TOWSON UNIVERSITY
COLLEGE OF GRADUATE STUDIES AND RESEARCH

AUTOPHAGY INDUCTION AND BACTERICIDAL ACTIVTY IN *DICTYOSTELIUM DISCOIDEUM*

By

KATHERINE PFLAUM

A thesis in partial fulfillment of the requirements for the degree of

Master of Science in Biology

December 2012

Department of Biological Sciences

Towson University

Towson, Maryland 21252

Advisor: Dr. Michelle Snyder

Committee Members: Dr. Brian Masters and Dr. Barry Margulies

TOWSON UNIVERSITY
COLLEGE OF GRADUATE STUDIES AND RESEARCH

THESIS APPROVAL PAGE

This is to certify that the thesis prepared by Katherine Pflaum entitled, "Autophagy induction and bactericidal activity in *Dictyostelium discoideum*" has been approved by the thesis committee as satisfactorily completing the thesis requirements for the degree of Master of Science in Biology.

Committee Chair: _____ Date: 7/20/12

Committee Member: _____ Date: 7/20/12

Committee Member: _____ Date: 7/20/12

Associate Dean, College of Graduate Studies and Research

_____ Date: 12-7-12

ACKNOWLEDGEMENTS

I sincerely thank my advisor and mentor Michelle Snyder for her wisdom, assistance, and guidance, both in the lab and in life. I thank her for dedicating her time and effort to my success. I would like to thank my committee members, Brian Masters and Barry Margulies for their help in my project, and their guidance and teachings during my graduate career. I would also like to thank the faculty and staff of the Department of Biological Sciences at Towson University for providing me with the opportunity of two years of learning and research. I also thank my lab members, Kimberly Gerdes, Kossi Yovo, and Bivor Shrestha for all of their help and support. I also thank the Graduate Student Association for travel funding to present my findings at national conferences. Finally, I thank the NIH for providing the funding to make this project possible.

ABSTRACT

The innate immune system is the body's first line of defense and uses pattern recognition machinery to identify conserved pathogen-associated molecular patterns (PAMPs) to detect and neutralize pathogens. *Dictyostelium discoideum* is a unique model organism that phagocytizes bacteria for nutritional purposes. We have shown that *D. discoideum* cells will clear intracellular bacteria faster in the presence of the PAMP, lipopolysaccharide (LPS) suggesting that they may detect and respond to bacterial pathogens through pattern recognition machinery. In this study we extend these findings showing that LPS pre-treatment will stimulate autophagosomal maturation in *D. discoideum* cells exposed to *Staphylococcus aureus*. The induction of autophagy by rapamycin causes *D. discoideum* cells to degrade intracellular bacteria at a faster rate. Here we also show that the increased rate of LPS-stimulated bacterial degradation is dependent upon the autophagy related proteins, Atg1 and Atg9, thus linking *D. discoideum* pattern recognition and the induction of autophagy.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I:

INTRODUCTION

In order to defend against invading pathogens, the mammalian immune system relies on both a general innate response as well as a specific adaptive response to the pathogen. The innate immune system is the body's first line of defense against invading pathogens and will respond immediately and in a non-specific manner to any invading pathogen. The adaptive immune system, consisting of highly specialized cells, will provide a delayed, targeted response to a particular pathogen. Both the adaptive and innate responses work together to eliminate the pathogen, and in the absence of an effective immune response, the organism may succumb to infection and eventually death.

Once an organism is initially exposed to a pathogen, the immediate innate response occurs, priming the adaptive immune system. The cells of the adaptive immune system form a specific memory response against those pathogens, so that upon a subsequent challenge, the immune system will respond quickly and effectively. This memory response is the basis of a successful vaccination (O'Hagan et al., 2001; Pichichero et al., 2008) and is most effective when there is a simultaneous innate immune response in the organism (O'Hagan et al., 2001). Conversely, an over-active immune system can result in severe autoimmune diseases and allergic reactions (Lang et al., 2007; Rosenstiel et al., 2008).

In order to mount an effective response against pathogens, while still avoiding autoimmunity, the immune system must be able to differentiate between cells that are self and non-self (Akira et al., 2006). Cells of the innate immune system differentiate between self and non-self by utilizing highly conserved pattern recognition machinery to recognize equally conserved pathogen associated molecular patterns (PAMPs) found on the invading pathogen, but absent from the host cells (Medshitov and Janeway, 2000). Some common PAMPs include peptidoglycan from the cell wall of all bacteria and lipopolysaccharide (LPS), a component of the Gram negative cell walls of bacteria. If we could better understand the innate immune system's ability to recognize these structural patterns on microbial pathogens, it could help provide the tools necessary to design drugs for the regulation of autoimmune disorders and extreme allergic responses. This could also aid in the development of more effective vaccines. For example, addition of a PAMP to a vaccine could increase the innate immune response which would then provide better stimulation of the adaptive response to offer greater protection to the host organism (Newell, 1971; Desjardin et al., 2005).

Autophagy is an essential cellular process that can be regulated by signaling at the cell surface. It is the process by which nutrient recycling during starvation and the degradation of cellular organelles that are no longer needed or functioning correctly occurs (Mizushima et al., 2008; Yang et al., 2010). Recently it has been demonstrated that this process is also a key component in the innate immune response (Sanjuan et al., 2007; Xu et al., 2007; Delgado et al., 2008; Shi and Kehrl et al., 2008; Shin et al., 2010)

as it plays an important role in the removal of intracellular bacterial pathogens (Tekinay et al., 2006; Deretic, 2011; Levine et al., 2011). Autophagy is a highly conserved downstream cellular process characterized by lysosomal degradation (Delgado et al., 2009; Jia et al., 2009). The exact mechanisms of pathogen-induced autophagy pathways are still unclear (Xu et al., 2007; Jia et al., 2009), but it is known that a double membrane bound autophagosome is formed, engulfing the foreign particle or microbe, and the autophagosome is delivered to and fuses with the lysosome for degradation (Tekinay et al., 2006; Yang et al., 2010). Jia at al. (2009) observed that genetic inactivation of certain autophagy related genes (*atg)* increases the replication and survival of intracellular bacterial pathogens, suggesting that the induction of autophagy will aid the cell in the clearance of those invading pathogens.

There are four known classes of mammalian pattern recognition receptors which are responsible for recognizing and responding to PAMPs on invading pathogens. Those classes include Toll-like receptors (TLR), NOD-like receptors (NLR), RIG-I-like receptors (RLR), and C-type lectin receptors (CLR). The TLRs are transmembrane proteins that recognize pathogens through the N-terminal leucine rich repeat (LRR) region (Takeuchi and Akira, 2010). Upon pathogen recognition, TLRs recruit adaptors, such as MyD88, through their cytoplasmic C-terminal toll/interleukin-1 (Tir) domains to signal for activation of NF-κB, MAP kinases and inflammatory caspase signaling pathways (Rosentiel, et al., 2008). There are 10 identified mammalian TLRs, each responding to different microbial patterns. The NLRs are cytoplasmic proteins with a C-

terminal LRR region, responsible for recognition of pathogen. The NLRs also contain a central nucleotide-binding domain and an N terminal domain, which mediates binding to downstream effector molecules, triggering signaling through NF-kB or through the inflammasome to promote a caspase-mediated inflammatory cascade. NLRs often work in synergy with TLRs to promote the production of inflammatory cytokines (Creaght and O'Neil, 2006). The cytoplasmic RLRs typically respond to a viral invasion by the recognition of both double stranded and single stranded RNA through a conserved helicase domain and subsequently transmit signals via their N-terminal caspase recruiting domains (Takeuchi and Akira, 2010). Finally, the CLRs are transmembrane receptors containing a carbohydrate binding domain responsible for the recognition of carbohydrates on the surface of many viruses, bacteria, and fungi (Takeuchi and Akira, 2010).

