

THESIS

ASSESSING ANTIMICROBIAL MECHANISMS IN LANGERHANS CELLS DURING A
MYCOBACTERIUM LEPRAE INFECTION

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Linda Fischbacher

Department of Microbiology, Immunology and Pathology

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Master's Committee

Advisor: John Belisle

Casey Gries
Dawit Tesfaye

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ABSTRACT

ASSESSING ANTIMICROBIAL MECHANISMS IN LANGERHANS CELLS DURING A *MYCOBACTERIUM LEPRAE* INFECTION

Langerhans cells are essential immune cells in the skin that maintain homeostasis and clear pathogens. Despite their importance, much is unknown about Langerhans cells, including their innate antimicrobial mechanism. Single-cell sequencing of leprosy skin lesions identified genes upregulated in Langerhans cells of reversal reaction lesions that may be associated with antimicrobial activity. CCL22, MPEG1, and IDO1 were selected to study further as potential effectors in Langerhans cells for killing *Mycobacterium leprae*. We hypothesized that altered metabolic processes in Langerhans cells impact antimicrobial effects on *M. leprae*.

An *in vitro* model was developed to induce antimicrobial gene expression in Langerhans cell-like dendritic cells (LCDCs). IL-1 β was identified as the best inducer of CCL22 and MPEG1, and IFN- γ as the best inducer of IDO1. Induction was measured by gene expression and protein production, as well as enzyme activity for IDO1 by measuring metabolites. The antimicrobial effect of IDO1 on intracellular *M. leprae* in LCDCs was assessed by inducing IDO1 with IFN- γ or inhibiting IDO1 activity with 1-methyltryptophan. Stimulation by this agonist or this antagonist modulated IDO1 expression and activity but did not affect *M. leprae* viability.

The changes of intracellular tryptophan catabolites in non-stimulated and *M. leprae*-infected LCDCs were measured. The *M. leprae* infection increased two kynurenine pathway catabolites after 24 and 48 hours, 3-hydroxyanthranilic acid and quinolinic acid. These data indicate that while *M. leprae* did not induce IDO1 expression, it did increase IDO1 and

kynurenine pathway activity. Neither metabolite has reported antimicrobial properties, but quinolinic acid may benefit *M. leprae* for synthesizing nicotinamide adenine dinucleotide.

A different tryptophan catabolite pathway leads to serotonin production. In *M. leprae*-infected LCDCs, serotonin was decreased, but 5-hydroxyindoleacetic acid, a breakdown product of serotonin, was increased. The implication of these changes for an *M. leprae* infection of LCDCs is unknown. 5-hydroxyindoleacetic acid is also increased in *Mycobacterium tuberculosis* patients. This metabolite may benefit these mycobacteria as it is reported to increase PPAR γ activity, which is known to support *M. leprae* and *M. tuberculosis* in macrophages.

The main antimicrobial mechanism of IDO1 is depleting tryptophan from tryptophan auxotrophic pathogens. Whether the tryptophan biosynthesis pathway in *M. leprae* is functional was assessed using ^{13}C -tracing, to determine if tryptophan depletion by IDO1 could kill *M. leprae*. In axenic media, *M. leprae* did not synthesize tryptophan from ^{13}C -glucose and ^{13}C -palmitic acid nor synthesize tryptophan from intracellular ^{13}C -glucose. *In vitro*, *M. leprae* only synthesized tryptophan from an intermediate, anthranilic acid. Using the same method, *M. tuberculosis* synthesis of tryptophan from ^{13}C -glucose was confirmed as a control. The functionality of the tryptophan biosynthesis pathway in *M. leprae* could not be confirmed. However, because of the homology between the *M. leprae* and *M. tuberculosis* genes for tryptophan biosynthesis, this pathway likely is functional, and *M. leprae* would not be killed by IDO1-mediated tryptophan depletion.

These findings indicate that IDO1 is not associated with antimicrobial activity towards *M. leprae* in LCDCs. Instead, increased IDO1 activity induced by *M. leprae* infection resulted in increased tryptophan catabolites likely to benefit rather than kill *M. leprae* in LCDCs. *M. leprae* likely evades the primary killing mechanism of IDO1, tryptophan depletion,

by possessing an intact pathway for tryptophan biosynthesis. Further studies to elucidate the importance of quinolinic acid and 5-hydroxyindoleacetic acid for *M. leprae* and validate that the *M. leprae* tryptophan biosynthesis pathway is functional will aid in identifying essential pathways for *M. leprae* that can be targeted with therapeutics. Other potential antimicrobial effectors in LCDCs, including CCL22 and MPEG1, will need to be assessed to study this innate mechanism in Langerhans cells further.

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CHAPTER 1: LITERATURE REVIEW

1.1 Leprosy

Mycobacterium leprae (ML) or *Mycobacterium lepromatosis* infection is the primary cause of leprosy, also known as Hansen's disease. Leprosy is reported in 120 primarily tropical low- and middle-income countries worldwide, with 174,087 new human cases reported in 2022. Brazil, India, and Indonesia reported the most new cases at more than 10,000 per year (Figure 1) [1-4]. In the United States, 136 new cases were reported in 2022 [3, 5]. In a recent case report, the Centers for Disease Control suggests that central Florida represents an endemic location for leprosy, with Florida being among the states with the most new cases [5, 6].

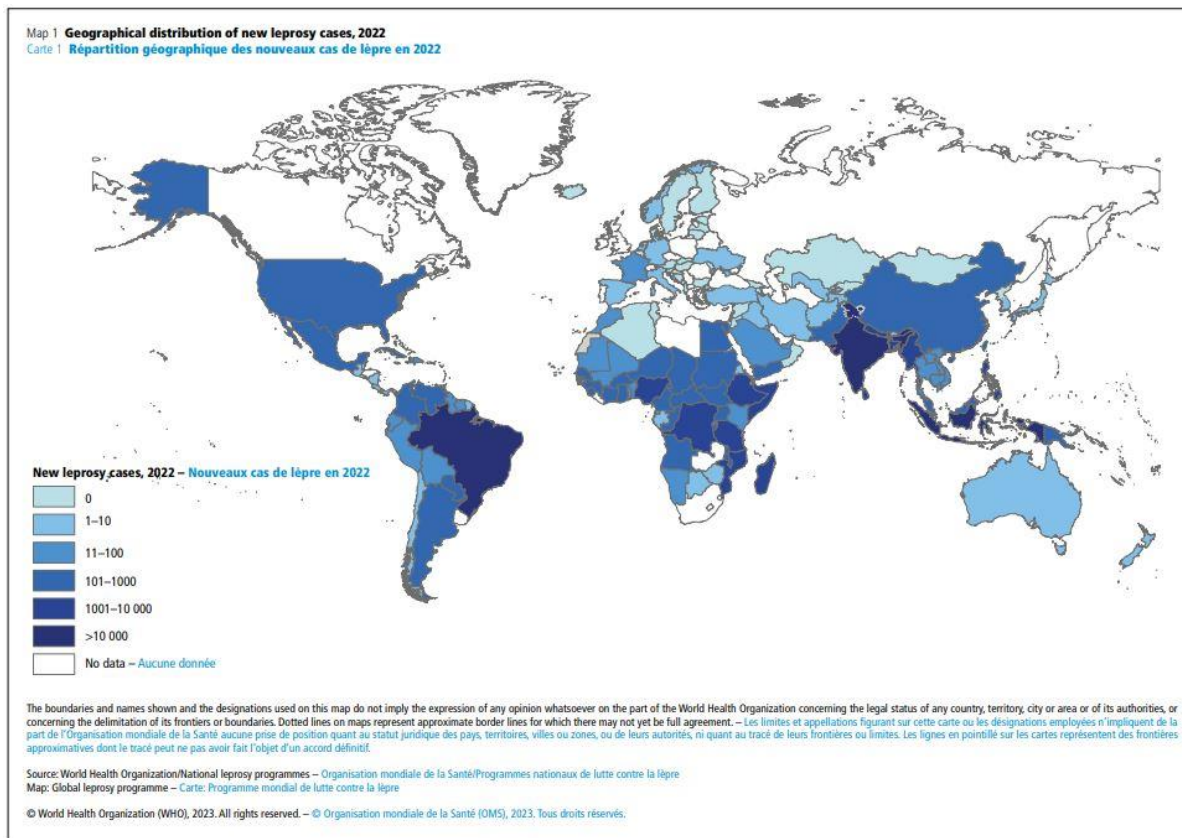


Figure 1: Geographical Distribution of New Leprosy Cases in 2022
from the World Health Organization global leprosy (Hansen disease) update, 2022 [1]

1.1.1 Leprosy Reservoirs and Transmission

Leprosy is a zoonotic disease [7-10]. The best-described reservoir of ML is the nine-banded armadillo (*Dasypus novemcinctus*). These armadillos are a large natural ML reservoir in the Americas and a primary cause of new human cases [7-10]. Susceptible to disease, the armadillos develop lesions (both in the skin and nerves) and can exhibit the entire disease spectrum (tuberculoid, lepromatous, and intermediate stages) similar to humans, both when they are naturally and experimentally infected [7, 8]. Infected armadillos naturally transmit ML to humans and other armadillos through direct contact [9]. Whether ML can be spread and survive in the environment before finding a new host is unknown. Survival of the bacterium in soil contaminated by infected armadillos, followed by infecting humans that handle the soil, is being studied [7-9, 11].

In addition to armadillos, various non-human primates have reportedly acquired leprosy, but no established reservoirs or models exist [7]. These non-human primates seem to have naturally acquired the disease. While non-human primates exhibit similar signs of leprosy in humans, they do not yet replicate the full spectrum of disease or develop all the features associated with human leprosy. So far, there are no reports of non-human primate-to-human transmission [7, 10].

Other potential natural zoonotic leprosy reservoirs are also being studied, such as the red squirrels (*Sciurus vulgaris*) in the British Isles [9]. However, in mammals, other leprosy-like diseases are caused by pathogens related to ML, including *Mycobacterium lepraefelis* and *Mycobacterium uberis*, which have convoluted studies of leprosy reservoirs [9, 12-14]. Amoebae are being studied as a potential refuge for the ML in the environment between hosts [7, 10] and ticks as a possible ML transmission route [10, 15, 16]. More insight into the life

cycle of ML outside of humans may be vital in controlling ML transmission. The involvement of animals and the environment in the transmission of ML has been suggested to contribute to a steady rate of new leprosy cases despite a widely used multi-drug regimen [9].

In addition to the poor understanding of the zoonotic and environmental spread of ML, human-to-human transmission still needs to be fully characterized. Close contact with infected individuals increases the risk of infection, with cases frequently occurring through household contact [9, 10, 17, 18]. The spread of aerosol droplets and contact with infected skin and tissues are thought to be the main modes of transmission [18-21]. Exactly how ML progresses from transmission to causing disease, including the dissemination of the bacteria, remains unknown. Upon respiratory transmission, ML seems to enter the new host through the nasal and alveolar epithelial cells and macrophages, although no respiratory tract lesions occur [19, 22-27]. The bacteria then likely spread to more favorable infection sites (i.e., the skin and peripheral nerves) through the vascular or lymphatic systems [26-31]. The bacteria are cleared, or the infection progresses to leprosy disease. Why the immune response to ML differs among patients, either clearing the infection or developing different forms of the disease with reactions also sometimes occurring, is not known. However, certain risk factors have been identified, including older age, specific genetic influences, nutritional factors, and immunosuppression [9, 10, 17, 18, 20, 32].

1.1.2 Leprosy Disease Immune Responses

Leprosy disease presents on a spectrum due to the varying immune responses if the bacteria are not cleared. A summary of the leprosy disease spectrum is presented in Figure 2. On one end of the spectrum is the development of a type 1, cell-mediated immune response with pro-inflammatory classically activated macrophages (M1-like), T-helper cells (e.g., Th1 cells), and cytokines (e.g., IFN- γ , TNF- α , IL-15) and well-formed granulomas. This response controls

the growth of the ML, resulting in few lesions with a no-to-low bacillary load and is known as paucibacillary (PB) leprosy by the World Health Organization (WHO) classification, or tuberculoid (TT) and borderline tuberculoid (BT) leprosy by the Ridley-Jopling classification. The other end of the leprosy disease spectrum is characterized by a type 2 humoral immune response with anti-inflammatory alternatively activated foamy macrophages (M2-like), T-helper cells (e.g., Tregs), and cytokines (e.g., IL-10 and IL-4). This response is inadequate to control ML, and the bacteria can persist and grow to high bacillary loads in numerous lesions. This end of the spectrum is known as multibacillary (MB) by the WHO or by the Ridley-Jopling classification as lepromatous (LL), borderline lepromatous (BL), and borderline borderline (BB) leprosy [17, 32-39].

Pathological immune reactions also occur during disease, treatment, or after treatment. About half of LL and BL patients experience the erythema nodosum leprosum (ENL) reaction due to complex and poorly understood immune interactions. ENL involves an acute inflammatory condition associated with an influx of neutrophils and the development of immune-complex-associated vasculopathy [32, 40, 41]. Lucio's phenomenon (erythema necroticans) is a rare and poorly understood reaction occurring in diffuse non-nodular lepromatous leprosy, resulting in widespread skin necrosis [40, 42]. Reversal reaction (RR) is a delayed hypersensitivity reaction caused by developing a robust cell-mediated pro-inflammatory immune response to ML antigens. It can occur in patients with any form of leprosy except TT leprosy, as these patients already exhibit the most robust pro-inflammatory response to ML [17, 43]. The exact trigger for this reaction is unknown. In the reversal reaction lesions, there is a switch from a Th2 to Th1 response with an increase of CD4⁺ T cells and Th1 cytokines such as IFN- γ , TNF- α , IL-1 β , and a switch from M2-like to M1-like macrophages [32, 40].

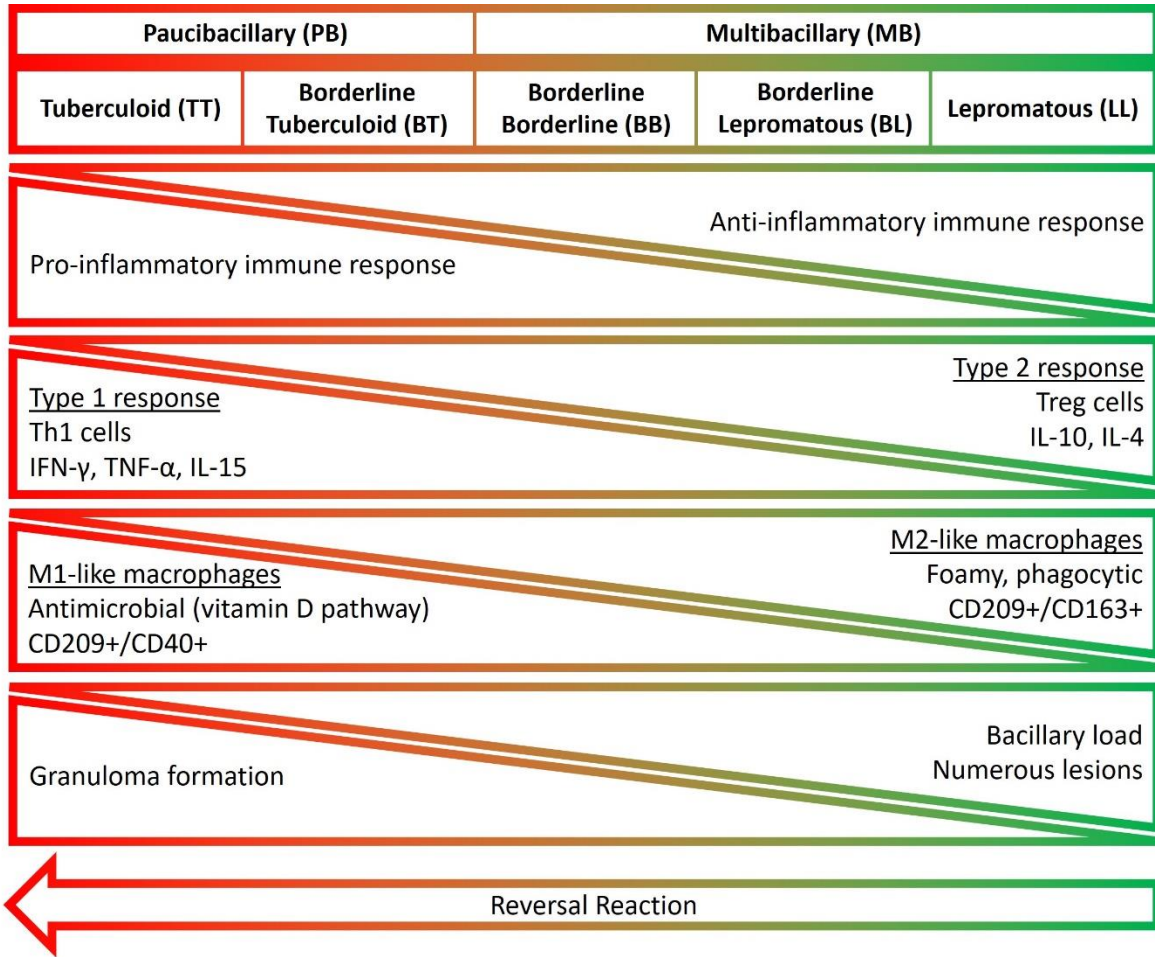


Figure 2: Leprosy Disease Spectrum

Leprosy primarily develops in the peripheral nerves and skin. Both sites have the cooler temperature the ML needs. The bacillus also has a tropism for peripheral nerve Schwann cells and skin macrophages [17, 32]. In the skin, circulating monocytes are recruited to the disease site. In MB lesions, unstimulated endothelial cells trigger the differentiation of macrophages to the M2-like anti-inflammatory phenotype. In contrast, IFN- γ stimulated endothelial cells cause the monocytes to become M1-like pro-inflammatory macrophages as found in PB lesions [36]. However, evidence is accumulating supporting the concept that a high percentage of tissue macrophages are independent of circulating monocytes and are a mix of embryonic and bone marrow-derived cells with different phenotypes and functions relative to their origin [17, 44-46].

Despite their unknown origin, the distinct phenotypic differences between macrophages of PB and MB leprosy can be induced by the cytokines associated with these two forms of the disease. IL-15, found in PB lesions, differentiates monocytes into CD209+/CD40+ expressing macrophages that have less binding and phagocytic ability but have a more active vitamin D-dependent antimicrobial pathway with more antimicrobial cathelicidin expression. Meanwhile, IL-10 stimulation results in CD209+/CD163+ expressing foamy macrophages with increased binding and phagocytic capacity but decreased vitamin D-dependent antimicrobial pathway activity. In leprosy lesion biopsies, there is an increase of foamy CD209+/CD163+ macrophages containing ML bacilli in LL compared to TT and RR patients correlating with the cytokine-induced macrophage profiles [37].

Like its relative *Mycobacterium tuberculosis* (Mtb) and other pathogenic mycobacteria, ML can grow in phagosomes and prevent phagosome-lysosome fusion to avoid degradation by the host [47, 48]. In LL, more binding and uptake of the bacilli into the phagosome occurs, supporting the continued growth of ML. The M2-like macrophages of LL disease also downregulate antigen-presentation and co-stimulatory molecules, poorly stimulating Th1 cells, and secrete IL-10, which induces Treg differentiation. In contrast, in TT and RR, the macrophage phenotype exhibits more antimicrobial mechanisms, like cathelicidin expression, controlling ML growth. These M1-like macrophages stimulate Th1 cells and secrete IL-23 and IL-12 [37, 49]. Overall, there is a clear difference in how macrophages behave, correlating to the different leprosy forms.

1.1.3 Leprosy as an Immune Response Model

The clear delineation in the immune response to ML has resulted in leprosy being studied as a pro- or anti-inflammatory immune response model [35, 48, 50, 51]. *In vitro*, macrophages

infected with a high multiplicity of infection (MOI) (e.g., 50:1 live ML) have the same response as infected macrophages in LL lesions, while lower MOIs (5:1 and 10:1) correlate with the response of TT-infected macrophages [52]. Leprosy is also an effective model for studying Langerhans cells (LCs) [48, 50]. Although LCs are not one of the cell types primarily infected by ML, LC numbers increase in BT and RR lesions compared to BL and LL lesions. LCs efficiently present ML antigens to T cells, thus participating in the immune response [48, 50, 53].

1.1.4 Leprosy Model Inducing an Antimicrobial Response in Langerhans Cells

LCs phagocytose ML bacilli, but prevention of phagosome-lysosome fusion allows pathogen survival in the host cell. In the environment of a Th1 response, it is suggested that LCs are activated to kill ML. An *in vitro* study revealed that IFN- γ treatment of LCs before ML infection activates antimicrobial mechanisms leading to ML killing [48]. Specifically, IFN- γ stimulation of LCs followed by infection results in autophagy of the ML-containing phagosome, forming a two-membrane autophagosome. This autophagosome can then fuse with the lysosome, forming an autophagolysosome in which the ML is degraded [48]. Furthermore, the IFN- γ stimulation enhances CD1a-restricted antigen presentation of live ML to CD4⁺ T cells derived from leprosy skin lesion T resident memory cells. These T cells secrete IFN- γ upon stimulation, creating a positive feedback loop [48]. The IFN- γ stimulation also increases the vitamin D-dependent antimicrobial pathway in LCs, increasing cathelicidin expression, which is found in the autophagolysosomes and is needed to kill the ML [48]. These data demonstrate that T cells can activate antimicrobial mechanisms in LCs. The autophagy and vitamin D-dependent pathways induced in the LCs result in the direct killing of the ML through the lysosomal contents (e.g., hydrolytic enzymes) and cathelicidin (e.g., disrupts bacterial membranes) [54-56]. Other antimicrobial mechanisms are also likely induced in LCs, but they remain to be elucidated.

1.2 Langerhans Cells

LCs are the main antigen-presenting cells in the epidermal layer of the skin, primarily characterized by their CD207 and CD1a expression and having endosomal compartments known as Birbeck granules [50, 57-59]. Traditionally, LCs are categorized as dendritic cells (DCs) because of their shape and function [60]. Recent studies, though, suggest that there are multiple LC subgroups. Resident, true LCs seem to originate from yolk sack and fetal liver monocytes, which seed the fetal skin as LC progenitors, developing into differentiated LCs. These resident LCs are self-renewed in a differentiated state without contributions from stem cell progenitors. These new data regarding LC ontogeny and replenishment suggest that LCs are actually tissue-resident macrophages that behave like dendritic cells [61-64].

In addition, there are monocyte-derived LC-like cells (moLCs) and inflammatory dendritic epidermal cells (IDECs). During inflammation, upon antigen recognition, the resident LCs drain to the lymph node. The skin inflammation recruits monocytes, which differentiate into short-term moLCs to replenish the loss of the resident LCs. When the inflammation is resolved, the moLCs are replaced by resident LCs. IDECs are recruited myeloid cells found in the inner part of the epidermis, while the resident LCs are in the outer part. How the behavior of these LC populations differs is still largely unknown, and most studies investigate total epidermal LCs as a population that expresses the characteristic LC markers CD1a and CD207 [61, 62, 65-67].

While much is still being learned about LCs, their primary role in the skin is maintaining homeostasis by regulating immune responses. LCs promote tolerogenic immune responses in non-dangerous conditions, expanding and activating skin Tregs. When sensing danger (e.g., pathogens), LCs use their own antimicrobial defenses and promote pro-inflammatory responses through T cell activation, such as skin resident memory T cells that secrete cytokines such as

IFN- γ [48, 68, 69]. After clearing the pathogens, the LCs switch to abrogating the inflammatory response by expanding Tregs to return the local environment to steady-state conditions. Because of their location, exposure, and interaction with the environment and microbiome, LCs must be tightly regulated to help prevent unrestrained and unneeded inflammation. The behavior of LCs seems to be programmed by the epidermal environment, primarily through cytokines from keratinocytes and T cells [61, 68, 69].

However, studies of LCs, especially human LCs, have been relatively scarce as the availability of LCs (which may be a mixed population) is very limited. Much needs to be elucidated about the innate function of LCs and their role in the skin [69]. There are LC experimental models, that also have numerous constraints, and whether they genuinely reflect LC function is controversial [69]. One model is to differentiate LC-like dendritic cells (LCDCs) from CD34+ stem cells, usually isolated from cord blood [48, 50, 59, 70-73]. Compared to isolated LCs, LCDCs have similar expression of CD1a and CD207 and contain birbeck granules. LCDCs and LCs can be infected by ML at similar levels, with a 10:1 MOI resulting in ~50% of the cells being infected by three to five bacilli per cell. Both cell types develop the same antimicrobial responses when stimulated by IFN- γ before ML infection and have similar antigen-presentation behavior [48, 50].

1.3 Langerhans Cell Antimicrobial Genes

By using sequencing and transcriptome mining, potential antimicrobial responses produced by LCs to fight ML infection have been identified [73-75]. Dr. Robert Modlin and colleagues identified potential antimicrobial genes with increased expression in LCs from RR patients compared to LL patients [73, 74, 76]. The genes and products CCL22, MPEG1, and IDO1 are of interest for further study.

1.3.1 CCL22

CCL22 codes the C-C motif chemokine ligand 22 (*CCL22*) or macrophage-derived chemokine (MDC) [77]. This chemokine primarily acts as a chemoattractant for the various cells that express its receptor (CCR4), including differentiated T cells and DCs. CCR4 also binds *CCL17* [78-80]. *CCL22* is constitutively expressed by DCs, B cells, and macrophages, though most strongly by mature DCs and macrophages. Other cells, such as natural killer cells and CD4⁺ T cells, express *CCL22* after stimulation [77, 79, 81]. This chemokine is important in recruiting CCR4⁺ Th2 and Treg cells, especially during allergic inflammation and atopic dermatitis [77, 78, 81]. During chronic infection, *CCL22* continuously recruits DCs and lymphocytes [79, 81]. LCs that have matured also secrete *CCL22*. LCs in the lymph node present antigens to T cells, which can differentiate into Tregs that are then recruited by mature skin LCs using *CCL22*. IL-10, produced by Tregs, suppresses *CCL22* secretion by LCs, creating a negative feedback loop [78]. *CCL22* expression can be altered by a variety of stimulants, including LPS, IL-1, CD40, IL-10, and IFN- γ and thymic stromal lymphopoietin (TSLP) from keratinocytes [68, 78, 81-87].

1.3.1.1 CCL22 – Antimicrobial Activity

CCL22 is considered an antimicrobial molecule because, in one direct chemokine antimicrobial activity study, 100% of *Escherichia coli* and 74% of *Staphylococcus aureus* were killed when directly treated with *CCL22* [88]. Whether *CCL22* directly kills these pathogens (e.g., lysing the bacteria) or influences pathogen processes that result in the death of these bacteria (e.g., inhibiting nutrients import from media) is unknown. There are no reports of direct involvement between this chemokine and mycobacteria. *CCL17*, but not *CCL22*, is elevated in the serum of active tuberculosis patients [89]. When stimulated with Mtb purified protein

derivative (PPD) or mannose-capped lipoarabinomannan (ManLAM), CCL22 secretion by peripheral lymph node mononuclear cells (PBMCs) increases non-significantly [90]. While its role and importance are still unclear, studies suggest that CCR4 may be involved in immunity to mycobacterial infections, specifically immune cell recruitment. However, this receptor does not seem essential for overall immunity to mycobacteria [89-92].

1.3.2 MPEG1

MPEG1 codes for the macrophage-expressed gene 1 protein (MPEG1), also known as perforin 2, and is an ancient, highly conserved, intronless gene [93]. MPEG1 is thought to be the oldest protein of the membrane attack complex/perforin (MACPF) branch of the MACPF/cholesterol dependent cytolyisin (CDC) superfamily of pore-forming proteins (PFPs). More modern relatives thought to have arisen from MPEG1 are the important immune pore-forming MACPF complexes, perforin 1 and membrane attack complex (MAC) [94, 95]. MPEG1 localizes in early endosomes (early endosome antigen 1 (EEA1)+ vesicles), phagosomes, and lysosomes, forming oligomeric pre-pore intermediates that create pores upon acidification. Exactly how this pore complex forms and functions remain unknown [94, 96].

MPEG1 is constitutively expressed by innate immune cells, primarily phagocytes, but can be induced in many other cell types. Known triggers of expression are pro-inflammatory mediators such as type 1 and type 2 interferons, LPS, TNF- α , and bacterial infections [93, 96, 97]. In cells stimulated with IFN- γ and LPS after pathogen engulfment, MPEG1 is monoubiquitinated. Subsequently, EEA1+/MPEG1+ vesicles fuse with LAMP+ vesicles (late phagosomes, lysosomes, and phagolysosomes), and MPEG1 is found to colocalize with phagosomes that engulf microbes [94, 98, 99].

1.3.2.1 MPEG1 – Antimicrobial Activity

Several studies using the (MPEG1(tm1Pod)) mouse mutant show that MPEG1 is bactericidal for Gram-positive, Gram-negative, and acid-fast bacteria [93, 96]. MPEG1-deficient macrophages and neutrophils from the (MPEG1(tm1Pod)) mutant cannot kill several different mycobacteria, including Mtb [96]. Additionally, multiple MPEG1-deficient cell lines succumb to bacterial infection [96]. MPEG1 forms large pores in the bacterial cell wall, including in *Mycobacterium smegmatis* but may not directly kill the bacteria [96]. Most likely, once MPEG1 forms pores in the bacterial cell wall, other bactericidal effectors have increased access to the cytoplasmic membrane and cytoplasm. This includes reactive oxygen species (ROS), nitric oxygen species (NOS), proteases, peptides, and the acidic environment of the phagolysosome [96, 100]. ROS and NOS can enhance MPEG1 bactericidal activity but have little effect without this pore-forming complex [96].

Bacteria have a mechanism to block MPEG1 activity. When host cells detect bacteria or pathogen-associated molecular patterns (PAMPs), the cullin-RING E3 ubiquitin ligase (CRL) complex monoubiquitylates MPEG1. This post-translational modification facilitates MPEG1 transfer to punctuate bodies (microtubule-associated protein 1A/1B-light chain 3 (LC3)+, a protein found on the membrane of phagosomes and autosomes), allowing the protein to target bacteria efficiently. Some bacteria use cell cycle inhibiting factor (CIF) to prevent CRL ubiquitination of MPEG1; however, CIFs have not been identified in mycobacteria [98, 101-104].

1.3.2.2 MPEG1 - Antigen Presentation Involvement

Some studies show that MPEG1 is essential for antigen presentation rather than killing bacteria. MPEG1 bactericidal activity was demonstrated using the (MPEG1(tm1Pod)) mutant

mouse [96, 98, 100, 105]. More recently, the mutant mouse strain (MPEG1(tm1.1Pib)) was created and is not abnormally susceptible to bacterial and viral infections and can kill bacteria *in vitro*. In macrophages and cross-presenting DCs, MPEG1 colocalizes with phagocytosed *M. smegmatis*, but the bacterial survival is not altered between (MPEG1(tm1.1Pib)) and wild-type (WT) mouse cells [105]. During an Mtb infection, there is no difference in weight loss or bacterial burden between the (MPEG1(tm1.1Pib)) mutant and WT mice [105]. Instead of killing, cross-presentation is impaired in the (MPEG1(tm1.1Pib)) MPEG1 mutant cells. While cell-associated protein antigen presentation is not affected, the presentation of exogenous soluble protein is [105]. The mechanism behind why the mutants ((MPEG1(tm1Pod)) and (MPEG1(tm1.1Pib))) responded differently is unknown.

A subsequent study found that MPEG1 mediates endocytic escape in cross-presenting DCs. Endogenous peptide antigens are typically presented on major histocompatibility complex (MHC) class 1, and exogenous peptide antigens on MHC class 2 [106]. During cross-presentation, exogenous antigens are presented on MHC class 2 [106]. Cross-presentation relies on permeable endocytic vesicles from which exogenous peptides can escape. It was shown that MPEG1 is needed for this endocytic escape; presumably by forming pores through which antigens can enter the cytosol. Additionally, it was shown that despite the MPEG1-mediated escape, the membrane and degradation ability of the phagosome are maintained. However, no infections were performed in this study [107].

