OREGON HEALTH AND SCIENCE UNIVERSITY
SCHOOL OF MEDICINE – GRADUATE STUDIES

The Role of Rab6 in Presenting *Mycobacterium tuberculosis* Antigens to CD8+ T Cells from Infected Epithelial Cells

by
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Certificate of Approval

This is to certify that the Master's thesis of
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ABSTRACT

MHCI molecules present antigens to CD8+ lymphocytes from intracellular pathogens. The mechanism by which antigens are processed and presented to classically restricted T cells has been described. However the mechanisms of antigen processing and presentation to non-classically restricted T cells are less well understood. Moreover, the vesicular trafficking molecules involved in antigen presentation and processing on all MHCI molecules are unknown. It was determined that Rab6 is involved in the presentation of *Mycobacterium tuberculosis* antigens to MR1 restricted T cells from infected epithelial cells and not to classically restricted or HLA-E restricted T cells. Rab6 is involved in exocytosis from the Golgi as well as in retrograde transport from the endosomes to the Golgi and from the Golgi to the Endoplasmic Reticulum (ER).

Knockdown of Rab6 using siRNA showed this protein is not involved in surface expression of MR1 in uninfected cells. MR1 translocates to the plasma membrane upon binding the ligand 6-Formyl Pterin (6-FP), causing the overall surface expression of MR1 to increase. Flow cytometry and fluorescence microscopy demonstrated that Rab6 is also not involved in this translocation of MR1 to the cell surface.

It was observed that Rab6 and MR1-GFP vesicles do not associate within epithelial cells. This suggests that the role of Rab6 in presenting Mtb antigens to MR1 restricted T cells is not related to vesicular trafficking of MR1 to the cell surface or within the cell. Further studies will analyze the role of Rab6 in trafficking of Mtb antigen to be loaded on MR1.

CHAPTER 1: Introduction

Tuberculosis Global Impact and Treatment

Tuberculosis (TB) remains a major cause of morbidity and mortality around the globe. It is the second largest killer due to infectious disease after HIV/AIDS. The disease is caused by the bacillus *Mycobacterium tuberculosis* (Mtb). Every year about 8 million people are newly infected with Mtb and over a million people die as a consequence of infection. These cases are added to the one third of the world's population that is presently infected with Mtb. In some regions such as sub-Saharan Africa it is estimated that as much as 50% of the total population has TB. Despite a dramatic decline in the rate of death due to disease over the last few decades, many problems continue to arise increasing the burden of the disease including the appearance of Multidrug-Resistant Tuberculosis (MDR-TB). Beyond the appearance of this newly drug-resistant TB, co-infection of Mtb with HIV remains a great complication of the disease where TB kills about a quarter of HIV infected patients (1). The rates of incidence of TB and of death due to the disease remain varied around the world but its impact is clearly experienced across regional borders. For an instance, patients presenting MDR-TB in the United States are mostly foreign born (2,3). The most striking characteristic of TB is that most patients remain asymptomatic with Latent TB Infection (LTBI). Only about 10% of all people that become infected with Mtb develop active disease. Individuals with LTBI have a 2-23% risk of progression to active TB during their lifetime and these rates are greatly increased in patients who are also infected with HIV (1).

Treatment for both LTBI and active disease lasts from six months for drug-susceptible TB to about 20 months for MDR-TB. The drugs administered to patients have a considerable level of toxicity, particularly those with MDR-TB, and require a considerable

adjustment in lifestyle, which, along with the long period of treatment add to the considerable burden the disease has on the individual (1,2). There has been considerable effort in developing prophylactic methods for treatment and prevention of TB. *Mycobacterium bovis* Calmette-Guérin, or BCG, is presently the only vaccine available for prevention of TB. This vaccine is widely administered among infants in many parts of the world. With billions having received BCG; it is the most used vaccine across the globe (4). The vaccine has shown to be effective in preventing TB in children; however its efficacy in preventing adult pulmonary TB has proven to be variable, if not dismal. Protection has ranged from 0-80% with the poorer results coming from endemic areas (2,4). The challenges presented by the appearance of MDR-TB, the severe effect of current chemotherapy to infection, and the inefficacy of the BCG vaccine make it more pressing to understand the mechanisms by which the human hosts controls Mtb infection. In order to develop more efficient and lasting treatments to control TB it is essential to understand the cellular host immune response to the disease. This thesis work is a study of the cellular mechanisms underlying antigen presentation to CD8+ T cells, in order to understand how Mtb infected cells report to lymphocytes so that they can promote elimination of the microbe.

Cellular Immunity to Tuberculosis

Tuberculosis is most typically characterized as a disease of the respiratory system where Mtb enters through aerosolized droplets. The mycobacteria are taken up by phagocytic immune cells in the alveolar epithelium. These cells transport the bacilli across the lung epithelium but the bacteria have also been reported to be able to cross this barrier on their own (5). Although phagocytic cells have been characterized as the primary cells where Mtb infection occurs, Mtb DNA has been found in a variety of cells across the body suggesting that other kinds of cells can be infected (6). Once in the

lung, Mtb does not remain in a specific subset of phagocytic cells; rather, the bacteria appear in different subsets of macrophages, dendritic cells (DCs) and neutrophils (7). Phagocytic cells are among the many components of the paramount structure characteristic of active Mtb infection, the granuloma. The granuloma is a macrocellular structure that is composed of numerous infected phagocytes that are contained to the site of infection by other immune cells including T and B lymphocytes (7,8). Dendritic cells that have been exposed to Mtb antigens by virtue of propagation of the infection or from sampling the granuloma environment migrate to the draining lymph node. At the lymph node DCs activate lymphocytes and trigger the adaptive immune response to Mtb (9). The stage during which the adaptive immune response has not yet been triggered is characterized by higher growth of the bacterial burden at the site of infection. The mycobacteria replicate within infected cells which eventually die. This allows transmission to other cells (7). During this stage of continued Mtb growth, it is important to have a strong immune response. Enhancing the response from early-acting innate immune cells can contribute in controlling infection in its initial phase. This thesis is particularly interested in the presentation of Mtb antigens to lymphocytes that are fast acting, innate-like cells that are triggered before the adaptive immune response occurs.

Once they are taken up by cells, mycobacteria inhabit a membrane bound compartment called a phagosome. The bacteria take advantage of the endosomal system inside macrophages. The endosomal system is composed of vesicles that move within the cell and carry materials that have entered the cell. In the case of most pathogen invasions, these vesicles progress from early endosomes, to late endosomes, and eventually become lysosomes. Lysosomes are organelles in charge of degrading and killing pathogens through use of reactive oxygen and nitrogen species and proteolytic enzymes. Mycobacteria are unusual in that they can co-opt endosomes of infected cells

and prevent them from maturing to lysosomes, thus avoiding degradation. The phagosome contains markers typical of early endosomes such as Rab5 and Lamp1 (10,11). All the while, Mtb prevents the phagosome from obtaining markers of later endosomes such as active GTP-bound Rab7 and prevents the conversion of phosphatidylinositol-3-phosphate, which allows the organelle to mature into a lysosome (10,12). Another way in which these vesicles mature into lysosomes and degrade bacteria is by progressive acidification. Mtb secretes effector molecules that inhibit the vacuolar H^+-ATPase, which is in charge of this acidification (13). The activation of macrophages promotes the killing of bacteria in membrane bound compartments and promotes the activation of iNOS. iNOS synthesizes nitric oxide which in turn can convert to free radicals that kill bacteria (14).

Macrophages can be activated by Th1 helper T cells. The secretion of IFN- γ and TNF-α by these cells activates macrophages and is essential for killing Mtb. Mice deficient in IFN-γ are not capable of controlling sub-lethal doses of Mtb and die following infection (15). Th1 helper T cells are typically a subset of CD4+ lymphocytes. CD4+ T cells are an integral component of the adaptive immune response to Mtb infection. This is reflected in HIV patients who suffer from CD4 T cell depletion and have a much higher likelihood of developing active TB (1). Mice deficient in CD4+ T cells succumb much faster to Mtb and have higher bacterial burdens (16). Defects in MHCII, which is responsible for antigen presentation to CD4+ cells, replicate these results (21).

Although CD4+ T cells are important to the control of Mtb, there are also subsets of CD8+ T cells that provide Th1 help. Additionally, CD8+ T cells specialize in detecting and killing cells infected with intracellular pathogens such as Mtb. There are Mtb antigen-specific IFN-γ producing CD8+ T cells that infiltrate the lungs and are present within granulomas (18). Also, adoptive transfer of CD8+ T cells was able to reduce

bacterial load in the lungs of mice following infection (16,19). MHCI molecules present antigens to CD8+ T cells. Disrupting the process by which MHCI presents Mtb antigens to CD8+ lymphocytes can increase the severity of Mtb infection. MHCI molecules form a heterodimer with β_2-microglobulin. β_2-microglobulin knockout mice showed higher bacterial burden and mortality when exposed to Mtb (20). Mice deficient in TAP1, an essential component for loading peptides to MHCI, present high mortality and bacterial burdens as well (21). This project concentrates on the response of CD8+ T cells following infection with Mtb.

Although much has been described on the role of T cells in the adaptive immune response to Mtb; comparably little has been described on the role of B cells and the humoral response. Activated B cells as well as IgG are found within granulomas. Mice deficient in B cells had higher bacterial burden within the lung and present symptoms of sickness as compared to wild type animals (22). However, most work on the cellular immunity to Mtb concentrates in the T cell response.

Antigen Presentation to CD8+ T Cells

When cells are infected by intracellular pathogens they present antigens to CD8+ T cells through the Major Histocompatibility Complex Class I (MHCI). Antigens presented on class I molecules often come from proteins that are processed in the cytosol by the proteasome. Peptides are then translocated to the ER lumen by TAP proteins. In the lumen peptides are loaded onto newly synthesized MHCI molecules. This process is carefully regulated by a multi-protein complex that includes tapasin, calnexin, calreticulin and Erp57. Additionally, peptides are further processed and trimmed in the lumen of the ER by peptidases such as ERAAP so that they can fit properly on MHCI. Once peptides are loaded to MHCI, the complex is shuttled to the cell surface through the secretory

pathway (23). The secretion of MHCI molecules is carefully regulated and improperly loaded MHCI are recycled and use retrograde transport from the plasma membrane and the later cisternae of the Golgi apparatus back to the ER (24). In cells infected with Mtb, the phagosome contains HLA-I, the human MHCI, and TAP indicating that it is an organelle competent for the loading of antigens to MHCI (11).

In humans, MHCI molecules are classified as HLA-Ia or HLA-Ib. HLA-Ia molecules are polygenic (HLA-A, B, and C) and polymorphic (multiple alleles within a population). These characteristics allow for the production of multiple HLA-Ia proteins which in turn allows for a large repertoire of CD8+ T cells (23). T cells restricted by HLA-Ia are said to be classically restricted and their corresponding antigens are processed and presented as described above.

