DISSERTATION

DEFINING INTERACTIONS BETWEEN *MYCOBACTERIUM LEPRAE* AND LANGERHANS CELLS

Submitted by

Darcy Fletcher

Department of Microbiology, Immunology, and Pathology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2021

Doctoral Committee:

Advisor: John Belisle

Marcela Henao-Tamayo
Mark Zabel
Ann Hess

ABSTRACT

DEFINING INTERACTIONS BETWEEN *MYCOBACTERIUM LEPRAE* AND LANGERHANS CELLS

Leprosy is a chronic infection that affects the skin and peripheral nerves. Written accounts of the disease date back to at least 600 BC. *Mycobacterium leprae*, the causative agent of leprosy was first discovered by Dr. Gerhard Armauer Hansen in 1873. Leprosy remains a major health problem in several low- and middle-income countries including Brazil, India, and Indonesia. There are numerous clinical presentations of the disease which presents many challenges for controlling the disease including diagnosis, treatment regimen and duration, and occasional instances of drug resistant cases. Further challenges exist in studying the disease, knowledge of the intricate interactions with innate immune cells has made advances in some cell subsets but is limited in others leaving an incomplete picture of the disease. These gaps limit advances in disease management.

M. leprae is an obligate, intracellular pathogen that grows preferentially between 33-35° C and selectively invades peripheral nerves and skin-resident innate immune cells including macrophages. Numerous host cells including macrophages and Schwann cells have been studied to understand their interaction with *M. leprae*, but other skin-resident immune cells like dendritic cells, specifically Langerhans cells, have not been studied as extensively. The findings that *M. leprae* antigens can be presented via CD1a on Langerhans cells has spurred interest in understanding how Langerhans cells interact and uptake *M. leprae* leading to downstream effects on T cell activation and overall immune responses. The hypothesis of this study is that *M. leprae*

interacts with Langerhans cells via various cell surface receptors that influence a Th1 or Th2 immune response.

This study interrogates the complex interactions between *Mycobacterium leprae* and Langerhans cells via multiple cell surface receptors. In Chapter 2, an *ex vivo* optical tissue clearing method was modified for fragile skin samples to analyze innate cell recruitment to the site of infection. Colocalization between Langerhans cells and a closely related mycobacterial spp. to *M. leprae, M. haemophilum,* was observed in a 3D optically cleared tissue. These observations indicate that wholistic insight of bacteria/innate immune cell interactions can be gleaned using experimentally infected tissues or human skin biopsies. Chapter 3 presents the contributions from multiple cell surface receptors present on Langerhans cells in recognizing and binding *M. leprae*. Langerin was found to play a role in binding *M. leprae*, however, was not the only cell surface receptor involved in recognition of *M. leprae*. $CD5^{+}$ Langerhans cells can be separated into $CD5^{high}$ and $CD5l^{ow}$ LCs that have differences in binding capacity for *M. leprae*.

This study builds the foundation to explore the wholistic contributions of Langerhans cells interactions and uptake of *M. leprae*. Further work should be conducted to identify *M. leprae* ligand(s) for CD5 and downstream effects on cytokine secretion and T cell activation.

ACKNOWLEDGEMENTS

First, I would like to acknowledge my advisor Dr. John Belisle for his incredible support. By accepting a “lab orphan” into his laboratory, he provided me the opportunity to work on a stimulating and adventurous project that allowed me to continue honing my skills as an immunologist. He was always willing to try to work through problems with me but pushed me to lean on the community of experts within our laboratory, our department, and our collaborators around the country. It pushed me to find my confidence and collaborative skills to solve challenging problems. I would also like to thank my graduate committee: Drs. Marcela Henao-Tamayo, Mark Zabel, and Ann Hess for going above and beyond to provide guidance on a challenging project that required expertise from multiple facets of our department. I am also exceptionally grateful to the past and present members of the Belisle lab for all of their guidance and friendship. The love and support found within the lab is unique and helped shape the person and scientist I have become. Last, but not least are all of the incredible members of the MIP department. I feel like I was “raised” by a village and I could not be more thankful for the time and energy each and every member of our department poured into my success.

I would like to thank all of my friends who have supported me through this journey and provided much needed distractions from science. Importantly, I would like to thank my husband, parents, and sister for providing endless amounts of encouragement on my lowest days and celebrating my accomplishments. Lastly, my dog Doug deserves recognition for providing endless hours of companionship and my goat, Hope, for being my reason for falling in love with science.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: LITERATURE REVIEW

1.1 LEPROSY

Leprosy, also known as Hansen's disease, was named after Gerhard-Henrik Armauer Hansen who discovered *Mycobacterium Leprae*, the causative agent, in 1873. Prior to his discovery, the disease was thought to be hereditary. Leprosy is a chronic infection that affects the peripheral nerves and skin (1). Typically, skin lesions are the first symptom that is recognized, appearing as nodules, discolored patches, thick or stiff skin, or painless ulcers. The damage to peripheral nerves caused by *M. leprae* results in numbness in affected areas, muscle weakness, or enlarged nerves. If the disease is left untreated, permanent and severe impairment and disability can result. Patients with untreated leprosy show signs of paralysis in hands and feet, shortening of toes and fingers due to reabsorption, non-healing ulcers, blindness, and/or nose disfigurement (4, 10, 36).

Leprosy has historically been associated with severe stigmas and accounts of individuals being ostracized due to the debilitating symptoms. Even though the causative agent has been known since 1873, progress in understanding the disease and developing treatments has been slow. Numerous countries still have endemic leprosy. In 2019, over 200,000 cases of leprosy were detected in 118 countries. Almost 80% of new cases reported come from Brazil, India, and Indonesia. An accurate incidence of leprosy is challenging to calculate. Typically, the number of new cases is used as a substitute for incidence, even though those numbers tend to be lower. Because it potentially takes years to cure patients using multidrug therapy, the overall number of cases is likely higher (3).

The World Health Organization (WHO) has put forth a new strategy to achieve interruption of transmission and zero new cases by 2030. There has been a long line of strategies and goals through the years set forth by the WHO to reduce new cases and minimize or eliminate ostracization due to the disease. The key to successful strategies has been the willingness of countries to implement recommendations put forth by the WHO. The current strategy has 4 pillars which include combatting stigma, managing leprosy complications and preventing new disability, increasing prevention and active case detection, and implementing zero-leprosy road maps in all endemic countries. This strategy and previous strategies put forth by the WHO have led to dramatic decrease in the incidence of leprosy. There has been a current leveling off to the current number of new cases (~200,000) per year which included approximately 5% of cases with visible deformities. This equated to 40% reduction from the 2014 report (5).

1.1.1 THE PATHOGEN AND IMPORTANT PATHOGENICITY FACTORS

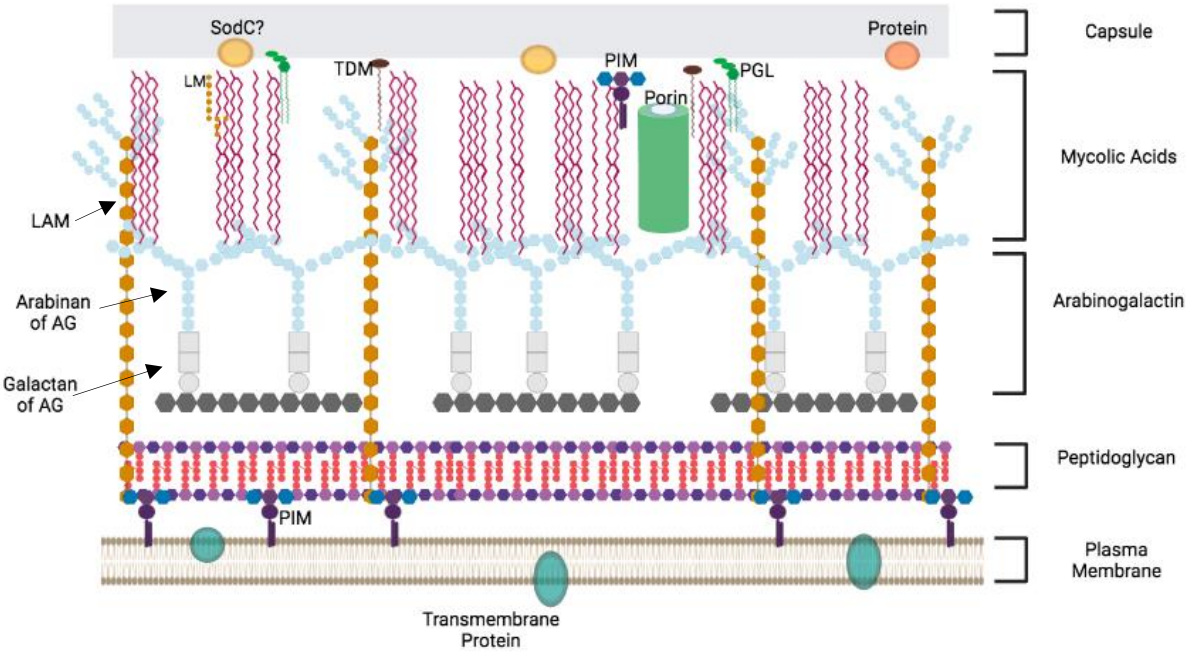

Figure 1: Mycobacterial cell envelope structure (Drawn based on Brennan,1995 (37) and Rotcheewaphan, 2016 (94))

M. leprae is an acid-fast, obligate intracellular pathogen and cannot be cultured in a laboratory. It has a doubling time of 14 days with an optimal growth temperature of 33° C which corresponds to the temperature in the peripheral nerves of the skin, hands, feet, and face. Since *M. leprae* cannot be cultured in a laboratory, the only source to obtain live bacilli is from the footpads of athymic *nu/nu* mice. A typical yield is up to 10^{10} bacilli per footpad. Armadillos are a source for large quantities of *M. leprae*, but typically the bacteria are dead and less useful for laboratory experiments. Other knockout mouse models are available, but typically have limitations only representing one or two presentations of the disease (33, 34, 66).

Aerosol transmission is thought to be the most likely mode of transmission with aerosol droplets taken up through nasal airway mucosa, but other modes of transmission like exposure to environmental sources including soil, nine-banded armadillos, and red squirrels are also possible

(2, 72-76). The incubation period ranges from months to years and several risk factors have been established for contracting the disease including genetic relatedness and close contact with lepromatous patients (i.e. those individuals with high bacterial loads). Other risk factors can include low education level, food insecurity, water exposure, armadillo exposure, lack of BCG vaccination, and soil exposure (47). Low education levels may be correlated to decreased health knowledge and behaviors. Low socioeconomic status may increase risk for contracting leprosy due to poor or crowded living conditions. Person-to-person contact inside households is one of the highest sources for leprosy transmission and may be exacerbated in crowded households (24, 65).

Multiple cell envelope components of *M. leprae* including PGL-1, LAM, and SodC (structural location depicted in figure 1) have been studied and are shown to be associated with various modes of entry into specific cells of the immune and nervous systems. The mycobacterial cell envelope is made up of the plasma membrane, a typical phospholipid bilayer. Exterior to the cell membrane is the cell wall composed of peptidoglycan that is covalently attached to arabinogalactan with mycolic acids esterified to the terminal arabinose units. A mycobacterial outer membrane (the mycomembrane) is associated with the mycolic acid layer. This mycomembrane is populated with glycolipids and highly hydrophobic aliphatic lipids (37). Phosphatidylinositol mannosides (PIMs), lipomannans (LM), and lipoarabinomannans (LAM) are the most abundant and highly conserved components in all M*ycobacterium* spp. and have been the subject of numerous studies to elucidate their role during infection (37, 38-45, 48-51, 78-79). Lipoarabinomannan, LAM, has been shown to induce complement activation which results in myelin loss and axonal damage (8, 77). Additionally, phenolic glycolipids are associated with the mycomembrane. Phenolic glycolipid 1, PGL-1, was discovered in the early

1980s as being unique to *M. leprae* (53). It is a highly antigenic lipid and evokes a high titer of IgM antibodies in lepromatous leprosy patients. PGL-1 has been implicated in the tropism of *M. leprae* for Schwann cells. It has also been shown to increase recruitment of macrophage subsets more permissible for infection by increasing expression of CCL2 on macrophages (7). It also modulates macrophage functions resulting in nerve demyelination. PGL-1 in cultured macrophages altered inflammatory mediator expression when used as a prestimulus, demonstrating inhibitory effects on cytokine induction in response to a second stimulus (8, 85). The structure of PGL-1 is based on the aliphatic lipid phthiocerol mycocerosic acid, an abundant cell mycomembrane component. However, the glycosylation observed in PGL-1 is essential for the biological activities of this molecule (8). Trehalose glycolipids that are based on the glycosylation of free mycolic acid are also present in the mycomembrane of *M. leprae* (86), however, their role in leprosy pathogenesis is not well defined.

1.1.2 SPECTRUM OF DISEASE

Classification of various forms of Leprosy utilizes the Ridley-Jopling scale (1, 67-71). Leprosy's clinical manifestations fall on a spectrum between two poles, tuberculoid (TT) and lepromatous (LL). There are several intermediate forms including indeterminate, borderline tuberculoid (BT), borderline borderline (BB), and borderline lepromatous (BL). Patients with lepromatous leprosy have a high burden of bacilli in skin lesions which leads to the classification as multibacillary (MB). These patients typically have multiple skin lesions, thickened peripheral nerves, loss of eyebrow hair, and thickened skin. The immune response for lepromatous leprosy is dominated by a humoral response. Patients with tuberculoid leprosy have a low burden of bacilli which leads to the classification of paucibacillary (PB) and few skin lesions. The immune

response for tuberculoid leprosy is dominated by a cell-mediated response. A small number of cases present as pure neural leprosy without skin symptoms. This creates a challenging clinical diagnosis due to the lack of skin lesions and negative skin smear. Often more advanced techniques are required to diagnose these individuals and they are misdiagnosed due to the lack of advanced techniques in clinics (23).

The World Health Organization (WHO) has classified leprosy into two groups, multibacillary and paucibacillary disease, for practical purposes and ease of diagnosis. Individuals with more than five skin lesions, more than one nerve involvement, or a positive skin smear are classified as having multibacillary disease. Paucibacillary disease is determined by fewer skin lesions, no nerve involvement, or a negative skin smear. These classifications become extremely important during treatment (5).

In addition to multiple classifications of the disease, there are multiple reaction episodes that can occur (1). Type 1 reactions, also known as reversal reaction, occurs from an increased cellular immune response leading to inflammation in the skin and nerves in BT, BB, and BL forms of leprosy. Type 1 reactions are diagnosed when erythematous patches appear over pre-existing lesions. These reactions can occur prior to treatment, during treatment or after the completion of successful treatment. Type 2 reactions, erythema nodosum leprosum (ENL), occur in lepromatous and borderline patients. This is characterized by painful erythematous nodules accompanied with fever, malaise, and arthritis. ENL can affect up to 50% of leprosy patients. Type 2 reactions are triggered by the release of mycobacterial antigens which trigger formation of immune complexes. These immune complexes deposit in tissues and activate the complement cascade leading to migration of neutrophils and activation of T-cells and macrophages (68).

1.1.3 TREATMENT

Leprosy is curable. If treatment is provided in the early stages of infection, it also aids in averting disability. Multidrug therapy which includes two to three antibiotics concurrently has been adopted by the WHO as the accepted treatment for leprosy. In accordance with the WHO's new strategy, treatment is free of charge through at least 2025. Tuberculoid leprosy (low bacilli) is treated with a daily dose of dapsone and a monthly dose of rifampicin. Lepromatous leprosy (high bacilli) is treated with a daily dose of clofazimine in addition to dapsone and rifampicin (1). Treatment for tuberculoid leprosy can take at least six months and lepromatous leprosy can take at least 12 months, but can last for up to two years, if necessary. Often, patients do not come forward for treatment due to continued discrimination and social stigma. The longer patients wait to be treated, the higher their chance of long-term disabilities (6, 23).

1.1.4 IN VITRO MODELS (MACROPHAGES, SCHWANN CELLS)

Numerous studies have demonstrated interactions between *M. leprae* and macrophages or Schwann cells. Schwann cells and macrophages share a few similar cell surface receptors including CD206 (mannose receptor), and CD209 (DC-SIGN) (11).

Schwann cells are the principal glial cell of the peripheral nervous system. They provide support and nutrition to the axons of neurons and myelinate peripheral nerves. One Schwann cell makes up one myelin sheath on a peripheral axon. This means that multiple Schwann cells are needed to myelinate the length of an axon. Schwann cells have extremely high lipid content with cholesterol being particularly important for assembling the myelin sheath (11, 62). *M. leprae* has a high affinity for Schwann cells and leads to eventual demyelination events. Initially, colonization results in loss of sensation including loss of temperature sensation and

decreased touch sensation. *M. leprae* binds to the globular domain of the α2 chain of laminin-2. Laminin-2, a laminin isotype, is a major component of the basal lamina that surrounds the Schwann cell/axon units (52). Two components of *M. leprae* have been identified to bind to Schwann cells, PGL-1 and Hlp. Hlp is the 21-kDa histone-like protein that is conserved among species of mycobacteria. PGL-I provides the main interaction with Schwann cells and Hlp acts in a secondary role to increase avidity. Furthermore, it has been shown that CD206 (a mannose receptor on macrophages and Schwann cells) is upregulated early time points after infection by *M. leprae* (12).

Macrophages have been extensively studied during *M. leprae* infections (17, 22, 54, 64). Each clinical presentation of leprosy seems to associate with a different macrophage population, M1 or M2. The proinflammatory M1 macrophages are present in paucibacillary or tuberculoid leprosy while M2 are found in lepromatous skin tissues (22). The infection of macrophages or Schwann cells by *M. leprae* results in the accumulation of lipid droplets and the characteristic "foamy" appearance that is observed in biopsies from leprosy patients. The accumulation of lipid droplets is also associated with increased cholesterol uptake and synthesis (91). *M. leprae* incorporates cholesterol and converts it to cholestenone (91). A process that is hypothesized to influence the pathology of leprosy. (18,22).

1.2 SKIN BIOLOGY

Skin is composed of two layers, epidermis and dermis, that contains innate immune cells, nerves, blood vessels, hair, and excretory glands. Typical adult skin thickness is 1-2 mm but can vary with anatomical location.

The epidermis is the outermost layer of the skin and provides a waterproof barrier and initial immune defense. It contains a population of keratinocyte stem cells at the basement membrane; the cells move outwards to repopulate the outer layer which is shed continuously. Migrating keratinocytes do not further divide and terminally differentiate. A few other cell types are also found in the epidermis including melanocytes, Merkel cells, and innate lymphoid cells. Melanocytes provide each individual with a unique skin color and represent a very small percentage of cells in the epidermis. Oxidation of the amino acid tyrosine produces melanin which is packaged into organelles and donated to neighboring keratinocytes. Merkel cells are neuro-sensory receptors that have synaptic connections to somatosensory afferent nerve axons. They enable perceptions of shape, sensation of light touch, and texture. Innate lymphoid cells are derived from bone marrow and lack B-cell or T-cell surface markers. They have key roles in maintaining homeostasis and inflammation. The epidermis also contains a unique dendritic cell subset, Langerhans cells, that will be discussed later in this chapter (15).

The dermis contains mesenchymal cells and numerous resident innate immune cells including many different subsets of dermal dendritic cells. It also is predominately comprised of an extracellular matrix containing proteins, proteoglycans, lipoproteins, and glycolipids. This matrix provides the mechanical stability to the skin and acts as a reservoir of blood, immune cells, metabolites, enzymes, growth factors, and cytokines. Numerous resident innate immune cell subsets are present in the dermis including many dermal dendritic cell subsets and mast cells.

Dermal dendritic cells have mixed phenotypes and function. Mast cells contribute to wound healing, angiogenesis, autoimmunity, and chronic inflammation (14).

The epidermis has numerous types of lipids. Sebaceous lipids are non-polar lipids such as triglycerides, wax esters and squalene. Epidermal lipids are a mixture of ceramides, free fatty acids, and cholesterol (20). Sphingolipids are also present and dysregulation of sphingolipid metabolism can lead to inflammatory skin diseases such as atopic dermatitis (21).

As a first layer of defense, the skin is susceptible to wounds that can range in severity. Superficial wounds only affect the epidermis and heal rapidly with no scarring. Partial-thickness wounds penetrate into the dermis and often heal with scarring and some loss of function. Full-thickness wounds penetrate beyond both the epidermis and dermis and typically have extensive scarring. Chronic wounds do not progress to healing and can be caused for numerous reasons including leprosy (13).

M. leprae has been demonstrated to interact with Langerhans cells (31). It has a lower growing temperature (30° C) and tropism for cells within the skin, including peripheral nerve cells, macrophages, and dendritic cells (4). Presentation of the disease is accompanied by the presence of skin lesions. Based on the cells *M. leprae* infects, it resides in both the epidermis and dermis of the skin resulting in partial-thickness to full-thickness wounds if disease progression occurs. Lesions are characterized by the presence (or absence) of *M. leprae* as well as infiltrating innate immune cells that will be discussed further in the following section.

1.3 INNATE IMMUNOLOGY

The innate immune system is the initial, general response to a foreign (non-self) target. It is made up of physical barriers such as the skin and general immune responses such as inflammation and complement. The innate immune system targets non-self particles such as bacteria, viruses, parasites, and the pathogen associated molecular patterns (PAMPs) produced by the pathogens. It can also target pollen and dust leading to allergies. *M. leprae* interacts with numerous innate immune cells including neutrophils and antigen-presenting cells (18, 35, 80). The activation of these cells can occur after interaction of PAMPs with pattern recognition receptors (PRRs). PRRs involved with *M. leprae* infections include C-type lectins, Nod-like receptors, RIG-1 receptors, and toll-like receptors. The innate immune system also utilizes molecules called cytokines and chemokines. Cytokines provide cell-to-cell communication and initiate an immune response and cell trafficking. Chemokines are released by infected cells to initiate an immune response, warn neighboring cells of the pathogen, and act as chemoattracts for inflammatory cells.

The complement system acts as a part of the innate immune system. It is made up of a variety of proteins that can be activated, come together and initiate the complement cascade through multiple steps. Opsonization is a process where foreign particles are marked for phagocytosis. Chemotaxis attracts macrophages and neutrophils to a chemical signal at a site of infection. Cell lysis destroys the membrane of a foreign cell and weakens their ability to proliferate and spread infection. Finally, agglutination uses antibodies to cluster and bind pathogens together where immune cells can mount an attack. These different steps complement the innate immune system and facilitate the "search" and removal of antigens. Studies have shown that *M. leprae* can enter some innate immune cells via complement receptors (9, 19, 63-

64). The complement component, C3, binds to PGL1 on *M. leprae* to form a three-component complex with C3, PGL1, and complement receptors on innate immune cells which mediates phagocytosis of *M. leprae* (63).

Another mechanism, autophagy, utilized by the innate immune system degrades and recycles organelles and proteins through the lysosomal system. This promotes cellular and organismal homeostasis and is utilized during infections to help degrade intracellular microbes. Autophagy has been demonstrated to play a role in leprosy polarization. In lepromatous macrophages, an inhibition of autophagy has been observed while in reversal reaction episodes, autophagy is restored (18). Further, autophagy contributes to an antimicrobial response in Langerhans cells which allows other dendritic cell subsets to process and present antigens to T-cells and subsequently instructing T-cells what type of response to exhibit (55).

There are numerous cell types that are classified as innate immune cells and include macrophages, mast cells, neutrophils, eosinophils, basophils, natural killer cells, and dendritic cells. Each cell type has a unique function and multiple phenotypes are observed within each cell type. Mast cells are found in mucous membranes and connective tissues. When activated, they release cytokines and granules that create an inflammatory cascade and recruit other immune cells like neutrophils and macrophages. Neutrophils are phagocytic cells that contain granules that are released when they explode and generate neutrophil extracellular traps (NETs) which immobilize and kill invading pathogens. Macrophages are also phagocytic cells that can release cytokines to signal and recruit other immune cells to a site of infection. Natural killer cells destroy infected host immune cells to stop the spread of an infection. Dendritic cells are antigen-presenting cells that are located in tissues such as the skin where they can identify pathogens and act as a messenger for the immune system via antigen presentation (18).