The exact role of MPEG1 still needs to be clarified. Whether involved in killing bacteria or cross-presentation, the protein is necessary for the immune response of phagocytic antigen-presenting cells. The function of MPEG1 in LCs has not been studied. Only in zebrafish has

MPEG1 been associated with LCs, for which MPEG1 is a marker of myeloid origin; zebrafish LCs seem to have a different ontogeny from mouse and human LCs [108-111].

1.3.3 IDO1

IDO1 and its protein indoleamine-2,3-dioxygenase 1 (IDO1) are the most well-studied of the three genes selected for study in this thesis project [112]. Additionally, IDO1 is an enzyme that has no evidence of direct effect, including in immune responses or antimicrobial. Instead, the metabolites associated with the enzymatic activity of IDO1 play many roles, including in immune responses and pathogen killing [113-115].

IDO1 is a heme enzyme that catalyzes the conversion of L-tryptophan to N-formylkynurenine in the first and rate-limiting step of tryptophan catabolism through the

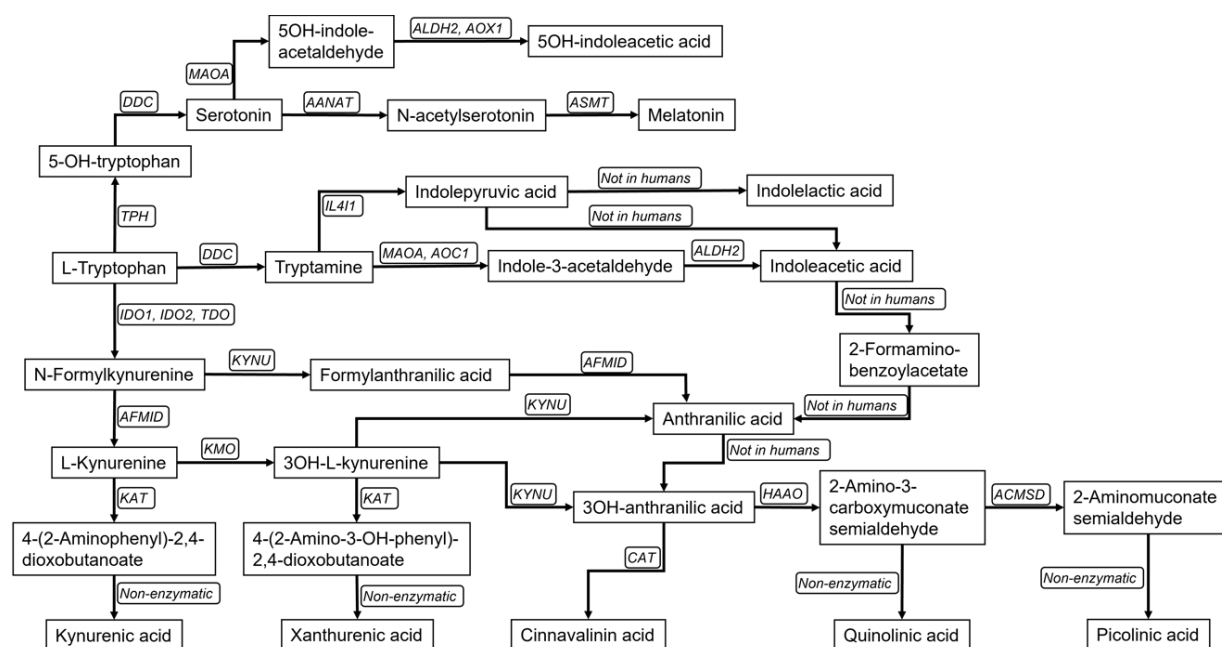


Figure 3: Tryptophan catabolism Pathways with Human Enzymes

Adapted from [110-112]. IDO1: indoleamine 2,3-dioxygenase; TDO: tryptophan 2,3-dioxygenase; AFMID: arylformamidase; KAT: kynurenine aminotransferase; KMO: kynurenine 3-monooxygenase; KYN: kynureninase; CAT: catalase; HAAO: 3-hydroxyanthranilate 3,4-dioxygenase; ACMSD: aminocarboxymuconate semialdehyde decarboxylase; TPH: tryptophan hydroxylase; DDC: aromatic-L-amino-acid decarboxylase; AANAT: aralkylamine N-acetyltransferase; MAOA: monoamine oxidase; ALDH2: aldehyde dehydrogenase (NAD⁺); AOX1: aldehyde oxidase; ASMT: acetylserotonin O-methyltransferase; AOC1: diamine oxidase; IL4I1: L-amino-acid oxidase

kynurenine pathway (Figure 3) [112, 116]. IDO1 can be induced in all cells. Only antigen-presenting cells have a constant IDO1 expression that stimulants can regulate [116, 117]. This same reaction is also catalyzed by IDO2 and tryptophan-2,3-dioxygenase (TDO). IDO2 has a very similar amino acid sequence to IDO1 but is primarily expressed in the liver. The activity of IDO2 is negligible *in vitro*, and IDO2 knockouts do not result in the same reduction of kynurenine as with an IDO1 knockout. The physiological role of IDO2 is not well understood [116, 118-120]. TDO activity is mainly found in the liver where it degrades dietary tryptophan. Circulating kynurenine seems to be produced by IDO1, with TDO-produced kynurenine being further degraded in the liver [116, 121, 122]. Only IDO1, referred to as IDO, will be discussed and assessed for this study.

The redox of the heme (ferrous) cofactor of IDO controls its enzymatic activity. The heme autoxidizes to the inactive ferric state during turnover. The supply of heme and reducing agents can control IDO activity. For example, polysulfides bind to inactive ferric IDO, reducing it to the ferrous oxygen-binding state [123]. After oxygen binds to the reduced heme, active IDO forms, and L-tryptophan binds to the catalytic pocket, where its indole ring is oxygenated to form N-formylkynurenine [123].

IFN- γ is the most potent inducer of IDO expression, but other stimulants such as type 1 interferons, TNF- α , and IL-10 also induce IDO expression in various cell types. PAMPS can also induce IDO expression, and cytotoxic T lymphocyte-associated protein 4 (CTLA4) on Tregs can stimulate IDO expression in DCs via co-stimulatory molecules CD80 and CD86 [124]. In summary, various conditions and stimuli control the expression of IDO, though IFN- γ remains the predominant stimulus [112, 116].

Tryptophan metabolism, including the kynurenine pathway and IDO activity, seems to be associated with nutritional immunity. Nutritional immunity is mainly related to trace minerals and iron, but similar patterns are described for tryptophan metabolism [125-127]. Nutritional immunity involves sequestering and depriving pathogens of nutrients (e.g., tryptophan), which can directly kill pathogens but can also weaken them, making them more susceptible to direct killing mechanisms [125-127]. Nutritional immunity also modulates the immune response through nutrient changes (e.g., a decrease in tryptophan and an increase in tryptophan catabolites) [116, 128-130]. While the direct treatment with some tryptophan metabolites (L-kynurenine and picolinic acid) has been bactericidal, how these metabolites kill the pathogens (directly or indirectly) is not known [131, 132]. In general, the bactericidal or immunoregulatory activity associated with tryptophan metabolism and IDO is due to modulation from changes in metabolites and nutrients by the host cell.

1.3.3.1 IDO – Antimicrobial Activity

IDO activation results in a marked change in the abundance of tryptophan metabolites. Increasing IDO expression and activation results in more of the biological form of tryptophan (L-tryptophan) being catabolized through the kynurenine pathway; tryptophan levels decrease as tryptophan catabolites increase [116, 124]. IDO is associated with antimicrobial activity because, when stimulated, the enzyme can deplete the cellular environment of tryptophan (an essential amino acid). As seen in a study with *Toxoplasma gondii*, IFN- γ stimulation depletes the lungs of tryptophan, and there is a corresponding increase in *IDO1* mRNA expression and kynurenine levels [133]. Tryptophan depletion kills tryptophan auxotrophic pathogens (Figure 4) [132-137]. Additionally, the unavailability of tryptophan restricts catabolism through other pathways, such as the serotonin pathway. There is also an antimicrobial effect for pathogens that rely on these

metabolites. For example, IDO strongly inhibits human parainfluenza virus type 3 (PIV3), which was found to need 5-hydroxytryptophan for replication [134, 138].

1.3.3.2 IDO – Immunoregulatory Involvement

For models not involving tryptophan auxotrophs, IDO expression and activation are associated with developing a tolerogenic immune response (Figure 4). In immune cells,

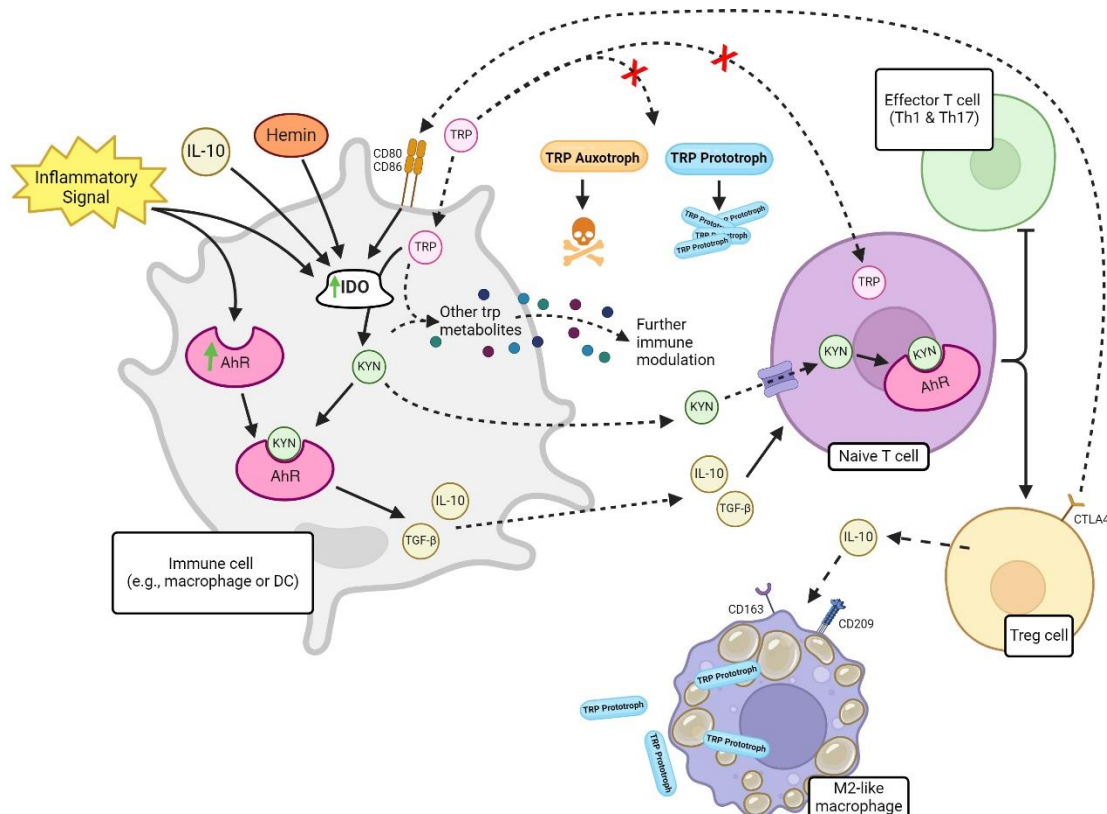


Figure 4: Antimicrobial and Immune Regulatory Involvement of IDO

adapted from [123], created with BioRender.com. In immune cells, IDO expression and enzymatic activity increase in response to various stimuli, depleting the intracellular and microenvironment tryptophan (TRP) by catabolism through the kynurenine pathway, in which tryptophan catabolites such as kynurenine (KYN) are produced. The unavailability of host tryptophan can kill tryptophan auxotrophic pathogens, while tryptophan prototrophs will survive. The kynurenine can be secreted or bind to the aryl hydrocarbon receptors (AhR), which results in TGF- β and IL-10 production and secretion. The secreted TGF- β , IL-10, and kynurenine and the depletion of tryptophan act on naive T cells, promoting differentiation to Tregs rather than effector T cells. Tregs can further modulate the microenvironment to facilitate an anti-inflammatory response. CTLA4 and CD80/CD86 interactions can increase IDO expression in immune cells. Secreted IL-10 promotes the M2-like anti-inflammatory foamy macrophage phenotype with CD163 expression. In this tolerogenic environment, tryptophan prototrophic pathogens evade immune killing mechanisms and survive.

inflammatory signals (e.g., IFN- γ) activate IDO, resulting in tryptophan depletion and kynurenine production. Additionally, inflammation stimulates the expression of the aryl hydrocarbon receptor (AhR). Kynurenine activates AhR, which results in cytokines such as IL-10 and TGF- β being secreted by the cell. These cytokines cause naive T cells to import kynurenine. The AhR activation by the kynurenine and the tryptophan depletion induces the naive T cell to differentiate into a Treg [139]. Also, the differentiation into Th1 and Th17 cells is blocked [116]. Metabolites downstream of kynurenine, including kynureninic acid and xanthurenic acid, also activate AhR and could contribute to the immune response regulation [124]. In LCs, IDO plays an anti-inflammatory role as a mediator for the LC tolerogenic response and has been used as a marker for tolerogenic LCs that induce Tregs. No studies have assessed an antimicrobial role for IDO in LCs [140-142].

The activation of IDO creates an anti-inflammatory environment that favors pathogen survival. Various pathogens, including uropathogenic *E. coli* and human immunodeficiency virus (HIV-1), activate IDO to induce an immune response permissive to survival [124, 143-145].

1.3.3.3 IDO and *M. tuberculosis*

In Mtb infections, CD4 cells and cytokines (IFN- γ and TNF- α) stimulate IDO activity in macrophages to deplete tryptophan. Mtb is a tryptophan prototroph, and the amino acid scarcity does not inhibit its growth. Rather, the immunotolerogenic environment that is created supports Mtb survival [146-148]. However, several studies have shown that tryptophan depletion inhibits the growth of tryptophan auxotrophic Mtb strains, even causing the mutant strains to become avirulent in an immunocompromised host (SCID mice). These results suggest that the tryptophan biosynthesis pathway in Mtb is essential for intracellular bacterial survival and growth [146, 149]. It is not entirely clear, however, whether WT Mtb actively induces CD4+ cells to activate

IDO in macrophages, which results in an anti-inflammatory response favoring Mtb survival, or if this is an active attempt by the host to starve the pathogen of tryptophan that fails.

1.3.3.4 IDO and *M. leprae*

ML also induces IDO expression and activity. In skin biopsies, LL lesions have the greatest amount of IDO protein, compared to BT and RR lesions, with most of the IDO being found in resident macrophages. Additionally, in LL patients, there is more IDO activity as measured by a greater kynurenine/tryptophan ratio in the sera. In PBMCs *in vitro*, ML also induces IDO expression and activity, a measurement of the tryptophan/kynurenine ratio in the supernatant. The percentage of IDO+ cells increases with IFN- γ stimulation but decreases with 1-methyltryptophan (1-MT) treatment, a known IDO inhibitor [150].

A subsequent study followed up on reports of interactions between IDO and CD163, a marker found on LL M2-like macrophages [37, 151]. Compared to BT lesions, LL lesions have more IDO+/CD163+ cells of the foamy macrophage phenotype, and there is more soluble CD163 in the sera of LL patients. *In vitro*, ML induces CD163+/IDO+/CD209+ macrophages, which also have increased TGF- β and IL-10 production. Blocking IL-10 reduces CD163 expression and IDO activity, while recombinant IL-10 increases IDO activity with and without ML. These data suggest that IDO is involved in the anti-inflammatory response of LL leprosy and that IDO could be induced by an IL-10 rather than an IFN- γ pathway in this disease state [151]. Further investigation found that a 1-MT pre-stimulation decreases ML survival in macrophages, suggesting that IDO activity is essential for ML survival. Also, IDO activity decreases in patients after treatment. This result could suggest that when ML is cleared, either the bacteria is no longer present to stimulate IDO expression and activity or the immune response is returning to normal [152].

The IDO expression response in DCs, however, is different than in macrophages. ML increases IDO expression and activity in human monocyte-derived DCs (mDCs). However, IDO activity is inhibited when TNF is neutralized, not when IL-10 is inhibited as previously seen in macrophages. In mDC-T cell cocultures, ML stimulation results in Treg differentiation, which depends on IDO activity. It should be noted that the ML used in this study was killed by γ -irradiation [153].

In LL macrophages, IDO is regulated by IL-10, likely forming a positive feedback loop, sustaining a tolerogenic environment upon which ML depends to survive [150-152]. In contrast, the TT leprosy cytokine TNF regulates IDO in DCs [153]. It could be that IDO is regulated differently depending on the cell type or that DCs are more responsive to pro-inflammatory signals. The induction of IDO by TNF could be associated with an antimicrobial response to clear ML, as seen in TT leprosy, or IDO is induced to control the pro-inflammatory response by inducing Tregs. IDO expression and activity are vital during ML infections, but the role IDO plays may vary depending on the model being studied.

1.4 *M. leprae*

The antimicrobial activity of the immune response, especially by effectors such as IDO, is highly dependent on the pathogen being assessed. ML is a slow-growing (12-14 day doubling time), Gram-variable, acid-fast bacillus that prefers cooler temperatures (27-33 °C) and is an obligate intracellular bacterium that only grows *in vivo*. Currently, ML is primarily cultured in armadillos and mice. Nine-banded armadillos have a lower core body temperature (32-35 °C) and a fully disseminated infection, resulting in large quantities of ML. In immunodeficient mouse strains, such as thymectomized and irradiated mice, congenitally athymic nude mice, and SCID mice, ML grows in the footpads where the temperature is cooler. Although ML can grow

in immunocompetent mice, fewer bacilli are collected from each footpad than from immunodeficient mouse strains when the growth plateaus at five-to-six months and the bacteria are harvested [11, 33].

Many researchers have attempted to culture ML either in axenic medium or cell cultures (cell lines and primary cells). Despite results showing metabolic activity, multiplication of the bacterium has not been achieved [11, 154]. In cell culture, the survival of ML can be extended, especially in macrophages treated with IL-10 [11]. Continued efforts are being made to culture ML *in vitro*, including using genomic analysis to help determine the potential composition of the medium, as *in vitro* cultivation would greatly aid ML research efforts [155, 156].

1.4.1 *M. leprae* Genome

The reason why ML cannot grow *in vitro* is still not wholly known, but it is assumed that ML requires host products (e.g., nutrients such as lipids). ML was discovered in 1873 as the first human pathogenic bacterium, with leprosy disease descriptions written in 600 BCE. It is an ancient pathogen with an extensive evolutionary history [157, 158]. Genome sequencing has shown that over this time, ML experienced an extensive reductive genome evolution, but it is very stable across strains. Compared to its well-studied relative Mtb, the ML genome is ~1.1 Mb smaller than the Mtb genome. Only 49.5% of the much smaller ML genome is protein-coding compared to 90.8% for Mtb, with 1,293 coding-sequence pseudogenes (12 for Mtb) [159, 160]. The role of pseudogenes and noncoding regions is still unknown but of interest, since ML kept these sequences and has a much higher proportion of pseudogenes than other bacteria and archaea. Additionally, there is some recent evidence that 43% of ML pseudogenes are transcriptionally active, some highly expressed, with expression levels changing upon macrophage infection. These data could indicate that these ML pseudogenes have regulatory

functions similar to eukaryotic noncoding RNAs, but further study is needed to elucidate their role [157].

1.4.1.1 *M. leprae* Metabolism Genes

Of the genes that are expected to produce functional products (non-pseudogenes), *Mtb* codes many redundant products. In comparison, in the smaller ML genome, proteins (and their genes) are often completely eliminated, a single protein is kept to perform a function, or a different protein is used for multiple purposes [159]. When comparing genes associated with carbon metabolism, this pattern becomes clear. Lipid catabolism is the primary source of energy for intracellular mycobacteria. *Mtb* has several fatty acid degradation pathways, while ML only has about one-third as many enzymes, such as the 22 lipase genes in *Mtb*, compared to the two in ML. However, ML has three times more FadD acyl-CoA synthases, which are involved in β -oxidation and anabolism, than FadE acyl-CoA dehydrogenases, only involved in beta-oxidation. In contrast, *Mtb* has equal proportions, highlighting the conservation of multi-functional enzymes in ML [159].

Despite the more limited number of enzymes encoded in ML, at least one enzyme for each step is present for most major carbon metabolism pathways, including glycolysis/gluconeogenesis, β -oxidation, the pentose phosphate pathway (PPP), and the tricarboxylic acid (TCA) cycle. All the necessary genes of these pathways being intact suggests that the bacterium can metabolize sugars such as glucose and lipids such as palmitate; acetate and galactose cannot be used as carbon sources [154, 155, 159]. Additionally, many anabolic processes remain intact in ML. For example, ML biosynthesis of all the amino acids is possible, including the essential amino acids, but with the exceptions of methionine and lysine. The

environment in which ML survives (i.e., the phagosome) may be depleted of various products, resulting in biosynthetic pathways remaining functional [154, 155, 159].

1.4.2 *M. leprae* Carbon Catabolism Studies

In addition to genetic analysis, various studies have shown that glycolysis, β -oxidation, the PPP, and the TCA cycle carbon metabolic pathways are functional in ML. Before the ML genome was first sequenced in 2001 [159], Saroj R. Khanolkar published some of the first results of ML carbon metabolism in 1982, showing that the bacteria takes up exogenous D-[U- ^{14}C]glucose, with uptake peaking at six days. Additionally, it was shown that ML also incorporates exogenous amino acids from [U- ^{14}C]protein hydrolysate [161].

Soon after, in 1983, Paul R. Wheeler published results of radiolabeled carbohydrate catabolism to CO_2 by ML. He found that glucose in an axenic medium is taken up and catabolized to CO_2 , with results suggesting that both glycolysis and the hexose monophosphate shunt (i.e., pentose phosphate pathway) are used [161, 162]. More recently, it was shown that ML only uses intracellular glucose as a carbon source, synthesizing amino acids from the [U- $^{13}\text{C}_6$] glucose only when inside Schwann cells [163]. CO_2 was not measured in this study. It is possible, though, that in this study, ML used exogenous glucose but not to make amino acids. All the cultures, including ML in medium without Schwann cells, were performed using RPMI 1640 medium with 100% [U- $^{13}\text{C}_6$] glucose added, which contains 20 amino acids [164], and ML can import exogenous amino acids [161]. Altogether, these studies demonstrate that ML takes up and catabolizes glucose as a carbon source.

Lipids are another important carbon source, especially for mycobacteria [159]. In 1988, ML was first shown to produce $^{14}\text{CO}_2$ from [U- ^{14}C] palmitic acid (a fatty acid), indicating that β -oxidation is functional in ML [165]. Since then, palmitic acid has frequently been used in ML

studies, corroborating the uptake and catabolism of this fatty acid by ML [166-169]. Measuring the rate of [^{14}C] palmitic acid oxidation to $^{14}\text{CO}_2$ by ML when this lipid is the sole carbon source is known as the radiorespirometry (RR) method. It is considered the most reliable biochemical method of assessing ML viability *in vitro* [11, 170].

Compared to ML, Mtb carbon use is much better characterized, with the discussed metabolic pathways functional *in vivo* and *in vitro*. Mtb can use palmitic acid and other lipids such as cholesterol as carbon and energy sources, as well as sugars such as glucose [163, 171-178]. *In vitro* cultivation of Mtb aids in the study of its metabolism, allowing for more bacteria for experiments, growth assessments under various conditions without host cell interference, and the creation of knockouts to assess the function of specific genes. As a well-studied ML relative, Mtb can be used as a control to design and optimize experiments before using ML [163].

1.4.3 *M. leprae* Amino Acid Biosynthesis Study

While ML catabolic pathways appear more disrupted, evaluating anabolic pathways, such as amino acid biosynthesis, is also essential to determine if the predicted enzymes are functional. In Mtb, biosynthesis of all the amino acids has been shown to occur, primarily through ^{13}C -labeling or knockout experiments [163, 179-183]. In comparison, little is known about the functionality of amino acid biosynthetic pathways in ML.

The one study showing *de novo* amino acid biosynthesis by ML used ^{13}C -glucose tracing to measure the ^{13}C -labeled amino acids produced by ML. In that study, only ML in infected Schwann cells used the ^{13}C -glucose to synthesize amino acids. The host cell material was separated from the intracellular ML by differential centrifugation before the amino acid extraction. Complete separation of the host and bacterial amino acid pools was confirmed since no ^{13}C -labeled essential amino acids (phenylalanine, threonine, valine, and isoleucine) were

measured in the host cell fractions. In addition to these four essential amino acids, serine, aspartate/asparagine, and glutamate/glutamine were determined to be synthesized by ML [163]. Not all the amino acids were evaluated in this study, and the functionality of the biosynthetic pathways of the remaining amino acids in ML still needs to be assessed. Tryptophan is particularly important, as IDO is primarily antimicrobial for tryptophan auxotrophs.

1.5 Research Aims

In summary, LCs are crucial in the immune response in the skin. These cells have innate antimicrobial mechanisms that can be activated by the local environment, such as IFN- γ , potentially during an ML infection. Other possible antimicrobial LC defenses remain to be elucidated, but CCL22, MPEG1, and IDO may be involved during an ML infection. The antimicrobial activity of IDO specifically depends on the pathogen's ability to biosynthesize tryptophan. Whether the tryptophan biosynthetic pathway is functional in ML is unknown. We hypothesize that altered metabolic processes in LCDCs impact antimicrobial effects on ML. The following aims were developed to address this hypothesis:

1. Establish an *in vitro* system that induces CCL22, MPEG1, and IDO in LCDCs.
2. Evaluate ML viability in LCDCs when IDO is induced or inhibited.
3. Measure LCDC intracellular tryptophan catabolite changes from a ML infection.
4. Assess whether ML synthesizes tryptophan.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials and Reagents

ML Thai-53 was purified from athymic *nu/nu* mouse footpads and donated by the National Hansen's Disease Program, Laboratory Research Branch, Louisiana State University, Baton Rouge, LA, USA. Mtb mc²6230 was gifted by William R. Jacobs, Albert Einstein College of Medicine, Bronx, NY [184].

The culture medium for all eukaryotic cell experiments was RPMI 1640 with L-glutamine + 10% heat-inactivated fetal bovine serum (HI-FBS) (Heat-inactivated for 30 minutes at 50 °C). Human Cord Blood CD34⁺ Stem/Progenitor Cells and THP-1 monocytes were used.

Table 1 lists all other materials and reagents used in the experimentation presented.

Table 1: Reagents and Materials

Item Name	Catalog Number	Vendor	Location
0.1 mm diameter zirconia/silica beads	11079101z	BioSpec Products	Bartlesville, Oklahoma
2-picolinic acid	P42800	Sigma-Aldrich	St. Louis, Missouri
2-picolinic acid-d4	D-5289	CDN Isotopes	Pointe-Claire, Quebec
3-hydroxy anthranilic acid	20512	Caymen Chemical	Ann Arbor, Michigan
3-hydroxy-DL-Kynurenine	27778	Caymen Chemical	Ann Arbor, Michigan
3-hydroxykynurenine (1,2,3-13C3; alpha-amino-15N)	CNLM-10399-PK	Cambridge Isotope Laboratories	Tewksbury, Massachusetts
3-indoleacetic acid	I3750	Sigma-Aldrich	St. Louis, Missouri
3-indoleacrylic acid	I2273	Sigma-Aldrich	St. Louis, Missouri
5-hydroxy indole-3-acetic acid	22889	Caymen Chemical	Ann Arbor, Michigan
5-hydroxy indole-3-acetic acid-d6	37199	Caymen Chemical	Ann Arbor, Michigan
5-hydroxy-tryptophan	sc-278210	Santa Cruz Biotechnology	Dallas, Texas
5-hydroxy-tryptophan-d3	H975702	Toronto Research Chemicals	North York, Ontario
7H9	271310	BD Bioscience	Franklin Lakes, New Jersey
95% pure 1-methyl-L-tryptophan	447439	Sigma-Aldrich	St. Louis, Missouri
98% pure 1-methyl-L-tryptophan	33313	Caymen Chemical	Ann Arbor, Michigan
Albumin Cohn fraction V fatty acid free	J64682	Alfa Aesar	Haverhill, Massachusetts
Albumin fraction V	A2134	Biomatix	Kitchener, Ontario
Alexa Fluor 647 Conjugation Kit (Fast) - Lightning-Link	ab269823	Abcam	Trumpington, Cambridge

Ampicillin	A9518	Sigma-Aldrich	St. Louis, Missouri
Anthranilic acid	A89855	Sigma-Aldrich	St. Louis, Missouri
Anthranilic acid (ring-13C6)	CLM-701-PK	Cambridge Isotope Laboratories	Tewksbury, Massachusetts
Atlantis T3 Column	186003719	Waters	Milford, Massachusetts
Autosampler vials and caps	186000307C	Waters	Milford, Massachusetts
Casamino acids	2240	EMD Millipore	Burlington, New Jersey
Casein hydrolysate	ic10129001	VWR	Radnor, Pennsylvania
Catalase	C1345	Sigma-Aldrich	St. Louis, Missouri
CCL22_PE antibody	565950	BD Bioscience	Franklin Lakes, New Jersey
D-Erythrose (U- ¹³ C ₄ , 98%) 1.2% in water	CLM-7863	Cambridge Isotope Laboratories	Tewksbury, Massachusetts
Dextrose (i.e., glucose)	D9434	Sigma-Aldrich	St. Louis, Missouri
D-Glucose (U-13C6)	CLM-1396-1	Cambridge Isotope Laboratories	Tewksbury, Massachusetts
DL-indole-3-lactic acid	I5508	Sigma-Aldrich	St. Louis, Missouri
Ethylenediaminetetraacetic acid (EDTA)	15575-038	Life Technologies Corporation	Carlsbad, California
Extraction tubes - 2 mL low-retention microcentrifuge tubes	02-681-321	Fisher Scientific	Waltham, Massachusetts
Fc Receptor Blocker	NB335	Innovexbio	Richmond, California
Fetal Bovine Serum	F-0500-D	Atlas Biologicals	Fort Collins, Colorado
Formic acid	533002	Sigma-Aldrich	St. Louis, Missouri
GM-CSF	572904	BioLegend	San Diego, California
GoScript Reverse Transcriptase - Random primers	A2801	Promega	Madison, Wisconsin
Hemin	H9030	Sigma-Aldrich	St. Louis, Missouri
Human Cord Blood CD34+ Stem/Progenitor Cells	2C-101 and 2C-101A	Lonza Bioscience	Morrisville, North Carolina
IDO1_BV421 antibody	567863	BD Bioscience	Franklin Lakes, New Jersey
IFN- γ	570204	BioLegend	San Diego, California
IL-10	571004	BioLegend	San Diego, California
IL-1 β	579404	BioLegend	San Diego, California
IL-2	589104	BioLegend	San Diego, California
InfinityLab Poroshell 120 EC-C8 column	693775-906T	Agilent Technologies	Santa Clara, California
Kynurenic acid (carbocyclic-13C6)	CLM-11139-PK	Cambridge Isotope Laboratories	Tewksbury, Massachusetts
Kynurenic acid (hydrate)	16792	Caymen Chemical	Ann Arbor, Michigan
LC-MS grade acetonitrile	LC0154	Honeywell Research Chemicals	Morris Plains, New Jersey
LC-MS grade methanol	LC2302.5	Honeywell Research Chemicals	Morris Plains, New Jersey
LC-MS grade water	LC3652.5	Honeywell Research Chemicals	Morris Plains, New Jersey
L-kynurenine	11305	Caymen Chemical	Ann Arbor, Michigan

L-Kynurenine sulfate:1/2H ₂ O (13C10, 99%)	CLM-9884-PK	Cambridge Isotope Laboratories	Tewksbury, Massachusetts
LPS	L4391	Sigma-Aldrich	St. Louis, Missouri
L-tryptophan	T0254	Sigma-Aldrich	St. Louis, Missouri
L-tryptophan-d5	34829	Caymen Chemical	Ann Arbor, Michigan
Melatonin	14427	Caymen Chemical	Ann Arbor, Michigan
MPEG1 antibody	LS-C680878	Lifespan Bioscience	Lynnwood, Washington
N-acetylserotonin (i.e., N-Acetyl-5-hydroxytryptamine)	sc-202716	Santa Cruz Biotechnology	Dallas, Texas
N-formylkynurenine	sc-490237	Santa Cruz Biotechnology	Dallas, Texas
Palmitic acid (U-13C16)	CLM-409-0.1	Cambridge Isotope Laboratories	Tewksbury, Massachusetts
Palmitic acid, [1-14C]-, 50 µCi	NEC075H250UC	Perkin Elmer	Waltham, Massachusetts
PE isotype antibody		BD Bioscience	Franklin Lakes, New Jersey
Phosphate-buffered saline (PBS)	21-040-CV	Corning Life Sciences	Tewksbury, Massachusetts
PrimeTime gene expression master mix with low reference dye added	155772	Integrated DNA Technologies (IDT)	Coralville, Iowa
Protein transport inhibitor cocktail	00-4980-93	Invitrogen	Waltham, Massachusetts
qPCR Probes	See in methods	Integrated DNA Technologies (IDT)	Coralville, Iowa
Quick-DNA/RNA Microprep Plus Kit	D7005	Zymo Research	Irvine, California
Quinolinic acid	14941	Caymen Chemical	Ann Arbor, Michigan
Quinolinic acid-4,5,6-d3	D-8099	CDN Isotopes	Pointe-Claire, Quebec
RPMI 1640 with L-glutamine	10-040-CV	Corning Life Sciences	Tewksbury, Massachusetts
SCF	572904	BioLegend	San Diego, California
Serotonin (hydrochloride)	14332	Caymen Chemical	Ann Arbor, Michigan
Serotonin-d4 (hydrochloride)	29415	Caymen Chemical	Ann Arbor, Michigan
Sodium azide	S2002	Sigma-Aldrich	St. Louis, Missouri
Sodium chloride	3624-05	JTBaker	Phillipsburg, New Jersey
TGF-β1	781804	BioLegend	San Diego, California
THP-1 cells	TIB-202	ATCC	Manassas, Virginia
TNF-α	570104	BioLegend	San Diego, California
TRIzol reagent	15596-018	Invitrogen	Waltham, Massachusetts
Tryptamine	20995	Caymen Chemical	Ann Arbor, Michigan
Tryptamine:HCl (A,A,B,B-D4)	DLM-6989-PK	Cambridge Isotope Laboratories	Tewksbury, Massachusetts
TURBO DNA-free kit	AM1907	Invitrogen	Waltham, Massachusetts
Tyloxapol	T8761	Sigma-Aldrich	St. Louis, Missouri
Vial inserts	WAT094171DV	Waters	Milford, Massachusetts
Xanthurenic acid	19191	Caymen Chemical	Ann Arbor, Michigan
Zombie NIR viability dye	423105	BioLegend	San Diego, California

2.2 Methods

2.2.1 Generation of LCDCs

CD34⁺ cells were differentiated as previously described [50]. The cells were cultured with 2.5 ng/mL TNF- α , 25 ng/mL SCF, and 100 ng/mL GM-CSF for the first five days and then with 1 ng/mL TGF- β and 100 ng/mL GM-CSF for seven days at 37 °C 5% CO₂ with humidity.

