

SURVIVAL OF *MYCOBACTERIUM AVIUM* SUBSPECIES PARATUBERCULOSIS IN THE
POLYMORPHONUCLEAR LEUKOCYTES OF A CROHN'S DISEASE PATIENT

by

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ABSTRACT

Mycobacterium avium subspecies *paratuberculosis* (MAP) is an intracellular pathogen that is known to parasitize macrophages and monocytes. MAP infiltrates gastrointestinal tract host tissue where it is the known etiological agent of Johne's disease in ruminants and implicated in the etiology of Crohn's disease in humans. MAP's ability to survive within macrophages enables it to disseminate throughout the rest of the host, possibly infecting other circulating blood leukocytes. In this study, the survival and fate of MAP strain ATCC 43015 (human isolate) following phagocytosis was determined using *in vitro* murine macrophage cell line J774A.1 and polymorphonuclear cells (PMNC's) from five Crohn's disease patients. PMNC's from three healthy individuals and two ulcerative colitis patients, as well as *Escherichia coli* (ATCC 11303) and *Mycobacterium tuberculosis* strain H37Ra (ATCC 25177), were included as controls (MOI 10:1). Maturation of the phagosome was determined by evaluating the presence of stage specific markers on the surface of the phagosomal membrane. The endosomal protein, transferrin receptor, and the lysosomal marker, Lamp-1, were then immunostained with Cy-5 conjugated secondary antibodies, and colocalization of bacteria with each marker was evaluated separately using confocal scanning laser microscopy (CSLM). In both tissue models, colocalization of viable MAP and *M. tuberculosis* with the early endosomal marker, transferrin receptor occurred with an estimated five fold higher frequency than did association with the late lysosomal marker, Lamp-1, as compared to live *E. coli*, and all dead bacterial species. Using differential live/dead staining and fluorescent microscopy, survival of *M. tuberculosis* and MAP was estimated at 85% and 79%, respectively compared to only 14% for *E. coli*. The outcome was similar for both tissue culture and PMNCs from all patients tested, suggesting that MAP and

M. tuberculosis can survive readily in both cell types, and regardless of the disease state of the host or the killing power of the cell. MAP's survival appears to mimic *M. tuberculosis*, suggesting the ability to resist phagolysosome fusion, by maintaining association with the early endosomes. Overall, the data confirms MAP virulence in host human blood leukocytes.

I would like to dedicate this work to Nicole T. Spiering and my family for always believing in my potential and motivating me to do my best.

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LIST OF ACRONYMS/ABBREVIATIONS

ATCC	American Type Culture Collection
CLSM	Confocal Laser Scanning Microscopy
DMEM	Dulbecco's Modified Eagle's Medium
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal Calf Serum
FITC	Fluorescein-5-isothiocyanate
GDP	Guanosine Diphosphate
GPI	Glycosylphosphatidylinositol
HeNe	Helium Neon
IgG	Immunoglobulin G
LAM	Lipoarabinomannan
Lamp-1	Lysosomal associated membrane protein-1
LSM	Laser Scanning Microscope
ManLAM	Mannosylated Lipoarabinomannan
<i>MAP</i>	<i>Mycobacterium avium</i> subspecies <i>paratuberculosis</i>
MOI	Multiplicity of Infection
OADC	Oleic acid-Albumin-Dextrose-Catalase
PBS	Phosphate Buffered Saline
PI	Phosphoinositol
PMNC	Polymorphonuclear Cells
RPMI-1640	Roswell Park Memorial Institute

TFR

Transferrin Receptor

TRITC

Tetramethylrhodamine Isothiocyanate

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

Mycobacterium avium subspecies *paratuberculosis* (MAP), the causative agent of Johne's disease, and a suspected agent of the inflammatory bowel disease, Crohn's disease, is a slow growing, intracellular pathogen of phagocytic cells (1,2). While survival mechanisms for *Mycobacterium tuberculosis* have been suggested, MAP's ability to evade the immune system is poorly characterized. Phagosomal maturation inhibition is one mechanism employed by many intracellular pathogens, including *M. tuberculosis*, to evade the host's immune response and maintain an environment conducive for continued virulence (3). Although the maturation process is not well understood, it is currently accepted that phagosomes mature by entering an endosomal like pathway (4). This is supported by the fact that Rab5 endosomal pathway protein and Rab7-GDP late endosomal / lysosomal protein have been detected on the membrane phagosomes (4,5). Therefore, as phagosomes cycle through a cell, they fuse with vesicles from the endosomal pathway (6,7). As a result of this fusion, proteins displayed on the phagosome are indicative of what stage endosomes it has bound. Consequently, the fate of the phagosome depends on the array of receptor proteins it displays (6,7). One important receptor of the early endocytic pathway is transferrin receptor (TFR) (8). Transferrin receptor continually recycles between the plasma membrane and early phagosomes, delivering transferrin, and other nutrients, to the vesicle. When transferrin binds to its receptor, it is internalized into the acidic phagosomal compartment where bound iron rapidly dissociates. Due to the iron dependence of mycobacterial species, a continued association with this receptor would provide a key nutrient for continued survival, as well as an evolutionary advantage for survival within cellular compartments. Conversely, if a phagosome proceeds through the maturation process, it fuses with late stage

