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Review Article

Autophagy in *Mycobacterium abscessus* Infection

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Autophagy is a self-degradative process that removes misfolded or aggregated proteins, clears damaged organelles, as well as eliminates intracellular pathogens playing a role in innate immunity. *Mycobacterium abscessus* (*M. abscessus*) has been reported as a causative organism in nearly 80% of the rapid growing mycobacteria (RGM) pulmonary disease. The strain exhibits two different colony types: the smooth (S) one which is considered wild-type and the rough (R) one which is the mutated strain. In accordance to the colony morphology, the S and R types display varying autophagic responses in the host cells with the R type inducing elevated autophagy compared to the S type. The major difference in the autophagy could be based on the bioactive molecules exposed on the surface of the S and R types. Though autophagy has a vital role to play in the clearance of intracellular pathogens, very little is known on the autophagy induced by *M. abscessus*. It has been known that the intracellular pathogens employ different strategies to evade the autophagic pathway and to survive within the host cells. This review summarizes the most up-to-date findings on autophagy induced by *M. abscessus* morphotypes and how *M. abscessus* evades the autophagic machinery to divide and thrive inside the host cells. In addition, the prospects of autophagic machinery in devising new anti-infective strategies against mycobacterial infection is also been discussed.

Key Words: *Mycobacterium abscessus*, Autophagy, Rough type, Smooth type, Mycobacterial infection

I. INTRODUCTION

Autophagy is a conserved catabolic process that delivers cytoplasmic contents to lysosomes for degradation via autophagosomes (1~5). Autophagy plays an important role in removing damaged organelles (mitochondria, endoplasmic reticulum, peroxisomes etc.), clearing misfolded or aggregate-prone proteins, as well as eliminating intracellular pathogens. Though autophagy was initially studied as a cellular process in response to starvation-induced stress, now it is clear that it is a critical regulator of cellular homeostasis (6). In addition to starvation, autophagy is induced by many other perturbations including hypoxia, metabolic, osmotic and oxidative stresses (7~9). Autophagy appears to be relevant in cell metabolism, growth control, the balance between cell survival and cell death as well as ageing (6). Autophagy plays a pivotal role in human health and disease by taking part in inflammation, immunity, neurodegeneration, tumor suppression and genome stability (10~14).

II. CELLULAR AND MOLECULAR MECHANISMS OF AUTOPHAGY

The classical autophagy pathway proceeds through a number of well-defined steps. In response to various stimuli,

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autophagy is induced by the formation of phagophore, an isolation membrane (IM) which is a lipid bilayer derived from the endoplasmic reticulum (ER) and/or the trans-Golgi and endosomes (15, 16). Although the importance of autophagy is well studied in mammalian systems, many of the molecular mechanism studies revealing how autophagy is regulated and executed at the molecular level have been conducted in yeast (*Saccharomyces cerevisiae*) (17, 18). Thirty two different autophagy-related genes (Atg) have been identified so far by the genetic screening in yeast and many of which are later found to be conserved in mammals. In yeast, the phagophore membrane is formed around a cytosolic structure known as the pre-autophagosomal structure (PAS) (18). However, there is no report for PAS in mammals. The yeast serine/threonine kinase Atg1 along with other Atg proteins, including Atg13 and Atg17 are known to be important for the expansion of phagophore (16, 18, 19). This step is regulated by TOR kinase which is responsible for the phosphorylation of Atg13 leading to the initiation of autophagy sensitive to growth factor and nutrient availability. The mammalian homologue of Atg1, ULK1 along with the closely associated protein ULK2 (a second Atg1 homologue) is reported to have a role in the induction of autophagy. There are two ubiquitin-like systems that are key to the formation of autophagosomes (20, 21). In the first system, Atg7 acts like a ubiquitin activating enzyme and activates Atg12 eventually contributing to the conjugation of Atg12 with Atg5. The Atg5-Atg12 conjugate along with Atg16L dimers forms a multimeric Atg5-Atg12-Atg16L complex that associates with the extending phagophore. The second ubiquitin-like system in autophagy is involved in the processing of microtubule-associated protein light chain 3 (LC3B). Upon induction of autophagy, LC3B is proteolytically cleaved by Atg5, a cysteine protease, to generate LC3B-I. LC3B-I is then conjugated to the phosphatidylethanolamine (PE) by the carboxyl glycine to form the processed LC3B-II. The LC3B-II is later on recruited and integrated into the growing phagophore depending on the presence of Atg5-Atg12 complex. The generation and processing of LC3 is elevated during autophagy, making it a key molecule for checking the autophagy levels in cells (22). LC3B-II is found on both internal and external surfaces of the phagophore and acting as a 'receptor', it interacts with 'adaptor' molecules on targets (e.g. protein aggregates, mitochondria) to boost their selective uptake and degradation. One such adaptor molecule is p62/SQSTM1 that promotes the degradation of ubiquitinated protein aggregates. p62 is able to bind to ubiquitin and LC3 at the same time, targeting the proteins to phagophore and facilitating their clearance. The fusion of the expanding phagophore membrane forms a double-membrane vesicle known as the autophagosome, which eventually fuses with the specialized endosomal compartment, lysosome to form autolysosome (20) where the captured material along with the inner membrane is degraded. Within the lysosome, cathepsin protease B, cathepsin protease D, Lamp-1 and Lamp-2 are critical for the maturation of autolysosome (23, 24).

