



January 2015

The Role Of Atg7 In The Host Defense Against Bacterial Infection

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THE ROLE OF ATG7 IN THE HOST DEFENSE AGAINST BACTERIAL
INFECTION

by

Yan Ye

Bachelor of Science, Anhui University of Traditional Chinese Medicine, 2005
Master of Science, Shanghai University of Traditional Chinese Medicine, 2009

A Dissertation
Submitted to the Graduate Faculty

of the

University of North Dakota

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

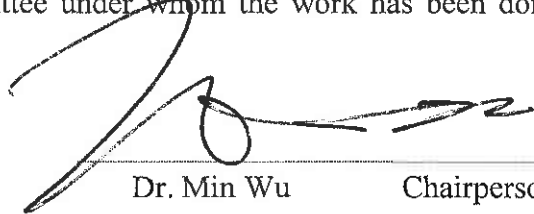
Grand Forks, North Dakota

August

2015

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This dissertation, submitted by Yan Ye in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.



Dr. Min Wu Chairperson



Dr. Katherine Sukalski



Dr. Archana Dhasarathy



Dr. Brij Singh



Dr. Colin Combs

This dissertation is being submitted by the appointed advisory committee as having met all of the requirements of the Graduate School at the University of North Dakota, and is hereby approved.



Dr. Wayne Swisher
Dean of the Graduate School



Date

Title The role of Atg7 in the host defense against bacterial infection

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ABBREVIATIONS

| | |
|--------|---|
| AM: | alveolar macrophage |
| ANOVA: | analysis of variance |
| ATG: | autophagy related gene |
| Atg7: | autophagy-related gene 7 |
| BAL: | bronchoalveolar lavage |
| CFU: | colony formation units |
| Co-IP: | co-immunoprecipitation |
| CPS: | capsule polysaccharide |
| DMEM: | dulbecco's modified eagle medium |
| IFN: | interferon |
| IL: | interleukin |
| KO: | knock out |
| Kp: | <i>Klebsiella pneumoniae</i> |
| LB: | luria-bertani |
| LPS: | lipopolysaccharide |
| MAPK: | mitogen-activated protein kinases |
| MLE: | murine lung epithelial |
| PBS: | phosphate buffered saline |
| ROS: | reactive oxygen species |
| RIPA: | radio immuno precipitation assay buffer |

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

siRNA: small interfering RNA

TBS: tris-buffered saline

TLRs: toll-like receptors

TNF: tumor necrosis factor

Ub: ubiquitin

UPS: ubiquitin-proteasome system

PAO1: Pseudomonas lab strain

p-I κ B α : phosphorylated-I κ B α

WT: wild type

ACKNOWLEDGEMENTS

I would like to acknowledge the many people who have given me guidance, support and help in completing my dissertation work at the University of North Dakota.

First and foremost, I would like to thank my supervisor, Dr. Min Wu, for his understanding, constant support and mentorship that have guided me through my research project. I am grateful for him giving me such an opportunity to work on this project, for trusting my judgment and helping me understand ideas. I also thank him for his spending many hours reading and editing my dissertation.

I would like to show my appreciation to all the advisory committee members. I truly thank Dr. Katherine Sukalski for her continuous encouragement, practical advice, and helping me explore ideas. To Dr. Colin Combs, thank you for providing insightful expertise, intelligent feedback and fundamental guidance regarding my research and life. To Dr. Brij Singh, I thank him for challenging my thinking by helping me question assumptions and furthering my thinking. I must also thank Dr. Archana Dhasarathy for her teaching me experiments and providing great ideas and numerous discussions in my current and future study that help me enrich my knowledge and view issues from multiple perspectives.

I would also like to thank the members of Dr. Min Wu's lab. They have greatly helped me with these projects by providing their suggestions, skills and friendship along the way. I thank them for making the workplace more fun and enjoyable.

Finally, I am especially thankful to my family with my heart. I thank my husband Liang Hui for his understanding and empathy through those difficult years. I am forever grateful to both our parents for their support. This dissertation is dedicated to them.

ABSTRACT

Klebsiella pneumoniae (Kp) is a Gram-negative bacterium that can cause serious infections in humans. Autophagy-related gene 7 (Atg7) has been involved in certain bacterial infections; however, the role of Atg7 in macrophage-mediated immunity against Kp infection has not been elucidated. To gain a better understanding of Kp-host interaction which may provide insight into the design of novel and effective therapeutics for this infection, we set out to investigate the potential immune role for autophagy in Kp infection *in vitro* and *in vivo*. We found that Atg7 was significantly induced in murine alveolar macrophages (MH-S) upon Kp infection, indicating that Atg7 participated in host defense in this infection. Knocking down Atg7 with siRNA increased bacterial burdens in MH-S cells. Using cell biology assays and whole animal imaging analysis, we found that compared to WT mice, *atg7* knockout (KO) mice exhibited increased susceptibility to Kp infection, with decreased survival rates, decreased bacterial clearance, and intensified lung injury. Moreover, Kp infection induced excessive proinflammatory cytokines and superoxide in the lung of *atg7* KO mice. Similarly, Atg7 silencing in MH-S cells markedly increased expression levels of proinflammatory cytokines. These findings indicate that Atg7 offers critical resistance to Kp infection by modulating both systemic and local production of proinflammatory cytokines.

We further investigated the molecular mechanism by which Atg7 regulates Kp-induced inflammatory responses. We found that Atg7 expression and p-I κ B α level were increased

in a time-dependent pattern in MH-S upon Kp infection. We also revealed an interaction between Atg7 and p-I κ B α , which was decreased upon Kp infection, whereas the interaction between ubiquitin (Ub) and phosphorylated-I κ B α (p-I κ B α) was increased in MH-S. We further demonstrated that knocking down Atg7 with siRNA increased p-I κ B α ubiquitylation and promoted NF- κ B translocation into the nucleus, and increased proinflammatory cytokine production (TNF- α). Moreover, overexpression of Ub in MH-S increased Kp infection-induced proinflammatory cytokines (TNF- α) and silencing ubiquitinase decreased cytokine production. In addition, infection of cells with lentivirus-shUb particles decreased binding of p-I κ B α to Ub and inhibited expression of TNF- α in the primary alveolar macrophage (AM) cells and lung tissue of *atg7* KO mice upon Kp infection. Thus, loss of Atg7 switched binding of p-I κ B α from Atg7 to Ub, resulting in an increased ubiquitylation of p-I κ B α , which facilitated NF- κ B nuclear translocation and intensified inflammatory responses against Kp. Our current findings reveal a regulatory role of Atg7 in ubiquitylation of p-I κ B α . Collectively, research of this dissertation provides new insight into the molecular detail of host-pathogen interaction for Kp, which may be beneficial for design of novel therapeutics to control Kp infection.

CHAPTER I

INTRODUCTION

Klebsiella pneumoniae

Klebsiella pneumoniae (**Kp**) is a Gram-negative bacterium that can infect various human organs such as lung and gut. When the bacterium invades the lung, it can cause pneumonia, which is a lung infection that primarily impacts on the lung alveoli. Pneumonia is one major cause of adult health care associated infections with a population of 15% or 20% for children (Figure 1). Although many factors can cause pneumonia (Wunderink & Waterer, 2014), the major causes are bacteria, viruses, or a combination of both (Campbell, Marrie, Anstey, Ackroyd-Stolarz, & Dickinson, 2003; Khasawneh et al., 2014).

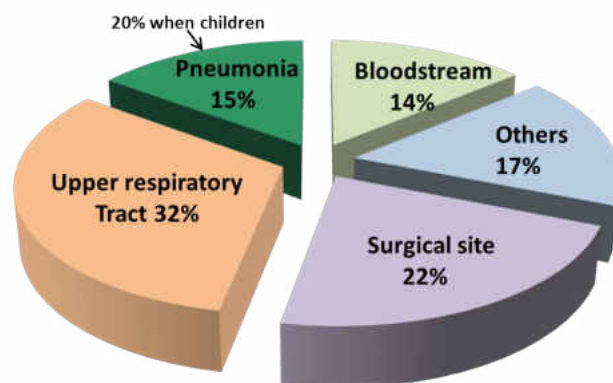


Figure 1. Major causes of health care-associated infections. Pneumonia affects a

population of 15% in the adult health care associated infections or 20% for children (Heller, 2010).

Most pneumonia infections (i.e., bacterial and viral pneumonia) usually share common symptoms such as cough, chest pain, fever, and difficulty in breathing (Watkins & Lemonovich, 2011), while certain types of bacterial pneumonia may exhibit specific symptoms. For example, *Legionella pneumoniae* may occur with abdominal pain, diarrhea, or neurological disorders (Darby & Buising, 2008; Mercante & Winchell, 2015); *Streptococcus pneumoniae* may have rusty colored sputum (Altiner et al., 2009); Kp can be associated with bloody sputum, described as currant jelly (Kawai, 2006). Pneumonia caused by Kp is commonly diagnosed by X-rays or culture of the sputum.

The treatment of pneumonia is mainly based on the underlying causes. Bacterial pneumonia is treated with antibiotics. Although pneumonia used to be called as "the captain of the men of death" in the 19th century, with the development of antibiotics, the incidence and corresponding mortality of bacterial pneumonia have been greatly reduced (Ruuskanen, Lahti, Jennings, & Murdoch, 2011). Unfortunately, overuse of antibiotics also stimulate antibiotics-resistant bacterial strains. Healthy people can usually defend against bacterial pneumonia, however, if they have health issues (e.g. immunocompromised individuals), their weakened immune systems greatly increase the susceptibility to bacterial pneumonia. Therefore, bacterial pneumonia is still one of the major infectious causes of hospitalization and death in the United States and worldwide, and incurs enormous costs in economic and human terms (Mizgerd, 2008; Moore &

Standiford, 1998). Given the above circumstances, the study of bacterial pneumonia is still of great importance.

Three types of bacterial pneumonia have been identified so far: Gram-negative, Gram-positive, and atypical pneumonia. Compared to the two other types of pneumonia, Gram-negative pneumonia is a less frequent one and has received less attention. To fill this gap in knowledge about bacterial pneumonia, Gram-negative pneumonia has been one of our long-term laboratory interests (Q. Guo et al., 2012; Wu et al., 2011; Yuan et al., 2011). Previous work focused on the mechanism of different Gram-negative pneumonia, such as how bacteria is delivered and degraded in phagocytes (Yuan et al., 2011). My dissertation project is focusing on one type of Gram-negative pneumonia, Kp. As an encapsulated Gram-negative bacterium, Kp normally resides in the flora of the mouth, skin, and intestines of human body as well as in natural environment (Y. Guo et al., 2012). Clinically, Kp is the third commonest organism isolated from intensive care units in the United States (Bedenic et al., 2005; Jones, 2010). Thus, Kp is a frequently encountered hospital-acquired opportunistic pathogen and often infects patients through indwelling medical devices. This pathogen is difficult to eradicate since it can rapidly develop resistance to multiple antibiotics, including broad-spectrum cephalosporins and β -lactams (Gouby et al., 1994), resulting in severe and persistent infections in immunodeficient or immunocompromised individuals. Kp can invade a variety of tissues and organs, such as lung, urinary tract, blood (sepsis), liver, etc. (Williams, Ciurana, Camprubi, & Tomas, 1990), which suggests that host environments and immune competency are important for modulating the outcomes of infection progression. Due to the clinical significance, a

variety of underlying virulence factors have been characterized and implicated in the pathogenesis of Kp (Ko, Chiang, Yan, & Chuang, 2005; Nassif, Fournier, Arondel, & Sansonetti, 1989).

Kp consists of two important virulence surface structural components, capsular polysaccharides (CPS) and lipopolysaccharides (LPS). Each component has its own antigen for serotyping of pathogenesis potency: K antigens for CPS structure and O antigens for LPS structure (Kubler-Kielb et al., 2013). In addition, non-structural components might assist invasion of Kp. Adherence factors, such as fimbriae, can increase the binding of bacteria to epithelial cells through mannose-sensitive attachment, thus facilitating invasion to host tissue. Biofilms that are often found in the respiratory or urinary tracts of chronically infected patients might aid Kp in subverting immune systems and establishing infection in adverse host environments.

Host-pathogen interaction

The human body employs a full spectrum of anti-infectious apparatus within the host defense system to combat bacterial invasion in a complex manner, which includes mechanical, phagocytic and immunologic components (Standiford, Kunkel, Greenberger, Laichalk, & Strieter, 1996).

The epithelial surfaces of our body, e.g., skin, gut and respiratory tract, are constantly challenged by pathogens since they are frontiers of the bodies to the environment. The

respiratory epithelial surface also encounters a great deal of microorganisms inhaled from the air due to its larger extension surface area (about 150 m²) (Figure 2). The epithelial cells in the small units of the lung, alveoli, have to be efficient in the host defense against inhaled pathogens. The alveoli have more concerns about the sterility compared to gut or skin, whose prime job responsibility is to maintain normal flora. On the other hand, the alveoli have increased risks of dissemination due to their localization between the environment and the bloodstream, compared to any other body boundary (Blasi, Tarsia, & Aliberti, 2005).

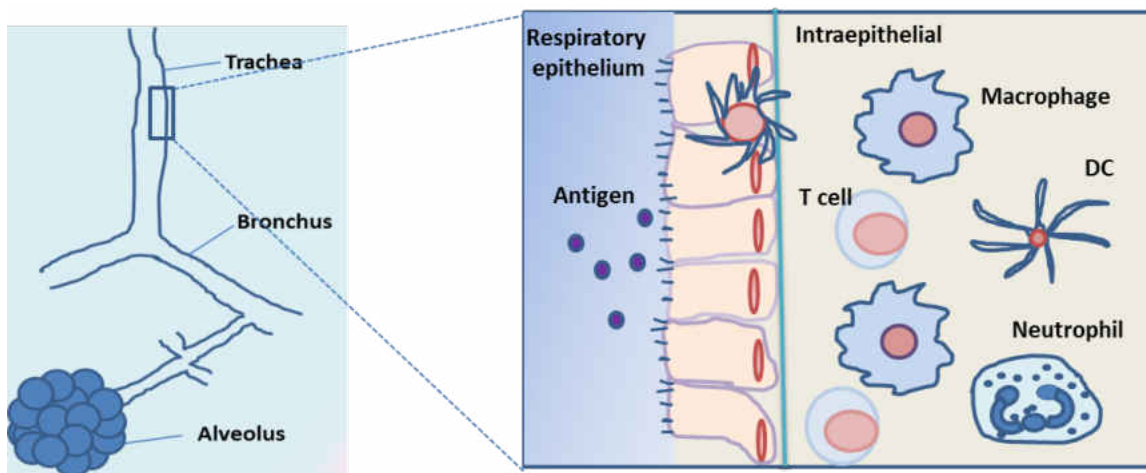


Figure 2. The immunological homeostasis in the respiratory tract (Holt, Strickland, Wikstrom, & Jahnsen, 2008) .

Innate immunity serves as the first line of host defense system to invading pathogens. Such defense includes recognition of the alien pathogens and executing function, e.g., release substances to directly kill or neutralize microbes, secrete cytokines and chemokines to recruit inflammatory cells to the infection site. In addition, it also activates adaptive immune response mediated by lymphocyte priming (Medzhitov & Janeway, 1997).

The innate response occurs very quickly (within minutes after the pathogen invasion) and to the same extent every time, no matter how many times the infectious agent is encountered, while the adaptive immunity responses are slower in developing and to a greater extent if repeatedly exposed to a given agent. Phagocytic cells (macrophages, monocytes and neutrophils) in the innate responses can cooperate or release inflammatory mediators (basophils, mast cells and eosinophils, and natural killer cells). There are also some other molecular components involved in innate responses including complement protein, acute phase protein, and cytokines (Delves & Roitt, 2000a, 2000b; Standiford et al., 1996).

The initial barriers including cough reflex, airway secretions and intact mucociliary system line the surface of the airways to fight off the invasion of bacteria (Nelson, Mason, Kolls, & Summer, 1995; Onofrio, Toews, Lipscomb, & Pierce, 1983). Once this first line of defense of airways is beaten, the phagocytic cells are recruited to the infected sites for the clearance of injurious agents of the lung. Macrophages are the primary resident phagocytic cells of the alveolus and constitute a critical cell type in the innate immune response (Chow, Brown, & Merad, 2011; Murray & Wynn, 2011). Macrophages express, on their membrane surface, pattern recognition receptors/sensors (PRRs) that help recognize various moieties from microbes. The microbial molecules being recognized by PRRs are termed pathogen-associated molecular patterns (PAMPs) and also danger-associated molecular patterns (DAMPs) (Kawai & Akira, 2010). PAMPs can be a variety of molecules, such as lipopolysaccharide (LPS), lipids, nucleic acids,

proteins, lipoproteins, oligosaccharides, glycans derived from various bacteria, viruses, parasites, and fungi. Once a specific receptor (PPR) for PAMP/DAMP is engaged, various downstream effectors/pathways are activated. Activation of the immune active cells in the immune system aids in the host cells combating the invading agents by activating degradation pathways and relaying signals such as cytokines to alert other cells of the innate and adaptive immune system in the proximal and distal sites (Blander & Sander, 2012; Mihalache & Simon, 2012). Many inflammatory cytokines (TNF- α , IFN- γ and IL-1 β) and chemokines (CXCL1, CXCL1 and CCL3) could be highly produced or secreted following phagocytosis by neutrophils and macrophages (J. Z. Liu, Pezeshki, & Raffatellu, 2009; Woodford, Turton, & Livermore, 2011). The recruitment and activation of other inflammatory cells, such as neutrophils, also contribute to the clearance of bacteria including Kp (Standiford et al., 1996; Toews, Gross, & Pierce, 1979). In addition, adaptive immune cells including dendritic cells, T and B lymphocytes, are activated to defend the body against Kp. These cells also play an important role in killing the pathogen or neutralizing the toxic components (K. Chen & Kolls, 2013). Recent studies suggest that a subset of novel cytokines in Th17 family, such as IL-17 and IL-22, is critical in the host defense against Kp. Both cytokines stimulate the expression of antimicrobial peptides and neutrophil chemoattractants to the infectious sites (Curtis & Way, 2009).

The lung progenitor epithelial cells responsible for infection and injury also play a crucial role in defense against Kp infection by contributing to host innate immunity. Research has also shown that murine alveolar macrophages (MH-S, a well-established cell line)

can kill Kp by recognizing their CPS either through the capsular structures by macrophage mannose receptors or opsonization by the lung surfactant protein (Keisari, Kabha, Nissimov, Schlepper-Schafer, & Ofek, 1997). Despite intense research on pathogenesis and host-pathogen interaction, the mechanism by which Kp is cleared from the lung by alveolar macrophages is largely unknown, thereby impeding the development of effective strategies for control of this infection.