Autophagy is a lysosomal cellular degradation process, directly linked to innate immunity through TLR pathways of the mammalian immune system (Xu et al., 2007, Delgado et al., 2008). The TLR4 receptor, located on the surface of immune cells, becomes active when bound by LPS, stimulating the immune response (Xu et al., 2007). Autophagy has been shown to be induced through the TLR4 in human macrophages and murine immune cells by the addition of LPS (Xu et al., 2007). Recent evidence has shown that signaling from the TLR enhances the function of Beclin-1, (the mammalian homologue of Atg6) an essential component of the PI3-Kinase complex that stimulates the formation of the autophagosome by helping to localize key autophagy related proteins

to the site of the developing autophagosome (Shi and Kehrl, 2008). This suggests that a potential link is present between LPS recognition pathways and autophagy in mammalian systems.

Dictyostelium discoideum is a unicellular amoeba that takes up bacteria for nutritional purposes (Cosson and Soldati, 2011) and can be induced to form a unique multi-cellular sporulating body upon starvation (Newell, 1971). The intracellular phagocytic system of *D. discoideum* parallels the mechanisms used by the mammalian innate immune system in removing an invading pathogen, making this organism an excellent model organism for studying the mammalian innate immune system. *D. discoideum* is already used as a common model organism for studying development, host pathogen interactions, and cell signaling pathways (Janssen and Schleicher, 2001).

The *D. discoideum* genome contains genes encoding Tir proteins and proteins containing leucine rich repeating (LRR) regions, which are known to be highly conserved in pattern recognition proteins (Eichinger et al., 2005; Benghezal et al., 2006; Chen et al., 2007; Sillo et al., 2008). *D. discoideum* cells deficient in TirA are unable to grow in the presence of Gram negative bacteria, unlike their wild type counterparts (Chen et al., 2007). Previously, our lab has reported that *D. discoideum* cells can clear *S. aureus* more efficiently in the presence of the known microbial pattern, LPS (Walk et al., 2011). This suggests that the organism can, in fact, respond to known pathogen-associated molecular patterns, making this a good model organism for innate immune pattern recognition mechanisms.

Autophagy can be induced in *D. discoideum* through a variety of stimuli including starvation, damaged organelles, and mechanical stimulation (Sanjuan et al., 2007). Recent evidence indicates that autophagy can also be induced upon pathogen invasion. During this process, conserved autophagy genes involved in producing the autophagosome and other structures that cause the digestion of intracellular pathogens are expressed (Sillo et al., 2008; Jia et al., 2009; Tung et al., 2010). Jia et al (2009) reported that the Gram negative bacteria, *Salmonella typhimurium* are able to replicate more efficiently in mutant *D. discoideum* cells lacking the critical autophagy related (Atg) proteins, Atg1, Atg6, or Atg7. It has also been demonstrated that intracellular *Legionella* species have a greater survival rate when *D. discoideum* cells are lacking the Atg9 protein (Tung et al., 2010).

In this study we demonstrate that autophagosomal maturation can be induced upon incubation with bacteria, corroborating previous studies that autophagy may play a role in the response of *D. discoideum* cells to bacteria. Furthermore, we show that LPS, a molecular pattern molecular found in the outer membrane of all Gram negative bacteria, can induce autophagy in *D. discoideum* cells. This LPS-induced autophagy leads to an enhanced ability of the amoeba cells to neutralize and degrade the intracellular bacterial species. Finally, we demonstrate that the previously described LPS-enhanced bactericidal activity of *D. discoideum* is dependent upon the expression of the autophagy related proteins, Atg1 and Atg9. For the first time, this study links together in *D. discoideum* microbial pattern recognition and the induction of autophagy.

CHAPTER II:

MATERIALS AND METHODS

Cell culture

Dictyostelium discoideum DH1 (wild type) , Atg1$^-$ (Atg 1 deficient), and Atg9$^-$ (Atg 9 deficient) cells, and HR87 cells (expressing GFP-Atg8) were received from the Dicty Stock Center and grown axenically at 22ºC in HL-5 media supplemented with vitamins (400 µg/L biotin, 100 µg/L cyanocobalamin, 4 mg/L folic acid, 8 mg/L lipoic acid, 10 mg/L riboflavin and 1 mg/L thiamine) and 20 µg/mL uracil. Media used for HR87 cells was also supplemented with 5 µg/mL G418.

Klebsiella pneumoniae and *Staphylococcus aureus* were obtained from Carolina Biological Supply Co. (Burlington, NC). *Salmonella enterica* serovar Typhimurium (ATCC 29631) was obtained from the American Type Culture Collection (Manassas, VA).

Immunoblotting

Dictyostelium discoideum HR87 cells expressing GFP-Atg8 were incubated at 22ºC in shaking culture at $3x10^6$ cells/mL with or without 1µg/mL *E. coli* O55:B5 LPS (Sigma, St. Louis, MO) or Kdo-2 lipid A from *E. coli* (Enzo Life Sciences, Plymouth Meeting, MA) for 1 h and mixed with bacteria harvested at log phase. In experiments observing inhibition, cells were pre-treated with 5 mM 3-methyladenine (Acros Organics, Geel,

Belgium) for 1 h. Cells were harvested 3 h after addition of bacteria and lysed with 1% NP-40 in TBS containing protease inhibitors (2.5 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin A). Lysates were subjected to SDS-PAGE and immunoblotting using rabbit anti-GFP (Life Technologies, Grand Island, NY), followed by HRP-conjugated goat anti-rabbit Ig (Jackson ImmunoResearch, West Grove, PA) detected by chemiluminescence (GE Heathcare, Buckinghamshire, UK).