Normal proteins are routinely turned over by different protein degradation systems including autophagy. It has been reported that diverse neurodegenerative diseases, including Alzheimer's diseases, transmissible spongiform encephalopathies, Parkinson's disease, and Huntington's disease are associated with impaired autophagy where the proteins are aggregated leading to neurodegeneration (25, 26). Tissue-specific knock out studies in mice have indicated the role of basal hepatocyte autophagy in preventing the most common genetic human liver disease, α1-antitrypsin deficiency which is linked carcinogenesis and chronic inflammation (27). The pathogenesis of myodegenerative diseases involves either the impairment of autolysosome formation or the aggregation of misfolded proteins that exceed the autophagic clearance capacity of the cells. Danon disease, a genetic disease is the result of a mutation in the lysosomal protein, LAMP-2 and is associated with enhanced accumulation of autophagosomes in the muscles (28). The accumulation of autophagosomes (due to the impaired autophagosome-lysosome fusion) has been noticed in cardiac biopsy tissues of patients with coronary artery disease, hypertension, aortic valvular disease, and congestive heart failure (29). The fact that the autophagy defects are closely associated with tumorigenesis provides increasing support for the concept that autophagy is a bona-fide tumor suppressor pathway (30, 31). The tumor suppressor genes in the autophagy machinery include *beclin 1*,

atg4c, *atg5*, *UVRAG*, *ambra1*, *bif-1* (required for autophagy), *P53* (regulates autophagy) etc. (32~37).

III. AUTOPHAGY IN INFECTION AND IMMUNITY

Autophagic machinery plays a central role in the clearance of invading intracellular pathogens through a process called xenophagy (38, 39). In this process, the phagophores engulf the invading microbes forming autophagosomes and guiding them toward lysosomal degradation. Thus, xenophagy is an important host defense mechanism in the elimination of intracellular pathogens, indicating that autophagy does have a role to play in innate immunity. Autophagy restricts the growth of diverse species of bacteria, including Group A Streptococcus (GAS), *Mycobacterium tuberculosis*, *Rickettsia conorii*, *Salmonella* Typhimurium, and *Shigella flexneri* (40, 41). In the autophagic pathway, the intracellular bacteria are targeted within phagosomes, (e.g. *M. tuberculosis*), in damaged vacuoles (e.g. *S. enterica* serovar Typhimurium), or in the cytosol (e.g. GAS), and kill them via the autolysosome. Once internalized, *M. tuberculosis* resides in early phagocytic compartments which avoid maturation and fusion with lysosomes (42). Induction of autophagy facilitates autophagosome-lysosome fusion and clearance of the pathogen (43). Furthermore, *M. tuberculosis* infection of autophagy -gene-deficient mice displayed increased bacterial burden and tissue inflammation compared to autophagy-proficient mice (44). Thus, autophagy seems to play a critical role not only in bacterial clearance but also in preventing host tissue destruction. *S. enterica* serovar Typhimurium is a facultative intracellular pathogen which is seen localized inside the host cells within membrane-bound compartments called the *Salmonella*-containing vacuoles (SCV) where it replicates, protected from the immune system (45). The damage of the SCV membrane later on causes the recognition of these vesicles by the autophagy pathway (46). GAS enters the cytosol of host cells when internalized into endosomes, which are then captured by autophagosomes. The GAS-containing phagosomes eventually fuse with lysosomes killing most intracellular GAS and preventing GAS replication (47).