2.2.2 LCDC Culture to Induce Gene Expression

Following differentiation, LCDCs were incubated at 37 °C 5% CO₂ with humidity with 10 ng/mL LPS, 20 ng/mL TNF- α , 20 ng/mL IFN- γ , 10 ng/mL IL-1 β , 10 ng/mL IL-10, 2 ng/mL IL-2, 10 μ M hemin in 0.325 % DMSO, or 0.325 % DMSO for 24 hours. LCDCs were also infected at 10:1 MOI with live or heat-killed (HK) ML for 24 hours. The Welch's t-test was used for statistical analysis.

2.2.3 Quantitative PCR (qPCR)

To assess the induction of gene expression, RNA was isolated from cells using TRIzol (Invitrogen), treated with DNase (TURBO DNA-free kit, Invitrogen), and used to synthesize cDNA (GoScript Reverse Transcriptase - Random primers, Promega) according to the manufacturers' instructions. To measure ML viability in the IDO1 inhibition assay, RNA and DNA were isolated using the Quick-DNA/RNA Microprep Plus Kit (Zymo Research), and cDNA (GoScript Reverse Transcriptase - Random primers, Promega) was synthesized, all according to the manufacturers' instructions. qPCR was performed using an Applied Biosystems QuantStudio 3 (Life Technologies Corporation, Carlsbad, California) following the PrimeTime gene expression master mix instructions (IDT) with fast cycling using the primers and probes listed in Table 2. LCDC gene expression levels were calculated by the $2^{-(\Delta\Delta C_t)}$ method, and ML

viability was calculated by comparing the 16S RNA to the 16S DNA expression as previously described [152, 185, 186].

Table 2: IDT Probe Sequences

Target (IDT Probe assay name)	Probe (5'-3')	Primer 1 (5'-3')	Primer 2 (5'-3')
IDO1 (Hs.PT.58.92 4731)	/56- FAM/CCAGTTTGC/ZEN/CAAGACACAGT CTGC/3IABkFQ/	CCTTACTGCCAAC TCTCAA	ACGTCCATGTTCTC ATAAGTCAG
CCL22 (Hs.PT.58.39 751181.g)	/56- FAM/CCCATTCCTCA/ZEN/CTTTCCCTGCCT CC/3IABkFQ/	CTCCAACCTCCA ATACCCA	CATAGCATGAATTT GATTGTCAGC
MPEG1 (Hs.PT.58.24 58425.g)	/56- FAM/CTTGCCTGA/ZEN/TTTAGCCCATG CTGC/3IABkFQ/	CTGTCTGAGCCAT GAACAAC	GAAGTCCAACTTCG TCCATCT
HPRT1 (Hs.PT.58v.4 5621572)	/56- FAM/AGCCTAAGA/ZEN/TGAGAGTTCA AGTTGAGTTTGG/3IABkFQ/	TTGTTGTAGGATA TGCCCTTGA	GCGATGTCAATAGG ACTCCAG
RPLP0 (Hs.PT.39a.2 2214824)	/56- FAM/CCCTGTCTT/ZEN/CCCTGGGCATC AC/3IABkFQ/	TCGTCTTTAAACC CTGCGTG	TGTCTGCTCCACA ATGAAAC
16S	/56- FAM/AGTGGCGAA/ZEN/CGGGTGAGTA ACAC/3IABkFQ/	TGAAGTGCAGGG CAGATTAC	GTCGAACGGAAAG GTCTCTAAA

2.2.4 Flow Cytometry

LCDCs were stimulated with 20 ng/mL IFN- γ or 10 ng/mL IL-1 β and incubated at 37 °C 5% CO₂ with humidity. After 19 hours, 1X protein transport inhibitor was added for five hours (24 hours total incubation). Cells were washed with 1X PBS + 2 mM EDTA and incubated with Zombie NIR viability dye (1/5000; 100 μ L/test) for 30 minutes. Cells were washed with FACS buffer (1X PBS + 2 mM EDTA + 2 % HI-FBS + 0.0013% sodium azide) and incubated with Fc block (200 μ L/test) for one hour. After washing with FACS buffer, the cells were incubated for 45 minutes in fixation/permeabilization buffer (1:3 fixation/permeabilization concentrate in diluent; 500 μ L/test) and washed with permeabilization buffer (1X in DI water). Titrated antibodies (0.067 μ g/mL anti-CCL22 and PE isotype, 2.5 μ g/mL anti-MPEG1, 0.313 μ g/mL anti-IDO1) were incubated with the cells for 16 hours. The cells were washed with

permeabilization buffer and resuspended in FACS buffer. A 4 laser Cytex Aurora spectral cytometer (16V-14B-10YG-8R) was used to analyze the cells with 250,000 threshold, 45 forward scatter (FSC) gain, 61 side scatter (SSC) gain, and 0.61 FSC scaling factor. 50,000 live events were acquired. Events were gated using the fluorescence-minus-one and isotope controls in FlowJo_v10.9.0. Of the live cells (viability dye negative) and protein-positive cells, the corresponding fluorophore intensity of each replicate's first 5000 acquired events was exported to excel. Each replicate's minimum and maximum intensity was found and used to perform a linear transformation with a min-max normalization for each event [187]. The Mann-Whitney t-test was used for statistical analysis.

2.2.5 Radiorespirometry

The direct treatment of ML with 1-(L)-MT and radiorespirometry was done by Dr. Fabricio Da Mota Ramalho Costa. As previously described [170], 1×10^7 ML/replicate was incubated for 24 hours at 33°C in 100 µL of medium (7H9 + 1 µCi/mL of [1- 14 C] palmitic acid + 0.1% casein hydrolysate + 0.5% fatty acid free albumin + 48 U/mL catalase + 0.05% tyloxapol + 50 µg/mL ampicillin) with 20 µM or 200 µM 95% pure 1-L-MT. The captured $^{14}\text{CO}_2$ was measured by a scintillation counter (300 SL, Hidex Oy, Turku, Finland). The Welch's t-test was used for statistical analysis.

2.2.6 Effect of IDO1 Inhibition on *M. leprae* Viability in LCDCs

LCDCs were stimulated with 20 ng/mL IFN- γ for four hours. The cells were treated for 30 minutes with 98% pure 1-(L)-MT (200 µM final) solubilized in water and infected with live ML at a 10:1 MOI for two days at 33 °C 5% CO $_2$ with humidity. RNA and DNA were extracted, and IDO1 expression and ML viability were determined using qPCR [152, 185, 186]. Statistical

analysis was performed by Barbara Graham. IDO1 expression was analyzed with Dunnett's adjustment [188] and ML viability was analyzed with Tukey's method [189].

2.2.7 THP-1 and LCDC Metabolite Extraction

THP-1 cells were grown and maintained in RPMI + 10% HI-FBS. 2.5×10^6 THP-1 cells/sample were incubated with 20 ng/mL IFN- γ , and/or 200 μ M 98% pure 1-(L)-MT at 37 °C 5% CO₂ with humidity. After 24 hours, the cells were harvested. 2.5×10^6 LCDCs were incubated with a 10:1 MOI of live ML for 24 or 48 hours at 33 °C 5% CO₂ and harvested. The harvested THP-1 cells or LCDCs were washed twice with PBS in extraction tubes. To the cell pellets, 1 mL of liquid chromatography-mass spectrometry (LC-MS) grade 70% methanol in water with internal standards (ISTD) was added, and the samples were vortexed for five minutes. ISTD concentrations are listed in Table 3. The cells were lysed with 15 rounds of 30 seconds in a water bath sonicator (T1.9C, Health Sonics Corporation, Algonquin, Illinois) followed by 30 seconds in ice. The samples were rocked at 4 °C for one hour before being centrifuged at 16,100 xg, 6 °C for 20 minutes. An aliquot of supernatant (850 μ L) was transferred to new extraction tubes. To the pellet, 1 mL LC-MS grade 70 % methanol in water (without ISTD) was added. The samples were vortexed for five minutes, rocked one hour at 4 °C and centrifuged. 1 mL of supernatant was combined with the previous 850 μ L of supernatant in new extraction tubes and dried in a savant (SPD2010, Thermo Fischer, Waltham Massachusetts) at room temperature. For analysis, the samples were resuspended in 100 μ L LC-MS grade 25 % methanol in water, vortexed for five minutes, centrifuged, and 60 μ L transferred to vial inserts in autosampler vials for liquid chromatography–mass spectrometry multiple reaction monitoring.

Table 3: Internal Standard Concentrations

Compound	Internal Standard Concentration (ng/mL)
Quinolinic acid (4,5,6-d ₃)	4
Picolinic acid (d ₄)	1
3-hydroxykynurenine (1,2,3- ¹³ C ₃ ; alpha-amino- ¹⁵ N)	1
5-hydroxytryptophan (d ₃)	1
Serotonin (d ₄)	4
Kynurenine (¹³ C ₁₀)	4
Tryptophan (d ₅)	70
Kynurenic acid (carbocyclic- ¹³ C ₆)	1
Tryptamine (A,A,B,B-d ₄)	1
5-hydroxyindoleacetic acid (d ₆)	7
Anthranilic acid (ring- ¹³ C ₆)	1

2.2.8 Tryptophan Biosynthesis

Mtb mc²6230 was grown in 7H9 + 0.5% albumin fraction V + 0.2% dextrose + 0.0004 % catalase + 0.085% sodium chloride + 0.05% tyloxapol + 0.2% casamino acids + 24 µg/mL pantothenate at 37 °C [184]. The OD600 of the culture was measured, and the CFU/mL was calculated (“an OD600 measurement of 1 is equivalent to either 3.13×10^7 cfu/mL or 3.66 McFarland units.” [190]). ML was received in RPMI 1640 at 1×10^9 bacteria/mL. 2×10^8 bacteria per sample were washed with 7H9 three times, pelleting for six minutes at 16,100 xg. The bacteria were incubated in 2 mL of the media listed preparations in Table 4 in a 12 well plate with humidity at 37 °C for Mtb and 33 °C, 5% CO₂ for ML.

After incubation, the bacteria were transferred to microcentrifuge tubes and washed three times with LC-MS grade water. To extract the metabolites, the bacteria were lysed by bead beating, using 0.1 mm zirconia/silica beads, for 10 rounds of 45 seconds bead beating at 4,000 rpm followed by two minutes in ice. LC-MS grade 70% methanol in water (1 mL) was added to the lysate in each extraction tube, and the samples were vortexed for five minutes, rocked at 4 °C for one hour, and centrifuged at 16,100 xg, 6 °C for 20 minutes. An aliquot of supernatant (1 mL) was transferred to a new extraction tube. To the pellet, 1 mL of LC-MS grade 70% methanol in water was added. The samples were vortexed, rocked, and centrifuged as before. A 1 mL aliquot

of supernatant was combined with the previous 1 mL of supernatant aliquot in new extraction tubes and dried at room temperature in a savant (Thermo Scientific Savant SPD2010). To analyze, the samples were resuspended in 100 μ L LC-MS grade 25% methanol in water, vortexed for five minutes, centrifuged, and 60 μ L transferred to vial inserts in autosampler vials for untargeted liquid chromatography–mass spectrometry.

Table 4: Tryptophan Biosynthesis Conditions Tested

Condition	Bacteria used	Media composition	Heavy labeled starting material	Incubation time
Anthranilic acid	Mtb and ML	7H9 + 0.5 % albumin fraction V + 0.2 % dextrose + 0.0004 % catalase + 0.085 % sodium chloride	250 μ M (ring- $^{13}\text{C}_6$) anthranilic acid	24 hours
Glucose with fraction V albumin	Mtb and ML	7H9 + 0.5 % albumin fraction V + 0.0004 % catalase + 0.085 % sodium chloride	0.2 % (U- $^{13}\text{C}_6$) glucose or unlabeled glucose	Mtb (1-5 days); ML (5-13 days)
Glucose with fraction V albumin	3x the amount of ML (6.075×10^8 bacterium/replicate)	7H9 + 0.5 % albumin fraction V + 0.0004 % catalase + 0.085 % sodium chloride	0.2 % (U- $^{13}\text{C}_6$) glucose	5 days
Glucose with fatty acid free albumin	ML	7H9 + 0.5 % fatty acid free albumin + 0.0004 % catalase + 0.085 % sodium chloride	0.2 % (U- $^{13}\text{C}_6$) glucose	5 days
Erythrose with fatty acid free albumin	ML	7H9 + 0.5 % fatty acid free albumin + 0.0004 % catalase + 0.085 % sodium chloride	0.2 % (U- $^{13}\text{C}_4$) erythrose	5 days
Palmitic acid with fatty acid free albumin	ML	7H9 + 0.5 % fatty acid free albumin + 0.0004 % catalase + 0.085 % sodium chloride	4.9511 μ g/mL (U- $^{13}\text{C}_{16}$) palmitic acid (same molarity as 1 μ Ci of (1- ^{14}C) palmitic acid with 55 mCi/mmol activity) [170]	5 days

2.2.9 Untargeted Liquid Chromatography–Mass Spectrometry Analysis

The untargeted liquid chromatography–mass spectrometry (LC–MS) metabolite measurements, using a method created by Dr. Nurul Islam, were performed using a 6546A Quadrupole Time of Flight LC/MS system with a Dual Jet Stream ion source and a 1290 Infinity II LC (Agilent, Santa Clara, California). The reversed-phase (RP) chromatography was carried out with a Waters Atlantis T3 Column (C18, 100 $\text{\AA}$, 3 μ m, 2.1 mm X 150 mm) and a mobile

phase consisting of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) at a flow rate of 0.280 mL/min. The gradient program was as follows: 0-3 min, 0% B; 3-18 min, 0-50% B; 18-24 min, 50-97% B; 24-30 min, 97% B; 30-30.5 min 97%-0% B, and 6 min column conditioning at 0% B. LC eluent from 0-1.5 min was diverted to waste with the remainder introduced into the MS. The autosampler and column were kept at 4 °C and 35 °C respectively. Sample (5 µL) was injected into the LC. Positive electrospray ionization (ESI) mode was used for molecule detection. The acquisition mode was set to a 60-1500 *m/z* range with a 1.25 spectra/sec scan rate. The MS instrument parameters were: 320 °C gas temperature, 10 L/min gas flow, 38 psig nebulizer pressure, 315 °C sheath gas temperature, and 11 L/min sheath gas flow. The scan source parameters were: capillary voltage at 3500 V, nozzle voltage at 1000 V, fragmentor at 125 V, Skimmer1 at 65 V, and octupole radio-frequency (RF) peak at 750 V. Agilent Qualitative Analysis software 10.0 were used for data processing.

2.2.10 Targeted Liquid Chromatography–Mass Spectrometry Multiple Reaction Monitoring Analysis

The targeted liquid chromatography–mass spectrometry multiple reaction monitoring (LC-MS MRM) method was created by Dr. Paul Soma. The metabolite measurements were performed using an 6470B Triple Quadrupole LC/MS system with a Jet Stream ion source and a 1290 Infinity II LC (Agilent, Santa Clara, California). The RP chromatography was carried out with an Agilent InfinityLab Poroshell 120 EC-C8, 2.1 x 150 mm, 2.7 µm, narrow bore LC column and a mobile phase consisting of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) at a flow rate of 0.300 mL/min. The gradient program was as follows: 0-1 min, 1% B; 1-6 min, 1%-30% B; 6-8 min, 30%-60% B; 8-9.50 min, 60% B; 9.50-9.60 min, 60%-95% B; 9.60-11 min, 95% B; 11-11.10 min, 1% B; 11.10-14 min, 1% B. LC eluent from 0-1 min was

diverted to waste with the remainder being introduced into the MS. The autosampler and column were kept at 4 °C and 10 °C respectively. Sample (3 µL) was injected into the LC. Positive electrospray ionization (ESI) mode and dynamic multiple reaction monitoring (dMRM) scan type were used for molecule detection. The MS ion source parameters were: drying gas temperature 300 °C, drying gas flow 11 L/min, nebulizer pressure 55 psi, sheath gas temperature 400 °C, sheath gas flow 12 L/min, and capillary voltage 3200 V. MRM transitions, collision energies and fragmentation energies for all targeted compounds are presented in Supplementary Table 1 and were optimized using the Agilent MassHunter Optimizer. Agilent MassHunter Quantitative QQQ Analysis (Quant-My-Way) 10.1 and Qualitative Analysis software 10.0 were used for data processing.

A mix of all the unlabeled metabolites at 78.125 ng/mL was made in LC-MS grade 25% methanol in water. A 1:1 serial dilution was made from this highest calibrator concentration down to 0.1526 ng/mL. Internal standards were added to all the calibrators at the same concentration as in the samples (Table 3). The calibrators were injected five times, from lowest to highest concentration, whenever samples were analyzed.

The limit of detection (LOD) and limit of quantification (LOQ) was calculated for each metabolite. The LOD was set as the mean peak area of the blanks + 3X the standard deviation of the peak area of the blanks. The LOQ was set at the peak area of the lowest calibrator concentration that had a mean peak area $\geq$ the mean peak area of the blanks + 10X the standard deviation of the peak area of the blanks and where the final concentration calculated for the calibrator from its peak area was < 25% different than the expected concentration. The LOD and LOQ for each compound are presented in Supplementary Table 1.

To account for experiment-to-experiment differences with the LCDCs, the peak area and picomole amount of the samples were normalized per metabolite. For each experiment, the sum peak area between the non-stimulated and ML-stimulated samples for the time point was calculated. The peak area of each sample was then divided by the total peak area of the experiment. For metabolites over the LOQ, the metabolite concentration (ng/mL) was converted to picomoles using the 1 mL volume of ISTD added during the extraction and the monoisotopic mass (g/mol) listed in Supplementary Table 1. For the LCDC samples, the same normalization was done for the picomole amounts of the metabolites over the LOQ. A Welch's t-test analysis was only done on normalized peak area and picomole amounts for LCDCs. Tukey's test was used for comparing the metabolite peak area in the THP-1 cells.

All LC-MS MRM specifications are listed in Supplementary Table 1. These specifications include for each compound the: monoisotopic mass, precursor ion, product ions (quantifier and qualifier), collision energy, fragmentation energy, LC retention time, LOD and LOQ.

2.2.11 Statistical Analysis Summary

GraphPad Prism 9 and Excel were used for all graphing and statistical analyses. Welch's t-test, the Mann-Whitney t-test and Tukey's test for the THP-1 cell metabolite analysis were performed in GraphPad Prism. R 4.3.3 was used for Dunnett's test and Tukey's test for assessing the effect of IDO1 inhibition on ML viability in LCDCs.

CHAPTER 3: RESULTS

3.1 IL-1 β and IFN- γ Induce Antimicrobial Gene and Protein Expression in LCDCs

To test the antimicrobial activity of *CCL22*, *MPEG1*, and *IDO1* in LCDCs, a model that induces these genes had first to be developed. The expression of these genes by LCDCs was assessed after 24 hours of stimulation. Various stimulants identified from an ingenuity pathway analysis by Dr. Robert Modlin and colleagues and from literature as possible regulations were tested. *CCL22* expression was most greatly increased by IL-1 β but also by TNF- α (Figure 5A). *MPEG1* expression only increased in response to IL-1 β , but was decreased by LPS and hemin (Figure 5B). *IDO1* expression was increased by IFN- γ and decreased by IL-1 β , IL-10, and hemin (Figure 5C). Live and heat-killed ML only affected *CCL22* expression, increasing *CCL22* expression (Figure 5A). 10 ng/mL IL-1 β and 20 ng/mL IFN- γ , as the most

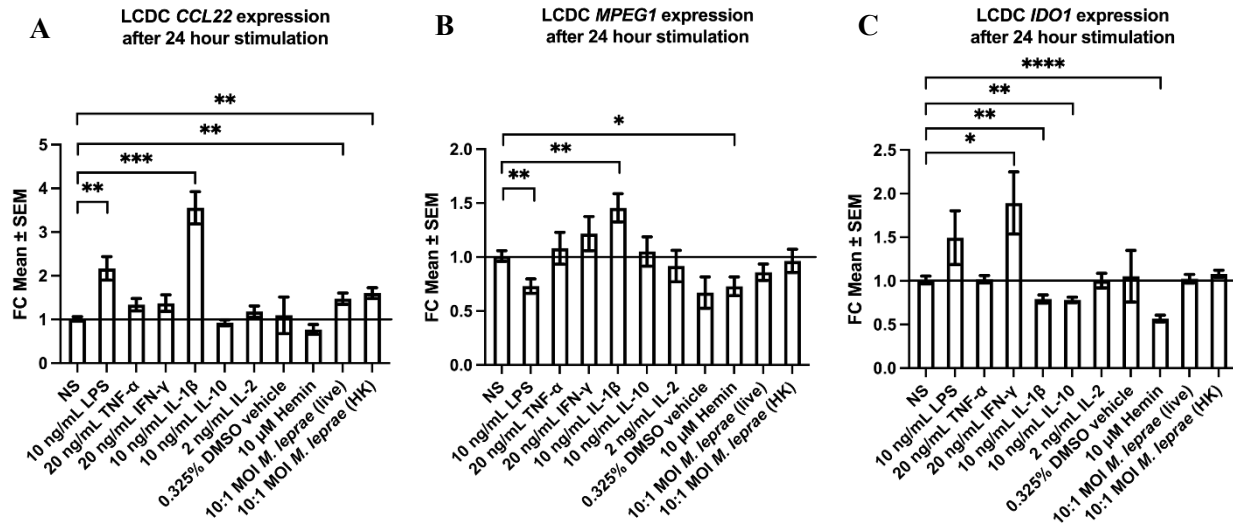


Figure 5: Antimicrobial Gene Expression Induction

LCDCs were stimulated for 24 hours by: LPS, TNF- α , IFN- γ , IL-1 β , IL-10, IL-2, DMSO vehicle and hemin, and live and heat-killed (HK) *M. leprae* at 10:1 MOI. *CCL22* (A), *MPEG1* (B), and *IDO1* (C) gene expression was assessed by qPCR. Data are represented as mean fold change $\pm$ SEM, * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001. Welch's t-test. Three experiments, three replicates per experiment.

potent inducers of *CCL22*, *MPEG1*, or *IDO1* expression in LCDCs, were selected to induce these genes in further studies.

The CCL22, MPEG1, and IDO1 protein abundance in LCDCs in response to IL-1 β or IFN- γ was measured by flow cytometry. The gating strategy is presented in Supplementary Figure 1. Briefly, in the gating strategy, the cells, single cells, live cells (Zombie NIR viability dye negative), and protein-positive cells (CCL22, MPEG1, or IDO1 positive) were sequentially selected. The percentage of cells positive for CCL22, MPEG1, or IDO1 (protein-positive cells) increased with stimulation (Figures 6A, D, G). The change in abundance levels per cell was also analyzed. In both the live cells (Figures 6B, E, H) and the protein-positive cells (Figures 6C, F, I), there was an increase in fluorophore intensity for the stimulated cells compared to the non-stimulated cells. This increase in intensity can be seen in the increased median intensity and the change in the shape of the violin plot. The fluorophore intensity corresponds to the amount of protein in the cell. These data indicate IL-1 β increased the number of cells that produced CCL22 and MPEG1 protein and increased the amount of protein in each cell. The same increase was seen for IDO1 with IFN- γ stimulation. Overall, IL-1 β induced CCL22 and MPEG1 gene and protein expression, while IFN- γ induced IDO1 gene and protein expression.

3.2 IFN- γ and 1-Methyltryptophan Modulate IDO1 Activity in THP-1 Cells

Because of the high number of cells needed to test the effects of IFN- γ and 1-MT on IDO activity, THP-1 monocytes were used instead of LCDCs. Not enough LCDCs were differentiated to perform this experiment with 2.5×10^6 cells per replicate, which is needed to measure the low abundant tryptophan catabolites. All the compounds in the LC-MS MRM were analyzed with the THP-1 cells, with data from select tryptophan metabolites presented in Figure 7.

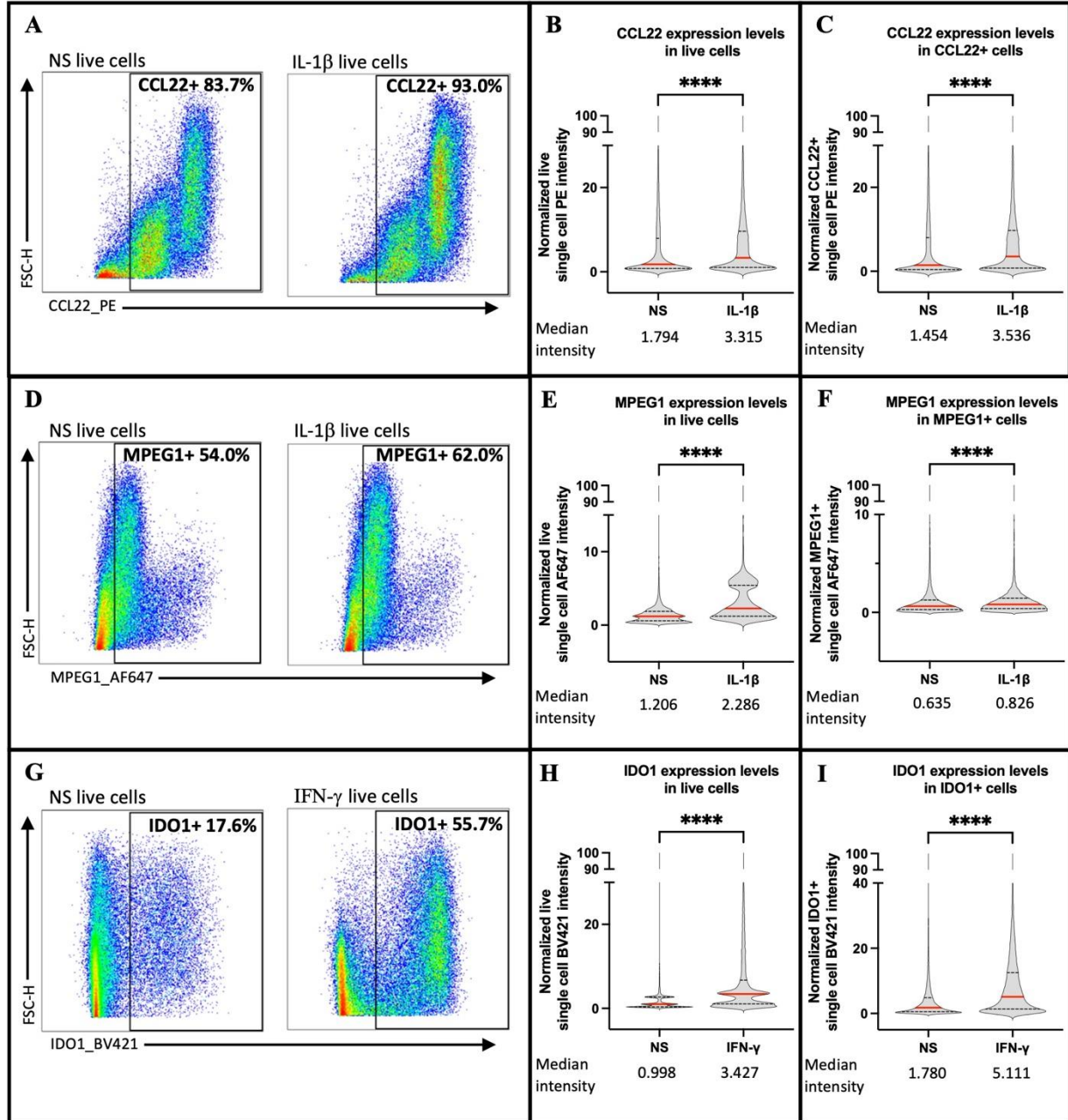


Figure 6: Antimicrobial Protein Expression Induction

LCDCs were stimulated for 24 hours, stained with either anti-CCL22 (A-C), anti-MPEG1 (D-F), or anti-IDO1 (G-I) and analyzed by flow cytometry. A, D, G show the percentage of protein-positive cells in the non-stimulated (NS) and stimulated (IL-1β or IFN-γ) conditions in a representative pseudocolor plot. B, E, H show the protein expression levels in live cells and C, F, I show the protein expression levels in protein-positive cells. For the expression levels, the fluorophore intensity of the first 5000 per replicate either live cells or protein-positive cells was min-max normalized. **** $P < 0.0001$. Mann-Whitney t-test. Two experiments with two staining replicates per experiment, total of 20,000 cells normalized.

THP-1 cells treated with IFN- γ for 24 hours had increased IDO activity since intracellular tryptophan catabolites kynurenine (Figure 7B) and quinolinic acid (Figure 7C) increased, and tryptophan (Figure 7A) decreased compared to non-stimulated (NS) cells. 1-MT decreased the agonistic effect of IFN- γ on IDO activity. When IFN- γ and 1-MT co-stimulated the THP-1 cells, IDO activity increased compared to NS cells, but IDO activity was significantly reduced compared to IFN- γ alone. 1-MT alone did not significantly affect IDO activity and kynurenine pathway catabolite production. These data show that in addition to inducing IDO1 gene expression and protein abundance, IDO activity was also increased by IFN- γ stimulation. Additionally, 1-MT abrogated the IFN- γ stimulation.