endosomes, then lysosomes and will display lysosomal-associated membrane protein-1 (Lamp-1) losing association with early endosomal markers in the process (6,7,8). Lamp-1 is a stage specific protein marker indicative of the formation of the phagolysosome (8,9). Within the phagolysosome, toxic compounds such as acid hydrolases, defensins, nitric oxide, superoxide radicals and hypochlorite degrade the phagocytosed bacteria. Current models of bacterial survival in phagolysosomes consider in vitro conditions using hybridoma cells lines, or extracted bovine macrophages. These studies show that MAP, like *M. tuberculosis*, resists phagosome-lysosome fusion (10,11). While the exact methods employed by *M. tuberculosis* to resist this fusion within phagocytes are unknown, one proposed method is mediated by the virulence factor mannosylated lipoarabinomannan (ManLAM) (12). LAMs are lipoglycans that are found only in the mycobacterial species (13). All LAMs are structurally similar, composed of two homopolysaccharides that make up the carbohydrate backbone, D-mannan and D-arabinan, the mannan core is terminated by a GPI-anchor, and the arabinan domain is capped by either mannosyl (14,15) or phosphoinositol (PI) residues (16,17). Mannosylated LAMs can be found in the pathogenic mycobacterial species *M. tuberculosis*, *M. leprae*, *M. bovis*, and *M. avium*, while PI capped residues are found in the nonpathogenic species *M. smegmatis* (18-22). Mycobacterial LAMs, in addition to arresting phagosomal maturation (18-22), have also been shown to inhibit microbicidal activities of macrophages (23) and neutralize reactive oxygen species (24). Due to virulent mycobacterial species' ability to survive within macrophages, these bacilli have been detected in the periphery of animals as well as humans (2, 25-28). Recently, MAP was isolated and cultured from the blood of patients with Crohn's disease (2). These findings raise an important question about the ability of blood leukocytes to combat a MAP infection, because after subverting the first line defense of tissue macrophages, in a disseminated infection,

circulating leukocytes play an important role in host defense (29,30). Specifically, these cells attack invaders extracellularly and within phagocytic vesicles (30). In this in vivo study, the ability of *Mycobacterium avium* subspecies *paratuberculosis* to survive within polymorphonuclear leukocytes extracted from a healthy individual, an ulcerative colitis patient and a Crohn's disease patient was investigated.

CHAPTER TWO: MATERIALS AND METHODOLOGY

Macrophage cell line

The mouse macrophage cell line J774A.1 (ATCC TIB-67) was maintained in a humidified, 37°C, 5% CO₂ incubator. Cells were cultured in DMEM supplemented with 10% FCS, 4mM L-glutamine, 4.5 g/l glucose, and 1.5 g/l sodium bicarbonate (ATCC, Rockville, MD, USA). Macrophages were grown until confluent in tissue culture plates, detached from the plates using trypsin-EDTA, pelleted by centrifugation, washed twice with PBS and resuspended in DMEM containing 10% FCS. The cells were then counted in the presence of trypan blue, adjusted to 1x10⁶ viable cells/ml with DMEM containing 10% FCS and distributed in 1ml aliquots in 24-well tissue culture plates.

Human PMNCs

Fresh blood samples assigned with a code were immediately processed in a Biosafety Cabinet Class II as follows: PMNCs were isolated using Polymorphoprep density gradient separation medium following the manufacturer's procedure (Greiner Bio One, Inc). Initially, blood samples were diluted 1:1 (v/v) with room temperature RPMI culture media (Invitrogen, CA). For PMNCs isolation, a volume of 5ml of the whole blood-RPMI mixture was layered on 5ml Polymorphoprep solution in 15ml polypropylene tubes. Tubes were centrifuged at 1,550 rpm (300g) and room temperature for 30 min. This results in formation of the density gradient interface, which is followed by isolation of bands containing PMNCs cells. Purified cells then were transferred into sterile tubes for further use. The polymorphonuclear cells were counted in

the presence of trypan blue, adjusted to 1×10^6 viable cells/ml with RPMI + 10% human serum, and distributed in 1ml aliquots in 24-well tissue culture plates.

Bacterial strains and growth conditions

M. avium subspecies *paratuberculosis*, (ATCC 43015) was originally obtained from a patient. MAP was grown in flasks containing modified Middlebrook 7H9 broth supplemented with mycobactin J and OADC (oleic acid-albumin-dextrose-catalase) in an incubator at 37°C. After 4 weeks of growth, the cell concentration was determined using spectroscopic analysis. *M. tuberculosis* strain H37Ra (ATCC 25177) was grown, and concentration determined, in BACTEC bottles in the BACTEC 460 TB Analyzer (Becton Dickinson, Franklin Lake, NJ) as described previously (2). *E. coli* (ATCC 11303) was grown in LB broth (Miller) in an incubator at 37°C. For all live bacterial samples, aliquots were taken from the cultures and suspended in PBS to give a final concentration of 1×10^7 cells/ml in 1.5ml tubes. For all dead bacterial samples, the remainder of each culture was killed by autoclaving for 20 minutes at 252°F and 20 psi. The dead bacteria were then pelleted and suspended in PBS and their concentrations were determined using spectroscopic analysis. Aliquots were then taken and suspended in PBS to give a final concentration of 1×10^7 cells/ml.

