THE ROLE OF NONCANONICAL AUTOPHAGY IN PERIODONTAL DISEASE PATHOGENESIS

Sing-Wai Wong

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in Curriculum of Periodontology

Chapel Hill
2021

Approved by:

Jennifer Martinez

Patricia Miguez

Kimon Divaris

ABSTRACT

Sing-Wai Wong: The role of non-canonical autophagy in periodontal disease pathogenesis
(Under the direction of Jennifer Martinez and Patricia Miguez)

Periodontal disease is a multifactorial disease that is associated with local infection and chronic inflammation in the tooth supporting tissues. In addition to the local etiologic factor, oral biofilm, host susceptibility also contributes to the disease pathogenesis and severity. Susceptible individuals may present with exaggerated inflammatory reactions in the periodontium, leading to alveolar bone resorption and eventually to loss of teeth. My dissertation research focuses on investigating the molecular mechanisms that underlie host susceptibility to periodontal disease.

Originated from the Greek for "*self-eating*", autophagy is a lysosome-mediated catabolic system that facilities the digestion of intracellular components to generate nutrients, which substantiate cell survival in the adverse environment. Emerging evidence has indicated that autophagy proteins can function beyond bulk degradation called non-canonical autophagy, which could regulate inflammation both *in vivo* and *in vitro*. Recent genome-wide association studies (GWAS) have identified genetic variations in multiple non-canonical autophagy genes that may be linked to periodontal disease. However, the role of non-canonical autophagy in periodontal disease pathogenesis remains unknown.

In Chapter 1, we introduce the background of non-canonical autophagy (LC3-associated phagocytosis, LAP) and periodontal disease. In Chapter 2, we investigate the role of Rubicon, a key LAP protein in the pathogenesis of periodontal disease. Our results demonstrate that LAP could be activated by different periodontal pathogens *in vitro*. The absence of Rubicon results in

accelerated periodontal disease progression and bone loss *in vivo*, suggesting that LAP may have a protective role in periodontal inflammation. Intrinsically, young Rubicon deficient mice exhibited normal skeletal phenotype and this deficiency does not interfere with osteoclast differentiation. In the last chapter, we discuss the significance of our findings and future research directions.

Taken together, my research results add knowledge to our current understanding of the etiologies of periodontal diseases –non-canonical autophagy may be involved in the pathogenesis of periodontal disease, and the alterations of its function might contribute to periodontal disease susceptibility and severity.

ACKNOWLEDGEMENTS

I want to give my deepest gratitude to my master's thesis co-supervisors, Jennifer Martinez, PhD and Patricia Miguez, DDS, MS, PhD. Drs. Martinez and Miguez were my PhD thesis committee members, who guided me through my PhD study. During residency, they have continued to provide me great research mentorship and support for this exciting and novel periodontal research project, which is critical for the completion of periodontology residency training.

The pandemic has created unprecedented challenges in life and many difficulties in research and studying. Thank you, Dr. Miguez, for overcoming the difficulties to provide me a lab space for performing cell experiments of this project during the school's lockdown. Thank you, Dr. Martinez, for helping me run a large and complex animal experiment such as the ligature-induced periodontitis model and your mental support during that rough time. Besides my research advisors, I would also like to thank Dr. Divaris for joining my thesis committee and mentor team. You'd offered tremendous help for this study and I wish I will be able to continue to work with you during my early research career development.

I am extremely fortunate to be enrolled in the UNC Periodontology Residency Program, by which I can build a clinical portfolio that matches my research background in bone biology and innate immunity, and can become a clinician-scientist. Thank you to the program directors, Drs. Antonio Moretti and Ryan Cook, for giving me flexible research time to support my research as well as the clinical instructions during my residency training. Thank you, Dr. Cristiano Susin for

your great clinical mentorship and unconditional help on my career development, and for your help on my first clinical case report manuscript. Last by not least, I want to thank Dr. Steven Offenbacher, who introduced me to the world of periodontology and gave me a chance to join the US's most competitive postgraduate periodontology and dental implant surgery program. I am very fortunate to stand on the shoulders of the giant to further explore this unknown but important research topic of periodontal disease pathogenesis based on your profound and everlasting discoveries.

Again, I am very appreciative of my former master's thesis mentor in China, Dr. Hailan Feng, who opened me a door to the life of an educator, clinician, and scientist.

Finally, yet importantly, I would like to thank my co-residents and friends during residency training – I enjoyed every moment with you and cherish our friendship.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATG	Autophagy
A.a	*Aggregatibacter actinomycetemcomitans*
BOP	Bleeding on probing
BSA	Bovine serum albumin
CAL	Clinical attachment loss
DMEM	Dulbecco's modified eagle medium
ECM	Extra cellular matrix
FBS	Fetus bovine serum
F.n	*Fusobacterium nucleatum*
FIP200	FAK-interacting protein of 200 kDA
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GWAS	genome-wide association studies
IL	Interleukin
IL6	Interleukin 6
LAP	LC3-associated phagocytosis

LPS	Lipopolysaccharide
MMPs	Matrix metalloproteases
OPTN	Optineurin
P.g	*Porphyromonas gingivalis*
PBS	Phosphate buffered saline
PE	Phosphatidylethanolamine
PI(3)P	phosphatidylinositol 3-phosphate
PI3K	phosphoinositide 3-kinase
SNP	single nucleotide polymorphism
TNF-α	Tumor necrosis factor alpha
ULK1/2	Unc-51-like autophagy activating kinase
UVRAG	UV radiation resistance-associated gene
VPS34	Vacuolar protein sorting 34

CHAPTER 1: INTRODUCTION [1]

LC3 associated phagocytosis

During environmental challenges, the immune system of the body must mount an appropriate response that can eliminate the stimulus, but finally resolve inflammation and return to homeostasis. Dysregulation of homeostasis of the immune system can lead to destructive inflammatory effects at the local tissue and systemic levels, resulting in sustained chronic inflammation (Kotas and Medzhitov 2015). As the prevalence of chronic inflammatory diseases is increasing worldwide (Kotas and Medzhitov 2015), understanding the molecular mechanisms that govern the induction and resolution of inflammation is required for developing more efficacious treatment. Macrophages and dendritic cells are a group of phagocytes serving as frontline sentinels in the local environment, as they could recognize and eliminate pathogenic components. The ability of these phagocytes to degrade dangerous cargos in an immunologically appropriate way is the key for both elimination of the threat and the final return to its basal state. Largely controlled by the ATG family members, autophagy is an evolutionarily-conserved, catabolic, cell survival mechanism activated during nutrient deprivation status that involves the degradation and recycling

[1] This chapter is partially adapted from the author's book chapter and publication.
The original citations are as follows:
Giulia Petroni and Lorenzo Galluzzi. "Non-Canonical Autophagy: Mechanisms and Pathophysiological Implications", United States, Elsevier, In press (Wong SW, Upadhyay S, and Martinez J. Chapter 4. LC3-associated phagocytosis (LAP)
Wong SW, Sil P, Martinez J. "Rubicon: LC3-associated phagocytosis and beyond." *FEBS J* 285(8) (April 2018): 1379-1388.

of cytosolic components (Levine 2005). While canonical autophagy is considered a non-specific bulk degradation system for cell survival during starvation, we now appreciate that the function of autophagy protein extends far beyond nutrient deprivation, notably including host defense against internalized pathogens (i.e., xenophagy) and cellular quality control (e.g., mitophagy) (Sil, Muse, and Martinez 2018).

LC3-associated phagocytosis (LAP) is a type of newly discovered non-canonical autophagy pathway. It can be triggered by the engagement of different extracellular receptors (Toll-like receptors for pathogens (Sanjuan, Milasta, and Green 2009), Fc receptors immune complexes (Henault et al. 2012), or the phosphatidylserine receptor for dying cells (Martinez et al. 2011)) from a hazardous particle, leading to the engulfment of the cargo and partially recruitment of autophagy proteins to the single membraned, cargo-containing vesicle, termed the LAPosome (Martinez et al. 2015; S. W. Wong, Sil, and Martinez 2018). Though the autophagy conjugation system, cytosolic LC3-I is processed and converted to lipidated LC3-II, which is bounded to the outer surface of the LAPosome membrane, in which cargo degradation and immunomodulatory response is initiated (Martinez 2018). While LAP shares many molecular commonalities with the canonical autophagy pathway, it has unique functions and exhibits molecular distinction. For example, canonical autophagy requires the formation of the pre-initiation complex (FIP200, ATG13 and ULK1/2) to induce autophagy, but LAP does not (Martinez et al. 2015; Sanjuan, Milasta, and Green 2009; Henault et al. 2012). LAP, however, exclusively employs the Rubicon containing class III PI3K complex for the localized production of PI(3)P at the LAPosome formation site, depending on the LC3-PE and ATG5-12 ubiquitin-like conjugation systems (Martinez et al. 2015). The conjugation of ATG5 to ATG12 further forms a large multimeric complex with ATG16 (S. W. Wong, Sil, and Martinez 2018). On the other hand, ATG4 mediates

the conversion of cytosolic LC3-I into lipidated LC3-II, which subsequently is conjugated to the phosphatidylethanolamine (PE) at the LAPosome membrane surface, through the activity of ATG7 and ATG3 (Martinez et al. 2015). In addition, the above-mentioned ATG5-12/16L1 protein complex also participates in the transformation of LC3. From a global view, Rubicon acts as the a hub protein since it is involved in the two important processes of LAP (S. W. Wong, Sil, and Martinez 2018): First, Rubicon serves as a bridging molecule that engages with Beclin 1 and UVRAG (through its CCD domain) (Zhong et al. 2009), and VPS34 (through it RUN domain) (Sun et al. 2011) to form type 3 class III PI3K complex, which is essential for the production of PI(3)P signal in response to LAP stimuli. Second, after its translocation to the LAPosome, Rubicon interacts with and stabilizes the membraned form p22phox via its S-R domain (Yang et al. 2012); with the concomitant TLR or FcR stimulation, cytosolic components Rac1, p67phox, p40phox and p47phox move to the phagosome to form the active form of NOX2 protein complex - this active NOX2 complex in turn generates reactive oxygen species (ROS) in the phagosomal cavity (Ueyama et al. 2011; Yang et al. 2012; S. W. Wong, Sil, and Martinez 2018). Therefore, Rubicon is also required for the generation of an optimal level of ROS for the effective killing of the pathogens (S. W. Wong, Sil, and Martinez 2018). The Rubicon-NOX2 dependent ROS production could further facilitate the translocation of downstream LAP components, like ATG16L1, LC3-II and ATG7 to the LAPosome (Martinez et al. 2015). The lipidated LC3-II is required for the maturation of the LAPosome, and the LC3-II decorated LAPosome fuse to the lysosome for final digestion (S. W. Wong, Sil, and Martinez 2018).

