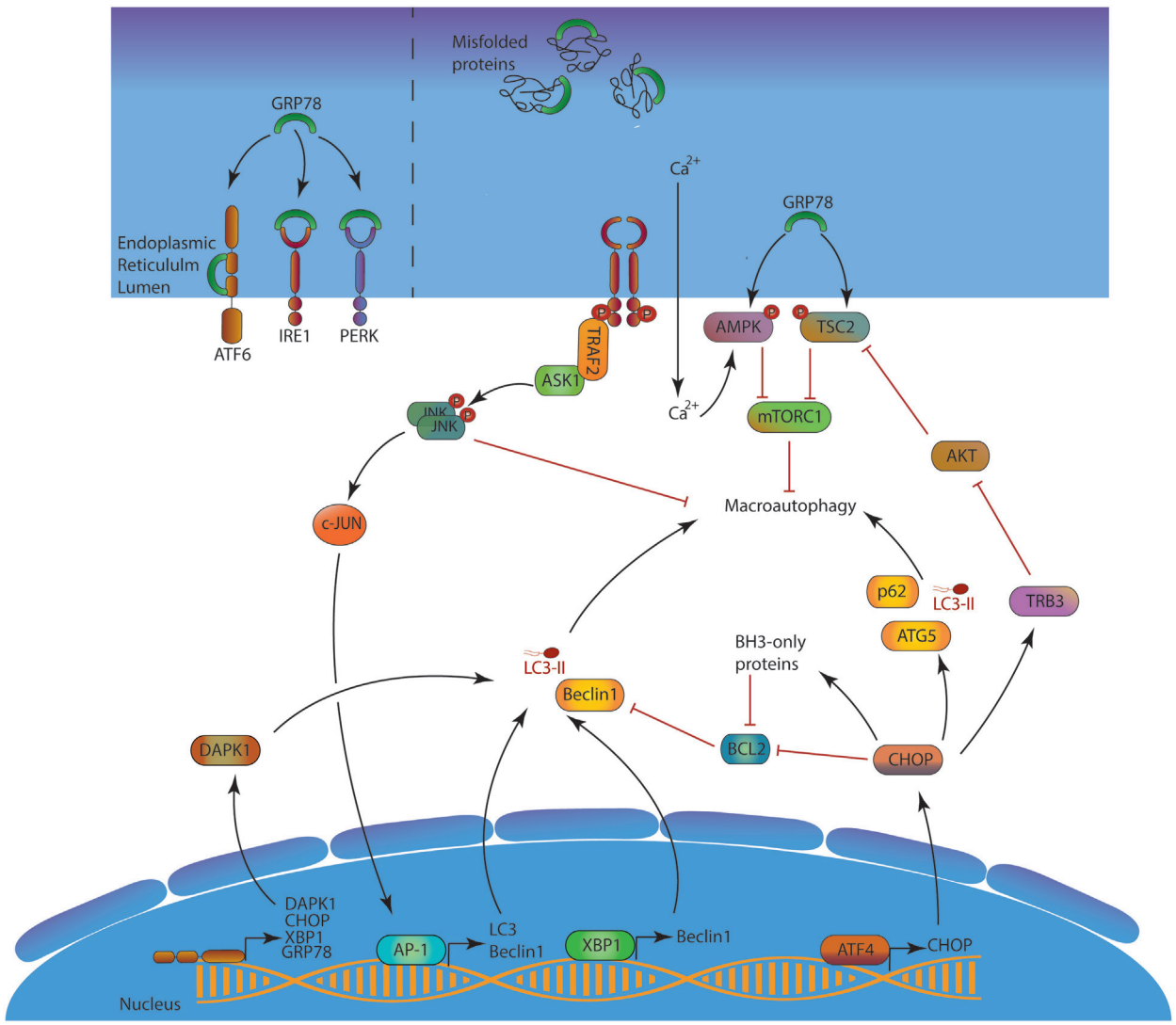

FIGURE 3 | ER stress and macroautophagy induction. In the absence of stress, GRP78 neutralizes the three UPR activation pathways ATF6, IRE1, and PERK. Misfolded proteins accumulation in the lumen creates a competition for GPR78, leading to UPR response activation. Consequently, ATF6 can reach the nucleus, IRE1 splices XBP-1 mRNA to form a functional XBP-1 protein and indirectly activates AP-1, and PERK activates ATF4. ATF6, XBP-1 AP-1, and ATF4 act as transcription factor and induce the expression of several mechanisms to alleviate the ER stress. Among them, autophagy is directly enhanced at two different levels first by the transcription of several *Atgs*, and second at a protein level by inhibiting several autophagy modulators such as BCL-2 or AKT. The blue/purple structures represent, respectively, the nucleus and the ER membranes. Abbreviations: AKT, *protein* kinase B; AMPK, adenosine monophosphate-activated protein kinase; AP-1, activator protein 1; ASK1, apoptosis signal-regulating kinase 1; ATF4/6, activating transcription factor 4/6; BCL-2, B-cell lymphoma 2; CHOP, C/EBP homologous *protein*; DAPK1, death-associated protein kinase 1; GRP78, 78-kDa glucose regulated protein; IRE1, inositol-requiring enzyme 1; JNK, c-Jun N-terminal kinases; mTORC1, mTOR complex 1; PERK, protein kinase RNA-like endoplasmic reticulum kinase; TRAF2, TNF receptor-associated factor 2; TRB3, tribbles pseudokinase 3; TSC2, *tuberous sclerosis complex 2*; *XBP1*, X-box binding protein 1; UPR, unfolded protein response; ATGs, autophagy-related.

THE PLASTICITY OF LYMPHOCYTES IMPLIES METABOLIC AND STRUCTURAL ADAPTATIONS

All lymphocyte subtypes originate from a progenitor named common lymphoid progenitor (CLP), derived from the hematopoietic stem cell (HSC). Although sharing the same origin, these cells display very diverse functions and localizations. Most of them belong to adaptive immunity, while some subsets rely on innate-type detection of danger for activation. Naive lymphocytes of the adaptive immune system are quiescent cells, with low metabolic and transcriptional activity (26, 27). After activation by the antigen receptors, namely B cell receptor (BCR) and T cell receptor (TCR), a dramatic change in both parameters occurs. Naïve cells relying on OXPHOS for survival switch to glycolysis to ensure energy for rapid proliferation and cytokine production. *In vivo*, after an immune response peak, most reactive lymphocytes undergo apoptosis. However, some cells remain as memory cells differentiated from the beginning of the response, or from later effector cells. Memory B and T lymphocyte clones persist for several months or years after immune priming. Most studies point toward a major role for OXPHOS fueled by FAO for long-term survival. Long-lived cells, like memory cells, need to maintain a healthy pool of mitochondria to ensure energy supply, without being affected by damages related to oxidation. During the immune response itself, lymphocytes become effector cells with various functions. Plasma cells differentiated from activated B cells secrete high amounts of antibodies. Helper T cells secrete large quantities of cytokines, and CD8 T cells synthetize lytic granules and become cytotoxic T cells. These changes are possible thanks to high transcriptional activity and capacity for protein synthesis. Cells must then cope with high levels of stress linked to protein synthesis. Finally, nutrient supply and exposure to environmental stress depend on the localization of cells. Naive cells are mainly located in secondary lymphoid organs, while memory cells and innate-like lymphocytes can reside in peripheral tissues, leading them to face different stresses. Consequently, lymphocytes must be very plastic in terms of adaptation to availability of energy source and production, to oxidative stress, and must modulate their synthesis machinery to be able to produce large amounts of proteins. Autophagy is central in these cells, at precise stages of their life, since it regulates ER stress, protein homeostasis, mitochondria function, cell signaling, metabolism, and antigen presentation.

MACROAUTOPHAGY IN EARLY HEMATOPOIESIS AND LYMPHOCYTE HOMEOSTASIS

Studies delineating the role of autophagy in particular cell populations are complicated by the fact that most ATG knockout mouse models are lethal after birth (28). Transfer experiments or conditional deletion models are then required to investigate in detail the role autophagy plays in lymphocyte biology (**Table 1**). Early studies had evidenced basal macroautophagic activity and its induction upon stimulation in lymphocytes (29, 30).

B and T cells are differentiated from HSC localized in the bone marrow, during hematopoiesis. HSC mainly rely on anaerobic metabolism pathways, thus limiting their need for OXPHOS and ROS production. However, their differentiation in CLP requires a metabolism shift to OXPHOS, leading to an increase in energy and ROS production. As a consequence, several studies reported a strong loss of HSC and CLP numbers in the absence of several autophagy genes in these cells (31–33). HSC loss is the result of defective mitochondrial function and DNA damage, leading to an over-activation of caspase-3 and apoptosis (34). These findings are corroborated by the demonstration of the existence of a protective autophagy transcriptional program in HSCs, which lowers their sensitivity to apoptosis (35). These impairments in early hematopoiesis have been reported to induce a blockade after the pro- to pre-B cells transition step and to impair both B and T cell peripheral populations (36, 37). In addition, chimeric mouse models, generated by transfers of *Beclin-1* or *Atg5* KO fetal HSC in *Rag1* KO mice, demonstrated a strong impairment in CLP number and mature B and T cell populations (38, 39). However, in Beclin-1 KO chimeric mice, a decrease of thymocyte populations was described, that was not observed in mice reconstituted with *Atg5* KO cells. This observation suggests that non-autophagic functions of Beclin-1 are crucial during thymic maturation. A role for macroautophagy from thymic development is, however, likely. Later studies demonstrated that macroautophagy impairment leads to an abnormal accumulation of mitochondria in thymocytes that is maintained in peripheral T cells (40). Consequently, ROS are more abundantly produced and are responsible for thymocyte apoptosis, leading to a decrease in thymic and peripheral populations (39). Since Beclin-1 is involved in both mitophagy and apoptosis regulation, its deletion might act at both levels leading to thymocyte death (41).

The study published by Pua et al. gave first insight into the function of autophagy in mature lymphocyte lineage (39). This work showed that lymphocytes from chimeric mice transferred with ATG5-deficient fetal liver hematopoietic progenitors exhibited profound survival and proliferation defects, in both B and T cell populations. This group, and several others, then generated conditional knockout mice with autophagy deficiency after invalidation of several genes: *Atg5*, *Atg7*, *Atg3*, *Beclin-1*, and *Vps34* (36, 42–54). Of note, all studies used promoters highly engaged during early lymphopoiesis: *CD4* or proximal *Lck*, to delete in developing T cells, or *CD19* for B cell lineage-specific deletion. All studies pointed toward a major role for autophagy in naive T cell homeostasis (45, 46, 48, 50, 51). In all models considered, autophagy-deficient thymocytes were reduced in number, but the most striking phenotype is the reduced proportions of T cells in secondary lymphoid organs. As mentioned above, experiments involving fetal liver chimeras demonstrated that B cell differentiation was blocked after the transition between pro to pre-B cell stage, explaining the decrease in B cell number in the periphery (36). These results were discordant with the absence of similar phenotype in *CD19*-cre *Atg5*$^{f/f}$ mice, allowing the deletion of this essential autophagy gene from the pro-B cell stage (36, 42–44, 47). Moreover, our work showed that deleting autophagy very early in *Mb1*-cre *Atg5*$^{f/f}$ *mice*, with autophagy invalidation since the pro-B cell stage, only led to a mild decrease in peripheral

TABLE 1 | Proteins related to autophagy and related functions in lymphocyte populations.

Autophagy-related gene (ATG)	Cell type	Lymphocyte functions	Reference	Pathologies	Reference
Beclin1	Development	B and T cell development	(38)		
	T cells	Caspase degradation after T cell receptor (TCR) engagement	(46)	Experimental autoimmune encephalomyelitis	(46)
		T CD4$^+$ apoptosis after HIV infection	(30)		
Atg7	Development	Hematopoietic stem cell (HSC) survival	(32, 37)		
		Natural killer (NK) cell development and viral clearance	(76)		
		iNK development and mitochondrial homeostasis	(77)		
	T cells	CD4$^+$ T cell apoptosis after HIV infection	(30)		
		Survival and metabolic homeostasis of regulatory T (Treg) cells	(73)		
		Regulation of mitochondrial load in mature T cells	(48)		
		Memory T cell maintenance	(52, 57)		
		ER and calcium homeostasis	(45)		
		Degradation of CDKN1B following TCR activation	(65)		
		Energy supply following TCR activation	(60)		
	B cells	Plasmablast differentiation	(85)	Systemic lupus erythematosus (SLE) and lupus-prone mice	(85)
		B-1a B cells survival and metabolic homeostasis	(36, 53, 84)		
		Memory B cell survival	(16, 17)		
		Medullar recirculating memory B cell maintenance	(36, 53)		
	iNK cells	Proliferation and survival of developing iNK cells	(78)		
Atg5	Development	B cell medullar development	(36)		
		Innate lymphocyte development and survival	(75)		
	T cells	CD8$^+$ T cell survival	(39, 56)	SLE and lupus-prone mice, multiple sclerosis	(95, 115)
		T cell proliferation following TCR stimulation	(39)		
		Memory CD4$^+$ and CD8$^+$ T cell maintenance	(52, 56, 57)		
		Mitochondrial load regulation in mature T cells	(25, 50)		
		Survival and metabolic homeostasis of Treg cells	(73)		
		Repression of Th9 differentiation and function	(63)		
	B cells	Plasma cell survival	(47, 53)	SLE and lupus-prone mice	(53, 85, 106)
		Mature B cell survival	(53)		
		Toll-like receptor 7 signaling	(106)		
		ER homeostasis and antibody secretion in plasma cell	(47)		
	iNK cells	Proliferation and survival of developing iNK cells	(78)		
Atg3	T cells	ER and mitochondria homeostasis	(58)		
		Modulation of Nf-κB activation following activation	(64)		
		Repression of Th9 differentiation and function	(63)		
Atg12	B cells	Major histocompatibility class II presentation the nuclear viral antigen Epstein–Barr Virus nuclear antigen 1	(87)		
Atg16L1	T cells	Survival of intestinal Foxp3$^+$ Treg cells	(74)	Crohn's disease	(74, 92)
Vps34	T cells	Reactive oxygen species and mitochondrial load regulation in mature T cells	(51)		
		Peripheral maintenance and function of CD4$^+$ Foxp3$^+$	(40)		
WIPI2	B cells	Mitochondria homeostasis in B cells and germinal center reaction	(54)		
FOXO1	Other lymphocytes	NK cell development and viral clearance	(76)		
FOXO3A	Development	HSC maintenance	(35)		
FIP200	Development	HSC maintenance	(31)		
LAMP2a	T cells	Regulation of activation	(79)		
	B cells	Endogenous antigen presentation	(89)	Lupus-prone mice	(108)

Impact on inflammatory pathologies when described are indicated.

B cell populations, without developmental abnormalities (55). Interestingly, subsequent studies have shown that some *Atg5* expressing cells still remain in models with ATG deletion driven by *CD19* promoter, which could explain that these mice do not phenocopy chimera models (47). Alternatively, one could argue that hematopoietic cells from chimeric mice harbor a defect in autophagy since the very early steps of hematopoietic differentiation. The work by Simon's group showed that HSC needed autophagy for survival, and that early differentiation of both lymphoid and myeloid lineages are hampered under ATG7 deficiency (34). It is thus possible that defects in the lymphoid compartments reported in first studies, could be linked to defects accumulated during hematopoiesis and not to intrinsic roles in later developmental stages or in mature peripheral T or B cells.

AUTOPHAGY IN PERIPHERAL T CELLS

Concomitantly with thymic cellularity reduction observed in first mouse models, several works suggest that autophagy deficiency in T cells is associated with peripheral T cell homeostasis perturbation as well as proliferative defects and increased apoptosis after TCR stimulation. This could be explained by autophagy impact on energy mobilization and organelle homeostasis.

Macroautophagy in Naïve T Cell Survival

In the periphery, mitophagy might be involved in the maintenance of resting T cell, since naive T cells exhibit a lower mitochondrial load compared with thymocytes isolated from the same mice. Several *Atg* deficiencies lead to an abnormally high mitochondrial load in peripheral T cells (45, 48). Naive T cells produce more ROS, correlated with a higher cell death rate in absence of macroautophagy. In addition, *Atg3*-deficient naive T cells present a more intense rate of apoptosis in long-term culture than wild-type counterparts (45). However, the view of mitophagy as central was challenged, since Beclin-1-deficient T cells do not exhibit any increase in mitochondrial load (46). In this study, Kovacs and colleagues suggest that Beclin-1 might be rather involved in pro-apoptotic protein regulation. However, if mitophagy regulation is truly Beclin-1-independent in T cells remain to be assessed.

Several reports demonstrated a higher sensitivity of $CD8^+$ T cells to macroautophagy loss, compared to CD4 T cells (39, 45, 56). Interestingly, CD8 T cells possess a lower mitochondrial load than their CD4 counterpart. This mitochondrial load was reported as increased and associated with higher ROS levels and cell death in *Atg7- or vps34*-deficient CD8 T cells (40, 45). However, the previously quoted papers were based on mouse models presenting an early deletion of *Atgs* (**Table 1**). In such models, as mentioned before, one could argue that the higher ROS production and cell death observed in mature populations could be for part due to defects accumulated during their developmental phases. After deletion occurring only in mature T cells (56), we confirm $CD8^+$ T cells sensibility to ROS linked to defective mitophagy, which is not shared by $CD4^+$ T cells. Altogether, these data demonstrate a superior need for mitophagy prior to their activation in $CD8^+$ than in $CD4^+$ T cells. This requirement for mitophagy could be one of the reasons why $CD8^+$ T cells deficient for macroautophagy are globally unable to properly differentiate into memory cells after an immune challenge, as developed later (49, 52, 57).

The importance of proteostasis regulation in peripheral T lymphocytes has first been shown in ATG3- and ATG7-deficient T cells, which harbor an abnormally expanded ER (45, 58). Inducible deletions demonstrated *in vitro* that the progressive accumulation of ER membranes correlated with an increased apoptosis. The ER expansion is accompanied by a defect in calcium storage that could account for part of the apoptotic-prone phenotype and activation defects observed in autophagy-deficient T cells. Thus, autophagy can regulate the amount of ER membranes in T cells.

Macroautophagy in T Cell Activation

Following T cell activation by TCR engagement, energetic demand dramatically increases. An initial ATP burst is necessary to sustain an increased energetic demand (59). It is mediated by the increase in glucose uptake and glycolysis induction, generating energy and leading to lactate production. 3-methyladenine-mediated blockade of macroautophagy induces a decrease of lactate and ATP generation after T cell activation (60). Furthermore, this blockage can be partially restored by the addition of an exogenous source of energy in lymphocytes. Interestingly, after T cell activation, mitochondria and other organelles are spared from autophagic degradation (60). This suggests that T cells use autophagy as a mean to increase energy supply, while maintaining its preexisting production capacity. Moreover, after lymphocyte activation, several signaling pathways are activated. Mitochondrial ROS are themselves responsible for the proper activation of NFAT, notably needed for interleukin-2 (IL-2) production (61). To balance this ROS production, mitogen-activated protein kinase extracellular signal-regulated kinases pathway is responsible for mitophagy induction (62).

The specific degradation of proteins by autophagy also modifies lymphocyte fate. Kovacs and colleagues showed in Beclin-1-deficient T cells that helper T cell subtypes are differentially affected (46). Macroautophagy could also contribute to define helper T cell polarization, through specific protein degradation. IL-9 production by T cells, characteristic of the TH9 profile endowed with antitumoral properties, is under the control of the PU.1 transcription factor. Interestingly, macroautophagy targets PU.1 for degradation, regulating its levels. As a consequence, autophagy-deficient T cells show enhanced TH9-dependent anti-tumor responses (63).

Autophagy can also modulate inflammatory signals in T cells through degradation of signaling intermediates. Indeed, in the absence of autophagy, an accumulation of Bcl-10 mediating NF-kB activation downstream of TCR activation is observed (64). This lowers the threshold for T cell activation. Cell cycle regulators might also be regulated by macroautophagy-mediated degradation. CDKN1B/p27Kip1 can be selectively targeted to lysosomes by macroautophagy (65). After TCR stimulation, macroautophagic degradation of this negative regulator of the cell cycle is increased. Thus, autophagic activity contributes to progression into the cell cycle after T cell stimulation, which could explain proliferative defects described in several ATG-deficient models.

Lipid droplets can also act as modulators of nuclear events, by regulating the nuclear translocation of transcription factor. NFAT-5 is a key transcription factor involved in response to osmotic stress and pro-inflammatory cytokine production. In T-cells, NFAT-5 is activated following TCR stimulation (66, 67). Its knockdown causes a severe decrease of T-cell number (68). Ueno et al. demonstrated that LDs are able to sequester NFAT-5 at their surface thus, blocking its translocation to the nucleus (69). The expending importance of LDs in the immune system and proteins/pathways regulating their formation and degradation, make them interesting targets to modulate the immune system activity. It is elegant to think that *via* lipophagy, macroautophagy could directly modulate the activity of transcription factor such as NFAT-5. However, no proof of this concept has yet been reported.

The role of autophagy early after T cell activation has been controverted after important findings from Ahmed's team (52). Previously described studies had postulated that TCR engagement led to macroautophagy activation to support energetic demand. By rigorously assessing autophagic flux *in vivo* during the dynamics of CD8 T cell responses, Xu and colleagues found instead that the accumulation of autophagic markers previously observed in T cells early after activation is mainly due to autophagic blockade at final stages. This assumption makes sense, as naive cells possess, before activation, few energetic stores. Extensive degradation of these stocks could then be detrimental to cell survival. Thus, T cells activated trough the TCR would limit autophagic degradation while requiring exogenous energy source, quickly mobilized through glycolysis. Nevertheless, other reports focused on $CD4^+$ T cells show that autophagy can be induced in particular settings. Botbol and colleagues showed a crucial role for gamma-chain-associated cytokine receptor in macroautophagy induction, through a post-transcriptional mechanism (70). Cytokines like IL-2, IL-4, IL-7, and IL-15 are potent inducers of helper T cell activation. It is possible that $CD4^+$ and $CD8^+$ T cell differ in their autophagy requirement and sensitivity to cytokines stimulation at first steps of activation. It is also possible that $CD8^+$ T cells sensitivity to cytokine-induced autophagy is visible later during immune response. Interestingly, IL-2 is not mandatory for primary CD8 responses but essential for the generation of memory cells. We can thus speculate that autophagy activation in response to IL-2 occurs several days after initial $CD8^+$ T cell activation, at the effector phase, supporting memory T cell generation.

In accordance with a non-essential role for autophagy in early T cell activation, several works using the previously cited models suggest that deficiency of autophagy in mature T lymphocytes poorly impact their function at short term (42, 43, 52, 57). Among them, our work studying dLck-cre *Atg5*$^{f/f}$ mice showed that autophagy was dispensable for short-term $CD4^+$ T cell activation.

Macroautophagy in Memory T Cells

The most recent studies suggest a preferential role for autophagy in certain lymphocyte subtypes. It is now clear that autophagy allows the persistence of both CD4 and CD8 memory T cells (42, 43, 52, 57). Using transfer mouse models, or granzyme-cre *Atg7*$^{f/f}$ mice allowing the suppression of autophagy only in effector $CD8^+$ T cells, three groups reported a major role for autophagy in memory T cell survival.

Autophagy requirement for memory maintenance could be explained by the switch that T cells operate toward OXPHOS linked to high FAO activity, at the transition from effector to memory phase (71). Metabolomic and transcriptomic studies revealed that autophagy-deficient T cells exhibit aberrant lipid content profile as well as deregulated enzymatic pathways related to lipid metabolism (52). The carnitine shuttle and di-unsaturated fatty acid β-oxidation are impaired in autophagy-deficient T cells. The authors suggest that defects in metabolite generation under macroautophagy deficiency selectively impair memory T cell survival. Autophagy could support $CD8^+$ T cell lipid metabolism trough lipophagy, as recently shown for neutrophil development (72). This could also be true for memory $CD4^+$ T cells. We have described that autophagy-deficient CD4 T^+ cell accumulate neutral lipids, which could be linked to defective lipophagy (56).

Macroautophagy in Regulatory T (Treg) Cells and Innate Lymphoid Cells

Other lymphocyte subsets might rely on autophagy for mobilization of lipids. Treg-specific autophagy invalidation leads to glycolysis over-activation, compromising their functions (73). Autophagy might thus contribute to metabolic balance in these cells. Other reports showed that Treg cells (73, 74), innate lymphoid cells that include natural killer cells (75, 76), NKT cells (40, 77, 78) strongly rely on autophagy for survival and differentiation.

CMA in Peripheral T Cells

Chaperone-mediated autophagy has also been shown to participate in T lymphocyte homeostasis. Macian's group reported that T cells deficient for LAMP2a exhibited a reduced responsiveness upon stimulation, limiting the magnitude of T cell-related immune responses (79). CMA can also modulate the extent of T cell activation, through selective degradation of the negative regulators ITCH and regulator of calcineurin (RCAN) (79). In that case, CMA deficiency in T cells compromises activation after stimulation.

AUTOPHAGY IN PERIPHERAL B CELLS

Macroautophagy in Peripheral B Cell Survival and Activation

First studies were discordant regarding the role of autophagy in the maintenance and activation of peripheral B cells. Some studies showed that autophagy was not required for proper B cell survival or activation (36, 80). Another study showed, however, that BCR associated with costimulation was able to activate autophagy although the physiological relevance of this phenomenon was not clear (81). Some studies suggest that primary immune responses are poorly impacted by autophagy deletion (42), while decrease in T cell-independent and -dependent responses have been described in others (44). A defect in plasma cell generation was also reported in the latter study after infections in the absence of autophagy in B cells. These discrepancies could be due to the model used (deletions of ATG5 or ATG7)

and the immunization protocol (infection, model antigens). For example, the use of Mb1- and CD21-cre mediated ATG5 deletion, leads to a small decrease of peripheral B cell populations in contrast to previous models (53).

Several arguments point toward a role for autophagy in B cell function and survival restricted to some activation stages. Recently, Batista's group observed that germinal center (GC) B cells were the most active in processing autophagy. They reported alterations of GC when WD repeat domain, phosphoinositide-interacting protein 2 (WIPI2) was deleted in B cells. Interestingly, deficiency in ATG16L1 did not lead to such a defect. WIPI2 is needed for terminal B cell differentiation and negatively regulates non-canonical forms of autophagy (82). Other non-canonical autophagic pathways such as LC3-assisted phagocytosis (LAP) are described, with components common with canonical autophagy, some unshared with autophagy, and specific ones like Rubicon (83). Similar pathways could also be activated in GC B cells. In the latter work, Martinez-Martin and co-authors also found more antibody-secreting cells under WIPI2 deficiency. The authors postulate that WIPI2 is an important regulator of mitophagy in B cells, regulating GC organization and the outcome of B cells. Mitophagy in B cells could then require a particular machinery. Additional studies are required to elucidate the contribution of different ATGs and their partners in B cell mitophagy.

Indeed, little is known concerning the role of macroautophagy in mitochondrial homeostasis in B lymphocytes. We and others did not detect any impact of core autophagy gene deletion (*Atg5* and *Atg16l1*) in mitochondrial content in naive B cells (53, 54).

As for mitophagy, only few is known about lipophagy in B-2 B cells. In a recent report, B-1a B cells were described as relying on lipophagy for their metabolic homeostasis and their self-renewal (84). This could explain the preferential decrease in this cell population among B cells, under ATG5 deficiency (36, 44, 47, 53). Mature recirculating B cells in the bone marrow (Fraction F according to Hardy's nomenclature) also seem dependent on autophagy for their maintenance, although the mechanisms explaining the preferential role for autophagy in that population remains to be defined (36, 53). NFAT-5 activity modulation by lipophagy could also play a role in B cell activation, in particular in response to B cell activation factor, as NFAT-5 KO leads to defects in immunoglobulins G production (68).

Macroautophagy in Memory B Cell and Plasma Cell Survival

In contrast to the discussed role of autophagy in naïve B cells, the high dependence on autophagic activity of memory B cells (42, 43) and plasma cells (44, 47, 53) is well described.

Mice with ATG7-deficient B cells infected by influenza virus are able to mount a normal primary immune response (42). They, however, fail to generate a protective secondary response upon a second viral challenge leading to increased mortality rates. Another article from the same group showed later that autophagy is involved in the maintenance of memory cells and not on their generation in that context (43). Chen et al. (42) showed that autophagy in memory B cells limits mitochondrial ROS production and toxicity of peroxidized lipids. It is also possible that mobilization of lipids through lipophagy might be required for the survival of both memory B and T cells.

Plasma cells, another late differentiation stage in the B cell lineage is also dependent on autophagy. Indeed, plasma cells are characterized by a large ER compartment compared with their B cell precursor (47). As previously mentioned, the high secretory activity of these cells exposes them to elevated levels of ER stress. These cells thus highly express several effectors of the UPR, some of which are known to induce macroautophagy. Several reports highlighted the particular role played by autophagy in the maintenance of plasma cell compartment (44, 47, 53, 85). Among them, Pengo and collaborators, showed an ER expansion in autophagy-deficient plasma cells. Although leading to an increased IgM secretion at short term, this expansion might lead to an apoptotic-prone phenotype, consequently to an uncompensated UPR response. It is also possible that mitophagy contributes to plasma cell survival. We indeed observed a slight increase in mitochondrial load and decreased membrane potential in plasmablasts differentiated after lipopolysaccharide stimulation (53). It is thus possible that plasma cells need macroautophagy to optimize their mitochondrial pool. Whereas several authors agree on the role of macroautophagy in plasma cell maintenance, if autophagy impacts early events, leading to plasma cell fate still remains unclear. Conway and collaborators report early defects in plasma cell markers after immunization (44). Batista's group shows that the balance between canonical and non-canonical autophagy in the GC affects B cell terminal differentiation (54). Further studies are needed to fully understand the role of autophagy in early plasma cell differentiation.

Autophagy and Antigen Presentation by B Cells

B cells are antigen-presenting cells (APCs) expressing high basal levels of major histocompatibility class II (MHC-II) molecules. They are poorly competent at activating naive T cells. They need, however, to present antigens to primed $CD4^+$ T cells in so-called T cell-dependent responses, for terminal differentiation into memory or plasma cells, and generation of high affinity antibodies. One major source of presented antigens comes from the ones internalized after recognition *via* the BCR. Presentation to cognate T cells allows the final maturation of B cells in GC. Early studies had shown that B cell lines also presented significant amounts of peptides coming from intracellular sources, on MHC-II molecules (86). Interestingly, B cell starvation led to increased proportions of such antigens, suggesting a contribution of macroautophagy in that process. Munz's team provided a functional insight in the presentation of endogenous antigens, by demonstrating that the nuclear antigen Epstein–Barr Virus nuclear antigen 1 is efficiently processed on MHC-II for presentation to T cells *via* macroautophagy (87). The relevance of basal or induced presentation of other viral antigens for the induction of immune responses by B cells remains to be investigated. It has also been shown on other APC, like dendritic cells, that autophagy is integral for the initiation of immune response during herpes simplex virus infection (88). CMA has also been shown to contribute to the

presentation of intracellular antigens. Blum's team showed that peptides derived from GAD enzyme were translocated *via* CMA to lysosomes and MHC-II compartments for processing and presentation (89). However, the role played by CMA in B cells at the onset of humoral response, remains to be established.

Aside the presentation of endogenous antigens, ATGs have been proposed to play a role in the processing of antigens internalized after recognition by the BCR. Chaturvedi and colleagues described that vesicles with an autophagosomal morphology colocalize with the internalized BCR (90). They postulated that macroautophagy was necessary for trafficking of BCR-containing endosomes toward toll-like receptor 9 (TLR9) positive compartments. A few years later, Unanue's group showed that LC3 colocalize with the internalized BCR (91). They also found that 3-MA treatment in B cells impaired the citrullination of antigens. They hypothesized that macroautophagy allows the trafficking of internalized BCR to protein arginine deaminase-containing compartments, which mediate citrullination. They also noticed a small decrease in non-citrullinated antigen presentation. It is thus possible that macroautophagy contributes to BCR trafficking for signaling or antigen processing. It could, however, appear paradoxical that most mouse models with B-cell-specific autophagy deficiency show weak impairment of primary humoral responses after T cell-dependent antigen challenges or infection. Indeed, presentation of antigens acquired through BCR internalization is needed to require help by T cells to enter GC. Martinez-Martin and colleagues show a balance between canonical form of autophagy on WIPI2 and non-canonical autophagy (54). The latter form could involve LC3 recruitment at BCR sites of endocytosis. The authors propose that only part of the autophagy machinery would contribute to BCR trafficking after endocytosis for optimal antigen processing. This process would be reminiscent of processes like LAP. Alternatively, our recent experiments with ATG5-deficient B cells show that a Beclin-1-Vps34-dependent pathway is integral to centrosome relocalization after BCR engagement (Arbogast et al., in press). This B cell polarization is needed for optimal acquisition of immobilized antigens *in vivo* and *in vitro*. Thus, macroautophagy, or part of its machinery, might tune BCR trafficking to optimize antigen processing under particular circumstances.

LYMPHOCYTE AUTOPHAGY IN CHRONIC INFLAMMATORY DISEASES

First indications about an involvement of macroautophagy in inflammatory diseases came from genome-wide association studies underlining a link between proteins of the autophagy machinery and Crohn's disease. In this chronic gut autoinflammatory disorder, variant forms of ATG16L1 are among the highest susceptibility marker. Deficiencies in this core ATG protein could lead to impaired Paneth cells secretions of antibacterial peptides, hyperactivation of the inflammasome, and impaired antigen presentation by APCs as reviewed in Ref. (92). The T300A variant, found in a subgroup of patients, does not impair all macroautophagy processes, but leads to hyporesponsiveness to NOD2 stimulation, and decrease in the capacity to degrade invasive bacteria through macroautophagy. This variant can also increase ATG16L1 degradation by caspases. Finally, it could lead to defects not linked to autophagy deregulation. Regarding T cells, a recent study by Maloy's team showed that T cell-specific ATG16L1 deficiency led to an aberrant type 2 inflammatory response toward bacterial antigens (74). Moreover, specific autophagy ablation in Treg cells leads to metabolic defects, which could explain the observed hyper-inflammation. Later studies identified polymorphisms in other genes linked to macroautophagy such as IRGM and ULK1, as recently reviewed in Ref. (93). Their precise impact on T cell biology in the intestine remains to be investigated.

The search for a role of autophagy during lupus has gained in interest in recent years. Treatments like rapamycin or hydroxychloroquine (HCQ), modulating lupus activity, showed beneficial effects. Moreover, polymorphisms in *IRGM* and *ATG5* polymorphisms, and variations in *PRDM1-ATG5* intergenic region have been associated with systemic lupus erythematosus (SLE) (94, 95). SLE is characterized by the activation of auto reactive lymphocytes, which induce the production of autoantibodies mainly directed against nuclear antigens. These antibodies induce local inflammation damaging several tissues, like blood vessels, skin, kidney, and central nervous system (CNS). The functional consequences of these polymorphisms need to be addressed, as they are not located in coding regions. They could translate the existence of other polymorphisms in particular alleles in subgroups of patients, with detrimental effects in terms of susceptibility to SLE. One study showed that *ATG5-PRDM1* allelic variant was associated with increased *ATG5* expression (95). An earlier work showed that addition of antibodies purified from serum of SLE patients to cell lines lead to an increase in autophagic markers (96). Recent important findings endowed non-canonical forms of autophagy with a role in cell clearance by phagocytes (97), proposed to be defective during SLE.

First studies investigating in detail the autophagic activity of lymphocytes in SLE patients reported an increase in the autophagic marker LC3-II (98–100). These studies diverge in their explanation for the accumulation of autophagic markers. Alessandri and colleagues conclude about a blockade of the autophagic flux, which could sensitize cells to apoptosis, a common feature of lupus T cells. They also reported in a later study the accumulation of α-synuclein linked to autophagy impairment in lupus T cells (101). Another work showed that impaired autophagy in lupus T cells leads to an apoptosis-prone phenotype due to increased ER stress (102). On the contrary, other studies (85, 98), and a more recent one (103), hypothesized that autophagy could contribute to the survival of activated T cells. Indeed, they found that the flux is not totally blocked in lupus T cells. It is clear, however, that aberrant macroautophagy occurs in lupus T cells, leading to an imbalance between the generation of autophagosomes and their degradation. Alternatively, macroautophagy could be impaired in some T cell subtypes and not in others. Kato and Perl recently found that autophagy was suppressed in Treg cells from SLE patients, in response to mTOR activation triggered by IL-21 signaling (104). A previous study had shown that suppression of DEF-6 and SWAP200 in lupus-prone mice allow an increased expression in interferon-regulated factor 4, leading to an augmentation of the Treg compartment and amelioration of

their function, mitigating the disease (105). This improved function was associated with an increased autophagy gene expression. To reconcile these different views, we could argue that it is possible that in some populations, like memory cells, macroautophagy contributes to survival and chronic activation. In some others like naive cells, or Treg cells, the continuous generation of autophagosomes is not balanced by their degradation and leads to cell death.

In B cell lineage from SLE patients and lupus-prone mice, a higher autophagic activity was observed in precursors and naive B cells (85). Macroautophagy was shown to favor survival of plasmablasts and plasma cells, contributing to the production of autoantibodies (53, 85). It was further shown that autophagy in B cells was integral to lupus development in a TLR7 overexpression mouse model (106). The authors postulate that macroautophagy might allow the translocation of RNA-containing antigens to TLR7 positive compartments, in a similar way to what was observed for TLR9 (90). The contribution of macroautophagy to memory B cell survival in chronic inflammation, although plausible, remains to be assessed during lupus. Macroautophagy could also contribute to autoantigens presentation by B cells during SLE. However, to date, no study precisely addressed this question. CMA has also been proposed to contribute to autoantigen presentation. The phosphopeptide P140, which efficacy was recently tested in phase 3 clinical trial for SLE, inhibits both CMA and macroautophagy in this context (107, 108). The authors of this work propose that limiting these autophagy pathways could limit autoantigen presentation. They indeed report a decrease in MHC-II molecules expression by B cells (103, 107, 109). Further studies are needed to confirm efficacy shown in phase IIb and to precisely define which types of antigens are concerned.

The role of autophagy has been addressed in several cell types in another systemic autoimmune disease: rheumatoid arthritis (RA). Macroautophagy has been involved in the deregulation of fibroblast, chondrocyte, macrophage, and osteoclast homeostasis (110). Most studies point toward a detrimental role for macroautophagy protecting, for example, inflammatory fibroblasts from ER stress-induced cell death and favoring osteoclastogenesis. In addition to previously discussed potential roles in memory lymphocyte survival, these variations might affect antigen presentation. Indeed, one hallmark of RA is the development of antibodies directed against citrullinated epitopes. As mentioned before, presentation of citrullinated antigens by B cells need the contribution of macroautophagy (91). The physiopathological relevance of this phenomenon is not known. Other studies reported that macroautophagy was impaired in T cells from RA patients (111). Macroautophagy inhibition was linked to the insufficient induction of phosphofructokinase, an enzyme favoring glycolysis. Thus, RA T cells are prone to apoptosis and senescence due to insufficient energy supply through glycolysis and macroautophagy mobilization. A recent study seems to contradict the previous findings, showing that autophagy is increased in $CD4^+$ T cells isolated from RA patients and mouse models (112). The authors argue that in their settings, $CD4^+$ T cells are assessed for autophagic activity directly after isolation, whereas in previous study cells were cultured for 48 h before macroautophagy assessment. Moreover, increased autophagic activity is found in total T cells in contrast to the study by Yang and colleagues who worked with naive cells. It is thus possible that, as for lupus, deregulation of macroautophagic activity is more linked to certain lymphocytes subtypes like memory cells.

Macroautophagy involvement has also been proposed in an organ-specific autoimmune pathology. Multiple sclerosis (MS) is characterized by chronic inflammation and demyelination in the CNS. An initial inflammation leads to leukocyte infiltration into the CNS. There, residents APCs present myelin-derived antigens, probably originate from dying oligodendrocytes, leading to the priming of T cells. Autoreactive T cells then contribute to the pathology by significantly contributing to inflammation and inducing further oligodendrocytes death (113). In MS context, autophagy roles have been notably assessed in neurons and APCs, however, only few is currently known in lymphocyte populations. However, an elevated autophagic flux has been reported in these autoreactive T cells, both in patients and in the mouse model of experimental autoimmune encephalomyelitis (EAE) (114). It might play a non-negligible role in the pathology onset, since mice deficient for *Beclin-1* in T cells are resistant to EAE development (46). Such a resistance might be link to a decrease in CD8 T cell number. Furthermore, Th1 cells that can contribute to the pathology are more susceptible to Beclin-1 loss than other subtypes. It might further limit CD8 T cells priming. The limited impact of autophagy invalidation on TH17 could appear surprising, as these cells are major actor in EAE pathology. It is thus possible that the global decrease in T cell response under Beclin 1 deficiency is sufficient to limit the disease. A recent report demonstrated that *ATG5* mRNA is increased in $CD4^+$ T cells isolated from MS patients (115). Interestingly, they demonstrated that this increase is independent on autophagic activity, but rather correlated with other inflammatory cytokine levels, such as tumor necrosis factor-alpha. Taken altogether, these results suggest a non-canonical role for autophagic machinery in the onset of MS. However, further studies are necessary to fully understand how autophagy in T cells might contribute to MS severity. As B cells are proposed as important actors in MS (116), assessing autophagic activity in these lymphocytes would also be of interest.

MODULATING LYMPHOCYTE AUTOPHAGY AS A THERAPEUTIC STRATEGY FOR INFLAMMATORY DISEASES

In the last decade, several pharmacological agents were reported as modulators of the autophagic activity. However, a large majority of these compounds were elaborated and tested *in vitro*, and only few succeeded to pass or are currently in clinical trials. In this last part, we will discuss those that are currently used, or in trial, to either activates or inhibits autophagy in the previously mentioned pathologies. Even if not proven, several actions of described compounds could be linked to autophagy impairment.

Inhibiting autophagy could appear beneficial in some settings. Chloroquine (CQ) and HCQ are two synthetic agents blocking autophagy used notably as anti-malaria treatment. CQ and HCQ, as lysosomotropic agent, raise the intralysosomal pH, thus impairing autophagosome degradation. Since years CQ and HCQ

are used during SLE and RA. Among their many actions, they impair MHC-II-mediated presentation, thus diminishing antigen presentation to CD4 T cells. This impairment seems to favor cross-presentation, and thus the priming of naive CD8 T cells (117). However, in humans CQ usage seems to induce a systemic reduction of both CD4 and CD8 T cells (118). In addition, CQ inhibits endosomal TLR responses, which are particularly implied in SLE and RA, notably in DNA and RNA recognition (119) CQ was also used during early studies about mechanism of lipid degradation in lysosomes, which were impaired during treatment (120). A recent report demonstrated that CQ induces apoptosis *via* ER stress (121). Even if all the effects induced by CQ/HCQ treatment are not currently known, they might act on several levers to modulate lymphocyte overreactions. First, by inhibiting T cell activation, and second potently by inducing several homeostatic challenges that might be damageable for B and T memory cells and plasma cells. However, due to its severe unwanted effects, CQ and HCQ are not fully satisfying for after long-term treatment for some patients. Thus, researches for novel autophagy inhibitors are still needed. Sphingosine-1-phosphate and its analog, fingolimod (FTY720) have been demonstrated to be mTOR/p70S6K pathway activators, thus indirect inhibitors of macroautophagy. Used in MS, it impairs CD8 T cells functions (122). However, its exact mechanism of action on these cells remains currently unknown.

Activating autophagy could also appear as a suitable strategy in several settings. Autophagy activators might be discriminated on two main classes. Some are activating autophagy by an inhibition of mTOR, and some others, which are mTOR-independent. Among the mTOR inhibitors, several drugs are currently envisaged for therapy, such as rapamycin, resveratrol, metformin, chlorpromazine, lithium, minocycline, and valproic acid. Even if these drugs do have potential as autophagy inducers, they also possess plenty of side effects. Rapamycin and Rapalogs are notably identified as immuno-suppressors (123). Therefore, even if their usage on mouse models has been rather conclusive, their relevance in human health remains to be fully investigated. mTOR-independent autophagy activators act on several other levers, such as Clonidine, a K^+ ATP channel opener or Verapamil a Calcium channel antagonist. Their mechanisms of action on autophagy is then probably more relying on AMPK–ULK pathway. However, these indirect actions on autophagy might also induce complications, notably in tissues highly relying on ATP and calcium such as the nervous and cardiac systems. Comparably to autophagy inhibitors, the lack of specificity and the spectrum of side effects, complicate their potential *in vivo* usage. Autophagy inducers might enhance notably memory cells functions and long-term survival which could be detrimental to disease progression. It could in contrast favor Treg cell survival and restore naïve cell homeostasis which would limit inflammation.

CONCLUSION

Important progresses have been made in our understanding of autophagy involvement in distinct lymphocyte subtypes. Several fundamental questions remain unanswered. First, why naïve CD8 T cells seem to be more dependent on autophagy than CD4 T cells? Moreover, is autophagy really induced at the beginning of T cell activation or rather inhibited as alternatively proposed in Ref. (52)? What are the signaling pathways downstream of the TCR that are responsible for autophagy modulation and how can they collaborate with cytokine-induced signaling? On the B cell side, it is still not known by which mean autophagy mediates memory B cell survival. It has been proposed that autophagy is responsible for survival of plasma cells sensitive to ER stress. Is it the only contribution of autophagy? Does mitophagy participate in plasma cell survival, or in metabolism regulation? In these cells, a very complex picture is emerging regarding autophagy regulation after activation, involving both canonical and non-canonical mechanisms. Their respective contributions to cell survival, receptor trafficking, antigen presentation remains to be assessed and the signaling pathways involved to be determined. Finally, the contribution of ATG proteins in intracellular trafficking, synapse formation, especially in cytotoxic mechanisms could be investigated. In any case, the first discoveries unraveling a major role for autophagy in secondary responses could help to optimize vaccination strategies.

Regarding autoimmune pathologies, it is complicated to draw a general picture of lymphocyte autophagy deregulation during autoimmunity. It can be over-activated or impaired according to the context and the cell subtype studied. Inhibiting autophagy to deplete memory autoreactive cells, or antibody producing cells like plasma cells is a seducing idea. Indeed, such approaches are envisaged for plasma cell depletion with bortezomib, leading to increased ER stress in this sensitive population. This kind of strategy needs to take into account a very narrow therapeutic window to avoid toxicity toward other cells. It appears also crucial to understand if current therapies modulating autophagy like HCQ exert their effect at least in part through autophagy regulation. Monitoring of autophagic activity under treatment, in different lymphocyte subtypes would be important to define the contribution of autophagy modulation. One must keep in mind that inhibiting autophagy can have deleterious effects in certain pathologies. It could increase the apoptotic phenotype of autoimmune cells like in SLE and contribute to the generation of new cell debris fueling inflammation. Moreover, broad inhibition of autophagy mechanisms could impact LAP and thus efferocytosis, which would increase inflammation. LAP relevance in SLE patients remains to be assessed but if its contribution is confirmed, developing agents targeting specific autophagy effectors, unnecessary for LAP could be an option. Finally, some autoimmune pathologies like systemic sclerosis are linked to polymorphisms in *ATG* genes. No information is available to date on autophagic activity in lymphocytes. It appears very important to define new strategies for this pathology as treatment options are scarce. These studies will be fundamental to envisage the use of more specific autophagy modulators for each autoimmune pathology, where autophagy deregulation in lymphocytes is a described feature. Targeting autophagy in lymphocytes is a unique occasion to target precisely memory or effector lymphocytes, to limit chronic inflammation.

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CORRECTION
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Corrigendum: Lymphocyte Autophagy in Homeostasis, Activation, and Inflammatory Diseases

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A Corrigendum on

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In the original article, two clarifications about cited references are necessary.

First, the sentence "As a consequence, autophagy-deficient T cells show impaired TH9 differentiation and antitumor responses (63)" should be "As a consequence, autophagy-deficient T cells show enhanced TH9-dependent anti-tumor responses (63)". A correction has been made to the section *Autophagy in Peripheral T Cells*, *Macroautophagy in T Cell Activation*, paragraph 2.

Moreover, even if mechanisms are not totally understood, Chen et al. indeed found experimental evidence in (42), for a role played by autophagy in limiting lipid peroxidation toxicity induced by reactive oxygen species. The sentence "To date, no mechanism linking autophagy and memory B cell survival has been proposed. It is possible that mitophagy and mobilization of lipids through lipophagy might be important, as for T cells" has been corrected to "Chen et al. (42) showed that autophagy in memory B cells limits mitochondrial ROS production and toxicity of peroxidized lipids. It is also possible that mobilization of lipids through lipophagy might be required for the survival of both memory B and T cells". A correction has been made to the section *Autophagy in peripheral B Cells*, *Macroautophagy in Memory B Cell and Plasma Cell Survival*, paragraph 2.

The authors apologize for these errors and state that they do not change the scientific conclusions of the article in any way. The original article has been updated.

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MINI REVIEW
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Epigenetic Regulation of Autophagy: A Path to the Control of Autoimmunity

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Autoimmune diseases are a significant cause of debilitation and mortality globally and are in need of cost-effective therapeutics. Autophagy is a cellular pathway that facilitates immune modulation involved in both pathogen control and autoimmunity. Regulation is multifactorial and includes a number of epigenetic pathways which can involve modification of DNA-binding histones to induce autophagy-related mRNA synthesis or microRNA and decapping-associated mRNA degradation which results in autophagy suppression. Appreciation of epigenetic-based pathways involved in autophagy and autoimmunity may facilitate application of a burgeoning group of epigenetic pharmaceuticals to these important diseases.

Keywords: histone, epigenetics, autoimmunity, autophagy, mRNA degradation, histone deacetylase inhibitors, miRNA

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INTRODUCTION

Autoimmunity-related diseases are a common cause of debilitation globally. The search for treatments is an active area of research, in no part due to the fact that three of the top six best-selling prescription drugs in 2015 were for the control of autoimmune disorders. In addition to the widespread prevalence of these diseases, the compelling economic benefit of these agents is borne out by a recent study showing that maintenance of even a mild degree of inflammation in patients resulted in comparative employee productivity to that of unaffected employees (1).

FDA approved epigenetic drugs include the histone deacetylase inhibitors romidepsin (cutaneous and peripheral T-cell lymphomas), belonostat (refractory peripheral T-cell lymphoma), panobinostat (refractory multiple myeloma), and vorinostat (refractory T-cell lymphoma) as well as a number of histone acetyltransferase inhibitors such as azacitidine and decitabine (both for chronic myelomonocytic leukemia and myelodysplastic syndrome) (2). As development progresses, it is likely that pharmaceutical epigenetic therapies will be adapted to other diseases including autoinflammatory diseases and small molecule inhibitors, such as these, may prove cost-effective. While this could have tremendous implications for patients with these diseases, it is important to identify regulatory pathways inherent to epigenetic regulation including autophagy to minimize side effects that are unexpected only because of ignorance of a relevant pathway(s). "On target" treatment toxicity is common. For example, the increased risk of *Aspergillus* infections in patients taking the B-cell-directed Bruton's tyrosine kinase (BTK) inhibitor, ibrutinib (3), due to an unexpected role of these inhibitors in a TLR9-BTK-calcineurin-nuclear factor of activated T-cells pathway in innate immunity to the fungus (4). Thus, a thorough understanding of the impact of epigenetic pathways may be key to avoiding unexpected toxicities of these agents.

THE BALANCE OF AUTOPHAGY DURING INFECTION AND AUTOIMMUNITY

Autophagy was first described in yeast as a mechanism of intracellular recycling during nutrient stress (5). During cellular stress, specific autophagy-related proteins (designated Atg) orchestrate the sequestration of cytosolic materials to be recycled into a double-membraned structure, called the autophagosome (**Figure 1D**). Recently, the role of autophagy in mammalian immune modulation has been demonstrated in both innate and adaptive immunity (6, 7). Autophagy plays a direct role in eliminating invading pathogens by phagocytic processes (8), as well as MAP1LC3-associated phagocytosis (LAP) and sequestosome-like receptor recruitment (9). Autophagy also limits excessive inflammation during pathogen control by: removing residual microbial debris, known to activate the inflammasome pathway; digesting dysfunctional mitochondria, which typically mediate production of reactive oxygen species (ROS); or through direct removal of inflammasome complexes (10). These residual "mop up operations" of autophagy can also be induced by secondary "danger" signals (11) typically mediated by the mTOR pathway that harkens back to the role of this pathway in the yeast nutrient response. In adaptive immunity, autophagy also facilitates effective major histocompatibility complex presentation for T-cell activation (12), serving to control pathogens and remove inflammatory microbial products. Indeed, the importance of autophagy has been recognized by the pathogens themselves in that many utilize host autophagy to protect themselves against killing and support survival within host cells. The fungus *Cryptococcus neoformans*, which causes lethal meningoencephalitis (13), as well as certain bacterial pathogens, such as *Mycobacterium tuberculosis* (*Mtb*), have co-opted autophagic vesicles to conceal their intracellular residence and prevent lysosomal fusion and microbial killing (14, 15).

Although a direct link between autophagy, autoimmunity, and infectious disease is still under investigation, monogenic primary immune deficiencies in humans highlight the growing evidence for their interconnection. Activated PI3K-delta syndrome (APDS), for example, whose dysregulation results in immune-mediated cytopenias treatable by the PI3K inhibitor leniolisib (18)—has been associated with higher risk for developing autoimmune diseases (19). Similarly, chronic granulomatous disease (CGD), resulting from defects in the NADPH oxidase complex is not only associated with reduced ROS production, multiple recurrent infections (20), and chronic inflammation in patients—whose inflammatory colitis can be successfully treated with the IL-1 receptor antagonist, anakinra (21), but also autoimmunity (22). Interestingly, the increased IL-1β production in CGD was linked to a reduction in autophagy that also resulted in defects in phagocyte killing of internalized bacteria and fungi (23), demonstrating a link between autoimmunity, autophagy, and infectious disease.

Therapeutic interventions against autoimmune diseases are also strongly associated with susceptibility to infection. This is exemplified in patients undergoing treatment for multiple sclerosis (MS) who are at an increased risk of life-threatening *Histoplasma capsulatum* infections with the use of TNFα inhibitors, such as infliximab and etanercept (24), or increased risk of CNS infections with the very late antigen 4 (VLA-4) inhibitor, natalizumab, used for minimized autoimmune inflammation (25, 26). A number of "off-target" epigenetic side effects have also been described that associate autophagy and epigenetics. For example, the psychotropic drug lithium acts to downregulate HDAC1 translation, leading to decrease in histone deacetylation and upregulation of autophagy (27). Clearly, an appreciation of regulatory pathways related to autophagy and immunity will be useful to anticipate side effects of epigenetic modifying pharmaceuticals.

mRNA TRANSCRIPT SYNTHESIS: ROLE OF HISTONE MODIFICATION

The field of epigenetics has been a slowly evolving and often controversial concept in genetics. Indeed, some of the first epigenetic molecular work was published in 1964 by Allfey et al. who proposed a role for histone modifications in gene regulation. However, the field progressed slowly until ignited by the synthesis of histone epigenetic studies by Strahl and Allis (28) and Turner (29). Since that time, epigenetic studies have identified a number of covalent histone post-translational modifications, including acetylation, methylation, phosphorylation (30, 31), ADP-ribosylation (32), ubiquitination (33), SUMOylation (30), citrullination (34), glycosylation (35), hydroxylation (36), and isomerization (37, 38). Prominent among these are acetylation and methylation with a number of these histone modifications related directly to the regulation of autophagy and will therefore be the focus of this review. However, since the field of epigenetic regulation of autoimmunity is still in its infancy, many areas remain to be elucidated.

Histone post-translational modifications control gene expression by a number of mechanisms including altering the electrostatic associations between nucleosomes, modulating interactions between nucleosomes and DNA, interfering with transcription factor binding to promoter/enhancer regions, or recruiting either activating or repressing protein complexes to the specific histone modification (39). Typical modifications occur at the epsilon amino group of lysine sidechains within the polypeptide and serve to reduce the electrostatic charge of histones by acetylation and methylation or reverse this by phosphorylation. Some of the best-known modifications affecting autophagy are exhibited in **Figure 1A**. For example, the histone acetyltransferase hMOF/KAT8 acts to add an acetyl group to H4K16 facilitating chromatin decondensation, which sterically allows transcriptional machinery and enhancers access to DNA facilitating expression of autophagy-related genes (40). Conversely, overexpression of the NAD-dependent histone deacetylase sirtuin 1 (SIRT1) antagonizes H4K16 acetylation reducing basal levels of autophagy, which can be inhibited by the drug valproic acid (41). However, the relationship is complicated by a feedback loop whereby SIRT1 acts on non-histone targets in an mTOR-dependent fashion to induce autophagy, which subsequently inhibits hMOF/KAT8 activity (40).

SIRT1 may also play a critical role in regulating the immune system by modulating the activity of essential transcriptional

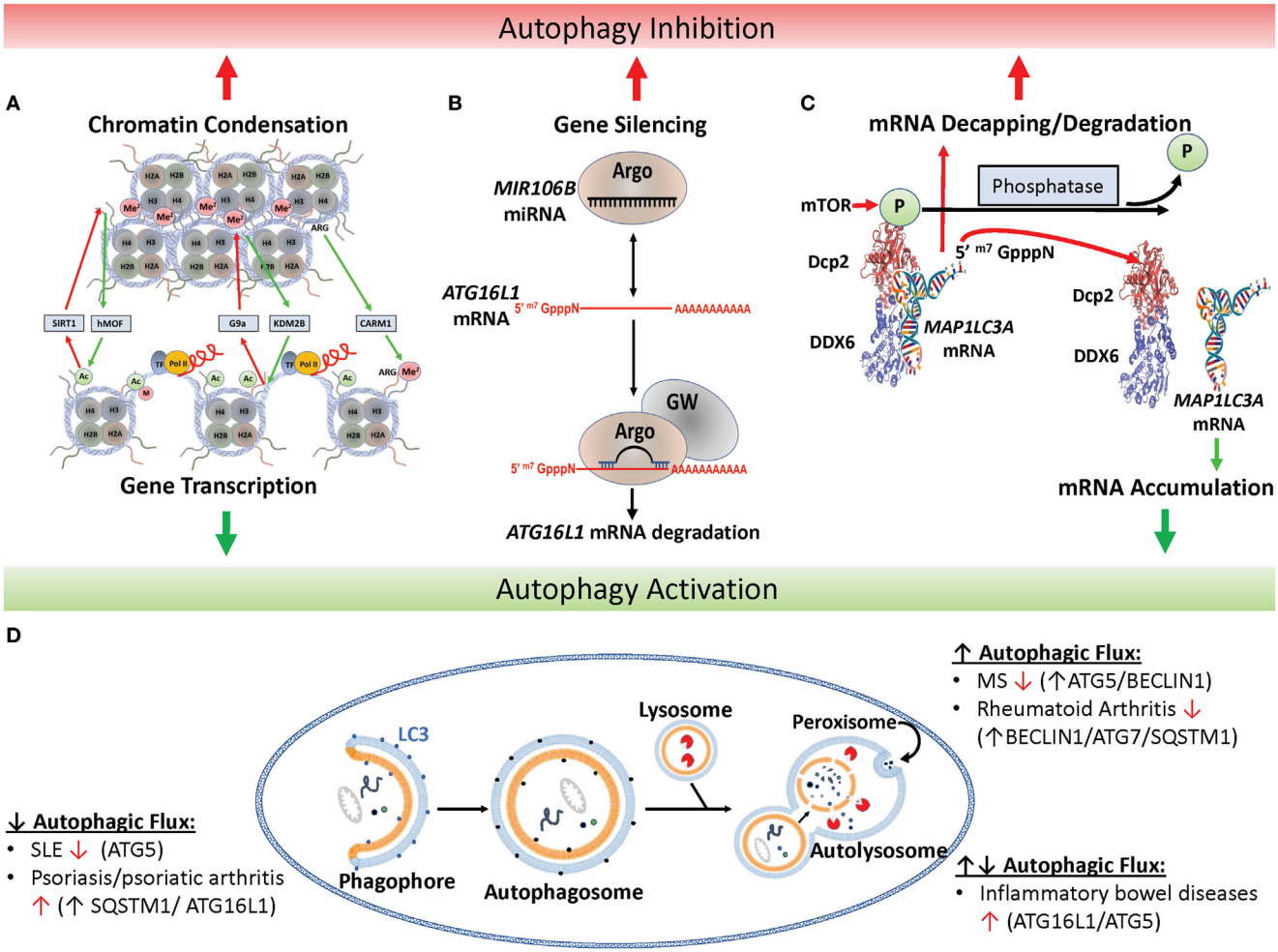

FIGURE 1 | Epigenetic regulation of autophagy. **(A)** Histone marks facilitate either chromatin condensation (top panel) or an open matrix which facilitates transcription (lower panel). Repressors include the histone deacetylase SIRT1, the H3 histone methyltransferase G9a and activators include the H3 histone acetyltransferase hMof, the H3 demethylase KMS2B and the H3 arginine methyltransferase co-activator-associated arginine methyltransferase 1 (CARM1). **(B)** Canonical microRNAs (miRNAs), such as MIR106B, is recruited to Argonaut members (ARG) which recruits target mRNAs, such as the autophagy-related ATG16L1 mRNA, for degradation and gene silencing in concert with GW-motif proteins (GW). **(C)** Mechanism of mTOR-dependent decapping/degradation. mTOR-dependent phosphorylation of the decapping protein DCP2 facilitates recruitment of target mRNA molecules for decapping followed by degradation. Conversely, low mTOR activities in the presence of phosphatases result in dissociation of the decapping complex from the mRNA target with resultant accumulation of target transcripts, illustrated here with the autophagy-associated transcription, *MAP1LC3A*. [Model of putative mammalian DCP2-DDX6 interaction with *MAP1LC3A* mRNA adapted from Ref. (16).] **(D)** Illustration of autophagy and autoimmune diseases associated with alterations in autophagic flux. In autophagy, protein aggregates, misfolded proteins, and pathogens are recruited to the phagophore and then enclosed by a double-membrane vesicle to form the autophagosome. Following lysosome fusion with the autophagosome, proteinaceous material undergoes degradation in the autolysosome. Red arrow found next to autoimmune disease names indicates whether disease improves (↑) or declines (↓) following pharmacological inhibition of autophagy (17).

regulators. Specifically, SIRT1 deacetylates RAR-related orphan receptor gamma promoting its transcriptional activity and Th17 cell differentiation (40). In thymic epithelial cells, SIRT1 is an essential regulator of AIRE-mediated expression of tissue-restricted antigens, a critical step for immunological self-tolerance (42, 43). Interestingly, polymorphisms in *SIRT1* are associated with autoimmune thyroiditis and high titers of anti-thyroid antibodies (44), suggesting a link between epigenetic regulators and autoimmunity. Immune consequences for overlapping regulation of autophagy and immunity can be seen with other related histone deacetylases. SIRT6 potentiates autophagy activation through effects on autophagy-related genes (*ATG12*, *ATG3*, and *ATG7*), as well as the well-known Crohn's colitis-associated autophagy gene, *IRGM* (45, 46). Broad spectrum deacetylases, such as those found within the HDAC family—exemplified by HDAC4's ability to deacetylate H3K9, 14, 18, and 23 and H4K5, 8, 12, and 16—are well known for their role in cancer biology prompting development of the HDAC inhibitors described above and in **Table 1** (47). But they also show promise for the treatment of autoimmune diseases, as HDAC4 inhibitors have been shown to alleviate vascular inflammation resulting from activation of autophagy (48). Similarly, HDAC6 has been shown to modulate *ATG6*

TABLE 1 | Epigenetic regulators associated with autophagy and immunity.