Infection of macrophages and human PMNCs for confocal microscopy slides

All 1ml bacterial aliquots were suspended in FITC at a final concentration of 167µg/ml for 30 minutes in the dark. Bacteria were then washed twice with PBS and finally suspended in 100µl of RPMI. Bacterial samples were then added to the cells in the 24-well tissue culture

plates with a multiplicity of infection of 10:1. The cultures or PMNCs with bacteria were then incubated for 3 hours in a humidified, 37°C, 5% CO₂ incubator

Immunofluorescent labeling

After the 3 hour infection period, the cells were resuspended by tapping the plate and 100µl aliquots were allowed to adhere to poly-lysine coated slides (Sigma-Aldrich) for 30 minutes in a Biosafety cabinet. The cells were then fixed with 4% paraformaldehyde for 20 minutes, rinsed with PBS, and permeabilized with PBS + 0.1% Triton-X 100 for 10 minutes. The cells were then incubated with either mouse anti-transferrin receptor or mouse anti-Lamp-1 primary antibody at a final concentration of 1µg/ml overnight at 4°C (BD Biosciences Pharmingen). Following the incubation, the slides were washed with PBS + 0.1% Triton-X 100 and incubated with Cy-5 conjugated goat anti-mouse secondary antibody at a 1:100 dilution for 3 hours at room temperature. The slides were then rinsed with PBS + 0.1% Triton-X 100, post-fixed with 4% paraformaldehyde for 10 minutes, rinsed with PBS and allowed to dry under a Biosafety cabinet. Finally, the slides were treated with 1 drop of Vectashield anti-fading mounting medium (Vector Laboratories, Burlingame, CA) and coverslipped.

Laser confocal microscopy and colocalization studies

Images were generated and captured with a Zeiss LSM 510 confocal microscope (Carl Zeiss, Inc.) using a 40x objective, equipped with argon (488nm excitation wavelength) and HeNe (633 excitation wavelength) lasers. Bacteria were then viewed as being colocalized with a membrane protein (yellow) or noncolocalized (green).

Differential live/dead assay - Intracellular survivability

Polymorphonuclear leukocytes were extracted as described previously, infected with viable MAP, *M. tuberculosis* and *E. coli* (MOI 10:1) and incubated in 24-well tissue culture plates for 3 hours in a humidified, 37°C, 5% CO₂ incubator. The cells were then suspended and a 700µl aliquot was transferred to a 1.5ml tube. The samples were then centrifuged for five minutes at 500g to pellet the leukocytes. The supernatant was removed and the cells were washed with PBS and pelleted twice to remove residual bacteria. The leukocytes were finally suspended in 800µl PBS and lysed with 0.1% deoxycholate (w/v) for five minutes. The lysate was then incubated for 15 minutes with the stains from the live/dead BacLight bacterial viability kit (Molecular Probes, Inc., Eugene, OR) as recommended by the manufacturer. A 30µl sample was then mounted and coverslipped. The bacteria were then counted using a Leica fluorescent microscope (40x objective) with a dual band (FITC/TRITC) filter set. The green fluorescing viable bacteria and the red fluorescing dead were counted in each of three fields and shown as an average. The results are expressed as the percentage of live bacteria.

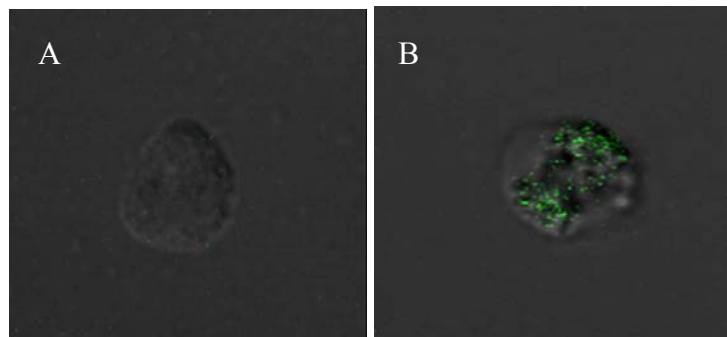
CHAPTER FOUR: FINDINGS

To evaluate MAP's ability to resist phagolysosome fusion, we labeled the bacterium with FITC and infected tissue culture macrophages and human polymorphonuclear leukocytes for three hours. Slides were prepared and probed with mouse anti-transferrin receptor antibodies to evaluate continued association with the early endosomal pathway, or with mouse anti-Lamp-1 antibodies to evaluate lysosomal fusion. All slides were immunostained with anti-mouse IgG-Cy5 secondary antibody. The bacteria were judged to be colocalized if a yellow signal was detected, and judged as not colocalized if the red and green signals could be seen together.

Generation of control slides

The absence of fluorescence in the PMNC without any treatment verifies the fluorescence seen in experimental slides is a result of immunostaining and not the auto-fluorescence of other cellular proteins as shown in Figure 1. The absence of red fluorescence in the PMNC treated only with FITC labeled bacteria and secondary antibody conjugated to Cy-5 verifies that the secondary antibody is not binding non-specifically as shown in Figure 1.

Figure 1: Evaluation of auto-fluorescence within PMNCs and non-specific binding of the secondary antibody. PMNCs from a healthy individual with no treatment or Cy-5 conjugated goat anti-mouse secondary antibodies and FITC-labeled bacteria for control images visualized with confocal microscopy. (A) PMNC with no immunofluorescent treatment showing no auto-fluorescence of intracellular structures. (B) PMNC treated with FITC-labeled MAP and only secondary IgG treatment showing no nonspecific binding (no red fluorescence) of the secondary IgG-Cy5. CLSM 40x objective. Green fluorescence represents FITC-labeled bacteria.