Several innate immune cells including macrophages, dendritic cells, and neutrophils have been recognized as key players in the pathogenesis of leprosy as well as other cells in the skin including keratinocytes and Schwann cells. Macrophages present in both an inflammatory M1 phenotype and anti-inflammatory M2 phenotype. Each clinical presentation of the disease typically associates with different macrophage phenotypes, however; multiple phenotypes can be found in all classifications of the disease. M1 macrophages are observed in paucibacillary lesions while M2 macrophages are often observed in lepromatous skin tissues (18). Dendritic cells may be one of the first innate immune cells to interact and encounter *M. leprae* and can effectively present antigens to T-cells (92). Dendritic cells can be found in both the epidermis (Langerhans cells) and dermis (dermal dendritic cells). Langerhans dendritic cells express both langerin which is unique to Langerhans cells and CD1a which can also be found on other dendritic cell phenotypes. $CD1a^{+}$ dendritic cells were found predominately in tuberculoid leprosy. Another dendritic cell marker, CD209, may function as a receptor for *M. leprae* where *M. leprae* can subsequently decrease the capacity of dendritic cells to induce T-cell responses (18). Other cells in the skin are also involved including keratinocytes and Schwann cells. Keratinocytes expressing ICAM-1 were found in tuberculoid leprosy, but not in lepromatous leprosy indicating local inflammatory responses. Other inflammatory cytokines are also present in the epidermis including TNF, IL-6, and IL-12 in tuberculoid leprosy. Further, involvement of infected Schwann cells can result in peripheral neuropathy and detrimental nerve damage (18). Subsequent sections in this chapter will dive further into dendritic cells and specifically, Langerhans cells.

1.3.1 DENDRITIC CELLS

There are numerous subsets of dendritic cells in humans. DCs were first discovered in 1973 by Ralph Steinman (25). DCs are professional antigen presenting cells that bridge that gap between the innate immunity and adaptive immunity by processing antigens for presentation to T cells. They have the capacity to activate naïve T-cells and induce effector differentiation and are involved in immune tolerance under homeostatic conditions. Human DCs have high expression of MHC-II and CD11c on their cell surface. They also express numerous other cell surface receptors that allow them to be classified into subtypes (29).

There are two functional states, mature and immature (16). There are many features that distinguish these states, but the main characteristic of a mature DC is the ability to activate antigen-specific naïve T-cells. The transition from an immature to mature DC is triggered by the recognition of pathogen associated molecular patterns (PAMP). Once DCs mature, they lose adhesive structures and increase motility ensuring access to naïve T-cells which trigger antigen-specific immune responses. The differentiation of T-cells is influenced by their interaction with DCs and result in various T helper subsets including Th1, Th2, and Th17 cells. Each subset of T-cells has different functions and secrete different cytokines. Immature DCs are not passive bystanders in the immune system, but instead are efficient at antigen capture due to their high endocytic capacity. They act as tissue scavengers to detect invading pathogens. DCs are also capable of cross-presentation (28). This allows DCs the ability to prime the other branch of T-cells, $CD8^+$ T-cells, via presentation of acquired exogenous antigens on MHC-I molecules.

Under steady-state tissue conditions, DC progenitors derive from the bone marrow and give rise to tissue specific DCs that include DCs like Langerhans cells and dermal DCs. Dermal DCs express CD14+ and CD1a+ cell surface receptors (27). Along with Langerhans cells,

dermal DCs continuously survey for foreign antigens under steady-state conditions. Under inflammatory conditions, DCs change their phenotype and upregulate cell-surface markers. Functional changes also occur including their ability to efficiently prime T cells and cross-present (27).

Human DCs arise from $CD34^+$ stem cells that give rise to myeloid and lymphoid precursors of which myeloid precursors can differentiate into DCs (27). Specific subsets like plasmacytoid DC (pDC) and conventional DC (cDC) are influenced by signals derived from the microenvironment. cDCs can be further divided into two subpopulations, cDC1 and cDC2. The cDC1 subpopulation efficiently primes $CD8^+$ T-cells. The cDC2 subpopulation induces Th1, Th2, and Th17 responses. Typically, cDC2 have regulatory roles. These two cDC subtypes differ in TLR expression which results in their different roles. pDC secrete high levels of IFN-α/β and are extremely important in viral infections.

DCs express various C-type lectin receptors that are crucial for tailoring immune responses to pathogens. These receptors include, but are not limited to, DC-SIGN, mannose receptor, Dectin 1, Dectin 2, Mincle, and Langerin. DC-SIGN is expressed on myeloid DCs and has a high affinity for mannose and fucose. It has been associated with numerous pathogens including *Mycobacterium tuberculosis, M. leprae,* and HIV-1 along with numerous others (26). When pathogens bind via DC-SIGN, upregulation of TLR induced IL-10 occurs and induction of Th1, Th2, and Th17 T-cell differentiation. Dectin 1 is expressed on myeloid DCs, monocytes, macrophages, and B-cells (26). Binding via Dectin 1 induces Th1 and Th17 cell differentiation via IL-1β, IL-6, IL-12, and IL-23 production. It also influences induction of TNF and CXCL2 production. Dectin 2 is also expressed on myeloid DCs as well as pDCs. It has a high affinity for mannose and induces TNF and IL-6 production. The mannose receptor is expressed on

myeloid DCs and macrophages. It facilitates phagocytosis and antigen presentation (26). Dectin 1 recognition of *M. leprae* causes secretion of IL-1β (88). Mincle, a macrophage receptor, recognizes trehalose dimycolate (TDM) and trehalose dibehenate (TDB) associated with the mycolic acid layer of *M. leprae* (87). Mincle interacts with the Fc receptor common γ-chain and triggers an inflammatory response through the SKY-CARD9 pathway (26).

The cell surface receptor, CD5, is known for its role in B-cell-to-B-cell interactions, but is also present as a scavenger receptor on dendritic cells recognizing beta-glucan (93). It is found on dendritic cell subsets including Langerhans cells and dermal DCs. CD5 does not appear to be an activation marker but is instead part of different subsets (84). Furthermore, dendritic cells can express varying levels of CD5 on their cell surface (high vs low) which has been shown to have differences in gene expression, cytokine production, antigen presentation, and T-cell polarization (83, 90).

1.3.2 LANGERHANS CELLS

Langerhans cells (LCs) are a subset of dendritic cells that are present in the epidermal layer of the skin (56). While there are a broad range of interstitial dendritic cells that populate the dermal layer of the skin, LCs are the only subset of dendritic cells to populate the epidermal layer as well. Along with their unique location within the skin, they have two additional unique characteristics, a type II C-type lectin cell surface marker, langerin, that has been shown to have a role in the uptake of several pathogens and tennis-racket shaped internal endosomal compartments called Birbeck granules that are not well characterized but thought to participate in antigen presentation.

LCs were named after Paul Langerhans who first discovered their presence in the epidermis and reported dendritic, non-pigmentary cells. After well over a century, it was elucidated that they are leukocytes that are derived from the bone marrow and are a unique DC population found in the epidermis. In human skin, LCs are the only population in the epidermal layer. Cell surface expression allows for easy identification of LCs. Markers in decreasing order of expression level include langerin, CD1a, MHC-II, CD45, CD11c, CD103, CD205 and EpCAM (56-58). LCs also express another cell surface receptor CD5 (59-61).

Langerin is a type II transmembrane protein classified as a C-type lectin receptor. Langerin recognizes and binds carbohydrates such as mannose and fucose as well as N-acetylglucosamine, oligosaccharides mannan, and β-glucan (59). LCs are closely related to macrophages, but their function is similar to cDCs. Langerin has a short intracellular domain. The extracellular domain has a neck-region consisting of alpha-helixes and a carbohydrate recognition domain that is similar to other C-type lectins. Langerin only has one binding site for $Ca2^{+}$ in contrast to other C-type lectins like CD209. The other binding sites on langerin are not $Ca2^{+}$ dependent which enables the binding of negatively charged carbohydrates as well. Langerin has been shown to bind to pathogens such as HIV-1 (59-60) and *Mycobacterium leprae* (31, 55). Specifically, LAM and the glycoprotein, SodC, of *M. leprae* have been shown to bind to langerin. Mannan has also been shown to bind to langerin and provides a positive control for binding in laboratory experiments (81).

Multiple polymorphisms of LCs exist and have different capacities to bind various sugars. Other C-type lectins, like the mannose-binding protein, have multiple well characterized polymorphisms as well. These polymorphisms are associated with susceptibility to severity of various diseases. Polymorphisms with langerin seem to instead reduce sugar binding activity

and not downstream effects for killing pathogens. These polymorphisms may have implications for susceptibility to infections of individuals with different haplotypes (82, 89). Further work needs to be conducted to understand how various polymorphisms may contribute to binding and uptake of pathogens known to associate with langerin.

Previous work has demonstrated that langerin specifically interacts with various pathogens including HIV-1 (59-61) and *Staphylococcus aureus* (81). HIV-1 can be internalized into Birbeck granules via langerin-mediated uptake which prevents HIV-1 infection of LCs and therefore blocks transmission to T-cells. This functions as an effective barrier against HIV-1 infection, but they also uniquely are able to cross-talk to other subsets of DCs via glycosaminoglycan (GAG) hyaluronic acid (HA) which facilitates LC-DC clustering. Langerin binds to HA expressed on the surface of DCs allowing cross-talk to occur. This leads to LC-DC cross-talk (59). In *Staphylococcus aureus* infections, langerin binds to the β-GlcNAc epitope. LCs induce a Th17 response that helps to contain the *S. aureus* infection (81).

There have been a few studies that demonstrate a role for LCs during *M. leprae* infection (30 -32). In TT patients' lesions, high numbers of Langerhans cells are found and can present antigens to T-cells via CD1a. $CD1a^+$ cells are associated with reactional episode outcomes. LCs are predominately present in tuberculoid leprosy whereas lepromatous leprosy patients have weak induction of CD1 proteins (18). Langerin has been shown to have a high binding capacity for the *M. leprae* cell wall glycoprotein, SodC, in surface plasmon resonance assays suggesting a potential uptake route (32, 55). *M. leprae* has also been shown to induce IL-10 production in lepromatous leprosy via CD209. To add to the complex nature of *M. leprae* infection of DCs, PGL-1 mediated binding to CD209 may impair DC maturation and facilitate *M. leprae* survival (35).

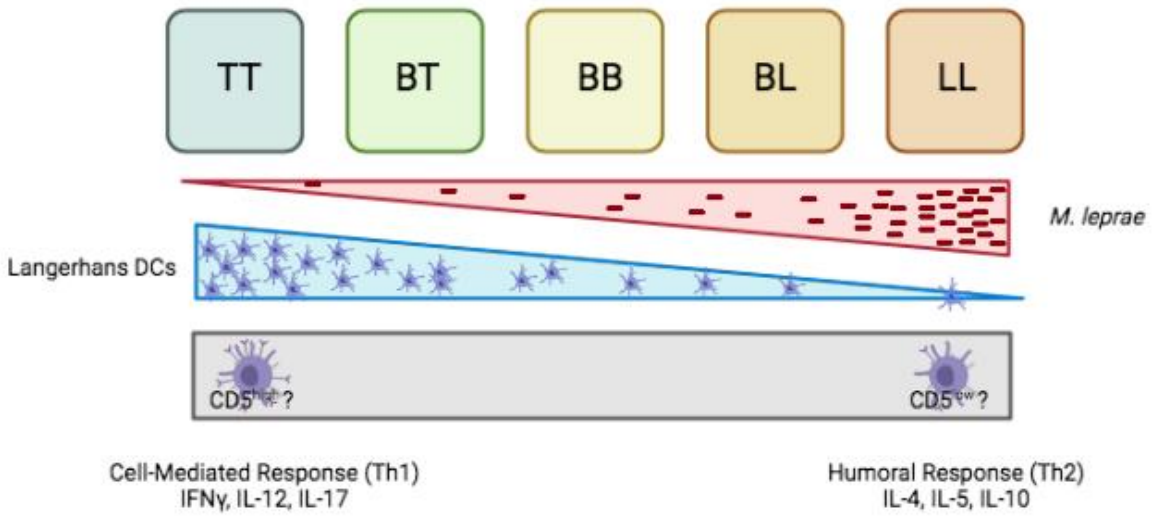

Figure 2: Clinical manifestations of leprosy and immune responses. Bacterial load is lower on the Tuberculoid pole and high on the Lepromatous pole. Numerous Langerhans dendritic cells are found in Tuberculoid lesions. A cell-mediated, Th1, immune response is associated with the Tuberculoid pole while a humoral, Th2, immune response is associated with the Lepromatous pole potentially correlating with the presence of $CD5^{high}$ vs $CD5^{low}$ dendritic cells, respectively. (Drawn based on Ridley, D.S. & Jopling, W.H. (1966) (69))

1.4 RESEARCH RATIONALE AND SUMMARY OF AIMS

A more in-depth understanding of the interactions between *M. leprae* and dendritic cells, specifically Langerhans cells is needed to understand initial host-pathogen interactions that may affect disease outcome. Figure 2 presents known disease classifications based on work by Ridley and Jopling. Questions remain in understanding the role $CD5^{+}$ dendritic cell subsets play in directing the T-cell response and subsequent disease manifestation. Kim *et al.* discovered that langerin recognized a glycoprotein on the cell wall of *M. leprae* (32) while Hunger *et al* sought to understand CD1a presentation of nonpeptide antigens (31). There are many cell surface receptors including CD207 and CD5 on Langerhans cells that can bind and facilitate uptake of *M. leprae*. After uptake and processing of *M. leprae via* potentially different pathways, the Langerhans cell may promote polarization of the adaptive immune response. Further, Yin *et al.* discussed dendritic cell subpopulations, $CD5^{high}$ and $CD5^{low}$, can elicit different Th1 vs Th2 T-cell responses (83). Taken together, these studies provide evidence that Langerhans cells are interacting with *M. leprae* and may affect the adaptive immune response. This led to the hypothesis that interactions between *M. leprae* and Langerhans cells via various cell surface receptors may influence a Th1 vs. Th2 immune response.

The following specific aims were developed to address this hypothesis and continue advancement of understanding initial interactions between *M. leprae* and the innate immune system.

I. Develop methodologies to better evaluate *M. leprae* infections in an infection model (Chapter 3).

II. Determine the interaction and uptake of *M. leprae* with Langerhans cells (Chapter 2 and 4).

REFERENCES

1. Alemu Belachew, W., & Naafs, B. (2019). Position statement: LEPROSY: Diagnosis, treatment and follow-up. *Journal of the European Academy of Dermatology and Venereology : JEADV*, *33*(7), 1205–1213. https://doi.org/10.1111/jdv.15569
2. Ploemacher, T., Faber, W. R., Menke, H., Rutten, V., & Pieters, T. (2020). Reservoirs and transmission routes of leprosy; A systematic review. *PLoS neglected tropical diseases*, *14*(4), e0008276. https://doi.org/10.1371/journal.pntd.0008276
3. Sarode, G., Sarode, S., Anand, R., Patil, S., Jafer, M., Baeshen, H., & Awan, K. H. (2020). Epidemiological aspects of leprosy. *Disease-a-month : DM*, *66*(7), 100899. https://doi.org/10.1016/j.disamonth.2019.100899
4. Centers for Disease Control and Prevention. (2017, February 10). *What is Hansen's Disease? | Hansen's Disease (Leprosy) | CDC*. https://www.cdc.gov/leprosy/about/about.html
5. World Health Organization. (2021, April 26). *Leprosy (Hansen's disease): interrupting transmission and achieving zero autochthonous cases*. https://www.who.int/news/item/26-04-2021-leprosy-(hansen-s-disease)-interrupting-transmission-and-achieving-zero-autochthonous-cases
6. Sarode, G., Sarode, S., Anand, R., Patil, S., Jafer, M., Baeshen, H., & Awan, K. H. (2020). Epidemiological aspects of leprosy. *Disease-a-month : DM*, *66*(7), 100899. https://doi.org/10.1016/j.disamonth.2019.100899
7. Spencer, J. S., & Brennan, P. J. (2011). The role of Mycobacterium leprae phenolic glycolipid I (PGL-I) in serodiagnosis and in the pathogenesis of leprosy. *Leprosy review*, *82*(4), 344–357.
8. Madigan, C. A., Cambier, C. J., Kelly-Scumpia, K. M., Scumpia, P. O., Cheng, T. Y., Zailaa, J., Bloom, B. R., Moody, D. B., Smale, S. T., Sagasti, A., Modlin, R. L., & Ramakrishnan, L. (2017). A Macrophage Response to Mycobacterium leprae Phenolic Glycolipid Initiates Nerve Damage in Leprosy. *Cell*, *170*(5), 973–985.e10. https://doi.org/10.1016/j.cell.2017.07.030
9. Bahia El Idrissi, N., Das, P. K., Fluiter, K., Rosa, P. S., Vreijling, J., Troost, D., Morgan, B. P., Baas, F., & Ramaglia, V. (2015). M. leprae components induce nerve damage by complement activation: identification of lipoarabinomannan as the dominant complement activator. *Acta neuropathologica*, *129*(5), 653–667. https://doi.org/10.1007/s00401-015-1404-5
10. Misch, E. A., Berrington, W. R., Vary, J. C., Jr, & Hawn, T. R. (2010). Leprosy and the human genome. *Microbiology and molecular biology reviews : MMBR*, *74*(4), 589–620. https://doi.org/10.1128/MMBR.00025-10
11. Fallon, M., & Tadi, P. (2021). Histology, Schwann Cells. In *StatPearls*. StatPearls Publishing.
12. Díaz Acosta, C. C., Dias, A. A., Rosa, T., Batista-Silva, L. R., Rosa, P. S., Toledo-Pinto, T. G., Costa, F., Lara, F. A., Rodrigues, L. S., Mattos, K. A., Sarno, E. N., Bozza, P. T., Guilhot, C., de Berrêdo-Pinho, M., & Pessolani, M. (2018). PGL I expression in live bacteria allows activation of a CD206/PPARγ cross-talk that may contribute to successful Mycobacterium leprae colonization of peripheral nerves. *PLoS pathogens*, *14*(7), e1007151. https://doi.org/10.1371/journal.ppat.1007151

13. Zeng, Q., Macri, L. K., Pasrad, A., Clark, R. A. F., & Zeugolis, D. I. (2011). Skin Tissue Engineering [E-book]. In C. Hanley, Y. Garcia, & A. Pandit (Eds.), *Comprehensive Biomaterials II* (Vol. 5, pp. 467–499). Elsevier. https://doi.org/10.1016/B978-0-12-803581-8.10157-2
14. Kanitakis J. (2002). Anatomy, histology and immunohistochemistry of normal human skin. *European journal of dermatology : EJD*, *12*(4), 390–401.
15. Agarwal, S., & Krishnamurthy, K. (2021). Histology, Skin. In *StatPearls*. StatPearls Publishing.
16. Patente, T. A., Pinho, M. P., Oliveira, A. A., Evangelista, G., Bergami-Santos, P. C., & Barbuto, J. (2019). Human Dendritic Cells: Their Heterogeneity and Clinical Application Potential in Cancer Immunotherapy. *Frontiers in immunology*, *9*, 3176. https://doi.org/10.3389/fimmu.2018.03176
17. de Sousa, J. R., Lucena Neto, F. D., Sotto, M. N., & Quaresma, J. (2018). Immunohistochemical characterization of the M4 macrophage population in leprosy skin lesions. *BMC infectious diseases*, *18*(1), 576. https://doi.org/10.1186/s12879-018-3478-x
18. Pinheiro, R. O., Schmitz, V., Silva, B., Dias, A. A., de Souza, B. J., de Mattos Barbosa, M. G., de Almeida Esquenazi, D., Pessolani, M., & Sarno, E. N. (2018). Innate Immune Responses in Leprosy. *Frontiers in immunology*, *9*, 518. https://doi.org/10.3389/fimmu.2018.00518
19. Bahia El Idrissi, N., Das, P. K., Fluiter, K., Rosa, P. S., Vreijling, J., Troost, D., Morgan, B. P., Baas, F., & Ramaglia, V. (2015). M. leprae components induce nerve damage by complement activation: identification of lipoarabinomannan as the dominant complement activator. *Acta neuropathologica*, *129*(5), 653–667. https://doi.org/10.1007/s00401-015-1404-5
20. Pappas A. (2009). Epidermal surface lipids. *Dermato-endocrinology*, *1*(2), 72–76. https://doi.org/10.4161/derm.1.2.7811
21. Kleuser B., Japtok L. (2013) Sphingolipids and Inflammatory Diseases of the Skin. In: Gulbins E., Petrache I. (eds) Sphingolipids in Disease. Handbook of Experimental Pharmacology, vol 216. Springer, Vienna. https://doi.org/10.1007/978-3-7091-1511-4_18
22. Leal-Calvo, T., Martins, B. L., Bertoluci, D. F., Rosa, P. S., de Camargo, R. M., Germano, G. V., Brito de Souza, V. N., Pereira Latini, A. C., & Moraes, M. O. (2021). Large-Scale Gene Expression Signatures Reveal a Microbicidal Pattern of Activation in *Mycobacterium leprae*-Infected Monocyte-Derived Macrophages With Low Multiplicity of Infection. *Frontiers in immunology*, *12*, 647832. https://doi.org/10.3389/fimmu.2021.647832
23. Santos, D., Mendonça, M. R., Antunes, D. E., Sabino, E., Pereira, R. C., Goulart, L. R., & Goulart, I. (2017). Revisiting primary neural leprosy: Clinical, serological, molecular, and neurophysiological aspects. *PLoS neglected tropical diseases*, *11*(11), e0006086. https://doi.org/10.1371/journal.pntd.0006086
24. Pescarini, J. M., Strina, A., Nery, J. S., Skalinski, L. M., Andrade, K., Penna, M., Brickley, E. B., Rodrigues, L. C., Barreto, M. L., & Penna, G. O. (2018). Socioeconomic risk markers of leprosy in high-burden countries: A systematic review and meta-analysis. *PLoS neglected tropical diseases*, *12*(7), e0006622. https://doi.org/10.1371/journal.pntd.0006622
25. Klechevsky E. (2015) Functional Diversity of Human Dendritic Cells. In: Schoenberger S., Katsikis P., Pulendran B. (eds) Crossroads Between Innate and Adaptive Immunity V. Advances in Experimental Medicine and Biology, vol 850. Springer, Cham. https://doi.org/10.1007/978-3-319-15774-0_4

26. Brown, G. D., Willment, J. A., & Whitehead, L. (2018). C-type lectins in immunity and homeostasis. *Nature reviews. Immunology*, *18*(6), 374–389. https://doi.org/10.1038/s41577-018-0004-8
27. Boltjes, A., & van Wijk, F. (2014). Human dendritic cell functional specialization in steady-state and inflammation. *Frontiers in immunology*, *5*, 131. https://doi.org/10.3389/fimmu.2014.00131
28. Artyomov, M. N., Munk, A., Gorvel, L., Korenfeld, D., Cella, M., Tung, T., & Klechevsky, E. (2015). Modular expression analysis reveals functional conservation between human Langerhans cells and mouse cross-priming dendritic cells. *The Journal of experimental medicine*, *212*(5), 743–757. https://doi.org/10.1084/jem.20131675
29. Klechevsky E. (2013). Human dendritic cells - stars in the skin. *European journal of immunology*, *43*(12), 3147–3155. https://doi.org/10.1002/eji.201343790
30. Hanske, J., Schulze, J., Aretz, J., McBride, R., Loll, B., Schmidt, H., Knirel, Y., Rabsch, W., Wahl, M. C., Paulson, J. C., & Rademacher, C. (2017). Bacterial Polysaccharide Specificity of the Pattern Recognition Receptor Langerin Is Highly Species-dependent. *The Journal of biological chemistry*, *292*(3), 862–871. https://doi.org/10.1074/jbc.M116.751750
31. Hunger, R. E., Sieling, P. A., Ochoa, M. T., Sugaya, M., Burdick, A. E., Rea, T. H., Brennan, P. J., Belisle, J. T., Blauvelt, A., Porcelli, S. A., & Modlin, R. L. (2004). Langerhans cells utilize CD1a and langerin to efficiently present nonpeptide antigens to T cells. *The Journal of clinical investigation*, *113*(5), 701–708. https://doi.org/10.1172/JCI19655
32. Kim, H. J., Brennan, P. J., Heaslip, D., Udey, M. C., Modlin, R. L., & Belisle, J. T. (2015). Carbohydrate-dependent binding of langerin to SodC, a cell wall glycoprotein of Mycobacterium leprae. *Journal of bacteriology*, *197*(3), 615–625. https://doi.org/10.1128/JB.02080-14
33. Krahenbuhl J, Adams LB. 2000. Exploitation of gene knockout mice models to study the pathogenesis of leprosy. *Lepr Rev* 71 Suppl:S170-175.
34. Adams LB, Scollard DM, Ray NA, Cooper AM, Frank AA, Orme IM, Krahenbuhl JL. 2002. The study of *Mycobacterium leprae* infection in interferon-gamma gene-disrupted mice as a model to explore the immunopathologic spectrum of leprosy. *J Infect Dis* 185 Suppl 1:S1-8.
35. Montoya D, Modlin RL. 2010. Learning from leprosy: insight into the human innate immune response. *Adv Immunol* 105:1-24.
36. Walker SL, Lockwood DN. 2007. Leprosy. *Clin Dermatol* 25:165-172.
37. Brennan PJ, Nikaido H. 1995. The envelope of mycobacteria. *Annu Rev Biochem* 64:29- 63.
38. Brennan PJ. 2003. Structure, function, and biogenesis of the cell wall of *Mycobacterium tuberculosis*. *Tuberculosis (Edinb)* 83:91-97.
39. Dmitriev BA, Ehlers S, Rietschel ET, Brennan PJ. 2000. Molecular mechanics of the mycobacterial cell wall: from horizontal layers to vertical scaffolds. *Int J Med Microbiol* 290:251-258.
40. Mahapatra S, Scherman H, Brennan PJ, Crick DC. 2005. N Glycolylation of the nucleotide precursors of peptidoglycan biosynthesis of *Mycobacterium* spp. is altered by drug treatment. *Journal of Bacteriology* 187:2341-2347.
41. Mahapatra S, Crick DC, McNeil MR, Brennan PJ. 2008. Unique structural features of the peptidoglycan of *Mycobacterium leprae*. *Journal of Bacteriology* 190:655-661.
42. Mahapatra S, Yagi T, Belisle JT, Espinosa BJ, Hill PJ, McNeil MR, Brennan PJ, Crick DC. 2005. Mycobacterial lipid II is composed of a complex mixture of modified muramyl and peptide moieties linked to decaprenyl phosphate. *Journal of Bacteriology* 187:2747-2757.