Histone modification

Histone modification	Regulator	Effect on autophagy	Immune phenotype	Disease implicated	Reference
H3K9Ac	SIRT6	↑ATG5	Inhibition of NOTCH/NF-κB signaling	Proteinuric kidney disease	(50–52)
H4K16Ac (H1.2 variant)	SIRT1/HDAC1	↑Autophagy	Inflammation	Diabetic retinopathy	(53)
H3K9me	HIF-1α, KDMs	↑BNIP3	Reactive oxygen species response	Traumatic brain injury/tumors	(51, 54)
H3R17me2	TFEB/co-activator-associated arginine methyltransferase 1	↑ATG14	Myeloid differentiation, SWI/SNF	Unknown	(39, 55, 56)
H4R3me2	C/EBPβ/PRMT5	Unknown	IL-8, TNFα expression	Unknown	(57)
Multiple	HDAC6	SQSTM1 autophagic clearance	Interferon response pathway	Viral/bacterial clearance	(58, 59)

Histone deacetylase inhibitors (HDACi)

Drug	Regulator	Effect on autophagy	Immune phenotype	Diseases treated with HDACi	Reference
Vorinostat	HDACs	↑Autophagosome formation (ATG5)	Viral myocarditis	Cutaneous T-cell lymphoma	(60)
Vorinostat	HDACs	Unknown	CD4 and CD8 tumor immunity	Metastatic colorectal cancer	(61)
Vorinostat	HDACs	↑Autophagy (ATG5)	NF-κB signaling, VSV oncolysis	See diseases treated above	(62)
Tubastatin A	HDAC6	↑Autophagy (ATG7)	TNFα, IL-6 cisplatin toxicity	Acute kidney injury/pancreatic cancer	(49, 63)
Panobinostat	HDACs	↑Autophagy (LC3)	Lymphocyte tumor killing, TNFα	Hodgkin lymphoma/multiple myeloma	(64, 65)
Multiple	HDACs	↑Autophagic flux (ULK1/ATG7)	Reverse HIV-1 latency	Peripheral T-cell lymphoma	(66)
Multiple	HDACs	↓Autophagy (ATG7)	Apoptosis induction	DS-AMKL (proposed)	(67)

microRNA (miRNA) regulation of autophagy

miRNA	Effect on autophagy	Immune phenotype	Disease implicated	Reference
miR-30a	↓BECN1 (↓autophagy)	Unknown	Cancer	(68)
miR-30b	↓Autophagy (↓ATG12, BECN1)	Intracellular survival of *Helicobacter pylori*	Cancer	(69, 70)
miR-106b, miR-93	↓Autophagy (↓ATG16L1)	Defects in bacterial clearance, inflammation	Crohn's disease	(71)
miR-142-3p	↓ATG16L1	Intestinal inflammation	Crohn's disease	(72)
miR-30c, miR-130a	↓Autophagy (↓ATG5, ATG16L1)	Invasive *Escherichia coli*, NF-κB activation, inflammation	Crohn's disease	(73)
miR-196	↓IRGM (↓autophagy)	Mitochondrial function, ineffective *Mycobacterium tuberculosis* (*Mtb*) and *E. coli* control	Crohn's disease	(74, 75)
miR-210	↓Bcl-2	HIF-1α pathways, hypoxia-induced apoptosis, TH_{17} differentiation	Traumatic brain injury	(76, 77)
miR-21	↓IL-12p35, ↓Bcl-2	NF-κB activation, impaired anti-mycobacterial T cell responses	*Mtb* infection, asthma	(78, 79)
miR-17, -20, -93, -106	↓SQSTM1	Elevated P-ERK levels, enhanced hematopoiesis	Acute myeloid leukemia	(80)
miR-155, -31	↓PPP2R5A (↓autophagy)	↓JAK-STAT ↑WNT-SHH, Th2 polarization	Mycobacteria, *Shigella*, *Listeria* infection	(81)
miR-UL148d (HCMV)	↓ERN1 (↓autophagy)	Inhibition of apoptosis, impaired anti-viral response	HCMV infection	(82)
miR-1303	↓ATG2B (↓autophagy)	Suppression of mycobacteria-induced autophagy, ↓TNF-α	*Mtb* infection	(83)
miR-471-5p	↓LC3, ↓ATG12, ↓BECN1	LC3-associated phagocytosis, apoptotic germ cells	Male infertility	(84)
miR-155	↓ATG3 (↓autophagy)	Suppression of anti-*Mtb* dendritic cell response	*Mtb* infection	(85)
miR-155	↓RHEB (↑autophagy)	Enhanced killing of intracellular *Mtb*	*Mtb* infection	(86)

(BECN1) and *ATG7* and the inhibitor tubastatin A was found to potentiate autophagy with inhibition of the pro-inflammatory cytokine, IL-6 (49). These experiences suggest that further studies may identify epigenetic pathways relevant to HDAC inhibition that may prove useful in autoinflammatory disorders.

A second set of histone modifiers are those involved in H3K9 methylation by the euchromatic histone-lysine N-methyltransferase 2 (G9a/EHMT2) and GLP/EHMT1 methyl transferases which form heteromeric complexes via their Su(var)3-9-Enhancer of zeste-trithorax (SET) domains (87). These lysine methyltransferases transfer one to three methyl groups from *S*-adenosyl-L-methionine to the target lysine e-amino group, causing a similar charge disruption as acetylation described above. In addition, H3K9me3 moieties (marks) recruit histone mark "readers" such as heterochromatin protein 1 (HP1) whose unique chromodomain mediates protein binding, heterochromatin formation (a tight lattice of DNA bound to histones), and transcriptional repression (**Figure 1A**, top panel). The methyltransferase activities of G9A/EHMT2 and the polycomb-repressive complex member, enhancer of zeste homolog 2 (EZH2), converge onto H3K27, whose methylation is associated with derepression of mTOR (88) and autophagy repression (89). Pharmacological inhibition of G9a/EHMT2 with BIX01294 results in induction of autophagy demonstrated by increased LC3B-positive autophagic vesicles (90), but autoimmunity was not studied in this context. Long non-coding RNAs (lncRNA) add an additional layer of regulation to this pathway that might hint at a connect to autoimmunity. Specifically, the lncRNA HOTAIR acts as a scaffold to recruitment the histone methyltransferase EZH2 to target genes, facilitating H3K27me-mediated gene repression (91). Aberrant expression of HOTAIR is associated with various cancers (92) and MS (93).

Interestingly, the hypoxia-inducible transcription factor (HIF1α)—recently shown to impact T-cell differentiation (94) and B-cell-related autoimmune disease (95)—was also found to regulate the H3K9 lysine (K)-specific demethylase, KDM2B, as well as the related demethylase KDM1A. This epigenetic regulation leads to activation of autophagy as well as mTOR, NF-κB, and TGF-β pathways important in T-cell adaptive signaling (96). In addition, HP1 located at H3K9me3 marked histones can recruit DNA methylases, such as DNA (cytosine-5)-methyltransferase 1 (DNMT1), which provide more permanent heterochromatin formation. DNA methylation carried out by DNMT1 have been implicated in susceptibility to endogenous retrovirus-induced systemic lupus erythematosus (97) related to increased antigen processing of hypomethylated DNA (98) or altered gene expression of inflammatory genes directly, including IL-17 (99).

An unusual histone modifying enzyme associated with autophagy is the arginine-specific H3R17 methyltransferase, co-activator-associated arginine methyltransferase 1 (CARM1), which collaborates with the transcription factor, TFEB, to enhance histone methylation allowing access of transcription factors in the AMP-SKP-CARM1 signaling cascade to autophagy-related genes (55). Previous studies have also shown a role for CARM1 as a promoter-specific regulator of NF-κB signaling (100), important for a number of innate and adaptive immune responses, demonstrating the intimate relationship between autophagy activation and autoimmunity.

mRNA TRANSCRIPT DEGRADATION: ROLE OF microRNAs (miRNAs)

The complexity of miRNAs has been quite daunting, but its complexity is important to the programmatic modulation of autophagy and its effect on inflammation. miRNAs are the shortest of the non-coding RNAs at approximately 22 nucleotides in length. Most miRNAs are considered "canonical" in that they undergo primary miRNA processing in the nucleus by the "microprocessor" complex, which contains the RNase III enzyme Drosha and the dsRNA-binding protein Dgcr8, and further maturation in the cytoplasm by the ribonuclease DICER. The resulting ~22 nucleotide duplex is loaded into the Argonaute-containing RNA-induced silencing complex (RISC), which then recruits mature mRNAs for degradation (**Figure 1B**). Interestingly, the complete structure of RISC is still unresolved and reports of size and component variability suggest complex adaptability to induction conditions or the passengers in question. In addition to "canonical" miRNAs, various non-canonical miRNAs appear to remain dependent on DICER, but are processed independent of Drosha or Dgcr8 (101). The best known of these are mirtrons, which are processed through a unique intronic splicing mechanism and have been recently associated with the pathophysiology of IgA deficiency *via* regulation of immunoglobulin heavy constant alpha 1 (*IGHA1*) and *IGHA2* (102). It is likely that this intricate web of miRNA regulation furnishes an important modulating capacity for the immune system to optimize survival against a range of pathogenic organisms under strong evolutionary pressure. But when inappropriately triggered may result in the "off-target" consequence of autoimmunity.

One of the first miRNAs identified to play a role in immunity was the global regulator miR-155. Prominently associated with IFN-γ expression and germinal center function (103–105), it was not determined until much later that some of the direct regulatory targets of miR-155 were autophagy-related (86). One of the best-known targets of miRNA-regulated autophagy is the immunity-related GTPase family M protein (IRGM) clinically associated with inflammatory bowel disease (106, 107). The canonical miRNA miR-196 targets and regulates *IRGM* whose levels confer either autophagic protection or cell death in target cells, implicated in both defense against the intracellular pathogen *Mtb* and damaging inflammation caused by Crohn's disease. Such studies are an important demonstration of the importance of the exquisite immunological balance necessary to provide both microbiological protection and avoid autoimmune pathology. Pertinent to the importance of epigenetic mechanisms of regulation, clinical genetic studies identified a disease variant that was originally felt to be dispensable due to a lack of effects on either IRGM protein sequence or splice site selection, but later found to result in down-regulation of an *IRGM* protective variant, but not a risk-associated allele due to a miRNA-based alteration in *IRGM* regulation (74).

Another prominent target of miRNA degradation is the autophagy-associated gene autophagy-related 16 like 1 (*ATG16L1*). Typically, ATG16L1 interacts with ATG12-ATG5 facilitating the phosphatidylethanolamine lipidation of the vesicular shaping protein, MAP1LC3A, and elongation of the nascent autophagosomal membrane (108, 109). In one study, levels of *MIR106B* were found to be elevated in the intestinal epithelium of patients with

active Crohn's disease along with decreased levels of *ATG16L1* transcripts (**Figure 1B**) (71). This was found in human cell lines to be associated with defects in autophagy-dependent eradication of intracellular bacteria. Studies in the same year identified a role for *MIR142-3p* in the same target gene (72) and others also found that Crohn's disease-associated adherent *Escherichia coli* were able to modulate *MIR30C* and *130A* to effect changes in *ATG16L1* transcripts as well as the autophagy-conjugation gene, *ATG5* (73). Interestingly, ATG16L1 has recently been found to play a role in NOD2 inflammasome activation (110) and is associated with inflammatory bowel disease (111). Recently, *ATG16L1* gene polymorphisms were found to be associated with necrotizing enterocolitis in premature infants, again stressing the potential importance of this regulatory pathway to autoimmunity (112).

mRNA TRANSCRIPT DEGRADATION: ROLE OF REGULATED mRNA DECAPPING/DEGRADATION

A more recently identified mechanism of post-transcriptional regulation implicated a well-characterized mRNA decay pathway, characterized as a housekeeping function to remove RNA in yeast (113) and mammalian systems (114). In this process, mRNA undergoes a reversible poly-A tail deadenylation followed by an irreversible 5′-decapping by the decapping enzyme DCP2 and subsequent XRN1-exonuclease mediated degradation of the RNA in the 5′–3′ direction. More recently, studies in the yeasts *Saccharomyces cerevisiae* as well as *C. neoformans* identified an RNA-binding protein, ATP-dependent RNA helicase Dhh1/Vad1, as an RNA chaperone that binds and recruits targeted autophagy-related mRNA to the decapping complex resulting in suppression of autophagic flux (**Figures 1C,D**). Regulation of this process by mTOR was demonstrated by a specific mTOR-dependent phosphorylation of the DCP2 protein in humans, without which mRNA recruitment and decapping was prevented. These studies were extended to patients with monogenic dominant-activating mutations in a PI3K p110δ subunit who were further characterized by increased mTOR activity and autoimmune-associated leukopenia (115). Increased mTOR activity in these patients resulted in accelerated decapping and degradation of relevant autophagy mRNA transcripts with resultant decreased autophagy activity. Further studies, prompted by the recent finding of a role for autophagy in modulation of inflammasome-related IL-1β levels (116) demonstrated that the reduced autophagy activity in these patients resulted in elevated levels of IL-1β, suggesting an etiology of the patient's autoimmunity. Conversely, knockdown of DDX6 by siRNA was successful in pseudonormalization of IL-1β levels, suggesting both a pathway for rapamycin-treatment of this disorder and new targets for pharmacological intervention against autophagy-related IL1β-associated autoimmunity.

A QUESTION OF BALANCE AND THE ROLE OF TRANSCRIPTIONAL "FUTILE CYCLES"

As suggested by the parallel pathways described in **Figure 1**, epigenetic mechanisms have the ability to modulate each pathway's activity. This coupling of mRNA synthesis with mRNA degradation is exemplative of transcriptional "futile cycles" first describe in yeast (117). Futile cycles were first described in energy metabolism with the classic example concerning gluconeogenesis, where regulated inhibition of a degradative phosphatase resulted in a rapid accumulation of fructose 1,6-bisphosphate required for *de novo* glucose synthesis during the "fight or flight" response (118, 119). These cycles were termed futile, because it was not yet understood why energy would be exerted to simultaneously synthesize and degrade a required cellular precursor. However, maintenance of basal levels of synthesis (metabolic intermediate or mRNA) even during periods of disuse allows more rapid induction of synthetic enzymes without the need to start from zero. It also allows rapid adaptation to newly required steady states by simultaneous modulations in both synthesis and degradation. The concept is well suited for immune mechanisms, demonstrated by the induction of inflammasome activation by TLR4, accompanied by the simultaneous induction of autophagy to degrade IL-1β, resulting in mechanisms to optimize pathogen control and yet avoid autoimmunity (116).

While relationships between epigenetic regulation, autophagy, and immunity are just now being elucidated, the study of HDAC inhibitors in cancer demonstrates some of the complexity of epigenetic manipulations. For example, studies of breast cancer carcinogenesis in the presence of the chemopreventative DNMT1 inhibitor, 3,6-dihydroxyflavone (3,6-DHF), demonstrated reduced DNA methylation with resulting activation of autophagy, as well as epigenetic activation of the *MIR21* promoter, resulting in an unexpected induction of the NOTCH-1 pathway (120). Applying some of the known pathways in **Figure 1** may help to anticipate some side effects. For example, mTOR-dependent signaling of SIRT1-dependent H3K9 acetylation would be expected to increase autophagy activity (121). However, high mTOR activities would be expected to phosphorylate the S249 amino acid of DCP2, resulting in increased autophagy-related transcript suppression, which modulates the effect of autophagy on IL-1β and autoimmunity with potential reductions in pathogen clearance. However, without experimental probing of these relationships, it is difficult to discern which effects would predominate under a given condition of autoimmunity or infection. Clearly, the study of the role of epigenetic networks in autophagy and autoimmunity is in its infancy, but is critical to the application of a developing repertoire of epigenetic pharmaceuticals to autoimmunity as well as for the anticipation of their potential side effects.

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REVIEW
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Exploring the Role of Autophagy-Related Gene 5 (*ATG5*) Yields Important Insights Into Autophagy in Autoimmune/Autoinflammatory Diseases

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Autophagy is a highly conserved process that degrades certain intracellular contents in both physiological and pathological conditions. Autophagy-related proteins (*ATG*) are key players in this pathway, among which *ATG5* is indispensable in both canonical and non-canonical autophagy. Recent studies demonstrate that *ATG5* modulates the immune system and crosstalks with apoptosis. However, our knowledge of the pathogenesis and regulatory mechanisms of autophagy in various immune related diseases is lacking. Thus, a deeper understanding of *ATG5*'s role in the autophagy mechanism may shed light on the link between autophagy and the immune response, and lead to the development of new therapies for autoimmune diseases and autoinflammatory diseases. In this focused review, we discuss the latest insights into the role of *ATG5* in autoimmunity. Although these studies are at a relatively early stage, *ATG5* may eventually come to be regarded as a "guardian of immune integrity." Notably, accumulating evidence indicates that other *ATG* genes may have similar functions.

Keywords: autophagy, ATG5, autoimmune disease, apoptosis, immunity

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INTRODUCTION

Autophagy is a highly conserved homeostatic process from yeast to mammals. Derived from Greek "self" and "eating", autophagy is the regulated cellular degradation of certain intracellular molecules and organelles (1). This process initiates from the engulfment of the unwanted cytoplasmic content, followed by fusion with the lysosome, and degradation. Generally, baseline (constitutive) autophagy in mammals occurs under physiological conditions, but can be increased by starvation or by various pathologies, including ischemic, toxic, immunological, and oxidative insults (2).

Autophagy is a tightly regulated process and the key players in this pathway are the AuTophaGy-related (ATG) proteins. To date, at least 41 *ATG* genes have been identified (3). The ATG core proteins are classified in five functional groups: (1) The ULK kinase (Unc-51 like autophagy activating kinase) complex (ULK1 or ULK2, ATG13, RB1CC1/FIP200, and ATG101); (2) the class III phosphatidylinositol 3-kinase (PtdIns3K) complex (BECN1/Beclin 1, ATG14,

PIK3C3/VPS34, and PIK3R4/p150/VPS15); (3) the ATG12 conjugation system (ATG7, ATG10, ATG12, ATG16L1, and ATG5); (4) the microtubule-associated protein 1 light chain 3 (LC3) conjugation system (ATG4, ATG7, ATG3, WIPI2, and LC3 protein family); and (5) the ATG9 trafficking system (ATG2A and ATG2B, WIPI4, and the transmembrane protein ATG9A) (4).

Among these 41 proteins, ATG5 is indispensable for autophagic vesicle formation. Knocking down or knocking out ATG5 can result in downregulation or total inhibition of autophagy, suggesting that ATG5 plays a central role in autophagy. Thus, *ATG5* is one of the most commonly targeted genes in autophagy gene editing assays. In addition, ATG5 has other functions, including mitochondrial quality control after oxidative damage; negative regulation of the innate antiviral immune response through direct association with retinoic acid receptor responder 3 (RARRES3) and mitochondrial antiviral signaling protein (MAVS); lymphocyte development and proliferation; MHC II antigen presentation; adipocyte differentiation; and apoptosis (5).

The direct association between *ATG5* and autoimmunity was identified in hypothesis-free genome-wide association study (GWAS) data. Several GWASs for systemic lupus erythematosus (SLE) confirmed genetic associations between common variants in/near *ATG5* and SLE, in Caucasians and Asians. Similar associations were identified in other autoimmune diseases, including rheumatoid arthritis, systemic sclerosis, and multiple myeloma (6, 7). *ATG5* alleles were associated with blood pressure, insulin sensitivity, glucose homeostasis, and age-related macular degeneration using GWAS (5, 8). These data strongly supported a genetic role in the development of immune related diseases, metabolism, and cancer.

Considering *ATG5*'s intimate association with immune related diseases as introduced above, we chose ATG5 as an example to discuss the importance of autophagy in immune related diseases. We also discuss ATG5's structure, function and related phenotypes. We hypothesize that the selective restoration of ATG5 function could be used to treat systemic autoimmune diseases.

STRUCTURE OF ATG5

Formerly known as apoptosis specific protein (ASP), ATG5 was first identified in Burkitt's lymphoma apoptotic cells. *ATG5* locates to human chromosome 6q21. Several transcript variants encoding protein isoforms have been identified. It can be transcribed from an open reading frame of 828 bp in yeast cells, encoding a protein of 276 amino acids. Human ATG5 comprises 275 amino acids, with an estimated molecular weight of ∼32.4 kDa. Western blotting shows a band of ∼56 kDa, representing the ATG5-ATG12 complex (9).

To date, there has been no direct X-ray crystallography study of ATG5 and thus little information is available for the structure of ATG5. One major reason is that ATG5 frequently binds with other proteins to form multiprotein complex and thus the isolated form is difficult to obtain. Furthermore, ATG5 is a soluble protein, and is prone to aggregation during purification (10). However, many studies have revealed the crystal structure of complexes involving ATG5, where the crystal structure of ATG5 is also obtainable. Matsushita et al. studied the ATG5-ATG16 complex and reported that ATG5 comprises three domains, including two ubiquitin-like (Ubl) domains flanking a helix rich domain (11). The two Ubl domains (UblA and UblB) both include a five-stranded β-sheet and two α-helices, exhibiting similar structures. The helix-rich domain comprises three long and one short α-helix. These three domains fold into the unique overall architecture of ATG5, where many protein interactions take place. Understanding the structure and binding sites of ATG5 complexes is important for the further determination of its functions.

ATG5 commonly binds with ATG12, catalyzed ATG7 and ATG10 (12, 13). ATG12's C-terminal glycine residue forms a covalent conjugation with a lysine residue of ATG5, forming the ATG5-ATG12 complex (14). According to the crystallization analysis of ATG5-ATG12 in *Saccharomyces cerevisiae*, a covalent conjugation of ATG5 Lys149 and ATG12 C-terminal Gly 186 was observed (11, 14). The ATG5-ATG12 complex also has several non-covalent interactions, including hydrophilic and hydrophobic interactions (14). After ATG5 binds covalently with ATG12, the complex further interacts with ATG16 via non-covalent linkages. The ATG16 α-helix interacts with UblA, UblB, and the short helix in helix-rich domain of ATG5, and its loop interacts with ATG5 UblA exclusively (11).

Tectonic β-propeller repeat containing 1 (TECPR1) is another key component in autophagy that promotes the fusion of lysosomes and autophagosomes (15, 16). TECPR1 includes several repeating tectonin β-propeller repeats, two dysferlin domains, an internal ATG12-ATG5 interacting region (AIR) domain, and a pleckstrin homology (PH) domain. TECPR1 functions by binding to the ATG5-ATG12 complex (17). Human ATG5 interacts with TECPR1 and binds non-covalently to the two ubiquitin-fold domains and N-terminal helix α-1 (10).

As stated above, current structural analysis is restricted to ATG5 complexes, because isolated human ATG5 is difficult to obtain. In the future, a more advanced structural analysis technique, i.e., cryogenic electronic microscopy (cryoEM), might be used to analyze the detailed structure of ATG5, which will increase our understanding of the function and pathogenic mechanisms of autophagy proteins.

ROLES OF ATG5 IN AUTOPHAGY

Canonical Autophagy

Canonical autophagy includes macroautophagy, microautophagy, and chaperone-dependent autophagy. Macroautophagy (hereafter referred to as autophagy), the classical pathway of autophagy, is initiated by the formation of an omegasome from the endoplasmic reticulum. The omegasome then forms an isolated membrane that further undergoes elongation, simultaneously engulfing intracellular components. Finally, the isolated membrane is closed as a complete autophagosome that then fuses with a lysosome, forming an autolysosome, in which the contents are degraded by lysosomal hydrolases, completing autophagy (2).

ATG5 is important in the context of autophagy (**Table 1**). Absence of ATG5 in mice causes neonatal lethality, possibly by disrupting autophagy and thus inhibiting the engulfment of lipid droplets (19, 44, 45). Furthermore, ATG5 can modulate autophagy. For example, under the conditions of starvation or rapamycin blockage, receptor activated C-kinase 1 (RACK1), a scaffold protein, binds with ATG5 to initiate autophagosome formation (46). The key role of RACK1-ATG5 in autophagy was further demonstrated by knockdown of RACK1, which demonstrated attenuated autophagy, and that blocking the interaction between ATG5 and RACK1 inhibits autophagy (47). Although the exact role of RACK1 is not fully understood, it is evident that RACK1 interacts with ATG5 and regulates autophagy. Another example is Calpain 1, a calcium dependent ubiquitous non-lysosomal cysteine protease that digests ATG5 (48). Low intracellular calcium levels downregulated the cleavage activity of Calpain 1, increasing the level of ATG5 and ATG12-ATG5 and ultimately upregulating autophagy (49). Thus, ATG5 levels can be regulated via Calpain 1 cleavage, which has a marked influence on autophagy. Similarly, microRNA miR181a interacts with an miRNA response element in the 3′ untranslated region (UTR) of *ATG5*, which inhibits its transcription. Overexpression of miR181a significantly attenuates *ATG5* mRNA and protein levels, resulting autophagy inhibition (50).

The ATG5-ATG12-ATG16 complex serves as a ubiquitin-like conjugation system that contributes to the elongation of the isolated membrane and autophagosome maturation (**Figure 1**). The conjugation of ATG5 with ATG12 is catalyzed by ATG7 and ATG10. The complex is termed as "ubiquitin-like conjugation system" because of the similar behavior of these proteins to ubiquitin enzymes. First, ATG7 activates ATG12, which resembles an E1 ubiquitin enzyme. ATG12 is then transferred to ATG10, an E2-like enzyme, and finally conjugates to ATG5 (2). However, no E3-like enzyme for conjugating ATG12-ATG5 has been identified.

TABLE 1 | Phenotypes of cells or organisms lacking ATG5 in different species.

Species	ATG5-absent cell/organ	Phenotype
L. major	Whole	Reduced flagellum, reduced virulence (18)
Mouse	Whole	Neonatal death (19, 20); more susceptible to liver fibrosis (21)
	B lymphocyte	Significant defect in B cell development at the pro- to pre-B cell transition (22); decreased antigen secretion (23); increased cell death (24)
	T lymphocyte	Increased CD8+ T cell death; decreased CD4/8+ T cell proliferation (25)
	Dendritic cell	Defect in processing and presentation of phagocytosed antigens (26)
	Macrophage	Increased plaques in artery wall (27); impaired restriction of pathogen L. Major (28)
	Embryo fibroblast	Higher level of apoptosis (29); higher migrating activity (30)
	Neuron	Progressive deficits in motor function and degeneration of some neural cells (31)
	Purkinje cell	Degenerate early and axonal swelling (32)
	Liver	Decreased survival in sepsis (33); higher hepatocyte proliferation (34)
	Lung	Alveolar epithelial cells are unable to mobilize internal glycogen stores independently of surfactant maturation (35)
	Renal proximal tubule cell	Accelerated cell death (36); G2/M arrest (37); decreased renal function (38, 39)
	Renal podocyte	Glomerular filter barrier damage, accelerated glomerulosclerosis (40)
Human	Adult-generated neuron	Reduced survival, delay in cell maturation (41)
	Cardiac myocyte	Increased ischemia/reperfusion cell injury (42)
	Fibroblast	Greater migration ability (43)

Membrane Formation

The ATG12-ATG5/ATG16 complex helps to form the autophagosome membrane in two ways: By involving in the LC3-PE conjugation pathway, or by directly binding with the membrane. Although the LC3- phophatidylethanolamine (PE) conjugation system was discovered earlier, these two pathways have equally important roles in autophagosome membrane formation. The major function of ATG12-ATG5/ATG16 is to serve as an E3-like enzyme in another ubiquitin-like conjugation system, the LC3 system (51), which mainly links LC3 with PE, and thus is termed the lipidation conjugation system. In this system, ATG7 serves as an E1-like enzyme to activate LC3 (52). LC3 is then transferred to ATG3, another E2-like enzyme, and conjugates with PE with the help of the ATG12-ATG5/ATG16 complex (13). Although the ATG12 complex helps the conjugation of LC3-PE, each component of the complex serves different roles (53). Recent studies showed that ATG16 is essential for the efficient conjugation of LC3-PE; however, previous studies, using different methods, disagreed with this conclusion. ATG5 facilitates direct membrane binding, while ATG12 inhibits it. ATG12-ATG5/ATG16 also binds directly to the autophagosome membrane to accomplish its formation, independently of LC3. ATG5 alone is able to bind with the autophagosome membrane, while conjugation with ATG12 inhibits this binding (54). After the non-covalent linkage with ATG16, ATG5 regains its membrane binding function. ATG16 might possess a coiled-coil domain that functions to dimerize ATG5-localized membrane binding sites (55). This hypothesis is supported by the observation that ATG5 alone cannot induce clustering of the membrane, while the ATG5/ATG16 complex can form giant unilamellar vesicles (54). Furthermore, inactivation of the ATG5-ATG12/ATG16 complex inhibits autophagosome formation to a greater extent than inhibiting LC3-PE conjugation (56–59).

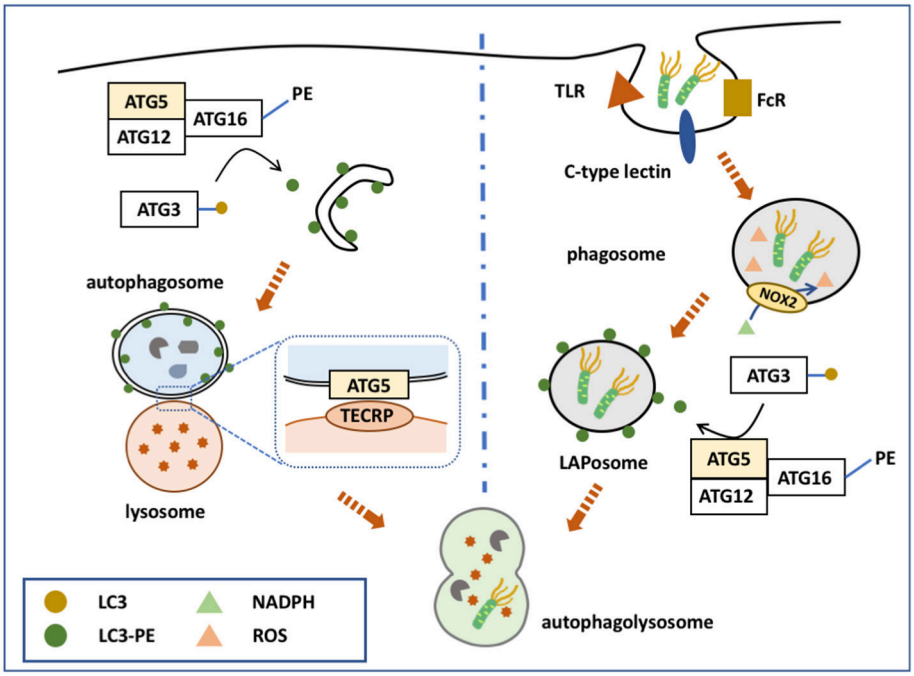

FIGURE 1 | ATG5 in canonical and non-canonical autophagy. ATG5 is involved in both canonical and non-canonical autophagy processes. The left side of the figure shows how ATG5 is implicated in the process of macroautophagy. Upon binding with ATG12 and ATG16, ATG5 forms a complex to conjugate PE with LC3, which is deposited on the membrane of the autophagosome. ATG5 is also expressed on the autophagosomal membrane that binds with TECRP, and therefore promotes the fusion of autophagosomes and the lysosomes. The right side of the figure presents ATG5 involved in the process of LAP. Similarly, ATG5 helps the conjugation of LC3 and PE by forming a complex of ATG5-ATG12-ATG16, and thus accomplishes the deposition of LC3-PE on the phagosome.

Fusion

ATG5-ATG12 is also involved in autophagosome-lysosome fusion. TECPR1, localized on the lysosomal membrane, binds with the ATG5-ATG12 complex to regulate autophagosome-lysosome fusion (16). When not involved in autophagy, the TECPR1 AIR domain occupies the PH domain to interrupt its binding with the autophagosome membrane (16). However, during autophagy, ATG5-ATG12 is generated to bind with the TECPR1 AIR domain, making the PH domain available to bind with phosphatidylinositol 3-phosphate of the autophagosomal membrane. TECPR1 is located on the lysosome membrane and ATG5 is localized on the autophagysomal membrane; therefore, TECPR1 binding with ATG5-ATG12 induces autophagosome and lysosome fusion (15, 60).

Non-canonical Autophagy

Non-canonical autophagy is characterized by either generating autophagosomes without the macroautophagy pathways, or involves canonical autophagy pathways without autophagosome formation. Currently, several pathways are identified in non-canonical autophagy, including LC3-associated phagocytosis (LAP); Beclin-1 independent autophagy; autophagosome formation from multiple phagophores and pathogen-specific autophagy modification; autophagy-associated unconventional protein secretion; and defective ribosomal products-containing autophagosome-rich blebs (61).

During pathogen-associated molecular pattern (PAMP) receptor activation [e.g., Toll-like receptor (TLR), Fc receptor, and C-type lectin], a PI3PK complex is recruited to the phagosomal membrane (62–64). Unlike in macroautophagy, this complex lacks ATG14, but consists of Rubicon and UVRAG (65). Subsequently, NADPH oxidase 2 (NOX2) is recruited to the phagosome with the help of Rubicon. PI3PK initiates lipidation of the phagosomal membrane, while NOX2 is responsible for the production of reactive oxygen species (ROS) (66). Together, these two events activate the two conjugation system to deposit LC3-PE on the outer membrane of the phagosome, forming a LAPosome. Thereafter, the LAPosome fuses with the lysosome, and LAPosome constituents are degraded. Complete LAPosomes may also fuse with endosomal vesicles, including major histocompatibility complex (MHC) class II-containing compartments, to present peptides to T helper cells (67).

ATG5, via its lipidation of LC3, plays a key role in the two conjugation systems of the LAP pathway (**Figure 1**). It is speculated that LC3 lipidation might alter the activity of lysosome fusion. Indeed, several studies demonstrated that ATG5 could regulate the fusion of LAPosomes and lysosomes by initiating LC3 lipidation. During TLR activation, phagosomes ligated with LC3-PE exhibited more rapid fusion with lysosomes compared with LC3-free phagosomes (64). Moreover, knockdown of ATG5 inhibited this promoting effect, suggesting that ATG5-dependent LAP promotes the fusion process (64). Consistently, blocking LAP by knocking down ATG7 attenuated the fusion between

LAPosomes and lysosomes, while LC3 recruitment to the phagosome is suggested to accelerate its maturation and fusion (68, 69).

However, other studies suggested that the deposition of LC3-PE was a delaying factor during fusion. LC3-PE-positive phagosomes are longer lived and mature later. Delayed phagosome-lysosome fusion also leads to prolonged MHC II antigen presentation. Other studies focused on the relationship between antigen presentation and ATG5-dependent LC3-PE deposition on phagosomes, which confirmed the delaying effect of LC3-PE on fusion. Lysosomal protease activity was related to the internalized antigen degradation speed (70). Macrophages with higher levels of lysosome protease maintained intracellular antigens for a shorter time. This suggested that delaying phagosome and lysosome fusion could delay the degradation of the antigens inside the phagosome. This finding was confirmed by another study demonstrating that ATG8/LC3-positive phagosomes could prolong antigen presentation, while Atg5-deficient macrophages failed to present extracellular antigens onto MHC class II molecules (63). Prolonged antigen presentation resulted in longer or continuous CD4+ T cell activation, and a more permanent humoral immune response.

ATG5 IN IMMUNITY REGULATION

Studies suggest that the major roles of autophagy in the immune system include elimination of microbes, control of inflammation, lymphocyte homeostasis, and the secretion of immune mediators. Thus, it is reasonable to infer that ATG5 could regulate certain aspects of the immune system, which has been confirmed by extensive research. In this section, we simply summarize the roles of ATG5 in innate and adaptive immunity, including regulating immune cell activation, cytokine secretion, and pathogen secretion.

Innate Immunity

In response to different stimuli, macrophages can be polarized into proinflammatory M1 or anti-inflammatory M2 (71). ATG5 regulates autophagic activity to alter the polarization of macrophages, subsequently modifying the extent of inflammation. ATG5 knockout hepatic macrophages hyperpolarized to the M1 phenotype, and therefore secreted more cytokines [interleukin (IL)-6 and tumor necrosis factor (TNF)] to increase the inflammatory response (72). Thus, ATG5-dependent autophagy is responsible for regulating macrophage polarization.

ATG5 activates neutrophils indirectly. In the presence of ATG5, lipopolysaccharide stimulates the secretion of mitochondrial proteins and autophagosomal luminal proteins, further activating polymorphonuclear leukocytes (73). Thus, lipopolysaccharide (LPS)-stimulated extrusion of mitochondrial contents provokes an inflammatory response of immune cells in an ATG5-dependent manner.

ATG5 regulates MyD88-dependent signaling to regulate innate immune responses (**Figure 2**). MyD88 is an important signaling adaptor molecule for TLRs and the IL-1 receptor, ultimately activating nuclear factor (NF)-κB signaling and mitogen-activated protein kinase (MAPK) signaling cascades, which leads to the transcription of many genes involved in innate and adaptive immunity (74, 75). ATG5 interacts with MyD88 and interrupts the downstream pathways, thereby suppressing NF-κB signaling (76). Thus, ATG5-mediated NF-κB signaling suppression might be involved in immune regulation.

The growth of intracellular bacteria can be restricted by autophagy, either canonical or non-canonical, in which ATG5 is essential. *Mycobacterium tuberculosis* is one of the pathogens in vacuoles that is eliminated by autophagy (77) and a double membrane structure was observed in tuberculosis infected type II alveolar epithelial cells (78). Atg5 knockout mice presented with a heavier *M. tuberculosis* burden, more severe inflammation, and higher levels of IL-1 (79). Autophagy also targets cytosolic bacteria, such as Group A Streptococcus (GAS). Mouse embryonic fibroblasts infected with GAS presented GAS-containing autophagosome-like vacuoles, while ATG5-deleted cells failed to produce such structures (80).

Recently, ATG5-mediated restriction of microbial infection via LAP was confirmed, and silencing or inactivation of ATG5 inhibited LAP activity and increased the survival of pathogens, including adherent and invasive *Escherichia coli*, *Shigella flexneri*, *M. tuberculosis*, *Aspergillus fumigatus*, and HIV (81–83). In particular, MORN2 recruits LC3 in macrophages to eliminate *M. tuberculosis* infection, and ATG5 is implicated in the process (84). Moreover, certain pathogens, such as *S. flexneri*, could interact with ATG5 to interrupt LAP and evade elimination. By binding with IcsB, an *S. flexneri* effector, ATG5 failed to bind with IcsA, another effector, thereby halting the LAP process and the elimination of the pathogen (85). However, recruiting ATG5 to promote LAP does not always help pathogen clearance. After HIV-1 infection, phagocytosis of, Vpu recruits ATG5 and LC3 to promote fusion with lysosomes to accelerate the degradation of the HIV-1 capsid protein, and thus favoring the dissemination of HIV into the cell (86).

Intriguingly, ATG5 also eliminates pathogens in an autophagy-independent manner. ATG5 regulates cytokine secretion through crosstalk with various pathways, and ATG5-mediated cytokine secretion achieved elimination of the pathogens. In addition, ATG5 recruited IFN-γ-inducible p47 GTPase IIGP1 (Irga6), which triggered IFN-γ-mediated clearance of *Toxoplasma gondii* (87). However, classical characteristics of autophagy, such as autophagosomes enveloping *T. gondii*, were not detected, further proving the autophagy-independent nature of ATG5-mediated *T. gondii* clearance. Similarly, *Atg5*-deleted mice vaginal cells expressed lower levels of cytokines involved in the anti-*Candida albicans* response, resulting in a lower *Candida* clearance rate (88).

Adaptive Immunity

ATG5 assists antigen presentation through autophagy, and thus is responsible for indirect lymphocyte activation by promoting the interaction between T or B cells and antigen presenting cells (APCs) (89). ATG5 is also directly responsible for regulating lymphocytes. ATG5-deleted CD8+ T lymphocytes were prone to cell death, and ATG5-deleted CD4+ and CD8+ T cells failed to undergo efficient proliferation after T-cell receptor

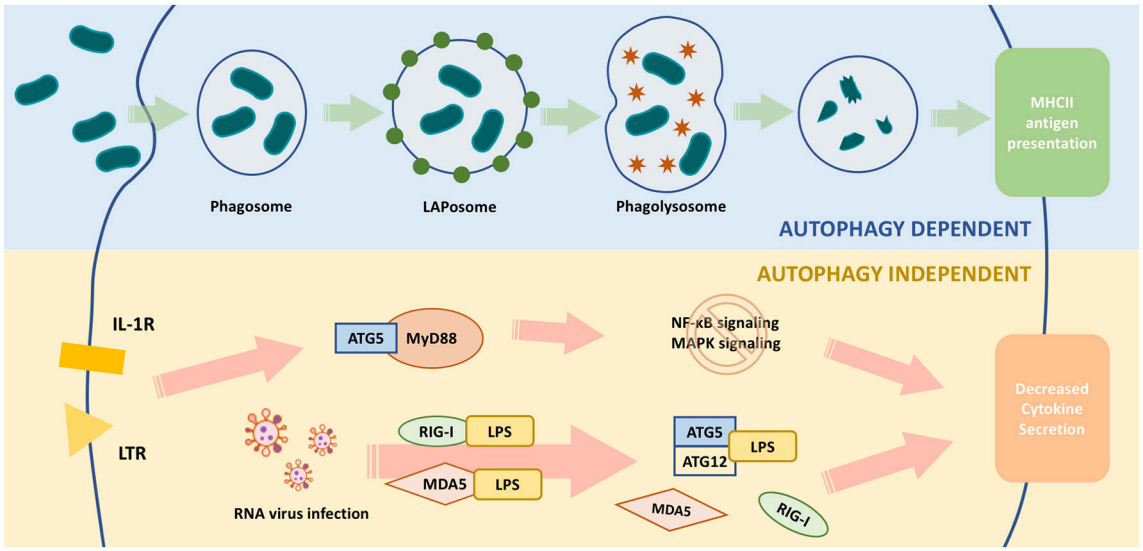

FIGURE 2 | Functions of ATG5 in macrophage. The upper part of the figure illustrates ATG5, as an important part of autophagy, is involved in the process of pathogen clearance and antigen presentation. The lower part illustrates ATG5 regulates macrophage cytokine secretion in an autophagy-independent manner. During RNA virus infection, ATG5-ATG12 binds with IPS to block the conjugation of IPS with RIG-1 or MDAS, and finally inhibits the expression and secretion of IFN. When TLR or IL-1R is activated, the downstream pathways, such as NF-κB signaling and MAPK signaling, are activated through MyD88. ATG5 is able to bind with MyD88 to block these pathways and eventually attenuates the production of certain cytokines.

(TCR) stimulation (90). The decreased survival of ATG5-deleted T cells was caused by the accumulation of abnormal autophagic structures and dysregulation of mitochondrial and ER homeostasis (25). ATG5-deleted progenitors failed to successfully transit from pro- to pre-B-cells. Knocking out Beclin-1 in B cells also resulted in differentiation failure (22). Thus, ATG5 might regulate lymphocyte development in an autophagy-dependent manner (**Figure 3**).

During RNA virus infection, retinoid acid-inducible gene I (RIG-I) or melanoma differentiation associated gene 5 (MDA5) are activated to bind with interferon-β promoter stimulator 1 (IPS-1) through their caspase recruitment domains (CARDs), eventually upregulating the production of type I interferon (91, 92). Type I interferon is an important cytokine responsible for enhancing antigen presentation and activating certain subtypes of immune cells, such as natural killer cells, cytotoxic T cells, B cells, and memory T cells (93). The ATG5-ATG12 complex bound with the CARD of RIG-I or MDA5 to inhibit the production and the secretion of interferon (94). Thus, ATG5 could regulate the production of type I interferon and the elimination of RNA viruses by influencing adaptive immunity activity.

In summary, ATG5 is responsible for the activation and the differentiation of various immune cells in innate and adaptive immunity. Evidence suggests that ATG5 regulates these immune cells via autophagy.

ATG5 IN CELL DEATH

Apoptosis is a programmed cell death process (95), whose pathways are distinct according to different stimulations (96). DNA damage generally triggers the intrinsic apoptosis pathway, where Bax and Bak induce the secretion of cytochrome c, leading to apoptosis (97, 98). Death receptor activation triggers the extrinsic apoptosis pathway, where a death-induced signaling complex (DISC) is formed to bind with Fas associated protein with death domain (FADD), leading to apoptosis (99, 100). By contrast, autophagy is regarded as a cytoprotective process in cell survival. Interestingly, evidence demonstrates crosstalk between autophagy and apoptosis (**Figure 4**).

ATG5 can regulate the extrinsic apoptosis pathway. ATG5 could bind with FADD to interrupt the interaction between FADD and DISC, halting the extrinsic apoptosis pathway (101). However, downregulation of ATG5 did not influence FADD-dependent cell death, and inhibiting caspase, the key component in apoptosis, did not block autophagosome formation (102), suggesting that apoptosis and autophagy are distinct processes. However, recent studies have challenged this conclusion. Yousefi et al. provided direct evidence that an apoptosis-related protease cleaves ATG5 to regulate the apoptosis and autophagy balance. Calpain mediated N-terminal cleavage product of ATG5 makes several different cell types more responsive to apoptotic stimuli. Apoptosis is associated with the translocation of this ATG5 fragment from the cytosol to mitochondria, in which it associates with the anti-apoptotic molecule Bcl-xL and triggers cytochrome c release and caspase activation, without activating autophagy (48). Similarly, the administration of Trichokonin VI (TK VI), an antimicrobial peptide, triggered the influx of extracellular calcium, which induced calpain-mediated ATG5 cleavage (103). TK VI administration also generated ROS, whose accumulation damages mitochondria, leading to ATG5-dependent autophagy (103). Thus, ATG5 has

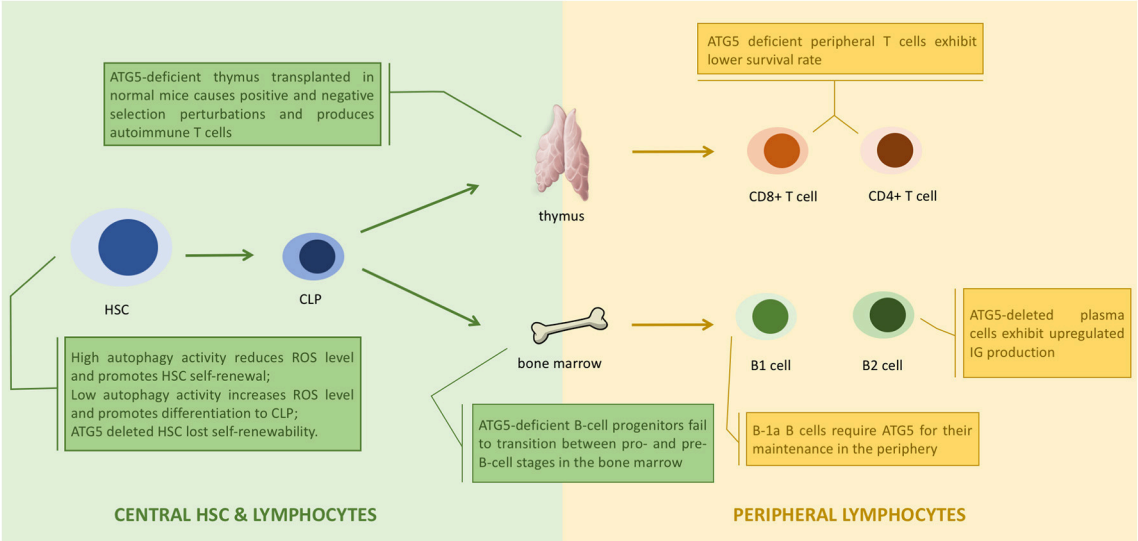

FIGURE 3 | ATG5 in the development of lymphocytes. ATG5 regulates the proliferation and differentiation of lymphocytes in each stage (1). Autophagy regulates the status of hematopoietic stem cell (HSC) through modulating the level of reactive oxygen species (ROS). Deletion of ATG5 in HSC inhibits autophagy and results in loss of self-renewability (2). ATG5 regulates lymphocyte maturation via autophagy in both bone marrow and thymus. Loss of ATG5 in this stage results in maturation failure and autoimmunity (3). ATG5 is responsible for the homeostasis of peripheral lymphocytes. Deletion of ATG5 in peripheral lymphocytes exhibit cell potency like autoimmunity.

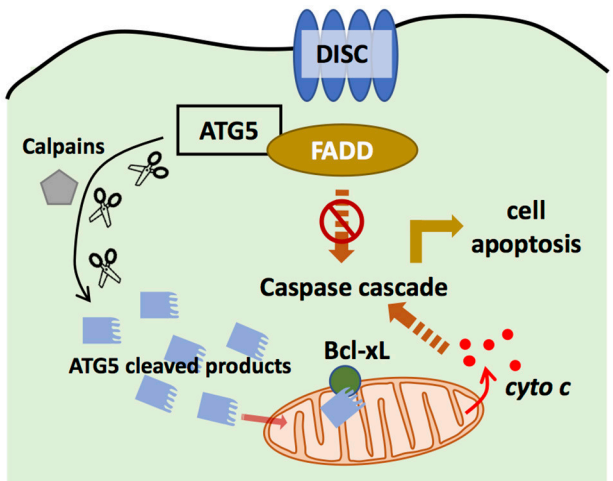

FIGURE 4 | ATG5 in apoptosis. ATG5 is able to bind with Fas associated protein with death domain (FADD) to block the binding of death-induced signaling complex (DISC) and FADD, and thus inhibits extrinsic apoptosis. ATG5 can also be cleaved by Calpains. The cleaved products then are translocated to the mitochondria to bind with Bcl-xL, and promote the secretion of cytochrome c, which activates intrinsic apoptosis.

an important role in the crosstalk between autophagy and apoptosis.

ATG5 is considered to exert its function as a key component in autophagy, which takes place in the cytoplasm, where ATG5 is commonly localized. However, ATG5 is also localized and functions in the nucleus. *ATG5* expression is upregulated after DNA damage, and ATG5 is recruited to the nucleus through a leucine-rich nuclear export signal (NES) (104). Inside the nucleus, ATG5 competes with aurora kinase B (AURKB) to bind with survivin, which inhibits the formation of the chromosome passenger complex responsible for chromosome segregation (105, 106). Failure to generate the chromosome passenger complex causes mitotic catastrophe, an oncosuppressive phenomenon occurring during or after defective mitosis, leading to death or senescence, and eventually resulting in G2/M arrest and the cessation of mitosis (107). This nuclear role of ATG5 in mitotic catastrophe shed it to be another spotlight in a separate form of cell death.

In summary, ATG5 is responsible for crosstalk among different forms of cell death. ATG5 also interrupts mitosis and promotes cell death triggered by DNA damage. Apoptosis is implicated in the pathogenesis of several autoimmune diseases, and thus we cannot rule out that the association of ATG5 with certain diseases is solely linked with autophagy or apoptosis (108). Further research is needed to determine whether apoptosis plays a role in ATG5-induced autoimmune diseases.

ATG5 AND AUTOINFLAMMATORY DISEASE

The term "autoinflammatory" was coined in 1999 to define a newly discovered family of recurrent fever syndromes (109). Compared with autoimmune diseases, autoinflammatory diseases are characterized by a lack of provocation for inflammation and the absence of high-titer autoantibodies or antigen-specific T lymphocytes (110). In the pathogenesis of

the traditionally defined autoimmune diseases, such as SLE, antigen receptor rearrangement and mutation play significant roles (111). In contrast, autoinflammatory diseases mainly involve aberrant innate immunity instead of adaptive immunity. During the last decade, genetic studies have identified a large number of gene mutation loci associated with abnormalities of innate immunity in several autoinflammatory diseases. Among them, several genes related to autophagy affect innate immunity associated with the development of autoinflammatory diseases. The following section introduces two autoinflammatory diseases, Crohn's disease and type 2 diabetes mellitus, and their association with autophagy.

Crohn's Disease (CD)

Crohn's disease is a non-specific chronic inflammatory disorder of the gastrointestinal tract. Its pathogenesis has several mechanisms, although the exact process remains unclear. CD is not traditionally defined as an autoimmune disease; however, its development involves the immune system attacking certain organs of the body. Therefore, CD is reasonably regarded as an "autoinflammatory" disease (112). Similar to SLE, the development of CD involves the interaction of genetic predisposing factors and environmental stimulation. The basis of CD pathophysiology is a classical Th1 cell reaction, in which TNF-α has a central role (113).

GWAS studies revealed that *ATG16L1* and immunity-related GTPase family M (*IRGM*) polymorphisms increase CD susceptibility (114–118), leading to extensive exploration of the association between autophagy and CD pathogenesis. IGRM initiates autophagy to eliminate invasive pathogens (119). Deletion of IRGM in human intestinal epithelial cells and macrophages caused defective autophagy (120, 121). Moreover, a CD-related *IRGM* single nucleotide polymorphism (SNP) (rs10065172, c.313C>T) produces an *IRGM* variant that fails to bind with miR-196, resulting in defective autophagy and attenuated pathogen clearance (122). In addition, miR-196 is overexpressed in the inflammatory intestinal epithelia of patients with CD, confirming that IRGM might play a role CD pathogenesis of CD by interfering with autophagy (123). It is observed that exposure to microbial products or bacterial invasion increases IRGM expression. And IRGM physically interacts with 2 other CD risk factors, ATG16L1 and NOD2, and additional pattern recognition receptors such as NOD1, RIG-I, and select TLRs. This explains how polymorphisms altering expression or function of autophagy in pathogenesis of infection and CD.

The *ATG16L1* risk allele causes defective lysozyme secretion of intestinal Paneth cells and autophagy dysfunction in intestinal macrophages, resulting in pathogen elimination failure (124). Previous studies showed that Paneth cells from mice carrying *Atg16l1*^{T300A} cannot secrete lysozyme through secretory autophagy when infected with *S. typhimurium*. Mice with selective ablation of autophagy in intestinal epithelial cells (IECs) (*Atg16l1*^{VC} mice) exhibit severely exacerbated intestinal pathology, characterized by increased accumulation of CD4^{+} T cells in the lamina propria and elevated levels of pro-inflammatory cytokines. *Atg16l1*-deficient IECs show increased induction of apoptosis following exposure to pro-inflammatory cytokines (TNF + IFNG/IFNγ) compared to wild-type IECs. These findings confirm that the exacerbated pathology in *Atg16l1*^{VC} mice is largely driven by TNF-induced IEC apoptosis (125, 126). More recent data also suggested an interaction between smoking and *ATG16L1*^{T300A} triggers Paneth cell defects in Crohn's disease (127).

ATG16L1 contributes to autophagy by forming the ATG5-ATG12-ATG16 complex; therefore, it unsurprising that similar morphological abnormalities are observed in ATG5 deleted Paneth cells, which were indistinguishable from ATG16L1 deleted cells (128). GWAS did not identify any CD pathogenesis-related *ATG5* SNPs; however, a prospective pharmacogenomic study of patients with CD treated with anti-TNF-α drugs reported several significant SNPs in *ATG5* and *ATG12* as associated with positive response to therapy (129). In addition, ileal biopsy samples of patients with CD revealed an inverse correlation between levels of microRNAs miR30C and miR130A and those of ATG5 and ATG16L1 (121). The possible mechanism was revealed by *in vitro* experiments, showing that inhibiting the two microRNAs in cultured mice intestinal epithelial cells upregulated the expression of *Atg5* and therefore restored autophagy function (128). Downregulation of ATG5 by miRNAs, leading to defective autophagy and inflammation, might be involved in the pathogenesis of CD.

ATG5 is associated with maintaining the regular functioning of Paneth cells and intestinal macrophages. Despite GWAS showing that *ATG5* is not directly related, *ATG5* is associated with CD patients' response to therapy, suggesting that it might serve as a downstream player in CD pathogenesis or some certain phenotypes. However, further studies are needed to determine the detailed mechanism of ATG5 in the development of CD.

Type 2 Diabetes Mellitus

Diabetes is an extensively investigated disease, characterized by elevated serum glucose, which might result in internal organ damage when not carefully controlled, such as diabetic nephropathy and diabetic retinopathy. Type 1 diabetes (T1DM) is an autoimmune disease, whose pathogenesis involves the production of pancreatic β cell antibodies, while type 2 diabetes (T2DM) centers around the state of insulin resistance (IR). However, inflammation and abnormal cytokines secretion from fat tissues are believed to play a major role in the development of IR, placing it in the spectrum of "autoinflammatory" diseases. Recent studies explored the relationship between ATG5 and IR, implying a possible role of ATG5 in T2DM development.

Significant evidences links obesity and T2DM with autophagy, where ATG5 plays a certain role disease development. Ultrastructural analysis of adipose tissue (AT) adipose tissue in obese and T2DM patients revealed increased numbers of autophagosomes and increased immunofluorescence signal of marker LC3 (130–132). Furthermore, several autophagy markers, including ATG5, are increased in visceral AT as well as subcutaneous AT of obese and T2DM patients, suggesting ATG5-dependent autophagy might be involved in the development of obesity-induced T2DM (130). Moreover, increased activity of ATG5-dependent autophagy is also linked with higher TNF-α

and IL-6 expression, suggesting that autophagy serves as a pro-inflammatory factor to enhance AT inflammation (130). In addition, levels of autophagy marker genes were increased in insulin resistant compared with insulin sensitive patients (132). ATG5 also regulates pancreatic β cell homeostasis by regulating autophagy (133). A substantial amount of proinsulin is rapidly delivered to autophagosomes and directed to lysosomal degradation, and deletion of ATG5 results in increased proinsulin, suggesting that ATG5-dependent autophagy might play a critical role in the production and secretion of insulin (133). Thus, ATG5 is responsible for regulating insulin production homeostasis in pancreatic β cells and for enhanced inflammation and IR in AT, revealing the possibility that ATG5 is closely related to the pathogenesis in T2DM.

However, ATG5 does not always appear to exacerbate the development of T2DM. The administration of dihydromyricetin (DHM), a natural flavonoid that exerts various bioactivities, including anti-oxidative effects, attenuated IR severity by promoting AMPK-induced autophagy, which also upregulated ATG5 (134). Similarly, AMPK signaling was also activated in amepelopsin-treated endothelial cells, which triggered ATG5-dependent autophagy (135).

Although there is some evidence demonstrating the involvement of ATG5 in T2DM, its exact role in the development of T2DM remains unclear. Whether the increase in ATG5 in certain tissues has as a protective role from inflammation and IR requires further research.

ATG5 AND AUTOIMMUNE DISEASES

Given the multi-faced function of ATG5, it is reasonable to speculate that it might be involved with other diseases whose pathogenesis interferes with autophagy or apoptosis; for example, the large spectrum of autoimmune diseases. Genetic predisposition and environmental stimulation both contribute to disease development. Generally, autoimmune diseases are characterized by immune cells or molecules attacking tissues or organs of the human body, resulting from false activation of immune cells by "self-derived" components. Therefore, aberrant autophagy or apoptosis might expose intracellular contents to the matrix, which could activate immune cells to trigger an autoimmune response.

Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease that affects multiple organs, including the skin, muscles, joints, kidney, and heart (136). The etiology of SLE is complex and not fully understood. Infection, UV exposure, certain drugs, and imbalanced hormone levels are risk factors for SLE that would undermine the immune system and provoke autoimmunity (136). Aberrant autoimmunity in SLE includes defects in clearing apoptotic cells, and abnormal antigen presentation and autoantibody production (137). These autoantibodies can directly target organs or form immune complexes to further damage tissues (137).

Studies have shown that mammalian target of rapamycin complex 1 (mTORC1) inhibition increases autophagy, whereas stimulation of mTORC1 reduces this process. And it have revealed that mTORC1 represses autophagy by phosphorylating and repressing ULK1 and ATG13. Activation of the mTOR pathway might induce abnormalities in lymphocytes of patients with SLE (138, 139). Depletion of glutathione, and increased nitric oxide and mitochondria in T cells are observed in patients with SLE (140–142). Consistently, low glutathione and high nitric oxide trigger mTOR signaling, which subsequently induced persistent mitochondrial hyperpolarization (MHP), presenting as a mitochondrial mass in T cells (140–142). Moreover, mTOR activation upregulates the expression of small GTPases to promote the recycling of TCR-associated signaling proteins (143). In patients with SLE, small GTPase-dependent lysosome degradation of CD3, a TCR-associated signaling protein, is observed, suggesting activation of the mTOR pathway (144). Therefore, autophagy might play an important role in SLE pathogenesis via mTOR signaling.

Moreover, ATG5 is implicated in SLE through LAP. Mice with *Atg5*-deleted myeloid cells exhibited LAP deficiency. Repeated injection of dying cells into these LAP-deficient mice induced the development of an SLE-like disease, including increased serum levels of autoantibodies and creatinine. Intriguingly, knocking out other autophagy genes, *Atg14* and *Fip200*, undermined canonical autophagy but not LAP, and did not induce SLE-like disease, which suggested that ATG5-dependent LAP plays a definite role in the pathogenesis of SLE.

The genetic association between *ATG5* alleles and SLE provides strong evidence of the role of autophagy in SLE. Several GWASs have identified SNPs in *ATG5* that are genetic predisposing factors for SLE. The first GWAS study (2008) reported the association of *ATG5* with SLE in females with European ancestry. Further evidence showed that *ATG5* SNPs are related to SLE in Caucasian and Chinese populations (145–148). However, other studies identified important SNPs in the intergenic region of *PRDM1-ATG5*, such as rs548234 and rs6568431. Moreover, GWAS from a Chinese population reported the association between SLE and *PRDM1-ATG5* instead of *ATG5*, which further obscured the role of ATG5 in SLE (147). Thirty-one genes involved in NF-κB signaling, IFN and IL-12 production, and apoptosis pathways are regulated by ATG5 genotypes (148). In addition, several SNPs, including rs548234, rs693612, rs9480642, rs6937876, rs548234, and rs6937876, exhibiting significant correlations with ATG5 expression [*cis* and *trans*- expression quantitative trait loci (eQTLs)] were also associated with SLE susceptibility. In addition, further follow-up study also suggested that rare variants (mutations) apart from SNPs were also associated with SLE (149). And SNPs that affect ATG5 expression (ATG5-trans eSNPs) also showed genetic associations with SLE (150). By *in-silico* analysis, all these associated SNPs were regulatory SNPs for ATG5 expression, suggesting a significant role of deregulated ATG5 expression in mediating SLE. And it was indeed observed ATG5 was increased in patients with SLE. A pilot study also observed significant gene-gene interactions between *ATG5*, *ATG7,* and *IRGM* (148). And a genetic pathway based study

not only confirmed these associations, but also identified novel associations with LC3 (148). Of note, most genetic studies focusing on autophagy were mainly conducted in Chinese populations, thus more wide-spread replications are still warranted.

ATG5 acts on the immune system to accelerate the inflammatory response, including NF-κB and interleukin production, and functions in antigen presentation. SLE-related *ATG5* SNPs influence these key pathways; therefore, it is reasonable to speculate that ATG5 initiates the development of SLE by disrupting antigen presentation or causing a cytokine imbalance. Tested genotypes of *ATG5* also showed changes in apoptosis-related protein expression, which also revealed ATG5's role in apoptosis in the pathogenesis of SLE.

A more recent study observed that the administration of shATG5-lentivirus ameliorated proteinuria and decreased the level of serum anti-dsDNA antibody in lupus-prone mice, suggesting promising therapeutic innovations targeting ATG5; however, more investigation is needed to evaluate its side effects (151).

Central Nervous System (CNS) Autoimmunity

Central nervous system autoimmune diseases comprise a large spectrum of diseases, each of which requires extensive research and investigation. Recent studies have revealed the role of autophagy and CNS autoimmunity, in which ATG5 might play an important role. ATG5 in dendritic cells is regarded as a possible autoimmune response driver, according to a study in which *Atg5*-deleted mice exhibited lower degree of demyelination in the CNS (152). In the absence of ATG5, CD4+ T cell presentation of endogenous myelin peptides was inhibited, which restricted the downstream autoimmune response (152, 153).