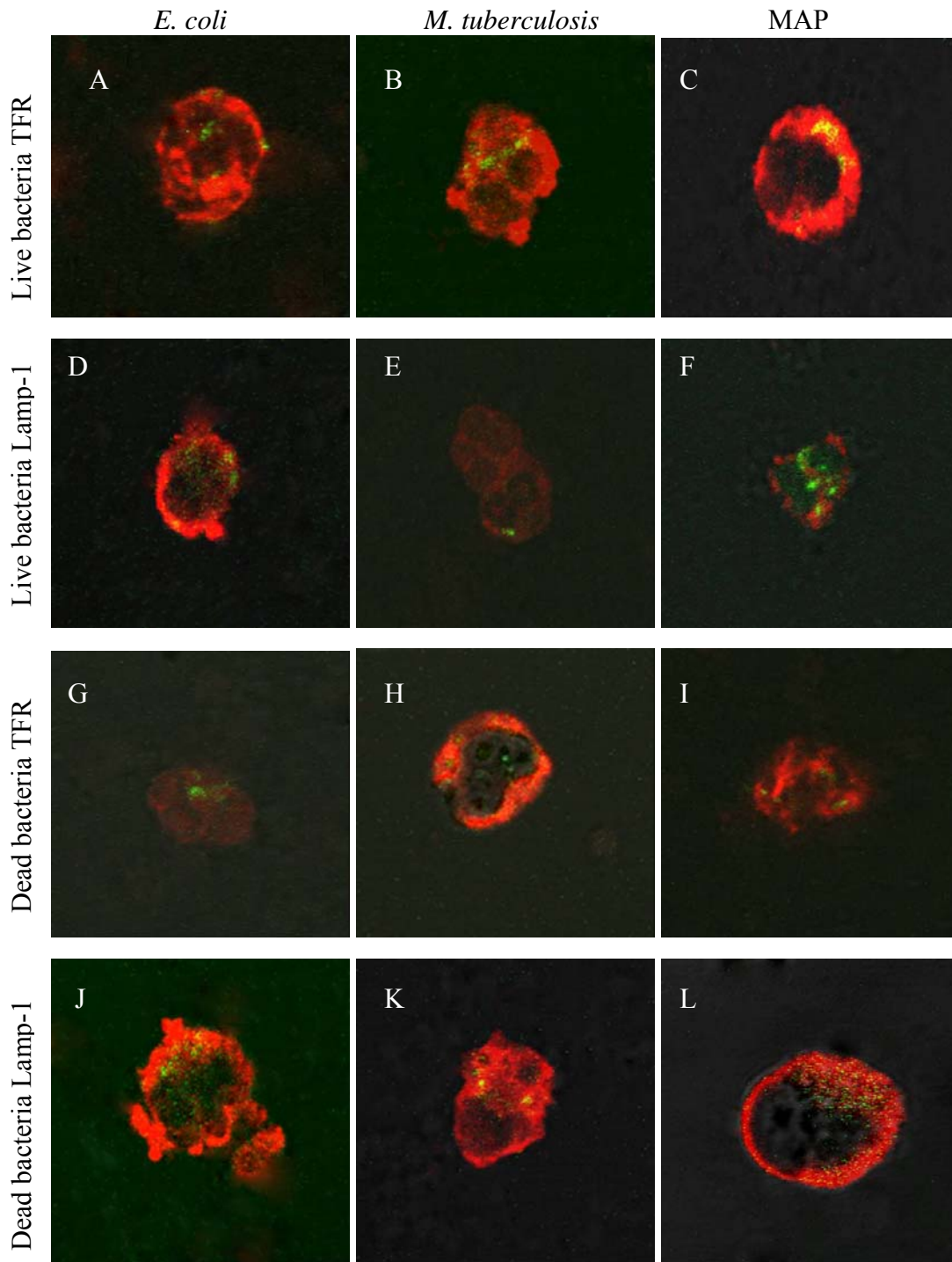
Colocalization of bacteria in tissue culture murine macrophages

The visible colocalization of live and dead *E. coli* with Lamp-1 protein indicates that the phagosomes containing these bacteria have fused with lysosomes. Supporting these observations is the lack of colocalization with the early endosomal marker, transferrin receptor. Dead *M. tuberculosis* and dead MAP show similar results shown in Figure 2.

Colocalization of live *M. tuberculosis* with the transferrin receptor can be seen, as it can with live MAP. These results, as shown in Figure 2, indicate a continued interaction with the early endosomal pathway. A lack of colocalization with the Lamp-1 protein supports the continued association with the early pathway.

Figure 2: *In vitro* survival of MAP, *M. tuberculosis* and *E. coli* in tissue culture murine macrophages.

J774 cells treated with FITC-labeled bacteria and indirectly immunostained with primary anti-TFR or anti-Lamp-1 antibodies and secondary Cy-5 conjugated goat anti-mouse antibodies. (A) Infected with viable FITC-labeled *E. coli* retaining the green signal indicating no colocalization. (B) Infected with viable FITC-labeled *M. tuberculosis* giving a yellow signal indicating colocalization. (C) Infected with viable FITC-labeled MAP giving a yellow signal indicating colocalization. (D) Infected with viable FITC-labeled *E. coli* showing a yellow signal indicating colocalization. (E) Infected with viable FITC-labeled *M. tuberculosis* retaining green signal indicating no colocalization. (F) Infected with viable FITC-labeled MAP retaining green signal indicating no colocalization. (G) Infected with dead FITC-labeled *E. coli* retaining a green signal indicating no colocalization. (H) Infected with dead FITC-labeled *M. tuberculosis* retaining a green signal indicating no colocalization. (I) Infected with dead FITC-labeled MAP retaining a green signal indicating no colocalization. (J) Infected with dead FITC-labeled *E. coli* showing a yellow signal indicating colocalization. (K) Infected with dead FITC-labeled *M. tuberculosis* showing a yellow signal indicating colocalization. (L) Infected with dead FITC-labeled MAP showing a yellow signal indicating colocalization. CLSM 40x objective. Green fluorescence represents FITC-labeled bacteria, red fluorescence represents labeled TFR or Lamp-1, yellow fluorescence represents colocalization of bacteria with labeled marker. Figure images are representative of all cells examined.