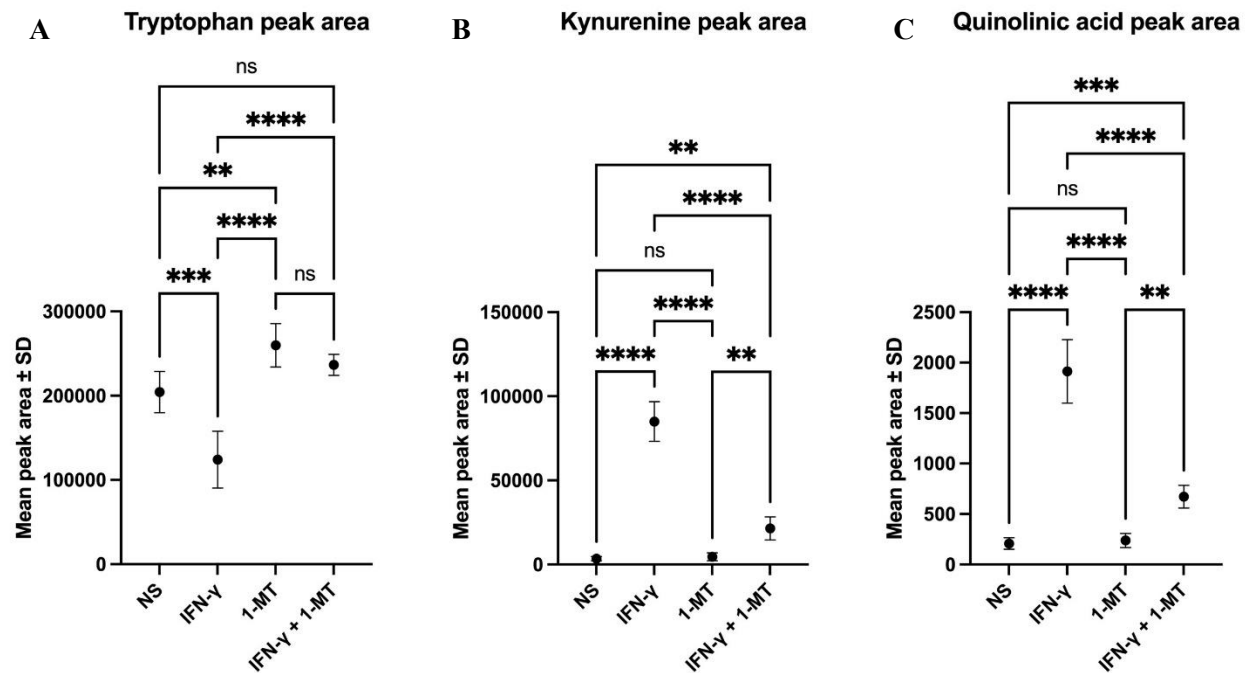


Figure 7: Induction by IFN- γ and Inhibition by 1-Methyltryptophan of IDO Activity

THP-1 cells were non-stimulated (NS) or stimulated for 24 hours with IFN- γ , 1-MT, or IFN- γ + 1-MT. Cells were harvested, and intracellular metabolites were extracted and analyzed by LC-MS MRM. Data are represented as the mean peak area $\pm$ SD of select metabolites from the kynurenine metabolic pathway, tryptophan (A), kynurenine (B), and quinolinic acid (C). ^{ns} $P > 0.05$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. Tukey's test. Two experiments, three replicates per experiment.

3.3 Direct Treatment with 1-(L)-Methyltryptophan is Not Bactericidal Against *M. leprae*

To test that 1-MT does not directly affect ML viability, ML was incubated with 20 μ M or 200 μ M of 95% pure 1-(L)-MT for 24 hours. Using radiorespirometry to measure ML viability (Figure 8A), 200 μ M 1-MT slightly increased ML viability, and 20 μ M 1-MT did not affect ML viability. The radiorespirometry work was by Dr. Fabricio Da Mota Ramalho Costa. 95% pure 1-(L)-MT was analyzed by untargeted LC-MS. In addition to the 1-MT peak, tryptophan was also present in this standard (Figure 8B). The tryptophan peak on the extracted ion chromatograph from 1-MT had the same retention time as a tryptophan standard (Figure 8C).

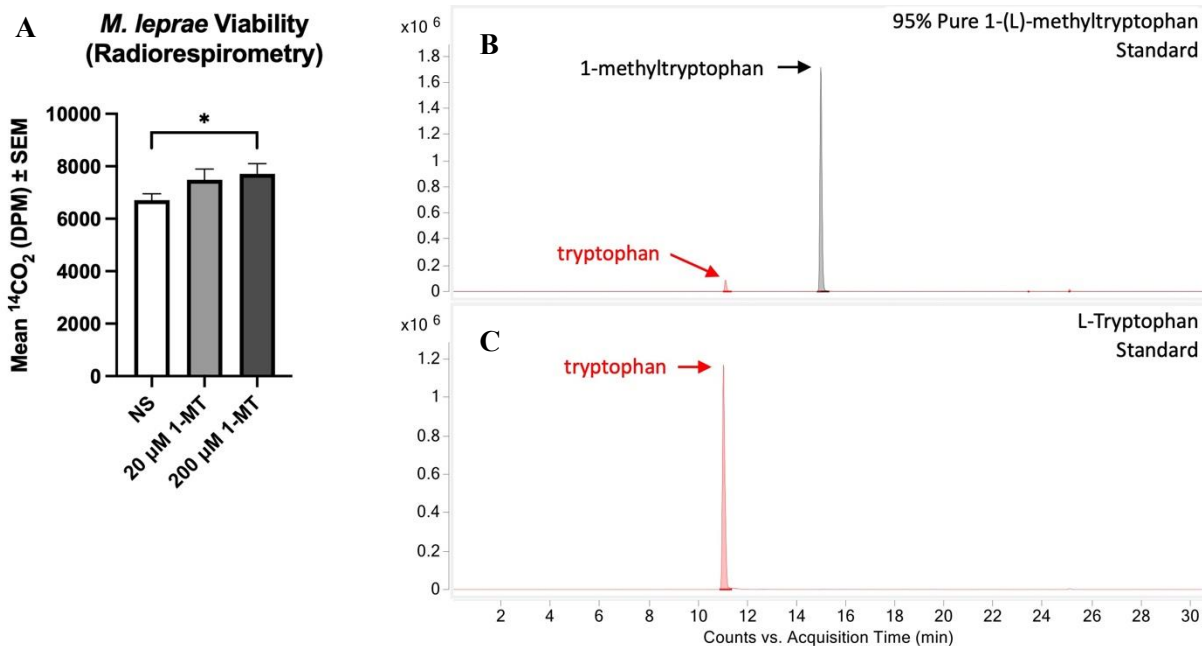


Figure 8: Direct Treatment of *M. leprae* with 1-(L)-Methyltryptophan

ML was incubated for 24 hours with 95% pure 1-(L)-MT and the bacterial viability was measured by radiorespirometry (A), $*P < 0.05$, Welch's t-test, three experiments, three replicates per experiment. 95% pure 1-(L)-MT (B) was analyzed by untargeted LC-MS and compared to L-tryptophan (C). Data are shown as the extracted ion chromatograms of 1-MT (m/z 219.1128) and tryptophan (m/z 205.0972).

3.4 IDO1 Activity Does Not Affect *M. leprae* Viability in LCDCs.

LCDCs were pretreated for four hours with IFN- γ . 1-MT was added for 30 minutes, and the cells were infected with 10:1 MOI live ML and incubated for 48 hours. All stimulation

condition with IFN- γ had significantly increased *IDO1* gene expression (Figure 9A). 1-MT and ML did not affect *IDO1* gene expression after 48 hours of stimulation (Figure 9A). ML viability was not altered when IDO1 expression and activity were induced by IFN- γ or inhibited by 1-MT (Figure 9B).

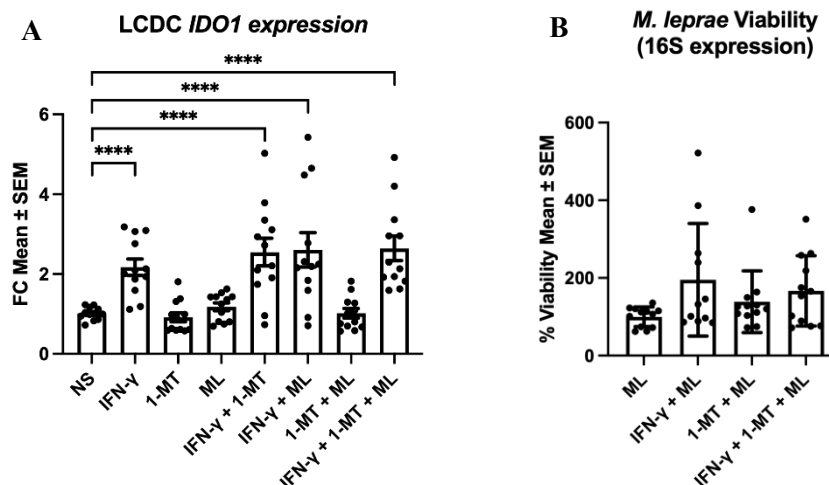
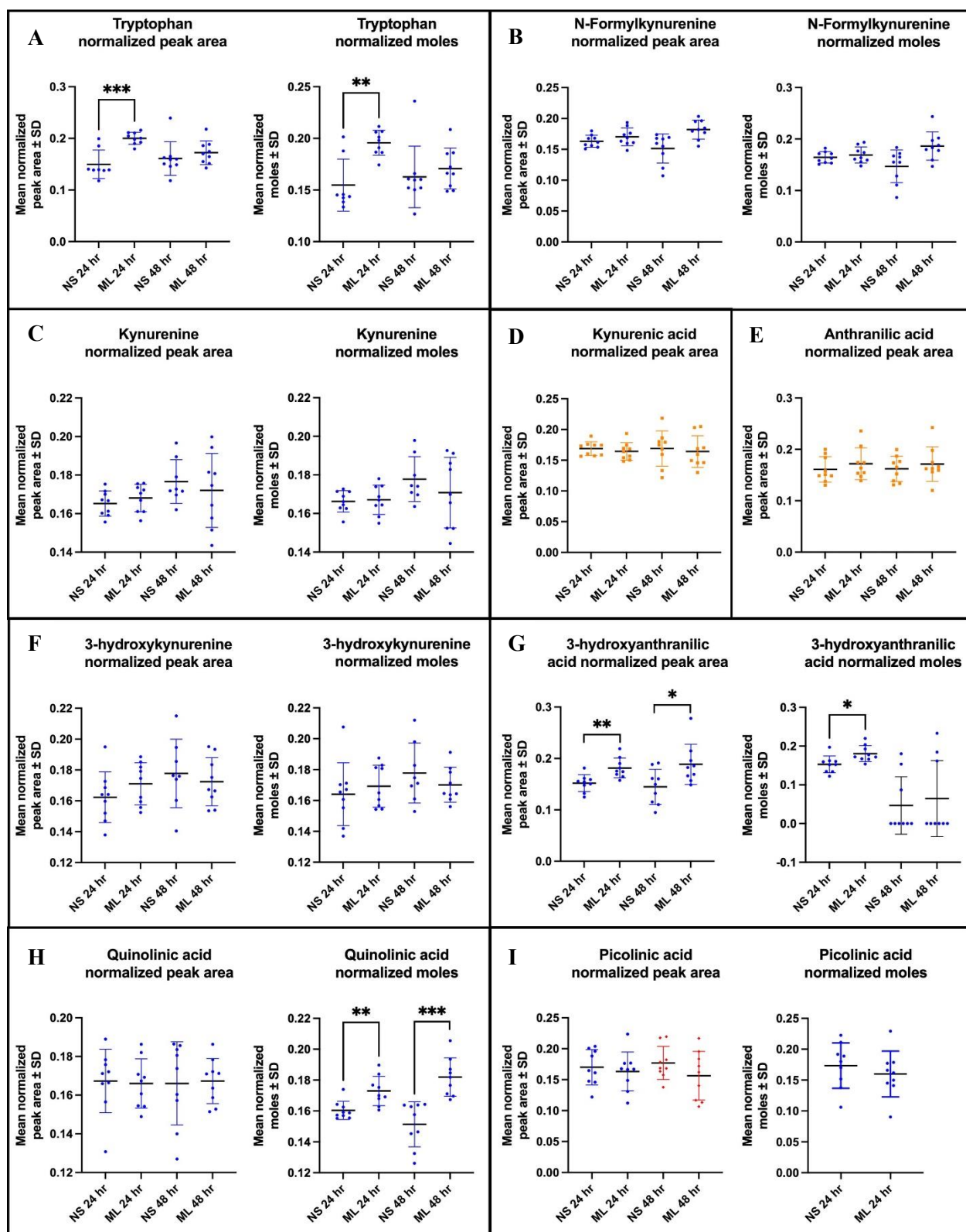


Figure 9: *M. leprae* Viability After Inhibiting or Inducing IDO1 in LCDCs

LCDCs incubated for four hours with IFN- γ , followed by adding 1-MT and infecting with live *M. leprae* at a 10:1 MOI. After 48 hours, *IDO1* gene expression (A) and *M. leprae* viability (16S RNA/DNA) (B) were assessed by qPCR. *IDO1* gene expression data are represented as mean fold change $\pm$ SEM using Dunnett's test, and *M. leprae* viability as mean % viability $\pm$ SEM using Tukey's test. **** P < 0.0001. Three experiments, three replicates per experiment.

3.5 Some Tryptophan Metabolites Have Altered levels in *M. leprae*-Infected LCDCs

The effect of ML infection on IDO1 activity and intracellular tryptophan catabolite changes were assessed. LCDCs were non-stimulated or infected by live ML at a 10:1 MOI for 24 or 48 hours, and the extracted metabolites were measured by LC-MS MRM. The normalized peak area for each metabolite over the limit of detection was calculated (Figure 10). For metabolites over the limit of quantification, the normalized moles amount was also calculated (Figure 10). The mean and range of the picomoles amount of the metabolites before normalization are also presented (Table 5). Nearly all the metabolites measured were detectable



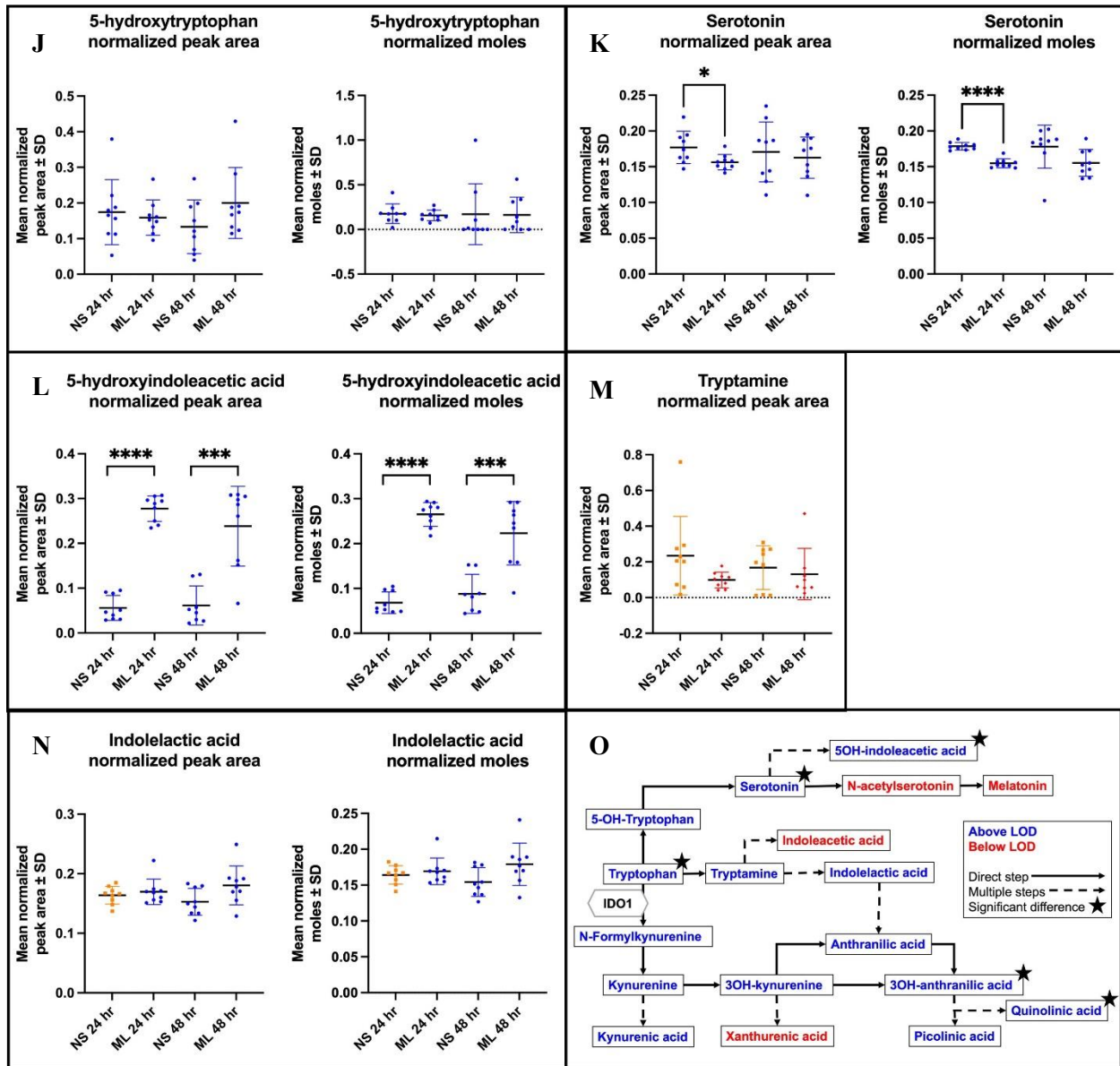


Figure 10: *M. leprae* Modulation of LCDC Tryptophan Metabolite Levels

LCDCs were non-stimulated (NS) or infected by 10:1 MOI *M. leprae* (ML) for 24 or 48 hours. Cells were harvested and intracellular metabolites were extracted and analyzed LC-MS MRM. Data are represented as mean normalized peak area $\pm$ SD, and for metabolites above the limit of quantification, also as mean normalized moles $\pm$ SD (A-N). Blue circles: peak area > limit of quantification (LOQ); Orange squares: limit of quantification (LOQ) $\geq$ peak area > limit of detection (LOD); Red diamonds: peak area $\leq$ limit of detection (LOD). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, Welch's t-test, three experiments, three replicates per experiment. (O) A pathway summary of the metabolites measured in LCDCs.

in at least one condition. N-acetylserotonin, melatonin, and xanthurenic acid were below the limit of detection for all the conditions (Figure 10O). Most metabolites did not have significantly different levels between NS and ML-infected at 24 or 48 hours (Figure 10B-F, I, J, M, N).

Table 5: LCDC Tryptophan Metabolite Amounts in Picomoles

Compound	NS 24 hours		ML 24 hours		NS 48 hours		ML 48 hours	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Tryptophan	387.786	8.632-3412.657	12.104	9.866-14.813	48.539	11.076-80.346	48.437	14.482-82.404
N-formylkynurenine	19.997	17.326-22.247	20.585	16.690-24.211	6.515	2.458-14.195	8.102	2.820-14.156
Kynurenine	239.284	216.882-266.704	241.339	203.161-282.680	55.360	22.150-93.760	58.414	21.929-91.308
3-hydroxykynurenine	17.942	8.383-25.839	18.756	9.451-28.450	1.509	0.914-2.370	1.598	0.962-2.319
3-hydroxyanthranilic acid	4.447	1.414-8.288	5.572	1.427-11.412	0.143	0.000-0.550	0.196	0.000-0.712
Quinolinic acid	5004.394	3072.981-8342.704	5458.117	3218.858-9964.021	1492.619	737.8026-2455.697	1737.891	1079.95-2796.573
Picolinic acid	1.257	0.624-2.480	1.236	0.487-2.312	Below LOQ	Below LOQ	Below LOQ	Below LOQ
5-hydroxytryptophan	1.415	0.189-3.853	1.352	0.514-2.986	0.035	0.000-0.141	0.566	0.000-2.815
Serotonin	32.074	20.463-46.599	27.608	17.621-38.575	14.682	9.902-17.728	13.096	9.984-18.269
5-hydroxyindoleacetic acid	10.973	5.622-17.346	41.487	33.540-54.434	7.407	4.288-27.226	16.680	5.948-28.427
Indolelactic acid	Below LOQ	Below LOQ	18.326	16.721-23.166	20.442	15.433-23.950	23.757	19.130-34.758

LCDCs were non-stimulated (NS) or infected by 10:1 MOI *M. leprae* (ML) for 24 or 48 hours. Cells were harvested and intracellular metabolites were extracted and analyzed by MRM LC-MS. Metabolite amounts were determined using internal standards and calibration curves. Data are represented as the amount (picomoles) mean and range for metabolites above the limit of quantification from three experiments, three replicates per experiment.

Tryptophan peak area and moles amount were significantly increased in ML-infected cells compared to NS at 24 hours but not 48 hours (Figure 10A). 3-hydroxyanthranilic acid peak area was increased in ML at 24 and 48 hours, and the moles amount was increased after 24 hours (Figure 10G). At 24 and 48 hours, the moles amount, but not the peak area, of quinolinic acid was increased in ML (10H). When assessing the extraction efficiency of each metabolite, the extraction efficiency of quinolinic acid was lower than that of most other metabolites (data not shown). Likely, the correction of the peak area by the internal standard and calibration curve when calculating the amount of metabolite was more critical for quinolinic acid than other metabolites, resulting in the dramatic difference in trend between the peak area and moles

amount of quinolinic acid. Serotonin peak area and moles amount were decreased in 24-hour ML (Figure 10K). 5-Hydroxyindoleacetic acid had significantly increased peak area and moles amount in ML at 24 and 48 hours (Figure 10L). These data show that ML infection caused slight differences in the levels of a few metabolites from the serotonin and kynurenine catabolic pathways. The increase in kynurenine pathway catabolites indicates that ML-infection activated IDO1.

3.6 Intact Metabolic Pathways in *M. leprae*

The ML orthologue of known Mtb functional enzymes for carbon metabolism and aromatic amino acid biosynthesis was identified, and whether the ML orthologue is a pseudogene or expected to be functional was assessed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Mycobrowser Portal databases [113-115, 191]. A network of the carbon metabolic and tryptophan biosynthesis pathways was constructed, comparing the Mtb and ML orthologue genes (Figure 11). Each step of aromatic amino acid biosynthesis in ML and key carbon metabolic pathways, including β -oxidation, glycolysis, gluconeogenesis, and the PPP, is expected to have at least one functional gene. ML has the genes to metabolize even-chain fatty acids (e.g., palmitic acid) and sugars (e.g., glucose).

The source of the carbons used to synthesize each aromatic amino acid was also identified by tracking each carbon through each enzymatic reaction (Figure 11). Tryptophan has 11 carbons, made from three carbons from D-erythrose 4P, three from phosphoenolpyruvate, two from 5-phospho- α -D-ribose 1-diphosphate (PRPP), and three from serine (serine biosynthesis is also intact in ML but not shown here). Phenylalanine and tyrosine have the same carbon structure, the difference being that tyrosine has a hydroxy group at position 4 (C₇ overall)

of the aromatic ring. Both of these amino acids have nine carbons. Three carbons are from D-erythrose 4P, and six are from two phosphoenolpyruvates, each contributing three carbons.

3.7 *M. tuberculosis* and *M. leprae* Synthesize $^{13}\text{C}_6$ Tryptophan from the (ring- $^{13}\text{C}_6$) Anthranilic Acid Precursor

Mtb and ML were incubated for 24 hours with (ring- $^{13}\text{C}_6$) anthranilic acid. The aromatic ring of this anthranilic acid isotope was composed of six ^{13}C . This aromatic ring remains intact throughout the conversions of anthranilic acid to tryptophan [113-115]. Therefore, this isotope's tryptophan product is also expected to have six ^{13}C . When Mtb was incubated with or without (ring- $^{13}\text{C}_6$) anthranilic acid, unlabeled, naturally occurring $^{12}\text{C}_{11}$ ($^{13}\text{C}_0$) tryptophan was successfully extracted and measured (Figure 12A, C). $^{13}\text{C}_6$ tryptophan was only measured in samples where (ring- $^{13}\text{C}_6$) anthranilic acid had been added (Figure 12B, D). When ML was incubated with (ring- $^{13}\text{C}_6$) anthranilic acid, $^{12}\text{C}_{11}$ tryptophan and $^{13}\text{C}_6$ tryptophan were extracted and measured (Figure 12E, F). These data demonstrate that $^{13}\text{C}_6$ tryptophan is only made from its ^{13}C -labeled precursor (ring- $^{13}\text{C}_6$) anthranilic acid, and both Mtb and ML have the enzymes to perform this conversion.

3.8 Synthesis of Tryptophan from Glucose or Palmitic Acid *In Vitro*

Mtb was incubated with (U- $^{13}\text{C}_6$) and unlabeled glucose for one to five days. The 12 different tryptophan isotopologues (0-11 ^{13}C) were searched for in the data. After five days, the greatest abundance of each isotopologue was measured. In the samples of Mtb incubated with unlabeled glucose, only $^{13}\text{C}_0$ tryptophan, $^{13}\text{C}_1$ tryptophan, and $^{13}\text{C}_2$ tryptophan were measured (Figure 13A). The relative abundance of these isotopologues was calculated and is very close to the naturally occurring abundances of these isotopologues for tryptophan (Table 6). For Mtb incubated with (U- $^{13}\text{C}_6$) glucose, all the isotopologues measured were within a mass error of $\pm$

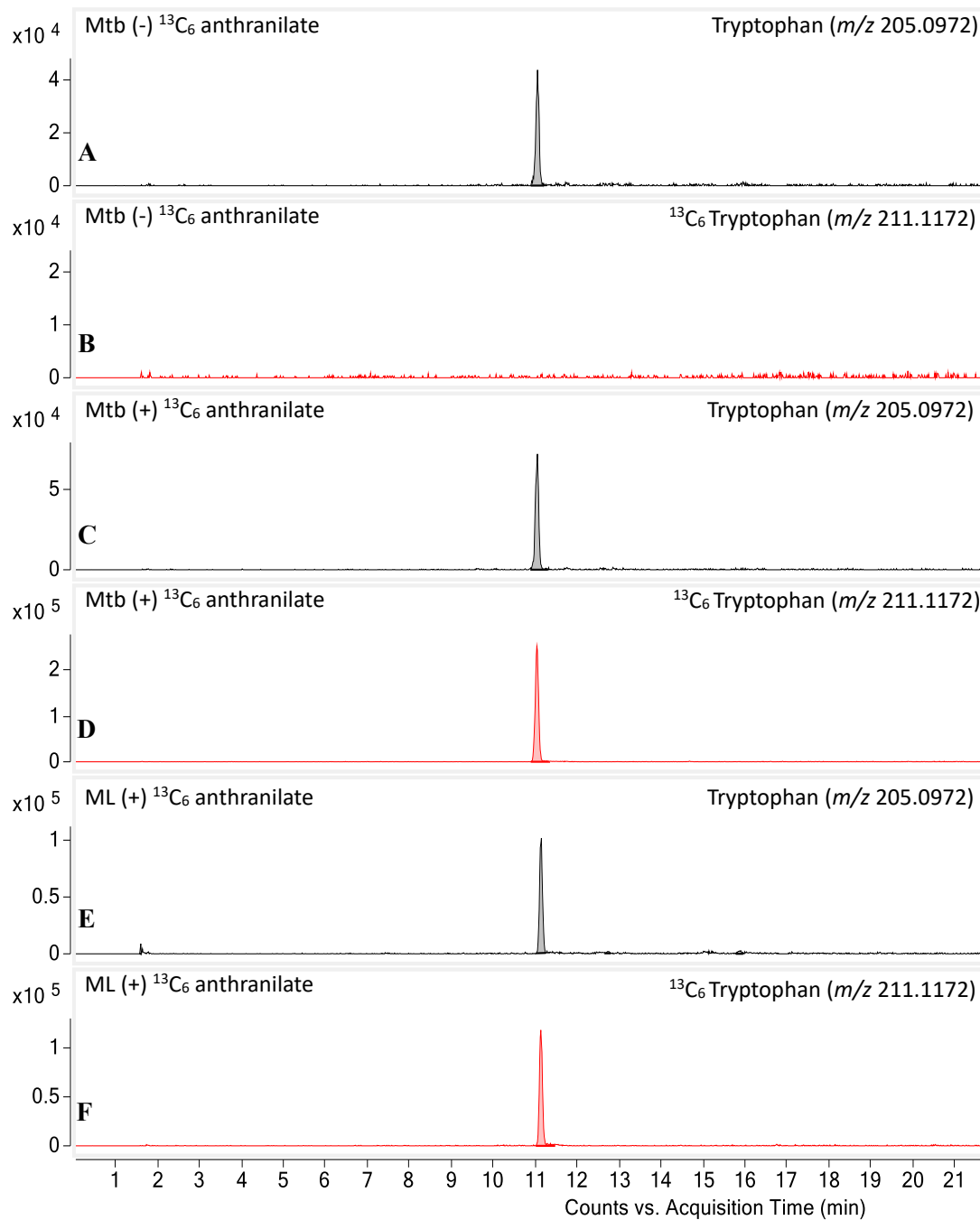


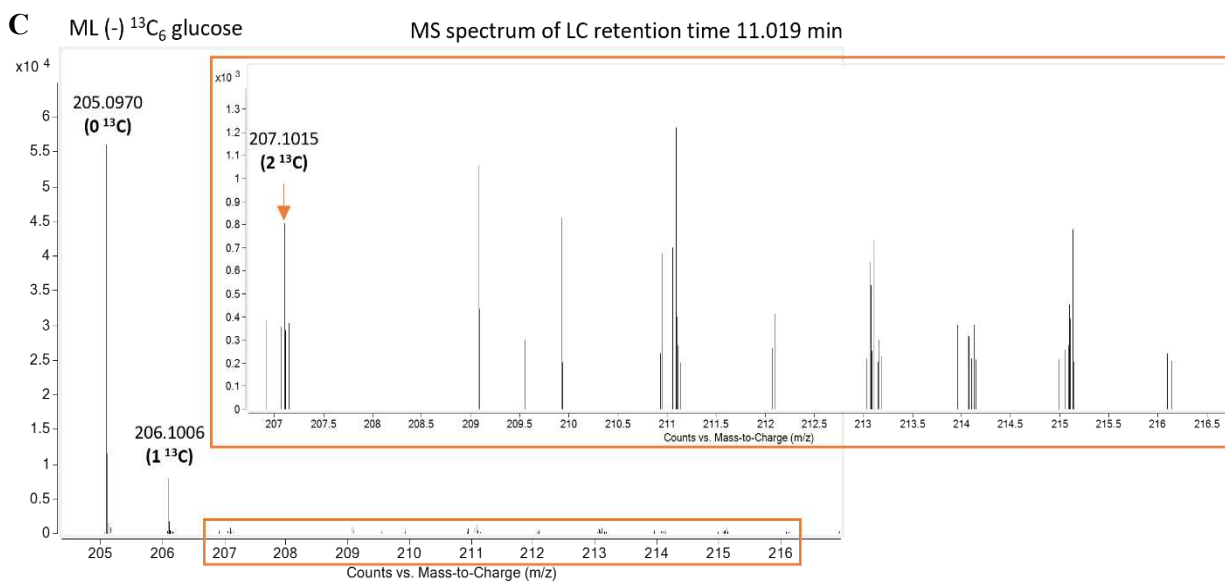
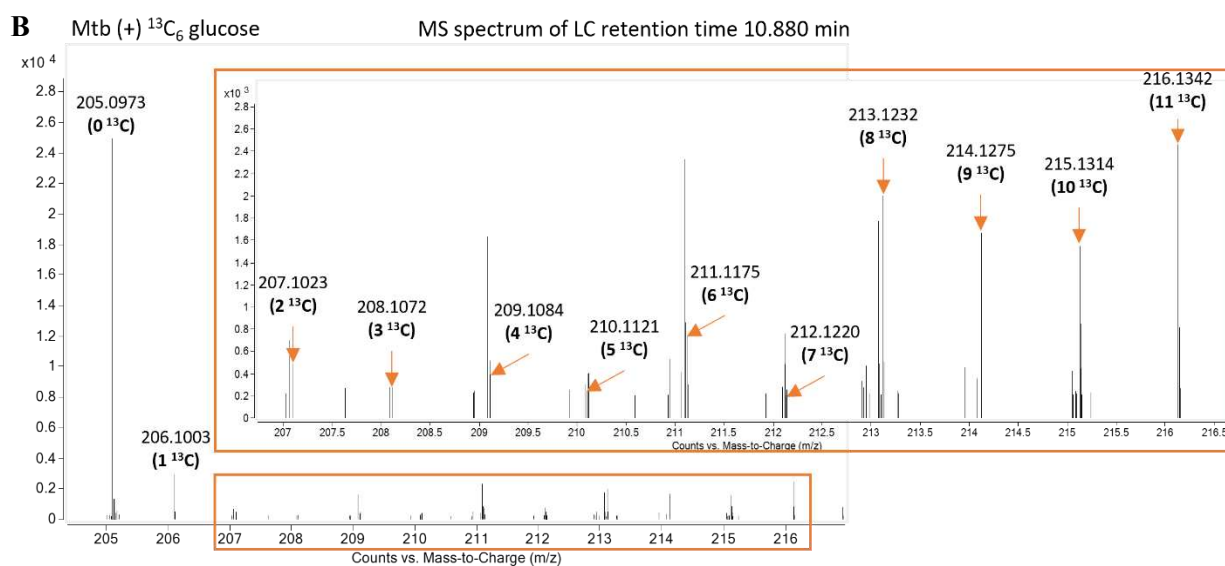
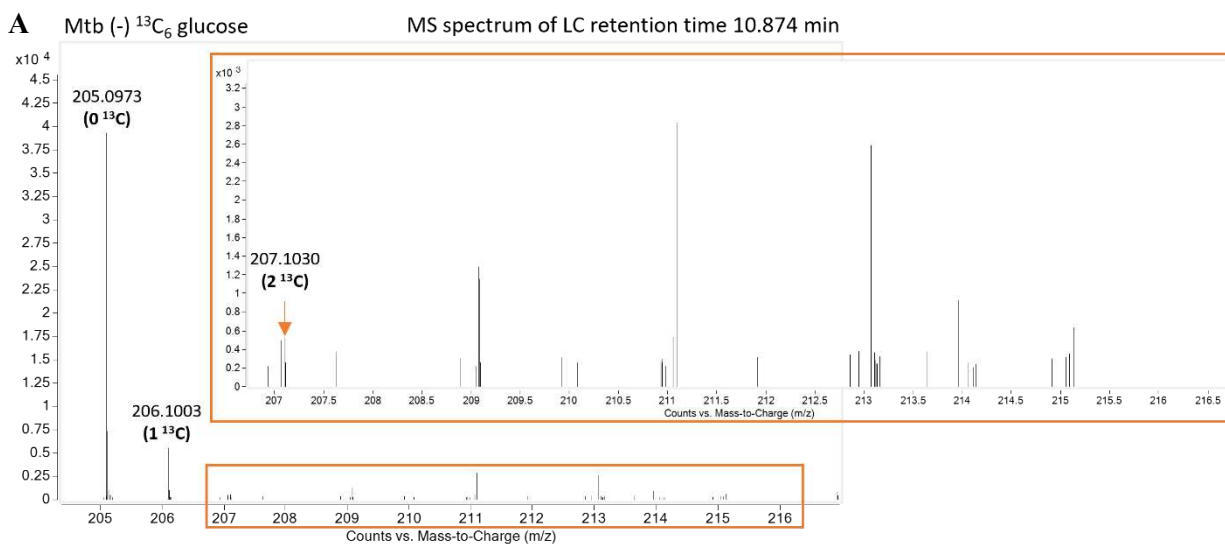
Figure 12: $^{13}\text{C}_6$ Tryptophan Synthesis from $^{13}\text{C}_6$ Anthranilate by *M. tuberculosis* and *M. leprae*

Mtb was incubated without (A, B) and with (C, D) and ML was incubated with (E, F) (ring- $^{13}\text{C}_6$) anthranilate for 24 hours. The metabolites were extracted and measured by untargeted LC-MS. (A-F) Representative extracted ion chromatograms (EIC) from one replicate. A, C, E are the EIC for unlabeled tryptophan (m/z 205.0972) and B, D, F are the EIC of $^{13}\text{C}_6$ tryptophan (m/z 211.1172). Two experiments, three replicates per experiment.

