EPITHELIAL CELL AUTOPHAGY IN ANTIBACTERIAL DEFENSE OF THE SMALL INTESTINE

APPROVED BY SUPERVISIORY COMMITTEE

_____
Lora V. Hooper, Ph.D.

_____
Beth Levine, M.D.

_____
Vanessa Sperandio, Ph.D.

_____
Felix Yarovinsky, M.D.

ACKNOWLEDGEMENTS

First, I would like to thank my mentor, Dr. Lora Hooper, for taking me into her lab and under her tutelage. I have learned a staggering amount from her and have grown scientifically in ways I never imagined possible. Her passion, insight and audacity inspires the entire lab to ask bold scientific questions. I thank her for exposing me to epithelial cell biology and innate immunity and for creating a fantastic laboratory environment where creative thinking is fostered. I would also like to thank the Hooper lab members, who have all contributed to this work in some fashion. Specifically, I would like to thank Dr. Shipra Vaishnava; she is an excellent scientist and teacher and taught me many of the critical microscopy skills used in my thesis. I thank post-doctoral fellows, Drs. Sohini Mukherjee and Breck Duerkop, for the guidance and expertise in biochemical or microbiological, respectively, studies. I would also like to thank Kelly Ruhn, Cassie Boyd and Charmaine Clements for their tireless efforts in maintaining the Hooper lab mouse colony. The other lab members have all contributed to a lively and thought-provoking lab environment.

I would like to thank other individuals at UT Southwestern who have collaborated on this work. Most of all I thank Dr. Beth Levine and former and current members of her lab Dr. Anthony Orvedahl and Dr. Rhea Sumpter. This work would not have been possible without the autophagy knowledge, expertise and generous time investment provided by Dr. Levine and her group members.

Additionally, the core facilities of UT Southwestern have been incredible vital and crucial to the development of this work. I would like to thank Dr. James Richardson and John Shelton of the Molecular Pathology Core and Laurie Mueller of the Electron Microscopy Core.

I am thankful for my wife, Dr. Kimberly Thomas Benjamin. I am lucky to have you as a colleague and confidant. I thank you for your constant support and encouragement. I love you very much.

Finally, I would like to thank my family. To my brothers, Kareem and Zaheer, I thank you both for your support and love. To my mother-in-law, Dorothy Thomas, I thank you for welcoming me into your family and your wisdom. Lastly, I would like to thank my parents. The constant love and support of my parents allowed me to believe this would be possible; I will always strive to live up to the shining examples set by both of you.

EPITHELIAL CELL AUTOPHAGY IN ANTIBACTERIAL DEFENSE OF THE SMALL INTESTINE

by

JAMAAL LOUIS BENJAMIN

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

July, 2013

EPITHELIAL CELL AUTOPHAGY IN ANTIBACTERIAL DEFENSE OF THE SMALL INTESTINE

Jamaal Louis Benjamin, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2013

Lora V. Hooper, Ph.D.

The intestines of all mammals are colonized with a diverse microbiota that provide metabolic benefits to their hosts. However, this symbiotic relationship can break down when resident bacteria opportunistically invade the intestinal barrier, leading to pathologies such as inflammatory bowel disease (IBD), and bacteremia. As a result, epithelial cell innate immune responses play an essential role in preventing bacterial invasion of host tissues and maintaining a symbiotic host-bacterial relationship. Autophagy is emerging as an important component of innate immunity. Mounting evidence suggests that dysregulation of the autophagy-independent function of autophagy genes can lead to inflammatory bowel disease. However, little is known about the role of autophagy-dependent gene function in controlling interactions between intestinal bacteria

and the intestinal epithelium *in vivo*. In this study, I have demonstrated that small intestinal epithelial cell autophagy is essential for protection against tissue invasion by intestinal pathogens and opportunistically invasive commensals. I have shown that small intestinal autophagy is an early innate immune response that functions in an epithelial cell-intrinsic MyD88-dependent, NOD2-independent manner. Utilizing mice deficient in small intestinal epithelial cell autophagy (*Atg5*$^{\Delta IEC}$), I have determined that epithelial cell autophagy is required to limit pathogen dissemination to extraintestinal sites. This study thus shows that autophagy is a critical mechanism of innate immune defense that protects intestinal epithelial surfaces from bacterial invasion. My findings may lead to new insights into how autophagy protects against gastrointestinal infections and maintains homeostasis with the intestinal microbiota.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS ..... **ii**

ABSTRACT ..... **vi**

TABLE OF CONTENTS ..... **viii**

LIST OF PUBLICATIONS ..... **xi**

LIST OF FIGURES ..... **xiii**

LIST OF ABBREVIATIONS ..... **xvi**

CHAPTER ONE: THE INTESTINAL MICROBIOTA 1

INTRODUCTION 1

FUNCTIONS OF INTESTINAL MICROBIOTA 3

ENHANCING DIGESTIVE EFFICIENCY 3

PROMOTING IMMUNE SYSTEM DEVELOPMENT 4

PROVIDING PROTECTION AGAINST PATHOGENS 7

CELLULAR MAKEUP OF THE INTESTINAL EPITHELIAL BARRIER 9

HOST MECHANISMS FOR SENSING BACTERIA 11

MECHANISMS LIMITING BACTERIAL INVASION

OF INTESTINAL TISSUES 17

CHAPTER TWO: AUTOPHAGY 22

INTRODUCTION 22

THE AUTOPHAGY MACHINERY 24

METABOLIC REGULATION OF AUTOPHAGY 26

MTOR AND STARVATION 26

EXERCISE 28

AUTOPHAGY IN INNATE IMMUNITY AND INFECTION 29

BACTERIAL SUBVERSION AND EVASION OF AUTOPHAGY 33

AUTOPHAGY IN INTESTINAL DISEASE 35

AIMS OF THIS STUDY 38

CHAPTER THREE: METHODOLOGY 40

ANIMALS 40

BACTERIAL STRAINS 40

ANTIBODIES AND REAGENTS 41

IMMUNE DETECTION OF AUTOPHAGOSOMES 41

ISOLATION OF SMALL INTESTINAL EPITHELIAL CELLS 42

ELECTRON MICROSCOPY 43

MOUSE INFECTION 43

FLUORESENCE IN SITU HYBRIDIZATION 43

CHAPTER FOUR: INTESTINAL EPITHELIAL AUTOPHAGY IS ESSENTIAL FOR HOST DEFENSE AGAINST INVASIVE BACTERIA.....45

INTRODUCTION 45

BACTERIAL INDUCTION OF AUTOPHAGY IN SMALL INTESTINAL EPITHELIAL CELLS 48

AUTOPHAGY ACTIVATION BY *ENTEROCOCCUS FAECALIS*, A NATIVE MEMBER OF THE MICROBIOTA 52

ULTRASTRUCTURE OF INTESTINAL EPITHELIAL AUTOPHAGOSOMES ...55

INVASIVE BACTERIA ELICIT INTESTINAL EPITHELIAL AUTOPHAGY 56

EPITHELIAL CELL INTRINSIC MYD88 IS REQUIRED FOR AUTOPHAGY ACTIVATION 58

AUTOPHAGY INDUCTION IN THE INTESTINAL EPITHELIUM IS NOT TRIF-DEPENDENT 61

NOD2 INDEPENDENT ACTIVATION OF EPITHELIAL AUTOPHAGY.....61

EPITHELIAL AUTOPHAGY LIMITS BACTERIAL DISSMEMINATION TO EXTRAINTESTINAL TISSUES.....63

CHARACTERIZATION OF ATG5$^{\Delta IEC}$ MICE.....64

DISCUSSION.....69

CHAPTER FIVE: CONCLUSIONS AND FUTURE DIRECTIONS 75

GENERAL DISCUSSION 75

REGULATION OF BACTERIA-INDUCED AUTOPHAGY 77

MECHANISM OF MYD88 REGULATION OF AUTOPHAGY 78

BCL-2: BECLIN 1 FUNCTION IN INTESTINAL EPITHELIAL AUTOPHAGY....79

IDENTIFICATION OF AUTOPHAGY ADAPTORS FOR COMMENSAL BACTERIA....83

AUTOPHAGY IN IMMUNODEFICIENCY....90

CONCLUSIONS....91

BIBLIOGRAPHY....93

PRIOR PUBLICATIONS

Benjamin, J.L., Sumpter, Jr., R., Levine, B. and Hooper, L. V. (2013) Intestinal Epithelial Autophagy is Essential for Host Defense against Invasive Bacteria. Cell Host Microbe. *13*, 723-34.

Ismail, A.S., Severson, K.M., Vaishnava, S., Behrendt, C.L., Yu, X., **Benjamin, J.L**., Ruhn, K.A., Hou, B., DeFranco, A.L. Yarovinsky, F. Hooper, L.V. (2011) Gammadelta intraepithelial lymphocytes are essential mediators of host-microbial homeostasis at the intestinal mucosal surface. Proc. Natl Acad Sci USA. *108*, 8743-8.

LIST OF FIGURES

CHAPTER ONE

FIGURE 1. BACTERIAL SENSING IN THE INTESTINAL EPITHELIUM 13

FIGURE 2. IMMUNE MECHANISMS MINIMIZING EPITHELIAL-BACTERIAL CONTACT 17

CHAPTER TWO

FIGURE 1. AUTOPHAGOSOME FORMATION AND MATURATION.....24

FIGURE 2. UBIQUITIN-LIKE CONJUGATIONS SYSTEM FOR AUTOPHAGY PROTEINS 26

CHAPTER THREE

FIGURE 1. ISOLATION OF EPITHELIAL CELL PROTEINS FROM INTESTINAL TISSUE 42

CHAPTER FOUR

FIGURE 1. *SALMONELLA TYPHIMURIUM* TRIGGERS AUTOPHAGOSOME FORMATION IN SMALL INTESTINE EPITHELIAL CELLS 49

FIGURE 2. WESTERN BLOT ANALYSIS OF ILEAL EPITHELIAL CELL LYSATES FOLLOWING *S. TYPHIMURIUM* INFECTION.....51

FIGURE 3. COLOCALIZATION OF AUTOPHAGOSOMES AND *S. TYPHIMURIUM* – GFP 52

FIGURE 4. *ENTEROCOCCUS FAECALIS* INVADES SMALL INTESTINAL EPITHELIAL CELLS 53

FIGURE 5. *ENTEROCOCCUS FAECALIS* INDUCES FORMATION OF AUTOPHAGOSOMES IN SMALL INTESTINAL EPITHELIAL CELLS 54

FIGURE 6. WESTERN BLOT ANALYSIS OF ILEAL EPITHELIAL CELL LYSATES FOLLOWING *E. FAECALIS* INFECTION.....55

FIGURE 7. ULTRASTRUCTURE OF AUTOPHAGOSOMES INDUCED BY BACTERIA IN SMALL INTESTINAL EPITHELIAL CELLS 56

FIGURE 8. INVASIVE BACTERIA ELICIT AUTOPHAGY IN INTESTINAL EPITHELIAL CELLS 57

FIGURE 9. INTESTINAL COLONIZATION LEVELS OF WILD-TYPE AND MUTANT *S. TYPHIMURIUM* STRAINS 58

FIGURE 10. MYD88 IS REQUIRED FOR INTESTINAL EPITHELIAL AUTOPHAGY.....59

FIGURE 11. EPITHELIAL CELL-INTRINSIC MYD88 IS REQUIRED FOR INTESTINAL EPITHELIAL CELL AUTOPHAGY 60

FIGURE 12. WESTERN BLOT ANALYSIS OF LC3 FROM ILEAL EPITHELIAL CELL LYSATES FOLLOWING *S. TYPHIMURIUM* INFECTION.....60

FIGURE 13. AUTOPHAGY INDUCTION IN THE INTESTINAL EPITHELIUM IS NOT TRIF-DEPENDENT.....61

FIGURE 14. INTESTINAL EPITHELIAL AUTOPHAGY DOES NOT REQUIRE NOD2.....62

FIGURE 15. INCREASED BACTERIAL INVASION INTO THE EPITHELIAL CELLS OF *NOD2*$^{-/-}$ MICE.....63

FIGURE 16. CHARACTERIZATION OF ATG5$^{\Delta IEC}$ MICE 64

FIGURE 17. INTESTINAL EPITHELIAL CELL AUTOPHAGY REQUIRES ATG5.....65

FIGURE 18. WESTERN BLOT AND EPITHELIAL INVASION ANALYSIS FOLLOWING *S. TYPHIMURIUM* INFECTION 66

FIGURE 19. EXTRAINTESTINAL DISSEMINATION OF *S. TYPHIMURIUM* IN ATG5$^{FL/FL}$ AND ATG5$^{\Delta IEC}$ MICE.....67

FIGURE 20. BACTERIAL BURDEN OF *S. TYPHIMURIUM* AFTER INTRAPERITONEAL INJECTION 68

FIGURE 21. PARACELLULAR PERMEABILITY ASSAY. 68

CHAPTER FIVE

FIGURE 1. MODEL OF MYD88 INTERACTION WITH AUTOPHAGY MACHINERY 79

FIGURE 2. AUTOPHAGY ACTIVATION BY *S.TYPHIMURIUM* IN BCL-2^{AAA} MICE..... 80

FIGURE 3. *PROTEUS MIRABILIS* TRIGGERS AUTOPHAGOSOME FORMATION IN SMALL INTESTINAL EPITHELIAL CELLS 84

FIGURE 4. PROTEOMIC DISCOVERY OF NOVEL ANTIBACTERIAL AUTOPHAGY ADAPTORS 87

FIGURE 5. EPITHELIAL CELL PROTEOME LIBRARY FOR PROTEIN DISCOVERY.....89

FIGURE 6. CONVENTIONAL MICROBIOTA ACTIVATE AUTOPHAGY IN REGIII GAMMA KNOCKOUT MICE 91

LIST OF ABBREVIATIONS

α – anti

Ab – antibody

Abx – antibiotics

Atg – Autophagy-related gene

Bcl-2 – B-cell lymphoma 2

B. theta – *Bacteroides thetaiotaomicron*

BSA – bovine serum albumin

Cfu – colony forming unit

C. rodentium – *Citrobacter rodentium*

CV – conventional

Cy3 – cyanine 3

ECB – epithelial cell border

Ef – *Enterococcus faecalis*

EM – electron microscopy

FITC – fluorescein isothiocyanate

GF – germ free

GFP – green fluorescent protein

HRP – horseradish peroxidase

H&E – hematoxylin and eosin

IBD – Inflammatory Bowel Disease

IEL – intraepithelial lymphocyte

IL-1R – Interleukin-1 receptor

IL-18R – Interleukin-18 receptor

InvA – Invasion gene A

Ig – immunoglobulin

IL – interleukin

iNKT – invariant natural killer T cell

LC3 – microtubule-associate protein light chain 3

LPS – lipopolysaccharide

LRR – Leucine-rich repeat

LS – *Lactobacillus salivarius*

MAMP – microbe-associated molecular pattern

MAPK – Mitogen associated protein kinase

MHC – major histocompatibility complex

MLN – mesenteric lymph node

mRNA – messenger RNA

MT – mitochondria

mTOR – mammalian Target of rapamycin

MNV – mouse Norovirus

MyD88 – Myeloid differentiation primary response gene 88

NFκB – nuclear factor κB

NOD – nucleotide-binding oligomerization domain

OCT – optimal cutting temperature compound

PAGE – polyacrylamide gel electrophoresis

PAMP – pathogen associated molecular pattern

PBS – phosphate buffered saline

PE – phosphatidyl ethanolamine

PRR – pattern recognition receptor

PVDF – polyvinylidene difluoride

RegIIIɣ - Regenerating islet-derived protein 3 gamma

rRNA – ribosomal RNA

SFB – segmented filamentous bacteria

SPI-I – *Salmonella* pathogenicity island 1

St – *Salmonella typhimurium*

T-PER – tissue protein extraction reagent

TBS-T – Tris-buffered Saline and Tween 20

TJ – tight junction

TRIF – Toll-interleukin-1 receptor domain containing adaptor-inducing interferon-β

TLR – Toll-like receptor

WT – wild-type

CHAPTER ONE

The Intestinal Microbiota

Introduction

Bacteria represent some of the most ancient inhabitants of our planet and have been members of the Earth's biota from at least the past 2700 million years (Brocks et al., 1999). These bacteria colonize a wide array of environments and ecological niches. The remarkable adaptability of bacteria is best exemplified by the extreme range of environments which bacteria call home, ranging from deep-sea geothermal vents at the bottom of the ocean floor to arsenic laden lakes. Bacteria are capable of not only surviving but thriving in staggering wide variety of conditions (Petersen et al., 2011) (Wolfe-Simon et al., 2011).

The mammalian intestine is home to a complex and diverse population of bacteria. Astonishingly, the total number of bacterial cells within the human host exceeds the total number of human cells in the body by more than tenfold (Savage, 1977). Prior to the advent of high-throughput sequencing, some of the most basic questions about the constituents of the intestinal microbiota were unanswerable. These questions include, 1) What are the bacterial species present? 2) What is the normal ratio of these constituents? 3) Is there diversity in the genetic background of intestinal bacteria? However, in recent years, massive projects such as the US Human Microbiome Progect (HMP) and the European Metagenomics of the Human Intestinal Tract (MetaHIT) aimed at understanding the relationship between gut microbes and the human host have made significant progress towards answering some of these basic questions.

Culture dependent analysis of the microbiota initially suggested that the majority of healthy adults had a ‘core’ microbiota that could be consistently identified in most people. However, further investigation via culture independent methods revealed a more nuanced picture. The more sensitive high-throughput genotyping strategies rely on identification of bacterial species through rDNA genotyping of sequences in their small subunit (16S) of ribosomal RNA genes. Utilizing this rDNA genotyping strategy, it was demonstrated that each person has more than 1,000, species-level phylotypes. The vast majority of these phylotypes belong to a small number of phyla. In adults, Firmicutes and Bacteroidetes are the primary components of the microbiota and the Proteobacteria, Verrucomicrobia and Actinobacteria typically representing minor members (Claesson et al., 2009).

The members of this microbial community are essential to host metabolism and digestive efficiency, promotion of immune system development and protection against pathogenic bacterial infection, thereby creating a functionally mutualistic relationship. Although the overall host-microbial relationship is symbiotic, the bacterial components of this community span a wide spectrum of lifestyles, encompassing benign commensals, opportunistically pathogenic pathobionts, and frank pathogens (O'Hara et al., 2006) (Eckburg et al., 2005). Additionally, the proximity of such a vast number of microbes to the intestinal epithelial surface poses an added challenge to the host, which must limit bacterial invasion of intestinal tissue without eliciting damaging inflammatory responses. In my thesis, I have explored the role of autophagy as an epithelial cell-intrinsic cellular process that limits bacterial persistence in epithelial cells and prevents their dissemination to extraintestinal sites.

Functions of Intestinal Microbiota

Enhancing Digestive Efficiency

The human intestine is home to 10 to 100 trillion bacteria. A major driver of the evolution of commensalism between humans and bacteria was the enhanced metabolic capabilities endowed upon humans by their microbial communities, including the capability of liberating nutrients and energy from portions of our diet that, without bacteria, would be inaccessible.

The metabolic relationship between the host and the intestinal microbiota is spotlighted when examining the lifestyle of the bacterium *Bacteroides thetaiotaomicron*. *B. thetaiotaomicron* is a protypical member of the phylum Bacteroidetes, a highly abundant obligate anaerobe found in the intestinal microbiota of most normal adult humans. Interestingly, *B. thetaiotaomicron* has a very large percentage of its genome related to carbohydrate uptake and utilization (Xu et al., 2003). Additionally, greater than 50 percent of the polysaccharide-degrading enzymes are predicted to be secreted into the extracellular or periplasmic space, allowing the bacterium to access both dietary and mucus based polysaccharides and break them into simpler oligo or monosaccharides that can then be absorbed by the bacterium or human host. Indeed, following monoassociation in the small intestines of male, germ-free mice, *B. thetaiotaomicron* was found to assemble on food particles and mucus. Once *B. thetaiotaomicron* colonizes the gut, it then induces production of glycoside hydrolases and outer-membrane polysaccharide-binding proteins.

In the intestines of fed germ-free mice, the most abundant monosaccharides were found to be arabinose, glucose and xylose, however following monocolonization with *B. thetaiotaomicron*, significant reductions in the same hexoses, arabinose, glucose and xylose, were noticed while no changes in pentose sugar levels were noted. The depletion of abundant hexoses following bacterial colonization is caused by the combination of increased host uptake and utilization of the liberated monosaccharide and microbial utilization (Sonnenburg et al., 2005). The presence of the bacterium allows the host to access sugars previously unavailable and simultaneously gives the bacterium continuous nutrient exposure and an environmental niche. Examining the relationship between the host and intestinal bacteria highlights a key function of the intestinal microbiota: enhancing digestive efficiency. Both sides win, establishing a mutually-beneficial host-microbial relationship.

Promoting immune system development

Initial exposure to microbes during early life has a profound impact on the normal development of the mammalian immune system. The intestine represents the largest immunological compartment of the body. The perpetual exposure of the host immune system to microbial antigens is critical for normal development and encompasses part of the immune system known as the gut – associated lymphoid tissues, or GALT.

IgA production

As mammals pass through the birth canal, they come into contact with the mother's fecal matter and are thus able to acquire her intestinal bacteria. These bacteria

then become major drivers of immune system development. Indeed, a similar transition and change to the immune system is seen as germ-free mice undergo conventionalization. When germ-free mice are moved from their isolators to specific pathogen-free conditions, increases in all immunoglobulin classes, except IgM, are seen (Horsfall et al., 1978). Of these changes in immunoglobulin levels, the most striking change is in the production of IgA, and more specifically, secretory IgA, an antibody class specific to mucosal surfaces.

Subsequent work has demonstrated that the development of the gut-associated lymphoid tissues (GALT) is initiated by the colonization of the intestines with bacteria. The role for intestinal microbiota in driving immune function is highlighted by the fact that at least 80 percent of all immunoglobulin producing plasma cells are found in the intestinal lamina propria, of which most produce secretory IgA. A particularly interesting finding, was that monoassociation of germ-free mice with a commensal bacteria, *Morganella morganii*, resulted in the production of broad-specturum IgA in an antigen-nonspecific manner. Amazingly, greater than 85% of the IgA produced by these animals was not reactive to the *M. morganii* with which the mouse was colonized (Shroff et al., 1995). Therefore, colonization of the intestine with the intestinal microbiota endows the mouse with protection against unseen antigens. The ability and importance of the innate IgA or natural IgA, as they are termed, to bind multiple antigens and protect against pathogenic bacterial invasion is further emphasized when considering mice deficient in the polymeric immunglobulin receptor (pIgR).

The polymeric immunoglobulin receptor mediates the active transport of secretory IgA and IgM across mucosal surfaces. Mice deficient in the pIgR are unable to bind and actively transport dimeric IgA and pentameric IgM and completely lack active

external IgA and IgM (Johansen et al., 1999). When pIgR deficient mice are orally challenged with *S. typhimurium*, they are profoundly more sensitive to disease than their wild-type controls, exhibiting increased epithelial invasion by *S. typhiumurium*, a lower median lethal dose of *S. typhimurium* and increased fecal-oral spread of *S. typhimurium* infection (Wijburg et al., 2006). Taken together, these data emphasize the crucial role of the intestinal microbiota in development of IgA production as well as the critical role of sIgA production in maintaining intestinal homeostasis.

Invariant natural killer T cells (iNKT cells)

Invariant natural killer T cells are important immune mediators throughout the body but are especially important in the gut. They are thought to play an important role in the pathogenesis of inflammatory bowel disease. iNKT cells recognize antigens presented to them by the major histocompatibility complex (MHC) class I. In response to activation, iNKT cells produce and secrete large amounts of proinflammatory cytokines such as interleukin-4 (IL-4) and interleukin-13 (IL-13).

A recent study showed that intestinal microbiota have a profound influence over the development of iNKT cells during a specific developmental window. Analysis of iNKT cell populations revealed that germ-free (GF) mice had higher numbers of iNKT cells in the lamina propria of the colon both after weaning and later in life. Utilizing the oxazolone-induced colitis model, a model of intestinal inflammation that is dependent on IL-13 production by iNKT cells, GF mice were found to be more sensitive to colitis than specific pathogen-free (SPF) mice. However, if pregnant GF mice were colonized with

the SPF microbiota just prior to delivery, their pups exhibited completely normal iNKT cell levels that persisted for months.

The expression of the chemokine receptor CXCR6 and its ligand CXCL16 play an important role in iNKT cell homeostasis (Germanov et al., 2008). Expression of *Cxcl16* was significantly increased in the colons of GF mice when compared to SPF mice. Indeed, it was demonstrated that hypermethlyation of the 5' region of the Cxcl16 gene occurs in germ-free mice, but neonatal colonization of GF mice with SPF microbiota was capable of decreasing the hypermethylation of Cxcl16 to SPF levels. Interestingly, the reversibility of the hypermethylation did not occur when adult GF mice were conventionalized, highlighting the importance of the microbiota in normal immune system and iNKT cell development during early life (Olszak et al., 2012).

Providing protection against pathogens

In addition to enhancing digestive efficiency and promoting immune system development, the intestinal microbiota also plays an important role in providing protection against invasive intestinal pathogens. Perhaps one of the best studied examples of a beneficial space-filling commensal is the bacterial genus *Bifidobacterium*. One of the first demonstrations of the protective effects of *Bifidobacterium* was seen when researchers were examining the effects of different *Bifidobacteria* strains on the mouse response to Shiga toxin-producing *E. coli* O157:H7 (STEC/EHEC). In the absence of beneficial, commensal bacterial strains, mice infected with STEC demonstrate profound weight loss and ultimately die. However, it was noticed that when the mice were pre-colonized with the *Bifidobacterium breve* stain Yakult, they were almost completely

protected from the deleterious effects of Shiga-Toxin producing *E. coli*. Interestingly, not all strains of *Bifidobacterium* were capable of conferring protection, as *B. bifidum* and *B. catenulatum* were unable to protect mice from *E. coli* infection even after colonizing the intestines to high density. In a finding that set the foundation for subsequent discoveries, it was noted that the difference between the protective and non-protective strains included a high production of acetic acid by the protective strains (Asahara et al., 2004). Subsequent experiments with additional strains have confirmed this initial finding and also identified additional strains such as *Bifidobacterium longus,* that also protect mice from a fatal *E. coli* 0157:H7 infection (Yoshimura et al., 2010). Although the phenomenon of protection against pathogens by a commensal had been seen, the mechanism behind the protective effects of *Bifidobacterium* remained unclear.

A significant advancement in our understanding of the commensal protection against pathogens came when researchers began to build upon the observation that protective *Bifidobacterium* strains produced more acetate than non-protective strains. Utilizing a proteomics approach, researchers examined the differential host response to protective and non-protective strains. They found that in the presence of non-protective strains, *E. coli* O157 elicits epithelial cell apoptosis and mucosal inflammation, but in the presence of protective strains like *B. longus*, such inflammation does not occur. Additionally, some of the most striking differences were found in the fecal metabolite composition, with byproducts of carbohydrate metabolism, such as acetate, as the primary difference. They found a correlation between the efficiency of carbohydrate uptake by *Bifidobacterium* and their ability to protect against STEC. They continued by showing that the difference in carbohydrate metabolism was due to an ATP-binding-

cassette carbohydrate transporter. The carbohydrate transporter allowed the protective strains to produce more acetate, and production of acetate was critical in the ability of *B. longus* to protect against fatal Shiga-Toxin producing *E. coli* infections (Fukuda et al., 2011).

Cellular Makeup of the Intestinal Epithelial Barrier

The mucosal surface of the intestinal epithelium represents a vast frontier that serves as the gateway to entry into the human body. In the terminal ileum, the lumen of the small intestine is filled with more than of 10^9 to 10^{10} colony forming units of bacteria per microliter of intestinal contents. The epithelial cell barrier is the primary interface with the intestinal microbiota. The epithelial cell border represents a single cell layer separating outside from inside. There are several different cell types that contribute to the maintenance of a functional epithelial layer, including enterocytes, goblet cells and gammadelta (γδ) T cells.