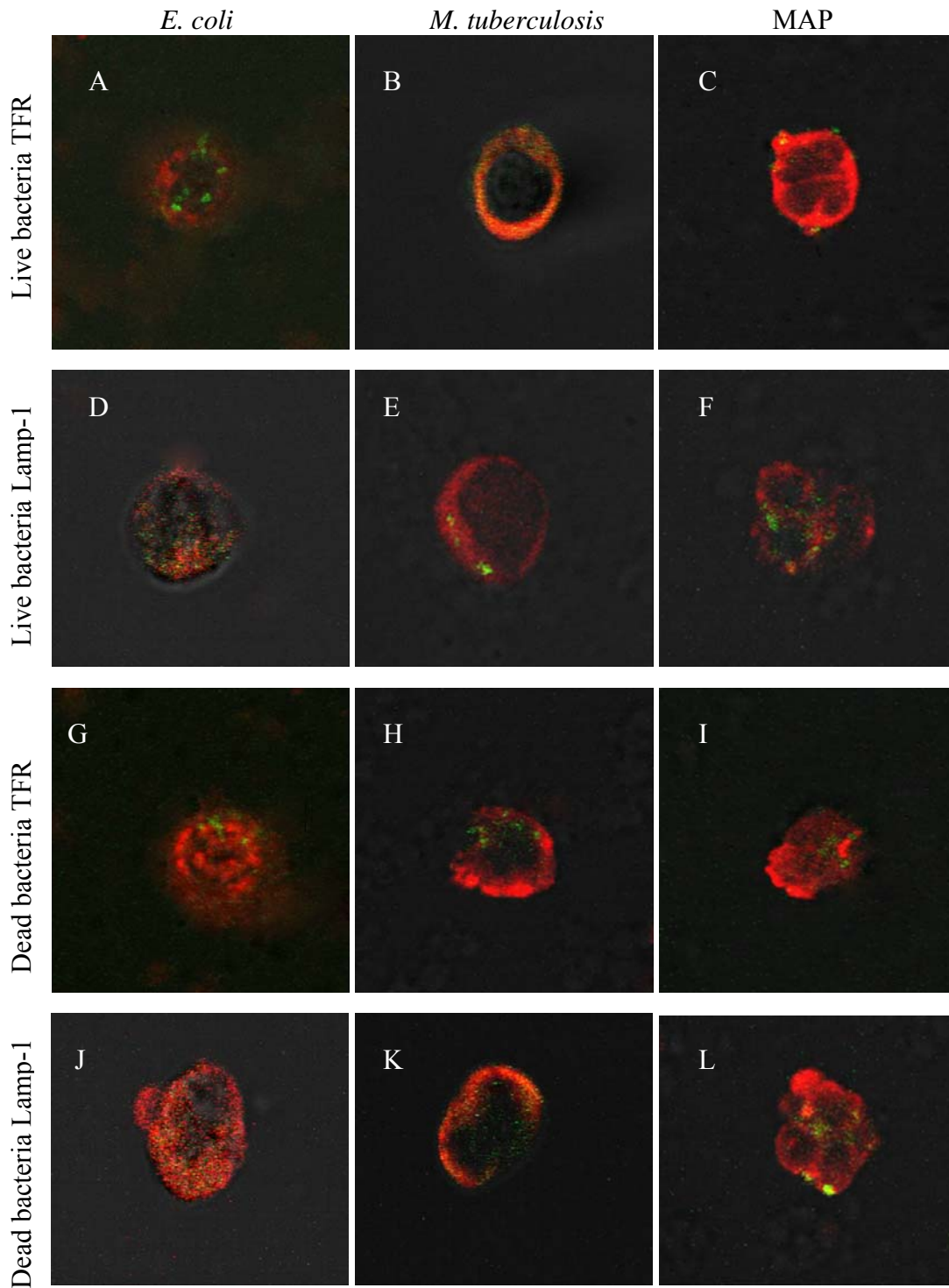
Colocalization of bacteria in healthy PMNCs

Polymorphonuclear leukocytes from a healthy individual infected with viable FITC-labeled MAP and viable FITC-labeled *M. tuberculosis* show colocalization with the transferrin receptor, while viable FITC-labeled *E. coli* and all dead bacteria show colocalization with the Lamp-1 marker (Figure 3).

The visible colocalization of live and dead FITC-labeled *E. coli* with Lamp-1 protein indicates that the phagosomes containing these bacteria have fused with lysosomes. Supporting these observations is the lack of colocalization with the early endosomal marker, transferrin receptor. Dead FITC-labeled *M. tuberculosis* and dead FITC-labeled MAP show colocalization with the Lamp-1 marker (Figure 3).

Figure 3: *In vivo* survival of MAP, *M. tuberculosis* and *E. coli* in healthy PMNCs.

Healthy individual PMNCs treated with FITC-labeled bacteria indirectly immunostained with primary anti-TFR or anti-Lamp-1 antibodies and secondary Cy-5 conjugated goat anti-mouse antibodies. (A) Infected with viable FITC-labeled *E. coli* retaining the green signal indicating no colocalization. (B) Infected with viable FITC-labeled *M. tuberculosis* giving a yellow signal indicating colocalization. (C) Infected with viable FITC-labeled MAP giving a yellow signal indicating colocalization. (D) Infected with viable FITC-labeled *E. coli* showing a yellow signal indicating colocalization. (E) Infected with viable FITC-labeled *M. tuberculosis* retaining green signal indicating no colocalization. (F) Infected with viable FITC-labeled MAP retaining green signal indicating no colocalization. (G) Infected with dead FITC-labeled *E. coli* retaining a green signal indicating no colocalization. (H) Infected with dead FITC-labeled *M. tuberculosis* retaining a green signal indicating no colocalization. (I) Infected with dead FITC-labeled MAP retaining a green signal indicating no colocalization. (J) Infected with dead FITC-labeled *E. coli* showing a yellow signal indicating colocalization. (K) Infected with dead FITC-labeled *M. tuberculosis* showing a yellow signal indicating colocalization. (L) Infected with dead FITC-labeled MAP showing a yellow signal indicating colocalization. CLSM 40x objective. Green fluorescence represents FITC-labeled bacteria, red fluorescence represents labeled TFR or Lamp-1, yellow fluorescence represents colocalization of bacteria with labeled marker. Confocal images are representative of all PMNCs examined for the three healthy individuals.