Multiple sclerosis (MS) is an autoimmune disease characterized by spatial and temporal dissemination (154). The pathophysiology of MS lies in the demyelination of the white matter of the CNS. Patients with MS present with intermittent episodes of neurological dysfunction (155). The exact mechanism of MS is not completely understood; however, an autoimmune reaction is regarded as the central mechanism. CD4+ Th1 cell dependent cell-mediated immunity is suggested as the dominant autoimmune reaction damaging the white matter. Activated T cells, along with certain B cells, cross the blood brain barrier and provoke inflammation that induces demyelination. Meanwhile, macrophage, IFN γ, and TNF-α are also involved in the process of demyelination (156).

Experimental autoimmune encephalomyelitis (EAE) is a widely used animal model of MS, in which elevated ATG5 mRNA levels are detected in blood and brain tissue (157). The ATG5 level also correlates positively with EAE clinical severity, suggesting a possible role of ATG5 in inflammatory demyelination. The ATG5 mRNA level is elevated in patients with active relapsing-remitting MS (RRMS) compared with those in quiescent RRMS (158). Strong ATG5 immunoreactivity is also observed in postmortem brain tissue of patients with secondary progressive MS (158). However, the involvement of ATG5 in MS is obscured by a gene analysis showing that MS is not associated with *ATG5* variants (159). Therefore, to further investigate ATG5 function in MS demyelination, the post-translational state of ATG5 was analyzed. The level of the ATG5-ATG12 complex increased significantly in EAE mice, while the level of cleaved ATG5 was lower than that in control mice, which possibly represents a pro-survival role of ATG5 in T cells by enhancing autophagy and blocking apoptosis (158). The role of ATG5 in autophagy in MS was supported by the altered expression of BECN1 and LC3, two autophagy pathway components, in blood from patients with MS (160).

Neuromyelitis optica (NMO) is a CNS autoimmune disease that is associated with ATG5. Similar to MS, the major characteristic pathology of NMO is demyelination; therefore, ATG5 might exert the same function in the pathogenesis of NMO. In a Chinese Han population, *ATG5* variants were found to be associated with NMO, among which SNP rs548234 increased susceptibility, while rs548234 and rs6937876 have protective roles in NMO (159).

Evidence supporting ATG5's association with MS or NMO is limited. Elevated levels of Atg5 in MS animal models and postmortem brain tissue might not suffice to conclude that ATG5 is responsible for the pathogenesis of MS. Likewise, further investigation is needed to provide direct evidence of the underlying mechanism of how ATG5 variants result in abnormal demyelination and the development of MS and NMO.

CONCLUSION AND PERSPECTIVES

ATG5 is an extensively investigated protein, most characteristics of which, including its gene, structure and functions, are gradually unveiling its mysterious mask. ATG5 initiates the formation of the autophagosome membrane and the fusion of autophagosomes and lysosomes, functioning in both canonical and non-canonical autophagy. ATG5 also functions in the immune system, regulating innate and adaptive immune responses, including macrophage polarization, cytokine secretion, antigen presentation, and the activation of certain immune-related cells. ATG5 is also involved in both intrinsic and extrinsic apoptosis. Lastly, ATG5 can also translocate into the nucleus and induce mitotic catastrophe. Based on its multi-faceted function, ATG5 could not only relate to MS, NMO, and SLE, as traditionally defined autoimmune diseases, but also shows association with CD and T2DM, which were considered as diseases related to autoinflammation recently.

However, our concept of the functions of ATG5 might be incomplete, and many details are lacking. In terms of the known and speculated the functions, some conclusions were simply drawn based on observing the phenotypes of ATG5 deletion or overexpression. Similarly, several diseases, such as SLE and CD, are associated with ATG5 according to GWAS, yet there have been few studies examining the exact functional role of ATG5 in

these diseases. While the importance of ATG5 has only emerged, it is possible ATG5 might eventually be regarded as a "guardian of immune integrity". An improved mechanistic understanding of the autophagy machinery could lead to treatments for human diseases. However, it is essential to further investigate the molecular mechanism of ATG5 in disease development and in executing certain functions, allowing the development of potential therapeutic innovations targeting ATG5. Notably, accumulating evidence also indicates that other ATG genes (i.e., ATG16L1, ATG7, and IRGM) may have similar functions. Further investigations are required to facilitate mechanism, biomarker and novel therapeutic intervention findings.

AUTHOR CONTRIBUTIONS

XY collected data and conceived and wrote the manuscript. X-JZ conceived and revised the manuscript critically for important intellectual content, supervised the research group, and has given the final approval of the version to be published. HZ revised the manuscript critically, supervised the research group, and has given the final approval of the version to be published.

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More Than Skin Deep: Autophagy Is Vital for Skin Barrier Function

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The skin is a highly organized first line of defense that stretches up to 1.8 m^2 and is home to more than a million commensal bacteria. The microenvironment of skin is driven by factors such as pH, temperature, moisture, sebum level, oxidative stress, diet, resident immune cells, and infectious exposure. The skin has a high turnover of cells as it continually bares itself to environmental stresses. Notwithstanding these limitations, it has devised strategies to adapt as a nutrient-scarce site. To perform its protective function efficiently, it relies on mechanisms to continuously remove dead cells without alarming the immune system, actively purging the dying/senescent cells by immunotolerant efferocytosis. Both canonical (starvation-induced, reactive oxygen species, stress, and environmental insults) and non-canonical (selective) autophagy in the skin have evolved to perform astute due-diligence and housekeeping in a quiescent fashion for survival, cellular functioning, homeostasis, and immune tolerance. The autophagic "homeostatic rheostat" works tirelessly to uphold the delicate balance in immunoregulation and tolerance. If this equilibrium is upset, the immune system can wreak havoc and initiate pathogenesis. Out of all the organs, the skin remains under-studied in the context of autophagy. Here, we touch upon some of the salient features of autophagy active in the skin.

Keywords: autophagy, skin autoimmunity, selective autophagy, skin diseases, skin cancers

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INTRODUCTION

Skin architecture is designed to shield against physical as well as immunological damage by environmental assaults [such as pathogens, ultraviolet radiation (UVR), allergens, oxidative stress, and various chemical toxins like hexavalent chromium, zinc, titanium oxide, and silver nanoparticles] (1–4). The skin is a nutrient-poor environment, which exposes itself to various environmental stressors regularly and therefore, requires recycling of limited resources *via* the autophagy machinery to maintain homeostasis (5, 6). Nonetheless, skin has a potent arsenal of weapons at its disposal to ward off potential threats from external aggressors. The cells populating the skin have both immune and non-immune components (1, 2). The skin is comprised of the epidermis, dermis, and hypodermis (subcutaneous fat) (**Figure 1A**) (1, 2, 7, 8). Skin also has several appendages (adena), such as nails, sweat glands, sebaceous glands, and hair follicles, which allow sensation, lubrication, and restriction of heat loss (9). Epidermis is comprised of keratinocytes, Langerhans cells (LCs), dendritic epidermal γδ T cells (DETC), melanocytes, and merkel cells (10). Dermis is comprised of fibroblasts, immune cells [dermal DCs (dDCs), innate lymphoid cells (ILCs), NK cells, B cells, macrophages, and T cells], endothelial cells, and neurons, which build up the extracellular matrix (1, 2). The hypodermis is comprised of adipocytes, nerves, blood, and lymphatic vessels.

Apart from the innate and adaptive immune cells present in the skin, the complement systems, antibodies, and antimicrobial peptides (AMPs) aid the immune system in clearing out pathogens and

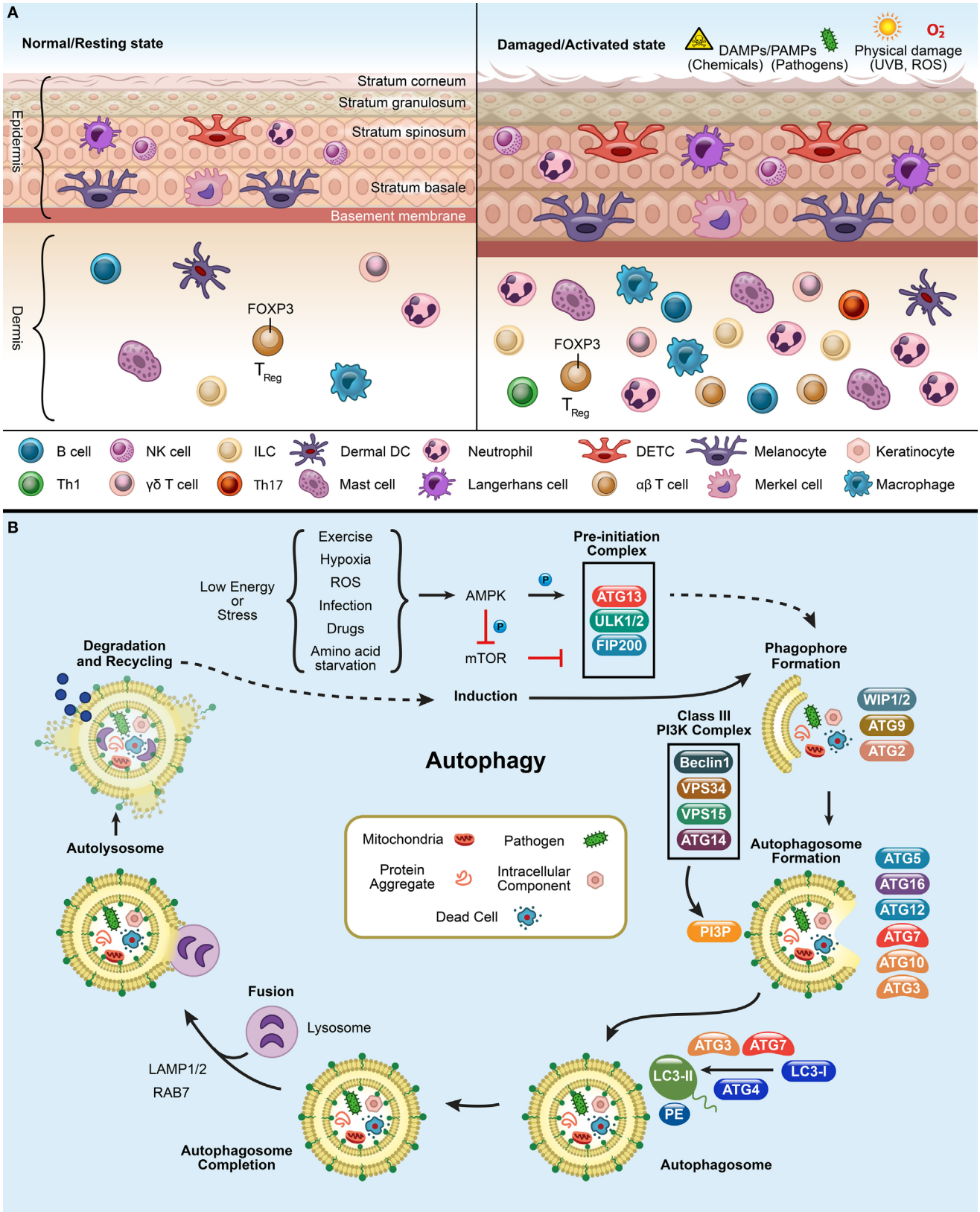

FIGURE 1 | Continued

FIGURE 1 | Panel **(A)** depicts the resting and activated state of the skin. In a normal or resting state, epidermis and dermis have circulating immune cells [DETCs, αβ T cells, γd T cells, macrophages, neutrophils, LCs, dermal DCs (dDCs), NK cells, B cells, innate lymphoid cells (ILCs)] and non-immune cells (melanocytes, keratinocytes, and merkel cells). Upon exposure to pathogens, chemicals, UV, or reactive oxygen species (ROS), the immune cells infiltrate at the site of infliction to defend the host and finally to resolve the inflammation after damage. Panel **(B)** shows the process of autophagy. mTOR inhibition triggers the activation of AMPK and initiates an autophagy-inducing signals during a low energy state such as starvation, ROS, exercise, infection, drugs, and hypoxic stress. This initiates the formation of pre-initiation complex (ULK1/2, ATG13, and FIP200) in the presence of unwanted cargo (such as, mitochondria, pathogens, protein aggregates, and intracellular components). This will, in turn activates the Class III phosphatidylinositol-3-kinase (PI3K) complex, composed of ATG14 (UVRAG)-VPS15-VPS34-Beclin1. The Class III PI3K complex completes the autophagosome formation by producing PI3P which recruits downstream ubiquitin-like conjugation systems (ATG5–12) and converts LC3-I to form LC3-PE. Finally, lysosome fuses with the autophagosome to form the autolysosome to degrade the enclosed cargo. The degraded cargo is finally assimilated and recycled.

foreign particles. Autophagy participates in various physiological activities to ensure the smooth and quiescent operation of the immunotolerant environment and to maintain skin integrity. These activities include maintaining homeostasis, performing efferocytosis, as well as determining skin color, host defense, longevity, antigen presentation, and survival (11).

AUTOPHAGY AS A CELL SURVIVAL MECHANISM

Autophagy means *self* (auto) *eating* (phagy) and is a highly conserved cellular process across eukaryotes, which allows cells to recycle cytoplasmic materials *via* the lysosome and survive periods of nutrient deprivation (11). The term autophagy is derived from ancient Greek, but the word first garnered attention when Christian de Duve not only coined it but also won the Nobel prize in Physiology or Medicine in 1974 for his work on lysosomes (12–16). More recently Dr. Yoshinori Oshumi, described the autophagy-related genes (ATG) in yeast in 1993 and received the Nobel prize in 2016 (11, 12, 17). His pioneering work led to the discovery of other ATG genes and its human orthologs.

Autophagy pathways include macroautophagy (canonical autophagy/autophagy), microautophagy, and chaperone-mediated autophagy (CMA) (11, 13, 18). Traditionally, autophagy is orchestrated by the group of ATG proteins, which precisely control the autophagic process (11, 19). The process kickstarts the formation of the pre-initiation complex, followed by generation of the phagophore, autophagosome, and autolysosome, leading to cargo degradation (**Figure 1B**) (11, 19, 20). Mammalian target of rapamycin complex 1 inhibition leads to the induction of autophagy and assembles ULK1/2, ATG13, and FIP200 to form the pre-initiation complex at the phagophore (**Figure 1B**). Once activated, it targets the Class III phosphatidylinositol-3-kinase (PI3K) complex (Beclin1, VPS34, VPS15, and ATG14) which recruits downstream conjugation ATG proteins. During autophagosome elongation, E3(Ubiquitin)-ligase ATG7 mediates ATG5–ATG12–ATG16L1 complex formation and is recruited to the autophagosome membrane. Ubiquitin-conjugating/E2-like enzyme ATG10 mediates covalent conjugation of the ubiquitin-like ATG12–ATG5 (21). E2-like enzyme ATG3 forms ATG12–ATG3 conjugate, controls mitochondrial homeostasis (21). ATG7 can recruit ATG3 and ATG10 forming ATG7–ATG3 and ATG10–ATG3, respectively (22). Mice lacking ULK1/2, ATG3, ATG5, ATG7, ATG12, or ATG16L1 are embryonic lethal mutations (23). ATG12-conjugation is essential for the formation of preautophagosomes (24). ATG3 aids in conjugation of LC3-I with phosphatidylethanolaminie (PE) required for the formation of autophagosomes (21, 24). This facilitates the LC3 lipidation with PE and forms LC3-PE (or LC3-II). LC3-PE embeds into the mature autophagosome which finally fuses with the lysosome, wherein the cargo is degraded and recycled. The autophagy pathway is not only limited to the processes of degradation and survival during starvation but is also active in regulating other cellular functions (11). This bolsters the need for investigating autophagy's widespread influence on different biological mechanisms.

To ensure proper scrutiny, the autophagy machinery takes on specialized roles that selectively targets and digests intracellular components and is called selective or non-canonical autophagy (25–27). Depending on the cargo engulfed, it can be classified into CMA (heat-shock cognate 70 stress protein mediated target of the substrate), aggrephagy (clearance of protein aggregates), macrolipidophagy (the degradation of lipids), pexophagy (autophagic degradation of peroxisomes), ER-phagy (endoplasmic reticulum autophagy), mitophagy (damaged mitochondria), xenophagy (intracellular pathogens), and LC3-associated phagocytosis (LAP) (efferocytosis and pathogen phagocytosis) (28–31). Selective autophagy receptors/adaptors p62/Sqstm1 (*Sequestome1*), OPTN (*Optineurin*), TAX1BP1 (T-cell leukemia virus type I binding protein 1), NDP52/CALCOCO2 (calcium binding and coiled-coil domain 2), and NBR1 (neighbor of BRCA1 gene 1) coordinate and mediate degradation of ubiquitinated cargos by delivering them to LC3-containing phagophores (**Figure 1B**) (11, 32–41). Mitophagy involves degradation of redundant and distressed mitochondria and normally occurs in a Parkin-PINK1-dependent manner (42, 43). After ubiquitination, autophagy adaptors, OPTN and NDP52, can recognize and deliver them to LC3-positive autophagosomes for degradation (44). Similarly, in xenophagy, cytosolic pathogens or pathogen-contained vacuole can be ubiquitinated by ubiquitin ligases (45–47). Subsequently, ubiquitinated pathogens or their substrates are recruited by autophagy receptors for autophagosomal degradation (11). However, when an extracellular pathogen is phagocytosed and it engages pathogen recognition receptor (PRR), as a result it activates a specialized autophagy process called LAP (27). The LAP pathway is also utilized for the clearance of dead cells triggered by wounds, pathogen exposure, or environmental triggers (26, 27, 48).

AUTOPHAGY IN SKIN IMMUNE CELLS

Skin inflammation induced by environmental irritants and pathogens requires autophagy as well as a crosstalk between

TABLE 1 | Lists the pertinent autophagy components active in skin cells.

	Cell types	Autophagy mediator	Processes that require the autophagy machinery	Reference
Non-immune cells	Keratinocytes	Phosphatidylinositol-3-kinase (PI3K)–AKT–mTOR pathway, ATG5, ATG7	Pigmentation, differentiation, hyperkeratosis	(2, 49–55)
	Melanocytes	ATG5, ATG7	Aging, oxidative stress damage, UV protection, melanin production	(56–65)
	Merkel cells	ATG7	Differentiatation, removal of damaged proteins/organelles	(66, 67)
Innate Immune cells	Neutrophils	ULK1, Beclin1, ATG16L1	Accelerated apoptosis in leprosy patients, degranulation, reactive oxygen species production	(68, 69)
	Macrophages	ULK1, Beclin1, ATG14, ATG16L1	Pigmentation, removal of damaged proteins/organelles	(70–73)
	Langerhans cells	*Maplc3b, Atg3,* ATG7	Removal of melanosomes, control of inflammation, antigen presentation	(74–76)
	Dermal DCs	*Maplc3b, Atg3, ATG7*	Removal of melanosomes, control of inflammation, antigen presentation	(74–76)
	Mast cells	ATG7	Degranulation of mast cells	(77, 78)
	NK cells	Beclin1	Melanoma	(79)
Adaptive immune cells	B cells	ATG5	Differentation, autoimmune disorders	(80)
	FOXP3 αβ T cells	ATG5, ATG7	Inflammation, expansion of T_{regs}, and skin homeostasis	(81–85)
	γd T cells	mTOR	Survival and wound repair	(70, 86)

immune and non-immune skin cells (listed below and in **Table 1**) to effectively alleviate the damage (4).

Non-Immune Components

- *Melanocytes* reside in the epidermis between keratinocytes, and they contain melanin or biochrome, a natural pigment found in hair, skin, and eyes (49). The process of producing melanin pigment continuously is called melanogenesis (87). Hair follicular melanogenesis (FM), unlike epidermal melanogenesis, is cyclic (88). FM starts at the anagen (active growth phase) stage of the hair cycle and is turned off by catagen (transition stage) and telogen (shedding stage) (88). Upon aging, the number of melanocytes in hair follicles is reduced and dendritic cells (including Langerhans cells) move from the upper to lower hair follicles as a response to age-related degradation of melanocytes (88).

  Stress can stimulate melanocyte to activate neurotransmitters, neutropeptides, and hormones which aids in regulating skin homeostasis (89). Neuroendocrine has been shown to govern the production and secretion of L-tyrosine and L-dihydroxyphenylalanine (L-DOPA), or its derivatives, during melanogenisis (89). Melanocytes are often called "neurons of the skin" and possesses melanocytes-stimulating hormone (MSH) receptors (89, 90). Certain substrates of melanogenesis like L-tyrosine and L-DOPA can also regulate cell (melanocyte) functions and cellular metabolism through non-receptor-mediated processes (91).

  Melanocytes develop into melanosomes, which can perform lysosomal degradation as seen in retinal epithelial cells (49, 56, 87). Autophagy is also involved in melanin synthesis in melanosomes (92) *via* FGF7/FGF7R-initiated AMPK/mTOR pathway (93–96). *ATG5* and *ATG7*-deficiency causes premature aging and accumulation of oxidative stress-induced damage (57–60). Upon undergoing UV exposure, melanocytes can undergo photosensitization by generating superoxide radicals in cells (97). In addition, p62 is upregulated upon phototherapy (UVA radiation and light-emitting ode 585 nm) in the melanocyte (61, 62). Melanin confers protection from UV-induced DNA damage, maintains skin homeostasis, modulates the immune environment, and regulates skin color

by autophagy (56, 63–65). Melanocytes can also phagocytose, present antigen *via* major histocompatibility complex (MHC) class II and produce cytokines like IL-1, IL-6, TNF-α, IL-3, and G-MCSF (98).

- *Merkel cells* populate broadly across the epidermis. They have high turnover and differentiate terminally (2, 99). Merkel cells have a longer life span than keratinocytes and require autophagy for differentiation (66). *Atg7*-deficient Merkel cells show accumulation of p62 (66, 67).

Keratinocytes are abundant and form the foundation of the epidermis. Out of all skin cells, they have been studied the most. Keratinocytes protect the skin cells by phagocytosing damaged melanosomes (49). Human keratinocytes also induce inflammasomes upon either UVB irradiation or viral infection (100). Autophagy in keratinocytes contributes to skin pigmentation, as it depends on the melanin from phagocytosed melanocytes engulfed by the keratinocytes (49, 92). Keratinocyte differentiation results in lysosomal enzyme activation, LC3 expression, and intracellular components degradation to form corneocytes (2, 50–52). Epidermal permeability barrier, mitophagy, and autophagy defects are observed in comparative gene identification-58 (CGI-58) (a co-activator of adipose triglyceride lipase)-deficient mice (51, 101). CGI-58 deficiency causes Chanarin–Dorfman syndrome (neutral lipid storage dysfunction) and chronic and excessive build up of keratin leading to ichthyosis (51, 101). *Atg7*-deficient keratinocytes are smaller, with outer root sheath thickening, acanthosis, and hyperkeratosis. They also have less keratohyalin, trichohyalin granules, and filaggrin (49, 53, 54, 102). Moreover, *Atg7*-deficient mice have more corneocytes (53). *Atg7*-deficiency leads to cellular aging and accumulation of p53 and p21 upon treatment with N,N′-dimethyl-4,4′-bipyridinium dichloride (paraquat) treatment (54). Keratinocyte growth factor (FGF7/KGF) controls human keratinocyte differentiation and induces autophagy *via* the PI3K–AKT–mTOR pathway (99, 103). Hence, *Atg7*-deficient mice demonstrate the importance of autophagy in epidermal keratinization and hair growth (53, 99, 102). Autophagy inhibition either *via* 3-MA treatment or *Atg5*-deficiency also impairs keratinocytes differentiation (49, 55).

Immune Components

- *Neutrophils* are a most abundant granulocytes and are considered the foot soldiers of the immune system (68). Autophagy-deficient neutrophils show impaired degranulation, NADPH-oxidase-mediated reactive oxygen species (ROS) production, and inflammatory responses (68). Neutrophils from the skin of leprosy patients release TNF-α, show increased autophagy and exhibit accelerated apoptosis *in vitro* (69).
- *Macrophages* are skin resident phagocytes surveilling the epidermis and dermis (2). They help in maintaining the immunotolerant environment of the skin (2, 92). Specialized macrophages in the skin called melanophages can engulf melanocyte fragments and melanin. These melanocyte fragments and melanin are processed by autophagy (70–72). In leprosy patients, skin-derived macrophages have significant upregulation of autophagy genes, including *Beclin1* and *Atg14* (73).
- *Epidermal DCs, also known as Langerhans cells (LCs)* are dendritic cells of the skin and are found among the keratinocytes in the epidermis (2, 104). The LCs surveil the epidermis and promote tolerance to environmental stressors (104–106). Once activated, LCs migrate to the draining lymph nodes and aid in T cell polarization *via* antigen presentation to define the adaptive immune environment (106). Cytosolic and endocytosed antigens are processing and presentation (or cross presentation) by DCs to either $CD4^+$ or $CD8^+$ T cells *via* MHC class II and MHC class I molecules, respectively, and this process requires the autophagy machinery (74). Macroautophagy (canonical autophagy) has been implicated in the intracellular antigen loading on MHC class II molecule, allows the autophagosomal membrane to fuse with the MHC class II-loading compartment and thus, allows efficient MHC class II presentation (75). Mintern et al. showed that $CD8^+$ splenic DCs from *ATG7-deficient* mice have impaired cross presentation of antigen *via* MHC class I pathway but can efficiently load antigen on MHC class II molecule (74). Thus, suggesting a role of autophagy in antigen presentation and cross presentation by DCs.

Both LCs and *dDCs* can induce interferon (IFN) and pro-inflammatory responses (107). Type I IFN can induce autophagy during viral infection, autoimmune disorders, and certain cancers (108, 109). ATG, *Map1lc3b* and *Atg*3 have been shown to be important in the production of hapten 2,4-dinitrofluorobenzene-induced (DNFB) ROS in skin DCs (76).

- *Mast cells* are granulocytes crucial for allergic inflammatory reactions (77). In the skin, they are triggered by UV or ROS-inducing irritants and undergo degranulation (77, 78). In addition, the degranulation process requires ATG7 (77).
- *NK cells* are cytotoxic innate immune lymphocytes present in the dermis. A recent study showed that targeting autophagy by inhibiting Beclin1 can increase NK cells resulting in melanoma tumor (79). Beclin1 inhibition reduces degradation of NK-derived Granzyme B as a result of which melanoma cells can thrive even in the presence of NK cells driven by CCL5 (79).
- *ILCs* are a new addition to the family of innate immune cells, and they are involved in shaping the immune system (110, 111). There are three types of ILCs defined by their cytokine profile: ILC1 produces IFN-γ, ILC2 produce IL-5 and IL-13, and ILC3 produces IL-17A and IL-22 (110). In human and murine skins, ILC2 are the most prelevant. The role of ILCs in skin autophagy is not well reported.
- Human skin contains approximately 20 billion skin *T cells or T lymphocytes* (112). *T cells* define the adaptive immune responses and are differentiated from other lymphocytes by the presence of surface T cell receptor (TCR) (112). TCR engagement activates autophagy, induces ROS, and causes activation of nuclear factor of activated T-cells (NFATC1) and lysosome-associated membrane protein 2 (LAMP-2) (113). Autophagy can alter the T cell repertoire through selection and aid in cross presentation by DCs (113–116).
  - ✓ *Alpha beta (αβ) T cells* ($CD8^+$ T cells and $CD4^+$ T cells) survive and persist in the skin long after the immune reac-

tion is over (113, 117–123). This ensures rapid protection from future exposures to pathogens or antigens (112, 124). Skin resident-memory T_{reg} cells mitigate inflammation and regulate immune response *via* autophagy (81, 82). T_{regs} (20–60% of $CD4^+$ T cells) have also been shown to alleviate autoimmune disorder by suppressing DC autophagy (81, 83). *Atg7*-deficient T_{regs} cannot establish skin homeostasis (84, 85).

- ✓ *Gamma delta (γd) T cells* comprise 1–5% of the circulating T cells found in both mice and humans (125). However, in skin about 50% of γd T cells are present among the T cells (126). They populate the skin epidermis and dermis and are named dendritic epidermal γd T cells (DETCs) and dermal γd T cells, respectively (125, 127). Like αβ T cells, they possess effector functions as well as mediate tissue repair (86, 125). γd T cells are the first responders to skin damage and are responsible for maintaining homeostasis, wound repair, and production of cytokines like insulin-like growth factor 1, TNF-α, and KGF-1 (86). DETCs are in close contact with keratinocytes and aid in wound healing by releasing KGF-7 and KGF-10 (128, 129). Skin γd T cells are adaptable under immunosuppression by rapamycin (inhibits mTOR pathway) or roseotoxin B (suppresses activated T cells but does not effect naïve T cells), and they undergo autophagy to survive in the absence of cytokines (70, 86).

- *B cells* are a component of the adaptive immune system populating the dermal layers and produces antibodies. In B cells, ATG5 seems to play an important role in differentiation and survival (80). Autoantibodies induce autophagy and can sometimes lead to autoimmune disorders (80).

SKIN EXPOSURES AND AUTOPHAGY

When the skin encounters pathogens, injury, or UVR, it deploys various defense mechanisms. Autophagy responds to these unwanted encounters to ensure inflammation resolution. Defects in autophagy can cause a hyperinflammatory skin reaction due to inflammasome activation (as seen in human keratinocytes), unpredicted ROS activation *via* UVR, and aberrant pro-inflammatory cytokine release (**Figure 1A**) (4, 113, 130–133).

Pathogens

Evolutionarily, epidermal pathogenic bacteria have devised multiple evasion mechanisms to avoid autophagic clearance (134, 135). Numerous instances of the autophagy machinery engaging skin pathogens during cutaneous infection have been described (**Table 2**). Autophagy is critical for the clearance of group A *streptococci* (GAS) which evades endosomal capture (13, 136). A recent study showed that the elimination of GAS is severely reduced in *Atg5-deficient* cells elucidating its vital role in pathogen clearance (13, 136). *Streptococcus pyogenes* is responsible for causing impetigo, a common skin infection in children (137, 138). Similarly, autophagy is essential in preventing infectious skin diseases such as *Staphylococcus epidermidis* infection, leprosy, and sepsis (**Table 1**).

The skin is susceptible to fungal infections, such as cutaneous candidiasis and *Candida* intertrigo caused by *Candida albicans* (168). Studies have shown that the intracellular clearance of *C. albicans* depends on both autophagy (ATG5) and LAP (168, 177). Autophagy in skin also plays a unique role in anti-viral function (**Table 2**). Herpes simplex virus (HSV) is a double-stranded DNA virus. It is categorized into HSV-1 and HSV-2, which causes oral herpes and genital herpes (13). Autophagy aids in processing and presentation of HSV-1 antigens on MHC class I molecule for effective viral elimination (178–180). HSV-2 is more susceptible to the host ATG5 (13, 179, 181). Furthermore, skin-related viruses, including human papillomavirus, varicella zoster virus, and Zika virus can induce autophagy to degrade viral capsid proteins in the skin cells and keratinocytes (172, 182).

Wound Healing

During wound healing after an injury or pathogen invasion, skin immune responses halt the ongoing inflammation to initiate the restoration process (2). In rats, autophagy heals burnt hair follicle epithelium (183). A recent study shows that the use of mesenchymal stem cells (MSCs) in skin repair requires autophagy (184). Rapamycin-induced autophagy in MSCs causes secretion of vascular endothelial growth factor (VEGF) and improves VEGF-mediated blood circulation which in turn promotes skin wound healing and tissue regeneration (184).

Ultraviolet Radiation

Sun-generated UVR is a mixture of UVA and UVB (185). Upon exposure to UVR, basal autophagy increases in keratinocytes (28) and causes epidermal thickening or hyperkeratosis (28). This in turn, leads to epidermal hyperplasia which prevents the UVR (UVA or UVB, depending on the wavelength) to penetrate the skin (28, 186). UVR-induced cell death in the skin can promote autoimmunity due to defective clearance of apoptotic keratinocytes (5, 108). Patients with systemic lupus erythematosus, an autophagy-related autoimmune disorder, are unable to clear dead cells and suffer from severe cutaneous lesions upon exposure to UVR as a result (187–191). UVR also inhibits antigen presentation by LCs and T_{reg} migration, as seen in cutaneous T cell-mediated dermatitis (70, 192–196).

UVA (long wavelength) irradiation penetrates the dermis, and its exposure induces autophagy to remove p62-associated protein aggregates in keratinocytes and melanocytes (5, 61, 197, 198). Chronic UVA exposure causes apoptosis of epidermal and dermal cells, photoaging, and skin pathogenesis, depending upon the presence of melanin (28, 185). Luteolin (3, 4, 5, 7-tetrahydroxyflavone), a flavonoid showing anti-cancer properties, has recently been shown to decrease UVA-induced autophagy in human skin fibroblasts by scavenging ROS (95, 191).

UVB irradiation entry is limited to the epidermis (198–200). UVB-induced autophagy involves glycogen synthase kinase signaling, which helps protect the epidermal cells from UVB-induced apoptosis (201). UVB-induced ROS downregulates mTOR in skin epidermal cells and induces autophagy (28, 187, 202). Thus, ROS inhibition prevents T cell-mediated dermatitis in mice (203). UVB-treated murine splenocytes show systemic

TABLE 2 | Showing the different autophagy markers involved in skin-related diseases.

Disease	Autophagy markers and associated skin cells	Reference
Autoimmune disorders and cancer		
Psoriasis	↓AP1S3 (keratinocytes), ↓ATG16L1 (keratinocytes)	(13, 139–141)
Systemic lupus erythematosus pathogenesis	↓ATG5 (human keratinocytes), ↓UVRAG (human keratinocytes)	(13)
Vitiligo	↓UVRAG (melanocytes), ↓Nrf2 (melanocytes)	(142–144)
	↑p62 accumulation (melanocytes), ↑unfolded protein response, ↑autophagy induction (melanocytes), Atg7↓(melanocytes)	
Diabetic skin disease/ulcer	↑Autophagy *via* AGEs (murine M1 macrophages)	(145–148)
Allergic contact dermatitis	↑Autophagy (murine skin)	(70)
Chanarin–Dorfman syndrome	↓Mitophagy (PINK1), ↓autophagy (murine keratinocytes and human skin)	(51, 101)
Merkell cell carcinoma (MCC)	↑mTOR, ↑p62 accumulation (human MCC cell lines)	(149–151)
Melanoma/malignant melanoma	↓*Beclin1*, ↓*Atg5*, and ↓*Atg7* [human melanoma cell lines (WM35, WM793, 451LU, A2058, and A375), SK-Mel-cell lines, and melanocytes]	(13, 152–161)
	miR-638 blockade—↑autophagy and ↑metastasis	
	Hydroxychloroquine-mediated autophagy inhibition therapy in clinical trials	
	↑miR-23a-ATG12 axis results in ↓ melanoma metastasis	
Infectious diseases		
Staphylococcus epidermidis (*S. aureus*)	Autophagosomal degradation diminishes bacterial accessory gene regulator activity (Hela cells and murine bone marrow-derived DCs)	(162)
Impetigo (*group A streptococci or Streptococcus pyogenes*)	↓ATG5, streptococcal cysteine protease (SpeB)-mediated ↓ubiquitin-LC3 adaptor proteins (HEp-2 epithelial cells)	(163)
Sepsis (MRSA)	↑Keratinocyte autophagy, ↓inflammasome	(164–167)
	↑ATG16L1 [human keratinocytes, murine ear skin, HAP1 fibroblast cells, ATG16L1 hypomorphic mice (*ATG16l1*^{HM})]	
Leprosy (*Mycobacterium leprae)*	↑BECN1, ↑ATG14, ↑LC3	(73)
	SQSTM1/p62, ↑NBR1, ↑autophagy by interferon-γ [skin macrophages (dead *M. leprae* induces autophagy and live *M. leprae* reduces it)]	
Cutaneous candidiasis/*Candida* intertrigo (invasive form) (*C. albicans*)	↓ATG5 (murine macrophages), ↓LC3-associated phagocytosis (murine macrophages)	(25, 168)
Warts [human papillomavirus (HPV)]	↑ATG5 (HPV16 infected primary human keratinocytes), ↑ATG7 (HPV16 infected primary human keratinocytes)	(99)
	Autophagosomal degradation of viral capsid proteins (HPV16 infected primary human keratinocytes)	
Oral and genital herpes simplex [herpes simplex virus (HSV)-1 and HSV-2]	↑Autophagy, ↑viral antigen processing and presenting (murine macrophages and dendritic cells), ↓ATG5 (HSV-2 infection in murine fibroblast, human foreskin fibroblasts), ↓Beclin1 and ↓autophagy (by HSV-1-encoded neurovirulence protein, ICP34.5)	(13, 169–171)
Chicken pox (VZV)	↑Autophagy induction (human keratinocytes) to protect	(172–174)
Zika virus infection (ZIKV)	↓Akt-mTOR signaling, ↑autophagy, ↑ATG16L controls ZIKV infection (fibroblasts, keratinocytes and skin DCs, pregnant *ATG16l1*^{HM} mice)	(175, 176)

immunosuprression by inhibiting both IFN-γ and IL-10 cytokine production 24 h post-irradiation (204).

UVB radiation also stimulates AMPs (psoriasin, RNase 7, human β-defensin [HBD 1–4]) in human keratinocytes (7, 8, 205). To protect against UVB-induced DNA damage, oxidative stress, cancer, and skin cells produce fat soluble vitamin D (206). Epidermal AMPs like cathelicidin (hBD18) expressed in keratinocytes is induced by 1,25 $(OH)_2$ vitamin D3 from 7-dehydrocholesterol and protects the skin against pathogens (8, 206, 207). Vitamin D promotes autophagy (ATG16L1 in autoimmune disorders like inflammatory bowel disorder) and suppresses pro-inflammatory pathways (such as p38 MAPK-mediated signaling pathway, prostaglandin pathway, nuclear factor kappa B signaling pathway) (206, 208, 209).

UV stimulates the central stress response center *via* hypothalamic–pituitary–adrenal axis, however, the mechanism is not well understood (204). UVR-induced β-endorphin and corticotropin-releasing hormone release from the skin causes soluble neuro-endocrine-immune factors to seep into the systemic circulation (204, 210). The skin immune cells stimulated by UV act as "second messengers" allowing crosstalk between neuroendocrine system and immune system (210). Certain neuropeptides, such as calcitonin gene-related peptide (CGRP), mediate anti-inflammatory environment by UVR (211). Steriods like proopiomelanocortin produced from α-melanocyte-stimulating hormone (α-MSH) of UVR-triggered epidermal, dermal cells and macrophages induce immunosuppression (210, 211).

SKIN CANCER AND AUTOIMMUNE DISORDERS

Autoimmune disorders are the result of the adaptive immune system generating autoreactive lymphocytes (T and B cells) that target self-antigens. Skin disorders often arise as a secondary complication, as reported in diabetes, cancer, and dermatitis. The role of autophagy reported in skin-related disorders (**Table 1**) is limited, yet nonetheless deserves recognition.

Atg5-deficiency, *Atg7*-deficiency, and *Beclin-1* partial deletion can spur spontaneous tumor growth commonly seen in most cancers (152, 212–214). Contrary to that, targeting Beclin-1 inhibits autophagy, overexpresses CCL5 and aids in recruit NK cells to the melanoma tumor (79). Melanoma is fatal in its aggressive form and is highly metastatic (153). *Atg5*-deficient melanoma cells *in vitro* have diminished survival (152, 154). Interestingly, in advanced stages, melanoma cells promote a tumor-suppressive environment by hijacking the autophagy machinery to ease stress induced by drugs (215). This suggests that cancer cells can manipulate the autophagy machinery to resist treatment (154, 216).

Diabetes mellitus (both type 1 and type 2) patients have defective insulin signaling in the keratinocytes and often suffer from skin lesions and foot ulcers (145–147, 217). In diabetic patients, IRF8 (an autophagy regulator) activation induces autophagy, poises the macrophages to permit inflammation and thus, impairs cutaneous wound healing (145, 217). Chronic hyperglycemia and hyperlipidemia disrupt ER homeostasis and resulted in increased unfolded protein burden (147, 217). Both autophagy and mitophagy are defective in diabetic patients and inhibit keratinocyte proliferation and migration that are requisite for wound healing (145–147).

Psoriasis is a chronic, polygenic autoimmune disease characterized by epidermal hyperplasia, defective keratinization, and infiltration of immune cells within the skin, causing dermatitis and thickened plaques formation. Genetic screening of psoriasis patients of Estonian origin reveals several single-nucleotide polymorphisms (SNPs) associated with *ATG16L1*, though the functional role of ATG16L1 variants in skin biology is unclear (139, 218). In pustular psoriasis cases, mutations in *AP1S3*, a gene encoding an autophagosome trafficking protein, result in the disruption of autophagy in keratinocytes and drive them to produce pro-inflammatory cytokines, including IL-1β, IL-8, and IL-36A (140). Furthermore, a recent study demonstrated that autophagy inhibition *in vivo* shows aberrations in keratinocyte differentiation, resulting in dysregulation of autophagy in psoriatic epidermis (219).

Vitiligo is a pigmentation disorder characterized by sharp demarcated white macules on skin due to the $CD8^+$ T cell-mediated destruction of melanocytes (220, 221). This results in localized (segmental vitiligo) and/or generalized (non-segmental vitiligo) partial loss of melanin. In addition, melanocytes show increased autophagy due to misfolding of *tyrosinase (tyr)* and *X-box binding protein 1 (Xbp1)* in the ER (220, 221). Individuals suffering from vitiligo may have defective epidermal permeability barrier functions which can be alleviated by the use of topical histamine treatment (222, 223). Vitiligo patients from Chinese Han population show abnormal antioxidant *Nrf2* [nuclear factor (erythroid-derived 2)-like 2] gene expressions in autophagy and associated pathways (142, 224, 225). Compared to normal melanocytes, vitiligo melanocytes *in vitro* show greater similarity with *Atg7-deficient* melanocytes (57, 60). Both exhibit impaired redox-sensitive Nrf2 activation and decreased activation of the antioxidant enzyme system in response to oxidative challenges induced by environmental stress and ROS (93, 226, 227). Multiple evidences indicate that autophagy controls melanosome degradation (92, 228). Furthermore, a Korean cohort study demonstrated an association between two *UVRAG* gene SNPs and non-segmental vitiligo (vitiligo vulgaris) (229). Skin autoimmune disorders are overly complicated, involve many aspects of the immune system and arise due to defects in autophagy machinery. Accumulating evidence displaying the importance of autophagy in skin disorders demonstrates the need for further research.

CONCLUDING REMARKS

Being at the forefront of the environmental defense system, a plethora of specialized cells reinforces the skin. It is a unique surface that is highly specialized in preserving immunotolerance. Therefore, autophagic clearance of senescent and damaged cells is necessary for the maintenance of specialized cellular machinery to effectively keep inflammatory triggers at bay. Several research groups have implicated the role of ATG5, ATG7, and ATG16L1 in keratinocytes, melanocytes, and immune cells in autoimmunity and cancer. Skin in the context of autophagy remains an uncharted territory and warrants further investigation. Autophagy dictates the immune response and resolution in skin cells to neutralize pathogens, clear senescent cells, or heal wounds. However, the autophagy machinery can either protect or cause autoimmune disorders. The physiological circumstances that govern this "balancing act" are not well understood. The cytokine microenvironment from dying immune cells, pathogens, or senescent cells can potentially direct the autophagic response in skin. In addition, there are evidences suggesting potential communication between autophagic machinery and skin cells (both immune and non-immune cells) in an inflammatory state that resonates in the literature; however, the mechanism is not yet reported (230). Adequate tools to examine the molecular response are lacking.

In addition, skin microbiota has been shown to shape the immune response by producing AMPs, complement, IL-1, and IL-17 as well as modulating the local T cell response (3, 231). The interaction with skin cells and microbiota may reveal underlying mechanisms to aid in conscientious operation of the autophagic machinery as seen in Crohn's disease and colorectal cancer (232–234). Overall, further research investigating the role of autophagy in barrier function will pave the way forward for therapeutic advancements in the field of skin inflammaging and dermatology.

AUTHOR CONTRIBUTIONS

PS and JM outlined the structure and theme of the paper. PS and S-WW performed the systemic literature search. S-WW helped with the disease section and table 2 of the manuscript.

PS developed and wrote the final version manuscript. PS also performed the revisions suggested by the reviewers. JM provided valuable guidance while formulating the manuscript and supervised the work.

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REVIEW
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Autophagy and Rheumatoid Arthritis: Current Knowledges and Future Perspectives

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Autophagy is a degradation mechanism by which cells recycle cytoplasmic components to generate energy. By influencing lymphocyte development, survival, and proliferation, autophagy regulates the immune responses against self and non-self antigens. Deregulation of autophagic pathway has recently been implicated in the pathogenesis of several autoimmune diseases, including rheumatoid arthritis (RA). Indeed, autophagy seems to be involved in the generation of citrullinated peptides, and also in apoptosis resistance in RA. In this review, we summarize the current knowledge on the role of autophagy in RA and discuss the possibility of a clinical application of autophagy modulation in this disease.

Keywords: autophagy, rheumatoid arthritis, autoimmunity, apoptosis, citrullination

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INTRODUCTION: OVERVIEW ON PHYSIOLOGICAL FUNCTIONS AND MOLECULAR MECHANISM OF AUTOPHAGY

Autophagy is a degradation pathway characterized by the isolation of targeted cytoplasmic material in a typical double-membrane vesicle, known as autophagic vacuole or autophagosome (1). The subsequent fusion of the autophagosome with the lysosome ensures the correct destruction of organelles, misfolded proteins, and microorganisms, carried inside the vesicle. Despite its emerging role in human pathology, autophagy is a physiological process involved in basal organelles turnover and in the removal of proteins aggregates (2, 3). In response to the condition of cellular stress, such as growth factors and nutrients deprivation, intracellular components degraded by autophagy are recycled in order to generate ATP and sustain essential cell functions (4). Autophagy is considered a pro-survival mechanism, allowing cells to respond to injury by degrading unnecessary and dysfunctional self-components; however, this ability may become a double-edged sword (5). Three types of autophagy can be distinguished: macroautophagy, microautophagy, and chaperone-mediated autophagy. In this review, we will focus on macroautophagy (hereafter referred to as autophagy), which is the most characterized type of autophagy. Considering the crucial role of autophagy in

Abbreviations: Abs, antibodies; AMBRA1, Beclin-1 regulated autophagy protein 1; anti-CCP, anti-cyclic citrullinated peptide; APC, antigen-presenting cells; Atg, autophagy-related genes; BMSCs, bone marrow mesenchymal stem cells; CIA, collagen-induced arthritis; ER, endoplasmic reticulum; FLS, fibroblast-like synoviocytes; HCQ, hydroxychloroquine; LC3, microtubule-associated protein 1 light-chain 3; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; MTX, methotrexate; OA, osteoarthritis; PAD, peptidylarginine deiminase; PCs, plasma cells; PE, phosphatidylethanolamine; PI3K-III, class III phosphatidylinositol 3-kinase; RA, rheumatoid arthritis; RANKL, receptor activator of nuclear factor kB (NF-kB) ligand; ROS, reactive oxygen species; SLE, systemic lupus erythematosus; TECs, thymic epithelial cells.

the maintenance of cellular homeostasis, it is not surprising that several signaling-related molecules are involved in the perfect functioning of this process. Genetic screens in yeasts allowed the discovery of at least 37 autophagy-related genes (Atg) (6). Many of these genes, encoding proteins involved in autophagy and its regulation, are evolutionarily conserved in humans (7). The mammalian target of rapamycin (mTOR) complex 1 (mTORC1) regulates the activation of autophagy machinery, acting as a sensor of energy levels and integrating upstream signals deriving from other pathways, including the phosphoinositide 3-kinase (PI3K)-Akt. As displayed in **Figure 1**, in the presence of aminoacids and growth factors, mTORC1 represses autophagy by inhibition of Vps34 and ULK1 complexes. On the contrary, in low nutrients state, defined as starvation, the dissociation of mTORC1 from the induction complex triggers autophagy (8, 9). The autophagosome derives from a double-membrane pre-autophagosome structure called phagophore, which seems to originate from different sources, including plasma membrane (10), endoplasmic reticulum (ER) (11), and Golgi complex, in mammalian cells (12). Phagophore nucleation requires the activity of class III phosphatidylinositol 3-kinase (PI3K-III) complex containing Beclin-1 (a mammalian homolog of yeast Atg6), hVps34, p150 (a mammalian homolog of yeast Vps15), and Atg14-like protein (Atg14L) (13). The autophagy

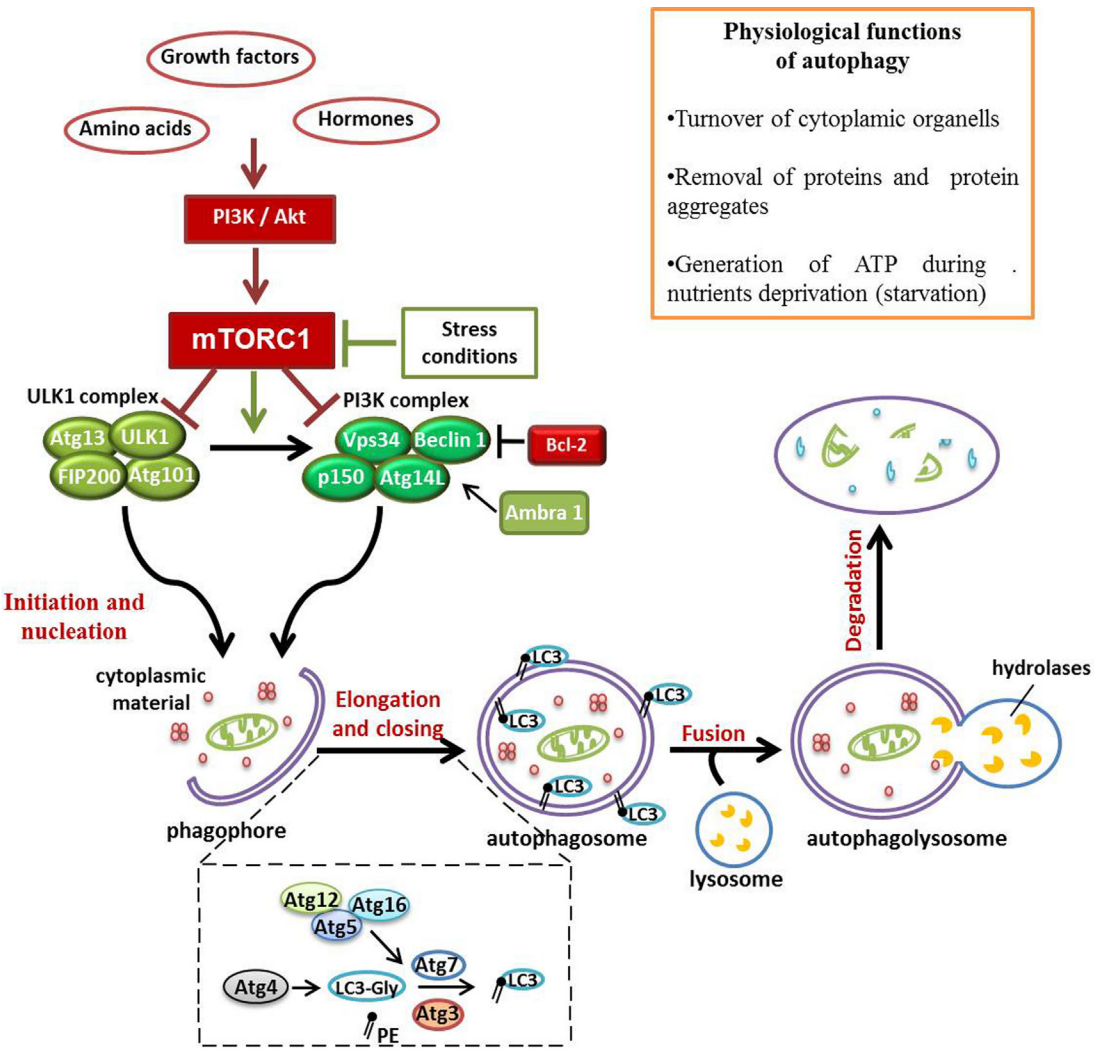

FIGURE 1 | Schematic representation of signaling pathways involved in autophagy multistep regulation. Autophagy represents a fundamental physiological process, considering its role in ATP production during condition of nutrients deprivation and in controlling organelles and proteins turnover. mTOR complex 1 (mTORC1), acting as a sensor of energy levels and integrating upstream signals [phosphoinositide 3-kinase (PI3K) and Akt], is one of the most important autophagy regulators in cells. In presence of growth factors and hormones, mTORC1 inhibits autophagy activation. On the contrary, in autophagy-activated conditions, the repression of mTORC1 activity allows autophagy initiation by ULK1 and PI3K complexes recruited to the just formed phagophore. This pre-autophagosomal structure begins to engulf cytoplasmic materials and, thanks to Atg12–Atg5–Atg16L and LC3–phosphatidylethanolamine conjugated systems, it grows up and closes to generate the autophagosome. The fusion of the autophagosome with lysosome exposes the cargo to the action of lysosomal hydrolases that degrade vesicular content for recycling.

promoting function of Beclin-1 is influenced by the antiapoptotic protein Bcl-2; in fact, when Beclin-1 is bound to Bcl-2, autophagy is inhibited; instead, the dissociation from Bcl-2 allows Beclin-1 to interact with PI3K-III complex, and to activate autophagy (14). On the contrary, Beclin-1 regulated autophagy protein 1 (AMBRA1) is a positive regulator of Beclin-1-dependent autophagy; thanks to its capacity to create a link between cytoskeletal motor proteins and class III PI3K complex (15, 16). Two ubiquitin-like conjugation systems, Atg12–Atg5–Atg16L and microtubule-associated protein 1 light-chain 3 (LC3)–phosphatidylethanolamine (PE), mediate the second step of autophagy, which concerns the expansion and closure of the autophagosome (17). In the first system, the enzymes E1-like Atg7 and E2-like Atg19 promote the covalent association of Atg12 to Atg5 (**Figure 1**). Subsequently, Atg16 binds to the complex to form the heterotrimer Atg16-Atg12-Atg5, this organization at the level of the outer portion of autophagosomal membrane, mediates the curvature of the growing membrane and also participates in the association of LC3 to PE (18). LC3 is cleaved by the cysteine protease Atg4 to produce the cytosolic form LC3-I, which, after being activated by Atg7, is transferred to Atg3 in order to be changed in the conjugate form with PE, named LC3-II (**Figure 1**). LC3-II is thus the most commonly used marker to test autophagic activity, being then the only protein that remains stably associated with the autophagosome in maturation (6). Upon being formed, the autophagosome fuses with the lysosome to generate the autophagolysosome, in which the vesicular content is degraded by lysosomal hydrolases. Finally, products of degradation, such as aminoacids and lipids, are exported from autophagy-related compartments to the cytoplasm to be recycled and generate new macromolecules (19).

EMERGING ROLES OF AUTOPHAGY IN HUMAN PATHOLOGY

Since the first observation of autophagy, more than 50 years ago, there has been a growing interest in studying this mechanism, and deregulated autophagy has been recently connected with the pathogenesis of several diseases. Aging is not properly considered a disease; however, it is associated with different pathological conditions. In the last period of human life, cells undergo several changes, including DNA mutations, damages at several other molecules, and accumulation of protein aggregates. Several studies have demonstrated that autophagy activation protects from aging. In fact, not only autophagy levels decrease with age but also overexpression of Atg proteins contributes to improve life span in a human model of aging *in vitro* and in mouse models *in vivo* (20–22). As already mentioned, one of the most important functions of autophagy is the degradation of misfolded proteins, so in neurons, the failure of autophagy can contribute to neurodegeneration (23). It can occur in Parkinson's Disease, a neurodegenerative disorder characterized by α-synuclein accumulation in the brain. In a study published by our group, we demonstrated that autophagy inhibition by 3-methyladenine (3-MA) and by Atg5 knocking down in lymphocytes lead to a significant increase of α-synuclein levels (24).

Moreover, autophagy seems to be linked also to cancer; however, this relationship is still controversial. The removal of mitochondria, source of reactive oxygen species (ROS), performed by autophagy, certainly protects cells from DNA mutations and prevents cellular transformation. It has also been demonstrated that deletion of the autophagic gene Beclin-1 may cause development of various malignancies in mouse models (25). Autophagy is also involved in the degradation of intracellular pathogens, which represent a source of proteins involved in tumorigenesis (26). Despite its role in the maintenance of genomic stability, many studies indicate a tumor-supporting function of autophagy, allowing tumor cells to respond to stress stimuli, such as nutrients deficiency and hypoxia, thus extending their lifespan. Furthermore, it has been demonstrated that autophagy-deficient tumors are more sensitive to several chemotherapeutic agents (27, 28). In this case, autophagy promotes the survival of cancer cells and protects them from the action of drugs that induce apoptosis. Although research in this field is just at the beginning, an encouraging number of works suggest that defects in the autophagy mechanism may be involved in the pathogenesis of autoimmune diseases (29). In our previous works, we focused on systemic lupus erythematosus (SLE), showing that factors present in the serum of SLE patients, probably antibodies, are able to induce autophagy in T lymphocytes from healthy donors, but not in T lymphocytes from patients with SLE. We speculated that chronic exposure to specific autoantibodies, as occurs in SLE, could lead to the selection of autophagy-resistant T lymphocytes (30). Some of these autoantibodies could be directed to D4GDI, a regulator of Rho proteins activation. More recently, we also identified IgGs directed to D4GDI in sera from patients with SLE (31). On the contrary, very little is known on the role of autophagy in the pathogenesis of rheumatoid arthritis (RA) and other autoimmune rheumatic diseases, thus making research on autophagy in autoimmune conditions a very intriguing field.

AUTOPHAGY IN AUTOIMMUNITY AFFAIR: ROLE OF AUTOPHAGY IN RA

Rheumatoid arthritis is a chronic autoimmune disease affecting not only the joints but also other organs including heart, vascular system, lungs, and skin. Environmental and genetic factors both lead to immune cells activation against self-antigens and production of autoantibodies, such as anticyclic citrullinated peptide (anti-CCP) antibodies (Abs), pathognomonic markers of this disease (32, 33). Several immune cells are involved: T and B cells, macrophages, synovial fibroblasts, chondrocytes, and osteoclasts, which lead to the release of different inflammatory mediators, sustaining the chronic inflammatory response of the disease (34).

Autophagy in Immunological Tolerance

Many studies demonstrated autophagy's contribution to the presentation of cytosolic antigens in association with MHC class II molecules, playing an important role not only in the acquired immune response but also in the maintenance of self-tolerance (35). Mechanisms of central (in the primary lymphoid organs)

and peripheral tolerance (in peripheral tissues) physiologically prevent immune responses to self-antigens (36). During T cells development in the thymus, the recognition of peptide–MHC molecules on the surface of thymic epithelial cells (TECs) ensures that only thymocytes restricted to MHC molecules, and specific for non-self (foreign) antigens, will survive and continue their maturation.

Emerging evidence indicates that autophagy contributes to the maintenance of the central tolerance mechanism (37). Mizushima and colleagues (38) found high autophagy levels in TECs, suggesting a possible involvement of autophagy in the formation of the lymphocytes repertoire during thymic selection. According to this hypothesis, it was recently revealed that there had been an alteration in the selection of the T cell receptor (TCR) restricted to MHC class II in mice transplanted with Atg5$^{-/-}$ thymus. Autophagy defects, in association with a consequent loss of self-tolerance, could be the reason of multiple signs of autoimmunity reported in these animals (39). However, Sukseree and colleagues demonstrated that autophagy suppression did not affect the selection of lymphocytes repertoire in TECs (40). These two opposite results probably depend on the different approach used to inhibit autophagy in the thymus, thus further investigations are necessary.

The involvement of autophagy in the presentation of self-antigens to immature T cells in the thymus was first analyzed by Kasai and colleagues, who showed a colocalization of LC3-II with the lysosomal compartment in which MHC–peptide complexes are formed (35). More recently, Aichinger et al. demonstrated that autophagy is essential for endogenous antigen-loading onto MHC class II of TECs for negative selection (41).

Autophagy in Joint Destruction

Although for many years the role of Th1 cells has been considered predominant in RA, recently, a crucial role of Th17 cells is also emerging (42). This cell subset is a primary source of the pro-inflammatory cytokine IL-17, which acts in synergy with TNF-α and IL-1, contributing to the bone destruction. In this context, receptor activator of nuclear factor kB (NF-kB) ligand (RANKL) produced by activated T and B cells and fibroblasts, by binding to its receptor RANK, expressed in monocyte–macrophage lineage, stimulates the differentiation of osteoclast precursor cells in mature osteoclasts (43). Most recent findings suggest a possible involvement of autophagy in osteoclastogenesis. Specifically, hypoxia, which is an autophagy-activating stimulus, seems to be able to stimulate maturation of osteoclasts (44); moreover, it has been demonstrated that the inhibition of autophagy blocked osteoclastogenesis in mouse monocyte/macrophage cell lines (45). In another work, it has been shown that treatment with RANKL caused up-regulation of autophagy markers and the knockdown of autophagy substrate p62 decreased the expression of genes involved in the osteoclastogenesis process (46). In experimental arthritis mouse models, the inhibition of autophagy reduced signs of bone erosion and the number of osteoclasts, suggesting a key role of autophagy in bone tissue degradation (47). In this regard, drugs that down-regulate autophagy may be used to prevent bone resorption in RA patients.

Autophagy as a Protective Mechanism Against Apoptosis

Autophagy acts promoting cell survival under conditions of nutrients deficiency, while apoptosis is a fundamental programmed cell death mechanism, thus the relationship between these two processes influences cell fate. Moreover, through the elimination of damaged mitochondria, autophagy also participates in the reduction of ROS and damaged DNA, thus preventing the development of apoptosis (48). In several studies in which autophagy was suppressed by knocking down autophagy genes, cell death was not inhibited, but increased, indicating the prominent role of autophagy as a cell survival mechanism (49).

Different molecules are common to both cellular mechanisms. As already discussed, family members of Bcl-2, well known as apoptosis regulators, are able to modulate also autophagy by inhibition of Beclin-1 (14). It has been demonstrated that caspase-dependent cleavage of Beclin-1 and its subsequent localization to mitochondria promotes the release of proapoptotic factors from these organelles (50). The balance between cell survival and cell death is essential in regulating immune cells destiny and it seems to have a crucial role in RA pathogenesis and progression. One of the most important apoptosis functions consists in the extinguishing of inflammation, by blocking an excessive immune cells activation and cytokines production. In this regard, a reduction of apoptosis rate and apoptotic mediators was found at the synovial level, indicating a downregulation of apoptosis in RA (51, 52). In fact, RA synovial fibroblasts are subjected to a complex pattern of molecular changes, including alterations in the expression of signaling pathways that lead to an aggressive and invasive phenotype (53). The progressive bone and cartilage destruction is attributable to resistance of synovial fibroblasts to apoptosis induction, and several intracellular processes, including autophagy, could take part in this phenomenon (54). As already discussed, there is a controversial crosstalk between autophagy and apoptosis; autophagy induction could be a potential mechanism by which RA cells protect themselves from apoptosis, increasing thus their lifespan. In support of this hypothesis, ER stress caused higher autophagy activation in synovial fibroblasts obtained from patients with RA than in those from osteoarthritis (OA) patients, and RA-fibroblast-like synoviocytes (FLS) appeared to be more resistant to cell death induction (55). Moreover, an inverse correlation between autophagy and apoptosis in synovial tissues from RA patients was found, indicating an involvement of autophagy in the apoptosis-resistant phenotype of RA synoviocytes (56, 57). Recently, immune-histochemical and molecular analysis of autophagy-related molecules on synovial biopsies showed increased levels of Beclin1, Atg5, and LC3-II in RA compared to OA patients (58). It is important to note that TNF-α is not only a potent modulator of inflammatory response in RA but also an apoptosis-activator molecule, inducing autophagy in different cell types including skeletal muscle, atherosclerotic vascular smooth cells, and also RA synoviocytes (59, 60). Connor and co-authors studied the effect of TNF-α on protein degradation, demonstrating that in RA synovial fibroblasts, TNF-α stimulates the conversion of LC3-I to LC3-II but not the activation of proteasome complex (61). A research work

by Xu and colleagues revealed a connection between autophagy hyperactivation and methotrexate (MTX) resistance, by showing that RA-FLS undergo higher levels of MTX-induced apoptosis when autophagy is inhibited (62). More recently, in a collagen-induced arthritis (CIA) rat model, it has been demonstrated that inhibition of autophagy alleviated synovial inflammation and promoted synovial cell apoptosis through the regulation of PI3K/AKT pathway (63). These data confer to autophagy an important protective role against apoptosis; for these reasons, therapy based on autophagy repression might have a beneficial effect in RA.

