The Use of Confocal Microscopy to Explore the Potential of the Mycobacterium Tuberculosis Phagosome as an Antigen Processing Organelle

By Andrew Goldstein

Background:

Tuberculosis (TB) is caused by the bacterium *Mycobacterium tuberculosis* (Mtb). It is estimated that this bacterium affects roughly a third of the world's population, both as latent and active infections. After individuals are infected, Mtb eventually survives within cells (such as dendritic cells) in a sub-cellular compartment known as the phagosome. This poses a challenge to the immune system because Mtb can evade or hinder usual immune responses within the phagosome.

$CD8^+$ T cells, an integral part of the immune response, are uniquely able to identify antigens in the cytosol of other cells, such as dendritic cells. This allows $CD8^+$ T cells to be in a specialized role to combat Mtb. These $CD8^+$ T cells recognize antigen in the context of HLA-I molecules. Published studies, using other intracellular organisms, indicate that the phagosome may contain HLA-I. I wish to determine whether the phagosome surrounding Mtb also possess HLA-I. In my study, both Mtb and inert particles (such as magnetic beads) were used to determine differences in the kinetics and markers surrounding phagocytosis of materials by dendritic cells. Magnetic beads were used because Mtb alters the phagosome – the magnetic beads follow the full process of normal phagosomal maturation. These differences in markers and kinetics of Mtb and magnetic beads could allow us to augment the immune function of DC's and $CD8^+$ T cells against Mtb.

It would be interesting to determine possible differences in uptake of Mtb in dendritic cells during infection as time increases. From a vaccine standpoint, the initial uptake is very important because early recognition would add to the efficiency of this vaccine. Mtb bacterial growth is thought to be exponential in media alone, but it would be valuable to determine whether this is the case in a cell population or a human host.

The study of phagosomes often involves lysing infected cells and retrieving phagosomes through the cellular debris. Findings using this method may represent contamination from other subcellular components during the lysis process rather than those specific to the isolated phagosome fraction. To circumvent this concern, I investigated the phagosome using whole cell microscopy. This technique allowed for the investigation of a desired process in a cell (such as the working of a phagosome) without disrupting the cell.

Confocal microscopy is a specific method of microscopy used to obtain images of very high resolution, particularly of intracellular processes. This is done by obtaining images at different levels ("Z-planes") within a specimen by eliminating "out-of-focus" images not within the plane of interest. *Optical sectioning* allows for the reconstruction of a three dimensional image by combining multiple Z-planes. This allows the investigator to obtain clear images of intracellular processes.

In order to study a limited number of proteins of interest in a cell with millions of components, these markers of interest need to be first identified and then visualized under the microscope. Antibodies, many of which are commercially developed, are used to identify markers. To easily visualize these markers under the microscope, these antibodies are conjugated to fluorescent molecules, which illuminated under specific wavelengths. Thus, the position of specific markers may be evaluated in the context of the position of other fluorescent markers. Using fluorescently-tagged antibodies to detect areas of interest within whole cells is called immuno-fluorescent microscopy.

I am interested in the associations of markers, namely the immune marker HLA-I – in two or three dimensions. It is difficult for the human eye to objectively determine these associations when looking at the image, but computer software can make these distinctions. Ideal

'co-localization' occurs when two or more molecules are interacting while occupying the same spot or it is possible to discern a membrane surrounding the TB inside the phagosome. Theoretically, this could be confirmed when the fluorescent tags attached to the molecules combine to form a different color from multiple angles in a high resolution image. Determining the reality of co-localization of three-dimensional material from measurements made on two-dimensional planar sections of the material is called 'stereology', an important quantitative device in applications of microscopy.

This project was designed to investigate phagosomal associations with immune markers within whole cells using immuno-fluorescent microscopy (Mtb versus magnetic beads). The results show that over time, an increasing number of cells were inhabited by *Mycobacterium tuberculosis* and magnetic beads. It was difficult to objectively define the associations between particles and the rate of cell penetration by Mtb and the magnetic beads due to the limitations of the computer programs, resolution of images, and the background. Despite its limitations at the current time, microscopy can be a very useful tool in further studies to objectively measure such associations and interactions between cells once co-localization can be strictly determined and small particles can be more readily defined.

