Carlin Miller for the degree of Honors Baccalaureate of Science in Microbiology presented on August 29,2008. Title: Identification of Autophagy Related Genes in *Mycobacterium tuberculosis*

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ABSTRACT

A primary target of *Mycobacterium tuberculosis* is the human alveolar macrophage. Infection by this bacterium can lead to a variety of responses, such as apoptosis, autophagy, and necrosis, which may be involved in controlling the infection. *M. tuberculosis* has evolved mechanisms to evade or use the host-mediated processes to its advantage. One of them, autophagy, has been shown to be suppressed by the bacterium. The goal of this study was to identify mycobacterial genes involved in autophagy inhibition. A transposon mutant bank was created using the *M. tuberculosis* virulent strain H37Rv and temperature-sensitive plasmid containing transposon Tn6753. U937 human macrophages were infected with the mutant library and individual clones were screened for attenuation. Once mutants showing impaired ability to grow within the host macrophage were identified, they were screened for an inability to inhibit autophagy. Fifty-four mutants exhibiting attenuation within the macrophage were identified. An LC3-staining assay was performed on eighteen clones that showed the greatest attenuation. Three of them were not associated with autophagy inhibition. The sequencing of the inactivated genes is in progress.

Key Words: *M. tuberculosis*, attenuation, LC3, autophagy

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Identification of Autophagy Related Genes in *Mycobacterium tuberculosis*

by

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My experience has been one of learning and growing and you all played a large part in it, thanks again.

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INTRODUCTION

It is estimated that approximately one-third of the world's population is infected with *Mycobacterium tuberculosis*. That computes to nearly 2 billion individuals. There are nearly nine million new cases of tuberculosis each year, and approximately two million tuberculosis-related deaths worldwide (24). Unfortunately, it is not a disease of the past but has become even more lethal under certain conditions. Recently, a relationship between HIV patients and tuberculosis has been established. It is known that a concomitant HIV infection increases the risk of sub-clinical *M. tuberculosis* infection becoming active disease (21).

To wage an effective battle against *M. tuberculosis*, it is necessary to understand the mechanism of bacterial pathogenesis, as well as the effective immune protection. Autophagy, a normal cellular process involving recycling of cellular organelles and degradation of pathogens via the autophagosome, is an important front line cellular defense. Many of the effector molecules of the autophagy process (the focus of this study) have been elucidated. In some cases, we know at what stage autophagy is being suppressed. What is unclear are the details of how *M. tuberculosis* inhibits autophagy. Many of the bacterial effectors involved in the autophagy process are yet to be elucidated. However, some bacterial genes and protein effectors have been implicated in controlling autophagy, regulating processes from phagocytosis by the macrophage to fusion of the lysosome with the phagosome. It is also possible that the bacteria may be able to directly regulate expression of host genes.

Our goal is to identify tuberculosis genes that are involved in inhibition of the autophagy process. We propose that attenuation of virulence as declared by lower CFU

counts due to inhibited replication within the macrophage as compared to wild-type (WT) bacteria, is indicative of mutants with deletion of genes that are necessary for infection and replication within the macrophage. Identification of mutants exhibiting attenuation and subsequent evaluation for deficiency in suppressing macrophage autophagy will unveil a set of genes important for the survival within macrophages. Once genes are characterized, they may offer some insight into their function and mechanism of inhibition of host pathways. This knowledge could then be useful in designing novel therapeutic modalities aimed at combating the pathogen.

Background. *M. tuberculosis* is an intracellular pathogen that preferentially infects the alveolar macrophage in the human lung. Common mode of transmission is person-to-person via aerosol. There are many individuals "infected," but only a fraction of them actually exhibit the active disease. Usually, in healthy individuals, *M. tuberculosis* bacteria is either eliminated or successfully walled off and controlled. In a susceptible host, the disease can progress through multiple stages. Even so, it is generally arrested prior to reaching the final stage of active tuberculosis, which often includes dissemination of the bacteria either in the lungs or circulatory system.

The infection process starts with the inhalation of droplet nuclei which are assumed to be taken up by the alveolar macrophages in the alveoli of the lungs. These macrophages may migrate to local lymph nodes and halt the infection, forming what is called a "Ghon complex." One alternative is that tubercles, consisting of high numbers of immune cells, may form at the initial site of infection. Some bacteria may also be phagocytosed by macrophages which then migrate though the blood stream to other areas of the lung forming tubercles there. Bacteria exist within the tubercle in a growth

arrested state for long periods of time. This is considered latent infection and the bacteria in this state may become sites of reactivation later on. For unknown reasons, the tubercle lesions may become necrotic and liquefy in the center while at the same time growing outward until they compromise an airway membrane seeding bacteria directly into the airway. The emptying of the necrotic tubercle leaves behind the characteristic cavity. In this stage of the disease, the patient is very contagious.