Classic inflammatory response pathways

The launch of inflammatory responses in humans to combat infection after bacterial challenge is a complex and dynamic process, which often involves the production and function of cytokines and chemokines. Innate immune cells residing in tissues, such as alveolar macrophages, are responsible for initiating inflammatory response and their function are dependent on the specific receptor-PAMP/DAMP to activate various downstream effectors/pathways. Activated pattern recognition receptors (PRRs) then assemble to large multi-subunit complexes that initiate signaling cascades to stimulate the release of factors and promote the recruitment of leukocytes to the infected sites. So far, several classical signaling pathways (see Figure 3) have been identified, including TLR, RIG-I, TNFR, NLRs (NOD-like receptors containing pyrin domain) signaling, etc. (Coggins & Rosenzweig, 2012; Newton & Dixit, 2012). In TLR signaling, the TLRs could be bound by various PAMPs- or DAMP to activate the intracellular signaling. The pathway shown in Figure 3 is typical of TLR4, whose ligand is LPS. In RIG-I (retinoic acid-inducible gene I) signaling, RIG-I or other members of the RIG-I-like receptor

family could recognize viral RNA and activated the transcription factor IFN regulatory factor (IRF)-3. In addition, RIG-I can also activate the inflammasome together with

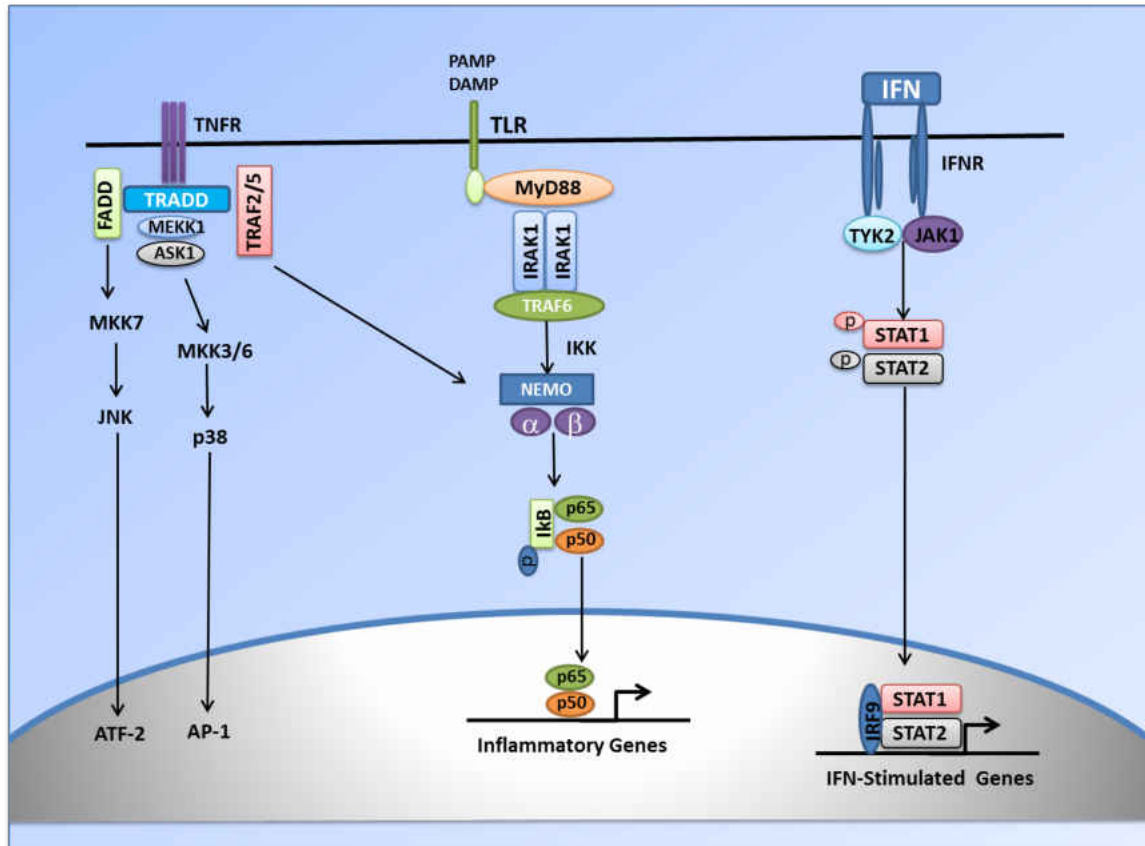


Figure 3. The signaling events involved in inflammation. Three classical signaling pathways including TLR signaling, TNFR signaling and IFN signaling have been identified (Coggins & Rosenzweig, 2012; Newton & Dixit, 2012).

apoptosis-associated speck-like protein containing C-terminal caspase recruitment domain [CARD] and procaspase-1. As for TNFR signaling, the binding of TNF- α to its receptor recruits the TNF receptor-associated death domain (TRADD) and activates the TNF receptor-associated protein complex. The type-1 IFNs bind to the two subunits of IFN- γ , leading to activation of tyrosine kinases JAK1 and TYK2, thus followed by the phosphorylation of transcription factors STAT1 and STAT2. The activated STAT proteins could regulate the expression of a large number of IFN-stimulated genes. Most of these signaling systems are associated with four major downstream transcriptional factors, such as NF- κ B, CREB, STATs, and IRF3 (Newton & Dixit, 2012), which promote transcription of target genes. Importantly, most of these target genes drive inflammation, while others regulate (generally inhibit) cell death. Many inflammatory pathways ultimately converge on NF- κ B signaling. In the present study, I focus on NF- κ B signaling pathway because the NF- κ B signaling pathway is thought to play an essential role in our Kp infection model (X. Li et al., 2014).

Nuclear factor- κ B (NF- κ B) pathway

Nuclear factor- κ B (NF- κ B) was discovered by Dr. Ranjan Sen in the lab of Nobel Prize laureate David Baltimore (Sen & Baltimore, 1986b). It is widely recognized as an essential transcription factor that is involved in a variety of physiological and abnormal processes such as cancer, inflammation, septic shock, bacterial infection and oxidative stress (Beutler & Cerami, 1989; Jing & Lee, 2014; Sha, 1998). In bacterial infection, the translocation of NF- κ B to the nucleus facilitates the transcription and translation of a

number of downstream pro-inflammatory cytokine genes, such as TNF- α and IL-6 (Beutler & Cerami, 1989; Sha, 1998). The NF- κ B signaling pathway is illustrated in Figure 4.

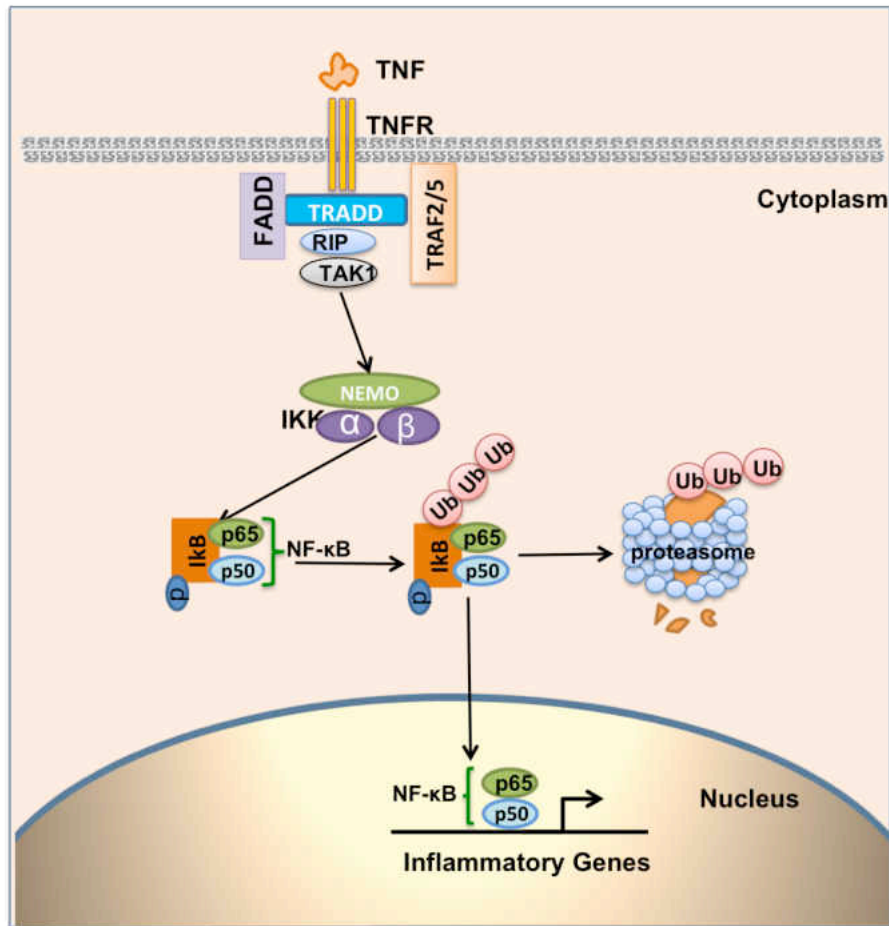


Figure 4. The NF- κ B signaling pathway (Oeckinghaus, Hayden, & Ghosh, 2011).

The discovery of I κ B, a cytoplasmic inhibitor of the NF- κ B (Sen & Baltimore, 1986a) is another remarkable achievement in NF- κ B research. These discoveries inspired studies to identify the mechanisms to liberate NF- κ B from the suppressing effects of I κ B. During that time, there were two major views regarding the NF- κ B liberation mechanism. One

group supported the idea that I κ B phosphorylation induced the release of NF- κ B from I κ B, thus activating NF- κ B (Baldwin, 1996; Ghosh & Baltimore, 1990), while other experiments demonstrated that I κ B phosphorylation itself was not adequate for NF- κ B activation, and that I κ B degradation was required for NF- κ B activation (Brown, Park, Kanno, Franzoso, & Siebenlist, 1993; Sun, Ganchi, Ballard, & Greene, 1993). Later on, several laboratories indicated that signal-induced ubiquitylation and proteasomal degradation of I κ B were necessary for NF- κ B activation (Z. Chen et al., 1995; Palombella, Rando, Goldberg, & Maniatis, 1994), which for the first time showed that ubiquitin-dependent proteolysis itself acted as a signal to induce transcriptional activation. Among the six highly conserved amino acids in the N-terminal of I κ B, two serine residues (32 and 36) can be phosphorylated upon phorbol ester stimulation. The mutations at S32 and S36 are resistant to phosphorylation, thus abrogating I κ B ubiquitylation and proteasomal degradation (Brown, Gerstberger, Carlson, Franzoso, & Siebenlist, 1995; Z. Chen et al., 1995). These findings led studies to characterize the degradation mechanisms of I κ B, especially via I κ B kinase and ubiquitin ligase. In addition to I κ B kinase and Ubiquitin-proteasome system (UPS), the degradation of I κ B kinase may also be regulated by an autophagy pathway (Sha, 1998). A recent study demonstrated that the liver of beclin1 mutant mice exhibited increased apoptosis and TNF- α production as well as NF- κ B activation due to the accumulation of p62 (Mathew et al., 2009). These findings suggest that autophagy may negatively impact inflammatory responses via NF- κ B signaling pathways. Herein, I will present a brief introduction to the two degradation systems, ubiquitin-proteasome system and autophagy, and especially to how they play a role in inflammatory responses.

Ubiquitylation and inflammatory responses

Ubiquitin-proteasome system (UPS) is an intracellular protein degradation system, which plays crucial roles in various basic cellular processes. Two successive steps are involved in targeting the protein to degradation through UPS: 1) conjugation of ubiquitin to the substrate; 2) degradation of proteins by 26S proteasome complex and recycling of ubiquitin. Ubiquitin is a highly conserved protein that is ubiquitously expressed in all eukaryotes (J. Chen & Chen, 2013). This 76-amino-acid protein is a crucial player for UPS, which is accomplished in a three step-cascade mechanism with three classes of enzymes, ubiquitin -activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin protein ligases (E3) (Figure 5) (S. Liu & Chen, 2011). Firstly, E1 activates ubiquitin in an ATP-requiring condition. Secondly, ubiquitin is transferred to E2. Finally, ubiquitin is added to the substrate, which is catalyzed by E3. The C-terminal glycine of ubiquitin can be attached to the epsilon amine of lysine on the substrate via an isopeptide bond. Additionally, the C-terminal of ubiquitin can bind to one of seven lysines (K6, K11, K27, K29, K33, K48, K63) or N-terminal methionine of another ubiquitin to form polyubiquitin chain. The linkage of polyubiquitin can influence the destination of the substrate by adding another layer of complexity to this modification. For instance, K48 or K29-linked ubiquitylation coordinates proteins for degradation in a proteasome-dependent way, while other polyubiquitylation, such as K63, K11 or K6-linked ubiquitylation functions are involved in the regulation of DNA damage repair, inflammation, vesicle trafficking and translation (Z. J. Chen & Sun, 2009). Ubiquitylation is a reversible process, which can be modulated by deubiquitylating enzymes. Proteins containing a ubiquitin binding domain can transmit signals from ubiquitylated substrates

to other proteins, which helps regulate specific signaling pathways in cells (Hicke, Schubert, & Hill, 2005).

Ubiquitylation accounts for 80% of the intracellular protein turnover and has been reported to play an important role in the development of inflammatory and autoimmune disease through various pathways including NF- κ B pathways. UPS regulates NF- κ B activity by promoting both scaffolding and degradation. In the resting state, NF- κ B binds to I κ B and is sequestered in the cytoplasm. Upon stimulation with various agents such as inflammatory cytokines or TLR ligands, I κ B is phosphorylated by I κ B kinase (IKK) complex (IKK α and IKK β) and one essential modulator (NEMO, also known as IKK γ). Phosphorylated I κ B is further ubiquitylated and degraded by 26S proteasome, thus releasing NF- κ B and allow its translocation to the nucleus, where it serves as a master regulator of the expression of a plethora of inflammatory response genes (Hayden & Ghosh, 2008).

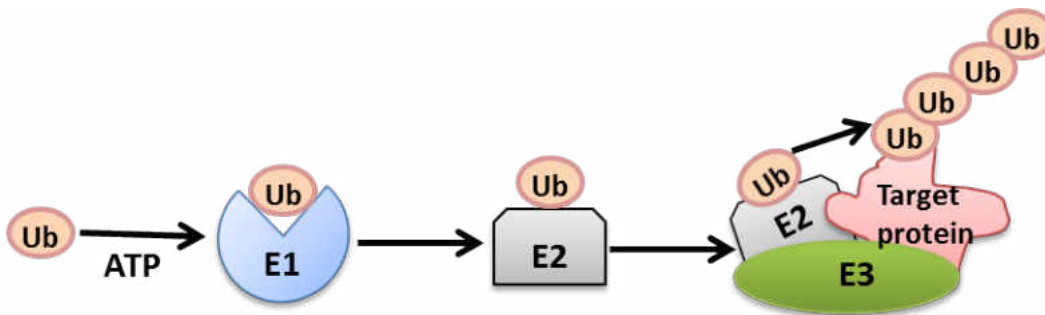


Figure 5. The ubiquitylation pathway. UPS is accomplished in three sequential steps with three classes of enzymes, ubiquitin -activating enzymes (E1), ubiquitin -conjugating enzymes (E2) and ubiquitin protein ligases (E3). (http://e3miner.biopathway.org/help_intro.html)

Autophagy and inflammatory responses

Autophagy, through a lysosomal degradation mechanism, is essential for cell survival, differentiation, development, and homeostasis (Marino & Lopez-Otin, 2004). Cellular homeostasis requires the degradation of long-lived stable proteins and recycling of organelles and foreign agents (Larsen & Sulzer, 2002; Marino & Lopez-Otin, 2004). At least three types of autophagy currently have been recognized according to their target molecules: 1) macroautophagy—degradation of bulky cytoplasmic components e.g., macromolecules and large organelles; 2) chaperone-mediated autophagy— degradation of cytosolic proteins with specific motifs; and 3) microautophagy—degradation of small organelles and molecules (Klionsky & Ohsumi, 1999).

Among these three types of autophagy, we focused on macroautophagy (hereafter autophagy) because it is the major type of autophagy that is responsible for the host defense against bacterial or viral infections (Gong, Devenish, & Prescott, 2012; L. T. Lin, Dawson, & Richardson, 2010).

Several critical steps including initiation of autophagy or the autophagosome, nucleation, maturation, transportation to lysosomes (autolysosome formation), and degradation and utilization of products (Mizushima, 2007) contribute to the autophagy process. The mechanisms of autophagosome formation have been well demonstrated while the exact mechanisms of the origin or initiation of the autophagosomal membrane remain unknown (Marino & Lopez-Otin, 2004). Such a process needs the coordination of autophagy-related gene encoded proteins (Atg), two ubiquitin-like (UBL) conjugation systems

(Atg12-Atg5 and Atg8-phosphatidylethanolamine (PE)), and Atg9 related membrane complex.

Currently, more than 32 Atgs have been identified and in this dissertation, I mainly focus on one of them, Atg7. Atg7 was discovered as a critical E1-like ubiquitin enzyme in yeast in 1998 by Dr. Ohsumi (Mizushima et al., 1998). The *atg7* gene encodes a 630-amino-acid protein and the Cys 507 is a putative active site cysteine. Atg7 participates in both canonical ubiquitin-like (UBL) conjugation systems (Atg12-Atg5-Atg16 and Atg8-PE conjugates). In the Atg12-Atg5 conjugation system, Atg7 activates the carboxy-terminal glycine of Atg12. The activated Atg12 is transferred to Atg10, an E2-like conjugating enzyme. Subsequently, Atg12 binds to Atg5 through an isopeptide bond. The Atg12-Atg5 conjugate then forms a large protein complex with Atg16. The Atg12-Atg5-Atg16 conjugates are essential for the elongation of the isolated membrane. Similarly, in the Atg8-UBL system, Atg8 is first processed by a protease, Atg4, to expose its glycine. Atg8 is also activated by Atg7 and then transferred to Atg3 (an E2). Ultimately, Atg8 forms a conjugate with PE. The Atg8-PE conjugation may also depend on the Atg12-Atg5 complex from the first UBL pathway. Atg8 could be recycled with the help of Atg4 once autophagosomes are completed, since Atg4 can deconjugate Atg8-PE (Ohsumi, 2001). Among the three mammalian orthologs of yeast Atg8, only (MAP-) LC3 (microtubule-associated protein light chain 3) is found in autophagosomes and in the small piece of membrane structure. LC3 is an important marker of autophagosomes and it has three isoforms: (1) ProLC3 represents the full-length molecule; (2) LC3-I is the proteolytic form; and (3) LC3II is the membrane bound form after PE-lipid conjugation

(Marino & Lopez-Otin, 2004). The lipid conjugation of LC3 to phagophore-membrane as LC3-II is useful as a mammalian autophagic marker. Consequently, Atg8 with additional factors contributes to the elongation and closure of the phagophore, thereby forming the double membrane autophagosome. Eventually, the autophagosomes fuse with lysosomes where their contents are digested by lysosomal hydrolytic enzymes (Settembre, Fraldi, Medina, & Ballabio, 2013) (see Figure 6).

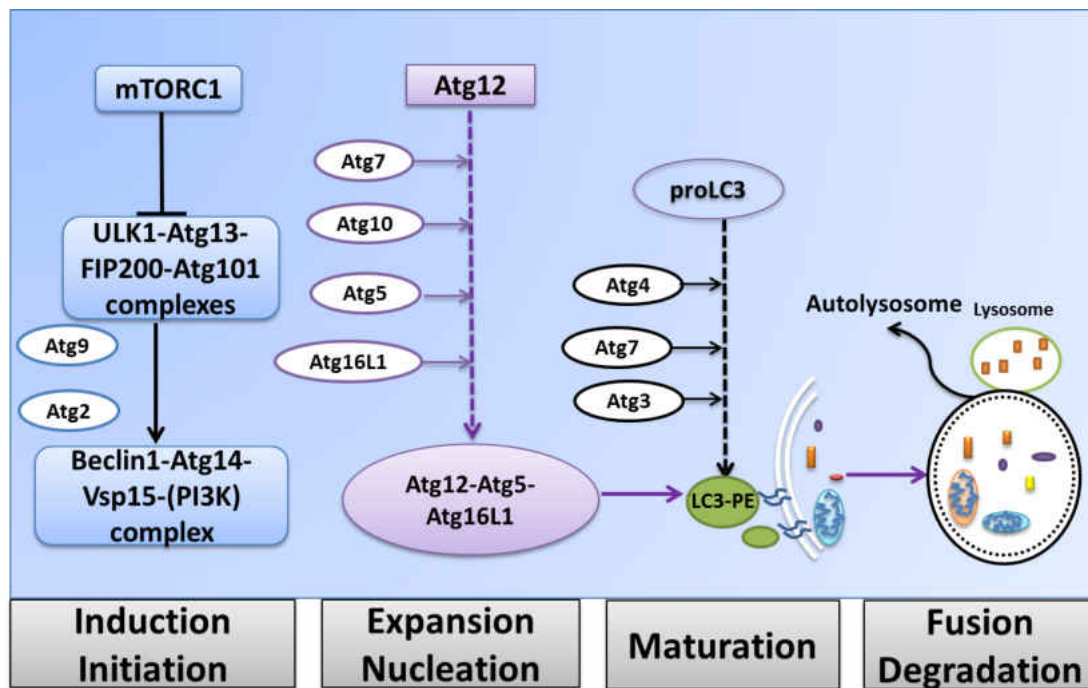


Figure 6. The main steps in autophagy and the main genes involved. Several steps include sequestration (induction of autophagy and autophagosome formation), nucleation (transportation to lysosomes), maturation (autolysosome formation), and degradation and utilization of products (Yang, Carra, Zhu, & Kampinga, 2013).

The autophagy response associates with a variety of general stress-response pathways and the close relationship between autophagy pathway and innate immune responses

have been well studied recently. For instance, autophagy could help to clear invading pathogens, regulate inflammasome-dependent signaling pathways, impact phagocytosis, etc. (Oh & Lee, 2012). Impaired autophagy pathway can lead to various infectious diseases and inflammatory syndromes (Deretic, 2012a). As a result of such considerable contributions to innate inflammatory responses, autophagy has been receiving growing attention in the immunology field (Deretic, 2012b).

Autophagy's involvement in bacterial infection has been studied as early as in 2004 by a group of scientists. Nakagawa et al suggested that *Streptococcus pyogenes* could trigger autophagy in HeLa cells (non-phagocytic) to defense against the infection (Nakagawa et al., 2004). In addition, Atg5 deficient cells had higher bacterial viability, suggesting the importance of Atg5 in bacterial killing activity (Nakagawa et al., 2004). At same time, another study observed that *Mycobacterium tuberculosis* is co-localized with LC3 and Beclin-1 in the phagolysosome and the degradation of *M. tuberculosis* is dependent on lysosome function (Gutierrez et al., 2004). After that, many studies suggest autophagy plays an essential role in immune response to viral or bacterial infection including bacterial pneumonia (Colombo, 2007; Levine & Klionsky, 2004; Levine & Kroemer, 2008; Ogawa et al., 2005). In A549 cells, autophagy is induced by *Streptococcus pneumoniae* via PI3K-I/Akt/mTOR pathway (Li et al., 2015). Use inhibitors of autophagy, such as 3-methyladenine and chloroquine, suppress the induction of inflammatory responses in TLR2 KO macrophages after challenging with *Mycoplasma pneumoniae* (Shimizu et al., 2014). Knock-down of the autophagy gene, Atg9a, induced the production of IFN- β in *Streptococcus pneumoniae* infected aged macrophages

(Mitzel, Lowry, Shirali, Liu, & Stout-Delgado, 2014). Mi et al demonstrated that using autophagy inhibitor reverses the therapeutic effect of IL-17A in bleomycin-induced pulmonary inflammation or fibrosis (Mi et al., 2011).