LAP in infectious diseases

Many studies have shown the association between LAP defects and disease. Because canonical autophagy and LAP share some overlapping components, it is unclear whether these associations are due to autophagic alterations *per se* or are caused by an impaired LAP function. As LAP exclusively utilizes Rubicon to induce its activities, the Rubicon knockout mouse represents an important tool to study LAP-associated pathologies and disorders without confounding deficiency in canonical autophagy(Martinez et al. 2016a; 2015). Furthermore, multiple lines of evidence from clinical studies have shown that LAP is involved in the pathogenesis of several human diseases that are independent of canonical autophagy (Assoum et al. 2013; Y. Wan et al. 2017).

LAP has been intensively investigated for its broad anti-microbial functions, as LAP is an effective pathway for the phagocytosis and degradation of microbial pathogens. LAP deficient mice (Rubcn-/- mice), could not sufficiently eliminate invaded bacteria or fungi, thus resulting in an overgrowth of pathogens, as well as high mortality and lethality after microbial infection (Martinez et al. 2015). Due to failed microbial control, the LAP deficient mice also elicited a heightened and exaggerated inflammatory response characterized by an increased concentration of pro-inflammatory cytokines (IL-6 and IL-1β) and a decreased level of anti-inflammatory cytokine (IL-10) upon challenges (Martinez et al. 2015), suggesting that LAP has an immunomodulatory role for the resolution of inflammation during the clearance of pathogens. Therefore, LAP is involved in the host innate immune response and serves as the first line of defense against microbial invasion.

Chronic granulomatous disease (CGD) is an inherited immunodeficiency disorder that affects phagocytes of the innate immune system and increases the risk of fungal (*A. fumigatus*)

infections. Etiologically, CGD patients harbor a genetic mutation in a subunit gene of the NOX2 complex (Debeurme et al. 2010), which is a key component in the LAP pathway. Similarly, the absence of LAP disinhibits the control of *A. fumigatus* proliferation both *in vivo* and *in vitro*. Furthermore, Rubicon deficient mice exhibit pulmonary granulomatous formation (Martinez et al. 2015), which is a hallmark pathology seen in CGD patients. In addition to *A. fumigatus*, LAP is also involved the clearance of *Candida albicans,* which depends on the receptor engagement of Dectin-1(Ma et al. 2012; Tam et al. 2014).

Besides preventing fungal infections, the innate immune system also recognizes and eliminates invading bacteria through LAP. Listeriosis is caused by the infection of *Listeria monocytogenes*, a type of gram-positive bacterium discovered by Dr. Lister. Upon *L. monocytogenes* infection, bacteria are recognized by the receptor β2 integrin Mac-1 in macrophages, triggering LAP activation via the recruitment of the LAPosome assembly, which enhances the clearance of bacterium and anti-listeral immunity of phagocytes (Gluschko et al. 2018; Herb, Gluschko, and Schramm 2018). *Legionella dumoffii* is a type of a gram-negative bacterium that can cause fatal pneumonia. *L. dumoffii* induces LAP through the Dot/IcmT4SS dependent manner to attract LAP proteins RUBCN and NOX2, and its cytosolic degradation exclusively depends on LAP rather than canonical autophagy or endocytic pathway (Hubber et al. 2017). Infection of *Burkholderia pseudomallei,* a gram-negative, aerobic, motile bacterium can cause melioidosis, a disease characterized by local and disseminated infections. During phagocytes' fighting against *B. pseudomallei*, the LC3-II positive, single-membraned phagosomes are formed at the engulfed cytosolic *B. pseudomallei* (Gong et al. 2011; Mulye et al. 2014), indicating of LAPosome formation and ongoing LAP activities. Phagocytes' defense against *Salmonella enterica*, a flagellate, gram-negative bacterium that can cause salmonellosis also

heavily depends on LAP. The phagocytosis of *S. enterica* triggers the recruitment of many LAP compartments including Rubicon and NADPH oxidase to the bacterium-containing vacuole *in vivo* (Masud et al. 2019). The absence of Rubicon, however, abolished this effect, suggesting LAP is an important defensive pathway in macrophages for restricting *Salmonella* infection (Masud et al. 2019). *Mycobacterium Tuberculosis* is causative agent of tuberculosis, a highly contagious disease that primarily affects the lungs (Köster et al. 2017). The absence of LRRK2, a negative regulator of the LAP pathway, results in an increased innate immunity and an effective clearance of *M. Tuberculosis in vivo* (Härtlova et al. 2018).

Genetics of periodontitis

Periodontitis is a polymicrobial disruption of host homeostasis, wherein an aberrant response to periodontal pathogens can lead to periodontal tissue destruction, alveolar bone loss and eventually loss of teeth (Divaris et al. 2013). It represents one of the most prevalent dysbiotic and inflammatory diseases in the world – about half of the adults in the United States have periodontitis (Eke et al. 2015) and approximately 9% are affected by its severe form (Kassebaum et al. 2014; Richards et al. 2014). Multiple lines of evidence have demonstrated that the microbial burden and the associated inflammation in periodontitis are associated with other systemic inflammatory diseases, including rheumatoid arthritis, stroke, cardiovascular disease, diabetes and preterm birth (J. D. Beck et al. 2019).

The current understanding of the etiology of periodontitis is that of a multifactorial disorder orchestrated by environmental and genetic factors (Morelli, Agler, and Divaris 2020). Local pathogenic factors such as specific pathogenic microorganisms in the subgingival dental plaque biofilm have been thought to initiate the periodontal infection and inflammation (Socransky et al.

1998). On the other hand, individuals could mount different degrees of innate and adaptive immune reactions in response to the periodontal microflora, leading to inconsistent disease severity and periodontitis phenotypes (Dutzan et al. 2018). Recent studies have shown that genetic variations that affect the function or the expression of the encoded protein in the genes of inflammatory pathways may contribute to the dissimilar host immune responses (Morelli, Agler, and Divaris 2020; Loos and Van Dyke 2020). Interestingly, the interactions between host responses and local microflora are reciprocal. For example, an increased, exacerbated inflammatory response caused by the genetic variation in inflammatory genes induces a shift to the dysbiotic microbiome in the gingival pockets, thus leading to the progression of periodontal disease (Morelli, Agler, and Divaris 2020; Loos and Van Dyke 2020).

Early genetic research from cohort studies showed that candidate genes or loci in Fc receptor, pattern recognition receptor, lnterleukin (IL)-1 and TNF-alpha were associated with periodontitis (Özer Yücel et al. 2015; Song, Kim, and Lee 2013; Trevilatto et al. 2011; Han, Ding, and Kyung 2015). However, due to a small number of tested loci, and limited sizes of patient and control samples, these results have not been widely replicated and should be treated with caution. Recent large-scale genome wide association studies (GWAS) have tested thousands of participants and millions of genetic markers, and have reported many novel candidate susceptibility genes and loci possibly associated with human periodontitis (Divaris et al. 2013; Offenbacher et al. 2016; Morelli, Agler, and Divaris 2020). Dr. Offenbacher's group at the University of North Carolina stratified the study population (Dental Atherosclerosis Risk in Communities - DARIC) (James D. Beck et al. 2001) based on participants' phenotypical and molecular profiles to create different periodontal complex traits (PCT), which were then carried forward to a GWAS aiming to identify new risk gene (Offenbacher et al. 2016). Based on that study's results, many genes in the LAP

pathways were found to be statistically associated with distinct PCTs. For example, the *ATG16L* genes, which encode a downstream conjugation protein of the LAP pathway, are strongly associated with PCT2, which resembles severe forms of periodontitis but has a reduced loading of *P. gingivalis* and IL-1β in gingival crevicular fluid (GCF) (Offenbacher et al. 2016). Similar associations were reported for PCT3, which has an increased loading of *A. actinomycetemcomitans* and a higher concentration of the GCF IL-1β (Offenbacher et al. 2016). The *DHX57* gene encodes the MORN2 protein, which is essential for the recruitment of LC3-II to LAPosome during LAP activation, and is associated with PCT5 (*P.g* trait) featured by the highest loading of *P. gingivalis*, and PCT1 (Socransky trait), featured by a uniformly high load of periodontal pathogens (Offenbacher et al. 2016).

These candidate susceptibility loci reported by Dr. Offenbacher's research group are also linked to other human systemic inflammatory diseases and defective bacterial clearance. The single-nucleotide polymorphism in *ATG16L1* gene (rs2241880) is strongly associated with the risk of developing Crohn's disease, an inflammatory disease featured by chronic inflammation and auto-inflammation in the gastrointestinal (GI) tract (Salem et al. 2015). Similarly, *in vivo* studies from ATG16L1 conditional knockout mice with gene deficiency in the myeloid cells compartment showed that these mice are more susceptible to dextran sulfate sodium-induced colitis(Saitoh et al. 2008). Mechanistically, ATG16L1 deficiency resulted in a heightened production of pro-inflammatory cytokines (IL-1beta and IL18) after bacterial stimulations (Saitoh et al. 2008), and in defects of the clearance of an intestinal pathogen, *C. rodentium* (Salem et al. 2015). Similarly, the absence the MORN2, an adaptor protein for LC3-II's recruitment during LAP activation, also results in a defective clearance of bacteria (*M. tuberculosis*, *L. pneumophila* and *S. Aureus*) *in vivo* (Abnave et al. 2014). Therefore, we believe that LAP is required for the phagocytic clearance of

periodontal pathogens, influences the dysbiotic shift of the oral microbiome, and restrains the local immune responses from the bacterial stimulation. Therefore, the objective of this project is to further investigate the role of LAP in periodontal disease pathogenesis.