Materials and Methods:

Growth of Dendritic Cells:

Human volunteers were recruited for obtaining peripheral blood, from which samples were frozen in liquid nitrogen. For growing dendritic cells, frozen PBMC from a healthy individual (not TB-infected) were retrieved, thawed at 37 degrees Celsius and spun down in RPMI +10%FBS. The pellet was resuspended in 20ml of RPMI + 2% Human Serum and then transferred into a T75 flask. DNAse was added (to allow single-cell suspension) and the flask

was incubated on its side for 60 minutes at 37°C degrees. After the flask was washed with Room Temperature Sterile PBS, RPMI + 10% Human Serum was added as well as 200 ul of the cytokines Granulocyte-Monocyte-Colony Stimulating Factor (GM-CSF) and Interleukin-4 (IL-4) to promote specific growth of DC's. The cells were incubated at 37 degrees for 4-5 days.

After growth, the Dendritic Cells were harvested by rinsing the flask wall with Room Temperature Sterile PBS and using Cell Dissociation Media to facilitate the disengagement of the cells from the plastic. The cells were spun down and resuspended in RPMI+10% Human Serum before being counted using a hemocytometer. Cells were then resuspended at $5x10^5$ cells per ml. Cytokines GM-CSF and IL-4 were added. 200ul of the DC suspension was added to each well in eight-well chamber slides as prescribed below and incubated at 37 degrees for four hours to allow cell adhesion to the slide.

Figure 1:

TB 5 min.	Magnetic 5 min.
TB 10 min.	Magnetic 10 min.
TB 15 min.	Magnetic 15 min.
TB 20 min.	Magnetic 20 min.

Figure 1: Representative layout of slide for microscopy. Cells were plated at $5x10^5$ cells/ml (or 100,000 cells/well). Various materials (TB, magnetic beads, or media alone as negative control) were added for different time courses.

Mtb Infection of Dendritic Cells:

An isolate of fluorescently-labelled Mtb (eGFP – enhanced Green Fluorescent Protein) was obtained from a collaborator. These bacteria were grown and then frozen in aliquots until they were used to infect cells.

In the BSL-3, the Dendritic Cells were infected with thawed eGFP-Mtb, washed with 300ul of RPMI and fixed with 500ul of 2% PFA (define – and why) at different time points. After moving out of the BSL-3, the PFA was washed off with PBS. The cells were blocked with FACS wash + 0.1% saponin. The chamber slides were incubated overnight at 4 degrees Celsius.