On the other hand, non-classically restricted CD8+ T cells recognize antigen presented on HLA-Ib molecules, including MR1 and HLA-E. These T cells are innate-like, fast acting lymphocytes that do not depend on the slower clonal expansion that HLA-Ia restricted T cells experience. The capacity of these cells to act faster than classically restricted T cells is of particular interest in Mtb infection. They can act during the early stages of infection when Mtb grows exponentially before the adaptive immune response is triggered. Non-classically restricted T cells recognize antigens with more general patterns found in multiple pathogens. Not all these antigens are peptides; as in the case of CD1 that presents bacterially produced lipids to Natural Killer T (NKT) cells (25). The processes by which infected cells process and present antigens through HLA-Ib molecules are less well understood than HLA-Ia. In humans HLA-Ib restricted T cells constitute a substantial fraction of Mtb-specific CD8+ lymphocytes in Mtb infected individuals (26). In this project we are interested in understanding the vesicular

trafficking pathways involved in presentation of antigen on non-classical class I molecules.

MR1 in Mtb Infection

Mucosal associated invariant T (MAIT) cells are CD8+ T cells that are restricted by the HLA-Ib molecule MR1. They comprise a substantial part of CD8+ T cells in the blood of individuals with LTBI and are significantly reduced in the blood of people with active infection. Furthermore, the Lewinsohn laboratory has shown that MAIT cells are enriched in the lung (26). These cells have antimicrobial capacity and they can detect bacteria and fungi that produce riboflavin, including many species of mycobacteria, but not viruses. MAIT cells are capable of detecting bacterially infected lung epithelial cells (26,27). They are characterized by having a semi-invariant T cell receptor with Vα7.2 heavy chain and they release IFN-γ and TNF-α (26,28). Not only are they restricted by MR1 but they also depend on MR1 for their selection in the thymus (28).

MR1 is a monomorphic MHCI-related molecule that is highly conserved across mammalian species (29). It is constitutively and ubiquitously expressed around the body. It is genomically and structurally similar to other MHCI molecules and there are multiple isoforms in human cells. Like MHCI, MR1 forms a heterodimer with β_2-microglobulin (30). Recently it was shown that Vitamin B metabolites are ligands for MR1 (31). It is not yet clear how these unusual ligands are processed and presented on MR1. We know that in contrast to classical class I molecules, processing of MR1 ligands does not require TAP activity (26).

MR1 uses an endocytic compartment to move within the cell (32). However, little is known about how MR1 and other MHCI molecules move within the cell and to the cell surface. In this project, we are interested in the vesicular trafficking pathways used during antigen presentation to CD8+ T cells.

Importance of Epithelial Cells in Immunity

Live Mtb can be found in phagocytic immune cells during active infection. However, Mtb DNA has been found in different tissues including epithelial cells. The mucosal epithelium is an important component in the immunity against pathogens and the tolerance towards non-damaging agents. It is the primary barrier that separates the sterile inner environment of the body with the outside environment from where pathogens originate. These are likely to be among the first cells that Mtb encounters upon entering the body. These surfaces are where the commensal microbiota live; particularly but not exclusively in the gut epithelium. The mucosal epithelium is the place where a delicate balance in the immune system is maintained. Here, tolerance is induced toward commensal microbes while the capacity to fight pathogens is maintained (33). The flora can induce certain subsets of lymphocytes like regulatory T cells which can contribute in tolerance while promoting the expansion of pathogen-fighting cells like MAITs (34,28).

In this location epithelial cells are of great importance. Not only do the cells themselves form a barrier against crossing of pathogens but they secrete mucus to include additional space between microbes, including commensals, and the cells. They also secrete antimicrobial substances to fight infection and promote the translocation of IgA antibodies to the outer environment (35). The barrier that they form is very tight and allows a very limited flow of substances between the inner and outer environments through the formation of tight junctions. Pathogens have to find different ways to cross this highly restrictive and regulated barrier. Some use immune cells as carriers. Monocytes can cross the epithelium to reach Mtb and they can cross back carrying the bacillus, although there is also evidence that the bacillus can cross epithelial cell monolayers by itself (5,35). .

Another way in which pathogens can cross this barrier is by infecting the epithelial cells, ensuring propagation upon cell death; this is a common way for mycobacteria to transmit from cell to cell (7). Mtb causes necrosis in A549 cells, an alveolar epithelial cell line, whereas BCG causes apoptosis in BEAS2B cells, the bronchial airway epithelial cell line that is used as a model in this thesis (35,36). It is important to consider the role of epithelial cells in presenting Mtb antigens to T cells. Different CD8+ T cell subsets can detect Mtb-infected epithelial cells. Hence, the presentation of Mtb antigens from epithelial cells can be instrumental in preventing the propagation of the disease early, before it disseminates beyond the mucosal surfaces. Epithelial cells also contribute to fighting infection, including Mtb infection in other cell types. For example, they secrete pro-inflammatory cytokines such as IFN-γ and IL-32 when they are infected with mycobacteria (38,39). These cytokines can in turn activate macrophages to degrade Mtb that may be living within.

The model epithelial cell used in this thesis, BEAS2B cells, can be infected by Mtb and secrete pro-inflammatory cytokines. Additionally, Melanie Harriff from our laboratory has described Mtb in these cells as inhabiting a membrane bound compartment that resembles a late endosomal compartment which has arrested acidification (Manuscript under review). These cells can efficiently present mycobacterial antigens on different MHCI molecules. In this thesis work, aspects of the process of antigen presentation on MHCI molecules in epithelial cells are described.

Presenting Antigens from the Mtb Phagosome

As described before, the antigens from intracellular pathogens are typically processed in the cytosol and translocated to the ER. However, the presentation of antigens from pathogens residing in membrane bound compartments within the cell, like the Mtb phagosome, requires mechanisms that are different to the one described before, since these antigens are not readily available in the cytosol. There are many mechanisms by which these antigens may be presented. Such mechanisms can differ depending on the class I molecule used. For example, the Lewinsohn laboratory has demonstrated that in DCs recycled HLA-E is used to present Mtb antigens, and that TAP translocation is not required for presentation on MR1(11,26). Mtb Phagosomes may be permeable or they may have transporters that allow mycobacterial proteins and other antigens to reach the cytosol. Some of the mycobacteria may be degraded in lysosomes and the resultant antigens can be loaded to MHCI molecules. Another possibility is that the phagosome may acquire the antigen processing and presentation machinery and become capable of loading antigens on MHCI (40). The Lewinsohn laboratory has shown that the Mtb phagosome from DCs contains HLA-I and TAP, and that the isolated phagosome alone is capable of eliciting a response from classically restricted and HLA-E restricted CD8+ T cells (11). Additionally, Melanie Harriff has shown that TAP in the phagosome is capable of translocating peptides (61). Other MHCI molecules such as CD1 have been located to the phagosome of BCG (41). Beyond being an immune competent organelle, antigens may be transported from the phagosome to other compartments where MHCI molecules or the antigen processing machinery are located.

The Lewinsohn laboratory has started to describe the mechanisms by which the presentation of antigens from Mtb occurs by using chemical and peptide blockers of certain cellular pathways. In order to keep elucidating these mechanisms, we chose to look at the vesicular trafficking pathways in the cell that may be used for antigen presentation. It is clear that the mycobacterial phagosome associates with certain Rab proteins, which are vesicular trafficking molecules (42). The association of different HLA-Ia and HLA-Ib molecules with Rab proteins or any other vesicular trafficking proteins, however, has not been described. By studying the vesicular trafficking pathways used in antigen presentation we hope to elucidate some of the mechanisms used to present antigens from Mtb.

Intracellular Vesicular Trafficking

Rabs are a large Ras-like family of GTP-ase proteins. They are the master coordinators of vesicular trafficking and transport around the cells. In their different GTPase cycle stages they can coordinate different events and effectors involved in vesicular trafficking. These events include, budding of vesicles, tethering to molecular motors and movement across the cell's cytoskeletal tracks, docking, and fusion of vesicles. They recruit diverse effectors that perform each specific task. Different Rabs coordinated specific kinds of movement and act as the markers of specific vesicles and other membrane bound compartments. There are over sixty Rab proteins identified in human cells (43). Many Rab proteins have been studied for their role in response to pathogen infection. Some proteins, such as Rab5, are known to associate with the Mtb phagosome (10). It is also known that Mtb actively prevents the association of active GTP-bound Rab7 with the phagosome, preventing its progression to a lysosome (10). Rab proteins are known to associate with other intracellular bacterial inclusions. Key to this thesis project, Rab6 localizes to the membrane bound compartment containing *Chlamydia trachomatis*

(44,45) and also to the phagosome of *Mycobacterium bovis* BCG (42). This association has not been found in Mtb infected cells. Very little is known about the association between Rabs, their effectors and antigen presentation and processing. This thesis works is part of an effort to determine how vesicular trafficking molecules influence these processes.

Rab6: Location and Function

As described in the results, our laboratory developed a project to determine which vesicular trafficking proteins are involved in presenting Mtb antigens to CD8+ T cells. Rab6 emerged as a candidate that specifically may affect presentation on MR1. Rab6 has four different isoforms Rab6a, Rab6a', Rab6b and Rab6c. The distinction between isoforms is not particularly clear. A significant part of the literature describes the function of Rab6 without alluding to which specific isoform is being described. Rab6a and Rab6a' are products from alternate splicing of the same gene and there is very little difference between both isoforms. They are different in three amino acid substitutions, only two of which are non-conservative (46). Rab6b and Rab6c are in separate genes but both have high identity to Rab6a (47,48). Rab6c is the most recently discovered isoform of the protein and it has been described as a retrogene of Rab6a' and contributes to progression through the cell cycle (47). Rab6b has similar functions to Rab6a and Rab6a' but its expression is limited to brain cells (48).

Rab6 is mainly located to the Golgi apparatus and it controls retrograde transport from early endosomes to the Golgi and from the Golgi to the ER. When HeLa cells are treated with GDP–bound Rab6 or siRNA targeting Rab6, molecules that use retrograde transport within the cell such as Ricin and Shiga toxin B are accumulated at the Golgi apparatus (49). Rab6 regulates retrograde transport in both clathrin coated and COPI

coated vesicles (50). Rab6 has also been shown to regulate transport in exocytosis from the Golgi to the plasma membrane (51,52). The capacity of Rab6 to regulate transport of cargo from the Golgi to the cell surface transcends beyond the direct transport through exocytosis. GDP-bound Rab6 and siRNA to Rab6 can slow down the secretion of influenza Hemagglutinin and Secreted Alkaline Phosphatase (53). Additionally, overexpression of wild type Rab6 or the constitutively active GTP-bound form prevents the secretion of these proteins. These proteins use the retrograde transport pathway of Rab6 rather than the exocytic one. Rab6 is also important to the structural integrity of the Golgi apparatus. When Rab6 is knocked down, the stacking of the Golgi cisternae is disrupted (46). This thesis project explores the role that Rab6 plays in the presentation of Mtb antigens on MHCI.