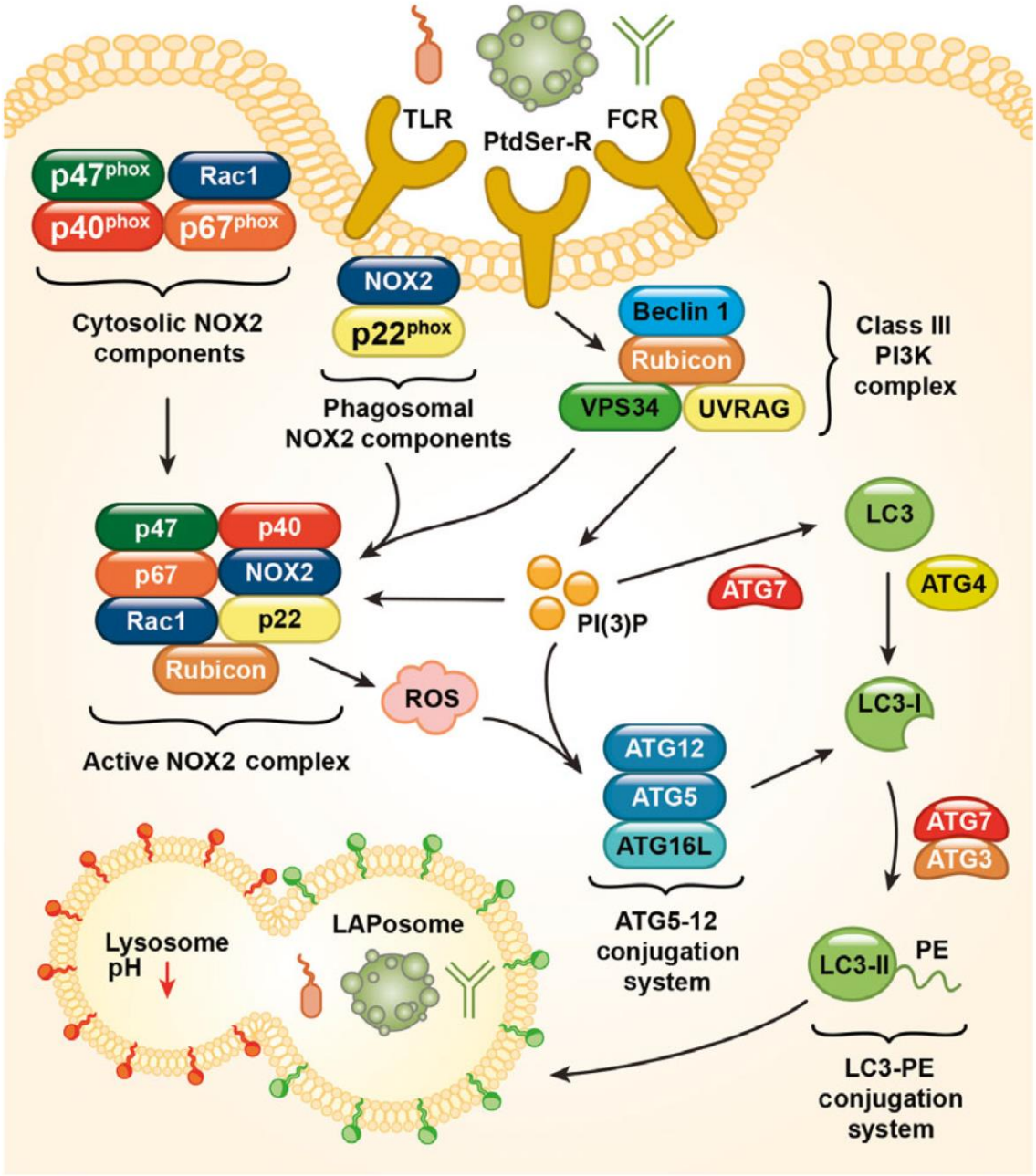


Figure 1.1 The molecular pathway of LC3 associated phagocytosis

(Figure from Wong SW et al. *FEBS*, 2018. Used with permission)

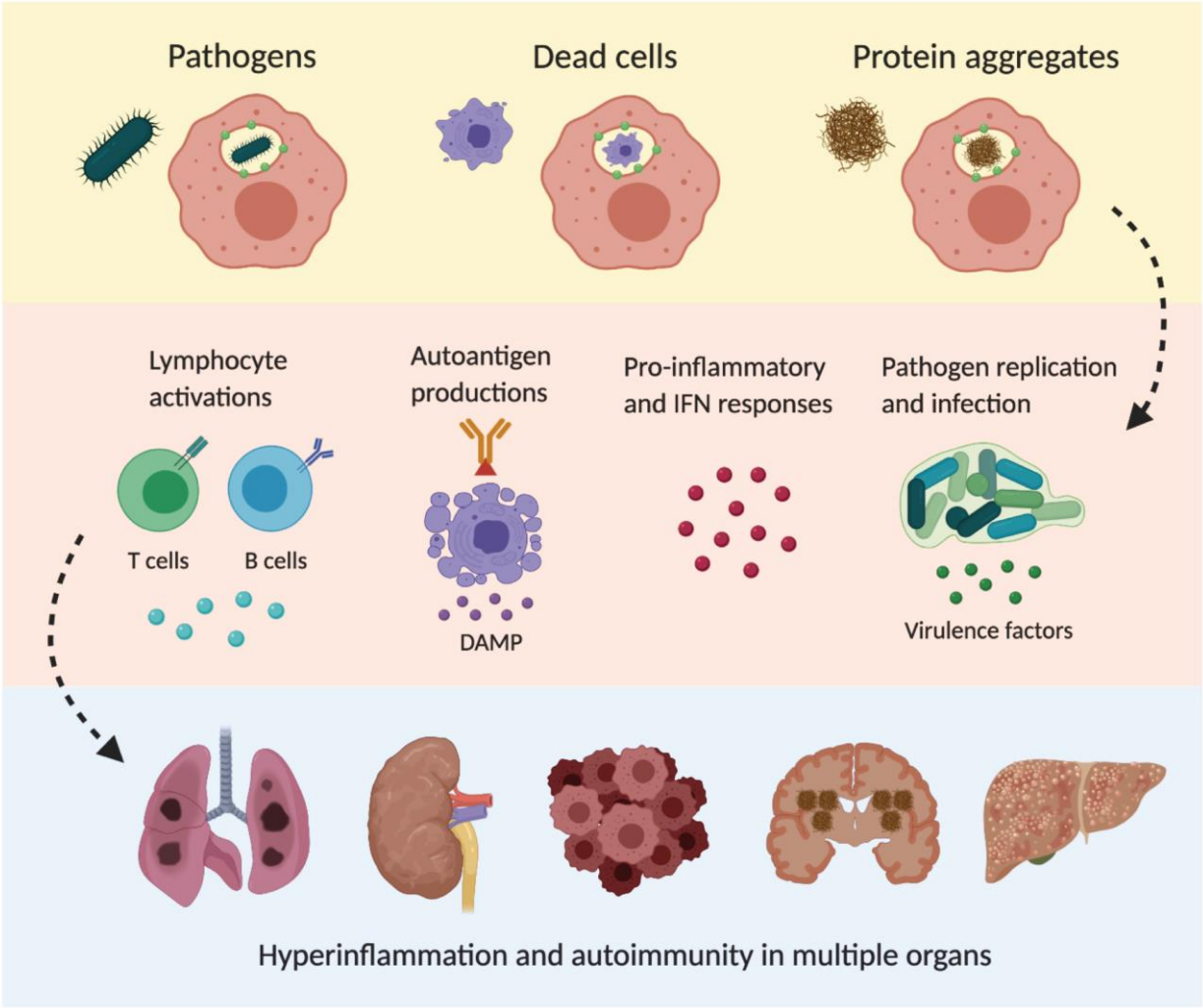


Figure 1.2 Biological mechanisms associated with LAP pathologies. An overview of the molecular mechanisms associated with aberrant LAP in inflammatory and autoimmune disorders. (Figure from Giulia Petroni and Lorenzo Galluzzi. "Non-Canonical Autophagy: Mechanisms and Pathophysiological Implications", Chapter 4, United States, *Elsevier*, In press. Used with permission)

Chapter 2: THE ROLE OF NON-CAONONICAL AUTOPHAGY IN THE PATHOGENESIS OF PERIODOTNAL DISEASE

Introduction

Periodontitis is a common chronic inflammatory disease of the tooth-supporting tissues in response to periodontal microbial pathogens. It affects over 60 million adults in the US (Eke et al. 2015), and its severe form affects 743 million people worldwide (Kassebaum et al. 2014). Susceptible individuals mount an exaggerated inflammatory response to biofilms, leading to immunopathology and alveolar bone resorption (Dutzan et al. 2018). However, the pathogenesis that underlies periodontal hyperinflammation in susceptible patients is not completely understood. Results from the genome wide association study (GWAS) from the Dental Atherosclerosis Risk in Communities (Dental ARIC – Offenbacher group) (Offenbacher et al. 2016) indicate that genetic variation in the genes of the LC3-associated pathway are implicated in chronic periodontitis and increased load of periodontal pathogens.

LC3-associated phagocytosis (LAP), a newly identified form of non-canonical autophagy, is critical for the clearance of microbial pathogens, as well as shaping the appropriate immune response to phagocytosed cargos . The molecular pathway that governs LAP has been well-described in a recent study. Studies demonstrated that Rubicon, in concert with other classical autophagic proteins, is required for the LAP (S. W. Wong, Sil, and Martinez 2018; Martinez et al. 2015). Thus, the Rubcn-deficient (Rubcn-/-) mouse could be used as a valuable tool to study LAP-dependent disease, without impairing the function of canonical autophagy (Martinez et al. 2016b).

LAP-deficient animals have been shown to exhibit increased inflammation and defective clearance of *A. fumigatus in vivo* (Martinez et al. 2015). We hypothesize that a dysregulation in LAP results in hyperinflammation in periodontal tissues during bacterial infection and contributes to increased periodontal disease severity. We aim to investigate the functionality of LAP in periodontal disease pathogenesis though complementary studies using the cell infection model and an experimental periodontitis rodent model. We anticipate that our study will demonstrate the relevance of LAP, illuminate its role in periodontitis, and improve the understanding of the factors driving increased disease severity and progression of periodontal disease.

Material and Methods

Animals

The mice used for this study were housed and bred in a specific pathogen-free animal facility at the National Institute of Environmental Health Sciences. Rubicon global knockout mice (Rubcn-/-) in C57BL/6 background were previously described (Martinez et al. 2015). For the *in vivo* ligature induced periodontitis experiment, 8- to 10-week-old female and male mice were used. For bone phenotyping experiments, 8-week-old female mice were used. For osteoclast primary culture in vitro experiments, 8- to 12-week-old female and male mice were used. For the wild-type control mice, age-matched littermates were used. This study was approved by the Animal Care and Use Committees at the National Institute of Environmental Health Sciences (ACUC #2015-0021; amendment approved on 12/16/2019).

Ligature-induced periodontitis model

The ligature-induced periodontitis model was used as previous described (Abe and Hajishengallis 2013). Briefly, a sterile 5-0 silk suture was tied around the maxillary second molar of the mice under anesthesia, and the control contralateral unligated tooth served as the baseline control (Ref). Eight days after ligature placement, the mice were euthanized by CO_2, and the maxillae were dissected, fixed in 10% formalin. The fixed samples were sent to University of North Carolina Small Animal Imaging for scanning by Scanco 40 micro-CT machine (Scanco Medical, Bassersdorf, Switzerland) in 20 μm resolution (E = 70kVa; I = 145 μA). The levels of alveolar bone loss were measured from 3-dimensional rendering pictures (distance from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC)).