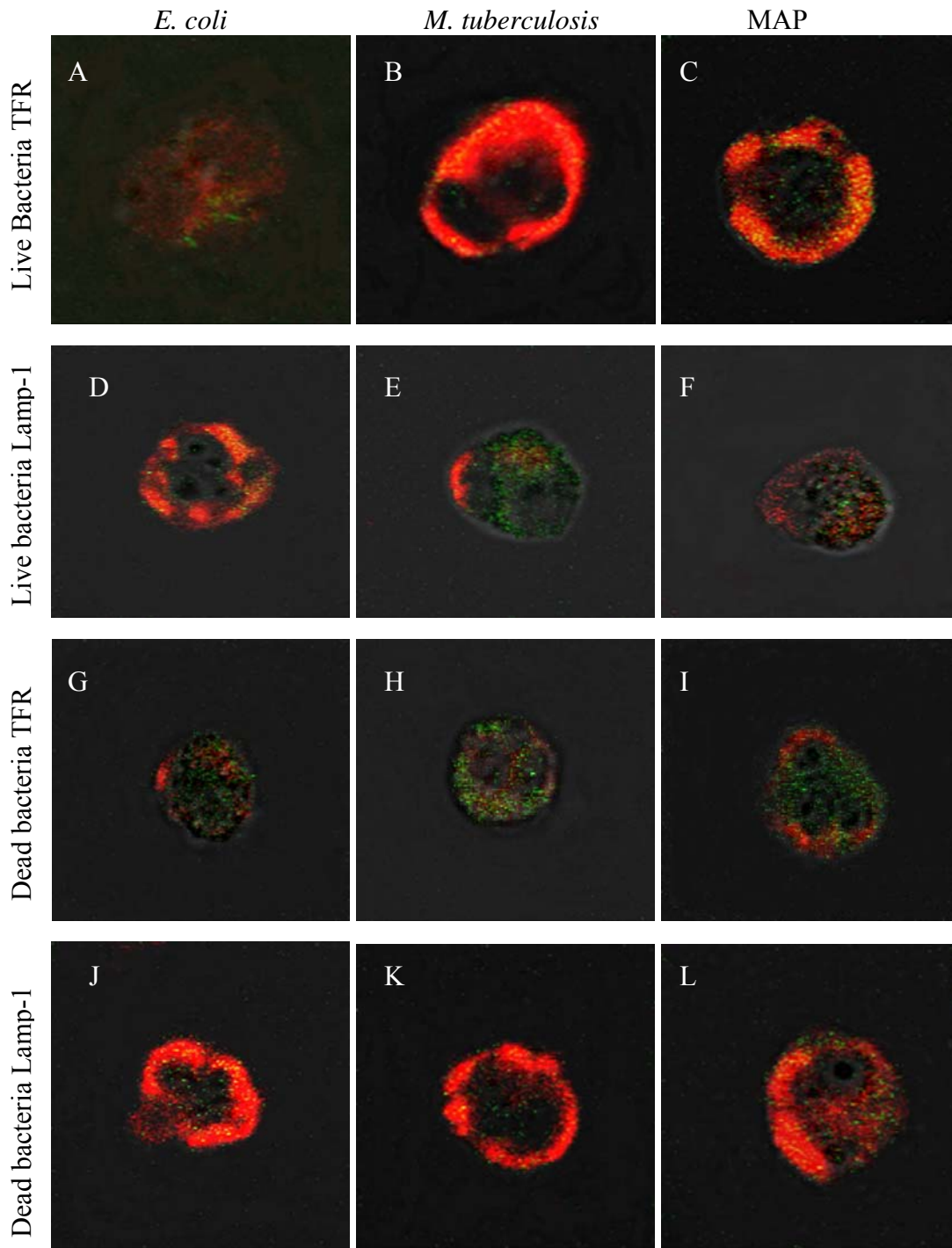
Colocalization of bacteria in Ulcerative Colitis PMNCs

Ulcerative colitis polymorphonuclear cells infected with viable MAP and viable *M. tuberculosis* show colocalization with the transferrin receptor, indicating continued association with early endosomes. Viable *E. coli* and all dead bacterial species show colocalization with the Lamp-1 marker, a hallmark of phagosome-lysosome fusion.

The visible colocalization of live and dead *E. coli* with Lamp-1 protein indicates that the phagosomes containing these bacteria have fused with lysosomes. Supporting these observations is the lack of colocalization with the early endosomal marker, transferrin receptor. Dead *M. tuberculosis* and dead MAP show colocalization with the Lamp-1 marker.

Figure 4: *In vivo* survival of MAP, *M. tuberculosis* and *E. coli* in the PMNCs of an ulcerative colitis patient.