Enterocytes

Enterocytes are the most numerous cell type in the intestine and they represent the most rapidly self-renewing tissue of adult mammals. These simple, columnar epithelial cells line the villi of the small and large intestines and are joined together by structures known as tight junctions (TJ), which form a critical physical barrier to bacterial entry into host tissues. In addition to the physical barrier presented by enterocytes, they also serve a critical innate immune function in their ability to produce antimicrobial peptides such as RegIIIɣ (Vaishnava et al., 2008). At the base of the small intestinal villus are structures

known as the crypts of Lieberkühn. A notable feature of the crypts is a cluster of specialized cells found at the base of the crypts of Lieberkühn called Paneth cells. Paneth cells are easily recognized by their prominent, apically oriented secretory granules. In contrast to enterocytes which live for only three to five days, Paneth cells in the crypts typically live approximately 30 days. Intestinal stem-cells, which replace old enterocytes that slough off from the villus tip, are thought to reside at the top of the crypts directly above the Paneth cells and are positive for Lgr5. After a new cell is born, it migrates upward while differentiating into enterocytes, goblets cells and tuft cells. Paneth cell secretory granules are antimicrobial and secrete antimicrobial peptides like Ang-4 and lysozyme (Hooper et al., 2003) (Deckx et al., 1967).

Goblet cells

Goblet cells are a secretory cell lineage of the intestinal epithelium. The goblet cells produce and secrete a thick mucus layer which covers the entire intestinal epithelium. The mucus produced by the goblet cells consists primarily of mucin glycoproteins. The mucin glycoproteins form a matrix of protection that extends up to 150 µm from the surface of the epithelium.

Gamma Delta (γδ) T cells

γδ T cells are a unconventional type of T cell, which express γδ T cell receptor (TCR) on their cell surface, and are thought to function in both innate and adaptive immunity. Numerically, γδ intraepithelial lymphocytes (IEL) T cells are the most abundant T cell population in the body, and they play an important role in maintaining

intestinal homeostasis. γδ T cells reside in the intestine, intercalate under epithelial tight junctions, and are constitutively present in the intestinal epithelia (Bandeira et al., 1990) . Work from the Hooper lab has been critical in understanding the function of γδ T cells. Ismail et al have shown that γδ T cells are a critical regulatory input in both the small and large intestine (Ismail et al., 2011). γδ have been shown to play two critical roles within the intestine. The first of these roles is to provide protection against invading commensals following mucosal damage. The second role of γδ T cells is to limit the extraintestinal dissemination of invasive pathogens. Combining these two functions of γδ T cells, γδ T cells are now considered to be a critical component of the immune response to invading bacteria early following infection.

Tuft Cells

Tuft cells are the most recent cell type to be discovered to exist within the small intestinal epithelium. Tuft cells are relatively rare cell types that are defined by their thick brush of long microvilli projecting from its apical surface. Additionally, tuft cells have been shown to be a major source of endogenous opioid production in the intestine with all tuft cells being positive for β-endorphin. Mature tuft cells express DCLK1 and are the only cells in the small intestinal epithelium that express cyclooxygenase enzymes (Gerbe et al., 2011) .

Host Mechanisms for Sensing Bacteria

Over millions of years, eukaryotes have evolved mechanisms that allow them to inspect their surroundings for signals indicating the presence of microbes. This constant surveillance allows the cells to maintain a sterile environment. The danger signals are

known as PAMPs (pathogen-associated molecular patterns) and come in a wide variety of forms, including lipopolysaccharide (LPS), double-stranded RNA, and flagellin. The sensing of PAMPs by eukaryotic cells is performed utilizing pattern recognition receptors (PRRs). PRRs can be cytoplasmic or transmembrane proteins. The ability of intestinal epithelial cells to monitor and sense bacteria is especially crucial because of the close contact with the intestinal microbiota. Therefore, bacterial sensing by intestinal enterocytes is a critical function in the maintenance of normal intestinal health. Furthermore, as I will discuss in Chapter Four, bacterial sensing is essential for the activation of antibacterial autophagy in the intestinal epithelium

TLRs

Among the best characterized pattern recognition receptors are the Toll-like receptors (TLRs). The name Toll-like receptor comes from the similarity of TLRs to the Toll protein initially discovered in *Drosophila melanogaster*. The gene products of TLRs encode two highly conserved motifs. In TLRs, the extracellular portion of the receptor contains a leucine-rich repeat (LRR) which is important in binding of the target ligand. The second conserved portion of the TLR is the intracellular or cytoplasmic portion of the receptor, known as the Toll / Interleukin-1 receptor (TIR) domain. The TIR allows the TLR to transduce its signal and activate downstream response following ligand binding. To date, the human genome has been shown to include at least 13 different TLRs. These TLRs interact with a wide variety of molecular targets, including lipospolysaccharide, a component of the bacterial cell wall of Gram-negative bacteria, unmethlyated CpG DNA, found in the genomes of both bacteria and DNA viruses and flagellin. Additionally, TLRs

can be positioned on the cell surface or within cellular compartments such as endosomes. Another important feature of TLRs is their requirement for adaptor proteins to transduce their signal. So far, there have been four adaptor proteins identified to be involved in TLR signaling, MyD88, Trif, Tirap and Tram. Of these four proteins, two of the best studied are MyD88 and Trif.

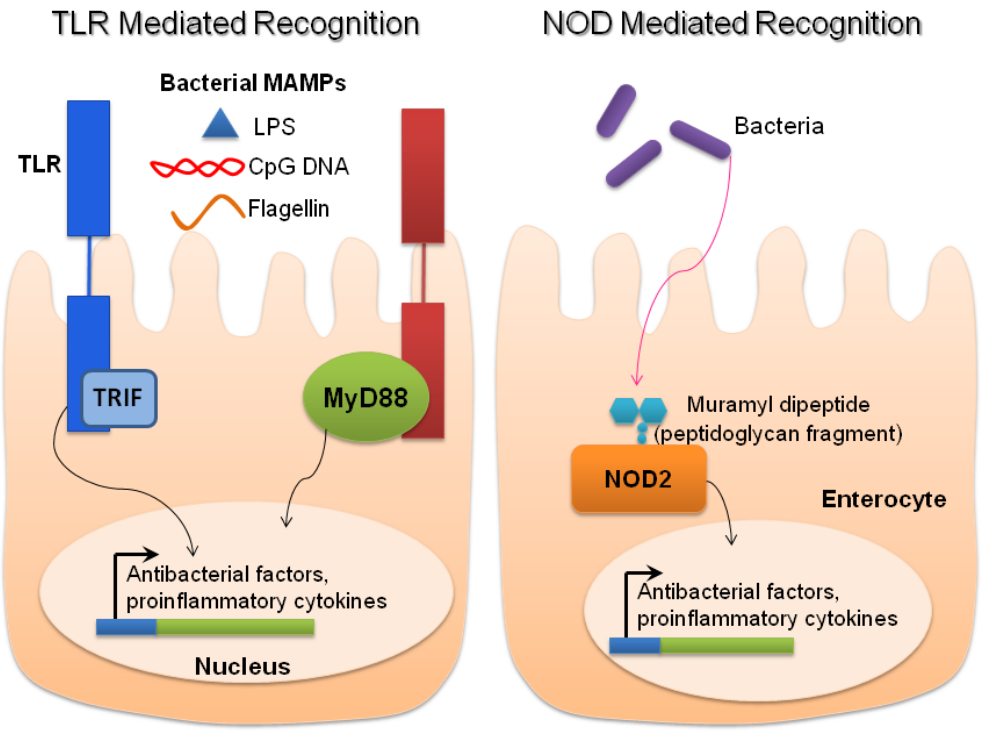

Figure 1: Bacterial Sensing in the Intestinal Epithelium. **(A)** TLR mediated recognition detect bacterial motifs through membrane bound receptors (B) Cytoplasmic Nod2 receptor detects muramyl dipeptide, a major fragment of peptidoglycan.

Myeloid differentiation primary response gene 88 (MyD88) is required for the signaling of all TLRs except TLR3. The primary effect of MyD88 activation is activation of both the mitogen-activated protein kinase (MAP kinase) and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathways. Following MyD88 activation, a series of events occurs which ultimately result in the phosphorlyation and

degradation of the Inhibitor of κB (IκB) which allows NFκB to enter the nucleus and activate the transcription of antibacterial peptides and pro-inflammatory cytokines.

TIR-domain-containing-adaptor-inducing-interferon-β (TRIF) is an adaptor protein involved in the signaling of TLR3 and TLR4. The primary responses following activation of TRIF are activation of two kinases, TANK-binding kinase 1 (TBK1) and receptor interacting protein 1 (RIP1). Activation of TBK1 results phosphorylation and translocation of Interferon Regulatory factor 3 (IRF3) into the nucleus were it turns on the production of type 1 Interferon. Meanwhile, the activation of RIP1 relieves repression of NFκB allowing it to enter the nucleus.

NODs

In addition to the membrane bound class of PRRs, the TLRs, there is also a class of PRRs that reside in the cytosol and are capable of recognizing intracytoplasmic PAMPs. An example of this type of cytoplasmic receptor is the nucleotide-binding oligomerization domain (NOD) family.

Nod proteins have a basic structure that is similar to the TLRs, in that one portion of the protein consists of LRR region which is thought to be responsible for the binding of the PAMP ligand. However, a recent report from the Baumler group at UC Davis has added additional insight into PAMP recognition by NOD receptors. Keestra et al. found that NOD1 senses the presence of cytosolic bacterial PAMPs by monitoring the activation state of small Rho GTPases (Keestra et al., 2013). They found that constitutive activation of RAC1 or CDC42, both Rho GTPases was sufficient to trigger NOD1 signaling. The N-terminal portion of the Nod proteins contains the caspase activation and

recruitment domains (CARDs) which, similarly to the TIR domain of TLRs, functions to activate the downstream response of NOD activation.

The initial observation that led to the discovery of the cytoplasmic PRR was that an invasive-bacterium, *Shigella flexneri*, was capable of inducing an inflammatory response in epithelial cells while a non-invasive strain of *S. flexneri* was not. Moreover, it was shown that microinjection of LPS into cells could induce both NFκB and c-Jun N-terminal kinase (JNK), leading to the production of pro-inflammatory cytokines like IL-8 (Girardin et al., 2001). These findings resulted in the discovery of Nod1/CARD4, a cytosolic protein involved in recognition of bacterial PAMPs.

Having established the existence and location of the Nod proteins, the next major question was the nature of the ligand bound by the LRR region of the Nod proteins. In this case, the initial finding of Nod1 protein activation by microinjection of LPS into epithelial cells was a red herring. Commercial preparations of LPS, which were used in the initial study, are often contaminated with bacterial cell wall components. When highly purified *E. coli* LPS was used to test activation of Nod1 proteins, no activation was seen. Additionally, it was observed that only peptidoglycan preparations from Gram-negative bacteria were could stimulate Nod1 signaling. Taken together, these findings indicated that PAMP activation of the Nod1 protein was unique to Gram-negative peptidoglycan and did not involve LPS or lipid A. After careful analysis of the peptidoglycan fragments released from Gram-negative bacteria, muropeptides were found to be the major peptidoglycan products released. These efforts ultimately resulted in the identification of *N*-acetylglucosamine (GlcNAc or "G") β-1,4 linked to *N*-acetylmuramic acid (MurNAc or "M") substituted with a tripeptide group as the ligand

for Nod1 and GM-dipeptide or M-dipeptide as the ligand for Nod2 (Girardin et al., 2003). These results established that Nod proteins sense similar but non-overlapping motifs that result in pathogen detection within the cytoplasm, with Nod1 sensing a structure unique to Gram-negative bacteria, and Nod2 sensing a structure present in the peptidoglycan of both Gram-negative and Gram-positive bacteria.

Having established the location of the Nod proteins as well as the nature of the ligands sensed by the PRR-domain of the proteins, the next major advance was the demonstration of an *in vivo* functional role for Nod proteins. Investigation of the immune response of wild-type and Nod2 deficient to the intracellular bacteria *Listeria monocytogenes* yielded very interesting results. The Flavell group found that both WT and Nod2 knockout mice demonstrated similar levels of *L. monocytogenes* dissemination to the spleen and liver when the bacteria were introduced via intravenous (IV) or intraperitoneal (IP) methods. However, when Nod2 knockout mice were challenged with *L. monocytogenes* via intragastric gavage, they demonstrated significantly more bacterial translocation to the spleen and liver than their wild-type counterparts. Additionally, it was observed that Nod2 deficient mice expressed significantly lower levels of a class of intestinal antimicrobial peptides known as cryptidins, called α-defensins in humans, especially defensin-related cryptdin 4 (Defcr4) and defensin-related cryptdin 10 (Defcr10) (Kobayashi et al., 2005). These results demonstrated that Nod2 is a critical component of innate immunity within the small intestine, recognizing M-dipeptide (MDP) and regulating antimicrobial peptide production.

Mechanisms Limiting Bacterial Invasion of Intestinal Tissues

For many years, it was accepted dogma that mammalian intestinal tissues are 'tolerant' to intestinal bacteria. In this context, tolerance means that the host tissues exhibit minimal recognition and/or responsiveness to the presence of bacteria. It was proposed that this lack of responsiveness to bacteria is how mammalian hosts maintained homeostasis with the microbiota (Sansonetti, 2004). Another concept that has emerged is that of "ignorance" which refers to the host limiting bacterial contact with intestinal tissues (Duerkop et al., 2009) (Figure 2). In this section, I will discuss the mechanisms that are critical for maintaining immune system ignorance of the microbiota.

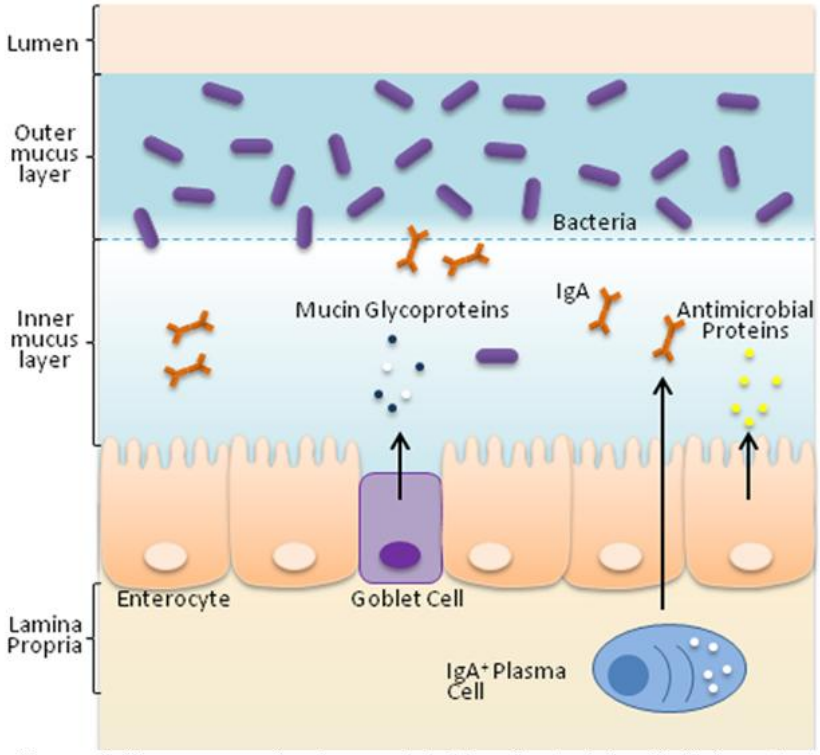

Figure 2: Immune mechanisms minimizing bacterial-epithelial contact The immune system has many mechanisms in place to sequester the microbiota in the lumen of intestine; These include mucus production, secretory IgA and antimicrobial peptides. (Adapted from Duerkop et al, *Immunity*, 2009)

Secretory IgA

IgA is the most abundant immunoglobulin produced in the human body. In fact, the body's total production of IgA exceeds that of all the classes of immunoglobulins combined (Pabst, 2012). Secretory IgA produced the intestine is a critical barrier separating the host from toxins and pathogens. Plasma cells residing in the lamina propria are responsible for the production of polymeric IgA (Figure 3 A-B). Most commonly, IgA is produced as a dimer with two IgA molecules joined with the joining (J) chain. The dimeric IgA molecule then binds the polymeric immunoglobulin receptor (pIgR) on the basolateral portion of the epithelial cell and is transcytosed to the apical side of the cell. When the transcytosed IgA reaches the apical membrane, the polymeric immunoglobulin receptor is cleaved from the IgA. However, not all of the pIgR is cleaved off, and a portion of the pIgR, called the secretory component, remains attached to the secretory IgA. In humans, there are two subclasses of IgA, IgA1 and IgA2. These two classes can be separated by tissue abundance – IgA1 is more abundant in the airways and serum, while IgA2 predominates in colon. Interestingly, the small intestine shows equal levels of both IgA1 and IgA2.

Given the considerable amount of energy expended in the production of secretory IgA, one could easily wonder whether it is efficient to transcytose IgA to simply lose it in the feces. Critical findings regarding the interaction between the intestinal microbiota and secretory IgA are yielding great insight into why secretory IgA is released in this way. A substantial portion of the commensal bacteria population is permanently coated with IgA (van der Waaij et al., 1996) (De Palma et al., 2010). The efficiency in binding to luminal targets by secretory IgA is due in part to utilization of unconventional binding sites. For

example, researchers have demonstrated that both the constant region and secretory component can facilitate binding to antigens (Mantis and Forbes, 2010).

Secretory IgA functions to capture antigens and maintain them at a safe distance from the mucosal surface. A growing body of work has now shown that secretory IgA is a critical portion of the innate immune response to invasive pathogens. Especially germane to this study, binding of secretory IgA to *S. typhimurium* has been shown to reduce *Salmonella* motility (Forbes et al., 2008). Utilizing a monoclonal IgA antibody to O antigen of lipopolysaccharide of *S. typhimurium*, it was seen that upon addition of IgA, *S. typhimurium*'s flagellar-based motility immediately ceased. Additionally, it was seen that the IgA blocked the SPI-I-dependent invasion of intestinal eptihelial cells by *S. typhimurium*. Invasion of intestinal epithelial cells *by S. typhimurium* is a Type 3 secretion system (T3SS), Spi-I-dependent process.

Finally, a major question surrounding IgA production in the intestine is the question of antigen specificity and the relationship between the two different types of IgA found in the intestine – classical IgA and natural IgA. Classical IgA, as it sounds, features IgA binding in a conventional manner, with a high-affinity antigen-specific interaction. Conversely, natural IgA features a low-affinity, non-specific antigen interactions. The idea of natural IgA when it was first observed was that a large portion of IgA produced in the intestine following monocolonization of germ free mice is not specific to the bacteria colonized the intestine (Shroff et al., 1995). The current accepted thinking is that the classical IgA develops in a T-cell dependent manner (Slack et al., 2012). In contrast, natural IgA is thought to originate in a T-cell independent manner, with the IgA positive plasma cell originating from structures known as isolated lymphoid follicles (ILFs).

Antimicrobial Peptides

Peptides and proteins that kill bacteria are a key mechanism by which the intestine limits physical interactions between the microbiota and host tissues. The intestinal epithelium produces a varied repertoire of antimicrobial proteins belonging to different protein families, including C-type lectins, ribonucleases and defensins.

The C-type lectins are proteins of approximately 15 kDa and utilize their lectin domains to recognize and bind to peptidoglycan of the bacterial cell wall. Of the C-type lectin family of antimicrobial peptides, regenerating islet-derived protein III gamma (RegIIIɣ) or HIP/PAP in humans is perhaps the best understood and well characterized. RegIIIɣ is expressed throughout the epithelium of the small intestine, in all epithelial lineages including enterocytes, goblet cells and Paneth cells. Biochemical and *in vivo* work on RegIIIɣ in the Hooper lab have provided considerable insight into how recognition of bacterial peptidoglycan by RegIIIɣ is mediated and into the physiological role of RegIIIɣ in the small intestine.

To uncover the *in vivo* functional role of RegIIIɣ in the intestine, studies were carried out on RegIIIɣ-deficient mice. In wild-type mice, there is a relatively bacteria free zone of approximately 50 µm immediately adjacent to the epithelial cell surface of the small intestine. However, RegIIIɣ-deficient mice showed extensive bacterial invasion into this normally protected zone. The bacteria were predominantly Gram-positive bacteria, which accords with the Gram-positive specificity of RegIIIɣ antibacterial activity. Additionally, RegIIIɣ deficient mice exhibitied increased numbers of IgA positive plasma cells in their lamina propria as well as higher levels of secretory IgA in the feces (Vaishnava et al., 2011). Taken together, these data highlight the importance of

antimicrobial peptide production and secretion in the maintenance of normal, healthy intestinal function.

Although RegIIIγ has clearly been shown to be an important innate immune effector maintaining a relatively bacteria free zone near the epithelial cell surface, there are some critical remaining questions. How does the host handle Gram-negative bacteria that are not killed by RegIIIγ? What happens to bacteria that manage to invade the epithelial cell cytoplasm? Are there epithelial cell-intrinsic innate immune responses that limit the persistence and dissemination of invading bacteria? These questions led me to investigate the role of epithelial cell autophagy in maintaining homeostasis with the intestinal microbiota. I hypothesized that epithelial cell autophagy would be uniquely positioned to handle the challenge of clearing invading bacteria in a cell-intrinsic manner. In the next chapter, I will discuss autophagy and its functions in immunity.

CHAPTER TWO

Autophagy

Introduction

The lysosomal degradation pathway is the only known mechanism for eukaryotic cells to target molecules too large for the proteasome for destruction. Autophagy is the general term used for the pathway of delivery of molecules to the lysosome for degradation.

The broad term "autophagy" generally refers to any process that involves the hallmark structure of the double-membrane autophagosome. However, there are at least three different types of autophagy – microautophagy, chaperone-mediated autophagy and macroautophagy. In macroautophagy or simply autophagy, a portion of the cytoplasm is engulfed by an isolation membrane. The isolation membrane subsequently matures into a double-membrane structure known as the autophagosome. There are a number of known targets for autophagy, including intracellular organelles such as the mitochondria, protein aggregates or intracellular microbes such as viruses, protozoa, fungi or bacteria. Additionally, autophagy has been shown to be an important cellular response to both starvation and inflammatory responses. Given the diverse functions of autophagy, understanding the function and regulation of autophagy in the intestine is critically important. By understanding how epithelial cell autophagy functions *in vivo*, we can learn a great deal about the central functions performed by epithelial cells – immunity and metabolism.

Autophagy is an evolutionarily ancient homeostatic process in which cytoplasmic materials are targeted to the lysosome for degradation. Portions of the cytoplasm are sequestered into double-membrane structures called autophagosomes which fuse with lysosomes, delivering their contents for degradation. It has long been known that autophagy is essential for cellular homeostasis by degrading cytoplasmic contents during starvation, and by recycling damaged organelles and proteins. More recently, autophagy has been shown to be critical for the recognition and degradation of intracellular pathogens, thus functioning as an innate barrier to infection. Anti-viral autophagy promotes the *in vivo* elimination of several viral pathogens including Sindbis virus and HSV-1. Autophagy also plays an important role in activating adaptive immune responses by promoting major histocompatibility complex class II (MHC II)-restricted antigen presentation.

Autophagy also functions in anti-bacterial innate immunity by limiting the replication of bacteria that invade host cells. Bacterial targets of autophagy include *Salmonella typhimurium* and *Listeria monocytogenes*. However, the importance of autophagy for anti-bacterial immunity in mammalian hosts *in vivo* remains underexplored. Such functions are likely to be especially important in the intestinal epithelium, which interfaces with a dense microbial community that harbors invasive bacteria, and which acts a critical barrier to bacterial penetration into deeper tissues. In this chapter, I will review the current state of knowledge about the functions of autophagy in both metabolic homeostasis and in immunity.

The Autophagy Machinery

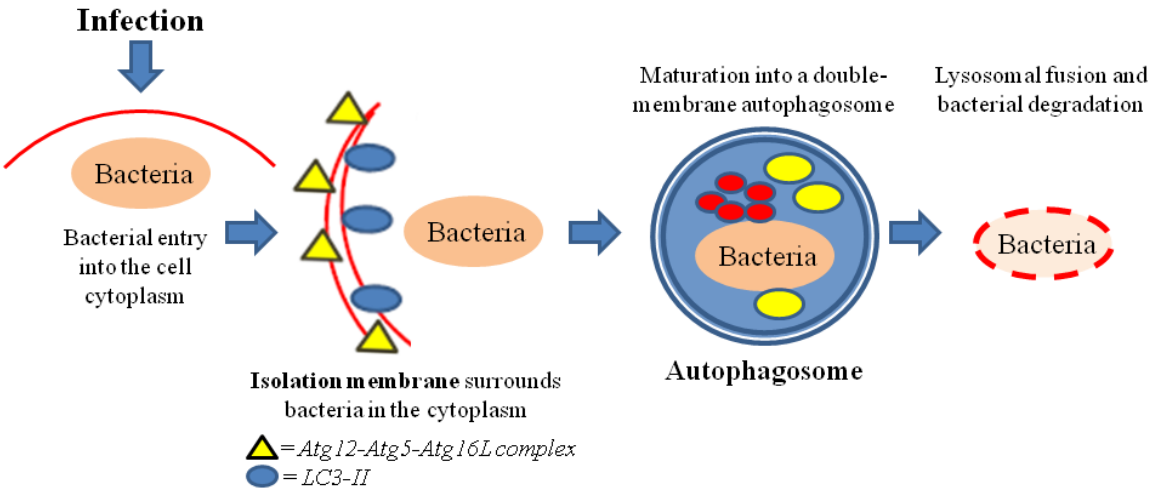

Figure 1: Autophagosome formation and Maturation. Isolation membrane formation (crescent membrane decorated on both sides with LC3-II and the Atg12-Atg5-Atg16 complex); Elongation (growth of isolation membrane and closure to form an autophagosome); and Maturation, which involves fusion of the autophagosome with the lysosome.

Autophagy is a highly conserved pathway in which eukaryotic cells are able to capture portions of their cytoplasm and ultimately deliver the cargo to the lysosome for destruction. The characteristic, trademark features of autophagy, separating it from other degradation pathways is the double-membrane structure, known as the autophagosome. In the first stages of autophagy, nucleation of the isolation membrane occurs. The nucleation event is followed by membrane elongation and closure around the cytoplasmic target resulting in a double membrane structure known as an autophagosome. Finally, the autophagosome fuses with a lysosome, destroying the contents of the autophagosome (Fig. 1). The origins of this membrane have yet to be definitively determined but current opinion suggests the endoplasmic reticulum or the Golgi apparatus to be the mostly likely sources. The proteins controlling the autophagy system utilize a series of conjugation and are all designated as "Atg" proteins.

The growth of the isolation membrane occurs via two series of ubiquitin-like conjugation reactions, with Atg7 functioning as the E1-like enzyme in both reactions. Briefly summarizing tremendous amounts of biochemical work, the first series of reactions involves the formation of the Atg12-Atg5-Atg16L complex. Following activation of the C-terminal glycine of Atg12 by Atg7, Atg12 is transferred by Atg10 to Atg5 where there a linked via an isopeptide bond. The Atg12-Atg5 complex then associates with Atg16L forming the Atg12-Atg5-Atg16L complex. Interestingly, the Atg12-Atg5-Atg16L protein complex then acts like a E3 ligase in the next set of conjugation steps. The second set of conjugation events involves the formation of LC3-II (Atg8-PE). In the formation of LC3-II, LC3 is first cleaved by Atg4, a cysteine protease. The cleaved LC3 is then conjugated to phosphatidylethanolamine (PE) by the actions of Atg7 and Atg3, both acting in a E2-like manner. Finally, the Atg12-Atg5-Atg16L complex, acts in a E3-like manner, directing the localization of LC3-II to the autophagosome. LC3-II associates with and remains on autophagosomal membranes until they fuse with lysosomes (Fig. 2) (Burman and Ktistakis, 2010).