Autophagy in Lymphocytes Homeostasis

Peripheral immune cells play an important role in the perpetuation of autoimmunity by sustaining systemic inflammation status and by participating in the extension of joint destruction mechanisms. Many studies demonstrated that autophagy allows T and B lymphocytes to survive in conditions of nutrients deprivation or during stress stimuli (64). Mice lacking Atg5 do not survive and have a reduction of peripheral T cells, showing how autophagy is essential for their survival (65). Since cytoplasmic calcium levels are essential for TCR-signaling pathways activation, autophagy-dependent calcium flux regulation could influence T lymphocytes activation. It has been demonstrated that $CD4^+$ and $CD8^+$ $Atg5^{-/-}$ cells are not able to properly proliferate following TCR stimulation (65). Moreover, the inhibition of autophagy causes defects in T cell activation. In fact, deletion of Atg7 results in decreased in IL-2 mRNA level and ATP generation, suggesting that autophagy is required to ensure appropriate energy level for T cell activation (66). Similar data were obtained also on B lymphocytes, demonstrating that autophagy is essential for the maturation process and for the subsequent maintenance of B lymphocytes repertoire in the periphery (67, 68).

Systemic autoimmune diseases such as RA are characterized by secretion of pathogenic autoantibodies by plasma cells (PCs), and an increase of this phenomenon seems to be associated with autophagy defects (69). First of all, autophagy seems to be involved in "PC differentiation program" since it has been found to be activated during this process (70). The absence of the autophagic gene Atg5 does not alter B lymphocytes differentiation, but these cells secrete a larger amount of Abs compared to the wild-type counterpart (71). On the other hand, the suppression of autophagy makes PCs more susceptible to cell death, stopping in this way the persistent Abs secretion. Conway and colleagues obtained similar results in the same mouse model, underlying a crucial role of autophagy in PCs homeostasis (72).

Studies on the role of autophagy in lymphocytes isolated from RA patients are scarce and yet contradictory. Yang and colleagues explored the metabolic activity of RA T cells, showing a defect of autophagy in these cells related to a deficiency of PFKFB3, a regulatory glycolytic enzyme (73). Opposite data were recently published by van Loosdregt and co-authors. They demonstrated that $CD4^+$ T cells from RA patients treated with hydroxychloroquine (HCQ) showed increased levels of LC3-II and autophagosomes number compared with cells isolated from healthy donors (74). Autophagy hyperactivation was found in CIA mouse model both in $CD4^+$ T cells and at inflammatory sites. Moreover, a reduction of arthritis signs was noticed after the animals were injected with the autophagy inhibitor HCQ (74).

In conclusion, autophagy could maintain autoreactive T and B cells populations sustaining RA chronic inflammatory response, but more experimental evidences are needed to confirm this hypothesis.

Autophagy and Citrullination

Citrullination, chemical conversion of arginine in citrulline by the action of peptidylarginine deiminase (PAD) enzymes (75), has a crucial role in RA pathogenesis and the presence of autoantibodies directed against citrullinated peptides is often associated with a poor prognosis. Anti-CCP Abs target certain epitopes of citrullinated autoantigens and have a crucial role in RA development due to their pathogenetic potential (76). In fact, anti-CCP Abs purified from RA patient is capable of causing not only *in vitro* differentiation of human osteoclasts but also bone loss, when they are injected into mice (77). The contribution of autophagy to the presentation of citrullinated peptides and to the generation of anti-CCP Abs seems to be relevant in RA. Ireland and colleagues showed that antigen-presenting cells (APCs) need autophagy to successfully perform citrullinated proteins presentation, but not the unmodified antigens presentation, and this process is stopped following autophagy inhibition (78). Moreover, since PAD enzyme was found to be expressed in autophagy compartment, and classical pro-autophagic stimuli, such as nutrients deprivation, promoted the presentation of citrullinated peptides in B cells, the authors thought that citrullination could represent a "biochemical marker of autophagy" (79). In a more recent study, in FLS from RA patients, the levels of some citrullinated protein, such as vimentin and α-enolase, increased after treatment with the autophagy inducer rapamycin (80). Furthermore, for the first time, a direct correlation between LC3-II levels and anti-CCP titers was found in monocytes from early active RA patients. These experimental evidences highlight that autophagy activation may participate to the break of self-tolerance by sustaining generation of citrullinated peptides.

Oxidative Stress

Production of ROS and reactive nitrogen species is triggered by different elements such as metabolism and endogenous inflammation, and exogenous factors including UV light and ionizing radiation. Despite the physiological production of these molecules, their accumulation can be deleterious for cell homeostasis, leading to DNA mutation and could stimulate different molecular pathways, including NF-kB activation, with consequent cytokines production and inflammation (81). Furthermore, protein structural changes induced by ROS are able to modify "primitive" antigen and to form new peptides that can trigger an autoimmune response (82). For these reasons, an imbalance in oxidative stress regulation plays a crucial role in inflammatory autoimmune disorders also by modulating cell fate mechanisms. Mitochondria are the most important source of

ROS and autophagy-mediated mitochondria degradation, called mitophagy, ensures correct balance of oxidative species levels in cells. However, the relationship between autophagy and oxidative stress seems to be very complex in the context of RA, where not only total oxidative status is higher in patients than in healthy control but also neutrophils ROS levels correlate positively with disease activity (83, 84). Since it has been demonstrated an involvement of ROS in autophagosome formation by regulation of Atg4 function (85), ROS-mediated autophagy induction could contribute to the resistance of apoptosis found in synovial and peripheral RA T cells and to the generation of citrullinated peptides in APCs, both these aspects will be important to deepen in future studies.

The possible roles of autophagy in RA pathogenesis and progression are summarized in **Figure 2**.

TARGETING AUTOPHAGY IN RA THERAPY

Considering the key role of autophagy not only in innate and adaptive immune regulation but also in immune system cells homeostasis, it is not surprising that autophagy modulation might be an attractive therapeutic choice in RA. As described in our previous review, rapamycin, an autophagy activator with mTOR inhibitory effects, led to a decrease of disease activity in a small group of SLE patients refractory to traditional treatments (29); at present, a clinical trial is testing the possible use of autophagy modulator in SLE treatment (https://clinicaltrials.gov/ct2/show/study/NCT00779194). Both in SLE and RA, drugs that are able to modulate autophagy, such as CQ and HCQ, are currently in use in the clinical practice showing a high effectiveness (86). It has been demonstrated that CQ is able to inhibit antigen presentation to T cells (87) and the differentiation of osteoclast precursors into mature osteoclasts *in vitro* and *in vivo* (88); in both these processes, autophagy is actively involved.

As previously discussed, although TNF-α was found to induce autophagy in different cell types directly associated with RA pathogenesis, till now, the effect of anti-TNF drugs on autophagy has not been investigated. TNF-mediated autophagy may have a role in the resistance to apoptosis, well documented in the disease, and the blocking of autophagy induction by anti-TNF drugs may reactivate apoptosis. This hypothesis is validated also by a study of Catrina and colleagues, who demonstrated an activation of synovial apoptosis after 8 weeks of treatment with either etanercept or infliximab (89, 90).

A future therapeutic approach based on autophagy suppression in RA might include 3-MA, a chemical compound that inhibits autophagy at an early stage of autophagosome formation, blocking the signaling of PI3K. This pathway has been used in experiments to analyze how autophagy inhibition can lead to beneficial effect in RA, including reduction

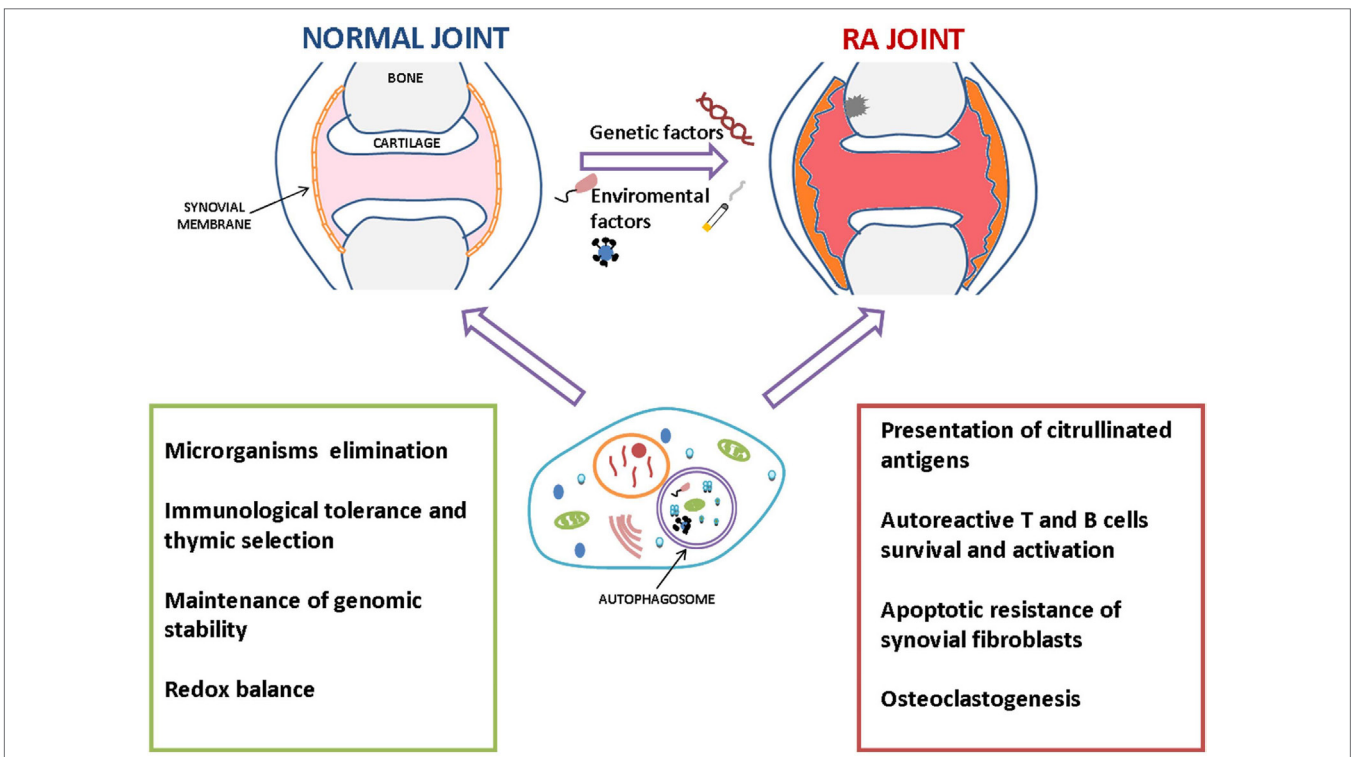

FIGURE 2 | Summary of the possible roles of autophagy in the pathogenesis and progression of rheumatoid arthritis (RA). Autophagy could prevent the development of autoimmunity scenario by eliminating intracellular pathogens and maintaining immune cells homeostasis, including regulation of immunological tolerance mechanisms. However, once it occurs, autophagy could take part in RA progression in several ways, e.g., participating in perpetuation of autoimmune response promoting the survival of inflammatory and autoreactive cells, cytokines production, and presentation of citrullinated antigens.

TABLE 1 | Autophagy modulators of clinical relevance in rheumatoid arthritis (RA).

Regulators of autophagy	Use/not in use in RA treatment	Effects on RA mediated by autophagy modulation	Reference
Inducers			
Rapamacin	Not in use	Promotion of cell survival; citrullination	(96–98)
Glucocorticoids	In use	Chondrocytes apoptosis protection	(93, 94)
Methotrexate	In use	Resistance to RA-FLS apoptosis	(62)
Inhibitors			
Chloroquine	In use	Inhibition of antigen presentation	(87, 88)
3-MA	Not in use	Reduction of citrullinated peptides presentation; apoptosis induction	(57, 91)
Anti-TNF drugs (?)	In use	Apoptosis reactivation	(89, 90)

of citrullinated peptides presentation and the reactivation of apoptosis pathways (57). Data on the systemic effect of 3-MA are limited, but a recent study showed an atheroprotective role of 3-MA, probably related to a downregulation of inflammation, in ApoE-deficient mice (91).

The importance of the balance between autophagy and apoptosis in the resistance to treatment in RA patients has been recently proposed by Xu and colleagues (62). The authors showed that MTX, commonly used in the treatment of RA, is able to induce autophagy in synovial cells protecting them from apoptosis. In fact, the inhibition of autophagy by Beclin-1 siRNA caused an increased death of these cells by apoptosis. Taking all of these considerations, a therapy based on combination of MTX and an autophagy inhibitor in RA has been proposed.

Glucocorticoids have been widely used in the treatment of autoimmune disorders for its anti-inflammatory and immunosuppressive action, although one of the dark effects of this therapy consists in a substantial risk of bone injury. A recent study demonstrated a pro-autophagic effect of glucocorticoid on bone marrow mesenchymal stem cells (BMSCs), concluding that autophagy activation sustained the proliferative potential of BMSCs by protecting them from apoptosis (92). Shen et al. showed that the induction of autophagy by rapamycin blocked the dexamethasone-induced apoptosis in meniscal cells, while the treatment with the autophagy inhibitor 3-MA increased the number of apoptotic cells (93). According to these results, other studies on condrocytes suggest a beneficial effect on bone loss by induction of autophagy contrasting glucocorticoid-induced apoptosis (94).

Abnormalities in PI3K/AKT/mTOR axis have been found in active RA patients and the activation of this pathway has been associated with an excessive activation, proliferation, and survival of T and B cells and apoptosis resistance in RA synoviocytes (95). The suppression of mTOR signaling may be another way to treat RA by modulating autophagy. In a multicenter study involving 121 RA patients, it was found that there was a greater response to the therapy in the group of patients treated with the mTOR inhibitor everolimus plus MTX than the MTX alone, suggesting that autophagy modulators may be added to standard therapy to increase the effectiveness of the therapy (96). This result was corroborated by Cejka and colleagues, who found a reduction in osteoclast number and bone erosions in TNF-transgenic mice treated with sirolimus or everolimus (97). Recently, a clinical trial compared the therapeutic response of temsirolimus (CCI-779) at three different concentrations with placebo in active RA patients (https://clinicaltrials.gov/ct2/show/record/NCT00076206). Considering the pleiotropic role of mTOR signaling in cell metabolism, it is important to note that the effects of mTOR inhibition might not be due as much to autophagy inhibition but rather to the shutdown of other related pathways. In fact, and not surprisingly, personalized pharmacological mTOR blockade has been proposed for the treatment of several non-immune and immune-related disorders (98). To conclude, there are several autophagy modulators in use or under investigation in the management of RA therapy (**Table 1**). This list includes both inducers and inhibitors of autophagy, reflecting the controversial role of this process in the pathogenesis of RA. Considering the intricate signaling pathways regulating autophagy, the pleiotropic activity of some of these drugs still represents the most enigmatic aspect. In fact, by acting on different substrates, they can produce opposite signals associated to autophagy activation. Moreover, *in vitro* studies revealed how experimental conditions, timing, and cell types can influence autophagy-associated results.

CONCLUSION AND PERSPECTIVES

The introduction of biologic agents has revolutionized the clinical approach to RA; however, research of new therapeutic targets appears to be essential to improve the response to therapy. Autophagy is a crucial physiological process and its functions are strictly related to tissue and environmental conditions. Increasing evidences point to autophagy as a driving mechanism of autoimmune diseases. In RA, autophagy activation was found to be essential for the survival of inflammatory cells such as synoviocytes and lymphocytes and has an important role in citrullination and osteoclastogenesis. However, repression of autophagy could expose patients to premature aging, infections, and development of malignancy. Currently, compounds that modulate autophagy pathway are approved for the management of the disease, but long-term effects must be evaluated in order to analyze whether autophagy modulation can interfere with other biological phenomena.

AUTHOR CONTRIBUTIONS

MV and CA designed and wrote the review. CB and TC finalized the table and figures and drafted the manuscript. CP, LN, FUC, FS, MF, FAC, and GV drafted the manuscript and provided useful suggestions. All authors read and approved the final manuscript.

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REVIEW
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The Therapeutic and Pathogenic Role of Autophagy in Autoimmune Diseases

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Autophagy is a complicated cellular mechanism that maintains cellular and tissue homeostasis and integrity *via* degradation of senescent, defective subcellular organelles, infectious agents, and misfolded proteins. Accumulating evidence has shown that autophagy is involved in numerous immune processes, such as removal of intracellular bacteria, cytokine production, autoantigen presentation, and survival of lymphocytes, indicating an apparent and important role in innate and adaptive immune responses. Indeed, in genome-wide association studies, autophagy-related gene polymorphisms have been suggested to be associated with the pathogenesis of several autoimmune and inflammatory disorders, such as systemic lupus erythematosus, psoriasis, rheumatoid arthritis, inflammatory bowel disease, and multiple sclerosis. In addition, conditional knockdown of autophagy-related genes in mice displayed therapeutic effects on several autoimmune disease models by reducing levels of inflammatory cytokines and autoreactive immune cells. However, the inhibition of autophagy accelerates the progress of some inflammatory and autoimmune diseases *via* promotion of inflammatory cytokine production. Therefore, this review will summarize the current knowledge of autophagy in immune regulation and discuss the therapeutic and pathogenic role of autophagy in autoimmune diseases to broaden our understanding of the etiopathogenesis of autoimmune diseases and shed light on autophagy-mediated therapies.

Keywords: autophagy, light-chain 3, Agt, autoimmunity, LAP

INTRODUCTION

Autophagy was discovered more than 40 years ago and has recently become a topic of increasing interest since the Japanese biologist Yoshinori Ohsumi won the Nobel Prize in 2016 for the discovery of this "self-eating" mechanism. Autophagy refers to a survival mechanism that cells use to degrade unwanted and useless organelles, proteins, and infectious agents to maintain homeostasis (1). There are three types of autophagy: macroautophagy, chaperone-mediated autophagy (CMA), and microautophagy. Among them, macroautophagy is the most intensively studied and is referred to as autophagy in general, and is the focus of this review. Macroautophagy occurs in all eukaryotic cells and initiates the recruitment of protein aggregates and misfolded proteins by phagophores. Then,

vesicles undergo elongation and form double-membraned vesicles, called autophagosomes, and then the cytoplasmic components are enclosed *via* cargo sequestration and fuse with lysosomes for degradation and recycling (2) (**Figure 1**). Macroautophagy is capable of constitutively delivering cytosolic proteins for MHC-II presentation. Autophagosomes are capable of fusing with multivesicular bodies or enodosomes and MHC-II loading compartments (3). CMA is another type of autophagy, which is involved with the direct recognition, targeting, and degradation of substrates by lysosomes, rather than by autophagosome formation (4). As a substrate of CMA, a protein should contain the amino acid sequence of the polypeptide motif KRERQ (5). Target proteins are selectively recognized by cytosolic heat shock cognate 70/co-chaperones and then delivered to the lysosomal membrane. Proteins then bind to the integral lysosomal membrane protein (LAMP-2A) and unfold and reach the lumen *via* a LAMP-2A-enriched translocation complex. Then proteins undergo degradation in the autolysosome (6). In addition, microautophagy is the process of lysosomal engulfment of cytoplasmic cargo with the formation of autophagic tubes and vesicles (7). Cytosolic and soluble proteins and particulate cellular constituents are directly internalized in single-membrane vesicles into lysosome by invaginating, protrusion, and or septation of the lysosomal limiting membrane (8). Microautophagy is a process of detect invagination and fusion of the vacuolar/lysosomal membrane under the limited nutrient status. Therefore, microautophagy is critical for cell survival, particularly for cells that are under stress such as nutrient starvation (9).

It is now widely accepted that macroautophagy (autophagy for short) is involved in several pathophysiological processes and complex diseases, such as autoimmune disorders, cancer, and metabolic disorders. Indeed, autophagy has been found to play four principal roles in immune responses: intracellular pathogen removal, lymphocyte development, pro-inflammatory signaling, and the secretory pathway (10–12). The combined data from genome-wide association studies (GWAS) and inhibition assays in mouse models have implicated autophagy in autoimmune diseases, especially in systemic lupus erythematosus (SLE), psoriasis, rheumatoid arthritis (RA), inflammatory bowel disease (IBD), and multiple sclerosis (MS). Therefore, this review will summarize the current understanding of the molecular regulation of autophagy and its roles in immunity and discuss the therapeutic and pathogenic role of autophagy in these autoimmune disorders, providing potential diagnostic targets and therapeutic strategies for autoimmune diseases. Although CMA and microautophagy has been found to be also related with immune responses (13), the regulations from these two pathways will not be discussed in this review.

MOLECULAR REGULATION OF AUTOPHAGY

It has been well established that autophagy is predominately regulated by the autophagy-related gene (*Atg*) family, which initiates the formation of autophagosomes (14). Energy depletion

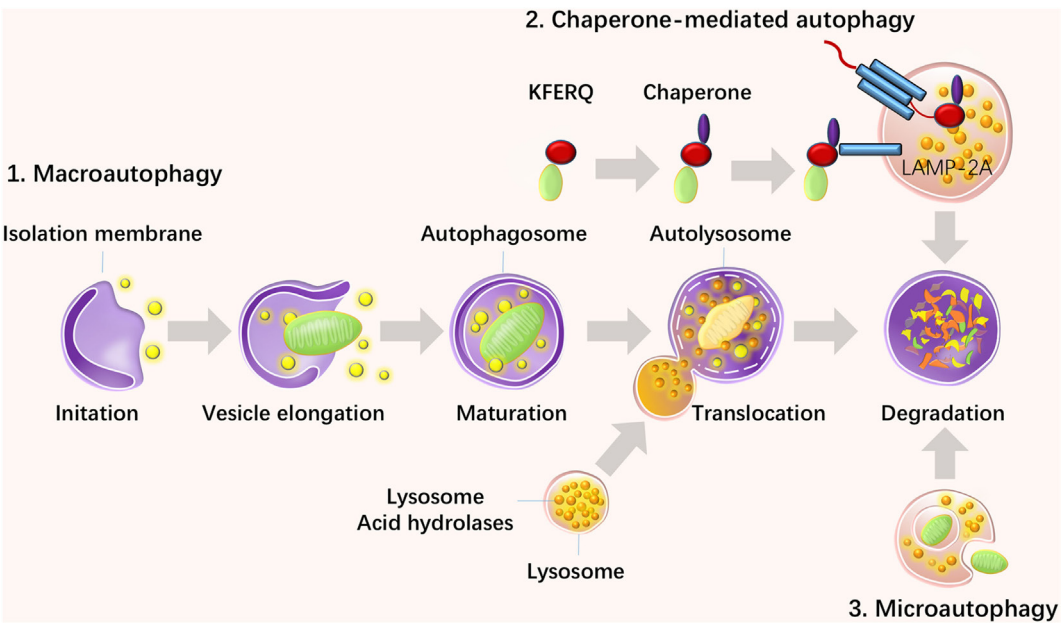

FIGURE 1 | Three types of autophagy and their steps. There are three types of autophagy: macroautophagy, chaperone-mediated autophagy (CMA), and microautophagy. Macroautophagy initiates with the recruitment of protein aggregates and misfolded proteins by phagophores. Then, vesicles undergo elongation and form double-membraned vesicles, called autophagosomes, and the cytoplasmic components are enclosed *via* cargo sequestration and fused with lysosomes for degradation and recycling. CMA is another type of autophagy, which is involved in the direct recognition, targeting, and degradation of substrates by lysosomes rather than autophagosome formation. Microautophagy is a process of lysosomal engulfment of cytoplasmic cargo with the formation of autophagic tubes and vesicles.

can activate AMP-activated protein kinase and further activate the mammalian target of rapamycin (mTOR) substrate complex, which consists of phosphorylated UNC-51-like kinase 1 (ULK1), ATG13, ATG101, and FIP200. In addition, this pathway positively regulates the formation of autophagosomes (15). Environmental signals, such as starvation, repress autophagy *via* inhibition of mTOR, which is located in the mTOR signaling complex 1, formed by the regulatory-associated protein of mTOR (Raptor), G protein beta subunit-like protein, and proline-rich Akt/PKB substrate 40 kDa (16). In addition, insulin and other growth factor signaling activates Class I PI3K–Akt, which inhibits autophagy *via* activation of the mTOR signaling complex 1 and inhibition of the Beclin 1 class III PI3K complex, which contains Beclin 1, class III phosphatidylinositol-3-kinase (PIK3C3), and ATG14L (17). It has been documented that autophagosomal elongation requires two ubiquitin-like conjugation systems: the ATG5–ATG12 conjugation system and light-chain 3 (LC3)–ATG8 conjugation system (16). When cells take up phagocytosed dead cells, LC3-associated phagocytosis (LAP) will take place to promote digestion (18). The molecular regulation of autophagy has been summarized in **Figure 2**.

AUTOPHAGY-MEDIATED REGULATION IN IMMUNITY

It has been reported that autophagy is initiated by different families of pathogen-recognition receptors [such as toll-like

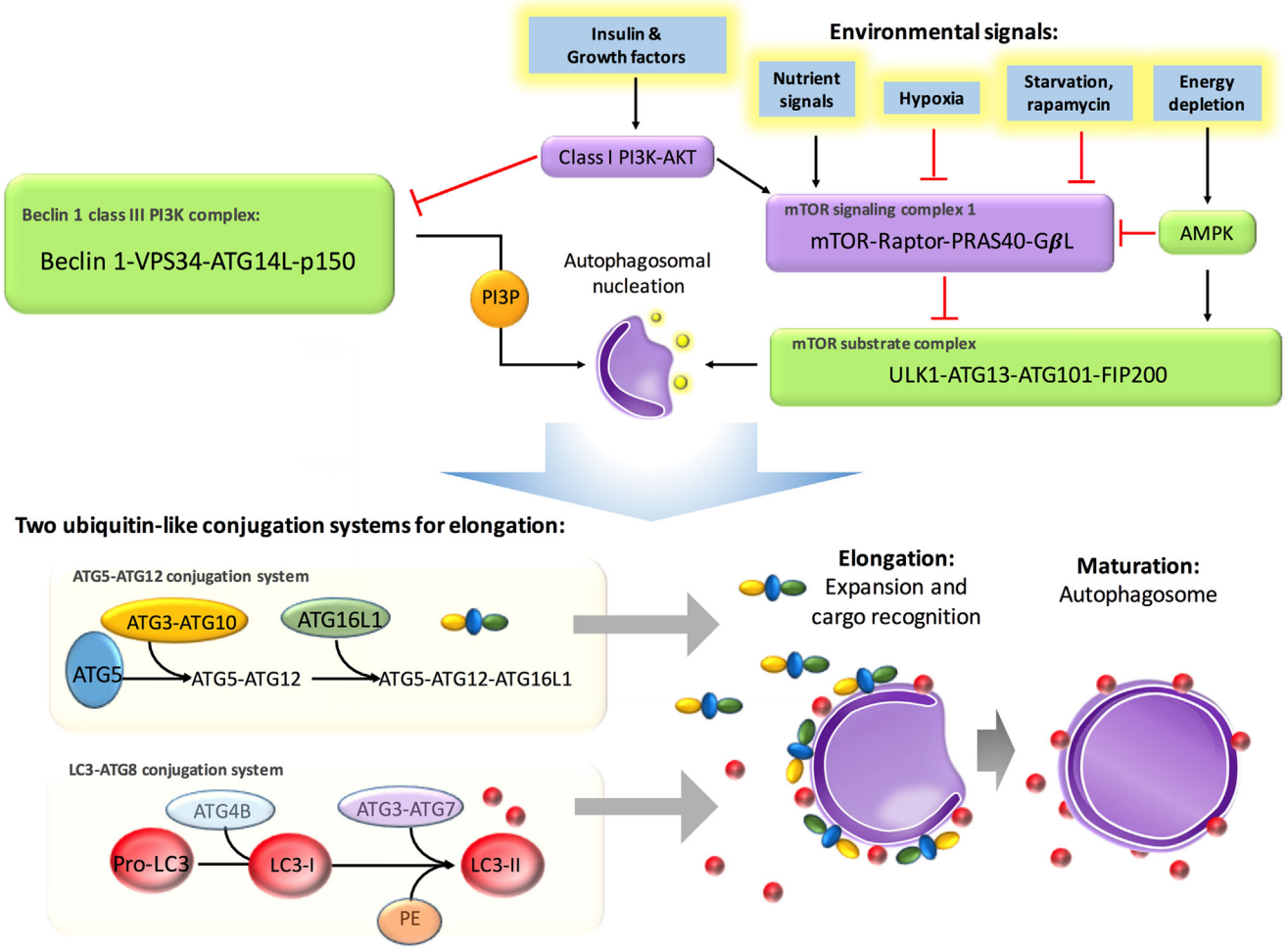

FIGURE 2 | Molecular regulation in autophagy. Autophagy is suppressed by insulin and other growth factor signaling, starvation, and nutrient signals but activated by energy depletion and AMBRAs, BIF1, and UVRAG. Energy depletion can activate AMP-activated protein kinase (AMPK) and further activate the mammalian target of rapamycin (mTOR) substrate complex, which consists of phosphorylated UNC-51-like kinase 1 (ULK1), ATG13, ATG101, and FIP200. In addition, this pathway positively regulates the formation of autophagosomes. Environmental signals, such as starvation, repress autophagy *via* inhibition of mTOR, which is located in the mTOR signaling complex 1, formed by the regulatory-associated protein of mTOR (Raptor), G protein beta subunit-like protein (GβL), and proline-rich Akt/PKB substrate 40 kDa (PRAS40). Insulin and other growth factor signaling activates class I PI3K–Akt, which inhibits autophagy *via* activation of mTOR signaling complex 1 and inhibition of the Beclin 1 class III PI3K complex, which contains Beclin 1, class III phosphatidylinositol-3-kinase (PIK3C3), and ATG14L. Autophagosomal elongation requires two ubiquitin-like conjugation systems: the ATG5–ATG12 conjugation system and light-chain 3–ATG8 conjugation system.

receptors (TLRs)], damage-associated molecular patterns (such as HMGB1 and misfolded proteins), pathogen receptors, IFN-gamma, DAP kinase, JNK, CD40, TNF-alpha, and NF-κB (19). And it is repressed by Th2 cytokines, Bcl-2, and canonical nutrient sensing insulin–AKT–TOR pathway. Therefore, it is no surprise that autophagy involves in innate and adaptive immune response.

Removal of Pathogens by Autophagy

Autophagy participates in innate immunity *via* removing intracellular microbial pathogens and protecting the cytosol. There are two major ways to remove intracellular pathogens by autophagy in dendritic cells (DCs), macrophages, and other phagocytes. One is called xenophagy, which eliminates pathogens by engulfing them in double-membrane autophagosomes. This process is activated during infection by TLRs (20). Many bacteria and parasites can be removed by xenophagy. The other way is mediated by microtubule-associated protein LC3 and named LAP, which encloses pathogens in single-membrane phagosomes and involves LC3 (18). LAP has been found to be activated by TLR agonists and immune complexes (21), which are abundant in SLE. LAP is triggered by bacteria *via* surface markers (TLRs, FcγR, NOD, and SLAM) expressed by host cells, or by cytosolic pathogen sensing signals *via* direct induction or binding to autophagy component proteins. LAP shows capacity in dead cell clearance, which requires PtdSer receptor TIM4 to induce the recruitment of LC3 to the phagosome (22).

However, several pathogens have been found to have some strategies to avoid autophagy process. *Listeria monocytogenes* and *Shigella flexneri* have been found to express several proteins which are modified to inhibit recognition by the autophagic machinery. Human immunodeficiency virus 1 (HIV-1) can express Nef protein to interact with Beclin-1, thereby blocking the fusion of autophagosomes with lysosomes (23). This is the reason why we can be infected with bacteria and viruses even though we have normal innate immune responses.

In physiological conditions, apoptotic cells, with ATG and Bclin-1expression, expresses phosphatidylserine on cell surfaces, which is referred to as "eat-me" signals. Then they release lysophosphatidylcoline, which is considered as "come-get-me" signals. Then, these apoptotic cells can be efficiently cleared by phagocytes. However, in ATG5 or Beclin-1-deficient cells, apoptotic cells cannot express "eat-me" and "come-get-me" signals, which will lead to impaired clearance of apoptotic bodies, thereby contributing to autoimmune disorders (24).

Antigen Processing for MHC Presentation by Autophagy

Antigen-presenting cells (APCs), including DCs, macrophages, and B cells, are the bridges between innate and adaptive immunity. MHC-I molecules present endogenous antigens to $CD8^+$ T cells *via* processing them by proteasome and translocating them into ER. Whereas MHC-II presents extracellular antigens on lysosome-derived organelles to $CD4^+$ T cells. Before presentation with MHC-II by APCs, extracellular pathogens need to be properly digested and processed by APCs *via* degradation pathways, which include the ubiquitin–proteasome system and autophagy. MHC-II molecules can also present intracellular antigens, such as cytosolic or nuclear antigens, *via* the fusion of autophagosomes with MHC-II rather than the lysosome (8). In addition, the process of MHC-I presentation can be enhanced by autophagy (25). This urgent MHC-I presentation which is promoted by autophagy seems to need more time and more antigens, which means that this alternative process in the "cellular emergency" situation occurs when the classical pathway is imparied.

Lymphocyte Development, Activation, and Polarization Regulated by Autophagy

It has been reported that autophagy plays a critical role for the thymic selection, T cell development, survival, and proliferation. $Atg5^{-/-}$ mice show reduced thymocyts and peripheral lymphocytes and increased cell death in $CD8^+$ T cells. And these $Atg5^{-/-}$ T cells fail to proliferate under TCR stimulation (26). $Atg7^{-/-}$ T cells show deficiency in cell survival, with the expanded ER content and mitochondria (27). The similar results have been observed in the $Atg3^{-/-}$ mice, which display impaired autophagy (28). On the other hand, some studies have claimed that autophagy promote the cell death during virus infection. For example, HIV Env-mediated autophagy induces apoptosis of $CD4^+$ T cells *via* CXCR4 (29), and autophagy is involved in RIPK1-dependent necroptotic cell death when Fas-associated death domain activity and caspase-8 is insufficient (30). In addition, Beclin-$1^{-/-}$ $CD4^+$ T cells, which are deficiency in autophagy, show preference in apoptosis under the TCR stimulation. Accumulation of pro-caspase-3, pro-caspase-3, and Bim might be one of the mechanisms (31). Therefore, the role of autophagy in T cell survival remains unclear. Future studies are needed to address this issue.

There are two subsets of conventional DCs (cDCs): CD8α ($CD103^+$) cDCs and $CD4^+$ ($CD11b^+$) cDCs. CD8α cDCs efficiently cross-present exogenous antigens on MHC-I to $CD8^+$ T cells, whereas $CD4^+$ cDCs more efficiently polarize $CD4^+$ T cells into Th1, Th2, Th17, or regulatory T cells (Tregs) by MHC-II-restricted presentation (32). Autophagy has been reported to be essential for the $CD4^+$ T-cell response by DCs (33, 34). Indeed, a deficiency of autophagy in DCs results in a mild EAE phenotype in mice (35), which is a Th17-mediated mouse model. Furthermore, autophagy has also been linked with DC-derived cytokine production, such as IL-6 and IL-12p40 (36), which is critical for T-cell activation and polarization. This evidence indicates autophagy might involve in the Th cell differentiation. Besides, Tregs have been reported to be regulated by autophagy. For example, during chronic hepatitis B virus infection, HMGB-1-induced autophagy maintains Treg cell functions (37). And *Agt16l1*gene has been found to differentially regulate Treg and Th2 cell and further control intestinal inflammation (38). However, autophagy can be also regulated by Treg cells. For example, $Foxp3^+$ Treg cells have been found to suppress immune response *via* inhibiting autophagic machinery in DCs depending on CTLA4. The binding of CTLA-4 promotes activation of PI3K/Akt/mTOR axis and FoxO1 nuclear exclusion in DCs, resulting in reduced

expression of autophagy component microtubule-associated protein 1 light chan 3 beta (39).

In addition, autophagy also regulates B cell survival and development. ATG5 has been identified to be critical for B-cell survival and subset maintenance, such as pre-B and mature B1a B cells (40). Autophagy has been found to be required for immunoglobulin production by plasma cells (41). Indeed, the degradation of misfolded protein is particularly important for antibody-secreting cells, in which protein synthesis and degradation must be balanced. It has been observed that increased autophagosome formation and degradation occur in activated mouse B cells during plasma cell differentiation (41), as well as in human B cells activated by CpG (42). Genetic studies have identified several autophagy-related genes essential for antibody responses and plasma cell homeostasis (43), and dysregulated autophagy contributes to the plasma cell pathology in antibody-mediated autoimmune diseases such as SLE (44, 45). In addition, autophagy has been found to contribute to IL-17-dependent plasma differentiation *via* regulating Blimp-1 expression and Beclin-1/p62-associated B cell apoptosis (46, 47). These findings show that autophagy in B cells might play a pathogenic role in antibody-mediated autoimmune disease, such as lupus.

Pro-Inflammatory Signaling Regulated by Autophagy

Increasing evidence has demonstrated the interplay between autophagy and the NF-κB signaling pathway (48). The NF-κB family of transcription factors regulates transcription of a broad range of genes, which are engaged in cell proliferation, survival, differentiation, and development. These transcription factors are also essential in inflammation and immune responses (49). The mammalian NF-κB family contains five members: RelA, c-Rel, Rel-B, p50, and p52 (50), while inhibitors of the NF-κB protein family consist of IκBα, IκBβ, IκBε, and the IκB-like inhibitors p100 and p105 (51). It has been well documented that activation of the inhibitor of NF-κB (IκBα) kinase complex is required for the induction of autophagy. Conversely, in an Atg5- and Atg7-deficient system, autophagy has been proven to be critical for the activation of NF-κB (52). In addition, the cross talk between NF-κB and autophagy has been observed in immune cells. The regulation of T-cell receptor-mediated NF-κB activation by B-cell lymphoma/leukemia 10 is associated with autophagy adaptor p62/SQSTM1 (53), which is also found to be a modulator of NLRP3-inflammasome activation and IL-1 beta production in macrophages (54). However, in tissue-specific macrophages, autophagy has been revealed to promote NF-κB activation to boost the antifungal immune response (55). This evidence indicates a pathogenic or therapeutic role of autophagy depending on different microenvironments and signals.

Interplay Between Cytokine Secretion and Autophagy

It is no surprise that autophagy-regulated cytokine secretion by the secretory pathway shares some common functions with phagocytosis, such as vesicle trafficking and membrane fusion, which facilitates the important role of autophagy in immune regulation. ATG5 deficiency, for example, results in elevated IL-1 alpha secretion by macrophages (56), while inhibition of autophagy leads to promotion of IL-1 beta *via* reducing degradation (57) by APCs and increases IL-23 secretion as a consequence (58), which can further promote Th17-mediated inflammatory responses. On the other hand, cytokines can also regulate autophagy. IL-10, which is an anti-inflammatory cytokine, has been found to inhibit autophagy in murine macrophages *via* activation of mTOR complex 1 (59). Another example is IL-6, which is a universal inflammatory cytokine involved in many autoimmune and inflammatory diseases. IL-6 has been illustrated to inhibit starvation-induced (60) and IFN-gamma-induced autophagy (61) by regulating Bcl-2 and Beclin1. However, IL-6 has also been found to be required for autophagy by promoting autophagosomal maturation (62, 63). The functions of autophagy have been summarized in **Figure 3**. Further studies are necessary to validate the interplay between autophagy and cytokines.

THE THERAPEUTIC AND PATHOGENIC ROLE OF AUTOPHAGY IN AUTOIMMUNE DISEASES

Based on its functions in the immune system, autophagy might display a pathogenic and/or therapeutic role in autoimmune diseases, depending on the pathogenesis of the disease and the key players in disease development. The detailed findings for each autoimmune disease are elaborated upon in the following paragraphs.

Autophagy in SLE

Systemic lupus erythematosus is a typical autoimmune disease, which is characterized by abnormal APCs and T and B cells, with abundant autoantibodies (64–66). Dysregulated production of IL-17 (67) and abnormally differentiated follicular helper T (Tfh) cells (68, 69), aberrant DCs, and plasma B cells (70) have been identified to play an essential role in the pathogenesis of SLE. Although autophagy negatively regulates IL-17 production, the inhibition of autophagy in a lupus mouse model reduced the disease phenotype by partially suppressing plasma cell differentiation and antibody production (44). In addition, GWAS in lupus cohorts have identified that several SNPs in the *Atg5* gene confer genetic susceptibility to lupus (71, 72). In addition, in a follow-up study, a SNP in the *Atg5* gene, rs573775, was identified to be related to IL-10 production and higher risk of lupus (73). In APCs, LAP has been found to be required for the trafficking of immune complexes and TLR9 into the interferon signaling pathway and to promote type 1 interferon production (21, 74), which is a key player in lupus pathogenesis. In addition, autophagy has been reported to deliver viral ligands to TLR7 in plasmacytoid DCs during vesicular stomatitis virus and Sendai virus infection and contribute to type 1 interferon production (75), which might be associated with lupus. However, defects in LAP, rather than canonical autophagy, can cause SLE-like phenotypes (76) with IL-10 production (77). Increased autophagy has been observed in T and B cells from a lupus mouse model, as well as in PBMCs from patients with SLE (44). In addition, autophagy-related genes, including mTOR, Beclin-1, LC3, and p62, have been found to be

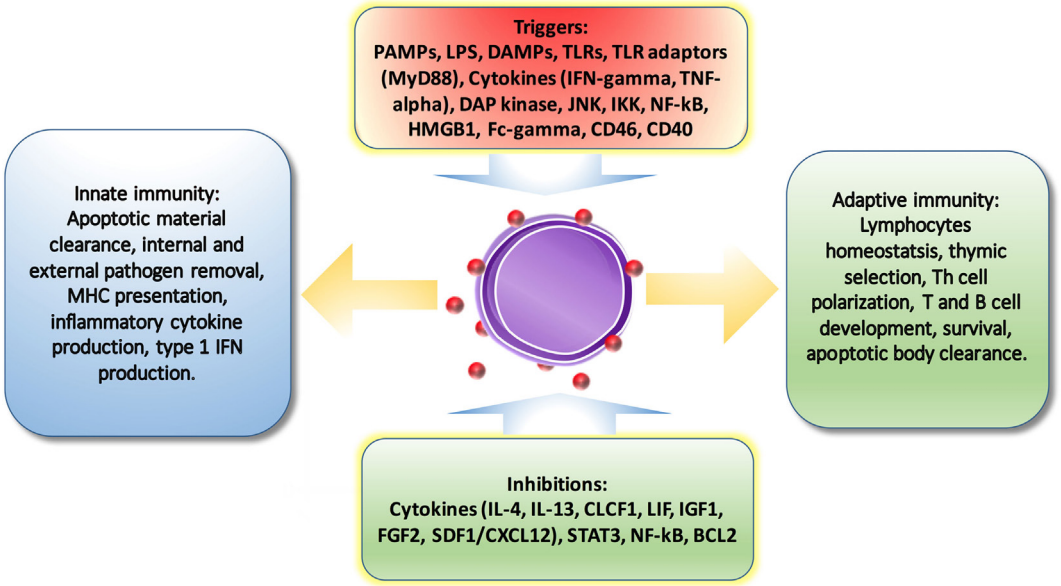

FIGURE 3 | The regulations of autophagy on immune system. Autophagy is triggered and inhibited by cytokines and molecules from immune system. And also, autophagy is involved in pathogen removal, cytokine secretion, lymphocyte survival and differentiation, MHC presentation, apoptotic cell clearance, and pro-inflammatory signaling.

expressed differentially by lupus PBMCs (45). Blockade of macrophage autophagy ameliorates activated lymphocyte-derived DNA-induced murine lupus possibly *via* inhibition of proinflammatory cytokine production, such as IL-6 and TNF-alpha (78). Recently, IL-21, which is a key cytokine produced by Tfh cells, has been found to induce mTOR activation and further eliminate autophagy and differentiation of Treg cells (79). To summarize, ATG5 deficiency and mTOR elevation in innate immunity leads to insufficient autophagy, which results in reduced dead cells clearance, enhanced levels of cytoplasmic nucleic acid and autoantigens. As a consequence, increased type 1 IFN by DCs can induce B cell hyper-differentiation and antibody production. In adaptive immunity, high LC3 and accumulation of autophagic vacuoles can increase autophagy and promote the survival of T and B cells (8).

In addition, environmental factors, such as UV light and Epstein–Barr virus (EBV) infection, which have been shown to contribute to the initiation of lupus, have been linked to autophagy. For example, UV-induced DNA damage has been found to result in decreased expression of AMBRA1 and ULK1, which are important mechanisms in autophagy (80). In addition, autophagy has been reported to be involved in MHC-II presentation of EB nuclear antigen 1 to T cells (81) and to participate in EBV infections (82). Furthermore, severe vitamin D deficiency affected the expression of ATGs in PBMCs and T-cell subsets in active SLE patients, indicating that vitamin D may affect T-cell subsets *via* regulating autophagy (83).

In SLE treatment, autophagy has been reported to be a therapeutic target. Rapamycin is a FDA approved immunosuppressive agent for organ transplantation. It excuses its inhibitory effects on T cells *via* blocking mTORC1 (84), which is also the key player of autophagy. Rapamycin has been shown to be efficient in treating lupus mice and patients, with the decreased levels of autoantibodies, proteinuria, and prolonged survival in mice and patients (85, 86). In an off-label clinical study, refractory SLE patients were treated with rapamycin. Compared with standard treatment, the rapamycin-treated group showed decreased disease activity and prednisone requirement (87). And this suppressive effect might be conducted through inhibition on HRES 1/Rab4 and Rab5A and limiting the production of type I IFN by DCs (88). Besides, other treatments also have effects on autophagy. In a clinical observation, hydroxychloroquine, which is the most common treatment for SLE, has been found to inhibit autophagy, particularly LAP-mediated autophagy (89). Glucocorticoids can induce autophagy *via* inhibiting IP3-mediated calcium signaling and mTOR (90). Anti-CD20 mAbs can trigger autophagy by caspase-independent cell death induction (91). And anti-TNF alpha mAbs can inhibit autophagy by limiting proautophagic cytokine production (92). In a recent study, inhibition of Treg cell differentiation and IL-21 has been found to repress rapamycin axis *via* suppression of autophagy in lupus patients (79). Although different drugs have various effects on autophagy in lupus, and the role of autophagy in lupus can be friend or foe, the balance between innate immunity and adaptive immune response should be considered when consider autophagy as therapeutic target.

Autophagy in Psoriasis/Psoriatic Arthritis

Psoriasis is an inflammatory autoimmune skin disease, which can also affect other organs. Approximately 6–42% of psoriasis patients will develop psoriatic arthritis (93). The pathogenesis of psoriasis is unclear. However, increased epithelial keratinocyte proliferation is an essential characteristic of psoriasis (94), and

IL-17 and other inflammatory cytokines have been revealed to play an important role in the development of psoriasis. There is no direct evidence to show whether ATG16L1 contributes to psoriasis. However, defects in autophagy have been found to result in proinflammatory cytokine production and keratinocyte proliferation *via* increased p62 expression (95). Enhanced expression of the autophagy-related gene *SQSTM1* has been observed in psoriatic skin lesions (95). In addition, mutation of the psoriasis risk gene *AP1S3* has been found to result in impaired autophagy and increased skin inflammation (96), and increased expression of ATG16L1 has been observed in DCs from psoriatic arthritic patients (97). The inhibition of autophagy *via* activation of PI3K/AKT/mTOR has been suggested as a therapeutic method for the treatment of IL-17a-mediated psoriasis (98). In addition, other studies have shown the therapeutic role of autophagy in psoriasis *via* inhibition of IL-17a production (58). In addition, the inhibition of autophagy by chloroquine may accelerate psoriasis *via* promoting IL-23 production (99). In addition, vitamin D, sirolimus, retinoids, and UVB therapy, which can promote psoriasis, can induce the activation of autophagy (100–103). Taken together, these findings indicate that autophagy shows a therapeutic role in this disease.

Autophagy in MS

Multiple sclerosis is an inflammatory disorder that is characterized by immune system reactivity against myelin in the central nervous system, resulting in varying degrees of either relapsing or progressive neurological degeneration. ATG5 (104) and immune-related GTPase M (IRGM) 1 are increased, while ATG16L2 is decreased, in autoreactive T cells in EAE and actively relapsing-remitting MS brains (105). Inhibition of autophagy by conditional knockout of Beclin-1 in $CD4^+$ T cells has shown a protective role in the EAE model (31). Similar effects have been observed in an ATG7 conditional knockout system (35). Administration of rapamycin reduces relapsing–remitting EAE *via* inhibition of autophagy (106), indicating a pathological role of autophagy in MS.

Autophagy in RA

Rheumatoid arthritis is a chronic and systemic inflammatory autoimmune condition that primarily affects the joints and is characterized by progressive destruction of the joints. The pathogenesis of RA remains unclear. However, dysregulated immune cells, such as Th17 cells, Tfh cells, macrophages, B cells, and fibroblast-like synoviocytes have been identified to contribute to this disorder (107). Fibroblasts are a key player, and their survival has been found to be regulated by autophagy induction and CHOP underexpression under endoplasmic reticulum stress (108). Increased levels of autophagy have been observed in the synovial tissues from patients with active RA and are correlated with disease activity (109). However, the effect of autophagy on the survival of RA synovial fibroblasts is controversial (110). In

TABLE 1 | The regulation of autophagy in autoimmune diseases.

Diseases	Cell types	Autophagy related genes and proteins	Effects	Reference
Systemic lupus erythematosus (SLE)	White blood cells	*Atg5*	Regulating IL-10 production	(71–73)
SLE	Plasma cells	*Atg7*	Regulating plasma cell differentiation	(43)
SLE	Antigen-presenting cells (APCs)	LAP	Regulating interferon and IL-6, TNF-alpha, and IL-10 production	(21, 74–77)
SLE	PBMCs	Mammalian target of rapamycin (mTOR), Becline-1, light-chain 3 and p62	Expressed differentially	(45)
SLE	APCs	AMBRA1 and UNC-51-like kinase 1 (ULK1)	UBV induced lower expression of AMBRA1 and ULK1	(80)
SLE	APCs and T cells	–	Involving in the Epstein–Barr virus infections	(81, 82)
SLE	PBMCs and T cells	–	Vitamin D affects T-cell subsets *via* regulating autophagy	(83)
Psoriasis	Keratinocytes	ATG16L1 *SQSTM1*	Regulating keratinocytes proliferation	(95)
Psoriatic arthritis	Dendritic cells	ATG16L1	–	(97)
Psoriasis	T cells	PI3K/AKT/mTOR	Regulating IL-17 production	(98)
Multiple sclerosis (MS)	T cells	ATG5, immune-related GTPase M (IRGM)1, ATG16L2	Expressed differentially	(104, 105)
MS	T cells	Beclin-1, ATG7, mTOR	Knockdown or inhibition shows protective role in EAE	(31, 35, 106)
Rheumatoid arthritis (RA)	RA synovial fibroblasts	ALFY, p62	Regulating survival of RA synovial fibroblasts	(111, 112)
RA	RA synovial fibroblasts	–	Impairing apoptosis	(113)
RA	T cells	ATG7, optineurin	Regulating pro-inflammatory cytokine production	(114, 115)
Inflammatory bowel disease (IBD)	–	ATG16L1, IRGM	SNPs, IL-17a, and IL-1beta production	(116, 117)
IBD	Macrophages	ATG16L1, nucleotide-binding oligomerization domain-containing protein 2	Regulating IL-1beta production	(118, 119)

another study, reduced expression of ALFY and the formation of p62-positive polyubiquitinated protein aggregates promote cell death in RA synovial fibroblasts under severe ER stress (111). Moreover, IL-17-mediated mitochondrial dysfunction has been found to impair apoptosis in RA synovial fibroblasts through activation of autophagy (112).

In immune cells, increased autophagy has been observed in RA $CD4^+$ T cells, resulting in T-cell hyperactivation and resistance to apoptosis (113). The inhibition of autophagy *via* an ATG7 knockdown system showed impaired bone destruction in TNF-mediated arthritis (114), partially resulting from the reduced production of IL-6 and IL-1 by inhibition of autophagy. The autophagy-related protein optineurin has also been found to negatively regulate osteoclastogenesis by modulating NF-κB and IFN-β signaling (115). Taken together, these findings indicate that autophagy shows a pathological role in RA by regulating inflammatory cytokines and bone destruction.

Autophagy in IBD

Inflammatory bowel disease refers to two different chronic conditions or diseases that may be related, Crohn's disease and ulcerative colitis, which consist of inflammation of the wall of the bowel or intestines. GWAS have identified ATG16L1 and immunity-related IRGM in Crohn's disease (116), indicating a role of autophagy in the pathogenesis of IBD. Altered expression of IRGM leads to Crohn's disease with defective autophagy (117). In addition, IBD is an IL-17a- and IL-1 beta-mediated disease. The deletion of ATG16L1 also leads to increased IL-1 beta production in macrophages (118), which might contribute to IBD. Moreover, ATG16L1 and nucleotide-binding oligomerization domain-containing protein 2 interact in an autophagy-dependent antibacterial pathway implicated in Crohn's disease pathogenesis (119).

CONCLUSION

The interplay between autophagy and the immune system emphasizes an important role of autophagy in the pathogenesis of autoimmune diseases. Autophagy is involved in pathogen removal, cytokine secretion, lymphocyte survival and differentiation, MHC presentation, apoptotic cell clearance, and pro-inflammatory signaling. In both *in vivo* and *in vitro* systems, inhibition of autophagy ameliorates diseases including SLE, MS, and RA. However, in other cases, it seems to exacerbate diseases such as psoriasis, psoriatic arthritis, and IBD. Even in RA, autophagy shows a therapeutic and pathogenesis role in the survival of RAFLS (**Table 1**). In addition, the regulation of autophagy varies in different tissues and cells. This evidence means that extreme care should be exercised if autophagy is to be utilized as a therapeutic target. Individual differences, even in the same types of diseases, should be considered. For example, SLE is a heterogeneous disease, and lupus patients might be either predominated by IL-17a and/or IL-21 expression. The outcome might be totally different if autophagy inhibition is applied in these two different types of patients. Therefore, further investigation is needed to clarify the regulation of autophagy in each autoimmune disease, and personalized therapy is strongly recommended in the future.

AUTHOR CONTRIBUTIONS

HY and HW wrote the manuscript. YC, JZ, MZ, LL, and GC edited the manuscript. QL revised the manuscript.

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The Mitochondrion-lysosome Axis in Adaptive and Innate Immunity: Effect of Lupus Regulator Peptide P140 on Mitochondria Autophagy and NETosis

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Mitochondria deserve special attention as sensors of cellular energy homeostasis and metabolic state. Moreover, mitochondria integrate intra- and extra-cellular signals to determine appropriate cellular responses that range from proliferation to cell death. In autoimmunity, as in other inflammatory chronic disorders, the metabolism of immune cells may be extensively remodeled, perturbing sensitive tolerogenic mechanisms. Here, we examine the distribution and effects of the therapeutic 21-mer peptide called P140, which shows remarkable efficacy in modulating immune responses in inflammatory settings. We measured P140 and control peptide effects on isolated mitochondria, the distribution of peptides in live cells, and their influence on the levels of key autophagy regulators. Our data indicate that while P140 targets macro- and chaperone-mediated autophagy processes, it has little effect, if any, on mitochondrial autophagy. Remarkably, however, it suppresses NET release from neutrophils exposed to immobilized NET-anti-DNA IgG complexes. Together, our results suggest that in the mitochondrion-lysosome axis, a likely driver of NETosis and inflammation, the P140 peptide does not operate by affecting mitochondria directly.

Keywords: NETosis, autophagy, mitochondrion, systemic lupus erythematosus, neuroinflammation, P140 peptide

INTRODUCTION

Mitochondria are specialized cytoplasmic organelles known to generate cellular energy, converting oxygen, and nutrients into adenosine triphosphate (ATP), that are now emerging as true metabolic sensors. Mitochondria are present in all nucleated cells and in platelets where they affect a wide array of vital cell functions, notably in cellular stress responses such as autophagy and apoptosis. They exert crucial roles in reactive oxygen species (ROS) signaling, which is important in hypoxia sensing, and in cellular differentiation during development (1–3). Mitochondria are also central to innate immunity (4).

Disruption of the mitochondrial genetic material or mitochondrial metabolic functions contributes to numerous pathologies. Even though primary genetic mitochondrial myopathies remain relatively rare, the prevalence of disorders due to secondary mitochondrial dysfunction (caused by pathological events originated outside mitochondria) is much higher. Defective mitochondrial functioning contributes to cardiac diseases (ischemia/reperfusion injury), metabolic syndrome (obesity), and neurodegenerative diseases, such as Huntington's, Parkinson's, and Alzheimer's diseases (5). Cells of the central nervous system and muscles impose high demands on energy supply and are therefore particularly susceptible to mitochondrial insufficiency. In systemic lupus erythematosus (SLE), a chronic inflammatory autoimmune syndrome, mitochondrial respiration is critical for neutrophil extracellular trap (NET) formation, and mitochondria released by neutrophils induce inflammatory cytokine production (6). Mitochondrial ROS inhibition was thus found to reduce disease severity and type I interferon responses in a mouse model of lupus (6). Although autoantibodies (autoAb) to citrullinated proteins suggest that NETosis makes an important contribution to autoimmunity, experiments in mice have led to inconsistent results. For example, the manifestations of autoimmunity are more severe in mice with deficiencies that are expected to reduce the ability of neutrophils to release NETs (7, 8). One possible explanation for the discordant results is the fact that mice and humans respond differently to citrullinated autoantigens (9).

NETosis, named so because it involves the release of NETs as part of a regulated, multi-step cell death characteristic of neutrophils, plays an important role in autoimmune diseases. In SLE, NETs released by activated neutrophils form lattices of decondensed chromatin fibers containing intact DNA filaments, histones, and neutrophil enzymes. Each of these macromolecules generate prominent (auto)immune Ab targets (10–13). Autoreactivity of NET components is enhanced by post-translational modifications, such as deimination of arginine residues (11, 12) in histones and other neutrophil proteins that enhance their immunogenicity (14, 15). Curiously, autophagy, a complex genetically-regulated mechanism involved in the cell survival/death balance, affects NETosis. It determines the efficiency of neutrophils for NET release, including in inflammatory disorders (16) and infectious diseases (17, 18). Despite clear evidence from different experimental systems, the precise autophagy regulators required for the execution of NETosis is somewhat controversial (19, 20).

Autophagy is a tightly regulated mechanism that allows cells to renew themselves through the lysosomal degradation of damaged organelles and of proteins, which are misfolded or produced in excess. It is functionally central in many compartments of the cell as it maintains homeostasis and plays important roles in the immune response that may be connected to NET formation (21–24). There are three major pathways that characterize bulk autophagy, namely macroautophagy, chaperone-mediated autophagy (CMA), and microautophagy. Other forms of autophagy exist that are more specific, for example mitophagy, which involves the selective degradation of mitochondria, lipophagy that results in degradation of lipids, and xenophagy, in elimination of invading pathogens. Recent publications describe multiple effects of autophagy defects in different autoimmune and inflammatory processes (25–30). At this stage, however, the data are still scarce and incomplete, and the molecular and cellular links between NETosis and autophagy remain largely unexplored (31–34). This question is central as inhibition of autophagy and ROS might prevent intracellular chromatin decondensation, a key step of NETosis.

To explore the relationships of NETosis, autophagy and mitochondria, we employed several biochemical and cellular approaches with both human cell lines and primary cells from patients. We exploited a peptide called P140 known to be rapidly endocytosed *via* a clathrin-mediated endocytosis (CME) pathway and ultimately accumulated into lysosomes of B cells after its intravenous administration into MRL/lpr lupus-prone mice (35). P140, a 21-mer phosphopeptide derived from the spliceosomal protein U1-70K, was found to act directly on CMA, which appears to be hyperactivated in MRL/lpr splenic B cells (35), and most likely indirectly, on the macroautophagy process (36–38), which also shows higher activity in both T and B cells in murine lupus (39–42). As a close functional link between lysosomes (where CMA is active) and mitochondria has been suggested (3, 43, 44), it was important to first examine whether P140 peptide could alter functions of mitochondria and modulate mitophagy. Our data show that P140 inhibits some of mitochondrial properties but has no effect on mitophagy, indicating the selectivity of P140 peptide for CMA (and macroautophagy). Regarding the effect of P140 on NETosis, we did observe that P140 suppresses NET release from neutrophils stimulated with autoimmune NET-IC (NICs). Thus, in an autoimmune context, P140 could decrease NET release and dampen the exposure of nuclear autoantigens, therefore attenuating immune responses to self-antigens, an observation we made previously both in mice and patients with lupus. Our results, therefore, highlight the importance of the mitochondrion-independent pathway in NETosis, which seems to be more specially modulated by P140. The exact target of P140 in this pathway remains to be identified.

MATERIALS AND METHODS

Peptides

The P140 (RIHMVYSKRpSGKPRGYAFIEY), scrambled (Sc) P140 (YVSRYFGpSAIRHEPKMKIYRG) phosphopeptides (pS standing for phosphoserine residues), and non-phosphorylated peptide 131–151 (RIHMVYSKRSGKPRGYAFIEY) were synthesized as described previously (45). Peptides homogeneity was checked by analytical high-performance liquid chromatography and their identity was assessed by mass spectrometry.

Live Imaging Analysis by Spinning Disk Confocal Microscopy

MRL/N-1 fibroblastoid cells established from the spleens of MRL/MpTn-*gld/gld* mice (46) were incubated with 10 μM AF633-P140 for 4 h followed by staining with 100 nM LysoTracker Green at 37°C for 5 min, or cells were incubated with 10 μM AF488-P140 for 4 h followed by staining with 50 nM

MitoTracker DeepRed at 37°C for 20 min. Stained cells were washed three times with phosphate-buffered saline (PBS) pH 7.4, and imaged immediately with a spinning-disc confocal microscope consisting of a CSU confocal spinning disk unit (Yokogawa), an EMCCD Evolve camera (Roper Scientific), mounted on an Axio Observer Z1 microscope (Zeiss) at 37°C with 5% CO_2 supply. Both LysoTracker Green and MitoTracker DeepRed were purchased from ThermoFisher Scientific.

Primary Fibroblast Cultures

For analyzing the effect of P140 on mitophagy, primary fibroblasts established from skin biopsies obtained from two symptomatic patients harboring different loads (both <40%) of the m.3243A > G "MELAS" (mitochondrial encephalopathy with lactic acidosis and stroke episodes) mutation (47) were tested with informed consent of patients and the approval from the UK National Research Ethics Service. These two patients are identified here as P1 and P2. MELAS is a rare progressive multisystemic disorder that particularly affects the brain and nervous system (associated to neurological and psychiatric manifestations), and muscles, with onset typically in childhood. Patients also develop endocrinopathy, heart disease, diabetes, and hearing loss. We also used a control from a panel of 22 anonymized control fibroblast cultures established either with parental consent from children undergoing diagnostic skin biopsy for karyotyping and whose cytogenetic markers were normal ($n = 10$) or from healthy consented adults aged 18–81 years ($n = 12$). The control used was close to the median for all functional tests carried out.