Intracellular pathogens have evolved mechanisms to evade (e.g. *Shigella flexneri*, *Listeria monocytogenes*) (48, 49), inhibit (e.g. *M. tuberculosis*, *Legionella pneumophila*) (50, 51), and subvert (*Coxiella burnetii*, *Staphylococcus aureus*) autophagy (52, 53). *Shigella* secretes factors, IcsB and IcsA which helps them to escape from phagosome into the cytoplasm where they can multiply and induce the formation of actin tails (54). These actin tails aid the bacteria to actively move and invade neighboring cells. *L. monocytogenes* like *Shigella* also escapes the autophagic machinery and replicates in the cytoplasm of host cells. This phagosomal escape is accomplished by a pore-forming toxin, listeriolysin O (LLO) and two bacterial phospholipase Cs (49). Once in the cytoplasm, the bacteria induce the generation of actin tails to assist them to invade the neighboring cells. It has been reported that *M. tuberculosis* is capable of persisting within the phagosomes by interfering with the stereotypical phagosomal maturation process by means of inhibition of phagosome-lysosome fusion (42). The varied expression of Rab5 on the phagosomes containing *M. tuberculosis* causes the phagosome maturation arrest at the early endosomal stage. Sulfatides and ammonium chloride (produced in high abundance) of *M. tuberculosis* are reported to have an anti-fusion effect (55, 56). In addition, it has been revealed that *M. tuberculosis* disrupts the delivery of V_0H^+-ATPase subunits and lysosomal hydrolases to *Mycobacterium*-containing phagosomal compartments preventing acidification of the same (57, 58). After internalization, *L. pneumophilia* evades fusion with lysosomes and interacts with ER-like structures to establish a replicative niche where it multiplies, before it eventually merges with the lysosomes after a delay of several hours (51, 59). Upon internalization into the host cells, *C. burnetii* is localized in early phagosomes which fuse with other vacuoles to form large and spacious parasitophorous vacuoles (PV) where the bacteria replicate (52, 60). This bacterium has the ability to withstand the harsh acidic environment of the phagolysosome-like vacuoles though the exact mechanism of resistance is not known (61, 62).

Autophagy can be considered as a form of innate immunity against invading microorganisms due to its role in intracellular killing of pathogens by means of phagosome-

lysosome fusion, pattern recognition receptor (PRR) recognition of pathogen components and in regulation of type I interferon (IFN) induction pathways (63, 64). Autophagy enhances delivery of pathogen-associated molecular patterns (PAMPS) to the PRR, triggering the production of cytokines (63, 64). In addition, a number of autophagy factors negatively regulate type I IFN induction. Besides, studies have shown that autophagy aids in the induction and execution of adaptive immune responses by governing MHC class II processing and presentation of various intracellular antigens to $CD4^+$ T cells and also by facilitating processing and loading of lysosomally processed viral proteins to MHC-I complexes and subsequent presentation to $CD8^+$ T cells (65, 66).