Long bone phenotyping

The entire femurs and tibiae were dissected from age-matched 8-week old female wild type and Rubcn-/- mice. The bones were fixed in 4% paraformaldehyde or 10% formalin for at least 48 hours. After fixation, bones were scanned by Scanco 40 micro computed tomography (μCT) machine in 10 μm resolution under E = 70kVa and I = 145 μA. We selected the regions of interest of the trabecular and cortical bones 0.7 mm (70 slices) distal to metaphysis of tibia, and 0.7 mm proximal to the distal tibiofibular junction.The bone morphometric and microarchitecture analyses were performed with the usage from reconstructed solid 3D pictures. The following trabecular and cortical bone parameters were measured: BV/TV (bone volume fraction), Tb.Th (trabecular thickness), Ct.Th (average cortical thickness), Tb.Sp (trabecular separation), Tt.Ar (total cross-sectional area inside the periosteal envelope), Ct. Ar. (cortical bone area), Ct.Ar/Tt.Ar (cortical area fraction) and Tb.N (trabecular number).

Macrophage primary culture

Bone-marrow-derived macrophages were isolated from mouse bone marrow progenitor cells. These bone marrow cells were cultured in DMEM cell culture medium supplemented with 10% heat-inactivated FBS (Cytiva, Marlborough, MA), 15-20% L929 supernatant, non-essential amino acid, 2mM L-alanyl-L-glutamine, penicillin/streptomycin, and non-essential amino acids for 6 days. Nonadherent cells were discarded on day 6, and adherent macrophages were detached by cell scraping and re-plated with cell culture medium that contains 5% L929 supernatant for experimental usage.

Osteoclast primary culture

The method employed for the osteoclast primary culture was previously described (S.-W. Wong et al. 2020). Briefly, tibiae and femurs were harvested from the CO_2-euthanatized mice Osteoclast basic culture medium made by free α-MEM medium with 10% FBS, penicillin/streptomycin, L-Glutamine and non-essential amino acids was used to flush bone marrow cells from collected bones. After culturing the bone marrow cells with the osteoclast basic culture medium, the floating, non-adherent cells were collected and re-seeded at a density of 1.5×10^5 cells/cm^2 with the supplement of 30 ng/mL M-CSF (R&D Systems, Minneapolis, MN USA). After the stimulation of M-CSF for two days, osteoclast precursors were formed and osteoclast differentiation medium was changed to osteoclast basic culture medium with 10 ng/mL RANKL (R&D Systems Minneapolis, MN USA) and 30 ng/mL M-CSF. Three days after the application of osteoclast differentiation medium, multinucleated osteoclasts were formed and were fixed with 10% formalin. Fixed cells were stained with tartrate-resistant acid phosphatase (TRAP) to detect osteoclasts according to a published method (S.-W. Wong et al. 2020).

Antibodies and Western Blot

The following antibodies were from Cell Signaling (Beverly, MA USA): α-Tubulin (Cat. #2144), Rubicon (D9F7) (Cat. #8465), #p62 (Cat. #5114), LC3B (Cat. #2775), c-Fos (9F6) (#2250). NFATc1 (Cat. #sc-7294) and Actin (Cat. #sc-1616) antibodies were from Santa Cruz Biotechnology Inc. (Dallas, TX USA). Customized polyclonal anti-MORN2 antibody against mouse MORN2 peptide (CNFNENRVEGEGEYT) was produced from GenScript (Piscataway, NJ, USA). The secondary antibodies were purchased from Jackson Immuno-Research (West Grove, PA, USA).

Macrophages infected with periodontal pathogens (*P.g*. and *F.n.*) were lysed in RIPA lysis buffer with protease and phosphatase inhibitor cocktails (Thermo Fisher, Waltham, MA, USA). Protein concentration was determined by the BCA Protein Assay. 5-20 μg of total protein lysate was subjected to Criterion TGX precast gel (Biorad, Hercules, CA, USA) and Western blotting. Proteins in the nitrocellulose membranes were visualized by enhanced chemiluminescence (ECL, Amersham Bioscience, Little Chalfont, UK). Protein band intensities were quantified using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

All data this study are presented as mean ± SD. We used student's t test determine statistical differences between groups. *P values* less than 0.05 were considered statistically significant.

Results

Periodontal pathogen may induce the activation of LC3-associated phagocytosis in macrophages

To explore the relevance of LC3-assciated phagocytosis in periodontal infection and periodontal disease development, we first examined if key periodontal pathogens activate LAP in phagocytes. We used an *in vitro* infection model by treating a macrophage cell line and primary macrophages with *P.g*. or *F.n*. During LAP activation, there is a conversion of the cytosolic form of LC3 (LC3-I) to the membrane-associated form of LC3 (LC3-II), which is recruited to the engulfed cargo to form the single-membraned LAPosome with orchestration by the autophagy conjugation system. Co-incubation of the macrophage cell line, RAW 264.7 cells with live and heat-killed (HK) *P.g*. both induced the conversion of LC3-I to LC3-II (Figure 2.1 A). The expression level of Rubicon, a key upstream protein in the LAP pathway was not apparently changed during the bacterial challenge. However, we noticed that the expression of MORN2, a downstream LAP was upregulated at all time points with *P.g.* treatment. To validate this result, we infected bone marrow derived macrophages with live and H.K *P.g. in vitro*. Similarly, the primary macrophages produced a drastic conversion of LC3-I to LC3-II, with the peak at 15 minutes post-infection. Moreover, infection with another periodontal pathogen, *F.n.* also induced the transformation of LC3-II (Figure 2.2). These data show that infection with pathogenic periodontal bacteria could induce LAP in macrophages, suggesting that the clearance of these pathogens might be dependent on LAP.

LAP deficient mice exhibited an increased inflammatory bone loss

To investigate the role of LAP in periodontitis pathogenesis *in vivo*, we used a ligature-induced periodontitis model using Rubicon global deficient mice (Rubcn-/-) and controlled littermates. 8 days after the ligature placement, we noticed that the alveolar bone resorption in the Rubcn-/- mice was significantly higher than that in control mice (Figure 2.2 A). The ligature

induced bone loss was similar between male and female mice within the same genotype (Figure 2.1 B). Therefore, these *in vivo* data suggest that LAP deficiency resulted in an increased disease severity (alveolar bone loss) and that LAP may protect against periodontal disease.

Rubicon deficient mice exhibited normal skeletal phenotype

Recent studies have implicated the functions of autophagy proteins in osteoblasts and osteoclasts (Ref). To investigate the role of Rubicon in bone biology *in vivo*, we performed bone phenotyping of 2-month-old Rubicon global knockout (Rubcn-/-) mice. The Rubcn-/- mice did not have any developmental defects and were phenotypically normal at the skeletally mature age of 2 months. Phenotypic analysis of the long bones using μCT scanning revealed that Rubcn-/- mice had identical trabecular (Figure 2.3D) and cortical bone (Figure 2.3D) morphometric parameters, compared to Rubcn+/+ littermates. Furthermore, no overt bony pathologies were found in Rubcn-/- mice. Therefore, these results suggest that Rubicon might not directly impact on bone remodeling *in vivo.*

Rubicon deficiency does not affect *in vitro* osteoclastogenesis

Our previous unpublished data indicated that some autophagy proteins are involved in osteoclast differentiation. To further confirm our *in vivo* results and to explore the function of Rubicon in osteoclastogenesis, we performed *in vitro* osteoclast primary culture using bone marrow cells as osteoclast precursors. TRAP staining revealed that the bone marrow osteoclast precursor cells from Rubcn-/- mice generate similar number of osteoclasts compared to Rubcn+/+ osteoclast precursors (Figure 2.5A). In addition, Rubcn-/- osteoclast precursors expressed similar levels of c-Fos and NFATc1, two important transcription factors related to osteoclast

differentiation after RANKL stimulation, compared to Rubcn+/+ differentiating osteoclasts. In addition to its pivotal role in LAP, Rubicon has been shown to negatively regulate canonical autophagy pathway. We next asked if Rubicon deficiency alters autophagic induction during osteoclastogenesis. We found that Rubcn-/- deficient osteoclast precursors exhibited similar degradation of autophagy marker p62, and conversion from LC3-I to LC3-II during osteoclast differentiation, compared to Rubcn+/+ osteoclast precursors. These results suggest that canonical autophagy is not affected in Rubcn-/- osteoclasts during cell differentiation (Figure 2.5D). Therefore, these results indicate that osteoclastogenesis *in vitro* is independent of Rubicon.

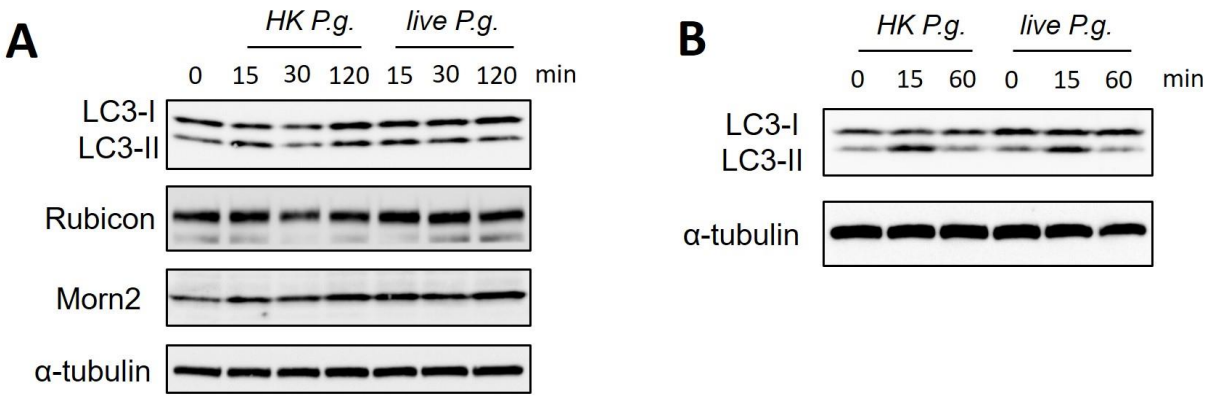


Figure 2.1 Periodontal bacteria P.g. induces conversion of LC3-I into LC3-II. (A) Macrophage cell line RAW cells were infected with HK and live *P.g.* at an MOI of 0.1. Cell protein lysates were collected for Western blot analysis of LC3, Rubicon, Morn2 and alpha tubulin to access LAP induction. (B) Murine primary macrophages were infected with HK and live *P.g.* at an MOI of 1. Transformation of LC3-I to LC3-II was analyzed by western blotting.