Confocal immunofluorescence microscopy

HR87 cells were pre-treated or not with 1 μg/mL LPS (*E. coli* O55:B5 LPS, Sigma) in shaking culture at 22ºC and mixed with *S. aureus* at a ratio of 7.5:1 *D. discoideum* cell. After 1.5 h, non-internalized bacteria were washed from cells with 5 mM sodium azide in PBS and the *D. discoideum* cells incubated on poly-lysine coated coverslips at 22ºC. After 1 h, *D. discoideum* cells were fixed with 4% PFA in PBS, permeabilized with 0.1% Triton X-100 in PBS, and blocked with 0.2% BSA in PBS. To label bacteria, samples were incubated with biotin-labeled polyclonal rabbit antibodies specific for *S. aureus* (Thermo Scientific, Waltham, MA), followed by incubation with Alexa Fluor (AF) 594-conjugated streptavidin (Invitrogen). To amplify signals from GFP-Atg8, samples were incubated with mouse F(ab)'$_2$ anti-GFP (MBL, Woburn, MA; F(ab)'$_2$ fragments generated with Pierce F(ab)'$_2$ micro preparation kit, Thermo Scientific) followed by incubation with AF488-conjugated F(ab)'$_2$ anti-mouse IgG (Jackson ImmunoResearch). Samples were visualized using a Zeiss 510 Meta confocal laser

scanning microscope (Jena, Germany) fitted with a 1.4 oil Plan-Apochromat. A 63x objective was used, and confocal sections acquired during channel mode, multi-track acquisition with Ex488/Em 505-530BP for AF488 and Ex543/Em 585LP for AF594. LSM software was used to calculate overlap coefficients (after Manders).Use of equipment was kindly provided by The National Institutes of Health (NIH).

Bacterial intracellular survival assay

Dictyostelium discoideum HR87 cells were incubated in 24-well plates at $3x10^5$ cells/mL with or without 100 nM rapamycin (LC Laboratories, Woburn, MA) for 1 hr at 22^oC. *Staphylococcus aureus,* harvested from an overnight culture, was added at ratio of 10:1 *D. discoideum* cell. After 15 min each at 22ºC and 4ºC, cells were washed 2 times with HL-5 media of non-internalized bacteria and treated with 30 µg/mL streptomycin to kill any remaining external bacteria. Survival of phagocytized *S. aureus* was assessed between 0-90 min by lysing *D. discoideum* cells with 0.1% Triton X-100 in PBS and plating on Luria-Bertani (LB) agar. Colonies of *S. aureus* were counted after 24 h at 37ºC and percent remaining bacteria at each time point were determined relative to bacteria present at 0 min.

Bacterial clearance assay

Dictyostelium discoideum DH1, $Atg1^-$ and $Atg9^-$ cells were incubated for 1 h at 22ºC in shaking culture at $2x10^6$ cells/mL with or without *E. coli* 055:B5 LPS (1

mg/mL). *Staphylococcus aureus*, taken from overnight cultures, were labeled with STYO-9® (Invitrogen, Carlsbad, CA) and added at a ratio of 15:1 *D. discoideum* cell. Cells were incubated 1 h at 22ºC in the dark to allow for phagocytosis. Non-internalized bacteria were removed by centrifugation at 150g, and *D. discoideum* cells were resuspended at $2x10^6$ cells/mL. Samples were harvested during an additional 45 min incubation in shaking culture at 22ºC, in the dark, washed, and resuspended in PBS containing 1% paraformaldehyde. Samples were analyzed for bacterial clearance, using flow cytometry to measure the loss of fluorescence from cells at 45 min compared with the levels at 0 min.

Statistical analysis

The confocal fluorescence microscopy of localization of *S. aureus*-containing vesicles and GFP-Atg8 data were analyzed using the overlap coefficient (after Manders) with LSM software. The intracellular *S. aureus* viability data were analyzed using a fixed-effects two-way analysis of variance (ANOVA) and significant differences at the specific time points were determined using Tukey's HSD multiple comparison with JMP software. The flow cytometry data determining the differences in LPS-enhance bacterial clearance were analyzed with a paired t-test.

CHAPTER III:

RESULTS

The Gram negative bacteria, Salmonella typhimurium *and* Klebsiella pneumoniae *stimulate autophagosomal maturation*

During the process of autophagy the essential autophagy related protein, Atg8 is trafficked along with the autophagosome to the lysosome for degradation. When the Atg8 protein is tagged with a GFP marker, the GFP-Atg8 protein complex is cleaved by the lysosomal enzymes at the completion of autophagy. Upon cleavage the Atg8 is degraded but the GFP remains stable, and easily detectable, making this a relatively simple and reliable method to test for the completion of autophagy by looking for the presence of free GFP in the cytoplasm (Klionsky et al., 2007).

To ensure this procedure was reliable in *D. discoideum*, we tested for the appearance of free cleaved GFP in *D. discoideum* cells expressing GFP-Atg8 (HR87 cells) that were grown in nutrient supplemented media and cells that were grown in starvation media devoid of amino acids. Since it is known that *D. discoideum* will undergo autophagy when faced with starvation, our results are consistent with previous findings, showing that free GFP is detected in a time dependent manner only in cells grown in the starvation media (Figure 1A). The longer the cells were cultured in the starvation media, the higher the amount of free GFP detected. The HR87 cells that were

growing in the nutrient supplemented media did not show any free GFP, suggesting that autophagy, as expected, was not occurring in these cells.

To determine if phagoctized Gram negative bacteria containing the common molecular pattern, LPS, can stimulate the autophagy pathway, we used this GFP-Atg8 cleavage assay to test for stimulation of autophagy in *D. discoideum* cells exposed to increasing concentrations of *Salmonella typhimurium* and *Klebsiella pneumoniae.* Exposure to either of these pathogens was sufficient to induce autophagosomal maturation in the amoeba cells in a dose-dependent manner (Figure 1B). The higher the concentration of bacteria, the stronger the GFP signal, suggesting an up-regulation of autophagy. The specificity of this assay was verified by the addition of the type III PI3-kinase inhibitor, 3 methyladenine (3MA), a drug known to inhibit the formation of the autophagosome (Seglen and Gordon, 1982). *D. discoideum* cells pre-treated with 3MA before exposure to *S. typhimurium* did not exhibit production of free GFP while those cells not pre-treated did display free GFP (Figure 1C), suggesting that the appearance of free GFP is dependent on the activity of the type III PI3-Kinase and subsequent induction of autophagy.

LPS stimulates the maturation of autophagosomes in D. discoideum *cells exposed to* S. aureus

To determine if phagocytized *S. aureus* were able to induce autophagosomal maturation, the GFP-Atg8 cleavage assay was again employed. Cells exposed to the

Gram positive bacterium, *S. aureus* alone did not show the presence of free GFP, suggesting they were unable to complete autophagy unless they were first pre-treated with LPS (Figure 2A). When the cells were pre-treated with LPS, free GFP was detected in the blots (Figure 2A). The ability of the lipid component alone, responsible for the toxicity of LPS, lipid A, was also tested for its ability to induce autophagy. Cells pre-treated with lipid A also showed an accumulation of free GFP, indicating that autophagy was induced (Figure 2B). LPS-induced autophagy is inhibited when cells are pre-treated with 3MA, as there is no accumulation of the free GFP cleaved from Atg8 in the presence of the drug (Figure 2C).