43. McNeil M, Daffe M, Brennan PJ. 1990. Evidence for the nature of the link between the arabinogalactan and peptidoglycan of mycobacterial cell walls. *Journal of Biological Chemistry* 265:18200-18206.
44. Ng V, Zanazzi G, Timpl R, Talts JF, Salzer JL, Brennan PJ, Rambukkana A. 2000. Role of the cell wall phenolic glycolipid-1 in the peripheral nerve predilection of *Mycobacterium leprae*. *Cell* 103:511-524.
45. Chatterjee D, Lowell K, Rivoire B, McNeil MR, Brennan PJ. 1992. Lipoarabinomannan of *Mycobacterium tuberculosis.* Capping with mannosyl residues in some strains. *J Biol Chem* 267:6234-6239.
46. Monot M, Honoré N, Garnier T, Araoz R, Coppée J-Y, Lacroix C, Sow S, Spencer JS, Truman RW, Williams DL, Gelber R, Virmond M, Flageul B, Cho S-N, Ji B, Paniz-Mondolfi A, Convit J, Young S, Fine PE, Rasolofo V, Brennan PJ, Cole ST. 2005. On the origin of leprosy. *Science* 308:1040-1042.
47. Wheat WH, Casali AL, Thomas V, Spencer JS, Lahiri R, Williams DL, McDonnell GE, Gonzalez-Juarrero M, Brennan PJ, Jackson M. 2014. Long-term survival and virulence of *Mycobacterium leprae* in amoebal cysts. *PLoS Negl Trop Dis* 8:e3405
48. Spencer JS, Dockrell HM, Kim HJ, Marques MA, Williams DL, Martins MV, Martins ML, Lima MC, Sarno EN, Pereira GM, Matos H, Fonseca LS, Sampaio EP, Ottenhoff TH, Geluk A, Cho SN, Stoker NG, Cole ST, Brennan PJ, Pessolani MC. 2005. Identification of specific proteins and peptides in *Mycobacterium leprae* suitable for the selective diagnosis of leprosy. *J Immunol* 175:7930-7938.
49. Spencer JS, Kim HJ, Wheat WH, Chatterjee D, Balagon MV, Cellona RV, Tan EV, Gelber R, Saunderson P, Duthie MS, Reece ST, Burman W, Belknap R, Mac Kenzie WR, Geluk A, Oskam L, Dockrell HM, Brennan PJ. 2011. Analysis of antibody responses to *Mycobacterium leprae* phenolic glycolipid I, lipoarabinomannan, and recombinant proteins to define disease subtype-specific antigenic profiles in leprosy. *Clin Vaccine Immunol* 18:260-267.
50. Sakamuri RM, Kimura M, Li W, Kim HC, Lee H, Kiran MD, Black WCt, Balagon M, Gelber R, Cho SN, Brennan PJ, Vissa V. 2009. Population-based molecular epidemiology of leprosy in Cebu, Philippines. *J Clin Microbiol* 47:2844-2854.
51. Monot M, Honore N, Garnier T, Zidane N, Sherafi D, Paniz-Mondolfi A, Matsuoka M, Taylor GM, Donoghue HD, Bouwman A, Mays S, Watson C, Lockwood D, Khamesipour A, Dowlati Y, Jianping S, Rea TH, Vera-Cabrera L, Stefani MM, Banu S, Macdonald M, Sapkota BR, Spencer JS, Thomas J, Harshman K, Singh P, Busso P, Gattiker A, Rougemont J, Brennan PJ, Cole ST. 2009. Comparative genomic and phylogeographic analysis of *Mycobacterium leprae*. *Nat Genet* 41:1282-1289.
52. Marques MA, Ant nio VL, Sarno EN, Brennan PJ, Pessolani MC. β001. Binding of αβ-laminins by pathogenic and non-pathogenic mycobacteria and adherence to Schwann cells. *J Med Microbiol* 50:23-28.
53. Cho SN, Cellona RV, Villahermosa LG, Fajardo TT, Jr., Balagon MV, Abalos RM, Tan EV, Walsh GP, Kim JD, Brennan PJ. 2001. Detection of phenolic glycolipid I of *Mycobacterium leprae* in sera from leprosy patients before and after start of multidrug therapy. *Clin Diagn Lab Immunol* 8:138-142.
54. de Lima, C. S., Marques, M. A., Debrie, A. S., Almeida, E. C., Silva, C. A., Brennan, P. J., Sarno, E. N., Menozzi, F. D., & Pessolani, M. C. (2009). Heparin-binding hemagglutinin (HBHA) of Mycobacterium leprae is expressed during infection and enhances bacterial

adherence to epithelial cells. *FEMS microbiology letters*, *292*(2), 162–169. https://doi.org/10.1111/j.1574-6968.2009.01488.x
55. Dang, A. T., Teles, R. M., Liu, P. T., Choi, A., Legaspi, A., Sarno, E. N., Ochoa, M. T., Parvatiyar, K., Cheng, G., Gilliet, M., Bloom, B. R., & Modlin, R. L. (2019). Autophagy links antimicrobial activity with antigen presentation in Langerhans cells. *JCI insight*, *4*(8), e126955. https://doi.org/10.1172/jci.insight.126955
56. Cotton, R. N., Wegrecki, M., Cheng, T. Y., Chen, Y. L., Veerapen, N., Le Nours, J., Orgill, D. P., Pomahac, B., Talbot, S. G., Willis, R., Altman, J. D., de Jong, A., Van Rhijn, I., Clark, R. A., Besra, G. S., Ogg, G., Rossjohn, J., & Moody, D. B. (2021). CD1a selectively captures endogenous cellular lipids that broadly block T cell response. *The Journal of experimental medicine*, *218*(7), e20202699. https://doi.org/10.1084/jem.20202699
57. Cernadas, M., Cavallari, M., Watts, G., Mori, L., De Libero, G., & Brenner, M. B. (2010). Early recycling compartment trafficking of CD1a is essential for its intersection and presentation of lipid antigens. *Journal of immunology (Baltimore, Md. : 1950)*, *184*(3), 1235–1241. https://doi.org/10.4049/jimmunol.0804140
58. Kissenpfennig, A., Aït-Yahia, S., Clair-Moninot, V., Stössel, H., Badell, E., Bordat, Y., Pooley, J. L., Lang, T., Prina, E., Coste, I., Gresser, O., Renno, T., Winter, N., Milon, G., Shortman, K., Romani, N., Lebecque, S., Malissen, B., Saeland, S., & Douillard, P. (2005). Disruption of the langerin/CD207 gene abolishes Birbeck granules without a marked loss of Langerhans cell function. *Molecular and cellular biology*, *25*(1), 88–99. https://doi.org/10.1128/MCB.25.1.88-99.2005
59. van den Berg, L. M., Cardinaud, S., van der Aar, A. M., Sprokholt, J. K., de Jong, M. A., Zijlstra-Willems, E. M., Moris, A., & Geijtenbeek, T. B. (2015). Langerhans Cell-Dendritic Cell Cross-Talk via Langerin and Hyaluronic Acid Mediates Antigen Transfer and Cross-Presentation of HIV-1. *Journal of immunology (Baltimore, Md. : 1950)*, *195*(4), 1763–1773. https://doi.org/10.4049/jimmunol.1402356
60. de Witte, L., Nabatov, A., Pion, M., Fluitsma, D., de Jong, M. A., de Gruijl, T., Piguet, V., van Kooyk, Y., & Geijtenbeek, T. B. (2007). Langerin is a natural barrier to HIV-1 transmission by Langerhans cells. *Nature medicine*, *13*(3), 367–371. https://doi.org/10.1038/nm1541
61. Yin, X., Yu, H., Jin, X., Li, J., Guo, H., Shi, Q., Yin, Z., Xu, Y., Wang, X., Liu, R., Wang, S., & Zhang, L. (2017). Human Blood CD1c+ Dendritic Cells Encompass CD5high and CD5low Subsets That Differ Significantly in Phenotype, Gene Expression, and Functions. *Journal of immunology (Baltimore, Md. : 1950)*, *198*(4), 1553–1564. https://doi.org/10.4049/jimmunol.1600193
62. Rambukkana A. 2004. *Mycobacterium leprae*-induced demyelination: a model for early nerve degeneration. *Current Opinion in Immunology* 16:511-518.
63. Schlesinger LS, Horwitz MA. 1991. Phenolic glycolipid-1 of *Mycobacterium leprae* binds complement component C3 in serum and mediates phagocytosis by human monocytes. *J Exp Med* 174:1031-1038.
64. Schlesinger LS, Horwitz MA. 1991. Phagocytosis of *Mycobacterium leprae* by human monocyte-derived macrophages is mediated by complement receptors CR1 (CD35), CR3 (CD11b/CD18), and CR4 (CD11c/CD18) and IFN-gamma activation inhibits complement receptor function and phagocytosis of this bacterium. *J Immunol* 147:1983- 1994.
65. Bezerra Da Cunha FM, Werneck MC, Scola RH, Werneck LC. 2006. Pure neural leprosy: diagnostic value of the polymerase chain reaction. *Muscle Nerve* 33:409-414.

66. Shepard CC. 1960. The experimental disease that follows the injection of human leprosy bacilli into foot-pads of mice. *J Exp Med* 112:445-454.
67. Walker SL, Lockwood DN. 2006. The clinical and immunological features of leprosy. *Br Med Bull* 77-78:103-121.
68. WHO. WHO Guidelines for the management of severe erythema nodosum leprosum (ENL) reactions.
69. Ridley DS, Jopling WH. 1966. Classification of leprosy according to immunity. A five-group system. *Int J Lepr Other Mycobact Dis* 34:255-273.
70. Britton WJ, Lockwood DN. 2004. Leprosy. *Lancet* 363:1209-1219.
71. Bhat RM, Prakash C. 2012. Leprosy: an overview of pathophysiology. *Interdiscip Perspect Infect Dis* 2012:181089.
72. Loughry WJ, Truman RW, McDonough CM, Tilak MK, Garnier S, Delsuc F. 2009. Is leprosy spreading among nine-banded armadillos in the southeastern United States? *J Wildl Dis* 45:144-152.
73. Meyers WM, Gormus BJ, Walsh GP, Baskin GB, Hubbard GB. 1991. Naturally acquired and experimental leprosy in nonhuman primates. *Am J Trop Med Hyg* 44:24-27.
74. Truman R. 2005. Leprosy in wild armadillos. *Lepr Rev* 76:198-208.
75. Truman RW, Singh P, Sharma R, Busso P, Rougemont J, Paniz-Mondolfi A, Kapopoulou A, Brisse S, Scollard DM, Gillis TP, Cole ST. 2011. Probable zoonotic leprosy in the southern United States. *N Engl J Med* 364:1626-1633
76. Truman R, Fine PE. 2010. 'Environmental' sources of *Mycobacterium leprae*: issues and evidence. *Lepr Rev* 81:89-95.
77. Nigou J, Gilleron M, Puzo G. 2003. Lipoarabinomannans: from structure to biosynthesis. *Biochimie* 85:153-166.
78. Mehaffy C, Belisle JT, Dobos KM. Mycobacteria and their sweet proteins: An overview of protein glycosylation and lipoglycosylation in M. tuberculosis. Tuberculosis (Edinb). 2019 Mar;115:1-13. doi: 10.1016/j.tube.2019.01.001. Epub 2019 Jan 14. PMID: 30948163.
79. Silva CAM, Belisle JT. Host Lipid Mediators in Leprosy: The Hypothesized Contributions to Pathogenesis. Front Immunol. 2018 Feb 2;9:134. doi: 10.3389/fimmu.2018.00134. PMID: 29472920; PMCID: PMC5810268.
80. Schenk M, Mahapatra S, Le P, Kim HJ, Choi AW, Brennan PJ, Belisle JT, Modlin RL. Human NOD2 Recognizes Structurally Unique Muramyl Dipeptides from Mycobacterium leprae. Infect Immun. 2016 Aug 19;84(9):2429-38. doi: 10.1128/IAI.00334-16. PMID: 27297389; PMCID: PMC4995902.
81. van Dalen, R., De La Cruz Diaz, J. S., Rumpret, M., Fuchsberger, F. F., van Teijlingen, N. H., Hanske, J., Rademacher, C., Geijtenbeek, T., van Strijp, J., Weidenmaier, C., Peschel, A., Kaplan, D. H., & van Sorge, N. M. (2019). Langerhans Cells Sense *Staphylococcus aureus* Wall Teichoic Acid through Langerin To Induce Inflammatory Responses. *mBio*, *10*(3), e00330-19. https://doi.org/10.1128/mBio.00330-19
82. Ward, E. M., Stambach, N. S., Drickamer, K., & Taylor, M. E. (2006). Polymorphisms in human langerin affect stability and sugar binding activity. *The Journal of biological chemistry*, *281*(22), 15450–15456. https://doi.org/10.1074/jbc.M511502200
83. Yin, X., Yu, H., Jin, X., Li, J., Guo, H., Shi, Q., Yin, Z., Xu, Y., Wang, X., Liu, R., Wang, S., & Zhang, L. (2017). Human Blood CD1c+ Dendritic Cells Encompass CD5high and CD5low Subsets That Differ Significantly in Phenotype, Gene Expression, and

Functions. *Journal of immunology (Baltimore, Md. : 1950)*, *198*(4), 1553–1564. https://doi.org/10.4049/jimmunol.1600193
84. Korenfeld, D., Gorvel, L., Munk, A., Man, J., Schaffer, A., Tung, T., Mann, C., & Klechevsky, E. (2017). A type of human skin dendritic cell marked by CD5 is associated with the development of inflammatory skin disease. *JCI insight*, *2*(18), e96101. https://doi.org/10.1172/jci.insight.96101
85. Manca, C., Peixoto, B., Malaga, W., Guilhot, C., & Kaplan, G. (2012). Modulation of the cytokine response in human monocytes by mycobacterium leprae phenolic glycolipid-1. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research*, *32*(1), 27–33. https://doi.org/10.1089/jir.2011.0044
86. Kai, M., Fujita, Y., Maeda, Y., Nakata, N., Izumi, S., Yano, I., & Makino, M. (2007). Identification of trehalose dimycolate (cord factor) in Mycobacterium leprae. *FEBS letters*, *581*(18), 3345–3350. https://doi.org/10.1016/j.febslet.2007.06.029
87. Bird, J. H., Khan, A. A., Nishimura, N., Yamasaki, S., Timmer, M., & Stocker, B. L. (2018). Synthesis of Branched Trehalose Glycolipids and Their Mincle Agonist Activity. *The Journal of organic chemistry*, *83*(15), 7593–7605. https://doi.org/10.1021/acs.joc.7b03269
88. Silva, L. M., de Sousa, J. R., Hirai, K. E., Dias, L. B., Jr, Furlaneto, I. P., Carneiro, F., de Souza Aarão, T. L., Sotto, M. N., & Quaresma, J. (2018). The inflammasome in leprosy skin lesions: an immunohistochemical evaluation. *Infection and drug resistance*, *11*, 2231–2240. https://doi.org/10.2147/IDR.S172806
89. Halbritter, F., Farlik, M., Schwentner, R., Jug, G., Fortelny, N., Schnöller, T., Pisa, H., Schuster, L. C., Reinprecht, A., Czech, T., Gojo, J., Holter, W., Minkov, M., Bauer, W. M., Simonitsch-Klupp, I., Bock, C., & Hutter, C. (2019). Epigenomics and Single-Cell Sequencing Define a Developmental Hierarchy in Langerhans Cell Histiocytosis. *Cancer discovery*, *9*(10), 1406–1421. https://doi.org/10.1158/2159-8290.CD-19-0138
90. Zani, I. A., Stephen, S. L., Mughal, N. A., Russell, D., Homer-Vanniasinkam, S., Wheatcroft, S. B., & Ponnambalam, S. (2015). Scavenger receptor structure and function in health and disease. *Cells*, *4*(2), 178–201. https://doi.org/10.3390/cells4020178
91. Marques, M. A., Berrêdo-Pinho, M., Rosa, T. L., Pujari, V., Lemes, R. M., Lery, L. M., Silva, C. A., Guimarães, A. C., Atella, G. C., Wheat, W. H., Brennan, P. J., Crick, D. C., Belisle, J. T., & Pessolani, M. C. (2015). The Essential Role of Cholesterol Metabolism in the Intracellular Survival of Mycobacterium leprae Is Not Coupled to Central Carbon Metabolism and Energy Production. *Journal of bacteriology*, *197*(23), 3698–3707. https://doi.org/10.1128/JB.00625-15
92. Scollard DM, Adams LB, Gillis TP, Krahenbuhl JL, Truman RW, Williams DL. 2006. The continuing challenges of leprosy. *Clinical Microbiology Reviews* 19:338-381.
93. Lemoine, S., Jaron, B., Tabka, S., Ettreiki, C., Deriaud, E., Zhivaki, D., Le Ray, C., Launay, O., Majlessi, L., Tissieres, P., Leclerc, C., & Lo-Man, R. (2015). Dectin-1 activation unlocks IL12A expression and reveals the TH1 potency of neonatal dendritic cells. *The Journal of allergy and clinical immunology*, *136*(5), . https://doi.org/10.1016/j.jaci.2015.02.030
94. Rotcheewaphan, S. (2016). *Characterization of mycobacterium leprae diguanylate cyclases* (dissertation).

CHAPTER 2: OPTICAL TISSUE CLEARING FOR SKIN

2.1 INTRODUCTION

Classical methods of tissue imaging like immunohistochemistry (IHC) require tissue sectioning and can only be probed with one set of labels (3). Special tissue handling may be required to prepare epitopes for antibody binding depending on the method of fixation. While IHC is still an extremely important tool for immunology, pertinent information about cell interactions can be better resolved by visualizing interactions in intact tissue.

Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging/Immunostaining/In-situ hybridization-compatible Tissue hYdrogel (CLARITY) is a technique used to transform 3D tissue sections into a 3D nanoporous hydrogel. Using this technique, the 3D hydrogel infused tissue becomes optically transparent, is relatively stable, and is permeable to macromolecular probes such as fluorescent antibodies (1). During the CLARITY process, lipids are removed to reduce light scatter that can occur with the imaging of intact tissue preparations (2). However, non-lipophilic structures such as proteins remain in their original location due to fixation and the formation of a hydrogel. This overall process allows for investigation of cellular relationships and protein complexes globally within the tissue. CLARITY was originally developed in 2013 to investigate whole brain tissue where sectioning could miss key relationships (1). Although CLARITY was originally developed for brain tissue, it has been adapted for almost every tissue in the body (4). However, this approach has not been thoroughly reported for use with skin tissue.

A modification on the CLARITY protocol, passive clarity technique (PACT), uses refractive index matching solution (RIMS) as an imaging medium. PACT was developed as a

milder, passive clearing process better suited for fragile tissues (8). The PACT method removed bisacrylamide and increased detergent concentration for lipid removal. The passive removal of lipids using 8% sodium dodecyl sulfate without electrophoresis reduced the risk of tissue damage or swelling. In addition to decreasing the costs associated with CLARITY, the high sorbitol concentration (70%) in RIMS makes it an effective storage buffer by reducing any potential bacterial or fungal growth during storage.

M. leprae is an obligate, intracellular pathogen with few animal models available (11-12). Most studies are accomplished *in vitro* using cell lines and *M. leprae* purified from athymic *nu/nu* mouse footpads (16-17) or *M. leprae* components (13-15). While these studies provide insight into *M. leprae* infection, they can be limited in their capacity to draw conclusions. Other techniques utilize human skin biopsies and immunohistochemistry (18-21) to visualize immune cells present and interactions with *M. leprae*, but immunohistochemistry relies on thin slices of tissue and once stained, slices cannot be re-stained for other cells of interest. This means important information can potentially be lost if cells are not uniformly present throughout the entire tissue biopsy and all slices. CLARITY utilizes the intact 3D biopsy for staining and can be de-stained and re-stained with new antibodies of interest. This becomes important to investigate paucibacillary skin biopsies where *M. leprae* may be sparse. We adapted the CLARITY protocol to clear skin samples and probe with antibodies of interest.

2.2 MATERIALS AND METHODS

2.2.1 Animal Model

Female Balb/c mice 4-6 weeks of age were gifted from the Henao-Tamayo laboratory at Colorado State University. All methods were carried out in accordance with relevant guidelines and regulations with respect to animal welfare. Mice were housed at the Pathology building biosafety level 2 animal facility under pathogen-free conditions and were handled in accordance with experimental protocols that were approved by IDRIs Institutional Animal Care and Use Committee (IACUC). Mice were infected by the intradermal route (i.d.) with 10^5 CFU of *Mycobacterium haemophilum* or *Mycolicibacterium smegmatis*. Twenty-four hours post challenge, the skin at the inoculation site was harvested and placed in ice-cold 4% paraformaldehyde.

2.2.2 *In vitro growth of bacterial cultures*

M. smegmatis was grown in 7H9 (VWR) medium supplemented with 5% glucose and OADC at 37° C while shaking at 200 rpm for 3 days to an OD_{600} of 0.6. *M. haemophilum* was grown in 7H9 (VWR, Radnor, PA) medium supplemented with 5% glucose, 60 μM Hemin (Sigma-Aldrich, St. Louis, MO), and OADC at 30° C protected from light while shaking for 7 days to an OD_{600} of 0.6.