Effect of P140 on Mitochondria Purified From Raji Cells

Raji B cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco) supplemented with 10% (v/v) fetal calf serum (FCS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and gentamycin. The medium was changed every 2–3 days and cells were split when reaching 2-3 $\times$ 10^6 viable cells/mL. The experiments were done using a cell density of 1 $\times$ 10^6 Raji B cells/mL. Cell survival was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) cell proliferation colorimetric assay kit from Abcam. Mitochondria from Raji cells were isolated as described previously (48) and re-suspended in homogenization buffer (300 mM sucrose, 5 mM 2-[Tris(hydroxymethyl)-methylamino]-ethanesulfonic acid/TES buffer pH 7.2, 0.2 mM ethylene glycol tetraacetic acid, 1 mg/mL BSA). Mitochondrial swelling and $\Delta\Psi$m were evaluated as described (48, 49) in the presence of succinate and rotenone. Mitochondria were incubated with or without P140 or ScP140 peptides during 5 min before absorbance at 545 nm (swelling) and Rh123 fluorescence ($\Delta\Psi$m, $\lambda_{Excitation}$ 485 nm, $\lambda_{Emission}$ 535 nm) recording during 45 min using a fluorescence multi-well plate reader (Infinite, Tecan®). Calcium ($CaCl_2$; 50 μM) and carbonyl cyanide 3-chlorophenylhydrazone (50 μM) were used as the 100% baseline for swelling and $\Delta\Psi$m loss respectively. Oxygen consumption was monitored as described (49) in the presence of the O_2 sensitive dye MitoXpress® (LUXCEL). Mitochondria were incubated with or without P140 or ScP140 during 5 min before the measure of oxygen consumption during 45 min in 96-well plates using a spectrofluorimeter (Infinite® 200; $\lambda_{Excitation}$ 380 nm, $\lambda_{Emission}$ 650 nm). Rotenone (2 μM) and oligomycin A (1 μM) were used as 0% baseline for oxygen consumption driven by complex I and complex II respectively. The areas under curve were used for calculations. For measuring ATP production, 2 μg of isolated mitochondria were pre-incubated with or without P140 or ScP140 during 5 min in the presence of 25 mM succinate, 2 μM rotenone and 1.65 mM ADP in oxygen consumption buffer (49) ATP produced during this time was monitored using the ApoSENSOR kit (BioVision) by spectrofluorimeter (Infinite® 200; luminescence detection). Results were expressed in percent of ATP production after normalization by positive (untreated cells; 100%) and negative (25 mM malonate; 0%) controls. For studying mitochondrial localization, 20 μg of isolated mitochondria were incubated with or without AF488-peptides P140 or ScP140 during 5 min at 37°C before washes with homogenization buffer at pH 11.6 and centrifugation at 10,000 g for 10 min. The pellet was next resuspended in homogenization buffer at pH 7.2 before analysis by flow cytometry (FACSCalibur; $\lambda_{Excitation}$ 488 nm, $\lambda_{Emission}$ 530 nm).

Evaluation of Mitophagy

Primary fibroblast from control individuals and patients (see above) were seeded in a 96-well plate at a density of 2,500 cells/well. Next day the media was changed for regular Dulbecco's Modified Eagle's medium (DMEM) containing 25 mM glucose or glucose-free galactose media (0 mM glucose, 10 mM galactose). P140 and control ScP140 peptide were given to cells at a concentration of 40 μM for 30 min, 2, 6, 16, and 24 h before the cells were fixed with 4% (v/v) paraformaldehyde (PFA) for 15 min at room temperature (RT). The cells were then immunostained using Abs raised against transporter outer membrane (TOM) 20 (mouse, 1/200; Santa Cruz, sc6341) and microtubule-associated protein 1A/1B-light chain 3B (MAP1LC3B; rabbit, 1/500; Caltag Medsystems, PM036) and revealed with secondary Abs anti-mouse AF546 and anti-rabbit AF488, diluted 1/200 and 1/400, respectively. Nuclei were stained using 4′,6-diamidino-2-phenylindole (DAPI). Cells were then imaged using the IN Cell 1000 analyzer (GE Healthcare), 9 fields of view per well and the images analyzed using a development of a specialized Developer toolbox protocol (50). For the mitochondrial DNA experiments, the fixation step was replaced by a staining using PicoGreen solution (Molecular Probes Inc.) diluted at 3 μL/mL directly into cell culture medium and tetramethyl rhodamine methyl ester, a cell-permeant, cationic, red-orange fluorescent dye (25 nM final concentration), for 45 min in an incubator. The media was washed away and replaced with fresh media and the cells imaged and analyzed using the IN Cell 1000 analyzer and Developer toolbox.

Evaluation of Autophagy Processes in Neuronal Cells

Adherent U-251 MG cells (ECACC, ref. 0906300) were selected for this study as a model. Cells were maintained in DMEM

(ThermoFisher, ref. 41965-039), containing 10% (v/v) FCS (ref. 26140-079), 100 units/mL of penicillin and 100 μg/mL of streptomycin (ref. 15140-122) all from ThermoFisher. Cells were subcultured after reaching 70–80% confluency and their doubling time was 22 h (51). For studying the effect of P140 and ScP140 peptides on autophagy levels, cells were seeded at 0.2×10^6 cells per 12-well plate well and incubated for 16 h at 37°C. They were treated or not for 8 h with 10 μM P140. To measure the autophagic flux, half of P140-samples and controls were incubated with 5 μg/mL of each pepstatin A, a potent inhibitor of aspartyl proteases (Merck, 5318) and E-64d/Aloxistatin, a pan-cysteine cathepsin inhibitor (Merck, E8640). Cells were lyzed in RIPA buffer, pH 7.6 (ThermoFisher, ref. 89900), transferred in Laemmli buffer for analysis on SDS-PAGE, and analyzed by western immunoblotting (30). Ten μg protein was loaded per lane as quantified by bicinchoninic acid assay. The conditions used to measure autophagy markers were as described (30) using Abs to MAP1LC3 (MBL, M186-3; 0.5 μg/mL), sequestosome-1/p62 (SQSTM1; Abcam, ab109012; 0.5 μg/mL), B-cell lymphoma (BCL)-2 interacting myosin/moesin-like coiled-coil protein 1 (BECLIN-1; Abcam, ab207612; 0.5 μg/mL), autophagy-related protein (ATG) 12/5 (Abcam, ab155589; 1 μg/mL), lysosome-associated membrane protein-2A (LAMP2A; Abcam, ab18528; 1 μg/mL) and phosphatase and tensin homolog deleted on chromosome 10 (PTEN)-induced putative kinase 1 (PINK1; Abcam, ab75487; 1 μg/mL). To avoid quantification mistakes resulting from the fact that a loading control protein could represent a substrate for autophagy, normalization of blots was done using stain-free technology (total protein lane content).

Isolation of Human Peripheral Blood Neutrophils

Neutrophils were isolated from heparinized blood from healthy donors, in accord with protocols approved by the University of Tennessee Institutional Review Board, and isolated following published methods (52). Briefly, neutrophils were purified at RT, enriched by Isolymph gravity sedimentation, and recovered in the pellet of an Isolymph density gradient (CTL Scientific Supplies; ref. 759050) under endotoxin-free conditions. The contaminating erythrocytes were lysed in ice-cold hypotonic (0.2%, w/v) sodium chloride solution for 30 s, at which point the solution was rendered physiologic saline by addition of hypertonic (1.6%, w/v) sodium chloride. The neutrophils were rinsed once in Hanks' balanced salt solution (HBSS; without calcium or magnesium and with 10 mM HEPES) and re-suspended at 2×10^6 cells/mL in the same buffer. Neutrophil viability was found to be 98% by Trypan blue dye exclusion.

Preparation of Immobilized Immune Complexes (ICS)

Plate-bound ICs were prepared by coating wells of 96-well black tissue culture plates (ThermoFischer; ref. 165305) with purified monoclonal Ab 3H9 at 5 μg/mL in PBS overnight at 4°C. The 3H9 mouse monoclonal IgG was grown and purified from culture supernatants by Protein A beads, as described previously (52). The next day, plates were washed once with PBS and blocked with 2% (w/v) IgG- and protease-free BSA (Jackson ImmunoResearch Labs; ref. 001-000-161) and 10 μg/mL poly-L-Lysine in PBS for 1 h at RT, followed by 2 washes with PBS. NETs, prepared from healthy human neutrophils by incubation with hydroxyapatite and isolated according to previously described procedures (53), were added to the wells at a concentration of 2 μg/mL protein and 4 μg/mL DNA. Following incubation for 1 h at RT, the plates were washed 3 times with HBSS.

Effect of P140 on NETS Release From Human Neutrophils

Freshly isolated human neutrophils were incubated for 15 min at RT with different concentrations of P140 or control peptides in HBSS without Ca/Mg but containing 10 mM HEPES. Neutrophil-peptide suspensions were then transferred to wells of IC-coated plates, and cells were allowed to settle at the bottom of the wells for 10 min at 37°C in a CO_2 incubator. Fifty μL of HBSS with HEPES and Ca/Mg were added to the wells. NET release was measured by fluorescence of 2.5 μM cell-impermeable Sytox Green (Molecular Probes; ref. S7020) in a Synergy plate reader (BioTek Instruments, set for excitation at 488 nm and emission at 510 nm).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 5.0. Depending on the number of samples that were included in the analyses, and the distribution of data, statistical significances were assessed using the parametric, Student's *t*-test or the non-parametric Mann-Whitney's test. For comparing the significance of data obtained with P140 and ScP140, *P* values were determined by using one-way ANOVA. $P < 0.05$ were considered significant.

RESULTS

Effect of P140 Peptide on Isolated Mitochondria

Mitophagy is essential for the degradation of damaged mitochondria and therefore occurs constantly at a basal level. Mitochondrial dysfunction is known in lupus, notably in patient's T cells (persistent mitochondrial hyperpolarization, cytoplasmic alkalization, increased reactive oxygen intermediates production, and diminished levels of intracellular glutathione and ATP) (54–56). Although the mammalian target of rapamycin (mTOR), a sensor of mitochondrial homeostasis in T cells, is activated in SLE patients, blockade of mTOR with rapamycin, for example, incompletely reverses mitochondrial hyperpolarization and fails to correct accumulation of mitochondria, suggesting that mitochondrial dysfunction occurs upstream of mTOR activation in SLE (57). In this context, we therefore performed a series of experiments to determine whether P140 peptide interacts with purified mitochondria and if it could have some effect on mitophagy, in parallel to its effects on lysosomes and CMA/macroautophagy (35, 36, 38).

In a dose-dependent manner, AF488-labeled P140 peptide was detectable at higher levels than the ScP140 control in

mitochondria purified from Raji cells (**Figure 1A**). This marked rise of fluorescence was observed even after washing at pH 11.6, which removes components non-specifically bound to the mitochondrial outer membrane, leading us to conclude that the binding of P140 to mitochondria was both strong and specific. P140 (but not ScP140) also affected respiratory capacity of mitochondria. It strongly increased oxygen consumption driven by both succinate oxidation (complex II) and malate and glutamate oxidation (complex I) (**Figures 1B,C**). This stimulation of oxygen consumption was associated with a strong perturbation of mitochondrial potential, without any swelling and significant modification of ATP production (**Figures 1D,E**). P140 may therefore perturb proton and/or electron transfer through mitochondrial inner membrane, leading to potential loss and oxidative phosphorylation uncoupling, hence triggering an important stimulation of oxygen consumption, while ATP production remains stable during the time of the measure. There was no impact of P140 tested in a concentration range of 0 to 100 μM on cell survival as measured by MTS cell proliferation colorimetric assay at 72 h on Raji B-cell lymphoma line (not shown).

Effect of P140 Peptide on Mitophagy

We then sought to assess the effect of P140 on mitophagy. This was done using a high-throughput fluorescence microscopy quantitative assay (IN Cell 1000), which was developed to screen drugs that might modulate mitophagy (50). Because baseline autophagy and mitophagy are increased in cells with mitochondrial dysfunction, we used skin fibroblasts from one control and two patients suffering from MELAS [rare syndrome associated with mitochondrial encephalomyoapthy and psychiatric disorders (47)], one who was mildly affected and the other who was significantly sick. Similar autophagosome counts were registered in the presence of P140 and ScP140 (**Figure 2**; **Supplementary Figures S1–S3**), leading to the conclusion that P140 peptide displays no specific effect on mitophagy. We also excluded a significant P140 effect on mitophagy, using co-localization of mitochondrial fragments and autophagosomes, or on mitochondrial length.

Taken together, our results support the view that P140 exerts some effects on isolated mitochondria from Raji B cells by influencing transmembrane potential and oxygen consumption but has no impact on mitophagy in patient's fibroblasts.

Effect of P140 on Macroautophagy and CMA Processes in Neuronal Cells

To extend our observation on macroautophagy and CMA of P140 in neuronal cells, we selected U-251 MG cells, formerly known as U-373 MG, as a model. This human astrocytoma cell line was initially derived from a malignant glioblastoma tumor by explant technique. These cells are supposed to display some immune functions, notably as antigen-presenting cells, and continuously express glial fibrillary acidic protein, a prototypical marker of astrocytes (58) and a CMA regulator (59). Astrocytes produce a wide range of pro- and anti-inflammatory cytokines and seem to play complex roles in autoimmune inflammatory disorders, e.g., in multiple sclerosis and neuropsychiatric lupus (58, 60, 61). Astrocyte-expressed cytokines can exert potent suppressive effects on inflammatory cells (62).

The autophagic flux as measured by the MAP1LC3-II expression in U-251 MG cells in the presence or absence of anti-proteases was moderate in the absence of any autophagy activator or conditions of nutrient deprivation (**Figures 3A,B**). It was found to depend on the number of cell divisions with a more active flux in cells generated after several division cycles compared to cells, which had a low number of divisions in culture medium (**Figure 3B**). In these basal conditions, and in both cases, P140 had no detectable effect on the autophagic flux. These results were confirmed by measuring the expression levels of another macroautophagy-linked protein, SQSTM1 (**Figure 3B**). P140 had also no effect on the accumulation of MAP1LC3-I (**Figure 3C**). Likewise, the expression of ATG5/12, which is involved in the extension of the phagophore membrane in autophagic vesicles, was not affected by P140 (**Figure 3C**). However, the expression level of BECLIN-1, which is involved in the very early stages of autophagosome formation (63) was significantly increased ($P = 0.0023$; **Figure 3C**).

The possible effect of P140 on other forms of autophagy was also examined in U-251 MG cells. CMA activity was evaluated by measuring the expression of LAMP2A by western blot (30) and found to be unaffected by P140 (**Figure 3C**). The expression of the mitochondrial membrane protein PINK1 was not affected either in U-251 MG cell cultures that were treated or not by P140 peptide (**Figure 4A**). This absence of P140 effect was confirmed by measuring PINK1 expression in MRL/lpr splenocytes (**Figure 4B**).

Together these results support the view that P140 is able to affect basal macroautophagy in astrocytoma cell line U-251 MG cells, while it has no effect on mitophagy. This conclusion was reinforced by live-cell imaging experiments using laser scanning confocal microscopy and fluorescent labeled P140. Confocal images of MRL/N-1 cells treated *in vitro* with fluorescent P140 for 4 h showed that the peptide readily co-localized with lysosomal vesicles revealed by LysoTracker Green staining (**Figure 5A**) but not with mitochondria visualized with mitoTracker DeepRed dye (**Figure 5B**).

Effect of P140 on NETosis

To examine the effect of P140 on NET release, we developed an assay in which healthy human neutrophils are stimulated by incubation with immune complexes formed between a murine anti-DNA monoclonal Ab, 3H9, and purified NETs. Immobilized NICs elicited NET release above the levels of 3H9 alone and the observed NET release approached levels elicited by lipopolysaccharide (LPS) and N-Formylmethionyl-leucyl-phenylalanine (fMLP), used here as internal controls (64). fMLP is a peptide that is generated by proteolysis of bacterial polypeptides and induces neutrophil chemotaxis and degranulation. Pre-incubation of neutrophils with P140 suppressed NIC-induced NET release in a dose-dependent manner, whereas the ScP140 showed no such effect over the same concentration range (**Figure 6**). The P140 peptide concentrations that inhibited NET release in response to NIC stimulation were ineffective as inhibitors of fMLP/LPS (not shown) or phorbol

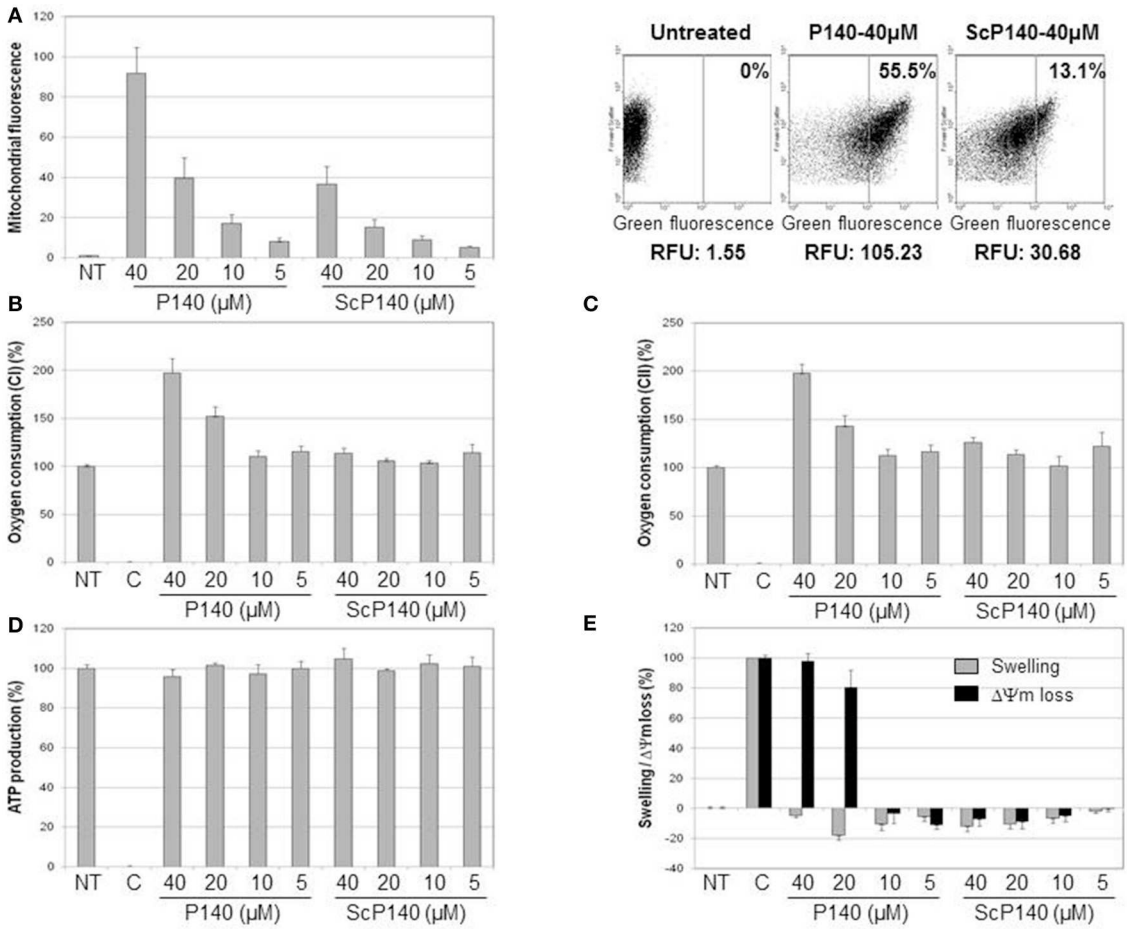

FIGURE 1 | Direct effect of P140 peptide on mitochondria isolated from Raji cell line. **(A)** Mitochondrial targeting was estimated by flow cytometry after incubation of increasing concentrations of AF488-P140 and ScP140 peptides with isolated mitochondria. Left panel: mitochondrial labeling was expressed in fluorescence ratio in function of mitochondrial basal fluorescence (Untreated; mitochondria without peptide). Right panel: relative mitochondrial fluorescence with or without incubation with AF488-peptides. The percentage of highly labeled mitochondria (right section) and relative fluorescence unit were indicated for each sample. **(B,C)** Oxygen consumption in the presence of malate, glutamate and ADP **(B)** or succinate **(C)** was measured with or without increasing concentrations of P140 and ScP140 peptides. Results were normalized on both untreated mitochondria (100% of oxygen consumption) and inhibition control (rotenone for complex I and oligomycin A for complex II; 0% of oxygen consumption). **(D)** ATP production of mitochondria in the presence of succinate and ADP was measured with or without increasing doses of P140 and ScP140 peptides. Results were normalized on both untreated mitochondria (100% of ATP production) and inhibition control (malonate; 0% of ATP production). **(E)** Mitochondrial swelling (gray bars) and potential loss ($\Delta\Psi$m loss; black bars) were measured with or without increasing concentrations of P140 and ScP140 peptides. Results were normalized on both untreated mitochondria (0% of swelling and $\Delta\Psi$m loss) and control (Triton for 100% optical density decrease and carbonyl cyanide 3-chlorophenylhydrazone for 100% $\Delta\Psi$m loss).

myristate acetate/ionomycin (33)-stimulated NET release under conditions used for the positive control (**Figure 6**).

DISCUSSION

Our previous studies demonstrated that following intravenous injection into lupus-prone MRL/lpr mice, P140 enters B cells through CME, and homes into lysosomes where it potentially blocks lupus-related hyperactivated CMA (35, 38). *In vitro*, P140 destabilizes the complex formed by CMA chaperones heat shock protein (HSP)A8 and HSP90, and cofactors (35). By reducing excessive CMA and macroautophagy, P140 peptide may alter antigen presentation (65) and lead to a reduced stimulation of autoreactive T cells. Reduction of MHC expression and autoreactive T cell hyporesponsiveness was effectively observed in P140-treated MRL/lpr mice and human P140-treated peripheral blood cells (36, 66–68). In the salivary glands of MRL/lpr mice, similar to what was observed in the spleen, the therapeutic CMA-regulator P140 peptide reduced the abnormally raised lysosomal pH and rescued the altered autophagy functions (69).

Our present findings show that P140 does not alter the autophagic flux measured in human astrocytoma U-251 MG cells in basal culture conditions and in the absence of any overt stress or stimulus. The expression levels of several components of the macroautophagy and CMA processes, namely MAP1LC3, SQSTM1, ATG12/5, and LAMP2A, were not affected by peptide

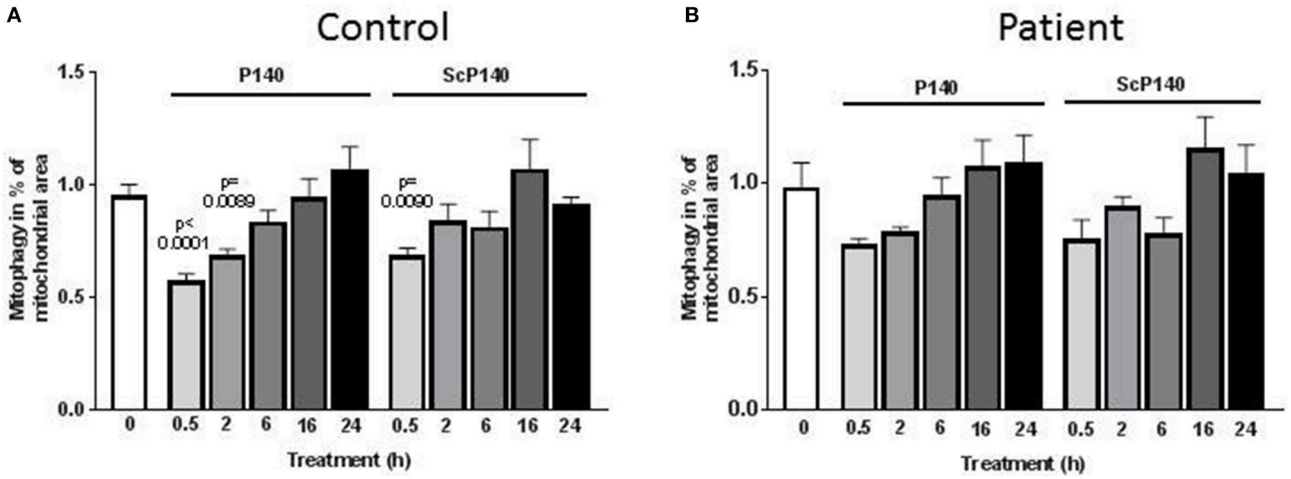

FIGURE 2 | Effect of P140 on mitophagy. Control **(A)** and patient's **(B)** fibroblasts (harboring the m.3243G>A mitochondrial DNA mutation) were cultured in 96-well plates in high-glucose media. They were exposed to P140 or ScP140 peptides (40 μM final concentration) for 30 min to 24 h. MAP1LC3-positive puncta counts were evaluated by imaging cells using the INCell 100 analyzer. Mitophagy was determined as the co-localization of mitochondria (stained with anti-TOM20 Ab) and autophagosomes (stained with anti-MAP1LC3 Ab). A minimum of 275 cells were analyzed per condition. Error bars are standard error of the mean (SEM). *P* values are expressed vs. the time 0.

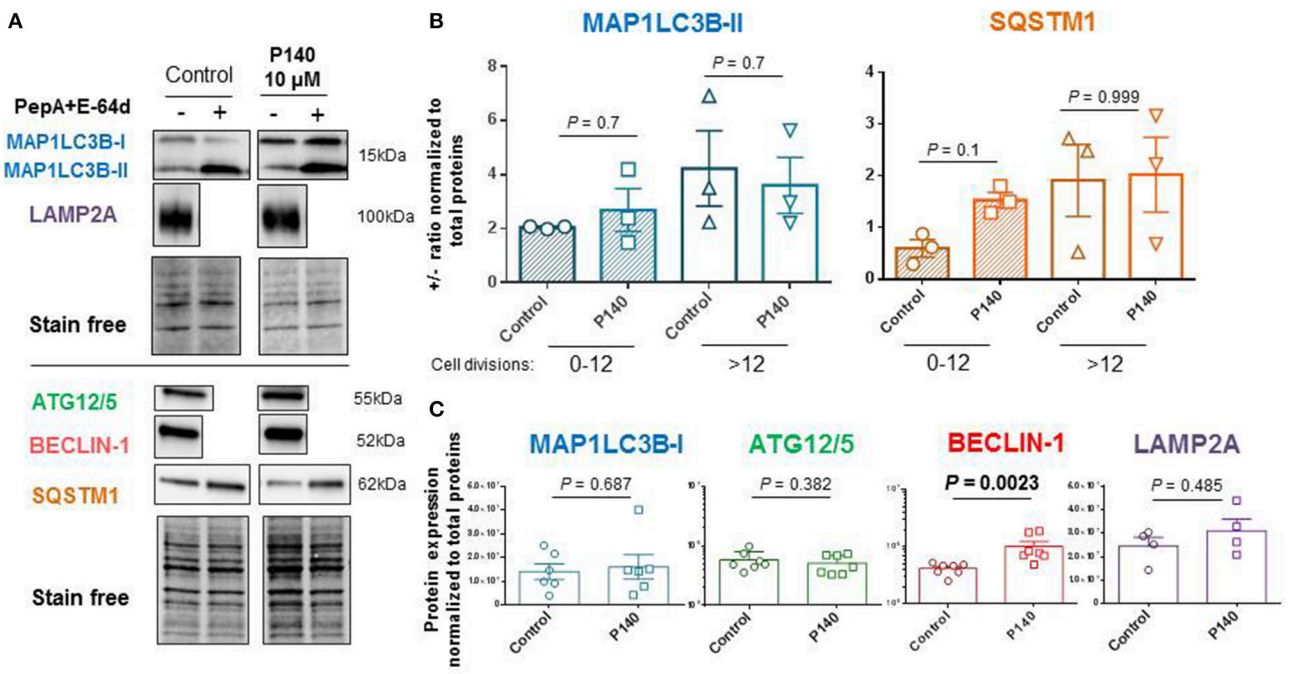

FIGURE 3 | Effects of P140 on autophagy in U-251 MG glioblastoma cells. U-251 MG cells were treated or not with 10 μM P140 for 8 h in the presence or absence of lysosomal enzyme inhibitors pepstatin A (PepA) and E64D. Protein lysates were separated on 4–20% SDS-gels and then transferred onto nitrocellulose membrane. **(A)** Proteins were revealed with Abs to ATG12/5, MAP1LC3B, SQSTM1, BECLIN-1, and LAMP2A. Comparisons were made between untreated and P140-treated U-251 MG cells. Normalization was performed by measuring total protein directly on the membrane (stain-free procedure). **(B)** Autophagic markers MAP1LC3B and SQSTM1 measured in the absence and presence of anti-proteases as indicators of flux. The effect of P140 peptide on the flux intensity is shown. The results are displayed with cells collected after 1–12 cycles of divisions or after a longer period of more than 12–20 cycles of division in culture medium. **(C)** Effect of P140 on ATG12/5, BECLIN-1, and LAMP2A protein expression. Error bars are SEM. Each sample was tested in triplicates or quadruplets in at least 3 independent experiments (1 point corresponds to the mean value of replicates). *P* values are from Mann-Whitney U tests.

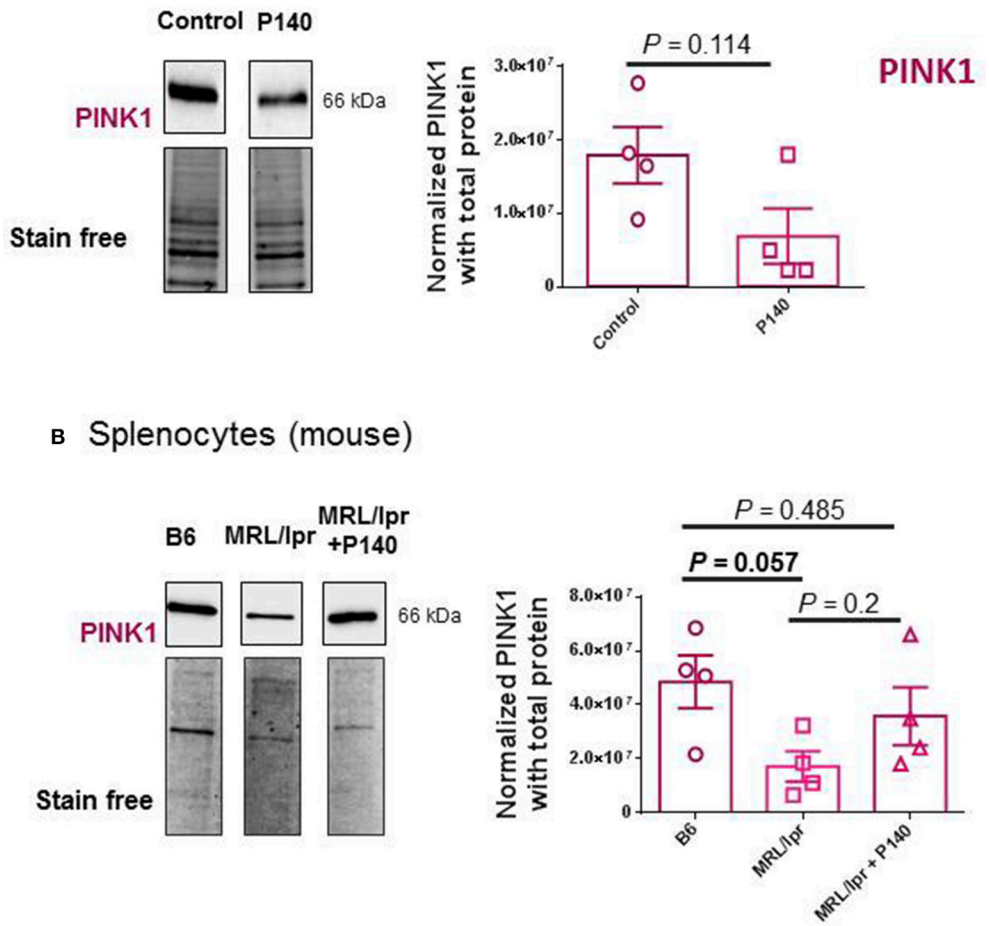

FIGURE 4 | Effects of P140 on PINK1 expression. PINK1 expression was measured by western blotting in P140-treated and non-treated human U-251 MG cells **(A)** and in splenocytes collected from untreated normal B6 mice and P140-treated or non-treated MRL/lpr mice **(B)**. The experimental conditions were as in **Figure 3** using PINK1 antibodies. Each sample was tested in quadruplets in 4 independent experiments. Error bars are SEM. *P* values are from Mann-Whitney U tests.

treatment. However, the expression of BECLIN-1, a mammalian homolog of yeast Vps30/Atg6, was raised in an impressive manner. This result is important because BECLIN-1, which forms part of the class III PtdIns3K complex that also contains VPS34, VPS15, and ATG14 is involved in the initial steps of autophagosome formation and is a central regulator of autophagy (70–72). BECLIN-1 deficiency has been characterized in several pathological conditions and enhancing autophagy, for example with the Tat-BECLIN-1 construct, has been presented as a potential route for valuable therapeutic applications, notably in neurodegenerative diseases (63, 73, 74).

The results presented here further suggest that in contrast to its effect on CMA (shown in MRL/lpr primary spleen cells) and macroautophagy, P140 does not affect mitophagy, although a significant functional effect of P140 could be visualized *in vitro* when isolated mitochondria were used. This finding is corroborated by microscopy observations indicating that labeled-P140 co-localizes with lysosomes but not with mitochondria.

To explore further the effects of P140 and control peptides on innate immune cells, we developed a fluorescence assay that measures NETs released into the culture supernatants in response to NICs formed with 3H9, a murine anti-DNA and anti-chromatin IgG (52). Healthy human neutrophils release NETs when exposed to NICs composed of purified NETs and 3H9. The NET release was attenuated in neutrophils incubated with P140, yet remained unperturbed by control ScP140 peptide, consistent with the view that NET release is regulated, in part, by autophagy. The observation that P140 drastically affects NETs release but has little or no detectable effect on mitochondria and mitophagy, highlights the importance of a NETosis pathway that would be mitochondrion-independent and modulated by the P140 peptide. The exact target of P140 in this mitochondrion-independent pathway remains to be identified. Hopefully, our

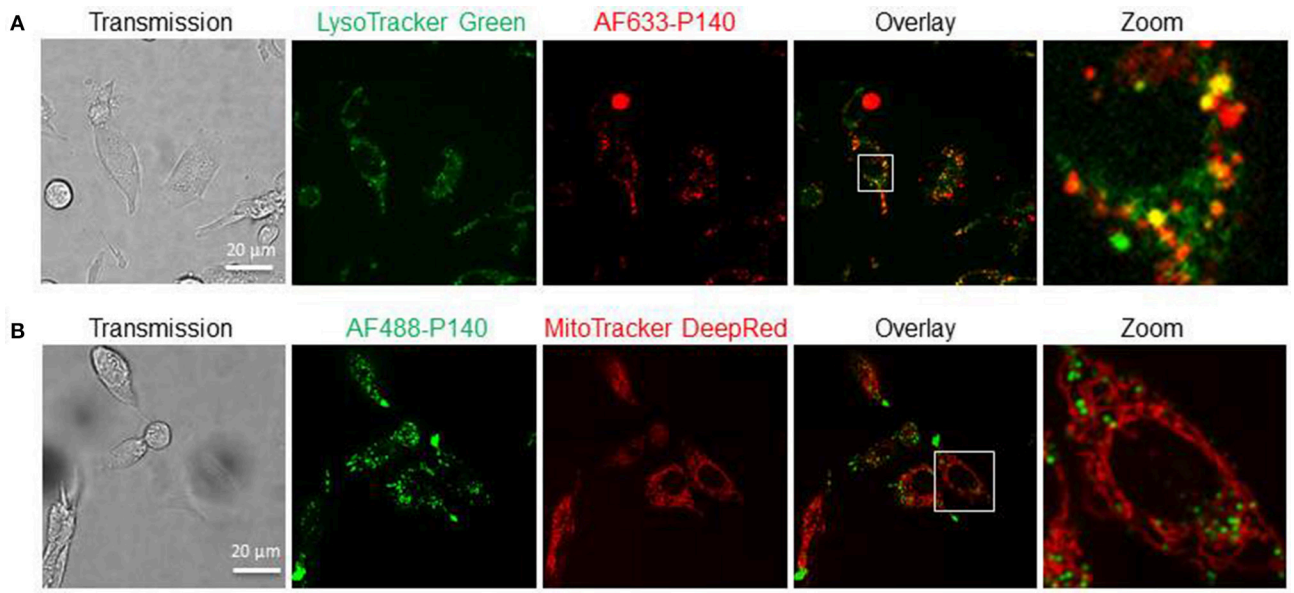

FIGURE 5 | Cellular localization of P140 peptide. Confocal images of MRL/N-1 cells treated with 10 µM AF633-P140 for 4 h and stained with LysoTracker Green **(A)**, or with 10 µM AF488-P140 for 4 h and stained with Mito Tracker DeepRed **(B)**. Images are analyzed in Image J (NIH). A magnification of the images is shown in right panels (zoom).

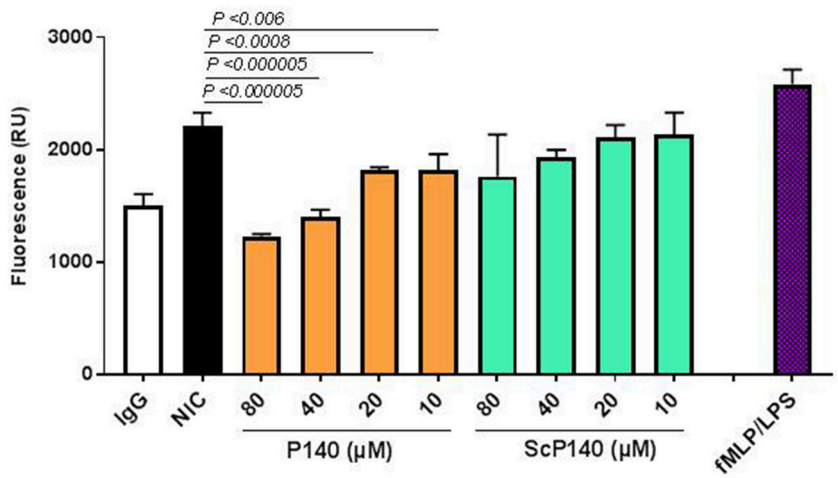

FIGURE 6 | NIC-induced NET release from neutrophils incubated with P140 or control peptides. The NETs released in response to immobilized anti-DNA IgG 3H9 and purified NETs were quantified by fluorescence (expressed as relative units, RU). Readings were recorded at 120 min from neutrophils incubated without peptide, neutrophils incubated with LPS and fMLP (as positive control), and neutrophils that were incubated with increasing concentrations of P140 or ScP140. This set of experiment was performed at least five times with samples tested in triplicates in 96-well plates with consistent results. Error bars are SD. *P* values indicate probability thresholds for significant differences between NIC-incubated cells vs. cells pretreated with either P140 or control ScP140. *P* values were determined by using one-way ANOVA.

future investigations will allow us to understand why P140, as possibly other molecules, apparently affects only certain forms of NETosis. This might depend on different modes of NET release, based on different stimuli and differential requirements for signaling pathways (52, 75).

Given that the P140 sequence is derived from the U1-70K ribonucleoprotein, the central component of the spliceosome and a prominent autoantigen in lupus, it is conceivable that neutrophils have developed a sensitive mechanism for detecting cellular damage and the release of nuclear contents, as may occur during NETosis. Interestingly, the phosphorylation of Ser^{140} is observed in apoptotic conditions, while the whole U1-70K protein is largely dephosphorylated by PP1-type phosphatases (76). It is therefore plausible that other modifications in the

nominal sequence encompassing the RNA binding motif (present in the P140 peptide) provide cellular signals, which may fine tune the neutrophil response to cellular distress.

AUTHOR CONTRIBUTIONS

MB performed the macroautophagy and CMA experiments. IN developed and performed the NET-IC-stimulated NET release assays. FW performed live imaging analyses by spinning disk confocal microscopy. SB performed western blotting experiments. NB and AB-S performed the experiments on isolated mitochondria. ED and JP performed the mitophagy experiments. SM and MR conceived this study, contributed the study design, and wrote the manuscript with input of all other authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02158/full#supplementary-material

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Quantitative Proteomic Analysis Reveals That Arctigenin Alleviates Concanavalin A-Induced Hepatitis Through Suppressing Immune System and Regulating Autophagy

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Concanavalin A-induced autoimmune hepatitis is a well-established experimental model for immune-mediated liver injury. It has been widely used in the therapeutic studies of immune hepatitis. The in-depth analysis of dysregulated proteins from comparative proteomic results indicated that the activation of immune system resulted in the deregulation of autophagy. Follow-up studies validated that some immune related proteins, including Stat1, Pkr, Atg7, and Adrm1, were indeed upregulated. The accumulations of LC3B-II and p62 were confirmed by immunohistochemistry and Western blot analyses. Arctigenin pretreatment significantly alleviated the liver injury, as evidenced by biochemical and histopathological investigations, whose protective effects were comparable with Prednisone acetate and Cyclosporin A. Arctigenin pretreatment decreased the levels of IL-6 and IFN-γ, but increased the ones of IL-10. Next, the quantitative proteomic analysis demonstrated that ARC pretreatment suppressed the activation of immune system through the inhibition of IFN-γ signaling, when it downregulated the protein expressions of Stat1, P-Stat1, Pkr, P-Pkr, Bnip3, Beclin1, Atg7, LC3B, Adrm1, and p62. Meanwhile, Arctigenin pretreatment also reduced the gene expressions of Stat1, Pkr, and Atg7. These results suggested that Arctigenin alleviated autophagy as well as apoptosis through inhibiting IFN-γ/IL-6/Stat1 pathway and IL-6/Bnip3 pathway. In summary, the comparative proteomic analysis revealed that the activation of immune system led to Concanavalin A-induced hepatitis. Both autophagy and apoptosis had important clinical implications for the treatment of immune hepatitis. Arctigenin might exert great therapeutic potential in immune-mediated liver injury.

Keywords: arctigenin, concanavalin A, IFN-γ, Stat1, immune system, autophagy, apoptosis, proteomics

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INTRODUCTION

Concanavalin A (ConA) is a plant lectin from seeds of Jack beans (*Canavalia ensiformis*). Intravenous injection of ConA leads to $CD4^+$ T cell-mediated hepatitis in mice (1). The model might allow the study of the pathophysiology of self- or foreign antigen-mediated hepatic failure such as autoimmune hepatitis (AIH) and viral hepatitis (2). Some cytokines, including interferon (IFN)-γ and tumor necrosis factor (TNF)-α (3, 4), might participate in this process (3, 4). It has been shown that the alleviation of Con A-induced hepatitis was observed in IFN-$\gamma^{-/-}$ mice but not in TNF-$\alpha^{-/-}$ mice, suggesting that IFN-γ rather than TNF-α is a key regulator in Con A-induced liver injury (5). During viral infections, the antiviral effect of IFN-γ was stronger and more durable than the ones of IFN-α and IFN-β (6). The class of genes and proteins, which were predominantly upregulated by IFN-γ, were directly involved in the activation of the immune system, including antigen processing and presentation, recruitment of T cells and attack against the virus-infected hepatocytes (7). The killing of virus is accompanied with the apoptosis of liver cells, and apoptosis is the main mechanism of hepatitis (8, 9).

IFN-γ mediates apoptosis *via* activation of Stat1 (10, 11). IFN-γ activates Stat1 to form homodimers that bind to IFN-γ activated sequence (GAS) on the promoter, so as to activate IFN-γ-induced gene expression (12). Transgenic mice overexpressing Stat1 showed elevated levels of IFN-γ and significantly aggravated liver injury after ConA administration, while Con A-induced liver injury hardly occurred in Stat1$^{-/-}$ mice (13). This is due to Stat1-mediated upregulation of IFN-γ, which enhances the production of chemokines, adhesion molecules, and ROS (14, 15). As reported, autophagy can facilitate IFN-γ-induced cellular inflammation *via* the activation of Jak2–Stat1 signaling (16). Atg5 deficiency extremely inhibited the IFN-γ-induced pro-inflammatory responses (17).

Autophagy is a highly conserved lysosomal degradation pathway that regulates cellular homeostasis and disposes intracellular pathogens in eukaryotic cells (18, 19). The stages of autophagy include induction, phagophore formation, autophagosome formation and maturation, autolysosome formation, and final degradation (20). Autophagy is initiated by the activation of the unc-51-like kinase 1 (ULK1; Atg1 in yeast) complex. Then, PI3K (Vps34 in yeast), beclin 1 (Atg6 in yeast), VPS15 (PIK3R4), and Atg14L (Atg14) form the class III phosphatidylinositol 3-kinase complex to trigger vesicle nucleation. The Atg12-Atg5-Atg16 complex promotes phagophore elongation by conjugation of Atg8 to phosphatidylethanolamine (PE). The process is mediated by Atg7 and Atg3. LC3 (Atg8 in yeast) is widely used a marker for autophagosomes. During autophagy, LC3 is processed by the removal of the C-terminal 22 amino acids to form LC3-I, followed by conjugation with PE to become LC3-II. The amount of LC3-II is widely used for the quantification of autophagosome formation (21). Once the autophagosome was formed or enhanced, a blockage of autophagic flux at late steps will downregulate the clearance of autophagosomes, as reflected by the accumulation of the autophagic substrate SQSTM1/p62. A blockage of autophagic flux finally results in autophagy-dependent cell death (22).

Evidences showed that accumulation of autophagosomes were easily observed under the electron microscope in ConA-induced hepatitis mice model, and the upregulation of Beclin1 and LC3 confirmed the observation stated above (23–25). Autophagic cell death can be observed in hepatocytes as well as endothelial cells (26). The deregulated autophagy was also found in biliary epithelial lesion in primary biliary cirrhosis (27). The inhibition of autophagy may represent a new therapy for AIH. Many natural products were reported to attenuate liver injury by suppressing autophagy as well as apoptosis in ConA-induced hepatitis, such as quercetin (24), astaxanthin (25), shikonin (28), epigallocatechin-3-gallate (29), and resveratrol (30).

Arctigenin (ARC), a phenylpropanoid dibenzylbutyrolactone lignin, is a biologically active lignan extracted from the seeds of *Arctium lappa* L. (Compositae). ARC was shown to have distinct antioxidant and anti-inflammatory properties. It exhibited protective properties on several inflammatory diseases, including brain damage (31), neurotoxicity (32–34), cardiovascular diseases (35), kidney injury (36), colitis (37), encephalomyelitis (38), asthma (39), and lung injury (40). ARC can inhibit the T lymphocyte proliferation, Th17 differentiation, macrophage activation and the release of pro-inflammation cytokines (37, 41, 42). The anti-inflammation mechanisms of ARC included the suppressions of MAPK, NF-κB, Stat1 and iNOS *via* the activation of AMPK (40, 42, 43). All these data suggest that ARC is likely to be used as a regulator of immune-mediated diseases. In addition, ARC has potent antivirus activity *in vitro* and *in vivo* (44–46). However, to our knowledge, the potential protective effects of ARC have not been evaluated in immune-mediated hepatitis *in vivo*. The autophagy involved in pathogenesis of ConA-induced hepatitis, there is still need evidence to confirm this. The proteomic methods were extremely valuable to study organ responses and to elucidate mechanism of disease and drugs by monitoring the changes of protein expressions (47). The quantitative proteomic analysis using ^{12}C6- or ^{13}C6-NBS (2-nitrobenzenesulfenyl) labeling followed by MALDI-TOF mass spectrometric analysis has shown its superiority for the scope and accuracy of mass spectrometry-based proteomics studies (48). Thus, quantitative proteomic analysis based on NBS labeling of peptides and nano-LC/MALDI-TOF MS were used to elucidate the mechanism of ConA-induced hepatitis. On the basis of proteomic data, the mechanisms hidden behind the protective effects of ARC on hepatitis were further investigated.

MATERIALS AND METHODS

Materials

Arctigenin was provided by State Key Laboratory, Generic Manufacture Technology of Chinese Traditional Medicine, Lunan Pharmaceutical Group, purity >99%, white powder. MCT, HS15, labrasol, and transcutol were provided by BASF. ConA was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-Apg7 antibody (ab133528), Anti-SQSTM1/p62 antibody (ab91526), and Anti-ADRM1 antibody [EPR11450(B)] (ab157218) was purchased from Abcam, USA. The antibodies against LC3B, Beclin1, Stat1, p-Stat1 (Y701), Pkr, p-Pkr (T446), and Bnip3 were

from Immunoway, USA. Cyclosporin A oral solution (CSA) was provided by Lunan Houpu Pharmaceutical Co., Ltd. Prednisone acetate (PS) was purchased from Zhejiang Xianju Pharmaceutical Co., Ltd.

Animals and Experimental Design

Female Balb/c mice weighing between 18 and 22 g (5–6 weeks old) were purchased from Vital River Laboratory Animal Co., Ltd. (Beijing, China). The mice were housed in a clean room at a temperature of 23 ± 2°C and a humidity of 50 ± 5% with a 12 h alternating light and dark cycle. They were permitted free access to food and water. All animal experiments were performed according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Nanjing University, China.

ConA was dissolved in pyrogen-free normal saline (NS) and injected intravenously at the dose of 10, 15, and 20 $mg \cdot kg^{-1}$ to monitor survival rate. ConA was injected intravenously at the dose of 15 $mg \cdot kg^{-1}$ to monitor the effects of different doses of ARC on survival rate. ARC was dissolved in emulsion (MCT:HS15:labrasol:transcutol 1:3:1:1) (12.5, 25, and 50 $mg \cdot ml^{-1}$) and diluted with distilled water to 0.25, 0.5, and 1 $mg \cdot ml^{-1}$ for use. 2% emulsion was used as vehicle. The ARC was administrated by intragastric at a dose of 2.5, 5, and 10 $mg \cdot kg^{-1}$. The model group was administered with vehicle, the drugs were given twice per day for 10 days. The survival rates of mice were monitored continuously for 7 days, but no more mice died after 24 h.

ConA was injected intravenously at the dose of 10 $mg \cdot kg^{-1}$ to study the protective mechanism of drug. The ARC and CSA were administered by intragastric at a dose of 10 $mg \cdot kg^{-1}$. PS was dissolved in distilled water and was administrated by intragastric at a dose of 5 $mg \cdot kg^{-1}$. Finally, the mice were killed at the indicated time points after ConA injection, and their serum and liver tissue samples were collected. The right lobe of liver was fixed in 10% formalin for morphological analysis. Remaining liver tissues were stored at −80°C for further analysis.

Serum Biochemical Analysis

The plasma alanine transaminase (ALT), aspartate transaminase (AST) activities, total bilirubin (TBIL), and lactate dehydrogenase (LDH) were detected by BS-800 automatic biochemistry analyzer (Shenzhen Mindray Bio-Medical Electronics CO., Ltd., China). The animals were killed after anesthetization with an intraperitoneal injection of sodium pentobarbital (50 $mg \cdot kg^{-1}$).

Measurements of Cytokines

The CBA Flex Set (BD Biosciences, USA) was used for simultaneous detection of serum IL-6, IFN-γ, and IL-10 concentration in serum according to the manufacturer's instructions. The samples were measured with the CytoFLEX flow cytometer (Beckman Coulter Life Sciences), and the data were analyzed by the CytExpert software (Beckman Coulter Life Sciences).

Protein Sample Preparation

The liver samples were subjected to proteomic analysis. Liver samples were prepared using Ready Prep Protein Extraction kit (Bio-Rad) at first. Extracted protein concentration was determined by BioSpec-nano (Shimadzu Biotech, Kyoto). Approximately 4 mg of protein/group was used for quantitative proteomic profiling. NBS tagging was performed according to the manufacturer's protocol (13CNBS stable isotope labeling kit; Shimadzu). Briefly, each solution (each containing 400 μg of protein) was labeled with isotopically ^{12}C6NBS or ^{13}C6NBS reagent. NBS-tagged proteins were then mixed, reduced, alkylated, and digested by trypsin. NBS-tagged peptides were enriched and separated by 2D-nano HPLC (Prominence HPLC, Shimadzu) as described previously (49). Eluates were automatically deposited onto MALDI target plates by the LC spotting system (AccuSpot; Shimadzu Biotech, Kyoto). These spotted samples were automatically analyzed by MALDI-TOF/TOF MS (MALDI-7090, Shimadzu Kratos, Manchester, UK).

Relative Quantification and Identification of Liver Tissue Proteome

Relative quantification between ^{12}C6NBS- or ^{13}C6NBS-tagged peptides was performed using the proteome analysis assistant software for relative quantification, TWIP Version 1.0 (DYNACOM, Chiba, Japan), referring to a monoisotopic mass list from MASCOT Distiller Ver. 1.1.2 (Matrix Science, London, UK). Threshold values of ^{13}C6/^{12}C6 ratios in NBS-tagged peptide pairs were set to either larger than 1.25 or less than 0.8. Candidate peptides having significant difference in peptide pair ratios were selected and further subjected to MS/MS analysis. Proteins were identified by MASCOT MS/MS Ion Search algorithm (Version 2.0; Matrix Science) using mass lists generated by MASCOT Distiller. The Mascot search parameters were as follows: trypsin digestion allowing up to 1 missed cleavages, fixed modifications of ^{12}C6NBS (or ^{13}C6NBS) (W) and carbamidomethyl (C), variable modifications of oxidation (M), peptide tolerance of 0.2 Da, and MS/MS tolerance of 0.8 Da. Search results with p values less than 0.05 were judged as positive identifications.

Histopathology and Immunohistochemistry

Liver injury was assessed by light microscopy. Fixed liver tissue slices were processed and embedded in tissue embedding rings (Tissue-Tek® TEC™ 5 Sakura Fine tek Japan CO., Ltd.). Sections of 4 μm in thickness were subjected to hematoxylin and eosin staining by automatic staining machine (Leica ST5020). Paraffin sections (4 μm) were carried out for immunohistochemistry in the liver. Briefly, sections were deparaffinized and rehydrated using automatic staining machine (Leica TP5020). Citric acid buffer (PH 6.0) in high pressure was used for antigen retrieval. Peroxidase blocking was performed using 3% hydrogen peroxide for 10 min. Sections were incubated with different antibodies respectively for 12 h at 4°C. Thereafter, sections were incubated using anti-murine/rabbit Universal Immunoperoxidase Polymer kit (Proteintech Group). Sections were counterstained with hematoxylin. Slides were viewed and captured by Pannoramic Viewer1.15.4 and analyzed with Image pro plus (IPP) 6.0. The IHC staining within each lesion was assessed by estimating the area of the objects plus the average intensity of per object, as the integrated optical density (IOD).

TABLE 1 | Nucleotide sequences of primers used for RT-PCR.

Gene		Primer sequence (5′→3′)
IL-6	Forward	GGCCTTCCCTACTTCACAAG
	Reverse	ATTTCCACGATTTCCCAGAG
IFN-γ	Forward	CAAGTGGCATAGATGTG GAA
	Reverse	CTGGACCTGTGGGTTGTT
IL-10	Forward	ATTTGAAT TCCCTGGGTGAGAAG
	Reverse	CACAGGGGAGAAATCGATGACA
Pkr	Forward	GTACAAGCGCTGGCAGAACTCAAT
	Reverse	AAGAGGCACCG GGTTTTGTAT
Stat1	Forward	TGGGGGAGGGGCCTTCTTGATG
	Reverse	TGGCCCCCTTAATGGATGTGCAA
Atg7	Forward	ATAT CGCTGCGCTGGTCGTC
	Reverse	TGATGGAGCAGGGTAAGACC
β-actin	Forward	ATAT CGCTGCGCTGGTCGTC
	Reverse	TGATGGAGCAGGGTAAGACC

Western Blot Analysis

Extracted samples containing 100 μg protein were separated by 10% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and electrophoretically transferred onto a polyvinylidene difluoride transfer membrane. The nonspecific sites were blocked with a solution containing 5% non-fat milk powder in TBS with 0.1% Tween 20 (TBST) for 2 h at room temperature and then incubated with antibodies in TBST containing 5% bovine serum albumin overnight at 4°C with gentle shaking. After the membrane was washed, it was incubated with horseradish peroxidase-conjugated second antibodies (anti-rabbit IgG at a dilution of 1/5,000) at room temperature for 1 h. Protein bands were visualized using chemiluminescence reagent. β-actin was utilized as a housekeeping protein here.

RT-PCR Analysis

The RT-PCR analysis was performed as described previously (50). Total RNA was extracted from liver tissue with Trizol reagent as described by the manufacturer (Gibco). RT-PCR was performed using the Access RT-PCR Introductory System (Promega) with indicated primers (**Table 1**). PCR was performed for 30 cycles in 25 μL of reaction mixture. PCR products were monitored using microchip electrophoresis system (MultiNA, Shimadzu Biotech, Kyoto). β-actin was utilized as a housekeeping gene here.

Data and Statistical Analysis

All experimental data obtained from mice were expressed as mean ± SD. Statistical significance was determined by Student's unpaired two-tailed *t*-test. $P < 0.05$ was considered statistically significant. Immunohistochemical quantitative analysis data were made correlation analysis by Origin 8.

RESULTS

The Time Course of Pathological, Blood Chemical, and Cytokine Changes in ConA-Induced Hepatitis

ConA (10, 15, and 20 mg·kg^{-1}) were injected intravenously to mice to induce the survival rates of 100, 30, and 0%, respectively (**Figure 1A**). ConA (10 mg·kg^{-1}) was selected to monitor the time course of pathological, blood chemical, and cytokine changes in ConA-induced hepatitis. The serum levels of ALT, AST were elevated from 6 h after ConA administration, got peak at 12 h and declined at 24 h (**Figures 1B,C**). The levels of IL-6 and IFN-γ reached peak at 6 h (**Figures 1D,E**) and decreased at 24 h. The levels of IL-10 kept the increase from 6 to 24 h (**Figure 1F**). Histological analysis showed that HE-stained hepatic sections from control group was microscopically normal. At 6 h after ConA challenge, serious congestion occurred in hepatic sinusoid, a great number of inflammatory cells were found in liver tissues, and the hepatocytes cell death appeared with the nuclei condensation. At 12 h, patches of hepatocytes death occurred around the central vein region with the cytoplasm red staining. The lesions were still serious at 24 h (**Figure 1G**).

Comparative Proteomic Analysis Between ConA-Induced Hepatitis Mice and Normal Mice

After the administration with ConA (10 mg·kg^{-1}), the proteomic analysis highlighted 57 differentially expressed proteins, including 41 upregulated ones and 16 downregulated ones. They were listed in **Table 2**. The current interaction network made on the basis of STRING was shown in **Figure 2**. A large number of proteins showed functional enrichments in immune system processes, including Cd7, B2m, Anln, Oas3, Eif2ak2 (Pkr), Isg15, Gbp1, Ifi30, Ifit2, Ifit3, Isg20, Atg7, Adrm1 and Stat1. The levels of B2m, T-cell antigen Cd7 were upregulated which was associated with the activation and proliferation of T lymphocytes (51, 52). The IFN-induced antiviral ISGs have direct antimicrobial and immunomodulatory effects (53–55). These proteins constructed a complex network which co-induced immune disturbance during the course of hepatitis.

The autophagy and UPS are two highly conserved degradative pathways that regulate protein homeostasis by the clearance of cytoplasmic materials in eukaryotic cells. Here, the proteomic analysis revealed that they might be disturbed by a variety of IFN-induced proteins. For example, the highly elevated Isg15 has been shown to antagonize the ubiquitin/proteasome pathway. The protein ISGylation interferes with ubiquitination *via* substrate competition at E2/E3 level (56). Ube2l6 (UbcH8) is a major E2 enzyme for Isg 15 conjugation (57). Here, it was also upregulated. The Isg15 conjugation pathway overlapped the ubiquitin conjugation pathway, leading to changes in degradation of cellular protein.

The impaired of the proteolytic capacity of the UPS can promote inflammation or induce cell death in autoinflammation (58). Here, the dysregulation of proteasome capacity was revealed by the upregulation of Adrm1. Adrm1 (also named Rpn13 or Arm-1) was previously identified as an adhesion-regulating molecule of T cells (59). Adrm1 has also been described as an IFN-γ-inducible, heavily glycosylated membrane protein of 110 kDa (Gp110) (60). Adrm1 (Rpn13 in yeast) is a subunit of 19S-regulatory particle (19S-RP). The 26S proteasome is a large, multi-protein consisting of the 20S catalytic core particles (20S

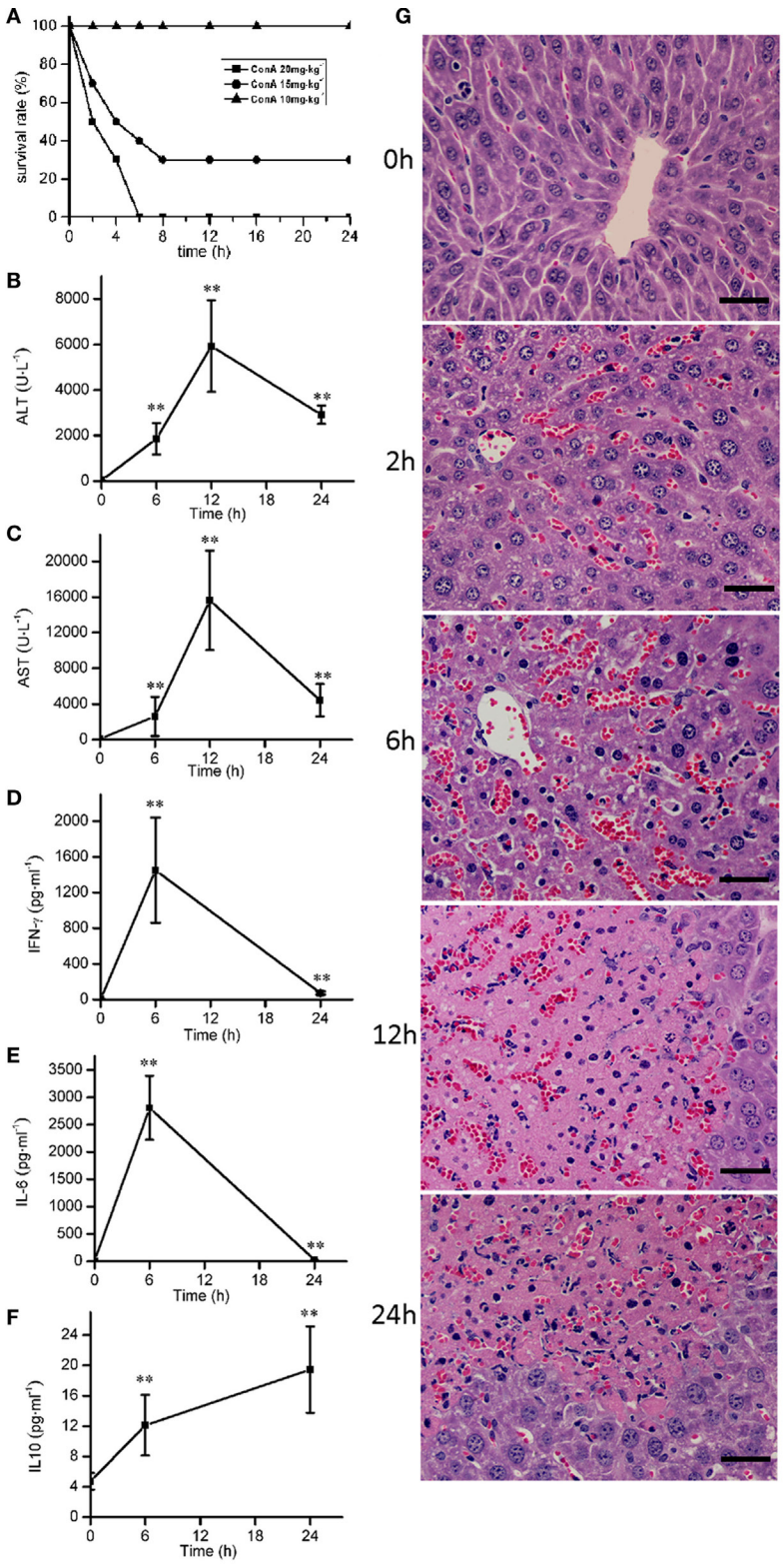

FIGURE 1 | The time course of pathological, blood chemical, and cytokine changes in ConA-induced hepatitis. **(A)** The survival rates were decreased with the increase of ConA. **(B,C)** The serum levels of alanine transaminase, aspartate transaminase after ConA (10 mg·kg^{-1}) administration. **(D–F)** The changes of serum levels of IFN-γ, IL-6, and IL-10 after ConA (10 mg·kg^{-1}) administration. **(G)** Representative micrographs depicting H&E stained hepatic sections observed under 40× objectives after ConA (10 mg·kg^{-1}) administration. Scale bars, 50 μm. Mice were administered with a single intravenous injection of saline containing ConA. The mice treated with intravenous injection of normal saline only were used as control (time 0 h). Error bars indicate mean ± SD. $^{**}P < 0.01$ compared with control group ($n = 6$).