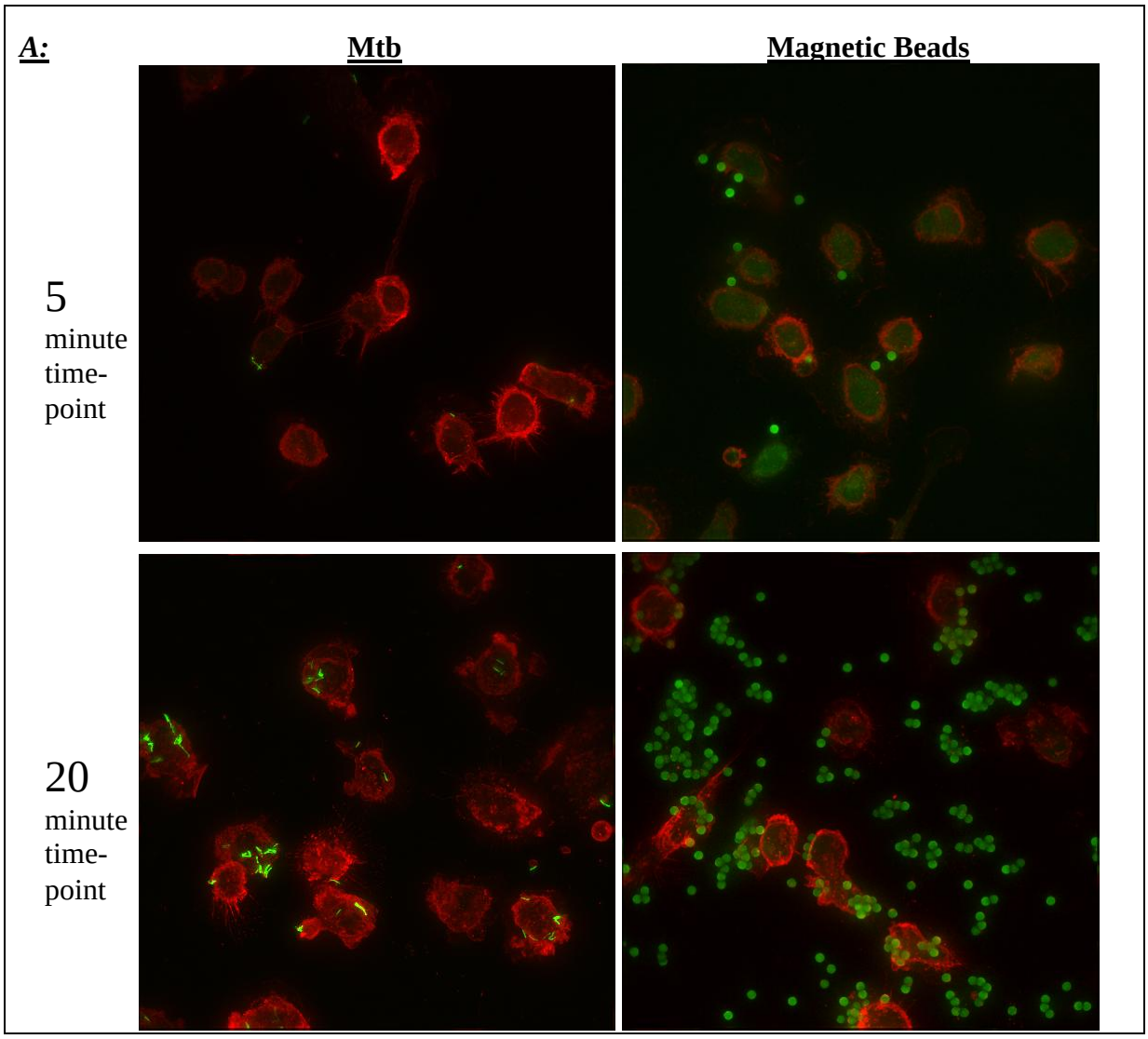
Fluorescent Staining of Cells:

The cells were stained with an HLA-I antibody. In order to detect and distinguish this marker from fluorescently-labeled Mtb, a secondary antibody (now linked with a fluorescent tag distinct from eGFP) was applied. These cells were washed before and after the staining with PBS+0.1% saponin multiple times. 300ul of Fluoromount-G was added to each well for better viewing of the cells during microscopy analysis.

Obtaining Microscopy Images:

Using proper techniques for immuno-fluorescent microscopy, ten sample spaces were investigated per well (5 for untreated wells). Each sample was chosen because it was determined to be representative of the well as a whole and easier to analyze. Each sample was chosen based on its inclusion of isolated cells (without too many overlapping sections) and whole cells that did not spread off the borders of the space. This allowed for less subjective readings at later stages in the process. These samples were chosen without viewing the Mtb, but images combining both the eGFP TB and the HLA-I stained phagosome where obtained using immuno-fluorescent microscopy. Aurelie Snyder and the MMI Core Microscopy lab (at Oregon Health and Science University) assisted in the acquisition and processing of the images, converting them from raw 2D images to 3D images through the process of deconvolution. Intelligent Imaging Systems' "Slidebook" computer software was used to analyze the data and view the images properly.

Figure 2:



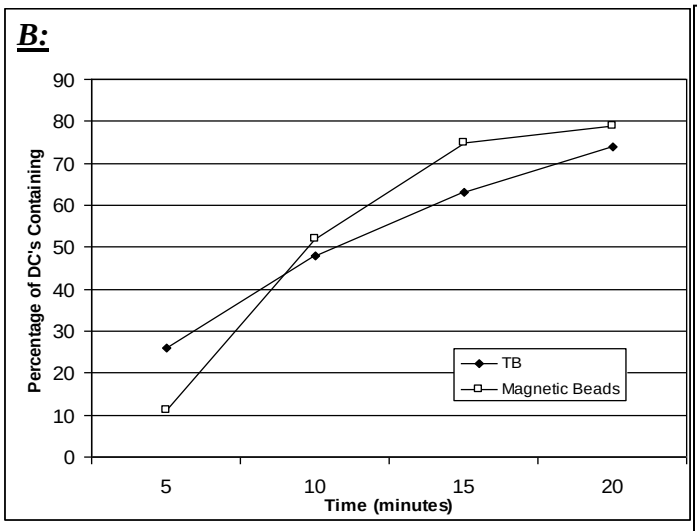
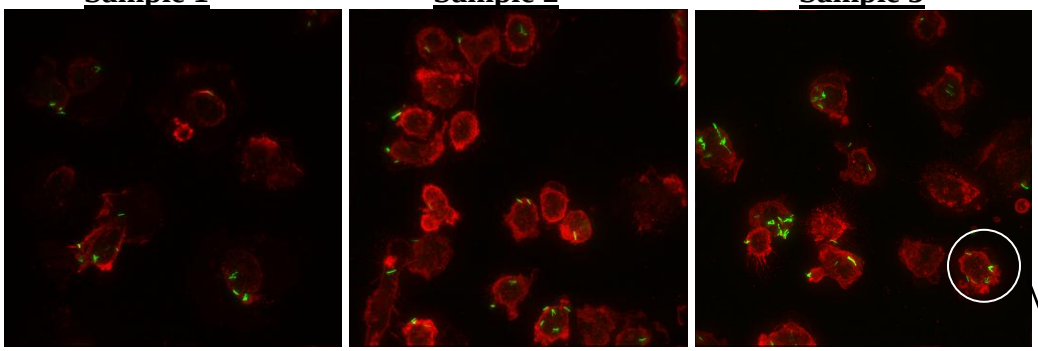


Figure 2: Entry of Mtb and magnetic beads into Dendritic Cells over time at a multiplicity of infection (MOI) of 10.
(A) Pictures from wells. The color on these images is optimized to lower background while illuminating co-localization. Representative of 10 samples per time point per experiment.
(B) The percentage of Dendritic Cells Containing either Mtb or Magnetic beads over time. Representative of two experiments.

Figure 3:

A:

Sample 1 **Sample 2** **Sample 3**



	Sample 1	Sample 2	Sample 3	Total
Infected	5	16	10	31
Total Cells	7	19	13	39

Percentage of DC's containing TB = ***79 percent***

B:

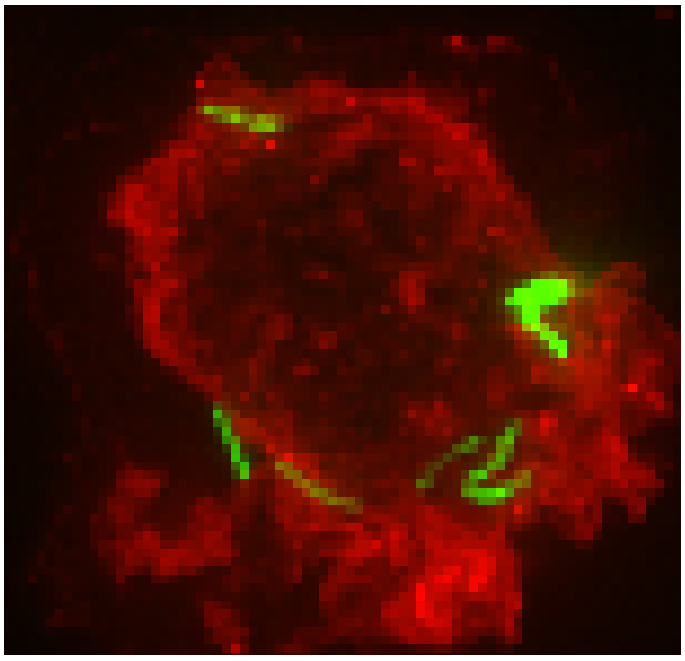


Figure 3: Explanation of process of image analysis. (A) A sample calculation of three of the 10 samples of the Mtb 20 minute time-point. The Average percentage of infected cells is calculated. (B) Enlarged cell which contains Mtb.