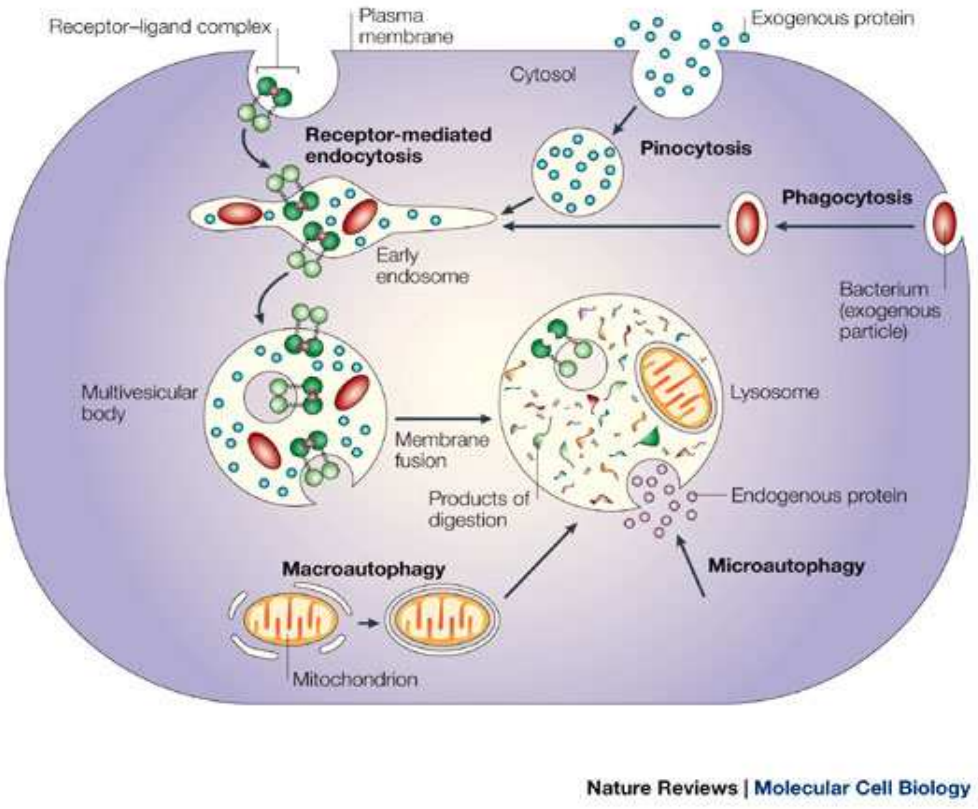
The specifics determining why some individuals are able to effectively fight the disease, while others succumb to it, are not known, but individual immune response is likely to be an important factor. The relationship between HIV and increased risk of tuberculosis highlights the importance of the health of the immune system in determining whether the individual will successfully fight the infection or develop active disease.

Mechanism of Infection. Macrophages are part of the innate and adaptive immune system and provide the front line defense to many invading pathogens. They recognize an array of conserved antigenic patterns (such as peptidoglycan, carbohydrates, and lipids) on the surface of bacteria, which are identified as foreign. Once the bacteria are taken up by phagocytic cells, they are sequestered inside a membrane-surrounded compartment called a phagosome. Phagocytic immune cells, including macrophages, have multiple methods of dealing with intracellular pathogens. After initial uptake, the phagosome becomes acidified killing most bacteria. Macrophages are also capable of producing toxic molecules (nitric oxide, superoxide anion, and hydrogen peroxide). These molecules are part of the macrophages front line innate response commonly known as the “respiratory or oxidative burst.” If the pathogen remains trapped within the phagosome, phagosome maturation may occur followed by lysosomal fusion and release

of bactericidal molecules into the phagosome environment, killing the bacteria. If these mechanisms fail to eliminate the pathogen the macrophage may undergo apoptosis in an effort to halt the infection.

Autophagy as a Defense Mechanism. Autophagy is a physiologic cellular process that involves the breakdown of intracellular organelles, for the purpose of recycling their contents. It is a mechanism that allows for cell survival under nutrient starvation. During the process, cellular proteins and organelles are enclosed in membrane vesicles (autophagosomes) originating from the endoplasmic reticulum. These vesicles then fuse with lysosomes which contain digestive enzymes that degrade the autophagosome contents and recycle the proteins for further use in the cell. The primary association between autophagy and bacterial degradation is that the macrophage uses much the same mechanism when dealing with intracellular pathogens. As seen in Figure 1, the bacterium is engulfed by the macrophage and contained within the phagosome. The phagosome then becomes acidified, following the acquisition of membrane ATPases, to approximately pH 5.