TABLE 2 | Protein changes in mice liver following ConA exposure for 12 h.

UniProt ID	Gene name	Protein description	Fold-change
			ConA/Normal saline
E9Q4J9	Adgrf2	Adhesion G-protein coupled receptor F2	16.37
Q8CEE6	Pask	PAS domain-containing serine/threonine-protein kinase	10.94
Q9JKV1	Adrm1	Proteasomal ubiquitin receptor ADRM1	7.91
P50247	Ahcy	Adenosylhomocysteinase	6.93
Q9QZU9	Ube2l6	Ubiquitin/ISG15-conjugating enzyme E2 L6	5.99
P62874	Gnb1	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	5.33
P50283	Cd7	T-cell antigen CD7	4.75
Q64339	Isg15	Ubiquitin-like protein ISG15	4.66
O08601	Mttp	Microsomal triglyceride transfer protein large subunit	4.57
Q9QUJ7	Acsl4	Long-chain-fatty-acid—CoA ligase 4	4.18
Q8R3B1	Plcd1	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase delta-1	4.16
Q0KK56	Fam184b	Protein FAM184B	3.62
Q03963	Eif2ak2/Pkr	Interferon-induced, double-stranded RNA-activated protein kinase	3.59
Q8BQZ5	Cpsf4	Cleavage and polyadenylation specificity factor subunit 4	3.54
P10126	Eef1a1	Elongation factor 1-alpha 1	3.53
P56380	Nudt2	Bis(5'-nucleosyl)-tetraphosphatase [asymmetrical]	3.42
P28740	Kif2a	Kinesin-like protein KIF2A	3.35
Q5SWU9	Acaca	Acetyl-CoA carboxylase 1	3.04
Q60936	Adck3	Atypical kinase COQ8A, mitochondrial	2.99
Q9QYJ3	Dnajb1	DnaJ homolog subfamily B member 1	2.89
P42225	Stat1	Signal transducer and activator of transcription 1	2.79
P35583	Foxa2	Hepatocyte nuclear factor 3-beta	2.78
P23506	Pcmt1	Protein-L-isoaspartate (D-aspartate) *O*-methyltransferase	2.76
P32233	Drg1	Developmentally regulated GTP-binding protein 1	2.74
Q8VI93	Oas3	2′-5′-oligoadenylate synthase 3	2.67
Q9D906	Atg7	Autophagy-related protein 7	2.63
Q64112	Ifit2	Interferon-induced protein with tetratricopeptide repeats 2	2.46
Q99LC5	Etfa	Electron transfer flavoprotein subunit alpha	2.45
Q9JII1	Inpp5e	72 kDa inositol polyphosphate 5-phosphatase	2.39
O08788	Dctn1	Dynactin subunit 1	2.35
P01887	B2m	Beta-2-microglobulin	2.34
P02469	Lamb1	Laminin subunit beta-1	2.18
O88587	Comt	Catechol *O*-methyltransferase	2.17
Q8K298	Anln	Anillin	2.13
Q01514	Gbp1	Guanylate-binding protein 1	2.08
P09922	Mx1	Interferon-induced GTP-binding protein Mx1	2.04
P14869	Rplp0	60S acidic ribosomal protein P0	1.98
Q02395	Mtf2	Metal-response element-binding transcription factor 2	1.98
Q9ESY9	Ifi30	Gamma-interferon-inducible lysosomal thiol reductase	1.97
Q9JL16	Isg20	Interferon-stimulated gene 20 kDa protein	1.88
Q64345	Ifit3	Interferon-induced protein with tetratricopeptide repeats 3	1.63
P02535	Krt10	Keratin, type I cytoskeletal 10	0.55
Q8R5M2	Wnt9a	Protein Wnt-9a	0.49
Q9WVR4	Fxr2	Fragile X mental retardation syndrome-related protein 2	0.47
Q99LB7	Sardh	Sarcosine dehydrogenase, mitochondrial	0.44
Q61166	Mapre1	Microtubule-associated protein RP/EB family member 1	0.39
F8WJD4	Sympk	Symplekin	0.39
Q99LD4	Gps1	COP9 signalosome complex subunit 1	0.38
P68373	Tuba1c	Tubulin alpha-1C chain	0.36
Q8BTI8	Srrm2	Serine/arginine repetitive matrix protein 2	0.34
Q8BHN3	Ganab	Neutral alpha-glucosidase AB	0.33
P19096	Fasn	Fatty acid synthase	0.29
P16675	Ctsa	Lysosomal protective protein	0.23
P01845	Iglc3	Ig lambda-3 chain C region	0.21
P29758	Oat	Ornithine aminotransferase, mitochondrial	0.18
Q99JZ7	Errfi1	ERBB receptor feedback inhibitor 1	0.09
P50446	Krt6a	Keratin, type II cytoskeletal 6A	0.08

Table showed the mean fold changes of proteins in model mice versus normal mice with p values less than 0.05 (n = 3), where ratios >1.25 were upregulation; ratios <0.8 were downregulation.

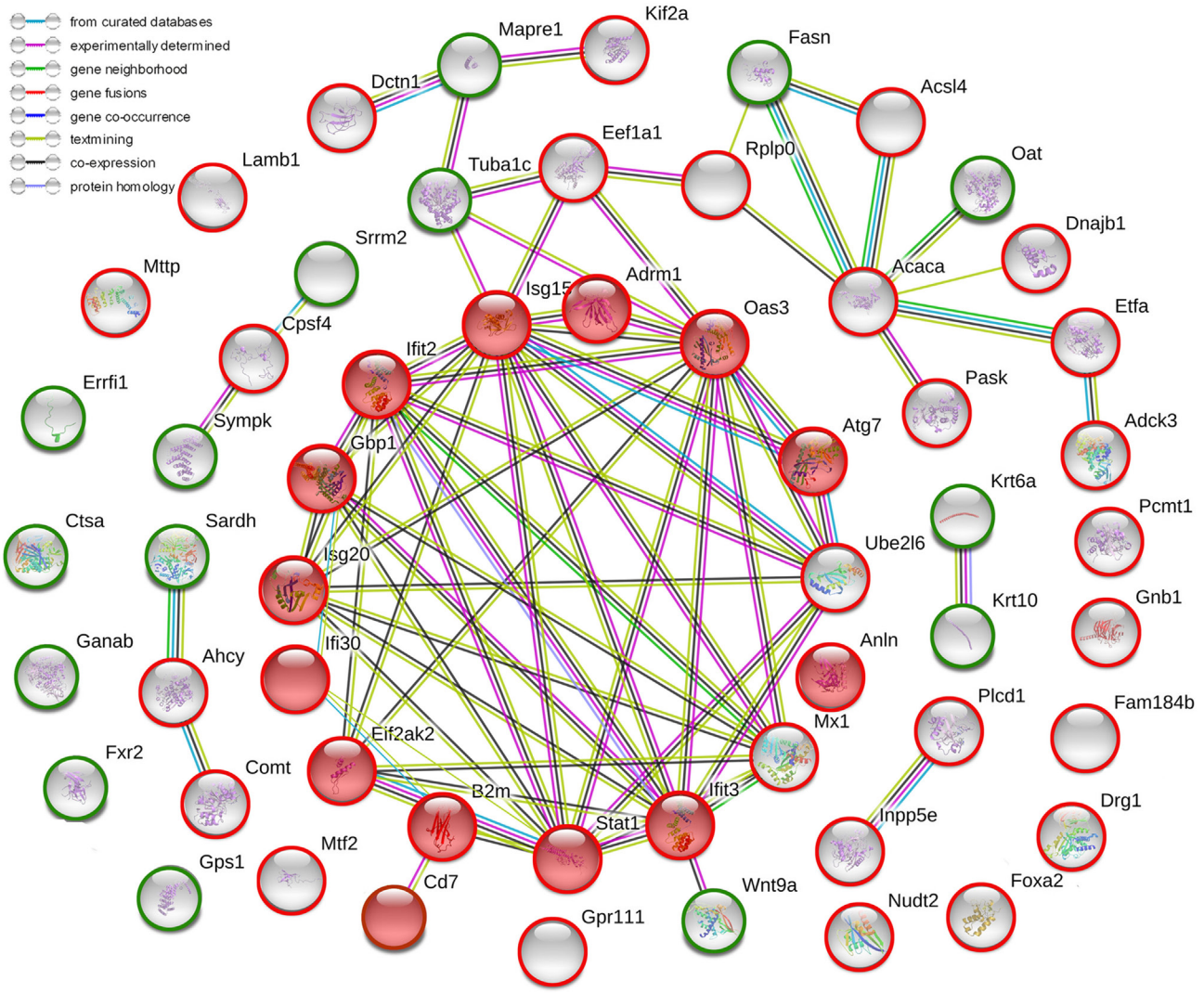

FIGURE 2 | The current interactions of differentially expressed proteins in liver tissues of ConA-induced hepatitis mice compared with that of normal mice. The red nodes represented the functional enrichments of proteins in immune system. The red circles represented upregulated proteins while the green circles represented downregulated proteins. The network was made on the basis of STRING.

CP) and 19S-RP. Adrm1 is a novel ubiquitin receptor, it is able to recruit ubiquitinated substrates to the 19S-RP, and then fed them into the proteasome's catalytic 20S-CP for degradation (61, 62). However, the overproduction of Adrm1 reduced the degradation of the most short-lived proteins, and the transfection of the C-terminal half of Adrm1 slowed proteolysis and induced cell death (63). The reason might be that Adrm1 was also the proteasomal receptor for Uch37, and it could recruit and activate Uch37, a deubiqitinating enzyme (63, 64). The Uch37 disassemble the poly-Ub chain and recycle the ubiquitin during proteasomal degradation (65). Interaction of Adrm1 with Uch37 increased the production of triubiquitin (Ub3), diubiquitin (Ub2), and monoubiquitin (Ub1) (66). Uch37 was activated by Adrm1 to form Uch37–Adrm1 complex, exhibiting 12-fold higher activity than Uch37 alone (67).

Multilple ubiquitins were released from changed degradation when the ubiquitin binding sites were occupied by Isg15. It was proposed that Adrm1 and Isg15 might work together to induce the overexpression of Ub in ConA-induced hepatitis. The p62 would undergo ubiquitylation under Ub stress and activate selective autophagy (68). The p62-dependent autophagic clearance of accumulated polyubiquitinated proteins is a compensatory process for the loss of proteasome ability (69). In addition, the Isg15 and ISGylation would lead to upregulation of p62 and LC3II, then the Isg15-congugated proteins were also degraded by lysosome through the conjugation with p62 (70).

Double-stranded RNA-dependent protein kinase (Pkr) is an IFN-inducible anti-viral protein. Pkr could participate in the inflammation and immune regulation by not only pro-apoptotic activity but also pro-autophagic activity (71). It is also known as

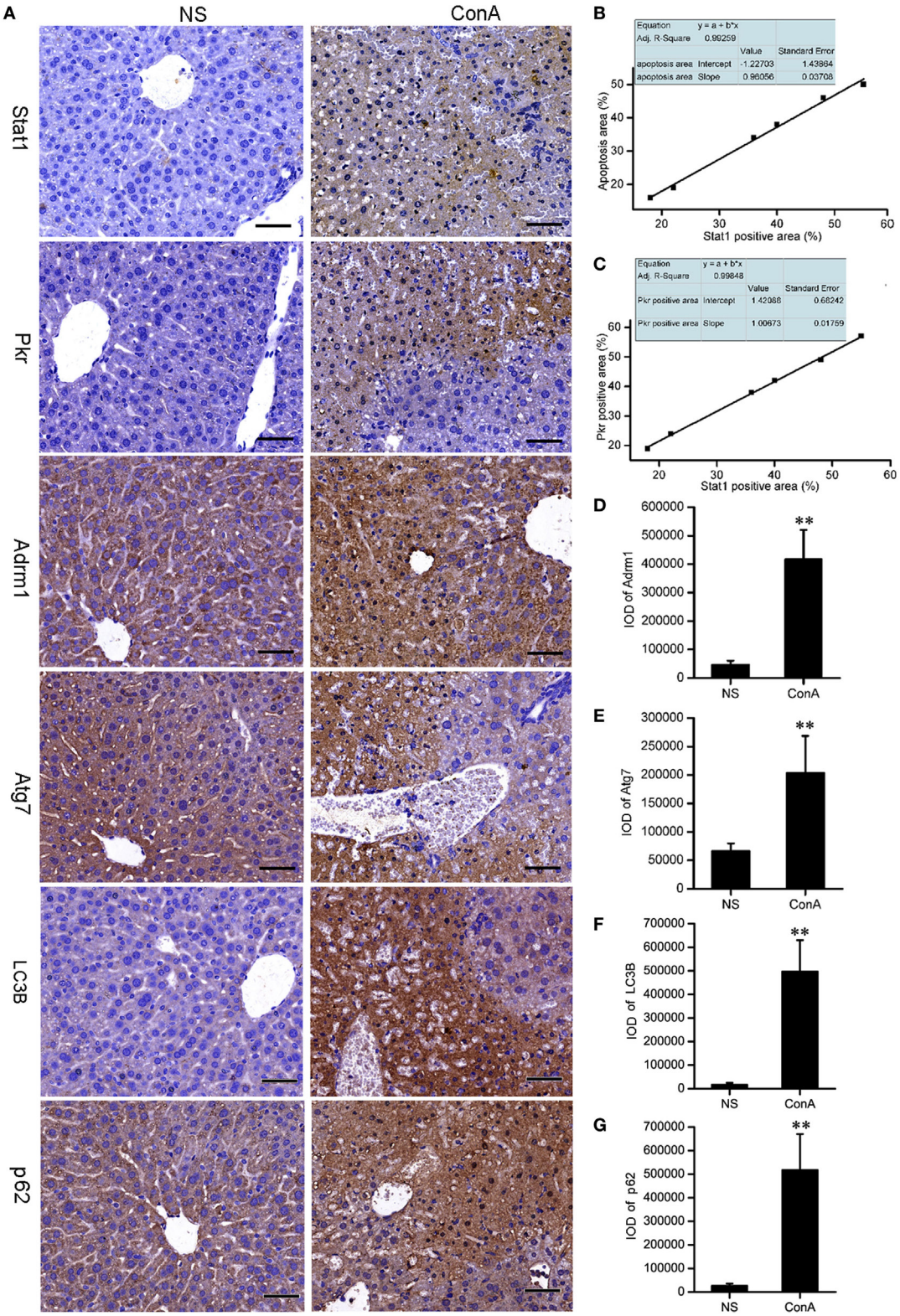

FIGURE 3 | The validation of the proteomic data of Stat1, Pkr, Atg7, and Adrm1 by IHC. The LC3B and p62 were detected by IHC. **(A)** The IHC of Stat1, Pkr, Atg7, Adrm1, LC3B, and p62 of control mice and model mice. Positive correlations of Stat1 with apoptosis **(B)**, Stat1 with Pkr **(C)** in the sections of ConA treatment group were shown. Integrated optical density of Adrm1 **(D)**, Atg7 **(E)**, LC3B **(F)**, and p62 **(G)** showed that they were upregulated after ConA treatment. $^{**}p < 0.01$ compared with the control group ($n = 6$).

eukaryotic translation initiation factor 2-alpha kinase 2 (eIF2α). EIF2α mediated the activation of the microtubule-associated protein LC3 (72). Atg7 is required by the induction of mammalian autophagy, which is one E1 enzyme for the activation of autophagy-essential ubiquitin-like protein LC3 (73). Thus, the upregulation of Pkr and Atg7 might lead to the increased expression of LC3II.

In summary, the overexpression of p62 would be associated with the accumulation of ISGylated proteins and autophagosomes. The ubiquitylation of p62 could liberate it to recognize polyubiquitylated cargoes for autophagy (68). It engulfed the aggregates in autophagosomes and delivered them to lysosome for degradation. The overexpression of p62 represented the accumulated autophagosome and a blockage of autophagic flux, eventually leading to autophagic cell death (21).

Validation of the Proteomic Analysis by Immunohistochemistry

It was proposed that LC3 and p62 might accumulate in the liver tissue after ConA challenge. Recent reports showed that IHC staining for LC3B and p62 might be the best approach to monitor autophagy in large human tissue sections, when electron microscopy is not feasible (74, 75). We performed IHC staining to validate them. The expressions of Stat1 and Pkr in control group were rarely seen, but they were increased sharply in model group and expressed at the apoptotic or necrotic areas at 12 h after ConA challenge. Correlation analysis showed that Stat1 expression was positively correlated with the apoptosis, and Pkr expression was positively correlated with Stat1 expression. Adrm1, Atg7, LC3B, and p62 were expressed in the cytoplasm of normal hepatocytes. They were overexpressed in patchy apoptotic areas together with Stat1 after ConA challenge. The overexpressions of LC3B and p62 were also verified by IHC analysis. The IOD of IPP were representative parameters to assess the immunostaining quantification, and provided a more accurate analysis of protein expression (76), so the IOD provided by IPP was performed to assess the IHC quantification here. The IHC results of Stat1, Pkr, Adrm1, and Atg7 were consistent with our proteomic analysis (**Figure 3**).

ARC Pretreatment Attenuated the ConA-Induced Hepatitis

ARC has distinct anti-inflammatory property. We proposed that it might extent protective effects on immune-mediated hepatitis. The protective effects of ARC pretreatment on ConA-induced hepatitis were tested in mice. Intravenous injection with ConA (15 mg·kg^{-1}) led to a survival rate of 30% in mice. ARC (2.5, 5, and 10 mg·kg^{-1}) pretreatment significantly reversed the survival rates, when there were no deaths in ARC (10 mg·kg^{-1}) group (**Figure 4A**). ConA (10 mg·kg^{-1}) was selected to investigate the protective mechanism of ARC on ConA-induced hepatitis. CSA and PS have been confirmed to be effective in the treatment of the AIH (77), so they were selected as positive parallel controls. The ARC or vehicle alone had no effects on normal liver functions (**Figure 4B**). CSA, PS, and ARC markedly decreased the serum levels of ALT, AST, TBIL, and LDH at 12 h after ConA administration (**Figures 4C–F**). Pathological sections showed that ARC or vehicle alone had no effects on morphology of liver section (**Figures 5A,B**). Serious liver damages were found in model group at 12 h after ConA (10 mg·kg^{-1}) administration (**Figure 5C**), including portal inflammation, hepatocytes edema, severe congestion in hepatic sinusoid around central veins, and focal or confluent necrosis. ARC significantly inhibited ConA-induced histological changes, which was comparable with effects of CSA and PS (**Figures 5D–F**). The scores of liver injury for each mouse were histopathologically evaluated using a semiquantitative scoring method revised previously described (78, 79). Liver injury was graded from 0 (normal) to 4 (severe) in four categories: inflammatory cell infiltration, congestion, edema, and necrosis. The total liver injury score (TLIS) was calculated by adding the individual scores for each category. The results showed that CSA, PS, and ARC all significantly alleviated ConA-induced hepatitis (**Table 3**). The TLIS was lowest in ARC pretreatment group. In PS treatment group, the inflammation was lightest, but the hepatocytes edema was rather obvious which might be attributed to the side effects of glucocorticoids.

ARC Impaired the Levels of Inflammatory Cytokines in ConA-Induced Hepatitis

The main role of IL-10 is to limit inflammatory responses and regulate activation of several immune cells (80). Systemic recombinant IL-10 administration is feasible as one therapy for AIH (77). ConA-induced hepatitis in mice is prevented by exogenous IL-10 and exacerbated by anti-IL-10 mAb or IL-10 KO (81). IL-6 exerts various effects on hepatocytes and lymphocytes in acute or chronic inflammatory diseases and anti-IL-6 receptor antibody was developed to treat arthritis and other refractory immune-mediated diseases (82). Thus, we selected IL-10 and IL-6 as well as IFN-γ to test the anti-inflammatory properties of ARC.

The level of IL-10 was elevated along with the progression of inflammation after ConA administration, which might be a feedback to suppress liver inflammation. ARC pretreatment further increased the mRNA level of IL-10 in liver tissue and the release of IL-10 to serum at 6 h after ConA administration. The levels of IL-10 were decreased at 24 h, which represented a resolution of inflammation in ARC pretreatment group at this time point. The serum levels of IL-6 and IFN-γ were shown in **Figure 6A**. ARC pretreatment markedly decreased the levels of IL-6 and IFN-γ at 6 and 24 h. The mRNA levels of IL-6 and IFN-γ in liver tissues were both elevated after ConA administration, but they were reversed by ARC pretreatment at 6 and 24 h (**Figure 6B**). The data demonstrated that ARC effectively suppressed the levels of pro-inflammatory cytokines, while it induced the ones of anti-inflammatory cytokines.

Comparative Proteomic Analysis Between ARC Pre-Treated and Vehicle Pre-Treated Mice Models

The proteomic analysis was carried out to investigate the effects of ARC on ConA-induced hepatitis. Compared to vehicle

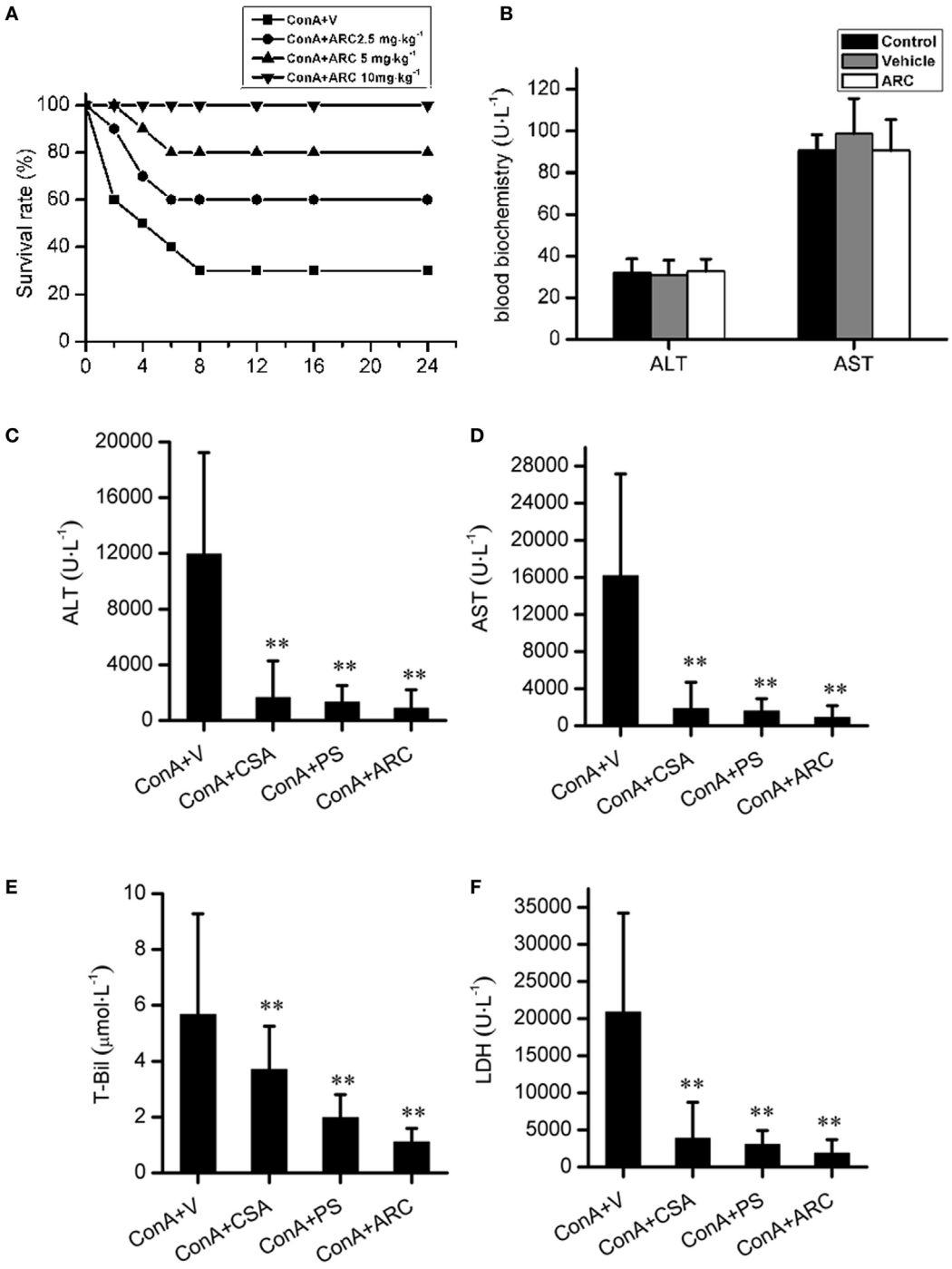

FIGURE 4 | The protective effects of ARC on survival rates and liver function in ConA-induced hepatitis. ARC (2.5, 5, and 10 mg·kg^{-1}) pretreatment significantly increased survival rates after ConA (15 mg·kg^{-1}) administration **(A)**. In the presence of drugs or vehicle treatments twice per day for 10 days, the blood biochemical measurements showed that ARC or vehicle alone had no effects on normal liver functions **(B)**. CSA, PS, and ARC markedly decreased the serum levels of alanine transaminase, aspartate transaminase, TBIL, and lactate dehydrogenase at 12 h after ConA (10 mg·kg^{-1}) administration **(C–F)**. $^{**}p < 0.01$ compared with model group ($n = 8$). The mice pre-treated with vehicle were used as model group.

pre-treated group, 37 changed proteins were highlighted in ARC pre-treated group, including 5 upregulated ones and 32 downregulated ones (**Table 4**). The STRING analysis showed that the proteins enriched in immune system were downregulated in ARC pre-treated group, including Cd7, B2m, Oas3, Eif2ak2 (Pkr), Isg15, Gbp1, Ifi30, Ifit2, Ifit3, Isg20, Atg7, Adrm1, and Stat1 (**Figure 7**). It indicated that the activation of immune system and autophagy were suppressed by ARC pretreatment.

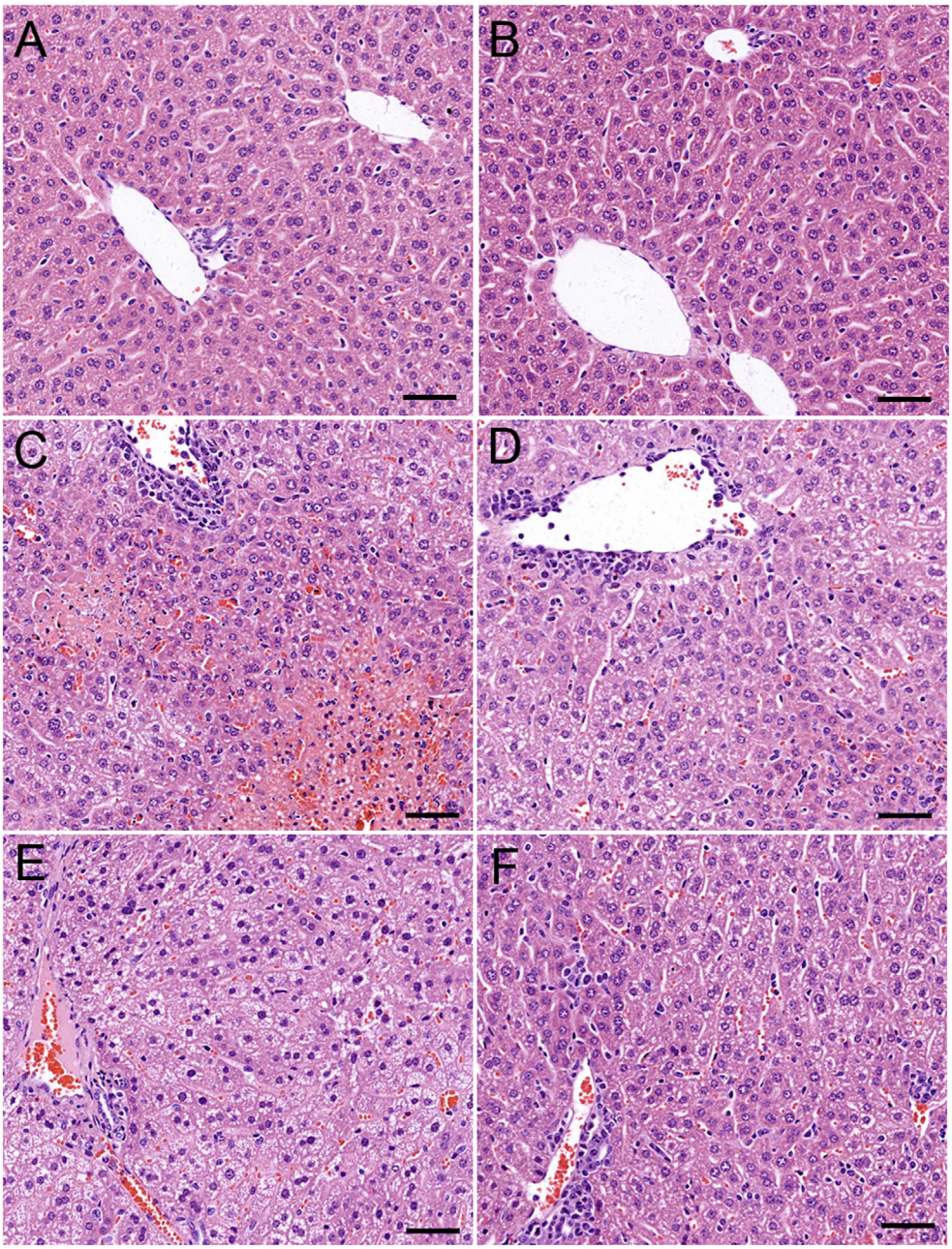

FIGURE 5 | The protective effects of ARC on liver pathology of ConA-induced hepatitis mice. ARC or vehicle alone had no effects on morphology of liver section **(A,B)**. Serious liver damage was found in model group at 12 h after ConA (10 mg·kg^{-1}) administration **(C)**. Portal inflammation, hepatocytes edema, severe congestion in hepatic sinusoid around central veins, and focal or confluent necrosis occurred significantly. ARC significantly inhibited ConA-induced histological changes, which was comparable with effects of CSA and PS **(D–F)**.

ARC Pretreatment Alleviated the Hepatocyte Apoptosis and Autophagy in ConA-Induced Hepatitis

The expression of Bnip3 (BCL2/adenovirus E1B 19 kDa interacting protein 3) was detected to evaluate the apoptosis. The Beclin1 represented the initiation of autophagy (83). Previous studies (23–25, 29) showed that overexpressions of Bnip3 and Beclin1 were involved in ConA-induced hepatitis. Present IHC analysis showed that Bnip3 and Beclin 1 were hardly seen in normal mice, and they were overexpressed in the apoptosis/necrosis regions. Previous proteomic analysis revealed the upregulation of Adrm1 mediated dysregulation of proteasome capacity and then influence autophagy, here WB and IHC results confirmed that Adrm1 was upregulated and overexpressed with LC3II and p62 in apoptotic/

TABLE 3 | Components and total values of liver injury score.

	Control	ConA + V	ConA + CsA	ConA + PS	ConA + ARC
Portal inflammation	0 ± 0	2.0 ± 0.89##	1.4 ± 0.48*	0.5 ± 0.65**	1.0 ± 0.64**
Edema	0.1 ± 0.33	2.8 ± 0.89##	1.4 ± 0.48**	0.8 ± 0.55**	0.5 ± 0.58**
Congestion	0.3 ± 0.43	2.3 ± 0.71##	1.3 ± 0.43**	2.4 ± 0.85	0.4 ± 0.97**
Necrosis	0 ± 0	2.5 ± 1.20##	0.4 ± 0.48**	0.3 ± 0.44**	0.3 ± 0.42**
Total liver injury score	0.4 ± 0.48	9.5 ± 2.20##	4.38 ± 0.70**	3.9 ± 1.23**	2.1 ± 0.54**

*Data are expressed as mean ± SD (n = 8), ##p < 0.01 compared with control; *P < 0.05 compared with model mice treated with vehicle; **P < 0.01 compared with model mice treated with vehicle.*

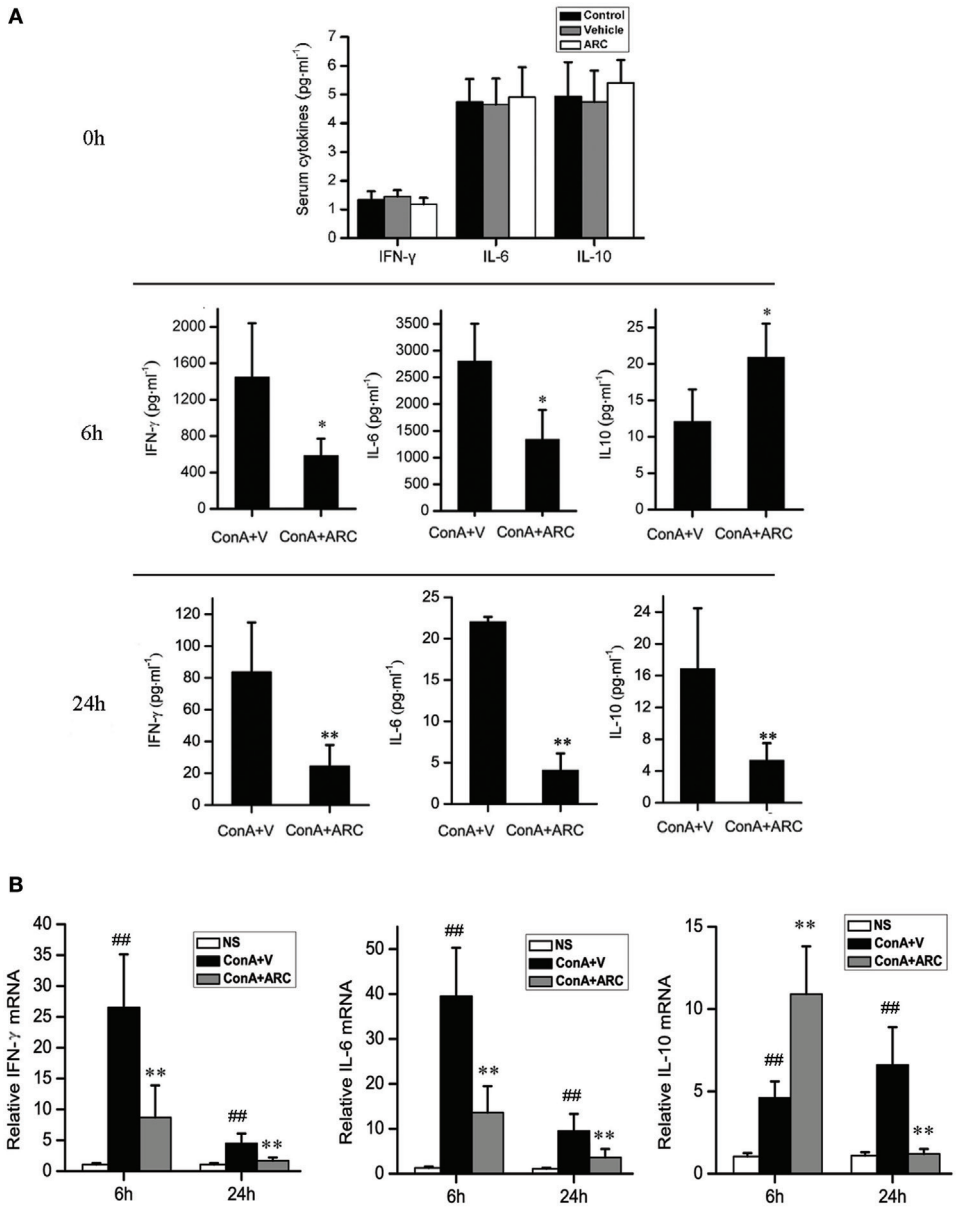

FIGURE 6 | The influence of ARC on the cytokines in serum and liver tissues after ConA (10 mg·kg⁻¹) administration with and without ARC pretreatment. **(A)** The influence of ARC on the release of cytokines. In the presence of drugs or vehicle treatments twice per day for 10 days, ARC or vehicle alone had no effects on the inflammatory responses in normal mice. The levels of IL-6 and IFN-γ were decreased in serum in ARC pretreatment group at 6 and 24 h, and the level of IL-10 was still kept increasing from 6 to 24 h at model group, ARC pretreatment further increased the level of IL-10 at 6 h, but the level of IL-10 in ARC pretreatment mice was lower than that in model mice at 24 h. **(B)** The influence of ARC on the mRNA level of cytokines in liver tissues. The mRNA levels of IL-6 and IFN-γ in liver tissues were both increased after ConA administration. They were decreased by ARC pretreatment at 6 and 24 h. ARC pretreatment further increased the mRNA level of IL-10 in liver tissue at 6 h after ConA administration. At 24 h, the mRNA level of IL-10 was further increased in model group, but it returned to normal level in ARC pretreatment group. ##$p < 0.01$ compared with normal group; *$p < 0.05$, **$p < 0.01$ compared with model group ($n = 8$). The mice pre-treated with vehicle were used as model group.

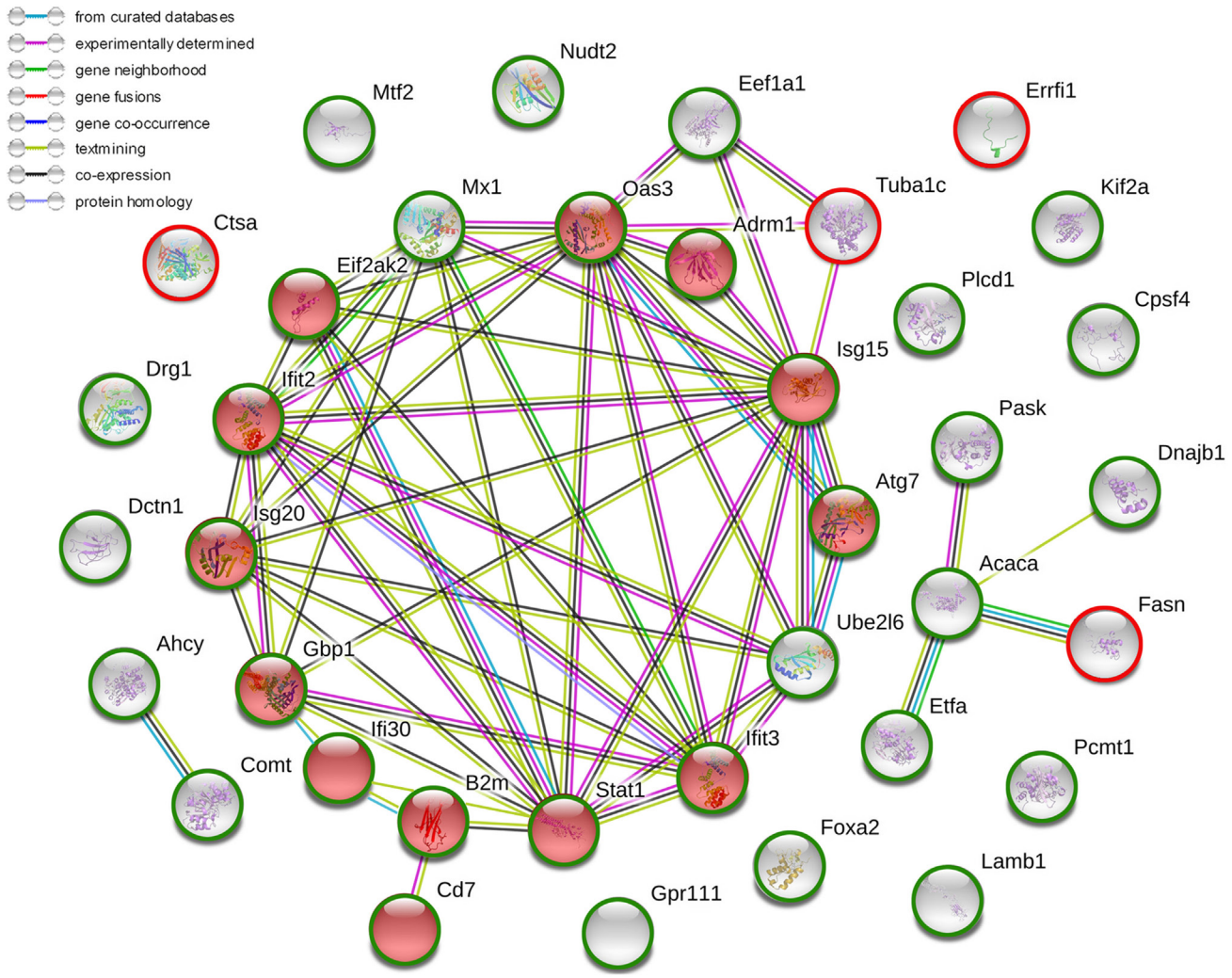

FIGURE 7 | The current interactions of changed proteins in liver tissues with ARC pretreatment compared with those with vehicle pretreatment at 12 h after ConA administration. The red nodes represented the functional enrichments of proteins in immune system. The red circles represented upregulated proteins while the green circles represented downregulated proteins. The network was made on the basis of STRING.

necrotic areas. All of their expressions were significantly reversed in ARC pre-treated mice (**Figure 8**).

Present proteomic analysis demonstrated that IFN-γ/Stat1 signaling played a major role in the activation of immune system. To verify the possible mechanisms hidden behind ARC pretreatment, we investigated the protective effects of ARC on the synthesis and activation of Stat1 in liver tissues. As an important factor involved in inflammation, apoptosis, and autophagy, Pkr was studied. The effect of ARC on the synthesis of Atg7 was also investigated. The results showed that Stat1, p-Stat1, Pkr, and p-Pkr were barely expressed in normal liver and Atg7 was weakly positive in liver section of normal mice. However, they were all overexpressed at apoptotic regions. Their expressions were decreased in ARC pre-treated mice (**Figures 9A,B**). Likewise, the mRNA levels of Stat1, Pkr, and Atg7 in liver tissues were all increased after ConA administration. They were reversed by ARC pretreatment (**Figure 9C**). All these data suggested that ARC indeed inhibited the autophagy and apoptosis in ConA-induced hepatitis.

DISCUSSION

The liver is not only a critical metabolic organ but also a lymphoid organ with unique immunological properties (84). Different phenotypic and functional lymphoid cells were discovered in liver that contribute to immune surveillance against toxins, pathogens, tumor cells and self-antigens of the liver (85). A peripheral break of tolerance against liver-expressed antigens is sufficient to induce an immune liver disease. Meanwhile, T-cell trapping plays an important role in the initiation and perpetuation of immune hepatitis, which can induce the expressions of specific

TABLE 4 | Protein changes in mice liver tissues with or without ARC pretreatments following ConA exposure for 12 h.

UniProt ID	Gene name	Protein description	Fold-change
			ConA + ARC/ ConA + vehicle
E9Q4J9	Adgrf2	Adhesion G-protein coupled receptor F2	0.09
Q8CEE6	Pask	PAS domain-containing serine/ threonine-protein kinase	0.13
Q9JKV1	Adrm1	Proteasomal ubiquitin receptor ADRM1	0.18
P50247	Ahcy	Adenosylhomocysteinase	0.21
Q9QZU9	Ube2l6	Ubiquitin/ISG15-conjugating enzyme E2 L6	0.23
P50283	Cd7	T-cell antigen CD7	0.29
Q64339	Isg15	Ubiquitin-like protein ISG15	0.32
Q9QUJ7	Acsl4	Long-chain-fatty-acid—CoA ligase 4	0.33
Q8R3B1	Plcd1	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase delta-1	0.34
Q03963	Eif2ak2/ Pkr	Interferon-induced, double-stranded RNA-activated protein kinase	0.39
P10126	Eef1a1	Elongation factor 1-alpha 1	0.43
P56380	Nudt2	Bis(5′-nucleosyl)-tetraphosphatase [asymmetrical]	0.41
P28740	Kif2a	Kinesin-like protein KIF2A	0.42
Q5SWU9	Acaca	Acetyl-CoA carboxylase 1	0.46
Q9QYJ3	Dnajb1	DnaJ homolog subfamily B member 1	0.48
P42225	Stat1	Signal transducer and activator of transcription 1	0.57
P35583	Foxa2	Hepatocyte nuclear factor 3-beta	0.55
P23506	Pcmt1	Protein-L-isoaspartate (D-aspartate) *O*-methyltransferase	0.51
P32233	Drg1	Developmentally-regulated GTP-binding protein 1	0.51
Q8VI93	Oas3	2′-5′-oligoadenylate synthase 3	0.52
Q9D906	Atg7	Autophagy-related protein 7	0.53
Q64112	Ifit2	Interferon-induced protein with tetratricopeptide repeats 2	0.57
Q99LC5	Etfa	Electron transfer flavoprotein subunit alpha	0.57
O08788	Dctn1	Dynactin subunit 1	0.61
P01887	B2m	Beta-2-microglobulin	0.64
P02469	Lamb1	Laminin subunit beta-1	0.64
O88587	Comt	Catechol *O*-methyltransferase	0.65
Q01514	Gbp1	Guanylate-binding protein 1	0.67
P09922	Mx1	Interferon-induced GTP-binding protein Mx1	0.69
Q02395	Mtf2	Metal-response element-binding transcription factor 2	0.71
Q9ESY9	Ifi30	Gamma-interferon-inducible lysosomal thiol reductase	0.71
Q9JL16	Isg20	Interferon-stimulated gene 20 kDa protein	0.74
Q64345	Ifit3	Interferon-induced protein with tetratricopeptide repeats 3	0.76
Q99LD4	Gps1	COP9 signalosome complex subunit 1	1.88
P68373	Tuba1c	Tubulin alpha-1C chain	1.98
Q8BHN3	Ganab	Neutral alpha-glucosidase AB	2.16
P19096	Fasn	Fatty acid synthase	2.46
P16675	CtsA	Lysosomal protective protein	3.11
P01845	Iglc3	Ig lambda-3 chain C region	3.41
Q99JZ7	Errfi1	ERBB receptor feedback inhibitor 1	7.94

Table showed the mean fold changes of proteins in ARC pre-treated model group verses vehicle group with p values less than 0.05 (n = 3), where ratios >1.25 were upregulation; ratios <0.8 were downregulation.

chemokines and adhesion molecules (86). Animal studies showed that ConA-induced hepatitis model was extremely suitable to mimic immune hepatitis. Here, the proteomic data on ConA-induced hepatitis model displayed a comprehensive view for the abnormal immune system and provided some new evidences for the ConA-induced hepatitis model to mimic autoimmune liver disease and virus hepatitis.

The upregulation of large amounts of IFN-induced proteins represented the activation of immune system in ConA-induced hepatitis. Although these proteins have direct antimicrobial effects (53–55), they also disrupt the immune system and induce hepatitis which is confirmed in present study. They not only cause inflammation and trigger apoptosis, but also affect cell function by inducing autophagy, such as Pkr. Pkr has multifaceted roles in inflammation and immune dysfunction. It interacted with inflammasome components by autophosphorylation (87). It was essential for the LPS-induced activation of Stat1 inflammatory signaling (88). Pkr mediated the IFN-γ-induced injurious effects through STAT1/IRF-1-dependent cell death signaling (89). The inhibition of Pkr stabilized Bax, in which stabilized Bax could not insert into the outer mitochondrial membrane and initiate stress-induced apoptosis (90). Pkr is essential for autophagy induced by herpes simplex virus infection (72), Moreover, Pkr participated in stress-related damages through generation of stress granules (91). Here, the overproduction and activation of Pkr contributed to the apoptosis and autophagy in ConA-induced hepatitis. ARC pretreatment impeded both the expression and activation of Pkr which was beneficial for restriction of liver injury.

As reported, Jak1–Stat1 signaling played an absolutely necessary role in IFN-α induces autophagy in the pathogenesis of autoimmune disease, and many autophagy-related proteins were upregulated in this process (92). In present study, Atg7 is one of upregulated proteins which showed functional enrichments in immune system process. The proteomic analysis showed the upregulation of Atg7 was along with the Stat1, and IHC results demonstrated the overexpression of Atg7 was distributed at apoptotic areas with the locations of Stat1 and p-Stat1. It was suggested that the Stat1 might also account for the upregulation of Atg7. Atg7 is a key pro-autophagic promoting gene which has a critical role in membrane elongation (73). In addition to its important role in autophagy, it also participated in the inflammation and apoptosis. Atg7 participated in the IFN-γ-induced recruitment of the immunity-related GTPases and guanylate-binding proteins (GBPs) to the intracellular pathogen (93), and GBPs could enable rapid activation of inflammasomes in infected macrophages (55). Atg7 deficiency decreased iNOS activity by downregulating Jak2/Stat1α signaling (94), and hyper-activated Atg7 could induce the cell death by modulating p53 activity (95). It was suggested that Atg7 might be the target of immune-related liver disease. The mRNA and protein levels of Atg7 were both decreased in ARC pretreatment group, thus indicating that ARC might protect liver from ConA-induced hepatitis through the inhibition of the upregulation of Atg7.

Among those upregulated proteins enriched in immune system, we speculated that the upregulated Adrm1, Isg15, and Ube2l6 contributed to the imbalance of autophagy and UPS in

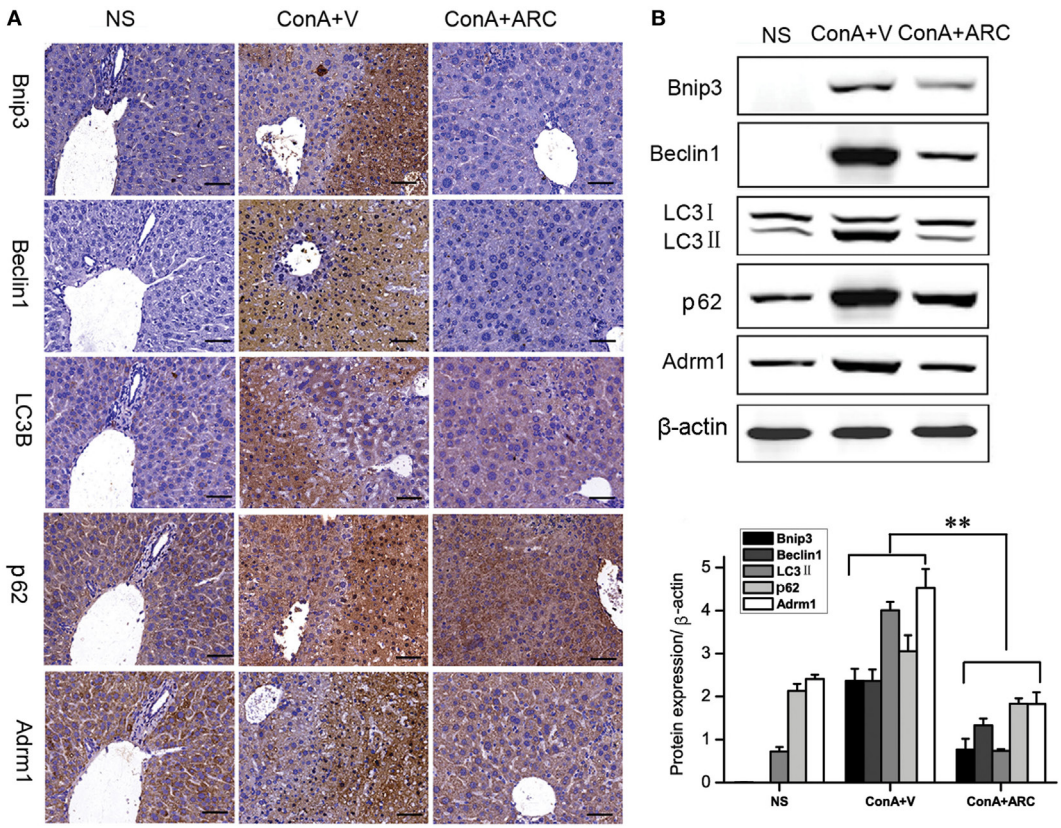

FIGURE 8 | ARC downregulated apoptosis and autophagy in ConA-induced hepatitis. **(A)** The expressions of Bnip3, Beclin1, LC3B, p62, and Adrm1 were measured by IHC. **(B)** The protein levels of Bnip3, Beclin1, LC3B, p62, and Adrm1 were measured by Western blot. The values are represented as mean ± SD. $^{**}p < 0.01$ compared with model group ($n = 8$). The mice pre-treated with vehicle were used as model group.

immune hepatitis. Considering their downregulations in response to ARC pretreatment, they may be the targets to treat immune hepatitis, but the correlation of Adrm1with Stat1 need further investigations. Adrm1, ubiquitin stress, ISGylation proteins, and LC3II jointly led to the overexpression of p62. The overexpression of LC3II and p62 were inhibited by ARC pretreatment. Present study suggested the inhibition of the accumulation of autophagosome by ARC might be due to the suppression of the activated immune system.

During ConA-induced hepatitis, IFN-γ not only induced IFN-induced proteins but also influenced the production of cytokines from other immune cells. As reported, IFN-γ could enhance IL-6 production in activated macrophages (96), and IFN-γ could stimulate the polarization of macrophages into pro-inflammatory M1 macrophages (97). As resident macrophages in liver, KCs can express pro-inflammatory M1 phenotype and the anti-inflammation M2 phenotype according to the immune and metabolic environment (98), and KC was reported to contribute to ConA-induced hepatitis through a Th1 type response-dependent pathway (99). IL-6 is one of indicators of M1-polarized KCs or macrophages (100). It was reported that IL-6 produced by KCs played a significant role in the pathogenesis of ConA-induced hepatitis (101). Further, IL-6 played a key role in $CD4^+$ T cell memory formation and contributed to the proliferation and survival of $CD4^+$ T cells (102, 103). KCs were crucial for IL-10 production in ConA or HBV tolerance (104, 105), and M2 cells could promote M1 death by IL10 releasing which was beneficial for inhibiting inflammation and hepatocyte injury (106). Previous studies showed that IL-10 not only inhibited Th1 cell producing IFN-γ but also inhibited the production of IL-6 by activated macrophages (96, 107). Previous studies demonstrated that ARC could inhibit IL-2 and IFN gene expression in T lymphocytes, it can also promote the transition of M1-like macrophages into M2-like macrophages (41, 108). The above findings suggested that the protective effects of ARC on ConA-induced hepatitis might be due to the promotion of IL-10 generation and the inhibition of IL-6 and IFN-γ production.

Stat1 was essential for M1 macrophages activation by IFN-γ (97), it also participated IL-6 induced $CD4^+$ T cell differentiation into follicular helper cells (Tfh) (109). Tfh cells were reported to correlate with autoantibody production in human autoimmune diseases due to its role in supporting the formation and differentiation of B cells into memory and plasma cells (110). A recent study showed that IL-6 mediated the IFN-α-induced cell apoptosis *via* the activation of Stat1 (111). Both IL-6 and IFN-γ were required for murine mercury-induced autoimmunity (112). They both interplayed with interferon regulatory factor 1 to

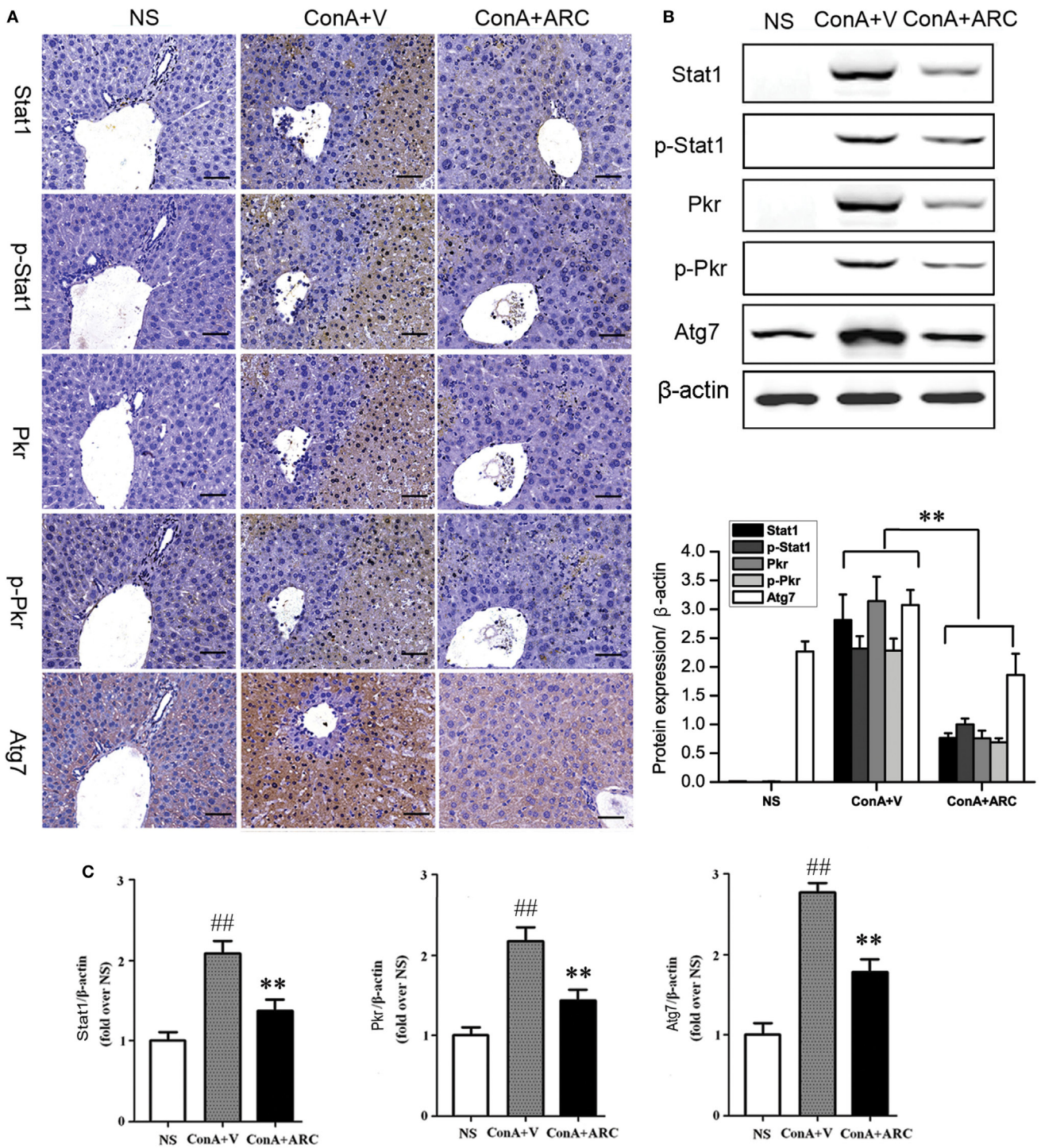

FIGURE 9 | ARC inhibited the expressions of Stat1, p-Stat1, Pkr, p-Pkr, and Atg7. **(A)** The expressions of Stat1, p-Stat1, Pkr, p-Pkr, and Atg7 were measured by IHC. **(B)** The protein levels of Stat1, p-Stat1, Pkr, p-Pkr, and Atg7 were measured by Western blot. **(C)** The mRNA level of Stat1, Pkr, and Atg7 were measured by RT-PCR. The values are represented as mean ± SD. $^{\#\#}p < 0.01$ compared with normal group; $^{**}p < 0.01$ compared with model group ($n = 8$). The mice pre-treated with vehicle were used as model group.

exacerbate the inflammatory responses. The expressions of Stat1 and p-Stat1 were both decreased in ARC pre-treated mice. It was concluded that ARC alleviated cell autophagy and apoptosis *via* the inhibition of IFN-γ/IL-6/Stat1 signaling in ConA-induced hepatitis. Further, the mRNA level of Stat1 was decreased by ARC pretreatment, thus suggesting that ARC attenuated the cell apoptosis and autophagy in ConA-induced hepatitis by suppressing the synthesis and activation of Stat1.

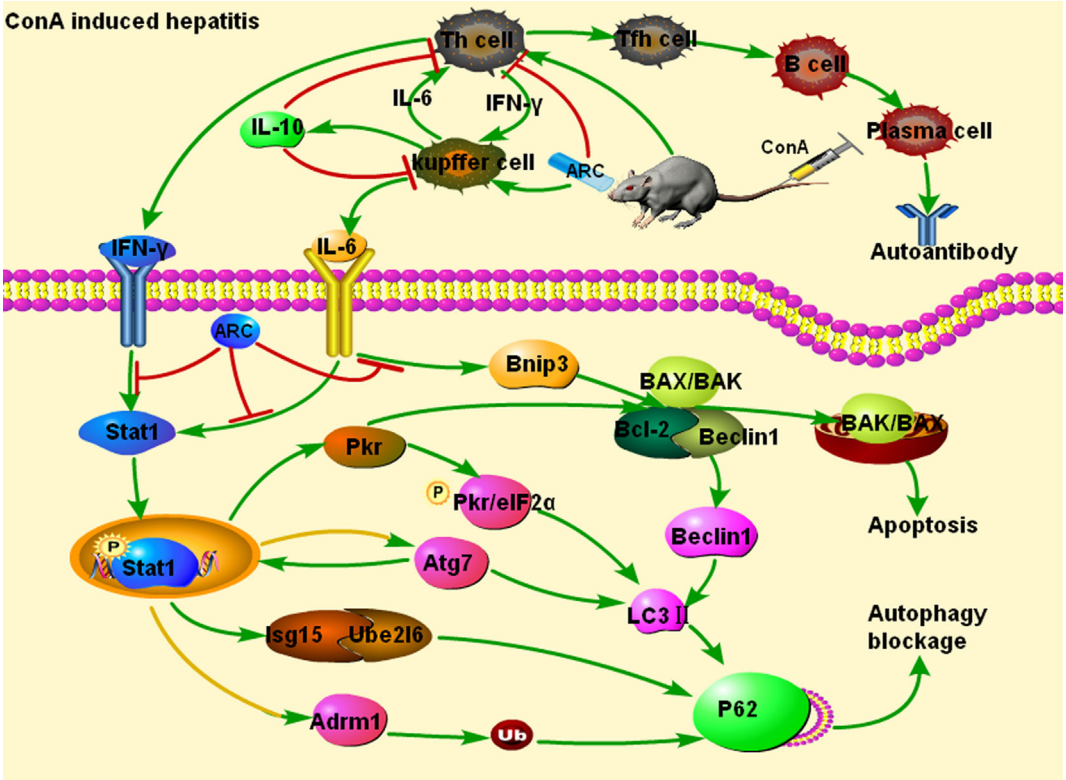

FIGURE 10 | Protective mechanism of ARC on ConA-induced hepatitis. ARC exhibited protective effects through decreasing the levels of pro-inflammatory IFN-γ and IL-6, and increasing the ones of IL-10. The upregulations of Atg7, Beclin 1, and Pkr induced the increase of the expression of LC3 II. The Adrm1, Isg15, Ube2l6, and LC3 II led to the increased expression of p62. ARC pretreatment inhibited autophagy as well as apoptosis by suppressing IFN-γ/IL-6/Stat1 signaling and IL6/Bnip3 signaling. But, whether the upregulations of Atg7 and Adrm1 depend on Stat1 need further study. The green arrows represent activation, the red lines represent inhibition, and the yellow lines with green arrows represent the predictive activation.