Figure 4:

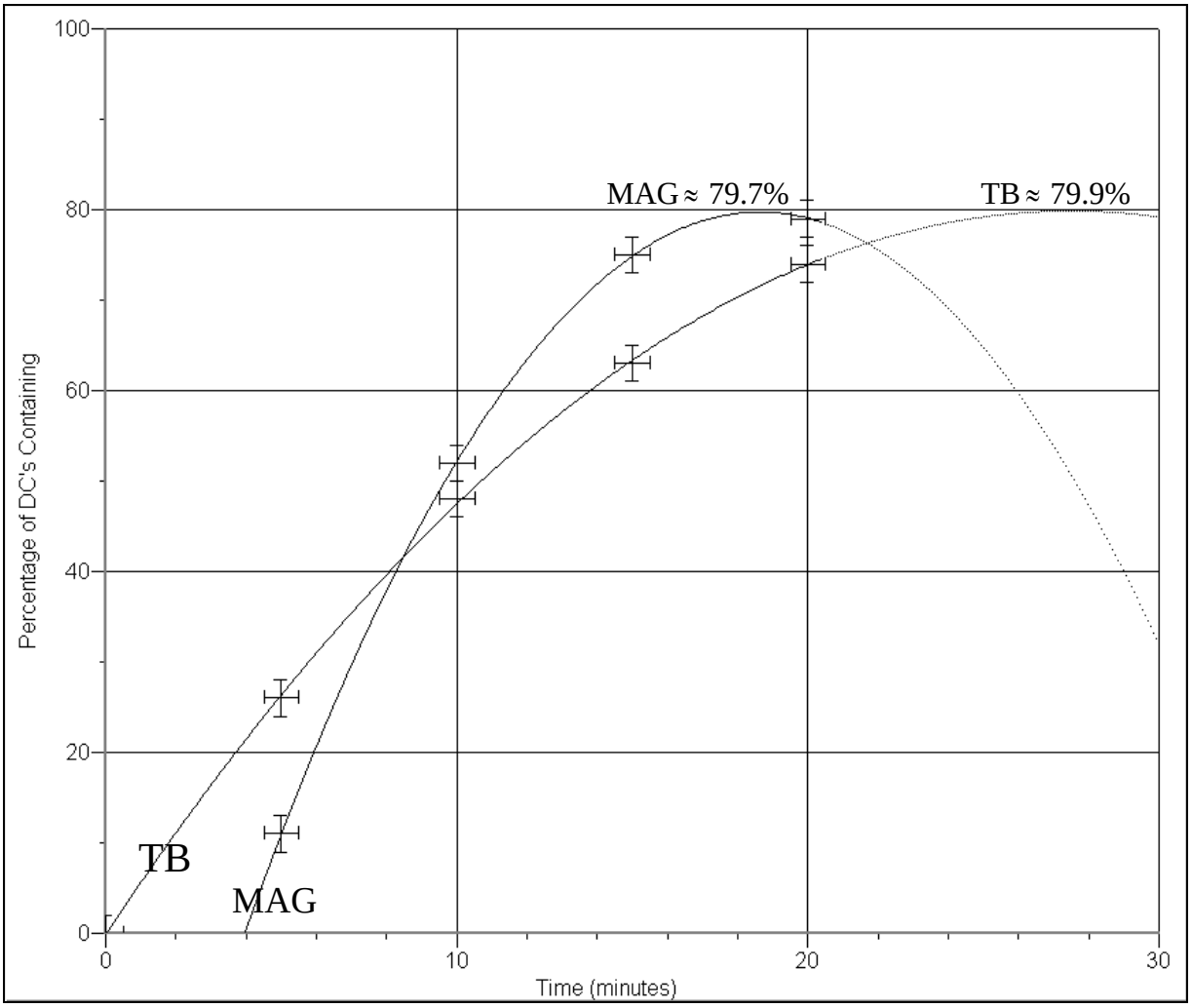


Figure 4: Projected Saturation Curves for Magnetic Beads and Mtb were found using a quadratic regression with graphing software LoggerPro (Vernier). See Figure 2(B) for explanation of where original data was derived from. The maximum points of the projected Saturation Curves are as follows:

MAG:(19 minutes, 79.7%)
TB: (28 minutes, 79.9 %)

NOTE: Time points past 20 minutes are just projections of maximum saturation – the experiments which I conducted lasted only 20 minutes. After examining this graph, it appears that the saturation would PLATEAU at around 80 percent of cells infected.