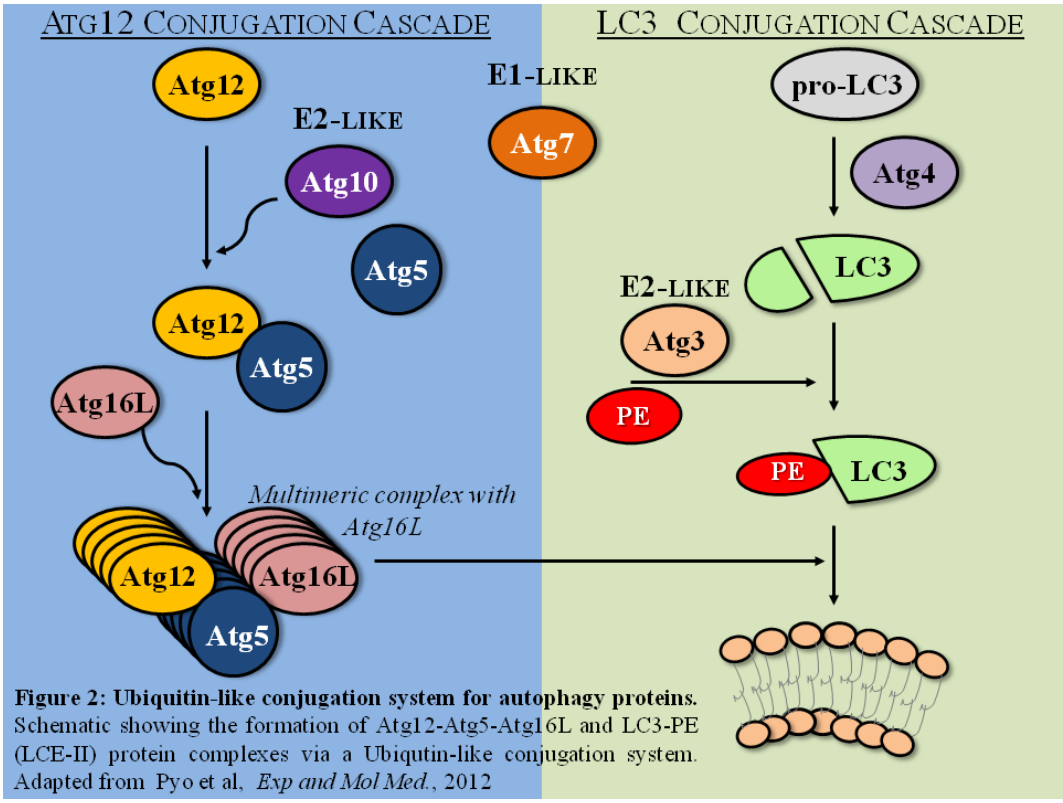

Figure 2: Ubiquitin-like conjugation system for autophagy proteins. Schematic showing the formation of Atg12-Atg5-Atg16L and LC3-PE (LCE-II) protein complexes via a Ubiqutin-like conjugation system. Adapted from Pyo et al, *Exp and Mol Med.*, 2012

Metabolic Regulation of Autophagy

mTor and Starvation

Autophagosomes were first described in 1966 by a group studying the tissues of stressed animals via electron microscopy. Under conditions of stress, such as amino acid deprivation, autophagy is strongly induced. Mounting evidence has suggested that starvation activates amino acid signaling pathways which involve the mammalian Target of rapamycin (mTor). mTor is a highly conserved, nutrient response regulator. It is a serine/threonine kinase that is known to be involved in the response of many cell types to nutrient deprivation and is found in all eukaryotes (Nobukuni et al., 2005). mTor exists in two independent complexes, mTor complex 1 (mTORC1) and mTor complex 2 (mTORC2). mTORC1 contains mTOR and other regulatory subunits like raptor which

are critical in controlling cellular metabolism. mTORC2 is important is regulating the actin cytoskeleton (Sengupta et al., 2010). mTor functions as an inhibitor of the autophagy pathway. Starvation inactivates mTORC1, thereby activating autophagy. More specifically, researchers have found that the mTor complex1 (mTORC1) requires three separate proteins, Vps34, Rheb and Rag GTPase, for the activation and ensuing repression of autophagy during starvation.

When there are low intracellular ATP concentrations and high intracellular AMP concentration, a key cellular enzyme, AMP-activated protein kinase (AMPK) is activated. Interestingly, AMPK and mTor demonstrate inverse type of regulation. AMPK is activated under low energy conditions, while mTor is activated under nutrient-rich conditions. Activation of AMPK results in the phosphorylation of raptor. Raptor is a scaffold protein which facilitates the recruitment of downstream substrates to the mTORC1 complex. The phosphorylation of raptor has been shown to be essential for the inhibition of mTORC1 complex by AMPK (Gwinn et al., 2008).

Recent work has begun to explain the mechanisms of how mTOR and autophagy are linked. Koroluchuk et al. demonstrated that lysosomal positioning was critical in the management of nutrient balance and autophagy activation. They found that during high-energy periods, mTORC1 was found to localize on peripheral lysosomes away from the nucleus. However, during periods of starvation, a change in the localization of mTORC1 was seen, with mTORC1 clustering in perinuclear lysosomes. They further found that the peripheral lysosomal localization resulted in increased mTOR activity which blocked autophagosome synthesis. Meanwhile, they found that perinuclear lysosome localization was associated with reduced mTOR activity and increased autophagosomal formation

(Korolchuk et al., 2011). These findings indicate that the cell carefully coordinates mTORC1 localization to orchestrate both the nutrient sensing and response mechanisms like autophagy induction.

Exercise

While starvation and cellular energy status are important regulators of autophagy, they are not the only activators of autophagy. Recent work from the Levine lab has shown that exercise can be a potent inducer of autophagy *in vivo*. When examining the role of autophagy in response to stimulus, they found that exercise was a powerful activator for autophagy in the muscle tissue of mice. Bcl-2 is an anti-autophagic protein that blocks autophagy through its interaction with the pro-autophagic proteins, Beclin 1. The dissociation of Bcl-2 from Beclin 1 is mediated by the phosphorylation of three separate residues on Bcl-2. Utilizing a knock-in mouse, engineered to replace Bcl-2 phosphorylation sites with alanine residues (Bcl-2^{AAA}), He et al demonstrated that the dissociation of Bcl-2 from Beclin 1 was essential for exercise induced autophagy to occur (He et al., 2012). Additionally, they showed that stimulus induced autophagy was critical for proper muscle glucose metabolism. Even more strikingly, they found that the inability to induce autophagy in Bcl-2^{AAA} mice meant that exercise no longer protected the mice from the negative effects of a high-fat, Western Diet, with the Bcl-2 mice demonstrating elevated levels of resting blood glucose and leptin. Given the critical role for Bcl-2 in regulating induced autophagy and energy homeostasis, it will be crucial to determine the role of Bcl-2: Beclin 1 complex in regulating other forms of induced autophagy such as

antibacterial autophagy. This may be especially relevant in small intestinal epithelial cells which must also function in glucose uptake.

Autophagy in Innate immunity and Infection

Autophagy was initially thought to be a non-selective degradation pathway. However, increasing evidence indicates that autophagy is an important innate immune response to intracellular pathogens. Selective autophagic degradation of intracellular pathogens is known as xenophagy. The exact organelle origin of the membranes involved in xenophagy still remains to be determined. However, there has been advancement in identifying the similarities and differences between xenophagy and bulk-degradative autophagy. Perhaps one of the most striking differences found in xenophagy is the size of the autophagosomes. Studies examining the xenophagy in response to group A *Streptococcus* found that the autophagosomes formed following group A *Streptococcus* infection could be as large as 10 μm (Nakagawa et al., 2004). In contrast, autophagosomes formed in response to starvation are typically much smaller, ranging from 0.5 to 1.0 μm, depending on tissue origin. Additionally, it has been shown that intracellular bacteria are commonly targeted for autophagosomes via interactions between a molecular tag found on the bacterial surface and adaptor proteins which recognize the tags and also contain an LC3-interacting region (LIR). The LIRs typically contain a conserved motif of WXXL or WXXI. Most commonly, intracellular bacteria are tagged with a polyubiquitin chain which mediates the recognition of the bacteria via adaptor protein containing LIRs. Examples of such adaptors include p62 and NDP52.

Bacteria

A key question in understanding xenophagy was determining how autophagy is activated when bacteria enter the cell cytoplasm. The initial clues into this link were provided when researchers began studying the fate of *S. typhimurium* once it had escaped the *Salmonella* containing vacuole (SCV). Following escape from the SCV, *S. typhimurium* was shown to be coated with Ubiquitin (Perrin et al., 2004). More recent work has provided even more detail into how *S. typhimurium* is detected for clearance via autophagy. Thurston et al have also shown that the human cells use a cytosolic lectin, galectin 8, to monitor endosomal integrity by binding glycans exposed on damaged SCVs. When *S. typhimurium* begins the process of escape from the *Salmonella* containing vacuole, holes in the SCV membrane are formed. The holes result in the exposure of foreign carbohydrates to the cell cytoplasm. These foreign carbohydrates are detected by galectin 8. Glycan binding by galectin 8 then recruits NDP52 to activate antibacterial autophagy (Thurston et al., 2012). Interestingly, they also showed that the recruitment of NDP52 by galectin 8 is independent of the Ub-dependent recruitment of NDP52 to *S. typhimuirium*. This same group also identified a novel adaptor protein, TBK1, which recognizes polyubiquitin chains on *S. typhimurium* and targets *S. typhimurium* for destruction via autophagy (Thurston et al., 2009). This indicates that the cell likely uses several distinct mechanisms to mark and recognize cytosolic bacterial invaders for autophagy. Similar mechanisms of Ubiquitin-mediated autophagy detection have been unraveled for *S. flexneri* and *L. monocytogenes*.

Several *in vitro* studies have demonstrated that autophagy plays a role in the clearance of bacteria that invade the cytoplasm. In mammalian cells, for example,

autophagy has been shown to be involved in innate defense against *Mycobacterium tuberculosis, S. flexneri, S. typhimurium* and group A streptococcus. Group A streptococcus is an extracellular, Gram-positive bacterium that is capable of invading the cytoplasm. Upon invasion, *Streptococcus* is rapidly sequestered into LC3 positive autophagosomes and subsequently degraded via autophagosomal fusion with the lysosome (Nakagawa et al., 2004). *S. typhimurium* is a Gram-negative rod that causes gastroenteritis and typhoid fever. An initial observation noted that following infection, a subset of intracellular *S. typhimurium* was found to be Ubiquitin positive, indicating that the bacterium had been exposed to the cytoplasm. Subsequent work by Birmingham et al, demonstrated in vitro that *S. typhimurium* uses its SPI-I pathogenicity island to damage the SCV. They went on to show that damage and escape from the SCV targeted *S. typhimurium* for autophagy. Additionally, they demonstrated that in Atg5-deficient cells, *S. typhimurium* proliferation was increased, thus indicating that autophagy was an important part of the cellular response to *S. typhimurium* (Birmingham et al., 2006).

There is significantly less data regarding an antibacterial role for autophagy at the organismal level. Studies in *Drosophila melanogaster* have shown that deletion of autophagy genes leads to increased susceptibility to *L. monocytogenes* (Yano et al., 2008). The work in *Drosophila* demonstrated that the innate immune receptor PGRP-LE, which is responsible for the detection of diaminopimelic acid-type peptidoglycan, was critical for the induction of autophagy by *L. monocytogenes*. Yano et al continued by showing that autophagy served to both limit *L. monocytogenes* replication as well as promote *Drosophila* survival following infection. Another major study involving antibacterial autophagy in an organism was performed in *Caenorhabditis elegans*. Using

RNAi to silence critical autophagy genes, Jia et al. demonstrated that autophagy was a critical mediator of host defense against *S. typhimurium* in *C. elegans*. When the researchers performed RNAi knockdown of the *C. elegans atg* genes, *bec-1* and *lgg-1*, the orthologs of yeast ATG6 and ATG8, they found that *S. typhimurium*-infected worms had significantly shorter lifespans than the control worms. Additionally, the RNAi treated worms demonstrated numerous examples of intact bacteria within the epithelial cells of the intestine while control worms very rarely had any bacteria visibly intact (Jia et al., 2009). Prior to 2013, no group had demonstrated an *in vivo* antibacterial role for autophagy in intestinal epithelial cells in a mammalian system.

Viruses

Relatively little is known about how viruses are targeted for autophagy. The first study linking autophagy genes and host response to infection was performed in the central nervous system of mice. The Levine group found that neuronal overexpression of Beclin 1, the mammalian ortholog of the yeast gene Atg6, protected mice from lethal Sindbis virus infection (Liang et al., 1998). Additional, studies in *Drosophila* have shown that loss of autophagy genes leads to increased susceptibility to Vesicular Stomatitis Virus (VSV-G) (Shelly et al., 2009). However, a recent study demonstrated that some of the mechanisms used to target bacteria for autophagy are also used to target viruses for destruction. Orvedahl et al. found that p62 interacts with a capsid protein of Sindbis virus during neuronal infection of the central nervous system. The interaction of p62 was shown to be required for the proper localization of Sindbis virus to the autophagosome (Orvedahl et al., 2010). Further confirming the importance of the host autophagy

response to viral infection, mice infected with Sindbis virus following genetic deletion of neuronal Atg5 demonstrated increased neuronal death and mortality and had increased p62 aggregates. Together, these findings show that both p62 and autophagy proteins are crucial in the host innate response to Sindbis virus infection. Another intriguing finding revealed by this study is that the adaptor protein p62 is capable of recognizing multiple molecular tags. This finding is implied by the fact that Sindbis virus has not been shown to be ubiquitinated. The concept of novel molecular tags as well as novel adaptors will be further considered in Chapter 5.

Bacterial Subversion and Evasion of Autophagy

Autophagy was initially thought to be a non-selective process; however, mounting experimental evidence has shown this not to be true. The process of autophagy evolved at the beginning of eukaryotic life. This process was likely driven by the ability of microbes – virus and bacteria – to invade the cell cytoplasm and cells' need to maintain a sterile cytosol. When viewed through this evolutionary prism, it is clear that autophagy was one of the first antimicrobial defenses evolved by eukaryotic cells. Given this ancient evolutionary pressure, it is not surprising that microbes, in turn, have developed mechanisms for subverting and avoiding detection by autophagy. Indeed, we find that many of the bacterial targets for autophagy, such as *Legionalla pneumophila*, *Shigella flexneri* and *Listeria monocytogenes* have evolved mechanisms to evade autophagy or hijack autophagy for their own benefit.

There are a number of bacteria that are capable of invading cells and these bacteria are often able to live within the vacuole or even an autophagosome. The real

danger in residing within a cell is when the vacuole or autophagosome is targeted to fuse with a lysosome. Therefore, it is not surprising that the majority of bacterial efforts to subvert destruction via autophagy center around avoidance of autophagic capture or suppression of autophagosomal maturation.

One example of suppression of autophagosomal maturation can be seen with *L. pneuomophila*. *Legionella* is a Gram–negative rod bacterium. The bacterium is ubiquitous although largely aquatic. *Legionella* is also the causative agent in Legionnaire's disease, a bacterial pneumonia like disease. During the initial stages of infection, *L. pneumophila* invades the epithelial cell and resides within an endosomal compartment that is Atg7-positive and ER marker-positive. Amer et al. have shown that *L. pneumophila* utilizes its Type 4 Secretion System (T4SS) to inject a soluble factor of between 10 and 30 kDa. This soluble factor causes a delay in the maturation of the bacteria containing vacuole/early autophagosome. They went on to show that this delay was critical for the survival of *L. pneumophila* (Amer and Swanson, 2005). The delay allows the bacteria to change their physiology to an acid-tolerant metabolic state which, in turn, allows *L. pneumophila* to survive more readily in acid conditions found in autophagolysosome and autolysosomes.

Another example highlighting the avoidance of autophagic capture strategy is seen with *Shigella flexneri*. *S. flexneri* is a Gram–negative rod that causes shigellosis or dysentery. During the infection of the epithelial cells of the colon, *Shigella* uses its Type 3 Secretion System (T3SS) to translocate the IcsB effector protein. VirG is required for the actin-based motility but VirG is also the surface protein targeting *Shigella* for autophagy. Researchers found that once in the cytoplasm, IcsB competitively inhibits the

binding of ATG5 to the *Shigella* surface protein VirG (Ogawa et al., 2005) (Kayath et al., 2010). By blocking the binding of ATG5, *Shigella* becomes capable of multiplying within the epithelial cells.

Autophagy in Intestinal Disease

Inflammatory bowel disease (IBD) is a serious chronic inflammatory disease that afflicts more than 1.4 million people in the United States alone. Crohn's disease (CD) is a specific type of IBD that affects any portion of the intestine from the mouth to the perianal area, with the ileum being the most common region of involvement. While the etiology of IBD and CD remain poorly understood, a number of studies have implicated dysregulated immune responses to the normal microbiota. Crohn's disease is commonly associated with increased bacterial adherence to the mucosa and abnormal microbiota composition (Sartor, 2010). Frontline therapies for CD are currently sulfasalazine and 5-aminosalicyate, which are thought to have anti-inflammatory and antibacterial properties. Ultimately, more potent treatments such as antibiotics, steroids, or immunosuppressants are often required. The poor treatment modalities reflect how little is known about how the body maintains homeostasis with the gut microbiota.

Mutations in autophagy genes have been shown to increase the susceptibility to intracellular infection in a variety of organisms ranging from *Drosophila* to mice. Interestingly, in humans the best characterized link between mutation in autophagy factors and disease has been Crohn's disease (CD). Crohn's disease is a type of Inflammatory Bowel Disease (IBD) that can affect any portion of the gastrointestinal tract. The terminal ileum, the most distal portion of the small intestine, is the region most

commonly affected in Crohn's disease. In Crohn's disease, there is often a loss in the spatial segregation of bacteria, meaning the 50 μm-wide bacteria-free zone immediately adjacent to the intestinal epithelium is no longer present. Bacterial invasion of the inner mucus layer brings bacteria into close contact with the intestinal epithelium, making them more likely to invade epithelial cells.

The first indication of a link between autophagy and Crohn's disease was found when the genome wide association studies found three different autophagy related genes – IGRM, NOD2 and ATG16L1 – linked to Crohn's disease (2007). Recent studies have begun to elucidate pathophysiological basis for the links between autophagy and Crohn's disease. Interestingly, some of these links appear to involve non-autophagy functions of the genes.

For example, mice expressing a hypomorphic variant of ATG16L1 exhibit distinctive defects in granule formation in Paneth cells, a specialized epithelial cell lineage that has key functions in mucosal defense. In addition to the Paneth cell abnormalities, ATG16L1 hypomorphic mice also displayed increased expression of leptin and adiponectin, two adipocytokines linked with the intestinal response to injury. Cadwell and colleagues went on to demonstrate that Crohn's disease patients who were homozygous for the same mutation in ATG16L1 also showed similar Paneth cell abnormalities and increased leptin expression (Cadwell et al., 2008).

More recently, Cadwell and colleagues demonstrated that viral infection with mouse norovirus (MNV) is required for the aberrant Paneth cell response in ATG16L1 hypomorphic mice, (Cadwell et al., 2010). They continued and found that ATG16L1 hypomorphic mice displayed an aberrant response to dextran sodium sulfate (DSS)-

induced colitis. In response to DSS, ATG16L1 mice showed ileal villus blunting, muscular hypertrophy and lymphoid aggregates, all hallmarks of Crohn's disease. Cadwell et al. concluded that both MNV infection as well as commensal bacteria were needed to induce the Crohn's-like phenotype in ATG16L1 hypomorphic mice, thus establishing a strong link between viral infection, commensal bacteria, autophagy, and Crohn's disease. Additionally, other groups have found that Crohn's disease patients who harbor a mutation in ATG16L1 gene have intestinal dysbiosis (Frank et al., 2011) as well as Paneth cell abnormalities that closely resemble that Paneth cell observation seen in mice by Cadwell and collaborators (Cadwell et al., 2010).

The discovery of a link between mutations in the gene encoding immunity-related GTPase family, M (IRGM) and Crohn's disease added another layer of intrigue to the relationship between autophagy and IBD. IRGM has been shown to be needed for clearance of an adherent, invasive strain of *E. coli* (AIEC) by autophagy (Brest et al., 2011) . Given that increased mucosal bacteria adherence in Crohn's disease is frequently observed (Sartor, 2008), the link between IRGM mutations and CD clearly hints at a role for antibacterial autophagy in maintaining normal intestinal homeostasis.

Even more recent work by Thachil and collaborators has reported that autophagy is specifically activated in the Paneth cells of Crohn's disease patients, independent of mutations in IGRM or ATG16L1. They found that the activation of Paneth cell autophagy in Crohn's patients ultimately led to a lower number of secretory granules (Thachil et al., 2012) . This finding and the preponderance of evidence linking autophagy, bacterial handling and Crohn's disease all point to the importance of

understanding how intestinal epithelial cell autophagy functions *in vivo* as a critical and necessary step in understanding and curing IBD.

Aims of the study

The intestines of all mammals are colonized with a dense and diverse bacterial flora. These microorganisms benefit their hosts by breaking down nutrients in the diet and by stimulating normal immune system development. However, this symbiotic relationship can break down when resident bacteria opportunistically invade the intestinal barrier, leading to pathologies such as inflammatory bowel disease (IBD) and bacteremia. As a result, epithelial innate immune responses play an essential role in preventing bacterial invasion of host tissues and maintaining a symbiotic host-bacterial relationship.

Autophagy is an evolutionarily ancient homeostatic process in which cytoplasmic materials are targeted to the lysosome for degradation. Recently, autophagy has emerged as a crucial element of the innate immune response to viruses as well as intracellular bacteria. Additionally, mounting evidence suggests that dysregulation of the autophagic pathway may be associated with inflammatory bowel disease. However, little is known about the role of autophagy in controlling interactions between intestinal bacteria and the host intestinal epithelium. The aims of this study were therefore to gain mechanistic insight into how intestinal epithelial autophagy is activated by bacteria, and to determine whether epithelial autophagy protects against bacterial invasion of the intestinal epithelium.

My specific aims were as follows:

1. Identify the enteric bacteria that activate autophagy in intestinal epithelial cells.
2. Determine the host factors that govern autophagy activation in the intestinal epithelium.
3. Define the role of epithelial autophagy in protecting against bacterial invasion of intestinal tissues.

Using gnotobiotic mouse models, I have shown that intestinal bacteria can induce autophagy in intestinal epithelial cells *in vivo.* I have further shown that this induction requires invasive bacteria, and depends on epithelial cell-intrinsic MyD88 signaling. On the basis of my findings, I conclude that autophagy is an important early component of the epithelial innate immune response to invasive members of the resident intestinal microbiota and overt intestinal pathogens.

CHAPTER THREE

Methodology

Animals

C57BL/6 wild-type, *Myd88*$^{-/-}$, *Myd88*$^{fl/fl}$, *Myd88*$^{\Delta IEC}$ and villin-*Cre* mice were maintained in the barrier at the University of Texas Southwestern Medical Center. *MyD88*$^{\Delta IEC}$ genotyping was described previously (Vaishnava et al., 2011). Germ-free C57BL/6 mice were maintained in isolators as described (Cash et al., 2006). *Atg5*$^{fl/fl}$ mice have been described previously (Hara et al., 2006). B6.SJL-Tg(Vil-cre)997Gum/J (villin-*cre*) mice were obtained from the Jackson Laboratory. Experimental mice were generated by mating *Atg5*$^{fl/fl}$ mice with *Atg5*$^{fl/fl}$ mice that were heterozygous for the villin-*Cre* transgene. Genotyping of the *Atg5*$^{fl/fl}$ and the *Cre* gene has been described previously (Miller et al., 2008). *Myd88*$^{fl/fl}$ mice were obtained from Dr. Anthony DeFranco of the University of California San Francisco. All experiments were performed using protocols approved by the Institutional Animal Care and Use Committees of the UT Southwestern Medical Center.

Bacterial Strains

Salmonella enterica Serovar Typhimurium (SL1344) and its isogenic mutants ΔSPI-1 (Eichelberg and Galan, 1999), Δ*invA (Galan et al., 1992)*, and *S. typhimurium*-GFP (a gift from Dr. Vanessa Sperandio, University of Texas Southwestern Medical Center) were cultured as described (Vaishnava et al., 2008). *Enterococcus faecalis* (V583) and its derivatives *E. faecalis* (V583 *pMV158gfp)* (Nieto and Espinosa, 2003)

were both cultured as described (Duerkop et al., 2012). *Lactobacillus salivarius* (ATCC 11741) was transformed with the plasmid *pMV158gfp* to create the strain ATCC 11741 *pMV158gfp*, and was cultured as described (Thompson and Collins, 1996).

Antibodies and Reagents

Rabbit polyclonal anti-ATG5 and anti-LC3 antibodies were from Novus Biologicals. Goat polyclonal anti-GFP and donkey polyclonal secondary antibody to Goat IgG (conjugated to DyLight 488) were from Abcam. Alexa Fluor 568-Phalloidin (Invitrogen) was used as a counterstain to identify epithelial cell borders of OCT-embedded frozen small intestine sections fixed in 4% paraformaldehyde/10% sucrose.

Immunodetection of Autophagosomes

Small intestines were fixed with Bouin's fixative overnight at 4^{o}C and then embedded in paraffin. Paraffin-embedded tissues were washed twice for 10 min in xylene, twice in 100% EtOH, twice in 95% EtOH, and then rinsed in deionzed water for 5 minutes. Sections were boiled in 10 mM sodium citrate, pH 6, for antigen retrieval and then washed in PBS. Sections were blocked in 1% BSA, 0.3% Triton-X-100 in PBS and incubated with a 1:200 dilution of rabbit polyclonal anti-LC3 antibody (Novus Biologicals) at 4^{o}C overnight. After washing in TBS-T (50 mM Tris, pH 7.6, 150 mM NaCl, 0.05% Tween-20), tissue sections were incubated with goat anti-rabbit IgG-Cy3 conjugate (1:400 Biomeda), for 30 min at 25°C. Tissues were counterstained with DAPI. After PBS washes, cover slips were added and images were captured on a Zeiss

AxioImager M1 microscope. LC3+ puncta were counted in 100 epithelial cells from well-oriented crypt/villus units unless otherwise indicated.

Isolation of Small Intestinal Epithelial cells

Distal small intestines from 6-8 week old mice were flushed with 4°C PBS. Epithelial cells were lysed by incubation with Tissue–Protein Extraction Reagent (T-PER, Thermo Scientific) with complete protease inhibitor cocktail (Roche) for 5 min (Fig. 1). Lysates were cleared of debris by centrifugation at 10,000 revolutions per minute at 4°C for 5 min and then boiled and analyzed by Western blotting. Immunoblots were visualized by chemiluminescence.

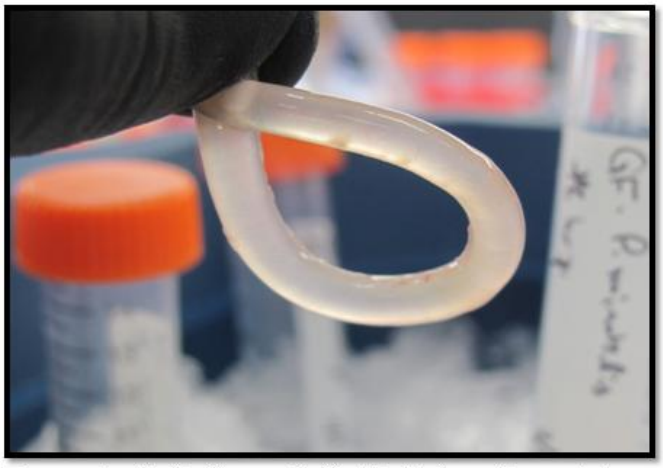

Figure 1: Isolation of Epithelial cell proteins. Example of a terminal ileal tissue filled with Tissue protein extraction reagent (T-PER). Sample should be filled maximally to ensure maximal mucosal epithelial exposure

Electron Microscopy

Distal small intestines from 6-8 week old mice were flushed with ice-cold PBS. The intestinal fragments were fixed for 24 h at 4°C in 2% paraformaldehyde, 2% glutaraldehyde, 0.1 M cacodylate (prepared in PBS), washed in PBS, post-fixed for 1 h in 2% osmium tetroxide, and stained with a solution containing aqueous uranyl acetate and lead. Samples were dehydrated in graded alcohols and embedded in Poly/BED 812 (Electron Microscopy Sciences). Sections were prepared and viewed with a TEM 2 JEOL 1200EX II or Scanning Electron microscope.