Autophagy also reportedly responds to a variety of intracellular pathogens including bacteria, viruses, fungi, etc. (Deretic, 2010, 2011), which is termed xenophagy. On the one hand, autophagy selectively uptakes invading microorganisms or aggregated protein via signals, adaptors (sequestosome 1/p62 like receptors, termed SLRs) or receptors, and then drives them to the autophagosomes. The outcome of autophagy is pathogen specific, indicating that subtle and varied mechanisms exist to counter intracellular bacteria (Ogawa et al., 2005). Autophagy potentially captures bacteria having escaped into the cytoplasm from phagosomes, and further delivers them into autophagosomes/autolysosomes for degradation (Campoy & Colombo, 2009). On the other hand, bacteria can escape from phagosomes and enter autophagosomes for survival and replication (Campoy & Colombo, 2009; Dorn, Dunn, & Progulsk-Fox, 2002). To date, whether autophagy plays a role in Kp pathogenesis is still poorly understood. We have studied autophagy in Kp-infected murine alveolar macrophage (MH-S) cells, and for the first time revealed the induction of autophagy by Kp through the important regulator Atg7. This observation may provide useful information for further understanding the role of autophagy in airway Kp infection.

Atg7 and bacterial infection

As a critical autophagy member, Atg7 is implicated in multiple physiological and pathological conditions (Komatsu et al., 2005; Kuma et al., 2004) including viral and bacterial infection (Inoue et al., 2012; Lenz, Vierstra, Nurnberger, & Gust, 2011; Shrivastava, Raychoudhuri, Steele, Ray, & Ray, 2011; Vural & Kehrl, 2014). Recent studies have delineated Atg7's function in cells challenged by bacteria or viruses. Japanese encephalitis virus replication is remarkably increased in neuronal cells where autophagy is impaired by Atg7 depletion (Sharma et al., 2014). Knockdown of Atg7 stimulates the innate immune response in hepatitis C virus-infected hepatocytes (Shrivastava et al., 2011). Mice with B cell-specific deletion of Atg7 fail to give rise to the protective secondary antibody responses, even though they showed normal primary antibody responses when infected with influenza viruses (M. Chen et al., 2014). T cell specific deletion of Atg7 results in enhanced apoptosis and decreased survival rate in a sepsis disease model (C. W. Lin et al., 2014). Atg7 deficient macrophages exhibit higher bacterial uptake when challenged with *M. tuberculosis* (Bonilla et al., 2013). In Atg7-depleted macrophages, protein expression of two types of macrophage scavenger receptors is enhanced by up-regulation of one nuclear factor, and the nuclear factor is caused by accumulated sequestosome 1 (SQSTM1 or p62). Using the same bacteria, Kim et al. demonstrate that Atg7 mutant drosophila exhibited decreased survival rates in *M. tuberculosis*-infected host cells (Kim et al., 2012). Atg7 deficiency impaired *Citrobacter rodentium* clearance in the intestinal epithelium (Inoue et al., 2012). Nevertheless, some studies also indicate that Atg7 may not be particularly critical in the inflammatory responses under some circumstances. For instance, at the early time point of post-

infection, virus or gene expression in host cells showed no significant changes with the depletion of Atg5 or Atg7 (McFarlane et al., 2011).

A few studies suggested that one possible mechanism for how Atg7 regulates inflammatory responses in the bacterial infection is the activation of inflammasome. Impaired autophagy promotes NLRP3 activation and IL-1 β release in murine sepsis models (Nakahira et al., 2011; van der Burgh et al., 2014). Bonilla et al. suggested that Atg7 regulated phagocytosis activity through accumulation of p62 and nuclear factor (erythroid-derived 2)-like 2 (NFE2L2) transcription factor, thus modulating the expression of scavenger receptors (Bonilla et al., 2013). Atg7 might dampen cytokine gene expression and thereby regulate *Citrobacter rodentium* infectious colitis (Inoue et al., 2012). Besides Atg7, other Atgs may regulate several inflammatory transcriptional responses. For example, increased levels of p62, the adaptor protein of autophagy, activates NF- κ B in autophagy-deficient cells (Moscat & Diaz-Meco, 2009). Lipopolysaccharide (LPS) could induce TNF- α production and IL-1 β mRNA, and nuclear translocation of NF- κ B in the intestines of autophagy conditional knockout mice (Fujishima et al., 2011), suggesting that a loss of autophagy enhances the immune response to bacteria through activation of the transcriptional factor NF- κ B (Inoue et al., 2012).

Although many studies reported the role of Atg7 in virus or bacterial infection, whether Atg7 or autophagy participates in host defense against Kp infection has not been elucidated. Previously, our lab was the first to report that *Pseudomonas aeruginosa* (PA)

infection could induce autophagy in alveolar macrophages, as evidenced by the dose- and time-dependently increased LC-puncta after PA infection (Yuan et al., 2012). Further, knocking down Atg7 or Beclin-1 downregulates autophagy and impairs bacterial clearance (Yuan et al., 2012). In this study we aim to demonstrate the involvement of Atg7 in the Kp infection disease model, and explore the mechanisms by which Atg7 regulates inflammatory responses.

Crosstalk between autophagy and ubiquitin-proteasome system (UPS)

UPS and autophagy are the two major protein independent degradation systems in the eukaryotic cell (Lilienbaum, 2013). There has been enormous interest in the protein degradation processes or mechanisms within the cells recently, considering its crucial roles in the modulation of many cellular activities, such as the cell cycle, cell differentiation and apoptosis (Wojcik, 2013). In addition, clinical evidence also strongly suggested an intimate relationship between ubiquitylation and autophagy in the pathogenesis of many diseases (Ding et al., 2007; Lilienbaum, 2013). Both UPS and autophagy play important roles in the modulation of inflammatory processes as well as in aiding cancer treatment. The failure to clear the misfolded or aggregated proteins in nerve and glial cells is a very typical pathogenic event in different neurodegenerative diseases. Despite intense research on both the UPS and autophagy pathways, the crosstalk of these two systems is not fully understood (Wojcik, 2013).

Several review articles summarized the interplay and crosstalk of the two systems, such as the activation of autophagy upon inhibition of UPS (Ding et al., 2007; Lilienbaum,

2013). For example, large protein aggregates, even ubiquitylated, cannot be degraded by UPS and can be degraded only through the autophagy pathway. One well-studied protein involved in both degradation systems is p62. With ubiquitin-associated domain, p62 binds to the ubiquitin of polyubiquitylated proteins, and they together eventually merge to cytosolic, nuclear or lysosomal aggregates. Also, p62 could bind to LC3 through specific regions and bridge ubiquitylated protein aggregates to autophagosomes (Komatsu et al., 2007; Zheng et al., 2009). Interestingly, p62 is known as an inhibitory factor of proteasomal degradation of LC3 as well (Gao et al., 2010). Wilde et al. confirmed that upon proteasome inhibition, p62 expression is highly induced and colocalized with ubiquitin in the large aggregates (Wilde, Brack, Winget, & Mayor, 2011). Deletion of p62 in $\Delta F508$ macrophages released Beclin1 from aggregates, which subsequently interacts with LC3 and improves *Burkholderia cenocepacia* clearance activity (Abdulrahman et al., 2013). Several proteasome inhibitors are also associated with autophagy. Recently, there has been growing interest in these inhibitors' therapeutic potential in the treatment of chronic inflammation and cancer. It has been shown that suppression of proteasomal activities stimulated autophagy pathway, eventually caused cell death (Ding et al., 2007; Zhang et al., 2014), which suggested that these two protein-degradation systems could coordinate and complement with each other.

An alternative relationship between UPS and autophagy pathways was proposed by Rubinsztein and his colleagues, who found blockage of autophagy might lead to the impaired degradation of UPS substrates or proteins (Korolchuk, Menzies, & Rubinsztein, 2009). They further demonstrated that long-term inhibition of autophagy caused an

accumulation of p62, which sequestered ubiquitylated substrates like p53, and impaired their delivery to the proteasome, thus preventing them from degradation. Overexpression of p62 inhibited UPS, which is dependent on the Ub binding domain. (Korolchuk, Mansilla, Menzies, & Rubinsztein, 2009). The Goldberg laboratory showed that FoxO3 stimulated UPS in muscle through the activation of autophagy, and that both UPS and autophagy pathways contribute to muscle atrophy (Zhao et al., 2007). Although much work has been done elucidating the link between these two degradation systems, much still remains to be explored.

Significance and rationale

When most bacterial pneumonia studies focus on pathogenesis and/or antibiotics against this bacterium, our study focuses on the host response mechanism. Here, we identify a novel role of Atg7 in the host defense against Kp in the lung by demonstrating that Atg7 deficiency potentiates an impaired bacterial killing, induces inflammatory responses, and lung injury. Another highlight of this work is the identification of the underlying mechanisms that Atg7 serves as an inhibitory regulator of ubiquitylation of p-I κ B α to limit NF- κ B-initiated inflammatory responses. These observations provide a new insight into the role of Atg7 in innate immunity against Kp and indicate a novel therapeutic target in Kp infection. Such regulatory mechanism of Atg7 is not restricted to Kp infection but also extended to other gram-negative bacterium, such as PA infection. Considering the awkward situation of antibiotics in bacterial infection because of the stimulation of antibiotic resistant strains, our study strongly suggests that Atg7 stimulation and/or UPS inhibition could be therapeutic strategies to uncontrolled

inflammatory responses induced by bacterial infections. The proposed studies are shown in Figure 7.

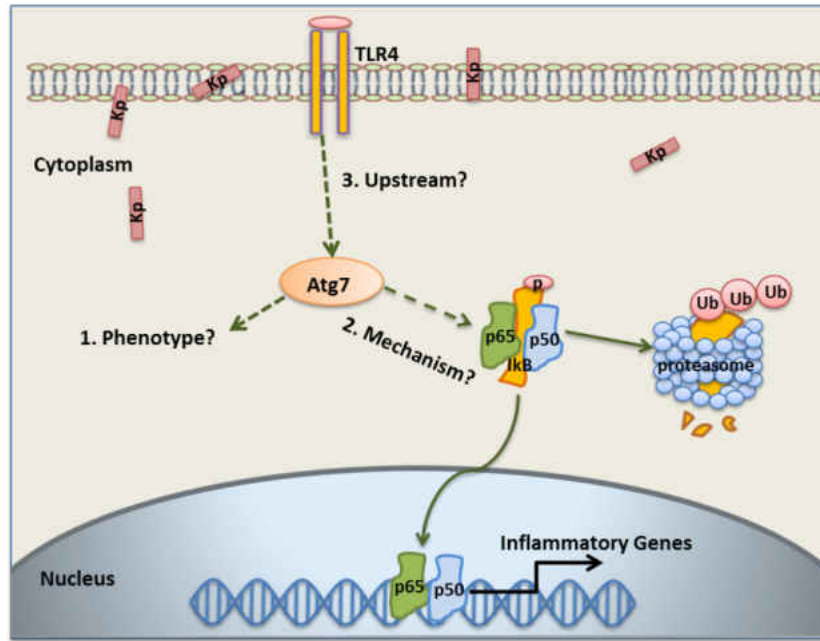


Figure 7. The proposed studies and rationale of this project.

CHAPTER II

MATERIALS AND METHODS

MATERIALS

Animals.

Atg7 wild type mice or control mice were C57BL/6J background with no health issues. Mice were kept and bred in the animal facility at the University of North Dakota, and the animal experiments were performed under the NIH guidelines and approved by the institutional animal care and use committee (IACUC).

***atg7* KO mice details:**

atg7 KO mice (in a C57BL/6J background) were kindly provided by Dr. Youwen He (Duke University) (W. Jia, Pua, Li, & He, 2011). *Atg7*^{Flox} (*Atg7*^{F/F}) mice were generated as reported (Komatsu et al., 2005). Mouse *atg7* gene has 17 exons that span 216-kb long genomic DNA. Exon 14 encoded the active site cysteine residue. *Cre-loxP* technology was used to conditionally disrupt the exon 14 by breeding *Atg7*^{F/F} mice with ER-cre mice (Vooijs, Jonkers, & Berns, 2001). *Atg7* deficiency was induced by intraperitoneally injecting tamoxifen 0.5 mg three times every other day.

Cell types. Murine alveolar epithelial cell line (MLE-12) and murine alveolar macrophage (AM) cell line (MH-S) were obtained from American Type Culture Collection (ATCC, Manassas, VA). HEK-BlueTMTLR4 cells were kindly provided by Dr. Matthew L. Nilles (University of North Dakota). The cells were originally bought from InvivoGen.

Bacterial strains. The Kp strain (ATCC 43816 serotype II strain) was provided by Dr. V. Miller (University of North Carolina, Chapel Hill) (Lawlor, Hsu, Rick, & Miller, 2005).

Reagents. All chemicals were obtained either from Sigma Aldrich or from Fisher Scientific Corp unless specified. Safety precautions were followed while handling toxic chemicals.

Antibodies. Antibodies to the following proteins, anti-Atg7, IL-6, NF- κ B, phospho-NF- κ B (ser536, sc-33020), GAPDH, p38, p-p38 (D-8, SC-7973), IL-1 β , and β -actin were purchased from Santa Cruz Biotechnology, Inc. Fluorescent secondary antibodies were bought from Molecular probes (Invitrogen).

Equipment. Equipment needed for the study was located primarily in the lab resources and Department of Basic Sciences Core facilities. Microscopy was performed at the UND Imaging facility.

Methods

Primary cell isolation and culture. Mouse AM cells were isolated by Bronchoalveolar lavage (BAL) as described (Yuan et al., 2011). Briefly, mice were anesthetized with 0.1 mg Ketamine and the trachea was exposed through a neck incision and cannulated using a sterile 20GA (BD angiocath, Becton Dickson, Utah) catheter. The lungs were lavaged with 5 ml of AM isolation buffer (140 mM NaCl, 5 mM KCl, 2.5 mM sodium phosphate buffer, 10 mM HEPES, 6 mM Glucose, 0.2 mM EGTA, pH 7.40) in 0.5 ml increments. BAL fluid was centrifuged to isolate AM cells and cultured in RPMI 1640 medium supplemented with 10% new born calf serum (NBS) and antibiotics. The cells were grown in culture overnight and used next day.

Cell line culture

MLE-12 and MH-S cells were maintained in F12/DMEM medium (1:1) and RPMI 1640 medium with 5% newborn calf serum and penicillin/streptomycin antibiotics in a 5% CO₂ incubator, respectively. HEK-BlueTMTLR4 cells maintained in DMEM medium with 10% fetal bovine serum, 50 U/ml penicillin, 50 mg/ml streptomycin, 100 mg/ml Normocim, and 2 mM L-glutamine.

Animal handling

Mice were given 45 mg/kg ketamine and intranasally infected with 5×10^5 CFU/mouse (six mice/group). The mice were killed when they became moribund to obtain survival curves (Yuan et al., 2011), while additional mice were used to attain data at designated

times. After BAL procedure, the trachea and lung were obtained for cell biology assays or fixed in 10% formalin for histological analysis (see Figure 8).

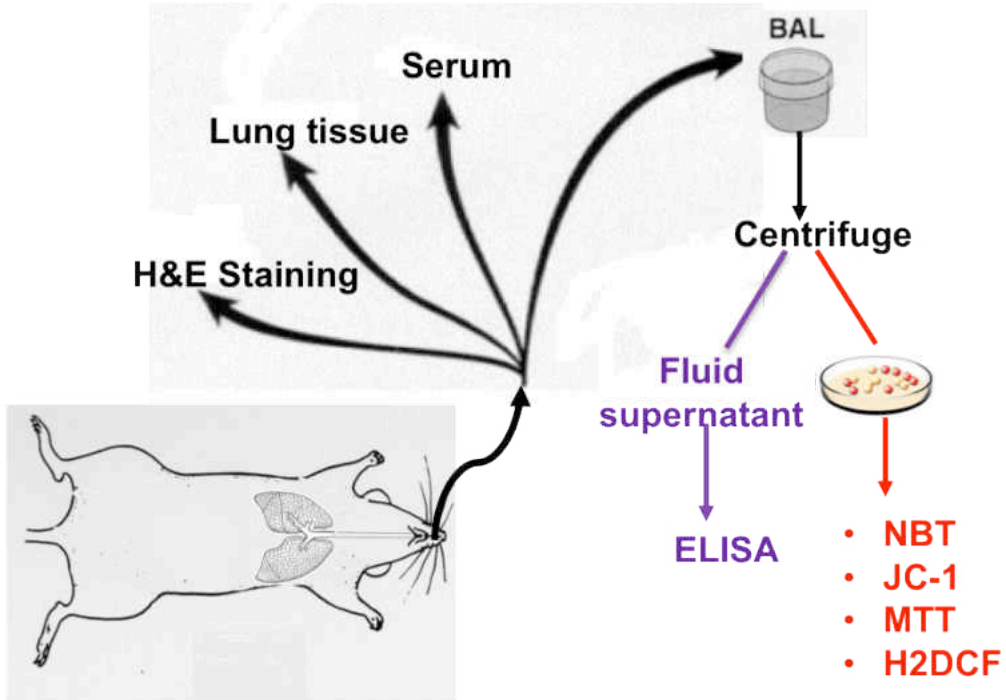


Figure 8. Schematic illustration of animal experiments in this research.

Bacterial culture and infection

Bacteria were grown overnight in Luria-Bertani (LB) broth at 37 °C with vigorous shaking. The next day, the bacteria were pelleted by centrifugation at 5000 × g and resuspended in 10 ml of fresh LB broth and allowed to grow until the mid-logarithmic phase. Optical density (OD) was measured at 600 nm, 0.1 OD=1×10⁸ cells/ml. Cells were washed once with PBS and changed to serum-free and antibiotic-free medium immediately before infection. Cells were infected by Kp with a 10:1 (bacteria-cell) ratio (Yuan et al., 2011) for indicated time points.

Cell transfection

Cells were transfected with Atg7 siRNA (Invitrogen) using LipofectAmine 2000 reagent (Invitrogen) in serum-free RPMI 1640 medium following the manufacturer's instructions. The cells were lysed after 24 h of transient transfection to evaluate the expression (Yuan et al., 2011).

Tandem GFP-RFP-LC3 plasmids were transfected to MH-S cells for 24 h as reported previously (Yuan et al., 2012). The tandem RFP-GFP-LC3 plasmid was generated and kindly provided by Dr. Tamotsu Yoshimori of Osaka University, Japan (Kimura, Noda, & Yoshimori, 2007). The RFP-GFP-LC3B plasmids allow enhanced dissection of the maturation of the autophagosome to the autolysosome. By combining an acid-sensitive GFP with an acid-insensitive RFP (TagRFP), the change from autophagosome (neutral pH) to autolysosome (acidic pH) can be microscopically determined by loss of GFP fluorescence but not red fluorescence, indicating that RFP-LC3 can label the autophagic

compartments both before and after fusion with lysosomes. After Kp infection, the cells were observed by confocal fluorescence microscopy.

In vivo transduction.

Mice were anesthetized using 45 mg/kg ketamine. The lentiviral reagent Ub shRNA (Santa Cruz, sc-36770-V) was delivered intranasally (Wilson et al., 2013). Thirty minutes prior to infection, all viral supernatants were mixed with LipofectAmine 2000 (5% final vol/vol; Invitrogen) to increase transduction efficiency (Wilson et al., 2010).

Inflammatory cytokine profiling

After infection, BAL fluid was collected to measure the cytokine concentrations using an ELISA kit (eBioscience Co., San Diego, CA). The trachea was surgically exposed and lungs were lavaged five times with 1.0 ml volume of lavage fluid to obtain BAL fluid except the first lavage 0.6 ml. The supernatant was collected after centrifugation. For ELISA assay, 96-well plates (Corning Costar 9018) were firstly coated with 100 µl/well capturing antibodies in coating buffer overnight at 4 °C (Yuan et al., 2011). 100 µl aliquots of serum samples were added to the coated wells. After incubating with corresponding detection-HRP-conjugated antibodies, the plate was read at 450 nm and analyzed to determine the cytokine concentrations using the known cytokine standards.

Western blotting assay

Cells or lung homogenates were collected and lysed with RIPA lysis buffer [50 mM Tris–Cl, 1% NP-40, 0.35% sodium-deoxycholate, 1 mM EDTA, 150 mM NaCl, 1 mM EGTA,

pH 7.4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM NaF, 1 mM Na₃VO₄] containing a protease inhibitor cocktail (ThermoFisher, Rockford, IL). Protein concentrations were determined by Bio-Rad Protein Assays (Bio-Rad, USA). Equal amounts of individual protein samples were resolved by SDS-PAGE and then electro-transferred onto the nitrocellulose membrane (10). Membranes were blocked for 30 min with 5% skim milk in TBST buffer composed of 50 mM Tris (pH 7.6), 150 mM NaCl and 0.1% Tween-20 and incubated with the primary antibody overnight at 4 °C. Antibodies against Atg7 was purchased from Invitrogen; Anti-IL-6, NF-κB, phospho-NF-κB (ser536, sc-33020), GAPDH, p38, p-p38 (D-8, sc-7973), IL-1β, and β-actin were purchased from Santa Cruz Biotechnology, Inc. GAPDH or β-actin was used as loading control. After incubation with secondary antibodies, ECL detection reagents (Santa Cruz Biotechnology, Inc.) were used to detect signals.