Figure 1. Relationship of autophagy to phagocytosis. Autophagy and phagocytosis are connected by lysosomal degradation. Autophagy is a mechanism of cell survival by degradation of intracellular contents while phagocytosis involves the degradation of pathogens. Figure adapted from Ciechanover (2).



Further maturation of the phagosome and fusion with lysosomes follows. Finally, the bacterium is degraded by the digestive enzymes in the phagolysosome. It is imperative that the phagosome is first acidified because many of the lysosome enzymes require an acid environment to be activated. In some cases, the acid environment in the phagosome is capable of killing invading bacteria without further phagosome maturation and lysosomal fusion. Lysosome enzymes include: proteases and lysozyme, which degrade cell surface components of bacteria; defensins that create pores in bacterial membranes; and myeloperoxidase, known to produce reactive oxygen species toxic to most bacteria.

The phagocytosis and breakdown of pathogens requires that the macrophage first senses the pathogen and engulfs it. Further maturing of the phagosome, docking of the lysosome, and delivery of toxic enzymes to the phagosome requires a complex system of sensing and regulation of phagosome membrane components. Some of the key effectors in the autophagy process have been identified. There are over 30 autophagy-related genes found in yeast, with eleven having orthologs in mammals (23). A few known regulators in autophagy maturation are PI3P, involving localization of trafficking proteins (16); P13 kinase/Akt, known to phosphorylate PI3P (25); small GTPases, used in traffic control; calcium signaling, involved in intracellular signaling; and inositol triphosphates (23). Many of these regulators seem to operate through Ser/Thr kinase Tor (mTOR), a potent inhibitor of autophagy (23). These regulatory pathways probably relieve mTOR inhibition when it is advantagious to do so. Therefore, since mTOR is an inhibitor of autophagy and many of these regulatory and trafficking mechanism inhibit mTOR to activate autophagy, *M. tuberculosis* inhibition of autophagy would likely involve interactions with these regulatory mechanisms upstream of mTOR. It might also be possible also that *M. tuberculosis* interacts directly with mTOR activating it and consequently inhibiting autophagy.

Researchers have also characterized a number of useful autophagy markers. There is: LC3, beclin-1 (a subunit of PI3K), cathepsin D, and LAMP-1 (a lysosomal-associated membrane protein). These four markers were found associated with mature phagosomes and lysosomes (10). The ability to test for markers is a useful research tool in determining where there is active autophagy taking place and to what stage it has proceeded.

***M. tuberculosis* Inhibition of Macrophage Killing.** Pathogenic bacteria possess the ability to survive and multiply within the host macrophage. They have strategies to circumvent the potent host mechanism of autophagy which is a mechanism of cell survival and pathogen degradation within macrophages. Many bacteria, such as *Staphylococcus aureus,* are sequestered within the phagosome and further degraded. Alternately, we know that bacterial pathogens can inhibit this phagosome-lysosome fusion, while others, such as *Listeria monocytogenes,* use listeriolysins, to disrupt the phagosome membrane, and are capable of escaping from the endocytic vacuole into the cytoplasm where they can replicate (9). Generally, the inhibition is achieved by regulating the surface proteins involved in the fusion process. *Salmonella typhimurium* and *Mycobacterium leprae* are examples of pathogens able to halt this degradation pathway by controlling phagosome membrane proteins and preventing lysosome fusion (7, 15). In the case of *M. tuberculosis,* the bacterium has an ability to regulate the acquisition of vacuole membrane proteins, with consequent impact on the maturation and environment within the phagosome. While parts of the pathogenic mechanisms are currently known, there is still much to be learned.

Past studies showed that the *M. tuberculosis* phagosome appears as if it has been arrested at an early stage of its maturing process and maintains a pH of 6.4. It has also been demonstrated that virulent *M. tuberculosis* is able to exclude the proton ATPases from its phagosome membrane (22). Under normal circumstances pathogen proteins degraded within macrophage phagolysosomes are loaded and displayed on MHC II complexes. Prevention of acidification of the vacuole and degradation of the bacteria would disrupt this antigen presentation on the macrophage and further activation of other

immune cells (22). Not only does *M. tuberculosis* disrupt this process but one laboratory also showed down regulation of the expression of MHC II molecules (19). Another study indicated that *Mycobacterium bovis* has the same inhibiting capabilities (8).

