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IL-32-Derived Dendritic Cells Cross-Present

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A thesis submitted in partial satisfaction of the

requirements for the degree Master of Science

in Microbiology, Immunology, and Molecular Genetics

By

Aaron Wonho Choi

2018

ABSTRACT OF THE THESIS

IL-32-Derived Dendritic Cells Cross-Present

Defined *Mycobacterium leprae* Antigens to CD8^{+} T Cells

By

Aaron Wonho Choi

Master of Science in Microbiology, Immunology, & Molecular Genetics

University of California, Los Angeles, 2018

Professor Robert L. Modlin, Chair

Leprosy is a human disease caused by the intracellular pathogen *Mycobacterium leprae*[3]. By investigating leprosy, we discovered a novel pathway involving NOD2-mediated induction of interleukin-32 (IL-32) triggering the differentiation of *M. leprae* infected monocytes into CD1^{+} dendritic cells (DCs) with professional antigen presenting cell function[13]. These IL-32-derived DCs were able to "cross-present" antigen, that is capture exogenous antigen via the endocytic pathway and then present processed peptides via MHC class I to CD8^{+} T cells. It was also discovered that key components of the NOD2→IL-32→CD1+ DC pathway were highly expressed only in lesions of leprosy patients with cell-mediated immunity. We hypothesized that cross-presentation of *M. leprae* antigens by IL-32-derived DCs resulted in the activation of MHC class I-restricted CD8^{+} T cells. To test our hypothesis, we established CD8^{+} T cell clones from leprosy patients to putatively secreted *M. leprae* antigens. IL-32-derived DCs were able to cross-present exogenous recombinant *M. leprae* protein as well as live *M. leprae* to the CD8^{+} T cell clones. We conclude that IL-32-induced cross-presentation may play a role in CD8^{+} T cell responses in leprosy.

The thesis of Aaron Wonho Choi is approved

Steven Bensinger

Philip O. Scumpia

Robert L. Modlin, Committee Chair

University of California, Los Angeles

2018

DEDICATION

This thesis is dedicated to Jiwon, Dad, Mom, Emo, Uncle Bill, Grandma Choi, and Grandma Ryu.

Your love and support inspired me to reach for the stars. Thank you.

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I would like to also thank all the current and past lab members of the Modlin lab. Their technical, emotional, and intellectual support have been priceless. A special thanks to Devika Roy for teaching me how to ficoll, Mirjam Schenk for the late night discussions with Lily in your apartment, and Elliot for keeping me grounded during the tough times in lab.

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CHAPTER 1

INTRODUCTION

LEPROSY AS AN IMMUNOLOGICAL MODEL

Leprosy, caused by the intracellular pathogen *Mycobacterium leprae*, is a disease that primarily affects the skin, peripheral nerves, mucosa of the upper respiratory tract and eyes. Although the mode of transmission is unclear, researchers suggest air droplets from nasal secretions of infected individuals could represent one route[1]. The disease also affects half a million people and approximately 200,000 new leprosy cases are registered globally every year and is a health and economic burden in developing countries[2].

Leprosy offers an attractive model for investigating the regulation of immune responses to infection. The disease presents as a spectrum in which the clinical manifestations correlate with the type of immune response mounted against the pathogen[3] (Figure 1.1). At one end of the disease spectrum, patients with tuberculoid leprosy (T-lep) display the resistant response that restricts the growth of the pathogen. The number of lesions is low and bacilli are rare, but tissue damage is frequent. At the opposite end of this spectrum, patients with lepromatous leprosy (L-lep) show susceptibility to disseminated infection. Skin lesions are numerous and growth of the pathogen is unceasing. These clinical presentations correlate with the type of acquired T cell–mediated immunity against *M. leprae*, including T helper type 1 (T_H1) cytokines, which are present in T-lep lesions and diminished in L-lep lesions[4,5]. Conversely, antibody responses against *M. leprae* and T helper type 2 (T_H2) cytokines are more prevalent in L-lep lesions[6].

INNATE IMMUNE RESPONSE IN LEPROSY

Macrophages (MΦs) and dendritic cells (DCs) play an important role in the innate host defense response against mycobacteria. Tissue MΦs in the dermis are the primary host cells for *M. leprae*. Therefore, the innate immune response can be mediated by these MΦs, resulting in receptor expression, cytokine and chemokine production, and initiation of host defense

mechanisms. Pattern recognition receptors (PRRs) are germ-line encoded receptors found on immune host cells that can recognize a broad spectrum of pathogen associated molecular patterns (PAMPs). Mycobacteria possess many PAMPs that are recognized by Toll-like receptors (TLRs), the nucleotide oligomerization domain-like receptors (NLRs), the C-type lectin recptors (CLRs), RIG- like receptors (RLR). These PRRs, along with complement are all involved in the innate recognition of mycobacteria[7,8]. Ligation of surface PPRs leads to phagocytosis, activation of receptor specific signaling pathways, production of cytokines and chemokines, and antimicrobial activity. The specific receptor engaged can determine the type of immune response to mycobacteria.

TLRs are a conserved family of membrane receptors that are present on the surface of antigen presenting cells, including MΦs. Mycobacterial antigens, such as lipoproteins and lipoglycans, are able to trigger TLRs, which leads to the activation of MΦs to mount an immune response against mycobacteria via the release of cytokines and induction of antimicrobial activity. TLR2 in association with TLR1 or TLR6, TLR4, and TLR9 have been shown to be ligated by mycobacterial PAMPs. One TLR of particular interest is TLR2 because it has been shown to be the primary mediator of cellular activation for mycobacteria and activation of TLR2/1 mediated killing of *M. leprae*[10]. Activation of monocytes or MΦs via TLR2/1 produces a pro-inflammatory cytokine response, including TNF-a, which is involved in granuloma formation[10] and IL-12[9,11,12] which is critical for instructing the adaptive immune response in the T_H1 direction.

ADAPTIVE IMMUNE RESPONSE IN LEPROSY

The adaptive immune response is initiated by the innate immune response through antigen presentation of mycobacterial components, co-stimulation, and cytokine production that instruct T-helper cells. T cell-mediated immunity plays an important role in protection against

disease during mycobacterial infection through the induction of immune modulating cytokines as well as the targeted cytotoxic killing of infected cells. T_H1 responses, including production of IFN-γ, are correlated with protection against mycobacterial infection[6,14,15]. In leprosy, IFN-γ expression is higher in T-lep lesions, the self-limited form, but not in L-lep lesions, the disseminated form which predominantly displays a T_H2 type of response[6,16,17].

Genetic studies showing defects in IFN-γ production and/or related signaling pathways lead to increased susceptibility to mycobacterial diseases[18]. Furthermore, CD8+ T cells also play a role in protection through cytotoxic killing of mycobacteria infected cells. Antigen-specific CD8+ T cells are able to lyse infected MΦs and restrict mycobacterial growth through the release of granulysin, an antimicrobial agent[19]. In addition, cytotoxic T cells are able to induce apoptosis in infected MΦs[20]. On the other hand, the humoral response characterized by a T_H2 immune profile with production of IL-4 and IL-10 and activation of T regulatory cells is robust, but not protective[21]. The production of *M. leprae*-specific antibodies results in the formation of immune complexes and fail to restrict *M. leprae* growth.