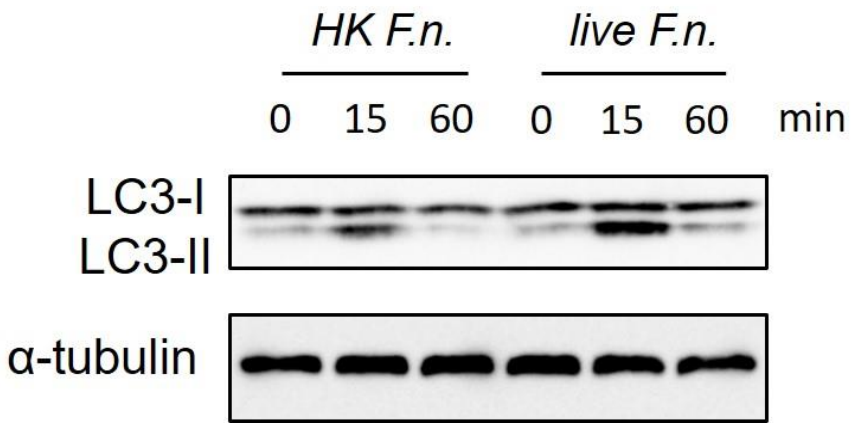


Fig 2.2. Periodontal pathogen *F.n.* induces conversion of LC3-I into LC3-II. Wild type murine bone marrow derived macrophages were infected with HK and live *F.n.* at an MOI of 1. Conversion of LC3-I into LC3-II was analyzed by immunoblotting.

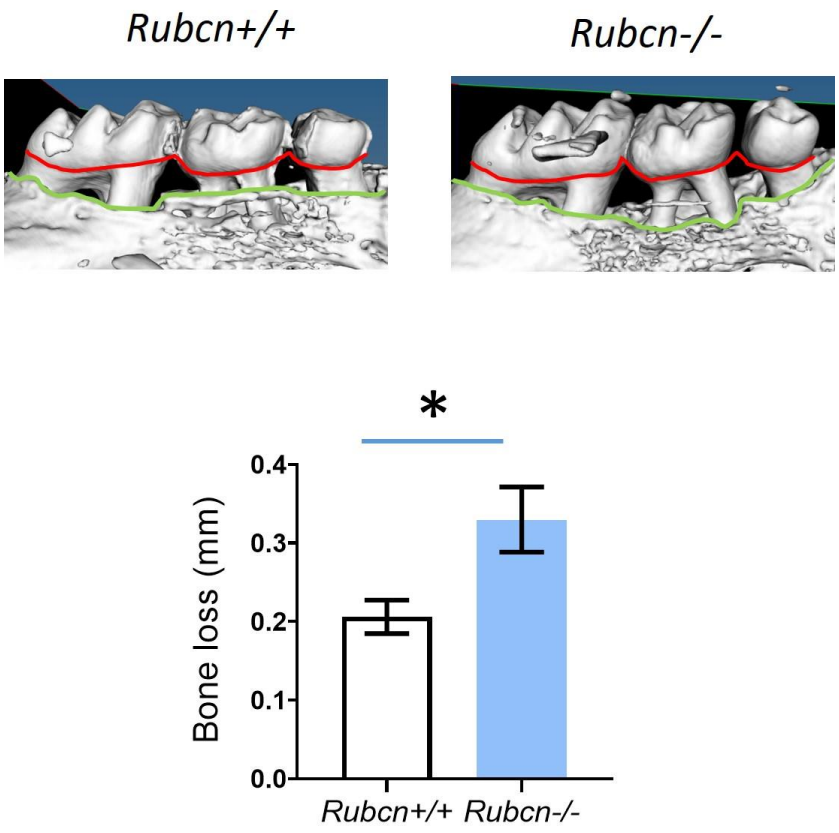


Fig 2.3. LAP deficient mice exhibited increased periodontal disease severity. Bone loss (mm) after ligature induced periodontitis in Rubicon deficient mice and control littermates.

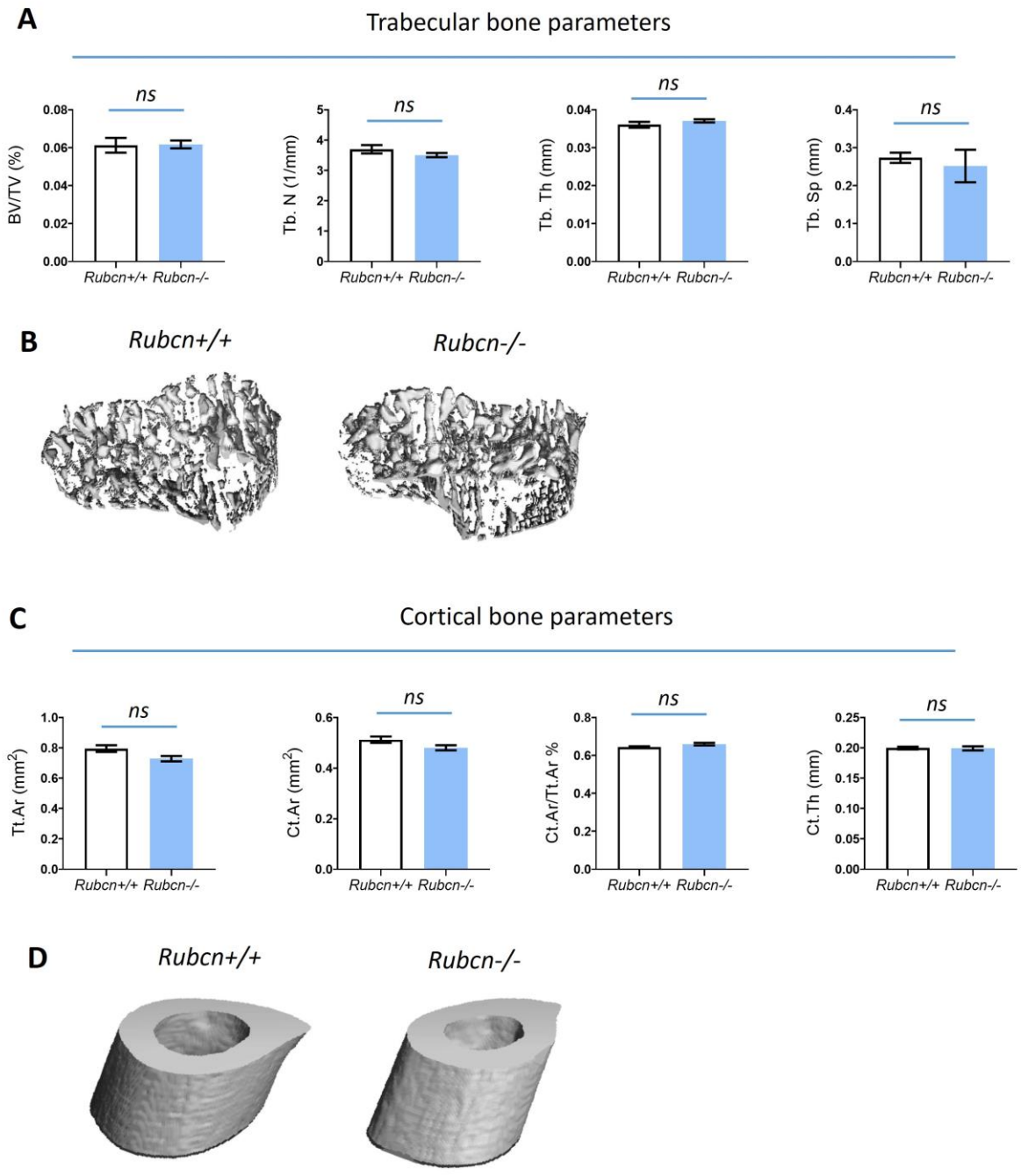
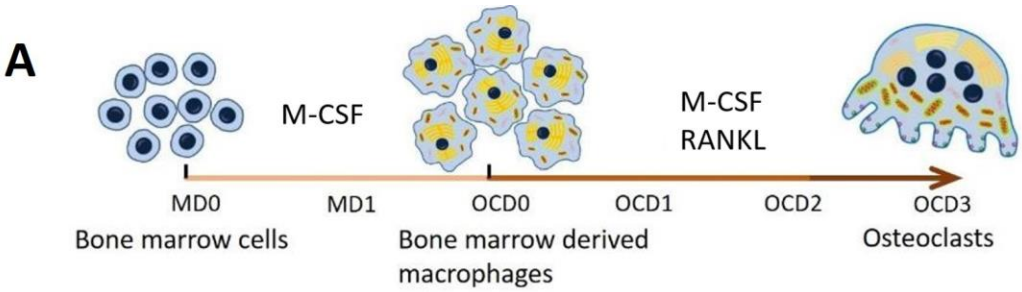
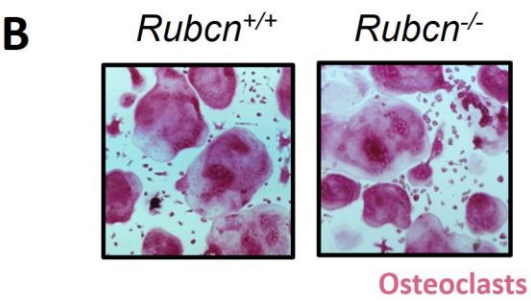
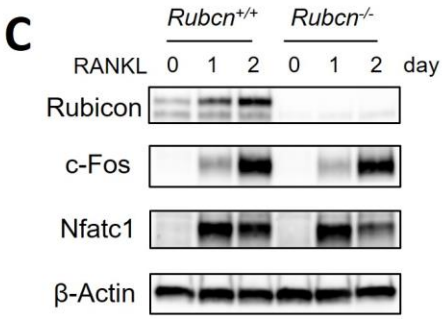


Figure 2.4. *Rubicon*$^{-/-}$ mice exhibited normal bone phenotype at 2 months of age. (A) Trabecular morphometric analysis of 2-month-old female *Rubcn*$^{+/+}$ (n=6) and *Rubcn*$^{-/-}$ (n=6) mice. BV/TV, bone volume / total volume; Tb. Th, trabecular thickness; Tb. N, trabecular number; Tb. Sp, trabecular spacing. (B) Representative 3 dimensional rendering pictures of the trabecular bones

in the proximal tibiae of *Rubcn*$^{+/+}$ and *Rubcn*$^{-/-}$ mice. (C) Cortical bone morphometric analysis of 2-month-old female wild type (*Rubcn*$^{+/+}$; n=6) and Rubicon deficient (*Rubcn*$^{-/-}$; n=6) mice. Tt. Ar, total area; Ct. Ar, cortical area; Ct. Th, cortical thickness; Ct. Ar/Tt. Ar, cortical area / total area. (D) Representative 3 dimensional reconstructed images of the cortical bones in the distal tibiae of *Rubcn*$^{+/+}$ and *Rubcn*$^{-/-}$ mice.