Co-immunoprecipitation (IP)

For co-immunoprecipitation, the supernatants were incubated with bare protein G-Sepharose beads (Invitrogen, Carlsbad, CA) for 2 h at 4 °C and then incubated with anti-Atg7 or IκBα antibodies (Santa Cruz Biotechnology) bound to protein G-Sepharose beads overnight at 4 °C. The beads were then washed three times in lysis buffer and boiled after resuspension in SDS sample loading buffer. The proteins were then resolved by SDS-PAGE electrophoresis and analyzed by Western blotting.

Confocal microscopy and indirect immunofluorescence staining

Alveolar macrophages or epithelial cells were grown in 3 cm glass-bottomed dishes (MatTek, Ashland, MA). After infection, the cells were fixed in 4% paraformaldehyde and then permeabilized with 0.2% Triton X-100 in PBS. After been incubated with the blocking buffer for 30 min, primary Abs were added at a 1:500 dilution in blocking buffer and incubated overnight. The next day, cells were washed three times with washing buffer (X. Chen, Hui, Geiger, Haughey, & Geiger, 2013; Wu, Pasula, Smith, & Martin, 2003). After incubation with appropriate fluorophore-conjugated secondary Abs, the cells were mounted on slides with mounting medium before taking images (Kannan, Pang, Foster, Rao, & Wu, 2006). The images were taken by an LSM 510 Meta confocal microscope (Carl Zeiss MicroImaging, Thornwood, NY).

Nitroblue tetrazolium (NBT) assay

NBT assay is used to detect the released superoxide. The color of NBT dye changes upon reduction by released superoxide. The dye is yellow in color, and after reduction by superoxide becomes a blue formazan product. After 24 h infection with Kp, the dye was added as previously described (Yuan et al., 2011).

Dihydrodichlorofluorescein diacetate assay

Dihydrodichlorofluorescein diacetate dye (Molecular Probes, Carlsbad, CA) only emits green fluorescence upon reaction with superoxide inside cells. Cells were seeded in the 96 well plates and treated as above. After 10-min incubation with the dye, fluorescence was measured using a fluorescence plate reader (BioTek, Winooski, VT) (Hui et al., 2012; Wu et al., 2011).

Lipid peroxidation assay

After infection, lungs were homogenized and lysed in 62.5 mM Tris-HCl (pH=6.8) supplemented with protease inhibitor (Thermofisher, Rockford, IL). Malondialdehyde could be measured in a colorimetric assay (Calbiochem) according to the manufacturer's instructions. Then the protein concentration is measured and normalized for the assay (Wu et al., 2009).

Phagocytosis assay

MH-S cells or primary AM were plated in 24-well plates and grown overnight. The cells were treated with the antibiotic-free medium immediately followed by Kp infection. After 1 h incubation at 37 °C, the wells were washed and treated with 100 µg/ml polymyxin B for 1 h to kill extracellular bacteria (Kannan, Audet, Huang, Chen, & Wu, 2008; Yuan et al., 2011). After washing with PBS three times, the cells were lysed in 1% Triton X-100. Then, colony-forming units (CFU) were counted to quantify phagocytosis.

Myeloperoxidase (MPO) assay

Lung tissue samples were homogenized in 50 mM hexadecyltrimethylammonium bromide, 50 mM KH₂PO₄, pH 6.0, 0.5 mM EDTA at 1 ml/100 mg of tissue and centrifuged for 15 min at 12,000 rpm at 4 °C. Precipitate was collected and 100 µl of reaction buffer (0.167 mg/ml O-dianisidine, 50 mM KH₂PO₄, pH 6.0, 0.0005% mM H₂O₂) were added to 100 µl of the sample. Absorbance was read at 460 nm at 2 min intervals. Triplicates were used for each sample and control (Kannan et al., 2008; Yuan et al., 2011).

Bacterial burden assay

AM cells from BAL fluid and lungs were homogenized in PBS and spread on LB plates to determine the number of bacteria (Yuan et al., 2011).

Luciferase reporter assay-NF- κ B activity

The p-NF- κ B Luciferase reporter plasmid was obtained commercially to measure NF- κ B activity. The plasmid encodes three repeats of NF- κ B binding consensus sequences followed by the Luciferase gene. If NF- κ B becomes activated then it will induce expression of luciferase protein in the transfected cells which can be measured using the substrate. Transient transfections were performed in 70% confluent MHS cells plated in 12-well plates with NF- κ B-reporter-luc plasmid following the manufacturer's instruction. 24 h after transfection, the cells were infected with Kp. Cell lysates were subjected to luciferase activity analysis by using the Luciferase Reporter Assay System (Promega) (Zhou et al., 2014). The Renilla Luciferase control construct commercially obtained from Promega was used as internal control for basal levels of expression.

Electrophoretic mobility-shift assay (EMSA)

Nuclear extracts from the cells with different treatment were isolated with Nuclear Extraction Kit according to the manufacture's introduction (Thermofisher). Oligonucleotide labeling and binding reactions were performed by using the reagent supplied in the NF- κ B EMSA Gel Shift Assay System (Thermofisher). After gel electrophoresis and transfer to a NC membrane, the signals were visualized using a digital

imaging system (Bio-Rad, CA, USA). The specificity of the bands has been confirmed by adding an excess amount of cold oligonucleotide to the reaction mixture.

Histopathology analysis

Lung tissues were fixed in 10% formalin in PBS using a routine histologic procedure. The fixed tissue samples were processed for obtaining standard H&E staining and examined for differences in morphology post infection (Wu et al., 2001).

Statistical analysis

All experiments were performed in triplicate and repeated at least three times. Data were presented as percentage changes compared to controls \pm S.D. from the three independent experiments. Group means were compared by one-way ANOVA (Tukey's post hoc) or Student t test using prism software, and a difference was accepted at $p < 0.05$ (Wu et al., 2011). The survival test results were represented by Kaplan-Meier survival curves using prism software.

CHAPTER III

RESULTS

SECTION 1: Atg7 suppresses inflammatory responses in Kp-infected mice

(Adopted from Ye et al. Atg7 deficiency impairs host defense against *Klebsiella pneumoniae* by impacting bacterial clearance, survival and inflammatory responses in mice. *American Journal of Physiology-Lung Cellular and Molecular Physiology*. 2014; 307 (5): 355-63.)

Atg7 was involved in Kp clearance in MH-S cells

MH-S and MLE-12 cells are widely used as models for studying molecular characteristics of murine lung macrophage and epithelial cells, respectively (Haranaga, Yamaguchi, Ikejima, Friedman, & Yamamoto, 2003; Mbawuike & Herscowitz, 1989). To study whether autophagy is involved in Kp infection, we first screened several autophagy related proteins including beclin-1, Atg5, and Atg7 and found that the expression of Atg7 was significantly increased upon Kp infection, especially in MH-S cells (about 2 fold) compared to uninfected controls (Figure 9A). Thus, we chose MH-S cells for the following experiments. We further observed that LC-3 was converted from LC3-I to LC3-II after Kp infection, suggesting that autophagy was induced by Kp infection (Figure 9A). To clearly identify the induction of autophagy by Kp, we

transfected a tandem RFP-GFP-LC3 plasmid into MH-S cells. RFP-GFP-LC3 plasmid was designed to differentiate the autophagosome and autolysosome. The tandem RFP-GFP-LC3 construct can form puncta after stimulation when transfected to target cells, suggesting autophagosome formation. Once an autophagosome fuses with a lysosome, the GFP moiety degrades while RFP-LC3 maintains the puncta and fuses with autolysosomes. After tandem construct transfection and followed by Kp infection, we observed an increase of LC3 puncta in both green and red fluorescence (Figure 9B and 9C). However, infected cells showed markedly increased RFP-puncta than control cells, confirming the induction of autolysosome formation. Taken together, these findings established that Kp infection could specifically induce autophagy in MH-S cells, because transfection of the tandem plasmid as a measure can exclude the possibility of lysosome deficiency-induced LC-3 accumulation (Yuan et al., 2012). These findings prompted us to further characterize the role of Atg7 in bacterial clearance. Bacterial burden assay demonstrated that a down-regulated Atg7 by siRNA silencing strategy led to decreased bacterial phagocytosis and clearance activity compared to scrambled siRNA transfection controls (Figure 10A and 10B). Successful knockdown about 50% of Atg7 was confirmed by measuring protein expression using western blot analysis (Figure 10C). To examine the effect of autophagy on inflammation, we measured cytokine levels after Atg7 siRNA silencing and found that suppressing Atg7 increased cytokine secretions (e.g., IL-6 and IL-1 β) in MH-S cells compared to uninfected and scrambled siRNA controls using ELISA (Figure 10D and E). Although IL-1 β was also increased, the change in IL-6 levels was more substantive, suggesting a possible IL-6 dominant inflammatory response. To assess which component of Kp is a major immune stimulator,

we also compared the potency of whole organism and LPS in inducing cytokine secretion in MH-S cells. We showed that both LPS and live Kp increased cytokine production, but live Kp (vs. LPS alone) induced higher IL-6 levels in Atg7 siRNA groups than scrambled siRNA groups (Figure 10F). These results suggest that the whole pathogen is required for inducing robust inflammatory responses despite the involvement of LPS (Deng et al., 2013).

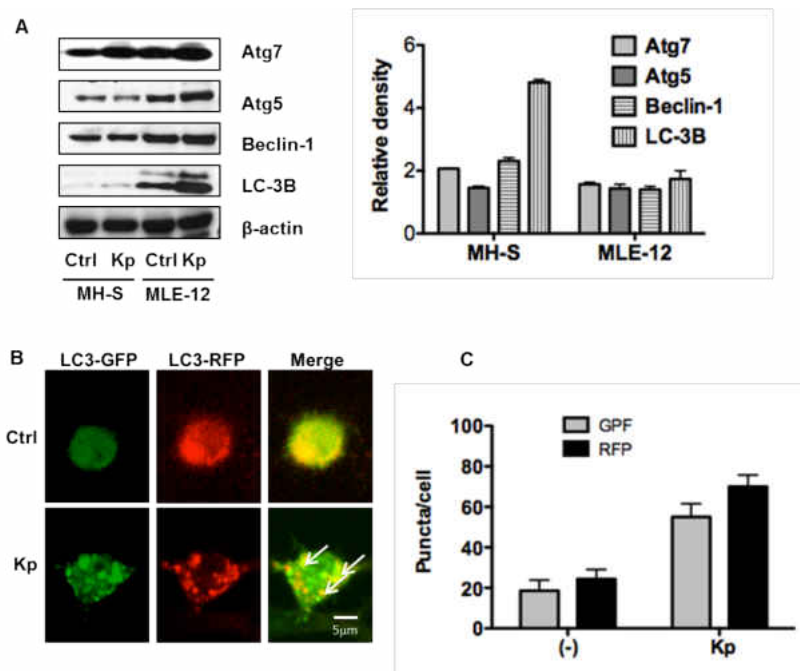


Figure 9. Atg7 and autophagy were involved in Kp infection in MH-S cells. (A) Increased expression of Atg7 in MH-S and MLE-12 cells 2 h post Kp infection as assessed by Western blot analysis. Gel data were quantified using densitometry with ImageJ software. The expressions of the proteins were quantified relative to β -actin. (B) Tandem GFP-RFP-LC3 plasmids were transfected into MH-S cells for 24 h. Then the cells were infected with Kp for 1 h (MOI=10:1). Arrows indicate LC3 puncta. (C) Puncta numbers (>10 in each cell) were considered as positive cells (at least 100 cells per group).

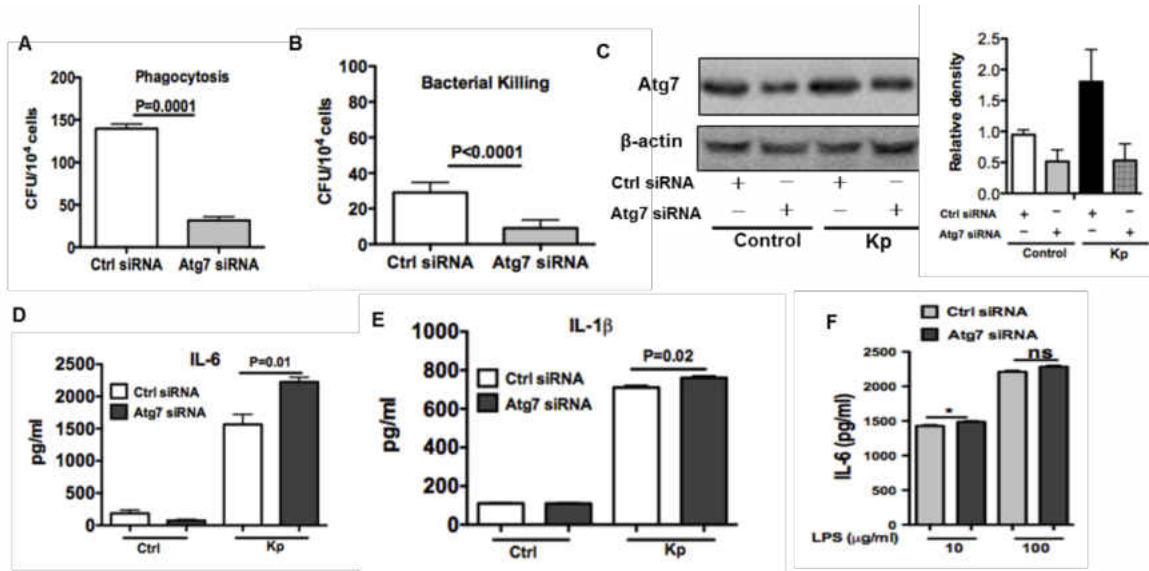


Figure 10. Atg7 contributed to bacterial clearance and inflammatory responses in MH-S cells. (A) and (B) Knocking down Atg7 with siRNA in MH-S cells decreased bacterial phagocytosis and clearance after Kp infection as determined by CFU assay with polymyxin B treatment (see methods). (C) Atg7 was knocked down with siRNA and followed by Kp infection detected by Western blotting. (D) and (E) Increased cytokine secretion in MH-S cells by knocking down Atg7 with siRNA silencing and Kp infection (ELISA). Data were representative of three experiments with similar results (student t-test). (F) IL-6 secretion in Atg7 siRNA silencing MH-S cells after LPS challenge. LPS was derived from Kp. Cells were treated with different doses of LPS (10 and 100 $\mu\text{g/ml}$) for 2 h and then the medium were collected to detect cytokine secretion by ELISA.

***atg7* KO mice exhibited decreased survival rates upon Kp infection**

To investigate the physiological relevance of Atg7 in Kp infection, we used *atg7* KO mice to examine outcomes following Kp invasion. After intranasally instilling luminescence emitting Kp (5×10^5 CFU/mouse), we noted an increased bacterial retention and dispersion in the lung of *atg7* KO mice compared to that of WT mice (Figure 11A and B) by a small animal imaging system (IVIS XRII, Caliper). The powerful *in vivo* imaging enabled the convenient determination of accumulated bacteria in a spatio-temporal manner without sacrificing animals. We found that *atg7* KO mice exhibited quicker spread, wider distribution (both left and right lung lobes vs. only left lung lobes), and longer persistence than those in WT mice, confirming the critical role of Atg7 in host defense against Kp infection. In addition, *atg7* KO mice exhibited increased lethality (40% *atg7* KO mice died within 24 h post-infection) as shown in Figure 11C. At 48 h, 80% of KO mice died, whereas 80% of WT control mice remained alive (n=6). This result was analyzed using Kaplan-Meier survival curves ($p < 0.05$, log-rank test). Taken together, these findings indicate that Atg7 is critically required for host defense against Kp infection in acute pneumonia models.

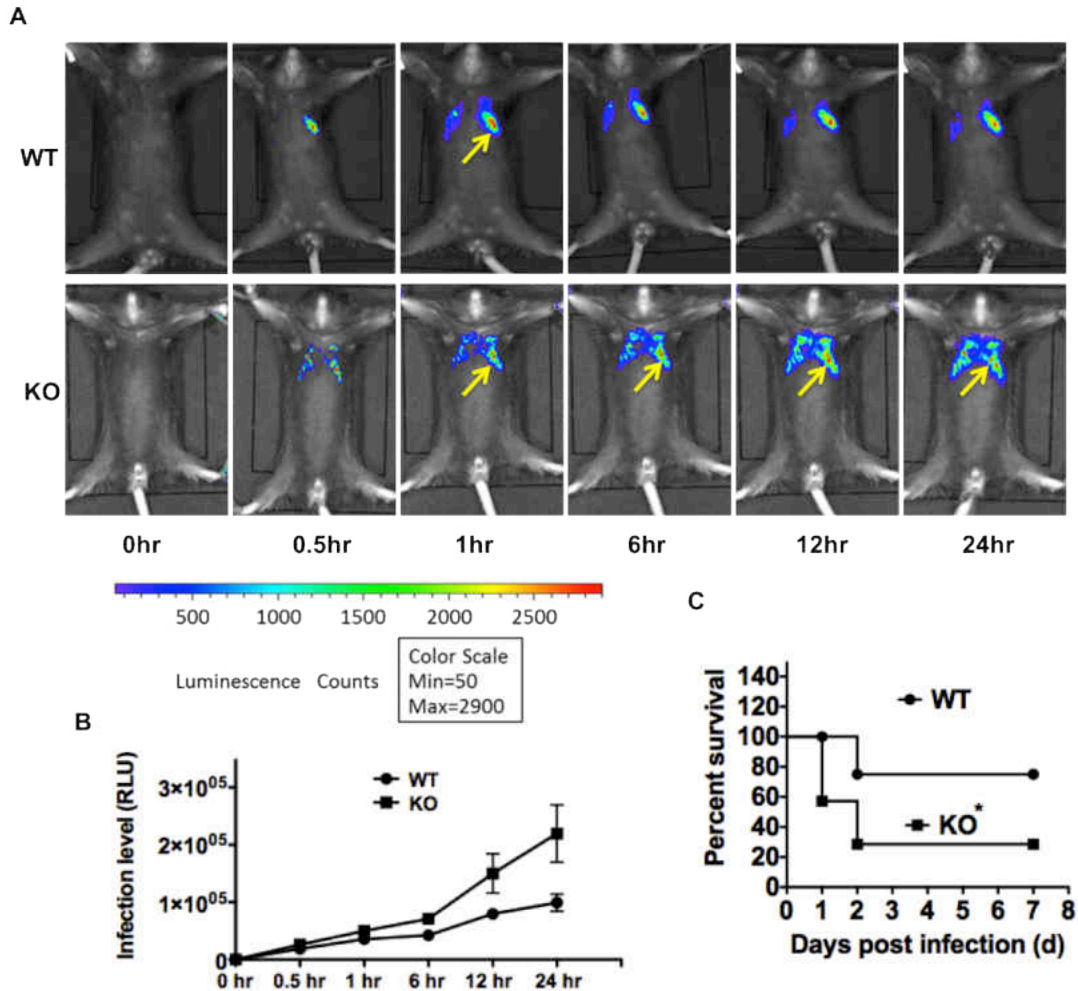


Figure 11. *atg7* KO mice displayed an increased susceptibility to *Kp* infection. (A and B) *in vivo* imaging (whole body) of *Kp* infection was taken in mice. *atg7* KO mice (n=6) and WT mice (n=6) were intranasally challenged with 5×10^5 CFU/mouse. Images showing bioluminescence of different time points using IVIS XRII small animal imaging machine (Caliper) (arrows indicating *Kp* spread region). Images are representative of 6 mice. Data are presented as mean \pm SDEVs. (C) Survival of *atg7* KO and WT mice was represented by Kaplan-Meier survival curves (n=6, $p=0.0195$; 95% confidence interval, log-rank test).

***atg7* KO mice showed increased bacterial burdens and oxidation**

As a direct measure of lung injury, we examined lung histology of mice at 24 h post infection. We found that both *atg7* KO mice and WT mice exhibited signs of pneumonia while exposed to Kp, whereas histological alterations and PMN infiltration were further intensified in the lungs of *atg7* KO mice compared to those of WT mice (see arrows, Figure 12A). These results indicated that the loss of Atg7 in mice exacerbated the lung tissue injury after infection.

We also noted that *atg7* KO mice showed significantly increased colony-forming units (CFU) of Kp in the lung tissue (Figure 12B; $p=0.001$) compared with WT mice, indicating more severe pneumonia occurring in *atg7* KO mice. We also found increased polymorphonuclear neutrophils (PMN) infiltration in both BAL fluid and serum of *atg7* KO mice compared to those of WT mice (Figure 12C and D).