INTERLEUKIN-32

NOD2 is a cytoplasmic receptor belonging to the NLR family, recognizing muramyl dipeptide (MDP), part of the peptidoglycan of the mycobacterial cell wall. Previous studies have shown that activation of NOD2 in monocytes induced the production of IL-32[13]. In turn, NOD2 activation induced the IL-32-dependent differentiation of monocytes into DCs (Figure 1.2 and Figure 1.3). TLR2/1-induced DC differentiation in monocytes was not IL-32-dependent (Figure 1.3). These IL-32-derived DCs differed from granulocyte-macrophage colony-stimulating factor (GM-CSF)-differentiated DCs in possessing the capacity to cross-present exogenous antigen (tetanus toxoid) via major histocompatibility complex (MHC) class I to CD8+ T cells (Figure 1.4). The biological relevance to leprosy was demonstrated by the high expression of NOD2, IL-32,

and CD1^{+} DCs in skin lesions of T-lep patients compared to that of L-lep patients (Figure 1.5). Furthermore, NOD2 polymorphisms were discovered to be associated with susceptibility to leprosy[13].

CROSS-PRESENTATION

All nucleated cells express MHC class I molecules, which present peptides derived from endogenous proteins that have been degraded in the cytosol by the proteasome and subsequently transported to the ER where they are loaded onto newly synthesize MHC class I molecules. Cross-presentation provides the means for MHC class I molecules to gain access to peptides from an exogenous source[13]. There are two main pathways that have been suggested for cross-presentation- the cytosolic and the vacuolar pathways (Figure 1.6). In the cytosolic pathway, exogenous antigens gain access to the cytosol, where proteasome dependent processing occurs. Subsequently, loading of processed peptides onto MHC class I molecules can occur via the ER or endocytic pathway. On the other hand, the vacuolar pathway does not require the proteasome for antigen degradation. Rather, both the antigen processing and the loading of peptides to MHC class I molecules occur in endocytic compartments, relying on lysosomal proteolysis for antigen degradation. Specific human DC subsets that cross-present efficiently have been identified- CD141^{+} DCs and IL-32-induced DCs[13,22,23].

AIMS & SIGNIFICANCE

The discovery that key components of the NOD2→IL-32→CD1^{+} DC pathway are highly expressed in T-lep vs. L-lep lesions supports a new avenue for CD8^{+} T cell activation in leprosy. The focus of this thesis is to determine whether IL-32-derived DCs can cross-present defined *M. leprae* epitopes via MHC class I to CD8^{+} T cells. Our goals are to 1) Identify *M. leprae* epitopes for CD8^{+} T cells, 2) generate CD8^{+} T cell clones that recognize *M. leprae* epitopes, and 3) test whether IL-32-derived DCs can cross-present recombinant *M. leprae* antigen and live *M. leprae*

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to the CD8^{+} T cell clones. Understanding cross-presentation will provide new information about antigen presentation in human infectious disease with relevance to induction of CD8^{+} T cell responses. Specifically, how CD8^{+} T cells recognize microbial pathogens that reside in the endocytic pathway. Insights gained from cross-presentation and activation of CD8^{+} T cells may provide new biomarkers for diagnosis, as well as targets for treatment of human infectious diseases.
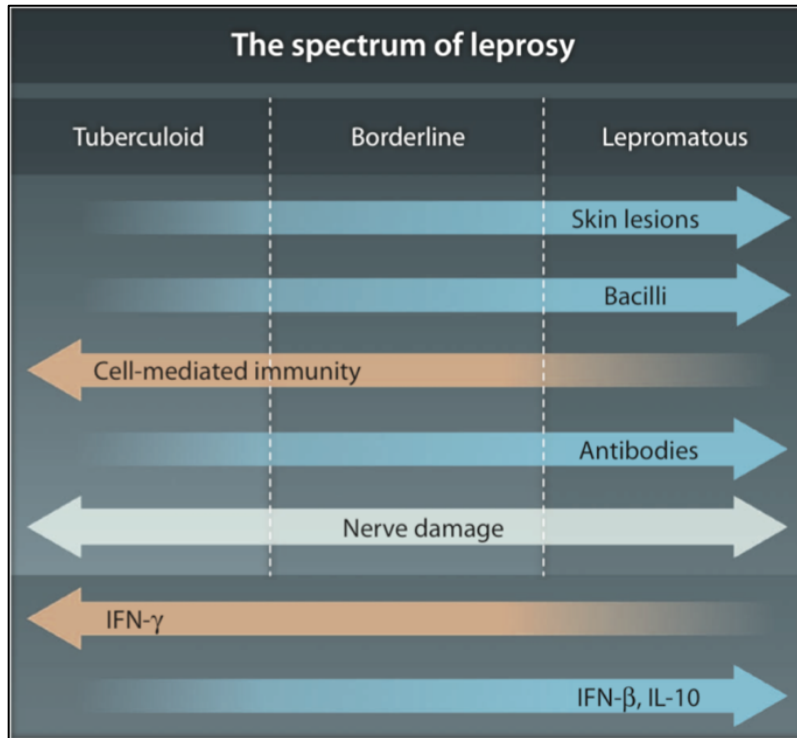
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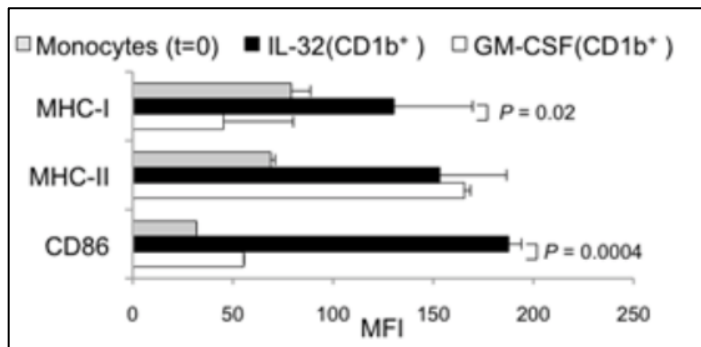