Mouse Infection

Mice were infected intraperitoneally with *S. typhimurium* at 2 x 10^3 CFU per mouse, or intragastrically by gavage with 10^9 CFU per mouse. Intestinal bacterial colonization levels were determined by serial dilution plating of 1 μl of intestinal contents taken directly from the terminal ileum. To monitor bacterial dissemination to liver and spleen, organs were homogenized in sterile PBS, and bacteria were quantified by dilution plating on Luria broth plates containing 100 μg/ml ampicillin.

FISH analysis

Small intestinal tissues were prepared for FISH analysis by fixation in Carnoy's fixative (Ricca Chemical), followed by embedding in paraffin. Tissues were sectioned at a thickness of 5 μm and hybridized to a universal bacterial probe directed against the 16*S* rRNA gene: 5'-Cy3GCTGCCTCCCGTAGGAGT-Cy3-3'. Hybridizations were carried

out as described (Vaishnava et al., 2011), and tissues were visualized using a Zeiss AxioImager M1 Microscope.

CHAPTER FOUR

Intestinal Epithelial Autophagy is Essential for Host Defense Against Invasive Bacteria

Introduction

The mammalian intestine is home to a complex and diverse population of bacteria. The members of this microbial community are essential for host metabolism and digestion, thereby creating a mutualistic relationship. Although the overall host-microbial relationship is symbiotic, the bacterial components of this community span a wide spectrum of lifestyles, encompassing benign commensals, opportunistically pathogenic pathobionts, and overt pathogens.

The intestinal epithelium interfaces directly with this diverse community of bacteria, and is the first line of defense against bacterial invasion of host tissues. The epithelium must therefore be equipped with a diverse array of defenses against bacterial attachment and invasion. One mechanism by which the epithelium defends against bacterial penetration is by secreting antimicrobial proteins, such as RegIIIɣ, that limit bacterial contact with the epithelial surface (Vaishnava et al., 2011). Such secreted antimicrobial factors are essential for limiting bacterial invasion of epithelial cells and maintaining immune homeostasis with the intestinal microbiota (Gallo and Hooper, 2012) (Salzman et al., 2010) However, certain intestinal pathogens, such as *Salmonella enterica* Serovar Typhimurium (*Salmonella typhimurium*), or opportunistically invasive commensal bacteria, such as *Enterococcus faecalis*, can evade this first line of innate defense and enter intestinal epithelial cells (Eichelberg and Galan, 1999) (Klare et al., 2001) (Muller et al., 2012). This raises the question of whether there are epithelial cell-

intrinsic immune mechanisms that detect invading bacteria and limit their further dissemination into deeper tissues.

Autophagy is an evolutionarily ancient process in which cytoplasmic materials are targeted to the lysosome for degradation. Portions of the cytoplasm are sequestered into double-membrane structures, called autophagosomes, which fuse with lysosomes, delivering their contents for degradation by lysosomal enzymes (Deretic and Levine, 2009). The process involves the concerted action of several cytoplasmic proteins. These include LC3, which becomes lipid-conjugated and associates with the autophagosome membrane, and ATG5, which is conjugated to ATG12 and associates with the elongating isolation membrane (Mizushima et al., 2002).

Autophagy plays a central role in cellular homeostasis by degrading cytoplasmic contents during cellular starvation, and by recycling damaged organelles and proteins (Rabinowitz and White, 2010). More recently, autophagy has been shown to be critical for the recognition and degradation of intracellular pathogens, thus functioning as an innate barrier to infection (Deretic and Levine, 2009) (Levine et al., 2011) . Bacterial targets of autophagy include the intestinal pathogens *S. typhimurium* (Jia et al., 2009) (Kuballa et al., 2008) (Rioux et al., 2007) (Wild et al., 2011) and *Listeria monocytogenes* (Py et al., 2007). Autophagy has been shown to limit the replication of both of these bacterial species in cell culture models (Py et al., 2007) (Wild et al., 2011). In the case of *S. typhimurium,* intestinal epithelial cell autophagy is critical for host resistance of the nematode *Caenhorhabditis elegans* (Jia et al., 2009). However, the importance of autophagy for intestinal immunity in mammalian hosts *in vivo* remains underexplored. Such functions are likely to be especially important in the intestinal epithelium, which

interfaces with a dense microbial community that harbors invasive bacteria, and which acts as a critical barrier to bacterial penetration into deeper tissues.

Genetic studies of inflammatory bowel disease (IBD) have revealed important roles for autophagy pathway proteins in intestinal immune homeostasis. IBD is a chronic inflammatory disease of the intestine that is thought to arise from dysregulated interactions with the resident intestinal microbiota (Xavier and Podolsky, 2007). Recent studies have uncovered polymorphisms in genes of the autophagy pathway that are linked to Crohn's disease, a type of IBD in which the inflammation is localized to the distal small intestine and variable regions in the colon. Mutations in the critical autophagy gene ATG16L1 are associated with a predisposition to Crohn's disease in humans (Hampe et al., 2007) (Rioux et al., 2007) (2007). However, recent studies have shown that the ATG16L1 polymorphisms associated with Crohn's disease do not confer autophagy defects in mice, suggesting that the inflammatory phenotypes arise from autophagy-independent functions of ATG16L1 (Cadwell et al., 2008). Rather, the mutations have been found to cause defects in granule formation in Paneth cells, a specialized epithelial cell lineage that secretes abundant antimicrobial proteins and has key functions in mucosal defense (Cadwell et al., 2008) (Cadwell et al., 2010). Thus, it is not yet clear whether *bona fide* autophagy plays a role in maintaining intestinal homeostasis *in vivo*.

Here we report that intestinal epithelial cell autophagy is essential for mammalian intestinal defense against invasive bacteria. We show that epithelial autophagy is activated in the mouse intestinal epithelium by an intestinal pathogen, *S. typhimurium*, as well as by *E. faecalis*, an opportunistically invasive member of the intestinal microbiota. Our data further suggest that activation of epithelial autophagy is specifically triggered by

bacterial invasion of epithelial cells and requires epithelial cell-intrinsic MyD88 signaling. Finally, we use mice with an epithelial cell-specific deletion of a critical autophagy factor to show that epithelial cell autophagy is critical for limiting extraintestinal spread of *S. typhimurium*. Our observations thus reveal that autophagy is a key epithelial cell-autonomous mechanism of antibacterial defense that protects against dissemination of intestinal bacteria. Our findings provide new insight into how the mammalian intestinal epithelium maintains homeostasis with a diverse intestinal microbiota and establish a key role for autophagy in innate immune defense of the intestine.

Bacterial Induction of Autophagy in Small intestine Epithelial cells

LC3 is an essential autophagy protein that is recruited from the cytoplasm to the autophagosome membrane (Mizushima et al., 2002). A classical assay for autophagy activation uses immunofluorescence to visually assess the recruitment of cytoplasmic LC3 to autophagosomes, which are readily detected as morphologically distinct punctate structures (Mizushima et al., 2010). To assess whether bacteria activate autophagy in the intestinal epithelium *in vivo*, we compared the distribution and localization of LC3 in the intestinal epithelial cells of mice of differing bacterial colonization status. Germ-free mice are microbiologically sterile, thus allowing us to compare LC3 localization in the presence and absence of intestinal bacteria. Both germ-free and conventionally-raised (conventional) mice exhibited a diffuse, cytoplasmic distribution of LC3, indicating that the presence of specified pathogen-free (SPF) microbiota was not sufficient to activate epithelial autophagosome formation as indicated by LC3 staining (Figure 1A).

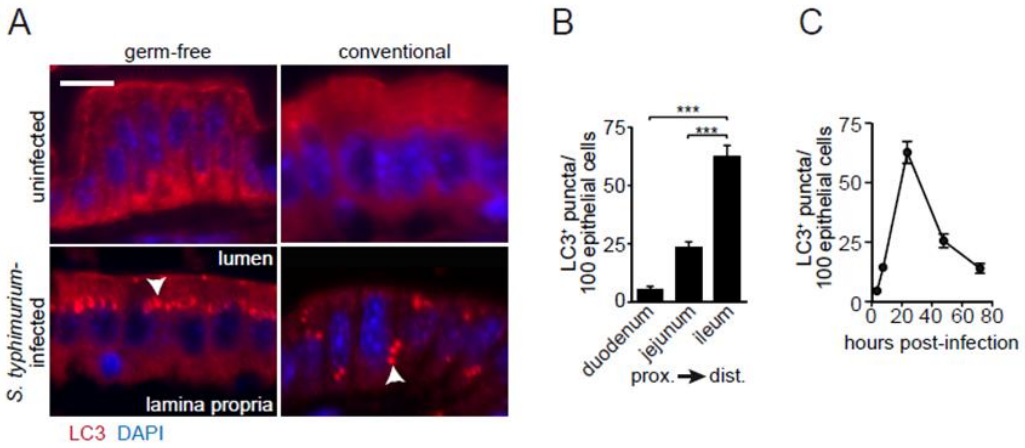

Figure 1: Salmonella typhimurium triggers autophagosome formation in small intestinal epithelial cells. (A) Germ-free and conventional mice were orally inoculated with 10^9 CFU of *S. typhimurium*. Sections of distal small intestine (ileum) were stained with anti-LC3 and anti-rabbit IgG-Cy3 (red). Uninfected mice show diffuse cytoplasmic LC3, while $LC3^+$ autophagosomes are visible as distinct puncta in the infected mice (arrowheads). Tissues were counterstained with DAPI (blue). Scale bar=10 μm. (**B**) Autophagosome number as a function of small intestinal location (proximal to distal). Tissue sections were from the experiment shown in (**A**). Data are represented as mean±SEM; ***, $p<0.001$, n=6 mice. (**C**)Time course of $LC3^+$ autophagosome formation after oral challenge with 10^9 CFU of *S. typhimurium*. Numbers of $LC3^+$ puncta in ileal sections were counted. Data are represented as mean±SEM; n=6 mice/time point).

To test whether an invasive bacterial pathogen could elicit autophagosome formation we orally infected mice with *S. typhimurium*. 24 hours after *S. typhimurium* inoculation into both germ-free and conventional mice, we observed punctate LC3+ structures in small intestinal epithelial cells, indicating autophagosome formation (Fig. 1A). The LC3+ puncta were positioned apically relative to the nucleus following *S. typhimurium* infection of germ-free mice,whereas they were positioned both apically and basolaterally relative to the epithelial cell nuclei in conventional mice (Fig. 1A). Although the cause of this difference is not clear, it may be due to different routes of *S. typhimurium* entry (e.g., apical versus basolateral) in the two different host settings (Chieppa et al., 2006) (Muller et al., 2012) (Niess et al., 2005). We also performed Z-stack reconstructions of the fluorescent images in multiple focal planes to verify that the LC3+ structures were located within epithelial cells.

We next characterized the location and timing of epithelial LC3+ autophagosome formation following *S. typhimurium* infection. Numbers of LC3+ puncta were highest in the distal small intestine (ileum) and diminished in the middle and proximal regions (jejunum and duodenum, respectively) (Fig. 1B). LC3+ autophagosomes were more abundant in epithelial cells inhabiting the ileal villus tips compared to the cells located closer to the crypts. LC3+ puncta were also observed in colonic epithelial cells following *S. typhimurium* infection but were rare relative to the numbers in the terminal ileum. Numbers of LC3+ autophagosomes were highest in the terminal ileum at ~24 hours following *S. typhimurium* infection, and diminished at 48 and 72 hours post-infection (Fig. 1C). Thus, autophagosome formation is a rapid and transient response of the intestinal epithelium to oral *S. typhimurium* infection. Drawing on these initial observations, all subsequent analysis was performed on ileal tissues, with epithelial cells visualized at the midpoint between the crypt base and villus tip.

During recruitment to the autophagosome, LC3-I is lipidated to yield LC3-II, which becomes associated with the autophagosome membrane (Pankiv et al., 2007). Western blot analysis of isolated ileal epithelial cells showed increased conversion of LC3-I to LC3-II at 24 hours following *S. typhimurium* infection, consistent with autophagy activation (Fig. 2A,B). This conversion was diminished after 72 hours (Figure 1A,B), which accords with the reduced numbers of LC3+ autophagosomes observed by immunofluorescence (Fig. 1C).

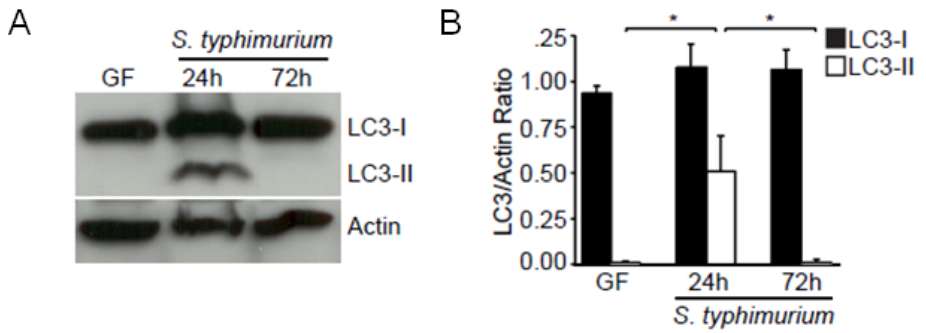

Figure 2: Western Blot Analysis of Ileal Epithelial Cell Lysates following *S. typhimurium* infection (A) Western blot of LC3 and actin (loading control) from ileal epithelial cells isolated from germ-free mice before and after colonization with *S. typhimurium* for 24 and 72 hr. Epithelial cell protein extracts were blotted and probed with anti-LC3 and anti-rabbit IgG-HRP. LC3-I and II denote the non-lipidated and lipidated forms of MAP1LC3, respectively. Results are representative of three independent experiments. (B) The LC3-I and LC3-II band intensities in (A) were quantified by scanning densitometry and normalized to the actin band intensity. Data are represented as mean±SEM; *, $p<0.05$; n=3 independent experiments.

To assess whether autophagosomes colocalized with intracellular bacteria, we orally challenged germ-free mice with *S. typhimurium* constitutively expressing green fluorescent protein (*S. typhimurium*-GFP). 24 hours after challenge, we could visualize S. *typhimurium* within enterocytes (Fig. 3A). Analysis of serially-cut sections with a no primary antibody control verified that the GFP signal was not due to nonspecific autofluorescence, which is frequently observed in fixed small intestinal tissues (Salzman et al., 2010; Vaishnava et al., 2011). The GFP signal was specific to *S. typhimurium*, as no signal was detected above background in uninfected germ-free tissues. Upon merging the LC3 and GFP channels, we found that some *S. typhimurium*-GFP were colocalized with LC3 (Fig. 3A), consistent with the targeting of *S. typhimurium* to autophagosomes. The colocalization of LC3 and GFP was determined to have a Pearson's coefficient of 0.89, indicating a strong, positive relationship between the two signals (Fig. 3B). Approximately 40% of intracellular *S. typhimurium* colocalized with LC3 (Fig. 3C),

while ~70% of the LC3+ puncta colocalized with *S. typhimurium*-GFP (Fig. 3D). These results demonstrate that autophagosome formation occurs in epithelial cells in response to *S. typhimurium* infection, and that the majority of autophagosomes colocalize with bacteria.

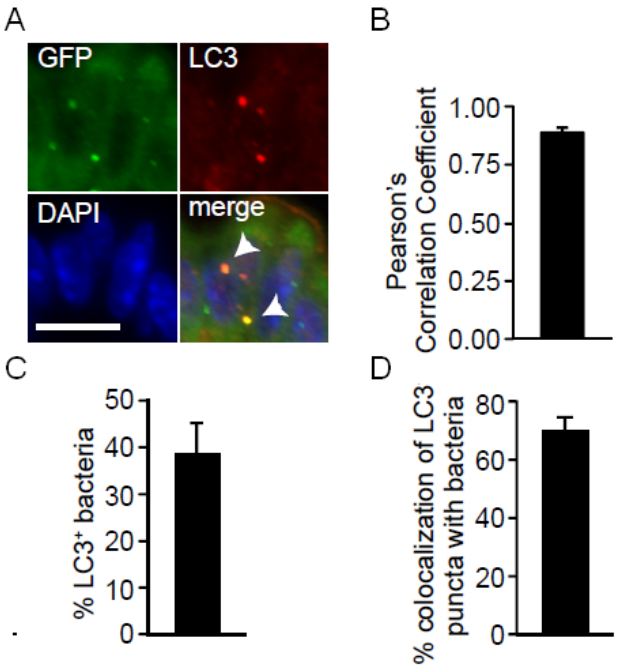

Figure 3: Colocalization of Autophagosomes and S. typhimurium – GFP . (**A**) Fixed tissue sections were stained with anti-GFP (green), anti-LC3 (red), and counterstained with DAPI (blue). The merged image shows GFP-labeled bacteria that colocalize with $LC3^+$ puncta in ileal epithelial cells (arrowheads). Scale bar=10 μm. (**B**) Pearson's colocalization coefficient for LC3 and *S. typhimurium*-GFP. The coefficient was calculated from three independent experiments with 3 fields per experiment, for a total of 9 fields. Data are represented as mean±SEM. (**C, D**) Quantification of LC3 and GFP colocalization in ileal epithelial cells from mice challenged with *S. typhimurium*-GFP and stained with anti-LC3. Results are the means ±SEM of three independent experiments.

Autophagy activation by *Enterococcus faecalis*, a native member of the microbiota

To determine whether the epithelial autophagic response to S. *typhimurium* represented a general response of epithelial cells to invading bacteria, we asked whether epithelial autophagy could also be activated by opportunistically-invasive members of the intestinal microbiota. To test this idea we orally challenged germ-free mice with *Enterococcus faecalis*, a Gram-positive, opportunistically invasive member of the human intestinal microbiota (Klare et al., 2001). Twenty-four hours after challenge, we detected

E. faecalis (harboring an episomal GFP-expressing plasmid) in epithelial cells of the small intestine (Fig. 4A,C).

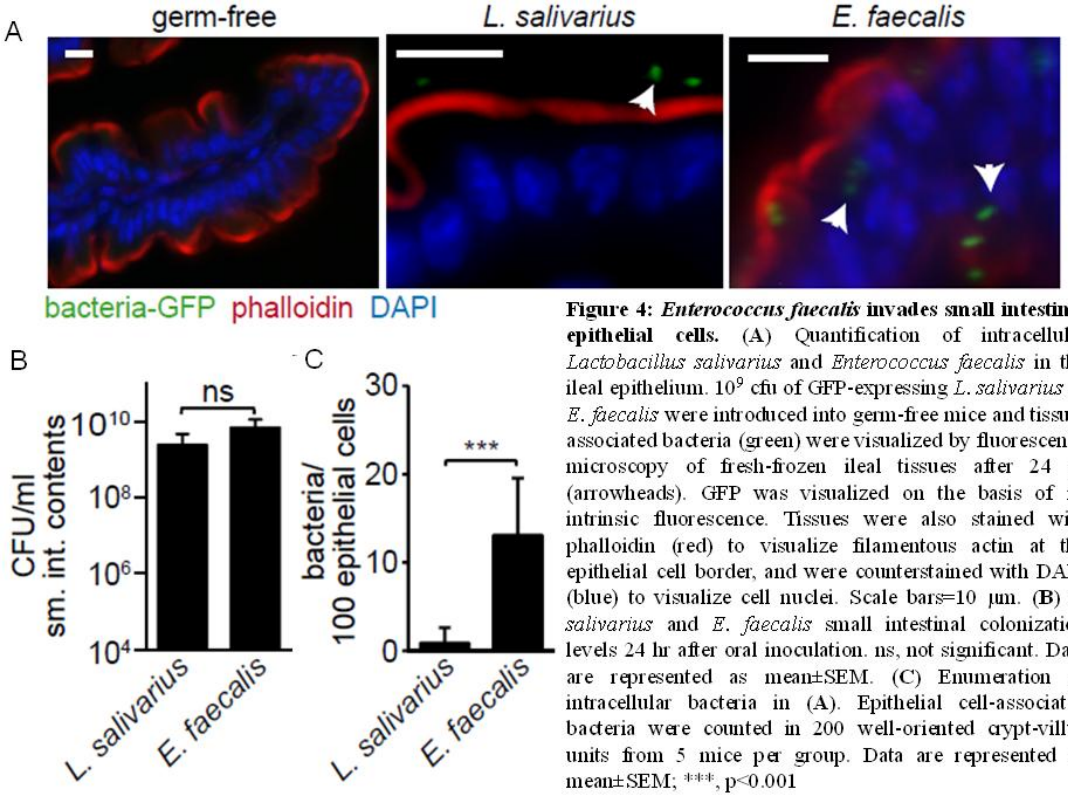

Figure 4: *Enterococcus faecalis* invades small intestinal epithelial cells. (A) Quantification of intracellular *Lactobacillus salivarius* and *Enterococcus faecalis* in the ileal epithelium. 10^9 cfu of GFP-expressing *L. salivarius* or *E. faecalis* were introduced into germ-free mice and tissue-associated bacteria (green) were visualized by fluorescence microscopy of fresh-frozen ileal tissues after 24 hr (arrowheads). GFP was visualized on the basis of its intrinsic fluorescence. Tissues were also stained with phalloidin (red) to visualize filamentous actin at the epithelial cell border, and were counterstained with DAPI (blue) to visualize cell nuclei. Scale bars=10 μm. (**B**) *L. salivarius* and *E. faecalis* small intestinal colonization levels 24 hr after oral inoculation. ns, not significant. Data are represented as mean±SEM. (**C**) Enumeration of intracellular bacteria in (**A**). Epithelial cell-associated bacteria were counted in 200 well-oriented crypt-villus units from 5 mice per group. Data are represented as mean±SEM; ***, $p<0.001$

Coincident with the ability of *E. faecalis* to invade epithelial cells, we observed the formation of numerous LC3+ autophagosomes (Fig. 5A,B). In contrast to the apical localization of the LC3+ puncta observed after *S. typhimurium* colonization of germ-free mice (Figure 1A), the majority of the *E. faecalis*-induced autophagosomes were localized on the basolateral side of the nucleus. As discussed above, we suggest that this may be due to differing routes of epithelial cell entry for *S. typhimurium* and *E. faecalis*.

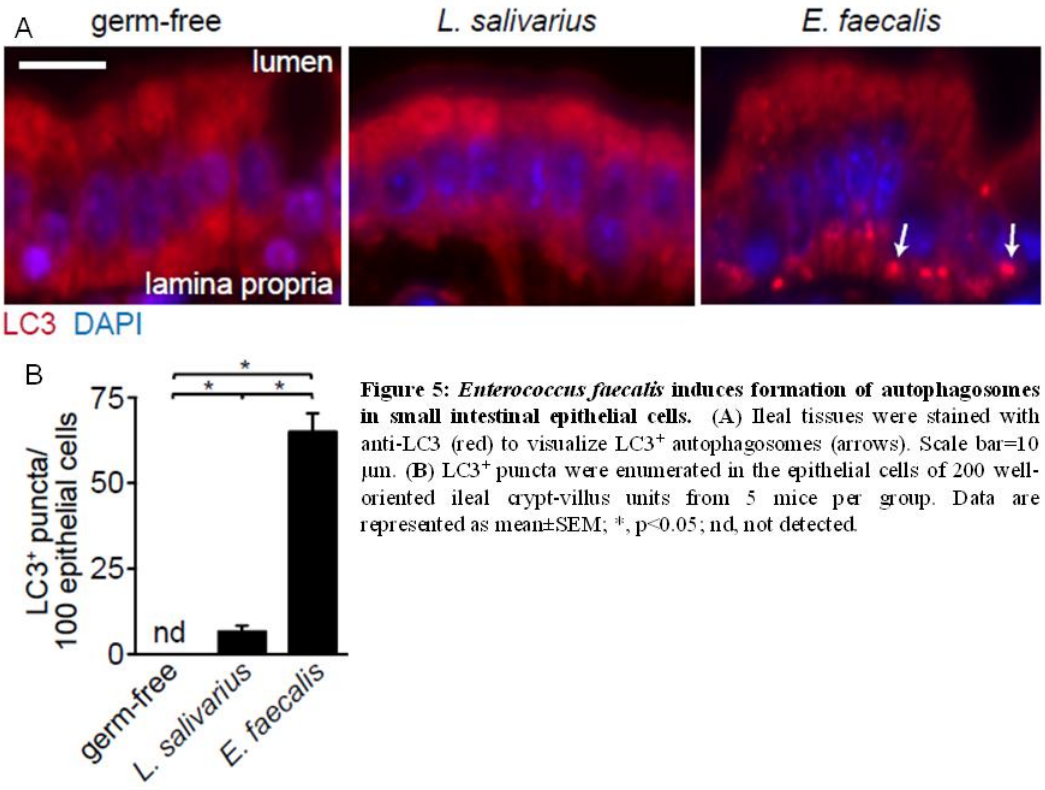

Figure 5: *Enterococcus faecalis* induces formation of autophagosomes in small intestinal epithelial cells. (A) Ileal tissues were stained with anti-LC3 (red) to visualize $LC3^+$ autophagosomes (arrows). Scale bar=10 μm. (B) $LC3^+$ puncta were enumerated in the epithelial cells of 200 well-oriented ileal crypt-villus units from 5 mice per group. Data are represented as mean±SEM; *, $p<0.05$; nd, not detected.

Western blot analysis of isolated ileal epithelial cells revealed increased conversion of LC3-I to LC3-II at 24 hours following *E. faecalis* colonization, consistent with autophagy activation (Fig 6A,B). In contrast, a non-invasive member of the microbiota, *Lactobacillus salivarius*, colonized the small intestine to approximately the same extent as *E. faecalis* (Fig. 4B) but was not detected within epithelial cells (Fig. 4A,C) and did not trigger formation of LC3+ autophagosomes (Fig. 5A,B). Together, these results suggest that epithelial cell autophagy can be activated by opportunistically invasive members of the microbiota such as *E. faecalis*.

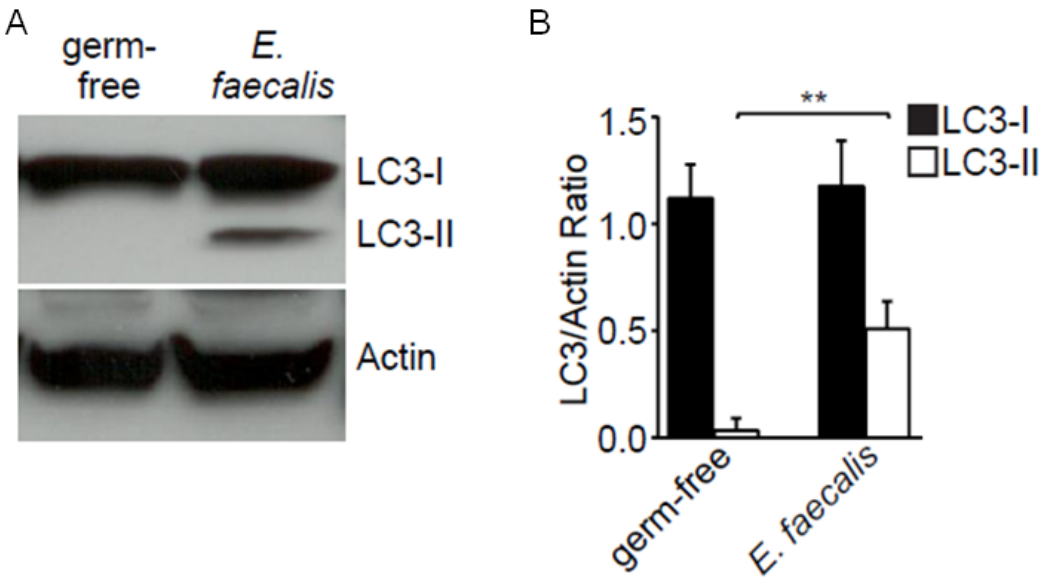

Figure 6: Western Blot Analysis of Ileal Epithelial Cell Lysates following *E. faecalis* infection (**A**) Western blot of LC3 and actin (loading control) from ileal epithelial cells isolated from germ-free mice before and after colonization with *E. faecalis* for 24hr. The experiment was performed as described for Figure 1D and results are representative of three independent experiments (**B**) The LC3-I and LC3-II band intensities in (**A**) were quantified by scanning densitometry and normalized to the actin band intensity. Data are represented as mean±SEM; *, $p<0.05$; n=3 independent experiments.