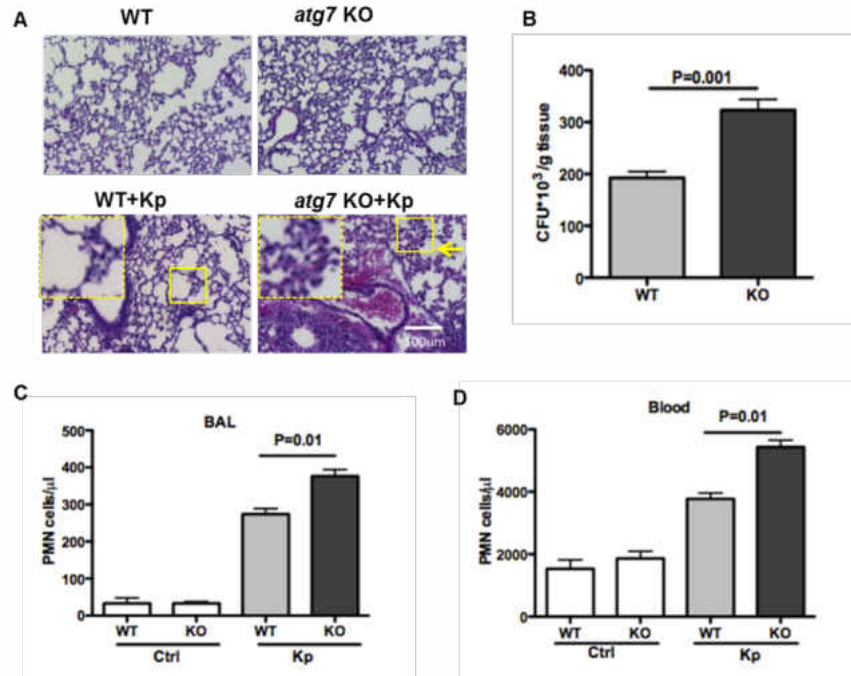


Figure 12. Increased bacterial burdens and PMN penetration in the lungs of *atg7* KO mice following Kp infection. (A) Increased lung injury and inflammation as detected by histology evaluation. *atg7* KO mice and WT mice were infected 5×10^5 CFU/mouse for 24 h. Mice were dissected, and lungs were embedded in formalin. Sections were analyzed by H&E staining. (B) *atg7* KO mouse lungs showed significantly increased bacterial burdens after infection with Kp compared to those of WT mice. After infection, tissues were homogenized in PBS (n=6). The same amounts of tissue were evaluated for bacterial colonies for CFU/g of tissue. (C) and (D) Increased PMN infiltration was observed in the lung and serum of *atg7* KO mice compared to that of WT mice. After HEMA-3 staining, PMN cell percentages were calculated *versus* total nuclear cells. The data were representative of 6 mice/group (Student t-test). RLU, relative luciferase units; RFU, relative fluorescence units.

Kp infection has been previously shown to induce the release of reactive oxygen species (ROS), whose accumulation may compromise lung injury and eventually lead to lung breakdown (Hickman-Davis et al., 2002). We also measured the superoxide production in AM cells of *atg7* KO mice and found it showed increased oxidative stress versus those of WT mice 24 h post infection assessed by an NBT assay (Figure 13A). To verify the data, we used a more sensitive and quantitative Dihydrodichlorofluorescein diacetate (H₂DCF) assay to further confirm the superoxide increase in *atg7* KO AM cells following Kp infection (data not shown). Together, these data suggest that increased ROS may hamper cell survival by increasing apoptosis and may significantly damage the lung and other vital organs. We next evaluated MPO activity in the lung tissue of *atg7* WT and KO mice to reflect neutrophil penetration, since MPO is a widely recognized influx for oxidation in physiological context. As expected, we noted increased MPO levels in the lung of *atg7* KO mice compared to those of WT mice (Figure 13B). Taken together, *atg7* KO mice exhibit much greater lung injury than WT mice, indicating that loss of Atg7 results in inflammatory responses, which may aggravate tissue injury.

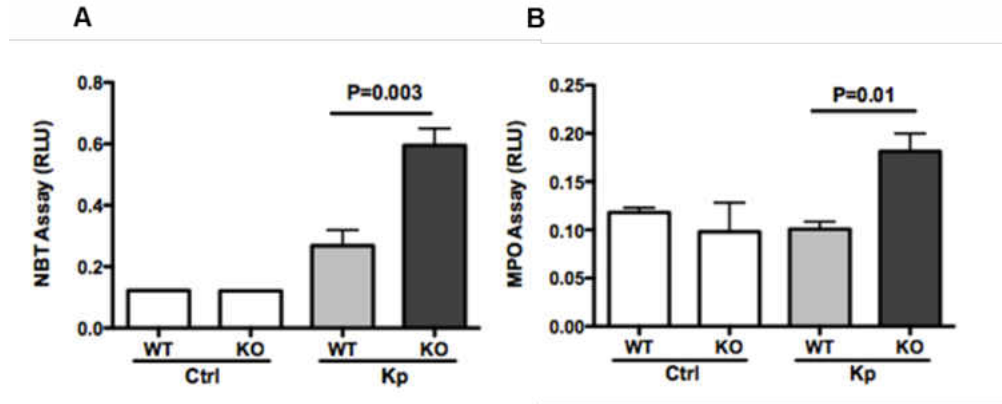


Figure 13. Increased oxidative injury in the lungs of *atg7* KO mice after Kp infection. (A) Superoxide production of AM cells was significantly increased in *atg7* KO mice compared to WT mice using an NBT assay (1 μ g/ml). AM cells were seeded in 96 well plates and infected with Kp at MOI of 10:1 for 1 h. The optical density for NBT was determined at 560 nm. (B) Increased MPO activity in *atg7* KO mouse lungs compared to WT mice (n=3). Absorbance was read at 460 nm.

***atg7* KO mice manifested altered inflammatory responses**

To reveal the inflammatory profile in our model, we determined some critical proinflammatory cytokines, an indicator of inflammatory responses, in BAL fluid and lungs at 24 h post infection. We found that BAL fluid of *atg7* KO mice showed no significant change in inflammatory responses compared to those of WT mice (Figure 14A-C). However, in *atg7* KO mouse lungs, we found that the levels of TNF- α , IL-6, and IL-1 β were significantly elevated compared to those of WT mice as assayed by ELISA ($p < 0.01$, Figure 14A-C). To validate these data, we measured the protein expression levels of these quantified cytokines by Western blot analysis, and found that expressions of TNF- α , IL-6, and IL-1 β protein were also be up-regulated, especially TNF- α and IL-1 β , with more than 10 fold increase (Data not shown). Collectively, these data demonstrated that *atg7* KO mice manifested more intense proinflammatory responses following Kp infection than WT mice.

***atg7* KO mice exhibited activated NF- κ B and p38 MAPK by Kp infection**

To investigate the underlying mechanism of the dysregulated inflammatory responses in *atg7* KO mice, we analyzed the cell signaling proteins in the lung tissue by Western blotting assay (Figure 14D). We showed that Kp infection significantly increased the phosphorylation (ser536) of NF- κ B p65 subunit. We then attempted to identify the upstream regulator of NF- κ B and found that phospho-p38 MAPK was greatly increased in *atg7* KO mice compared to WT mice (Figure 14D) after Kp infection. Hence, these results indicate that the activation of p38/NF- κ B signaling in *atg7* KO mice may be a contributing factor for the intensified inflammatory responses to Kp infection.

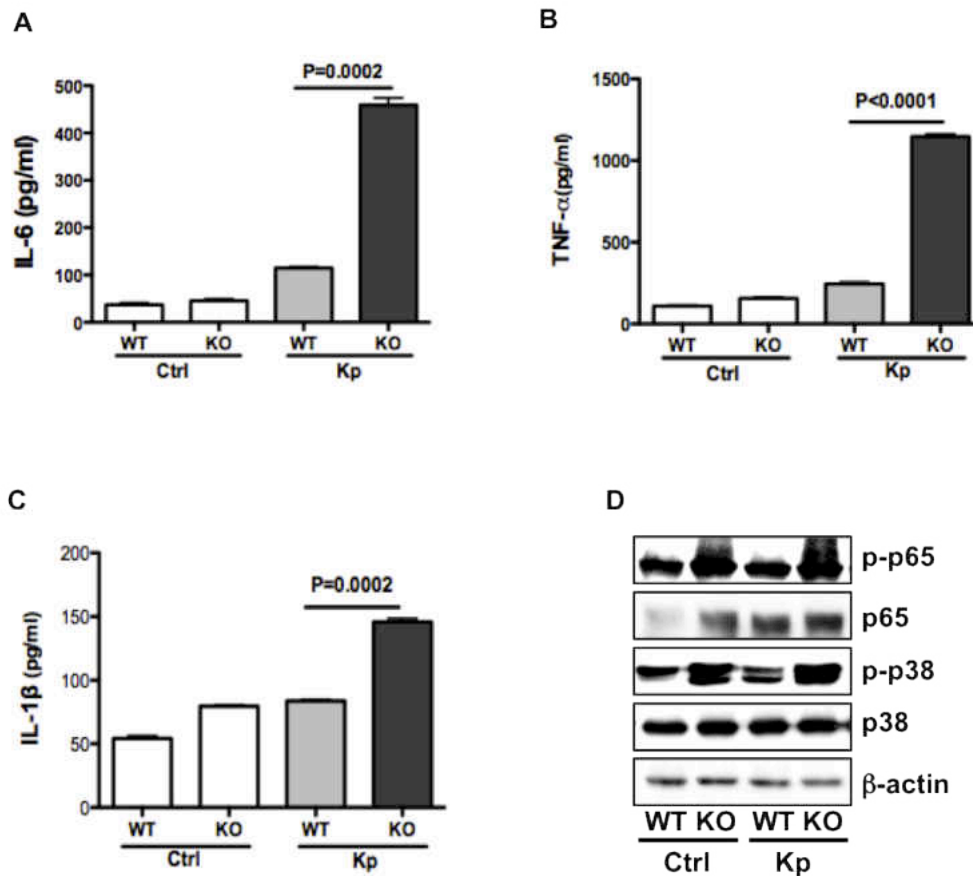


Figure 14. Kp induced intense inflammatory responses in *atg7* KO mice. (A–C) The inflammatory cytokines in BAL fluid of *atg7* KO mice was increased compared to those of WT mice as assessed by ELISA. Mice (n=6) were infected with 5×10^5 CFU/mouse of Kp for 24 h. BAL fluid was collected, and cytokines were measured by ELISA. (D) Increased p-p38 and p-NF- κ B of *atg7* KO mice was observed compared to WT mice as assessed by Western blotting. Frozen lung tissue of *atg7* KO mice and WT mice at 24 h post infection was lysed for protein assays. Data are representative of two experiments.

AM cells from *atg7* KO mice manifested intensified inflammatory responses

We further confirmed the role of Atg7 in bacterial killing and inflammatory responses in primary macrophages. Bacterial burden assay demonstrated that increased bacterial phagocytosis and decreased killing in AM cells of *atg7* KO mice compared to those of WT mice (Figure 15A and B). Bacterial killing in primary AM was similar to MH-S cells, whereas Kp phagocytosis in AM cells was not (Figure 15A). These differences may be due to different characteristics of cell lines vs. primary cells and indeed our recent studies showed that knocking down of another autophagy protein (FIP-200) also reduced MH-S cell phagocytosis (Y. Li et al., 2014). To examine the effect of Atg7 on inflammation, we measured cytokine levels by ELISA assays and found that AM cells of *atg7* KO mice had increased cytokine secretion (IL-6 and IL-1 β) compared to that of WT mice (Figure 15C and D). Collectively, *atg7* deficiency significantly altered macrophage host defense in mouse models, which may contribute to the worsened phenotype.

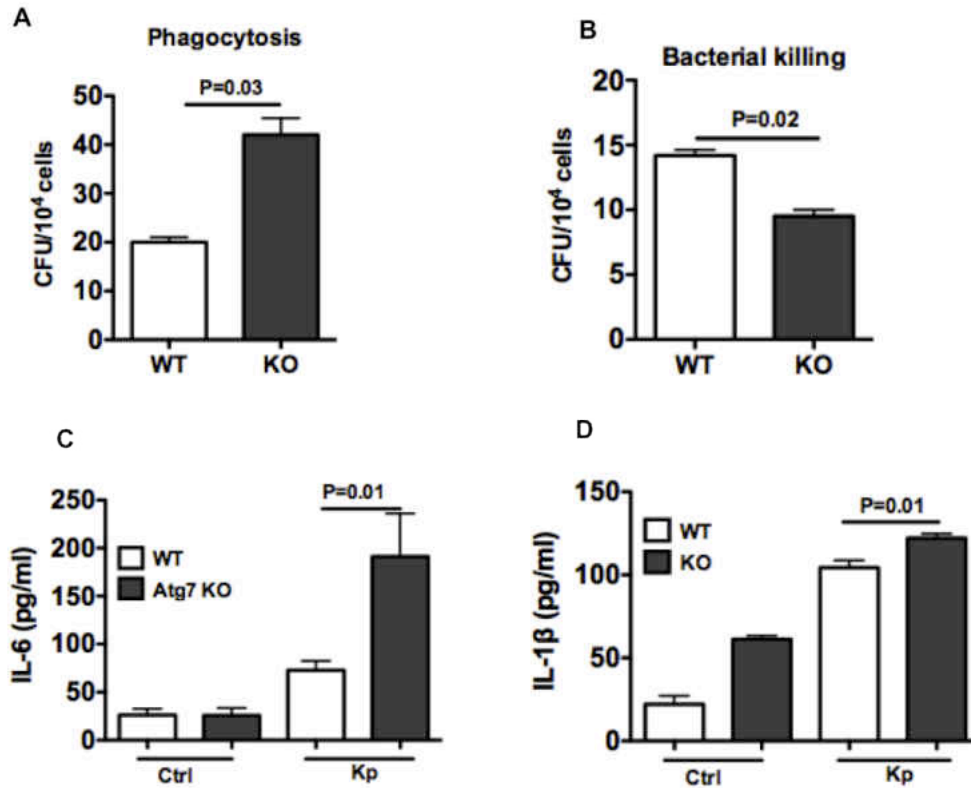


Figure 15. Host defense of AM cells from *atg7* KO mice was impaired.

(A) and (B), AM cells of *atg7* KO mice showed increased bacterial phagocytosis and decreased killing after Kp infection, respectively, using CFU assay following polymyxin B treatment. (C) and (D), Increased inflammatory cytokines in AM cells of *atg7* KO mice compared to those of WT mice after Kp infection as assessed by ELISA. Data represents three biological replicates.

SECTION 2: Atg7 inhibits ubiquitylation of p-I κ B α to regulate the inflammatory responses

Atg7 dissociates from p-I κ B α after Kp infection in MH-S Cells

To explore the regulatory role of Atg7 in the inflammatory responses during infection, we infected alveolar macrophage MH-S cells and murine lung epithelial MLE-12 cells with Kp at different time points (0, 1, 2, 5 h) and found that Atg7 expression and p-I κ B α activity were increased in a time dependent manner (Figure 16). Meanwhile, the expression of I κ B α total protein was not significantly increased except somewhat increase in MLE-12 cells at later time points (2, 5 h), indicating that I κ B α protein expression is not regulated upon infection. Then, we probed the molecular interaction between these two proteins using confocal laser scanning fluorescence microscopy (CLSM) and found that Atg7 was co-localized with p-I κ B α at resting, but this codistribution became less so after 1 hr of Kp infection (Figure 17A). We then identified the interaction between Atg7 and p-I κ B α by immunoprecipitation assay (Figure 17B). Similar to fluorescence microscopic results, the binding between these two proteins was decreased after Kp infection, proportional to lengths of infection time (5 h) (Figure 17B). These data indicate that Kp infection causes disassociation of Atg7 from p-I κ B α in a time dependent manner.

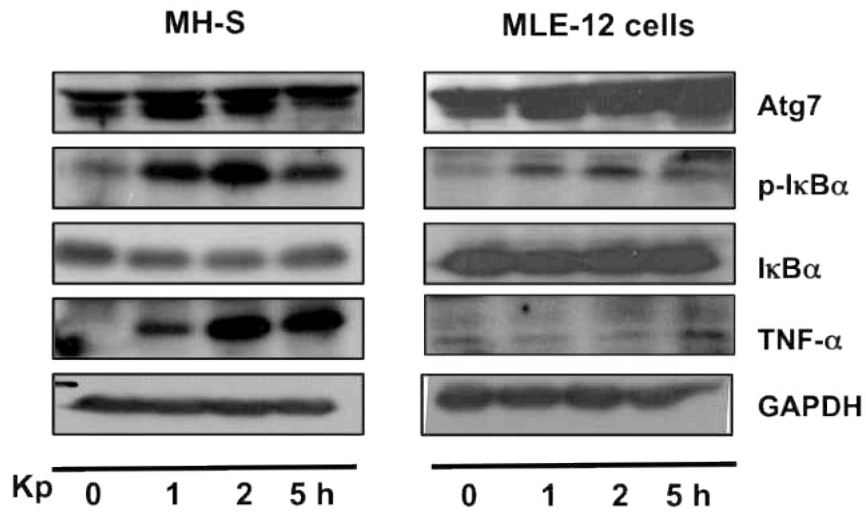


Figure 16. Increased expressions of Atg7, p-IκBα and TNF-α post Kp infection in MH-S and MLE-12 cells. Cells were infected with Kp at different time points (0, 1, 2, 5 h) and were collected to determine the protein expressions of Atg7, p-IκBα and TNF-α by Western blotting analysis. GAPDH was used as a loading control. Data were representative of three independent experiments.

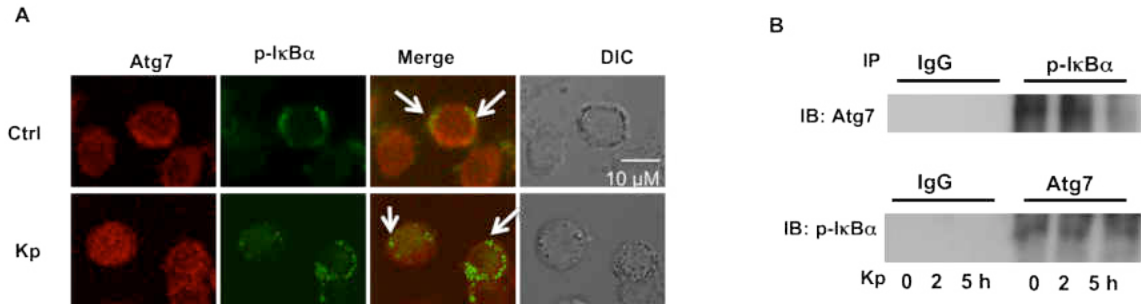


Figure 17. Interaction of Atg7 and p-IκBα was decreased after Kp infection. A, Co-localization of Atg7 and p-IκBα was observed under a fluorescence microscopy. MH-S cells were infected with Kp at MOI of 10:1 for 2 h. **B,** Interaction between p-IκBα and Atg7 as detected using Co-IP assay. IB: immunoblotting. IP: immunoprecipitation. MH-S cells were infected with Kp at MOI of 10:1 for 0, 2, and 5 h.

NF- κ B pathway was altered by silencing Atg7 or I κ B α in MH-S Cells

To unravel the role of Atg7 in NF- κ B signaling, we found that down-regulating Atg7 by siRNA silencing strategy led to an increased level of p-I κ B α after Kp infection (Figure 18A). To elucidate the impact of I κ B α in NF- κ B signaling, we transfected I κ B α siRNA to MH-S cells. After successful knockdown of I κ B α , the phosphorylation of NF- κ B subunit p65 (ser536) was increased upon Kp infection, suggesting that I κ B α serves as the upstream signal in NF- κ B pathway. In addition, we found that knocking down I κ B α increased protein expression of TNF- α assessed by Western blotting analysis (Figure 18B). These data indicated that I κ B α was critical for regulating host inflammatory response to Kp infection via NF- κ B signaling.

Moreover, the protein expressions of TNF- α was higher in Atg7 siRNA-transfected MH-S cells compared to those in scrambled siRNA-transfected controls as detected by Western blotting (Figure 18A). Thus, Atg7 silencing may be attributable to a dysregulated proinflammatory response through the Atg7/I κ B α /NF- κ B axis.