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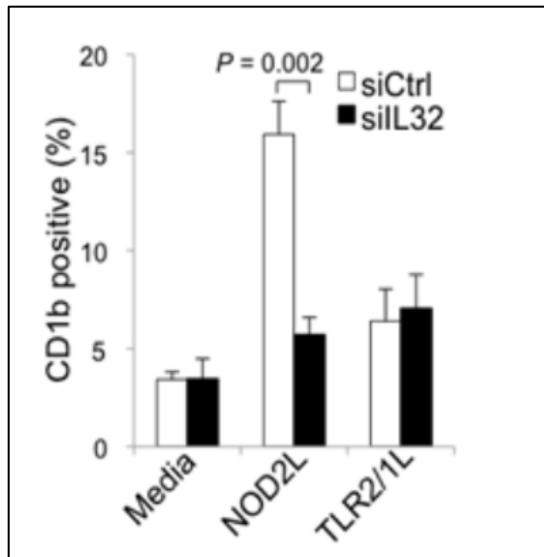
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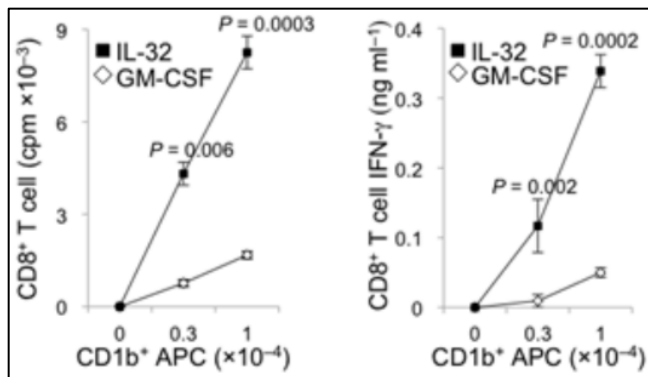
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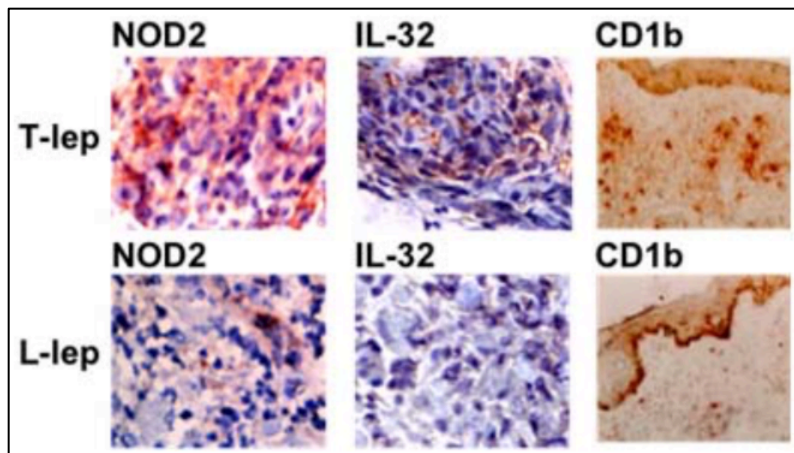
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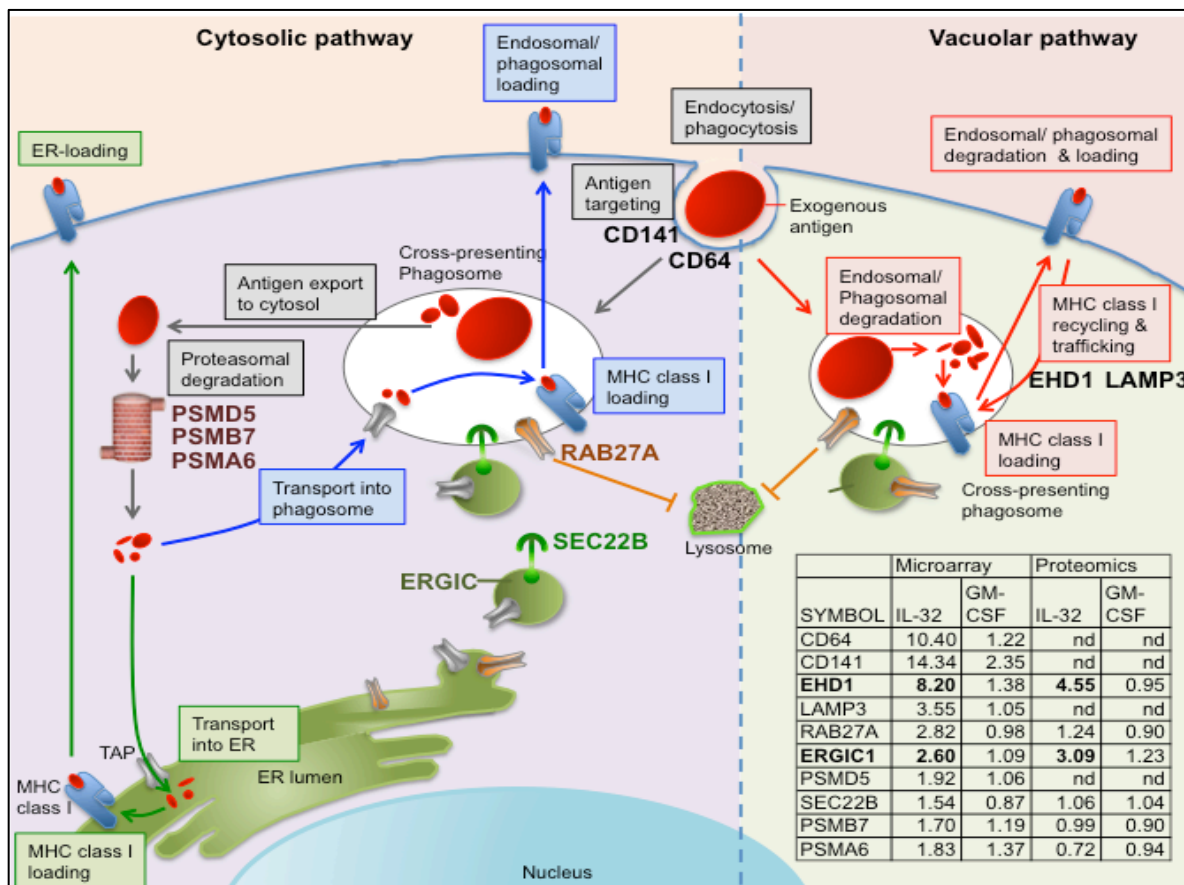Figure 1.6. The cytosolic and vacuolar pathways of cross-presentation and a list of genes involved in both pathways that are upregulated in IL-32-derived DCs vs. GM-CSF-derived DCs. Reprint from Mirjam Schenk (unpublished data).

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Chapter 2

Cross-Presentation of *M. leprae* Antigens to CD8$^+$ T cells

ABSTRACT

A novel pathway initiated by *Mycobacterium leprae* infection was discovered to trigger the NOD2-mediated induction of interleukin-32 (IL-32), triggering the differentiation of monocytes into CD1^{+} dendritic cells (DCs) with the ability to cross-present exogenous tetanus toxoid to CD8^{+} T cells via MHC class I molecules[13]. To investigate the role of cross-presentation in leprosy, we made peptide pools to putatively secreted *M. leprae* proteins and assessed their ability to stimulate IFN-γ production in CD8^{+} T cells isolated from leprosy patients. We then cloned CD8^{+} T cells to *M. leprae* proteins ML0049 (ESAT-6) and ML0411 (PPE). Of the six CD8^{+} T cell clones generated, J88.B.4 was used to show that IL-32-derived DCs could cross-present recombinant ML0049 and live *M. leprae*.

INTRODUCTION

Leprosy is a chronic intracellular infection caused by *M. leprae* that presents as a spectrum in which the clinical manifestations correlate with the level of cell-mediated immunity (CMI) to the bacteria[3]. At one end of the spectrum, tuberculoid patients (T-lep) are able to restrict and eliminate the pathogen, the number of skin lesions and bacteria are few, and patients mount a strong cell-mediated response to *M. leprae*. On the other end of the spectrum, Lepromatous patients (L-lep) are unable to contain the infection, the skin lesions are disseminated, and the bacteria are abundant- a result of the poor cell-mediated response to the pathogen.

The discovery that T-lep lesions highly express components of the NOD2→IL-32→CD1^{+} DC pathway compared to L-lep lesions as well as the cross-presenting function of these IL-32-derived DCs suggests that activation of CD8^{+} T cells via cross-presentation may play a role in the CMI characteristic of T-lep patients[13]. To investigate this hypothesis, we set out to define *M.*

leprae antigens that would possess epitopes recognized by CD8^{+} T cells in T-lep patients. L-lep patients were excluded due to their poor CMI.

The early secretory antigenic target 6 (ESAT-6) system (ESX) is a specialized secretion system essential for the virulence and disease pathogenesis of many mycobacteria[35]. In *Mycobacteria tuberculosis*, the ESX secretion system has been linked to virulence[24-26]. The ESX system is involved in the secretion of the "PE" and "PPE" proteins[25,27,28], which contain an N-terminal motif of highly conserved Proline-Glutamate or Proline-Proline-Glutamate, respectively, and are targets of T cell responses in tuberculosis (TB)[29,30]. *M. tuberculosis* secreted proteins and the secretion apparatus constitute the major group of proteins recognized by T cells in TB patients[31]. It is not known whether *M. leprae* contains a functional ESX complex because the pathogen cannot be grown in culture and there is no available genetic system to manipulate it. However, T cell responses to two of the *M. leprae* counterparts for the ESX system have been investigated, ESAT-6 and CFP-10[32,33]. In addition, *M. leprae* encodes only 13 PE/PPE family members[34]. Due to the success of using putatively secreted proteins to isolate and clone CD8^{+} T cells in TB patients[36], we employed a similar strategy to identify define *M. leprae* antigens in CD8^{+} T cells.