Hypothesis

A decreased response from T cells when Rab6 is knocked down indicates that there are fewer antigen-loaded MHCI molecules on the cell surface. There are many potential roles of Rab6 in presenting MHCI to the cell surface.

1. There may be fewer MHCI molecules on the cell surface because a) their transport has been hindered and they remain within the cell body; b) there are fewer MHCI molecules in the cell.
2. Molecules may be reaching the cell surface but not loaded with antigen. MHCI molecules may not reach the location where they encounter antigen. MHCI molecules loaded with antigen may not succeed in passing through quality control steps on their way to the surface, steps that typically ensure that folded class I molecules on the cell surface contain properly loaded ligand (24).

3. Mtb antigens may not reach MHCI: The Mtb antigens may not be reaching the place where they encounter MHCI molecules. Conversely antigens may not be available at all because of fewer bacteria present in the cell or mycobacteria are contained in aberrant phagosomes.

Because Rab6 plays a role in the exocytosis and surface expression of different molecules through multiple pathways, *I hypothesize that Rab6 is involved in transporting MHCI molecules, particularly MR1, to the cell surface*. Figure 2 highlights vesicular trafficking pathways for which Rab6 may be involved in antigen presentation of MHCI molecules. Green arrows show the pathways of transport used by some MHCI. On the left the arrow points at HLA-E, which can use recycled molecules to present Mtb antigens (11). In the center, the green arrows indicate how some class I molecules, particularly classical MHCI, shuttle in a secretory pathway through the ER and the Golgi to the plasma membrane (23). The right green arrow depicts how MR1 translocates to the cell surface upon encountering the 6-FP ligand (unpublished results by Melanie Harriff). The red arrows show the pathways in which Rab6 is involved. On the left side, Rab6 directs retrograde transport between endosomes and the Golgi, and between the Golgi and the ER. Altering the function of this pathway has demonstrated delayed or reduced secretion of proteins (53). Rab6 may alter MR1 surface expression in a similar manner. The red arrow to the right shows the role of Rab6 directing exocytic transport to the plasma membrane. This pathway may cross over with MHCI shuttling to the cell surface through the ER and the Golgi, as shown in the central green arrows. Additionally, Rab6 involvement in exocytic transport may also influence the pathway depicted by the green arrow on the right in which MR1 translocates to the cell surface.

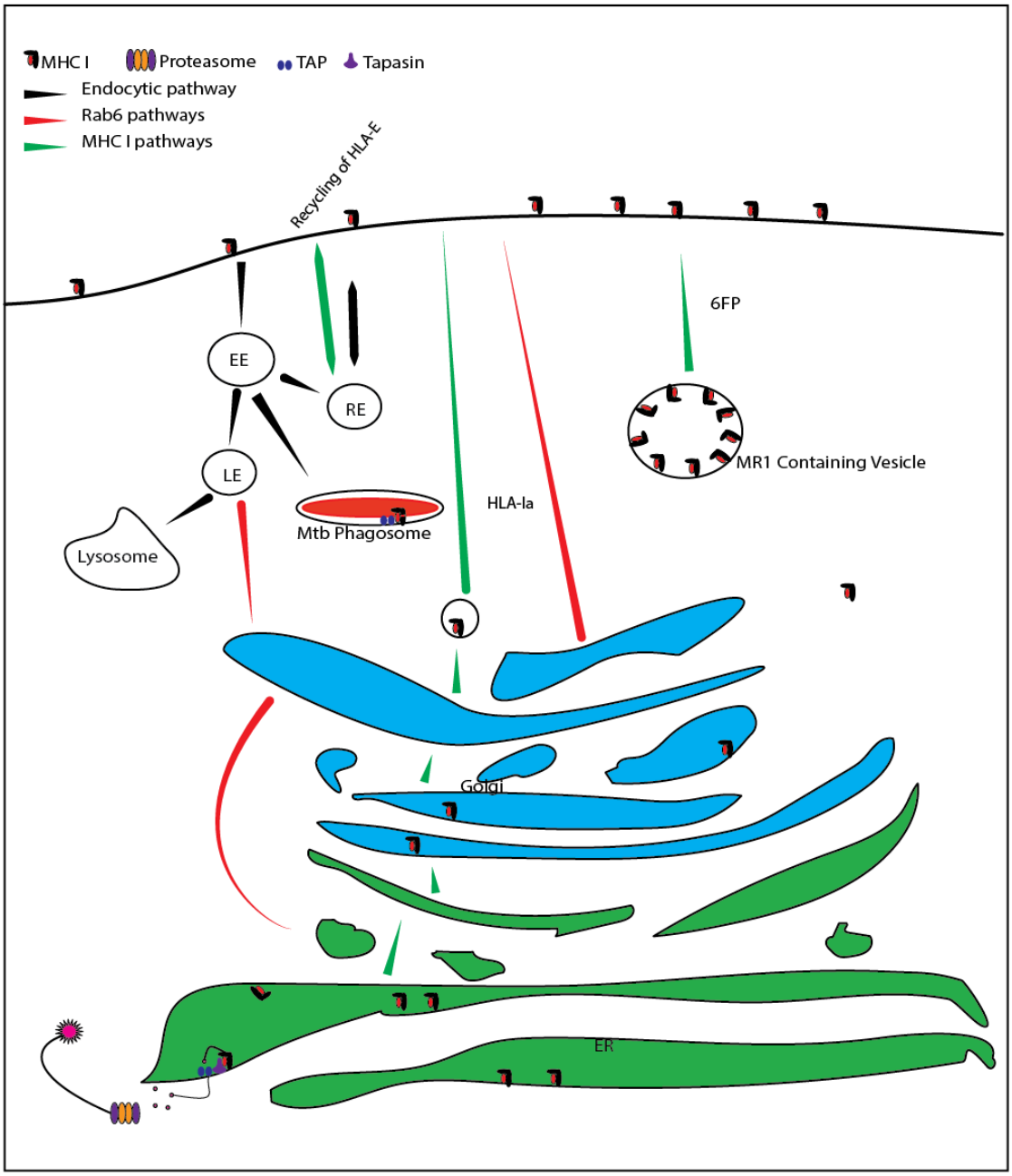

Figure 1: Cellular trafficking pathways. Described patterns of MHC I trafficking are shown in green arrows. Rab6 pathways of vesicular traffic are shown in red arrows. The endosomal system is presented with black arrows

CHAPTER 2: Materials and Methods

Cells:

BEAS2B is a cell line isolated from normal human bronchial epithelium of healthy non-cancerous individuals. The cells were transformed and made immortal with an adenovirus 12-SV40 hybrid virus. These cells did not produce tumors in immunosuppressed mice. The cells were obtained from ATCC. T cell clones were derived and expanded as previously described (11,54). D4261B1 is an MR1 restricted CD8+ T cell clone for which the specific antigen is unknown, D160 1-23 is an HLA-E restricted CD8+T cell clone that responds to a component of the cell wall of Mtb and D466 H4 is an HLA-B45 CD8+ T cell clone specific to the $CFP10_{2-9}$ peptide from Mtb protein CFP10.

Bacteria:

Mycobacterium tuberculosis (Mtb) H37Rv-dsRed and *Mycobacterium smegmatis* (Msmeg)-RFP were each grown in Middlebrook 7H9 broth (BD) supplemented with Middlebrook ADC (BD), 0.05% Tween-80, 0.5% glycerol.

Antibodies:

Anti-Class I W6/32 detects folded classical class I molecules including HLA-B45, as well as HLA-E (IgG2a, Serotec) ; 3D12 specifically detects HLA-E (IgG1,Biolegend) ; the 26.5 specifically detects folded MR1 (IgG2a). This last antibody was provided by Ted Hansen's laboratory and was biotinylated by Biolegend. The anti-Rab6 rabbit monoclonal IgG antibody was obtained from Cell Signaling. All other antibodies are monoclonal mouse antibodies. Goat anti-mouse (GAM) Alexa64 anti-IgG1, GAM

Alexa647 anti-IgG2a, Goat anti-rabbit Alexa488 anti-IgG, GAR Alexa568 anti-IgG and streptavidin Alexa 647 were all obtained from Invitrogen.

Other Reagents:

All siRNA, primer and probe pairs for Rab6 and negative control (scramble siRNA) were obtained from Life Technologies. The MR1-GFP expression vector was provided by Wilmon Grant who modified and optimized an MR1-GFP expression vector obtained from Origene. Briefly, cDNA encoding MR1-GFP was inserted into a pCI vector from Promega. 6-Formyl Pterin (6-FP) was obtained from Shircks Laboratories.

siRNA transfection:

Negative control or gene specific siRNA (200pmoles) mixed with 12 ul of HiPerfect transfection reagent and diluted in serum free DMEM were added to 100,000 BEAS2B cells in 6-well tissue culture plates. In some cases 50 pmoles of siRNA mixed with 3ul of HiPerfect and diluted in serum free DMEM were added to 20,000 BEAS2B cells in a 24 well plate. For experiments using fluorescence microscopy, 12.5 pmoles of siRNA were mixed with 0.75 ul of HiPerfect and diluted in serum free DMEM were added to 5,000 cells in an 8-chamber slide (Lab-Tek). Cells were allowed to incubate up to 96 hours following siRNA transfection.

qRT-PCR:

Messenger RNA was extracted from BEAS2B cells with an RNeasy kit (Qiagen). cDNA was reverse transcribed from mRNA using a High-Capacity cDNA Reverse Transcription kit (Invitrogen). After cleaning the cDNA with a QuiaQuick PCR purification kit (Qiagen) 11 pg of DNA were mixed with primer probes and TaqMan PCR Master Mix. The ΔCT of each reaction was measured in a StepOne Plus thermocycler (Applied Biosystems).

ELISPOT:

At 72 hours following siRNA transfection, cells were infected for 18 hours with Mtb-dsRed (Multiplicity of Infection, MOI 2.5) or Msmeg-RFP (MOI 1.4). After 18 hours the cells were collected, counted and placed at equivalent numbers in two-fold dilutions from 5,000 to 312 cells in an IFN-γ ELISPOT plate. Cells were allowed to incubate for an hour and then 10,000 T cell clones were added and allowed to incubate overnight. IFN-γ ELISPOT was performed as previously described (55). In the case of cells treated with $CFP10_{2-9}$, uninfected cells were incubated with 50 ng of peptide for 1 hour before adding T cells.

Fluorescent siRNA transfection:

Cy3-labelled control siRNA and unlabeled siRNA were transfected into BEAS2B seeded in a 24-well plate as described above. Cells were incubated overnight and fixed in 1% paraformaldehyde at 4˚C overnight. Cells were analyzed in a FACSCalibur flow cytometer.

Surface expression of MHCI:

For basal MHCI surface expression cells treated with siRNA were collected and stained on ice with biotinylated anti-MR1 26.5 (1:100), 3D12 (1:100) or W6/32 (1:1000) followed by streptavidin Alexa647 (1:1000), GAM Alexa647-IgG1 (1:1000) or GAM Alexa647-IgG2a (1:1000) respectively. Following staining cells were suspended in 1% paraformaldehyde and fixed overnight and analyzed in a FACSCalibur flow cytometer.