Other studies have implicated Rab GTPases in the signaling and trafficking that controls some of the phagosome-lysosome maturation and targeting. It appears as though some *Mycobacterium* species can halt the process between rab 5 (an early endosomes marker) and rab 7 (known as a GTP binding protein found on late endosomes) (26). Other proteins and ligands also appear to be involved in blocking phagosome maturation. These include cell wall lipids lipoarabinomannan (ManLAM) (6), and trehalose dimycolate (12) which have exhibited phagosome-lysosome fusion inhibiting capabilities. Phosphatase SapM, a Phosphatase which dephosphorylates P13P (25), and serine/threonine kinase PknG, a bacterial cell-wall component (3), were also found to be involved in regulation of phagosome lysosome maturation. *M. tuberculosis* mutants defective in production of these constituents are exposed to lower pH and are prevented from growth (20).

Gutierrez and colleagues investigated whether or not autophagy could be induced in the presence of *M. tuberculosis*. They found that nutrient starvation, artificial induction with rapamycin (A pharmacological agent capable of inhibiting mTOR) (17), and IFN-γ all induced autophagy and effectively overcame *M. tuberculosis* inhibition of phagosome maturation. This positive induction reduced the viability of the bacterium within the macrophage (10). Understanding what the bacteria are using to inhibit the immune response and knowing what can overcome this inhibition may have some potential in developing methods of stimulating immune responses in patients with active

disease. If bacterial inhibition can be overcome, successful destruction of the pathogen might be possible.

If the macrophage fails to eradicate the intracellular pathogen via autophagy or other front line mechanisms, it may attempt to induce apoptosis as another strategy to contain the infection. There is also evidence that *M. tuberculosis* has strategies for protecting itself against macrophage apoptosis. Studies have revealed that macrophages infected with the attenuated H37Ra bacterial strain exhibit greater apoptosis than macrophages infected with a mutant virulent H37Rv strain (14). Another work confirmed these findings showing that both H37Ra and H37Rv induced greater apoptosis, as compared to the control, but the apoptosis induced by the virulent strain was significantly decreased compared with the apoptosis induced by the attenuated strain (5). Zhang and colleagues used J774 macrophages and showed that *M. tuberculosis* may down-regulate the Fas/FasL signaling pathway and, thereby, reduce apoptosis. They also found that Bcl-2, an anti-apoptotic protein, was up-regulated by H37Rv strain (27). This allows the bacterium to prevent apoptosis in the early stages of infection.

There are also connections between regulation of apoptosis and autophagy. The picture is complex with some molecules shown to regulate both processes. The protein p53, commonly associated with apoptosis, is also known to induce autophagy (4). Alternately, phosphatidylinositol 3 kinase/protein kinase B (AKT/PKB), which inhibits apoptosis, can also inhibit autophagy (1). Beclin 1, an autophagy regulator, has also been shown to interact with Bcl-2, an anti-apoptotic regulator (18). This interaction suppresses both autophagy and apoptosis (23). Bcl-2 can also regulate autophagy by blocking calcium release from the endoplasmic reticulum (11). This leads to the inhibition of

mTOR and the activation of autophagy (23). In addition, in epithelial cells the FADD receptor (usually associated with apoptosis induction) has been implicated in the induction of autophagy; although, the mechanism is not yet known.

It appears that *M. tuberculosis* not only controls the process of macrophage autophagy and apoptosis, but in late infection, it may also induce necrosis as a strategy of dissemination. This would point to the possibility that apoptosis, autophagy and necrosis may be induced by some of the same signals, carried out simultaneously, and may all have a hand in cell death in a given situation. It might be useful to think about apoptosis, necrosis and autophagy as a continuum in which the mechanism observed is dependent up on the process that is dominating at the time. Finally, understanding the interplay between these processes could affect the way certain diseases are treated (23).

MATERIALS AND METHODS

Tissue Culture. The U937 human monocytes were maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco Laboratories) in T-25 flasks at 37°C and 5% CO_2.

Mutant Library. A temperature-sensitive (replication at 30°C) plasmid pTNGJC, based on a pUC19 plasmid with the addition of a mycobacterial origin of replication and the transposon Tn5367 (with a kanamycin- resistant cassette) cloned into it, was created. It was then transformed into *M. tuberculosis* H37Rv and the bacterium grown at 30°C in presence of kanamycin. After approximately three weeks of growth, the environmental temperature was raised to 42°C, resulting in "death of the plasmid" and transposition of Tn5365 at randomized sites in the bacterial chromosome. The microbial colonies were then screened for the presence of the transposon. Approximately 5,000 individual mutants were grown selectively on 7H9 Middlebrook broth-based media, with 200 µl/ml kanamycin. Wild-type (WT) virulent strain H37Rv was maintained on 7H10 Middlebrook agar base media supplemented with oleic acid, albumin, dextrose and catalase (OADC), 100 ml/l of 7H9 or 7H10 (Hardy Diagnostics).