20 ppm of the theoretical m/z value (Figure 13B), and the relative abundance of these tryptophan isotopologues was greater than the naturally occurring abundance (Table 6). These data indicate that Mtb used the $^{13}\text{C}_6$ -glucose to make tryptophan. For ML incubated with (U- $^{13}\text{C}_6$) glucose or unlabeled glucose (Figure 13C, D), only $^{13}\text{C}_0$ tryptophan, $^{13}\text{C}_1$ tryptophan, and $^{13}\text{C}_2$ tryptophan were measured, and their abundances were close to the naturally occurring abundances of these isotopologues (Table 6) indicating ML did not use the $^{13}\text{C}_6$ glucose to make tryptophan. The same labeling pattern of tryptophan for Mtb and ML was also measured for tyrosine and phenylalanine (data not shown).

Other medium conditions were also tested with ML. With ^{13}C -labeled glucose, erythrose, or palmitic acid all in fatty acid-free medium, only the naturally occurring tryptophan isotopologues were measured. Additionally, increasing the ML per replicate and extending the incubation time did not result in the synthesis and detection of ^{13}C -labeled aromatic amino acids (data not shown).

The previously published method [14] to assess ML amino acid biosynthesis from intracellular glucose in ST8814 Schwann cells was also attempted. The same method was used, except after separating the host cell and ML fractions, the extraction and analysis method previously used in this current study was used. The experiment was repeated with various infection times to increase ^{13}C amino acid yield. All the experiments produced the same results. In all the fractions, only naturally occurring tryptophan isotopologues were measured ($^{13}\text{C}_0$ tryptophan - $^{13}\text{C}_2$ tryptophan), the same as in Figure 13 (data not shown). Additionally, only the naturally occurring isotopologues of tyrosine and phenylalanine were measured (data not shown). These data show that Mtb used the ^{13}C -labeled glucose to make tryptophan. However,



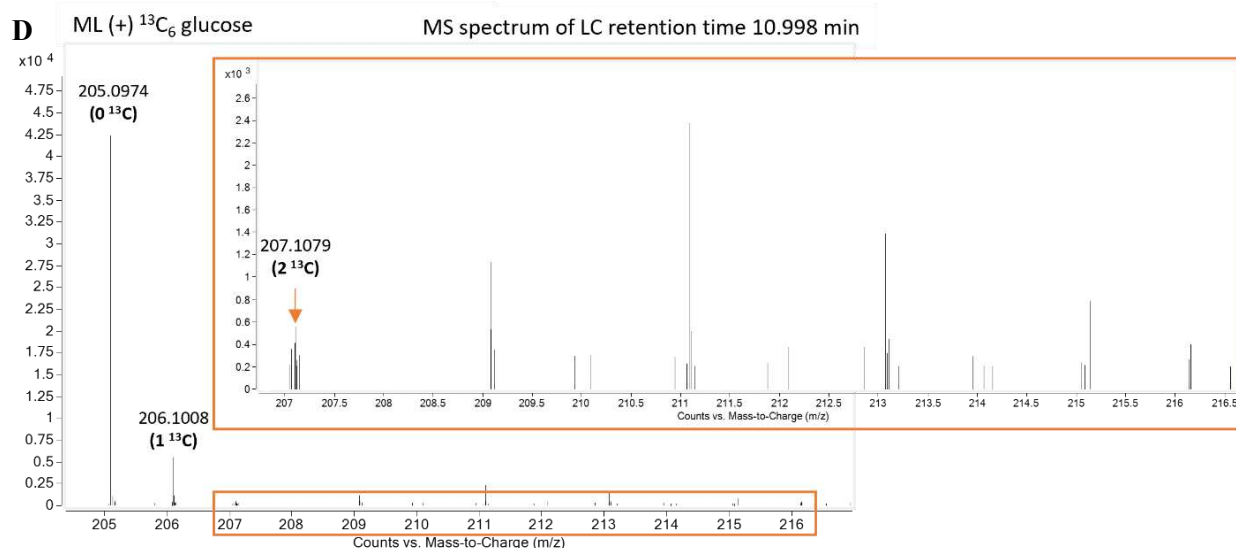


Figure 13: *M. tuberculosis* and *M. leprae* Biosynthesis of Tryptophan from (U- $^{13}\text{C}_6$) Glucose

Mtb (A, B) and ML (C, D) were incubated with unlabeled glucose (A, C) or (U- $^{13}\text{C}_6$) glucose (B, D) for five days. The metabolites were extracted and analyzed by untargeted LC-MS. Data are a representative MS spectrum at the LC retention time of greatest tryptophan abundance. The measured m/z of the tryptophan isotopologues was within a mass error of ± 20 ppm of the calculated theoretical m/z values. Three experiments, two replicates per experiment.

Table 6: Calculated Naturally Occurring and Experimental Tryptophan Isotopologue Abundances

		Calculated naturally occurring ^a	Mtb		ML	
			(+) $^{13}\text{C}_6$ glucose	(-) $^{13}\text{C}_6$ glucose	(+) $^{13}\text{C}_6$ glucose	(-) $^{13}\text{C}_6$ glucose
1 ^{13}C	(M + 1)	12.8921 %	11.83 %	14.12 %	13.06 %	14.14 %
2 ^{13}C	(M + 2)	1.1663 %	1.97 %	1.33 %	1.31 %	1.14 %
3 ^{13}C	(M + 3)	0.0792 %	1.102 %	ND	ND	ND
4 ^{13}C	(M + 4)	0.0040 %	1.561 %	ND	ND	ND
5 ^{13}C	(M + 5)	0.0001 %	1.61 %	ND	ND	ND
6 ^{13}C	(M + 6)		2.97 %	ND	ND	ND
7 ^{13}C	(M + 7)		1.94 %	ND	ND	ND
8 ^{13}C	(M + 8)		8.02 %	ND	ND	ND
9 ^{13}C	(M + 9)		6.69 %	ND	ND	ND
10 ^{13}C	(M + 10)		6.22 %	ND	ND	ND
11 ^{13}C	(M + 11)		9.88 %	ND	ND	ND

Using an online isotope distribution calculator, the expected natural tryptophan isotopologue abundance was determined. The relative experimental tryptophan isotopologue abundance for the representative replicates from Figure 13 was calculated (labeled isotope abundance / unlabeled isotope abundance) x 100 %.

ND = not detected

a: "Isotope Distribution Calculator, Mass Spec Plotter, Isotope Abundance Graphs." n.d.

<https://www.sisweb.com/mstools/isotope.htm>.

ML did not, so tryptophan biosynthesis by ML from glucose or palmitic acid does not occur in these conditions.

CHAPTER 4: DISCUSSION

4.1 Induction of Antimicrobial Genes

LCs play an essential role in skin immunity by maintaining epidermal homeostasis and clearing pathogens during skin infections [50, 57-59, 68, 69]. However, much is still unknown about LCs, particularly their antimicrobial mechanisms. Single-cell sequencing of LCs has identified 123 genes associated with antimicrobial activity that were more greatly expressed in LCs in RR leprosy lesions compared to LL leprosy lesions. CCL22, MPEG1, and IDO were selected for further study to identify if the gene products are involved in LC antimicrobial mechanisms, using LCDCs as a LC model. For this study, we hypothesized that altered metabolic processes in LCDCs impact antimicrobial effects on ML.

To evaluate the antimicrobial activity of these genes, an *in vitro* system that induces CCL22, MPEG1, and IDO in LCDCs was established. A change in gene expression in response to various stimulants was assessed (Figure 5). IL-1 β was chosen to induce CCL22 and MPEG1 (Figure 5A, B) and IFN- γ to induce IDO (Figure 5C). IDO gene expression was later also measured after 52 hours of IFN- γ stimulation (4-hour pretreatment + 48-hour infection) (Figure 9A). With longer stimulation, IDO was more significantly increased compared to after the 24-hour IFN- γ stimulation, further solidifying that IFN- γ induced IDO in LCDCs. The induction of the corresponding proteins by these cytokines was confirmed by flow cytometry (Figure 6). For IDO, enzyme activity was also increased by IFN- γ stimulation, as seen with the increase in kynurenine pathway metabolites and the decrease in tryptophan (Figure 7).

IL-1 β and IFN- γ have previously been reported to induce CCL22 and IDO, respectively. While IL-1 β is not a frequently described CCL22 inducer, these results support previous findings of this relationship [83]. IFN- γ is recognized as the main and most established IDO agonist and

can induce IDO expression through the JAK-STAT pathway [116, 192-197]. IDO has several IFN-stimulated response elements (ISREs) and gamma activation sequences (GAS) in its promotor region [192, 198-200]. The regulation of IDO, though, is much more complex than a single inducer and pathway. IFN regulator factor or non-canonical NF- κ B pathways can also regulate IDO gene expression, with the IDO promotor region also containing NF- κ B binding sites [199, 201-205].

In contrast to the expression in LCDCs, *MPEG1* gene expression in mouse embryonic fibroblasts does not increase in response to IL-1 β [97]. Type 1 and 2 interferons increase MPEG1 expression in the fibroblasts, with the expression greater when the interferons are used in combinations [97]. The MPEG1 protein expression in fibroblasts compared to macrophages is different. The IFN- γ induced MPEG1 in fibroblasts is rapidly degraded and is barely detected by western blot, while the constitutively expressed or IFN- γ + LPS-induced MPEG1 in macrophages is strongly measured by western blot [97]. McCormack, et al. [96] observed further differences in *MPEG1* expression between cell types in response to interferon stimulation. IL-1 β was not used in this study [96]. These comparisons between cell types indicate that MPEG1 regulation varies between different cell types, possibly explaining the differing MPEG1 induction results with the LCDCs.

4.2 Inhibition of IDO

An inhibitor also needed to be selected to assess the antimicrobial activity of IDO on ML in LCDCs. 1-MT is a frequently used inhibitor of IDO activity, with 1-L-MT a more potent inhibitor *in vitro* than 1-D-MT [206-211]. In the previous study assessing the effect of IDO inhibition on ML, 1-L-MT was also used [152]. 1-MT did not affect IDO expression in LCDCs (Figure 9A). With other cell types, 1-MT treatment decreases, increases, or has no effect on IDO

expression [212-215]. 1-MT did modulate IDO activity, however. Compared to non-stimulated THP-1 cells, 1-MT alone had little effect on IDO activity; the kynurenine and quinolinic acid levels were the same as in non-stimulated cells, though there was a slight increase in tryptophan with 1-MT treatment (Figure 7). However, in a co-stimulation with IFN- γ and 1-MT, 1-MT significantly reduced IDO activity as there was a significant reduction in kynurenine pathway metabolites and an increase in tryptophan compared to IFN- γ stimulation alone (Figure 7). The 1-MT treatment did not completely abrogate the IFN- γ effect, with the kynurenine and quinolinic acid levels still slightly elevated compared to non-stimulated and 1-MT treated cells (Figure 7). This effect of 1-MT with or without IFN- γ on IDO activity was also seen in previous studies [212, 216, 217]. Assessing the effect of IFN- γ and 1-MT stimulation on IDO activity in LCDCs will be performed in the future to ensure these stimuli have the same modulatory effects in LCDCs as they did in THP-1 cells.

1-MT is a competitive inhibitor [206]. 1-L-MT is an L-tryptophan analog with a methyl group replacing a hydrogen bound to the indole ring nitrogen atom [206]. 1-MT binds to the same catalytic site on IDO as L-tryptophan, but the methyl group prevents the conversion to N-formylkynurenine [206]. Likely, the IFN- γ induces the expression of so many IDO enzymes that despite the 1-MT, the still present tryptophan is also binding and being converted. This would explain why IDO activity is not inhibited in the IFN- γ and 1-MT co-stimulation to the levels of the non-stimulated and 1-MT stimulated conditions. Overall, IFN- γ stimulation increased IDO activity, but 1-MT can lessen this increase.

As a control, the direct killing of ML by 1-MT treatment was evaluated by radiorespirometry, a measurement of ML viability by assessing the bacteria's metabolic activity. Directly treating the ML with 95% pure 1-(L)-MT did not decrease the bacteria's viability

(Figure 8A). There was a slight increase in metabolic activity and viability with the 200 μ M 1-MT treatment. The 1-MT was analyzed by untargeted LC-MS, and residual tryptophan was detected. Schmidt, et al. [207] demonstrated that in tryptophan-depleted conditions, prokaryotic and eukaryotic cells can use the residual L-tryptophan in 1-(L)-MT. The radiorespirometry medium contains casein hydrolysate. However, the tryptophan concentration may be so low that adding the L-tryptophan from the 1-MT still had a beneficial effect, increasing ML metabolic activity. The 20 μ M 1-MT treatment likely had too little residual L-tryptophan to have a significant effect. These data showed, though, that 1-MT did not have a direct bactericidal effect on ML. 1-MT was determined to be a suitable IDO inhibitor.

4.3 LCDC IDO Antimicrobial Activity

With an *in vitro* system established to induce or inhibit IDO, the antimicrobial role of IDO in LCDCs during an ML infection was assessed. However, there was no significant change in ML viability when the cells were stimulated with IFN- γ and/or 1-MT (Figure 9B). There was a possible slight trend that when IFN- γ was present in the treatment, the viability was slightly increased. Nevertheless, with the variation between experiments, there were no conclusive differences in ML viability between the conditions.

4.3.1 Comparison of LCDC IDO Response to Previous Studies

These data contrast with previous studies that investigated the relationship between IDO and ML. In naïve monocytes or macrophages, 1-MT treatment substantially reduces the intracellular ML viability, demonstrating that IDO activity is necessary for ML survival [152]. In these cells, IDO expression also increases differently than in the LCDCs. In naïve monocytes or macrophages, live or γ -irradiated ML, IFN- γ , IL-10, and hemin increase IDO expression and activity. The IDO expression induced by ML is IL-10 dependent and is associated with the

CD163+, foamy, anti-inflammatory phenotype [150-152]. In monocyte-derived DCs (mDCs), dead ML induces IDO expression and activity, and blocking TNF decreases the ML-associated IDO induction, which seems to be regulated by the TNF-associated pathway, likely the NF- κ B pathway. In these mDCs, blocking IL-10 does not alter IDO expression or activity [153].

In comparison, IDO expression in LCDCs only increased in response to IFN- γ . IL-10 and hemin decreased IDO expression. Live and heat-killed ML did not affect IDO expression in LCDCs. IDO activity was increased after 24 and 48 hours of ML infection in LCDCs though; the increase of two kynurenine pathway metabolites, 3-hydroxyanthranilic acid and quinolinic acid, indicated that IDO activity was higher in the ML-infected cells than in non-stimulated LCDCs (Figure 10G, H).

The contrasting results of different studies highlights a common trend in the IDO response being model-dependent, in this case, dependent on the eukaryotic cell type. In macrophages, IDO expression seems to be intertwined with the anti-inflammatory macrophage phenotype, which favors ML survival and is prevalent in the MB leprosy lesions [37, 150-152]. IDO activity and the resulting anti-inflammatory environment are vital to ML survival in macrophages, and these associations also involve heme-iron metabolic pathways [152].

In mDCs, the intracellular ML viability was not assessed. However, the IDO expression and activity depends on TNF regulation instead of the IL-10 regulation seen with the macrophages. Interestingly, when the mDCs are stimulated with the dead ML and the ML cytosol and membrane fractions, different levels of IDO expression and activity are induced. The mDC phenotype also changes depending on the stimulus. Nevertheless, for all the stimuli, blocking TNF rather than blocking IL-10 decreases IDO activity. These data suggest again that the IDO response is not only different in mDCs compared to macrophages but also that different

ML antigens could affect the IDO response in mDCs differently. In the study with mDCs, no live ML was used and intracellular ML viability was not assessed [153].

4.3.2 Comparison to Previous IFN- γ -Induced LCDC Antimicrobial Activity

The previous results showing that IFN- γ stimulation of purified epidermal LCs and LCDCs has an antimicrobial effect on the intracellular ML could not be replicated [48]. Dang, et al. [48] positively selected the CD1a+ LCDCs after a slightly different differentiation process and found that the antimicrobial activity induced by IFN- γ is dependent on vitamin D in the media. With fetal calf serum (FCS), which is vitamin-D insufficient, IFN- γ stimulation results in only ~30% antimicrobial activity in CD1a+ LCDCs after four days of infection, compared to the ~70% antimicrobial activity observed with human vitamin-D sufficient serum or FCS supplemented with 25-hydroxyvitamin D (supplementary figure 15) [48]. Furthermore, the IFN- γ -induced antimicrobial activity in the LCDCs depends on cathelicidin induction, but IFN- γ did not induce cathelicidin in FCS media (supplementary figure 14A) [48].

Fetal bovine serum was used in the media to assess IDO antimicrobial activity in LCDCs. In these conditions, after 48 hours of infection (with the same four-hour pre-treatment previously used [48]), IDO expression was strongly induced, even without other additives that may enhance IFN- γ stimulation. However, experiment-to-experiment variation likely prevented the detection of the significant but low amount of antimicrobial activity previously measured in response to IFN- γ stimulation without sufficient vitamin D.

In the future, various co-stimulants present in a leprosy skin lesion could be explored to enhance IFN- γ stimulation of the LCDCs, such as adding vitamin D or other IDO inducers like TNF- α [48, 218]. However, replicating the *in vivo* environment of a leprosy skin lesion would pose a significant challenge with the complex and constantly changing environment and many

unknown molecules and cell-to-cell relationships in the skin. For such a goal, an *ex vivo* model may be better suited, as previously used for LC studies [219-221]. Nevertheless, this current study has shown that IFN- γ from Th1 cells in leprosy lesions could induce IDO in LCs.

4.3.3 LCDC Variability

There was too much variability between experiments to determine any significant changes in ML viability when IDO was induced or inhibited in LCDCs. For each experiment, a fresh batch of LCDCs was differentiated from a new vial of CD34+ stem cells. Each vial of stem cells was a different lot with a different donor, harvested, and cryopreserved on different days. With the differentiation process being as closely reproduced as possible, there was still variation, especially in the number of cells harvested, the CD1a and CD207 expression, and subsequently, the response to stimulation; the variation seems to be associated with the different lots of stem cells.

The exact profile of all the differentiated cells is unknown. However, it is not uniform. The purchased CD34+ stem cells were characterized only by CD34 expression and were positively selected using fluorescence-activated cell sorting (personal communication with Lonza Bioscience Scientific Support). Immunotyping of cord blood CD34+ cells identified a mix of CD34+ cell types [222]. The percentage of the different CD34+ stem cell types may differ between the purchased vials, resulting in the batch-to-batch variation of the LCDCs.

To reduce batch-to-batch variation of the LCDCs, isolating the CD1a+/CD207+ cells may be effective, as done in other studies using LCDCs [48]. However, fluorescence-activated cell sorting and magnetic-activated cell sorting have been shown to have varying effects on cell yield, purity, viability, and phenotype depending on the cell type and parameters. Sensitive cells, such as the LCDCs, are more prone to lasting adverse effects from cell sorting (personal

communication with Colorado State University Flow Cytometry Core and Dr. Darcy Deines (née Fletcher)) [223, 224]. Given the cost to properly optimize the cell sorting conditions, the lasting effects on the LCDCs, and then not having enough cells for experiments, it was decided not to isolate CD1a+/CD207+ cells, as was done in previous LCDC studies [50].

4.4 Altered Levels of LCDC Tryptophan Metabolites from *M. leprae* Infection

Previous studies assessing IDO activity in response to ML infection had measured the kynurenine/tryptophan ratio in the spent culture medium [150-153]. In the current study, a wide variety of intracellular metabolites in LCDCs were measured (Figure 10O). If inducing IDO decreased ML viability, one possible mechanism would be the direct killing by the tryptophan metabolites. For example, picolinic acid can directly kill *Mtb* in axenic media [131]. If inducing IDO increased ML viability, the tryptophan metabolites could directly benefit the ML. No other study was found that measured so many tryptophan metabolites inside of cultured cells.

4.4.1 Kynurenine Pathway Metabolites

While ML infection did not alter the IDO gene expression in LCDCs after 24 or 48 hours (Figure 5C, Figure 9A), there was an increase in IDO activity after 24 and 48 hours of ML infection. At both time points, 3-hydroxyanthranilic acid and quinolinic acid were increased in ML-infected LCDCs (Figure 10G, H). How ML infection activated IDO is not known. Given the increase in intracellular tryptophan after 24 hours in the ML-infected LCDC (Figure 10A), the ML infection may have caused an increase in intracellular tryptophan, possibly resulting in more IDO activity and production of kynurenine pathway metabolites. Alternatively, the ML infection may have activated IDO, resulting in the catabolism of tryptophan. IDO activity can be affected by various post-translational modifications, including phosphorylation, ubiquitination, binding of the heme cofactor, and the redox state of IDO [123, 225-230]. The LCDCs may have then

responded to the decrease in tryptophan by importing more tryptophan from the culture medium. Whatever the mechanism, ML infection caused an increase in IDO activity.

There was no significant change in most of the intracellular kynurenine pathway tryptophan catabolites between the non-stimulated and ML-infected conditions, possibly with the level of these metabolites being maintained to stay constant (Figure 10B-F, I). However, towards the end of the pathway, there were differences in catabolite levels between the conditions. The intermediate metabolites were potentially further catabolized through the pathway, accumulating at quinolinic acid.

The increase in 3-hydroxyanthranilic acid was more significant in the ML-infected LCDCs after 24 hours than after 48 hours compared to the non-stimulated LCDCs (Figure 10G). This is inversely correlated with the quinolinic acid. After 48 hours, more 3-hydroxyanthranilic acid had likely been catabolized to quinolinic acid. Further catabolism of quinolinic acid may have stalled or slowed, resulting in the accumulation of quinolinic acid. Compared to the non-stimulated, after 48 hours, there was a more substantial increase in quinolinic acid than at 24 hours in the ML-infected LCDCs (Figure 10H).

Quinolinic acid is not the only tryptophan catabolite at the end of the kynurenine pathway. Picolinic acid is also at the end of the pathway (Figure 10O). 3-hydroxyanthranilic acid is converted to 2-amino-3-carboxymuconate semialdehyde (ACMS) by 3-hydroxyanthranilate 3,4-dioxygenase (HAAO). ACMS is preferentially converted to 2-aminomuconate semialdehyde (AMS) by aminocarboxymuconate-semialdehyde decarboxylase (ASMSD) instead of to quinolinic acid in a non-enzymatic reaction. AMS is then further catabolized to picolinic acid in a non-enzymatic reaction or enters the glutarate pathway that leads to the TCA cycle. When ACMS saturates ASMSD, ACMS is non-enzymatically converted to quinolinic acid [113-115,

231-234]. Therefore, because the picolinic acid levels did not change in the LCDCs, ASMSD may have been saturated, with the rest of the ACMS being catabolized to quinolinic acid where the metabolic pathway ended and all the catabolized ACMS accumulated.

Quinolinic acid can have direct activity, primarily in the brain. Quinolinic acid is an agonist of the N-methyl-D-aspartate receptor and acts as an excitotoxin [235-237]; the neurotoxicity of this metabolite is associated with a variety of neurological diseases such as Parkinson's disease and Alzheimer's disease [235, 238-240]. The quinolinic acid in the brain is primarily produced by brain macrophages and astrocytes [241, 242]. The metabolite poorly crosses the blood-brain barrier, so quinolinic acid made elsewhere in the body, such as the skin, is less likely to have neurotoxic activity [243, 244].

Quinolinic acid is usually beneficial outside the brain as it is an essential precursor in the nicotinamide, nicotinic acid, and nicotinamide adenine dinucleotide (NAD) biosynthesis pathway [113-115]. The genes for this biosynthetic pathway are also present in ML and Mtb; these mycobacteria can also use L-aspartate as a precursor, which humans cannot [113-115]. The accumulated quinolinic acid in the LCDCs could be used for NAD biosynthesis either by the LCDCs or the ML.

In Mtb infections, quinolinic acid is also increased. IDO activity is increased in tuberculosis patients and Mtb-infected cells. In various metabolic profiles of tuberculosis patient samples, such as serum and urine, there is an increase in kynurenine pathway metabolites such as quinolinic acid [245-248]. Quinolinic acid is also increased in Mtb-infected macrophages, in which there is also an increase in NAD⁺ [131, 249]. This correlates with an increase of several host NAD biosynthesis genes, with the tryptophan metabolic pathway considered part of the NAD biosynthesis pathway by the study [249]. Mtb NAD biosynthesis genes were not evaluated.

ML can modulate host cell metabolism, shifting glucose metabolism towards the PPP to increase the production of nicotinamide adenine dinucleotide phosphate (NADPH), which further modulates the host for increased ML intracellular survival [250, 251]. ML can also generate NADPH (from NADP⁺) and NADH (from NAD⁺) during the conversion of cholesterol to cholestenone by the ML enzyme 3 β -hydroxysteroid dehydrogenase (3 β -HSD); inhibition of this enzyme decreases ML intracellular survival [252]. Whether the NADP⁺ and NAD⁺ used by 3 β -HSD are derived by the bacteria or imported from the host is unknown. However, ML clearly modulates the host and uses its own enzymes to increase reductive power, increasing the bacteria's survival.

The increase in host quinolinic acid may be part of the host modulation by the ML. With increased quinolinic acid, more NAD, NADH, nicotinamide, and nicotinic acid could be produced by the host cell or the ML. These molecules would then be available to affect the host further or for the bacteria to use, all to support the ML infection, growth, and survival. Whether ML can import quinolinic acid and use the metabolite to produce NAD, nicotinamide, and nicotinic acid has never been assessed. The biosynthesis of NAD, nicotinamide, and nicotinic acid from quinolinic acid by Mtb has been shown to occur using Mtb whole cell lysate. However, the import of quinolinic acid by Mtb is also untested [253, 254]. Future studies should assess mycobacterial and host use of quinolinic acid during infection and whether this metabolite is vital for the ML-associated reductive power change.

4.4.2 Serotonin Pathway Metabolites

The non-stimulated and ML-infected LCDCs also showed significant differences in the serotonin pathway metabolites. After 24 but not 48 hours, ML-infected LCDCs had a decrease in serotonin compared to non-stimulated LCDCs (Figure 10K). Additionally, 5-

hydroxyindoleacetic acid was increased in the ML-infected LCDCs at both time points (Figure 10L). Because the culture medium contained tryptophan and ML infection only slightly increased IDO activity, tryptophan was still available to be catabolized through the serotonin pathway. The decrease in serotonin inversely correlated with the increase in 5-hydroxyindoleacetic acid, most likely because serotonin was catabolized to 5-hydroxyindoleacetaldehyde and then further to 5-hydroxyindoleacetic acid [113-115].

No literature has been found discussing a connection between ML and 5-hydroxyindoleacetic acid, but previous studies have been conducted with *Mtb*. When metabolites in serum from active tuberculosis and healthy patients were assessed, 5-hydroxyindoleacetic acid was among the metabolites with significantly different levels; tuberculosis patients had higher 5-hydroxyindoleacetic acid levels, and 5-hydroxyindoleacetic acid was selected as one of the metabolites that could potentially be used as a biomarker for tuberculosis [248, 255]. In a longitudinal metabolomics study of urine metabolites throughout the six-month treatment of tuberculous meningitis (TBM) pediatric patients, 5-hydroxyindoleacetic acid was unique to the first sample at the start of treatment compared to the samples taken each following month. TBM is a severe form of extrapulmonary tuberculosis. 5-Hydroxyindoleacetic acid and the other seven metabolites only in this first sample represented TBM-induced metabolites [256].

Another study assessed the *Mtb* virulence factor Rv1987 using *M. smegmatis* overexpressing Rv1987 (MS1987) as a model. Mice infected with MS1987 exhibited reduced inflammatory responses and altered lung microbiomes. The dysbiosis contributed to an altered host metabolic profile and immune response, and the metabolites secreted by the altered microbiome were beneficial to MS1987. One of the metabolites that was significantly increased in the MS1987-infected mice was 5-hydroxyindoleacetic acid. 5-Hydroxyindoleacetic acid added

to the medium of empty-vector *M. smegmatis* significantly increased the growth of the bacteria, indicating that this host-made metabolite can be imported by and is beneficial to mycobacteria [257].