2.2.3 CLARITY

After IACUC approved humane-euthanasia, skin to be harvested was shaved. Shaved skin was harvested and placed in ice-cold 4% paraformaldehyde and incubated overnight at 4° C. Skin was transferred to ice-cold 4% acrylamide (BioRad, Hercules, CA) in a 15 mL conical tube and

incubated overnight at 4° C. The initiator, VA-044 (Wako), was added to 4% acrylamide solution to a final concentration of 0.25% and the cap was tightened on liquid so no head space remained, and zero oxygen was present in the tube. The tube was transferred immediately to 37° C for 4 h or until acrylamide polymerized. Skin was removed from polymerized acrylamide and washed five times with 1X phosphate-buffered saline (PBS). Skin-acrylamide hydrogel was transferred to a 50 mL conical tube with 8% SDS (Thermo Fisher, Waltham, MA) and placed on a rocking plate at 37° C. 8% SDS was replenished every 24 h until the solution was no longer cloudy. The deplipidated skin was washed five times with 1X PBS-tween 20 (Sigma Aldrich), and incubated for at least five days with appropriate antibodies for imaging (Table 2.1). Skin was washed five times with 1X PBS and transferred to sRIMS solution for at least five days.

Table 2.1: Fluorescent antibodies used to stain clarified mouse skin for confocal imaging.

Marker	**Clone**	**Fluorophore**	**Manufacturer**
CD207	eBioRMUL.2	eFluor660	eBioscience
MHC-II	M5/114.15.2	eFluor450	eBioscience
CD5	UCHT2	BV421	BioLegend
CD1a	O10	AF647	Novus
CD11b	Poly	AF700	Novus

2.2.4 Imaging

Confocal imaging was performed using an inverted Olympus FV1000-IX81 confocal imaging system. Images were analyzed using Volocity (Perkin Elmer, Waltham, PA).

2.3 RESULTS

2.3.1 Modified CLARITY protocol for skin samples

Female Balb/c mice were intradermally injected with 10^5 CFU of *Mycobacterium haemophilum* or *Mycolicibacterium smegmatis* or mock injection of phosphate buffered saline. Post challenge (24 h), mice were humanely euthanized and the skin at the injection site was shaved to remove all fur. The shaved skin at the inoculation site was harvested and placed in ice-cold 4% paraformaldehyde overnight at 4° C. Balb/c mice have white skin and the color of the skin remained the same after overnight incubation in 4% paraformaldehyde. Skin samples were transferred to ice-cold 4% acrylamide in a pre-chilled 15mL conical tube and incubated overnight at 4° C followed by an addition of an initiator, VA-044. The conical tube was overfilled, the cap was tightened, and extra acrylamide was allowed to leak out so no oxygen remained in the tube. The cold conical tube was immediately transferred to a 37° C incubator for three to four hours. The temperature change activated the initiator which polymerized the acrylamide. Once the acrylamide was visually polymerized (4% acrylamide does not look like a polymerized gel, but instead is extremely viscous),the skin was removed from the polymerized acrylamide and placed in a new 15mL conical tube containing PBS at room temperature on a rocker plate to wash. The PBS was replenished five times, with each wash allowed to rock for at least 30 minutes. Skin samples were then transferred to a new 50mL conical tube containing 8% sodium dodecyl sulfate (SDS) in PBS and placed in a 37° C incubator on a rocker plate. 8% SDS was replenished every 24 hours until the solution was clear after a 24 hours incubation and no more lipids were being removed from the tissue. Skin samples were washed similar to before, five times in PBS containing 0.1% tween to remove any excess SDS from the tissue. The skin samples were transferred to a PBS solution containing antibodies of interest and allowed to

incubate on a rocker plate at room temperature for five days. Antibodies were replenished at least two times during the five days for efficient staining (Figure 2.1.1). Skin samples remained relatively opaque during this entire protocol. Finally, the samples were transferred to sRIMS solution which transformed the tissue from opaque to translucent (Figure 2.1.2). Samples were placed in fresh sRIMS solution for imaging.

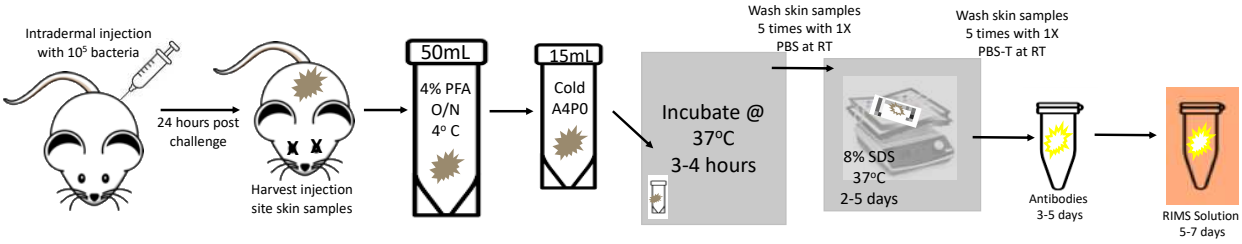

Figure 2.1.1: Modified PACT protocol adapted for skin samples.

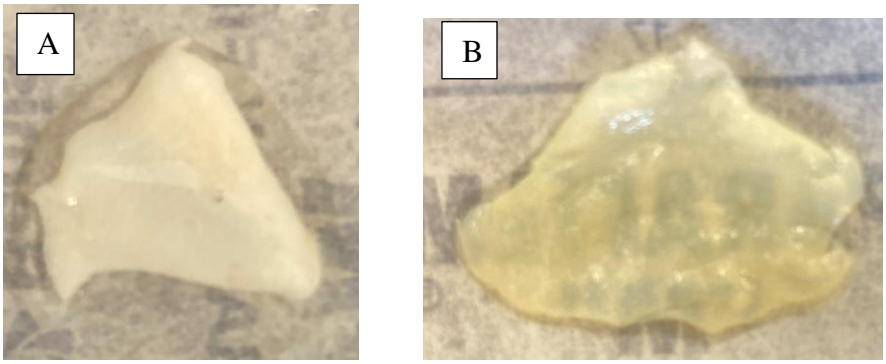

Figure 2.1.2: Mouse skin tissue prior to PACT clearing process is opaque (A) and after multiple days in sRIMS solution, the mouse skin tissue becomes transparent (B).

2.3.2 M. haemophilum and M. smegmatis infected skin samples

Mice were intradermally injected with 10^5 recombinant *M. haemophilum* or recombinant *M. smegmatis*, expressing mCherry. As a mock control, PBS was intradermally injected. Samples were harvested and stained for langerin (CD207), CD5, and CD1a. All three markers were detected and colocalized on cells in the PBS skin samples (Figure 2.2.1). In the *M. smegmatis* skin samples, CD5 and CD207 were detected and colocalized around *M. smegmatis* (Figure 2.2.3). CD5 and mCherry were detected in the *M. haemophilum* infected skin, but CD207 was not detected (Figure 2.2.2).

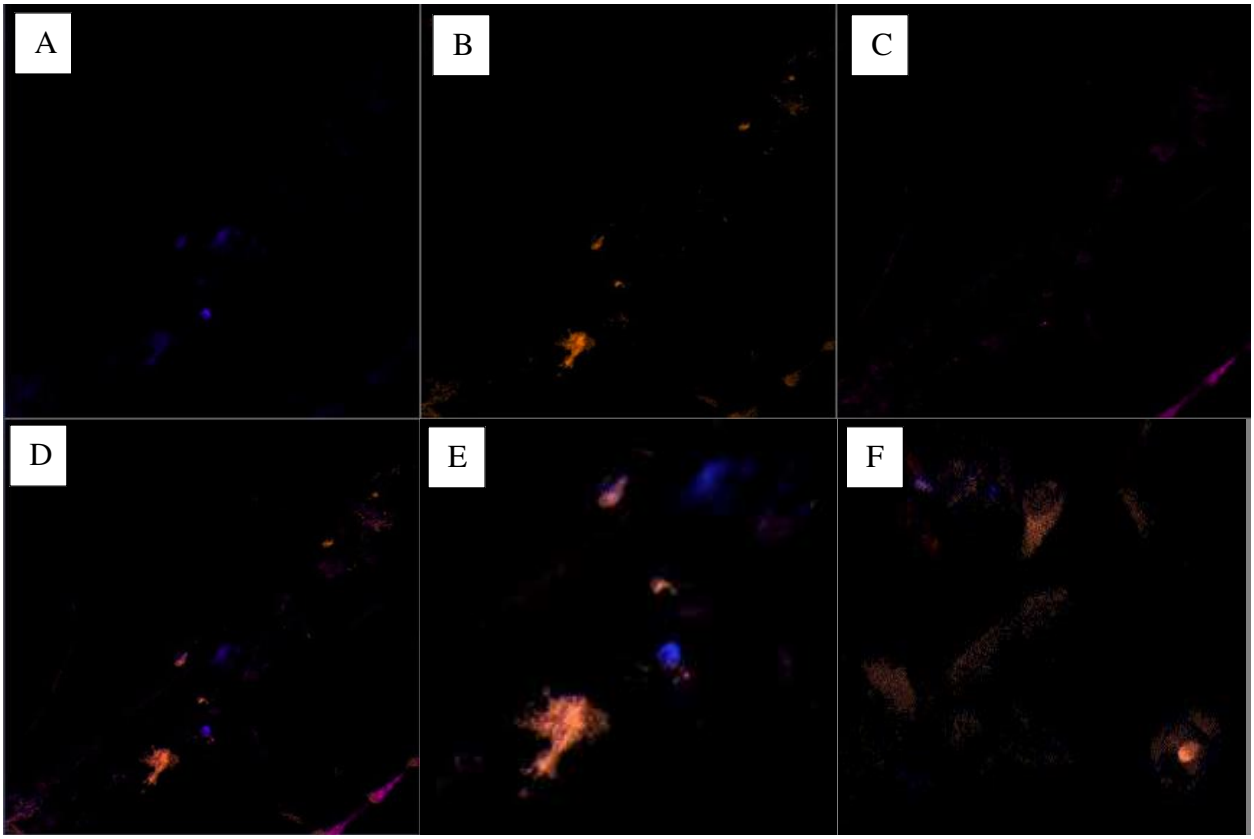

Figure 2.2.1: Confocal images of CLARITY treated mouse skin tissues following mock injection with PBS. CD5 (A), CD207 (B), and CD1a (C) were colocalized (D) on imaged cells in the skin sample indicating Langerhans cells were labeled. E and F show enlarged areas. n=1

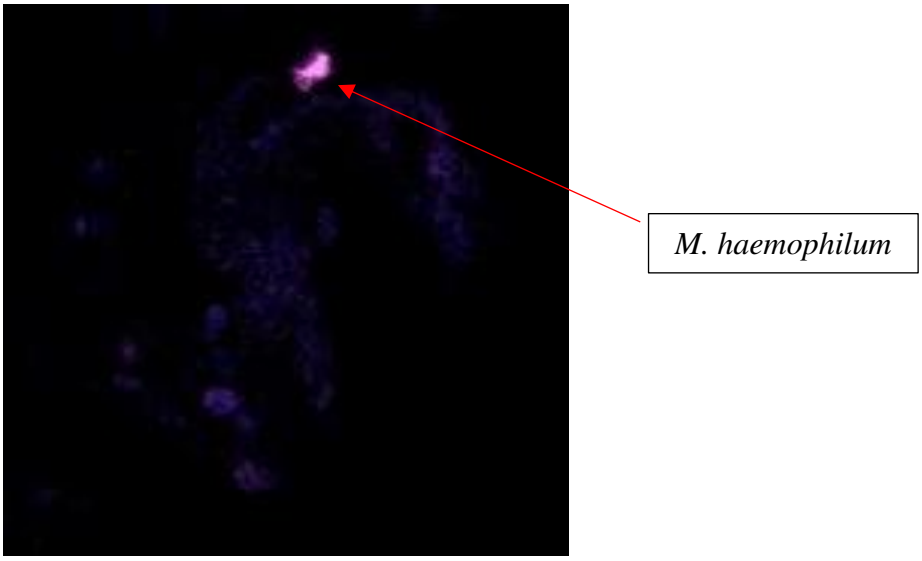

Figure 2.2.2: Confocal images of CLARITY treated mouse skin tissues following injection with *M. haemophilum. M. haemophilum* (red) colocalized with CD5 (blue) labeled cells indicating recruitment of dendritic cells to the site of infection. n=1

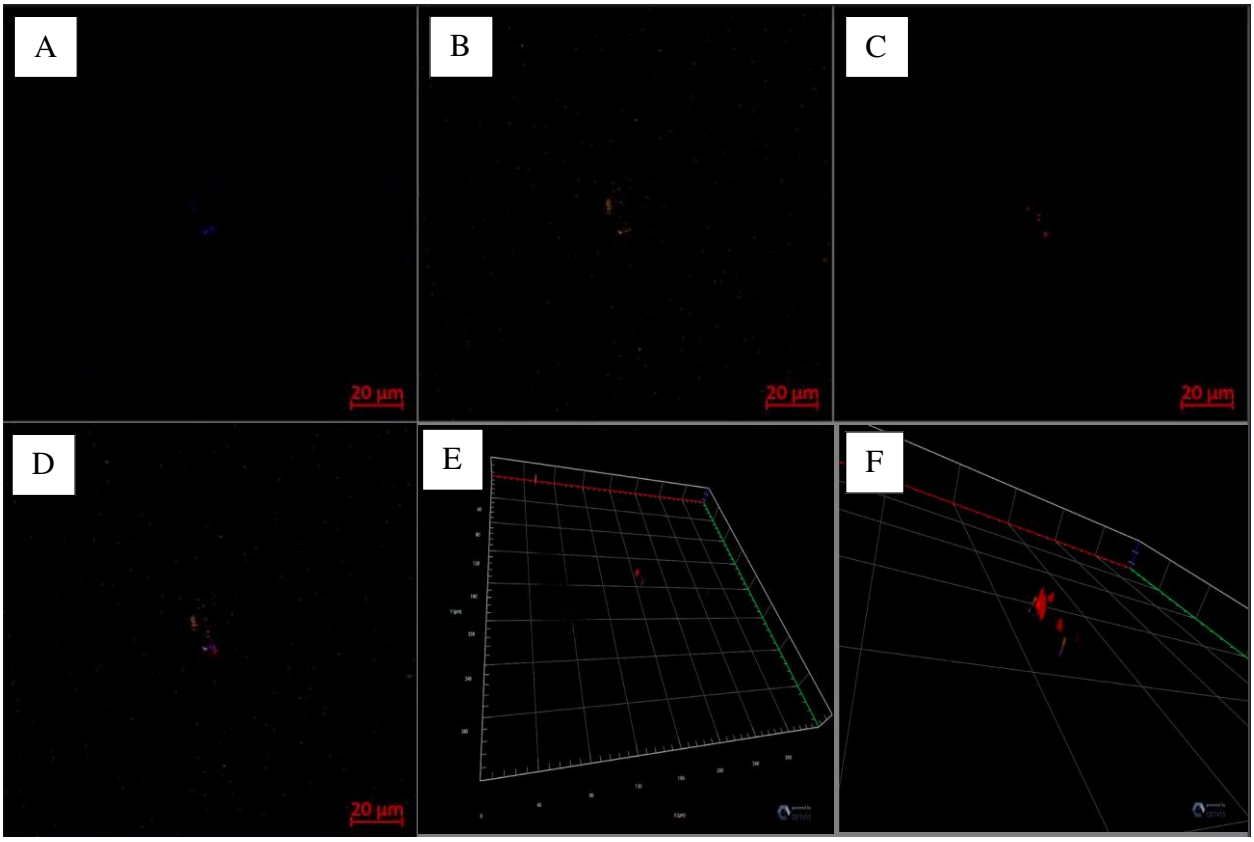

Figure 2.2.3: Confocal images of CLARITY treated mouse skin tissues following injection with *M. smegmatis.* CD207 (B), CD5 (A), and mCherry (C) labeled *M. smegmatis* were colocalized (D) indicating recruitment of Langerhans cells to the site of infection. 3D images (E, F) further visualize recruitment of cells to the site of infection. An enlarged section (F) visualizes colocalization from (D). Bacilli determined to be at the interface between the epidermis and dermis based on the orientation of the tissue on the slide. n=1

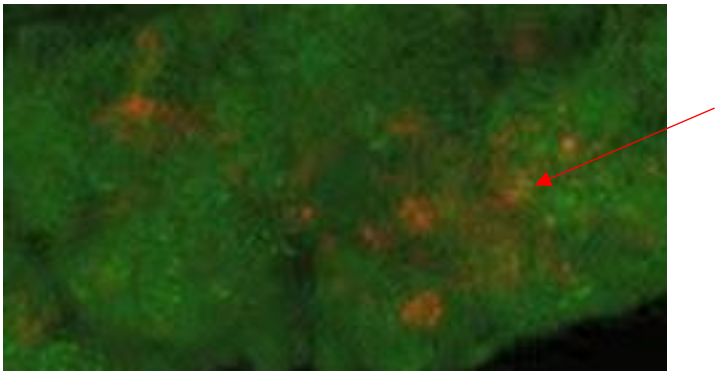

Figure 2.2.4: Confocal images of CLARITY treated mouse skin tissues following mock injection with PBS. Tissue was de-stained and re-stained with new fluorescent antibodies. MHC-II (bright green) and CD11b (red) were colocalized in the skin sample indicating macrophages were labeled. Higher autofluorescence was observed in the tissue after re-staining. n=1

2.4 DISCUSSION

Numerous tissue clearing techniques have been developed through the years to obtain information about processes and interactions that occur within biological tissues (6). There are many complicating factors that have made this effort difficult to achieve including penetration depth and light scattering. Different protocols used various dehydration methods, however, these methods caused severe tissue shrinkage which complicated any findings due to movement of structures within the tissue sample. In 2015 Schwarz et al. (7) proposed a method called FluoClearBABB to increase protein stability and reduce denaturation. This procedure still relied on hazardous materials and required specific mounting approaches to get optimal images. Developed in 2013, CLARITY remains the optimal clearing technique. Due to its ability to clear tissues efficiently, maintain relatively stable structures, and provide optimal images. The key improvement was removal of lipids which allows macromolecules to penetrate deep into tissues and allows higher resolution imaging due to decreased light scattering. One of the biggest modifications was utilizing laboratory prepped imaging solutions to lower expenses and provides preservation of endogenous fluorescence. Laboratory prepped imaging solutions also provide compatibility with aqueous refractive index matching mediums which allows the use of different microscopes and does not require specialized mounting apparatuses (6).

We aimed to contribute to the active field of optical tissue clearing by modifying the PACT protocol for use in skin samples. The skin differs from brain tissues in that there are more complex lipids that need to be removed and it is a thin, fragile tissue to put through the clearing process. The final protocol is extremely straightforward, but developing this modified method proved complicated at times due to the fragility of the skin samples. Samples were fixed in 4% paraformaldehyde as described in the original PACT method, but due to the thin and floppy

nature of skin, the skin would shrivel and curl. This was originally thought to pose issues in the final imaging, so we developed a plastic box to sew the skin samples into, so they would remain flat and not curl. The skin samples proved to be too fragile for this process and often the skin would tear or rip which would not be beneficial for any experimentally infected samples or human patient skin biopsies. We quickly abandoned this approach and continued without. While the skin still curled throughout much of the protocol, the final cleared tissue remained "relaxed" and uncurled.

Modifications in a few other steps of the protocol were also made specifically for skin samples. For mouse skin samples, we are able to obtain large areas of skin to clear. We modified the acrylamide polymerization step to avoid using N_2 to remove oxygen prior to placing the samples at 37° C. Instead, we overfilled the 15mL conical tubes prior to capping so no head space remained after the cap was tightened on the tube. Too much oxygen entered the solution after using N_2 before placing the samples at 37° C which caused suboptimal polymerization of the acrylamide and led to unstable tissues that had the potential to lose structures during delipidation with 8% SDS. This problem was exacerbated when additional days of 8% SDS treatment were added washes due to the complex nature of lipids present within the skin. By abandoning oxygen removal using N_2 and limiting head space, we were able to obtain consistent acrylamide polymerization resulting in a robust and stable tissue-hydrogel complex. The 8% SDS lipid removal step was repeated until the solution remained clear after 24 hours and no lipids were actively being removed from the tissues.

We aimed to modify the PACT method for skin samples to develop a robust *ex vivo* model to investigate immune cell interactions with *M. leprae* in human skin biopsies. We successfully tested experimentally infected skin samples for colocalization of innate immune

cells with various mycobacterial spp. including recombinant *Mycobacterium haemophilum* and *Mycolicibacterium smegmatis* that were both constitutively expressing mCherry. Both the *M. haemophilum* and *M. smegmatis* infected skin samples retained fluorescent bacteria that could be visualized during imaging, however, it was difficult to locate the small injection site in the tissue. A smaller tissue biopsy would allow more precise visualization of the injection site and a faster imaging timeline. While recombinant bacterial strains were used to validate this method, post-clearing labeling of the *M. leprae* will be necessary to identify bacteria in human skin biopsies. Post-clearing labeling allows visualization of specific structures of interest on the bacteria, providing more in-depth information about which bacterial structures are interacting with innate immune cells. These data provide evidence of effective clearing and fluorescent labeling of skin samples paving the way to investigate further interactions with other innate immune cells of interest in experimentally infected skin. For leprosy human skin biopsies, not only can interactions between immune cells and *Mycobacterium leprae* be investigated, but a more in-depth investigation into peripheral nerves (10) can be analyzed as well.