Surface expression of translocated MR1:

Cells were transfected with pCI MR1-GFP 48 hours after transfection with siRNA. After an additional 24hours, cells were treated with 15 µg 6-FP or NaOH. After 24 hours, cells were stained and analyzed for surface expression of MR1 as described above.

Fluorescence microscopy, surface expression of MR1:

Cells were seeded in an 8-chamber slide and treated with siRNA as described above. At 48 hours after transfection cells were transfected with pCI MR1-GFP. Following 72 hours of siRNA transfection cells were either treated with 7.5µg of 6-FP or NaOH. At 96 hours after the transfection cells were fixed with 4% paraformaldehyde at room temperature for 15 minutes Cells were stained with biotinylated ani-MR1 26.5 (1:100) followed by streptavidin Alexa647. Following surface staining of MR1, cells were permeabilized with 0.2% saponin for 25 minutes and stained with Rab6 antibody (1:200) followed by GAR Alexa488-IgG (1:1000). The cells were then stained with Nucblue Fixed Cell Stain (Life Technologies). Images were acquired on a high-resolution wide field Core DV system (Applied Precision ™) with a Nikon Coolsnap ES2 HQ. Each image was acquired as Z-stacks in a 256×256 format with a 100×1.42 NA Plan Apo N objective. Images were deconvolved with an optical transfer function using an iterative algorithm of 10 iterations.

Fluorescence microscopy, infected cells:

In an 8 chamber slide 40,000 BEAS2B cells were seeded and infected with 1.25 MOI of Mtb-dsRed and the infection was allowed to go overnight. Cells were fixed with 4% warm paraformaldehyde and then permeabilized with 0.2% saponin FACS buffer for 25 minutes. The cells were then stained with Rab6 antibody (1:200) following by GAR

Alexa647-IgG (1:1000) and finally with Nucblue. Images were acquired as described above.

Fluorescence microscopy, Rab6 knockdown:

Cells were seeded in an 8-well chamber and transfected with siRNA as described before. At 96 hours after transfection cells were fixed with warm 4% paraformaldehyde for 15 minutes. Cells were permeabilized with 0.2% saponin for 25 minutes. Cells were stained with Rab6 (1:200) followed by GAR Alexa488-IgG (1:1000) and finally with Nucblue. Images were acquired as described above.

CFU assay

Following siRNA knockdown and infection with Mtb 20,000 BEAS2B cells were lysed by resuspending them in 100ul 0.01% Tween-20 in PBS and incubating them for 20 minutes on ice. 10ul of the lysate was spot plated with multiple ten-fold dilutions on 7H10 medium (BD) supplemented with Middlebrook ADC Medium (BD) plates. The plates were incubated at 37˚C until colonies appeared and could be counted.

Image analysis:

Images from fluorescence microscopy were analyzed with the software package Imaris x64 version 7.6.3 by Bitplane.

Statistical analysis:

Statistical significance was determined using Student's two-tailed t-test in Graphpad Prism software.

CHAPTER 3: Results

Determining the Role of Vesicular Trafficking Molecules in Antigen Presentation and Processing

Through the Broad Institute, Dr. Luis F. Moita has developed an shRNA library that targets 150 vesicular trafficking molecules, including Rab proteins and the Rab effectors called VAMPs. VAMPs are the proteins in charge of the mechanical act of making membranes of vesicles fuse with one another (43). In this library, each gene is targeted by 5 different shRNAs. BEAS2B cells were transduced with lentiviruses containing the shRNA. Two days after transduction media was changed to select transduced cells with puromycin. Following selection cells were infected with Mtb for 18 hours and then plated in an IFN-γ ELISPOT plate. In the case of *Mycobacterium smegmatis* (Msmeg) transduced cells were infected 1 hour prior to adding T cells to the ELISPOT plate. T cell clones were added in excess. A classically restricted clone (D466 H4, HLA-B45) and two non-classically restricted clones to HLA-E (D160 1-23) and MR1 (D426 B1) were used. Genes where the response from the T cell clone was altered for two or more shRNAs were considered candidates for further inquiry on their role in antigen presentation.

Melanie Harriff in our laboratory performed the screening on the shRNA library. Figure 2 (kindly provided by Dr. Harriff) shows the results for three proteins. The response from T cell clones was normalized to the number of epithelial cells put in each well of the ELISPOT plate. These three proteins are isoforms of Rab6: Rab6a, Rab6b and Rab6c. Figure 1 shows that when all three target proteins are knocked down the response from the MR1 restricted T cell clone was decreased for at least 2 of the 5 shRNAs. The response of the MR1 restricted clone was not as prominently decreased when cells are infected with Msmeg. The counts from reading the ELISPOT plate are much higher when the MR1 restricted clone detects Msmeg as compared to Mtb. It was later

discovered that when Msmeg is used to infect cells in the ELISPOT plate the response of our MR1 clone are inconsistent and elevated. The MR1 restricted T cell clone can respond to Msmeg alone and even to the supernatant from bacterial culture. This may be the reason for the varied response from this clone observed in the screen.

The response from the HLA-B45 and HLA-E restricted T cell clones is decreased in some cases, but the knockdown of only one isoform, Rab6c, met the criteria of showing decreased response in at least 2 of 5 shRNAs. After the screen with this shRNA library the proteins considered hits were subjected to confirmation by knocking down protein expression with siRNA. Since all Rab6 isoforms were hits in this screen for at least the response from the MR1 restricted T cell clone, it was decided to make discovering the role of Rab6 in antigen presentation the central goal of this thesis project.

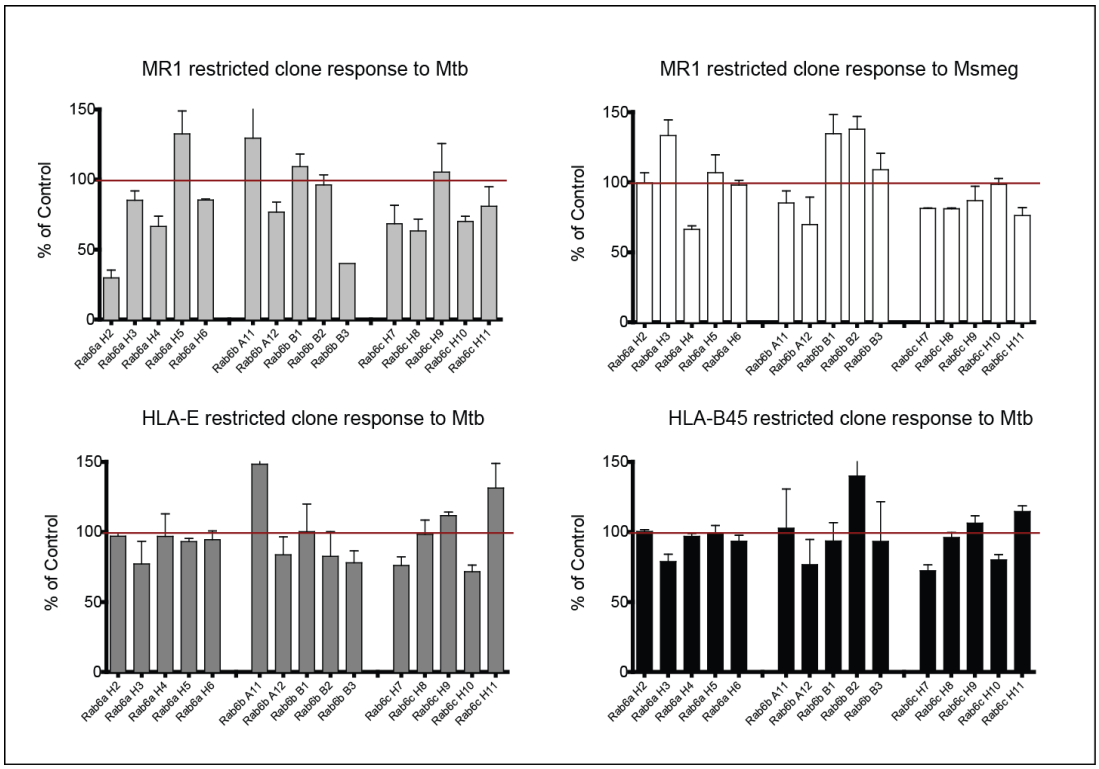

Figure 2: Results from shRNA library screen. Epithelial cells were transduced with shRNAs and infected with Mtb or Msmeg. The cells were set in an IFN-γ ELISPOT plate with T cell clones in excess. The response was measured as spot-forming units and normalized to the number of cells added per well (Provided by Melanie Harriff).

Rab6 is Involved in Presentation of Mtb Antigens by MR1 but no Other CD8+ T Cells

Rab6a, Rab6b, and Rab6c were considered candidates after completion of the shRNA screen of vesicular trafficking molecules affecting Mtb antigen presentation and processing to CD8+ cells. To confirm hits from the screen, siRNA was used to target Rab6. Initially, I generated custom siRNA that were specific for the different isoforms of Rab6 to determine which isoform specifically altered antigen presentation. However, I was unable to determine which specific isoform caused a functional decrease in the T cell response. As a result, I used an siRNA targeting Rab6a, Rab6a' and Rab6b in all subsequent experiments. To analyze the impact of Rab6 on presentation of Mtb antigens using siRNA, the protocol used in the shRNA library screen was modified. This protocol is briefly outlined in Figure 3a. I incubated BEAS2B cells with siRNA to Rab6 or a negative control for 72 hours. Then cells were infected with Mtb for 24 prior to using them as antigen presenting cells in an IFN- γ ELISPOT. The response from the MR1 restricted T cell clone to Mtb or Msmeg infected BEAS2B cells was significantly reduced when Rab6 is knocked down (Figure 3B). In contrast, the response from the classically restricted T cell clone and the HLA-E restricted T cell clone did not significantly differ from the control (Figure3C, 3D). As a control, uninfected BEAS2B cells were incubated with the cognate peptide for the HLA-B45 restricted T cell clone, $CFP10_{2-9}$. This suggests that Rab6 is involved specifically in antigen presentation through MR1.