MATERIALS AND METHODS

Patients and healthy subjects

Leprosy blood specimens were obtained through collaborations with Dr. Maria Theresa Ochoa at the Los Angeles County/University of Southern California Medical Center. The diagnosis of leprosy was established by means of clinical criteria according to Ridley[40]. Healthy donors served as controls and were used for baseline examination. We were blinded as to the race of the leprosy patients but based on epidemiology of the leprosy patients in Los Angeles, the majority of patients are of Hispanic or Asian descent; a large proportion of healthy donor blood

comes from donors in these ethnic/gender categories to best match the population of leprosy donors.

Peptides

All peptides were synthesized by Mimotopes (San Diego, CA).

HLA genotyping

All high-resolution HLA genotyping was conducted at the UCLA Immunogenetics Center (Los Angeles, CA)

PBMC isolation

PBMCs were isolated from the peripheral blood of healthy donors, or patients with a diagnosis of leprosy, using Ficoll-Paque gradients (Amersham Biosciences) per manufacturer's protocols.

Generation of T cell clones

T cell clones were generated as previously described[38,39]. Briefly, PBMCs were isolated from whole blood of T-Lep donors and DCs were generated by $CD14^+$ negative selection without CD16 depletion (Stemcell technologies) followed by culture in RPMI with 10% FCS, 800 IU/mL GM-CSF, and 1000 IU/mL IL-4. On day 5, DCs were harvested and cultured with RPMI-1640 medium supplemented with 12.5 mM HEPES, 4 mM L-glutamine, 100 U/mL penicillin, 100mg/mL streptomycin, 50μM ®-mercaptoethanol and 10% AB human serum and pulsed with 10 μg/mL of ML0049 peptide pool for 2 hours. These cells were then mixed with $CD8^+$ T cells isolated by negative selection (Stemcell technologies) from the same donor in 96 well U- bottom plates to create $CD8^+$ T cell lines. T cell lines were re-fed or split 1 to 2 every 3 to 4 days

depending on confluence, using the same media with 5ng/mL IL-7 (BioLegend) and 5ng/mL IL-15 (R&D systems) and 1nM IL-2 (Chiron). After 2 weeks, all cells were collected and positively selected CD8^{+} T cells were then cloned by limiting dilution in 96 well U bottom plates in the presence of 200,000 irradiated (7,500 rads) allogeneic PBMCs, 40,000 LCL (lymphoblastiod cell line – provided by David Lewinsohn), 10 ng/mL of anti-CD3 (clone OKT3, eBioscience), 5ng/mL IL-7, 5ng/mL IL-15, and 1nM IL-2. Clones were either split 1 to 2, or re-fed with fresh medium and cytokines every 3-4 days.

Generation and selection of CD1b^{+} IL-32 DCs

IL-32 DCs were generated as previously described[13]. Briefly, PBMCs were isolated from whole blood of healthy donors and monocytes were enriched by CD14+ negative selection without CD16 depletion (Stemcell technologies) followed by culture in RPMI 1640 with 10% FCS and 50 ng/mL of IL-32 for 48 hours. Adherent DCs were lifted off using a cell scraper and PBS 0.5% EDTA. CD1b+ DCs were selected using an anti-CD1b+ antibody (BioXcell, clone BCD1b.3) and anti-MigG1 microbeads (Miltenyi) passing through a magnetic column.

Cell sorting of viable CD8^{+} T cell populations

FACS was used to purify CD8^{+} T cells from other populations of T cells by labeling cells with CD3, CD4, CD8, and CD137 To sort, staining was performed in sterile PBS with 10% FCS and sorting was performed in complete media.

CD8^{+} T cell assay and IFN-γ measurement

All T cell assays were conducted in 96-well U-bottom plates. The media used was RPMI 1640 with 10% Human AB serum (Sigma H4522). 1×10^5 irradiated (50 Gy) PBMCs were pulsed for one hour with peptide or peptide pools at a concentration of 10 μg/mL. 1×10^4 CD8^{+} T cells

were added to each well to make the final volume 200 μL. Supernatants were collected after 24 hours and IFN-γ was measured using an ELISA kit from BD Biosciences (555142) per manufacturer's protocols

Recombinant ML0049

Recombinant ML0049 was gifted to us by Dr. John Belisle from Colorado State University.

RESULTS

Defining *Mycobacterium leprae* antigens against CD8+ T cells

Due to the similarities between *M. tuberculosis* and *M. leprae* in their genome, pathogenesis, and the immune response mounted against the pathogen, we elected to screen putatively secreted *M. leprae* proteins to screen CD8+ T cell recognition. We chose four *M. leprae* proteins for further analysis. These proteins include ML0049 (ESAT-6) from the ESX family and ML0411 (PPE), ML0538 (PE4), and ML0051 (PPE68) from the PE/PPE family. Peptide pools of overlapping 15mers were generated for each of the four proteins to be used to screen CD8+ T cell responses.

To test whether the four selected proteins contained epitopes recognized by CD8+ T, we used IFN-γ as a measure of antigen reactivity since it is a hallmark of CMI in leprosy infection and a marker for in infection in household contacts in T-lep patients[37]. PBMCs from 19 T-lep patients were isolated, frozen, and HLA-typed. 20 million PBMCs from each donor were thawed and pulsed with each peptide pool to promote expansion of reactive T cells. CD8+ T cells were then purified from the frozen cells and cocultured with autologous PBMCs pulsed with peptide pools of each of the four *M. leprae* proteins. Supernatants were collected at 24 hours and IFN-γ was measured by enzyme-linked immunosorbent assay (ELISA). Peptide pools that stimulated at least a 2-fold induction of IFN-γ compared to media control were considered to have stimulated CD8+ T cells. The cumulative data revealed that ML0411 and ML0049 stimulated

IFN-γ production in CD8$^+$ T cells isolated from 8/19 and 6/19 patients, respectively (Figure 2.1). ML0051 and ML0538 stimulated IFN-γ production in only 1/18 and 2/17 patient samples, respectively. Therefore, peptide pools of ML0049 and ML0411 were used to clone *M. leprae*-specific CD8$^+$ T cells.

Cloning *M. leprae*-specific CD8$^+$ T using CD137 as an activation marker

CD137, also known as 4-1BB, a member of the TNFR-family, is expressed on activated CD8$^+$ and CD4$^+$ T cells[38]. CD137 mediates costimulatory and anti-apoptotic functions, promoting T-cell proliferation and T-cell survival. Although undetectable on unstimulated CD8$^+$ T cells, it is up-regulated 24 hours after activation. This feature made it a favorable marker to isolate CD8$^+$ T cells that recognize *M. leprae*-specific epitopes.

CD8$^+$ T cell clones were established using CD137 as a marker for ML0049 and ML0411 peptide pool-reactive CD8$^+$ T cells. We selected 5 patients based on the IFN-γ production of expanded CD8$^+$ T cells to ML0049 and ML0411 peptide pools. PBMCs were isolated from blood samples of these patients and divided into 3 groups. The first group was frozen for use as autologous feeder cells in future steps. The second group was frozen and thawed at a later date for CD8$^+$ T cell isolation. The third group was used to differentiate mature DCs to be used as professional antigen presenting cells. To start the cloning process, mature DCs were pulsed with either ML0049 or ML0411 peptide pools and cultured with autologous CD8$^+$ T cells in the presence of IL-2, IL-7, and IL-15 for 2 weeks. CD8$^+$ T cell cultures were re-stimulated with autologous PBMCs pulsed with their respective peptide pool every two weeks for a total of 2 re-stimulations. At this point, the cells were collected and stained with anti-CD3, anti-CD4, anti-CD8, and anti-CD137. Using a fluorescence-activated cell sorter (FACS), CD3$^+$, CD8$^+$, and CD137$^+$ cells were sorted from the total population. The phenotype of the cells in culture prior to each re-stimulation and prior to the FACS show an increase in CD3$^+$, CD8$^+$, and CD137$^+$ cells,

demonstrating an expansion of activated CD8^{+} T cells during each stimulation (Figure 2.2). The sorted T cells were then expanded using Lewinsohn et al's method of rapid expansion[39].