5-Hydroxyindoleacetic acid seems to be associated with Mtb infections and beneficial to the bacteria. However, these previous studies assessed *in vivo* metabolites and attributed the metabolite changes to dysbiosis. The increase in 5-hydroxyindoleacetic acid in the ML-infected LCDCs did not occur because of a microbiome but rather in response to the ML. How 5-hydroxyindoleacetic acid benefits mycobacteria in axenic medium, intracellularly *in vitro* or *in vivo*, remains to be elucidated. In studies with microbiome species, one effect of 5-hydroxyindoleacetic acid treatment in mice was increased PPAR γ translocation into the nucleus, indicating more active PPAR γ [258]. ML or Mtb infection activates host PPAR signaling in macrophages, which is involved in intracellular lipid accumulation and supports bacterial survival [259-261]. Increasing host-derived 5-hydroxyindoleacetic acid to activate PPAR signaling may be one of the ways mycobacteria modulate host metabolism to support their survival. However, such interactions still need to be investigated.

4.5 *M. leprae* Tryptophan Biosynthesis

The most frequently described antimicrobial mechanism of IDO is the depletion of the local tryptophan through the kynurenine metabolic pathway. The lack of available tryptophan prevents protein synthesis for tryptophan auxotrophic species [132-137]. The tryptophan prototrophs are unaffected by this IDO antimicrobial activity, as they can synthesize their own tryptophan [134, 145, 146, 207]. For species that require host tryptophan, IDO activity can be deadly, while for tryptophan prototrophs, IDO activity and the modulation of the immune

response aid in pathogen survival [122-127, 135, 136, 197]. Therefore, knowing whether the pathogen of interest is a tryptophan prototroph or auxotroph is critical.

4.5.1 Tryptophan Biosynthesis from Anthranilic Acid

ML synthesized tryptophan *in vitro* from extracellular anthranilic acid in axenic media (Figure 12F). Anthranilic acid is a catabolite of host tryptophan catabolism through the kynurenine pathway [113-115]. These data spurred the idea that if IDO is activated to deplete tryptophan to kill ML, in doing so, anthranilic acid is made; the ML could import this host-derived anthranilic acid to synthesize tryptophan and evade tryptophan-depletion killing even if the rest of the tryptophan biosynthesis pathway is not functional. This idea has not been tested with ML but would be very challenging without the ability to create knockout mutants.

However, looking at an *Mtb* study, this idea has been proven unlikely to occur with ML. Yanjia, et al. [146] made a *Mtb* *trpE* knockout; *trpE* (anthranilate synthase) converts chorismate to anthranilic acid. This knockout could not grow in axenic media without tryptophan supplementation. The growth of this knockout was compared to WT *Mtb* in macrophages. When the macrophage IDO was induced, the *trpE* knockout could not grow, indicating that the intracellular anthranilic acid could not support this mutant *Mtb*'s survival. Only when the IDO induction was paired with inhibiting IDO by 1-MT, knocking out the macrophage IDO, or supplementing with tryptophan could the *trpE* knockout survive. This study showed that tryptophan depletion kills intracellular *Mtb* if tryptophan biosynthesis is not functional, despite all the genes to import and use host anthranilic acid being present.

While mycobacteria have the ability to import and use extracellular anthranilic acid to synthesize tryptophan, the host cell intracellular levels of anthranilic acid may be too low both for the transport mechanism and for *trpD* (anthranilate phosphoribosyltransferase which converts

anthranilic acid to N-(5-Phosphoribosyl)anthranilic acid) [113-115]. In LCDCs and THP-1 cells, there were only trace amounts of anthranilic acid, much lower than other kynurenine pathway catabolites such as kynurenine and quinolinic acid. The anthranilic acid may also not be present in the phagosome where the mycobacteria are. A potential lack of tryptophan and anthranilic acid in the phagosome may explain why the tryptophan biosynthesis pathway is essential for Mtb and other intracellular pathogens' survival [146, 262]. The metabolic contents of phagosomes are not well characterized but may be critical in understanding the lifestyle of intracellular pathogens such as mycobacteria [263]. As technology evolves and the technical challenges of studying the contents of phagosomes are overcome, such an analysis may soon be possible [264-267].

4.5.2 Tryptophan Biosynthesis from Glucose or Palmitic Acid

Pathway analysis primarily based on genome sequencing revealed that ML has intact genes to synthesize tryptophan (Figure 11). However, whether these enzymes are functional remains unknown. Using ^{13}C carbon tracing in axenic media, ML synthesized tryptophan from anthranilic acid (Figure 12F) but not from glucose, erythrose, or palmitic acid (Figure 13C, D). The methods were tested with Mtb as a control, and ^{13}C tryptophan was synthesized from glucose, indicating both that the assay works and further validating that Mtb is a tryptophan prototroph (Figure 13A, B). In all the assays for both ML and Mtb, unlabeled and naturally occurring aromatic amino acids (tryptophan, phenylalanine, and tyrosine) that were not from the ^{13}C starting material were measured. When tryptophan was made from a ^{13}C starting material, the other aromatic amino acids were also ^{13}C labeled.

ML takes up and metabolizes ^{14}C glucose and ^{14}C palmitic acid to ^{14}C CO_2 in axenic media [161, 162, 165, 170, 268]. The lack of aromatic amino acid biosynthesis from the ^{13}C glucose and palmitic acid was likely not because these carbon sources were not taken up and

metabolized by the ML. However, Borah, et al. [163] showed that ML only uses intracellular glucose and not glucose in axenic media. In this current study, the published infection, heavy labeling, and fraction separation protocol from Borah, et al. [163] was used, with a different metabolite extraction and detection method. The aromatic amino acids were measured in all the fractions, but no isotopes from the ^{13}C glucose were made (data not shown). We could not reproduce the amino acid biosynthesis results of Borah, et al. [163].

Borah, et al. [163] measured glucose use by measuring amino acids synthesized by the ML from the ^{13}C glucose, unlike the ^{14}C metabolic studies of ML that measured CO_2 production from the carbon source. Borah, et al. [163] used RPMI 1640 to assess intracellular glucose use in Schwann cells and the use of glucose in axenic media. RPMI 1640 contains all the amino acids, and ML can import amino acid in axenic media rather than having to spend energy synthesizing amino acids [161, 164]. For the intracellular glucose use, the ^{13}C enrichment of the few essential amino acids measured, one being phenylalanine, in the intracellular ML fraction was relatively low, and no statistically significant difference between the fractions was mentioned [163].

The ^{13}C labeling pattern of the phenylalanine and tyrosine is intriguing in this study. Borah, et al. [163] observed that in the bacterial fraction of the infected Schwann cell samples, the M+6 ($^{13}\text{C}_6$) phenylalanine isotopologue was very abundant compared to the other isotopologues. In the host fraction of these samples, the tyrosine isotopologue profile (supplementary data figure S3 g.i) is unexpectedly different compared to the phenylalanine. In humans, tyrosine is made by adding a hydroxy group on C_7 of phenylalanine with the carbon structure not changing [269]. For the Schwann cell made tyrosine to have ^{13}C labeling from the ^{13}C glucose, the ML would have to make ^{13}C phenylalanine, which the Schwann cell would use

to make tyrosine. The ^{13}C labeling between these two amino acids would be the same, but in Borah, et al. [163], they are not.

In all the conditions tested in the current study, unlabeled aromatic amino acids were always abundant in ML. The aromatic amino acids are the most energy-intensive amino acids to synthesize, with tryptophan having the greatest energy cost [270]. Likely, ML does not need to waste energy making additional amino acids in the unfavorable *in vitro* environment and with abundant amino acids present. Therefore, even though the glucose and palmitic acid are taken up and metabolized by ML, likely no amino acids are made *in vitro* from these carbon sources. With this study, the functionality of the entire tryptophan biosynthesis pathway could not be validated.

Despite being unable to prove that ML synthesizes tryptophan conclusively, literature on other uses for chorismate, though mainly in Mtb, adds confidence to the ML genome sequencing that the chorismate biosynthetic pathway is functional. In Mtb, chorismate is used for aromatic amino acid biosynthesis but is also a precursor for 4-hydroxybenzoic acid, folate biosynthesis, mycobactin biosynthesis, and menaquinone biosynthesis [113-115, 271, 272]. Among these alternate uses, pathway analysis shows ML only has the putative gene to use chorismate to make 4-hydroxybenzoic acid (Chorismate pyruvate lyase (Mtb RV2949C; ML ML0133) [113-115, 191, 271]. In Mtb strains that make phenolic glycolipids (PGLs) and glycosylated p-hydroxybenzoic acid methyl esters (p-HBADs), 4-hydroxybenzoic acid is used to make the glycosylated aromatic nucleus in the phenolphthiocerol chain of PGLs and p-HBADs [271-276]. ML also produces PGLs and p-HBADs, likely making the phenolphthiocerol chain in the same manner as Mtb, though the function of the putative ML genes has not been validated [276-279].

If ML shares the same biosynthesis pathways of the phenolphthiocerol chain of PGL and p-HBADs as Mtb, then ML would need to have a functional chorismate biosynthesis pathway to

produce these essential ML components without having to rely on the host [271, 275, 276, 278, 280, 281]. A 4-hydroxybenzoic acid synthesis pathway in humans has been suggested but remains putative, so in humans, this molecule may be acquired only from the microbiome or diet [113-115, 282]. Therefore, most likely, the shikimate pathway, along with the glycolysis and PPP steps required to make the shikimate pathway precursors (Figure 11), was conserved by ML and remains functional to produce chorismate for PGL, p-HBAD, and aromatic amino acid biosynthesis.

Because of its importance, the shikimate pathway poses an intriguing therapeutic target. A *Mtb* mutant that lacked a step in the shikimate pathway could not grow in tryptophan-depleted axenic media [283]. A *Mtb* *trpE* mutant could not survive in macrophages, and a *trpD* *Mtb* mutant was avirulent in SCID mice, indicating that the tryptophan biosynthesis pathway is essential to *Mtb* and likely also ML [146, 149]. Additionally, the presence of PGLs and p-HBADs in *Mtb* leads to more severe disease as these molecules seem to dampen the pro-inflammatory immune response [274, 275, 281]. PGLs from other mycobacterial species also downregulate the pro-inflammatory immune response [284]. For ML specifically, some immune response modulations by the ML-specific PGL (PGL-1) are increased ML phagocytosis by monocytes and a shift of the macrophage phenotype to M2-like with less antimicrobial activity [280, 285]. Therefore, targeting the shikimate pathway with therapeutics could have multiple adverse effects on ML and mycobacteria survival. In an effort to target this pathway in *Mtb*, several inhibitors have been synthesized and tested, but no studies were found in which the ML shikimate pathway was inhibited [286, 287].

4.6 Limitations

The main limitations of this study are the LCDCs and the ML. While the LCDCs have been previously shown to be an acceptable LC model, they have characteristics that make them challenging to use. While using primary cells is beneficial to account for the heterogeneity between people, the variability in cord blood CD34+ stem cell populations likely affected the number of differentiated LCDCs harvested and the percentage of CD1a+/CD207+ LCDCs. The variability in results between experiments with LCDCs, often preventing statistically significant differences between treatments, was likely a result of the variability in the stem cells from different donors. In addition, the stem cells were very expensive, and a low number of LCDCs differentiated in each batch, preventing numerous experiments or experiments requiring many cells. Finally, the LCDCs are very sensitive cells, prone to quickly dying with harsh handling or extended incubation times. This prevented longer incubation times, especially when testing the antimicrobial of LCDCs, and the selection of CD1a+ LCDCs for the experiments. However, despite their shortcomings, of all the LC models, LCDCs are one of the easiest to establish in the laboratory as no skin is needed either to purify LCs or as an *ex vivo* model.

The other most significant limitation of this study is the ML. Because ML only grows *in vivo*, all *in vitro* experimentations have the caveat of being performed in conditions where the ML is dying, the ML does not have essential host properties, and many ML attributes may not be functional, such as amino acid biosynthesis. ML currently cannot be genetically altered, and the amount of live ML available for experiments is minimal. These drawbacks must be taken into account when interpreting and comparing ML studies. Despite these limitations, many scientific advances have been made with ML *in vitro* that aid in understanding this bacterium and its pathogenicity and disease.

4.7 Future Directions

IDO was shown not to be associated with an antimicrobial mechanism against ML in LCDCs. More experimentation is needed to establish how LCs kill pathogens, including evaluating other genes potentially involved with LC antimicrobial activity, including CCL22 and MPEG1.

A bit more work is needed to finish evaluating the potential involvement of IDO in killing ML in LCs. IDO activity in response to IFN- γ and 1-MT treatments in LCDCs must be assessed. These experiments are necessary to validate that IDO activity was modulated in the experiments that assessed ML viability in response to IDO stimulation or inhibition. To perform these experiments, less than 2.5×10^6 LCDCs per replicate must be used. While the low-abundance tryptophan metabolites will likely not be measure or only be measured as trace amounts, differences in levels of high-abundance metabolites such as tryptophan, kynurenine, and quinolinic acid will determine if IDO activity changes in response to the treatments.

Future directions from this study include assessing the implications of the tryptophan catabolite levels altered by ML infection. Quinolinic acid is increased during Mtb and ML infections, but whether the bacteria can use this host metabolite is unknown. Assessing whether these bacteria can import exogenous quinolinic acid for nicotinamide, nicotinic acid, and NAD synthesis will give insight into whether the increase in quinolinic acid benefits the bacteria. Additionally, no other studies have assessed the relationship between 5-hydroxyindoleacetic acid and ML. However, the increase of 5-hydroxyindoleacetic acid may be crucial in the modulation of host lipid metabolism by mycobacteria and must be further studied to elucidate why ML infection increases this metabolite.

CHAPTER 5: CONCLUSION

In conclusion, to test if altered metabolic processes in LCDCs impact antimicrobial effects on ML, we developed an *in vitro* model that induces CCL22, MPEG1, and IDO in LCDCs. Using this model, there was no significant effect on intracellular ML when IDO was induced or inhibited in LCDCs, unlike a previous study in macrophages that showed IDO inhibition was detrimental to ML survival. However, the IDO expression induction in LCDCs was different overall from previous studies with macrophages and mDCs; for example, in LCDCs, IDO gene expression was not induced by ML. Despite these differences, ML infection did increase IDO activity as kynurenine pathway catabolites such as quinolinic acid were increased. Also, 5-hydroxyindoleacetic acid from the serotonin metabolic pathway was increased in ML-infected LCDCs. The increased quinolinic acid and 5-hydroxyindoleacetic acid may be important for ML survival based on their involvement in NAD biosynthesis and PPAR signaling, respectively. If these tryptophan catabolites are important for ML, IDO activity would be beneficial rather than antimicrobial for ML in LCDCs, complementing the study with macrophages. However, the role of these metabolites during an ML infection remains to be elucidated.

We also assessed whether the ML tryptophan biosynthetic pathway was functional. However, *in vitro*, ML only made tryptophan from the intermediate anthranilic acid but not from the known ML carbon sources glucose or palmitic acid. Nevertheless, given that chorismate is also used in PGL biosynthesis, a key ML virulence factor, the enzymes of the shikimate pathway and those of glycolysis and the PPP are most likely functional. Therefore, only the functionality of *trpE* would remain unknown in ML.

Overall, this study has provided new insights into the role of altered tryptophan metabolic processes in LCDCs during an ML infection and identified exciting avenues for future research.

Our findings suggest that rather than having an antimicrobial impact ML in LCDs, altered tryptophan metabolic processes are more likely to benefit ML. Many new hypotheses arose for the future, with assessing the importance of quinolinic acid, 5-hydroxyindoleacetic acid, and chorismate for ML among the more intriguing future directions. These potential research directions could significantly advance our understanding of ML survival mechanisms and pave the way for novel therapeutic strategies.

REFERENCES

1. "World Health Organization - Control of Neglected Tropical Diseases (NTD) Team: Global leprosy (Hansen disease) update, 2022: new paradigm – control to elimination," in "Weekly epidemiological record," 14 September 2023 2023, vol. 37 issue 98. [Online]. Available: <https://www.who.int/publications/i/item/who-wer9837-409-430>
2. "World Health Organization: Leprosy." <https://www.who.int/news-room/fact-sheets/detail/leprosy> (accessed 2024).
3. "World Health Organization - The Global Health Observatory: Leprosy (Hansen's disease)." <https://www.who.int/data/gho/data/themes/topics/leprosy-hansens-disease> (accessed 2024).
4. D. M. Scollard, "Infection with *Mycobacterium lepromatosis*," *The American Journal of Tropical Medicine and Hygiene*, vol. 95, no. 3, pp. 500-501, 2016, doi: 10.4269/ajtmh.16-0473.
5. "National Hansen's Disease (Leprosy) Program Caring and Curing Since 1894." <https://www.hrsa.gov/hansens-disease> (accessed 2024).
6. A. Bhukhan, C. Dunn, and R. Nathoo, "Case Report of Leprosy in Central Florida, USA, 2022," *Emerging Infectious Diseases*, vol. 29, pp. 1698-1700, 2023, Art no. 8, doi: <https://doi.org/10.3201/eid2908.220367>.
7. D. S. Walsh, "Chapter 10.1. Overview of Animal Models.," *International Textbook of Leprosy*, D. M. Scollard and T. P. Gillis, Eds., Greenville, SC.: American Leprosy Missions, 2020. [Online]. Available: [https://internationaltextbookofleprosy.org/chapter/overview#:~:text=The%20ideal%20experimental%20model%20of,peripheral%20neuritis%20with%20disability%20\(see](https://internationaltextbookofleprosy.org/chapter/overview#:~:text=The%20ideal%20experimental%20model%20of,peripheral%20neuritis%20with%20disability%20(see)
8. M. T. Pena, R. Sharma, and R. W. Truman, "Chapter 10.2. The Armadillo Model for Leprosy," *International Textbook of Leprosy*, D. M. Scollard and T. P. Gillis, Eds., Greenville, SC: American Leprosy Missions, 2016. [Online]. Available: <https://internationaltextbookofleprosy.org/chapter/armadillos>
9. T. Ploemacher, W. R. Faber, H. Menke, V. Rutten, and T. Pieters, "Reservoirs and transmission routes of leprosy; A systematic review," *PLOS Neglected Tropical Diseases*, vol. 14, no. 4, p. e0008276, 2020, doi: 10.1371/journal.pntd.0008276.
10. J. Bhandari, M. Awais, B. A. Robbins, and V. Gupta, "Leprosy," *StatPearls [Internet]*/NCBI Bookshelf: Treasure Island (FL): StatPearls Publishing, Updated Sep 15 2023. [Online]. Available: <https://www.ncbi.nlm.nih.gov/books/NBK559307/>
11. R. Lahiri and L. B. Adams, "Chapter 5.3. Cultivation and Viability Determination of *Mycobacterium leprae*," *International Textbook of Leprosy*, D. M. Scollard and T. P. Gillis, Eds., Greenville, SC: American Leprosy Missions, 2016. [Online]. Available: <https://internationaltextbookofleprosy.org/chapter/assays-determining-viability-mycobacterium-leprae>
12. C. R. O'Brien, R. Malik, M. Globan, G. Reppas, C. McCowan, and J. A. Fyfe, "Feline leprosy due to *Candidatus 'Mycobacterium lepraefelis'*: Further clinical and molecular characterisation of eight previously reported cases and an additional 30 cases," *Journal of Feline Medicine and Surgery*, vol. 19, no. 9, pp. 919-932, 2017, doi: 10.1177/1098612x17706470.

13. D. Pin *et al.*, "Mycobacterium Species Related to *M. leprae* and *M. lepromatosis* from Cows with Bovine Nodular Thelitis," *Emerging Infectious Diseases*, vol. 20, no. 12, pp. 2111-2114, 2014, doi: 10.3201/eid2012.140184.
14. A. Benjak *et al.*, "Highly Reduced Genome of the New Species *Mycobacterium uberis*, the Causative Agent of Nodular Thelitis and Tuberculoid Scrotoitis in Livestock and a Close Relative of the Leprosy Bacilli," *mSphere*, vol. 3, no. 5, 2018, doi: 10.1128/msphere.00405-18.
15. A. M. Palomar *et al.*, "Isolation of known and potentially pathogenic tick-borne microorganisms from European ixodid ticks using tick cell lines," *Ticks and Tick-borne Diseases*, vol. 10, no. 3, pp. 628-638, 2019, doi: 10.1016/j.ttbdis.2019.02.008.
16. N. Tongluan *et al.*, "*Mycobacterium leprae* Infection in Ticks and Tick-Derived Cells," *Frontiers in Microbiology*, vol. 12, 2021, doi: 10.3389/fmicb.2021.761420.
17. R. O. Pinheiro *et al.*, "Innate Immune Responses in Leprosy," *Frontiers in Immunology*, vol. 9, 2018, doi: 10.3389/fimmu.2018.00518.
18. J. C. Lastória and M. A. M. M. D. Abreu, "Leprosy: review of the epidemiological, clinical, and etiopathogenic aspects - Part 1," *Anais Brasileiros de Dermatologia*, vol. 89, no. 2, pp. 205-218, 2014, doi: 10.1590/abd1806-4841.20142450.
19. C. A. M. Silva *et al.*, "Interaction of *Mycobacterium leprae* with Human Airway Epithelial Cells: Adherence, Entry, Survival, and Identification of Potential Adhesins by Surface Proteome Analysis," *Infection and Immunity*, vol. 81, no. 7, pp. 2645-2659, 2013, doi: 10.1128/iai.00147-13.
20. K. Borah, "A Current Perspective on Leprosy (Hansen's Disease)," Springer International Publishing, 2023, pp. 29-46.
21. A. Ghorpade, "Inoculation (tattoo) leprosy: a report of 31 cases," *Journal of the European Academy of Dermatology and Venereology*, vol. 16, no. 5, pp. 494-499, 2002, doi: 10.1046/j.1468-3083.2002.00548.x.
22. L. G. Patrocã-nio, I. M. B. Goulart, L. R. Goulart, J. A. Patrocã-nio, F. R. Ferreira, and R. N. Fleury, "Detection of *Mycobacterium leprae* in nasal mucosa biopsies by the polymerase chain reaction," *FEMS Immunology & Medical Microbiology*, vol. 44, no. 3, pp. 311-316, 2005, doi: 10.1016/j.femsim.2005.01.002.
23. V. B. Fadlitha *et al.*, "The unique tropism of *Mycobacterium leprae* to the nasal epithelial cells can be explained by the mammalian cell entry protein 1A," *PLOS Neglected Tropical Diseases*, vol. 13, no. 3, p. e0006704, 2019, doi: 10.1371/journal.pntd.0006704.
24. A. A. Dias *et al.*, "TLR-9 Plays a Role in *Mycobacterium leprae*-Induced Innate Immune Activation of A549 Alveolar Epithelial Cells," *Frontiers in Immunology*, vol. 12, 2021, doi: 10.3389/fimmu.2021.657449.
25. A. C. McDougall, R. J. W. Rees, A. G. M. Weddell, and M. Wajdi Kanan, "The histopathology of lepromatous leprosy in the nose," *The Journal of Pathology*, vol. 115, no. 4, pp. 215-226, 1975, doi: 10.1002/path.1711150406.
26. M. C. Vidal Pessolani, M. A. De Melo Marques, V. M. Reddy, C. Loch, and F. D. Menozzi, "Systemic dissemination in tuberculosis and leprosy: do mycobacterial adhesins play a role?," *Microbes and Infection*, vol. 5, no. 7, pp. 677-684, 2003, doi: 10.1016/s1286-4579(03)00098-4.
27. S. Araujo, L. O. Freitas, L. R. Goulart, and I. M. B. Goulart, "Molecular Evidence for the Aerial Route of Infection of *Mycobacterium leprae* and the Role of Asymptomatic

- Carriers in the Persistence of Leprosy," *Clinical Infectious Diseases*, vol. 63, no. 11, pp. 1412-1420, 2016, doi: 10.1093/cid/ciw570.
28. P. E. M. Fine, "LEPROSY: THE EPIDEMIOLOGY OF A SLOW BACTERIUM," *Epidemiologic Reviews*, vol. 4, no. 1, pp. 161-188, 1982, doi: 10.1093/oxfordjournals.epirev.a036245.
 29. G. Coruh and A. C. McDougall, "Untreated lepromatous leprosy: histopathological findings in cutaneous blood vessels," (in eng), *Int J Lepr Other Mycobact Dis*, vol. 47, no. 3, pp. 500-511, 1979/09// 1979. [Online]. Available: <http://europepmc.org/abstract/MED/573750>.
 30. D. M. Scollard, G. McCormick, and J. L. Allen, "Localization of *Mycobacterium leprae* to Endothelial Cells of Epineurial and Perineurial Blood Vessels and Lymphatics," *The American Journal of Pathology*, vol. 154, no. 5, pp. 1611-1620, 1999, doi: 10.1016/s0002-9440(10)65414-4.
 31. Y. Wen, H.-Y. Li, J. Liu, Y. Zhang, Y. Xing, and L.-C. Yuan, "Whole-Blood Nested-PCR Amplification of *M. leprae*-Specific DNA for Early Diagnosis of Leprosy," *The American Journal of Tropical Medicine and Hygiene*, vol. 88, no. 5, pp. 918-922, 2013, doi: 10.4269/ajtmh.11-0253.
 32. E. A. Misch, W. R. Berrington, J. C. Vary, and T. R. Hawn, "Leprosy and the Human Genome," *Microbiology and Molecular Biology Reviews*, vol. 74, no. 4, pp. 589-620, 2010, doi: 10.1128/mmbr.00025-10.
 33. M. Sugawara-Mikami *et al.*, "Pathogenicity and virulence of *Mycobacterium leprae*," *Virulence*, vol. 13, no. 1, pp. 1985-2011, 2022, doi: 10.1080/21505594.2022.2141987.
 34. J. R. De Sousa, M. N. Sotto, and J. A. Simões Quaresma, "Leprosy As a Complex Infection: Breakdown of the Th1 and Th2 Immune Paradigm in the Immunopathogenesis of the Disease," *Frontiers in Immunology*, vol. 8, 2017, doi: 10.3389/fimmu.2017.01635.
 35. D. Montoya and R. L. Modlin, "Learning from Leprosy," Elsevier, 2010, pp. 1-24.
 36. J. Kibbie *et al.*, "Jagged1 Instructs Macrophage Differentiation in Leprosy," *PLOS Pathogens*, vol. 12, no. 8, p. e1005808, 2016, doi: 10.1371/journal.ppat.1005808.
 37. D. Montoya *et al.*, "Divergence of Macrophage Phagocytic and Antimicrobial Programs in Leprosy," *Cell Host & Microbe*, vol. 6, no. 4, pp. 343-353, 2009, doi: 10.1016/j.chom.2009.09.002.
 38. M. Yamamura *et al.*, "Defining Protective Responses to Pathogens: Cytokine Profiles in Leprosy Lesions," *Science*, vol. 254, no. 5029, pp. 277-279, 1991, doi: 10.1126/science.1925582.
 39. D. Jullien, P. A. Sieling, K. Uyemura, N. D. Mar, T. H. Rea, and R. L. Modlin, "IL-15, an immunomodulator of T cell responses in intracellular infection," *The Journal of Immunology*, vol. 158, no. 2, pp. 800-806, 1997, doi: 10.4049/jimmunol.158.2.800.
 40. S. Kamath, S. A. Vaccaro, T. H. Rea, and M. T. Ochoa, "Recognizing and managing the immunologic reactions in leprosy," *Journal of the American Academy of Dermatology*, vol. 71, no. 4, pp. 795-803, 2014, doi: 10.1016/j.jaad.2014.03.034.
 41. E. Negera *et al.*, "Clinico-pathological features of erythema nodosum leprosum: A case-control study at ALERT hospital, Ethiopia," *PLOS Neglected Tropical Diseases*, vol. 11, no. 10, p. e0006011, 2017, doi: 10.1371/journal.pntd.0006011.
 42. T. H. Rea, "Lucio's Phenomenon and Diffuse Nonnodular Lepromatous Leprosy," *Archives of Dermatology*, vol. 114, no. 7, p. 1023, 1978, doi: 10.1001/archderm.1978.01640190011004.

43. C. A. Renault and J. D. Ernst, "*Mycobacterium leprae* (Leprosy)," Elsevier, 2015, pp. 2819-2831.e2.
44. L. C. Davies and P. R. Taylor, "Tissue-resident macrophages: then and now," *Immunology*, vol. 144, no. 4, pp. 541-548, 2015, doi: 10.1111/imm.12451.
45. S. Gordon and A. Plüddemann, "Tissue macrophages: heterogeneity and functions," *BMC Biology*, vol. 15, no. 1, 2017, doi: 10.1186/s12915-017-0392-4.
46. D. Hashimoto *et al.*, "Tissue-Resident Macrophages Self-Maintain Locally throughout Adult Life with Minimal Contribution from Circulating Monocytes," *Immunity*, vol. 38, no. 4, pp. 792-804, 2013, doi: 10.1016/j.immuni.2013.04.004.
47. C. Frehel and N. Rastogi, "*Mycobacterium leprae* surface components intervene in the early phagosome-lysosome fusion inhibition event," *Infection and Immunity*, vol. 55, no. 12, pp. 2916-2921, 1987, doi: 10.1128/iai.55.12.2916-2921.1987.
48. A. T. Dang *et al.*, "Autophagy links antimicrobial activity with antigen presentation in Langerhans cells," *JCI Insight*, vol. 4, no. 8, 2019, doi: 10.1172/jci.insight.126955.
49. F. A. W. Verreck *et al.*, "Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria," *Proceedings of the National Academy of Sciences*, vol. 101, no. 13, pp. 4560-4565, 2004, doi: 10.1073/pnas.0400983101.
50. R. E. Hunger *et al.*, "Langerhans cells utilize CD1a and langerin to efficiently present nonpeptide antigens to T cells," vol. 113, no. 5, pp. 701-708, 2004, doi: 10.1172/jci19655.
51. A. T. Dang *et al.*, "IL-26 contributes to host defense against intracellular bacteria," *Journal of Clinical Investigation*, vol. 129, no. 5, pp. 1926-1939, 2019, doi: 10.1172/jci99550.
52. L. R. Batista-Silva *et al.*, "*Mycobacterium leprae*-induced Insulin-like Growth Factor I attenuates antimicrobial mechanisms, promoting bacterial survival in macrophages," *Scientific Reports*, vol. 6, no. 1, p. 27632, 2016, doi: 10.1038/srep27632.
53. A. Miranda *et al.*, "Increased Langerhans cell accumulation after mycobacterial stimuli," *Histopathology*, vol. 51, no. 5, pp. 649-656, 2007, doi: 10.1111/j.1365-2559.2007.02848.x.
54. N. Bouhamdani, D. Comeau, and S. Turcotte, "A Compendium of Information on the Lysosome," *Frontiers in Cell and Developmental Biology*, vol. 9, 2021, doi: 10.3389/fcell.2021.798262.
55. S. Alonso, K. Pethe, D. G. Russell, and G. E. Purdy, "Lysosomal killing of *Mycobacterium* mediated by ubiquitin-derived peptides is enhanced by autophagy," *Proceedings of the National Academy of Sciences*, vol. 104, no. 14, pp. 6031-6036, 2007, doi: 10.1073/pnas.0700036104.
56. R. Van Harten, E. Van Woudenberg, A. Van Dijk, and H. Haagsman, "Cathelicidins: Immunomodulatory Antimicrobials," *Vaccines*, vol. 6, no. 3, p. 63, 2018, doi: 10.3390/vaccines6030063.
57. E. Fithian, P. Kung, G. Goldstein, M. Rubinfeld, C. Fenoglio, and R. Edelson, "Reactivity of Langerhans cells with hybridoma antibody," *Proceedings of the National Academy of Sciences*, vol. 78, no. 4, pp. 2541-2544, 1981, doi: 10.1073/pnas.78.4.2541.
58. J. Valladeau *et al.*, "The monoclonal antibody DCGM4 recognizes Langerin, a protein specific of Langerhans cells, and is rapidly internalized from the cell surface," *European Journal of Immunology*, vol. 29, no. 9, pp. 2695-2704, 1999, doi: 10.1002/(sici)1521-4141(199909)29:09<2695::aid-immu2695>3.0.co;2-q.