Ultrastructure of intestinal epithelial autophagosomes

We further characterized the autophagic response of intestinal epithelial cells using transmission electron microscopy. 24 hours after introduction of *S. typhimurium* into germ-free mice we observed double-membraned autophagosomes within small intestinal epithelial cells (Fig. 7B,C) (Mizushima et al., 2010). The double membranes enclosed bacteria, and were absent from the intestinal epithelial cells of germ-free mice (Fig. 7A). We also observed double-membraned structures surrounding bacteria in the epithelial cells of mice colonized for 24 hours with *E. faecalis* (Fig. 7D,E). These results support the idea that invading bacteria activate autophagy within intestinal epithelial cells, and that bacteria are targeted to the autophagosomes.

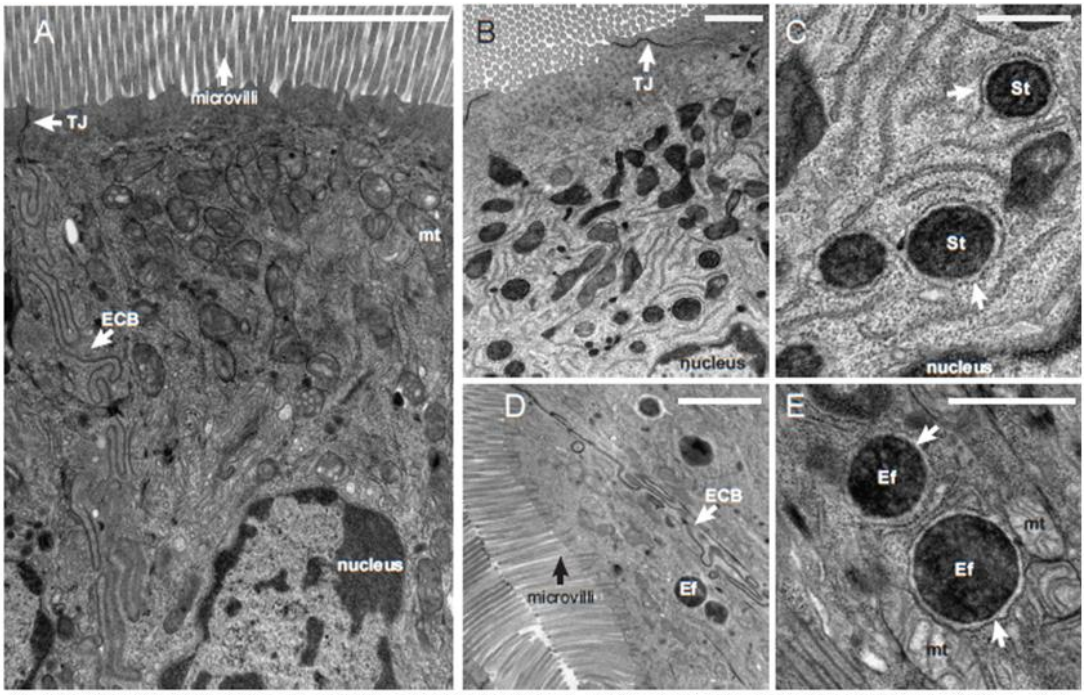

Figure 7: Ultrastructure of autophagosomes induced by bacteria in small intestinal epithelial cells. (**A**) Transmission electron microscopy of an ileal epithelial cell of a germ-free mouse. Key morphological features such as the nucleus, microvilli, tight junctions (TJ), epithelial cell border (ECB), and mitochondria (mt) are indicated. Scale bar=2 μm. (**B,C**) Lower and higher magnification images of an ileal epithelial cell 24 hr after *S. typhimurium* colonization of a germ-free mouse. Arrows indicate double membrane-bound compartments surrounding bacteria (St). Scale bars=1 mm (**B**) and 0.5 μm (**C**). (**D,E**) Ileal epithelial cell 24 hr after *E. faecalis* colonization of a germ-free mouse. Arrows indicate examples of double membrane-bound compartments enclosing bacteria (Ef). Scale bars=2 mm (**D**) and 1 μm (**E**).

Invasive bacteria elicit intestinal epithelial autophagy

Our findings above suggested that bacterial invasion of epithelial cells is required to activate intestinal epithelial autophagy. To further test this idea we used genetically altered *S.typhimurium*. The SPI-1 pathogenicity island encompasses genes essential for *S. typhimurium* entry into epithelial cells, and a *S. typhimurium* mutant engineered to lack this island (ΔSPI-1) is defective in its ability to invade gut epithelia (Eichelberg and Galan, 1999). The wild-type and ΔSPI-1 strains colonized germ-free mice to equivalent levels after 24 hours (Fig. 9) yet the numbers of LC3+ autophagosomes formed in response to the ΔSPI-1 mutant were dramatically reduced relative to the wild-type strain

(Figure 8A,B). This suggests that cellular invasion is required for *S. typhimurium* to activate epithelial autophagy.

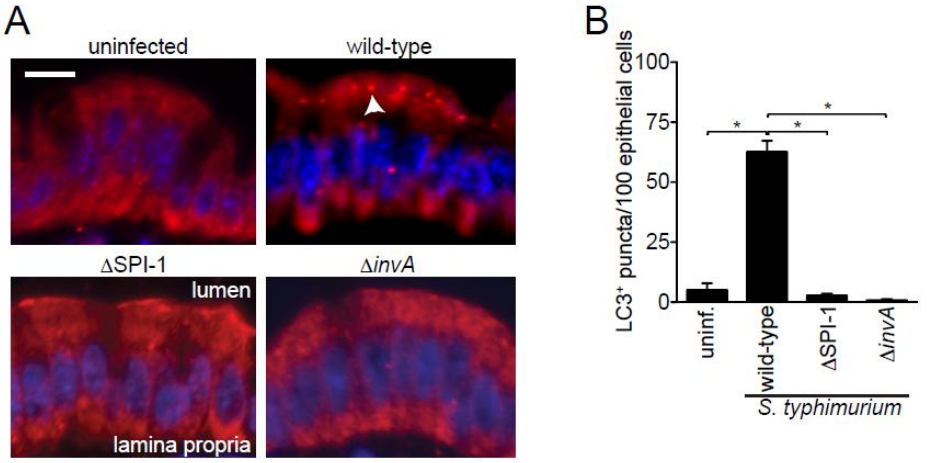

Figure 8: Invasive bacteria elicit autophagy in intestinal epithelial cells. (A) 10^9 CFU of wild-type or isogenic mutant *S. typhimurium* (ΔSPI-1 or Δ*invA*) were introduced into germ-free mice and LC3⁺ puncta in ileal epithelial cells were visualized by immunofluorescence 24 hr later. Scale bar=10 μm. **(B)** Quantification of LC3⁺ puncta in **(A)**. Data are represented as mean±SEM; *, $p<0.05$; n=4 mice/group.

To corroborate this finding we tested a second isogenic *S. typhimurium* mutant strain lacking a single component of the type III secretion apparatus, InvA. Deletion of *invA* also inhibits epithelial cell entry by *S. typhimurium* (Everest et al., 1999; Galan et al., 1992), and like the ΔSPI-1 strain, the *ΔinvA* mutant elicited reduced numbers of LC3+ autophagosomes in the small intestinal epithelium (Fig. 8A,B). These results support the idea that bacterial entry into epithelial cells is a prerequisite for autophagy activation.

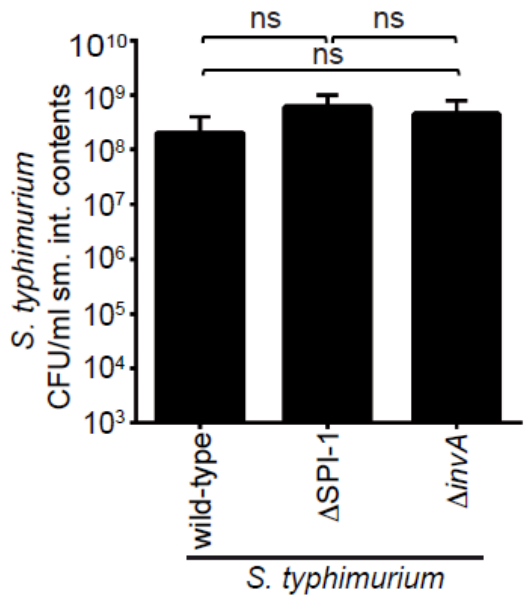

Figure 9: Intestinal colonization levels of wild-type and mutant *S. typhimurium* strains. There is no significant difference in the small intestinal colonization levels among the wild-type and mutant (ΔSPI-I and Δ*invA*) *S.typhimurium* strains.

Epithelial cell intrinsic MyD88 is required for autophagy activation

Prior studies have shown that bacterial induction of autophagy in macrophages requires activation of innate immune signaling pathways (Shi and Kehrl, 2008; Travassos et al., 2010). At the same time, other intestinal epithelial cell-intrinsic innate immune responses, such as expression of the antimicrobial protein RegIIIγ, are dependent on MyD88 (Brandl et al., 2007; Vaishnava et al., 2008), which signals downstream of Toll-like receptors (TLRs), the IL-1 receptor (IL-1R), and the IL-18 receptor (IL-18R) (Akira et al., 2006). We therefore tested whether bacterial induction of epithelial autophagy was similarly dependent on MyD88. As in conventional wild-type mice, epithelial LC3 showed a cytoplasmic distribution in conventional *Myd88*$^{-/-}$ mice (Fig. 10A,B). However,

unlike wild-type mice, *Myd88*$^{-/-}$ mice did not show detectable LC3+ autophagosome formation after oral challenge with *S. typhimurium* (Fig 10A,B), indicating that MyD88 is essential for bacterial activation of autophagy in small intestinal epithelial cells.

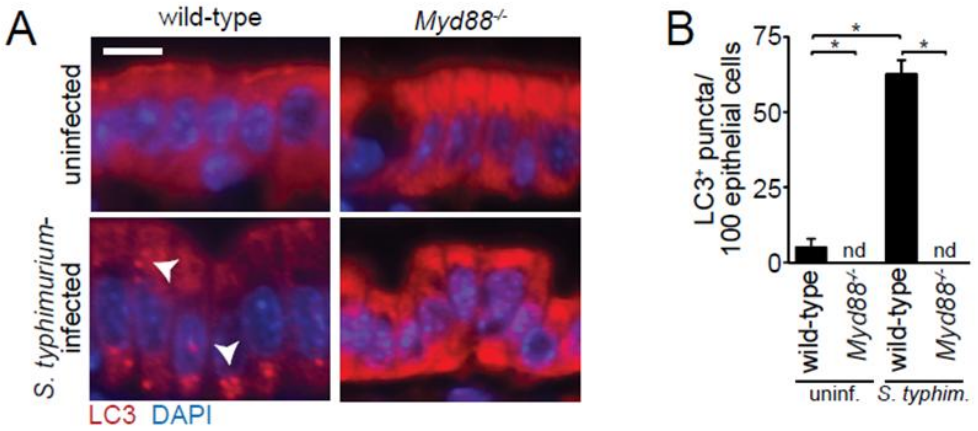

Figure 10: MyD88 is required for intestinal epithelial autophagy. Immunofluorescence detection of LC3 (red) in ileal epithelial cells from wild-type and *MyD88*$^{-/-}$ mice **(A)**. Tissues were counterstained with DAPI (blue). Mice were orally infected with *S. typhimurium* for 24 hr or were left uninfected. Arrowheads indicate examples of LC3^{+} puncta within intestinal epithelial cells. LC3^{+} autophagosomes were quantified in **(B)**. Data are represented as mean±SEM; *, $p<0.05$; n=5-8 mice/group; nd, not detected; scale bar=10 μm.

We next investigated the cellular origin of the MyD88 signals required to elicit epithelial autophagy. We generated mice with an epithelial cell–specific deletion of Myd88 (*Myd88*$^{\Delta IEC}$) (Ismail et al., 2011) by crossing mice carrying a *loxP*-flanked (floxed, fl) Myd88 allele (*Myd88*$^{fl/fl}$) (Hou et al., 2008) with mice expressing Cre recombinase under the control of the intestinal epithelial cell (IEC)–specific villin promoter (Madison et al., 2002). 24 hours after oral challenge with *S. typhimurium*, the *Myd88*$^{\Delta IEC}$ mice retained a cytoplasmic distribution of epithelial LC3 whereas their *Myd88*$^{fl/fl}$ littermates showed LC3+ autophagosome formation (Fig. 11A,B).

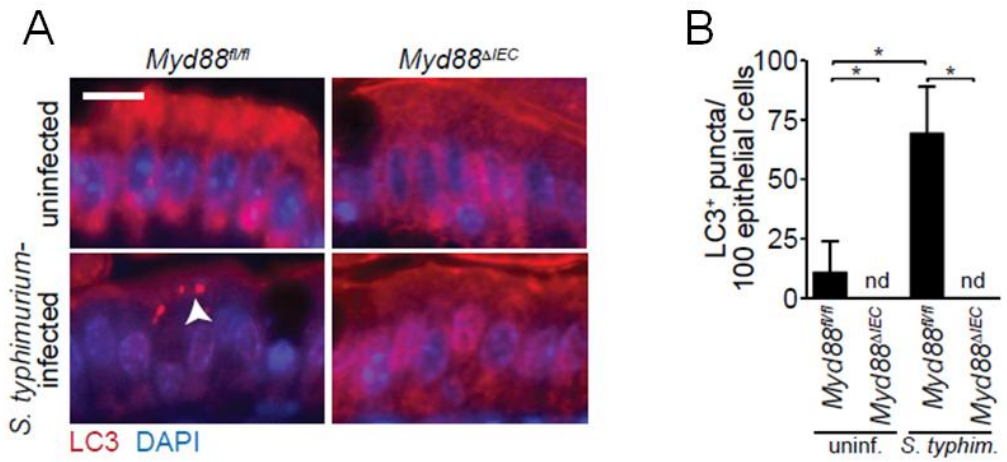

Figure 11: Epithelial cell-intrinsic MyD88 is required for intestinal epithelial cell autophagy. **(A)** *Myd88*$^{\Delta IEC}$ mice or *Myd88*$^{fl/fl}$ littermates were analyzed by immunofluorescence detection of LC3 in ileal epithelial cells. LC3^{+} puncta were quantified in **(B)**. Data are represented as mean±SEM; *, $p<0.05$; n=5-8 mice/group; nd, not detected; scale bar=10 μm.

Western blot analysis of isolated ileal epithelial cells revealed increased conversion of LC3-I to LC3-II at 24 hours following *S. typhimurium* colonization of the *Myd88*$^{fl/fl}$ mice but not the *Myd88*$^{\Delta IEC}$ mice (Fig. 12A,B), consistent with reduced autophagosome formation in the absence of epithelial MyD88. These results show that epithelial cell-intrinsic MyD88 signaling is required for bacterial induction of autophagy in intestinal epithelial cells.

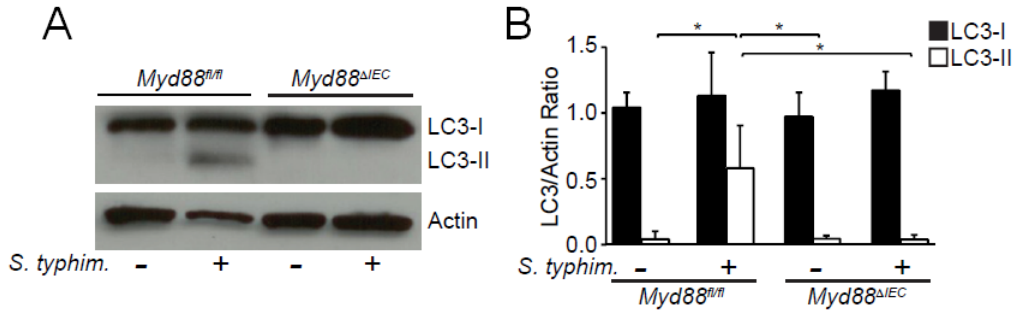

Figure 12: Western blot analysis of LC3 from ileal epithelial cell lysates following *S. typhimurium* infection. **(A)** Ileal Epithelial cell lysates were isolated from *Myd88*$^{fl/fl}$ or *Myd88*$^{\Delta IEC}$ mice before and after colonization with *S. typhimurium* for 24 hr **(B)**. The experiment was performed as described in Figure 1D, and band intensities from three independent experiments were quantified in **(F)**. Data are represented as mean±SEM; *, $p<0.05$.

Intestinal Autophagy activation is TRIF independent

We examined whether other innate immune signaling pathways are also required for intestinal epithelial autophagy. Prior studies have implicated signaling through the adaptor protein Toll-interleukin-1 receptor domain containing adaptor-inducing interferon-β (TRIF) in bacterially-activated autophagy in RAW macrophages (Xu et al., 2007). However, we observed that *TRIF*$^{-/-}$ mice exhibited autophagosome formation following oral *S. typhimurium* infection (Fig. 13), indicating that TRIF is not required for intestinal epithelial autophagy *in vivo*.

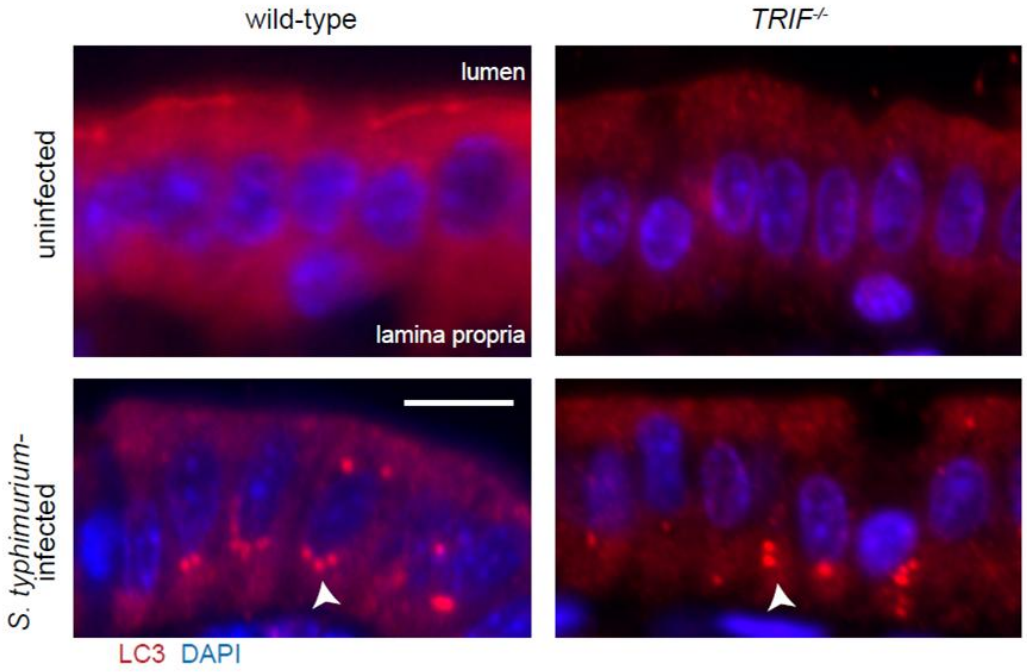

Figure 13: Autophagy Induction in the intestinal epithelium is not TRIF-dependent. *Trif* -/- and wild-type were analyzed by immunofluorescence detection of LC3 in ileal epithelial cells before and after colonization with *S. typhimurium* for 24 hr.

Nod2 Independent activation of the epithelial cell autophagy

Since the intracellular pattern recognition receptor Nod2 has been implicated in the autophagy pathway in macrophages (Travassos et al., 2010), dendritic cells (Cooney

et al., 2010), and cultured epithelial cells (Homer et al., 2010), we also examined *Nod2*$^{-/-}$ mice (Kobayashi et al., 2005). We observed numerous LC3+ puncta in conventional *Nod2*$^{-/-}$ mice, even in the absence of an additional bacterial challenge (Fig. 14A,B). Depletion of the microbiota with broad spectrum antibiotics reduced the number of LC3+ autophagosomes in the *Nod2*$^{-/-}$ mice (Fig. 14A,B), indicating that autophagosome formation was a response to the microbiota and was probably not due to blocked flux through the autophagy pathway (Mizushima et al., 2010).

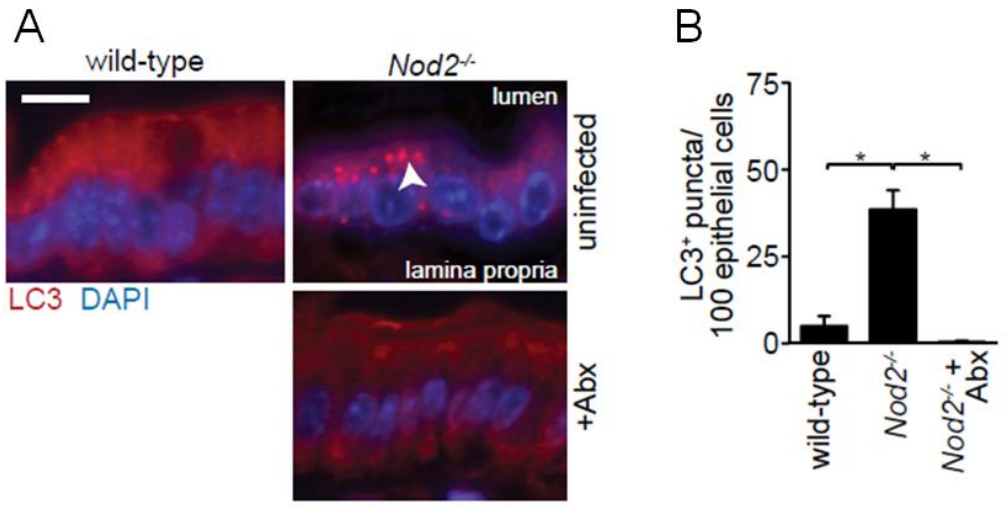

Figure 14: Intestinal epithelial autophagy does not require Nod2. (A) Ileal tissue sections from conventionally-raised *Nod2*$^{-/-}$ mice were analyzed by immunofluorescence detection of LC3. LC3^{+} puncta formation was reversible by antibiotic (Abx) treatment to reduce the microflora. LC3^{+} puncta were quantified in **(B)**. Data are represented as mean±SEM; *, $p<0.05$; n=5-8 mice/group; scale bar=10 μm.

Because epithelial autophagosome formation is associated with bacterial invasion into the cell cytoplasm (Fig. 1-8), we postulated that the increased autophagosome formation in *Nod2*$^{-/-}$ mice was due to increased invasion of epithelial cells by commensal bacteria. To test this idea we assayed for intracellular bacteria using fluorescence *in situ* hybridization with a universal 16S ribosomal RNA (rRNA) gene probe. We observed increased numbers of intracellular bacteria within the small intestinal epithelial cells of *Nod2*$^{-/-}$ mice as compared to wild-type mice (Fig. 15A,B). Numbers of intracellular

bacteria were reduced upon antibiotic treatment of the *Nod2*$^{-/-}$ mice (Fig. 15A,B). These findings support the idea that autophagy activation in the intestinal epithelium is associated with bacterial invasion of epithelial cells.

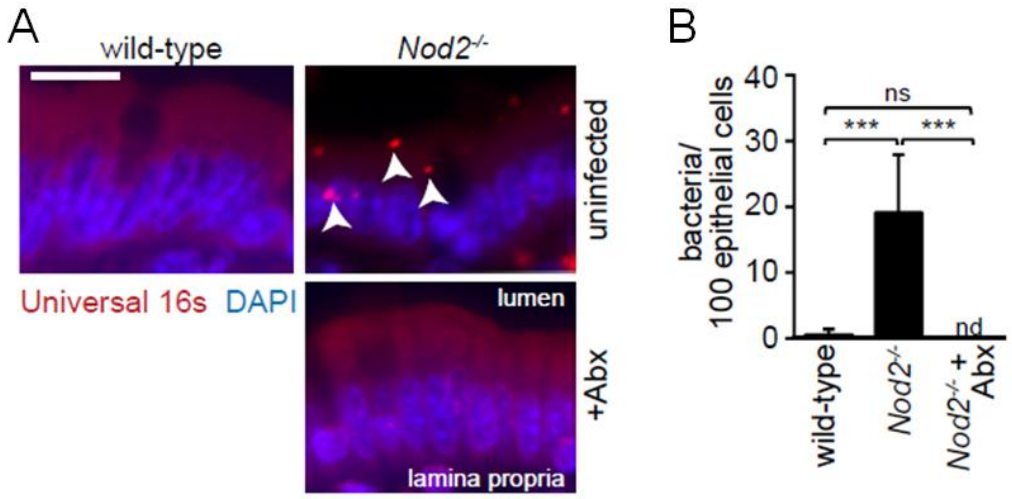

Figure 15: Increased bacterial invasion into the epithelial cells of *Nod2*$^{-/-}$ mice. (I) Bacteria were localized by FISH using a fluorescent probe against the 16S rRNA genes of all bacteria (universal 16S, red). **(A)** Cell-associated bacteria in **(B)** were counted. Data are represented as mean±SEM; ***, $p<0.001$; n=3 mice/group; scale bar=10 μm.

Together, our results establish that *in vivo* intestinal epithelial autophagy is MyD88-dependent, TRIF-independent and NOD2-independent, and suggest that commensal bacteria can elicit epithelial autophagy in certain immune deficient hosts where there is increased bacterial invasion of the intestinal epithelium.

Epithelial cell autophagy limits bacterial dissemination to extraintestinal tissues

We next sought to determine whether autophagy in intestinal epithelial cells contributes to host resistance to bacterial infection and dissemination. To assess this, we created mice with an intestinal epithelial cell-specific deletion of the essential autophagy gene *Atg5* by crossing mice with a loxP-flanked Atg5 allele (*Atg5*$^{fl/fl}$) (Hara et al., 2006; Tsukamoto et al., 2008) with villin-*Cre* transgenic mice (Madison et al., 2002) to produce

Atg5$^{\Delta IEC}$ mice. We verified that ATG5 expression was diminished in intestinal epithelial cells isolated from the terminal ileum (Fig 16A).

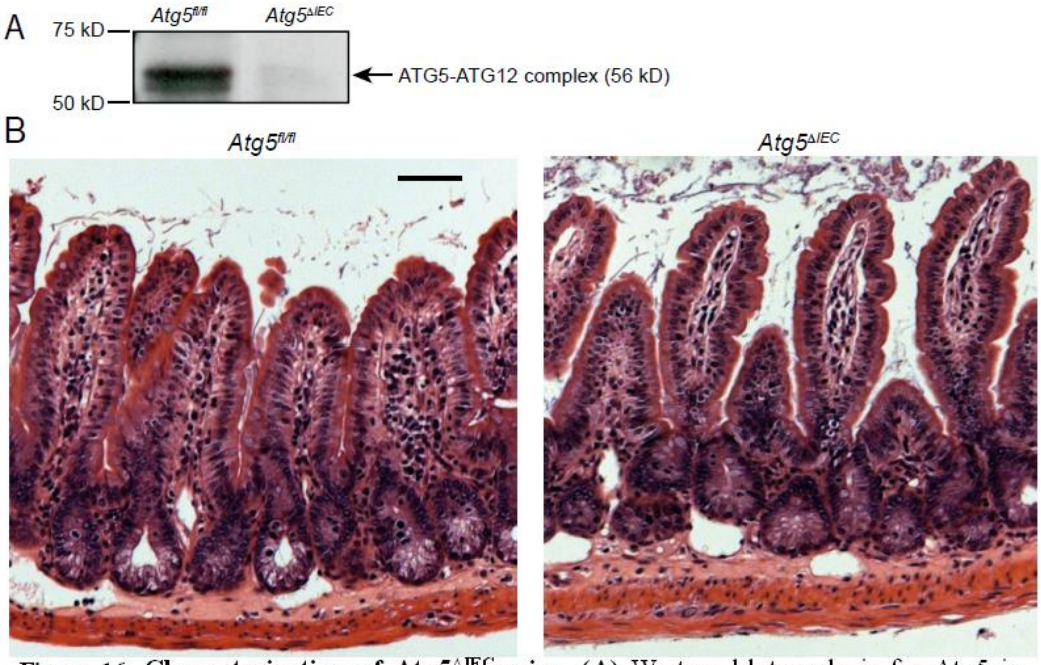

Figure 16: Characterization of Atg5$^{\Delta IEC}$ mice. (A) Western blot analysis for Atg5 in Ileal Epithelial cell lysates of Atg5$^{fl/fl}$ and Atg5$^{\Delta IEC}$ mice. **(B)** H & E stains of Ileal tissue of Atg5$^{fl/fl}$ and Atg5$^{\Delta IEC}$ mice scale bar=10 μm.