To ensure epitope specificity, expanded CD8^{+} T cells were tested with each individual peptide from their respective peptide pool to determine which 15mer possessed the specific epitope (Figure 2.3). Next, we needed to elucidate the HLA restriction of the CD8^{+} T cell and *M. leprae* peptide. Determining the HLA restriction would allow us to utilize healthy donors for generation of feeder cells and antigen presenting cells in future experiments. Having immunotyped each patient for their HLA expression, we cocultured the CD8^{+} T cells, the specific peptide, and PBMCs from healthy donors with a matched HLA molecule with the CD8^{+} T cell. Only the PBMCs that possessed the restricted HLA of the CD8^{+} T cell would be able to present the specific peptide to the CD8^{+} T cell and induce IFN-γ production (Figure 2.4). Once the HLA restriction was identified, the CD8^{+} T cell line was cloned by limiting dilution as described by Lewinsohn et al.[39.]. Individual epitope reactivity and HLA restriction were verified for each clone by IFN-γ production. Using this method of cloning, we were able to generate 6 CD8^{+} T cell clones from five leprosy patients that were CD3^{+} and CD8^{+} and responded to a single epitope specific to *M. leprae*. Figure 2.5 shows the phenotype and peptide response of CD8^{+} T cell clone J88.B.4 and figure 2.6 shows the cumulative list of all the CD8 T cell clones we have generated to date.

IL-32-derived DCs cross-present recombinant ML0049 and live *M. leprae* to CD8^{+} T cells

To test whether IL-32-derived DCs could cross-present recombinant *M. leprae* antigens to CD8^{+} T cells, we focused on CD8^{+} T cell clone J88.B.4. First, we generated IL-32-derived DCs from an HLA-matched healthy donor. J88.B.4 was cocultured with IL-32-derived DCs that were pulsed for 24 hours with either media alone, ML0049$_{(aa1-15)}$, recombinant ML0049, or live *M. leprae*. Supernatants were collected after 48 hours and IFN-γ was measured by ELISA. The

data revealed that ML0049$_{(aa1-15)}$, recombinant ML0049, and live *M. leprae* induced IFN-γ production in J88.B.4 (Figure 2.7), showing that IL-32 DCs could indeed cross-present *M. leprae* antigens to CD8$^+$ T cells.

DISCUSSION

The correlation between the clinical manifestation and the immune response against *M. leprae* demonstrates the importance of CMI in resistance vs. susceptibility in leprosy, offering a unique setting to identify mechanisms of CMI that gives rise to protection against the pathogen. By investigating leprosy, the novel pathway involving NOD2-mediated induction of IL-32, triggering differentiation of monocytes into CD1$^+$ DC with professional antigen presenting cell function was discovered[13]. Subsequent experiments revealed that these IL-32-derived DCs could not only present processed peptides via MHC class I and II, but cross-present exogenous antigen to CD8$^+$ T cells via MHC class I. Cross-presentation involves capturing exogenous antigen via the endocytic pathway and then loading processed peptides onto MHC class I molecules to be recognized by CD8$^+$ T cells. The fact that *M. leprae* also resides in the endocytic pathway prompted the hypothesis that cross-presentation could play a role in CD8$^+$ T cell activation in leprosy.

In order to study cross-presentation, we first set out to define *M. leprae* antigens to CD8$^+$ T cells. PE/PPE and ESAT-6 are major targets of T cell responses in TB, suggesting that homologs in *M. leprae* may also be targets of T cell responses in leprosy. Our hypothesis that putatively secreted proteins would be highly immunogenic to CD8$^+$ T cells was confirmed with the identification of ML0049 (ESAT-6) and ML0411(PPE) was found to induce IFN-γ production in CD8+ T cells isolated from 8/19 and 6/19 T-lep patients. Cloning of CD8$^+$ T cells that recognized ML0049 and ML0411 also revealed that 4/6 *M. leprae*'s CD8$^+$ T cell epitopes were restricted to HLA-B. A similar finding was reported for TB antigens by Lewinsohn et al. where

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out of the 15 CD8^{+} T cell clones, from 10 TB patients, 14 were restricted to HLA-B[41]. Although we must take into account the small sample size, the possibility that our cloning methods biased isolation of HLA-B-restricted CD8^{+} T cell clones, and the fact that only two *M. leprae* proteins were used, the observation of this phenomenon in leprosy adds to Kiepiela et al's report that HIV-specific T cell responses were found to be 2.5-fold more likely to be HLA-B than HLA-A-restricted and that vial load was more closely linked to HLA-B than HLA-A alleles[42]. Nonetheless, the data suggests that this link between HLA-B and *M. leprae* epitopes may be significant.

Dr. Schenk's discovery that components of the NOD2$\rightarrow$IL-32$\rightarrow$CD1^{+} DC pathway is highly expressed in T-lep vs. L-lep lesions coupled to our data that IL-32-derived DCs can cross-present defined *M. leprae* antigens to CD8^{+} T cells suggests that cross-presentation may play a role in the CMI that contributes to protective immunity[13]. Therefore, one can speculate that cross-presentation may play a role or could be utilized as part of a treatment for any disease where the antigenic source for CD8^{+} T cell activation resides in the endocytic pathway. The efficacy of inducing cross-presentation in cancer immunotherapy is a prime example. IL-32 can be supplemented to cancer vaccines and therapies to induce monocyte differentiation into IL-32-derived DCs to efficiently prime the CD8^{+} T cell response via cross-presentation.

Our data provides evidence to further investigate the mechanisms of cross-presentation and activation of CD8+ T cells, in the hopes of providing new insights into the human immune system as well as elucidating new targets and therapy strategies for the treatment of human diseases.

FIGURES

Donor ID	ML0049	ML0051	ML0411	ML0538	Reactivity
G03	+	+	+	+	4/4
A74	-	-	-	-	0/4
A03	+	-	+	-	2/4
B61	-	-	-	-	0/4
A12	+	-	-	-	1/4
F04	-	-	-	-	0/4
N08	-	-	-	-	0/4
C41	-	-	-	-	0/4
B05	-	-	-	-	0/4
G24	+	-	-	-	1/4
H06	-	-	-	-	0/4
R14	-	-	-	-	0/4
J44	+	-	+	+	3/4
G41	+	-	+	-	2/4
N56	-	-	-	-	0/4
A66	-	-	-	-	0/4
G11	+	-	+	-	2/4
J88	+	-	+	-	2/4
J42	-	-	-	-	0/4
Total	8/19	1/19	6/19	2/19	8/19