Mutant Screening. A total of 384 *M. tuberculosis* mutants were screened for attenuation. The U937 macrophages were seeded in duplicate 96-well plates (5×10^5 cells/well) and PMA 1 µl/ml 24 h prior to the infection. Macrophages were then infected with 10 µl of WT H37Rv or mutant bacteria (MOI of 10:1 or 5×10^6 bacteria/well). A 1-h and 5-day, plating was carried out for each well. At 1-h post infection, the supernatant was removed from each 96-well plate and each plate was washed twice with Hanks' buffered salt solution (HBSS). Lysing solution containing 0.25% SDS was added to each

well, and the resulting macrophages lysate was diluted in HBSS and plated (10^{-3}, 10^{-4}) onto 7H10 Middlebrook agar based media supplemented with OADC, 100 ml/l, containing 200 µl/ml kanamycin (Hardy Diagnostics). The other duplicate plate was refreshed with new RPMI and incubated at 37°C and 5% CO_2 until day five. On day five, the macrophages in the second 96-well plate were lysed and plated using the same dilutions and media as the 1 h infection. Bacteria were allowed to grow at 37°C and 5% CO_2 until there were visible colonies for both the 1-h and 5-day platings, Growth of each mutant from 1-h to 5-days was compared and those mutants showing attenuation (reduced growth) after five days of infection, as compared to the 1-h infection, were recorded.

Autophagy Assay. The U937 macrophages (5×10^5 cells/well) and PMA 1 µl/ml were placed on 8-chamber glass slides 24 h prior to infection with *M. tuberculosis* mutants. Infection with mutants (MOI of 10:1) was allowed to proceed for 2 h at 37°C and 5% CO_2. Wells were washed with HBSS, replaced with media (RPMI), and incubated for 3 days at 37°C and 5% CO_2. After 3 days of infection, macrophages were fixed with 4% paraformaldehyde for 1 h, followed by incubation with Triton X-100 0.1% (3-5 min on ice) for permeabilization. Then, 5% blocking solution in phosphate buffered saline (PBS-Tween) was added for 1 h, and anti-LC3 H-50 rabbit polyclonal IgG at a dilution of 1:500 (Santa Cruz Biotechnologies) was added for another hour. The primary antibody was removed, cells were washed with HBSS twice, and goat anti-rabbit IgG-FITC, mouse human adsorbed secondary antibody (Santa Cruz Biotechnologies) was added at a concentration of 1:2,000 for 1 h. In the positive control experiment, the U937 macrophages were seeded in 8-chamber glass slides as described above (5×10^5

cells/well and PMA 1 µl/ml) and treated with 50 or 150 µg/µl rapamycin for 4 h. Rapamycin solution was then removed and slides were processed for LC3 immuno-staining. The LC3 stained macrophages were viewed with a Leica fluorescent microscope.

RESULTS

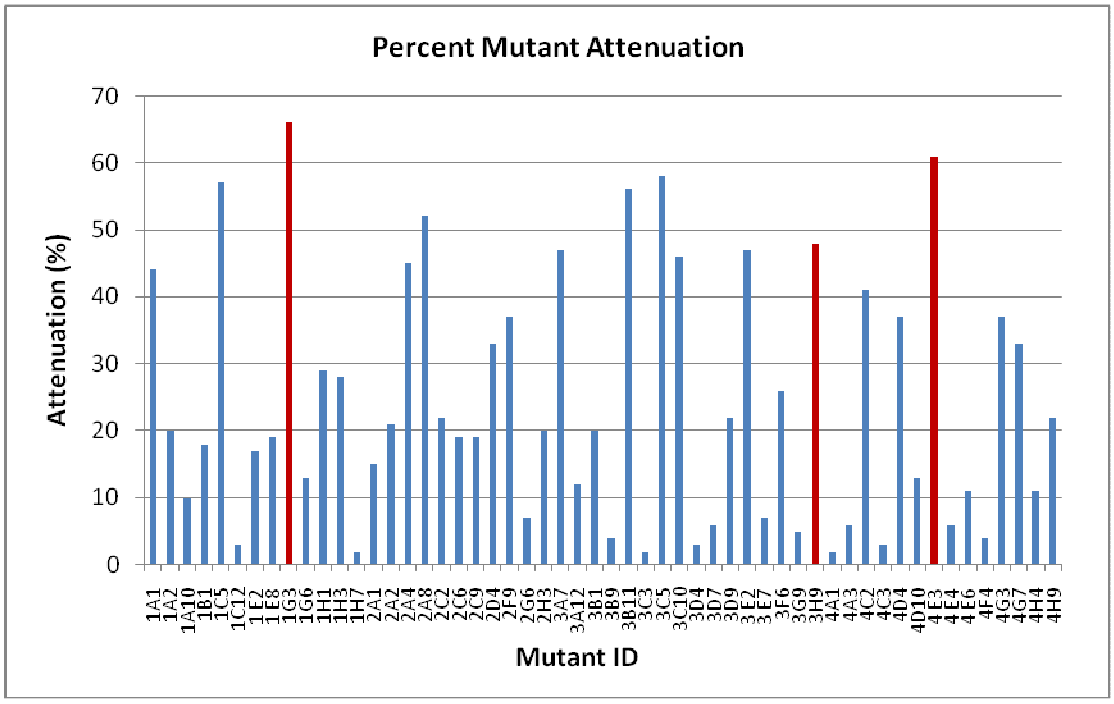
We screened 384 mutants for attenuation in U937 human macrophages. Of those screened, fifty-four clones, showing significant attenuation compared with WT infection, were identified (Table 1). The numbers in Table 1 were calculated assuming the growth exhibited by the WT is total possible growth. Numbers representing mutants represent attenuation as compared to the WT.