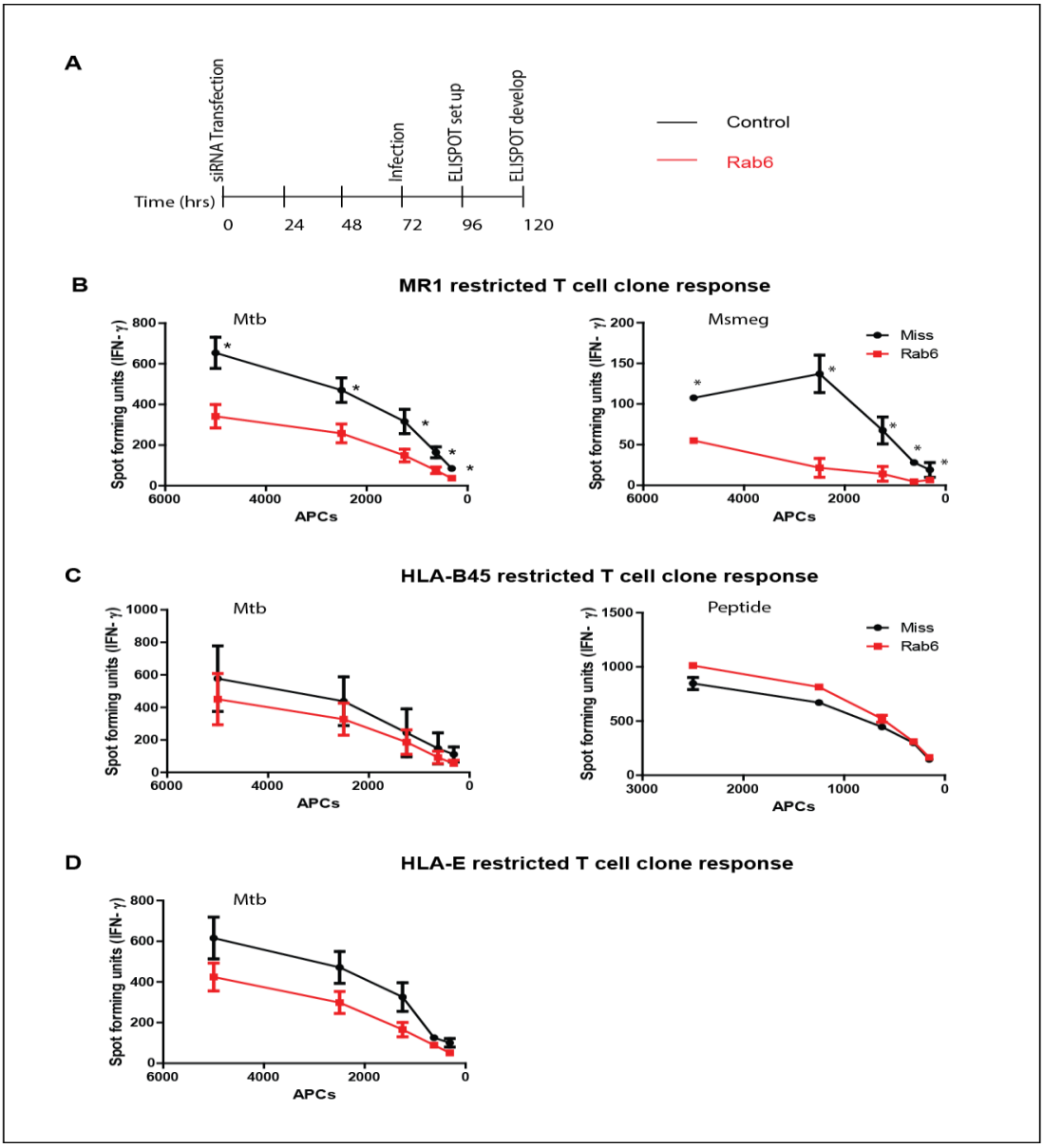

*Figure 3. A) BEAS2B cells were transfected with siRNA and 72 hours after transfection they were infected with Mtb or Msmeg and the infection was allowed to occur overnight. 96 hrs after transfection, cells were collected and set up in two fold dilutions in an ELISPOT plate with 10,000 T cell clones per well. Data shown are representative of two independent experiments. B) MR1 restricted T cell clone response to infection with both mycobacteria, where each point represents one two-fold dilution. (*p<0.05) C) Response from a classically-restricted T cell clone. The panel on the right shows uninfected siRNA treated cells incubated with $CFP10_{2-9}$ in the ELISPOT plate. D) The response from an HLA-E restricted T cell clone to infected BEAS2B cells. Two different aliquots of cells were separated to measure knockdown of Rab6 by qRT-PCR and to measure the bacterial content on each sample (see Figure 8). These are the results of two separate experiments where Rab6 expression was knocked down by 80% and 90%.*

Because there was not a complete inhibition of the T cell response, I further analyzed the efficiency of gene knockdown. First, BEAS2B cells were transfected with Cy3-labeled or unlabeled negative control siRNA and analyzed by flow cytometry. All cells take up the fluorescent siRNA overnight, suggesting that the transfection of these cells with siRNA is efficient (Figure 4A). Second, I determined the level of Rab6 knockdown by isolating RNA from cells treated with siRNA and measuring mRNA levels of Rab6 by qRT-PCR. The amount of Rab6 mRNA continuously decreased over the observed time (Figure 4B) with a maximum knockdown of 86% at 96 hours, the time that the ELISPOT assay is performed. Finally, decrease of Rab6 protein expression upon siRNA treatment was determined by immunofluorescence microscopy (Figure 4C). Rab6 positive and negative cells were counted by Imaris image processing software and there was a significant decrease of Rab6 positive cells after knockdown. The level of intensity of Rab6 staining in each image was measured and compared between both treatment groups. Interestingly, when comparing cells that stained positively with Rab6 antibody in both groups, there was no significant difference in the amount of Rab6 protein after siRNA knockdown. Rab6 positive cells were equally bright in both groups. This suggests that although all cells appear to be transfected with siRNA, some cells retain normal levels of Rab6 protein.

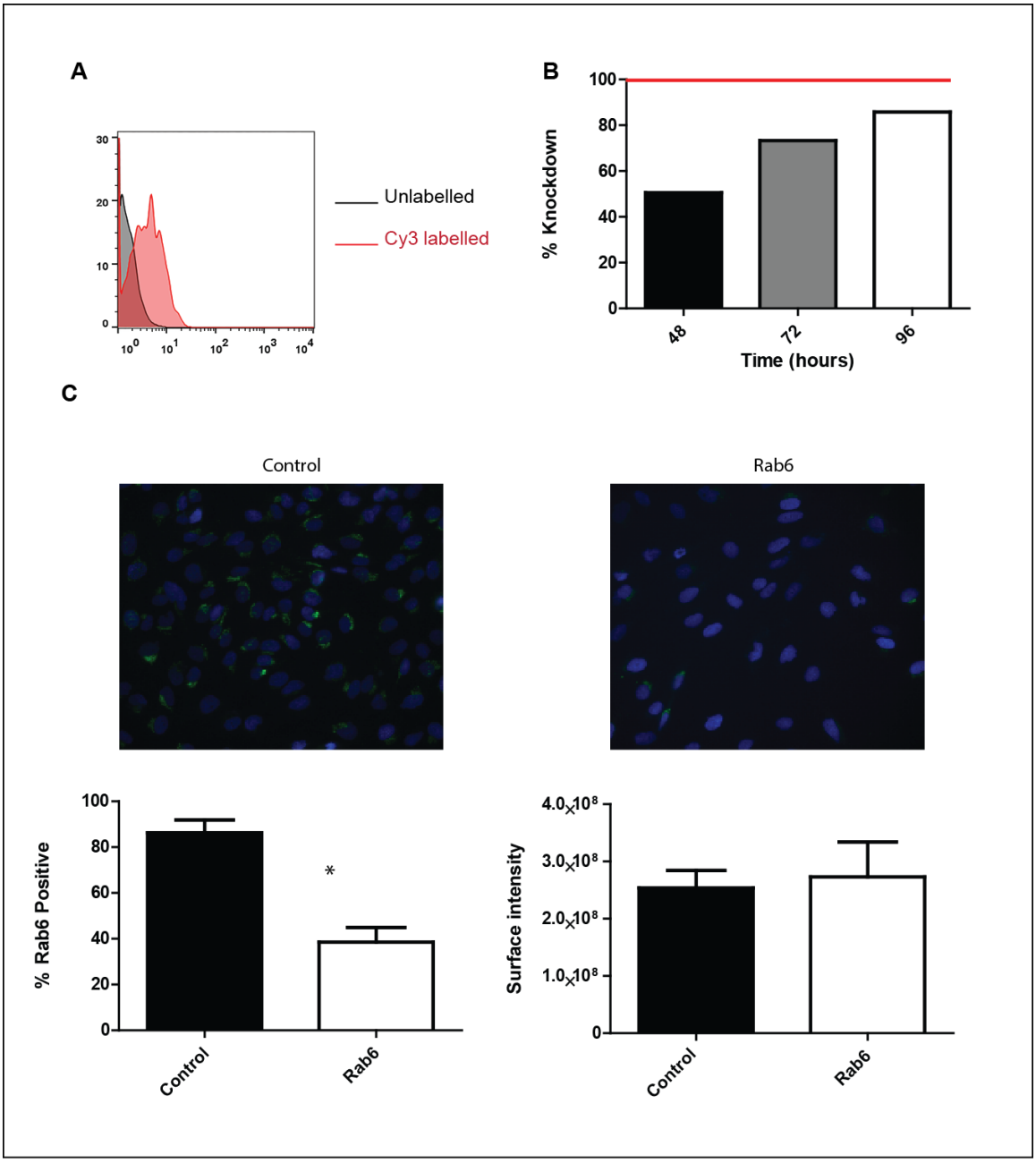

Figure 4. A) BEAS2B cells were transfected with either Cy3-labelled or unlabeled negative control siRNA and incubated overnight. Cells were fixed and analyzed in a FACSCalibur flow cytometer. B) BEAS2B cells were transfected with siRNA and collected at 48, 72 and 96 hours after transfection. RNA was isolated and measured by qRT-PCR. C) BEAS2B cells were transfected with siRNA for 96 hours. Cells were fixed in 4% PFA and stained for Rab6. 10x images of each treatment were analyzed ($p<0.05$).

Surface Expression of MHCI Molecules

One explanation for the decrease of MR1 antigen presentation after Rab6 knockdown is that Rab6 regulates the expression of MR1 on the surface of cells. I used surface staining of MHCI molecules and flow cytometry following Rab6 knockdown to address this possibility. Cells were treated with siRNA and allowed to incubate for 96 hours. Cells were stained with antibodies to either HLA-Ia (HLA-A/B/C), HLA-E or MR1. It is important to note that surface levels of MR1 expression are low in uninfected cells, particularly when compared to classical class I molecules. Furthermore, there was no noticeable change in the level of any of any of these MHCI molecules at the cell surface after Rab6 knockdown (Figure 5). mRNA was extracted from cells and Rab6 knockdown was measured by qRT-PCR with 85% Rab6 knockdown.