We further observed reduced numbers of LC3+ autophagosomes in the intestinal epithelial cells of the *Atg5*$^{\Delta IEC}$ mice after oral *S. typhimurium* challenge as compared to *Atg5*$^{fl/fl}$ littermates (Fig 17A,B).

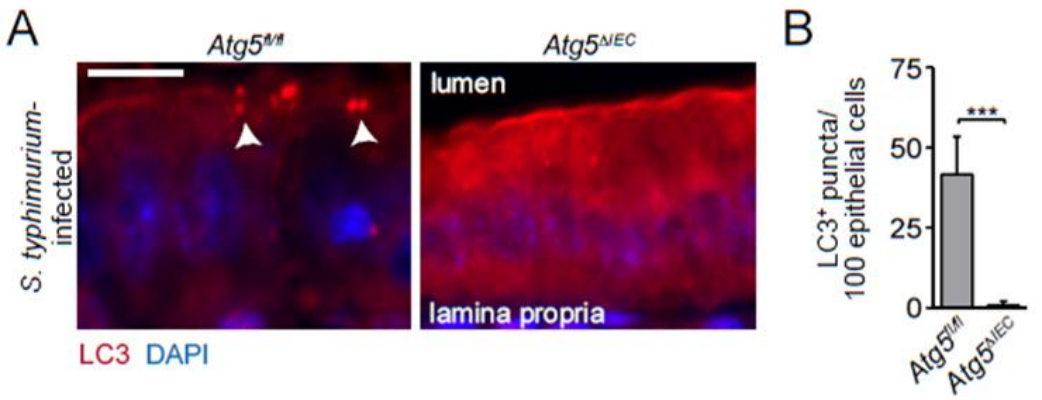

Figure 17: Intestinal epithelial cell requires Atg5. (A) *Atg5*$^{fl/fl}$ and *Atg5*$^{\Delta IEC}$ littermates were orally infected with *S. typhimurium*-GFP. Ileal sections were stained with anti-LC3 (red) to visualize LC3^{+} autophagosomes (arrows)**(A)**. Scale bar=10 μm. Numbers of LC3^{+} autophagosomes in **(A)** were counted **(B)**. Data are represented as mean±SEM; ***, $p<0.001$; n=5 mice/group.

The decreased numbers of autophagosomes accorded with reduced conversion of LC3-I to LC3-II in the *Atg5*$^{\Delta IEC}$ mice (Fig. 18A,B). While there was no histological evidence of overt inflammation or pathology in unchallenged *Atg5*$^{\Delta IEC}$ mice (Figure 16B), we saw that the decreased autophagosome formation in the *Atg5*$^{\Delta IEC}$ mice coincided with increased numbers of intracellular *S. typhimurium* 24 hours after oral challenge (Fig. 17A,B and Fig. 18C). These data are consistent with a role for intestinal epithelial autophagy as a cell autonomous mechanism for limiting the persistence of invading bacteria.

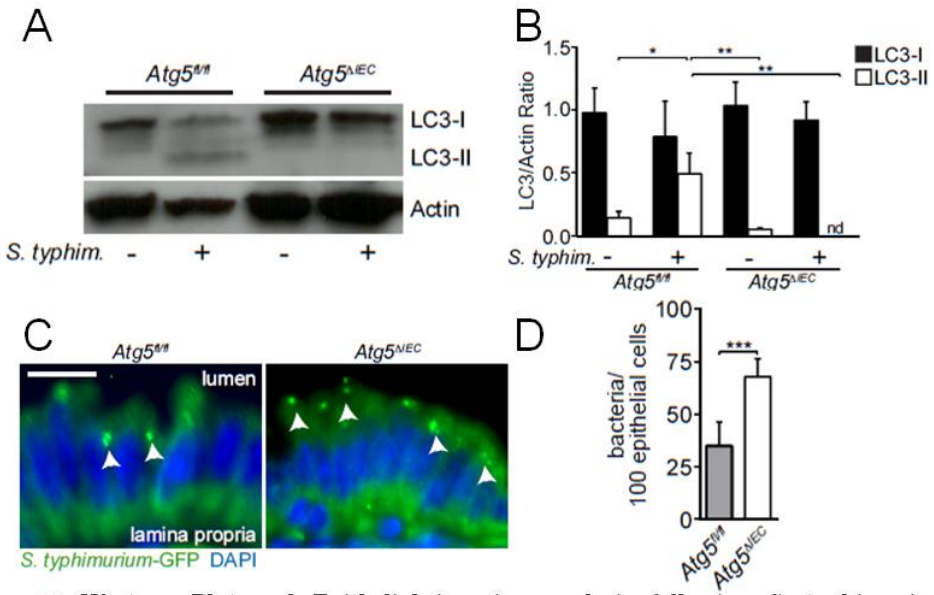

Figure 18: Western Blot and Epithelial invasion analysis following S. typhimurium infection. (A) Proteins from ileal epithelial cells isolated from *Atg5*$^{fl/fl}$ and *Atg5*$^{\Delta IEC}$ mice before and after oral infection with *S. typhimurium* for 24 hr. Band intensities from three independent experiments are quantified in (**B**). Data are represented as mean±SEM; *, $p<0.05$; **, $p<0.01$; nd, not detected. **(C)** Intracellular bacteria (green) were visualized by immunofluorescence microscopy in the ileums of *Atg5*$^{fl/fl}$ and *Atg5*$^{\Delta IEC}$ mice 24 hr post-infection with *S. typhimurium*-GFP. Tissue sections were counterstained with DAPI (blue) to visualize cell nuclei. Arrowheads point to examples of intracellular bacteria. Scale bar=10 μm. **(D)** Intracellular bacteria were counted. Data are represented as mean±SEM; ***, $p<0.001$; n=5 mice/group.

The *Atg5*$^{\Delta IEC}$ mice also showed increased *S. typhimurium* dissemination to extraintestinal tissues. 24 hours after oral *S. typhimurium* challenge, we recovered significantly higher numbers of bacteria from the spleens and livers of the *Atg5*$^{\Delta IEC}$ mice relative to their *Atg5*$^{fl/fl}$ littermates (Fig. 19A,B). This was not due to differences in overall small intestinal bacterial colonization levels, which were similar between the two groups (Fig. 19C).

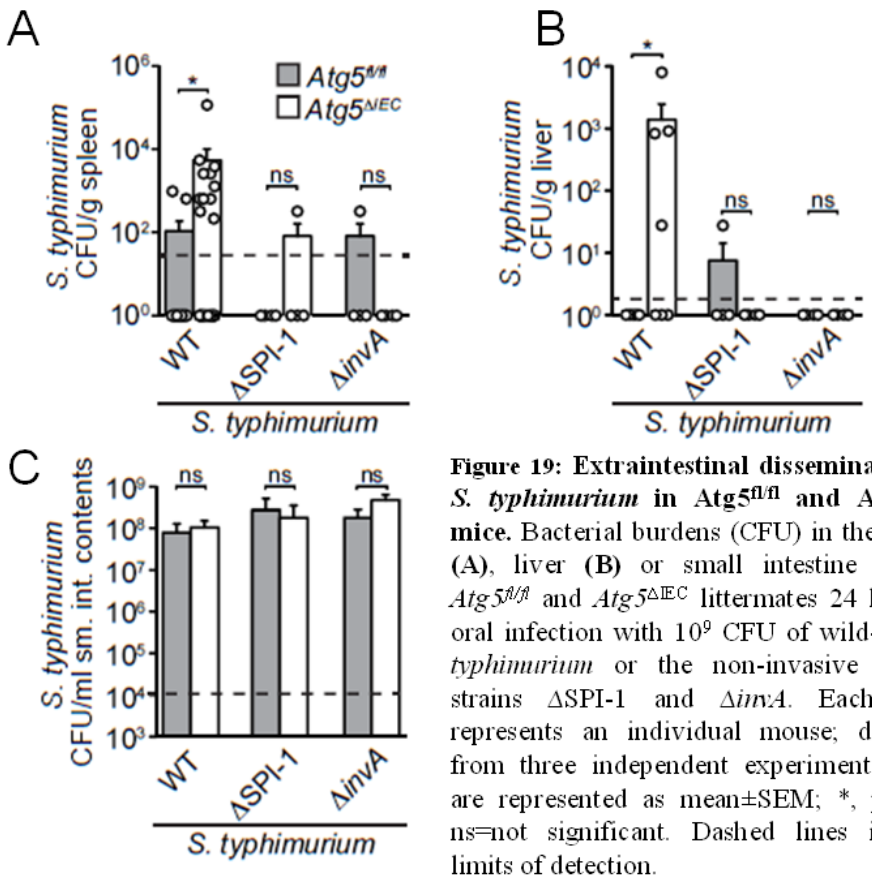

Figure 19: Extraintestinal dissemination of *S. typhimurium* in Atg5$^{fl/fl}$ and Atg5$^{\Delta IEC}$ mice. Bacterial burdens (CFU) in the spleen **(A)**, liver **(B)** or small intestine **(C)** of *Atg5*$^{fl/fl}$ and *Atg5*$^{\Delta IEC}$ littermates 24 hr after oral infection with 10^9 CFU of wild-type *S. typhimurium* or the non-invasive mutant strains ΔSPI-1 and Δ*invA*. Each point represents an individual mouse; data are from three independent experiments. Data are represented as mean±SEM; *, $p<0.05$, ns=not significant. Dashed lines indicate limits of detection.

The differences in dissemination to extraintestinal tissues were only observed after oral infection; no significant difference was observed in the numbers of *S. typhimurium* recovered from the spleens of *Atg5*$^{\Delta IEC}$ and *Atg5*$^{fl/fl}$ mice following intraperitoneal inoculation (Fig. 20). This indicates that the increased *S. typhimurium* dissemination was not due to a global immune defect in the *Atg5*$^{\Delta IEC}$ mice.

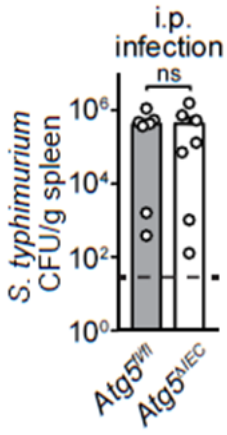

Figure 20: Bacterial Burden of *S. typhimurium*. Bacterial burden (CFU) in the spleen 24 hr after intraperitoneal infection of *Atg5*$^{\Delta IEC}$ and *Atg5*$^{fl/fl}$ littermates with 10^3 CFU of wild-type *S. typhimurium*. ns, not significant. Data are represented as mean±SEM.

We considered whether the increased dissemination of *S. typhimurium* in *Atg5*$^{\Delta IEC}$ mice might be due to autophagy-independent effects of ATG5-deficiency. The *Atg5*$^{\Delta IEC}$ mice did not exhibit increased permeability to orally-administered FITC-dextran (Fig. 21), suggesting that the increase in *S. typhimurium* dissemination did not arise from enhanced nonspecific barrier permeability. We also did not observe enhanced dissemination of the non-invasive *S. typhimurium* strains ΔSPI-1 and *ΔinvA* when introduced into *Atg5*$^{\Delta IEC}$ mice (Fig. 19A-B).

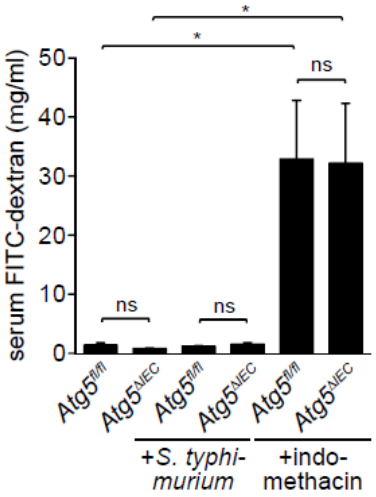

Figure 21: Paracellular Permeability Assay. Amount of serum FITC-dextran present before and after the addition of indomethacin of *Atg5*$^{\Delta IEC}$ and *Atg5*$^{fl/fl}$ littermates. Permeability also assessed before and after infection with 10^9 CFU of wild-type *S. typhimurium*. ns, not significant. Data are represented as mean±SEM.

This indicates that the increased dissemination of wild-type *S. typhimurium* in *Atg5*$^{\Delta IEC}$ mice is unlikely to arise from defects in Paneth cell granule exocytosis and

antimicrobial protein secretion that have been observed in these mice (Cadwell et al., 2008). Taken together, our findings show that intestinal epithelial autophagy plays an essential role in limiting the numbers of *S. typhimurium* within intestinal epithelial cells and in controlling its dissemination to extraintestinal tissues.

DISCUSSION

The mammalian intestine is colonized with thousands of bacterial species that include potential pathogens as well as symbionts. As the primary interface between the host and the microbiota, the epithelium must use multiple immune defense strategies that both prevent bacterial attachment to the epithelial surface and eliminate those microbes that manage to penetrate the epithelial barrier. Here, we have identified autophagy as an important component of epithelial cell-intrinsic innate immune defense in the intestine. We found that autophagy was activated in epithelial cells of the intestine by bacteria that invade the cell cytoplasm. These included *Salmonella typhimurium*, an overt pathogen, as well as *Enterococcus faecalis*, an opportunistically-invasive member of the microbiota. We found that activation of autophagosome formation required epithelial cell-intrinsic MyD88 signaling, and that epithelial autophagy was essential for limiting the numbers of epithelial intracellular bacteria and for preventing their spread to extraintestinal sites (Figure 6K). These findings provide essential new insight into how the mammalian intestinal epithelium maintains homeostasis with a large and diverse microbial community, and demonstrate the importance of autophagy for intestinal innate immunity *in vivo*.

The intestinal epithelium plays diverse roles in immune protection against commensal and pathogenic bacteria in the intestine. For example, epithelial cells produce a variety of antimicrobial proteins that are critical for maintaining homeostasis with the microbiota (Gallo and Hooper, 2012). α-defensins produced by Paneth cells play an important role in shaping the composition of luminal bacterial communities in the small intestine (Salzman et al., 2010). Other antimicrobial proteins protect the apical surface of the epithelium from excessive bacterial attachment. RegIIIγ, an antimicrobial lectin that is expressed throughout the intestinal epithelium, is regulated by bacterial signals through an epithelial cell-intrinsic MyD88 pathway (Brandl et al., 2007; Cash et al., 2006; Vaishnava et al., 2008). RegIIIγ interacts with the intestinal mucus layer to limit bacterial interactions with the intestinal epithelial surface and thus restrict bacterial invasion of host tissues (Vaishnava et al., 2011). In addition to directing antimicrobial responses to the epithelial surface and the lumen, the intestinal epithelium also orchestrates a complex array of subepithelial immune responses to intestinal bacteria through the secretion of cytokines and other factors (Artis, 2008).

Despite the presence of a formidable antimicrobial barrier at the epithelial surface, some bacteria are equipped to evade these defenses and invade epithelial cells. Our findings suggest that autophagy is a critical mechanism of epithelial cell-intrinsic innate immunity that functions to eliminate invading bacteria before they can access deeper tissues. Consistent with this idea, our results indicate that epithelial autophagy is specifically activated by invasive bacteria. These include S. *typhimurium*, an overt pathogen, and *E. faecalis*, an opportunistically-invasive pathobiont. In contrast, non-invasive bacteria, including *Lactobacillus salivarius* and S.*typhimurium* lacking the SPI-1

pathogenicity island, did not trigger autophagosome formation in the intestinal epithelium. This argues that autophagy is not activated as a general response to a SPF microbiota, but requires bacterial invasion into the cell cytoplasm.

The autophagy machinery has been shown to be involved in cellular functions that are distinct from classical autophagy involving the targeting of bacteria to autophagosomes (Zhao et al., 2008). Mice harboring genetic mutations or deletions in *Atg5* or *Atg16L1* exhibit abnormalities in the secretory pathway of Paneth cells, a specialized small intestinal epithelial lineage involved in antimicrobial protein secretion (Cadwell et al., 2008; Cadwell et al., 2010). It is possible that the *Atg5*$^{\Delta IEC}$ mice exhibit increased S. *typhimurium* invasion because of such defects in Paneth cell function, which could lead to increased accessibility of bacteria to the epithelial surface (Vaishnava et al., 2008). However, a role for classical autophagy would be consistent with our finding of prominent activation of autophagosome formation in the intestinal epithelium after S. *typhimurium* challenge. Additionally, we found that unlike wild-type *S. typhimurium*, non-invasive *S. typhimurium* strains do not show increased dissemination in *Atg5*$^{\Delta IEC}$ mice. This argues against the idea that the increased dissemination of wild-type *S. typhimurium* in Atg5$^{\Delta IEC}$ mice is due to defective Paneth cell secretory function. Finally, it is possible that *S. typhimurium* infection results in conditions of nutrient deprivation that initiate autophagy in order to sustain essential metabolic functions (Tattoli et al., 2012). However, our observation that *S. typhimurium*-induced autophagosome formation is also MyD88 dependent suggests that nutrient starvation does not entirely account for bacterially-induced autophagy in the intestinal epithelium *in vivo*.

Other types of intestinal bacterial pathogens may also be targets of epithelial cell autophagy. A prior study showed that epithelial cell expression of the autophagy protein ATG7 confers protection against the mouse intestinal pathogen *Citrobacter rodentium in vivo*, limiting its intestinal colonization levels and the pathogenic response of host tissue (Inoue et al., 2012). *C. rodentium* belongs to a family of enteric pathogens that includes the human pathogens enteropathogenic *Escherichia coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC). These organisms colonize the gastrointestinal tract by forming attaching and effacing lesions on the apical surface of the colonic epithelium but do not appear to enter epithelial cells in large numbers (Mundy et al., 2005). It is not yet clear whether bona fide autophagy is involved in limiting *C. rodentium* intestinal colonization and pathogenesis (Inoue et al., 2012). Thus, it will be interesting to determine whether *C. rodentium* triggers autophagy in colonic epithelial cells, and whether autophagy-dependent or -independent functions of ATG7 are involved in protecting against the pathogenic effects of *C. rodentium*.

Our analysis of the host factors involved in autophagy initiation in the intestinal epithelium revealed a requirement for epithelial cell-intrinsic MyD88. However, the host receptors that lie upstream of MyD88 remain to be defined. Given that autophagy activation requires invasive bacteria, it is possible that intracellular TLRs, such as TLR9, might be involved. It is also possible that invading bacteria could penetrate the epithelium via a paracellular route, or could traverse and exit epithelial cells via the basolateral surface, and thus might trigger basolaterally oriented epithelial TLRs such as TLR5 (Gewirtz et al., 2001). Given the dependence of epithelial autophagosome formation on bacterial invasion, it seems unlikely that these responses are stimulated through activation

of apically-oriented epithelial TLRs. It is also possible that autophagy activation could involve MyD88-dependent IL-1 or IL-18 signaling (Akira et al., 2006).

Another key question is how MyD88 integrates with the autophagy pathway to promote autophagosome formation. One possibility is that MyD88 signaling controls the expression of proteins involved in autophagosome formation in response to bacteria. Another possibility is that MyD88 acts as an adaptor for assembly of protein complexes that regulate autophagosome formation. In macrophages, MyD88 promotes autophagy by interacting with Beclin 1, a key autophagy protein that promotes autophagosome formation by recruiting other autophagy factors to the pre-autophagosomal membrane (Kihara et al., 2001a; Kihara et al., 2001b). Beclin 1 function is inhibited by binding to Bcl-2, an autophagy inhibitor (Pattingre et al., 2005). The MyD88-Beclin 1 interaction inhibits the binding of Beclin 1 to Bcl-2, thus relieving the Bcl-2 inhibition and promoting autophagy (Shi and Kehrl, 2008). It will be interesting to determine whether MyD88 plays a similar role in the intestinal epithelium.

A growing body of research supports a connection between autophagy gene mutations and IBD. Genome wide association studies have linked mutations in Atg16L1 to an increased risk for developing Crohn's disease. The polymorphisms were found to impact autophagy independent functions of Atg16L1, resulting in abnormal granule packaging and protein secretion in Paneth cells (Cadwell et al., 2008). These abnormalities were associated with increased susceptibility to Crohn's disease-like pathologies upon virus infection of mice (Cadwell et al., 2010). These findings suggested how defects in autophagy gene function can lead to inflammatory pathologies. Our results expand upon these findings by suggesting that autophagy defects could have a

broader role in inflammatory disease by interfering with the ability of the intestinal epithelium to clear invading bacteria. This could lead to ongoing immune activation, particularly in the presence of other immune system defects or perturbed intestinal microbial communities.

CHAPTER FIVE

Conclusions and Future Directions

General Discussion

The intestinal epithelium is tasked with the incredibly difficult yet extremely important task of absorbing the critical nutrients from the diet. To acieve maximal efficiency at extracting calories, mammals have evolved to coexist with trillions of bacteria within the lumen of our intestines. However, the normally beneficial, mutualistic relationship can go awry when the bacteria invade the host tissue. When considering the findings of the past decade, it becomes clear that the host expends considerable amounts of energy to limit the extent to which members of the intestinal microbiota invade mucosal tissue. Perhaps one of the most interesting things about the innate immune protection of the intestinal surface is that many of the different mechanisms used by the host to protect the mucosal surface are critically important, meaning that loss of any one of them results in a perturbation of homeostasis and ultimately a quantifiable phenotype.

For example, the goblet cells of the intestine are responsible for production of the mucus in the gut. One might think that loss of mucus production alone would probably not be enough to cause disease, but in fact that turns out not to be the case. Mice engineered to be deficient in the production of mucus via deletion of MUC2 gene show a significant phenotype relative to their littermate controls. At just 5 weeks of age, *Muc2* deficient mice demonstrated significant growth retardation. Additionally, they were also showed rectal prolapse, an increase in colonic crypt length with crypt hyperplasia and

enhanced lymphocyte infiltration into the colon, all signs of colitis (Van der Sluis et al., 2006).

The importance of all various arms of innate immunity is again highlighted when considering RegIIIɣ. RegIIIɣ is one of a number of antimicrobial peptides produced in the intestine. Again, when considering the variety and number of antimicrobial peptides, one would predict some level of redundancy with respect to their role in maintaining intestinal homestasis, but in fact, Vaishnava et al. have demonstrated RegIIIɣ to be essential in maintaining normal intestinal function (Vaishnava et al., 2011).

The work presented here has established that epithelial cell autophagy is a critical component of the innate immune defense on the small intestine. Without epithelial cell autophagy in the small intestine, hosts are more susceptible to extraintestinal dissemination of invasive bacteria. When we consider autophagy against the evolutionary backdrop upon which it evolved, it is not surprising that epithelial autophagy is critical in innate immunity of the intestine. Evolution is driven by natural selection which is determined by fitness. Over millions of years, adaptations in immune responses have occurred that have fine-tuned our immune response. This fine-tuning process undoubtedly included the loss of unessential functions and the maintenance of immune functions providing most benefit to the host. Therefore, I suggest that many of the antibacterial innate immune mechanisms will ultimately prove critical to some part of maintenance of intestinal homeostasis.

Regulation of Bacterial-Induced Autophagy

Moving forward, critical questions remain about the molecular mechanisms involved in the regulation of epithelial cell autophagy. A great deal of interest was generated in the NOD2 gene when it was first identified as having polymorphims associated with Crohn's disease (2007). This was especially intriguing because NOD2 had been shown to recognize bacterial muramyl–dipeptide (MDP). This was consistent with the fact that the etiology of Crohn's disease is thought to involve aberrant bacterial invasion of intestinal tissue (Sartor, 2008). Subsequent findings also indicated an interaction between the Nod proteins and the autophagy protein Atg16L1 following infection of tissue culture cells with invasive bacteria (Travassos et al., 2010). The researchers conducting that study suggested that a direct interaction between Nod2 and Atg16L1 might explain the link seen between Nod2 mutations, Atg16L1 mutations and Crohn's disease. However, following investigation of the role of Nod2 *in vivo* in the intestinal epithelium, I hypothesize a different mechanism linking Nod2 and autophagy.

When I analyzed the ability of *Nod2*$^{-/-}$ mice to form autophagosomes, I was surprised to find that conventionally-raised *Nod2*$^{-/-}$ mice, with no additional pathogenic bacterial challenge like *S. typhimurium* infection, demonstrated autophagy activation. Moreover, autophagosomal formation in the intestinal epithelium disappeared when the *Nod2*$^{-/-}$ mice were placed on antibiotic treatment. This led me to investigate the spatial distribution of the conventional microbiota in *Nod2*$^{-/-}$ mice versus wild-type or antibiotic treated *Nod2*$^{-/-}$ mice. To my surprise, I saw that the entire crypt-villus unit of conventionally-raised *Nod2*$^{-/-}$ mice was covered in bacteria, a strikingly different phenotype than that displayed by conventional-raised wild-type or antibiotic treated

Nod2$^{-/-}$ mice. This finding helped develop my suggested my hypothesis regarding Nod2 mutations and their link to Crohn's disease: NOD2 deficiency leads to an alteration, either in the composition or the control of conventional microbiota, and this alteration ultimately brings the microbiota much closer to the mucosal surface making invasion into epithelial cell much more likely.

MyD88 Integration into Autophagy Machinery

Given the requirement of epithelial MyD88 to activate autophagy in response to bacterial challenge, a critical question is how MyD88 integrates with the autophagy pathway in the intestinal epithelium. In cultured macrophages, MyD88 promotes autophagy by interacting with Beclin 1 (Shi and Kehrl, 2008), a key autophagy protein that initiates autophagosome formation and helps to localize other autophagy factors to the pre-autophagosomal membrane. The MyD88-Beclin 1 interaction inhibits the binding of Beclin 1 to BCL2, which suppresses autophagy. Based on these findings, I hypothesize that bacterial triggering of MyD88 signaling in the intestinal epithelium stimulates autophagy by promoting the dissociation of Beclin 1 from the BCL2 inhibitor (Fig. 1). Understanding the nature of this relationship should yield key insights into the mechanisms by which MyD88 induces intestinal epithelial autophagy.

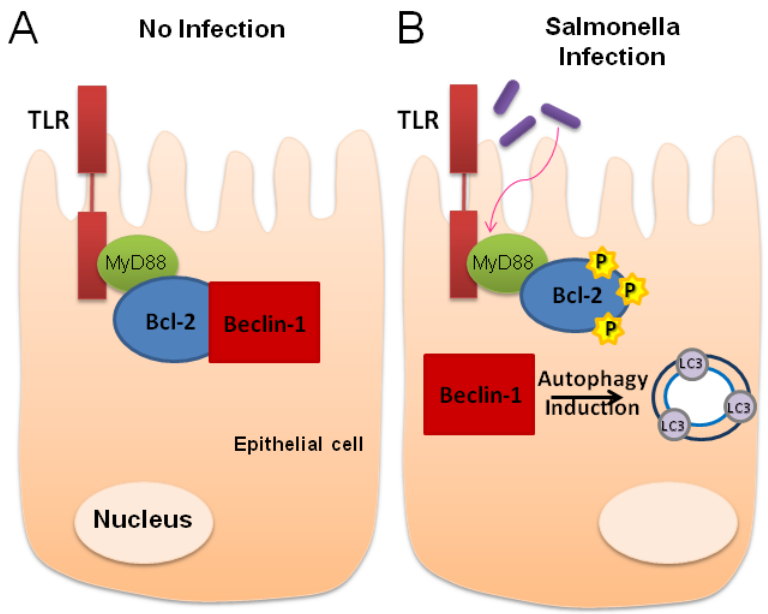

Figure 1: Model of MyD88 Interaction with Autophagy Machinery. (A) In the uninfected condition, epithelial cell Myd88 is not triggered, keeping the BCL2:Beclin 1 complex intact. **(B)** Following S. *typhimurium* infection, Myd88 is triggered, causing dissociation of BCL2:Beclin 1 complex and autophagy activation

Bcl-2:Beclin 1 Function in Intestinal Epithelium

An important question regarding epithelial cell autophagy is to identify the mechanisms regulating autophagy function and activation. Previous work has demonstrated that the Bcl-2:Beclin 1 protein interaction is critical in regulating inducible autophagy. Work from the Levine lab has shown that the dissociation of Bcl-2 from Beclin 1 is a critical part of the autophagic response to metabolic stimulus. Additionally, the proper regulation of the dissociation of BCL2: Beclin 1 complex was also shown to be required for proper glucose metabolism following exercise stimulus (He et al., 2012). The contribution of the Bcl-2:Beclin 1 complex to autophagy regulation as well as energy metabolism suggests that it may also regulate autophagy within the intestinal epithelium.