Discussion:

This project was designed to investigate phagosomal associations with immune markers within whole cells using immuno-fluorescent microscopy (Mtb versus magnetic beads). Microscopy is a valuable way to visualize interactions between protein molecules within intact cells. In this study, increasing numbers of total cells were infected with either Mtb or magnetic beads over time. The increase did not appear to be linear, for a linear regression did not fit the time points as well as a quadratic regression as is shown in the results section. Therefore, although the total number of infected cells increased with time, the rate of Mtb penetration appeared to decrease with time. The plotted points formed a concave down graph showing a clear decrease in the rate of change of the Mtb infection. This emphasizes that the dangerous process which Mtb undergoes to infect human cells occurs rather quickly – cells are susceptible to Mtb at the earlier time points. However, the rate may decrease because the cells were already becoming sick and thus unable to absorb any more environmental particles. In addition, during infection the continuous retrieval of particles was not constant. The non-linear rate could have been caused by the fact that certain cells are harder to infect than others. Perhaps certain cells are all but immune to Mtb because they are so resistant. Perhaps the rate decreases because Mtb have a way to slow down infection once a critical mass of infected cells has been reached.

Saturation curves can be used to model the data for both Mtb and magnetic beads. The peak of the saturation curve in this experiment is the maximum percentage of cells which would be infected – the symmetrical second half of the quadratic graph would be irrelevant. Interestingly enough, both saturation curves peaked at about 80 percent of the total number of dendritic cells. Although this furthers the idea that there is a maximum threshold that Mtb

reaches, more experimentation is necessary before this can be confirmed because of high uncertainties.

The determination of interactions (co-localization) between phagosomes and Mtb was difficult to complete due to certain limitations, which will be described in greater detail below. I recognize that this study (as any microscopy study) could benefit from continued optimization. These limitations can be broken down into three main areas:

Sample preparation:

The cells which were used were all fixed after a certain time point (in order to analyze Mtb outside the BSL-3) and so the same cell population could not be tracked through the four different time points. These fixed cells allowed for only one snapshot per time point into the interaction between Mtb and the dendritic cells. Ideally, I would like to have one population with four time points, but instead I had four populations with one time point.

A high background of the stain in areas without cell interactions limited the scope of the data. Therefore, it was difficult to determine positive or negative staining of a particular protein or cell. This made analysis difficult because some Mtb cells or magnetic beads fell within this band of uncertainty and their exact locations were a challenge to ascertain. In addition, the staining was not specific enough to allow complete certainty that, for example, only the phagosome was stained with HLA-I.

Data collection:

My selection of cells was not arbitrary, as my eyes were likely drawn to cells that appeared healthier within the microscope field. This may have created a bias toward healthier

cells and those which were not covered by other cells. This may have limited the results to apply to a healthier set of cells than in a real system.

In part, the resolution of my cell image depended on the number of Z-planes able to be collected per cell. I designated a limited number of Z-planes to collect per cell, in order to try to optimize data collection without overwhelming the storage capacity or time dedicated to data analysis. This likely resulted in less than optimal resolution of the images, which decreased my level of certainty in data analysis.

Data analysis:

The analysis of the data was centered on the viewing of a maximum projection image as well as scrolling through the individual z-planes. Both methods were used extensively. However, certain cells whose locations were more difficult to ascertain were given more careful scrutiny and detailed investigations of all z-planes. Co-localization was determined by the human eye from these pictures and the bias in this process is inherent. No computer program was used to determine these associations – only close examination of the images by eye.

Realistic Improvements:

Some general improvements would help to reduce uncertainties in the data and systematic error:

If an additional approach such as Flow Cytometry were to be used in conjunction with a form of microscopy, more quantitative analysis would be readily available. In terms of the signal

to noise discussion, Flow Cytometry would eliminate the noise (the cytosol) and quantitatively allow for a more statistically significant discussion of the remaining parts of the cell.