Atg8 is recruited to S. aureus *containing vesicles only in the presence of LPS*

Using confocal fluorescence microscopy, we assessed the location of the intracellular *S. aureus*-containing vesicles inside the *D. discoideum* cells in relation to the GFP tagged Atg8 protein. We found that the GFP-Atg8 co-localized with the vesicles containing *S. aureus* only when the cells were first pre-treated with LPS (Figure 3A). When cells were not pre-treated with LPS, the GFP-Atg8 was typically found in other locations of the *D. discoideum* cells, disperse from the *S. aureus*-containing vesicles. The overlap coefficients (after Manders) were calculated for the *S. aureus*-containing vesicles and the GFP-Atg8 based on the location of the green and red pixels. We found that when the cells were first pre-treated with LPS, there was a significant increase in overlap coefficients, which was not observed in the cells that were not pre-treated with LPS

(Figure 3B). The number of cells in which the GFP-Atg8 was co-localized (+), moderately co-localized (+/-), or not co-localized (-), was assessed and it was concluded that cells pre-treated with LPS had a significantly higher chance of exhibiting GFP-Atg8 co-localization then those cells that were not pre-treated with LPS prior to *S. aureus* exposure (Figure 3C).

Dictyostelium discoideum *cells are able to clear* S. aureus *faster when pre-treated with rapamycin*

We tested whether autophagosomal maturation is sufficient to increase the rate at which phagocytized intracellular bacteria are cleared from *D. discoideum* cells. Using the drug rapamycin, which is known to induce autophagy in mammalian cells by blocking the TOR signaling pathway (Cutlet et al., 1999), we tested the ability of the drug to induce autophagy in *D. discoideum* cells using the GFP-Atg8 cleavage assay. Free GFP accumulated in cells pre-treated with rapamycin (Figure 4A), suggesting that the drug does induce autophagy in *D. discoideum* cells. Cells that were not pre-treated with rapamycin did not display any free GFP cleaved from the GFP-Atg8 complex. Next, to determine if this rapamycin-induced autophagy increased bactericidal activity in *D. discoideum* cells, we assessed the survival of intracellular phagocytized *S. aureus* over time in cells pre-treated with the drug vs. survival in control cells that were not pre-treated before exposure to *S. aureus*. We found that at the 20 minute and 40 minute time point following phagocytosis, there was a significantly lower concentration of viable

bacteria remaining in samples treated with rapamycin as compared with those that were not treated with the autophagy-inducing drug (Figure 4B). These results suggest that the induction of autophagy causes *D. discoideum* cells to clear intracellular pathogens at a faster rate.

LPS enhanced degradation is dependent upon critical autophagy related proteins

The percent change in clearance of SYTO-9 fluorescently labeled intracellular *S. aureus* in cells pre-treated with LPS and cells that were not treated was assessed through flow cytometry. Wild type DH1 cells demonstrated a ten percent increase in the clearance of intracellular bacteria in cells treated with LPS compared with non-treated cells (Figure 5). However this change in the clearance of bacteria due to pre-treatment of LPS was not seen in mutant cells lacking the critical autophagy related proteins Atg1 or Atg9 (Figure 5). These results suggest that the observed LPS-enhanced clearance of intracellular bacteria by the wild type DH1 *D. discoideum* cells is dependent upon the critical autophagy related proteins, Atg1 and Atg9.

CHAPTER IV:

DISCUSSION

Cells of the innate immune system rely on highly conserved pattern recognition machinery to identify common molecular patterns on invading pathogens and to neutralize those pathogens. *Dictyostelium discoideum* genomic analysis has revealed the existence of putative genes encoding known pattern recognition components such as the leucine rich repeat-containing protein, SlrA, and the Tir domain-containing protein, TirA (Chen et al., 2005). If *D. discoideum* cells do in fact use these pattern recognition receptors to identify invading pathogens, then we would expect the cells to mount a response against known pathogen-associated molecular patterns.

In this study we extend our previous findings that pre-treatment of *D. discoideum* cells with LPS increases bacterial clearance, showing that LPS also stimulates autophagosomal maturation in *D. discoideum* cells that have phagocytized *S. aureus*. Induction of autophagy stimulates an increased rate of intracellular pathogen clearance by the cells. Examining this phenomenon further, we report here that LPS-induced up-regulation of bacterial clearance is dependent on the autophagy related proteins Atg1 and Atg9, suggesting that LPS-mediated induction of autophagy is responsible, at least in part, for the LPS-induced regulation of bacterial clearance. These findings thereby link pattern recognition and autophagy at a very early stage in evolution, in the unicellular amoeba, *D. discoideum.*

The LPS-induced autophagy response is a key component of the overall innate immune response of an organism. Previous studies have demonstrated that when autophagy is unable to be completed, due to a knockout of genes encoding certain autophagy related proteins, the invading pathogen has a much better survival and replication rate than it does in wild-type *D. discoideum* cells. For example, it has previously been shown that *S. typhimurium* will survive longer in *D. discoideum* cells deficient in the autophagy related proteins Atg1, Atg6, or Atg7 (Jia et al., 2009). It has also been demonstrated that intracellular *Legionella* species have a greater survival rate when *D. discoideum* cells are lacking the Atg9 protein (Tung et al., 2010). We have observed similar results, showing that LPS-induced clearance of intracellular phagocytized *S. aureus* is dependent upon the autophagy related proteins, Atg1 and Atg9.

It has recently been noted that some bacteria, namely *S. aureus,* have developed ways to evade the mammalian host autophagy response (Schnaith et al., 2007). Recent studies have shown that during the progression of the autophagy pathway in *S. aureus*-infected epithelial cells, the autophagosome forms and recruits the autophagy marker LC3, a mammalian protein homologous to Atg8; nonetheless, autophagy is never taken to completion as the autophagosome never fuses with lysosomes for digestion (Schnaith et al., 2007).We have found that autophagy maturation is similarly inhibited in *D. discoideum* cells exposed to *S. aureus*; however, we observed that Atg8 is not recruited to the *S. aureus* containing-vesicles, in the first place, unless the cells are first pre-treated with LPS. This suggests a possible evolutionary difference between mammalian and *D.*

discoideum cells. It is important to note, however, that the mammalian study was performed with epithelial cells and not professional phagocytes, such as macrophages, which may show further similarities to phagocytic *D. discoideum* cells.

The autophagy pathway stimulated by the exposure to pathogen-associated molecular patterns provides insight into medically relevant pathways essential in fighting and preventing infections. For example, knowing that *D. discoideum* cells will respond more robustly to *S. aureus* when stimulated with a microbial pattern like LPS and knowing that LPS also stimulates autophagosomal maturation in mammalian cells indicates potential success in the development of treatments for *S. aureus* infections including those involving methicillin resistant *S. aureus* (MRSA).

Recent evidence has linked singling from the TLR on the surface of mammalian cells to the induction of autophagy. Signaling in response to recognition of microbial particles by TLRs triggers the recruitment of LC3 to the site of the forming autophagosome in a manner dependent on the proteins Atg5 and Atg7 (Sanjuan et al., 2007). Prior to LC3 recruitment, TLR stimulation signals beclin-1 activation, which stimulates the formation of the autophagosome by helping to localize key autophagy related proteins (Shi and Kehrl, 2008). Our finding linking LPS recognition and the induction of autophagy in *D. discoideum* mirror findings that LPS induces autophagy in mammalian cells, indicating a potential evolutionary link between microbial pattern recognition and autophagy.