The $BCL2^{AAA}$ knock-in mice will be a critical tool in assessing whether dissociation of the BCL-2:Beclin 1 complex is important for driving autophagy in the

intestinal epithelium. BCL2^{AAA} knock-in mice harbor three point mutations that together render BCL2 nonphosphorylatable and thus incapable of dissociating from Beclin 1. Using fluorescent microscopy analysis, I asked whether the BCL2^{AAA} knock-in mice were capable of forming autophagosomes in response to *S. typhimurium* infection in a manner similar to that seen in wild-type mice. My hypothesis was that the BCL2^{AAA} mice would be unable to form autophagosomes following *S. typhimurium* infection, as Beclin 1 should be unable to dissociate from BCL2. Preliminary experiments performed on both conventional (CV) and *S. typhimurium* challenged BCL2^{AAA} mice indicate that, in contrast to wild-type mice which form autphagosomes in their epithelium following *S. typhimurium* challenge (Fig. 2A), BCL2^{AAA} mice are unable to form autophagosomes in their intestinal epithelial cells following bacterial stimulus (Fig. 2B). These preliminary data need to be confirmed with biochemically via Western blot analysis of small intestinal epithelial cell lysates and via electron microscopy.

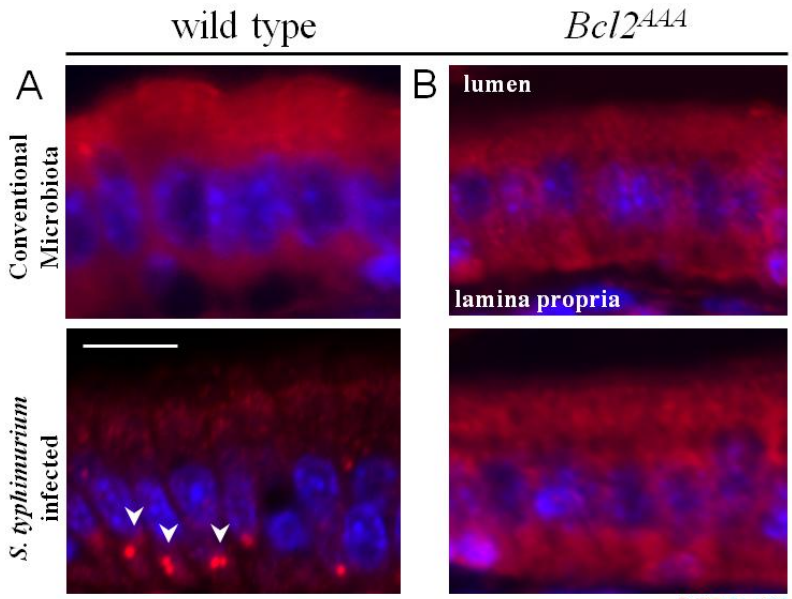

Figure 2: Autophagy activation by *S. typhimurium* in Bcl2^{AAA} mice. Conventional mice were left uninfected or were orally inoculated with 10^9 CFU of *S. typhimurium.* for 24hr. Sections of ileum were stained for LC3. **(A)** WT mice form autophagosomes in response to *S. typhimurim* **(B)** CV Bcl2^{AAA} mice show diffuse cytoplasmic LC3 staining. but Bcl2^{AAA} are unable to form autophagosomes following *S. typhimurium* infection. Tissues were counterstained with DAPI (blue). Scale bar = 10μm

If the phosphorylation of Bcl-2 does indeed prove to be a critical step in the initiation of autophagy, the next logical step is to determine if dissociation of BCL2 from Beclin 1 is under MyD88 control. Analysis and comparison of epithelial cell lysates via immunoprecipitation with an antibody specific for BCL2 and subsequent Western blot analysis for Beclin 1 will be critical experiments in determining the nature of the MyD88, BCL2:Beclin 1 relationship. In uninfected wild-type mice, Beclin 1 should remain complexed with BCL2 and should therefore be detectable via Western Blot analysis. However, in the *S. typhimurium* challenged wild-type mice, a MyD88 signal will be triggered activating epithelial autophagy and thus Beclin 1 should no longer co-precipitate with BCL2. Conversely, in *MyD88*$^{-/-}$ mice, Beclin1 should co-precipitate with BCL2 in both the uninfected and infected conditions.

The prior study of Shi and Kehrl showed that the disruption of the Beclin 1:BCL2 interaction was both *MyD88* and *Trif* dependent (Shi and Kehrl, 2008). Therefore, it is possible that the residual TRIF signal in the *MyD88*$^{-/-}$ mice will be sufficient to disrupt the Beclin 1:BCL2 complex. However, because Trif is only required for signaling through TLR3 and 4, it seems likely that MyD88 is the critical signal for autophagy activation by invading bacteria. Additionally, my previous work has demonstrated that TRIF is not required for autophagy activation following *S. typhimurium* challenge, making its requirement for Beclin 1:BCL-2 dissociation even less likely although not impossible.

It is also possible that dissociation of the Beclin 1:BCL2 complex will be unimportant for autophagy activation in the intestinal epithelium. However, such a scenario seems unlikely given the fundamental role of the Beclin 1: BCL-2 complex in

autophagy regulation; the nature of the BCL-2:Beclin 1 complex and bacterial activation of epithelial autophagy should be revealed by *Salmonella* challenge experiments on the $BCL2^{AAA}$ mice. However, preliminary immunofluorescence data indicates that dissociation of the Beclin 1:BCL-2 complex is important in activation of epithelial cell autophagy (Fig. 2B).

If further experimentation reveals dissociation of the BCL2:Beclin 1 complex unimportant, or if MyD88 does not regulate the dissociation another alternative but equally interesting hypothesis is that MyD88 regulates epithelial autophagy through activation of NFκB. To test this hypothesis, we will use mice with an epithelial cell-specific deletion of IKKβ (*IKKβ*$^{\Delta IEC}$), a component of the kinase complex that signals downstream of MyD88 and phosphorylates IκB, thus liberating NFκB for translocation into the nucleus. Analysis of *IKK*β$^{\Delta IEC}$ mice is preferable to analysis of individual NFκB subunit knockouts, as there are several different NFκB subunits that have redundant functions. Previous work from the Hooper lab has shown that the *IKK*β$^{\Delta IEC}$ mice lack expression of the MyD88-regulated antibacterial protein RegIIIɣ (Vaishnava et al., 2008), thus confirming that epithelial NFκB activation is defective in the small intestines of these mice. If NFκB activation is required to activate epithelial autophagy, then the intestinal epithelial cells of these mice should be unable to form autophagosomes in the face of bacterial challenge. From there, the goal should be to identify autophagy factors that are transcriptionally regulated in the intestinal epithelium by MyD88 and NFκB.

Identification of Autophagy Adaptors for Commensals

Understanding how bacteria are identified and ultimately targeted for destruction via the autophagy machinery is an area of intense investigation in the autophagy field. The vast majority of the work centers around the identification of molecules required for efficient target of bacteria to autophagosome. Because most of the studies are performed in tissue culture cells, the work usually involves the addition of known targets for autophagy – such as *S. typhimurium*, *S. flexneri* and *Mycobacterium tuberculosis* and subsequent monitoring of the cellular autophagy responses to the pathogen. Indeed, such studies have greatly enhanced our understanding of the various adaptors required for clearance of bacterial targets. Intracellular pathogens are commonly targeted for autophagosomes via interactions between a molecular tag found on the bacterial surface and adaptor proteins which recognize the tags and also contain an LC3-interacting region (LIR). The LIRs typically contain a conserved motif of WXXL or WXXI. Several different adaptors have been shown to be involved with clearance of *Salmonella typhimurium*. For example p62, NDP52 and most recently LRSAM1 have all been shown to be required for efficient delivery of *S. typhimurium* to autophagosome (Huett et al., 2012). Although these studies are quite informative, an equally interesting question is whether other adaptors play a role in the intestinal epithelium *in vivo*.

An example of one such bacterial activator of autophagy is *E. faecalis*. In this study, *E. faecalis* was shown to be capable of epithelial autophagy activation within intestine of ex-germ-free mice. Additionally, in unpublished data, I have identified a *Proteus mirabilis* strain present in the conventional microbiota of mice in the Hooper lab mouse colony that is also a newly discovered bacterial activator of epithelial cell

autophagy (Fig. 3B). *P. mirabilis* is a Gram-negative rod and native member of the intestinal microbiota. It is also one of the most common causes of urinary tract and nosocomial infections, leading to its designation as a pathobiont. Many have studies have sought to understand the nature of the interaction between *P. mirabilis* and the host (Coker et al., 2000) . Perhaps most relevant to this study was the finding that germ-free mice that had been monocolonized with *P. mirabilis* mice demonstrated evidence of *P. mirabilis* within their intestinal tissue (Wells and Erlandsen, 1991). This finding indicates that *P. mirabilis* is capable of crossing the intestinal epithelial barrier. An interesting question is whether or not there are novel adaptor proteins required for autophagy activation in response to *E. faecalis* and *P. mirabilis* or if the same adaptors previously characterized can account for autophagy activation by this bacterial species.

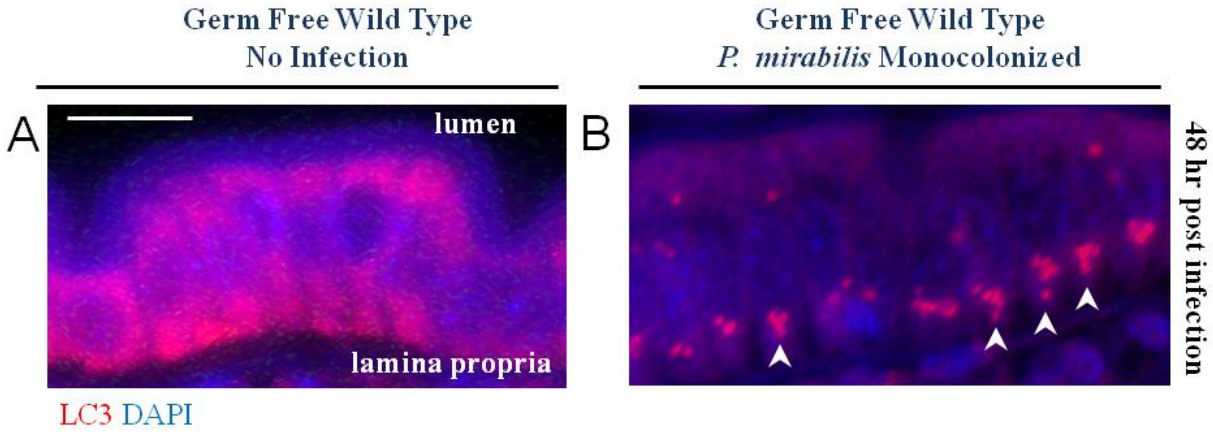

Figure 3: *Proteus mirabilis* triggers autophagosome formation in small intestinal epithelial cells. Germ-free mice were maintained germ-free or orally inoculated with 10^9 CFU of *P. mirabilis* Sections of distal small intestine (ileum) were stained with anti-LC3 and anti-rabbit IgG-Cy3 (red). **(A)** Germ-free no infection mice show cytoplasmic LC3 staining **(B)** 48 hrs post infection with *P. mirabilis* mice show $LC3^+$ autophagosomes visible as distinct puncta (arrowheads). Tissues were counterstained with DAPI (blue). scale bar=10 μm.

Although it is possible that no new proteins are involved in targeting newly discovered bacterial activators for destruction via autophagy, previous studies with different bacterial activators of autophagy suggest that this is unlikely. For example,

research investigating the relationship between *M. tuberculosis* clearance through the autophagy pathway has produced interesting findings regarding the possible identification of Parkin as critical adaptor protein and E3 ligase in the recognition of *M. tuberculosis* by the autophagy pathway. Preliminary studies from the Cox group at UCSF have shown that *Parkin*$^{-/-}$ mice are more susceptible to *M. tuberculosis* infection and that Parkin functions to mark cytosolic *M. tuberculosis* with a Lysine 63-linked Ubiquitin chain (Komatsu et al., 2013). Given that *E. faecalis* is Gram-positive bacterium, I hypothesize that recognition of bacteria molecularly tagged for autophagy by the host cell will differ in mechanism from Gram-negative bacteria like *S. typhimurium.*

There are a number of different strategies one could take to identify novel activators of autophagy, but these strategies become much more complicated with the added complexity of studying a specific cell type in mice. As previously demonstrated, *E. faecalis* is readily capable of invading epithelial cells. I propose to take an unbiased proteomics approach and utilize the ability to distinguish and separate *E. faecalis* infected epithelial cells from non-infected epithelial cells to identify proteins involved in the enterocyte response to *E. faecalis* infection. I also hypothesize that since autophagy is an important, early, enterocyte-intrinsic process, autophagy proteins and, therefore, novel antibacterial autophagy adaptors will be among the proteins most differentially expressed in infected versus non-infected epithelial cells.

First, epithelial cell preparations are made utilizing a protocol initially developed to isolate intestinal lamina propria lymphocytes (Ismail et al., 2011). Incidentally, it was noticed that a large contaminant in the preparations for lamina propria lymphocytes preparations were intestinal epithelial cells. In fact, if we use an anti-CD45.2 antibody

conjugated to magnetic beads, we can readily separate the epithelial cell preparations into two relatively pure fractions of CD45.2 positive and CD45.2 negative populations (Fig 2B). CD45.2 is a cell surface marker that expressed by all cells of hematopoietic origin. Therefore, by selecting for CD45.2 positive cells and depleting them, we have removed abundant contaminant cells such as lymphocytes and macrophages from our epithelial cells. Integrins are a class of transmembrane proteins that function in the attachment of the cell to the extracellular matrix. Within the intestinal epithelium, Integrins β1 and β4 classes are the most prominent integrins, mediating binding to Type IV collagen and laminins. Additionally, the Integrins β1 and β4 classes are required for the proper formation of adherent junctions between epithelial cells (Beaulieu, 1999). Therefore, by selecting for cells that are Integrin $\beta4^+$ and $CD45.2^-$ (Fig. 2B) we can be confident that we have isolated and purified ileal intestinal epithelial cells.

Next, we want to subdivide the infected epithelial cells from the non-infected cells. By utilizing the *E. faecalis*-GFP strain, we can sort the GFP positive cells away from the GFP negative cells. Once the different population have been created, comparative proteomic analysis will be conducted to identify newly expressed or highly enhanced proteins following *E. faecalis* infection (Fig. 2C). The proteomics approach is the preferred method for identifying adaptors for antibacterial autophagy because autophagy is regulated post-translationally.

Although autophagy is classically thought to be regulated post-translationally, it is possible that the regulation of antibacterial autophagy in the intestinal epithelium is transcriptionally regulated. If this is the case, the best experimental approach would be to use laser capture microdissection to specifically isolate the mRNA from the epithelial

cells of germ-free or *E. faecalis* monocolonized mice (Espina et al., 2006). Following the isolation of the mRNA, microarray studies can then be performed to look for genes whose expression is increased following *E. faecalis* infection.

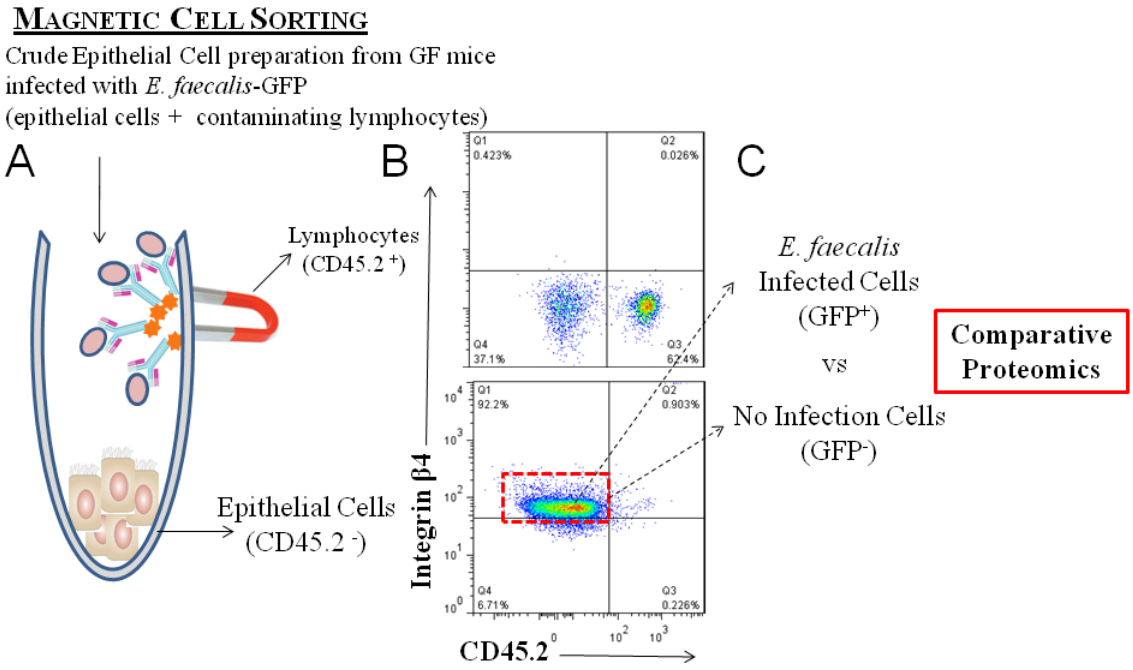

Figure 4: Proteomic Discovery of Novel Antibacterial Autophagy adaptors. Epithelial cell preparations are made from the terminal ileum. **(A)** Epithelial cells are negatively selected via MACS with an α-CD45.2 Ab. **(B)** Selection for Integrin β4+, CD45.2- indicates high enrichment of epithelial cell population. **(C)** GFP positive and negative epithelial cells are then separated and used to compare changes in epithelial cell protein expression with (GFP+) and without(GFP-) *E. faecalis* infection.

On initial consideration the proteomics approach to identification of adaptors might seem unfeasible; however, recent findings suggest otherwise. In fact, a recent publication has used the proteomics analysis approach to comprehensively identify 97% of the proteins produced by the 4,000 open reading frames found within the genome of *M. tuberculosis*. The advancement of this technology has become so sensitive that for over half of the *M. tuberculosis* proteins, researchers were able to estimate the absolute abundance (Schubert et al., 2013). The development of the complete proteome for *M. tuberculosis* has resulted in the identification of new proteins. Thus, proteomics is a valid approach for discovering previously unknown proteins.

The process of developing a proteome library can be broken into 3 phases – Proteome Mapping, Proteome Library Generation, Proteome Library Validation. During the proteome mapping phase, I will develop a library of the core proteins expressed by the epithelial cell utilizing discovery-driven mass spectrometry (MS). The benefits and details of discovery driven focus more on highly accurate and reproducible identification of the proteins. The next step involves the development of proteome libraries for three-different experimental conditions – germ-free no infection, *E. faecalis* monocolonized germ-free and *P. mirabilis* monocolonized mice (Fig. 5B). Finally, potential protein adaptors identified through proteomic analysis are validated and confirmed through *in vivo* and *in vitro* assays of autophagy activation. The proteome analysis of germ-free epithelial cells and epithelial cells following infection with both a Gram-negative and Gram-positive bacterium should yields incredible amounts of information. This information is easily categorized by category (Fig. 5C). Proteins in the Gram-negative category are specifically increased following infection with *P. mirabilis*, whereas proteins in the Gram-positive category are important in the epithelial cell response to *E. faecalis*. Initial categorization will make determination of function for previous unknown proteins considerably easier.

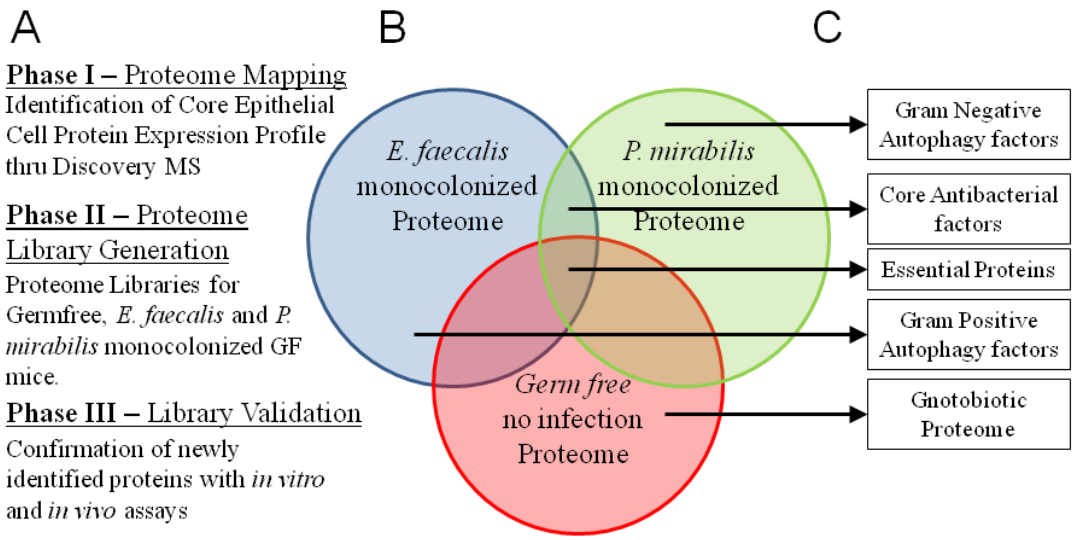

Figure 5: Epithelial Cell Proteome Library for Protein Discovery. Epithelial cell preparations are made from the terminal ileum. **(A)** A three phase workflow is used to develop the core proteome libraries. **(B)** Proteomes of different experimental sets are compared allowing for separation of proteins into distinct functional groups **(C)** Functional groups designation allows newly identified proteins to be quickly annotated and classified.

Utilizing the proteomics approach will likely yield large numbers of potential adaptor proteins. The next step in the adaptor protein identification pipeline will involve validation of the putative adaptor in MODE-K cells, an immortalized small intestinal cell line. One way to test the validity of the adaptor candidate is through RNAi studies. RNAi validation studies would first involve knockdown of the proposed adaptor followed by infection of MODE-K cells with *E. faecalis* or *P. mirabilis*. If the putative adaptor is involved in antibacterial autophagy, I would expect to see increased proliferation of *E. faecalis* or *P. mirabilis* in the RNAi knockdown cells relative to wild-type cells. If any of the candidate proteins show promise in the initial infection studies, the next step will be to determine if the potential adaptor protein directly interacts with the invading bacteria or LC3. Co-immunoprecipitation and Western blot studies using anti-*E. faecalis* and anti-*P. mirabilis* antibodies will be critical in determining interactions between the newly identified adaptor, invading bacteria and LC3.

Autophagy in Immunodeficient Mice

The ability of the conventional microbiota to invade epithelial cells and activate epithelial cell autophagy in *Nod2*$^{-/-}$ mice poses an interesting question; do other defects in innate immunity also result in activation of epithelial autophagy by the conventional microbiota? An initial observation made by the Hooper lab hinted at this possibility. Vaishnava et al. demonstrated that loss of the intestinal antibacterial peptide, RegIIIγ, resulted in a loss of the spatial segregation of the microbiota (Vaishnava et al., 2011). This paper furthered showed that loss of RegIIIγ also resulted in increased fecal IgA levels. I predicted that the loss in spatial segregation of the intestinal microbiota in RegIIIɣ knockout mice would make it more likely for intestinal bacteria to invade the epithelium and activate autophagy. When I examined autophagy activation within the intestinal epithelium of conventionally raised RegIIIγ deficient mice, I observed autophagosome formation within the intestinal epithelium (Fig. 6A-B). The observation of autophagy activation represents the second time I have observed a mouse genotype where the conventional, intestinal microbiota is capable of activating epithelial cell autophagy, *Nod2*$^{-/-}$ mice representing the first.

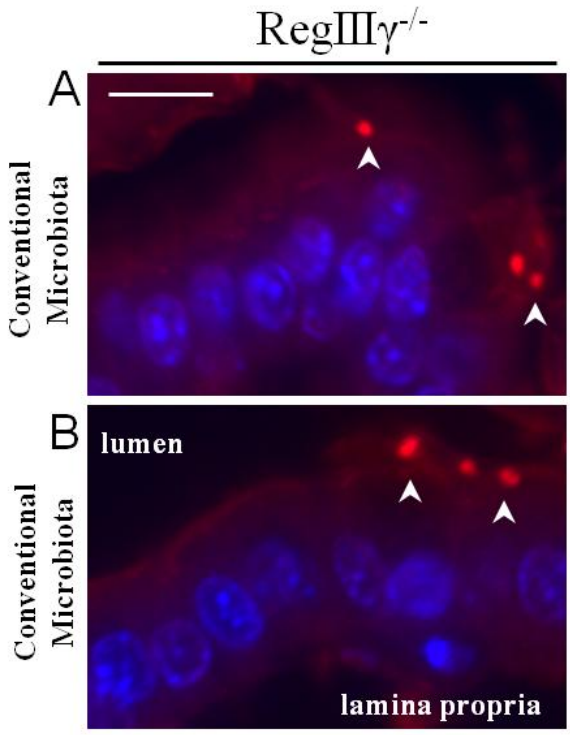

Figure 6: Conventional Microbiota activate autophagy in RegIIIγ knockout mice. (A-B) Ileal tissue sections from conventionally-raised *RegIIIγ*$^{-/-}$ mice were analyzed by immunofluorescence detection of LC3. LC3^{+} autophagosomes (arrowheads) were observed within the intestinal epithelium in the absence of pathogenic bacterial challenge. n=5 mice/group; scale bar=10 μm.

Given this link between mutation in innate immune effectors and the ability of the conventional microbiota to activate epithelial autophagy, I hypothesize that other mouse models with defects in innate immune mediators such as antimicrobial peptides will likely also have conventional microbiota capable of activating epithelial autophagy. Looming questions going forward will be analysis of the effect of losing both antimicrobial peptide production and epithelial autophagy, and how loss of innate immune proteins, like RegIIIɣ and NOD2, results in a more invasive conventional microbiota.

Conclusions

My findings indicate that epithelial cell autophagy is an important and essential response to invasive bacteria within the small intestine. Additionally, I have determined that epithelial cell autophagy is rapid and transient response to invasion of the epithelium.

This activation of epithelial cell autophagy occurs in an epithelial-cell intrinsic MyD88 dependent manner and can occur in the absence of the adaptor protein TRIF or NOD2, a cytosolic pattern recognition receptor. Although we have gained a great deal of insight into the bacterial targets of epithelial cell autophagy and the pattern recognition receptors involved, there is still a great amount of work to be done to fully understand the role of autophagy in maintaining homeostasis with intestinal microbiota. It will also be important to determine if the same mechanisms of autophagy regulation occurs in other intestinal cell types like macrophages and dendritic cells, professionally phagocytic cells that are abundant in the lamina propria of the intestine.

The ultimate goal of biomedical research is to achieve a better fundamental understanding of a disease process so that we can better readily intervene when needed. We are only now beginning to understand the complex interplay between the intestinal microbiota, innate immune responses and immunity. Mechanistic dissection of autophagy in the intestine will be critical in developing the next line of therapies for debilitating diseases like IBD or intestinal infectious diseases.

BIBLIOGRAPHY

(2007). Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature *447*, 661-678.

Akira, S., Uematsu, S., and Takeuchi, O. (2006). Pathogen recognition and innate immunity. Cell *124*, 783-801.

Amer, A.O., and Swanson, M.S. (2005). Autophagy is an immediate macrophage response to Legionella pneumophila. Cell Microbiol *7*, 765-778.

Artis, D. (2008). Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. Nat Rev Immunol *8*, 411-420.