If tagged proteins were used, the non-specific staining would not be an issue because no staining would be necessary. This would reduce background confusions and make exact cell location easier to determine. Multiple tags per eGFP-Mtb (Green Fluorescent Protein) would amplify the signal, which would only help in the later stages of analysis. Co-localization recognition would also be improved if it could be determined that the tagging proteins do not alter the properties of association of the Dendritic Cells.

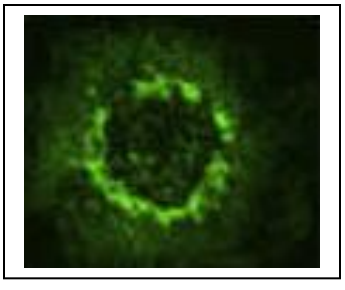
If more z-planes were used, more data would be collected and more detailed analysis could be attempted. This would require more time and lots of computer storage space, but this extra delineation could be the key to the further definition of co-localization of these cells and proteins.

Although Mtb is a BSL-3 pathogen, which is difficult to analyze, there is equipment on the OHSU campus to examine live Mtb cells through microscopy. This would allow for the same cells to be tracked through the four time points and the process to be analyzed with more accuracy. The confidence in the results would be enhanced by the video confirmation of Mtb penetrating the cell in real time. Once again, storage space would limit the amount of samples to be analyzed, but the co-localization in each sample could be determined with a much greater degree of certainty.

If the cells sample spaces were chosen randomly by a computer program, a true random cross-section of the dendritic cell response would be created. However, it must be examined what this random sampling phenomenon would truly mean for this set of chosen samples. This would create regions where only a few cells or a dense field of cells or half-cells (cells

overlapped the edge of the sample space) are present. Unless an objective, more quantitative approach was used for analysis of infection, this random sampling technique would create a nightmare for statistical relevance of the results. The number of cells would vary from sample to sample by greater margins and this would bias the overall DC infection rate towards the fewer selected areas of the well which contained more cells.

The use of a computer algorithm to assist in the analysis could increase the objectivity of determining whether co-localization is occurring. A fine grid could be laid down over all images and the distance between the cells could be determined more readily. This would give a quantitative analysis of not only how close the cells are together when apparent co-localization occurs, but a general idea of the rate of Mtb or Magnetic Beads penetration into the dendritic cell. The computer program data could be used to determine how far apart the particles are and criteria could be set on the maximum distance required for confirmation of co-localization. In addition, Slidebook could be adjusted so that all images have the same color scale. The magnitude of the color could then be obtained fairly easily and this would clarify whether or not the dendritic cell was any more built up directly around the Mtb or Magnetic Bead. A spike in the magnitude of fluorescence would indicate a greater structural density directly surrounding the particle, providing further evidence of co-localization. An example of an image with a ‘spike’ is shown below. This magnitude of fluorescence could also be used to determine the density of Mtb by examining fluorescence intensity or pixel brightness. The use of the grid method would greatly impact the amount of relevant, quantitative data collected and the general applicability of the results.



Further Experimentation:

Once these limitations have been addressed, there are many questions and ideas to explore in the future. Although Dendritic Cells are valuable in the representation of the human reaction, exploration of the phagosomal difference in DC's and other types of cells (such as macrophages) would allow for a more comprehensive view of the immune response to Mtb. Given that patients have varying amounts of Mtb per gram of tissue, it is likely that this would affect the cell function inside of human beings. Testing latent vs. active TB donors would allow for this direct investigation. At the Lewinsohn lab, there are a wide variety of TB donor samples available for examination, which would allow for a study into whether variations in human patients (ethnic background, clinical disease, immune status) would predict Mtb infection, disease outcome, and response to anti-mycobacterial therapy. There are many questions that still need to be answered in order to better understand Mtb and other pathogens and the versatility of the improved technology surrounding microscopy will allow for groundbreaking experiments in the future. It is clear that specific parameters (cell preparation, data collection, and analysis) need to be fully optimized before attempting a new microscopy project. This will separate the 'signal from the noise' and create more confidence in the validity of the results. It is important to use this valuable tool to create actual images of these microscopic interactions so that we may better understand the actions of Mtb and our own human immune response.

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