Figure 2.1. Data of the CD8^{+} T cell screen against *M. leprae* peptide pools.
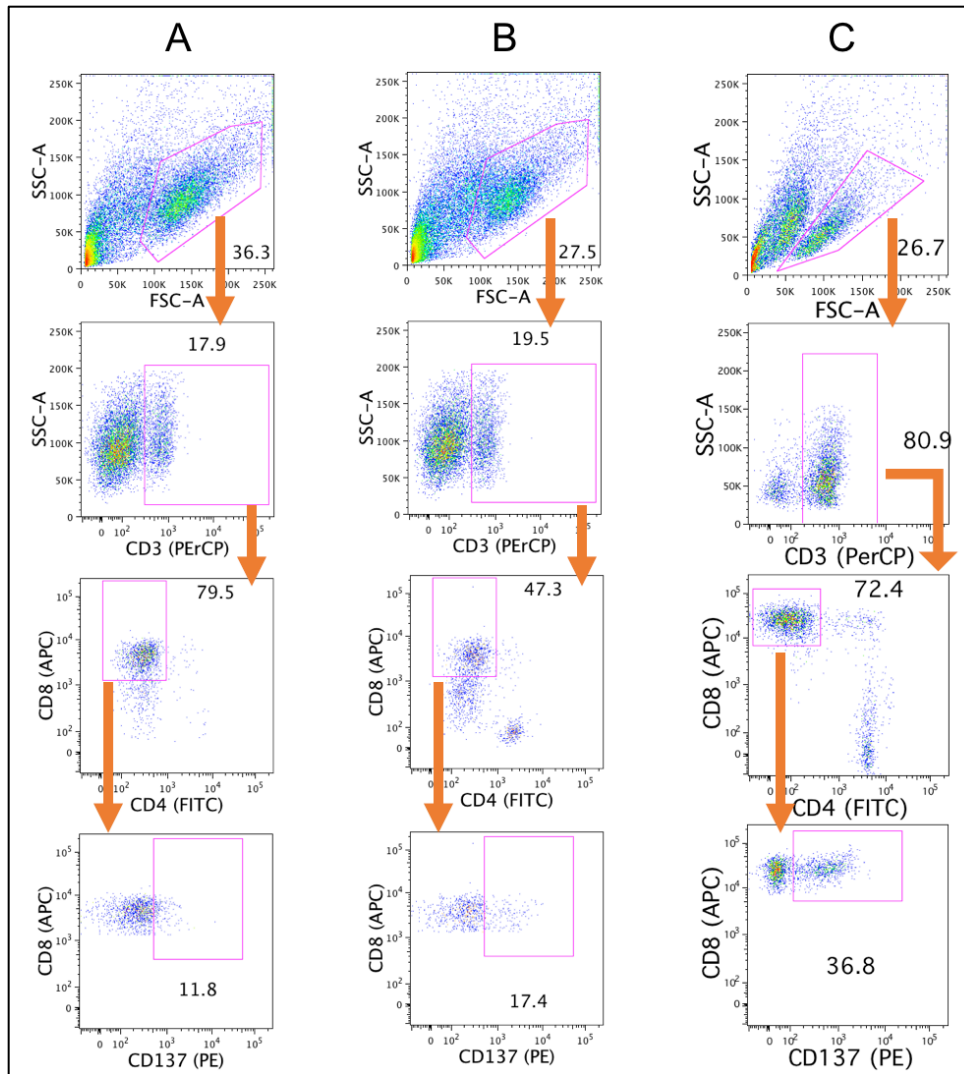
Figure 2.2. Confirmation of the expansion of CD3$^+$, CD8$^+$, and CD137$^+$ T cells during the cloning process. Each column of flow cytometry dots represents 14 days (A), 28 days (B), and 42 days(C) after CD8$^+$ T cells from pt. J88 was initially stimulated with mature DCs and ML0049 peptide pool.

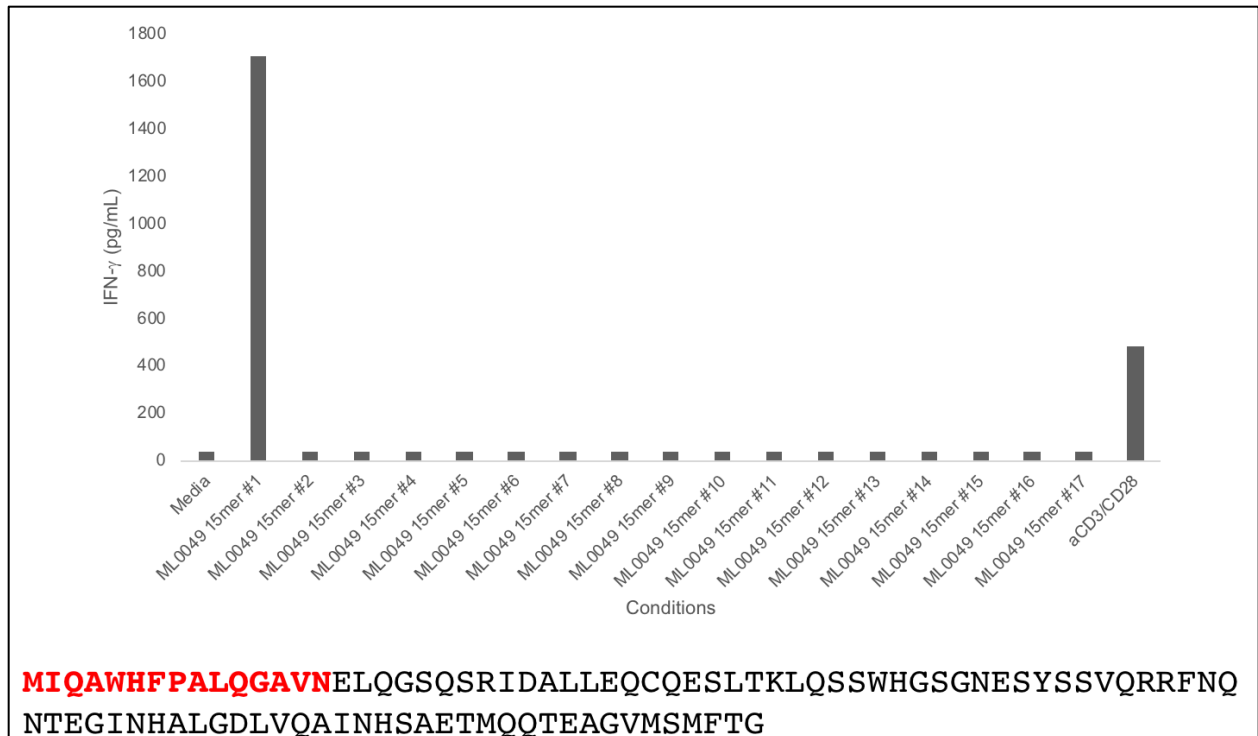Figure 2.3. Deconvolution of antigenic epitope for pt. J88 CD8^{+} T cell line. J88 CD8^{+} T cell line was incubated with autologous PBMCs pulsed with each 15mer within ML0049's peptide pool. IFN-γ production measured by ELISA. Anti-CD3/anti-CD28 beads used as a positive control and media only used as negative control. Amino acid sequence of ML0049 shows the location of the J88 CD8^{+} T cell line's epitope (in red).