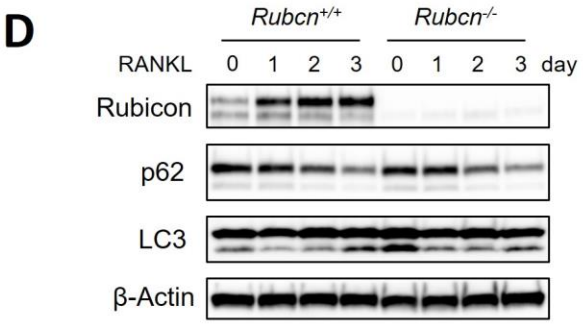


Figure 2.5 Rubicon deficiency does not affect osteoclast differentiation *in vitro*. (A) A schematic picture of *in vitro* osteoclast primary cell culture; after RANKL stimulation, osteoclast precursors differentiate and fuse with each other to form the multinucleated osteoclasts. (B) 3 days post-RANKL stimulations, representative images of TRAP positive, multinucleated osteoclasts generated from wild type (Rubcn+/+) and LAP deficient (Rubcn-/-) mice. (C) Cell lysates were collected from day 0 to 2 of *in vitro* osteoclastogenesis from Rubcn+/+ and Rubcn-/- osteoclast precursors, and were subjected to immunoblotting of Rubicon, c-Fos, NFATc and beta-actin to evaluate osteoclast differentiation capacity. (D) Cell lysates were collected from day 0 to 3 of *in vitro* osteoclastogenesis from Rubcn+/+ and Rubcn-/- osteoclast precursors, and were subjected to immunoblotting of Rubicon, p62, LC3 and beta-actin to access autophagic activities.

CHAPTER 3: DISCUSSION AND FUTURE DIRECTIONS

LAP has been implicated in many systemic inflammatory and auto-inflammatory diseases, such as systemic lupus erythematosus (Martinez et al. 2016b), liver fibrosis/cirrhosis (J. H. Wan et al. 2020), Alzheimer's disease (Heckmann et al. 2019) and contact dermatitis (Sil et al. 2020). Functionally, LAP has an anti-inflammatory and immunosuppressive role to restrict the inflammatory responses by the effective limitation of hazardous components, thus reducing disease activity and progression (S. W. Wong, Sil, and Martinez 2018). Although many LAP related genes are associated with periodontal infection (Offenbacher et al. 2016), the knowledge base regarding the function of LAP in periodontitis is scarce and remains unclear. Similar to the previous findings, we demonstrated that LAP could protect against the development of periodontal disease *in vivo*, which may be due its anti-inflammatory effect. To assess periodontal inflammation in LAP deficient mice, future work should examine the production of cytokines in infected periodontal tissues at the mRNA and protein levels. Since LAP primarily executes its function in the phagocytes, targeting examination of the LAP activity and transcriptional profiles in different types of immune cells can be achieved by single-cell sequencing from human periodontitis tissues.

LAP can be induced by the uptake of different microorganisms, including bacteria, fungi and parasites, thus facilitating their intracellular degradation in the ROS dependent manner (S. W. Wong, Sil, and Martinez 2018). In the present study, we demonstrated that two established periodontal pathogens (*P.g*, and *F.n*) could also induce the transformation of the LC3 protein, indicative of an ongoing LAP activation. These results suggest that the phagocytic function and

bacterial degradation of periodontal pathogens in phagocytes may be dependent of LAP. To validate the role of LAP in the degradation of periodontal pathogens, quantifying the abundance of these pathogens will be needed.

While we have completed some fundamental experiments, we need to perform more *in vivo* and *in vitro* experiments to explain the molecular mechanisms that underlie dysfunction of LAP and associated defects in periodontal pathogen clearance, pathogenic microbial induced dysbiosis, heightened inflammatory reaction and inflammatory bone loss in the periodontal tissues, and crosstalk between LAP and adaptive immunity (Figure 3.1).

In addition to LAP, another form of selective autophagy, xenophagy is also a required component of response to some bacterial species. For example, the autophagic clearance of cytosolic Salmonella relies on the autophagy receptor, Optineurin (Formstone et al. 2011; Slowicka et al. 2016). The molecular pathways of LAP and xenophagy also exhibit overlapping autophagy machinery and distinct components. For instance, both LAP and xenophagy need for the downstream conjugation proteins ATG16L for the development of LC3-II decorated membranes (Sil, Muse, and Martinez 2018). Xenophagy exclusively requires one of the autophagy receptors but not the class III PI3K complex, whereas LAP exclusively requires Rubicon containing class III PI3K complex (Sil, Muse, and Martinez 2018). Therefore, the usage of LAP deficient (Rubcn-/-), xenophagy deficient (Optn-/-) and LAP/xenophagy co-deficient mice will help us understand and elucidate the which type(s) of non-canonical is specifically involved in the periodontal infection and disease progression.

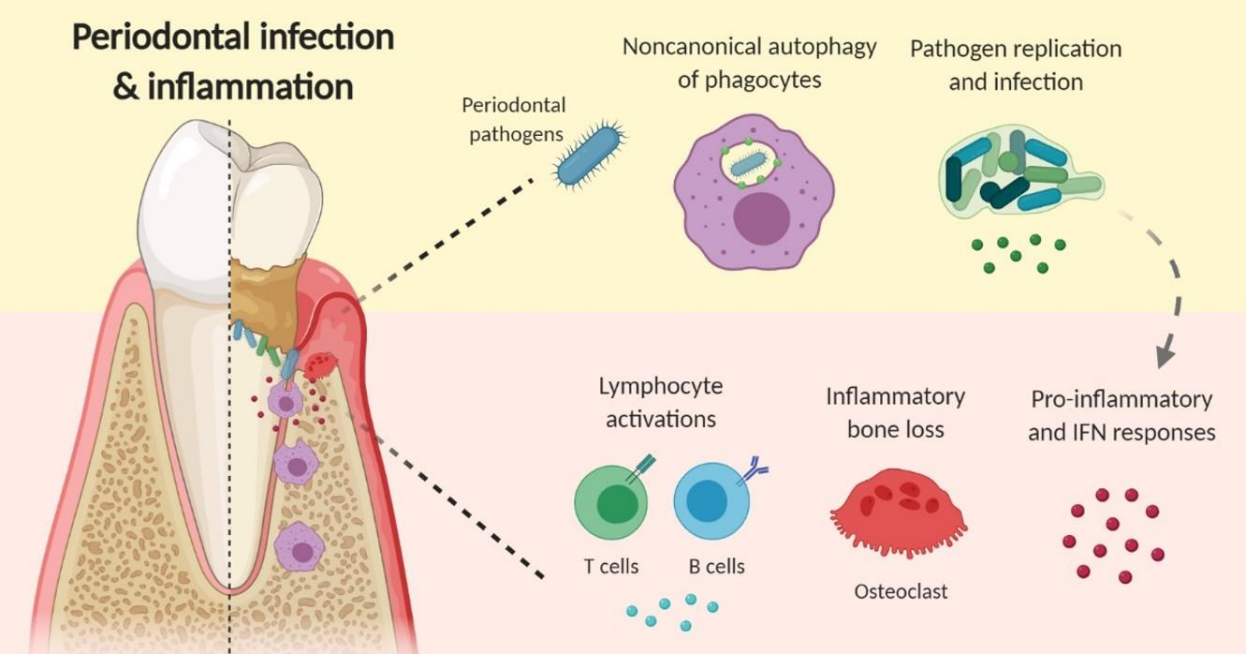


Figure 3.1 Possible role of LAP in periodontal disease pathogenesis

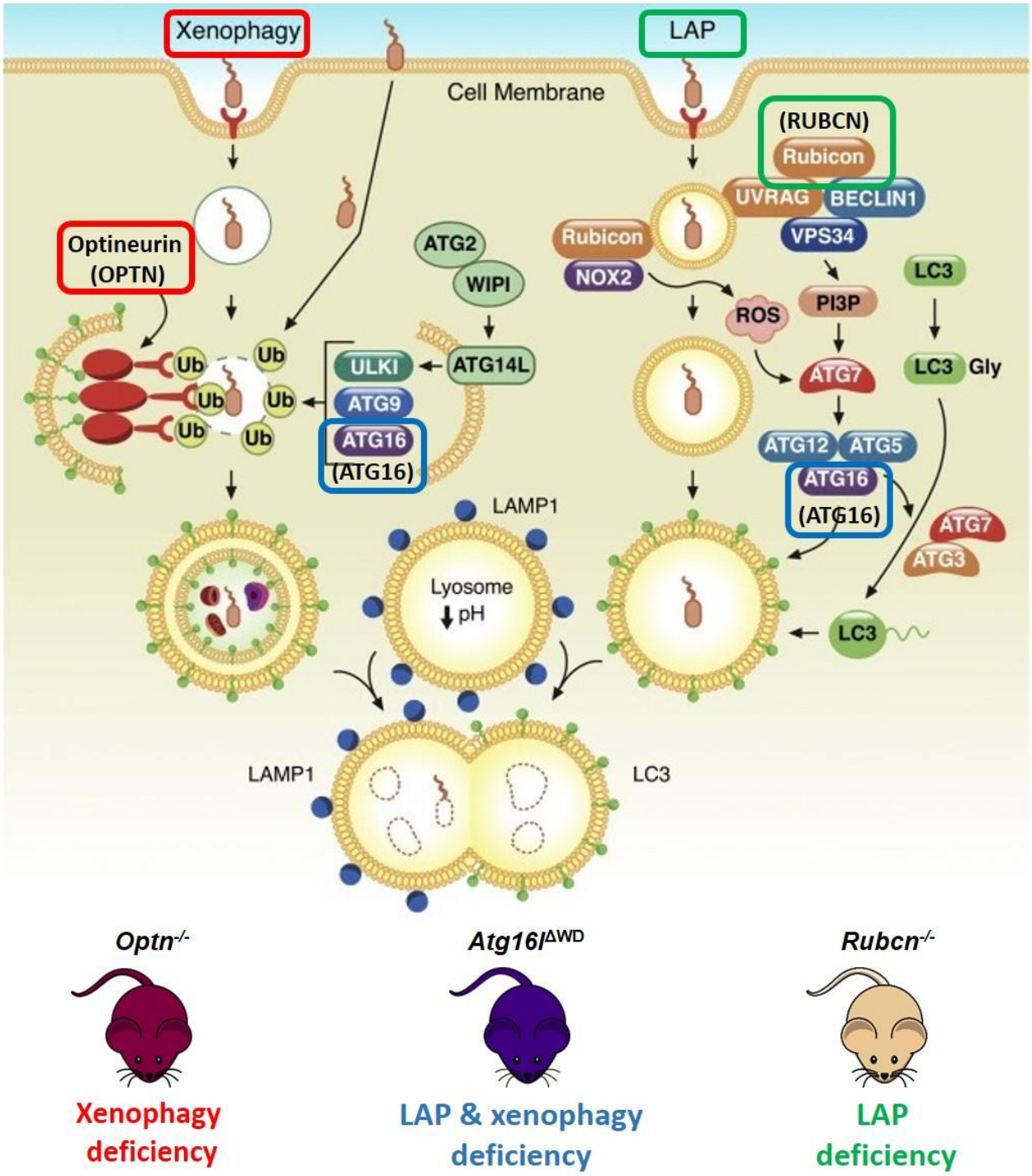


Figure 3.2 Differences of molecular signatures between LAP and xenophagy. During xenophagy, the intracellular pathogen or the microbial containing double-membraned vesicles is conjugated by the ubiquitin chains. After that, autophagy receptors, such as Optineurin

(highlighted in red) recognize the ubiquitinated cargo through the ubiquitin binding domain, and link it to the LC3-containing autophagosome. Meanwhile, the autophagy conjugation proteins such as ATG16L (highlighted in blue) are recruited to these pathogens to trigger the formation and maturation of autophagy membranes around the ubiquitinated pathogen substrates. During LAP, the engagement of a pattern recognition receptor by a pathogen leads to the recruitment Rubicon (highlighted in green) containing Class III PI3K complex to from a single membraned LAPosome. This Class III PI3K protein complex is required for the attraction of the downstream LAP conjugation proteins, including ATG16L (highlighted in blue) for the maturation of LC3-II decorated, cargo containing LAPosome. (Figure from Sil P et al. *Curr Opin Immunol*. 2018. (Sil, Muse, and Martinez 2018) Used with permission)

REFERENCES

Abe, Toshiharu, and George Hajishengallis. 2013. "Optimization of the Ligature-Induced Periodontitis Model in Mice." *Journal of Immunological Methods* 394 (1–2): 49–54. https://doi.org/10.1016/j.jim.2013.05.002.