REFERENCES

1. Chung, K., Wallace, J., Kim, S. Y., Kalyanasundaram, S., Andalman, A. S., Davidson, T. J., Mirzabekov, J. J., Zalocusky, K. A., Mattis, J., Denisin, A. K., Pak, S., Bernstein, H., Ramakrishnan, C., Grosenick, L., Gradinaru, V., & Deisseroth, K. (2013). Structural and molecular interrogation of intact biological systems. *Nature*, *497*(7449), 332–337. https://doi.org/10.1038/nature12107
2. Du, H., Hou, P., Zhang, W., & Li, Q. (2018). Advances in CLARITY-based tissue clearing and imaging. *Experimental and therapeutic medicine*, *16*(3), 1567–1576. https://doi.org/10.3892/etm.2018.6374
3. Chen, Y., Shen, Q., White, S. L., Gokmen-Polar, Y., Badve, S., & Goodman, L. J. (2019). Three-dimensional imaging and quantitative analysis in CLARITY processed breast cancer tissues. *Scientific reports*, *9*(1), 5624. https://doi.org/10.1038/s41598-019-41957-w
4. Gómez-Gaviro, M. V., Sanderson, D., Ripoll, J., & Desco, M. (2020). Biomedical Applications of Tissue Clearing and Three-Dimensional Imaging in Health and Disease. *iScience*, *23*(8), 101432. https://doi.org/10.1016/j.isci.2020.101432
5. Libard, S., Cerjan, D., & Alafuzoff, I. (2019). Characteristics of the tissue section that influence the staining outcome in immunohistochemistry. *Histochemistry and cell biology*, *151*(1), 91–96. https://doi.org/10.1007/s00418-018-1742-1
6. Costantini, I., Cicchi, R., Silvestri, L., Vanzi, F., & Pavone, F. S. (2019). In-vivo and ex-vivo optical clearing methods for biological tissues: review. *Biomedical optics express*, *10*(10), 5251–5267. https://doi.org/10.1364/BOE.10.005251
7. Schwarz, M. K., Scherbarth, A., Sprengel, R., Engelhardt, J., Theer, P., & Giese, G. (2015). Fluorescent-protein stabilization and high-resolution imaging of cleared, intact mouse brains. *PloS one*, *10*(5), e0124650. https://doi.org/10.1371/journal.pone.0124650
8. Yang, B., Treweek, J. B., Kulkarni, R. P., Deverman, B. E., Chen, C. K., Lubeck, E., Shah, S., Cai, L., & Gradinaru, V. (2014). Single-cell phenotyping within transparent intact tissue through whole-body clearing. *Cell*, *158*(4), 945–958. https://doi.org/10.1016/j.cell.2014.07.017
9. Zhang, M. D., Tortoriello, G., Hsueh, B., Tomer, R., Ye, L., Mitsios, N., Borgius, L., Grant, G., Kiehn, O., Watanabe, M., Uhlén, M., Mulder, J., Deisseroth, K., Harkany, T., & Hökfelt, T. G. (2014). Neuronal calcium-binding proteins 1/2 localize to dorsal root ganglia and excitatory spinal neurons and are regulated by nerve injury. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(12), E1149–E1158. https://doi.org/10.1073/pnas.1402318111
10. Chung, K., & Deisseroth, K. (2013). CLARITY for mapping the nervous system. *Nature methods*, *10*(6), 508–513. https://doi.org/10.1038/nmeth.2481
11. Krahenbuhl J, Adams LB. 2000. Exploitation of gene knockout mice models to study the pathogenesis of leprosy. *Lepr Rev* 71 Suppl:S170-175.
12. Adams LB, Scollard DM, Ray NA, Cooper AM, Frank AA, Orme IM, Krahenbuhl JL. 2002. The study of *Mycobacterium leprae* infection in interferon-gamma gene-disrupted mice as a model to explore the immunopathologic spectrum of leprosy. *J Infect Dis* 185 Suppl 1:S1-8.
13. Kim, H. J., Brennan, P. J., Heaslip, D., Udey, M. C., Modlin, R. L., & Belisle, J. T. (2015). Carbohydrate-dependent binding of langerin to SodC, a cell wall glycoprotein of

Mycobacterium leprae. *Journal of bacteriology*, *197*(3), 615–625. https://doi.org/10.1128/JB.02080-14

14. Spencer JS, Kim HJ, Wheat WH, Chatterjee D, Balagon MV, Cellona RV, Tan EV, Gelber R, Saunderson P, Duthie MS, Reece ST, Burman W, Belknap R, Mac Kenzie WR, Geluk A, Oskam L, Dockrell HM, Brennan PJ. 2011. Analysis of antibody responses to *Mycobacterium leprae* phenolic glycolipid I, lipoarabinomannan, and recombinant proteins to define disease subtype-specific antigenic profiles in leprosy. *Clin Vaccine Immunol* 18:260-267
15. Spencer JS, Dockrell HM, Kim HJ, Marques MA, Williams DL, Martins MV, Martins ML, Lima MC, Sarno EN, Pereira GM, Matos H, Fonseca LS, Sampaio EP, Ottenhoff TH, Geluk A, Cho SN, Stoker NG, Cole ST, Brennan PJ, Pessolani MC. 2005. Identification of specific proteins and peptides in *Mycobacterium leprae* suitable for the selective diagnosis of leprosy. *J Immunol* 175:7930-7938.
16. Marques MA, Ant nio VL, Sarno EN, Brennan PJ, Pessolani MC. β001. Binding of αβ-laminins by pathogenic and non-pathogenic mycobacteria and adherence to Schwann cells. *J Med Microbiol* 50:23-28.
17. de Lima, C. S., Marques, M. A., Debrie, A. S., Almeida, E. C., Silva, C. A., Brennan, P. J., Sarno, E. N., Menozzi, F. D., & Pessolani, M. C. (2009). Heparin-binding hemagglutinin (HBHA) of Mycobacterium leprae is expressed during infection and enhances bacterial adherence to epithelial cells. *FEMS microbiology letters*, *292*(2), 162–169. https://doi.org/10.1111/j.1574-6968.2009.01488.x
18. Jin, S. H., Kim, S. K., & Lee, S. B. (2019). M. leprae interacts with the human epidermal keratinocytes, neonatal (HEKn) via the binding of laminin-5 with α-dystroglycan, integrin-β1, or -β4. *PLoS neglected tropical diseases*, *13*(6), e0007339. https://doi.org/10.1371/journal.pntd.0007339
19. Khanolkar, S. R., Mackenzie, C. D., Lucas, S. B., Hussen, A., Girdhar, B. K., Katoch, K., & McAdam, K. P. (1989). Identification of Mycobacterium leprae antigens in tissues of leprosy patients using monoclonal antibodies. *International journal of leprosy and other mycobacterial diseases : official organ of the International Leprosy Association*, *57*(3), 652–658.
20. Morgado de Abreu, M. A., Roselino, A. M., Enokihara, M., Nonogaki, S., Prestes-Carneiro, L. E., Weckx, L. L., & Alchorne, M. M. (2014). Mycobacterium leprae is identified in the oral mucosa from paucibacillary and multibacillary leprosy patients. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases*, *20*(1), 59–64. https://doi.org/10.1111/1469-0691.12190
21. Bahia El Idrissi, N., Das, P. K., Fluiter, K., Rosa, P. S., Vreijling, J., Troost, D., Morgan, B. P., Baas, F., & Ramaglia, V. (2015). M. leprae components induce nerve damage by complement activation: identification of lipoarabinomannan as the dominant complement activator. *Acta neuropathologica*, *129*(5), 653–667.

CHAPTER 3: ELUCIDATING BINDING INTERACTIONS BETWEEN *MYCOBACTERIUM LEPRAE* AND LANGERHANS CELLS THROUGH DIFFERENT CELL SURFACE RECEPTORS

3.1 INTRODUCTION

Chapter 3 presented novel methodology for investigating wholistic interactions between innate immune cells and *Mycobacterium leprae* in intact experimentally infected skin samples. However, to obtain a more detailed characterization of the specific Langerhans cell surface receptors that are involved in binding with *M. leprae* as well as the specific *M. leprae* ligands involved, *in vitro* cell-based assays could prove useful.

The causative agent of human leprosy is *Mycobacterium leprae*. The disease is characterized by nerve damage, skin lesions, and permanent disability (15). Numerous studies have investigated the interactions between *M. leprae* and nerve cells (21-25) or macrophages (16,26-29), however, information regarding the interactions between *M. leprae* and dendritic cells is lacking. It is known that *M. leprae* is phagocytized by Langerhans dendritic cells (LCs) (7-8). Mannosylated macromolecules on the cell envelope of *M. leprae* including mannose-capped lipoarabinomannan (ManLAM), lipomannan (LM), phosphatidylinositol mannosides (PIM), and glycoproteins contribute to the interaction with LCs (9). C-type lectins on the cell surface of LCs have been shown to directly bind specific mannosylated macromolecules of *Mycobacterium spp* (20) including *M. leprae*. Studies to elucidate the contributions of various ligands with each C-type lectin in cell-based assays have not been performed.

C-type lectin receptors are important pattern recognition receptors on innate immune cells including dendritic cells. Langerin, a C-type lectin receptor, is expressed on the cell surface of Langerhans cells. Located in the epidermis of human skin, Langerhans cells are of interest for

M. leprae infections. *M. leprae* grows preferentially between 33-35° C and selectively invades peripheral nerves and skin-resident innate immune cells including Langerhans cells. Langerin recognizes and binds carbohydrates such as mannose and has been studied in other diseases such as HIV-1 (13-14). Another C-type lectin receptor, DC-SIGN, is expressed on the cell surface of dendritic cells has been shown to bind ManLAM and PIM_6 of *M. leprae* (9).

Another cell surface receptor found on dendritic cell subsets, including LCs, is CD5. CD5 is a scavenger receptor that is also expressed on B-cells and promotes cell-to-cell interactions. CD5 on dendritic cells has been shown to mark a terminally differentiated inflammatory dendritic cell subsets (5). Beta-glucan has been identified as a potential ligand for CD5 (31), but there is no confirmed ligand in *M. leprae* infections. A study by Yin et al. from 2017, presented evidence for $CD5^{high}$ and $CD5^{low}$ expressing DCs that differ significantly in cytokine production, antigen presentation, and T-cell polarization. This is of interest for *M. leprae* infections where polarization of the immune response occurs resulting in distinct forms of disease. Elucidating how LCs bind and process ligands through CD5 may provide insight into the mechanisms behind this polarization.

Previous work demonstrated that LCs present cell wall antigens of *M. leprae* to CD1a-restricted T-cells. The T-cells proliferated and produced gamma interferon (IFN-γ). Further work determined that the *M. leprae* cell wall glycoprotein, SodC, bound to langerin more than other mannosylated macromolecules in a cell-free surface plasmon resonance (SPR) assay. However, this has not been confirmed in a more biologically relevant model. Due to *M. leprae* being an obligate intracellular pathogen, using an animal model to study the initial interactions during infection poses challenges. Thus, a cell-based *in vitro* method is required to elucidate the binding characteristics of LCs with *M. leprae* that may be occurring in the skin. Utilizing lab

differentiated Langerhan-like DCs (LC-DCs) and a langerin expressing THP1 cell line, we evaluated the binding characteristics with mycobacterial lipoglycans and glycoproteins as well as live *M. leprae*.

Here we present findings that additional LC cell surface receptors interact with *M. leprae*. Additionally, we confirm the interaction between langerin and glycosylated SodC in a cell-based assay. We also present evidence that langerin directly interacts with whole *M. leprae* and binding can be inhibited with preincubation of blocking reagents.

3.2 MATERIALS AND METHODS

3.2.1 Expression and production of recombinant M. leprae SodC in Mycolicibacterium smegmatis and Escherichia coli

Recombinant *M. leprae* SodC (ML1925) was expressed in *M. smegmatis* to obtain the glycosylated form of the protein. It was purified as previously described (9) with slight modifications for higher protein yield. Briefly, the recombinant plasmid, pMRLB101, was transformed into *M. smegmatis* mc^2155 ΔGroEL using electroporation and maintained with the addition of kanamycin. Cultures were upscaled to 5L in 7H9 (VWR, Radnor, PA) medium supplemented with 5% glucose to obtain enough rSodC for downstream experiments. Cells were harvested by centrifugation and lysed via French press. The rSodC with a C-terminal 6×His tag was purified by immobilized metal affinity chromatography (IMAC) with nickel-nitrilotriacetic acid (Ni-NTA)-agarose resin (Qiagen, Hilden, Germany), also described previously (9). Eluted rSodC was desalted and concentrated in an Amicon Ultra-15 10 K centrifugal filter unit (Millipore, Bedford, MA). Purity of rSodC was determined by silver stain. Purified rSodC was stored in phosphate buffered saline at -20° C.

3.2.2 Preparation of mycobacterial fractions and ligands

M. leprae ManLAM and PIM_6 were generated at Colorado State University and supplied through the Biodefense and Emerging Infections (BEI) Research Resources Repository ((http://www.beiresources.org/TBVTRMResearchMaterials/tabid/1431/Default.aspx))

3.2.3 Bacterial Cultures

M. leprae purified from athymic *nu/nu* mouse footpads was kindly donated by the National Hansen's Disease Program (Laboratory Research Branch, Louisiana State University, Baton Rouge, LA, USA).

M. smegmatis mc^2155 was grown in 7H9 medium supplemented with 5% glucose and OADC at 37° C while shaking for 3 days until the culture reached an OD_{600} of 0.6. *M. haemophilum* was grown in 7H9 medium supplemented with 5% glucose, 60 μM Hemin (Sigma-Aldrich, St. Louis, MO), and OADC at 30° C protected from light while shaking for 7 days until the culture reached an OD_{600} of 0.6.

3.2.4 Cell Culture

Langerhans-like dendritic cells (LCDCs) were differentiated from $CD34^+$ stem cells (Lonza, Basel, Switzerland) for 12 days. Stem cells were quickly thawed in a 37° C water bath and placed in prewarmed RPMI medium (VWR) containing 10% FBS (Atlas Biologicals, Fort Collins, CO), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were washed two times with supplemented RPMI medium. After the final wash, cells were cultured in a T175 flask containing 30 mL supplemented RPMI medium with 100 ng/mL GM-CSF (BioLegend, San

Diego, CA), 2.5 ng/mL TNF-α (BioLegend), and 25 ng/mL SCF (Fisher Scientific) at 37° C with 5% CO_2. Cells were washed and split into two flasks with fresh supplemented RPMI containing GM-CSF, TNF-α, and SCF on day 3. On days 5, 7, and 9 cells were washed, split, and placed in fresh supplemented RPMI containing 100 ng/mL GM-CSF and 1 ng/mL TGF-β1 (BioLegend). Cells were collected via centrifugation at 1,200 rpm for 10 min, counted using a hemocytometer, and frozen at -80° C on day 12 when langerin and CD1a expression were the highest as determined by flow cytometry.

Transfected THP1 cells (THP-L) and empty vector THP1 (THP-EV) cells were kindly provided by Dr. Nina van Sorge and cultured as previously described (17). Briefly, cells were cultured in RPMI supplemented with 5% FBS, 1% GlutaMAX (Life Technologies, Carlsbad, CA), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37° C with 5% CO_2 until they reached 80% confluence.

Transfected HeLa cells (HeLa-Lang) and WT HeLa cells were kindly provided by Dr. Robert Modlin. Cells were cultured in DMEM (Fisher Scientific, Waltham, MA) supplemented with 10% FBS, and 1 μg/mL geneticin (Thermo Fisher, Waltham, MA) at 37° C with 5% CO_2 until they reached 80% confluence.

3.2.5 Bead Coating

Blue/green fluorescent polystyrene beads (Polysciences, Warrington, PA) (1 μm diameter) were coated with 100 μg of mannan, ManLAM, PIM_6, unglycosylated rSodC (previously produced in the Belisle laboratory) (9), or glycosylated rSodC in sodium bicarbonate buffer (pH 9.6).

Conditions for coating beads with ManLAM, PIM_6, or mannan were incubation for 3h at room temperature with rocking followed by an overnight incubation at 4°C. Unglycosylated rSodC and glycosylated rSodC coated beads were only incubated overnight at 4° C. Following incubation, beads were washed three times with 1X PBS and then blocked with 2% human serum albumin (HSA) (Sigma Aldrich) in PBS overnight and at 4° C. Coated beads were stored in PBS at 4° C (18).

3.2.6 Bacterial binding assays

M. leprae purified from athymic *nu/nu* mouse footpads was washed with 1X PBS and fluorescently labeled with 0.1 mg/mL FITC isomer 1 (Sigma Aldrich) as previously described (19). LCDCs,THP1-L or THP1-EV (10^5 cells) were incubated with FITC-labeled *M. leprae* (MOI 10:1) in RPMI containing 2% Human Serum Albumin (HSA) for 10 min at 4° C followed by an additional 30 min at 33° C. Binding was blocked with preincubation for 15 min at 4° C with blocking reagents (2.5 mg/mL mannan (Sigma Aldrich), α-CD207, α-rLangerin, α-CD5, α-MHC-II, or α-DC-SIGN). Cells were washed once with RPMI containing 2% HSA to remove unbound bacteria and resuspended in 2% paraformaldehyde in PBS and measured by flow cytometry.

Table 3.1: Blocking antibodies used to pre-block various receptors on LCDCs prior to binding experiments.

Marker	Clone	Manufacturer	Concentration/ Dilution
CD207	10E2	BioLegend	20 ug/mL
CD207	REA770	Miltenyi Biotec	1:50
HLA-DR (MHC-II)	Tu39	BD Biosciences	10 ug/mL
DC-SIGN	120507	AbCam	10 ug/mL
CD5	L17F12	BioLegend	10 ug/mL

3.2.7 Coated beads binding assays

LCDCs, HeLa-Lang or HeLa-WT (10^5 cells) were incubated with coated blue/green fluorescent polystyrene beads (as described above) in RPMI containing 2% HSA for 1 h at 37° C. Binding was blocked with preincubation for 30 min at 37° C with blocking reagents (2.5 mg/mL mannan, or α-CD207 (Miltyni Biotech, Auburn, CA). Cells were washed once with RPMI containing 2% HSA to remove unbound beads and resuspended in 2% paraformaldehyde in PBS. HeLa cells were measured by confocal microscopy and LCDCs were measured by flow cytometry.

3.2.8 Flow Cytometry

Flow cytometry was performed on a FACSCantoII (BD Biosciences, Franklin Lakes, NJ). Per sample, 10,000 events were collected. Data were analyzed using FlowJo 10 (FlowJo, LLC, Ashland, OR).

Table 3.2: Fluorescent antibodies used to stain LCDCs, THP cells, and HeLa cells.

Marker	Clone	Fluorophore	Company
CD207	DCGM4	PE	Beckman Coulter
CD1a	O10	Alexa Fluor 647	Novus
CD5	G043H7	BV421	BioLegend

3.2.9 Imaging

Confocal imaging was performed using an inverted Olympus FV1000-IX81 confocal imaging system. Images were analyzed with Volocity (Perkin Elmer, Waltham, MA).

3.2.10 Statistics

Two tailed paired t-tests were used to evaluate differences between groups.

3.3 RESULTS

3.3.1 Langerin expression on various cell lines

Langerin expression was determined via flow cytometry. LCDCs were determined to have suitable expression for further experiments if the percentage of cells expressing langerin was above 80% on day 12 of differentiation. Similarly, THP-L and HeLa-LANG cell lines were determined to have suitable expression if cells expressing langerin was above 80%. HeLa-LANG cells lost expression through culture over time, but the addition of geneticin maintained langerin expression.

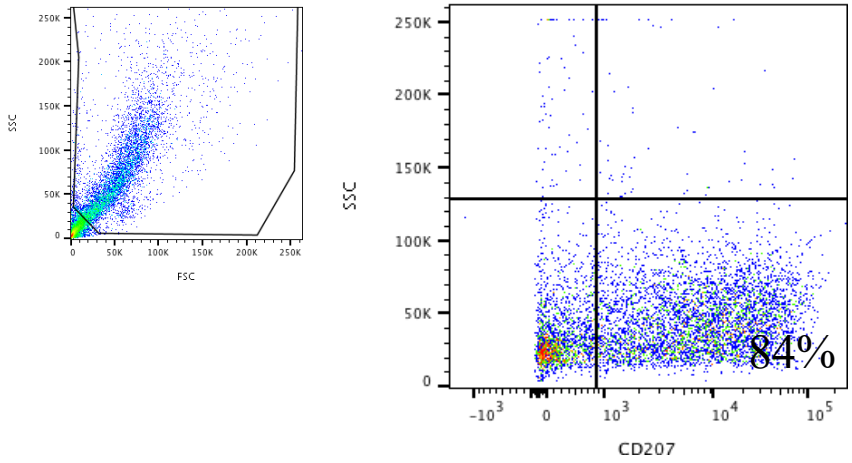

Figure 3.1.1: LCDCs were gated on FSC vs SSC to eliminate cell debris from analysis. 84% of LCDCs were CD207^{+} on day 12 of differentiation.

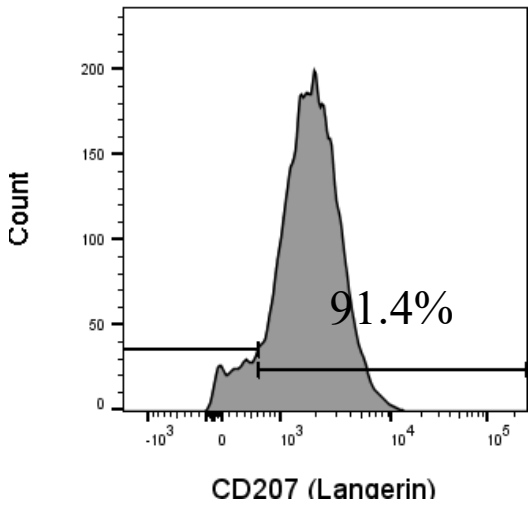

Figure 3.1.2: 91.4% of HeLa-Lang cells were CD207$^+$ as determined by flow cytometry.

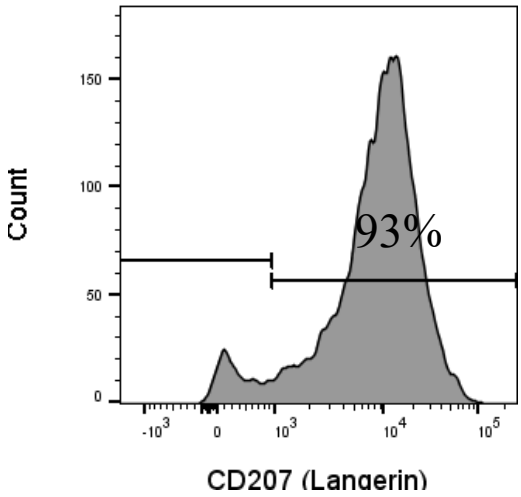

Figure 3.1.3: 93% of THP-L cells were CD207$^+$ as determined by flow cytometry.

3.3.2 Cell surface expression of LCDCs

Further elucidation of other cell surface receptors on day 12 differentiated LCDCs was conducted to understand what receptors may contribute to binding in downstream experiments. In table 3.2.1, cell surface receptors, clone, and isotype are outlined. LCDCs were determined to also express CD1a and CD5 (Figure 3.2.2).

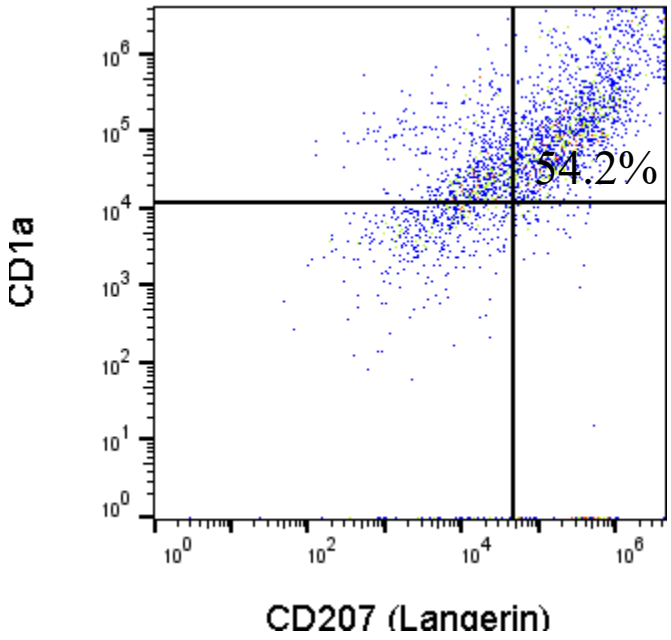

Figure 3.2.1: As determined by flow cytometry, 54.2% of LCDCs expressed $CD207^+$ and $CD1a^+$. Additional cells were $CD1a^+$ and $CD207^-$.

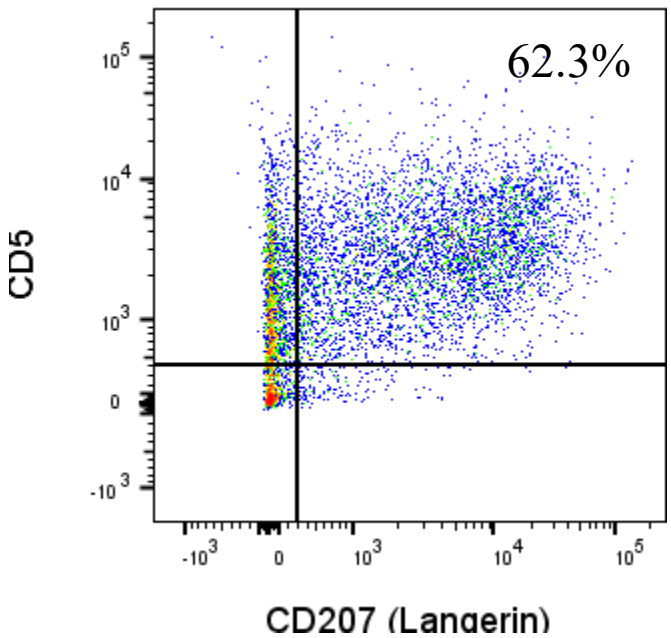

Figure 3.2.2: As determined by flow cytometry, 62.3% of LCDCs expressed $CD207^{+}$ and $CD5^{+}$. Additional cells were $CD5^{+}$ and $CD207^{-}$.

3.3.3 Binding capacity of CD5 sorted LCDCs with M. leprae

We first wanted to investigate if uptake of *M. leprae* by LCDCs was affected by CD5 cell surface expression level. Day 12 differentiated LCDCs were cell sorted via CD5 expression levels (high and low). In figure 3.3.1, 1.8% of LCDCs sorted were CD5 high and 66.8% were CD5 low.