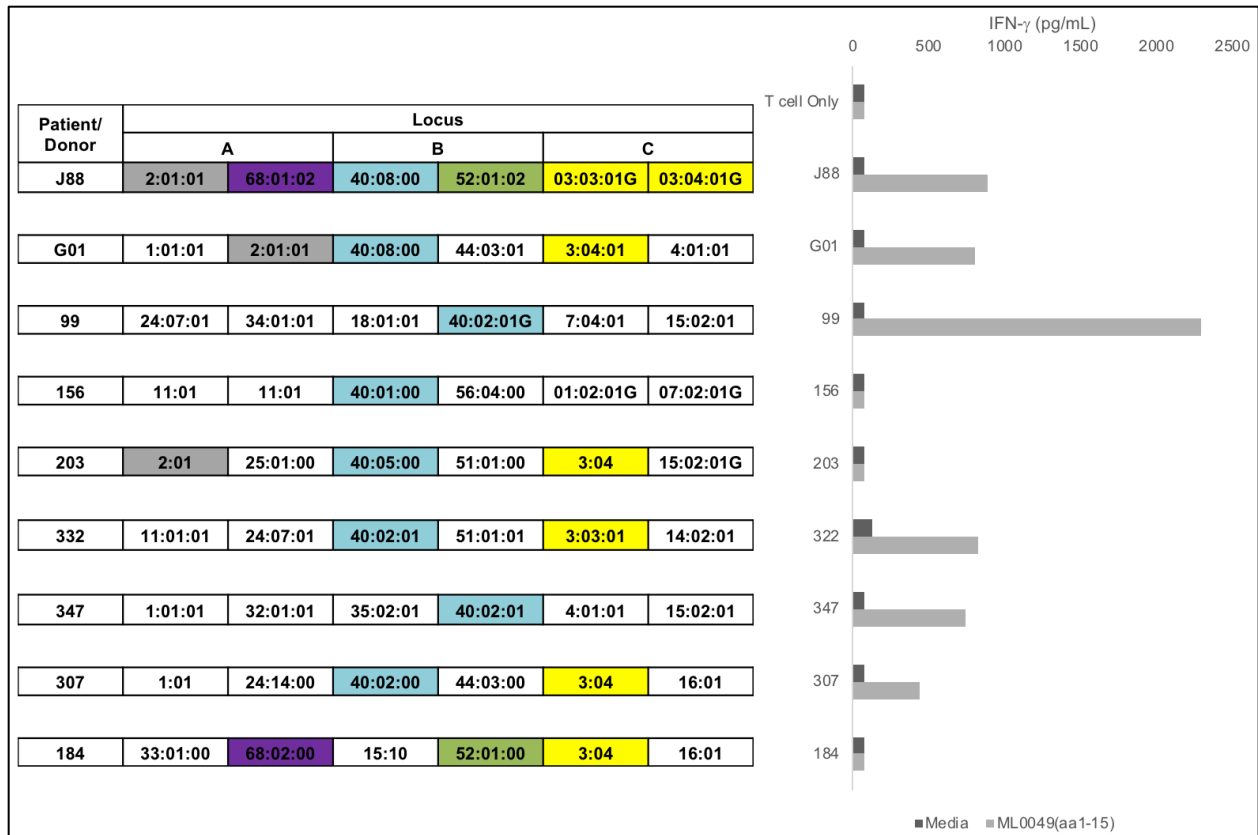| Patient/Donor	Locus					
	A		B		C	
J88	2:01:01	68:01:02	40:08:00	52:01:02	03:03:01G	03:04:01G
G01	1:01:01	2:01:01	40:08:00	44:03:01	3:04:01	4:01:01
99	24:07:01	34:01:01	18:01:01	40:02:01G	7:04:01	15:02:01
156	11:01	11:01	40:01:00	56:04:00	01:02:01G	07:02:01G
203	2:01	25:01:00	40:05:00	51:01:00	3:04	15:02:01G
332	11:01:01	24:07:01	40:02:01	51:01:01	3:03:01	14:02:01
347	1:01:01	32:01:01	35:02:01	40:02:01	4:01:01	15:02:01
307	1:01	24:14:00	40:02:00	44:03:00	3:04	16:01
184	33:01:00	68:02:00	15:10	52:01:00	3:04	16:01

Figure 2.4. Determining HLA restriction for J88 CD8^{+} T cell line. Colored boxes indicated HLA matches between the leprosy patient and the healthy donor. IFN-γ production was observed when the PBMCs possessed HLA-B 40:08 or HLA-B 40:02. Therefore, J88 CD8^{+} T cell line recognizes ML0049$_{(aa1-15)}$ in the context of HLA-B 40:08 or 40:02.
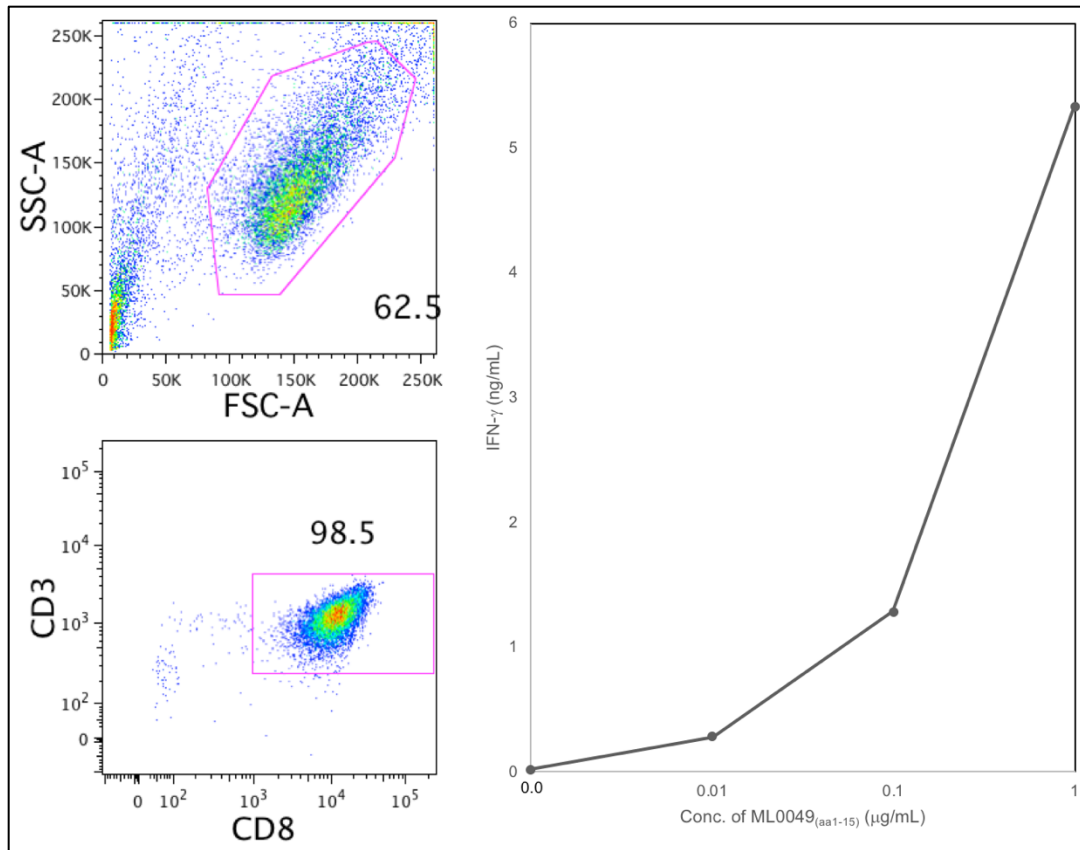
Figure 2.5. The phenotype and response to ML0049$_{(aa1-15)}$ for CD8$^+$ T cell clone J88.B.4.

Established CD8$^+$ T cell clones					
T cell ID	**Source**	**Patient ID**	**M. leprae Protein**	**Epitope**	**HLA Restriction**
A03.B.12	Blood	A03	ML0411	aa41-55	HLA-B 40:02
G11.B.5	Blood	G11	ML0411	aa6-20	HLA-A 02:01
G24.B.4.4	Blood	G24	ML0049	aa16-30	HLA-B 40:01/02/08
J88.B.4.1	Blood	J88	ML0049	aa1-15	HLA-B 40:02/08
A12.B.4.8	Blood	A12	ML0049	aa41-55	HLA-B 15:18/10
A12.B.4.8	Blood	A12	ML0049	aa71-85	HLA-A 33:03/01