IL-6 was also involved in the induction of BNIP3 through the activation of Jak/Stat3 signaling (113, 114), and the suppression of IL-6/Jaks/Stat3 signaling might contribute to the inhibition of Bnip3-mediated apoptosis and autophagy in ConA-induced hepatitis (29). Bnip3 is a pro-apoptotic BH3-only protein that mediates mitochondrial dysfunction and cell death *via* the heterodimerization with Bcl-2/Bcl-X(L) and the activations of Bak or Bax (115, 116). Bnip3 mediated the crosstalk between autophagy and apoptosis through the interactions with B-cell-lymphoma (Bcl2) (117). Bcl2 is an anti-apoptotic protein, and stabilized Bcl2 can inhibit ROS-induced apoptosis (118). Bcl2 was also able to inhibit cell autophagy through interaction with Beclin 1 and prevent cell death from elevated level autophagy (119). Bnip3 can disrupt the interaction between Bcl-2 and Beclin 1 to enhance the formation of autophagosome (120). The overexpression of Beclin1 increased the convention of LC3, and enhanced the cisplatin-induced apoptosis (121). ARC decreased apoptosis and autophagy in ConA-induced hepatitis might also by the inhibition of IL6/Bnip3 pathway.

All these data suggested that ARC exhibited protective effects through downregulating the levels of IFN-γ and IL-6, upregulating the ones of IL-10. The detailed mechanism of action was shown in **Figure 10**. The downregulations of Atg7, Beclin1, and LC3 II indicated that the ARC pretreatment inhibited the accumulation of autophagosome at the initial step, while the downregulation of LC3 II and p62 demonstrated that ARC pretreatment alleviated the blockage of autophagy flux. Pkr and Bnip3 mediated both apoptosis and autophagy, ARC pretreatment inhibited both of them. The expression and activation of Stat1 were decreased in ARC pretreatment group. ARC inhibited autophagy as well as apoptosis by suppressing IFN-γ/IL-6/Stat1 signaling and IL6/Bnip3 signaling. The previous study of luciferase activity test showed that ARC could inhibit the IFN-γ/Stat1 and IL-6/Stat3 signaling (122), while present study demonstrated the inhibitions of both IFN-γ/IL-6/Stat1 signaling and IL6/Bnip3 signaling contributed to the protective effects of ARC against ConA-induced hepatitis.

As reported, IFN-γ could induce activated but insufficient autophagy that contributed to p62-dependent apoptosis in epithelial cells (123). A blockage of autophagic flux at level of lysosome results in autophagy-dependent cell death in glioma cells (124). ConA/IFN-γ could trigger autophagy-related necrotic hepatocytes death through IFN-γ-related Irgm1-mediated lysosomal disruption (125). It was suggested that the lysosome might be disrupted in ConA-induced hepatitis. The decreased expression of LC3II and p62 demonstrated that ARC pretreatment alleviated

the blockage of autophagy flux by inhibition of the accumulation of autophagosome. However, the effects of ARC on degradation ability of lysosome need further study.

Recent evidences suggested that regulation of autophagy might be a potential strategy for viral hepatitis. HBV and HCV could induce accumulations of autophagosome and p62 but impair lysosomal acidification, leading to incomplete autophagy in autophagic degradation (126, 127). HCV-induced oxidative stress triggered p62-dependent autophagy and interplayed with exosomes to mediate the release of HCV particles (128, 129). Epigallocatechin-3-gallate inhibited HBV replication by resisting insufficient autophagy and enhancing lysosomal acidification (130). The inhibition of autophagosome formation by 3-MA or siRNA targeting Beclin1 and Atg5 markedly inhibited HBV replication (131). Downregulation of the autophagy-related gene expressions by mycophenolic acid could also inhibit HCV replication (132). The autophagy inducer, rapamycin enhanced HBV replication (133), while an autophagy inhibitor chloroquine reduced viral as well as ALT levels (134). In addition to the activation of immune system, the dysregulated autophagy could be a suitable proof for ConA-induced hepatitis model to mimic virus hepatitis. It might be a perfect model for forecasting the antivirus effect of agents. The agents that exerted protective effects on ConA-induced hepatitis through regulating autophagy might also have potentials to be antivirus drugs. Some previously reported agents seemed to support this hypothesis, such as quercetin (24, 135), Shikonin (28, 136), and epigallocatechin-3-gallate (29, 130).

The clinical treatments of the AIH and virus hepatitis are contradictory in clinical. The AIH need immunosuppressive therapy, but it will weaken the ability of antiviral immune suppression, increase the replication HBV or HCV (137). Among the treatments of viral hepatitis, IFN is the preferred one. It eliminates the virus by enhancing immune function. This process will induce or aggravate AIH, especially in HCV infection (138). Regulation of autophagy can simultaneously protect cells from apoptosis when killing the virus. It may be a new strategy for the treatment of immune hepatitis especially when the AIH and virus hepatitis occur simultaneously. Here, the protective mechanism of ARC on ConA-induced hepatitis indicated that it might be a candidate drug for both viral hepatitis as well as AIH. Furthermore, the antiviral effect of ARC has been already confirmed (44–46), but the effects on viral hepatitis need further validation.

With the help of proteomic analysis, it was demonstrated that both autophagy and apoptosis have important clinical implications for the treatment of liver disease. ARC exhibited protective effects on ConA-induced hepatitis by regulating both autophagy and apoptosis. This study indicated the therapeutic potential of ARC as a new strategy for the treatment of immune-mediated hepatitis.

ETHICS STATEMENT

This study was carried out according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and was approved by the Animal Care and Use Committee of Nanjing University, China.

AUTHOR CONTRIBUTIONS

JY, GZ and QF designed the study. QF, WX, GZ, JL, XL and TZ conducted the experiments. QF performed analysis and construction of network pharmacology. NZ performed proteomic analysis. QF and JY analyzed the data. QF, NZ and JY wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01881/full#supplementary-material.

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High Tidal Volume Induces Mitochondria Damage and Releases Mitochondrial DNA to Aggravate the Ventilator-Induced Lung Injury

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Objective: This study aimed to determine whether high tidal volume (HTV) induce mitochondria damage and mitophagy, contributing to the release of mitochondrial DNA (mtDNA). Another aim of the present study was to investigate the role and mechanism of mtDNA in ventilator-induced lung injury (VILI) in rats.

Methods: Rats were tracheotomized and allowed to breathe spontaneously or mechanically ventilated for 4 h. After that, lung injury was assessed. Inhibition of toll-like receptor 9 (TLR9), named ODN2088, was used to determine the involvement of TLR9/myeloid differentiation factor 88 (MyD88)/nuclear factor-κB (NF-κB) signaling pathway in VILI. The mitochondrial damage and release of mtDNA were assessed. Pharmacological inhibition of mtDNA (chloroquine) was used to determine whether mtDNA trigger inflammation *via* TLR9 in VILI. EDU-labeled mtDNA deriving from mitophagy was assessed by immunofluorescence. The role of mitophagy in VILI was shown by administration of antimycin A and cyclosporine A.

Main results: Rats subjected to HTV showed more severe pulmonary edema and inflammation than the other rats. The decreased expression of TLR9, MyD88, and NF-κB were observed following the use of ODN2088. Mechanical ventilation (MV) with HTV damaged mitochondria which resulted in dysfunctional ATP synthesis, accumulation of reactive oxygen species, and loss of mitochondrial membrane potential. Moreover, the results of distribution of fluorescence in rats upon HTV stimulation indicated that mtDNA cleavage was associated with mitophagy. The expression levels of mitophagy related genes (LC3B-II/LC3B-I, PINK1, Parkin, and mitofusin 1) in animals ventilated with HTV were significantly upregulated. Administration of antimycin A aggregated the histological changes and inflammation after MV, but these effects were attenuated when administered in the presence of cyclosporine A.

Conclusion: MV with HTV induces mitochondrial damage and mitophagy, contributing to the release of mtDNA, which may be induced VILI in rat *via* TLR9/MyD88/NF-κB signaling pathway.

Keywords: ventilator-induced lung injury, toll-like receptor 9/myeloid differentiation factor 88/nuclear factor-κB signaling pathway, mitochondrial DNA, mitophagy, mitochondrial damage

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INTRODUCTION

Acute lung injury/acute respiratory distress syndrome is characterized as acute onset, intractable hypoxemia, and bilateral lung infiltration with high morbidity and mortality for hospitalized patients (1). Mechanical ventilation (MV) is a life-saving therapy for these patients. Paradoxically, inappropriate use of MV especially high tidal volume (HTV) can directly exacerbate lung injury, a syndrome termed as ventilator-induced lung injury (VILI). Increased pulmonary and vascular permeability, infiltrated inflammatory cells (2), activated immune responses (3), or oxidative stress (4) are the main contributing factors for VILI. The main mechanism of VILI includes volutrauma, barotrauma, atelectrauma, and biotrauma which consists of proinflammatory cytokines released, leukocytes recruited, and local inhibition of inflammatory processes (5).

Toll like receptors (TLRs) are well-known toll-like pattern recognition receptors that play an important role in the induction of innate immune and inflammatory responses. It is reported that TLR9 receptors are normally involved in initiating innate immune responding to damage-associated molecular patterns (DAMPs) (6). In our previous study, signaling involving TLR9 and myeloid differentiation factor 88 (MyD88) contributes to inflammation associated with the use of HTV ventilation for 4 h (7).

Mitochondria, under a variety of critical conditions, especially after damaged and dysfunctional, were engulfed and eliminated by selective autophagy (mitophagy) (8). Mitochondrial autophagy (mitophagy) is activated by mitochondrial permeability transitions, accumulation of reactive oxygen species (ROS), and loss of mitochondrial membrane potential ($\Delta\psi m$) with regulation of phosphatase and tensin homolog (PTEN) inducing putative kinase 1 (PINK1)/Parkin and mitofusin 1 (Mfn1) (8, 9). Mitochondria possess DNA similar to bacterial DNA, containing inflammatogenic unmethylated CpG motifs (10).

Mitochondrial DNA (mtDNA) is considered to be one of the mitochondrial DAMPs (11). Recently, a growing body of researches show that mtDNA triggers the immune response directly *via* the activation of TLR9 as its ligand. For instance, scholars have found that mtDNA releasing into the circulation by shock could activate neutrophil (PMN) p38 mitogen-activated protein kinase, probably *via* TLR9, inducing an innate-immune stimulatory "danger" response (12). Back in 2012, Oka et al. showed that mtDNA escaped from autophagy was capable of inducing myocarditis, and dilated cardiomyopathy *via* TLR9-mediated inflammatory responses in cardiomyocytes (10).

Taken together, mitophagy may be involved in the physiopathologic process of VILI, which highlights the interplay of inflammation and oxidative or endoplasmic reticulum (ER) stress between mitophagy and VILI. This manuscript intends to demonstrate whether HTV induce mitochondrial damage to activate mitophagy, resulting in mtDNA release. And to investigate the role and mechanism of mtDNA in VILI in rats.

MATERIALS AND METHODS

Animals

Pathogen-free Sprague-Dawley rats were purchased from the Animal Center of Guangxi Medical University (Nanning, China), and approved by the Institutional Animal Care and Use Committee of Tumor Hospital of Guangxi Medical University. Rats were injected intravenously with 500 μg of CpG oligodeoxy nucleotides (ODN2088) 2.0 h before MV [tidal volume (VT) = 40 ml/kg] to inhibit the expression of TLR9 (10). Meanwhile, some rats were intraperitoneally pretreated with an inhibitor of mtDNA called chloroquine (CQ, 30 mg/kg) 2.0 h before MV [VT = 40 ml/kg] (13). EdU (5.0 mg/kg, 2.0 h/time and five times) was intraperitoneally injected to label mtDNA before MV (10). Antimycin A (AmA, 15 mg/kg, bw/day) and cyclosporine A (CsA, 5.0 mg/kg, bw/day) were intraperitoneally injected to inhibit mitochondrial electron transport and membrane permeability transition individually (14–16).

Reagents

Enzyme linked immunosorbent assay (ELISA) kits were used to test the levels of inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), interleukin-1 beta (IL-1β), and myeloperoxidase (MPO) (CUSABIO, Wuhan, China). DNase I (AMPD1), rat (I4131), and mouse IgG (I8765) were obtained from Sigma-Aldrich for the pretreatment of lung tissues; Percoll (P8370, Pharmacia), RPMI 1640-HEPES medium (22400097, Gibco), RT II-70 monoclonal antibody (gift from Professor Gonzalez, UCSF-Medical Center, San Francisco), goat anti-mouse IgG3 Secondary Antibody Alexa Fluor 488 conjugate (A-21151, Life Technologies) were used to isolate alveolar type II (AT-II) cells. Dulbecco's modified Eagle's medium (10567022, Gibco) with rat serum (D110-00-0050, Rockland), recombinant human keratinocyte growth factor (KGF/FGF-7, 251-KG-010, R&D Systems), 8-Bromoadenosine 3′, 5′-cyclic monophosphate (8-bromocyclic AMP, B5386, Sigma-Aldrich) and Engelbreth-Holmes-Swarm Matrix (EHS Matrix, Matrigel, 356234, BD) were employed for culturing AT-II cells. Then CQ and ODN2088 were purchased from Sigma-Aldrich (C6628) and InvivoGen (tlrl-2088), respectively. In addition, TRIzol (15596018, Invitrogen) and real time-quantitative polymerase chain reaction (RT-qPCR) kit (Takara) were used to test the TLR9, cytochrome *c* oxidase 4 (COX4), MyD88, nuclear factor (NF)-κB (NF-κB), and β-actin's mRNA levels. LC3B (L7543, Sigma, 1:1,000), PINK1 (BC100-494, Novus biological, 1:1,000), Parkin (SAB4502077, Sigma-Aldrich, 1:800), Mfn1 (M6319, Sigma-Aldrich, 0.7 μg/ml), TLR9 (NBP1-76680, Novus, 1:1,000), MyD88 (4283, Cell Signaling Technology, 1:1,000), COX 4 (NB110-39115, Novus, 1:2,000), NF-κB (4764, Cell Signaling Technology, 1:1,200), and β-actin (4970, Cell Signaling Technology, 1:1,000) were used as primary antibodies and horseradish peroxidase (HRP)-conjugated mouse anti-rabbit antibody (7074, Cell Signaling Technology, 1:1,000) was used as secondary antibody in immunoblot and immunofluorescence assays. Moreover, ATP (MAK190, Sigma-Aldrich), ROS (MAK144, Sigma-Aldrich), and $\Delta\psi m$ assay kits (V35116, Invitrogen) were used to evaluate the mitochondrial damage by MV. AmA and CsA were both obtained from Sigma (St. Louis, MO, USA) for regulation of mitophagy. An EdU from Click-iT EdU Alexa Fluor 488 Imaging Kit (C10337, Invitrogen) was applied to detect mtDNA in the section.

MV Model and Sample Collection

The animal model was established successfully based on the previous study (7, 17). Briefly, rats were anesthetized by

intraperitoneal injection of 60 mg/kg pentobarbital sodium and 80 mg/kg ketamine, and the rats were given with 15 mg/kg pentobarbital sodium every 30 min and 2 mg/kg/h pancuronium for muscle relaxation. After received 16-gauge tube by tracheotomy, all animals were allowed to breathe spontaneously or ventilated mechanically with room air (FiO_2 = 0.21%) by a small animal ventilator (TOPO, Kent Scientific, Torrington, CT, USA). The ventilation rate was 80 breaths/min and the fraction of inspired oxygen was approximately at 40–50%. The inspiration to expiration ratio was kept at 1:1 throughout the experiment, no positive end expiratory pressure was included, and VT was calculated as previously described. After MV or spontaneous breathing, all rats were sacrificed by carotid artery bleeding, and the bronchoalveolar lavage fluid (BALF), blood serum, and lung tissue were collected and stored at −80°C except the right lung, which was obtained for paraffin embedding, transmission electron microscope (TEM) examining, and wet/dry (W/D) ratio calculating. It should be noted that all animal procedures were performed with great care to minimize activation of an inflammation.

Inflammatory Responses

The W/D ratio was calculated to estimate the condition of lung edema during VILI. The middle lobe of right lung was weighed and then dried to a constant weight at 60°C for 48 h. Total protein of BALF was assessed for pulmonary permeability by bicinchonininc acid (BCA) assay, and cells were counted for inflammatory infiltration by hemocytometer. Moreover, IL-1β, IL-6, TNF-α, and MPO in plasma and BALF were detected by ELISA kits according to the manufacturer's instructions.

Histopathological Analysis

The right lower lung lobe was dissected and fixed with 10% formaldehyde. The lung tissues were embedded with paraffin and stained with hematoxylin and eosin. The degree of lung injury was estimated by scores according to four criteria: (1) alveolar congestion; (2) hemorrhages; (3) neutrophils' infiltration; and (4) incrassation of the alveolar wall. Criterion were scaled as five-point scores: 0, minimal injury; 1, mild injury; 2, moderate injury; 3, serious injury; and 4, maximal injury. The cumulative histology score for all of the parameters was calculated, then the overall score of VILI was obtained. A mean ± SD was generated from the cohort of spontaneous breathing or ventilated lungs to generate a cumulative histological VILI (7, 18). Simultaneously, lung samples were cut for TEM analysis to observe lung cell epithelial cells and other cell injuries.

RNA Extraction and RT-qPCR

Total RNA was isolated from lung tissue using TRIzol reagent. After RNA quality and quantity were determined by 260/280 nm absorbance, single-stranded cDNA was synthesized using the Takara RNA PCR kit. Total RNA was determined, and 1 μg of total RNA was reverse-transcribed into cDNA and amplified using SYBR Premix Ex Taq II and specific primers for TLR9, COX4, MyD88, NF-κB, and β-actin. The level of each target gene was normalized relative to that of β-actin in each sample using the ΔCt method. Relative differences in gene expression among groups of the lung tissues were determined using the comparative Ct (ΔΔCt) method and fold expression was calculated by the formula $2^{-\Delta\Delta Ct}$, where ΔΔCt represents ΔCt values normalized relative to the mean ΔCt of healthy control samples. The final data were shown as the ratio of the mean value of triplicate detection of the mtDNA samples (COX 4) and the average value of the nuclear gene (β-actin), namely mtDNA/β-actin (19).

Immunoblot Analysis

Total protein was extracted, and the concentrations were assessed by BCA assay. Then, the molecular weight marker and each sample were added to the lanes of sodium dodecyl sulfate (SDS)-polyacrylamide gel. The proteins were transferred onto a nitrocellulose membrane and then blocked. Subsequently, the membranes were incubated by LC3B, PINK1, Parkin, Mfn1, COX 4, TLR9, MyD88, NF-κB, and β-actin primary antibodies and HRP-conjugated mouse anti-rabbit antibody as a secondary antibody. The bands of each protein from different samples were scanned and detected *via* a West Pico enhanced chemiluminescence kit (Thermo Fisher Scientific).

Immunofluorescence Techniques

The slides from the paraffin-embedded lung tissues were dewaxed, hydrated, used for antigen retrieval, blocked, and cleared of endogenous peroxidases. Then, anti-LC3B was used as the primary antibody and anti-rabbit IgG (H + L), F(ab′)2 Fragment (Alexa Fluor 594 Conjugate) served as secondary antibody, along with DAPI for nuclear staining. Meanwhile, cells from the EdU-labeled rats were detected *via* Click-iT EdU Alexa Fluor 488 Imaging Kit according to the manufacturer's directions to observe mtDNA accumulation in lung tissues, as well as for analyzing the source of escaped mtDNA. The slides were coated with Prolong Gold quenching resistant reagent and preserved at 4°C for imaging with fluorescence microscopy. The whole process was kept out of the sun.

Cells Isolation and Purification

Alveolar Macrophages (AMs) Extraction

Alveolar macrophages were isolated and cultured according to the previous method (17). The collected BALF was centrifuged at 1,000 × *g* for 10 min and washed three times with pathogen-free PBS. The pellet was resuspended in RPMI 1640 media containing 10% fetal bovine serum (FBS) and 20 kU/l penicillin–30 kU/l streptomycin with 10% CO_2 in air at 37°C for 3.0 h.

AT-II Cells Isolation

Dr. Gonzalez's method (20) was used to isolate and culture AT-II cells. Briefly, the whitening lungs were minced by Mayo-Noble scissors and added to a 25 ml-beaker containing 20% FBS and 0.5 ml DNase I solution (2 mg/ml "Solution A": RPMI 1640-HEPES medium). An additional 10 ml of "Solution A" was added to this beaker and the minced lung tissues with solution were transferred to a 250 ml-Erlenmeyer flask. Then, rat IgG was added to a final concentration of 50 μg/ml. The flask was shaken vigorously with 130 cycles/min for 2 min at reciprocating water bath, applying shear force to the lung minces. As the water bath shakes from side-to-side, the liquid should move back-and-forth rather

than swirling. This liquid containing the lung minces was then filtered *via* 100, 40, and 20 μm nylon mesh and the cell suspension was transferred to a 50 ml-tube containing 50 μg/ml mouse IgG. Moreover, 1 ml of RT II-70 was added to this cell suspension and incubated for 10 min on ice, followed by centrifugation at 350 × *g* for 12 min at 4°C with 150 μl of Percoll cushion. The resulting pellet was resuspended with 50 μl of DNase I and 1 ml of "Solution A" in 20% FBS to a 1.3 ml total volume. 0.3 ml of the cell suspension was aliquot into three tubes, and to each 0.1 μl of RT II-70, 0.1 μl of PBS and 0.5 μl of goat anti-mouse IgG_3 secondary antibody Alexa Fluor® 488 conjugate with 0.5 μl of RTII-70 was added. In addition, the remaining 1 ml of cell suspension was added 5 μl of goat anti-mouse IgG3 secondary antibody Alexa Fluor 488 conjugate and 5 μl of RTII-70 for cell isolation. Both of these tubes were incubated for 10 min at room temperature and diluted into 15 ml/tube with "Solution A." These tubes were then added to 150 μl Percoll cushion on the bottom of each tube and centrifuged at 350 × *g* for 15 min at 4°C. The pellet was resuspended with 50 μl of DNase I and 0.5 ml of "Solution A" in 20% FBS for further flow cytometer sorting. AT-II cells were cultured in Dulbecco's modified Eagle's medium with 1% rat serum, 10 ng/ml KGF, 10^{-4} M 8-bromocyclic AMP, and 20 kU/l penicillin–30 kU/l streptomycin on an EHS matrix with 10% CO_2 in air at 37°C.

Mitochondrial Damage Evaluation

The MV-induced mitochondrial damage with mitophagy was evaluated *via* measuring ATP levels, ROS production, and Δψm. The ATP level, ROS production, and Δψm were assayed using corresponding assay kits from Sigma-Aldrich according to the manufacturer's instruction. Briefly, the ATP levels were determined *via* firefly luciferase-associated chemiluminescence and ROS production was detected by 2′7′-dichlorofluorescin diacetate using flow cytometry analysis. The Δψm was observed using a fluorescent probe JC-1 that assembles as J-aggregates with red fluorescence in the mitochondrial matrix during higher Δψm, but is depolymerized as monomer and results in green fluorescence.

DNase-II Expression

The single-phase enzyme diffusion (21) method was used to compare DNase-II expression in the serum. In brief, "Solution A" [1 ml of 6 g/l calf thymus DNA; 50 μl of 10 g/l ethidium bromide; pH 7.2, 0.05 M Tris–HCl (0.05 M $MgCl_2$) 8~10 μl; 40 μl of 1 M $CaCl_2$] and "Solution B" (10 ml of 2% agarose in diethyl pyrocarbonate water) were mixed to make agar board which was then bored with a 1.5 mm-diameter puncher. 4 μl of either serum samples or standard DNase-II were added to the agar board holes, followed by 36 h of incubation at 37°C. The agar board was then soaked with 0.1 M elhylene diamine tetraacetic acid (EDTA) to stop the reaction and the diameter of DNA hydrolyzed loop was measured using ultraviolet radiation.

mtDNA Extraction and Purification

The extraction and purification of mtDNA were modified from the previous study to improve the yield and quality of mtDNA (22). Briefly, a pellet composed of 10^7 cells were resuspended with 5.5 ml "Solution I" of (10 M Tris–HCl; 10 M NaCl; 5 M $MgCl_2$; pH = 7.5) and centrifuged at 2,000 rpm for 10 min. The pellet was resuspended in 5.5 ml of "Solution II" (10 mM Tris–HCl; 0.4 M NaCl; 2 mM EDTA; pH = 7.5) and centrifuged at 3,500 rpm for 10 min. Then the pellet was resuspended in 0.95 ml of "Solution II" and 50 μl of 20 mg/ml protease K and 10% SDS were added, followed by incubation at 4°C overnight. 0.3 ml of saturated sodium acetate was added to the suspension liquid and lightly shaken, followed by centrifugation at 15,000 rpm for 15 min at 4°C. The supernatant was obtained, and the last step was repeated. A phenol:chloroform:isopropanol (25:24:1) solution was added to the supernatant, agitated by light shaking and centrifuged at 10,000 rpm for 10 min at 4°C. The upper water phase was transferred to a new tube, and the previous step was repeated. Two volumes of ice-cold ethanol were added to the upper water phase and incubated on ice for 30 min, followed by centrifugation at 15,000 rpm for 10 min at 4°C. 70% ice-cold ethanol was next used to wash the pellet, centrifuging the sample at 15,000 rpm for 2 min at 4°C. The pellet was dried for 25 min at room temperature and finally dissolved by recombinational trypsin/EDTA solution at storage of 4°C.

Statistical Analysis

The analyses of data were conducted by SPSS 13.0 software. All quantitative data were demonstrated as mean ± SD. One-way ANOVA was operated in order to analyze multiple comparisons of each groups, followed by the LSD-*t* test and the SNK test for pair-wise comparisons. Chi-square test was used for analysis of categorical variables. The statistical difference was defined by *P* value less than 0.05.

RESULTS

MV With HTV Induces Lung Injury and Inflammation

Anesthetized animals were randomly allocated into three groups ($n = 12$) using table of random number: non-ventilated (CON), ventilated with normal VT (10 ml/kg, NTV), and ventilated with high VT (40 ml/kg, HTV). All animals survived the 4-h period of spontaneous breathing or MV at normal or high VT. The rats treated with high VT exhibited significantly severe pulmonary edema and higher BALF total protein levels than the rats treated with spontaneous breathing and normal VT by determining the lung W/D ratios (**Figures 1A,C**). The lung histopathology score was higher in high VT rats as compared with the CON and NTV group. However, no differences were noted between the CON and NTV group (**Figure 1B**). In the HTV group, significantly more cells were infiltrated than in the NTV group and non-ventilated animals (**Figure 1D**). In addition, the levels of cytokine profiles, including IL-1β, IL-6, TNF-α, and MPO, in BALF and plasma were significantly higher in the HTV group than that in the CON and NTV groups. And these cytokine profiles were similar in animals ventilated with normal VT and control animals (**Figures 1E–H**).

Lungs from animals ventilated with high VT showed acute inflammatory infiltration, perivascular edema, and more alveolar

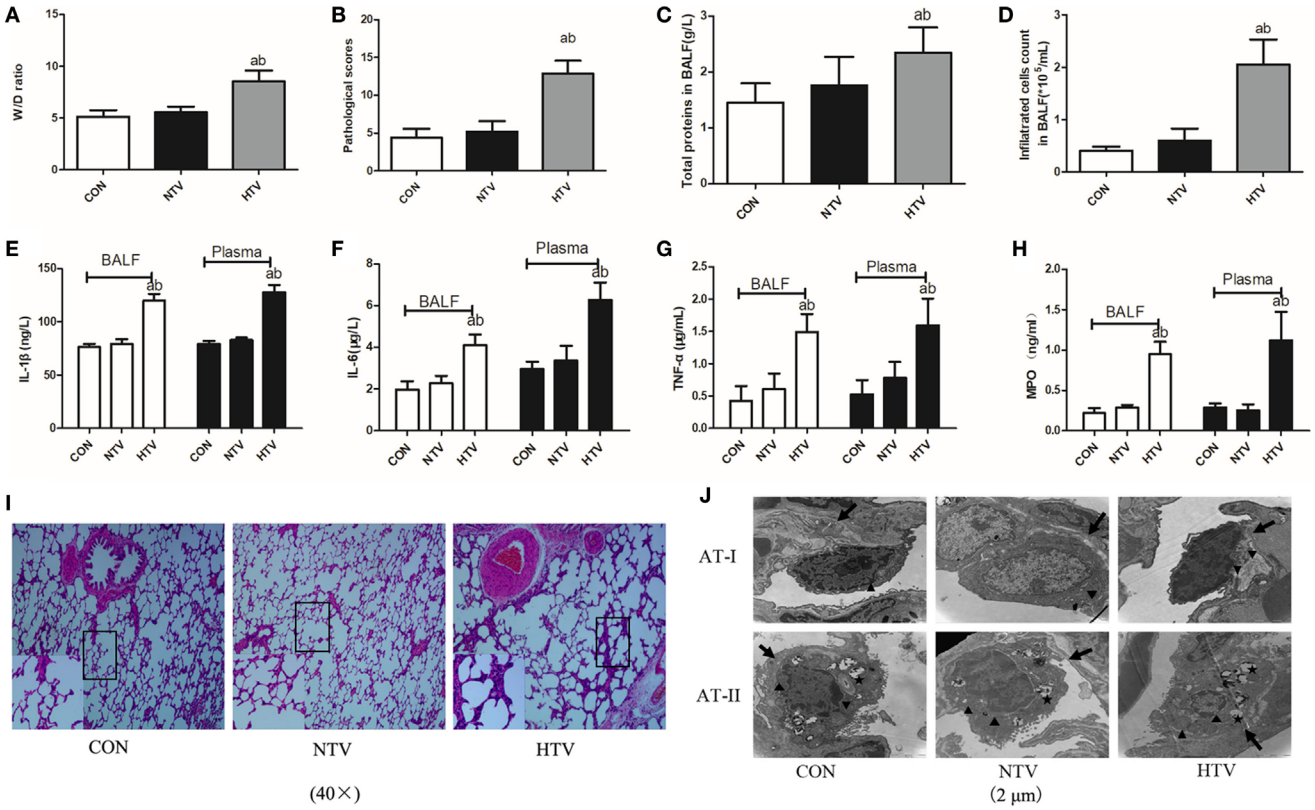

FIGURE 1 | Lung injury and inflammation in animals with spontaneous breathing (CON) or mechanical ventilation at normal tidal volume (NTV) or high tidal volume (HTV). **(A)** Lung edema was assessed by determining the weight ratio between wet and dry lung (W/D). **(B)** Pathological scores were assessed by results of hematoxylin and eosin (HE) staining. **(C)** Total protein concentration in bronchoalveolar lavage fluid (BALF). **(D)** Infiltrated cell counts in BALF. **(E)** Levels of interleukin (IL)-1β in BALF and plasma. **(F)** Levels of IL-6 in BALF and plasma. **(G)** Levels of tumor necrosis factor (TNF)-α in BALF and plasma. **(H)** Levels of myeloperoxidase (MPO) in BALF and plasma. **(I)** Histology of lung tissue was stained with HE. Magnification, 40×. **(J)** Transmission electron micrographs of alveolar cells. Areas marked with arrows signify continuous membrane and triangles mark areas of disrupted cytoplasmic and nuclear structure. Areas marked with stars represent the osmilphilic multilamellar bodies, the unique structure of AT-IIs. Tissue from CON and NTV groups appeared normal, but tissue from HTV group exhibited a disrupted cytoplasmic and nuclear structure, as well as cell membrane discontinuities. In addition, the osmilphilic multilamellar bodies show characteristic vacuolation in the HTV group. Magnification, 20,000×. Both of these experiments were in triplicate. $^{a}P < 0.05$ vs. CON group; $^{b}P < 0.05$ vs. NTV group.

septal thickening, whereas no major histological differences were observed between animals ventilated with normal VT and spontaneous breathing control animals (**Figure 1I**). Using TEM to examine alveolar histopathology in greater detail, we found that, as expected, alveolar cells in the tissue of the rats ventilated with high VT exhibited a disrupted cytoplasmic and nuclear structure, as well as cell membrane discontinuities. However, tissue from rats ventilated with normal VT and spontaneous breathing control animals showed a normal cytoplasmic and nuclear structure and continuous cell membrane for types I and II alveolar epithelial cells. The osmilphilic multilamellar body is the unique structure of AT-IIs. In addition, the osmilphilic multilamellar bodies show characteristic vacuolation in the AT-IIs of HTV group (**Figure 1J**).

MV With HTV Induced TLR9 Expression and Upregulated Protein Levels of MyD88 and NF-κB

The protein levels of TLR9, MyD88, and NF-κB were significantly higher in the HTV group as compared with the other groups. Moreover, pretreated with ODN2088 could significantly ameliorated the HTV-induced these proteins in the lungs. It might suggest that HTV induces inflammatory response in the lungs *via* TLR9/MyD88/NF-κB signaling pathway (**Figure 2**).

MV With HTV Favors the Damage of Mitochondrial and the Release of mtDNA

In order to explore whether MV with HTV can favor the damage of mitochondrial and the release of mtDNA, ATP levels, ROS production, Δψm variation, and expression level of mtDNA were measured. The level of ATP in the HTV was decreased in comparison to the rats treated with spontaneous breathing and pretreated CQ could alleviate the result (**Figure 3A**). Then, the production of ROS in HTV group was significantly increased in comparison to the rats treated with spontaneous breathing, but pretreated with CQ could reduce these ROS induced by MV with HTV, especially in AT-IIs (**Figures 3B,G**). Moreover, the Δψm in the HTV group was decreased in comparison to the rats treated with spontaneous breathing, while compared with the animals over-ventilated with CQ (**Figure 3C**).

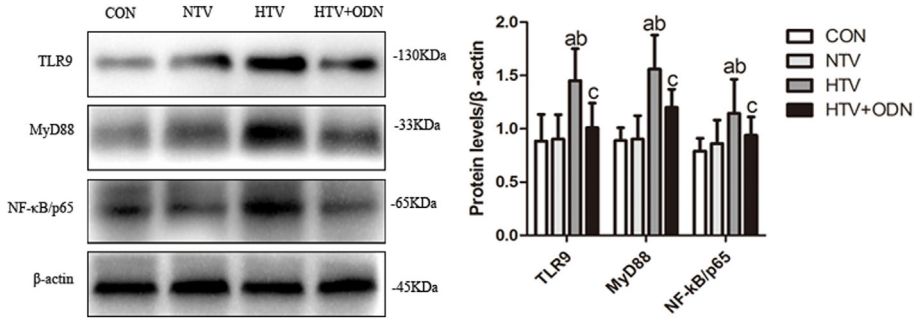

FIGURE 2 | Levels of toll-like receptor (TLR) 9, myeloid differentiation factor 88 (MyD88), and nuclear factor (NF)-κB in lung tissues from animals with spontaneous breathing (CON), mechanical ventilation at normal tidal volume (NTV), high tidal volume (HTV), and ODN2088 pretreatment upon HTV stimulation (HTV + ODN). $^{a}P < 0.05$ vs. CON group; $^{b}P < 0.05$ vs. NTV group; and $^{c}P < 0.05$ vs. HTV group.

The level of serum DNase-II (a main enzyme for degradation of mtDNA) in the HTV was decreased in comparison to the rats treated with spontaneous breathing or normal VT (**Figure 3D**). The outcome of RT-qPCR in isolated mtDNA in the HTV group was significantly higher than that in CON group, but level of mtDNA was significantly lower in the CQ pretreatment group compare with the HTV group (**Figures 3E,F**).

Inhibition of COX4 Regulates the Expression of COX4, TLR9, MyD88, and NF-κB

The mRNA and protein levels of COX4, TLR9, MyD88, and NF-κB were significantly higher for animals ventilated with high VT than animals in the CON and NTV groups. Expectedly, after pretreated with mtDNA inhibitor CQ, the mRNA and protein expression of COX4, TLR9, MyD88, and NF-κB in animals treated with high VT were significantly inhibited (**Figures 4A–C**).

mtDNA Escaped From a Selective Autophagy Named Mitophagy During MV With HTV

The expression of LC3B conjugated with a fluorescence moiety was recommended in order to observe the autophagy state and the EdU was used for mtDNA distribution. The fluorescence of mtDNA and LC3B, which specifically marked mitophagy, turned up in same places, indicating that mtDNA could be released due to autophagic lysosomes. The fluorescence intensity showed that the expression of LC3B and mtDNA in the HTV was increased compared to CON group. More importantly, the expression of LC3B and mtDNA in the HTV + CQ group was lower than that in the HTV group (**Figure 5**).

Regulation of Mitophagy Plays an Important Role in VILI *via* TLR9–MyD88–NF-κB Pathway

The role of mitophagy in VILI was shown by presence of antimycin A (inhibitor of mitochondrial electron transport) and cyclosporine A (inhibitor of mitochondrial membrane permeability transition). AmA pretreatment aggravated lung edema (**Figure 6A**) and morphological injuries (**Figures 6B,I,J**), but CsA pretreatment attenuated the lung edema and injury. Total protein level for pulmonary permeability in BALF was significantly increased in the AmA group compared to the HTV group and CON group (**Figure 6C**), while infiltrated cells counted in BALF was consistent with the variation of total proteins (**Figure 6D**). The concentrations of inflammatory factors, including IL-1β, IL-6, TNF-α, and MPO, in the AmA group (plasma and BALF) were higher than those in the HTV and control groups, and CsA pretreatment could attenuate these inflammatory factors (**Figures 6E–H**).

The expression levels of mitophagy related genes (LC3B-II/LC3B-I, PINK1, Parkin, and Mfn1) in animals ventilated with high VT were significantly upregulated (**Figures 7A–D**). AmA favored the higher ratio of LC3B-II/LC3B-I, expression of PINK1, Parkin, and Mfn1, while CsA inhibited the expression of these mitophagy related proteins (**Figures 7A–D**). What is more, AmA pretreatment upregulated the mRNA and protein level for COX4, TLR9, MyD88, and NF-κB, but CsA pretreatment attenuated the expression of these genes (**Figures 7E–I**).

DISCUSSION

Mechanical ventilation is life saving during the perioperative period, but inappropriate MV can lead to the development of VILI (23, 24). VILI is the result of a complex systematic inflammation associated with release of various proinflammatory mediators and activation of inflammatory signaling pathways (25). A better understanding of the mechanisms of VILI in lungs is needed if protective ventilator strategies are to be developed further.

In this study, rats subjected to HTV developed marked pathologic changes, ultrastructure changes in alveolar and release of various inflammatory cytokines (**Figure 1**). Pretreatment with ODN2088 significantly ameliorated the phenomenon HTV-induced described above, which was further explored that HTV ventilation caused the release of various mediators *via* TLR9/MyD88/NF-κB signaling pathway, leading to VILI in rats (**Figure 2**). The result is in accordance with our previous results (7). Meanwhile, MV with HTV damaged mitochondria,

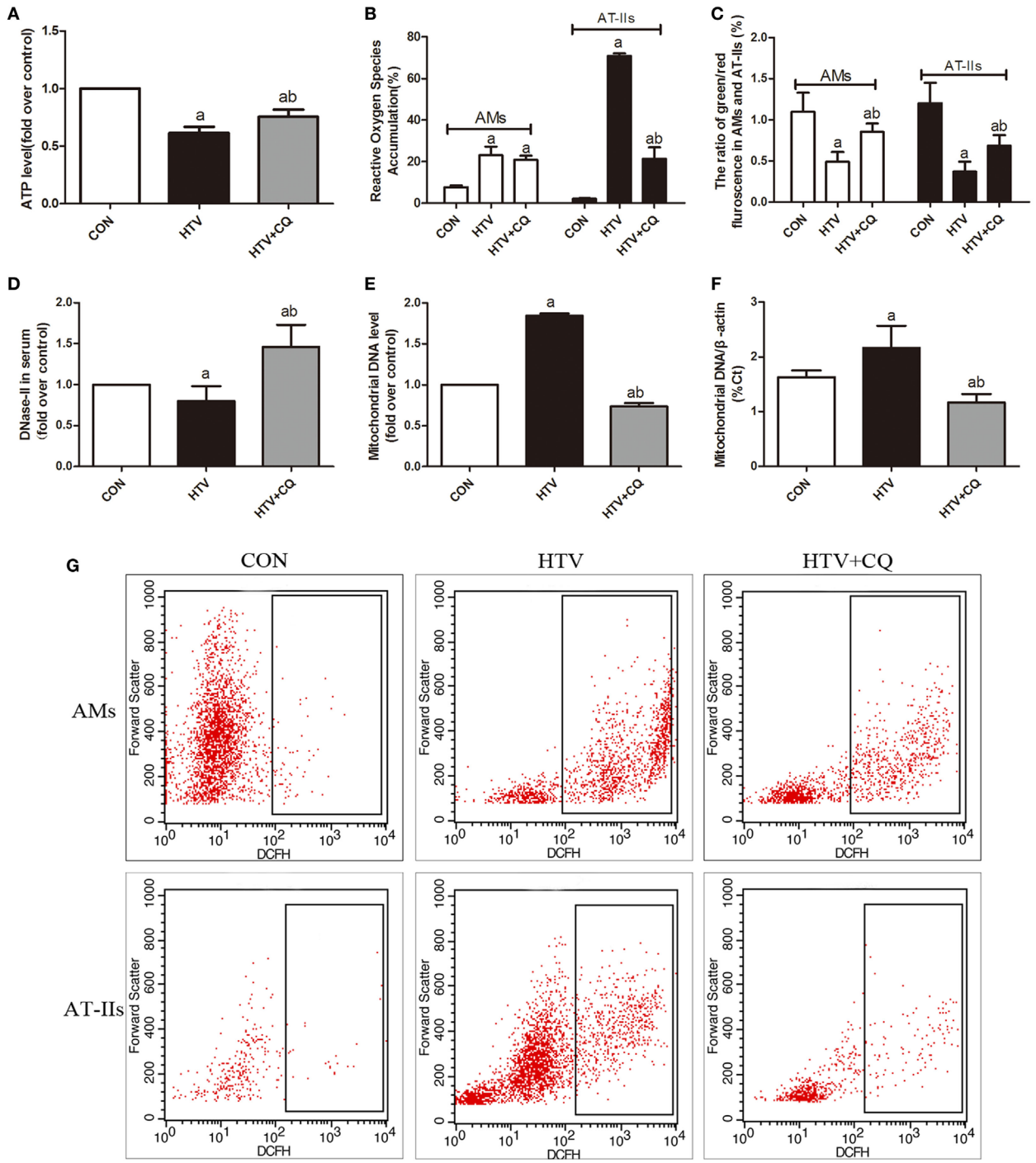

FIGURE 3 | Mechanical ventilation with high tidal volume (HTV) favors the damage of mitochondrial and the release of mitochondrial DNA (mtDNA). **(A)** Levels of ATP. **(B)** Levels of reactive oxygen species (ROS) in alveolar macrophages (AMs) and AT-IIs. **(C)** Levels of $\Delta\psi m$ were noted by the decreased ratio of green (JC-1 monomer for low $\Delta\psi m$)/red (J-aggregates for high $\Delta\psi m$) fluorescence in AMs and AT-IIs. **(D)** Levels of DNase-II in plasma. **(E)** Levels of isolated mtDNA (fold over control). **(F)** Relative levels of mtDNA by measurement of the β-actin gene. **(G)** The flow cytometry analysis of ROS. Both of these experiments were in triplicate. $^aP < 0.05$ vs. CON; $^bP < 0.05$ vs. HTV group.

causing dysfunctional ATP synthesis (**Figure 3A**), accumulation of ROS (**Figures 3B,G**) and loss of $\Delta\psi m$ (**Figure 3C**), especially in AT-IIs. The level of DNase-II and isolated mtDNA revealed that MV with HTV could cause the release of mtDNA (**Figures 3D–F**). Moreover, pretreatment with CQ reduced the protein and mRNA levels of COX4, TLR9, MyD88, and NF-κB

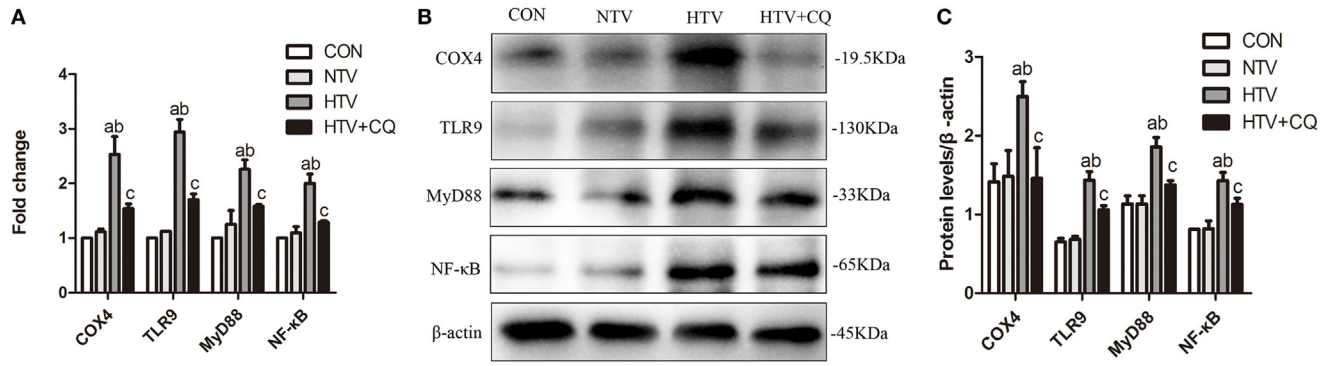

FIGURE 4 | The mRNA and protein expression of cytochrome *c* oxidase 4 (COX4), toll-like receptor (TLR) 9, myeloid differentiation factor 88 (MyD88), and nuclear factor (NF)-κB in lung tissues from spontaneous breathing group (CON group), normal tidal volume (NTV) group, high tidal volume (HTV) group, chloroquine (CQ) pretreatment upon HTV stimulation group (HTV + CQ). **(A)** Levels of COX4, TLR9, MyD88, and NF-κB mRNA. **(B)** Levels of COX4, TLR9, MyD88, and NF-κB protein by Western blot. **(C)** Relative expression of COX4, TLR9, MyD88, and NF-κB protein. Fold expression for target genes was normalized to that measured for the β-actin gene. Both of these experiments were in triplicate. $^{a}P < 0.05$ vs. CON group; $^{b}P < 0.05$ vs. NTV group; and $^{C}P < 0.05$ vs. HTV group.

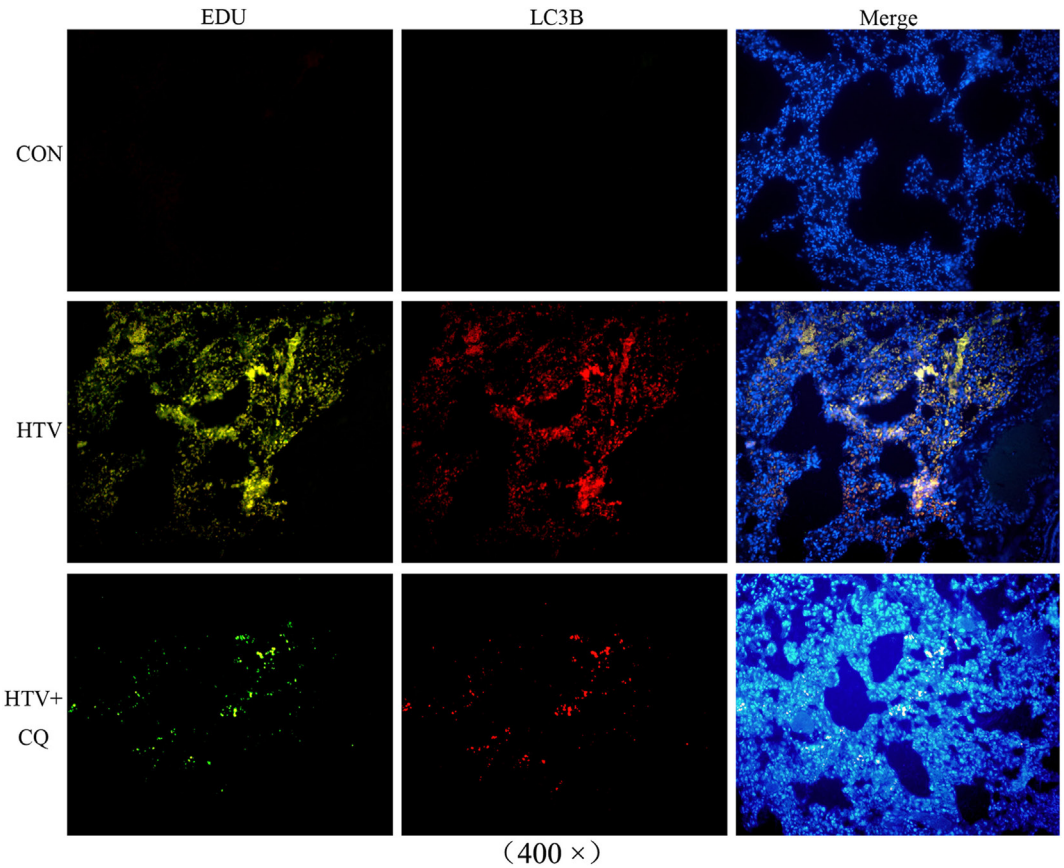

FIGURE 5 | Immunofluorescence studies of EdU-labeled mitochondrial DNA (mtDNA) (green) and crucial autophagic protein LC3B (red) in lung tissues from animals with spontaneous breathing (CON) and mechanically ventilated rats pretreated with saline [high tidal volume (HTV)], CQ (HTV + CQ). Immunofluorescence studies confirmed that the EdU-labeled mtDNA (green) was similarly distributed and accumulated with the crucial autophagic protein LC3B, which was labeled with Alexa Fluor 594 (red). Magnification, 400×. Both of these experiments were in triplicate.

(**Figure 4**). These findings suggested that mtDNA (COX4) may, at least in part through TLR9/MyD88/NF-κB signaling pathway, initiate an immunological response characteristic of VILI.

Interestingly, HTV-induced accumulation of ROS was observed in AT-IIs rather than AT-Is in the present study. AT-Is cover approximately 90–95% of the alveolar surface and involve in

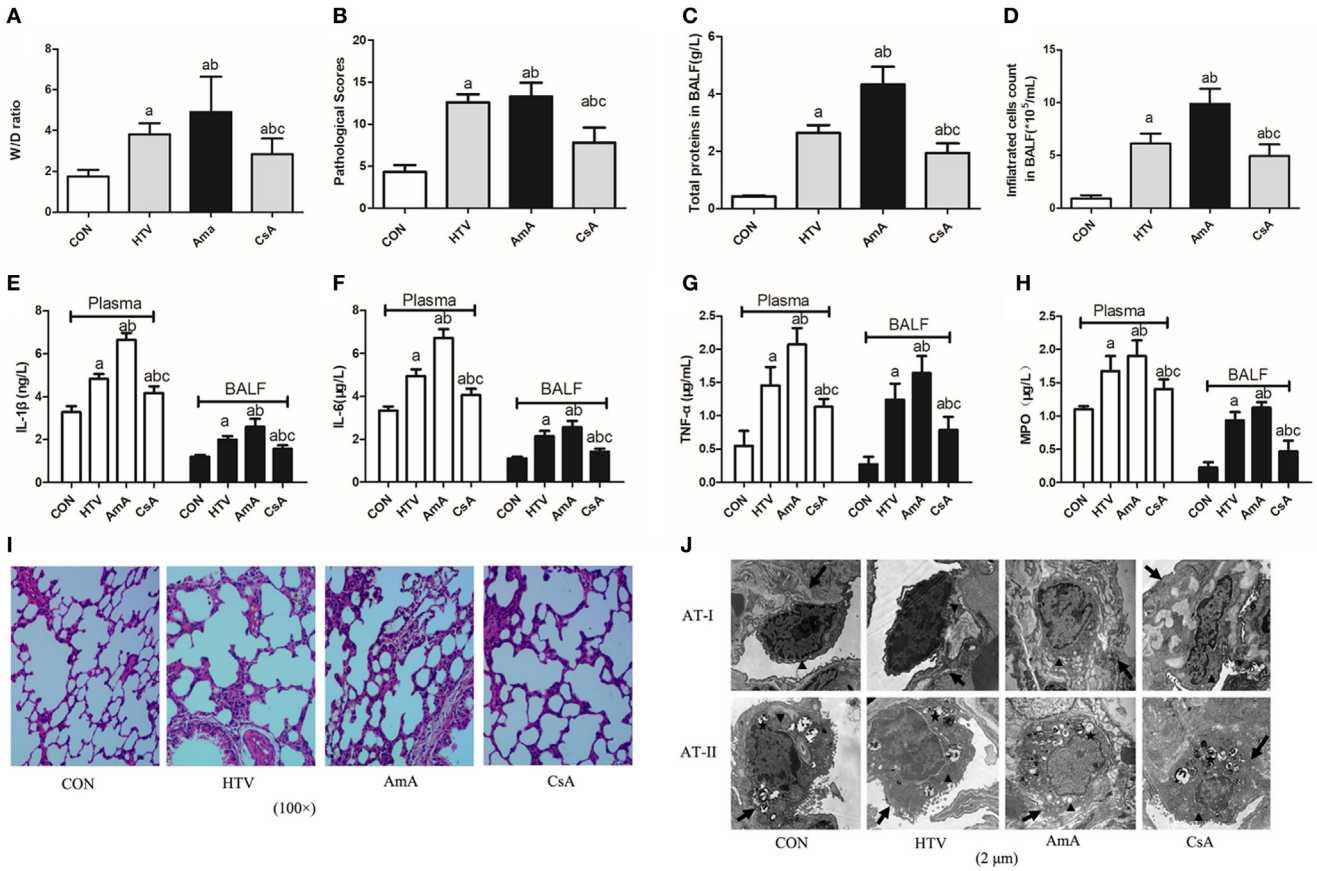

FIGURE 6 | Lung injury and inflammation in animals with spontaneous breathing (CON) or mechanical ventilation at high tidal volume (HTV) with saline, antimycin A (AmA), or cyclosporine A (CsA). **(A)** Lung edema was assessed by determining the weight ratio between wet and dry lung (W/D). **(B)** Pathological score was assessed by result of hematoxylin and eosin (HE) staining. **(C)** Total protein concentration in bronchoalveolar lavage fluid (BALF). **(D)** Infiltrated cell counts in BALF. **(E)** Levels of interleukin (IL)-1β in BALF and plasma. **(F)** Levels of IL-6 in BALF and plasma. **(G)** Levels of tumor necrosis factor (TNF)-α in BALF and plasma. **(H)** Levels of myeloperoxidase (MPO) in BALF and plasma. **(I)** Histology of lung tissue was stained with HE. Magnification, 100×. **(J)** Transmission electron micrographs of alveolar cells. Areas marked with arrows signify continuous membrane and triangles mark areas of disrupted cytoplasmic and nuclear structure. Areas marked with stars represent the osmilphilic multilamellar bodies, the unique structure of AT-IIs. Tissue from CON group appeared normal, but tissue from HTV and AmA groups exhibited a disrupted cytoplasmic and nuclear structure, as well as cell membrane discontinuities. Moreover, the osmilphilic multilamellar bodies show characteristic vacuolation in the HTV and AmA groups. But these effects were attenuated when administered in the presence of CsA. Magnification, 20,000×. Both of these experiments were in triplicate. $^{a}P < 0.05$ vs. CON group; $^{b}P < 0.05$ vs. HTV group; and $^{c}P < 0.05$ vs. AmA group.

the process of gas exchange between the alveoli and blood; AT-IIs favor the secretion of pulmonary surfactant and differentiation to AT-Is. At the cellular level, AT-IIs play the more important role in regulation of inflammation, apoptosis, oxidative, and ER stress during MV with HTV (26, 27). It showed that pulmonary autophagy or mitophagy mainly occurred in macrophages and AT-IIs during MV, *Mycobacterium tuberculosis*, and *S. aureus* infection (10, 28, 29).

In order to confirm the possible release of mtDNA during MV with HTV, immunoblot analysis (**Figure 7B**) and immunofluorescence (**Figure 5**) were measured. Results indicated that MV with HTV could activate mitophagy, causing damaged mitochondria to engulf as a result of mtDNA release from damaged mitochondria. Previous researches have shown that increased ROS and reduced ATP levels can be accompanied with mtDNA damage, NF-κB and MyD88 upregulation, triggering inflammation through the TLR9 pathway in cells and mouse models (9, 30), which was consistent with our studies described above.

In the current study, the role of mitochondrial damage in VILI was shown by presence of antimycin A (inhibitor of mitochondrial electron transport) and cyclosporine A (inhibitor of mitochondrial membrane permeability transition) (**Figures 6** and **7**). AmA pretreatment aggravated lung injuries, favored the higher ratio of LC3B-II/LC3B-I, expression of PINK1, Parkin, and Mfn1, and upregulated the mRNA and protein level of COX4, TLR9, MyD88, and NF-κB. But CsA pretreatment showed opposite results. The results indicated that regulation of mitophagy plays an important role in VILI.

Autophagy is a cytoprotective process that is activated for the turnover and metabolism of proteins, as well as in eliminating damaged organelles or pathogens (31, 32). Mitophagy, a selective autophagy, was activated to remove damaged mitochondria

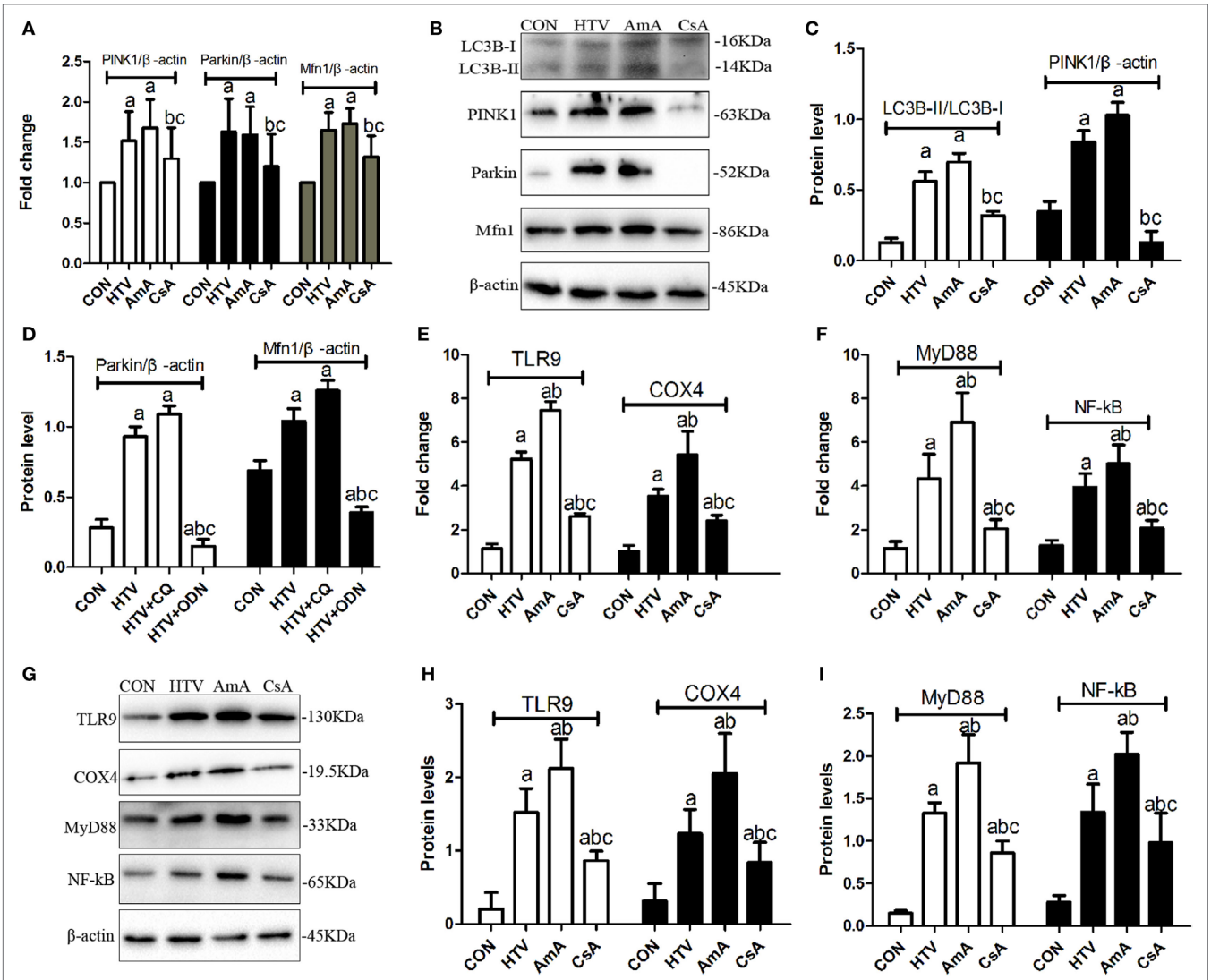

FIGURE 7 | The expression levels of microtubule protein light chain 3 (LC3B), PTEN inducing putative kinase 1 (PINK1), Parkin, mitofusin 1 (Mfn1), toll-like receptor (TLR) 9, cytochrome *c* oxidase 4 (COX4), myeloid differentiation factor 88 (MyD88), and nuclear factor (NF)-κB in lung tissues from animals with spontaneous breathing (CON) or mechanical ventilation at high tidal volume (HTV) with saline, antimycin A (AmA) or cyclosporine A (CsA). **(A)** Levels of PINK1, Parkin, and Mfn1 mRNA. **(B)** Levels of LC3B, PINK1, Parkin, and Mfn1 protein by Western blot. **(C)** Relative expression of LC3B-II/LC3B-I and PINK1 protein. **(D)** Relative expression of Parkin and Mfn1 protein. **(E)** Levels of TLR9 and COX4 mRNA. **(F)** Levels of MyD88 and nuclear factor-κB (NF-κB) mRNA. **(G)** Levels of TLR9, COX4, MyD88, and NF-κB protein by Western blot. **(H)** Relative expression of TLR9 and COX4 protein. **(I)** Relative expression of MyD88 and NF-κB protein. Fold expression for target genes was normalized to that measured for the β-actin gene. Both of these experiments were in triplicate. $^{a}P < 0.05$ vs. CON group; $^{b}P < 0.05$ vs. HTV group; and $^{c}P < 0.05$ vs. AmA group.

and maintain mitochondrial quality control. The recruitment of damaged mitochondria to the autophagosome is initiated by the phosphatase and tensin homolog deleted in chromosome 10-induced PINK1 that is stabilized on depolarized or damaged mitochondria *via* Parkin (33). After loss of $\Delta\psi m$, PINK1 recruits Parkin from the cytosol to the mitochondria, at which point it interacts with the GTPase Mfn, and then Parkin polyubiquitates the chains that marks the depolarized mitochondria for degradation. Parkin ubiquitinates many mitochondrial outer membrane proteins, such as porin, Mfn, and Miro, which are subsequently recognized and combined with the autophagic cargo adaptor protein p62 to deliver to autophagosomes (34). Confronted with oxidative stress, starvation, and pathogens, mitophagy is activated to eliminate redundant or damaged components, consequently causing inflammation. Mitophagy in Atg5 deficiency model facilitate cardiac inflammation and injury in response to angiotensin II (35).

Mitochondria DAMPs induce "sepsis-like" inflammation and mediate organ damage. It reported that mitochondrial DAMPs triggered inflammation and recruited inflammatory factors, including TNF-α, IL-6, and IL-10, under hepatic ischemia/reperfusion injury (36). Mitochondrial DAMPs act as potential proinflammatory mediators that participate in innate cell inflammation of non-hemolytic transfusion reactions (37). Moreover,

mitochondrial DAMPs in circulation are major arbiters for the systematic inflammatory response syndrome during trauma (38). But there was study demonstrated that mitochondrial DAMPs released from damaged tissues by trauma also suppress immune responses (39). Finally, combining with our study, results indicate that released mtDNA could act as DAMPs to mediate inflammation during MV.