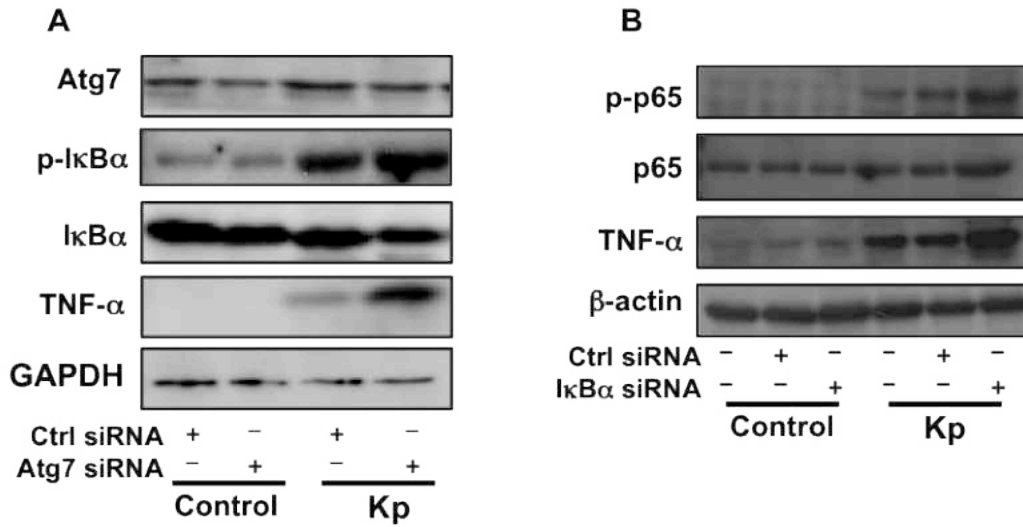


Figure 18. NF- κ B pathway was altered by silencing Atg7 or I κ B α in MH-S Cells. A, p-I κ B α level and TNF- α expression were determined by Western blotting. MH-S cells were transfected with Atg7 siRNA or control (scrambled) siRNA. After 24 h, cells were infected with Kp at MOI of 10:1 for 1 h. **B,** NF- κ B and TNF- α expression was determined by Western blotting. MH-S cells were transfected with I κ B α siRNA or control siRNA. After 24 h, cells were infected with Kp at MOI of 10:1 for 1 h. Data were representative of three independent biological experiments.

NF- κ B is a master transcriptional factor for initiating inflammatory responses. Here, we isolated the nuclear fraction of cells at 1 or 5 h after Kp infection and found that NF- κ B expression is significantly increased in Atg7 siRNA-silenced cells compared to controls after Kp infection (Figure 19A). We then carried out an electrophoretic mobility shift assay (EMSA) to study potential NF- κ B activation, and noticed that an ostensible shift of NF- κ B occurred in Atg7 siRNA cells after Kp challenge (Figure 19B). In addition, we showed that the NF- κ B luciferase reporter activity was significantly increased in Atg7 siRNA-transfected MH-S cells vs. control siRNA-transfected cells (Figure 19C). Further, we used an NF- κ B inhibitor (SN50, 1.8 μ M) to validate the activation and function of NF- κ B and found that SN50 inhibited levels of IL-6 and TNF- α induced by Kp infection with Western blotting (Figure 19D). To further confirm the critical role of Atg7 in NF- κ B activation, we used Atg7 siRNA-transfected MH-S cells to determine nuclear translocation. We found that Atg7 siRNA transfection markedly increased nuclear translocation of NF- κ B vs. control siRNA (Figure 20). To validate this observation, NF- κ B inhibitor SN50 (1.8 μ M) was used to pretreat the cells, which also abolished NF- κ B translocation (Figure 20). Our findings demonstrate that NF- κ B activity is modulated by Atg7.

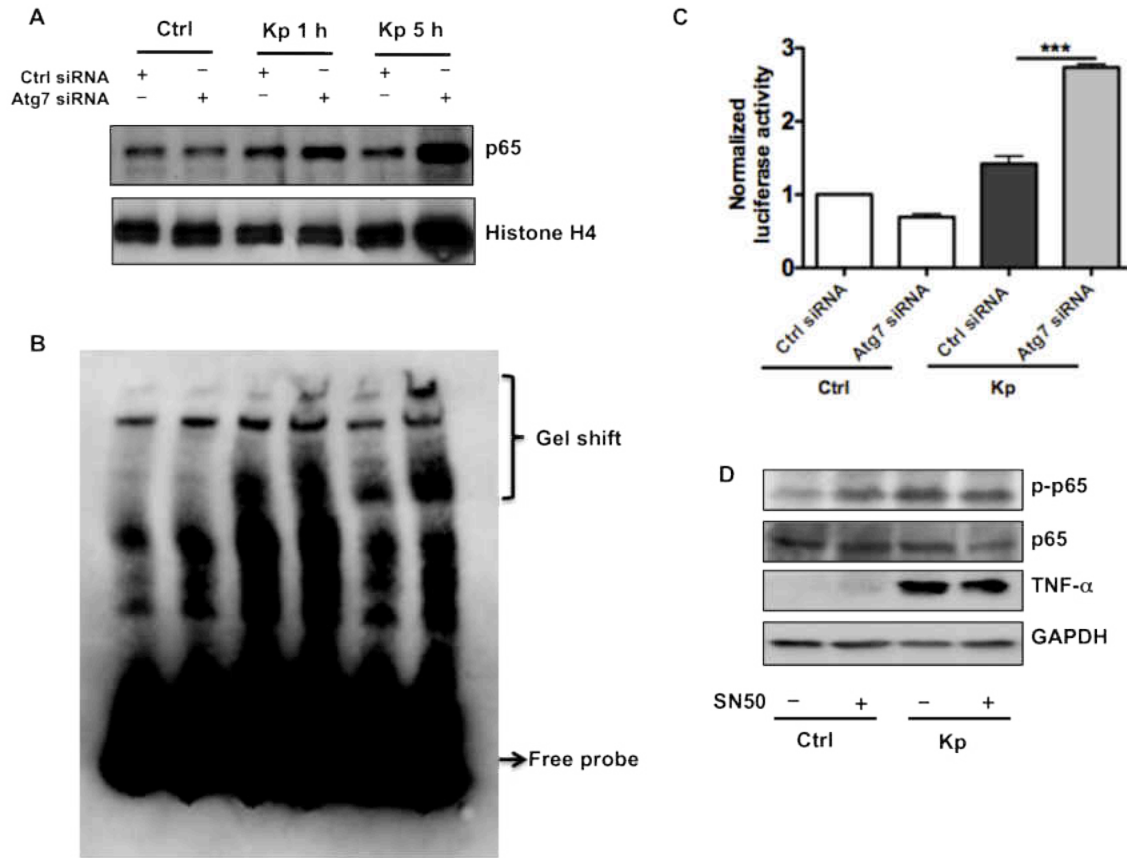


Figure 19. NF- κ B translocated into nuclei in Atg7-silenced cells. **A**, Nuclear NF- κ B levels were increased in the Atg7 silencing cells. MH-S cells were transfected with Atg7 siRNA or control (scrambled) siRNA. After 24 h, the cells were infected with Kp for 1 h and 5 h. Nuclear fractions were isolated from cells by a nuclear extraction kit (Thermofisher). **B**, EMSA was performed in MH-S cell nuclear extracts using the biotin-labeled probe (Thermofisher), which contains only a single copy of the 21-bp element. **C**, Increased luciferase reporter activity of NF- κ B in Atg7 siRNA-transfected MH-S cells. MH-S cells were transfected with Atg7 siRNA or control siRNA. After 24 h, cells were transfected with luciferase reporter NF- κ B plasmid. After another 24 h transfection, cells were infected with Kp at MOI of 10:1 for 1 h. SN50 (1.8 μ M) was used to pretreat cells for 0.5 h before infection. *** p <0.001 (One way ANOVA and Bonferroni's selected Multiple Comparison Test). **D**, TNF- α expression was decreased after inhibiting NF- κ B with SN50 (1.8 μ M). MH-S cells were infected with Kp at MOI of 10:1 for 1 h. Data were representative of three experiments.

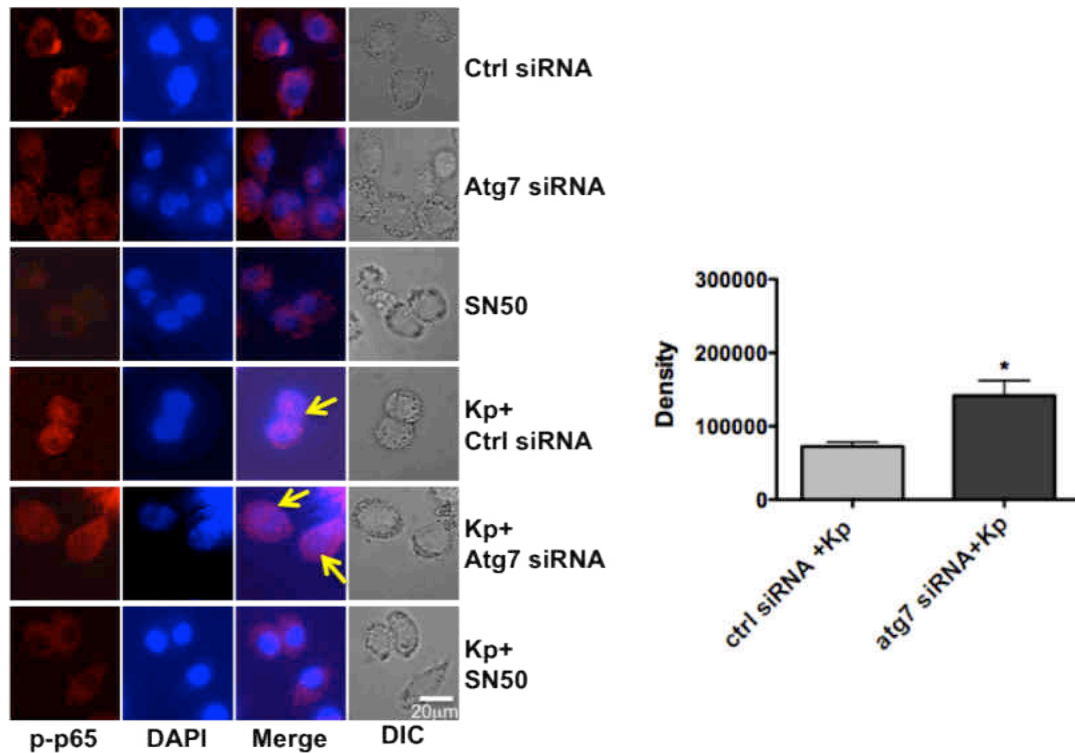


Figure 20. Decreased nuclear translocation of NF- κ B in Atg7 siRNA-transfected MH-S cells. Cells were transfected with Atg7 siRNA or control siRNA. After 24 h, cells were infected with Kp at MOI of 10:1 for 1 h. The localization of NF- κ B was visualized by indirect immunofluorescence staining (arrows show the nuclear translocation). SN50 (1.8 μ M) was used to pretreat the cells for 0.5 h before infection. Data were representative of three experiments. p-p65 was shown in red, and DAPI was shown in blue. * $p < 0.05$ (student t-test).

Ubiquitylation of p-I κ B α

Within the eukaryotic cell there are two main intracellular protein degradation pathways: the ubiquitin-proteasome system (UPS) and autophagy. To clearly establish the role of autophagy in inflammatory response, we set out to dissect whether UPS also contributes to the degradation of p-I κ B α and observed that expression of Ub was not significantly influenced after Kp infection at different time points in either murine lung epithelial MLE-12 cells or macrophage MH-S cells (Figure 21A). However, association between p-I κ B α and Ub became significantly increased after Kp infection as detected using immunoprecipitation assay in MH-S cells (Figure 21B). In addition, co-localization (see arrows) of p-I κ B α and Ub was revealed by fluorescence imaging assay (Figure 21C), suggesting a potential interaction between p-I κ B α and Ub. Together, these data suggest that after Kp infection, the interaction between p-I κ B α and Ub became significantly increased.

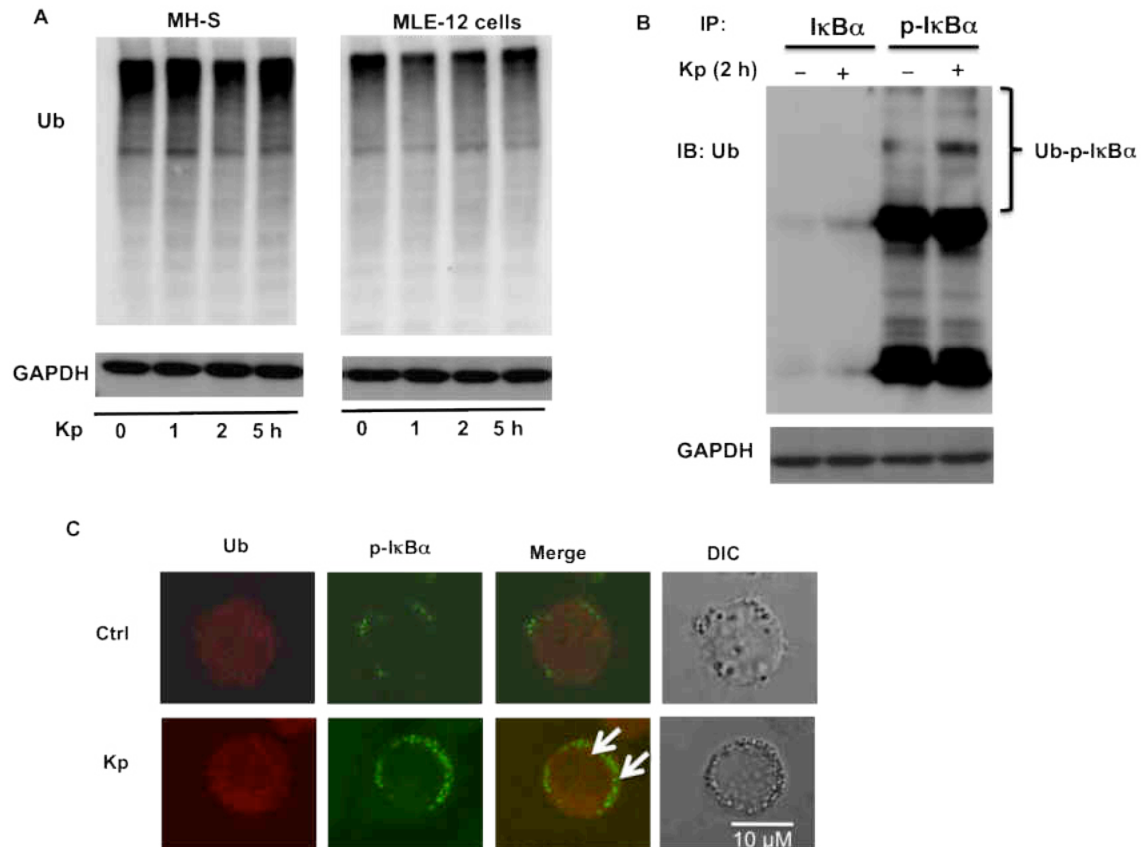


Figure 21. p-IκBα was ubiquitylated after Kp infection. **A**, Expression of Ub protein was not significantly changed after Kp infection at different time points in either MLE-12 or MH-S cells. Cells were infected with Kp at 1, 2 or 5 h. **B**, Interaction between p-IκBα and Ub was significantly increased after Kp infection. MH-S cells were infected with Kp for 2 h and then were collected for Co-IP assay. **C**, Co-localization of Ub and p-IκBα was observed by fluorescence microscopy. MH-S cells were infected with Kp for 2 h. Data were representative of three experiments.

Atg7 silencing increased the ubiquitylation of p-I κ B α

Since our results showed that p-I κ B α could interact with either Atg7 or Ub, we sought to elucidate whether there exists competition between Atg7 and Ub during their interaction with p-I κ B α . To approach this question, we knocked down Atg7 with specific siRNA and observed an increased interaction between p-I κ B α and Ub in the MH-S cells upon Kp infection (Figure 22A). To further confirm the role of Ub in Kp-infected cells, we transfected Ub plasmid (Ub-HA) to overexpress Ub and found an increased expression of TNF- α (Figure 22B), however the change was not significant. Moreover, we also observed a significantly increased ubiquitylation of p-I κ B α following Atg7 silencing in another Gram-negative bacterium *Pseudomonas aeruginosa* PAO1 strain or LPS derived from Kp treated MH-S cells (Figure 23).

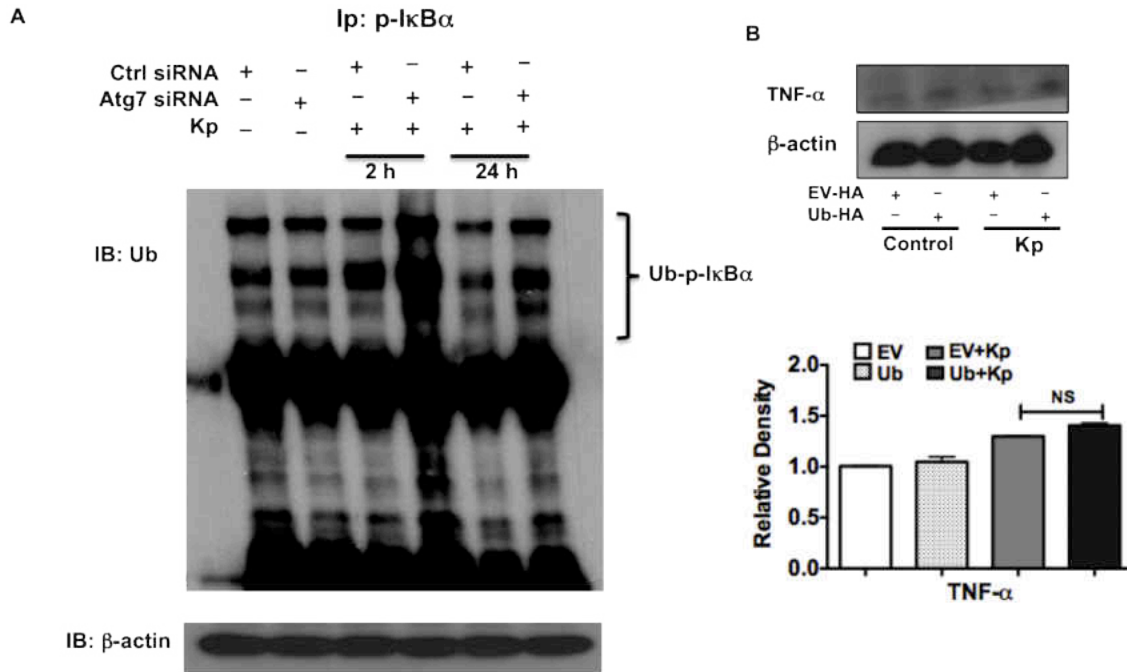


Figure 22. Atg7 silencing increased the ubiquitylation of p-I κ B α . **A**, Knocking down Atg7 with specific siRNA increased the interaction between p-I κ B α and Ub compare to control siRNA silenced cells after Kp infection. MH-S cells were infected with Kp for indicated hours. **B**, Overexpression of Ub increased the expression of TNF- α . The Ub-HA plasmid was kindly provided by Dr. Ron Hay (University of Dundee). MH-S cells were transfected with Ub-HA. After 24 h, cells were infected with Kp for 2 h. Data are representative of three experiments. No significance (ns) (One way ANOVA and Bonferroni's selected Multiple Comparison Test).

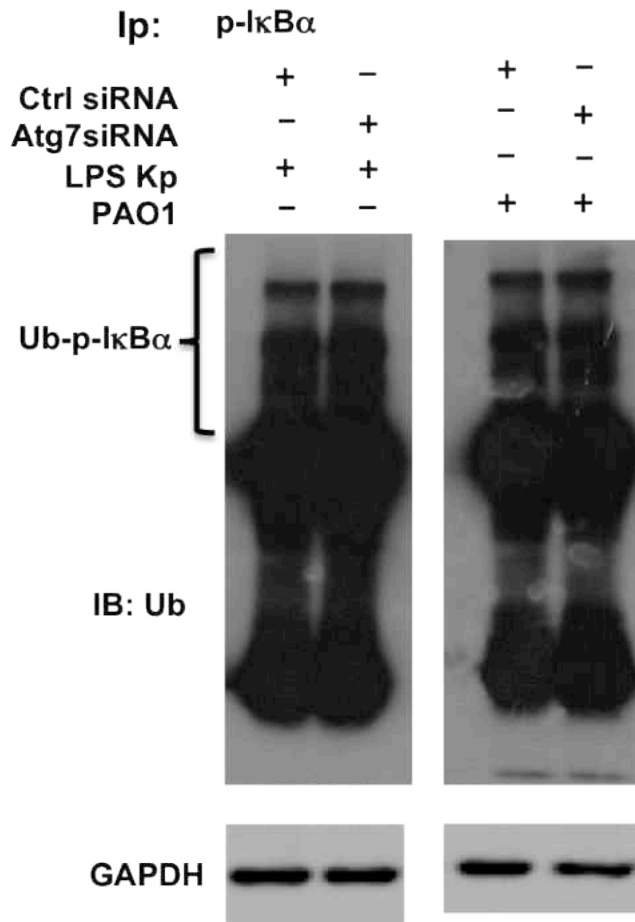


Figure 23. Atg7 silencing increased the ubiquitylation of p-IκBα after Lps or PAO1 infection. Knocking down Atg7 with specific siRNA transfection increased the interaction between p-IκBα and Ub after infection with LPS derived from Kp (left panel) or PAO1 (right panel). MH-S cells were infected with PAO1 for 1 h or LPS (100 ng/ml) for 3 h. Data were representative of three experiments with similar results.

To examine whether *Atg7* plays a specific role in p-I κ B α ubiquitylation, we knocked down two other autophagy related genes, *Atg5* and *Beclin-1*. Importantly, knockdown of *Atg5* and *Beclin-1* by siRNA did not significantly alter the ubiquitylation of p-I κ B α (Figure 24A), suggesting a major role of *Atg7* in regulating the ubiquitylation of p-I κ B α . However, 3-methyladenine (3-MA) pretreatment increased ubiquitylation of p-I κ B α after Kp infection (Figure 24B).