Ulcerative colitis patient PMNCs treated with FITC-labeled bacteria and indirectly immunostained with primary anti-TFR or anti-Lamp-1 antibodies and secondary Cy-5 conjugated goat anti-mouse antibodies. (A) Infected with viable FITC-labeled *E. coli* retaining the green signal indicating no colocalization. (B) Infected with viable FITC-labeled *M. tuberculosis* giving a yellow signal indicating colocalization. (C) Infected with viable FITC-labeled MAP giving a yellow signal indicating colocalization. (D) Infected with viable FITC-labeled *E. coli* showing a yellow signal indicating colocalization. (E) Infected with viable FITC-labeled *M. tuberculosis* retaining green signal indicating no colocalization. (F) Infected with viable FITC-labeled MAP retaining green signal indicating no colocalization. (G) Infected with dead FITC-labeled *E. coli* retaining a green signal indicating no colocalization. (H) Infected with dead FITC-labeled *M. tuberculosis* retaining a green signal indicating no colocalization. (I) Infected with dead FITC-labeled MAP retaining a green signal indicating no colocalization. (J) Infected with dead FITC-labeled *E. coli* showing a yellow signal indicating colocalization. (K) Infected with dead FITC-labeled *M. tuberculosis* showing a yellow signal indicating colocalization. (L) Infected with dead FITC-labeled MAP showing a yellow signal indicating colocalization. CLSM 40x objective. Green fluorescence represents FITC-labeled bacteria, red fluorescence represents labeled TFR or Lamp-1, yellow fluorescence represents colocalization of bacteria with labeled marker. Confocal images are representative of all PMNCs examined from both ulcerative colitis patients.



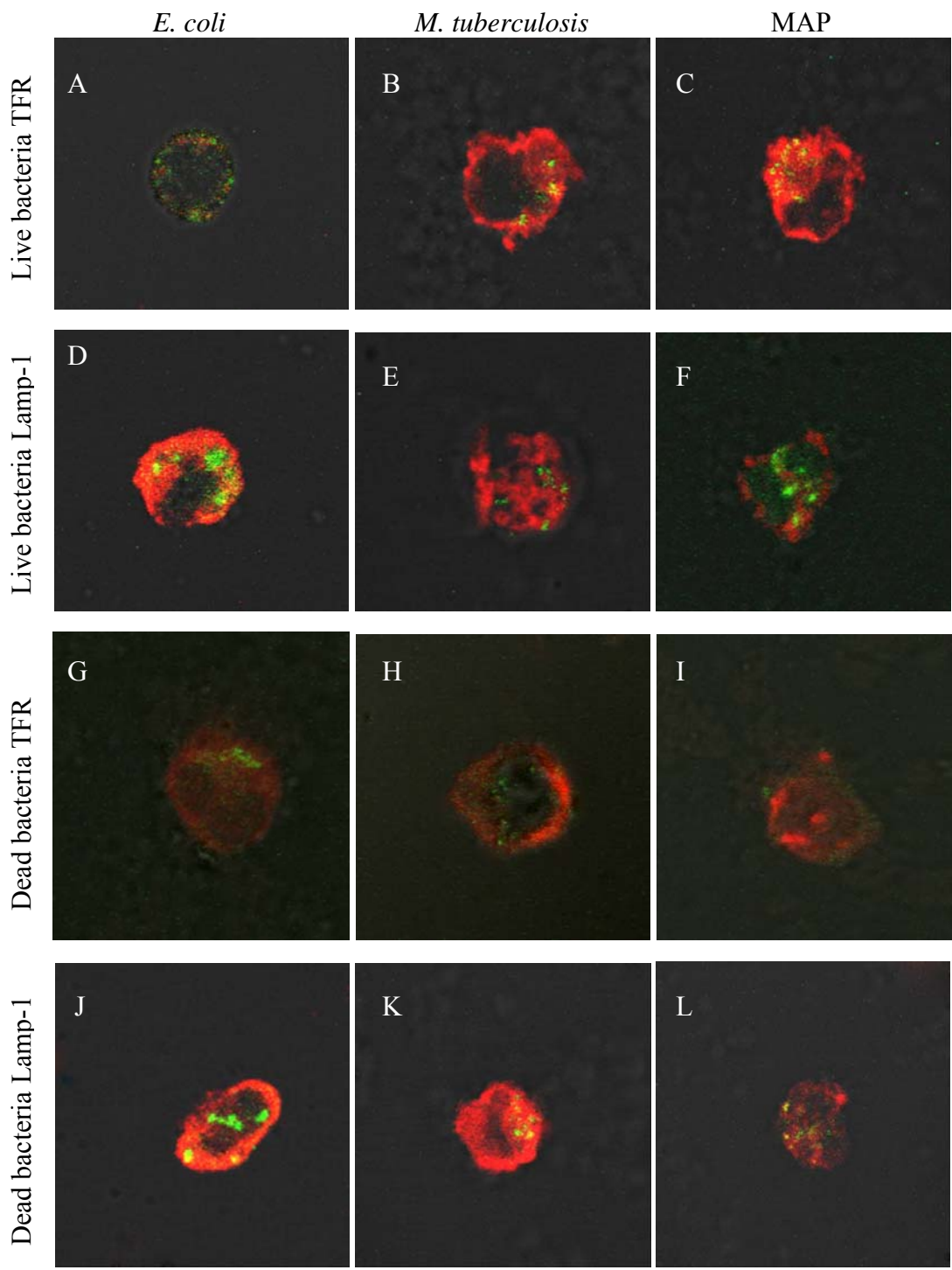
Colocalization of bacteria in Crohn's disease PMNCs

In Crohn's disease polymorphonuclear cells, colocalization of viable MAP and viable *M. tuberculosis* with the transferrin receptor can be seen (Figure 5). These findings are similar to the results of the tissue culture experiment, suggesting that these bacteria can resist intracellular degradation in polymorphonuclear leukocytes that have been treated with the host's serum. Conversely, viable *E. coli* as well as all dead bacteria show colocalization with Lamp-1, indicating the phagosomes they reside in fused with lysosomes.