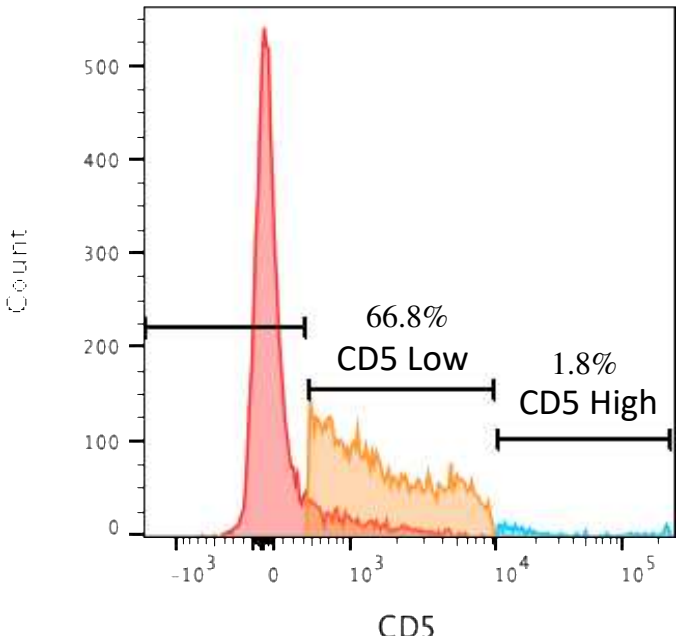

Figure 3.3.1 Multiple populations of CD5 LCs were detected via flow cytometry. $CD5^{high}$ (blue), $CD5^{low}$ (orange), and CD5 negative (red). Cells were subsequently sorted to obtain isolated populations of $CD5^{high}$ and $CD5^{low}$ for further studies. n=3

Sorted cells were placed in culture to recover and assessed in a binding assay with live *M. leprae* (MOI 10:1) on the following day. ~10% of $CD5^{high}$ cells bound to *M. leprae* compared to 16.7% of $CD5^{low}$ cells. However, the analysis of langerin contribution were inconclusive. Binding was not significantly inhibited by pre-blocking the langerin receptor with either mannan or a recombinant blocking antibody. Blocking multiple cell surface receptors using blocking antibodies also did not inhibit binding (Figure 3.3.2).

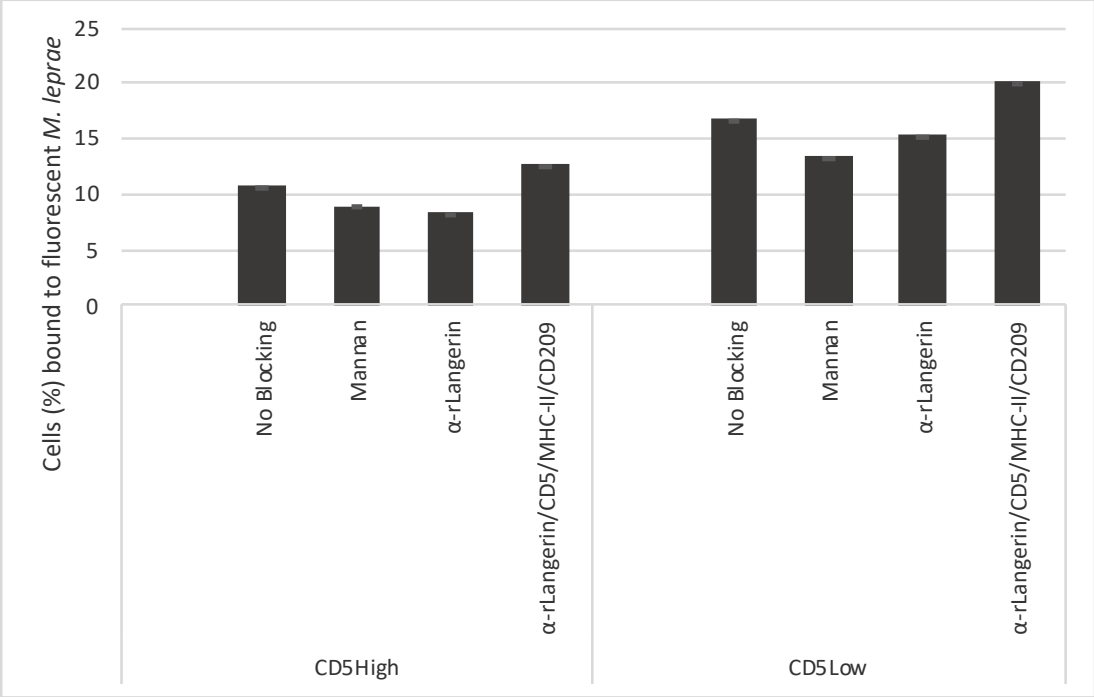

Figure 3.3.2: 10.7% of CD5high LCs bound to *M. leprae*. Binding was not significantly inhibited with any blocking reagents (mannan, α-rLangerin, or multiple receptors). 16.7% of CD5low LCs bound to *M. leprae*. Binding was not significantly inhibited with any blocking reagents (mannan, α-rLangerin, or multiple receptors). n=3

Utilizing remaining unsorted LCDCs, various receptors were blocked to assess contribution of binding with *M. leprae*. While the non-specific blocking reagent, mannan, inhibited binding, further inhibition was observed when combined with blocking antibodies specific for CD5; however, in contrast, CD209 did not appear to contribute to the binding of *M. leprae* to the LCDCs (Figure 3.3.3).

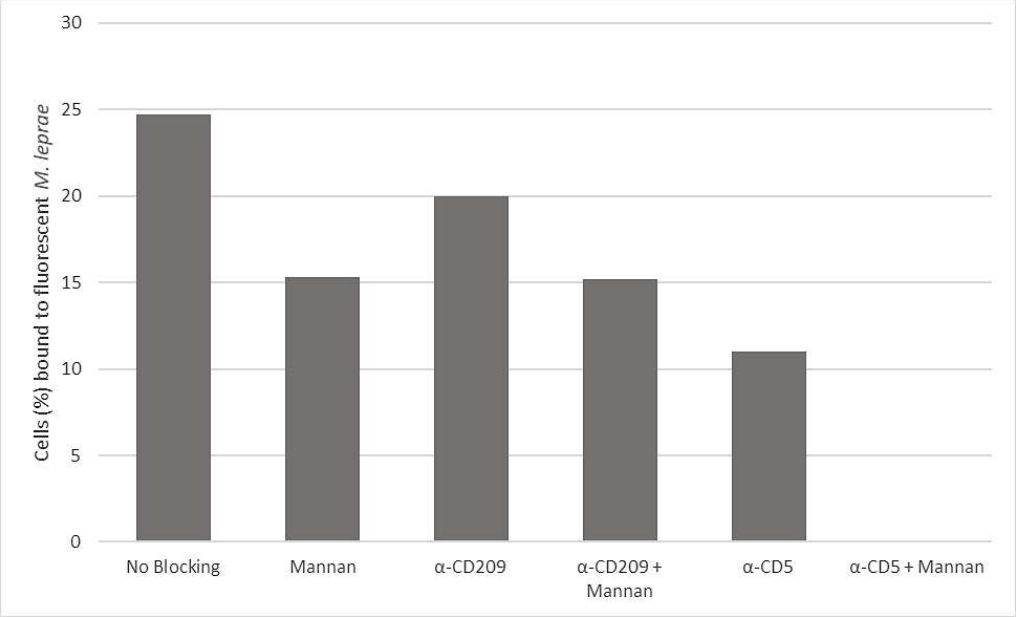

Figure 3.3.3: 24.9% of LCDCs bound to *M. leprae* when langerin was not blocked. Addition of mannan inhibited binding through langerin. Pre-incubation with α-CD209 or α-CD5 inhibited binding with *M. leprae* as compared to the unblocked condition indicating each receptor binds to *M. leprae*. Combinations of α-CD209/mannan or α-CD5/mannan inhibited binding further indicating both langerin and other receptors bind *M. leprae*. n=1

3.3.4 Elucidation of the binding contribution of langerin with M. leprae

We further elucidated the role of langerin in binding of *M. leprae*. LCDCs bind to *M. leprae* and binding can be partially inhibited when langerin is pre-blocked. Pre-blocking with free mannan inhibited binding by 10%. Similar inhibition was observed using a langerin blocking antibody, however, the inhibition was not consistent most likely due to potential glycosylation of the antibody which contributed to additional binding of *M. leprae*. As expected, the negative control isotype antibody did not inhibit binding.

Taken together, these data confirm that langerin binds to *M. leprae*, but is not the exclusive cell-surface receptor involved in initial binding.

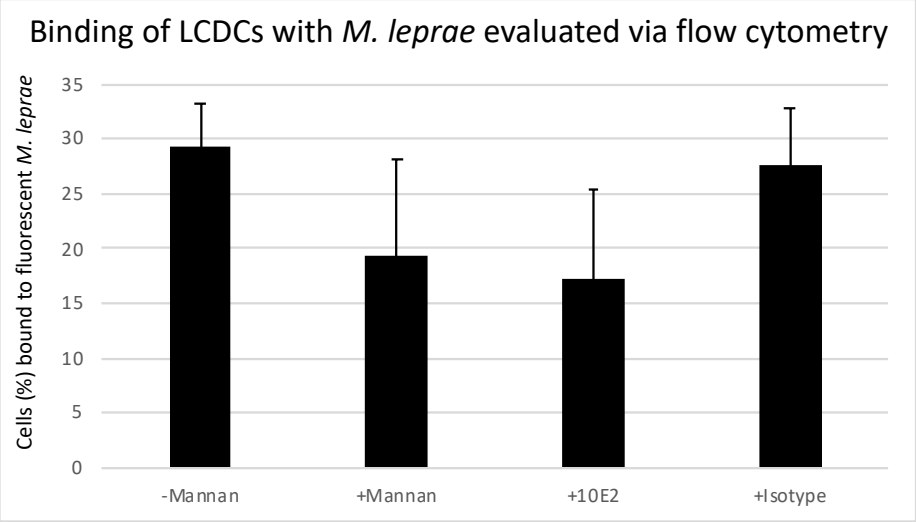

Figure 3.4.1: 29.4% of LCDCs bound to *M. leprae*. Blocking langerin with mannan, inhibited binding to 19.3%, and blocking with the blocking antibody, 10E2, inhibited binding to 17.2%. 27.6% of LCDCs bound to *M. leprae* after preincubating LCDCs with the negative control isotype antibody. No significance was determined (two tailed paired t-test). n=3

While other receptors appear to be contributing to binding *M. leprae* in LCDCs, we sought to understand langerin exclusively. Our collaborators at the University of California, Los Angeles (UCLA), provided us with transfected THP1 cells expressing langerin (THP-L) and the negative control cell line THP-empty vector (THP-EV). 18% of THP-L cells bound *M. leprae* without blocking reagents. When langerin was blocked with mannan or a recombinant langerin blocking antibody, inhibitable binding was observed. Specifically, binding was reduced to 9% when cells were preincubated with mannan and 10% when preincubated with the recombinant blocking antibody. Another blocking antibody, 10E2, that had been used previously with LCDCs did not block binding with *M. leprae.* We hypothesize there are glycosylated structures on the 10E2 antibody that non-specifically bind with *M. leprae* since it is produced in animals. As expected, the negative control isotype antibody did not inhibit binding. The THP-EV cells had low level background binding but did not show a similar decrease in binding with blocking reagents, suggesting non-specific binding (Figure 3.4.2A). Mannan-coated polystyrene beads were used as a positive control to confirm binding and inhibition was directly correlated with langerin binding (Figure 3.4.2B)

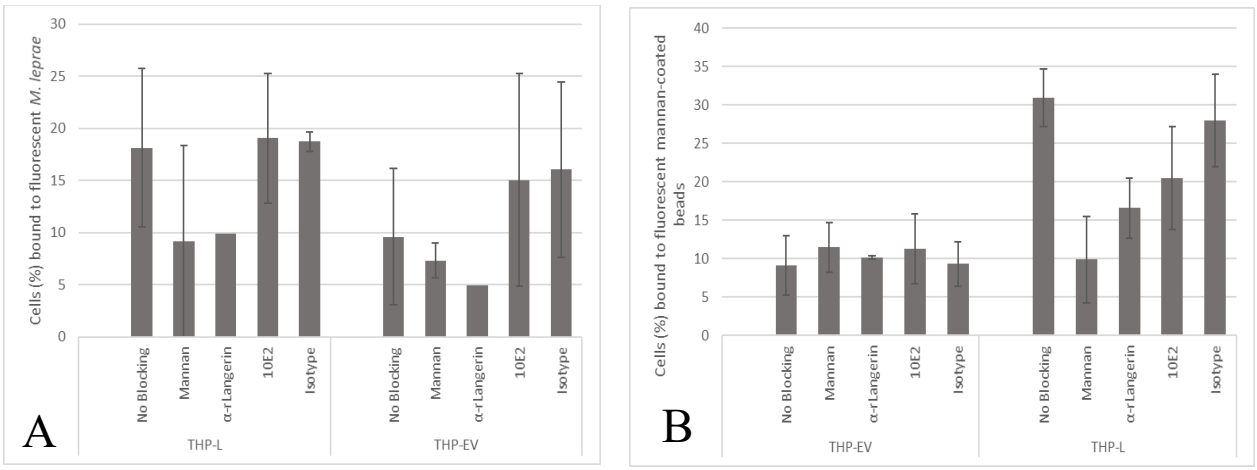

Figure 3.4.2: (A) 18.1% of THP-L cells bound to *M. leprae* when langerin was not blocked. Binding was inhibited to 9.2% with preincubation of mannan and 9.9% with α-rLangerin. Binding was not inhibited using the blocking antibody, 10E2. (B) Mannan-coated beads were used a control to ensure mannan-inhibitable binding could be observed with THP-L cells. n=3

3.3.5 Binding contribution of various mycobacterial cell wall components with langerin

Previous work (9) determined that the glycosylated cell wall protein, SodC, predominately interacted with langerin as compared to other cell wall components such as LAM or PIMs in an SPR assay. We utilized mycobacterial cell wall components bound to fluorescent polystyrene beads to elucidate if the findings were similar in a cell-based binding assay. HeLa-Lang and WT HeLa cells were used due to their adherent nature and binding was determined via confocal microscopy. *M. leprae* recombinant glycosylated SodC, produced in *M. smegmatis* showed higher binding affinity to HeLa-LANG cells in comparison to other mycobacterial cell wall components or the unglycosylated form of SodC produced in *E. coli*. Glycosylated SodC still bound to WT HeLa cells, most likely in a non-specific manner, but the interaction could not be inhibited with known blocking reagents (Figure 3.5.1).

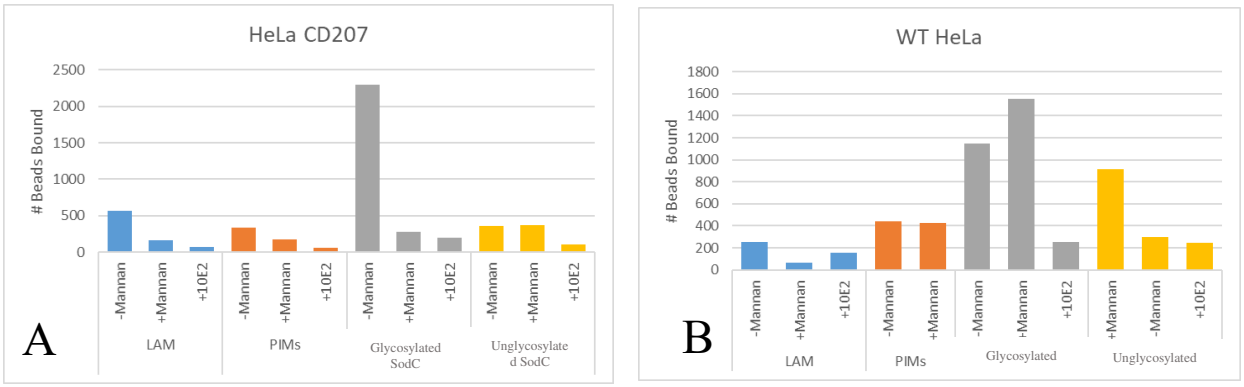

Figure 3.5.1: HeLa-Lang (HeLa CD207) cells showed inhibitable binding with glycosylated SodC (A). WT HeLa cells bound to glycosylated SodC similarly to HeLa-Lang but binding could not be inhibited with mannan (B) suggesting non-specific binding due to lack of langerin expression on WT HeLa cells. n=1

Replicating the experiment from figure 3.5.1, proved that the system is more complicated than the cell-free SPR assay suggested. Other cell wall components like LAM and PIMs bound as well or better than glycosylated SodC; however, the other components did not show inhibitable binding with blocking reagents. These data suggested that glycosylated SodC specifically interacts with langerin and the other cell wall components are non-specifically interacting with the HeLa cells (Figure 3.5.2).

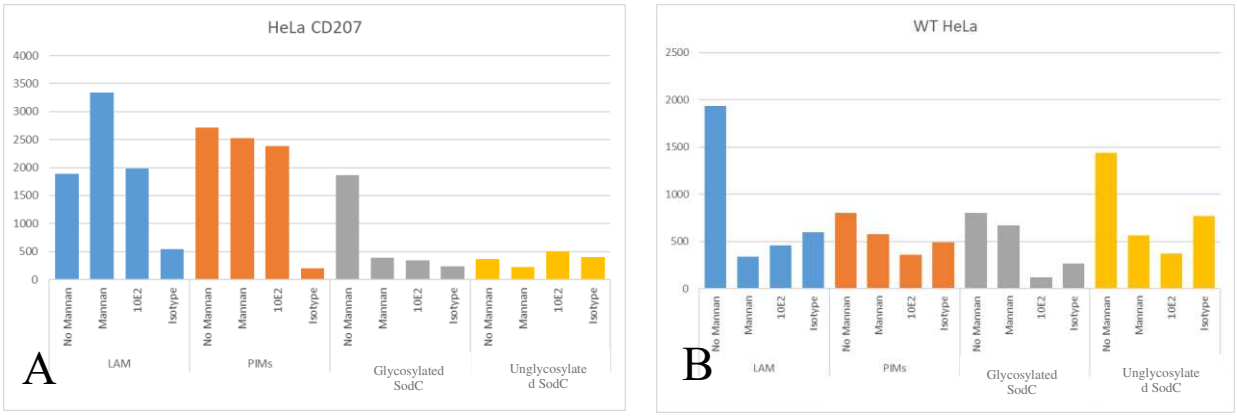

Figure 3.5.2: HeLa-Lang (HeLa CD207) cells showed inhibitable binding with glycosylated SodC (A). LAM and PIM_6 bound to HeLa-Lang cells similarly to glycosylated SodC, but binding could not be inhibited. WT HeLa cells bound to coated beads (B) suggesting non-specific binding due to lack of langerin expression on WT HeLa cells. n=1

3.3.6 Determining binding with other mycobacterial species

Since *M. leprae* cannot be cultured in a laboratory and has shown to be highly variable in cell-based binding assays (Figure 3.4.1), we explored if other mycobacterial spp., specifically *Mycobacterium haemophilum*, could be utilized as a model in *in vitro* assays. *M. haemophilum* is the most closely related mycobacterial spp. to *M. leprae* that can be cultured in a laboratory. Binding between *M. haemophilum* and LCDCs was observed, but inhibiting with mannan or the langerin blocking antibody did not show similar results to *M. leprae* (Figure 3.6.1). More work is needed to understand whether *M. haemophilum* could serve as a model during *in vitro* binding assays and if the native SodC glycoprotein is expressed similarly.

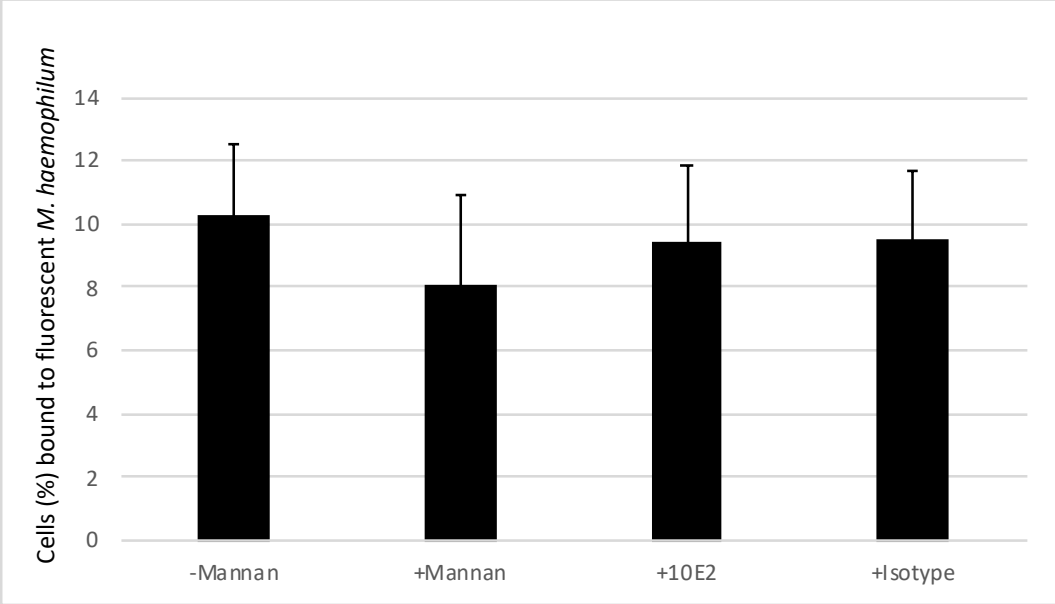

Figure 3.6.1: 10.2% of LCDCs bound to *M. haemophilum* when langerin was not blocked. Inhibition was slightly observed with the addition of mannan, but not significantly. Preincubation with the langerin blocking antibody, 10E2, did not significantly inhibit binding. No significance observed (Two-tailed paired t-test) n=3

3.4 DISCUSSION

Previous work in our laboratory provided biochemical evidence that langerin directly interacts with the glycoprotein, SodC, of *M. leprae* and other dominant lipoglycans including ManLAM and PIM_6. In cell-free binding assays, they found that SodC reacted with langerin more than the other dominant lipoglycans. In our current study using cell-based assays to elucidate binding characteristics, we found that while glycosylated SodC binds to langerin with stem cell derived LCDCs, the interactions of *M. leprae* with this cell type appears more complex than a simple langerin mediated interaction.

We investigated how various mannosylated macromolecules of *M. leprae* bound to langerin in cell-based assays. Using beads coated with the various mannosylated macromolecules and a simplified cell system (HeLa-Lang), we found that while glycosylated SodC was the only ligand that could be inhibited from binding to langerin in binding assays, ManLAM and PIM_6 still bound to langerin, sometimes as efficiently as glycosylated SodC. We also tested binding with LCDCs and found that glycosylated SodC bound to a higher percentage of cells than ManLAM and PIM_6, but not significantly. This indicates that all of these components contribute to binding at some level.

We confirmed that glycosylation is important for LCs to recognize and bind to *M. leprae* by globally disrupting glycosylation on the cell surface of *M. leprae* prior to binding experiments. Binding was reduced when glycosylation was disrupted confirming previous results in cell-free assays (9).

The previous results indicated that other LC receptors are involved in recognition and binding of *M. leprae*, thus we sought to understand how recognition of *M. leprae* was affected when langerin was blocked. Langerin is an important piece of the puzzle and we demonstrated

that blocking langerin prior to adding *M. leprae* to the culture system inhibited binding by up to 50%. However, inhibitable binding was not always reproducible. We regularly checked our cell lines for langerin expression via flow cytometry to ensure the cells had proper langerin available for binding, but *M. leprae* is a complicated bacterium that may not perform exactly the same in binding assays due to necessary growth conditions within a *nu/nu* mouse footpad.