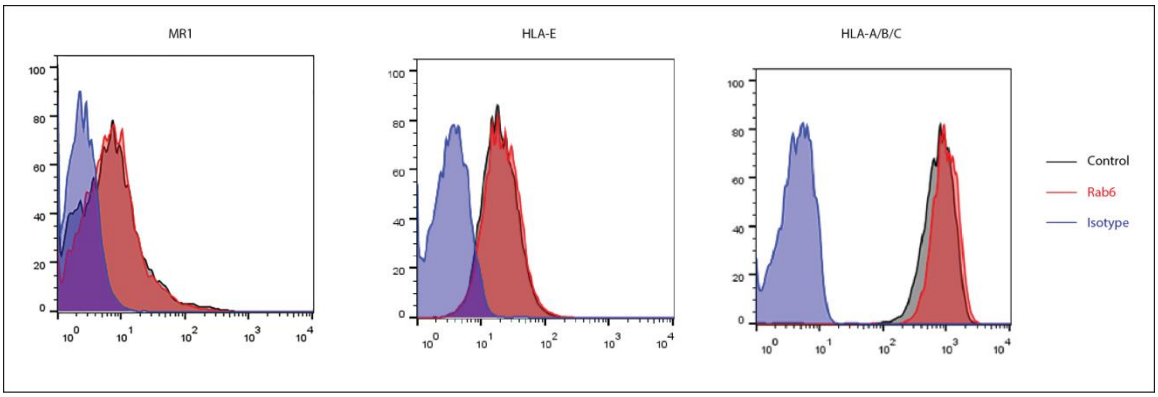

Figure 5. Surface expression of MHCI molecules in siRNA treated BEAS2B cells. Cells were stained with W6/32 antibody to HLA-A, B and C, 3D12 antibody to HLA-E or biotinylated anti-MR1 26.5 antibody. Rab6 mRNA expression was knocked down 85%.

Translocation of MR1 to the Cell Surface

Kjer-Nielsen et al. (31) described how vitamin B metabolites are ligands to MR1. 6-Formyl Pterin (6-FP, Figure 6A), a derivative of folic acid (vitamin B9), was used to determine the crystal structure of MR1. In our laboratory, Melanie Harriff discovered that when BEAS2B cells are expressing human MR1 fused with GFP (MR1-GFP); the molecule localized to vesicles in the cytosol. Upon treatment of cells with 6-FP, MR1 translocates from these vesicles to the cell surface enhancing the amount of MR1 detectable on the plasma membrane. I have replicated these results, as shown in Figure 6B. The increase of surface expression of MR1 upon 6-FP treatment is also measureable in cells expressing endogenous levels of MR1. BEAS2B cells were treated with 6-FP overnight and stained for surface MR1. Although this increase is not visible through fluorescence microscopy as in the case of MR1-GFP overexpression (Figure 6B), it is visible through flow cytometry (Figure 6C).

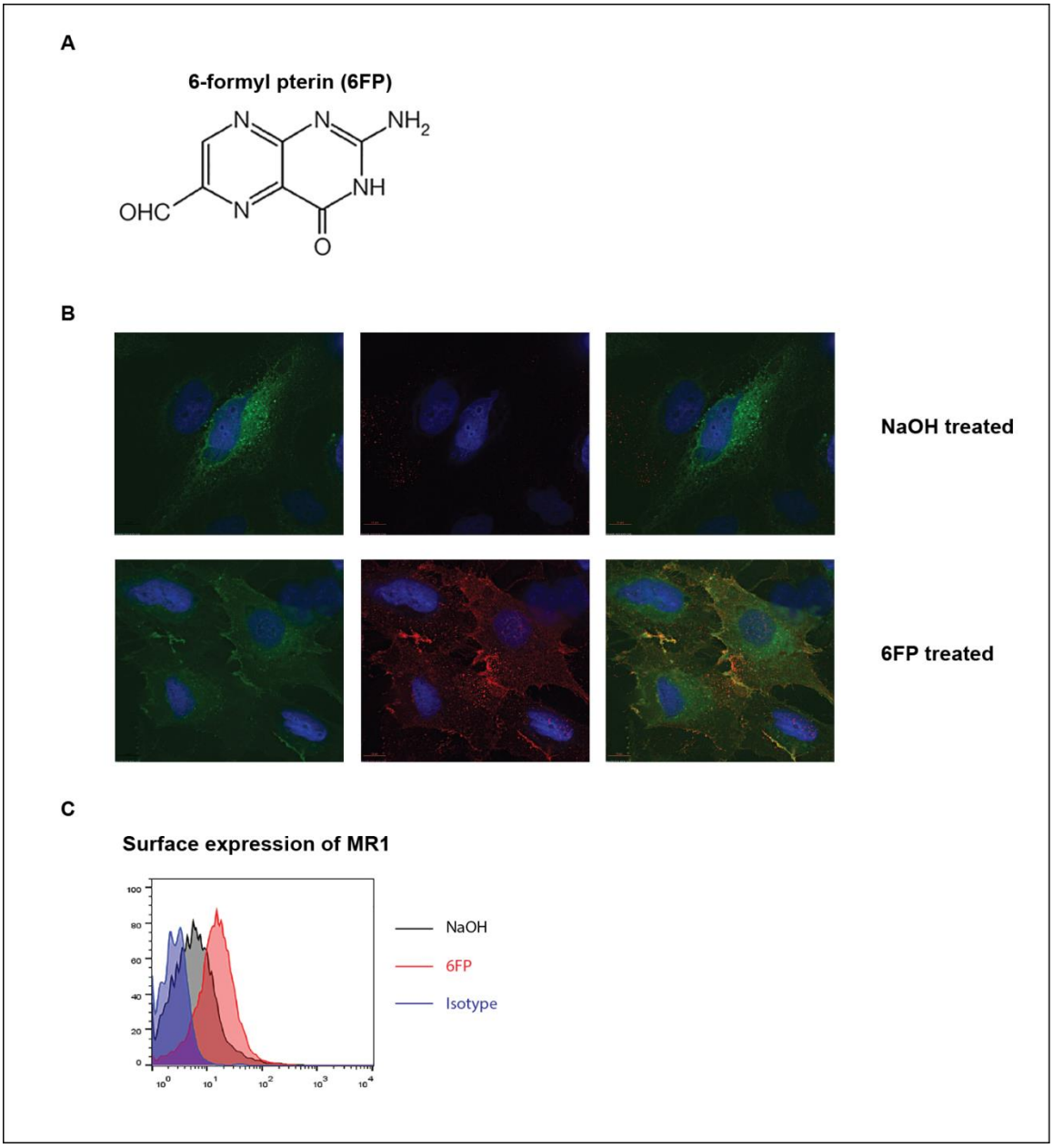

Figure 6. A) Structure of 6-FP. B) Translocation of MR1-GFP vesicles from the cytoplasm to the cell surface. Cells were stained on the surface with anti-MR1 26.5 antibody. C) Surface expression of endogenous MR1. Cells were treated with 6-FP overnight and stained for surface MR1.

The Role of Rab6 in the Translocation of MR1 to the Cell Surface

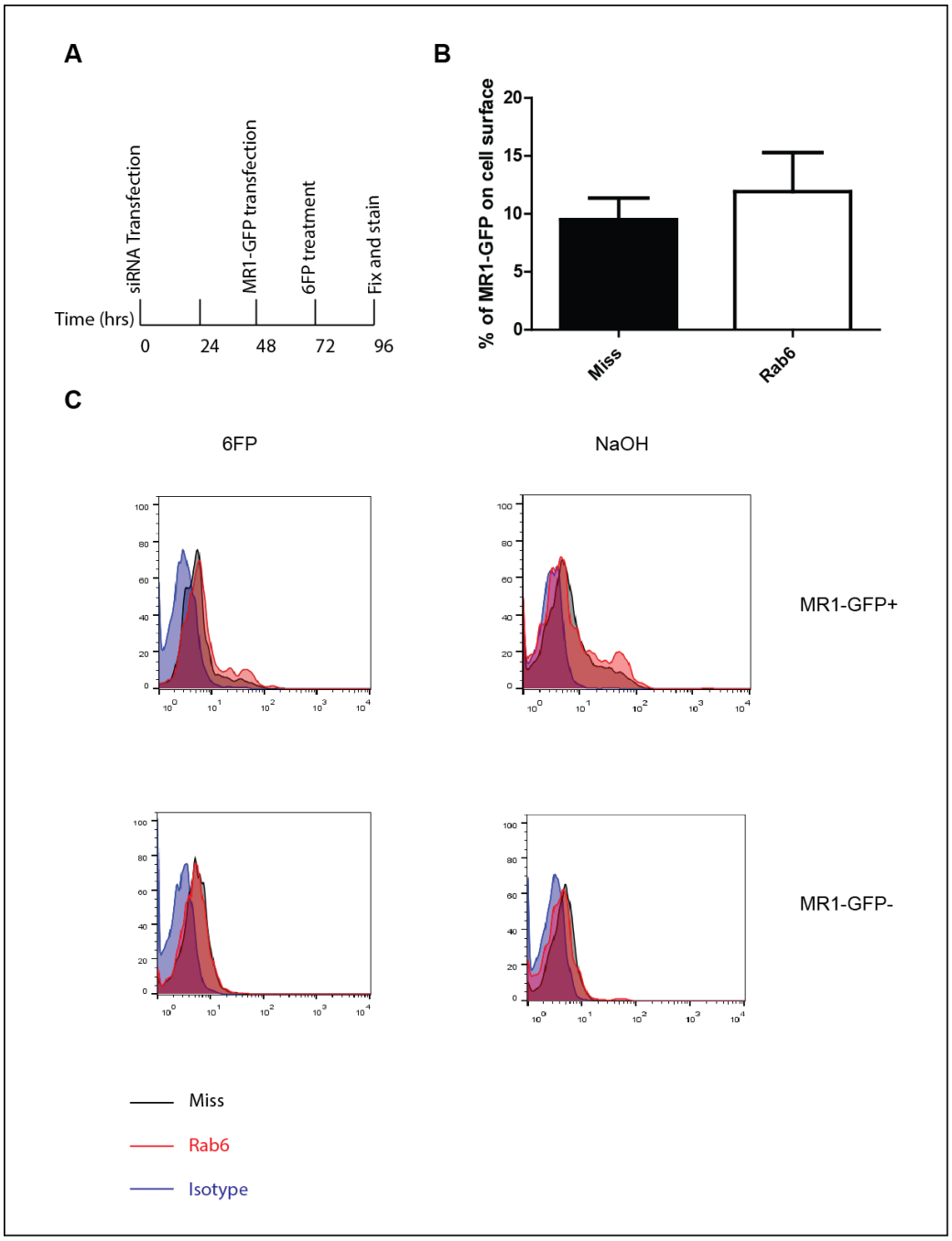

Figure 7. A) Cells were treated with siRNA and transfected 48 hours later with pCI MR1-GFP. At 72 hours cells were treated with 6-FP and incubated overnight. Cells were fixed and stained for both fluorescence microscopy and flow cytometry. B) Cells were stained for surface MR1 and intracellular Rab6. The amount of MR1-GFP colocalized to MR1 antibody on the cell surface was measured in Rab6 positive cells in the control and Rab6 negative in the cell group treated with siRNA directed at Rab6. These are results of two independent experiments. C) Cells were stained for surface MR1. The level of surface MR1 was measured by flow cytometry. Rab6 mRNA was reduced 78%.