IV. AUTOPHAGY INDUCTION BY *M. ABSCESSUS*

M. abscessus is one of the predominant pathogens belonging to non-tuberculosis *Mycobacterium* (NTM) causing approximately 80% of the pulmonary disease caused by rapidly growing mycobacteria (RGM) (67~69). *M. abscessus* infection occurs in individuals with no previous lung disease and the multidrug resistant nature of the strain makes it difficult to treat (68, 70). Like the *M. smegmatis* or *M. avium* (71), *M. abscessus* displays two colony morphologies on solid agar media; the rough type (R type) and the smooth type (S type) (72). The S type is non-cording, motile and biofilm forming while the R variant is coding, non-motile and non-biofilm forming. The major difference between R type and S type is that the S type expresses glycopeptidolipids (GPLs) which are componenets of cell wall (73, 74). GPLs are surface-glycolipids produced by NTM strains such as *M. avium* complex, *M. smegmatis*, and *M. abscessus* (72, 75). It is reported that the colony morphotype plays a role in virulence as the R type causes more severe infections in mice (76). A rough type of *M. abscessus* (UC22) isolated from patients with upper lobe fibrocavity form of pulmonary disease induced severe lung inflmmation in mice and elevated production of cytokines in macrophages (77). The GPL in the S type facilitates the initial colonization of *M. abscessus* strains but masks the underlying bioactive cell wall lipids involved in virulence (78). The ability of *M. abscessus* to transform between R and S types brings particular attention in terms of lung infection and the R type is reported to arise during the course of infection in the host organisms (76, 79). It has been suggested that the S type initially colonizes the airways and forms biofilms, with subsequent transition to R form leading to severe infections (72). However, the exact trigger by which the S type transforms into R type *in vivo* and the factors for the increased virulence in the R type is currently not known.

Since the R and S variants of *M. abscessus* displays different morphotypes and induce different cellular response, it can be hypothesized that these variants differentially affect the phagocytic pathway. The S variant is efficiently phagocytized by macrophages than the R variant and the majority of the phagosomes harbouring the S variant contain single bacterium in contrast to the R variant (80). In the case of the R variant, majority of the phgosomes were social phagosomes with at least 2 bacilli. The number of phagosomes formed within 24 hr of infection was high in the case of S variant. Meanwhile the R variant displayed less number of phagosomes and it could be very well connected to the aggreagative nature of the R variant. The cording of the R variants contributes to gathering of long chains of bacteria at the close vicinity of the cell surface or in phagocytic cups. This makes it difficult for the tips of the pseudopods to fuse together to give rise to nascent phagosomes. It has been reported that the R varaint displays increased virulence due to the massive production of serpentine cords that grow too large to be phagocytized by macrophages or neutrophils (81). As the cords cannot be phagocytized, uncontrolled bacterial replication leads to abscess formation, tissue damage and death. Thus, cording has a crucial role in the pathophysiology of *M. abscessus* infection as it is a mechanism of immune evasion. The intraphagosomal R and S strains display distinct morphology as the S strain exhibits a thick outermost electron transluscent zone (ETZ) which is apposed to the phagosome membrane all around while the R form produces a very thin ETZ (80).