Langerin is not the only cell surface receptor that may contribute to recognizing and internalizing *M. leprae*. Our findings indicate that CD5, a scavenger receptor, also contributes towards recognition and binding. Based on previous evidence of $CD5^{high}$ and $CD5^{low}$ dendritic cell sub-populations having distinct differences in cytokine profiles and antigen presentation (1, 3-6), we investigated if these populations would have differences in binding capacity for *M. leprae*. Our findings showed that fewer $CD5^{high}$ LCs bound *M. leprae* in comparison to $CD5^{low}$ LCs indicating that they do have distinct properties including binding characteristics. Further work needs to be completed to determine if cytokine secretion after *M. leprae* binding is different between the two subpopulations. $CD5^{high}$ cells have been shown to migrate more efficiently and induce IL-10-producing T cells. $CD5^{low}$ cells have been shown to induce IFN-γ producing T cells (2). This could have important implications for the polarization of disease seen in leprosy patients and how their immune system handles initial binding and uptake events. We hypothesize that if binding of *M. leprae* occurs via $CD5^{high}$ LCs, a Th2 primed immune response could occur and shift towards the lepromatous pole. On the other hand, there is a much higher percentage of $CD5^{low}$ LCs within the skin and if binding of *M. leprae* occurs via $CD5^{low}$ LCs, a Th1, cell-mediated response would be activated.

These studies had shortcomings due to the challenging nature of Langerhans cells and *M. leprae*. Binding assays were performed with fewer cells per condition than ideal due to limited

proliferation and differentiation of LCDCs. A 24-hour time window of optimal langerin expression on LCDCs meant that cells needed to be frozen or used fresh on that day. Due to the limitations surrounding obtaining live *M. leprae*, often LCDCs would be frozen on day 12 of differentiation and used in subsequent experiments when shipments of *M. leprae* would arrive. Cells were rested overnight after thawing and utilized in a binding assay the following day. Other complicating factors included identifying optimal blocking antibodies and reagents to observe meaningful inhibition of binding between *M. leprae* and LCDCs. *Mycobacterium leprae* is difficult to work with *in vitro* due to its limited viability outside of a host and combined with the finicky nature of LCDCs as well as the need for high quality blocking reagents meant that slotting all of the intricate pieces of the puzzle together was complicated and required precise timing and patience.

Future studies to elucidate all the pieces of the puzzle should include developing a transfected cell line expressing CD5 to investigate *M. leprae* specific ligands. Our data present strong evidence that CD5 contributes to recognition and binding of *M. leprae*. Uptake via CD5 may alter which cytokines are released and modify the microenvironment leading to differences in T cell activation. More work also needs to be conducted to develop *M. haemophilum* as a model for *M. leprae* in cell-based binding assays to reduce bacterial variability and develop robust conclusions about the effects of inhibiting, binding and uptake via langerin.

REFERENCES

1. Yin, X., Yu, H., Jin, X., Li, J., Guo, H., Shi, Q., Yin, Z., Xu, Y., Wang, X., Liu, R., Wang, S., & Zhang, L. (2017). Human Blood CD1c+ Dendritic Cells Encompass CD5high and CD5low Subsets That Differ Significantly in Phenotype, Gene Expression, and Functions. *Journal of immunology (Baltimore, Md. : 1950)*, *198*(4), 1553–1564. https://doi.org/10.4049/jimmunol.1600193
2. Miranda, A., Amadeu, T. P., Schueler, G., Alvarenga, F. B., Duppré, N., Ferreira, H., Nery, J. A., & Sarno, E. N. (2007). Increased Langerhans cell accumulation after mycobacterial stimuli. *Histopathology*, *51*(5), 649–656. https://doi.org/10.1111/j.1365-2559.2007.02848.x
3. Korenfeld, D., Gorvel, L., Munk, A., Man, J., Schaffer, A., Tung, T., Mann, C., & Klechevsky, E. (2017). A type of human skin dendritic cell marked by CD5 is associated with the development of inflammatory skin disease. *JCI insight*, *2*(18), e96101. https://doi.org/10.1172/jci.insight.96101
4. Burgueño-Bucio E, Mier-Aguilar CA, Soldevila G. The multiple faces of CD5. J Leukoc Biol. 2019 May;105(5):891-904. doi: 10.1002/JLB.MR0618-226R. Epub 2019 Jan 24. PMID: 30676652.
5. Canton J, Neculai D, Grinstein S. Scavenger receptors in homeostasis and immunity. Nat Rev Immunol. 2013 Sep;13(9):621-34. doi: 10.1038/nri3515. Epub 2013 Aug 9. PMID: 23928573.
6. Zani IA, Stephen SL, Mughal NA, Russell D, Homer-Vanniasinkam S, Wheatcroft SB, Ponnambalam S. Scavenger receptor structure and function in health and disease. Cells. 2015 May 22;4(2):178-201. doi: 10.3390/cells4020178. PMID: 26010753; PMCID: PMC4493455.
7. Dang, A. T., Teles, R. M., Liu, P. T., Choi, A., Legaspi, A., Sarno, E. N., Ochoa, M. T., Parvatiyar, K., Cheng, G., Gilliet, M., Bloom, B. R., & Modlin, R. L. (2019). Autophagy links antimicrobial activity with antigen presentation in Langerhans cells. *JCI insight*, *4*(8), e126955. https://doi.org/10.1172/jci.insight.126955
8. Hunger, R. E., Sieling, P. A., Ochoa, M. T., Sugaya, M., Burdick, A. E., Rea, T. H., Brennan, P. J., Belisle, J. T., Blauvelt, A., Porcelli, S. A., & Modlin, R. L. (2004). Langerhans cells utilize CD1a and langerin to efficiently present nonpeptide antigens to T cells. *The Journal of clinical investigation*, *113*(5), 701–708. https://doi.org/10.1172/JCI19655
9. Kim, H. J., Brennan, P. J., Heaslip, D., Udey, M. C., Modlin, R. L., & Belisle, J. T. (2015). Carbohydrate-dependent binding of langerin to SodC, a cell wall glycoprotein of Mycobacterium leprae. *Journal of bacteriology*, *197*(3), 615–625. https://doi.org/10.1128/JB.02080-14
10. Krahenbuhl J, Adams LB. 2000. Exploitation of gene knockout mice models to study the pathogenesis of leprosy. *Lepr Rev* 71 Suppl:S170-175.
11. Adams LB, Scollard DM, Ray NA, Cooper AM, Frank AA, Orme IM, Krahenbuhl JL. 2002. The study of *Mycobacterium leprae* infection in interferon-gamma gene-disrupted mice as a model to explore the immunopathologic spectrum of leprosy. *J Infect Dis* 185 Suppl 1:S1-8.
12. Mahapatra S, Crick DC, McNeil MR, Brennan PJ. 2008. Unique structural features of the peptidoglycan of *Mycobacterium leprae*. *Journal of Bacteriology* 190:655-661.
13. de Witte, L., Nabatov, A., Pion, M., Fluitsma, D., de Jong, M. A., de Gruijl, T., Piguet, V., van Kooyk, Y., & Geijtenbeek, T. B. (2007). Langerin is a natural barrier to HIV-1

transmission by Langerhans cells. *Nature medicine*, *13*(3), 367–371. https://doi.org/10.1038/nm1541
14. van den Berg, L. M., Cardinaud, S., van der Aar, A. M., Sprokholt, J. K., de Jong, M. A., Zijlstra-Willems, E. M., Moris, A., & Geijtenbeek, T. B. (2015). Langerhans Cell-Dendritic Cell Cross-Talk via Langerin and Hyaluronic Acid Mediates Antigen Transfer and Cross-Presentation of HIV-1. *Journal of immunology (Baltimore, Md. : 1950)*, *195*(4), 1763–1773. https://doi.org/10.4049/jimmunol.1402356
15. Centers for Disease Control and Prevention. (2017, February 10). *What is Hansen's Disease? | Hansen's Disease (Leprosy) | CDC*. https://www.cdc.gov/leprosy/about/about.html
16. Díaz Acosta, C. C., Dias, A. A., Rosa, T., Batista-Silva, L. R., Rosa, P. S., Toledo-Pinto, T. G., Costa, F., Lara, F. A., Rodrigues, L. S., Mattos, K. A., Sarno, E. N., Bozza, P. T., Guilhot, C., de Berrêdo-Pinho, M., & Pessolani, M. (2018). PGL I expression in live bacteria allows activation of a CD206/PPARγ cross-talk that may contribute to successful Mycobacterium leprae colonization of peripheral nerves. *PLoS pathogens*, *14*(7), e1007151. https://doi.org/10.1371/journal.ppat.1007151
17. van Dalen, R., De La Cruz Diaz, J. S., Rumpret, M., Fuchsberger, F. F., van Teijlingen, N. H., Hanske, J., Rademacher, C., Geijtenbeek, T., van Strijp, J., Weidenmaier, C., Peschel, A., Kaplan, D. H., & van Sorge, N. M. (2019). Langerhans Cells Sense *Staphylococcus aureus* Wall Teichoic Acid through Langerin To Induce Inflammatory Responses. *mBio*, *10*(3), e00330-19. https://doi.org/10.1128/mBio.00330-19
18. Kang, P. B., Azad, A. K., Torrelles, J. B., Kaufman, T. M., Beharka, A., Tibesar, E., DesJardin, L. E., & Schlesinger, L. S. (2005). The human macrophage mannose receptor directs Mycobacterium tuberculosis lipoarabinomannan-mediated phagosome biogenesis. *The Journal of experimental medicine*, *202*(7), 987–999. https://doi.org/10.1084/jem.20051239
19. Drevets, D. A., & Campbell, P. A. (1991). Macrophage phagocytosis: use of fluorescence microscopy to distinguish between extracellular and intracellular bacteria. *Journal of immunological methods*, *142*(1), 31–38. https://doi.org/10.1016/0022-1759(91)90289-r
20. Brown, G. D., Willment, J. A., & Whitehead, L. (2018). C-type lectins in immunity and homeostasis. *Nature reviews. Immunology*, *18*(6), 374–389. https://doi.org/10.1038/s41577-018-0004-8
21. Mietto, B. S., de Souza, B. J., Rosa, P. S., Pessolani, M., Lara, F. A., & Sarno, E. N. (2020). Myelin breakdown favours Mycobacterium leprae survival in Schwann cells. *Cellular microbiology*, *22*(1), e13128. https://doi.org/10.1111/cmi.13128
22. Jin, S. H., An, S. K., & Lee, S. B. (2017). The formation of lipid droplets favors intracellular Mycobacterium leprae survival in SW-10, non-myelinating Schwann cells. *PLoS neglected tropical diseases*, *11*(6), e0005687. https://doi.org/10.1371/journal.pntd.0005687
23. Silva, T. P., Silva, A. C., Baruque, M., Oliveira, R. B., Machado, M. P., & Sarno, E. N. (2008). Morphological and functional characterizations of Schwann cells stimulated with Mycobacterium leprae. *Memorias do Instituto Oswaldo Cruz*, *103*(4), 363–369. https://doi.org/10.1590/s0074-
24. Lucineia Alves, Leila de Mendonça Lima, Elisa da Silva Maeda, Laís Carvalho, Jon Holy, Euzenir Nunes Sarno, Maria Cristina Vidal Pessolani, Lucia P. Barker, *Mycobacterium leprae* infection of human Schwann cells depends on selective host kinases and pathogen-modulated endocytic pathways, *FEMS Microbiology Letters*, Volume 238, Issue 2, September 2004, Pages 429–437, https://doi.org/10.1111/j.1574-6968.2004.tb09786.x

25. Mattos, K. A., Lara, F. A., Oliveira, V. G., Rodrigues, L. S., D'Avila, H., Melo, R. C., Manso, P. P., Sarno, E. N., Bozza, P. T., & Pessolani, M. C. (2011). Modulation of lipid droplets by Mycobacterium leprae in Schwann cells: a putative mechanism for host lipid acquisition and bacterial survival in phagosomes. *Cellular microbiology*, *13*(2), 259–273. https://doi.org/10.1111/j.1462-5822.2010.01533.x
26. Ma, Y., Pei, Q., Zhang, L., Lu, J., Shui, T., Chen, J., Shi, C., Yang, J., Smith, M., Liu, Y., Zhu, J., & Yang, D. (2018). Live *Mycobacterium leprae* inhibits autophagy and apoptosis of infected macrophages and prevents engulfment of host cell by phagocytes. *American journal of translational research*, *10*(9), 2929–2939.
27. Madigan, C. A., Cambier, C. J., Kelly-Scumpia, K. M., Scumpia, P. O., Cheng, T. Y., Zailaa, J., Bloom, B. R., Moody, D. B., Smale, S. T., Sagasti, A., Modlin, R. L., & Ramakrishnan, L. (2017). A Macrophage Response to Mycobacterium leprae Phenolic Glycolipid Initiates Nerve Damage in Leprosy. *Cell*, *170*(5), 973–985.e10. https://doi.org/10.1016/j.cell.2017.07.030
28. Pinheiro, R. O., Schmitz, V., Silva, B., Dias, A. A., de Souza, B. J., de Mattos Barbosa, M. G., de Almeida Esquenazi, D., Pessolani, M., & Sarno, E. N. (2018). Innate Immune Responses in Leprosy. *Frontiers in immunology*, *9*, 518. https://doi.org/10.3389/fimmu.2018.00518
29. Yang, D., Shui, T., Miranda, J. W., Gilson, D. J., Song, Z., Chen, J., Shi, C., Zhu, J., Yang, J., & Jing, Z. (2016). Mycobacterium leprae-Infected Macrophages Preferentially Primed Regulatory T Cell Responses and Was Associated with Lepromatous Leprosy. *PLoS neglected tropical diseases*, *10*(1), e0004335. https://doi.org/10.1371/journal.pntd.0004335
30. Lindeboom, J. A., Bruijnesteijn van Coppenraet, L. E., van Soolingen, D., Prins, J. M., & Kuijper, E. J. (2011). Clinical manifestations, diagnosis, and treatment of Mycobacterium haemophilum infections. *Clinical microbiology reviews*, *24*(4), 701–717. https://doi.org/10.1128/CMR.00020-11
31. Lemoine, S., Jaron, B., Tabka, S., Ettreiki, C., Deriaud, E., Zhivaki, D., Le Ray, C., Launay, O., Majlessi, L., Tissieres, P., Leclerc, C., & Lo-Man, R. (2015). Dectin-1 activation unlocks IL12A expression and reveals the TH1 potency of neonatal dendritic cells. *The Journal of allergy and clinical immunology*, *136*(5), . https://doi.org/10.1016/j.jaci.2015.02.030

CHAPTER 4: OPTIMIZATION OF CELL GROWTH, EXPERIMENTATION CONDITIONS AND SodC PURIFICATION

4.1 INTRODUCTION

Optimization of experiments is critically important, but explanations of trials and tribulations are often lacking in scientific literature. The final method used to collect the presented data is recorded in the "materials and methods" section of a manuscript and modifications from previously published work may be briefly mentioned. However, detailed information about methods that did not work are often excluded to keep the story concise and relevant only to the presented data. This lack of troubleshooting information makes it challenging to reproduce the results or move forward with additional research questions and projects. It does not mean that the methods described are incomplete, but instead that nuances and subtle tricks or tips that could help others are missing or assumed to be common knowledge. The projects described in this dissertation had many techniques that required substantial optimization and this chapter serves to address many of the technical challenges that were overcome and to provide a roadmap of how we were able to generate the data.

We hypothesized that interactions between *Mycobacterium leprae* and Langerhans cells via various cell surface receptors would influence a Th1 vs. Th2 immune response. To design experiments that would address this hypothesis, we needed a reliable source of functional Langerhans dendritic cells and a large yield of the protein of interest from *M. leprae*, as well as, a reproducible experimental binding assay design that catered to dendritic cells' semi-adherent properties.

Functional Langerhans dendritic cells can be generated from $CD34^+$ stem cells. A protocol to generate Langerhans-like dendritic cells (LCDCs) was kindly provided by

collaborators at the University of California at Los Angeles and was performed as published (1). Generating LCDCs *in vitro* resulted in a narrow time window for optimal cell surface expression when utilized in functional assays. Further, midway through the differentiation process, cells exhibit adherent qualities similar to other antigen-presenting cells like macrophages, however; on the final day of differentiation, the cells behave more similarly to DCs and become semi-adherent and can be easily disturbed as is characteristic for dendritic cells. Adapting protocols to accommodate the short window of opportunity and difficulty of handling these cells, required patience and creativity.

Our laboratory has previously (2) purified cell wall components from *M. leprae* including SodC, a glycoprotein. Adaptations to various aspects of the protocol were required to achieve quantities of recombinant protein needed for cell-based assays. Purifying recombinant protein required knowledge of nuances in many different techniques to ensure successful isolation for downstream experimentation.

Much of the background information pertinent to this chapter was discussed in chapter 3, but presentation and discussion of the optimization process required its own chapter. The difficulty of generating and working with LCDCs, as well as producing protein in the quantity required to address our research questions was a significant challenge that required several iterations of protocols and seemingly endless optimization. This chapter will address the optimization that was done to answer our research questions to inform the research community about what did not work. Hopefully, the work presented here will be a resource to others who attempt similar protocols and enable faster production of meaningful results.

4.2 *OPTIMIZED* MATERIALS AND METHODS

4.2.1 Expression and production of recombinant M. leprae SodC in Mycolicibacterium smegmatis and Escherichia coli

Recombinant *M. leprae* SodC (ML1925) was expressed in *M. smegmatis* to obtain the glycosylated form of the protein. It was purified as previously described (2) with slight modifications for higher protein yield. Briefly, the recombinant plasmid, pMRLB101, was transformed into *M. smegmatis* mc^2155 ΔGroEL using electroporation and maintained with the addition of kanamycin. Cultures were upscaled to 5L in 7H9 medium supplemented with 5% glucose to obtain enough rSodC for downstream experiments. Cells were harvested by centrifugation and lysed via French press. The rSodC with a C-terminal 6×His tag was purified by immobilized metal affinity chromatography (IMAC) with nickel-nitrilotriacetic acid (Ni-NTA)-agarose resin (Qiagen, Hilden, Germany), also described previously (9). Eluted rSodC was desalted and concentrated in an Amicon Ultra-15 10 K centrifugal filter unit (Millipore, Bedford, MA). Purity of rSodC was determined by silver stain. Purified rSodC was stored in phosphate buffered saline at -20° C.

4.2.2 Cell Culture

Langerhans-like dendritic cells (LCDCs) were differentiated from $CD34^+$ stem cells (Lonza, Basel, Switzerland) for 12 days. Stem cells were quickly thawed in a 37° C water bath and placed in prewarmed RPMI medium (VWR, Radnor, PA) containing 10% FBS (Atlas Biologicals, Fort Collins, CO), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were washed two times with supplemented RPMI medium. After the final wash, cells were cultured in a T175 flask containing 30 mL supplemented RPMI medium with 100 ng/mL GM-CSF

(BioLegend, San Diego, CA), 2.5 ng/mL TNF-α (BioLegend), and 25 ng/mL SCF (Fisher Scientific, Waltham, MA) at 37° C with 5% CO_2. Cells were washed and split into two flasks with fresh supplemented RPMI containing GM-CSF (100 ng/mL), TNF-α (500 ng/mL), and SCF (25 ng/mL) on day 3. On days 5, 7, and 9 cells were washed, split, and placed in fresh supplemented RPMI containing 100 ng/mL GM-CSF and 1 ng/mL TGF-β1 (BioLegend). On day 12, cells were collected via centrifugation at 1,200 rpm for 10 min, counted using a hemocytometer, and frozen at -80° C. Day 12 was chosen because langerin and CD1a expression was the highest as determined by flow cytometry (data not shown).

4.3 RESULTS

4.3.1 Optimization of LCDC Growth and Experimental Conditions

To determine the importance of the interaction of the LCDC cell-surface protein, langerin, and *M. leprae*, we hoped to block langerin. Because DCs are semi-adherent, numerous culture conditions were tested to be able to answer our research question, including:

I. Dendritic cells were cultured in 12-well plates and allowed to adhere overnight. Cells were washed three times with 1X PBS to remove serum-containing RPMI. Serum-free RPMI was added to each well containing cells. Fluorescently labeled *M. leprae* was added to the wells and incubated for a minimum of 30 minutes at 33° C. Cells were washed three times with 1X PBS to remove unbound bacteria. Visual observation of cells using a light microscope indicated most cells had been washed away and too few cells remained for analysis via flow cytometry.

II. Dendritic cells were harvested from culture flasks, counted using a hemocytometer, and aliquoted into microcentrifuge tubes. Cells were washed three times with 1X

PBS to remove serum-containing RPMI. Cells lysed during the washing process and clumped together. Further binding with *M. leprae* was not possible.

III. Dendritic cells were harvested from culture flasks, counted using a hemocytometer, and aliquoted into 15mL conical tubes. Serum-free RPMI was added to each well containing cells. Fluorescently labeled *M. leprae* was added to the wells and incubated for a minimum of 30 minutes at 33° C. Cells were washed three times with 1X PBS to remove unbound bacteria. Binding was assessed via flow cytometry. Binding was not observed indicating bound and unbound bacteria had been washed away from the cells.

IV. Dendritic cells were harvested from culture flasks, counted using a hemocytometer, and aliquoted into 15mL conical tubes. Serum-free RPMI was added to each well containing cells. Fluorescently labeled *M. leprae* was added to the wells and incubated for a minimum of 30 minutes at 33° C. Cells were washed one time with RPMI containing 2% Human Serum Albumin to remove unbound bacteria and resuspended in 2% paraformaldehyde in PBS. Successful binding was assessed via flow cytometry.

4.*3.2 SodC Purification*

Purified recombinant SodC was needed to advance our knowledge of binding with Langerhans dendritic cells in a cell-culture based assay and further the work completed by Kim *et al*. (2). Initial purification attempts resulted in low protein yield and multiple steps in the process required optimization (Figure 4.2.1) to achieve a higher protein yield for subsequent experiments. Bacterial cultures were upscaled to a minimum of 5L and the breaking buffer was

modified to destabilize the bacterial membrane further which allowed more efficient breaking of the cells and subsequently more recombinant protein available for purification in the lysate.

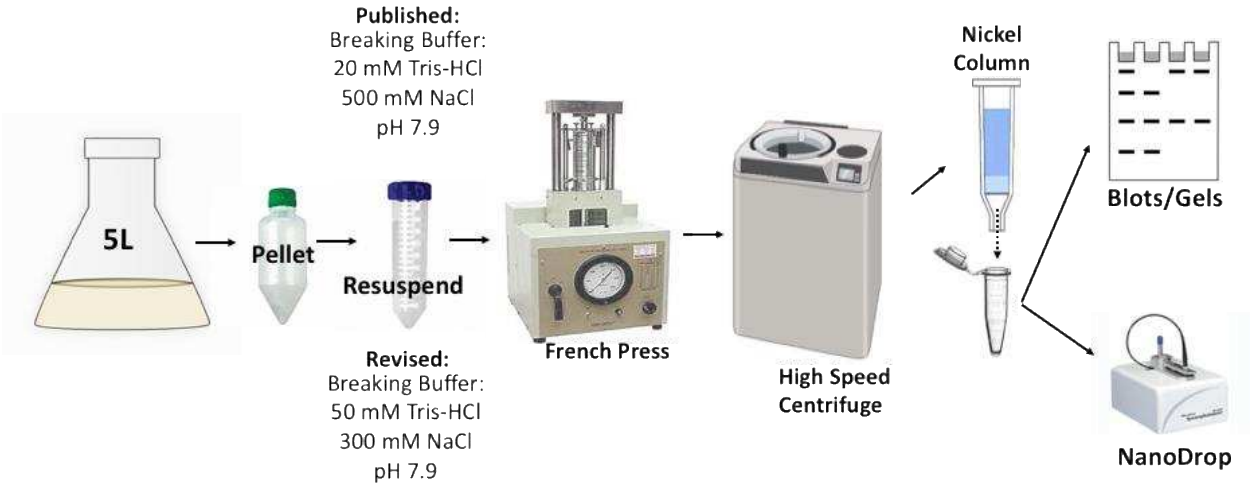

Figure 4.2.1: Graphical protocol for generation of sufficient SodC to be used in subsequent experiments.