I used fluorescence microscopy and flow cytometry to determine the role Rab6 plays in the ability of MR1 to translocate to the cell surface upon binding to the 6-FP ligand. Figure 7A describes how these experiments were performed. Briefly, cells were transfected with siRNA and 48 hours later they were transfected with MR1-GFP. I treated the cells with 6-FP 24 hours later. Finally, the cells were stained and fixed after being treated with 6-FP for 24 hours. For fluorescence microscopy, cells were surface stained for MR1, then permeabilized and stained for Rab6 prior to imaging. MR1 surface expression on cells that were treated with siRNA directed at Rab6 and did not stain with the Rab6 antibody was compared with cells treated with control siRNA and expressed Rab6. The colocalization between MR1-GFP and surface stain of MR1 antibody was measured using Imaris. There was no significant difference ($p=0.5$) in MR1 surface expression between cells where Rab6 was knocked down and cells treated with the negative control (Figure 7B). The surface expression of MR1 was also measured by flow cytometry. Similar to what was observed by microscopy, there was no shift in the surface expression of MR1 in cells treated with siRNA to Rab6 (Figure 7C). This was true for cells overexpressing MR1-GFP (Figure 7C top panels) as well as cells expressing endogenous MR1 (Figure 7C, lower panels). The level of Rab6 knockdown was measured by qRT-PCR. Rab6 expression was reduced 78%. These results suggest that Rab6 is not involved in translocation of ligand-bound MR1 to the cell surface.

Colocalization of MR1 and Rab6

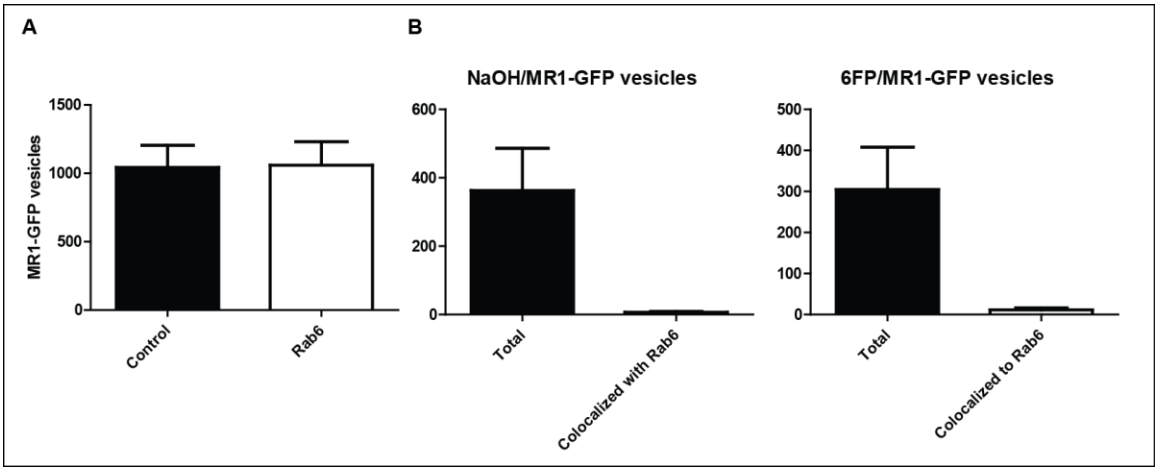

Figure 8. A) The number of MR1-GFP vesicles per cell from images in Figure 7B was counted with Imaris. B) Cells were transfected with MR1-GFP, 24 hours later they were treated with 6-FP and 24 hours later they were fixed, permeabilized and stained for Rab6. Imaris was used to measure the colocalization of MR1-GFP to Rab6.

Rab6 may be regulating the transport of MR1 within the cell. From the analysis of images used in Figure 7B we could not observe any gross changes in the distribution of MR1-GFP around the cell when Rab6 was knocked down and we did not detect a decrease in the amount of MR1-GFP vesicles per cell (Figure 8A). Further analysis on the morphology of MR1-GFP vesicles to determine any other changes cause by Rab6 knockdown in MR1 traffic is recommended. Additionally, I wanted to examine if Rab6 is directly located near MR1 within the cell. Cells were transfected with MR1-GFP and treated with 6-FP and then stained for intracellular Rab6. The colocalization of MR1-GFP with Rab6 in vesicles was analyzed by Imaris. As shown in Figure 8B, there were very few MR1-GFP vesicles colocalized with Rab6. These results suggest that Rab6 does not impact the overall transport of MR1 within the cell, if such effects occur, they are few and they could be transitory.

Rab6 Association with Intracellular Mtb

Because Rab6 knockdown did not affect the ability of MR1 vesicles to translocate to the cell surface upon encountering 6-FP or alter the formation of these vesicles, I next looked at the possibility that Rab6 plays a role in the availability of antigen from the Mtb phagosome. Rab6 may regulate egress of antigen from the phagosome or its transport within the cell. A basic explanation for reduced availability of Mtb antigen after Rab6 knockdown would be an impaired ability of Mtb to infect these cells. To address this possibility, aliquots of 20,000 cells from experiments in Figure 3 were lysed and the lysate was plated. When colonies were counted, equivalent numbers of CFU of Mtb was detected in cells where Rab6 was knocked down and the control (Figure 9A). The number of Rab6 containing vesicles in the proximity of Mtb in infected cells was measured with Imaris software. A small number of vesicles associated with Mtb bacteria in all infected cells (Figure 9B). Further analysis of live cells expressing a fluorescent Rab6 protein will need to be performed to determine whether these Rab6 vesicles are actually interacting with the mycobacteria in the cells.

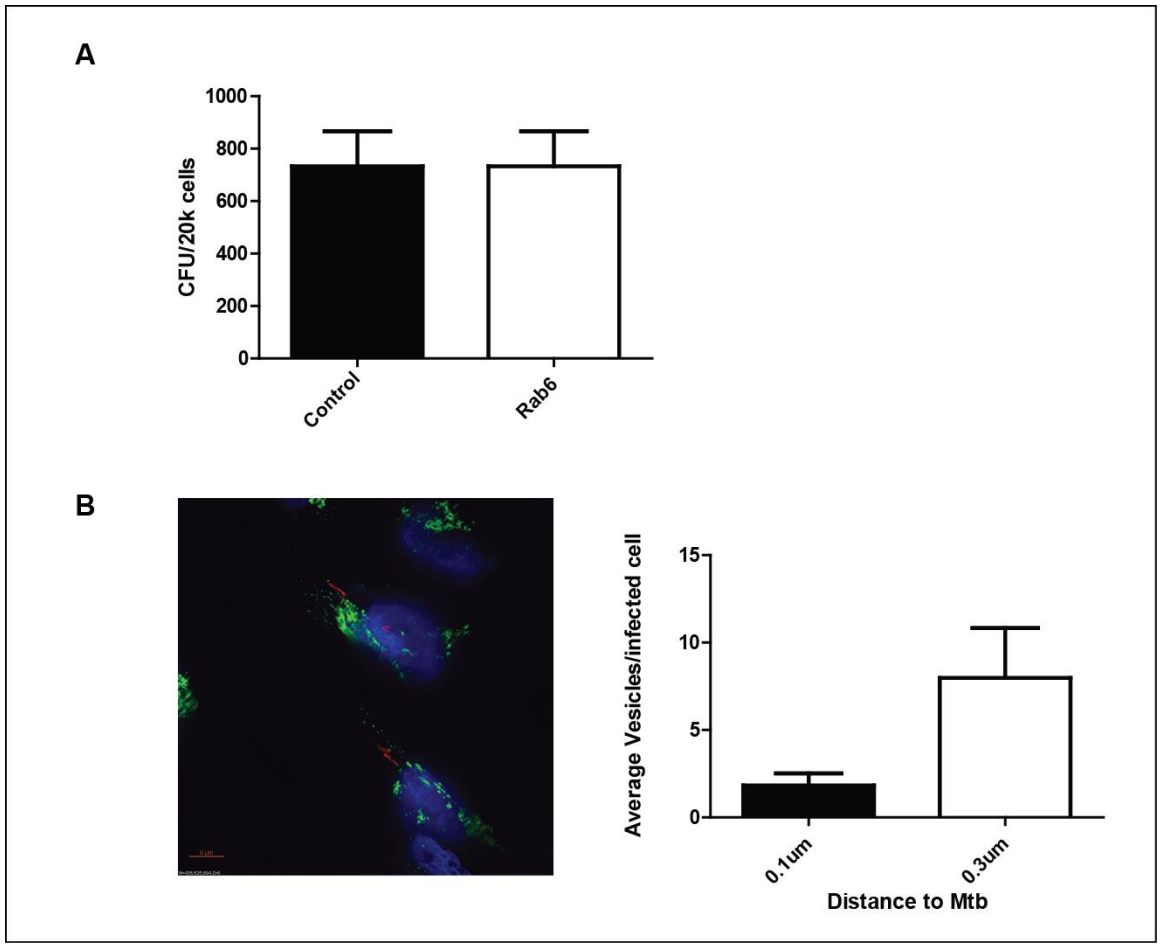

Figure 9. A) Cells from a single experiment in Figure 3 were lysed in 0.01% Tween-20 on ice for 20 minutes. The lysates were plated in 10 fold dilutions in triplicate in 7H10 agar medium plates. B) Cells were seeded in an 8-chamber slide and infected with Mtb overnight. They were then fixed, permeabilized in 0.2% saponin and stained for Rab6. Imaris was used to measure colocalization of Rab6 with Mtb.

CHAPTER 4: Discussion

Rab6 is a vesicular trafficking molecule that regulates retrograde transport from endosomes to the Golgi apparatus and from the Golgi to the endoplasmic reticulum. Additionally, it is involved in exocytic transport from the Golgi to the plasma membrane.

Using both shRNA and siRNA knockdown of Rab6, we determined that Rab6 is specifically involved in the presentation of Mtb antigens on MR1. Our initial observations from the shRNA screen of vesicular trafficking molecules was that three Rab6 isoforms affect antigen presentation to MR1 restricted T cells (Figure 2) while the responses from classically restricted and HLA-E restricted clones did not change. The results in Figure 3 using siRNA confirmed that Rab6 knockdown significantly decreases antigen presentation to MR1 restricted T cells. From these results we can assert that there is less Mtb antigen displayed by MR1 on the cell surface of BEAS2B cells. The classically restricted cell clone utilized in Figure 1 detects the $CFP10_{2-9}$ peptide from the Mtb protein CFP10. Upon incubating epithelial cells with the $CFP10_{2-9}$ peptide and observing the T cell response when Rab6 is knocked down, we can see no significant change in the T cell response. This demonstrates that Rab6 does not play a role in antigen presentation on classically restricted MHCI. $CFP10_{2-9}$has already been processed, so when its presentation is not affected it indicates that only the presentation of this antigen is not affected by Rab6 knockdown. On the other hand, Mtb antigens that come from the bacillus will need to be processed. When cells are infected with Mtb, the antigens it presents on HLA-B45 need to be both processed and presented. Since Rab6 knockdown in the antigen presenting cells infected with Mtb did not affect the response of the HLA-B45 clone, this suggests that the processing of Mtb antigens was not affected either.