It has been known that once inside the host cells, the R

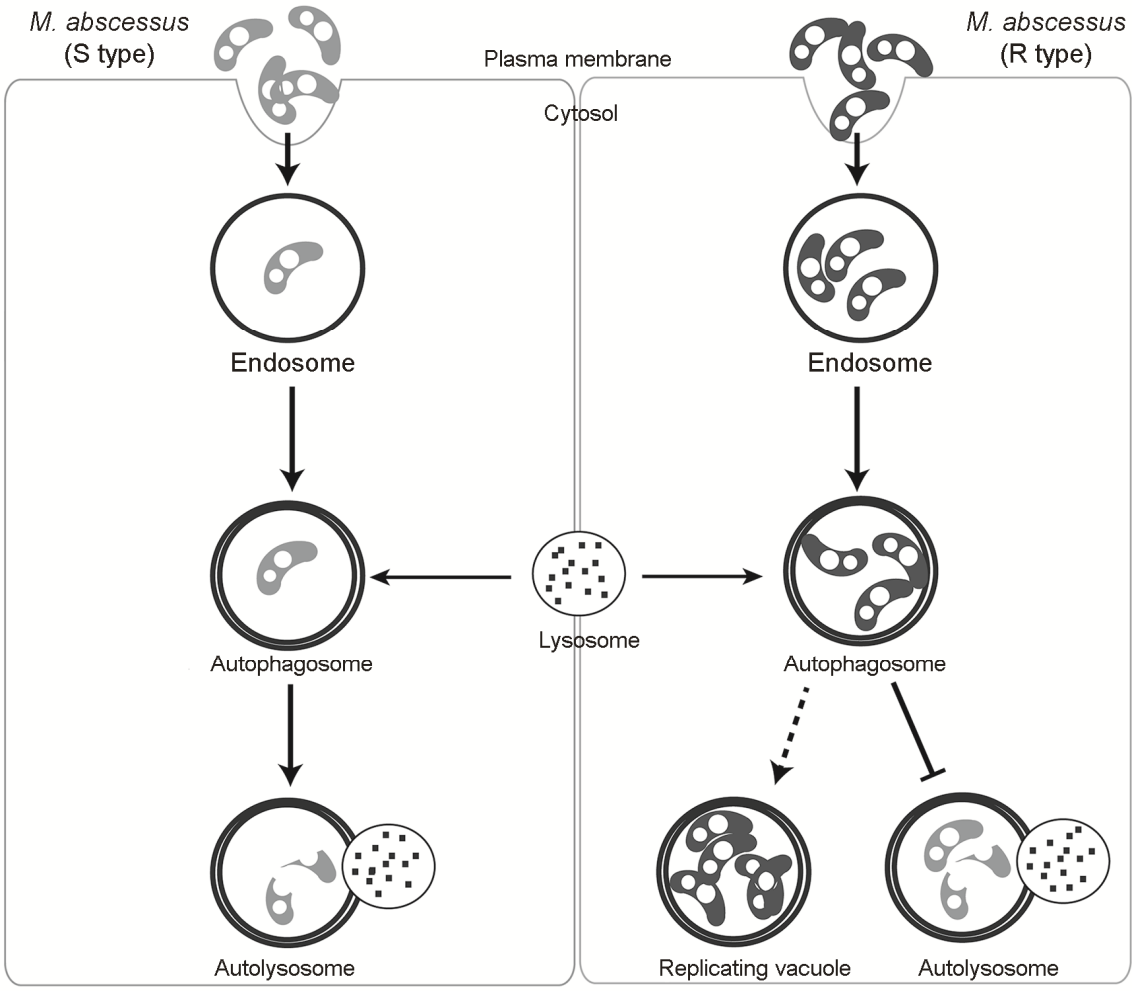

Figure 1. Proposed model of the autophagic pathway in response to S and R type *M. abscessus* infiltration. After invasion, the bacteria are contained in endosomes. The R type endosomes are characterized by the presence of multiple bacterial cells in contrast to the S type. The endosomes fuse with early autophagosomes which later matures into late autophagosomes. The late autophagosomes eventually fuse with the lysosomes to become autolysosomes in which the bacteria are killed. The R type *M. abscessus* inhibits lysosomal fusion and replicate within replicating vacuoles that resemble late autophagosomes.

and S types are present in morphologically distinct phagosomes (Fig. 1). However, there is not much information on the machanisms by which R and S types induce distinct autophagosome formation. Recently, it was demonstrated that the R type induces more autophagy than the S variant, as evidenced by the increased percentage of LC3 formation in infected cells (80). This was in accordance with the study conducted in our lab using the R type clinical isolate, UC22 where the R type induced high level of autophagy response compared to the S type, ATCC 19977 (unpublished data). It has been reported that p62 is required for the autophagic clearance of bacteria even though it doesn't play a role in autophagosome-lysosome fusion (82). Since, p62 is degraded along with LC3 through the autophagy-lysosomal pathway (83), the enhanced p62 level in UC22-infected macrophages indicated that high autophagy induction didn't cause increased lysosomal degradation and elimination of bacteria (unpublished data). The increased level of p62 due to block