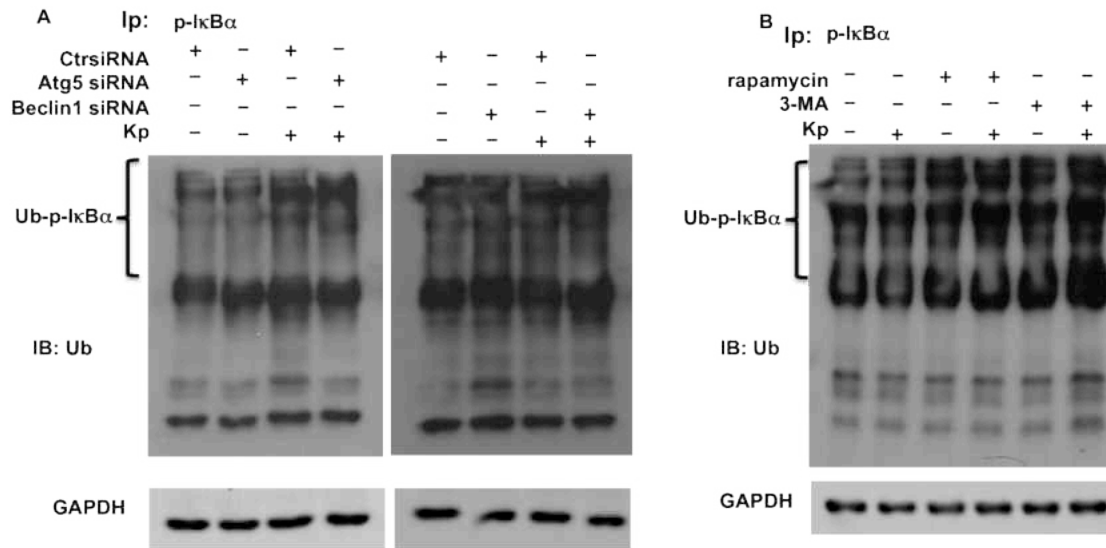


Figure 24. Potential roles of other autophagy related proteins in the ubiquitylation of p-IκBα. **A**, Knocking-down Atg5 or beclin1 with specific siRNA did not significantly alter the interaction between p-IκBα and Ub after infection with Kp. MH-S cells were infected with Kp for 2 h. **B**, Autophagy contributed (albeit not in a dramatic manner) to the ubiquitylation of p-IκBα. MH-S cells were pre-treated with rapamycin (10 nM) for 12 h or 3-methyladenine (3-MA) (5 mM) for 3 h before Kp infection. After 2 h infection, cells were collected for Co-IP assay. Data were representative of three experiments.

Deubiquitylation of p-I κ B α

To further analyze the role of ubiquitylation at the molecular level, we employed siRNA of ubiquitin to confirm the role of Ub in Kp infection in MH-S cells. We found that after knock-down of ubiquitin, the ubiquitylation of p-I κ B α (Figure 25A) was decreased as well as the expression of TNF- α cytokine (Figure 25B).

In addition, we used a deubiquitinase (usp30) to confirm the role of Ub in Kp infection *in vitro* models. We found that usp30 expression was significantly decreased after Kp infection in MH-S cells, but not in MLE-12 cells (Figure 26A). We further transfected MH-S cells with usp30 siRNA and found that TNF- α expression was significantly decreased upon Kp infection (Figure 26B).

We used the proteasome inhibitor (MG132) to confirm the downstream effects of UPS system. MG132 treatment alone could not alter the ubiquitylation status of p-I κ B α (Figure 27A). However, after knocking down Atg7, pre-treatment with MG132 increased the ubiquitylation of p-I κ B α (Figure 27B).

Collectively, these data suggest that knockdown of ubiquitin, the ubiquitylation of p-I κ B α was decreased as well as the expression of TNF- α cytokine.

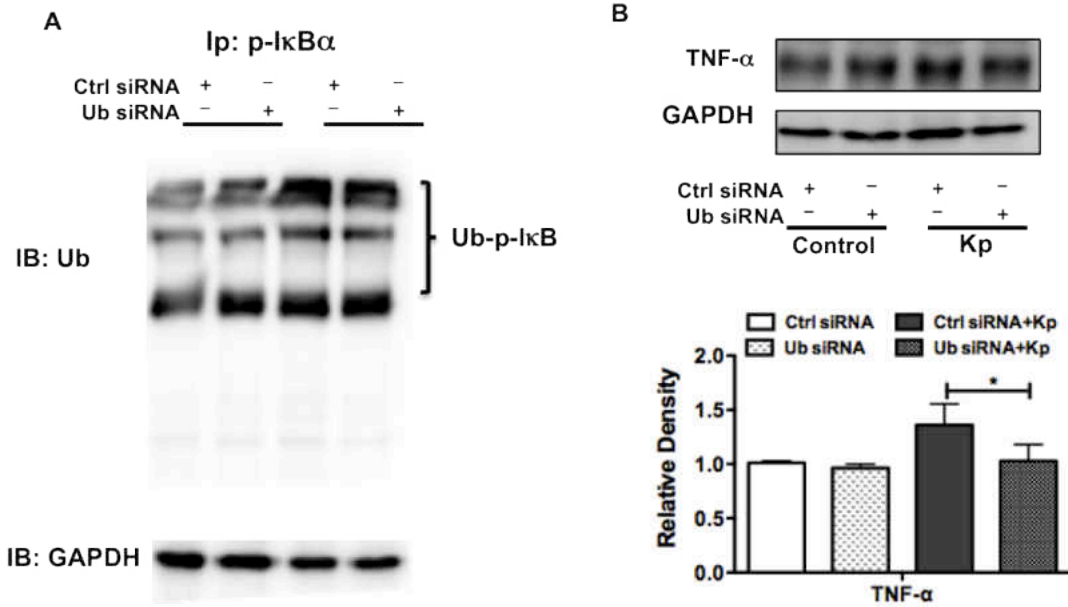


Figure 25. Silencing Ub with siRNA inhibited inflammatory responses. **A**, Interaction between p-IκBα and Ub was significantly decreased after Kp infection after knocking down Ub. Cells were transfected with Ub siRNA. 24 h after transfection, MH-S cells were infected with Kp for 2 h and then were collected for Co-IP assay. **B**, Silencing Ub followed by Kp infection decreased the expressions of TNF-α. MH-S cells were transfected with Ub siRNA. After 24 h, the cells were infected with Kp for 2 h. Data were representative of three experiments. *p<0.05 (One way ANOVA and Bonferroni's selected Multiple Comparison Test).

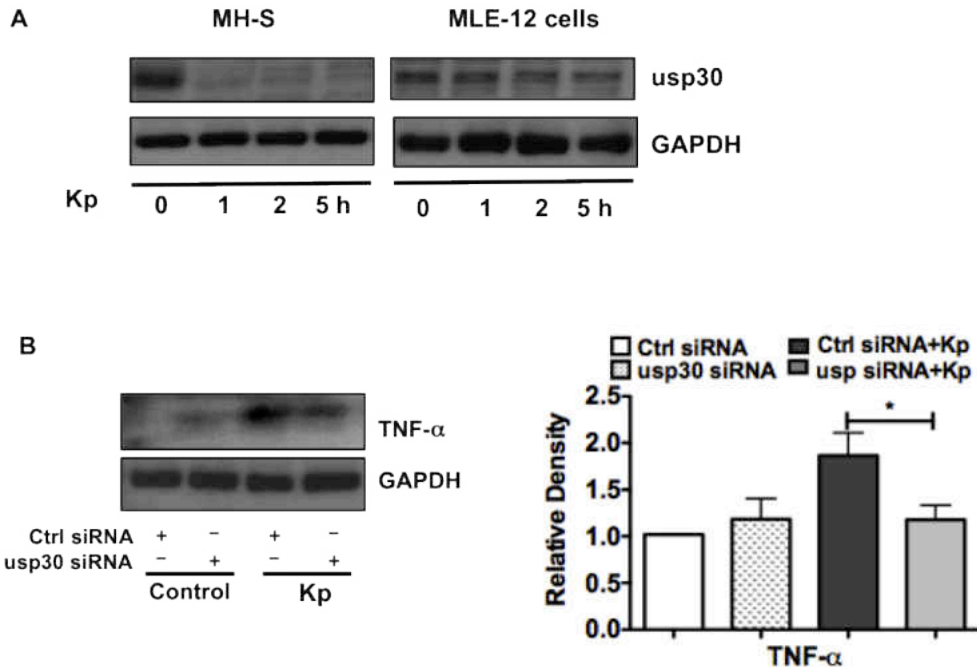


Figure 26. Silencing deubiquitinase (usp30) with siRNA inhibited inflammatory responses. **A**, usp30 was significantly decreased after Kp infection in MH-S cells but not in MLE-12 cells. Cells were infected with Kp at indicated time points. Data were representative of two experiments. **B**, Silencing usp30 followed by Kp infection decreased the expressions of TNF- α . MH-S cells were transfected with usp30 siRNA. After 24 h, the cells were infected with Kp for 1 h. Data were representative of three experiments. * $p < 0.05$ (One way ANOVA and Bonferroni's selected Multiple Comparison Test).

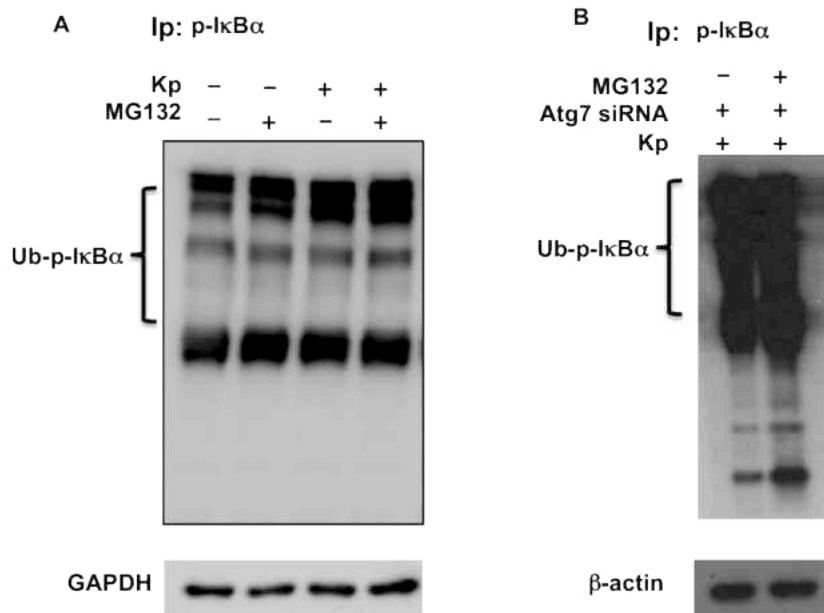


Figure 27. Inhibiting UPS by proteasome inhibitor (MG132) increased the ubiquitylation of p-IκBα in the Atg7 silencing cells. **A**, Pretreatment with MG132 did not influence the ubiquitylation status of p-IκBα. MH-S cells were pretreated with MG132 (40 μM) for 1 h, and followed by Kp infection for 2 h. Cells were collected to detect the ubiquitylation of p-IκBα by IP assay. **B**, MG132 increased ubiquitylation of p-IκBα in the Atg7 silenced cells. MH-S cells were transfected with Atg7 siRNA. After 24 h, cells were treated with or without MG132 (40 μM) for 1 h, and followed by Kp infection for 2 h. Cells were collected to detect the ubiquitylation of p-IκBα by Co-IP assay.

Ub silencing decreased the ubiquitylation of p-I κ B α and inflammatory responses in vitro and in vivo

To confirm the critical role of ubiquitylation of p-I κ B α in the inflammatory responses, we knocked down both Ub and Atg7 with specific siRNA in MH-S cells. We found that the increased ubiquitylation of p-I κ B α following Kp infection was decreased in the dual knock-down Ub and Atg7 group compared to single gene knock down or control groups (Figure 28A). Furthermore, the expression of TNF- α was significantly decreased when knock down both Ub and Atg7 (Figure 28B). To convincingly verify the regulatory role of Atg7 in p-I κ B α *in vivo*, we infected WT or Atg7 KO mice with lentivirus-shUb particles. We used histology assay to evaluate lung injury. After Kp infection for 24 h, we observed decreased inflammatory cell infiltration in the lentivirus-shUb infected Atg7 KO mice compared to control mice (Figure 29A). The secretion of TNF- α in the BAL fluid was significantly decreased in lentivirus-shUb-infected Atg7 KO mice in comparison to Atg7 KO mice (Figure 29B). We also isolated the primary AM cells from different groups of mice, and found that ubiquitylation of p-I κ B α and expression of TNF- α were significantly decreased in lentivirus-shUb-infected Atg7 KO mice while ubiquitylation of p-I κ B α and expression of TNF- α were significantly increased in Atg7 KO mice (Figure 30A). Similar results were also found in the lung tissue (Figure 30B).

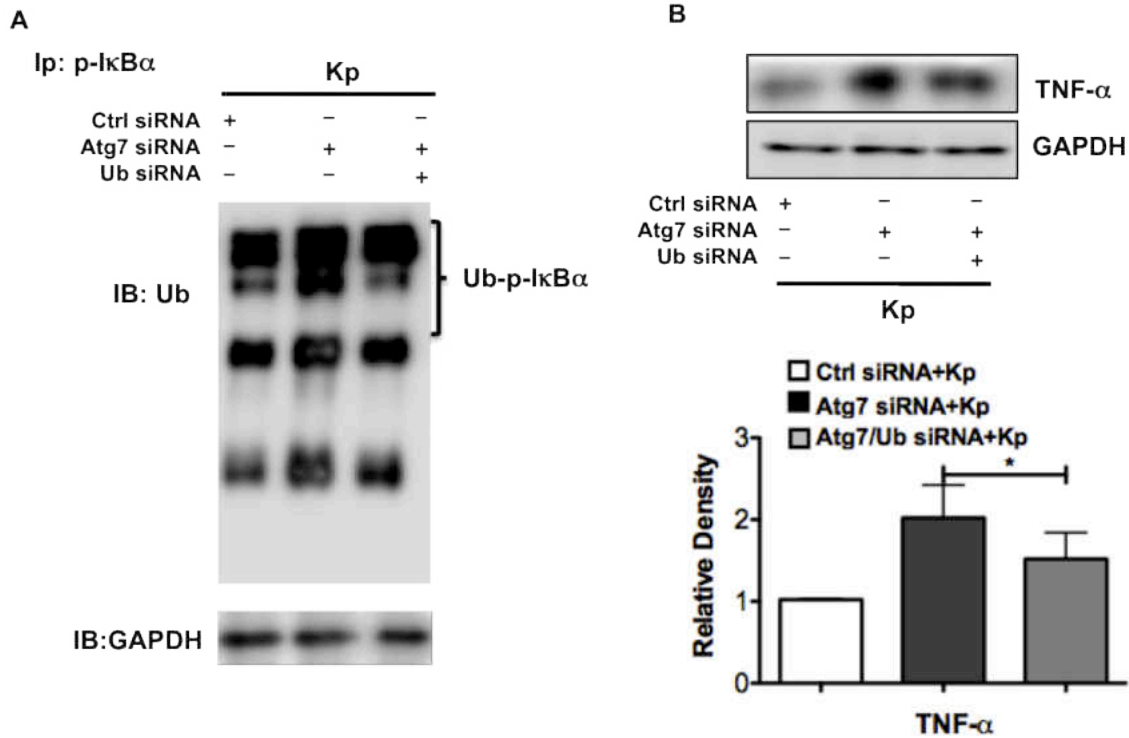


Figure 28. Dual knockdown of Ub and Atg7 decreased the ubiquitylation of p-I κ B α and inflammatory responses in MH-S cells. **A**, Interaction between p-I κ B α and Ub is significantly decreased after Kp infection after knocking down both Ub and Atg7 comparing to Atg7 siRNA silencing cells. Cells were transfected with Atg7 siRNA for 24 h. 24 h after transfection, MH-S cells were transfected with Ub siRNA. After another 24 h post transfection, the cells were infected by Kp for 2 h and then were collected for Co-IP assay. **B**, Dual knocking down Ub and Atg7 followed by Kp infection decreased the expressions of TNF- α comparing to Atg7 siRNA group. After 24 h, the cells were infected with Kp for 2 h. Data were representative of three experiments. * $p < 0.05$ (One way ANOVA and Bonferroni's selected Multiple Comparison Test).

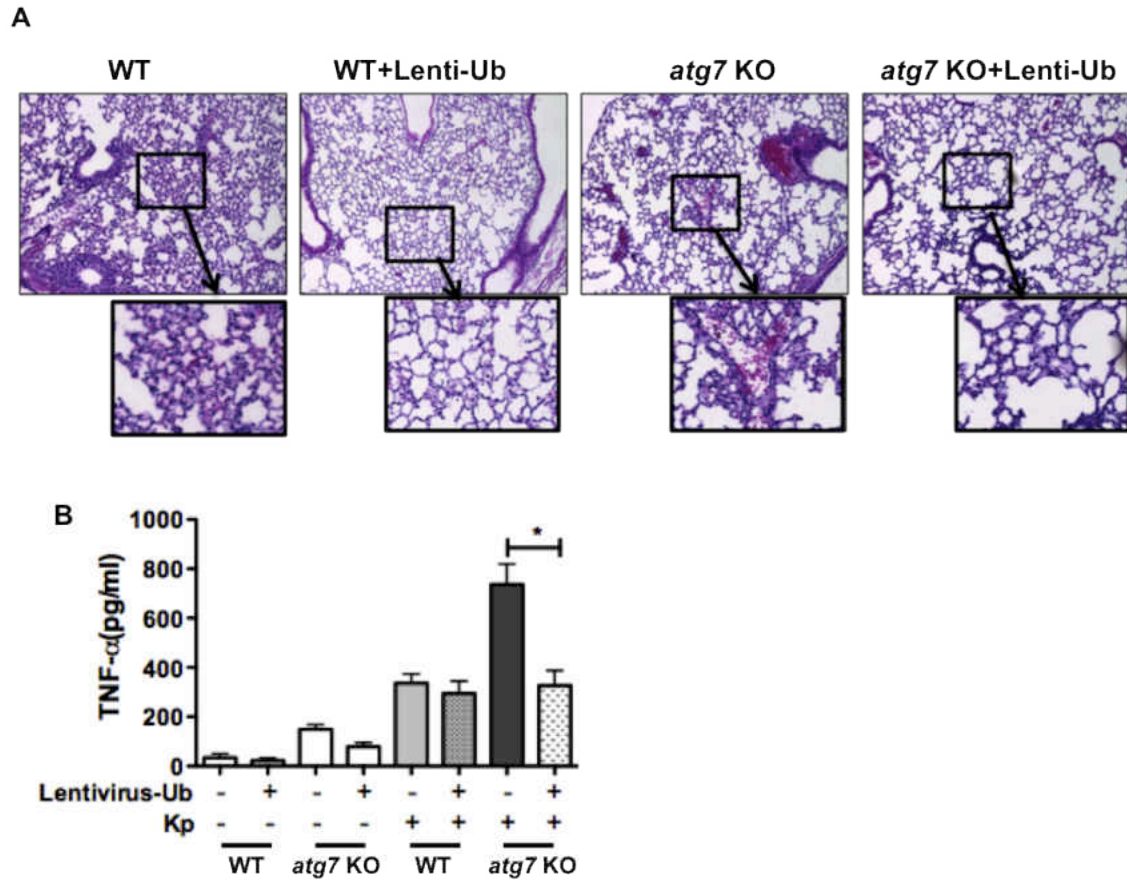


Figure 29. Lentivirus-shUb infected Atg7 KO mice exhibited decreased inflammatory responses after Kp infection. A, Decreased lung injury and inflammation as detected by histology evaluation. Mice were infected with lentivirus-shUb. After 3 days, mice were infected 5×10^5 CFU/mouse for 24 h. Mice were dissected, and lungs were embedded in formalin. Sections were analyzed by H&E staining. The data were representative of 6 mice/group. **B,** TNF- α in the BAL fluid was significantly decreased in lentivirus-shUb-infected Atg7 KO mice as detected by ELISA. * $p < 0.05$ (One way ANOVA and Bonferroni's selected Multiple Comparison Test).