Abnave, Prasad, Giovanna Mottola, Gregory Gimenez, Nicolas Boucherit, Virginie Trouplin, Cedric Torre, Filippo Conti, et al. 2014. "Screening in Planarians Identifies MORN2 as a Key Component in LC3-Associated Phagocytosis and Resistance to Bacterial Infection." *Cell Host and Microbe* 16 (3): 338–50. https://doi.org/10.1016/j.chom.2014.08.002.

Assoum, M., M. A. Salih, N. Drouot, K. Hnia, A. Martelli, and M. Koenig. 2013. "The Salih Ataxia Mutation Impairs Rubicon Endosomal Localization." *Cerebellum* 12 (6): 835–40. https://doi.org/10.1007/s12311-013-0489-4.

Beck, J. D., P. N. Papapanou, K. H. Philips, and S. Offenbacher. 2019. "Periodontal Medicine: 100 Years of Progress." *Journal of Dental Research* 98 (10): 1053–62. https://doi.org/10.1177/0022034519846113.

Beck, James D., John R. Elter, Gerardo Heiss, David Couper, Sally M. Mauriello, and Steven Offenbacher. 2001. "Relationship of Periodontal Disease to Carotid Artery Intima-Media Wall Thickness: The Atherosclerosis Risk in Communities (ARIC) Study." *Arteriosclerosis, Thrombosis, and Vascular Biology* 21 (11): 1816–22. https://doi.org/10.1161/hq1101.097803.

Debeurme, Franck, Antoine Picciocchi, Marie Claire Dagher, Didier Grunwald, Sylvain Beaumel, Franck Fieschi, and Marie José Stasia. 2010. "Regulation of NADPH Oxidase Activity in Phagocytes: Relationship between FAD/NADPH Binding and Oxidase Complex Assembly." *Journal of Biological Chemistry* 285 (43): 33197–208. https://doi.org/10.1074/jbc.M110.151555.

Divaris, Kimon, Keri L. Monda, Kari E. North, Andrew F. Olshan, Lindsay M. Reynolds, Wen Chi Hsueh, Ethan M. Lange, et al. 2013. "Exploring the Genetic Basis of Chronic Periodontitis: A Genome-Wide Association Study." *Human Molecular Genetics* 22 (11): 2312–24. https://doi.org/10.1093/hmg/ddt065.

Dutzan, Nicolas, Tetsuhiro Kajikawa, Loreto Abusleme, Teresa Greenwell-Wild, Carlos E. Zuazo, Tomoko Ikeuchi, Laurie Brenchley, et al. 2018. "A Dysbiotic Microbiome Triggers TH17 Cells to Mediate Oral Mucosal Immunopathology in Mice and Humans." *Science Translational Medicine* 10 (463). https://doi.org/10.1126/scitranslmed.aat0797.

Eke, Paul I., Bruce A. Dye, Liang Wei, Gary D. Slade, Gina O. Thornton-Evans, Wenche S.

Borgnakke, George W. Taylor, Roy C. Page, James D. Beck, and Robert J. Genco. 2015. "Update on Prevalence of Periodontitis in Adults in the United States: NHANES 2009 to 2012." *Journal of Periodontology* 86 (5): 611–22. https://doi.org/10.1902/jop.2015.140520.

Formstone, A, J Errington, C Baida, C L White, J W Gober, A H Crevenna, T Freisinger, et al. 2011. "Phosphorylation of the Autophagy Receptor Optineurin Restricts Salmonella Growth." *Science* 333 (July): 228–33.

Gluschko, Alexander, Marc Herb, Katja Wiegmann, Oleg Krut, Wolfram F. Neiss, Olaf Utermöhlen, Martin Krönke, and Michael Schramm. 2018. "The B2 Integrin Mac-1 Induces Protective LC3-Associated Phagocytosis of Listeria Monocytogenes." *Cell Host and Microbe* 23 (3): 324-337.e5. https://doi.org/10.1016/j.chom.2018.01.018.

Gong, Lan, Meabh Cullinane, Puthayalai Treerat, Georg Ramm, Mark Prescott, Ben Adler, John D. Boyce, and Rodney J. Devenish. 2011. "The Burkholderia Pseudomallei Type III Secretion System and BopA Are Required for Evasion of LC3-Associated Phagocytosis." *PLoS ONE* 6 (3). https://doi.org/10.1371/journal.pone.0017852.

Han, Min xuan, Cheng Ding, and Hee Moon Kyung. 2015. "Genetic Polymorphisms in Pattern Recognition Receptors and Risk of Periodontitis: Evidence Based on 12,793 Subjects." *Human Immunology* 76 (7): 496–504. https://doi.org/10.1016/j.humimm.2015.06.006.

Härtlova, Anetta, Susanne Herbst, Julien Peltier, Angela Rodgers, Orsolya Bilkei-Gorzo, Antony Fearns, Brian D Dill, et al. 2018. " LRRK2 Is a Negative Regulator of Mycobacterium Tuberculosis Phagosome Maturation in Macrophages ." *The EMBO Journal* 37 (12): 1–17. https://doi.org/10.15252/embj.201798694.

Heckmann, Bradlee L., Brett J.W. Teubner, Bart Tummers, Emilio Boada-Romero, Lacie Harris, Mao Yang, Clifford S. Guy, Stanislav S. Zakharenko, and Douglas R. Green. 2019. "LC3-Associated Endocytosis Facilitates β-Amyloid Clearance and Mitigates Neurodegeneration in Murine Alzheimer's Disease." *Cell* 178 (3): 536-551.e14. https://doi.org/10.1016/j.cell.2019.05.056.

Henault, Jill, Jennifer Martinez, Jeffrey M. Riggs, Jane Tian, Payal Mehta, Lorraine Clarke, Miwa Sasai, et al. 2012. "Noncanonical Autophagy Is Required for Type I Interferon Secretion in Response to DNA-Immune Complexes." *Immunity* 37 (6): 986–97. https://doi.org/10.1016/j.immuni.2012.09.014.

Herb, Marc, Alexander Gluschko, and Michael Schramm. 2018. "LC3-Associated Phagocytosis Initiated by Integrin ITGAM-ITGB2/Mac-1 Enhances Immunity to Listeria Monocytogenes." *Autophagy* 14 (8): 1462–64. https://doi.org/10.1080/15548627.2018.1475816.

Hubber, Andree, Tomoko Kubori, Cevayir Coban, Takeshi Matsuzawa, Michinaga Ogawa, Tsuyoshi Kawabata, Tamotsu Yoshimori, and Hiroki Nagai. 2017. “Bacterial Secretion System Skews the Fate of Legionella-Containing Vacuoles towards LC3-Associated Phagocytosis.” *Scientific Reports* 7 (July 2016): 1–17. https://doi.org/10.1038/srep44795.

Kassebaum, N. J., E. Bernabé, M. Dahiya, B. Bhandari, C. J.L. Murray, and W. Marcenes. 2014. “Global Burden of Severe Periodontitis in 1990-2010: A Systematic Review and Meta-Regression.” *Journal of Dental Research* 93 (11): 1045–53. https://doi.org/10.1177/0022034514552491.

Köster, Stefan, Sandeep Upadhyay, Pallavi Chandra, Kadamba Papavinasasundaram, Guozhe Yang, Amir Hassan, Steven J. Grigsby, et al. 2017. “Mycobacterium Tuberculosis Is Protected from NADPH Oxidase and LC3-Associated Phagocytosis by the LCP Protein CpsA.” *Proceedings of the National Academy of Sciences of the United States of America* 114 (41): E8711–20. https://doi.org/10.1073/pnas.1707792114.

Kotas, Maya E., and Ruslan Medzhitov. 2015. “Homeostasis, Inflammation, and Disease Susceptibility.” *Cell* 160 (5): 816–27. https://doi.org/10.1016/j.cell.2015.02.010.

Levine, Beth. 2005. “Eating Oneself and Uninvited Guests: Autophagy-Related Pathways in Cellular Defense.” *Cell* 120 (2): 159–62. https://doi.org/10.1016/j.cell.2005.01.005.

Loos, Bruno G., and Thomas E. Van Dyke. 2020. “The Role of Inflammation and Genetics in Periodontal Disease.” *Periodontology 2000* 83 (1): 26–39. https://doi.org/10.1111/prd.12297.

Ma, Jun, Courtney Becker, Clifford A. Lowell, and David M. Underhill. 2012. “Dectin-1-Triggered Recruitment of Light Chain 3 Protein to Phagosomes Facilitates Major Histocompatibility Complex Class II Presentation of Fungal-Derived Antigens.” *Journal of Biological Chemistry* 287 (41): 34149–56. https://doi.org/10.1074/jbc.M112.382812.

Martinez, Jennifer. 2018. “LAP It up, Fuzz Ball: A Short History of LC3-Associated Phagocytosis.” *Current Opinion in Immunology* 55: 54–61. https://doi.org/10.1016/j.coi.2018.09.011.

Martinez, Jennifer, Johann Almendinger, Andrew Oberst, Rachel Ness, Christopher P. Dillon, Patrick Fitzgerald, Michael O. Hengartner, and Douglas R. Green. 2011. “Microtubule-Associated Protein 1 Light Chain 3 Alpha (LC3)-Associated Phagocytosis Is Required for the Efficient Clearance of Dead Cells.” *Proceedings of the National Academy of Sciences of the United States of America* 108 (42): 17396–401. https://doi.org/10.1073/pnas.1113421108.