59. J. Valladeau *et al.*, "Langerin, a Novel C-Type Lectin Specific to Langerhans Cells, Is an Endocytic Receptor that Induces the Formation of Birbeck Granules," *Immunity*, vol. 12, no. 1, pp. 71-81, 2000, doi: 10.1016/s1074-7613(00)80160-0.
60. F. Ronchese, K. L. Hilligan, and J. U. Mayer, "Dendritic cells and the skin environment," *Current Opinion in Immunology*, vol. 64, pp. 56-62, 2020, doi: 10.1016/j.coi.2020.03.006.
61. M. Otsuka, G. Egawa, and K. Kabashima, "Uncovering the Mysteries of Langerhans Cells, Inflammatory Dendritic Epidermal Cells, and Monocyte-Derived Langerhans Cell-Like Cells in the Epidermis," *Frontiers in Immunology*, vol. 9, 2018, doi: 10.3389/fimmu.2018.01768.
62. T. Doebel, B. Voisin, and K. Nagao, "Langerhans Cells – The Macrophage in Dendritic Cell Clothing," *Trends in Immunology*, vol. 38, no. 11, pp. 817-828, 2017, doi: 10.1016/j.it.2017.06.008.
63. G. Hoeffel *et al.*, "Adult Langerhans cells derive predominantly from embryonic fetal liver monocytes with a minor contribution of yolk sac-derived macrophages," *Journal of Experimental Medicine*, vol. 209, no. 6, pp. 1167-1181, 2012, doi: 10.1084/jem.20120340.
64. G. Hoeffel *et al.*, "C-Myb+ Erythro-Myeloid Progenitor-Derived Fetal Monocytes Give Rise to Adult Tissue-Resident Macrophages," *Immunity*, vol. 42, no. 4, pp. 665-678, 2015, doi: 10.1016/j.immuni.2015.03.011.
65. K. Seré *et al.*, "Two Distinct Types of Langerhans Cells Populate the Skin during Steady State and Inflammation," *Immunity*, vol. 37, no. 5, pp. 905-916, 2012, doi: 10.1016/j.immuni.2012.07.019.
66. M. Haniffa, M. Gunawan, and L. Jardine, "Human skin dendritic cells in health and disease," *Journal of Dermatological Science*, vol. 77, no. 2, pp. 85-92, 2015, doi: 10.1016/j.jdermsci.2014.08.012.
67. K. Yoshida *et al.*, "Distinct behavior of human Langerhans cells and inflammatory dendritic epidermal cells at tight junctions in patients with atopic dermatitis," *Journal of Allergy and Clinical Immunology*, vol. 134, no. 4, pp. 856-864, 2014, doi: 10.1016/j.jaci.2014.08.001.
68. J. Seneschal, Rachael, A. Gehad, Clare, and Thomas, "Human Epidermal Langerhans Cells Maintain Immune Homeostasis in Skin by Activating Skin Resident Regulatory T Cells," *Immunity*, vol. 36, no. 5, pp. 873-884, 2012, doi: 10.1016/j.immuni.2012.03.018.
69. K. Clayton, A. F. Vallejo, J. Davies, S. Sirvent, and M. E. Polak, "Langerhans Cells—Programmed by the Epidermis," *Frontiers in Immunology*, vol. 8, 2017, doi: 10.3389/fimmu.2017.01676.
70. C. Caux *et al.*, "CD34+ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to GM-CSF+TNF α ," *The Journal of experimental medicine*, vol. 184, no. 2, pp. 695-706, 1996, doi: 10.1084/jem.184.2.695.
71. S. Jaksits, E. Kriehuber, A. S. Charbonnier, K. Rappersberger, G. Stingl, and D. Maurer, "CD34+ Cell-Derived CD14+ Precursor Cells Develop into Langerhans Cells in a TGF- β 1-Dependent Manner," *The Journal of Immunology*, vol. 163, no. 9, pp. 4869-4877, 1999, doi: 10.4049/jimmunol.163.9.4869.

72. M. Chopin *et al.*, "Langerhans cells are generated by two distinct PU.1-dependent transcriptional networks," *Journal of Experimental Medicine*, vol. 210, no. 13, pp. 2967-2980, 2013, doi: 10.1084/jem.20130930.
73. A. Oulee *et al.*, "Identification of Genes Encoding Antimicrobial Proteins in Langerhans Cells," *Frontiers in Immunology*, vol. 12, 2021, doi: 10.3389/fimmu.2021.695373.
74. F. Ma *et al.*, "The cellular architecture of the antimicrobial response network in human leprosy granulomas," *Nature Immunology*, vol. 22, no. 7, pp. 839-850, 2021, doi: 10.1038/s41590-021-00956-8.
75. F. Ma *et al.*, "Single Cell and Spatial Transcriptomics Defines the Cellular Architecture of the Antimicrobial Response Network in Human Leprosy Granulomas," Cold Spring Harbor Laboratory, 2020.
76. A. K. Abbas, A. H. Lichtman, and S. Pillai, *Cellular and Molecular Immunology*, 9 ed. Elsevier, 2017.
77. NCBI.NLM.NIH.gov. CCL22 C-C motif chemokine ligand 22 [Homo sapiens (human)] [Online] Available: <https://www.ncbi.nlm.nih.gov/gene/6367#gene-expression>.
78. O. Y. Elhussein Mohamed, A. Elazomi, M. S. Mohamed, and F. B. Abdalla, "Local elevation of CCL22: A new trend in immunotherapy (skin model)," *Journal of Cellular Immunotherapy*, vol. 2, no. 2, pp. 79-84, 2016, doi: 10.1016/j.jocit.2015.12.001.
79. Z. R. Korobova, N. A. Arsentieva, and A. A. Totolian, "Macrophage-Derived Chemokine MDC/CCL22: An Ambiguous Finding in COVID-19," *International Journal of Molecular Sciences*, vol. 24, no. 17, p. 13083, 2023, doi: 10.3390/ijms241713083.
80. H. L. Tang and J. G. Cyster, "Chemokine Up-Regulation and Activated T Cell Attraction by Maturing Dendritic Cells," *Science*, vol. 284, no. 5415, pp. 819-822, 1999, doi: 10.1126/science.284.5415.819.
81. D. P. Andrew *et al.*, "STCP-1 (MDC) CC Chemokine Acts Specifically on Chronically Activated Th2 Lymphocytes and Is Produced by Monocytes on Stimulation with Th2 Cytokines IL-4 and IL-13," *The Journal of Immunology*, vol. 161, no. 9, pp. 5027-5038, 1998, doi: 10.4049/jimmunol.161.9.5027.
82. T. Hirota *et al.*, "Variants of C-C Motif Chemokine 22 (CCL22) Are Associated with Susceptibility to Atopic Dermatitis: Case-Control Studies," *PLoS ONE*, vol. 6, no. 11, p. e26987, 2011, doi: 10.1371/journal.pone.0026987.
83. Y. H. Huang *et al.*, "Cancer-associated fibroblast-derived interleukin-1 β activates protumor C-C motif chemokine ligand 22 signaling in head and neck cancer," *Cancer Sci*, vol. 110, no. 9, pp. 2783-2793, Sep 2019, doi: 10.1111/cas.14135.
84. G. M. Wiedemann *et al.*, "Cancer cell-derived IL-1 α induces CCL22 and the recruitment of regulatory T cells," (in eng), *Oncoimmunology*, vol. 5, no. 9, p. e1175794, 2016, doi: 10.1080/2162402x.2016.1175794.
85. B. Liu *et al.*, "Affinity-coupled CCL22 promotes positive selection in germinal centres," *Nature*, vol. 592, no. 7852, pp. 133-137, 2021/04/01 2021, doi: 10.1038/s41586-021-03239-2.
86. L. Lin, S. Nonoyama, A. Oshiba, Y. Kabasawa, and S. Mizutani, "TARC and MDC are produced by CD40 activated human B cells and are elevated in the sera of infantile atopic dermatitis patients," (in eng), *J Med Dent Sci*, vol. 50, no. 1, pp. 27-33, Mar 2003.
87. M. Inoue *et al.*, "SCRG1 suppresses LPS-induced CCL22 production through ERK1/2 activation in mouse macrophage Raw264.7 cells," *Mol Med Rep*, vol. 15, no. 6, pp. 4069-4076, 2017/06/01 2017, doi: 10.3892/mmr.2017.6492.

88. D. Yang *et al.*, "Many chemokines including CCL20/MIP-3 α display antimicrobial activity," *Journal of Leukocyte Biology*, vol. 74, no. 3, pp. 448-455, 2003, doi: 10.1189/jlb.0103024.
89. R. Domingo-Gonzalez, O. Prince, A. Cooper, and S. A. Khader, "Cytokines and Chemokines in *Mycobacterium tuberculosis* Infection," *Microbiology Spectrum*, vol. 4, no. 5, pp. 33-72, 2016, doi: 10.1128/microbiolspec.tb2-0018-2016.
90. M. A. Arias *et al.*, "*Mycobacterium tuberculosis* Antigens Specifically Modulate CCR2 and MCP-1/CCL2 on Lymphoid Cells from Human Pulmonary Hilar Lymph Nodes," *The Journal of Immunology*, vol. 179, no. 12, pp. 8381-8391, 2007, doi: 10.4049/jimmunol.179.12.8381.
91. S. R. Slight and S. A. Khader, "Chemokines shape the immune responses to tuberculosis," *Cytokine & Growth Factor Reviews*, vol. 24, no. 2, pp. 105-113, 2013, doi: 10.1016/j.cytogfr.2012.10.002.
92. C. Wu, Q. Zhou, X. J. Qin, S. M. Qin, and H. Z. Shi, "CCL22 is involved in the recruitment of CD4⁺CD25^{high} T cells into tuberculous pleural effusions," *Respirology*, vol. 15, no. 3, pp. 522-529, 2010, doi: 10.1111/j.1440-1843.2010.01719.x.
93. R. M. McCormack *et al.*, "MPEG1/perforin-2 mutations in human pulmonary nontuberculous mycobacterial infections," *JCI Insight*, vol. 2, no. 8, 2017, doi: 10.1172/jci.insight.89635.
94. C. Bayly-Jones, S. S. Pang, B. A. Spicer, J. C. Whisstock, and M. A. Dunstone, "Ancient but Not Forgotten: New Insights Into MPEG1, a Macrophage Perforin-Like Immune Effector," *Frontiers in Immunology*, vol. 11, 2020, doi: 10.3389/fimmu.2020.581906.
95. M. E. D'Angelo, M. A. Dunstone, J. C. Whisstock, J. A. Trapani, and P. I. Bird, "Perforin evolved from a gene duplication of MPEG1, followed by a complex pattern of gene gain and loss within Euteleostomi," *BMC Evolutionary Biology*, vol. 12, no. 1, p. 59, 2012, doi: 10.1186/1471-2148-12-59.
96. R. M. McCormack *et al.*, "Perforin-2 is essential for intracellular defense of parenchymal cells and phagocytes against pathogenic bacteria," *eLife*, vol. 4, 2015, doi: 10.7554/elife.06508.
97. R. McCormack, L. R. de Armas, M. Shiratsuchi, J. E. Ramos, and E. R. Podack, "Inhibition of intracellular bacterial replication in fibroblasts is dependent on the perforin-like protein (perforin-2) encoded by macrophage-expressed gene 1," (in eng), *J Innate Immun*, vol. 5, no. 2, pp. 185-94, 2013, doi: 10.1159/000345249.
98. R. M. McCormack, K. Lyapichev, M. L. Olsson, E. R. Podack, and G. P. Munson, "Enteric pathogens deploy cell cycle inhibiting factors to block the bactericidal activity of Perforin-2," *eLife*, vol. 4, 2015, doi: 10.7554/elife.06505.
99. P. Xiong *et al.*, "Regulation of expression and trafficking of perforin-2 by LPS and TNF- α ," *Cellular Immunology*, vol. 320, pp. 1-10, 2017, doi: 10.1016/j.cellimm.2017.07.001.
100. F. Bai, R. M. McCormack, S. Hower, G. V. Plano, M. G. Lichtenheld, and G. P. Munson, "Perforin-2 Breaches the Envelope of Phagocytosed Bacteria Allowing Antimicrobial Effectors Access to Intracellular Targets," *The Journal of Immunology*, vol. 201, no. 9, pp. 2710-2720, 2018, doi: 10.4049/jimmunol.1800365.
101. D. J. Klionsky, E.-L. Eskelinen, and V. Deretic, "Autophagosomes, phagosomes, autolysosomes, phagolysosomes, autophagolysosomes... Wait, I'm confused," *Autophagy*, vol. 10, no. 4, pp. 549-551, 2014, doi: 10.4161/auto.28448.

102. I. Tanida, T. Ueno, and E. Kominami, "LC3 and Autophagy," Humana Press, 2008, pp. 77-88.
103. F. Taieb, J.-P. Nougayrède, and E. Oswald, "Cycle Inhibiting Factors (Cifs): Cyclomodulins That Usurp the Ubiquitin-Dependent Degradation Pathway of Host Cells," *Toxins*, vol. 3, no. 4, pp. 356-368, 2011, doi: 10.3390/toxins3040356.
104. A. Pisano *et al.*, "Revisiting Bacterial Ubiquitin Ligase Effectors: Weapons for Host Exploitation," *International Journal of Molecular Sciences*, vol. 19, no. 11, p. 3576, 2018, doi: 10.3390/ijms19113576.
105. S. Ebrahimnezhaddarzi *et al.*, "Mpeg1 is not essential for antibacterial or antiviral immunity, but is implicated in antigen presentation," *Immunology & Cell Biology*, vol. 100, no. 7, pp. 529-546, 2022, doi: 10.1111/imcb.12554.
106. K. L. Rock and L. Shen, "Cross-presentation: underlying mechanisms and role in immune surveillance," *Immunological Reviews*, vol. 207, no. 1, pp. 166-183, 2005, doi: 10.1111/j.0105-2896.2005.00301.x.
107. P. Rodríguez-Silvestre *et al.*, "Perforin-2 is a pore-forming effector of endocytic escape in cross-presenting dendritic cells," *Science*, vol. 380, no. 6651, pp. 1258-1265, 2023, doi: 10.1126/science.adg8802.
108. G. Ferrero *et al.*, "The macrophage-expressed gene (*mpeg 1*) identifies a subpopulation of B cells in the adult zebrafish," *Journal of Leukocyte Biology*, vol. 107, no. 3, pp. 431-443, 2020, doi: 10.1002/jlb.1a1119-223r.
109. X. Lin, Q. Zhou, C. Zhao, G. Lin, J. Xu, and Z. Wen, "An Ectoderm-Derived Myeloid-like Cell Population Functions as Antigen Transporters for Langerhans Cells in Zebrafish Epidermis," *Developmental Cell*, vol. 49, no. 4, pp. 605-617.e5, 2019, doi: 10.1016/j.devcel.2019.03.028.
110. E. Peterman *et al.*, "Zebrafish cutaneous injury models reveal that Langerhans cells engulf axonal debris in adult epidermis," *Disease Models & Mechanisms*, vol. 16, no. 4, 2023, doi: 10.1242/dmm.049911.
111. E. Peterman, E. J. A. Quitevis, C. E. A. Goo, and J. P. Rasmussen, "Rho-associated kinase regulates Langerhans cell morphology and responsiveness to tissue damage," Cold Spring Harbor Laboratory, 2023.
112. NCBI.NLM.NIH.gov. IDO1 indoleamine 2,3-dioxygenase 1 [Homo sapiens (human)] [Online] Available: [https://www.ncbi.nlm.nih.gov/gene/3620#:~:text=Indoleamine%20%2C3%2Ddioxygenase%2D1%20\(IDO1\)%20enhances,the%20pathogenesis%20of%20pre%2Declampsia](https://www.ncbi.nlm.nih.gov/gene/3620#:~:text=Indoleamine%20%2C3%2Ddioxygenase%2D1%20(IDO1)%20enhances,the%20pathogenesis%20of%20pre%2Declampsia).
113. M. Kanehisa, "KEGG: Kyoto Encyclopedia of Genes and Genomes," *Nucleic Acids Research*, vol. 28, no. 1, pp. 27-30, 2000, doi: 10.1093/nar/28.1.27.
114. M. Kanehisa, "Toward understanding the origin and evolution of cellular organisms," *Protein Science*, vol. 28, no. 11, pp. 1947-1951, 2019, doi: 10.1002/pro.3715.
115. M. Kanehisa, M. Furumichi, Y. Sato, M. Kawashima, and M. Ishiguro-Watanabe, "KEGG for taxonomy-based analysis of pathways and genomes," *Nucleic Acids Research*, vol. 51, no. D1, pp. D587-D592, 2023, doi: 10.1093/nar/gkac963.
116. M. T. Pallotta *et al.*, "Indoleamine 2,3-dioxygenase 1 (IDO1): an up-to-date overview of an eclectic immunoregulatory enzyme," *The FEBS Journal*, vol. 289, no. 20, pp. 6099-6118, 2022, doi: 10.1111/febs.16086.

117. I. Théate *et al.*, "Extensive Profiling of the Expression of the Indoleamine 2,3-Dioxygenase 1 Protein in Normal and Tumoral Human Tissues," *Cancer Immunology Research*, vol. 3, no. 2, pp. 161-172, 2015, doi: 10.1158/2326-6066.cir-14-0137.
118. H. J. Ball *et al.*, "Characterization of an indoleamine 2,3-dioxygenase-like protein found in humans and mice," *Gene*, vol. 396, no. 1, pp. 203-213, 2007, doi: 10.1016/j.gene.2007.04.010.
119. R. Metz *et al.*, "IDO2 is critical for IDO1-mediated T-cell regulation and exerts a non-redundant function in inflammation," *International Immunology*, vol. 26, no. 7, pp. 357-367, 2014, doi: 10.1093/intimm/dxt073.
120. F. F. Jusof *et al.*, "Investigation of the Tissue Distribution and Physiological Roles of Indoleamine 2,3-Dioxygenase-2," *International Journal of Tryptophan Research*, vol. 10, p. 117864691773509, 2017, doi: 10.1177/1178646917735098.
121. M. Kanai *et al.*, "Tryptophan 2,3-dioxygenase is a key modulator of physiological neurogenesis and anxiety-related behavior in mice," *Molecular Brain*, vol. 2, no. 1, p. 8, 2009, doi: 10.1186/1756-6606-2-8.
122. F. Schramme *et al.*, "Inhibition of Tryptophan-Dioxygenase Activity Increases the Antitumor Efficacy of Immune Checkpoint Inhibitors," *Cancer Immunology Research*, vol. 8, no. 1, pp. 32-45, 2020, doi: 10.1158/2326-6066.cir-19-0041.
123. M. T. Nelp, V. Zheng, K. M. Davis, W. K. J. Stiefel, and J. T. Groves, "Potent Activation of Indoleamine 2,3-Dioxygenase by Polysulfides," *Journal of the American Chemical Society*, vol. 141, no. 38, pp. 15288-15300, 2019, doi: 10.1021/jacs.9b07338.
124. M. Platten, E. A. A. Nollen, U. F. Röhrig, F. Fallarino, and C. A. Opitz, "Tryptophan metabolism as a common therapeutic target in cancer, neurodegeneration and beyond," *Nature Reviews Drug Discovery*, vol. 18, no. 5, pp. 379-401, 2019, doi: 10.1038/s41573-019-0016-5.
125. S. R. Hennigar and J. P. McClung, "Nutritional Immunity: Starving Pathogens of Trace Minerals," *American Journal of Lifestyle Medicine*, vol. 10, no. 3, pp. 170-173, 2016, doi: 10.1177/1559827616629117.
126. G. Núñez, K. Sakamoto, and M. P. Soares, "Innate Nutritional Immunity," *The Journal of Immunology*, vol. 201, no. 1, pp. 11-18, 2018, doi: 10.4049/jimmunol.1800325.
127. C. Healy *et al.*, "Nutritional immunity: the impact of metals on lung immune cells and the airway microbiome during chronic respiratory disease," (in eng), *Respir Res*, vol. 22, no. 1, p. 133, Apr 29 2021, doi: 10.1186/s12931-021-01722-y.
128. S.-K. Seo and B. Kwon, "Immune regulation through tryptophan metabolism," *Experimental & Molecular Medicine*, vol. 55, no. 7, pp. 1371-1379, 2023/07/01 2023, doi: 10.1038/s12276-023-01028-7.
129. C. Munteanu and B. Schwartz, "The relationship between nutrition and the immune system," *Frontiers in Nutrition*, vol. 9, 2022, doi: 10.3389/fnut.2022.1082500.
130. S. Cunningham-Rundles, D. F. McNeeley, and A. Moon, "Mechanisms of nutrient modulation of the immune response," *Journal of Allergy and Clinical Immunology*, vol. 115, no. 6, pp. 1119-1128, 2005, doi: 10.1016/j.jaci.2005.04.036.
131. A. Blumenthal *et al.*, "M. tuberculosis Induces Potent Activation of IDO-1, but This Is Not Essential for the Immunological Control of Infection," *PLOS ONE*, vol. 7, no. 5, p. e37314, 2012, doi: 10.1371/journal.pone.0037314.

132. A. Niño-Castro *et al.*, "The IDO1-induced kynurenines play a major role in the antimicrobial effect of human myeloid cells against *Listeria monocytogenes*," *Innate Immunity*, vol. 20, no. 4, pp. 401-411, 2014, doi: 10.1177/1753425913496442.
133. S. Fujigaki *et al.*, "L-tryptophan-L-Kynurenine Pathway Metabolism Accelerated by *Toxoplasma gondii* Infection Is Abolished in Gamma Interferon-Gene-Deficient Mice: Cross-Regulation between Inducible Nitric Oxide Synthase and Indoleamine-2,3-Dioxygenase," *Infection and Immunity*, vol. 70, no. 2, pp. 779-786, 2002, doi: 10.1128/iai.70.2.779-786.2002.
134. S. V. Schmidt and J. L. Schultze, "New Insights into IDO Biology in Bacterial and Viral Infections," *Frontiers in Immunology*, vol. 5, 2014, doi: 10.3389/fimmu.2014.00384.
135. J. M. Carlin, E. C. Borden, and G. I. Byrne, "Interferon-Induced Indoleamine 2,3-Dioxygenase Activity Inhibits *Chlamydia psittaci* Replication in Human Macrophages," *Journal of Interferon Research*, vol. 9, no. 3, pp. 329-337, 1989, doi: 10.1089/jir.1989.9.329.
136. K. Peng and D. M. Monack, "Indoleamine 2,3-Dioxygenase 1 Is a Lung-Specific Innate Immune Defense Mechanism That Inhibits Growth of *Francisella tularensis* Tryptophan Auxotrophs," *Infection and Immunity*, vol. 78, no. 6, pp. 2723-2733, 2010, doi: 10.1128/iai.00008-10.
137. C. R. Mackenzie, C. B. Willberg, and W. Däubener, "Inhibition of group B streptococcal growth by IFN γ -activated human glioblastoma cells," *Journal of Neuroimmunology*, vol. 89, no. 1-2, pp. 191-197, 1998, doi: 10.1016/s0165-5728(98)00138-6.
138. M. A. G. Rabbani and S. Barik, "5-Hydroxytryptophan, a major product of tryptophan degradation, is essential for optimal replication of human parainfluenza virus," *Virology*, vol. 503, pp. 46-51, 2017, doi: 10.1016/j.virol.2017.01.007.
139. F. J. H. Sorgdrager, P. J. W. Naudé, I. P. Kema, E. A. Nollen, and P. P. D. Deyn, "Tryptophan Metabolism in Inflammation: From Biomarker to Therapeutic Target," *Frontiers in Immunology*, vol. 10, 2019, doi: 10.3389/fimmu.2019.02565.
140. J. Davies *et al.*, "Transcriptional programming of immunoregulatory responses in human Langerhans cells," *Frontiers in Immunology*, vol. 13, 2022, doi: 10.3389/fimmu.2022.892254.
141. J. Davies *et al.*, "Langerhans cells immunocompetency is critical for IDO1-dependent ability to induce tolerogenic T cells," Cold Spring Harbor Laboratory, 2019.
142. A. Staudacher, T. Hinz, N. Novak, D. Von Bubnoff, and T. Bieber, "Exaggerated IDO1 expression and activity in Langerhans cells from patients with atopic dermatitis upon viral stimulation: a potential predictive biomarker for high risk of Eczema herpeticum," *Allergy*, vol. 70, no. 11, pp. 1432-1439, 2015, doi: 10.1111/all.12699.
143. J. A. Loughman and D. A. Hunstad, "Induction of Indoleamine 2,3-Dioxygenase by Uropathogenic Bacteria Attenuates Innate Responses to Epithelial Infection," *The Journal of Infectious Diseases*, vol. 205, no. 12, pp. 1830-1839, 2012, doi: 10.1093/infdis/jis280.
144. A. Boasso and G. M. Shearer, "How Does Indoleamine 2,3-Dioxygenase Contribute to HIV-Mediated Immune Dysregulation," *Current Drug Metabolism*, vol. 8, no. 3, pp. 217-223, 2007, doi: DOI: 10.2174/138920007780362527.
145. A. Boasso *et al.*, "HIV inhibits CD4⁺ T-cell proliferation by inducing indoleamine 2,3-dioxygenase in plasmacytoid dendritic cells," *Blood*, vol. 109, no. 8, pp. 3351-3359, 2007, doi: 10.1182/blood-2006-07-034785.

146. Yanjia *et al.*, "Tryptophan Biosynthesis Protects Mycobacteria from CD4 T-Cell-Mediated Killing," *Cell*, vol. 155, no. 6, pp. 1296-1308, 2013, doi: 10.1016/j.cell.2013.10.045.
147. J. S. Lott, "The tryptophan biosynthetic pathway is essential for *Mycobacterium tuberculosis* to cause disease," *Biochemical Society Transactions*, vol. 48, no. 5, pp. 2029-2037, 2020, doi: 10.1042/bst20200194.
148. U. S. Gautam *et al.*, "In vivo inhibition of tryptophan catabolism reorganizes the tuberculoma and augments immune-mediated control of *Mycobacterium tuberculosis*," *Proceedings of the National Academy of Sciences*, vol. 115, no. 1, pp. E62-E71, 2018, doi: 10.1073/pnas.1711373114.
149. D. A. Smith, T. Parish, N. G. Stoker, and G. J. Bancroft, "Characterization of Auxotrophic Mutants of *Mycobacterium tuberculosis* and Their Potential as Vaccine Candidates," *Infection and Immunity*, vol. 69, no. 2, pp. 1142-1150, 2001, doi: 10.1128/iai.69.2.1442-1150.2001.
150. J. De Souza Sales *et al.*, "The role of indoleamine 2,3-dioxygenase in lepromatous leprosy immunosuppression," *Clinical and Experimental Immunology*, vol. 165, no. 2, pp. 251-263, 2011, doi: 10.1111/j.1365-2249.2011.04412.x.
151. D. F. Moura *et al.*, "CD163 favors *Mycobacterium leprae* survival and persistence by promoting anti-inflammatory pathways in lepromatous macrophages," *European Journal of Immunology*, vol. 42, no. 11, pp. 2925-2936, 2012, doi: 10.1002/eji.201142198.
152. M. G. De Mattos Barbosa *et al.*, "Indoleamine 2,3-dioxygenase and iron are required for *Mycobacterium leprae* survival," *Microbes and Infection*, vol. 19, no. 11, pp. 505-514, 2017, doi: 10.1016/j.micinf.2017.06.006.
153. J. A. P. Oliveira *et al.*, "*Mycobacterium leprae* induces a tolerogenic profile in monocyte-derived dendritic cells via TLR2 induction of IDO," *Journal of Leukocyte Biology*, vol. 110, no. 1, pp. 167-176, 2021, doi: 10.1002/jlb.4a0320-188r.
154. V. D. Vissa and P. J. Brennan, "The Genome of *Mycobacterium Leprae*: A Minimal Mycobacterial Gene Set," *Genome Biology*, vol. 2, no. 8, p. reviews1023.1, 2001, doi: 10.1186/gb-2001-2-8-reviews1023.
155. K. Borah *et al.*, "GSMN-ML- a genome scale metabolic network reconstruction of the obligate human pathogen *Mycobacterium leprae*," *PLOS Neglected Tropical Diseases*, vol. 14, no. 7, p. e0007871, 2020, doi: 10.1371/journal.pntd.0007871.
156. O. Ojo, D. L. Williams, L. B. Adams, and R. Lahiri, "*Mycobacterium leprae* Transcriptome During In Vivo Growth and Ex Vivo Stationary Phases," *Frontiers in Cellular and Infection Microbiology*, vol. 11, 2022, doi: 10.3389/fcimb.2021.817221.
157. P. Singh and S. T. Cole, "*Mycobacterium leprae*: genes, pseudogenes and genetic diversity," (in eng), *Future Microbiol*, vol. 6, no. 1, pp. 57-71, Jan 2011, doi: 10.2217/fmb.10.153.
158. S. Pfengle *et al.*, "*Mycobacterium leprae* diversity and population dynamics in medieval Europe from novel ancient genomes," (in eng), *BMC Biol*, vol. 19, no. 1, p. 220, Oct 5 2021, doi: 10.1186/s12915-021-01120-2.
159. S. T. Cole *et al.*, "Massive gene decay in the leprosy bacillus," *Nature*, vol. 409, no. 6823, pp. 1007-1011, 2001/02/01 2001, doi: 10.1038/35059006.
160. F. J. Silva, D. Santos-Garcia, X. Zheng, L. Zhang, and X. Y. Han, "Construction and Analysis of the Complete Genome Sequence of Leprosy Agent *Mycobacterium*

- lepromatosis*," (in eng), *Microbiol Spectr*, vol. 10, no. 3, p. e0169221, Jun 29 2022, doi: 10.1128/spectrum.01692-21.
161. S. R. Khanolkar, "Preliminary studies of the metabolic activity of purified suspensions of *Mycobacterium leprae*," (in eng), *J Gen Microbiol*, vol. 128, no. 2, pp. 423-5, Feb 1982, doi: 10.1099/00221287-128-2-423.
 162. P. R. Wheeler, "Metabolism of carbon sources by *Mycobacterium leprae*: a preliminary report," (in eng), *Ann Microbiol (Paris)*, vol. 133, no. 1, pp. 141-6, Jul-Aug 1982.
 163. K. Borah *et al.*, "Intracellular *Mycobacterium leprae* Utilizes Host Glucose as a Carbon Source in Schwann Cells," *mBio*, vol. 10, no. 6, pp. 10.1128/mbio.02351-19, 2019, doi: 10.1128/mbio.02351-19.
 164. "Thermo Fisher Scientific - Medium Formulation: 11879 - RPMI 1640, no glucose." <https://www.thermofisher.com/us/en/home/technical-resources/media-formulation.116.html> (accessed).
 165. S. G. Franzblau, "Oxidation of palmitic acid by *Mycobacterium leprae* in an axenic medium," (in eng), *J Clin Microbiol*, vol. 26, no. 1, pp. 18-21, Jan 1988, doi: 10.1128/jcm.26.1.18-21.1988.
 166. S. G. Franzblau and E. B. Harris, "Biophysical optima for metabolism of *Mycobacterium leprae*," (in eng), *J Clin Microbiol*, vol. 26, no. 6, pp. 1124-9, Jun 1988, doi: 10.1128/jcm.26.6.1124-1129.1988.
 167. M. Ishaque, "Direct evidence for the oxidation of palmitic acid by host-grown *Mycobacterium leprae*," *Research in Microbiology*, vol. 140, no. 2, pp. 83-93, 1989, doi: 10.1016/0923-2508(89)90042-9.
 168. P. R. Wheeler, K. Bulmer, and C. Ratledge, "Fatty acid oxidation and the β -oxidation complex in *Mycobacterium leprae* and two axenically cultivable mycobacteria that are pathogens," *Journal of General Microbiology*, vol. 137, no. 4, pp. 885-893, 1991, doi: 10.1099/00221287-137-4-885.
 169. S. G. Franzblau, E. B. Harris, and R. C. Hastings, "Axenic incorporation of [U- ^{14}C]palmitic acid into the phenolic glycolipid-I of *Mycobacterium leprae*," *FEMS Microbiology Letters*, vol. 48, no. 3, pp. 407-411, 1987, doi: 10.1111/j.1574-6968.1987.tb02633.x.
 170. M. A. M. Marques *et al.*, "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*, vol. 197, no. 23, pp. 3698-3707, 2015, doi: 10.1128/jb.00625-15.
 171. D. Schnappinger *et al.*, "Transcriptional Adaptation of *Mycobacterium tuberculosis* within Macrophages," *The Journal of Experimental Medicine*, vol. 198, no. 5, pp. 693-704, 2003, doi: 10.1084/jem.20030846.
 172. B. C. Vandervan *et al.*, "Novel Inhibitors of Cholesterol Degradation in *Mycobacterium tuberculosis* Reveal How the Bacterium's Metabolism Is Constrained by the Intracellular Environment," *PLOS Pathogens*, vol. 11, no. 2, p. e1004679, 2015, doi: 10.1371/journal.ppat.1004679.
 173. E. V. Nazarova *et al.*, "Rv3723/LucA coordinates fatty acid and cholesterol uptake in *Mycobacterium tuberculosis*," *eLife*, vol. 6, 2017, doi: 10.7554/elife.26969.
 174. A. D. Baughn and K. Y. Rhee, "Metabolomics of Central Carbon Metabolism in *Mycobacterium tuberculosis*," *Microbiology Spectrum*, vol. 2, no. 3, 2014, doi: 10.1128/microbiolspec.mgm2-0026-2013.