Table 1. List of attenuated mutants. Fifty-four mutants were identified as having significant attenuation. The (+) indicates significant attenuation, (-) indicates minimal attenuation, and (+* in bold) were the mutants showing the greatest attenuation and were consequently the ones selected for the LC3 assay. All infections were performed with U937 macrophages and an MOI of 10.

Mutant ID	1 h CFU 1×10^5 bacteria	5 day CFU 1×10^7 bacteria	Mutant ID	1 h CFU 1×10^5 bacteria	5 day CFU 1×10^7 bacteria
WT	2.4±0.7	11±0.9	3B1+	4.8±0.5	8.8±0.3
1A1+*	**1.9**±0.2	**6.2**±0.3	3B9+	3.54±0.4	10.6±0.3
1A2+	3.5±0.9	8.8±0.3	**3B11+***	**7.8**±0.2	**4.8**±0.6
1A10+	2.7±0.3	9.9±0.4	3C3+	3.8±0.6	10.8±0.4
1B1-	1.6±0.2	8.9±0.6	**3C5+***	**5.8**±0.3	**4.6**±0.2
1C5+*	**2.2**±0.4	**4.7**±0.2	**3C10+***	**3.6**±0.3	**5.9**±0.2
1C12-	4.1±0.3	10±0.2	3D4+	2.5±0.4	10.7±0.2
1E2-	3.6±0.9	9.2±0.4	3D7+	6.5±0.3	10.3±0.3
1E8+	1.4±0.6	8.9±0.8	3D9+	2.3±0.7	8.5±0.4
1G3+*	**2.2**±0.5	**3.8**±0.4	**3E2+***	**1.4**±0.6	**5.7**±0.9
1G6-	1.6±0.2	9.6±0.3	3E7-	3.7±0.5	10.2±0.3
1H1+	3.1±0.3	7.8±0.3	**3F6+***	**3.6**±0.3	**8.1**±0.4
1H3+	3.0±0.4	7.8±0.2	3G9+	2.3±0.6	10.4±0.5
1H7+	1.8±0.3	10.8±0.4	**3H9+***	**5.3**±0.2	**5.8**±0.4
2A1+	3.3±0.4	9.4±0.6	4A1+	2.3±0.3	10.7±0.2
2A2-	2.1±0.4	8.7±0.3	4A3+	4.0±0.3	10.3±0.3
2A4+*	**3.1**±0.9	**6.1**±0.2	**4C2+***	**1.8**±1.0	**6.5**±0.9
2A8+*	**1.7**±0.7	**5.3**±0.2	4C3+	2.5±0.5	10.6±1.0
2C2+	2.2±0.5	8.5±0.5	4D4+	1.7±0.7	6.8±0.6
2C6+*	**3.4**±0.2	**8.9**±0.3	4D10+	2.6±0.4	9.5±0.4
2C9+	1.2±0.7	8.9±0.5	**4E3+***	**2.9**±0.5	**4.3**±0.3
2D4+*	**2.3**±0.4	**7.3**±0.4	4E4+	1.6±0.7	10.2±0.5
2F9+	1.4±0.3	6.9±0.2	4E6+	3.7±0.9	9.8±0.7
2G6+	6.7±0.2	10.3±0.2	4F4+	2.1±0.9	10.5±0.8
2H3+	3.1±0.3	8.7±0.3	4G3+*	**2.7**±0.5	**6.9**±0.3
3A7+*	**3.5**±0.8	**5.8**±0.4	**4G7+***	**3.6**±0.7	**7.4**±0.5
3A12+	9.7±0.3	9.7±0.7	4H4+	2.3±0.4	9.7±0.2
			4H9+	1.1±0.8	8.5±0.5

Eighteen mutants showing the greatest attenuation were selected for an LC3-staining assay to determine if autophagy was an active process in the macrophages when infected with these mutants. The degree of attenuation is indicated below (Figure 2), where the percent of attenuation was calculated based on numbers obtained from bacterial CFU counts.