Figure 2.6. A cumulative list of CD8$^+$ T cells cloned from leprosy patients and their epitopes.
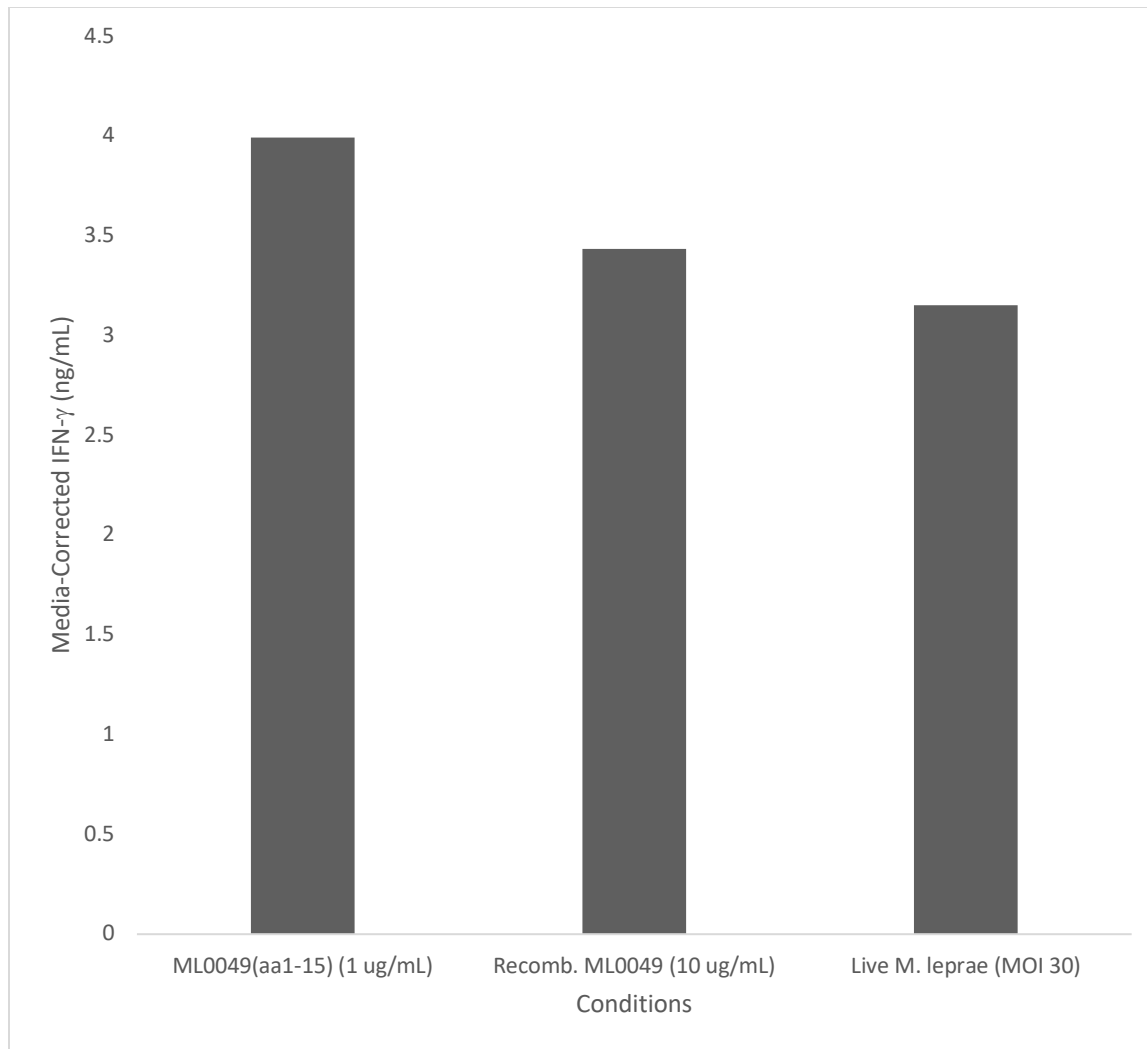
Figure 2.7. IL-32 DCs cross-present *M. leprae* antigens to CD8^{+} T cell clone J88.B.4.

Chapter 3

Conclusion

SUMMARY

Investigation of leprosy has led to the discovery that *Mycobacterium leprae* infection can trigger the NOD2-mediated induction of interleukin-32 (IL-32), triggering the differentiation of monocytes into CD1^{+} dendritic cells (DCs) with the ability to cross-present exogenous antigen to CD8^{+} T cells via MHC class I molecules[13]. Since *M. leprae* is an intracellular pathogen that resides in the endocytic pathway, we hypothesized that IL-32-derived DCs could also cross-present *M. leprae* antigens to CD8^{+} T cells. To investigate this hypothesis, we screened CD8^{+} T cells from 19 T-lep patients for their ability to produce IFN-γ in response to peptide pools of 5 putatively secreted *M. leprae* proteins. Of the four proteins, ML0049 (ESAT-6) and ML0411 (PPE) stimulated IFN-γ production in CD8+ T cells in 8/19 and 6/19 patients, respectively. Peptide pools of these two proteins were used to clone CD8^{+} T cell clones, resulting in six CD8^{+} T cell clones from five patients. We then showed that IL-32-derived DCs could cross-present recombinant ML0049 and live *M. leprae* to CD8^{+} T cell clone J88.B.4.

FUTURE PERSPECTIVES

Elucidation of the mechanism by which IL-32 induces cross-presentation of *M. leprae* will provide insight into how CD8^{+} T cells recognize pathogens taken up by the endocytic pathway and trigger an antimicrobial response, relevant to understanding the link between innate and adaptive immunity. The identification of *M. leprae* epitopes recognized by CD8^{+} T cells in leprosy provides a model to investigate the role of cross-presentation in leprosy.

The next step would be to elucidate the mechanism by which IL-32 induces cross-presentation of *M. leprae* antigen to CD8^{+} T cells. The two main pathways for cross-presentation of exogenous antigen are the cytosolic and vacuolar pathways. Chemical inhibitors and gene knockdowns can be utilized to dissect the pathways involved in cross-presentation. One key difference between the cytosolic and vacuolar pathway of cross-presentation is the

location of antigen processing. Therefore, cytosolic proteasome inhibitors (lactacystin, epoxomicin), lysosomal protease inhibitors (leupeptin), and cathepsin S inhibitor (Z-FL-COCHO) and be used to pre-treat IL-32 DC prior to culture with antigen and subsequent CD8$^+$ T cell activation. Furthermore, specific genes can be knocked down to determine their relevance during cross-presentation. A preliminary study of gene signatures involved in MHC class I and cross-presentation that were selectively induced by IL-32 vs. granulocyte-macrophage colony stimulating factor (GM-CSF) identified ten candidate proteins that have been implicated in cross-presentation pathway (Figure 1.6). These genes are differentially involved in either the cytosolic or vacuolar pathways. Investigating the effects of cross-presentation due to knocking down these genes in IL-32-derived DCs may provide evidence for the mechanism of cross-presentation of *M. leprae* antigens.

The generation of CD8$^+$ T cells that recognize *M. leprae* epitopes also provide the means to determine whether MHC class I-restricted CD8$^+$ T cells recognize *M. leprae* infected monocytes and MΦs and whether recognition is dependent on IL-32 production or enhanced by IL-32 addition. Although *M. leprae* is an intracellular pathogen, it is phagocytosed and enters the endosomal/lysosomal pathway, thereby bypassing the cytosol. Cross-presentation provides a pathway for antigens acquired by the endocytic pathway to gain access to MHC class I molecules. Since infection of monocytes with *M. leprae* induces IL-32 and differentiation into CD1$^+$ DCs, it would be interesting to investigate whether CD8$^+$ T cells recognize *M. leprae*-infected monocytes and macrophages (MΦs) and whether this process is dependent on IL-32 production or can be enhanced by addition of IL-32. IL-32 dependence of cross-presentation can be assayed utilizing siRNA against IL-32 prior to *M. leprae* infection and CD8$^+$ T cell activation. The enhancement of cross-presentation due to IL-32 can be tested by infecting monocytes and MΦs with *M. leprae* in the presence or absence of exogenous recombinant IL-

32. These experiments may elucidate the role of endogenous IL-32 in mediating cross-

presentation.

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