Although we know that *D. discoideum* contains pattern recognition machinery such as Tir domains, and we known that the cells will mount a response against known pathogen- associated molecular patterns like LPS, we do not know the exact mechanism by which the *D. discoideum* cells identify and react to the pathogen. Previous studies have demonstrated that TirA-deficient *D. discoideum* cells are unable to grow in the presence of Gram negative bacteria, unlike their wild type counterparts (Chen et al., 2007). It would be beneficial to investigate if the signaling downstream of TirA is responsible for stimulation of pathogen-induced autophagy and whether this induction is necessary for the ability of wild-type cells, but not TirA deficient cells, to grow in the presence of Gram negative bacteria.

In conclusion we report here that the Gram negative bacteria, *S. typhimurium* and *K. pneumoniae,* stimulate autophagy in *D. discoideum,* and that the Gram positive bacteria, *S. aureus* will only stimulate autophagosomal maturation when the *D. discoideum* cells are pre-treated with the pathogen-associated molecular pattern, LPS. We also show that the critical autophagy related protein Atg8 is recruited to *S. aureus*-containing vesicles only when the cells are pre-treated with LPS. This LPS-induced autophagy is inhibited by the addition of 3MA, a drug known to block the formation of the autophagosome. Autophagosomal processes may contribute to bacterial killing, as rapamycin-induced autophagy promotes enhanced bactericidal activity in *D. discoideum* cells. Finally, extending these findings, we report here that LPS- induced bactericidal activity of the *D. discoideum* cells is dependent upon Atg1 and Atg9. These findings

suggest that LPS-mediated bacterial clearance is mediated, in part, by the induction of autophagy. These findings link autophagy and pathogen-associated pattern recognition very early in evolution, in the unicellular amoeba, *Dictyostelium discoideum* as previously linked in mammalian cells.

FIGURE LEGENDS

Figure 1: Autophagosomal maturation is induced in *D. discoideum* exposed to *S. typhimurium* and *K. pneumoniae*.

A. HR87 cells were incubated in complete or amino-acid free media, harvested and lysed. Autophagosomal maturation was assayed using the GFP-Atg8 cleavage assay, immunoblotting with anti-GFP to detect GFP-Atg8 and free GFP, which is cleaved from GFP-Atg8 upon delivery of autophagosomes to lysosomes.

B. The effects of incubation with *S. typhimurium* and *K. pneumoniae* on autophagosomal maturation in HR87 cells were assessed using the GFP-Atg8 cleavage assay.

C. Cells incubated with or without *S. typhimurium* were treated with or without the autophagy inhibitor 3MA, and inhibition of autophagosomal maturation was assessed using the GFP-Atg8 cleavage assay. Shown are representative blots from at least three independent experiments.

Figure 2: Autophagosomal maturation is induced in cells incubated with *S. aureus* only upon treatment with LPS.

A. HR87 cells were treated with or without 1 μg/mL LPS and incubated with varying ratios of *S. aureus* (SA) or *S. typhimurium* (ST). The GFP-Atg8 cleavage assay was used to detect autophagosomal maturation.

B. HR87 cells were untreated, treated with LPS (1 μg/mL) or Lipid A (1 μg/mL), and incubated with *S. aureus* at a ratio of 3: 1 HR87 cell. Autophagosomal induction was tested using the GFP-Atg8 cleavage assay.

C. HR87 cells incubated with or without LPS (1 μg/mL) and *S. aureus* at a ratio of 3 bacteria: 1 cell. HR87 cell were pre-treated with or without the autophagosomal inhibitor 3MA (5 mM). The GFP-cleavage assay was used to detect autophagosomal maturation. Shown are representative blots from at least three independent experiments.

Figure 3: Autophagosomes are recruited near *S. aureus*-containing vesicles upon treatment with LPS.

A. HR87 cells were pretreated with or without LPS (1 μg/mL) and incubated with *S. aureus*.

Cells were fixed on poly-lysine slides and permeabilized. *S. aureus* was detected using biotin-labeled antibodies specific for *S. aureus*, which were detected with AF594-conjugated streptavidin (red), while GFP-Atg8 was detected using mouse F(ab)'$_2$ fragments specific for GFP. These were detected with AF488-conjugated F(ab)'$_2$ anti-

mouse IgG (green). Images were acquired using a Zeiss LSM 510 laser scanning microscope. Shown are the merged images of the optimal single planes at a magnification of 63x. Shown are representative images from five independent experiments.

B. The overlap coefficients (after Manders) of red and green pixels in cells treated with or without LPS were calculated using LSM software. Shown are means and SEM calculated from five independent experiments, analyzing at least 15 cells per condition for each experiment.

C. Depicted are the percentage of cells showing no co-localization (-), moderate juxtaposition (-/+), and almost complete juxtaposition or co-localization between *S. aureus* and GFP-Atg8 (+), as determined by a blinded observer using images acquired in 3B.

Figure 4: Induction of autophagy is linked with bactericidal activity.

A. HR87 cells incubated with *S. aureus* at a ratio of 3 bacteria: 1 cell were treated with 100 nM rapamycin and subjected to the GFP-Atg8 cleavage assay to test for induction of autophagosomal maturation.

B. HR87 cells were treated with or without rapamycin (100 nM) and incubated with *S. aureus*. At various times following phagocytosis, cells were lysed and released

bacteria plated on nutrient agar to test for viability. Shown are mean percent survival and SEMs from four independent experiments. The overall effects of rapamycin on clearance of bacteria were significant ($p<0.0001$) as measured by fixed-effects two-way ANOVA. * denotes significance at specific time points as measured by Tukey's HSD multiple comparison.

Figure 5: LPS-enhance bacterial clearance is dependent upon the autophagy related proteins (Atg).

DH1, Atg1$^-$ or Atg9$^-$ cells were pre-treated with or without 1 μg/mL LPS and incubated with SYTO9-labeled *S. aureus*. Cells were washed of non-phagocytized bacteria and clearance of internalized bacteria was assessed by measuring the loss of fluorescently-labeled bacteria, indicative of degraded *S. aureus*, in cells after 45 min. The percent differences in clearance in cells treated with vs. without LPS for each cell type were calculated. Shown are mean differences and SEMs from five independent experiments. * and ** denote significance ($p<0.05$ and $p<0.01$, respectively) as measured by the paired t-test.