Initially, *M. smegmatis* mc^2155 was used as an expression vector, however; in addition to purifying SodC, a second cell-wall protein from the expression vector co-purified with SodC and contaminated our preparations. The protein was identified as GroEL, which has multiple histidine residues that bind to the nickel column and elute similarly to SodC making it difficult to separate our protein of interest. We subsequently switched to a new expression vector that had previously been developed in our laboratory that lacked GroEL, *M. smegmatis* mc^2155 ΔGroEL, and allowed us to efficiently purify our protein of interest, SodC (Figure 4.2.2).

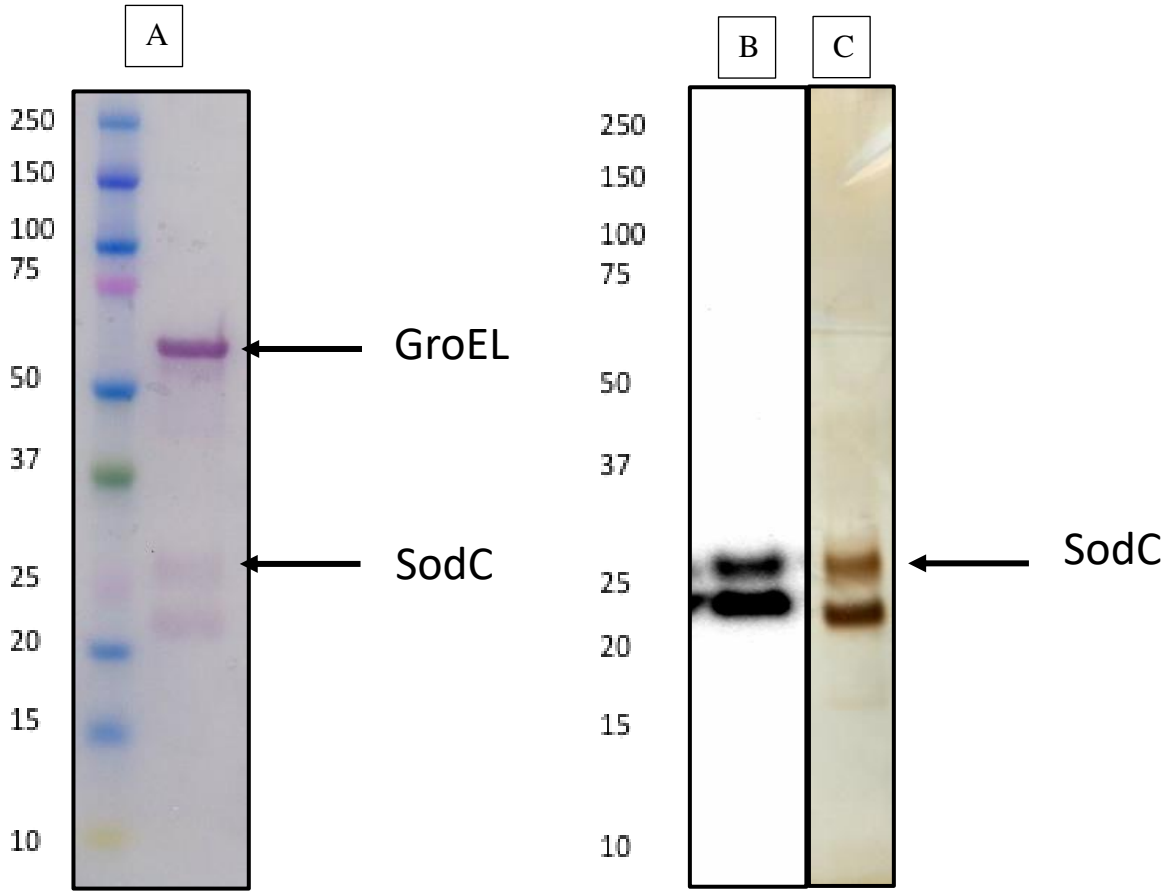

Figure 4.2.2: A) Anti-histidine western blot visualizing GroEL co-purified with SodC purification from *M. smegmatis* mc^2155. B) Anti-histidine western blot visualizing purified SodC from *M. smegmatis* mc^2155 ΔGroEL. C) Silver stain visualizing purified SodC from *M. smegmatis* mc^2155 ΔGroEL.

Further, it was later determined that we had been utilizing the incorrect expression vector. A member of the laboratory, Linda, Fischbacher, sequenced our *M. smegmatis* mc^2155 and compared the sequence to the Mycobrowser sequence to determine that our strain differed in numerous points from the published sequence (Figures 4.2.3 and 4.2.4). Utilizing a strain that had numerous mutations resulted in lower recombinant protein yield and large bacterial culture volumes. Due to time constraints, a new expression vector was not utilized; however future work in the laboratory will proceed with the correct expression vector.

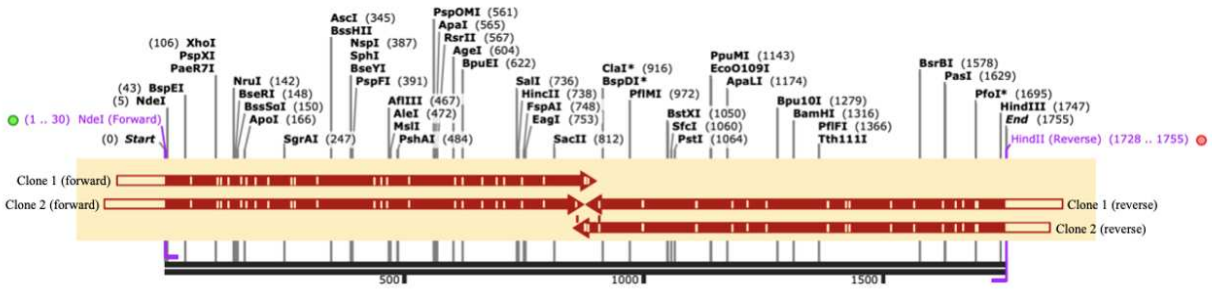

Figure 4.2.3: Sequencing of mWT *msmeg1604* in pGEM-T Easy aligned to the Mycobrowser sequence of *MSMEG1604* in *M. smegmatis* mc^{2}155 using Snapgene. Red section: sequence align; Break in the red section: sequences do not align.

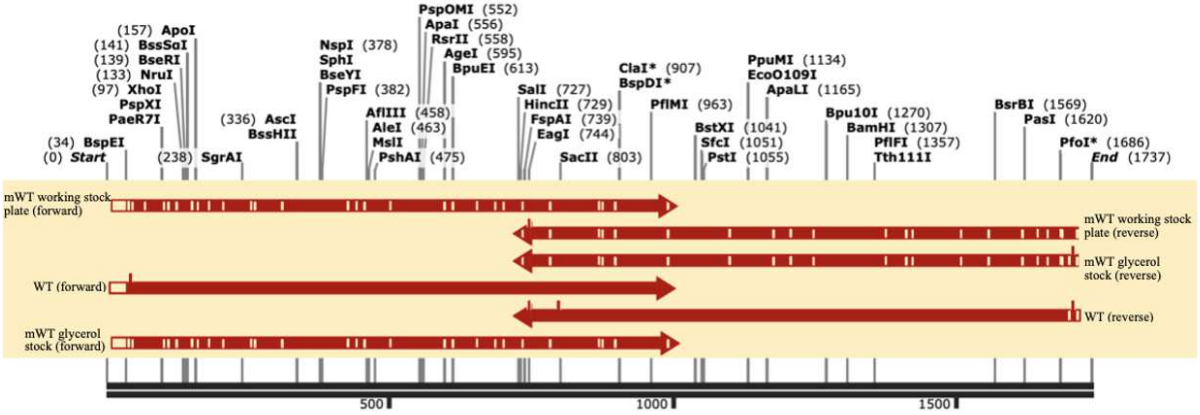

Figure 4.2.4: Sequencing of PCR amplified *msmeg1604* from mWT working stock plate, mWT glycerol stock and aWT aligned to the Mycobrowser sequence of *MSMEG1604* in *M. smegmatis* mc^{2}155 using Snapgene. Red section: sequence align; Break in the red section: sequences do not align.

4.4 DISCUSSION

Experiments often are packaged and presented in neatly prepared nuggets of data that make a well-crafted story, but rarely do scientists describe the weeks, months, or even years of optimization that are necessary to obtain meaningful data. While all projects have aspects that need optimized, this project had numerous angles that needed optimized before meaningful experiments could be conducted.

Dendritic cells in an *in vitro* cell-culture system are semi-adherent meaning they will adhere loosely to the culture vessel but can be easily disturbed and float in suspension. Due to the semi-adherent nature of LCDCs, developing a binding assay with *M. leprae* proved more complicated than other systems that utilize adherent cells, such as macrophages or Schwann cells (4). Adherent cells attach to the culture vessel, can be repeatedly washed, and can be analyzed via microscopy or flow cytometry. To mimic adherent properties with dendritic cells, a few attachment solutions were tested (including poly-lysine and collagen); however, *M. leprae* also bound to the coated plates, making it difficult to remove bacteria that was not bound to dendritic cells and made downstream analyses extremely challenging. Binding analyzed via confocal microscopy did not provide meaningful data due to difficulties deciphering bacteria bound to dendritic cells versus bacteria bound to the slide (data not shown). Similar difficulties existed when binding was analyzed via flow cytometry. Cells lifted from the coated culture vessel using trypsin disrupted binding with *M. leprae* and scraping cells led to significant cell death and lysis. Adapting the binding assay to a microcentrifuge tube proved too harsh for dendritic cell survival and led to significant cell lysis and death during wash steps. Final optimization of the binding experiment led to an experimental setup where dendritic cells remained in suspension after being harvested from their culture flasks which removed the issue of their semi-adherent properties.

All reagents and bacteria were added to the cells while in suspension and cells with bound bacteria were fixed in 2% formaldehyde so dendritic cells did not uptake and process bound bacteria prior to analysis by flow cytometry. Binding was enhanced when dendritic cells were pre-activated with IFNγ which allowed the dendritic cells to exhibit a mature phenotype. Achieving an experimental design where meaningful data could be collected and analyzed relied on adapting to the unique properties of both dendritic cells and *M. leprae*.

Designing a binding experiment adapted to dendritic cells and *M. leprae* was not the only hurdle. Purified cell wall components of *M. leprae* were also evaluated for binding efficiency with Langerhans dendritic cells in an *in vitro* system, however; purifying the *M. leprae* cell wall glycoprotein, SodC, was not simple. We modified multiple aspects of the purification protocol to achieve a protein yield that was large enough and contained only our protein of interest for cell culture assays including bacterial culture volume, breaking buffer, and expression vector. An additional confounding factor was inadvertently using the incorrect expression vector. Further work conducted by Linda Fischbacher allowed us to identify our strain of *M. smegmatis* mc^2155 was not correct and had been an inefficient expression vector leading to low protein expression and subsequent protein purification. Due to unknowingly using the incorrect expression vector initially, we increased our bacterial culture volume to over 5L to lyse and purify our protein of interest. We also modified our breaking buffer to extract as much protein of interest as possible. Increasing our concentration of Tris-HCl destabilized the bacterial membrane further releasing more protein into the lysate for purification. The NaCl concentration was decreased. NaCl weakens non-specific binding in the purification column, but too much potentially inhibited our protein of interest from binding as well leading to low protein yield and insufficient purification.

Adapting widely used protocols to our system presented significant hurdles that were more challenging to overcome than initially anticipated. Not only are DCs, LCDCs especially, difficult to generate and culture, isolating SodC also proved to be extremely difficult. Through numerous protocol changes, meaningful data was collected, but plenty of negative data was generated in the process. While none of the data discussed here is particularly exciting, the work and optimization that went in to generating these protocols and collecting the data presented in chapter 3 was not trivial. Although somewhat unconventional, the scientific process was shown and discussed in this chapter to share the (negative) results we did collect to help similar protocols move forward more effectively for others. Showing this process and negative data is a great first step towards a more transparent and effective scientific discourse that will facilitate *M. leprae* research.

REFERENCES

1. Dang, A. T., Teles, R. M., Liu, P. T., Choi, A., Legaspi, A., Sarno, E. N., Ochoa, M. T., Parvatiyar, K., Cheng, G., Gilliet, M., Bloom, B. R., & Modlin, R. L. (2019). Autophagy links antimicrobial activity with antigen presentation in Langerhans cells. *JCI insight*, *4*(8), e126955. https://doi.org/10.1172/jci.insight.126955
2. Kim, H. J., Brennan, P. J., Heaslip, D., Udey, M. C., Modlin, R. L., & Belisle, J. T. (2015). Carbohydrate-dependent binding of langerin to SodC, a cell wall glycoprotein of Mycobacterium leprae. *Journal of bacteriology*, *197*(3), 615–625. https://doi.org/10.1128/JB.02080-14
3. Korenfeld, D., Gorvel, L., Munk, A., Man, J., Schaffer, A., Tung, T., Mann, C., & Klechevsky, E. (2017). A type of human skin dendritic cell marked by CD5 is associated with the development of inflammatory skin disease. *JCI insight*, *2*(18), e96101. https://doi.org/10.1172/jci.insight.96101
4. Díaz Acosta CC, Dias AA, Rosa TLSA, Batista-Silva LR, Rosa PS, Toledo-Pinto TG, et al. (2018) PGL I expression in live bacteria allows activation of a CD206/PPARγ cross-talk that may contribute to successful *Mycobacterium leprae*colonization of peripheral nerves. PLoS Pathog 14(7): e1007151. https://doi.org/10.1371/journal.ppat.1007151

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

5.1 FINAL DISCUSSION

Leprosy is a disease that has been around since at least 600 BC from early written records, but anecdotal history describes disease similar to leprosy around 350 BC. If left untreated, severe deformities and disfigurement can occur which often led to stigma and ostracization. Social stigma and alienation still occur today. In 1873, Dr. Gerhard Armauer Hansen identified rod-shaped bacilli under a microscope. In the 1940s, sulfone therapy was used to treat the disease and marked the first successful treatment. By the late 1940s, dapsone was used as a monotherapy until drug resistance was noted in the 1970s. The 1960s marked the time when Dr. William Jopling, Dr. Dennis Ridley, and Dr. Olaf Skinsnes classified the clinical presentation into the current disease spectrum. They also classified the immunological response in different presentations of the disease. Research into new treatments and therapies advanced in 1960 when Dr. Charles Shepard discovered that *M. leprae* could be cultivated in the footpads of mice and by 1968, Drs. Eleanor Storrs and Dr. Waldemar Kirchheimer discovered that large quantities of *M. leprae* could be harvested from nine-banded armadillos. In 1982, the World Health Organization recommended multidrug therapy protocols that are still in place today (1). Since then, many other major advances have including the discovery of PGL-1 which has become an important tool in serological diagnosis as well as discoveries of its tropism for Schwann cells, downregulation of inflammatory immune responses, and inhibition of dendritic cell maturation.

Previous work with collaborators identified that *M. leprae* antigens could be presented via a cell surface marker, CD1a, on Langerhans dendritic cells to CD1a-restricted T cells.

Subsequently, the T-cells proliferated and produced IFN-γ (2). This opened an avenue to understand the role Langerhans cells during *M. leprae* infections. Further work previously performed in our lab, identified the *M. leprae* ligand for langerin (3). Langerhans cells reside in the epidermis of human skin and are capable of migrating from the epidermis, through the dermis, and into draining lymph nodes to present antigens. Biologically relevant models were needed to further elucidate the interactions between *M. leprae* and Langerhans cells. The studies presented here identified additional Langerhans cell surface receptors involved in binding *M. leprae* and modified an *ex vivo* imaging system to observe binding events.

Chapter 3 presented a modified CLARITY protocol developed for skin samples to investigate interactions between innate immune cells and mycobacteria spp. Modifications were made to properly allow acrylamide polymerization to occur, so the tissue-hydrogel matrix was stable. Additional time was added to the passive 8% SDS lipid removal step to ensure all complex lipids were removed from the skin which required the tissue-hydrogel complex to be robust so additional structures were not lost. Final images show colocalization of *M. haemophilum* with Langerhans cells at the site of infection. This indicates that the method was modified successfully for the skin. It also demonstrates that recruitment of innate immune cells can be visualized in an acute infection model. The colocalization observed between Langerhans cells and *M. haemophilum* indicate a potential for *M. haemophilum* to be investigated further as a model for *M. leprae* using this method to understand which innate immune cells and potentially nerve cells are associated with acute infection.

Chapter 4 elucidates the role of langerin interactions with *M. leprae*. Data indicated that in a cell-based assay using both LCDCs and a more simplified cell line system, THP-L, that langerin bound *M. leprae* and could be inhibited with the addition of blocking reagents, both

specific for langerin and general for C-type lectins. Further investigation of the affinity of langerin for glycosylated SodC, provided a more complicated story. Glycosylated SodC was found to bind langerin and binding could be inhibited with the addition of blocking reagents, however, unlike in the cell-free SPR assay, other mannosylated macromolecules including ManLAM and PIM_6 bound to langerin almost as efficiently as glycosylated SodC. This indicated that the straightforward hypothesis we had set forth was complicated by additional cell surface receptors on Langerhans cells indicating complex interactions during *M. leprae* infections in patients.

Further probing into other cell surface markers on Langerhans cells provided evidence for the interaction of CD5 with *M. leprae*. Fewer $CD5^{high}$ LCs bound M. leprae in comparison to $CD5^{low}$ LCs. $CD5^{+}$ DCs had previously (6) been found to have distinct differences in cytokine profiles and antigen presentation. Our findings confirm that the two subpopulations indeed have distinct characteristics including binding characteristics. Taken together with the langerin binding experiments, a complex picture appears. Multiple LC cell surface receptors are responsible for recognizing and binding to *M. leprae*. The specific receptor that interacts with *M. leprae* may influence antigen presentation and downstream T cell activation. While glycosylated SodC interacts with langerin, other mannosylated macromolecules present on *M. leprae* also interact with langerin and LCs in general.

5.2 FUTURE DIRECTIONS

The work presented here provides preliminary evidence that Langerhans cells play an important, but complex role during *M. leprae* infection. Leprosy presents with various forms of

disease. While there are numerous factors that contribute to the outcome of disease, Langerhans cells may play a pivotal role.

The evidence provided that $CD5^+$ LCs interact with *M. leprae* require further investigation to understand how the different subpopulations of LCs may secrete cytokines and eventually influence their microenvironment. To evaluate the interaction between *M. leprae* and CD5 further, SPR assays would provide information about specific *M. leprae* ligands recognized by CD5. Like in this study, further investigation utilizing biologically relevant *in vitro* models would be necessary to understand the more complex interactions between *M. leprae* and CD5 including any newly discovered ligands. A transfected cell line expressing CD5 would be necessary to isolate interactions with CD5. LCDCs or Langerhans cells isolated from human skin samples should be utilized to understand the more complex story including uptake, trafficking, and antigen presentation.

Human skin biopsies from leprosy patients could be analyzed utilizing the modified CLARITY protocol to investigate immune cell interactions with *M. leprae*. Multiple rounds of staining on the same tissue sample would alleviate the need for a constant supply of biopsy tissues. CLARITY has been adapted to investigate nerves (7,8) opening an avenue to investigate *M. leprae* interactions with peripheral nerves in an *ex vivo* system. Biopsies are invasive in nature and reduction of the number of tissues needed for laboratory investigation is ideal. Further, the modified CLARITY method could be utilized in mouse models (4,5) to investigate initial innate immune cells recruited to the site of infection and which cells interact with *M. leprae*. Additionally, *M. leprae* interactions with peripheral nerve cells like Schwann cells could provide additional information.

As intriguing as the details of a project and the intricate data can be, it is important to remember how these findings can affect patients. While we have an effective treatment, it is a long and arduous regiment that requires patients have access to clinics regularly and stay committed to finishing the full course of treatment which can seem overwhelming. Understanding the complicated nature of the innate immune response may provide information for how disease may progress in certain individuals. This would require more advanced techniques and integrated medical information; however, the benefits may include starting individuals on treatment earlier in the onset of disease.

REFERENCES

1. Bennett, B. H., Parker, D. L., & Robson, M. (2008). Leprosy: steps along the journey of eradication. *Public health reports (Washington, D.C. : 1974)*, *123*(2), 198–205. https://doi.org/10.1177/003335490812300212
2. Hunger, R. E., Sieling, P. A., Ochoa, M. T., Sugaya, M., Burdick, A. E., Rea, T. H., Brennan, P. J., Belisle, J. T., Blauvelt, A., Porcelli, S. A., & Modlin, R. L. (2004). Langerhans cells utilize CD1a and langerin to efficiently present nonpeptide antigens to T cells. *The Journal of clinical investigation*, *113*(5), 701–708. https://doi.org/10.1172/JCI19655
3. Kim, H. J., Brennan, P. J., Heaslip, D., Udey, M. C., Modlin, R. L., & Belisle, J. T. (2015). Carbohydrate-dependent binding of langerin to SodC, a cell wall glycoprotein of Mycobacterium leprae. *Journal of bacteriology*, *197*(3), 615–625. https://doi.org/10.1128/JB.02080-14
4. Krahenbuhl J, Adams LB. 2000. Exploitation of gene knockout mice models to study the pathogenesis of leprosy. *Lepr Rev* 71 Suppl:S170-175.
5. Adams LB, Scollard DM, Ray NA, Cooper AM, Frank AA, Orme IM, Krahenbuhl JL. 2002. The study of *Mycobacterium leprae* infection in interferon-gamma gene-disrupted mice as a model to explore the immunopathologic spectrum of leprosy. *J Infect Dis* 185 Suppl 1:S1-8.
6. Yin, X., Yu, H., Jin, X., Li, J., Guo, H., Shi, Q., Yin, Z., Xu, Y., Wang, X., Liu, R., Wang, S., & Zhang, L. (2017). Human Blood CD1c+ Dendritic Cells Encompass CD5high and CD5low Subsets That Differ Significantly in Phenotype, Gene Expression, and Functions. *Journal of immunology (Baltimore, Md. : 1950)*, *198*(4), 1553–1564. https://doi.org/10.4049/jimmunol.1600193
7. Chung, K., & Deisseroth, K. (2013). CLARITY for mapping the nervous system. *Nature methods*, *10*(6), 508–513. https://doi.org/10.1038/nmeth.2481
8. Chung, K., Wallace, J., Kim, S. Y., Kalyanasundaram, S., Andalman, A. S., Davidson, T. J., Mirzabekov, J. J., Zalocusky, K. A., Mattis, J., Denisin, A. K., Pak, S., Bernstein, H., Ramakrishnan, C., Grosenick, L., Gradinaru, V., & Deisseroth, K. (2013). Structural and molecular interrogation of intact biological systems. *Nature*, *497*(7449), 332–337. https://doi.org/10.1038/nature12107

LIST OF ABBREVIATIONS

Acronym	Term
BB	borderline borderline
BL	borderline lepromatous
BT	borderline tuberculoid
cDC	conventional dendritic cell
CLARITY	Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging/ Immunostaining/In-situ hybridization-compatible Tissue hYdrogel
DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-grabbing nonintegran
ENL	erythema nodosum leprosum
GAG	glicosaminoglycan
GM-CSF	granulocyte-macrophage colony-stimulating factor
HA	hyaluronic acid
IFN-γ	gamma interferon
IL	interleukin
IMAC	immobilized metal affinity chromatography
LAM	lipoarabinomannan
LCDCs	Langerhan-like dendritic cells
LCs	Langerhans dendritic cells
LL	lepromatous
LM	lipomannan
ManLAM	mannose-capped lipoarabinomannan
MB	multibacillary
MOI	multiplicity of infection
NETs	neutrophil extracellular traps
Ni-NTA	nickel-nitrilotriacetic acid
PACT	passive clarity technique
PAMP	pathogen associated molecular patterns
PRR	pathogen recognition receptor
PBS	phosphate buffered saline
PB	paucibacillary
pDC	plasmacytoid dendritic cell
PGL-1	phenolic glycolipid - 1
PIM	phosphatidylinositol mannosides
RIMS	refractive index matching solution
SCF	stem cell factor
SodC	*M. leprae* glycoprotein
SPR	surface plasmon resonance assay
sRIMS	sorbitol refractive index matching solution
TGF	transforming growth factor

TNF	tumor necrosis factor
TT	tuberculoid