Asahara, T., Shimizu, K., Nomoto, K., Hamabata, T., Ozawa, A., and Takeda, Y. (2004). Probiotic bifidobacteria protect mice from lethal infection with Shiga toxin-producing Escherichia coli O157:H7. Infect Immun *72*, 2240-2247.

Bandeira, A., Mota-Santos, T., Itohara, S., Degermann, S., Heusser, C., Tonegawa, S., and Coutinho, A. (1990). Localization of gamma/delta T cells to the intestinal epithelium is independent of normal microbial colonization. J Exp Med *172*, 239-244.

Beaulieu, J.F. (1999). Integrins and human intestinal cell functions. Front Biosci *4*, D310-321.

Birmingham, C.L., Smith, A.C., Bakowski, M.A., Yoshimori, T., and Brumell, J.H. (2006). Autophagy controls Salmonella infection in response to damage to the Salmonella-containing vacuole. J Biol Chem *281*, 11374-11383.

Brandl, K., Plitas, G., Schnabl, B., DeMatteo, R.P., and Pamer, E.G. (2007). MyD88-mediated signals induce the bactericidal lectin RegIII gamma and protect mice against intestinal Listeria monocytogenes infection. J Exp Med *204*, 1891-1900.

Brest, P., Lapaquette, P., Souidi, M., Lebrigand, K., Cesaro, A., Vouret-Craviari, V., Mari, B., Barbry, P., Mosnier, J.F., Hebuterne, X., *et al.* (2011). A synonymous variant in IRGM alters a binding site for miR-196 and causes deregulation of IRGM-dependent xenophagy in Crohn's disease. Nat Genet *43*, 242-245.

Brocks, J.J., Logan, G.A., Buick, R., and Summons, R.E. (1999). Archean molecular fossils and the early rise of eukaryotes. Science *285*, 1033-1036.

Burman, C., and Ktistakis, N.T. (2010). Autophagosome formation in mammalian cells. Semin Immunopathol *32*, 397-413.

Cadwell, K., Liu, J.Y., Brown, S.L., Miyoshi, H., Loh, J., Lennerz, J.K., Kishi, C., Kc,

W., Carrero, J.A., Hunt, S., *et al.* (2008). A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. Nature *456*, 259-263.

Cadwell, K., Patel, K.K., Maloney, N.S., Liu, T.C., Ng, A.C., Storer, C.E., Head, R.D., Xavier, R., Stappenbeck, T.S., and Virgin, H.W. (2010). Virus-plus-susceptibility gene interaction determines Crohn's disease gene Atg16L1 phenotypes in intestine. Cell *141*, 1135-1145.

Cash, H.L., Whitham, C.V., Behrendt, C.L., and Hooper, L.V. (2006). Symbiotic bacteria direct expression of an intestinal bactericidal lectin. Science *313*, 1126-1130.

Chieppa, M., Rescigno, M., Huang, A.Y., and Germain, R.N. (2006). Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. J Exp Med *203*, 2841-2852.

Claesson, M.J., O'Sullivan, O., Wang, Q., Nikkila, J., Marchesi, J.R., Smidt, H., de Vos, W.M., Ross, R.P., and O'Toole, P.W. (2009). Comparative analysis of pyrosequencing and a phylogenetic microarray for exploring microbial community structures in the human distal intestine. PLoS One *4*, e6669.

Coker, C., Poore, C.A., Li, X., and Mobley, H.L. (2000). Pathogenesis of Proteus mirabilis urinary tract infection. Microbes Infect *2*, 1497-1505.

Cooney, R., Baker, J., Brain, O., Danis, B., Pichulik, T., Allan, P., Ferguson, D.J., Campbell, B.J., Jewell, D., and Simmons, A. (2010). NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. Nat Med *16*, 90-97.

De Palma, G., Nadal, I., Medina, M., Donat, E., Ribes-Koninckx, C., Calabuig, M., and Sanz, Y. (2010). Intestinal dysbiosis and reduced immunoglobulin-coated bacteria associated with coeliac disease in children. BMC Microbiol *10*, 63.

Deckx, R.J., Vantrappen, G.R., and Parein, M.M. (1967). Localization of lysozyme activity in a Paneth cell granule fraction. Biochim Biophys Acta *139*, 204-207.

Deretic, V., and Levine, B. (2009). Autophagy, immunity, and microbial adaptations. Cell Host Microbe *5*, 527-549.

Duerkop, B.A., Clements, C.V., Rollins, D., Rodrigues, J.L., and Hooper, L.V. (2012). A composite bacteriophage alters colonization by an intestinal commensal bacterium. Proc Natl Acad Sci U S A *109*, 17621-17626.

Duerkop, B.A., Vaishnava, S., and Hooper, L.V. (2009). Immune responses to the microbiota at the intestinal mucosal surface. Immunity *31*, 368-376.

Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., Gill,

S.R., Nelson, K.E., and Relman, D.A. (2005). Diversity of the human intestinal microbial flora. Science *308*, 1635-1638.

Eichelberg, K., and Galan, J.E. (1999). Differential regulation of Salmonella typhimurium type III secreted proteins by pathogenicity island 1 (SPI-1)-encoded transcriptional activators InvF and hilA. Infect Immun *67*, 4099-4105.

Espina, V., Wulfkuhle, J.D., Calvert, V.S., VanMeter, A., Zhou, W., Coukos, G., Geho, D.H., Petricoin, E.F., 3rd, and Liotta, L.A. (2006). Laser-capture microdissection. Nat Protoc *1*, 586-603.

Everest, P., Ketley, J., Hardy, S., Douce, G., Khan, S., Shea, J., Holden, D., Maskell, D., and Dougan, G. (1999). Evaluation of Salmonella typhimurium mutants in a model of experimental gastroenteritis. Infect Immun *67*, 2815-2821.

Forbes, S.J., Eschmann, M., and Mantis, N.J. (2008). Inhibition of Salmonella enterica serovar typhimurium motility and entry into epithelial cells by a protective antilipopolysaccharide monoclonal immunoglobulin A antibody. Infect Immun *76*, 4137-4144.

Frank, D.N., Robertson, C.E., Hamm, C.M., Kpadeh, Z., Zhang, T., Chen, H., Zhu, W., Sartor, R.B., Boedeker, E.C., Harpaz, N., *et al.* (2011). Disease phenotype and genotype are associated with shifts in intestinal-associated microbiota in inflammatory bowel diseases. Inflamm Bowel Dis *17*, 179-184.

Fukuda, S., Toh, H., Hase, K., Oshima, K., Nakanishi, Y., Yoshimura, K., Tobe, T., Clarke, J.M., Topping, D.L., Suzuki, T., *et al.* (2011). Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature *469*, 543-547.

Galan, J.E., Ginocchio, C., and Costeas, P. (1992). Molecular and functional characterization of the Salmonella invasion gene invA: homology of InvA to members of a new protein family. J Bacteriol *174*, 4338-4349.

Gallo, R.L., and Hooper, L.V. (2012). Epithelial antimicrobial defence of the skin and intestine. Nat Rev Immunol *12*, 503-516.

Gerbe, F., van Es, J.H., Makrini, L., Brulin, B., Mellitzer, G., Robine, S., Romagnolo, B., Shroyer, N.F., Bourgaux, J.F., Pignodel, C., *et al.* (2011). Distinct ATOH1 and Neurog3 requirements define tuft cells as a new secretory cell type in the intestinal epithelium. J Cell Biol *192*, 767-780.

Germanov, E., Veinotte, L., Cullen, R., Chamberlain, E., Butcher, E.C., and Johnston, B. (2008). Critical role for the chemokine receptor CXCR6 in homeostasis and activation of CD1d-restricted NKT cells. J Immunol *181*, 81-91.

Gewirtz, A.T., Navas, T.A., Lyons, S., Godowski, P.J., and Madara, J.L. (2001). Cutting

edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. J Immunol *167*, 1882-1885.

Girardin, S.E., Boneca, I.G., Carneiro, L.A., Antignac, A., Jehanno, M., Viala, J., Tedin, K., Taha, M.K., Labigne, A., Zahringer, U., *et al.* (2003). Nod1 detects a unique muropeptide from gram-negative bacterial peptidoglycan. Science *300*, 1584-1587.

Girardin, S.E., Tournebize, R., Mavris, M., Page, A.L., Li, X., Stark, G.R., Bertin, J., DiStefano, P.S., Yaniv, M., Sansonetti, P.J., *et al.* (2001). CARD4/Nod1 mediates NF-kappaB and JNK activation by invasive Shigella flexneri. EMBO Rep *2*, 736-742.

Gwinn, D.M., Shackelford, D.B., Egan, D.F., Mihaylova, M.M., Mery, A., Vasquez, D.S., Turk, B.E., and Shaw, R.J. (2008). AMPK phosphorylation of raptor mediates a metabolic checkpoint. Mol Cell *30*, 214-226.

Hampe, J., Franke, A., Rosenstiel, P., Till, A., Teuber, M., Huse, K., Albrecht, M., Mayr, G., De La Vega, F.M., Briggs, J., *et al.* (2007). A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. Nat Genet *39*, 207-211.

Hara, T., Nakamura, K., Matsui, M., Yamamoto, A., Nakahara, Y., Suzuki-Migishima, R., Yokoyama, M., Mishima, K., Saito, I., Okano, H., *et al.* (2006). Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature *441*, 885-889.

He, C., Bassik, M.C., Moresi, V., Sun, K., Wei, Y., Zou, Z., An, Z., Loh, J., Fisher, J., Sun, Q., *et al.* (2012). Exercise-induced BCL2-regulated autophagy is required for muscle glucose homeostasis. Nature *481*, 511-515.

Homer, C.R., Richmond, A.L., Rebert, N.A., Achkar, J.P., and McDonald, C. (2010). ATG16L1 and NOD2 interact in an autophagy-dependent antibacterial pathway implicated in Crohn's disease pathogenesis. Gastroenterology *139*, 1630-1641, 1641 e1631-1632.

Hooper, L.V., Stappenbeck, T.S., Hong, C.V., and Gordon, J.I. (2003). Angiogenins: a new class of microbicidal proteins involved in innate immunity. Nat Immunol *4*, 269-273.

Horsfall, D.J., Cooper, J.M., and Rowley, D. (1978). Changes in the immunoglobulin levels of the mouse gut and serum during conventionalisation and following administration of Salmonella typhimurium. Aust J Exp Biol Med Sci *56*, 727-735.

Hou, B., Reizis, B., and DeFranco, A.L. (2008). Toll-like receptors activate innate and

adaptive immunity by using dendritic cell-intrinsic and -extrinsic mechanisms. Immunity *29*, 272-282.

Huett, A., Heath, R.J., Begun, J., Sassi, S.O., Baxt, L.A., Vyas, J.M., Goldberg, M.B., and Xavier, R.J. (2012). The LRR and RING domain protein LRSAM1 is an E3 ligase crucial for ubiquitin-dependent autophagy of intracellular Salmonella Typhimurium. Cell Host Microbe *12*, 778-790.

Inoue, J., Nishiumi, S., Fujishima, Y., Masuda, A., Shiomi, H., Yamamoto, K., Nishida, M., Azuma, T., and Yoshida, M. (2012). Autophagy in the intestinal epithelium regulates Citrobacter rodentium infection. Arch Biochem Biophys *521*, 95-101.

Ismail, A.S., Severson, K.M., Vaishnava, S., Behrendt, C.L., Yu, X., Benjamin, J.L., Ruhn, K.A., Hou, B., DeFranco, A.L., Yarovinsky, F., *et al.* (2011). Gammadelta intraepithelial lymphocytes are essential mediators of host-microbial homeostasis at the intestinal mucosal surface. Proc Natl Acad Sci U S A *108*, 8743-8748.

Jia, K., Thomas, C., Akbar, M., Sun, Q., Adams-Huet, B., Gilpin, C., and Levine, B. (2009). Autophagy genes protect against Salmonella typhimurium infection and mediate insulin signaling-regulated pathogen resistance. Proc Natl Acad Sci U S A *106*, 14564-14569.

Johansen, F.E., Pekna, M., Norderhaug, I.N., Haneberg, B., Hietala, M.A., Krajci, P., Betsholtz, C., and Brandtzaeg, P. (1999). Absence of epithelial immunoglobulin A transport, with increased mucosal leakiness, in polymeric immunoglobulin receptor/secretory component-deficient mice. J Exp Med *190*, 915-922.

Kayath, C.A., Hussey, S., El hajjami, N., Nagra, K., Philpott, D., and Allaoui, A. (2010). Escape of intracellular Shigella from autophagy requires binding to cholesterol through the type III effector, IcsB. Microbes Infect *12*, 956-966.

Keestra, A.M., Winter, M.G., Auburger, J.J., Frassle, S.P., Xavier, M.N., Winter, S.E., Kim, A., Poon, V., Ravesloot, M.M., Waldenmaier, J.F., *et al.* (2013). Manipulation of small Rho GTPases is a pathogen-induced process detected by NOD1. Nature *496*, 233-237.

Kihara, A., Kabeya, Y., Ohsumi, Y., and Yoshimori, T. (2001a). Beclin-phosphatidylinositol 3-kinase complex functions at the trans-Golgi network. EMBO Rep *2*, 330-335.

Kihara, A., Noda, T., Ishihara, N., and Ohsumi, Y. (2001b). Two distinct Vps34 phosphatidylinositol 3-kinase complexes function in autophagy and carboxypeptidase Y sorting in Saccharomyces cerevisiae. J Cell Biol *152*, 519-530.

Klare, I., Werner, G., and Witte, W. (2001). Enterococci. Habitats, infections, virulence

factors, resistances to antibiotics, transfer of resistance determinants. Contrib Microbiol *8*, 108-122.

Kobayashi, K.S., Chamaillard, M., Ogura, Y., Henegariu, O., Inohara, N., Nunez, G., and Flavell, R.A. (2005). Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. Science *307*, 731-734.

Komatsu, M., Kuma, A., and Okamoto, K. (2013). No ATG is an island--the connection of autophagy with diverse pathways and functions. EMBO Rep *14*, 219-221.

Korolchuk, V.I., Saiki, S., Lichtenberg, M., Siddiqi, F.H., Roberts, E.A., Imarisio, S., Jahreiss, L., Sarkar, S., Futter, M., Menzies, F.M., *et al.* (2011). Lysosomal positioning coordinates cellular nutrient responses. Nat Cell Biol *13*, 453-460.

Kuballa, P., Huett, A., Rioux, J.D., Daly, M.J., and Xavier, R.J. (2008). Impaired autophagy of an intracellular pathogen induced by a Crohn's disease associated ATG16L1 variant. PLoS One *3*, e3391.

Levine, B., Mizushima, N., and Virgin, H.W. (2011). Autophagy in immunity and inflammation. Nature *469*, 323-335.

Liang, X.H., Kleeman, L.K., Jiang, H.H., Gordon, G., Goldman, J.E., Berry, G., Herman, B., and Levine, B. (1998). Protection against fatal Sindbis virus encephalitis by beclin, a novel Bcl-2-interacting protein. J Virol *72*, 8586-8596.

Madison, B.B., Dunbar, L., Qiao, X.T., Braunstein, K., Braunstein, E., and Gumucio, D.L. (2002). Cis elements of the villin gene control expression in restricted domains of the vertical (crypt) and horizontal (duodenum, cecum) axes of the intestine. J Biol Chem *277*, 33275-33283.

Mantis, N.J., and Forbes, S.J. (2010). Secretory IgA: arresting microbial pathogens at epithelial borders. Immunol Invest *39*, 383-406.

Miller, B.C., Zhao, Z., Stephenson, L.M., Cadwell, K., Pua, H.H., Lee, H.K., Mizushima, N.N., Iwasaki, A., He, Y.W., Swat, W., *et al.* (2008). The autophagy gene ATG5 plays an essential role in B lymphocyte development. Autophagy *4*, 309-314.

Mizushima, N., Ohsumi, Y., and Yoshimori, T. (2002). Autophagosome formation in mammalian cells. Cell Struct Funct *27*, 421-429.

Mizushima, N., Yoshimori, T., and Levine, B. (2010). Methods in mammalian autophagy research. Cell *140*, 313-326.

Muller, A.J., Kaiser, P., Dittmar, K.E., Weber, T.C., Haueter, S., Endt, K., Songhet, P.,

Zellweger, C., Kremer, M., Fehling, H.J., *et al.* (2012). Salmonella gut invasion involves TTSS-2-dependent epithelial traversal, basolateral exit, and uptake by epithelium-sampling lamina propria phagocytes. Cell Host Microbe *11*, 19-32.

Mundy, R., MacDonald, T.T., Dougan, G., Frankel, G., and Wiles, S. (2005). Citrobacter rodentium of mice and man. Cell Microbiol *7*, 1697-1706.

Nakagawa, I., Amano, A., Mizushima, N., Yamamoto, A., Yamaguchi, H., Kamimoto, T., Nara, A., Funao, J., Nakata, M., Tsuda, K., *et al.* (2004). Autophagy defends cells against invading group A Streptococcus. Science *306*, 1037-1040.

Niess, J.H., Brand, S., Gu, X., Landsman, L., Jung, S., McCormick, B.A., Vyas, J.M., Boes, M., Ploegh, H.L., Fox, J.G., *et al.* (2005). CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. Science *307*, 254-258.

Nieto, C., and Espinosa, M. (2003). Construction of the mobilizable plasmid pMV158GFP, a derivative of pMV158 that carries the gene encoding the green fluorescent protein. Plasmid *49*, 281-285.

Nobukuni, T., Joaquin, M., Roccio, M., Dann, S.G., Kim, S.Y., Gulati, P., Byfield, M.P., Backer, J.M., Natt, F., Bos, J.L., *et al.* (2005). Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. Proc Natl Acad Sci U S A *102*, 14238-14243.

O'Hara, A.M., O'Regan, P., Fanning, A., O'Mahony, C., Macsharry, J., Lyons, A., Bienenstock, J., O'Mahony, L., and Shanahan, F. (2006). Functional modulation of human intestinal epithelial cell responses by Bifidobacterium infantis and Lactobacillus salivarius. Immunology *118*, 202-215.

Ogawa, M., Yoshimori, T., Suzuki, T., Sagara, H., Mizushima, N., and Sasakawa, C. (2005). Escape of intracellular Shigella from autophagy. Science *307*, 727-731.

Olszak, T., An, D., Zeissig, S., Vera, M.P., Richter, J., Franke, A., Glickman, J.N., Siebert, R., Baron, R.M., Kasper, D.L., *et al.* (2012). Microbial exposure during early life has persistent effects on natural killer T cell function. Science *336*, 489-493.

Orvedahl, A., MacPherson, S., Sumpter, R., Jr., Talloczy, Z., Zou, Z., and Levine, B. (2010). Autophagy protects against Sindbis virus infection of the central nervous system. Cell Host Microbe *7*, 115-127.

Pabst, O. (2012). New concepts in the generation and functions of IgA. Nat Rev Immunol *12*, 821-832.

Pankiv, S., Clausen, T.H., Lamark, T., Brech, A., Bruun, J.A., Outzen, H., Overvatn, A.,

Bjorkoy, G., and Johansen, T. (2007). p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J Biol Chem *282*, 24131-24145.

Pattingre, S., Tassa, A., Qu, X., Garuti, R., Liang, X.H., Mizushima, N., Packer, M., Schneider, M.D., and Levine, B. (2005). Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell *122*, 927-939.

Perrin, A.J., Jiang, X., Birmingham, C.L., So, N.S., and Brumell, J.H. (2004). Recognition of bacteria in the cytosol of Mammalian cells by the ubiquitin system. Curr Biol *14*, 806-811.

Petersen, J.M., Zielinski, F.U., Pape, T., Seifert, R., Moraru, C., Amann, R., Hourdez, S., Girguis, P.R., Wankel, S.D., Barbe, V., *et al.* (2011). Hydrogen is an energy source for hydrothermal vent symbioses. Nature *476*, 176-180.

Py, B.F., Lipinski, M.M., and Yuan, J. (2007). Autophagy limits Listeria monocytogenes intracellular growth in the early phase of primary infection. Autophagy *3*, 117-125.

Rabinowitz, J.D., and White, E. (2010). Autophagy and metabolism. Science *330*, 1344-1348.

Rioux, J.D., Xavier, R.J., Taylor, K.D., Silverberg, M.S., Goyette, P., Huett, A., Green, T., Kuballa, P., Barmada, M.M., Datta, L.W., *et al.* (2007). Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. Nat Genet *39*, 596-604.

Salzman, N.H., Hung, K., Haribhai, D., Chu, H., Karlsson-Sjoberg, J., Amir, E., Teggatz, P., Barman, M., Hayward, M., Eastwood, D., *et al.* (2010). Enteric defensins are essential regulators of intestinal microbial ecology. Nat Immunol *11*, 76-83.

Sansonetti, P.J. (2004). War and peace at mucosal surfaces. Nat Rev Immunol *4*, 953-964.

Sartor, R.B. (2008). Microbial influences in inflammatory bowel diseases. Gastroenterology *134*, 577-594.

Sartor, R.B. (2010). Genetics and environmental interactions shape the intestinal microbiome to promote inflammatory bowel disease versus mucosal homeostasis. Gastroenterology *139*, 1816-1819.

Savage, D.C. (1977). Microbial ecology of the gastrointestinal tract. Annu Rev Microbiol *31*, 107-133.

Schubert, O.T., Mouritsen, J., Ludwig, C., Rost, H.L., Rosenberger, G., Arthur, P.K.,

Claassen, M., Campbell, D.S., Sun, Z., Farrah, T., *et al.* (2013). The Mtb Proteome Library: A Resource of Assays to Quantify the Complete Proteome of Mycobacterium tuberculosis. Cell Host Microbe *13*, 602-612.

Sengupta, S., Peterson, T.R., and Sabatini, D.M. (2010). Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. Mol Cell *40*, 310-322. Shelly, S., Lukinova, N., Bambina, S., Berman, A., and Cherry, S. (2009). Autophagy is an essential component of Drosophila immunity against vesicular stomatitis virus. Immunity *30*, 588-598.

Shi, C.S., and Kehrl, J.H. (2008). MyD88 and Trif target Beclin 1 to trigger autophagy in macrophages. J Biol Chem *283*, 33175-33182.

Shroff, K.E., Meslin, K., and Cebra, J.J. (1995). Commensal enteric bacteria engender a self-limiting humoral mucosal immune response while permanently colonizing the gut. Infect Immun *63*, 3904-3913.

Slack, E., Balmer, M.L., Fritz, J.H., and Hapfelmeier, S. (2012). Functional flexibility of intestinal IgA - broadening the fine line. Front Immunol *3*, 100.

Sonnenburg, J.L., Xu, J., Leip, D.D., Chen, C.H., Westover, B.P., Weatherford, J., Buhler, J.D., and Gordon, J.I. (2005). Glycan foraging in vivo by an intestine-adapted bacterial symbiont. Science *307*, 1955-1959.

Tattoli, I., Sorbara, M.T., Vuckovic, D., Ling, A., Soares, F., Carneiro, L.A., Yang, C., Emili, A., Philpott, D.J., and Girardin, S.E. (2012). Amino acid starvation induced by invasive bacterial pathogens triggers an innate host defense program. Cell Host Microbe *11*, 563-575.

Thachil, E., Hugot, J.P., Arbeille, B., Paris, R., Grodet, A., Peuchmaur, M., Codogno, P., Barreau, F., Ogier-Denis, E., Berrebi, D., *et al.* (2012). Abnormal activation of autophagy-induced crinophagy in Paneth cells from patients with Crohn's disease. Gastroenterology *142*, 1097-1099 e1094.

Thurston, T.L., Ryzhakov, G., Bloor, S., von Muhlinen, N., and Randow, F. (2009). The TBK1 adaptor and autophagy receptor NDP52 restricts the proliferation of ubiquitin-coated bacteria. Nat Immunol *10*, 1215-1221.

Thurston, T.L., Wandel, M.P., von Muhlinen, N., Foeglein, A., and Randow, F. (2012). Galectin 8 targets damaged vesicles for autophagy to defend cells against bacterial invasion. Nature *482*, 414-418.

Travassos, L.H., Carneiro, L.A., Ramjeet, M., Hussey, S., Kim, Y.G., Magalhaes, J.G.,

Yuan, L., Soares, F., Chea, E., Le Bourhis, L., *et al.* (2010). Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. Nat Immunol *11*, 55-62.

Tsukamoto, S., Kuma, A., Murakami, M., Kishi, C., Yamamoto, A., and Mizushima, N. (2008). Autophagy is essential for preimplantation development of mouse embryos. Science *321*, 117-120.

Vaishnava, S., Behrendt, C.L., Ismail, A.S., Eckmann, L., and Hooper, L.V. (2008). Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface. Proc Natl Acad Sci U S A *105*, 20858-20863.

Vaishnava, S., Yamamoto, M., Severson, K.M., Ruhn, K.A., Yu, X., Koren, O., Ley, R., Wakeland, E.K., and Hooper, L.V. (2011). The antibacterial lectin RegIIIgamma promotes the spatial segregation of microbiota and host in the intestine. Science *334*, 255-258.

Van der Sluis, M., De Koning, B.A., De Bruijn, A.C., Velcich, A., Meijerink, J.P., Van Goudoever, J.B., Buller, H.A., Dekker, J., Van Seuningen, I., Renes, I.B., *et al.* (2006). Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. Gastroenterology *131*, 117-129.

van der Waaij, L.A., Limburg, P.C., Mesander, G., and van der Waaij, D. (1996). In vivo IgA coating of anaerobic bacteria in human faeces. Gut *38*, 348-354.

Wells, C.L., and Erlandsen, S.L. (1991). Localization of translocating Escherichia coli, Proteus mirabilis, and Enterococcus faecalis within cecal and colonic tissues of monoassociated mice. Infect Immun *59*, 4693-4697.

Wijburg, O.L., Uren, T.K., Simpfendorfer, K., Johansen, F.E., Brandtzaeg, P., and Strugnell, R.A. (2006). Innate secretory antibodies protect against natural Salmonella typhimurium infection. J Exp Med *203*, 21-26.

Wild, P., Farhan, H., McEwan, D.G., Wagner, S., Rogov, V.V., Brady, N.R., Richter, B., Korac, J., Waidmann, O., Choudhary, C., *et al.* (2011). Phosphorylation of the autophagy receptor optineurin restricts Salmonella growth. Science *333*, 228-233.

Wolfe-Simon, F., Switzer Blum, J., Kulp, T.R., Gordon, G.W., Hoeft, S.E., Pett-Ridge, J., Stolz, J.F., Webb, S.M., Weber, P.K., Davies, P.C., *et al.* (2011). A bacterium that can grow by using arsenic instead of phosphorus. Science *332*, 1163-1166.

Xavier, R.J., and Podolsky, D.K. (2007). Unravelling the pathogenesis of inflammatory bowel disease. Nature *448*, 427-434.

Xu, J., Bjursell, M.K., Himrod, J., Deng, S., Carmichael, L.K., Chiang, H.C., Hooper,

L.V., and Gordon, J.I. (2003). A genomic view of the human-Bacteroides thetaiotaomicron symbiosis. Science *299*, 2074-2076.

Xu, Y., Jagannath, C., Liu, X.D., Sharafkhaneh, A., Kolodziejska, K.E., and Eissa, N.T. (2007). Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. Immunity *27*, 135-144.

Yano, T., Mita, S., Ohmori, H., Oshima, Y., Fujimoto, Y., Ueda, R., Takada, H., Goldman, W.E., Fukase, K., Silverman, N., *et al.* (2008). Autophagic control of listeria through intracellular innate immune recognition in drosophila. Nat Immunol *9*, 908-916.

Yoshimura, K., Matsui, T., and Itoh, K. (2010). Prevention of Escherichia coli O157:H7 infection in gnotobiotic mice associated with Bifidobacterium strains. Antonie Van Leeuwenhoek *97*, 107-117.

Zhao, Z., Fux, B., Goodwin, M., Dunay, I.R., Strong, D., Miller, B.C., Cadwell, K., Delgado, M.A., Ponpuak, M., Green, K.G., *et al.* (2008). Autophagosome-independent essential function for the autophagy protein Atg5 in cellular immunity to intracellular pathogens. Cell Host Microbe *4*, 458-469.