Figure 1:

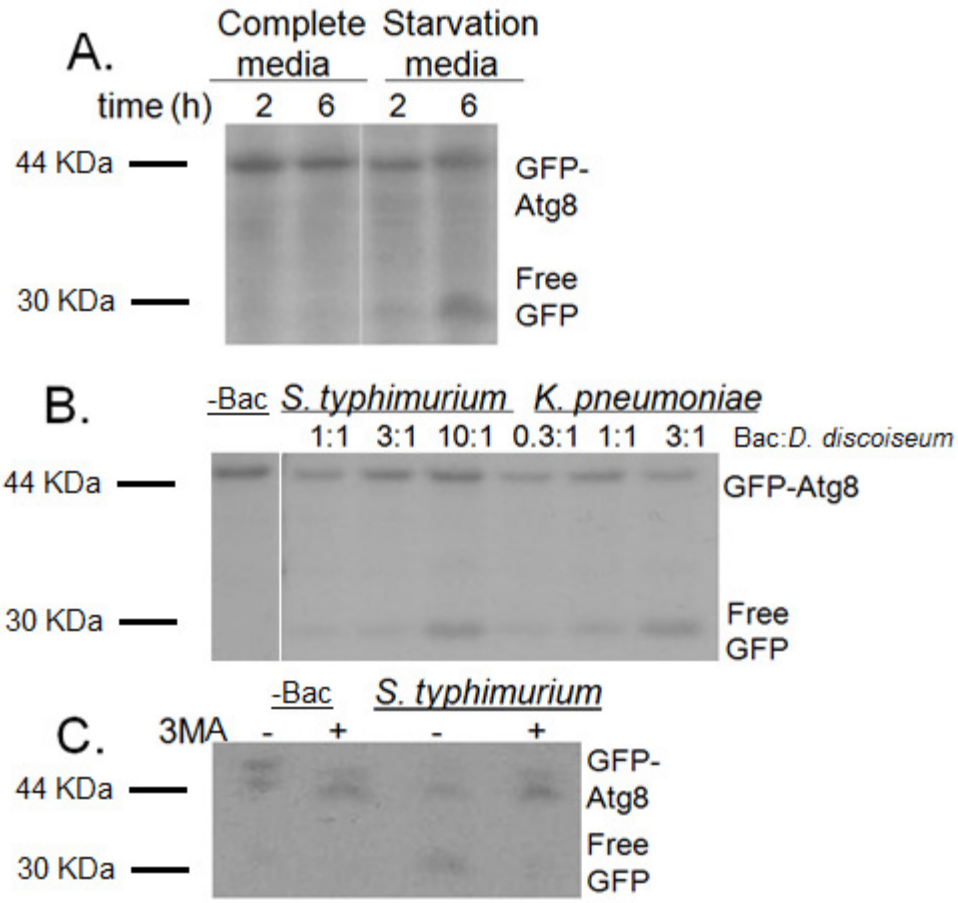

A.
Complete media
Starvation media
time (h) 2 6 2 6
44 KDa
30 KDa
GFP-Atg8
Free GFP
B.
-Bac
S. typhimurium
K. pneumoniae
1:1 3:1 10:1 0.3:1 1:1 3:1
Bac:D. discoiseum
44 KDa
30 KDa
GFP-Atg8
Free GFP
C.
-Bac
S. typhimurium
3MA - + - +
44 KDa
30 KDa
GFP-Atg8
Free GFP

Figure 2:

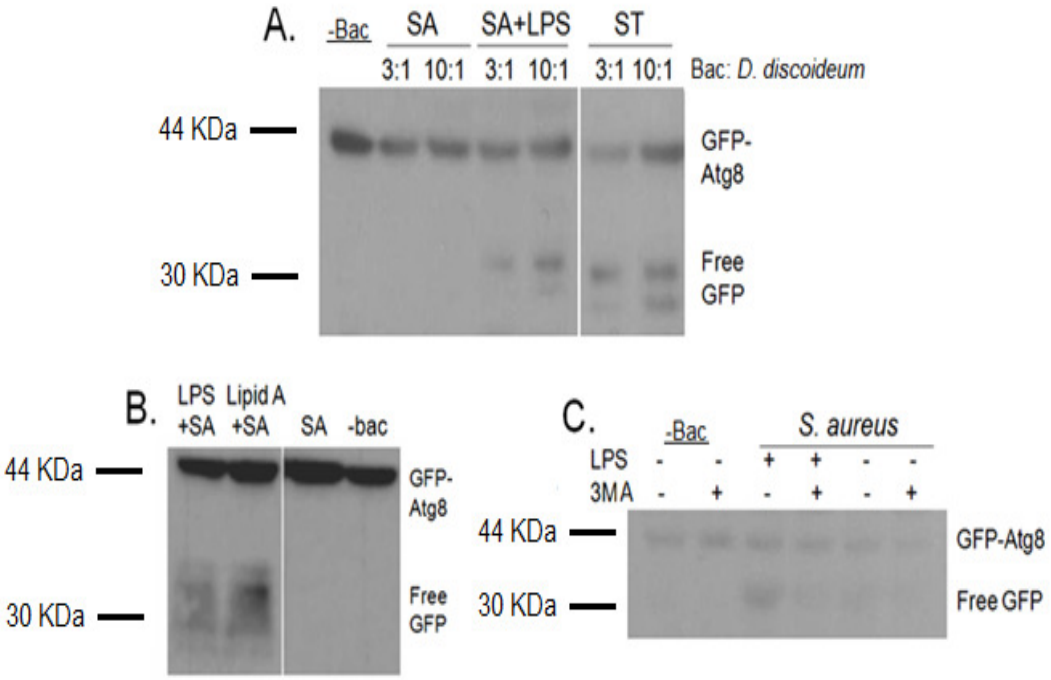

A.
-Bac
SA
SA+LPS
ST
3:1 10:1 3:1 10:1 3:1 10:1
Bac: D. discoideum
44 KDa
30 KDa
GFP-Atg8
Free GFP
B.
LPS +SA
Lipid A +SA
SA
-bac
44 KDa
30 KDa
GFP-Atg8
Free GFP
C.
-Bac
S. aureus
LPS - - + + - -
3MA - + - + - +
44 KDa
30 KDa
GFP-Atg8
Free GFP

Figure 3:

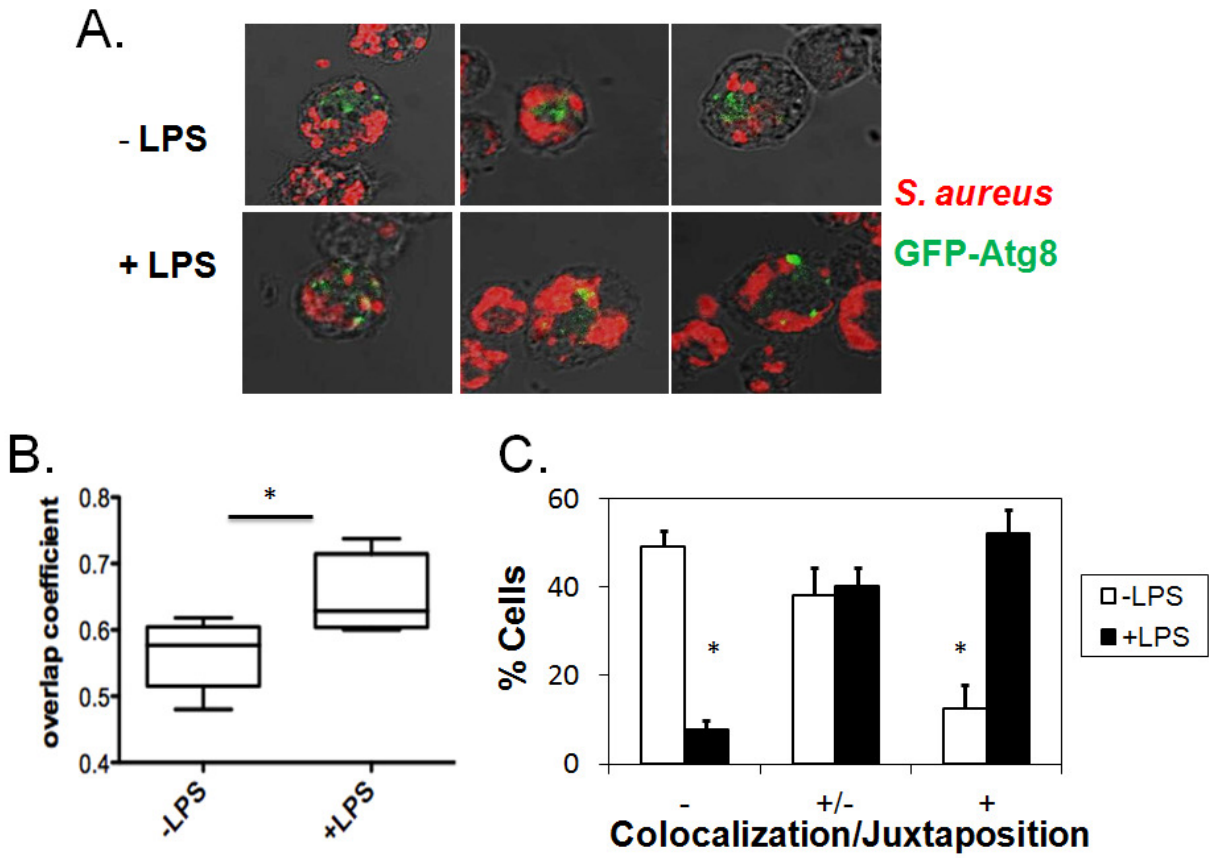

A.
- LPS
+ LPS
S. aureus
GFP-Atg8
B.
overlap coefficient
0.8
0.7
0.6
0.5
0.4
*
-LPS
+LPS
C.
% Cells
60
40
20
0
*
*
-
+/-
+
Colocalization/Juxtaposition
-LPS
+LPS

Figure 4:

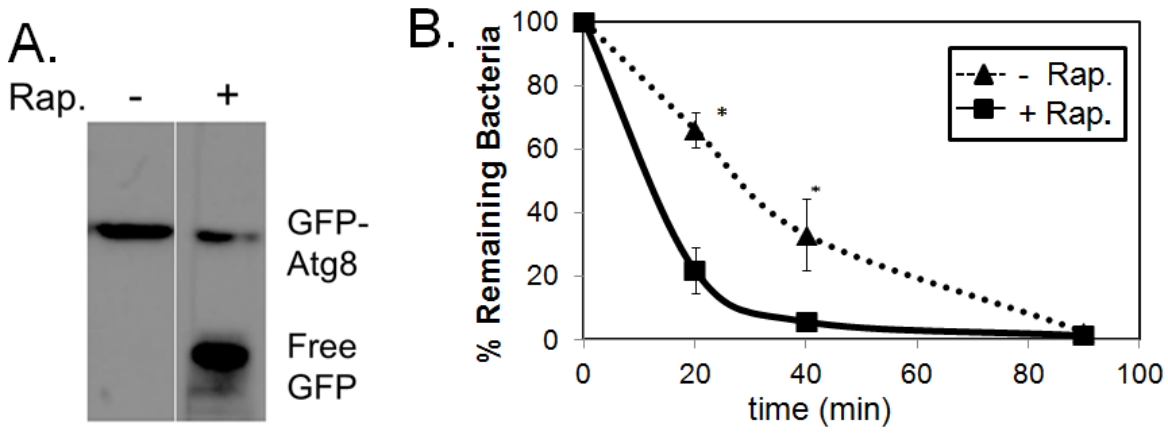
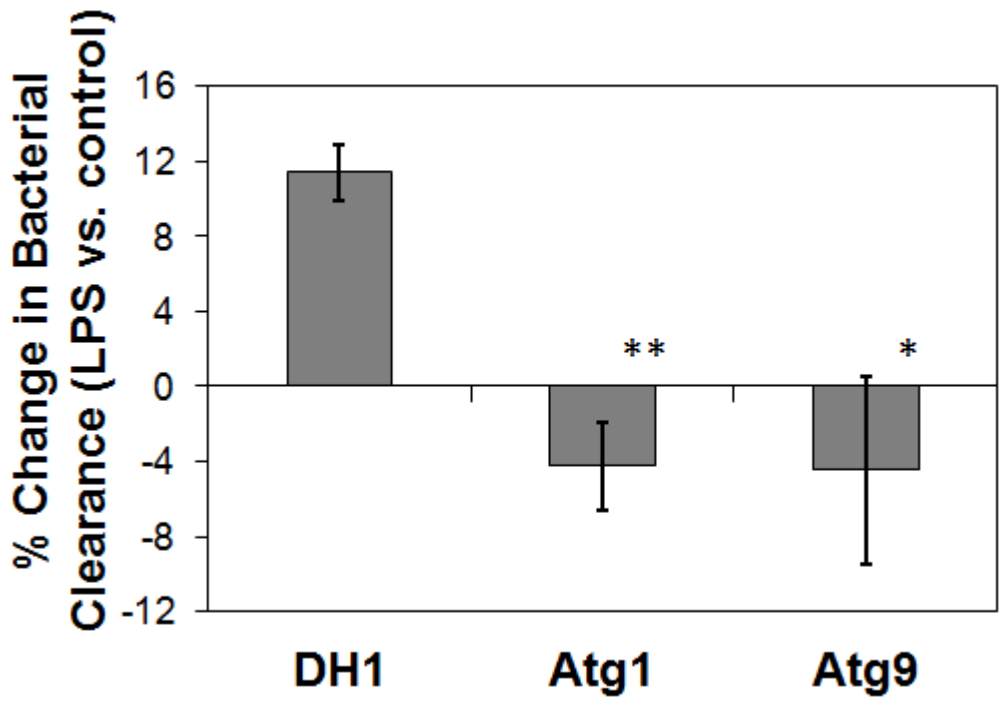

A.
Rap.
-
+
GFP-
Atg8
Free
GFP
B.
% Remaining Bacteria
100
80
60
40
20
0
0
20
40
60
80
100
time (min)
- Rap.
+ Rap.
*
*

Figure 5:


% Change in Bacterial Clearance (LPS vs. control)
16
12
8
4
0
-4
-8
-12
**
*
DH1
Atg1
Atg9

LITERATURE CITED

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

Benghezal, M., M.O.Fauvarque, R.Tournebize, et al., 2006. Specific host genes required for the killing of *Klebsiella* bacteria by phagocytes. Cell Microbiol. 8:139-148.

Chen, G., O.Zhuchenko, A.Kuspa.2007. Immune-like phagocyte activity in the social amoeba, Science 317: 678-681.

Cosson, P., and T.Soldati. 2011. Eat, kill or die: when amoeba meets bacteria. Curr. Opin. Microbiol. 11: 271-276.

Creaght and O'Neil. 2006. TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity. Trends in Immunology. 27:352-357

Cutlet, N.S., J. Heitman, and M.E. Cardenas. 1999. TOR kinase homologs function in a signal transduction pathway that is conserved from yeast to mammals. Mol.Cell.Endocrinol. 155:135-142

Delgado, MA., R.A.Elmaoued, A.S.Davis, et al. 2008. Toll-like receptors control autophagy, EMBO J. 27:1110-1121.