The visible colocalization of live and dead *E. coli* with Lamp-1 protein indicates that the phagosomes containing these bacteria have fused with lysosomes. Supporting these observations is the lack of colocalization with the early endosomal marker, transferrin receptor. Dead *M. tuberculosis* and dead MAP show colocalization with the Lamp-1 marker.

Figure 5: *In vivo* survival of MAP, *M. tuberculosis* and *E. coli* in the PMNCs of a Crohn's disease patient.

Crohn's disease patient PMNCs treated with FITC-labeled and indirectly immunostained with primary anti-TFR or anti-Lamp-1 antibodies and secondary Cy-5 conjugated goat anti-mouse antibodies. (A) Infected with viable FITC-labeled *E. coli* retaining the green signal indicating no colocalization. (B) Infected with viable FITC-labeled *M. tuberculosis* giving a yellow signal indicating colocalization. (C) Infected with viable FITC-labeled MAP giving a yellow signal indicating colocalization. (D) Infected with viable FITC-labeled *E. coli* showing a yellow signal indicating colocalization. (E) Infected with viable FITC-labeled *M. tuberculosis* retaining green signal indicating no colocalization. (F) Infected with viable FITC-labeled MAP retaining green signal indicating no colocalization. (G) Infected with dead FITC-labeled *E. coli* retaining a green signal indicating no colocalization. (H) Infected with dead FITC-labeled *M. tuberculosis* retaining a green signal indicating no colocalization. (I) Infected with dead FITC-labeled MAP retaining a green signal indicating no colocalization. (J) Infected with dead FITC-labeled *E. coli* showing a yellow signal indicating colocalization. (K) Infected with dead FITC-labeled *M. tuberculosis* showing a yellow signal indicating colocalization. (L) Infected with dead FITC-labeled MAP showing a yellow signal indicating colocalization. CLSM 40x objective. Green fluorescence represents FITC-labeled bacteria, red fluorescence represents labeled TFR or Lamp-1, yellow fluorescence represents colocalization of bacteria with labeled marker. Confocal images are representative of all PMNCs examined from the five Crohn's disease patients.



Intracellular survival of bacteria

The intracellular survival of *MAP* and *M. tuberculosis* compared to *E. coli* was substantially higher (Table 1). After the three hour infection period, 79% of MAP recovered from the lysate of healthy PMNCs was viable as was 85% of the *M. tuberculosis*. Conversely, only 14% of the *E. coli* recovered was viable after a three hour infection period. This experiment's data support colocalization findings that report a greater association of MAP and *M. tuberculosis* with the transferrin receptor compared to *E. coli*.

Table 1: Percent viable *E. coli*, *M. tuberculosis* and MAP recovered from healthy PMNC lysate after three hour infection.

<u>Microorganism</u>	<u>Percentage of viable bacteria recovered</u>
<i>E. coli</i>	13.9 ± 3.16
<i>M. tuberculosis</i>	84.5 ± 3.22
<u>MAP</u>	<u>79.2 ± 2.08</u>

Data expressed as the mean of three microscopic fields. Two standard deviations from the mean of the three microscopic fields are shown.

CHAPTER FIVE: DISCUSSION AND CONCLUSION

Intracellular mycobacterial pathogens possess several strategies for surviving innate host immune responses (31). The survival of *Mycobacterium avium* subspecies *paratuberculosis* within phagocytic vesicles depends on its ability to inhibit the maturation process while at the same time maintaining access to cellular nutrients. It has been shown that ManLAM residues on the surface of the cell wall of pathogenic mycobacterial species are at least partly responsible for these activities, allowing it to overcome host innate immune response (12). Due to MAP's ability to avoid these critical first line defenses, these bacilli have been detected in the breast milk of lactating women with Crohn's disease as well as milk from infected cattle (25,27,28). Therefore, the ability of circulating polymorphonuclear cells to phagocytose and kill MAP becomes of critical importance. The results of the tissue culture experiment are consistent with the findings of others' experiments that show MAP survives intracellular killing by maintaining association with the early endosomal pathway (10). In vitro experiments with tissue culture macrophages provide a good model for *Mycobacterium avium* subspecies *paratuberculosis* survival. One downfall of these cells is that they have not been activated by IFN- γ or other cytokines. Cells extracted from a Crohn's disease patient, and incubated with serum, could potentially be activated by IFN- γ , although a study showed that macrophages heavily burdened by ManLAM were refractory to IFN- γ activation in vitro (23,32). Consequently, infecting human polymorphonuclear cells with MAP provides good insight into this bacterium's ability to persist in the human host after it overcomes the resident tissue macrophages. Here we show that MAP, like *M. tuberculosis*, is capable of survival within human PMNCs extracted from blood of a

diagnosed Crohn's disease patient, an ulcerative colitis patient and a healthy individual, in a setting that more closely mimics the natural host environment. MAP's ability, like *M. tuberculosis*, to resist phagosome-lysosome fusion suggests that maybe a similar mechanism is at work. Evidence indicating other intracellular, pathogenic mycobacterial species, including *M. tuberculosis* and *M. avium*, contain ManLAM residues suggests that MAP could also contain mannosylated LAMs (14,24). If ManLAMs are present at high levels in the MAP cell wall, this highly potent virulence factor load could play a role in Crohn's disease pathogenesis. Elucidating the mechanism by which *Mycobacterium avium* subspecies *paratuberculosis* survives within the phagosomal compartment of host cells could provide insight into treatment regimes for both Johne's and Crohn's disease.

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