Figure 2. Percent attenuation of mutants. Levels of attenuation of the 54 mutants are indicated as percent attenuation as compared to the wild-type (WT data is not included). Three of these 18 mutants (1G3, 3H9, and 4E3), indicated by red bars, were positively identified as exhibiting autophagy.

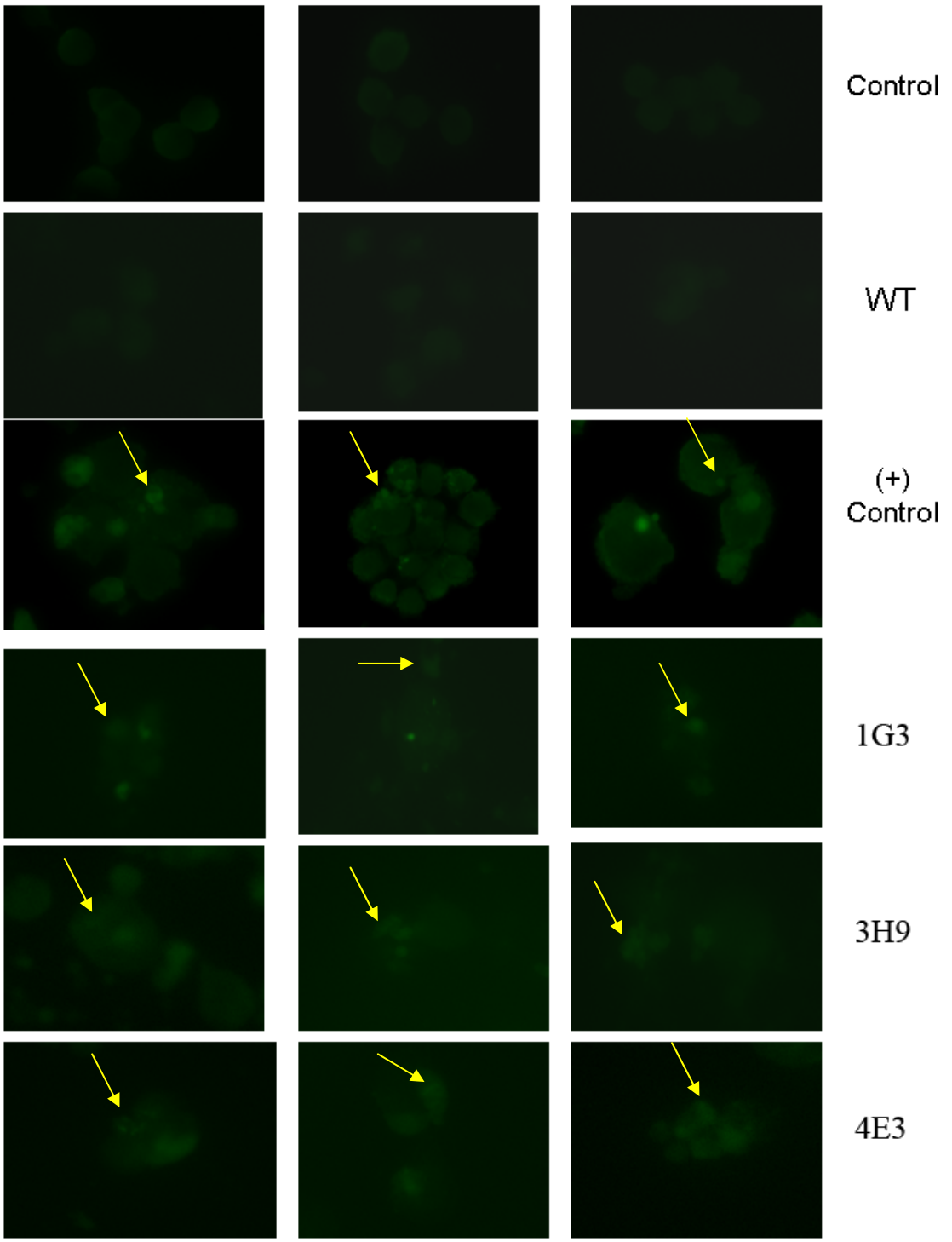


Prior to performing the LC3 assay on the clones, we performed a positive control LC3 stain on U937 cells induced with rapamycin. Macrophages showed strong autophagy induction as is seen in Table 2, and Figure 3 positive Control. The LC3 assay performed with the mutant strains did not show as strong an induction as the (+) Control.