I hypothesized that Rab6 impacts Mtb presentation by playing a role in the transport of MR1 to the cell surface. Using fluorescence microscopy and flow cytometry, I determined that Rab6 knock down did not lead to significant changes in the translocation of MR1 to the cell surface in the presence of the ligand 6-FP. Furthermore, Rab6 knockdown did not affect the intracellular composition of MR1 containing vesicles (Figure 8A). Based on these data, future experiments should focus on the role of Rab6 in transport of Mtb ligands from the Mtb phagosome to MR1 vesicles.

In the functional assays to assess the impact of Rab6 knockdown on MR1 antigen presentation, we cannot see a complete inhibition of the T cell response (Figure 3B). Although we had 80% and 90% knockdown of mRNA expression, the reduction in mRNA levels of a target protein does not mean there is a corresponding decrease in expression at the protein level. While siRNA is readily taken up by 100% of the BEAS2B cells (Figure 4A) and relative Rab6 mRNA progressively decrease until the time cells are collected for the ELISPOT assay (4B), fluorescence microscopy indicated that protein knockdown was not complete (4C). Unexpectedly, analysis of Rab6 using fluorescence microscopy indicated that approximately 40% of the cells did not have Rab6 knockdown, while the remainder of the cells had a high level of knockdown. The continued presence of Rab6 protein in some of these cells may be the reason why we do not see a full inhibition of response by the MR1 restricted T cell clone to cells treated with siRNA targeting Rab6. Alternately, there may be multiple mechanisms by which Mtb antigens are processed and presented on MR1. To analyze this possibility in future experiments, cells could be sorted based on Rab6 expression using flow cytometry, to determine whether there is a complete inhibition of the T cell response when these cells are used in an ELISPOT assay. So far, our attempts at detecting Rab6 protein expression by flow cytometry have not been successful. Another possibility is that the fourth isoform of

Rab6, Rab6c, is responsible for the continued presentation of Mtb antigens on MR1 observed in Figure 3B. The siRNA used in this thesis does not target this isoform. Future studies should include co-transfection of the Rab6 siRNA used in this work with an siRNA directed at Rab6c. Such co-transfected cells could then be tested using ELISPOT assays to determine if including Rab6c knockdown decreases the response of MR1 restricted T cells further.

Rab6 is responsible for shuttling cargo out of the Golgi apparatus. It is associated with exocytic vesicles transporting cargo from the Golgi. Separately, it is involved in the retrograde transport between endosomes and the Golgi and from the Golgi to the endoplasmic reticulum (ER). It appears to act in different pathways of retrograde transport including COPI dependent and COPI independent transport. Rab6 can hence be involved in the recycling pathways of proteins that are secreted from the Golgi. The secretion of ligand bound MHCI molecules is carefully regulated by tapasin and it utilizes retrograde transport to recycle these molecules until they are properly loaded with ligand (24). Hence, Rab6 may be involved in transporting MHCI molecules in this quality control pathway. Altering Rab6 function affects the secretion and surface expression of some molecules in the cell (53). Thus, I hypothesized that Rab6 is involved in transporting MR1 to the plasma membrane. To analyze this possibility, the levels of endogenous MR1, HLA-E or HLA-Ia at the cell surface were measured when Rab6 is knocked down, and I found that there was no difference between the control and cells where Rab6 was knocked down (Figure 5). The antibody used to detect MR1 at the cell surface, biotinylated anti-MR1 26.5, is specific to properly folded MR1 and has been described to have some non-specific binding to Fc receptors (56). Hence, it is possible that some of the surface MR1 detected by flow cytometry is actually binding to Fc receptors. Furthermore, there are very low levels of cell surface MR1 in uninfected cells and we do

not know whether these molecules are bound to ligand. To address these issues, our laboratory has recently characterized a way to measure the ligand-dependent translocation of MR1 to the cell surface. When MR1 is overexpressed as MR1-GFP it is localized into vesicles in the cytosol (Figure 6C, upper panels). Upon the discovery that vitamin B metabolites can be ligands to MR1, Melanie Harriff determined that when cells are incubated with 6-FP; MR1 translocates from these vesicles to the cell surface. The increase in cell surface expression of MR1 occurs both with endogenous MR1 (Figure 6D) and with overexpression of MR1-GFP (6C, lower panels). Figure 6D shows a clear shift in the histogram showing surface MR1 when cells are treated with 6-FP as compared to cells treated with NaOH. If Rab6 was playing a role in the translocation of ligand-bound MR1 to the cell surface, we would expect to see a decrease in cell surface MR1 following Rab6 knockdown. I showed however, that knockdown of Rab6 did not affect translocation of as measured by flow cytometry (Figure 7C). The use of an MR1-specific ligand to stabilize MR1 on the cell surface supports the argument that any changes in surface staining identified between control and Rab6 knockdown cells using the 26.5 anti-MR1 antibody are due to changes in MR1 and not as a result of the off-target Fc receptor binding. We used fluorescence microscopy to measure the colocalization of MR1-GFP with the surface staining using the 26.5 antibody to further validate the role of Rab6 on cell surface localization of MR1. This technique also allowed us to specifically analyze the localization of MR1 in cell where Rab6 protein was clearly silenced. Fluorescence microscopy confirmed the flow cytometry results that the fraction of MR1-GFP localized to the cell surface was not significantly different in Rab6 positive and Rab6 negative cells (Figure 7B). Additionally, by measuring colocalized MR1-GFP to the 26.5 antibody, we can assert that we are measuring MR1 molecules with the antibody and not off-target Fc staining.

These results suggested that Rab6 does not play a role in the translocation of MR1 to the cell surface. Another possibility is that Rab6 participates in transporting MR1 between compartments within the cell. This may include bringing MR1 to the site where it meets its antigen. Rab6 does not come in close contact with MR1-GFP vesicles (Figure 8) which serves as an initial observation that Rab6 may not be directly involved in the transport of MR1 within the cell. The images used to produce Figure 7B did not show any observable changes in the morphology or number of MR1 vesicles in Rab6 negative cells further suggesting that Rab6 may not be directly involved in the movement of MR1 containing vesicles. To confirm the lack of an effect of Rab6 knockdown on MR1 vesicles, additional characterization of differences in MR1 vesicles is needed. This could include an analysis of changes in the phenotype of the vesicle. For example, it has been shown that the MR1 vesicle has characteristics of a late endosome (32). Fluorescence microscopy could be used to determine whether Rab6 knockdown changes the association of MR1 with late endosomal markers such as Rab7.

The apparent lack of differences in MR1 vesicles or MR1 translocation to the cell surface raises the possibility that Rab6 may instead be involved in transport of antigen to MR1 vesicles. In Figure 9 we show that Rab6 does not reduce the amount of Mtb entering epithelial cells. Hence we can assume Rab6 does not play a role in the ability of Mtb to enter the cell. Rab6 may be involved in transporting antigens to MR1 or it may be in charge of moving the MR1 antigen processing and loading machinery.

The discovery that MR1 binds vitamin B metabolites suggests that MR1 likely needs the help of a specialized antigen loading and processing complex to properly fold and be presented at the cell surface. These small molecules may not be the only component of an antigenic complex that binds to MR1; they are, however, an important part. MAITs, an MR1 restricted CD8+ T cell subset, can only be stimulated by bacteria and fungi that

synthesize riboflavin, also know vitamin B2 (57). From the observations in Figure 6 we know that there is MR1 loaded with ligand at the cell surface when it binds 6-FP because the shift in MR1 at the cell surface measured by flow cytometry. It is important to note, however, that 6-FP is a metabolite of folic acid or vitamin B9, not riboflavin. It may be the reason why 6-FP is not stimulating of MAITs as compared to riboflavin metabolites. Both 6-FP and riboflavin metabolites do bind to MR1 however (31).

If MR1 possesses such singular ligands, it is possible that the antigen processing and loading machinery for MR1 antigens is different to other MHCI molecules. It has been already established that MR1 does not use the TAP peptide transporter for antigen presentation (11). There may be specialized molecules in charge of transporting small particles like these metabolites. We can hypothesize that Rab6 may be involved in transporting this processing and loading machinery to the location where MR1 encounters its antigen, and that this may account for decreased antigen presentation when Rab6 is knocked down. These MR1 ligands may need to be compartmentalized and MR1 may arrive to these places to exchange endogenous ligands on folded MR1, or bind the pathogenic ligands on unfolded MR1. Rab6 may be involved in creating or maintaining these compartments too.

The process of antigen presentation on MHCI is carefully regulated and surveyed by several molecules including molecular chaperones. Expression of MHCI molecules on the cell surface depends on MHCI being properly folded, bound to β_2-microglobulin and bound to a ligand such as a pathogen-derived antigen (23). Class I molecules bound to suboptimal ligands are not allowed to leave the ER and be brought to the cell surface. As described above, the protein tapasin acts as a chaperone and regulates the binding of high affinity peptides onto MHCI. When the binding to a ligand is not optimal, tapasin directs the retrograde transport of class I so that it can be recycled and antigen loading

can be attempted anew (24). The singularity of MR1 and its ligands can imply that Rab6 may participate in the transport of other chaperones to direct the proper folding of MR1 when ligand binds, to direct the arrival of properly folded MR1 to the cell surface, or direct the recycling of MR1 loaded with suboptimal antigens.

If Rab6 is directly involved in transporting Mtb antigens or the antigen processing and loading machinery from the phagosome, it may mean that Rab6 directly associates with the Mtb phagosome. Proteomic analysis of the Mtb compartment in bone marrow-derived BMA3.A3 macrophages did not indicate the presence of Rab6 associating with the Mtb phagosome (58). Interestingly, however, Rab6 and its effector, BICD1, have been localized to inclusions of *Chlamydia trachomatis*, another pathogen that inhabits a membrane bound compartment (44,45). The *Mycobacterium bovis* BCG phagosome does associate with Rab6 for a long period of time (59). Although not in large numbers, Rab6 vesicles were observed in close proximity to Mtb in this thesis (Figure 9B). However, these images are representative of a dynamic process that has been fixed in time. The association of Rab6 with the phagosome, if any, may be rapid and/or transient similar to other Rab proteins such as Rab8 and Rab22 (60). Additional imaging experiments using live cells are needed to capture dynamic cell trafficking events to determine whether these vesicles are specifically associating with Mtb.

Summary and Conclusions

Rab6 plays a role in the presentation of antigen from *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* to MR1 restricted T cells. However, Rab6 is not involved in the formation of MR1 vesicles in the cell or in the translocation of MR1 to the cell surface in the presence of the known ligand 6-Formyl Pterin. Rab6 does not appear to associate directly with vesicles containing MR1, or associate in large numbers with the

mycobacterial phagosome. In conclusion, Rab6 is likely to be involved in processes other than transporting MR1 to the cell surface or moving MR1 vesicles within the cell. Future studies should address the role of Rab6 in transport of mycobacterial antigens to MR1 vesicles.

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