in autophagic pathway was further confirmed by transfecting cells with siRNA targeting autophagy-related gene *Atg5* (siAtg), an inhibitor of the autophagic pathway. Thus, it was concluded that the R type induces autophagy and inhibits autophagy flux in murine macrophages. The decreased co-localization of LC3 or bacteria with lysosomal markers pointed out that the virulent UC22 is not delivered into acidified lysosomal compartments where the bacteria can be eliminated (unpublished data). Moreover, the intracellular survival of UC22 was significantly increased compared to the S type suggesting that autophagy plays a crucial role in the intracellular survival of UC22 by inducing autophagosome formation and preventing autophagy flux thereby evading the clearance from host cells. It was reported that the the rapamycin-induced autophagy increased intracellular survival of *M. smegmatis* in macrophages (84). However, the autophagy induction via starvation or treatment with the drug rapamycin contributed to significant killing of intracellular bacteria in *M. bovis* bacillus Calmette-Guérin- and *M. tuberculosis*-infected macrophages (43). The virulent *Brucella abortus* was seen distributed within the autophagosome by preventing the lysosome-phagosome fusion (85). The high virulence of the R type could be considered for the elevated autophagy and intracellular survival implying interesting links between virulence, autophagy and intracellular survival.

Known that the R type *M. abscessus* induces potent autophagy response, it would be better to understand whether any of the individual molecules of R type has the ability to induce autophagy. Lipids are major structural component of Mycobacteria (86) and induce autophagy response (84). Lipids isolated from UC22 induced elevated level of autophagy response compared to that from ATCC 1977, which was evidenced by significant increase in LC3 formation in UC22 lipid-treated cells (unpublished data). Recently, it has been reported that autophagy plays a role in the clearance of lipid droplets, thus regulating lipid metabolism in host cells (87). The surface-exposed lipids of mycobacteria differ from species to species and has a crucial part to play in the pathogenesis of *M. tuberculosis* (88). These lipids are accounted as important virulence factors of mycobacteria and they play relevant roles during infection via diverse mechanisms. Thus, it is possible that the loss of GPLs in UC22 could unmask the lipids which are capable of inducing autophagy, thereby allowing elevated response to be observed with intact mycobacteria. Further investigation on fractionated lipids from UC22 would help in comparing autophagy induction with respect to different classes of lipids. In addition, the surface-exposed lipids might be inhibiting the autophagy response in the S type (80).

It has been reported that some pathogens induce elevated autophagy response as a strategy to evade the immune response of the host cells (89). Once inside the cells, the pathogens survive and thrive within the replicating vacuoles that resemble late autophagosomes by inhibiting lysosomal fusion. In contrast, the GPL in the S type *M. abscessus* inhibits autophagy (80) and the failure to enter the autophagic pathway dooms these bacateia to the phagocytic/endocytic pathway, resulting in their deposition into phagolysosomes for certain death while the R type enters the autophagic pathway. The R type phagosomes contain mulple bacteria compared to the S type (80) and the R type strain has shown increased replication ability at the cellular level (81). It can be proposed that the R type might be thriving inside the replicating vacuoles which is evidenced by the decreased co-localization of LC3 or bacteria and lysosomal markers, and the increased intracellular survival in the infected macrophages (unpublished data). However, further studies need to be carried out in order to understand the exact mechanisms by which the rough type depicts increased autophagy response and intracellular survival.

V. CONCLUSION

Autophagy plays a central role in the clearance of invading intracellular pathogens and thus has a part to play in innate immunity. The current review discusses autophagy in terms of infection and immunity with special emphasis on autophagy response by *M. abscessus*. Autophagy has been described as a protective mechanism employed by some pathogenic bacteria including *M. abscessus* to evade the host cell defences, especially the lysosome. The present

knowledge of autophagy in *M. abscessus* is still in its infancy and many interesting questions remain unsolved. In this review, we have discoursed the current understanding of the autophagy response induced by *M. abscessus* with respect to different morphotypes, particularly based on the study conducted by our group. *M. abscessus* depicts varying autophagy response and intracellular fate depending on the morphotype, with the R type inducing increased autophagic response with higher intracellular survival. It seems like GPL is a determining factor in the fate of bacteria inside the host cells. However, the underlying mechanisms by which the rough type favours the autophagic pathway remains to be elucidated. Additional studies understanding the interactions between mycobacteria and specific molecules with the host autophagic machinery would help in devising new anti-infective strategies against mycobacterial infection and consequently improving immune response.

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