Table 2. Percent autophagy exhibited by three mutants isolated in LC3 assay. Numbers indicate percent autophagy comparing control, WT, rapamycin-treated, and the three mutants (indicated by red bars in Figure 2) exhibiting positive autophagy.

	% Autophagy/200 cells
Infections and Treatment	**U937**
No bacteria	9 ± 2
Infected with wild-type *M. tuberculosis*	18 ± 3
Treated with rapamycin	94.3 ± 3
Mutant # IG3	57 ± 4
Mutant # 3H9	74 ± 2
Mutant # 4E3	82 ± 1

Figure 3. LC3 antibody staining. The top two rows include control, and WT macrophages that exhibit no autophagy. Positive control was induced with (50 or 150 µg/µl) rapamycin for 4 h prior to LC3 staining. The last three rows show positive induction of autophagy by three of the previous 18 mutants selected for the LC3 assay. Infection was done with an MOI of 10. Arrows indicate active autophagy.



DISCUSSION

Considering the fact that the human macrophage has multiple methods of eliminating phagocytosed bacterial, we must also consider the variety of inhibition mechanisms that *M. tuberculosis* may have. As previously mentioned, *M. tuberculosis* has been implicated in inhibition of proton ATPase acquisition and in production of proteins involved in inhibition of phagosome-lysosome fusion. It must also have some method of avoiding death from the respiratory burst which is generally initiated within the macrophage soon after phagocytosis. *Mycobacterium tuberculosis'* unique cell wall, largely composed of lipids, provides some protection, but it may also be actively inhibiting this oxidative response. Likely the bacterium has a means of regulating macrophage mechanisms including: recognition and uptake by the macrophage, the respiratory burst, phagosome maturation, fusion with lysosomes, and eventual inducement of apoptosis and necrosis.

Even though 54 mutants showed attenuation, this does not mean that all of these mutants had mutations in autophagy related genes. Attenuation, lower CFU compared to WT, may simply mean that uptake of the bacteria was inhibited in some way, while replication within the macrophage may not have been affected at all. The LC3 assay was necessary to confirm the mutated genes were connected to the autophagy process.

Researchers have shown interaction between the *M. tuberculosis* surface lipoglycan, lipoarabinomannan , and the macrophage mannose receptor (13)**.** This is one possibility of a gene that could be implicated in attenuation that is not associated with replication ability. For these reasons it was necessary to isolate attenuated mutants that

specifically showed autophagy actively taking place. These other possibilities provide potential areas of research that could be pursued using other investigative methods.

We identified mutants that allowed autophagy to proceed by phagosome maturation and lysosome fusion. This is just one small step in drawing connections between effectors that are produce by *M. tuberculosis* and their function relating to inhibition. To this point, of the 384 mutants screened, only three have been identified as lacking autophagy inhibiting capabilities. It is also evident from Figure 3 that the mutants did not exhibit as high a level of autophagy as the (+) Control. This is to be expected and is likely because there was still some autophagy inhibition by the bacteria.

The next step is to sequence the genes we have isolated and attempt to determine the individual functions. Our understanding of *M. tuberculosis* autophagy inhibition is limited, and there are multiple possibilities of proteins these *M. tuberculosis* genes may encode. We are still looking for genes involved in inhibiting phagosome acidification and protein effectors involved in inhibition of phagosome-lysosome fusion. Phosphatase SapM and PknG, both produced by *M. tuberculosis,* are thought to inhibit or regulate phagosome maturation. It is possible that the genes we have isolated may be other cell wall components involved in this inhibition mechanism. Another mechanism we know little about is how *M. tuberculosis* regulates cytosolic proteins when it lacks a Type III secretion system. It has also been shown that close association of *Mycobacterium avium* with the phagosome membrane is necessary for it to carry out inhibition of phagosome-lysosome maturation (20). Not all the bacterial and phagosome membrane proteins involved in this bacteria-phagosome association are known either.

The three mutants showing autophagy in macrophages were three of the mutants showing some of the highest attenuation in the screening experiment. This is not necessarily a direct correlation, but it would indicate (and agree with other research) that shows bacterial survival within the macrophage is closely related to regulation of autophagy. Even though only eighteen of the fifty-four mutants selected after attenuation screening were tested with the LC3 assay, the other 36 mutants may well provide more mutants shown to be repressed in their autophagy inhibition capabilities.

CONCLUSIONS

There is little direct information from this study as of yet. Further sequencing and characterization of the isolated genes may yield useful information and greater understanding of *M. tuberculosis'* pathogenicity and autophagy inhibition. Assays looking for connections to apoptosis, necrosis, or bacterial recognition and uptake mechanism may also be reasonable directions to proceed.

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