The released mtDNA was recently considered to be mitochondrial DAMPs to induce inflammation. Mitochondrial damage and mtDNA release is not favored the inflammation in myocardium, atherosclerosis (40), and pulmonary diseases (41), but is triggered during the ER stress and autophagy induced by palmitate in skeletal muscle cells (42). Moreover, mitochondria are involved in initiating inflammasomes and inflammatory pathways, including excess ROS generation that can result in mtDNA mutations to initiate a vicious cycle of mitochondrial collapse (43), as well as activating the NLRP3 inflammasome, the adapter protein ASC, and caspase-1 (44). What is more, some studies have shown that mtDNA, containing unmethylated CPG motifs, which can be recognized by intracellular TLR9, has been shown to have powerful immunostimulatory effects (10, 12, 45, 46). Furthermore, TLR9 regulated by mtDNA can activate NF-κB signaling, which thereby induces transcription of many proinflammatory cytokines levels, such as TNF-α, IL-6, and IL-1β (12, 47). These data are consistent with the results of our present study.

Limitations

Although mtDNA is clearly involved in inflammation through the TLR9/MyD88/NF-κB pathway in regulating inflammatory cytokines during MV, there are several limitations that warrant further discussion. First of all, there are some other factors can affect injury severity, such as positive end expiratory pressure and variations in pressure support (48). For minimizing these impacts, we continuously monitored hemodynamic stability and oxygen saturation in the anesthetized rats. Second, according to the literature, the type and dose of anesthetic (e.g., sevoflurane, ketamine, or protofol) use can also affect animal studies on acute lung injury (49, 50). In addition, our study, however, were done only in living animals. We cannot say whether the results would be the same *in vitro* experiments.

CONCLUSION

Excessive MV with HTV triggers mitochondria damage to activate mitophagy, resulting in mitochondrial membrane fracturing and mtDNA release, which was recognized by TLR9 that modulate inflammatory factor expression through TLR9/MyD88/NF-κB signaling pathway during VILI.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of "the Animal Center of Guangxi Medical University (Nanning, China)." The protocol was approved by the "Institutional Animal Care and Use Committee of Tumor Hospital of Guangxi Medical University."

AUTHOR CONTRIBUTIONS

L-hP designed and directed the overall study. J-YL and RJ carried out the main experiments, contributed to the collection and analysis of data, and wrote the paper. FL, H-jD, and W-yG carried out experiments and collected and analyzed data with RJ. All authors read and approved the final manuscript.

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Defective Autophagy in T Cells Impairs the Development of Diet-Induced Hepatic Steatosis and Atherosclerosis

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Macroautophagy (or autophagy) is a conserved cellular process in which cytoplasmic cargo is targeted for lysosomal degradation. Autophagy is crucial for the functional integrity of different subsets of T cells in various developmental stages. Since atherosclerosis is an inflammatory disease of the vessel wall which is partly characterized by T cell mediated autoimmunity, we investigated how advanced atherosclerotic lesions develop in mice with T cells that lack autophagy-related protein 7 (Atg7), a protein required for functional autophagy. Mice with a T cell-specific knock-out of Atg7 (Lck-Cre $Atg7^{f/f}$) had a diminished naïve $CD4^+$ and $CD8^+$ T cell compartment in the spleen and mediastinal lymph node as compared to littermate controls ($Atg7^{f/f}$). Lck-Cre $Atg7^{f/f}$ and $Atg7^{f/f}$ mice were injected intravenously with rAAV2/8-D377Y-mPCSK9 and fed a Western-type diet to induce atherosclerosis. While Lck-Cre $Atg7^{f/f}$ mice had equal serum Proprotein Convertase Subtilisin/Kexin type 9 levels as compared to $Atg7^{f/f}$ mice, serum cholesterol levels were significantly diminished in Lck-Cre $Atg7^{f/f}$ mice. Histological analysis of the liver revealed less steatosis, and liver gene expression profiling showed decreased expression of genes associated with hepatic steatosis in Lck-Cre $Atg7^{f/f}$ mice as compared to $Atg7^{f/f}$ mice. The level of hepatic $CD4^+$ and $CD8^+$ T cells was greatly diminished but both $CD4^+$ and $CD8^+$ T cells showed a relative increase in their IFNγ and IL-17 production upon Atg7 deficiency. Atg7 deficiency furthermore reduced the hepatic NKT cell population which was decreased to <0.1% of the lymphocyte population. Interestingly, T cell-specific knock-out of Atg7 decreased the mean atherosclerotic lesion size in the tri-valve area by over 50%. Taken together, T cell-specific deficiency of Atg7 resulted in a decrease in hepatic steatosis and limited inflammatory potency in the (naïve) T cell compartment in peripheral lymphoid tissues, which was associated with a strong reduction in experimental atherosclerosis.

Keywords: autophagy, Atg7, T cells, steatosis, atherosclerosis

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INTRODUCTION

Atherosclerosis is an autoimmune-like disease of the vessel wall in which local accumulation of (modified) lipoproteins elicits an inflammatory response, which is among others T cell-mediated (1). The progression and stability of an atherosclerotic lesion largely depends on the subtype of the T cell, as $CD4^+$ T helper cells are generally considered atherogenic, while T regulatory (Treg)

cells predominantly act atheroprotective (1). T helper 1 (Th1) cells represent a major fraction of the T cells which drive local inflammation through the secretion of inflammatory cytokines such as interferon-gamma (IFNy) (2, 3). In contrast, Treg cells are immunosuppressive T cells, which can inhibit effector T cells and other immune cells in lymphoid tissues, and atherosclerotic lesions. Treg cells act via direct cell-cell interactions and via secretion of cytokines such as interleukin (IL)-10 and transforming growth factor β (4, 5). Other T helper cell subsets besides Th1 and Treg cells have a less dichotomous contribution to atherosclerosis. Th17 cells represent another subset of T helper cells, which are functionally characterized by the secretion of the interleukin IL-17. Th17 cells are involved in mucosal immunity where these cells help clearance of extracellular pathogens. Interestingly, their contribution to the ongoing inflammatory response in atherosclerotic lesions is context-dependent as Th17 cells have been described to have both atheroprotective as atherogenic functions (6). Cytotoxic CD8$^+$ T cells exert their inflammatory function by secreting cytokines, by performing cell-lysis via perforin, or granzyme-B and by inducing cell-death of their target cells through Fas-FasL interactions (7). CD8$^+$ T cells might diminish the development of atherosclerotic lesions in early stages of the disease by killing macrophages and other antigen-presenting cells but they actually might promote lesion development by secreting pro-inflammatory cytokines (8). Thus, the contribution of CD8$^+$ T cells to atherosclerosis seems context-dependent and remains to be elucidated. One particular process which has gained interest in T cells but has not been studied extensively yet in the context of atherosclerosis is macroautophagy.

Macroautophagy (from henceforth called autophagy) is a well-conserved cellular process in which cytoplasmic cargo is (non-)selectively isolated in double-membrane vesicles called autophagosomes and subsequently transported to lysosomes for lysosomal degradation. Various autophagy-related proteins (Atg) contribute to consecutive phases of the autophagic process (9).

The engulfment of cytoplasmic cargo by autophagosomes is mediated by two ubiquitin-like conjugation systems which are involved in the expansion and closure of the autophagosomal membranes. In the Atg12 conjugation system, Atg12 is activated by the E1 enzyme Atg7 after which Atg12 forms a conjugate with Atg5, and forms a complex with Atg16L (10, 11). In mammalian cells, the Atg12-Atg5-Atg16L is bound to the isolation membrane from which it dissociates after its maturation to an autophagosome (12). The other conjugation system involves microtubule-associated protein 1 light chain 3 (LC3) which is activated by Atg7 and subsequently transferred to Atg3 (13). The Atg12-Atg5-Atg16L complex is required for the adequate conjugation of LC3 to the phospholipid phosphatidylethanolamine (PE) by Atg3 (9, 13). Finally, the LC3-PE conjugate subsequently facilitates the tethering and fusion of the autophagosome membrane (14), thus closing the autophagosome.

Under homeostatic conditions, autophagy is important for the quality control of key organelles for example by degrading and recycling damaged or dysfunctional mitochondria (9). Accordingly, genetic blockade of the Atg5 and Atg7 proteins impact the (functional) stability of CD8$^+$ T cells (15), Th1 (16), and regulatory T cells (17).

In CD8$^+$ T cells for example, deficiency of Atg5 or Atg7 does not affect clonal expansion, but does impair memory formation and survival, which was also associated with an altered metabolic phenotype in Atg7 deficient T cells (15). Genetic blockade of Atg7 decreases the proliferation of activated naïve CD4$^+$ T cells whereas pharmacological blockade of autophagy in differentiated Th1 cells blocks proliferation and IFNγ secretion (16). Treg cell-specific genetic blockade of Atg7 does not affect Treg cell development but severely disrupts their immunosuppressive phenotype under activating conditions. Atg7 deficient Treg cells are apoptotic, lose expression of FoxP3, and gain an inflammatory phenotype characterized by high levels of glycolysis and IFNγ secretion (17).

Thus, genetic or pharmacological inhibition of autophagy modulates the inflammatory phenotype of various CD4$^+$ and CD8$^+$ T cell subsets albeit in different stages, and through different mechanisms. Given the contribution of the aforementioned T cell subsets to the development of atherosclerosis, their reliance on functional autophagy and the therapeutic implication of certain autophagy inhibitors (such as chloroquine) to treat cardiovascular disease (18), we aimed in this study to determine how atherosclerosis is affected by T cell-specific deletion of Atg7.

Here, we show that atherosclerosis development is severely hampered in mice with T cell-specific Atg7 deficiency and that this is associated with decreased hepatic steatosis and by decreased frequencies of CD4$^+$, CD8$^+$ T cells, and natural killer T (NKT) cells.

MATERIALS AND METHODS

Mice

All animal work was performed according to the guidelines of the European Parliament Directive 2010/63EU and the experimental work was approved by the Animal Ethics committee of Leiden University. *B6.Cg-Atg7<tm1Tchi>* (Atg7$^{f/f}$) and *B6.Cg-Tg(Lck-cre)1Jtak* (Lck-Cre) mice were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. To generate mice with T cell-specific deficiency of Atg7, Atg7$^{f/f}$ mice were crossed with mice expressing Cre recombinase under control of the *Lck* promotor (Lck-Cre), thus creating Lck-Cre Atg7$^{f/f}$ mice. Atg7$^{f/f}$ littermates served as controls. 18 week old Lck-Cre Atg7$^{f/f}$ mice and their littermates were used to examine the effects of Atg7 deficiency on the T cell populations in the blood, spleen, and mediastinal lymph nodes (medLN) under normolipidemic conditions.

Flow Cytometry

Spleens and mediastinal lymph nodes (medLN) were isolated and mashed through a 70 μm cell strainer. Erythrocytes were subsequently eliminated from the blood and spleen by incubating the cells with ACK erythrocyte lysis buffer to generate a single-cell suspension prior to staining of surface markers. To isolate hepatic lymphocytes, non-parenchymal cells from the liver were first separated from parenchymal cells by centrifugation at low

speed. Subsequently, the non-parenchymal cells were put on a Lympholyte gradient (Cedarlane) to isolate hepatic lymphocytes prior to staining of surface markers. For analysis of surface markers identifying $CD4^+$, $CD8^+$, and NKT cells, splenocytes, or lymphocytes were stained at 4°C for 30 min. in staining buffer [phosphate buffered saline with 2% (vol/vol) fetal bovine serum (FBS)]. All antibodies used for staining of surface markers or transcription factors were from Thermo Fischer and BD Biosciences (**Supplementary Table 1**). To identify NKT cells, an allophycocyanin labeled α-GalCer/CD1d tetramer kindly provided by the NIH tetramer core facility (Atlanta, GA) was used.

For staining of intracellular cytokines, splenocytes, or liver-derived lymphocytes were incubated for 4 h with 50 ng/mL phorbol myristate acetate (PMA) (Sigma), 500 ng/mL ionomycin (Sigma), and Brefeldin A (ThermoFisher). Extracellular staining was then performed with subsequent fixation and permeabilization with Cytofix/Perm and Perm Wash buffer (both from BD Biosciences). Staining for intracellular cytokines was performed in Perm Wash Buffer after which the cells were washed with staining buffer prior to flow cytometric analysis.

Flow cytometric analysis was performed on a FACSCantoII (BD Biosciences) and data was analyzed using Flowjo software (TreeStar).

T Cell Proliferation

Splenocytes were isolated from Lck-Cre $Atg7^{f/f}$ or $Atg7^{f/f}$ mice and activated with anti-CD3e (1 μg/mL) and anti-CD28 (0.5 μg/mL) (both from ThermoFischer) for 72 h and incubated with 0.5 μCi/well ^{3}H-thymidine (Perkin Elmer, The Netherlands) for the last 16 h. The amount of ^{3}H-thymidine incorporation was measured using a liquid scintillation analyzer (Tri-Carb 2900R). Responses are expressed as the mean disintegrations per minute (dpm). The stimulation index (s.i.) was defined by dividing the dpm under activated conditions by the dpm under non-activated conditions per mouse.

Atherosclerosis

To investigate atherosclerosis in Lck-Cre $Atg7^{f/f}$ and $Atg7^{f/f}$ mice, 18 to 20-week old female mice were administered rAAV2/8-D377Y-mPCSK9 (5×10^{11}genome copies/mouse) by i.v. injection (19), which results in overexpression of PCSK9 and subsequent development of atherosclerosis. After 1 day, mice were switched from a normal chow diet to a Western-type Diet (WTD, Special Diet Services) containing 0.25% cholesterol and 15% cocoa butter. The weight of the mice was monitored regularly. After 22 weeks, the mice were anesthetized by subcutaneous injections with ketamine (100 mg/mL), sedazine (25 mg/mL), and atropine (0.5 mg/mL) after which their vascular system was perfused with PBS at a continuous low flow via heart puncture in the left ventricle. Next, the spleen, liver, and inguinal white adipose tissue (iWAT) were collected for further processing. The hearts were collected, embedded in O.C.T. compound (Sakura), and then snap-frozen using dry-ice and stored at −80°C until further use.

Histology

To examine atherosclerotic lesions in the aortic root, the hearts were sectioned horizontally to the aortic axis, and toward the aortic arch. Upon identification of the aortic root, defined by the trivalve leaflets, 10 μm sections were collected. In order to visualize hepatic steatosis, a small piece of liver was dissected upon sacrifice, and fixed using Zinc Formal-Fixx solution. Subsequently, the livers were embedded in O.C.T. compound after which 8 μm sections were prepared. After fixation with Zinc Formal-Fixx solution (Thermo Fischer) the neutral lipids in both aortic root and liver were stained using Oil-red-O (Sigma). Collagen content of the plaques was stained using a Mason's Trichrome staining kit (Sigma). Monocytes and macrophages were detected using a Moma2 primary antibody (Serotec) and biotinylated secondary antibody and visualized using the VECTASTAIN® Avidin-Biotin Complex Staining Kit (Vector Labs). After dissection of the iWAT, the tissue was fixed, dehydrated, and subsequently embedded in paraffine and sectioned in 8 μm sections. After this, iWAT sections were deparaffinized and rehydrated prior to staining with Gill No. 3 hematoxylin solution (Sigma). Adipocyte size was quantified using the Adiposoft plugin in Fiji software (20). The liver sections were examined visually to assess the degree of Oil-red-O staining as a measure for hepatic steatosis. For morphologic and morphometric analysis of the aortic root, the slides were analyzed using a Leica DM-RE microscope, and LeicaQwin software (Leica Imaging Systems). Mean plaque size (in μm^2) was calculated from five sequential sections, displaying the highest plaque content. (Immuno)histochemical stainings were expressed as the percentage of positive stained area of the total lesion area. All morphometric analyses were performed by blinded independent operators.

Confocal Microscopy

Fresh frozen livers of $Atg7^{f/f}$ and Lck-Cre $Atg7^{f/f}$ mice were sectioned on a cryostat in 8 μm thick sections. Sections were fixed in an ice-cold 75% acetone/25% ethanol mix and blocked in 2% normal goat serum and 3% bovine serum albumin (Sigma). Next, the sections were incubated overnight at 4°C with antibodies detecting CD68 and CD3e (both from Abcam). Subsequently, the sections were washed and incubated with antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 647 (both from Abcam). Nuclei were visualized using Fluoroshield mounting medium with DAPI (Sigma). T cells were manually quantified while blinded to the genotype using Fiji software. Three images were analyzed of every liver section and three liver sections were analyzed per mouse. Confocal images were acquired using a 20x objective on a Nikon TiE 2000 confocal microscope.

Real-Time Quantitative PCR

RNA was extracted from mechanically disrupted livers by using Trizol reagent per the manufacturer's instructions (Invitrogen) after which cDNA was generated using RevertAid M-MuLV reverse transcriptase according to the manufacturer's protocol (Thermo Scientific). Quantitative gene expression analysis was performed using Power SYBR Green Master Mix on a 7500 Fast Real-Time PCR system (Applied Biosystems).

Gene expression was normalized to housekeeping genes (**Supplementary Table 2**).

Immunoblot

Immunoblot was performed as described previously (21) with minor modifications. Briefly, CD4$^+$ T cells were isolated using MACS microbeads (Miltenyi Biotec). For protein isolation, cells were lysed with 1xRIPA (Cell Signaling Technology) supplemented with cOmplete™ Protease Inhibitor Cocktail (Sigma) and 0.1% SDS for 30 min on ice. Proteins were detected using rabbit-anti-mouse Atg7 (Abcam) and rabbit-anti-mouse β-actin (Novus Biologicals) antibodies and visualized using chemiluminescence.

Serum Analysis

The serum PCSK9 concentrations were determined using the Mouse Proprotein Convertase 9 DuoSet ELISA kit (R&D Systems) per the manufacturer's instructions. Concentration of total cholesterol in the serum was determined by an enzymatic colorimetric assay (Roche Dagnostics). Precipath (standardized serum, Roche Diagnostics) was used as an internal standard in the measurements for cholesterol.

Statistical Analysis

For statistical analysis, a two-tailed Student's *T*-test was used to compare individual groups with Gaussian distributed data. Non-parametric data was analyzed using a Mann-Whitney *U*-test. Data from three or more groups were analyzed using a one-way ANOVA whereas data from three groups with more than one variable were analyzed by a two-way ANOVA, both with a subsequent Sidak multiple comparison test. Correlation was assessed using Pearson's correlation coefficient. A p-value below 0.05 was considered significant. Throughout the manuscript a *indicates $p < 0.05$, **indicates $p < 0.01$, ***indicates $p < 0.001$, and ****indicates $p < 0.0001$.

RESULTS

Atg7 Deficiency Affects T Cell Populations in medLN and Spleen

To confirm that Cre recombinase expression in the Lck-Cre Atg7$^{f/f}$ mice was sufficiently high to induce Atg7 deficiency in T cells, we isolated splenic CD4$^+$ T cells from Lck-Cre Atg7$^{f/f}$ mice and Atg7$^{f/f}$ littermates, and analyzed Atg7 expression on a protein level by immunoblot. Atg7 was successfully knocked out as Atg7 could not be detected by immunoblot in CD4$^+$ T cells from Lck-Cre Atg7$^{f/f}$ mice whereas the control showed a clear Atg7 signal (**Figure 1A**). As described previously, T cell-specific deficiency of Atg7 compromises single positive T cell generation in the thymus and induces peripheral lymphopenia (22). We identified CD4$^+$ and CD8$^+$ T cells from peripheral tissues by flow cytometry using the gating strategy described in **Figure S1A**. The spleens of Lck-Cre Atg7$^{f/f}$ mice indeed contained significantly fewer CD4$^+$ and CD8$^+$ cells both in percentage and numbers (**Figure 1B**). We assessed the naïve CD4$^+$ T cell compartment in blood, spleen, and mediastinal lymph node (medLN, the lymph node draining from the trivalve area) as CD4$^+$ T cells can be activated by lipoprotein-derived antigens during atherosclerosis (3). The percentage of CD4$^+$ naïve T (Tn) cells as defined by CD4$^+$CD44$^-$CD62L$^+$ was significantly decreased in the spleen and medLN of Lck-Cre Atg7$^{f/f}$ mice as compared to Atg7$^{f/f}$ mice (**Figure 1C**). Accordingly, the number of CD4$^+$ Tn cells was decreased in the spleen of Lck-Cre Atg7$^{f/f}$ mice as compared to Atg7$^{f/f}$ mice (**Figure S1B**). Similar to the percentage of CD4$^+$ Tn cells, the percentage of CD8$^+$ Tn cells was decreased in the same tissues in Lck-Cre Atg7$^{f/f}$ mice (**Figure 1D**) and the number of CD8$^+$ Tn cells in the spleen was decreased as well in Lck-Cre Atg7$^{f/f}$ mice (**Figure S1C**). Next, we assessed whether the proliferative capacity of Atg7 deficient T cells is impaired which was confirmed using a proliferation assay measuring ^{3}H-thymidine incorporation with/without anti-CD3 and anti-CD28 stimulation (**Figure 1E**). The stimulation index was calculated and this parameter showed the proliferation of Atg7 deficient T cells was lower than Atg7 competent T cells (**Figure 1F**). The percentage of IFNγ, IL-17, and IL-10 was measured in splenic CD4$^+$ T cells using flow cytometry to assess the inflammatory capacity of the diminished T cell population in Lck-Cre Atg7$^{f/f}$ mice. Atg7 deficiency significantly increased the percentage of IFNγ-producing CD4$^+$ T cells (**Figure 1G**), causing the absolute number of IFNγ producing CD4$^+$ T cells to be unaltered between both genotypes (**Figure S1D**). The percentages of IL-17 and IL-10 producing T cells were unaltered.

Together, these results indicate that the generation of mice with Atg7 deficient T cells was successful from a genotypic and phenotypic perspective.

Atg7 Deficiency in T Cells Decreased Hepatic Steatosis and Dyslipidemia During WTD-Induced Atherosclerosis

As we were interested in the impact of Atg7 deficiency in T cells on the development of diet-induced experimental atherosclerosis, we injected Lck-Cre Atg7$^{f/f}$ and Atg7$^{f/f}$ mice with an adenoassociated virus encoding an active variant of PCSK9 (rAAV2/8-D377Y-mPCSK9) and fed them a WTD for 22 weeks to induce advanced atherosclerosis. As a result of overexpression of murine PCSK9 in the liver, the LDL receptor is targeted for lysosomal degradation and upon WTD-feeding circulating cholesterol levels are significantly elevated, to a level which is comparable to WTD-fed LDL receptor deficient mice (19). After 4 weeks of WTD, the levels of mPCSK9, and cholesterol in the serum were measured to confirm that the viral transduction was successful. In general, the levels of serum PCSK9 exceeded 10,000 ng/mL which is sufficiently high to induce atherosclerosis upon prolonged WTD feeding (19). Moreover, the levels of serum PCSK9 did not differ between both genotypes (**Figure 2A**). Interestingly, despite the serum PCSK9 levels being equal between both genotypes, serum cholesterol was significantly lower after 4 weeks of WTD in Lck-Cre Atg7$^{f/f}$ mice as compared to Atg7$^{f/f}$ mice (**Figure 2B**). The lowest serum PCSK9 levels in Lck-Cre Atg7$^{f/f}$ (∼8,000 ng/mL) in this study were sufficiently high to be associated with serum cholesterol levels comparable to what we observed in Atg7$^{f/f}$ mice (19), suggesting that Atg7 deficiency results in decreased

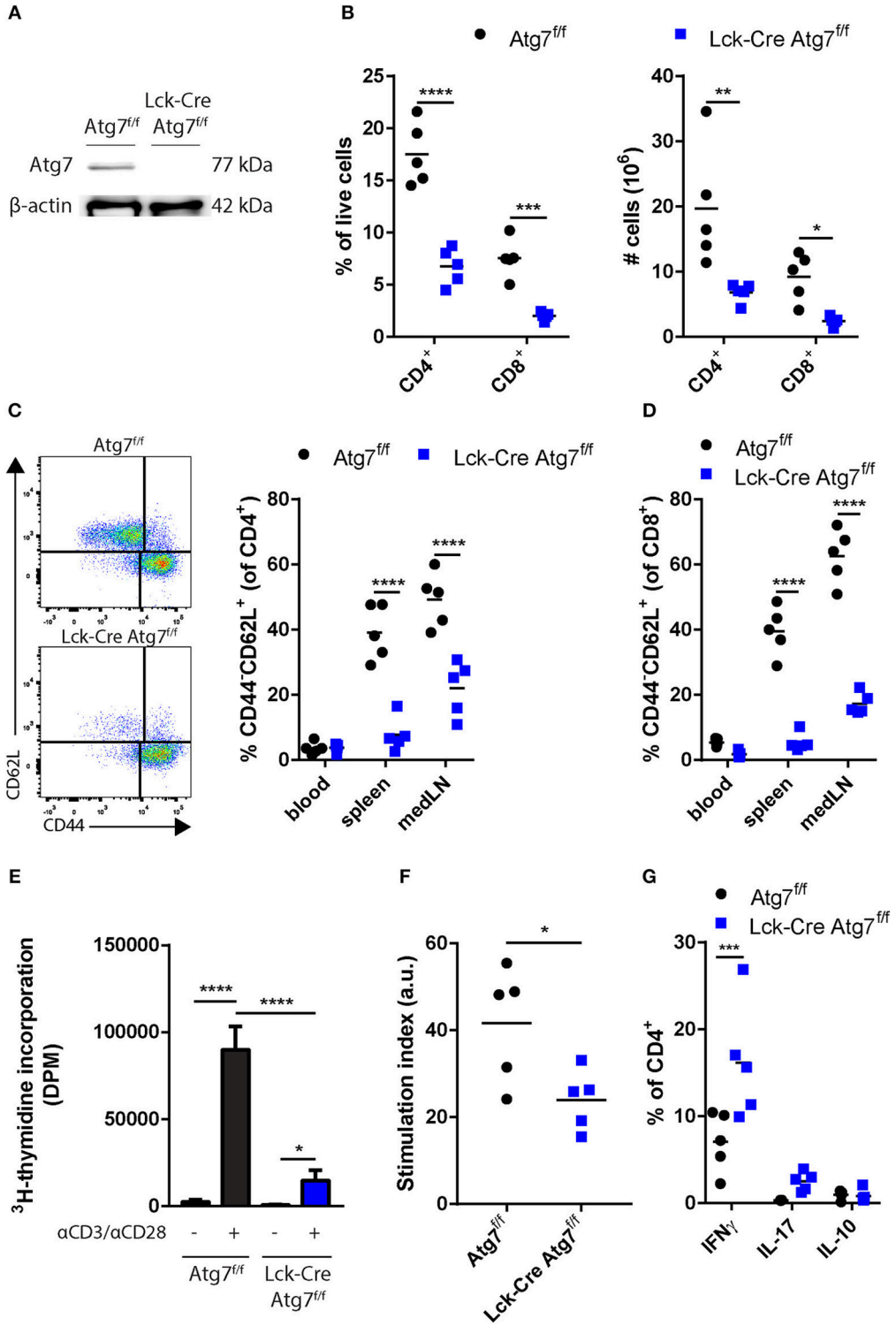

FIGURE 1 | Effect of Atg7 deficiency on CD4$^+$ and CD8$^+$ T cells. **(A)** Immunoblot of Atg7 in CD4$^+$ T cells. β-actin was used as a loading control. **(B)** Percentage and numbers of CD4$^+$ and CD8$^+$ cells in the live lymphocyte fraction of spleens of indicated genotypes. **(C)** Gating and percentages of naive T cells in the CD4$^+$ compartment. **(D)** Percentages of naive T cells in the CD8$^+$ compartment. **(E)** T cell proliferation in stimulated or unstimulated splenocyte cultures. Expressed as mean ± standard deviation. **(F)** Stimulation index as calculated by dividing DPM of anti-CD3/anti-CD28 stimulated splenocytes by the DPM of non-stimulated splenocytes for each genotype. **(G)** Quantification of cytokine producing CD4$^+$ T cells. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

serum cholesterol levels after WTD feeding. Furthermore, after prolonged WTD feeding, the weight of Lck-Cre Atg7$^{f/f}$ mice was lower compared to Atg7$^{f/f}$ mice (**Figure 2C**) and in line, the weight of the iWAT was lower in Lck-Cre Atg7$^{f/f}$ mice (**Figure 2D**) also when this was corrected for body weight at sacrifice (**Figure 2E**). Accordingly, the mean adipocyte size was decreased in Lck-Cre Atg7$^{f/f}$ mice as compared to Atg7$^{f/f}$ mice, although this did not reach significance ($p = 0.06$, **Figures 2F,G**). As compared to Atg7$^{f/f}$ mice, the livers of Lck-Cre Atg7$^{f/f}$ mice appeared less steatotic (**Figure 2H**), which is consistent with a decrease in total serum cholesterol levels. Next, we measured the expression of genes that are associated with hepatic steatosis (23) and the expression of a number of additional genes involved in lipid metabolism was decreased in livers of Lck-Cre Atg7$^{f/f}$ mice. The expression of CD36, for example, a scavenger receptor known to mediate the uptake of native and modified lipoproteins, was decreased in Lck-Cre Atg7$^{f/f}$ mice (**Figure 2I**). Furthermore, the expression of the transcription factor peroxisome proliferator activated receptor gamma (*Pparg*), which is known to be associated with hepatic steatosis (24), was decreased while the expression of sterol regulatory element binding protein 2 (*Srebp2*) was increased in the liver of Lck-Cre Atg7$^{f/f}$ mice (**Figure 2J**). On the other hand, the mRNA expression of genes involved in fatty acid synthesis in the liver was decreased, including *Acaa2*, *Scd1,* and *Fas* (**Figure 2K**). In line with *Srebp2* expression being increased, the expression of *Fdft1*, which is involved in cholesterol synthesis, was elevated in Lck-Cre Atg7$^{f/f}$ mice (**Figure 2L**). The macrophage content in livers of Lck-Cre Atg7$^{f/f}$ mice was also decreased as suggested by the decreased mRNA expression of the macrophage lineage marker *CD68* and the chemokine *Mcp1* (**Figure 2M**). The decreased CD68 expression in livers of Lck-Cre Atg7$^{f/f}$ mice was confirmed on protein level in confocal images (**Figure 2N**).

Altogether, T cell-specific Atg7 deficiency hampered WTD-induced hepatic steatosis and dyslipidemia despite successful viral transduction with rAAV2/8-D377Y-mPCSK9.

Atg7 Deficiency in T Cells Decreases T Cell Abundance in the Liver but Increases Inflammatory Cytokine Production

As inflammation is one of the drivers of hepatic steatosis (25), we postulated that Lck-Cre Atg7$^{f/f}$ mice developed less severe hepatic steatosis during our experiments as the inflammatory capacity of the hepatic T cell population was impaired. Therefore, we characterized the CD4$^+$ and CD8$^+$ T cell populations in the liver. In line with the observations in the spleen, the percentage of CD4$^+$ T cells in the lymphocyte fraction of the liver was decreased as a result of Atg7 deficiency (**Figure 3A**). Additionally, the percentage of CD8$^+$ T cells was decreased as well, albeit to a lesser extent (**Figure 3A**). Quantification of the number of T cells per mm^2 liver tissue using confocal microscopy showed that the absolute number of T cells was decreased in livers of Lck-Cre Atg7$^{f/f}$ mice as compared to Atg7$^{f/f}$ mice (**Figure 3B**). We measured the inflammatory capacity of CD4$^+$ and CD8$^+$ T cells through flow cytometry by measuring the percentage of IFNγ and IL-17 producing cells in both genotypes (**Figure 3C**). The percentage of IFNγ$^+$ cells in the hepatic CD4$^+$ T cells showed a 2-fold increase in Atg7 deficient CD4$^+$ T cells whereas the percentage of IL-17 producing cells was also increased by Atg7 deficiency going from ∼0.4% in Atg7 wildtype CD4$^+$ T cells to ∼6.3% in Atg7 deficient CD4$^+$ T cells (**Figure 3D**). Similarly, the IFNγ and IL-17 production was increased in Atg7 deficient CD8$^+$ T cell compartment as compared to Atg7 wildtype CD8$^+$ T cells (**Figure 3E**). However, this did not result in increased mRNA levels of IFNγ or IL-17 (not detectable) in liver homogenate (**Figure S2A**). To confirm that the total amount of IFNγ producing T cells was not increased as a result of Atg7 deficiency, we isolated hepatic lymphocytes from normocholesterolemic Atg7$^{f/f}$ and Lck-Cre Atg7$^{f/f}$ mice and quantified the total number of activated IFNγ producing cells. The total number of IFNγ producing lymphocytes was unaltered between Atg7$^{f/f}$ and Lck-Cre Atg7$^{f/f}$ mice (**Figure S2B**), suggesting that Atg7 deficiency in T cells causes an increase in the percentage of hepatic IFNγ producing T cells but not in the total number of IFNγ producing cells prior to steatosis development. Despite a previous report describing Atg7 deficient Treg cells to express more IFNγ than their Atg7 competent counterparts (17) barely any IFNγ producing T cell in livers of normocholesterolemic mice are FoxP3$^+$ (**Figure S2C**). As the results described above suggested Atg7 deficiency induced the skewing of the diminished T cell population toward an inflammatory phenotype and IL-10 is an anti-inflammatory cytokine, we measured *Il10* expression in the livers of Lck-Cre Atg7$^{f/f}$ and Atg7$^{f/f}$ mice. In line with an inflammatory phenotype and Atg7 deficiency disrupting Treg cell function and stability, the expression of *Il10* was decreased in livers of Lck-Cre Atg7$^{f/f}$ mice as compared to Atg7$^{f/f}$ control mice (**Figure 3F**). Whether this decrease in *Il10* expression is truly T cell dependent or whether it is due to the less advanced stage of hepatic steatosis in Lck-Cre Atg7$^{f/f}$ mice remains to be determined.

In conclusion, although Atg7 deficiency resulted in a relative increase in T cells which produce inflammatory cytokines, the decrease in CD4$^+$ and CD8$^+$ T cells in the liver likely impairs the development of hepatic steatosis in Lck-Cre Atg7$^{f/f}$ mice.

Lack of NKT Cells in Mice With T Cell-Specific Atg7 Deficiency

In mice, natural killer T (NKT) cells represent a relatively large fraction of hepatic lymphocytes (up to 35%). NKT cells recognize lipid-derived antigens when presented on the major-histocompatibility complex-resembling protein CD1d. The most common type of NKT cells in mice is the type I NKT cells, also called invariant NKT cells. Upon stimulation, NKT cells secrete a plethora of cytokines, including Th1-like (IFNγ, TNFα) cytokines and IL-10. As NKT cells have a functional TCRαβ and express one of its proximal signaling kinases Lck (26), we hypothesized that Atg7 deficiency disrupted NKT cell function. Using flow cytometry, we observed that the percentage of NKT cells was severely diminished in the hepatic lymphocyte fractions of Lck-Cre Atg7$^{f/f}$ mice from ∼18% to ∼0.1% (**Figure 4A**). Similarly, in the spleens of Lck-Cre Atg7$^{f/f}$, only ∼0.1% of the

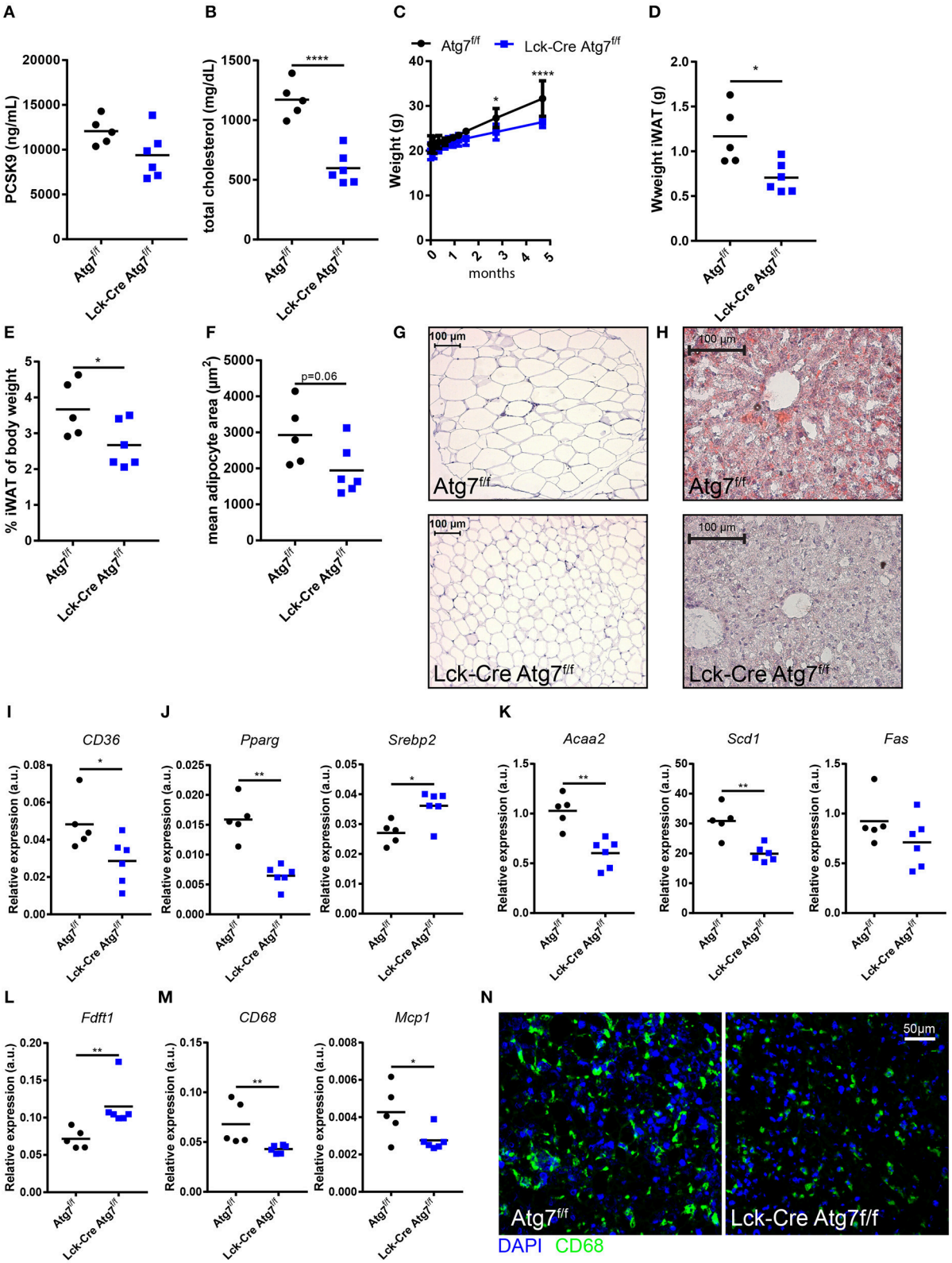

FIGURE 2 | Atg7 deficiency in T cells decreased hepatic steatosis and dyslipidemia. **(A)** PCSK9 levels in serum. **(B)** Total cholesterol levels in serum. **(C)** Weight of the mice over the course of the experiment. **(D)** Weight inguinal white adipose tissue. (iWAT). **(E)** Weight iWAT as a percentage of body weight at sacrifice.

(Continued)

FIGURE 2 | (F) Quantification of adipocyte area in iWAT. **(G)** Representative sections of iWAT used for adipocyte size quantification in **(F)**. **(H)** Representative Oil-Red-O stained sections of liver. **(I)** Gene expression in liver of scavenger receptor CD36. **(J)** Gene expression in liver of transcription factors Pparg and Srebp2. **(K)** Gene expression in liver of fatty acid synthesis genes Acaa2, Scdl, and Fas. **(L)** Gene expression in liver of enzyme involved in cholesterol synthesis, Fdftl. **(M)** Gene expression in liver of macrophage lineage marker CD68 and monocyte chemoattractant Mcp 1. **(N)** Representative confocal images of CD68 expression in liver sections. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

lymphocyte population consisted of NKT cells compared to ∼1% in the control (**Figure 4B**).

Thus, T cell-specific Atg7 deficiency not only diminished the CD4$^+$ and CD8$^+$ T cell populations but also severely reduced the percentage of hepatic NKT cells, which may have contributed to impaired hepatic steatosis development as suggested by literature (25).

T Cell Specific Atg7 Deficiency Decreases Atherosclerosis

Since we were interested in the effect of T cell-specific Atg7 deficiency on the development of diet-induced advanced atherosclerosis we quantified atherosclerotic lesion size in the aortic root after 22 weeks of WTD. Lck-Cre Atg7$^{f/f}$ mice developed 50% smaller lesions than Atg7$^{f/f}$ control mice (**Figure 5A**, $p < 0.01$). Interestingly, the correlation between lesion size and serum total cholesterol levels was stronger in Lck-Cre Atg7$^{f/f}$ mice as compared to Atg7$^{f/f}$ mice (**Figures S3A**,**B**), suggesting that T cell-specific Atg7 deficiency renders serum cholesterol to be a stronger driver of atherogenesis in Lck-Cre Atg7$^{f/f}$ mice. Additionally, T cell-specific Atg7 deficiency reduced the collagen content by ∼50% (**Figure 5B**). Lastly, the relative amount of monocytes and macrophages in the lesions was quantified using a MOMA-2 staining. No differences were observed in terms of macrophage content between Lck-Cre Atg7$^{f/f}$ and Atg7$^{f/f}$ mice (**Figure 5C**).

DISCUSSION

Different T cell subsets which are crucially involved in the development of atherosclerosis depend on autophagy for their functional integrity and survival. Systemic administration of autophagy inhibitors such as chloroquine or hydroxychloroquine has therapeutic potential to treat atherosclerosis as it shows anti-inflammatory effects in other autoimmune diseases such as rheumatoid arthritis. In mice, systemic low-dose administration of chloroquine inhibits diet-induced atherosclerosis in ApoE-deficient mice (27). To gain more insight in the T cell-specific contribution of the anti-inflammatory effect of autophagy blockade, we genetically blocked autophagy in T cells and studied the impact on diet-induced atherosclerosis in experimental models of disease.

In this model, knock-out of Atg7 in T cells significantly decreased the percentage and numbers of CD4$^+$ and CD8$^+$ T cells. The percentage of naïve T cells was also decreased in lymphoid tissues, the mediastinal lymph nodes and spleen, in which naïve T cells respond to lesion and lipoprotein-derived antigens. These findings are in line with data that naïve T cells go into apoptosis without functional autophagy (28), which is highly relevant for atherosclerosis research as this would result in relatively fewer T cells to respond to atherosclerosis derived antigens and thus to the ongoing inflammation in atherosclerotic lesions. Atg7 deficiency in T cells impaired their proliferative capacity in a splenocyte culture under anti-CD3 and anti-CD28 antibody induced stimulation, which is in line with a previous report describing that Atg7 deficient naïve CD4$^+$ T cells proliferate less after antibody-mediated TCR stimulation (16). TCR stimulation also activated CD8$^+$ T cells in the splenocyte culture but as autophagy is not induced upon activation of CD8$^+$ T cells (15) and their proliferative capacity is not affected by Atg5 or Atg7 deficiency (15) it is unlikely that the decrease in T cell proliferation we observed in our experiments was CD8$^+$ T cell-mediated. Under normolipidemic conditions, the spleens of Lck-Cre Atg7$^{f/f}$ mice contained fewer CD4$^+$ T cells although a relatively higher percentage of these CD4$^+$ cells secreted IFNγ. Although we did not observe a difference in total numbers of CD4$^+$ IFNγ$^+$ T cells between Lck-Cre Atg7$^{f/f}$ and Atg7$^{f/f}$ mice, Atg7 deficient T cells have been described to secrete lower amounts of IFNγ and IL-10 and also lower amounts of other T helper cell cytokines including IL-4 and IL-17 (29). These results suggested that Atg7 deficiency severely compromised the inflammatory capacity of all the T helper cell populations in the medLN and spleen in our studies. Therefore, the fact that Atg7 deficient CD4$^+$ and CD8$^+$ T cells had a higher percentage of IFNγ producing cells than their Atg7 competent counterparts is negated by the diminished number of CD4$^+$ and CD8$^+$ T cells and their capacity to not only produce but also secrete cytokines in Lck-Cre Atg7$^{f/f}$ mice as compared to Atg7$^{f/f}$ mice.

As LDL receptor competent mice barely develop atherosclerosis we injected Atg7$^{f/f}$ and Lck-Cre Atg7$^{f/f}$ mice with a single injection of rAAV2/8-D377Y-mPCSK9. Compared to Atg7$^{f/f}$ mice, Lck-Cre Atg7$^{f/f}$ mice had lower serum cholesterol levels and less hepatic steatosis under dyslipidemic conditions based on histological evaluation and gene expression of genes associated with hepatic steatosis. As Lck-Cre Atg7$^{f/f}$ mice gained less weight, and had a lower iWAT weight, the decreased extent of hepatic steatosis in these mice had effects beyond just the decrease in circulating cholesterol levels. However, given the role of T cells in the development of obesity-associated inflammation of WAT (30–32), the impaired weight gain could also be explained by impairments in T cell-mediated inflammation of WATs. This indicates that, under dyslipidemia conditions, Atg7 deficiency has impact on the immunometabolic phenotype of mice on a systemic level which is relevant for further research examining autophagy blockade in models of dyslipidemia *in vivo*.

Since hepatic steatosis was decreased upon T cell-specific deletion of Atg7, we hypothesized that this was due to a reduction in the numbers and profile of cytokine secretion of CD4$^+$ and

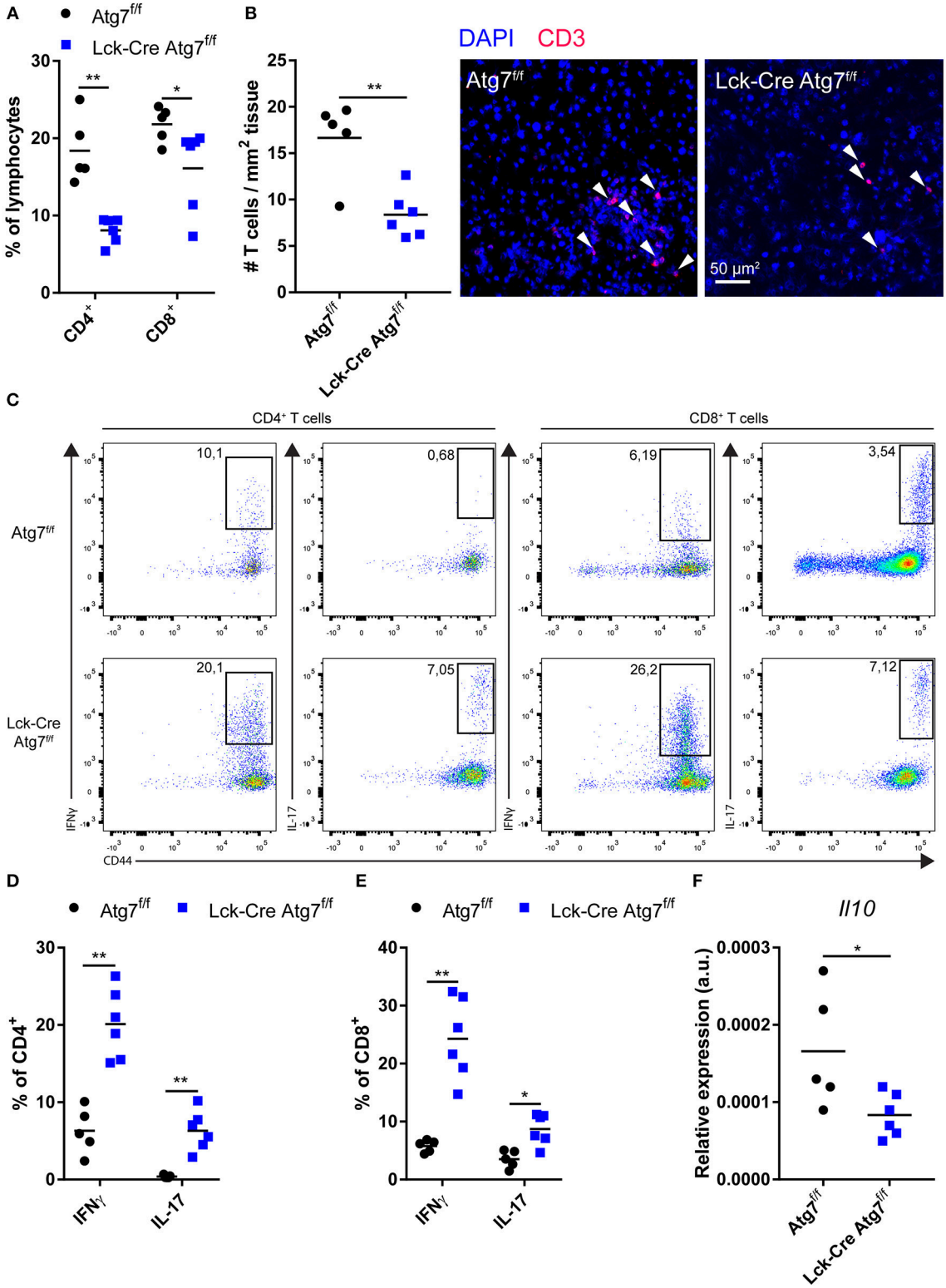

FIGURE 3 | Atg7 deficiency in T cells diminishes T cells in liver but increases relative inflammatory cytokine production. **(A)** Percentage of CD4$^+$ and CD8$^+$ T cells in live hepatic lymphocyte fraction. **(B)** Quantification and representative confocal images of T cells in liver **(C)** Gating strategy to identify IFNγ and IL-17 producing T cells in liver. **(D)** Percentage of IFNγ and IL-17 producing hepatic CD4$^+$ T cells. **(E)** Percentage of iFNγ and IL-17 producing hepatic CD8$^+$ T cells. **(F)** *Il10* expression in liver. $^*p < 0.05$, $^{**}p < 0.01$.

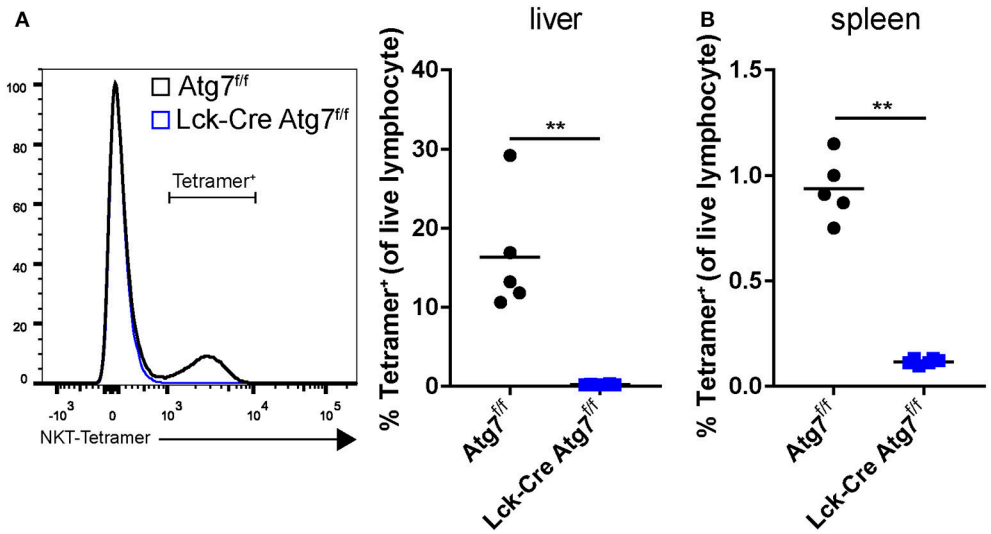

FIGURE 4 | Atg7 deficiency in T cells reduces the amount of NKT cells in liver and spleen. **(A)** NKT cells as detected by an α-GalCer/CDld tetramer staining and presented as a percentage of the lymphocyte fraction in the liver. **(B)** Percentage ofNKT cells in spleen. $^{**}p < 0.01$.

CD8$^+$ T cells in the livers of Lck-Cre Atg7$^{f/f}$ mice. Similar to the spleens and medLNs during normolipidemia, the livers of mice with Atg7 deficient T cells contained fewer CD4$^+$ and CD8$^+$ T cells. Also in the hepatic T cell populations, the percentages of IFNγ and IL-17 secretion were increased but since the reduction in the percentages of CD4$^+$ and CD8$^+$ T cells is considerable, the total amount of IFNγ and IL-17 which is produced by T cells throughout the process of hepatic steatosis development was likely lower in Lck-Cre Atg7$^{f/f}$ mice. It is unclear why the relatively few CD4$^+$ and CD8$^+$ T cells in the liver had a higher level of inflammatory cytokine secretion. Inhibition of autophagy in Th1 cells using 3-methyladenine or NH_4Cl and leupeptin impairs IFNγ secretion (16), suggesting that it is unlikely that Atg7 deficiency increases Th1 differentiation resulting in enhanced IFNγ secretion. Interestingly, Treg cell-specific Atg7 deficiency has been described to induce a loss of FoxP3 and enhance their production of IFNγ and IL-17 (17). Likewise, knock out of Atg16L, another essential protein in autophagy, mimics the effects of Atg7 deficiency in Treg cells as Atg16L1 deficient Treg cells have increased IFNγ and IL-17 production (33). Though Atg7 deficiency in Treg cells impairs their survival and immunosuppressive capacity it also increases their homeostatic proliferation (17), suggesting that Atg7 deficient Treg cells are more resilient to defective autophagy as compared to Atg7 deficient conventional T cells. It is likely that the increase in IFNγ and IL-17 producing CD4$^+$ T cells in the liver and spleen of Lck-Cre Atg7$^{f/f}$ mice is partly due to Atg7 deficiency in Treg cells, which produce IFNγ and IL-17 under specific inflammatory conditions (17). In line, Treg cells with a Th1- or Th17-like phenotype have been described before in (models for) cardiovascular disease (34, 35). However, since barely any IFNγ producing CD4$^+$ T cells were FoxP3$^+$ in livers of normocholesterolemic Lck-Cre Atg7$^{f/f}$ mice, the Treg cells which might have contributed to the increased percentage of IFNγ and IL-17 producing CD4$^+$ T cells in the liver no longer express FoxP3 and are actually "former-Treg cells." Another explanation for the increased percentage of IFNγ and IL-17 producing T cells which we observed in the liver is that Atg7 deficient Treg cells have impaired immunosuppressive capacity, which causes IFNγ and IL-17 producing CD4$^+$ T cells to be improperly inhibited by Treg cells. Since Treg cells represent a relatively small percentage of the total T cell population it is unlikely that the cells which caused the increased percentage of IFNγ and IL-17 producing CD4$^+$ T cells are exclusively (former) Treg cells. Further research is required to examine what the functional effects of autophagy deficiency in Th17 cells are and what their contribution to the observed T cell phenotype might have been.

Hepatic steatosis (or fatty liver) can develop when hepatocytes accumulate dietary lipids, potentially resulting in lipotoxicity. When this persists, immune cells such as Kupffer cells are activated and monocytes can be recruited when damaged or dead cells release danger signals such as damage-associated molecular patterns, leading to the development of non-alcoholic steatohepatitis (NASH) (25). *Ldlr*$^{-/-}$ mice which are fed a high fat, high cholesterol diet are a suitable model to study the onset of inflammation in hepatic steatosis (32), suggesting the development of hepatic steatosis in virus-induced LDL receptor deficient mice is physiologically relevant. In the hepatic lymphocyte population, it is mainly Th17 cells which drive the development of steatosis (36). Through the secretion of IL-17, Th17 cells directly drive sinusoidal cells such as fat storing cells to produce type 1 collagen and activate macrophages to secrete inflammatory cytokines (37). Patients with hepatic steatosis have increased intrahepatic IL-17 expressing CD4$^+$ T cells while in the blood, more IFNγ secreting CD4$^+$ T cells are detected as compared to healthy controls (36). In line, morbidly obese patients with NASH have higher intrahepatic gene expression of Th1-associated genes and a decreased ratio

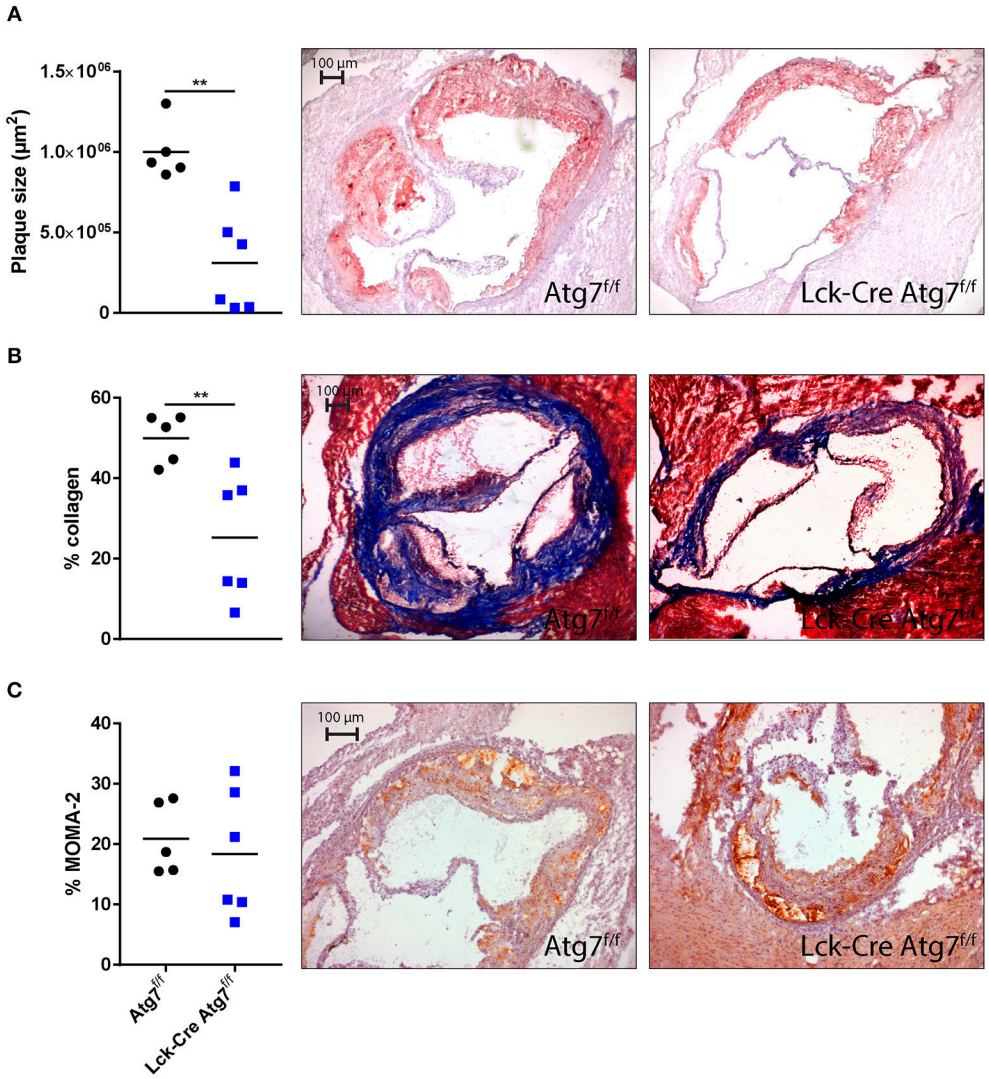

FIGURE 5 | Histological analysis of atherosclerosis in the aortic root. **(A)** Quantification of mean plaque size using Oil-red-O staining. **(B)** Quantification of collagen content using a Masson's Trichrome staining. Collagen fibers are indicated in blue. **(C)** Quantification of monocyte-macrophage content using a MOMA-2 antibody. $^{**}p < 0.01$.

of IL-10/IFNy as compared to patients with non-alcoholic fatty liver disease (38). Whereas, Th17 and Th1 cells appear to drive NASH, Treg cells presumably inhibit its development as Treg cell deficient mice with WTD-induced atherosclerosis have more severe hypercholesterolemia due to impaired clearance of chylomicron remnants and very low density lipoproteins (39). Taken together, in our study we deem it most likely that the diminishment in the amount of hepatic T cells impaired the development of hepatic steatosis.

The contribution of NKT cells to the development of hepatic steatosis remains to be elucidated. In high-fat diet induced obesity, hepatic NKT cell numbers diminish, possibly contributing to the development of hepatic steatosis as the cytotoxicity-mediated killing of hepatocytes, which are under lipotoxicity-induced stress, is impaired (40, 41). The expansion of NKT cells during diet-induced steatosis development using probiotics actually protects against hepatic steatosis and insulin resistance (42). In contrast, expansion of hepatic NKT cells through the Hedgehog-pathway contributes to hepatic fibrosis (43), suggesting that the contribution of NKT cells to the pathogenesis of steatosis and NASH depends on the dynamics and inflammatory phenotype of hepatic NKT cells.

Studies describing the abundance of NKT cells in different organs during experimental atherosclerosis show contradictory results (44). Both an increase and a decrease in NKT cell number in atherosclerosis has been reported. In our experiments, the abundance of NKT cells was severely diminished in the livers of WTD-fed Lck-Cre Atg7$^{f/f}$ mice, which is relevant as the liver contains the highest number of NKT cells in mice. This effect of Atg7 deficiency on hepatic NKT cell abundancy is

due to reduced thymic NKT cell output as Atg7 deficiency inhibits the progression of NKT cells through the cell cycle and increases NKT cell apoptosis (45). Interestingly, Atg5 deficiency primarily hampers the secretion of IFNy by Th1-like NKT cells and Atg7 deficiency presumably has the same effect (45). Given their modulatory role in steatosis development, the lack of inflammation competent NKT cells diminished the development of hepatic steatosis. As LDLr$^{-/-}$CD1d$^{-/-}$ mice, which lack NKT cells, have similar cholesterol levels when fed a WTD as compared to LDLr$^{-/-}$ mice (46) it is likely that in Lck-Cre Atg7$^{f/f}$ mice, the combined effect of the lower numbers of inflammatory CD4^{+}, CD8^{+}, and of NKT cells inhibited the development of WTD-induced hepatic steatosis.

Atherosclerosis development was severely impaired in mice with T cell-specific Atg7 deficiency which can be explained by low levels of serum cholesterol and low numbers of CD4^{+} T cells, CD8^{+} T cells and NKT cells. Additionally, the lesions of Lck-Cre Atg7$^{f/f}$ mice contained less collagen as compared to lesions from Atg7$^{f/f}$ mice which is most likely due to more advanced and stabilized lesions in the latter group. The lack of a proper NKT cell population in Lck-Cre Atg7$^{f/f}$ mice likely contributed to decreased lesion growth as NKT cells can drive atherogenesis through various mechanisms, including through perforin and granzyme-B mediated cytotoxicity and cytokine secretion (47–49). The fact that activated iNKT cells can decrease lesion stability by reducing collagen content (47) was likely overruled by their low abundancy in our study.

In this study we determined the effect of genetic blockade of autophagy in T cells on late stages of atherosclerosis in a mouse model where T cells lack functional autophagy from an early developmental stage and subsequently induced atherosclerosis. For a translation into a clinical setting in which cardiovascular patients could be treated with pharmacological autophagy inhibitors, it would be interesting to knock-out Atg7 using an inducible Cre mouse model in mice with pre-developed atherosclerosis. In addition it would have been highly interesting to dissect the effect of Atg7 deficiency in specific T cell subsets using mice with Cre recombinase under control of the promotor of T-bet, RORγt, and FoxP3 to induce Atg7 deficiency in Th1-, Th17-, and Treg cells, respectively.

In conclusion, T cell-specific Atg7 deficiency decreased the degree of diet-induced hepatic steatosis and atherosclerosis due to a decrease in numbers of CD4^{+}, CD8^{+}, and NKT cells. These results suggest that autophagy inhibition in T cells is feasible to diminish atherosclerosis. Further research focusing on the effect systemic administration of pharmaceuticals such as chloroquine has on non-T cells could contribute to its applicability to inhibit inflammation and potentially prevent cardiovascular disease.

AUTHOR CONTRIBUTIONS

JA and JK designed the experiments. JA, HD, FS, AF, MK, PvS, GvP, and IB performed the experiments. JA, IB, and JK wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.02937/full#supplementary-material

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