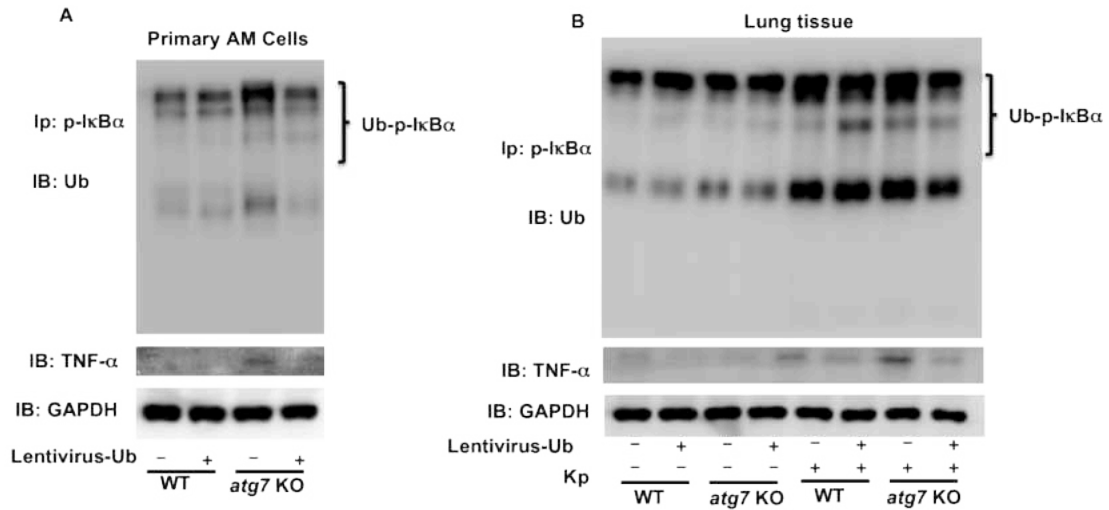


Figure 30. Lentivirus-shUb infected *Atg7* KO mice exhibited decreased ubiquitylation of p-IκBα after *Kp* infection. **A and B,** Ubiquitylation of p-IκBα and the expression of TNF-α were significantly decreased in the lentivirus-shUb particles infected AM cells (**A**) and lung tissue (**B**) of *atg7 KO* mice compared to that of *atg7 KO* mice after *Kp* infection. After infection, AM cells or lung tissue were isolated and lysed in IP lysis buffer. Half of the lysate was used for Co-IP assay and the rest was used to determine the protein expression level of TNF-α and GAPDH. Data were representative of three experiments.

TLR4 plays a role in controlling autophagy pathway after Kp infection

To probe the upstream signals of Atg7, we evaluated several innate immunity participants and identified that TLR4 was involved in the autophagy pathway during Kp infection (Data not shown). After knocking down TLR4 with specific siRNA, we found that Atg7 expression and LC3 conversion from LC3-I to LC3-II were significantly decreased (Figure 31A, left panel). These results are further confirmed by employing TLR4 neutralizing antibodies (Ab) (Figure 31A, right panel). To further evaluate the effect of TLR4 on autophagy, we co-transfected a tandem RFP-GFP-LC3 plasmid and TLR4 siRNA into MH-S cells and found that TLR4 silencing significantly decreased the formation of LC3B puncta (Figure 31B), indicating that blocking TLR4 weakened autophagy induction after Kp infection.

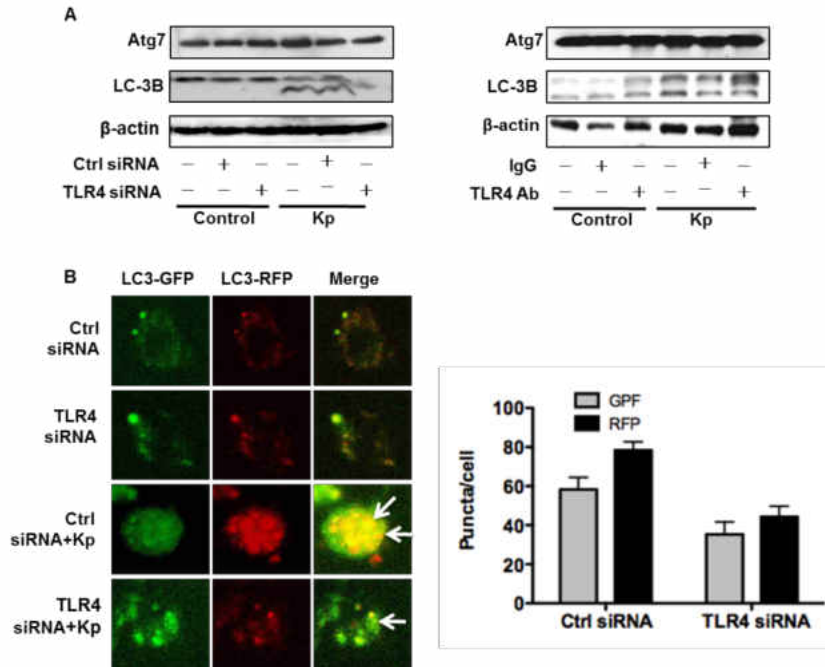


Figure 31. TLR4 silencing in MH-S cells blocked Kp infection-induced autophagy.

A, Decreased expressions of autophagy markers in MH-S cells were observed by TLR4 siRNA silencing or neutralizing Ab blocking. MH-S cells were transfected with siRNA or pretreated with TLR4-Ab (1 μ g/ml) for 3 h, and followed by Kp infection. **B**, Tandem GFP-RFP-LC3 plasmids and TLR4 siRNA were simultaneously transfected into MH-S cells for 24 h. Then the cells were infected with Kp for 1 h (MOI=10:1). Puncta number in each cell (>10 as positive cells) was determined in 100 cells per group.

CFU counting demonstrated that down-regulated levels of TLR4 led to decreased bacterial phagocytosis and bactericidal activity (Figure 32A and B). To further confirm TLR4's impact on Atg7, we used a HEKblue-TLR4 cell line, which stably over-expressed TLR4 (InvivoGen, San Diego, CA). We found that Atg7 activation by Kp infection was partially blocked by TLR4 silencing or neutralizing antibody (Figure 32C). More importantly, we found that *tlr4* KO mice also manifested decreased Atg7 expression 24 h post Kp infection (Figure 32D). To summarize the findings of this study, we presented a simplified signaling pathway using *atg7* KO mice and siRNA silenced cells (Figure 33). In this model, *atg7* deficiency induces the phosphorylation of I κ B, thus release NF- κ B in the cytoplasm after Kp infection. The translocation of NF- κ B to the nucleus continuously induces cytokine production.

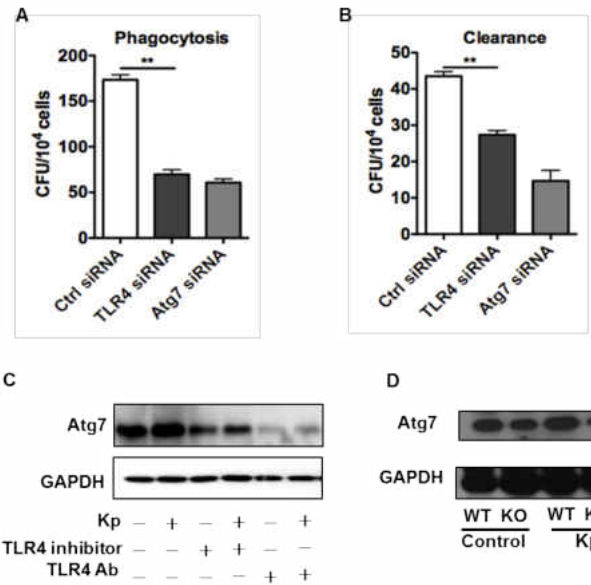


Figure 32. The bacterial clearance and Atg7 expression were decreased after blocking TLR4. (A and B), Knocking down TLR4 with siRNA in MH-S cells decreased bacterial phagocytosis and clearance after Kp infection, respectively. C, Atg7 expression was decreased after blocking TLR4. HEK-TLR4 cells were pretreated with E5564 (1 μ M), or TLR4-Ab (1 μ g/ml) for 3 h, and followed by Kp infection. D, *tlr4* KO mice showed decreased autophagy pathway after Kp infection at 24 h. Data are representative of three experiments with similar results (Student t-test, * $p < 0.05$).

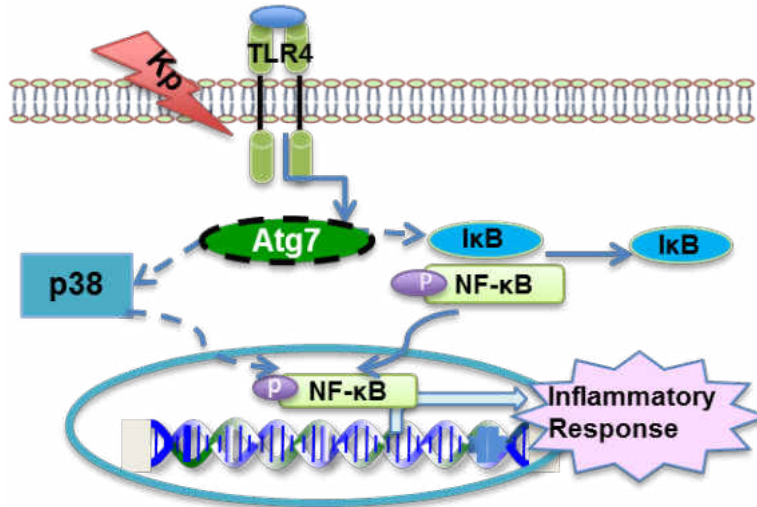


Figure 33. Illustration of the signaling pathways involved in *atg7* knock down cells after *Kp* infection. *atg7* deficiency induces the phosphorylation of NF-κB in the cytoplasm during *Kp* infection. The translocation of NF-κB to the nucleus continuously induces cytokine production.

CHAPTER IV

DISSCUSSION

We demonstrated a typical phenotype of Kp infection in *atg7* KO mice, including decreased survival, increased inflammatory response, and more severe lung injury compared to WT mice. Despite a variety of virulence factors that cause varying tissue abnormalities, we proposed that severely dysregulated cytokine responses and increased superoxide release by host cells are strongly associated with this serious pathophysiology. Previously, similar phenotypes (increased susceptibility, increased production of cytokines, and elevated bacterial burdens) have been observed in *atg7* KO mice infected with other pathogens, such as fungi and viruses (Hwang et al., 2012; Lenz et al., 2011; Pei et al., 2011). The current study suggests that Atg7 may contribute to critical host immune defense against Kp infection.

Atg7 plays a role in host responses to pathogens

Atg7 has been demonstrated to impact host defense against various pathogens (Amer, Byrne, & Swanson, 2005; K. Jia et al., 2009; Tanida et al., 2009) and is widely expressed in alveolar epithelial and lung macrophage cells (Ryter & Choi, 2010). Previous studies have implicated the involvement of Atg7 in host responses to pathogen virulence factors (Amer et al., 2005; K. Jia et al., 2009; Tanida et al., 2009); however, the role of Atg7 in inflammatory responses has not been well elucidated. Our laboratory previously showed

that Atg7 could influence the phagocytosis activity of another Gram-negative bacterium *P. aeruginosa* in macrophage cells (Yuan et al., 2012). In the present study, using animal and cell culture models, we showed for the first time, that Atg7 is indispensable in host defense against Kp infection, and autophagy defects could lead to a worsened outcome in affected animals due to dysregulated inflammatory responses. Thus, our studies indicate that Atg7 may provide broad roles in immunity against Gram-negative bacterial infection.

We found that a differential role of Atg7 in bacterial phagocytosis killing by macrophages, showing that increased bacterial phagocytosis and decreased killing in primary AM cells of *atg7* KO mice compared to those of WT mice. Interestingly, phagocytosis of Kp by primary AM was contradictory to cell line data (Fig. 15A and Fig. 10A). However, these results are in agreement with our recent studies in MH-S cells showing that knocking down of another critical autophagy protein FIP-200 also reduced phagocytosis (Y. Li et al., 2014). Similarly, a previous study showed that knocking down Atg7 in monocytes showed decreased phagocytosis activity after challenging with CSF-1 (Jacquel et al., 2012). siRNA strategies may not completely and stably deplete the protein expression, whereas KO mice may exhibit complex phenotypes resulted from compensatory activities. Nevertheless, combination of these two models generates relatively unbiased data, thus closely modeling physiological relevance of our observations.

Involvement of ROS in lung injury

To probe the underlying mechanism, we determined the change in ROS that may be responsible for bacterial clearance by phagocytes. Previous studies have shown that Atg7 in tetradrine-induced autophagy is ROS dependent during human hepatocellular carcinogenesis (K. Gong et al., 2012). Our results also showed significantly increased ROS levels in AM cells of *atg7* KO mice, which may impair mitochondrial membrane potential as compared to those in WT mice. These data suggested that Atg7 may act as a down-regulator in superoxide production and release, whereas an elevated oxidative stress in *atg7* KO mice may be associated with lung injury and systemic bacterial infection. We also detected the superoxide production in MH-S cells as well as MLE-12 cells after silencing Atg7 with siRNA, and the superoxide production was increased along with an increased bacterial burden under Atg7 knockdown conditions, which is consistent with the animal AM data (data not shown). Together, these observations demonstrate that autophagy machinery in bacterial clearance is markedly impaired in *atg7*-deficient mice in the case of Kp infection. However, Atg7's role in human cells has not been described and warrants future investigations. Since we have relevant experience in human lung epithelial cells, we will further evaluate the relevant pathway in cell lines such as A549 (He et al., 2001) and primary human lung epithelial cells.

Another major contributing factor to lung injury and mortality is a heightened inflammatory response. Significantly, lungs and BAL fluid of *atg7* KO mice exhibited an increase in proinflammatory cytokines. We found that although IL-1 β was also increased, the change of IL-6 was more significant, suggesting that an IL-6 dominant inflammatory response may be related to Atg7 function. Indeed, a previous study also revealed that IL-

6 is involved in the Atg7 pathway (Pastore et al., 2013). Previously, similar results (increased pro-inflammatory cytokines) have also been observed in the intestine in *atg7* KO mice (Inoue et al., 2012). It has been increasingly recognized that lung epithelial cells can contribute to the production of cytokines (Amano et al., 2004; Thorley, Goldstraw, Young, & Tetley, 2005), which may be a topic of investigation in the future. Furthermore, phagocytes (e.g., macrophages and neutrophils) are traditionally regarded as the main players of inflammatory responses (Ozaki et al., 1989). Thus intensified superoxide or cytokines in both epithelia and phagocytes together led to exceedingly heightened inflammatory responses in *atg7* KO mice, which warrants further investigation.

Activation of NF- κ B pathway increased inflammatory responses

To illustrate the mechanism for the dysregulated inflammatory responses in *atg7* KO mice, we assessed potential cell-signaling pathways in the lung tissue following infection. Interestingly, we observed marked activation of NF- κ B that has been widely recognized as a major transcription factor for cytokine production in alveolar epithelial cells. We further explored the mechanism by which Atg7 regulates NF- κ B signaling, thus impacting on cytokine secretion during Gram-negative bacterial infection. We revealed a new mechanism wherein Atg7 regulates inflammation via inhibition of ubiquitylation degradation systems with p-I κ B α in both cells and mice after Kp infection. NF- κ B activation may be triggered to augment inflammatory response after the normally suppressed p-I κ B α to be disassociated from an Atg7-p-I κ B α complex. Finally this

signaling is governed by TLR4 whose critical regulatory role is validated by using cell lines stably overexpressing TLR4 and knockout mice.

Ubiquitylation and autophagy regulate degradation of I κ B α

Ubiquitylation and autophagy are two most important protein degradation systems in the eukaryotic cells involved in a variety of cellular processes. Both of them play essential roles in cellular protein homeostasis and control many cell functions, including cell growth, proliferation, apoptosis, and immune response (Knecht et al., 2009). They are usually considered independent of one another because of their differences in constituents and degradation mechanisms. However, there is also evidence that immune response may be associated with both degradation systems to impact different disease processes in various models (Errafiy et al., 2013; Korolchuk, Menzies, & Rubinsztein, 2010). Inhibition of proteasomal activities that are used to induce cell death has been previously shown to activate autophagy, indicating a coordinated and complementary relationship between these two protein-degradation systems (Wojcik, 2013; Zhang et al., 2014). On the other hand, in U87MG glioma cells, increasing autophagy may decrease the activity of the UPS (Errafiy et al., 2013). In our Kp infection model, we found that Atg7 deficiency disrupted the balance between UPS and autophagy system, skewed the reaction towards the UPS system. We observed that p-I κ B α ubiquitylation was increased in Atg7 silenced cells, suggesting that p-I κ B α degradation was augmented. The degradation of p-I κ B α allowed the release of NF- κ B to initiate transcriptional activity of proinflammatory cytokines (e.g., TNF- α).

The degradation of I κ B α kinase is a key regulatory mechanism in NF- κ B activation and may be modulated either by an autophagy pathway (Sha, 1998) or by a ubiquitylation pathway (Alkalay et al., 1995). Autophagy's inhibitory impact on inflammatory responses by influencing I κ B α /NF- κ B signaling has also been documented in other disease models. For instance, the liver of beclin1 mutant mice exhibits increased apoptosis and NF- κ B activation due to the accumulation of p62 (Mathew et al., 2009). Defective Atg7 contributes to the pathogenesis of obesity via the activation of IKK pathway (Meng & Cai, 2011). Ub is necessary for the phosphorylation and degradation of I κ B α both *in vitro* and *in vivo* (Z. J. Chen, Parent, & Maniatis, 1996). Here, we reveal a role of Ub in Atg7-modulated inflammation during bacterial infection, which explains the critical regulation of inflammatory responses by Atg7. At present, we cannot exclude another possibility i.e., regulation of IKK-I κ B α complex. I κ B α could be phosphorylated via activation of IKK and recognized by β -TrCP following p-I κ B α ubiquitylation (Kanarek, London, Schueler-Furman, & Ben-Neriah, 2010). However, our data still suggest a critical role for Ub in Atg7-modulated inflammation with bacterial infection.

Other possible signaling molecules

p38 MAPK is one of the major signaling molecules, which plays a pivotal role in most types of cytokine production in various cells and different conditions (Adcock, Chung, Caramori, & Ito, 2006; Yoshizumi et al., 2010). In the Kp model, we also found that both p38 and NF- κ B were significantly activated in *atg7* KO mice following Kp infection. Atg7 has been shown to interact with NF- κ B directly through molecular interactions and

Atg7 deficiency may impact NF- κ B activity (Fujishima et al., 2011). To date, no studies have linked Atg7 with NF- κ B pathway in response to any respiratory pathogen. To this end, we used Atg7 siRNA to determine nuclear translocation of NF- κ B and found that Atg7 knockdown markedly increased nuclear translocation of NF- κ B vs. controls (Figure 20). Furthermore, NF- κ B inhibitor SN50 (1.8 μ M) also reduced NF- κ B translocation (Figure 20). Our findings indicate that NF- κ B activity may be modulated by Atg7, which may be relevant to the altered inflammatory response. The detailed mechanism is unclear and is worth further investigation.

In our present study, we specifically focused on autophagy protein Atg7 and particularly addressed its potential role in Kp infection instead of discussing the effect of general loss of autophagy in Kp infection. However, this doesn't exclude the contribution of other autophagy related genes to the Kp infection. It has been reported that mouse cells lacking Atg5 or Atg7 can still form autophagosomes/autolysosomes and perform autophagy-mediated protein degradation after challenge with certain stressors (Nishida et al., 2009). We also found other autophagy related genes are involved in Kp infection (manuscript in preparation).

There is initial evidence reporting the linkage between TLRs and autophagy (Xu et al., 2007), and suggesting that TLRs might induce autophagosome formation in macrophages. TLR4, as one of TLRs family, is also reported to be associated with autophagy in bacterial infectious disease model. TLR4 has been shown to be coimmunoprecipitated with Beclin-1, TRIF, IRAK4, and MyD88 (Shi & Kehrl, 2008). In addition, previous reports have shown that TLR4 signaling can induce autophagy in

leukocytes that can positively influence microbial clearance and NF- κ B signaling (Neal et al., 2013; Xu et al., 2007), and these studies led us to evaluate the role of TLR4 in autophagy after Kp infection. In probing upstream immune molecules of Atg7, we identified that TLR4, the receptor of Gram-negative bacterial LPS, is critically involved in Atg7 signaling. We also found that knocking down TLR4 led to an increased superoxide production after Kp infection (data not shown), which might be a link between TLR4 and the autophagy pathway. Our findings indicate that TLR4 is a potential sensor of autophagy in Kp infection.

Conclusion and overall significance

We present the first disease phenotype of Kp infection in *atg7* KO mice, and our data identify an important role for Atg7 in innate immunity against Gram-negative bacterium Kp in KO mice. *atg7* deficiency impaired the phagocytic ability in alveolar macrophages and other immune functions, resulting in sustained infiltration of PMN cells into the lung and an intense inflammatory response. Importantly, we identify a critical mechanism for Atg7-associated innate immunity against the Gram-negative Kp bacterium. *atg7* deficiency intensified a proinflammatory response via a ubiquitylation mechanism. Collectively, these observations provide new insight into the role of Atg7 in innate immunity against Kp, unraveling ubiquitylation of p-I κ B α as a critical molecular process by which Atg7 negatively regulates inflammatory response to Kp. Since Kp is an extremely important human pathogen, imposing significant healthcare burdens and mortality, the mechanism indicated in the current work may have unprecedented bearing in our understanding of Kp invasion behaviors and host defense patterns. Further, Kp is

increasingly becoming resistant to conventional antibiotics, our options to combat the multi-drug resistant super-bug is exceedingly limited as stated by the Center of Disease Control and Prevention. In addition to a series of discoveries in molecular mechanisms,, my studies may also implicate novel targets for potential therapeutic interventions in Kp and potentially other Gram-negative bacteria.

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