Martinez, Jennifer, Larissa D. Cunha, Sunmin Park, Mao Yang, Qun Lu, Robert Orchard, Quan Zhen Li, et al. 2016a. “Noncanonical Autophagy Inhibits the Autoinflammatory, Lupus-like Response to Dying Cells.” *Nature* 533 (7601): 115–19. https://doi.org/10.1038/nature17950.

———. 2016b. “Noncanonical Autophagy Inhibits the Autoinflammatory, Lupus-like Response to Dying Cells.” *Nature* 533 (7601): 115–19. https://doi.org/10.1038/nature17950.

Martinez, Jennifer, R. K.Subbarao Malireddi, Qun Lu, Larissa Dias Cunha, Stephane Pelletier, Sebastien Gingras, Robert Orchard, et al. 2015. “Molecular Characterization of LC3-Associated Phagocytosis Reveals Distinct Roles for Rubicon, NOX2 and Autophagy Proteins.” *Nature Cell Biology* 17 (7): 893–906. https://doi.org/10.1038/ncb3192.

Masud, Samrah, Tomasz K. Prajsnar, Vincenzo Torraca, Gerda E.M. Lamers, Marianne Benning, Michiel Van Der Vaart, and Annemarie H. Meijer. 2019. “Macrophages Target Salmonella by Lc3-Associated Phagocytosis in a Systemic Infection Model.” *Autophagy* 15 (5): 796–812. https://doi.org/10.1080/15548627.2019.1569297.

Morelli, Thiago, Cary S. Agler, and Kimon Divaris. 2020. “Genomics of Periodontal Disease and Tooth Morbidity.” *Periodontology 2000* 82 (1): 143–56. https://doi.org/10.1111/prd.12320.

Mulye, Minal, Michael P. Bechill, William Grose, Viviana P. Ferreira, Eric R. Lafontaine, and R. Mark Wooten. 2014. “Delineating the Importance of Serum Opsonins and the Bacterial Capsule in Affecting the Uptake and Killing of Burkholderia Pseudomallei by Murine Neutrophils and Macrophages.” *PLoS Neglected Tropical Diseases* 8 (8). https://doi.org/10.1371/journal.pntd.0002988.

Offenbacher, Steven, Kimon Divaris, Silvana P. Barros, Kevin L. Moss, Julie T. Marchesan, Thiago Morelli, Shaoping Zhang, et al. 2016. “Genome-Wide Association Study of Biologically Informed Periodontal Complex Traits Offers Novel Insights into the Genetic Basis of Periodontal Disease.” *Human Molecular Genetics* 25 (10): 2113–29. https://doi.org/10.1093/hmg/ddw069.

Özer Yücel, Ozlem, Ezel Berker, Lütfiye Mesci, Kenan Eratalay, Eser Tepe, and Ilhan Tezcan. 2015. “Analysis of TNF-α (-308) Polymorphism and Gingival Crevicular Fluid TNF-α Levels in Aggressive and Chronic Periodontitis: A Preliminary Report.” *Cytokine* 72 (2): 173–77. https://doi.org/10.1016/j.cyto.2015.01.001.

Richards, Derek, W. Marcenes, N. J. Kassebaum, E. Bernabé, M. Dahiya, B. Bhandari, and C. J. Murray. 2014. “Review Finds That Severe Periodontitis Affects 11% of the World Population.” *Evidence-Based Dentistry* 15 (3): 70–71. https://doi.org/10.1038/sj.ebd.6401037.

Saitoh, Tatsuya, Naonobu Fujita, Myoung Ho Jang, Satoshi Uematsu, Bo Gie Yang, Takashi Satoh, Hiroko Omori, et al. 2008. “Loss of the Autophagy Protein Atg16L1 Enhances Endotoxin-Induced IL-1β Production.” *Nature* 456 (7219): 264–68. https://doi.org/10.1038/nature07383.

Salem, Mohammad, Mette Ammitzboell, Kris Nys, Jakob Benedict Seidelin, and Ole Haagen Nielsen. 2015. “ATG16L1: A Multifunctional Susceptibility Factor in Crohn Disease.” *Autophagy* 11 (4): 585–94. https://doi.org/10.1080/15548627.2015.1017187.

Sanjuan, Miguel A., Sandra Milasta, and Douglas R. Green. 2009. “Toll-like Receptor Signaling in the Lysosomal Pathways.” *Immunological Reviews* 227 (1): 203–20. https://doi.org/10.1111/j.1600-065X.2008.00732.x.

Sil, Payel, Ginger Muse, and Jennifer Martinez. 2018. “A Ravenous Defense: Canonical and Non-Canonical Autophagy in Immunity.” *Current Opinion in Immunology* 50: 21–31. https://doi.org/10.1016/j.coi.2017.10.004.

Sil, Payel, Jutamas Suwanpradid, Ginger Muse, Artiom Gruzdev, Liwen Liu, David L. Corcoran, Cynthia J. Willson, et al. 2020. “Noncanonical Autophagy in Dermal Dendritic Cells Mediates Immunosuppressive Effects of UV Exposure.” *Journal of Allergy and Clinical Immunology* 145 (5): 1389–1405. https://doi.org/10.1016/j.jaci.2019.11.041.

Slowicka, Karolina, Lars Vereecke, Conor Mc Guire, Mozes Sze, Jonathan Maelfait, Annasaheb Kolpe, Xavier Saelens, Rudi Beyaert, and Geert van Loo. 2016. “Optineurin Deficiency in Mice Is Associated with Increased Sensitivity to Salmonella but Does Not Affect Proinflammatory NF-KB Signaling.” *European Journal of Immunology* 46 (4): 971–80. https://doi.org/10.1002/eji.201545863.

Socransky, S. S., A. D. Haffajee, M. A. Cugini, C. Smith, and R. L. Kent. 1998. “Microbial Complexes in Subgingival Plaque.” *Journal of Clinical Periodontology* 25 (2): 134–44. https://doi.org/10.1111/j.1600-051X.1998.tb02419.x.

Song, Gwan Gyu, Jae Hoon Kim, and Young Ho Lee. 2013. “Toll-like Receptor (TLR) and Matrix Metalloproteinase (MMP) Polymorphisms and Periodontitis Susceptibility: A Meta-Analysis.” *Molecular Biology Reports* 40 (8): 5129–41. https://doi.org/10.1007/s11033-013-2616-1.

Sun, Qiming, Jing Zhang, Weiliang Fan, Kwun Ngok Wong, Xiaojun Ding, She Chen, and Qing Zhong. 2011. “The RUN Domain of Rubicon Is Important for HVps34 Binding, Lipid Kinase Inhibition, and Autophagy Suppression.” *Journal of Biological Chemistry* 286 (1): 185–91. https://doi.org/10.1074/jbc.M110.126425.

Tam, Jenny M., Michael K. Mansour, Nida S. Khan, Michael Seward, Sravanthi Puranam, Antoine Tanne, Anna Sokolovska, et al. 2014. "Dectin-1-Dependent LC3 Recruitment to Phagosomes Enhances Fungicidal Activity in Macrophages." *Journal of Infectious Diseases* 210 (11): 1844–54. https://doi.org/10.1093/infdis/jiu290.

Trevilatto, Paula C., Ana Paula De Souza Pardo, Raquel M. Scarel-Caminaga, Rui B. De Brito, Fabiano Alvim-Pereira, Claudia C. Alvim-Pereira, Christian M. Probst, Gustavo P. Garlet, Antonio W. Sallum, and Sergio R.P. Line. 2011. "Association of IL1 Gene Polymorphisms with Chronic Periodontitis in Brazilians." *Archives of Oral Biology* 56 (1): 54–62. https://doi.org/10.1016/j.archoralbio.2010.09.004.

Ueyama, Takehiko, Junya Nakakita, Takashi Nakamura, Takeshi Kobayashi, Toshihiro Kobayashi, Jeonghyun Son, Megumi Sakuma, Hirofumi Sakaguchi, Thomas L. Leto, and Naoaki Saito. 2011. "Cooperation of P40phox with P47phox for Nox2-Based NADPH Oxidase Activation during Fcγ Receptor (FcγR)-Mediated Phagocytosis: Mechanism for Acquisition of P40phox Phosphatidylinositol 3-Phosphate (PI(3)P) Binding." *Journal of Biological Chemistry* 286 (47): 40693–705. https://doi.org/10.1074/jbc.M111.237289.

Wan, Jing Hong, Emmanuel Weiss, Sanae Ben Mkaddem, Morgane Mabire, Pierre Marie Choinier, Olivia Picq, Tristan Thibault-Sogorb, et al. 2020. "LC3-Associated Phagocytosis Protects against Inflammation and Liver Fibrosis via Immunoreceptor Inhibitory Signaling." *Science Translational Medicine* 12 (539): 1–13. https://doi.org/10.1126/scitranslmed.aaw8523.

Wan, Yushun, Wei Cao, Tao Han, Sheng Ren, Jian Feng, Tie Long Chen, Jun Wang, Ruth Broering, Mengji Lu, and Ying Zhu. 2017. "Inducible Rubicon Facilitates Viral Replication by Antagonizing Interferon Production." *Cellular and Molecular Immunology* 14 (7): 607–20. https://doi.org/10.1038/cmi.2017.1.

Wong, S.-W., B.-W. Huang, X. Hu, E. Ho Kim, J.P. Kolb, R.J. Padilla, P. Xue, et al. 2020. "Global Deletion of Optineurin Results in Altered Type I IFN Signaling and Abnormal Bone Remodeling in a Model of Paget's Disease." *Cell Death and Differentiation* 27 (1). https://doi.org/10.1038/s41418-019-0341-6.

Wong, Sing Wai, Payel Sil, and Jennifer Martinez. 2018. "Rubicon: LC3-Associated Phagocytosis and Beyond." *FEBS Journal* 285 (8): 1379–88. https://doi.org/10.1111/febs.14354.

Yang, Chul Su, Jong Soo Lee, Mary Rodgers, Chan Ki Min, June Yong Lee, Hee Jin Kim, Kwang Hoon Lee, et al. 2012. "Autophagy Protein Rubicon Mediates Phagocytic NADPH Oxidase Activation in Response to Microbial Infection or TLR Stimulation." *Cell Host and Microbe* 11 (3): 264–76. https://doi.org/10.1016/j.chom.2012.01.018.

Zhong, Yun, Qing Jun Wang, Xianting Li, Ying Yan, Jonathan M. Backer, Brian T. Chait, Nathaniel Heintz, and Zhenyu Yue. 2009. “Distinct Regulation of Autophagic Activity by Atg14L and Rubicon Associated with Beclin 1-Phosphatidylinositol-3-Kinase Complex.” *Nature Cell Biology* 11 (4): 468–76. https://doi.org/10.1038/ncb1854.