Delgado,M., S.Singh, H.S.De, et al. 2009. Autophagy and pattern recognition receptors in innate immunity, Immunol.Rev. 227: 189-202.

Deretic,V. 2011. Autophagy in immunity and cell-autonomous defense against intracellular microbes, Immunol.Rev. 240: 92-104.

Desjardins, M., M. Houde, and E. Gagnon. 2005. Phagocytosis: the convoluted way from nutrition to adaptive immunity. Immunology Review 207:158-165

Eichinger, L., J.A.Pachebat, G.Glockner, et al .2005. The genome of the social amoeba *Dictyostelium discoideum*, Nature 435:43-57.

Janeway, CA., Jr., and R.Medzhitov. 2002. Innate immune recognition, Annu.Rev.Immunol. 20 : 197-216.

Janssen, K., and M. Schleicher. 2001. Dictyostelium discoideum: a genetic model system for the study of professional phagocytes. Profilin, phosphoinositides and the lmp gene family in Dictyostelium. Biochim.Biophys.Acta 1525:228-233.

Jia, K., C. Thomas, A. Akbar, et al. 2009. Autophagy genes protect against *Salmonella typhimurium* infection and mediate insulin signaling-regulated pathogen resistance. PNAS. 106:14564-14569

Klionsky, D.J., A.M. Cuerco, and P.O. Seglen. 2007. Methods for monitoring autophagy from yeast to human. Autophagy. 3:181-206.

Kumar,H., T.Kawai, S.Akira. 2011. Pathogen recognition by the innate immune system, Int.Rev.Immunol. 30 : 16-34.

Lang, K., A. Burow, M. Kurrer, et al. 2007. The role of the innate immune response in autoimmune disease. J.Autoimm. 29:206-212.

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

Mestre, M.B., C.M.Fader, C.Sola et al. 2010. Alpha-hemolysin is required for the activation of the autophagic pathway in *Staphylococcus aureus*-infected cells, Autophagy 6. 110-25.

Mizushima, N., B.Levine, A.M.Cuervo, et al. 2008. Autophagy fights disease through cellular self-digestion, Nature 451:1069-1075.

Newell, P.1971. The development of the cellular slime mould Dictyostelium discoideum: a model system for the study of cellular differentiation. Essays in Biochemistry 7:87-126

O'Hagan, D., M. MacKichan, and M. Singh. 2001. Recent developments in adjuvants for vaccines against infectious diseases. Biomolecular Engineering 18:69-85.

Pichichero, M., 2008. Improving vaccine delivery using novel adjuvant systems. Human Vaccines 4:262-270.

Rosenstiel, P., G. Jacobs, A. Till, and S. Schreiber. 2008. NOD-like receptors: ancient sentinels of the innate immune system. Cell Molecular Life Science 65:1361-1377.

Sanjuan, MA., C.P.Dillon, S.W.Tait, et al. 2007. Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis, Nature 450: 1253-1257.

Schnaith, A., H.Kashkar, S.A.Leggio, et al. 2007. *Staphylococcus aureus* subvert autophagy for induction of caspase-independent host cell death, J.Biol.Chem. 282:2695-2706

Shi,CS. and J.H.Kehrl 2008. MyD88 and Trif target Beclin 1 to trigger autophagy in macrophages, J.Biol.Chem. 283:33175-33182.

Shin, DM., J.M.Yuk, H.M.Lee, et al. 2010. Mycobacterial lipoprotein activates autophagy via TLR2/1/CD14 and a functional vitamin D receptor signalling, Cell Microbiol. 12:1648-1665.

Sillo, A., G.Bloomfield, A.Balest, et al. 2008. Genome-wide transcriptional changes induced by phagocytosis or growth on bacteria in *Dictyostelium*, BMC.Genomics 9 :291.

Segle, P.O. and P.B. Gordon. 1982. 3-Methylandenine: specific inhibitor of autophagic/Lysosomal protein degradation. PNAS. 79:1889-1892

Takeuchi, O. and S. Akira. 2010. Pattern recognition receptors and inflammation. Cell. 140:805–820

Tekinay,T., M. Yu, G. Otto, O. Anderson, and R. Kessin. 2006. Function of the *Dictyostelium discoideum* Atg1 kinase during autophagy and development. Eukaryotic Cell 1797-1806.

Tung, SM., C.Unal, A.Ley, et al. 2010. Loss of *Dictyostelium* ATG9 results in a pleiotropic phenotype affecting growth, development, phagocytosis and clearance and replication of *Legionella pneumophila*, Cell Microbiol. 12:765-780.

Walk,A., J.Callahan, P.Srisawangvong, et al. 2011. Lipopolysaccharide enhances bactericidal activity in *Dictyostelium discoideum* cells, Dev.Comp Immunol. 35:850-856

Xu, Y., C. Jagannath, X. Liu, et al. 2007. Toll like receptor 4 is a sensor for autophagy associated with innate immunity. Immunity 27:135-144

KATHERINE PFLAUM

404 Newburg Ave 410-371-4175
Baltimore, MD 21228 kpflau1@students.towson.edu

EDUCATION

M.S.: Biology, Towson University, Towson, MD. Expected December 2012
Awards: *Graduate Student Association Travel Award*

B.S.: Biology, Salisbury University, Salisbury, MD. May 2010
Awards: *Honors, Guerrieri summer research program scholar*

Relevant Coursework: Gene Expression and Regulation, Virology, Immunology, Molecular Biology, Biostatistics, Microbiology, Genetics

SELECTED PUBLICATIONS

Pflaum, K., Gerdes, K., Yovo K., Callahan J., and Snyder, ML. 2012. Lipopolysaccharide induction of autophagy is associated with enhanced bactericidal activity in *Dictyostelium discoideum*, Biochem. Biophys. Res. Commun. 422:417-22.

SELECTED PRESENTATIONS

Pflaum, K., Gerdes, K., Yovo, K., and Snyder, ML. Cellular mechanisms linking pattern recognition and autophagy in *Dictyostelium discoideum.* Poster at the American Association of Immunologists in Boston, MA in May 2012.

Snyder,M.D., **Pflaum, K**., Gerdes ,K. Cellular mechanisms underlying LPS-induced bactericidal activity in *Dictyostelium discoideum*. Poster (and talk) at the American Association of Immunologists in San Francisco, CA in May 2011.

PROFESSIONAL EXPERIENCE

Graduate Teaching Assistant 2010-2011

Towson University, Towson, MD
Led laboratory sections of undergraduates each semester in Microbiology. Prepared and graded exams.

Master's Student

Towson University, Towson, MD 2009-2011

Utilized functional assays to investigate the ability of *D. discoideum* to recognize and respond to common molecular patterns on pathogens.

Lab Assistant 2010

USDA Agricultural Research Center, Beltsville, MD

Assisted in agricultural and plant pathology research related to crop management and disease resistance.

General Assistant 2009-2010

University of Maryland Research and Education Center, Salisbury, Maryland

Research related to environmental relationships between soil fertility, crop management systems, and disease resistance.