175. J. Marrero, K. Y. Rhee, D. Schnappinger, K. Pethe, and S. Ehrt, "Gluconeogenic carbon flow of tricarboxylic acid cycle intermediates is critical for *Mycobacterium tuberculosis* to establish and maintain infection," *Proceedings of the National Academy of Sciences*, vol. 107, no. 21, pp. 9819-9824, 2010, doi: 10.1073/pnas.1000715107.
176. K. Y. Rhee *et al.*, "Central carbon metabolism in *Mycobacterium tuberculosis*: an unexpected frontier," *Trends in Microbiology*, vol. 19, no. 7, pp. 307-314, 2011, doi: 10.1016/j.tim.2011.03.008.
177. L. P. S. De Carvalho, S. M. Fischer, J. Marrero, C. Nathan, S. Ehrt, and K. Y. Rhee, "Metabolomics of *Mycobacterium tuberculosis* Reveals Compartmentalized Co-Catabolism of Carbon Substrates," *Chemistry & Biology*, vol. 17, no. 10, pp. 1122-1131, 2010, doi: 10.1016/j.chembiol.2010.08.009.
178. A. Serafini *et al.*, "*Mycobacterium tuberculosis* requires glyoxylate shunt and reverse methylcitrate cycle for lactate and pyruvate metabolism," *Molecular Microbiology*, vol. 112, no. 4, pp. 1284-1307, 2019, doi: 10.1111/mmi.14362.
179. D. J. V. Beste *et al.*, "¹³C-Flux Spectral Analysis of Host-Pathogen Metabolism Reveals a Mixed Diet for Intracellular *Mycobacterium tuberculosis*," *Chemistry & Biology*, vol. 20, no. 8, pp. 1012-1021, 2013, doi: 10.1016/j.chembiol.2013.06.012.
180. S. Tiwari *et al.*, "Arginine-deprivation-induced oxidative damage sterilizes *Mycobacterium tuberculosis*," *Proceedings of the National Academy of Sciences*, vol. 115, no. 39, pp. 9779-9784, 2018, doi: 10.1073/pnas.1808874115.
181. K. Gokulan, B. Rupp, M. S. Pavelka, W. R. Jacobs, and J. C. Sacchettini, "Crystal Structure of *Mycobacterium tuberculosis* Diaminopimelate Decarboxylase, an Essential Enzyme in Bacterial Lysine Biosynthesis," *Journal of Biological Chemistry*, vol. 278, no. 20, pp. 18588-18596, 2003, doi: 10.1074/jbc.m301549200.
182. J. Meng *et al.*, "Identification and Validation of Aspartic Acid Semialdehyde Dehydrogenase as a New Anti-*Mycobacterium Tuberculosis* Target," *International Journal of Molecular Sciences*, vol. 16, no. 10, pp. 23572-23586, 2015, doi: 10.3390/ijms161023572.
183. T. Parish, "Starvation Survival Response of *Mycobacterium tuberculosis*," *Journal of Bacteriology*, vol. 185, no. 22, pp. 6702-6706, 2003, doi: 10.1128/jb.185.22.6702-6706.2003.
184. V. K. Sambandamurthy *et al.*, "A pantothenate auxotroph of *Mycobacterium tuberculosis* is highly attenuated and protects mice against tuberculosis," *Nature Medicine*, vol. 8, no. 10, pp. 1171-1174, 2002, doi: 10.1038/nm765.
185. A. N. Martinez *et al.*, "Molecular Determination of *Mycobacterium leprae* Viability by Use of Real-Time PCR," *Journal of Clinical Microbiology*, vol. 47, no. 7, pp. 2124-2130, 2009, doi: 10.1128/jcm.00512-09.
186. J. D. S. Ferreira *et al.*, "Ticks as potential vectors of *Mycobacterium leprae*: Use of tick cell lines to culture the bacilli and generate transgenic strains," *PLOS Neglected Tropical Diseases*, vol. 12, no. 12, p. e0007001, 2018, doi: 10.1371/journal.pntd.0007001.
187. B. Burke *et al.*, "Regulatory T cell-like response to SARS-CoV-2 in Jamaican fruit bats (*Artibeus jamaicensis*) transduced with human ACE2," *PLOS Pathogens*, vol. 19, no. 10, p. e1011728, 2023, doi: 10.1371/journal.ppat.1011728.
188. C. W. Dunnett, "A Multiple Comparison Procedure for Comparing Several Treatments with a Control," *Journal of the American Statistical Association*, vol. 50, no. 272, pp. 1096-1121, 1955/12/01 1955, doi: 10.1080/01621459.1955.10501294.

189. J. W. Tukey, "Comparing Individual Means in the Analysis of Variance," *Biometrics*, vol. 5, no. 2, pp. 99-114, 1949, doi: 10.2307/3001913.
190. K. Peñuelas-Urquides, L. Villarreal-Treviño, B. Silva-Ramírez, L. Rivadeneyra-Espinoza, S. Said-Fernández, and M. B. D. León, "Measuring of *Mycobacterium tuberculosis* growth: a correlation of the optical measurements with colony forming units," *Brazilian Journal of Microbiology*, vol. 44, no. 1, pp. 287-290, 2013, doi: 10.1590/s1517-83822013000100042.
191. A. Kapopoulou, J. M. Lew, and S. T. Cole, "The MycoBrowser portal: A comprehensive and manually annotated resource for mycobacterial genomes," *Tuberculosis*, vol. 91, no. 1, pp. 8-13, 2011/01/01/ 2011, doi: <https://doi.org/10.1016/j.tube.2010.09.006>.
192. M. W. Taylor and G. S. Feng, "Relationship between interferon-gamma, indoleamine 2,3-dioxygenase, and tryptophan catabolism," (in eng), *Faseb j*, vol. 5, no. 11, pp. 2516-22, Aug 1991.
193. K. Tang, Y.-H. Wu, Y. Song, and B. Yu, "Indoleamine 2,3-dioxygenase 1 (IDO1) inhibitors in clinical trials for cancer immunotherapy," *Journal of Hematology & Oncology*, vol. 14, no. 1, p. 68, 2021/04/21 2021, doi: 10.1186/s13045-021-01080-8.
194. L. Zhai *et al.*, "Molecular Pathways: Targeting IDO1 and Other Tryptophan Dioxygenases for Cancer Immunotherapy," *Clinical Cancer Research*, vol. 21, no. 24, pp. 5427-5433, 2015, doi: 10.1158/1078-0432.Ccr-15-0420.
195. P. Puccetti, "On watching the watchers: IDO and type I/II IFN," *European Journal of Immunology*, vol. 37, no. 4, pp. 876-879, 2007, doi: <https://doi.org/10.1002/eji.200737184>.
196. I. D. Jung *et al.*, "Differential regulation of indoleamine 2,3-dioxygenase by lipopolysaccharide and interferon gamma in murine bone marrow derived dendritic cells," *FEBS Letters*, vol. 581, no. 7, pp. 1449-1456, 2007/04/03/ 2007, doi: <https://doi.org/10.1016/j.febslet.2007.02.073>.
197. A. Hosseini-Tabatabaei, R. B. Jalili, Y. Li, R. T. Kilani, A. Moeen Rezakhanlou, and A. Ghahary, "Mechanism Underlying Defective Interferon Gamma-Induced IDO Expression in Non-obese Diabetic Mouse Fibroblasts," *PLOS ONE*, vol. 7, no. 5, p. e37747, 2012, doi: 10.1371/journal.pone.0037747.
198. S. Y. Chon, H. H. Hassanain, R. Pine, and S. L. Gupta, "Involvement of Two Regulatory Elements in Interferon- γ -Regulated Expression of Human Indoleamine 2,3-Dioxygenase Gene," *Journal of Interferon & Cytokine Research*, vol. 15, no. 6, pp. 517-526, 1995, doi: 10.1089/jir.1995.15.517.
199. A. Meireson, M. Devos, and L. Brochez, "IDO Expression in Cancer: Different Compartment, Different Functionality?," (in English), *Frontiers in Immunology*, Review vol. 11, 2020-September-24 2020, doi: 10.3389/fimmu.2020.531491.
200. K. V. Konan and M. W. Taylor, "Importance of the Two Interferon-stimulated Response Element (ISRE) Sequences in the Regulation of the Human Indoleamine 2,3-Dioxygenase Gene *," *Journal of Biological Chemistry*, vol. 271, no. 32, pp. 19140-19145, 1996, doi: 10.1074/jbc.271.32.19140.
201. T. H. Mogensen, "IRF and STAT Transcription Factors - From Basic Biology to Roles in Infection, Protective Immunity, and Primary Immunodeficiencies," (in English), *Frontiers in Immunology*, Review vol. 9, 2019-January-08 2019, doi: 10.3389/fimmu.2018.03047.

202. G.-N. Zhao, D.-S. Jiang, and H. Li, "Interferon regulatory factors: at the crossroads of immunity, metabolism, and disease," *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, vol. 1852, no. 2, pp. 365-378, 2015/02/01/ 2015, doi: <https://doi.org/10.1016/j.bbadis.2014.04.030>.
203. O. Manches, M. V. Fernandez, J. Plumas, L. Chaperot, and N. Bhardwaj, "Activation of the noncanonical NF- κ B pathway by HIV controls a dendritic cell immunoregulatory phenotype," *Proceedings of the National Academy of Sciences*, vol. 109, no. 35, pp. 14122-14127, 2012, doi: [doi:10.1073/pnas.1204032109](https://doi.org/10.1073/pnas.1204032109).
204. J. Yu *et al.*, "Noncanonical NF- κ B activation mediates STAT3-stimulated IDO upregulation in myeloid-derived suppressor cells in breast cancer," (in eng), *J Immunol*, vol. 193, no. 5, pp. 2574-86, Sep 1 2014, doi: [10.4049/jimmunol.1400833](https://doi.org/10.4049/jimmunol.1400833).
205. M. T. Pallotta *et al.*, "Indoleamine 2,3-dioxygenase is a signaling protein in long-term tolerance by dendritic cells," *Nature Immunology*, vol. 12, no. 9, pp. 870-878, 2011/09/01 2011, doi: [10.1038/ni.2077](https://doi.org/10.1038/ni.2077).
206. S. G. Cady and M. Sono, "1-methyl-dl-tryptophan, β -(3-benzofuranyl)-dl-alanine (the oxygen analog of tryptophan), and β -[3-benzo(b)thienyl]-dl-alanine (the sulfur analog of tryptophan) are competitive inhibitors for indoleamine 2,3-dioxygenase," *Archives of Biochemistry and Biophysics*, vol. 291, no. 2, pp. 326-333, 1991/12/01/ 1991, doi: [https://doi.org/10.1016/0003-9861\(91\)90142-6](https://doi.org/10.1016/0003-9861(91)90142-6).
207. S. K. Schmidt *et al.*, "Influence of Tryptophan Contained in 1-Methyl-Tryptophan on Antimicrobial and Immunoregulatory Functions of Indoleamine 2,3-Dioxygenase," *PLOS ONE*, vol. 7, no. 9, p. e44797, 2012, doi: [10.1371/journal.pone.0044797](https://doi.org/10.1371/journal.pone.0044797).
208. K. Heseler, K. Spekker, S. K. Schmidt, C. R. MacKenzie, and W. Däubener, "Antimicrobial and immunoregulatory effects mediated by human lung cells: role of IFN- γ -induced tryptophan degradation," *FEMS Immunology & Medical Microbiology*, vol. 52, no. 2, pp. 273-281, 2008, doi: [10.1111/j.1574-695X.2007.00374.x](https://doi.org/10.1111/j.1574-695X.2007.00374.x).
209. F. Qian *et al.*, "Efficacy of Levo-1-Methyl Tryptophan and Dextro-1-Methyl Tryptophan in Reversing Indoleamine-2,3-Dioxygenase-Mediated Arrest of T-Cell Proliferation in Human Epithelial Ovarian Cancer," *Cancer Research*, vol. 69, no. 13, pp. 5498-5504, 2009, doi: [10.1158/0008-5472.Can-08-2106](https://doi.org/10.1158/0008-5472.Can-08-2106).
210. S. Lob, A. Konigsrainer, R. Schafer, H.-G. Rammensee, G. Opelz, and P. Ternes, "Levo- but not dextro-1-methyl tryptophan abrogates the IDO activity of human dendritic cells," *Blood*, vol. 111, no. 4, pp. 2152-2154, 2008/02/15/ 2008, doi: <https://doi.org/10.1182/blood-2007-10-116111>.
211. D.-Y. Hou *et al.*, "Inhibition of Indoleamine 2,3-Dioxygenase in Dendritic Cells by Stereoisomers of 1-Methyl-Tryptophan Correlates with Antitumor Responses," *Cancer Research*, vol. 67, no. 2, pp. 792-801, 2007, doi: [10.1158/0008-5472.Can-06-2925](https://doi.org/10.1158/0008-5472.Can-06-2925).
212. T. Okamoto, S. Toné, H. Kanouchi, C. Miyawaki, S. Ono, and Y. Minatogawa, "Transcriptional regulation of indoleamine 2,3-dioxygenase (IDO) by tryptophan and its analogue : Down-regulation of the indoleamine 2,3-dioxygenase (IDO) transcription by tryptophan and its analogue," (in eng), *Cytotechnology*, vol. 54, no. 2, pp. 107-13, Jun 2007, doi: [10.1007/s10616-007-9081-4](https://doi.org/10.1007/s10616-007-9081-4).
213. H. C. Lewis, R. Chinnadurai, S. E. Bosinger, and J. Galipeau, "The IDO inhibitor 1-methyl tryptophan activates the aryl hydrocarbon receptor response in mesenchymal stromal cells," (in eng), *Oncotarget*, vol. 8, no. 54, pp. 91914-91927, Nov 3 2017, doi: [10.18632/oncotarget.20166](https://doi.org/10.18632/oncotarget.20166).

214. R. B. O. Brito *et al.*, "1-Methyl-D-Tryptophan Potentiates TGF- β -Induced Epithelial-Mesenchymal Transition in T24 Human Bladder Cancer Cells," *PLOS ONE*, vol. 10, no. 8, p. e0134858, 2015, doi: 10.1371/journal.pone.0134858.
215. C. A. Opitz *et al.*, "The Indoleamine-2,3-Dioxygenase (IDO) Inhibitor 1-Methyl-D-tryptophan Upregulates IDO1 in Human Cancer Cells," *PLOS ONE*, vol. 6, no. 5, p. e19823, 2011, doi: 10.1371/journal.pone.0019823.
216. J.-W. Lee *et al.*, "Co-treatment with interferon- γ and 1-methyl tryptophan ameliorates cardiac fibrosis through cardiac myofibroblasts apoptosis," *Molecular and Cellular Biochemistry*, vol. 458, no. 1, pp. 197-205, 2019/08/01 2019, doi: 10.1007/s11010-019-03542-7.
217. M. S. F. Branquinho *et al.*, "1-Methyl-tryptophan enantiomers differently affect the cross-talking between mononuclear and tumor cells and interferon- γ production," *bioRxiv*, p. 578369, 2019, doi: 10.1101/578369.
218. T. A. Babcock and J. M. Carlin, "TRANSCRIPTIONAL ACTIVATION OF INDOLEAMINE DIOXYGENASE BY INTERLEUKIN 1 AND TUMOR NECROSIS FACTOR α IN INTERFERON-TREATED EPITHELIAL CELLS," *Cytokine*, vol. 12, no. 6, pp. 588-594, 2000/06/01/ 2000, doi: <https://doi.org/10.1006/cyto.1999.0661>.
219. K. W. Ng *et al.*, "Development of an ex vivo human skin model for intradermal vaccination: tissue viability and Langerhans cell behaviour," (in eng), *Vaccine*, vol. 27, no. 43, pp. 5948-55, Oct 9 2009, doi: 10.1016/j.vaccine.2009.07.088.
220. A. Blauvelt, S. Cohen, T. Kawamura, A. Neurath, and J. Orenstein, "Ex vivo HIV-1 infection of human immature langerhans cells within epithelial tissue explants: a novel model for sexual transmission of HIV-1," *AIDS*, vol. 15, 2001. [Online]. Available: https://journals.lww.com/aidsonline/fulltext/2001/02001/ex_vivo_hiv_1_infection_of_human_immature.58.aspx.
221. E. C. da Silva Carlos, G. A. Cristovão, A. A. Silva, B. C. de Santos Ribeiro, and B. Romana-Souza, "Imiquimod-induced ex vivo model of psoriatic human skin via interleukin-17A signalling of T cells and Langerhans cells," (in eng), *Exp Dermatol*, vol. 31, no. 11, pp. 1791-1799, Nov 2022, doi: 10.1111/exd.14659.
222. S. Mantri *et al.*, "CD34 expression does not correlate with immunophenotypic stem cell or progenitor content in human cord blood products," *Blood Adv*, vol. 4, no. 21, pp. 5357-5361, Nov 10 2020, doi: 10.1182/bloodadvances.2020002891.
223. G. Pfister, S. M. Toor, V. Sasidharan Nair, and E. Elkord, "An evaluation of sorter induced cell stress (SICS) on peripheral blood mononuclear cells (PBMCs) after different sort conditions - Are your sorted cells getting SICS?," *Journal of Immunological Methods*, vol. 487, p. 112902, 2020/12/01/ 2020, doi: <https://doi.org/10.1016/j.jim.2020.112902>.
224. H. Yan *et al.*, "Magnetic cell sorting and flow cytometry sorting methods for the isolation and function analysis of mouse CD4⁺ CD25⁺ Treg cells," (in eng), *J Zhejiang Univ Sci B*, vol. 10, no. 12, pp. 928-32, Dec 2009, doi: 10.1631/jzus.B0920205.
225. E. Albini *et al.*, "Distinct roles of immunoreceptor tyrosine-based motifs in immunosuppressive indoleamine 2,3-dioxygenase 1," (in eng), *J Cell Mol Med*, vol. 21, no. 1, pp. 165-176, Jan 2017, doi: 10.1111/jcmm.12954.
226. C. Orabona *et al.*, "SOCS3 drives proteasomal degradation of indoleamine 2,3-dioxygenase (IDO) and antagonizes IDO-dependent tolerogenesis," (in eng), *Proc Natl*

- Acad Sci U S A*, vol. 105, no. 52, pp. 20828-33, Dec 30 2008, doi: 10.1073/pnas.0810278105.
227. H. Fujigaki *et al.*, "Nitration and Inactivation of IDO by Peroxynitrite1," *The Journal of Immunology*, vol. 176, no. 1, pp. 372-379, 2006, doi: 10.4049/jimmunol.176.1.372.
 228. S. R. Thomas *et al.*, "Post-translational Regulation of Human Indoleamine 2,3-Dioxygenase Activity by Nitric Oxide*," *Journal of Biological Chemistry*, vol. 282, no. 33, pp. 23778-23787, 2007/08/17/ 2007, doi: <https://doi.org/10.1074/jbc.M700669200>.
 229. A. Lewis-Ballester, S. Karkashon, D. Batabyal, T. L. Poulos, and S.-R. Yeh, "Inhibition Mechanisms of Human Indoleamine 2,3 Dioxygenase 1," *Journal of the American Chemical Society*, vol. 140, no. 27, pp. 8518-8525, 2018/07/11 2018, doi: 10.1021/jacs.8b03691.
 230. M. T. Nelp *et al.*, "Immune-modulating enzyme indoleamine 2,3-dioxygenase is effectively inhibited by targeting its apo-form," (in eng), *Proc Natl Acad Sci U S A*, vol. 115, no. 13, pp. 3249-3254, Mar 27 2018, doi: 10.1073/pnas.1719190115.
 231. L. Pucci, S. Perozzi, F. Cimadamore, G. Orsomando, and N. Raffaelli, "Tissue expression and biochemical characterization of human 2-amino 3-carboxymuconate 6-semialdehyde decarboxylase, a key enzyme in tryptophan catabolism," *The FEBS Journal*, vol. 274, no. 3, pp. 827-840, 2007, doi: <https://doi.org/10.1111/j.1742-4658.2007.05635.x>.
 232. R. S. Grant, S. E. Coggan, and G. A. Smythe, "The Physiological Action of Picolinic Acid in the Human Brain," *International Journal of Tryptophan Research*, vol. 2, p. IJTR.S2469, 2009, doi: 10.4137/ijtr.S2469.
 233. M. Salter, R. G. Knowles, and C. I. Pogson, "Quantification of the importance of individual steps in the control of aromatic amino acid metabolism," *Biochemical Journal*, vol. 234, no. 3, pp. 635-647, 1986, doi: 10.1042/bj2340635.
 234. D. A. BENDER and G. M. McCREANOR, "Kynurenine hydroxylase: a potential rate-limiting enzyme in tryptophan metabolism," *Biochemical Society Transactions*, vol. 13, no. 2, pp. 441-443, 1985, doi: 10.1042/bst0130441.
 235. A. Rahman, K. Ting, K. M. Cullen, N. Braidy, B. J. Brew, and G. J. Guillemin, "The Excitotoxin Quinolinic Acid Induces Tau Phosphorylation in Human Neurons," *PLOS ONE*, vol. 4, no. 7, p. e6344, 2009, doi: 10.1371/journal.pone.0006344.
 236. N. Braidy, R. Grant, S. Adams, B. J. Brew, and G. J. Guillemin, "Mechanism for quinolinic acid cytotoxicity in human astrocytes and neurons," (in eng), *Neurotox Res*, vol. 16, no. 1, pp. 77-86, Jul 2009, doi: 10.1007/s12640-009-9051-z.
 237. T. W. Stone, "Neuropharmacology of quinolinic and kynurenic acids," *Pharmacological reviews*, vol. 45, no. 3, pp. 309-379, 1993.
 238. A. Zinger, C. Barcia, M. T. Herrero, and G. J. Guillemin, "The Involvement of Neuroinflammation and Kynurenine Pathway in Parkinson's Disease," *Parkinson's Disease*, vol. 2011, p. 716859, 2011/06/03 2011, doi: 10.4061/2011/716859.
 239. G. J. Guillemin, B. J. Brew, C. E. Noonan, O. Takikawa, and K. M. Cullen, "Indoleamine 2,3 dioxygenase and quinolinic acid Immunoreactivity in Alzheimer's disease hippocampus," *Neuropathology and Applied Neurobiology*, vol. 31, no. 4, pp. 395-404, 2005, doi: <https://doi.org/10.1111/j.1365-2990.2005.00655.x>.
 240. G. J. Guillemin, K. R. Williams, D. G. Smith, G. A. Smythe, J. Croitoru-Lamoury, and B. J. Brew, "QUINOLINIC ACID IN THE PATHOGENESIS OF ALZHEIMER'S DISEASE," in *Developments in Tryptophan and Serotonin Metabolism*, G. Allegri, C. V.

- L. Costa, E. Ragazzi, H. Steinhart, and L. Varesio Eds. Boston, MA: Springer US, 2003, pp. 167-176.
241. R. Lugo-Huitrón, P. Ugalde Muñiz, B. Pineda, J. Pedraza-Chaverri, C. Ríos, and V. Pérez-de la Cruz, "Quinolinic acid: an endogenous neurotoxin with multiple targets," (in eng), *Oxid Med Cell Longev*, vol. 2013, p. 104024, 2013, doi: 10.1155/2013/104024.
 242. G. J. Guillemín, G. Smythe, O. Takikawa, and B. J. Brew, "Expression of indoleamine 2,3-dioxygenase and production of quinolinic acid by human microglia, astrocytes, and neurons," (in eng), *Glia*, vol. 49, no. 1, pp. 15-23, Jan 1 2005, doi: 10.1002/glia.20090.
 243. S. Fukui, R. Schwarcz, S. I. Rapoport, Y. Takada, and Q. R. Smith, "Blood–Brain Barrier Transport of Kynurenines: Implications for Brain Synthesis and Metabolism," *Journal of Neurochemistry*, vol. 56, no. 6, pp. 2007-2017, 1991, doi: <https://doi.org/10.1111/j.1471-4159.1991.tb03460.x>.
 244. F. Cathomas, K. Guetter, E. Seifritz, F. Klaus, and S. Kaiser, "Quinolinic acid is associated with cognitive deficits in schizophrenia but not major depressive disorder," *Scientific Reports*, vol. 11, no. 1, p. 9992, 2021/05/11 2021, doi: 10.1038/s41598-021-89335-9.
 245. S. Feng, Y.-Q. Du, L. Zhang, L. Zhang, R.-R. Feng, and S.-Y. Liu, "Analysis of serum metabolic profile by ultra-performance liquid chromatography-mass spectrometry for biomarkers discovery: application in a pilot study to discriminate patients with tuberculosis," (in eng), *Chin Med J (Engl)*, vol. 128, no. 2, pp. 159-68, Jan 20 2015, doi: 10.4103/0366-6999.149188.
 246. S. Mason, A. M. T. van Furth, R. Solomons, R. A. Wevers, M. van Reenen, and C. J. Reinecke, "A putative urinary biosignature for diagnosis and follow-up of tuberculous meningitis in children: outcome of a metabolomics study disclosing host–pathogen responses," *Metabolomics*, vol. 12, no. 7, p. 110, 2016/06/10 2016, doi: 10.1007/s11306-016-1053-2.
 247. N. K. Dutta *et al.*, "Integration of metabolomics and transcriptomics reveals novel biomarkers in the blood for tuberculosis diagnosis in children," *Scientific Reports*, vol. 10, no. 1, p. 19527, 2020/11/11 2020, doi: 10.1038/s41598-020-75513-8.
 248. Y. Yu, X.-X. Jiang, and J.-C. Li, "Biomarker discovery for tuberculosis using metabolomics," (in English), *Frontiers in Molecular Biosciences*, Review vol. 10, 2023-February-20 2023, doi: 10.3389/fmolb.2023.1099654.
 249. F. Vrieling *et al.*, "Analyzing the impact of Mycobacterium tuberculosis infection on primary human macrophages by combined exploratory and targeted metabolomics," *Scientific Reports*, vol. 10, no. 1, p. 7085, 2020/04/27 2020, doi: 10.1038/s41598-020-62911-1.
 250. R. C. A. Medeiros *et al.*, "Subversion of Schwann Cell Glucose Metabolism by Mycobacterium leprae*," *Journal of Biological Chemistry*, vol. 291, no. 41, pp. 21375-21387, 2016/10/01/ 2016, doi: <https://doi.org/10.1074/jbc.M116.725283>.
 251. M. F. Oliveira *et al.*, "Reduction of host cell mitochondrial activity as Mycobacterium leprae's strategy to evade host innate immunity," (in eng), *Immunol Rev*, vol. 301, no. 1, pp. 193-208, May 2021, doi: 10.1111/imr.12962.
 252. T. Rosa *et al.*, "Reductive Power Generated by Mycobacterium leprae Through Cholesterol Oxidation Contributes to Lipid and ATP Synthesis," (in eng), *Front Cell Infect Microbiol*, vol. 11, p. 709972, 2021, doi: 10.3389/fcimb.2021.709972.

253. L. B. Kasârov and A. G. Moat, "Metabolism of Nicotinamide Adenine Dinucleotide in Human and Bovine Strains of *Mycobacterium tuberculosis*," *Journal of Bacteriology*, vol. 110, no. 2, pp. 600-603, 1972, doi: 10.1128/jb.110.2.600-603.1972.
254. K. Konno, K. Oizumi, and S. Oka, "Biosynthesis of Niacin Ribonucleotide from Quinolinic Acid By Mycobacteria," *Nature*, vol. 205, no. 4974, pp. 874-875, 1965/02/01 1965, doi: 10.1038/205874a0.
255. J. Jiang, Z. Li, C. Chen, W. Jiang, B. Xu, and Q. Zhao, "Metabolomics Strategy Assisted by Transcriptomics Analysis to Identify Potential Biomarkers Associated with Tuberculosis," (in eng), *Infect Drug Resist*, vol. 14, pp. 4795-4807, 2021, doi: 10.2147/idr.S330493.
256. S. Isaiah *et al.*, "Urinary markers of Mycobacterium tuberculosis and dysbiosis in paediatric tuberculous meningitis cases undergoing treatment," *Gut Pathogens*, vol. 16, no. 1, p. 14, 2024/03/12 2024, doi: 10.1186/s13099-024-00609-9.
257. Y. Liu *et al.*, "Mycobacterium tuberculosis Rv1987 protein attenuates inflammatory response and consequently alters microbiota in mouse lung," (in eng), *Front Cell Infect Microbiol*, vol. 13, p. 1256866, 2023, doi: 10.3389/fcimb.2023.1256866.
258. M. Wu *et al.*, "Gut microbiota-derived 5-hydroxyindoleacetic acid from pumpkin polysaccharides supplementation alleviates colitis via MAPKs-PPAR γ /NF- κ B inhibition," *International Journal of Biological Macromolecules*, vol. 264, p. 130385, 2024/04/01/ 2024, doi: <https://doi.org/10.1016/j.ijbiomac.2024.130385>.
259. Y. Luo, K. Tanigawa, A. Kawashima, Y. Ishido, N. Ishii, and K. Suzuki, "The function of peroxisome proliferator-activated receptors PPAR- γ and PPAR- δ in Mycobacterium leprae-induced foam cell formation in host macrophages," (in eng), *PLoS Negl Trop Dis*, vol. 14, no. 10, p. e0008850, Oct 2020, doi: 10.1371/journal.pntd.0008850.
260. M. V. S. Rajaram, M. N. Brooks, J. D. Morris, J. B. Torrelles, A. K. Azad, and L. S. Schlesinger, "Mycobacterium tuberculosis Activates Human Macrophage Peroxisome Proliferator-Activated Receptor γ Linking Mannose Receptor Recognition to Regulation of Immune Responses," *The Journal of Immunology*, vol. 185, no. 2, pp. 929-942, 2010, doi: 10.4049/jimmunol.1000866.
261. S. Mahajan *et al.*, "Mycobacterium tuberculosis Modulates Macrophage Lipid-Sensing Nuclear Receptors PPAR γ and TR4 for Survival," *The Journal of Immunology*, vol. 188, no. 11, pp. 5593-5603, 2012, doi: 10.4049/jimmunol.1103038.
262. Q. Shen, A. Gonzalez-Mireles, S. C. Ray, and C. A. Rappleye, "Histoplasma capsulatum Relies on Tryptophan Biosynthesis To Proliferate within the Macrophage Phagosome," (in eng), *Infect Immun*, vol. 91, no. 6, p. e0005923, Jun 15 2023, doi: 10.1128/iai.00059-23.
263. O. Nüsse, "Biochemistry of the phagosome: the challenge to study a transient organelle," (in eng), *ScientificWorldJournal*, vol. 11, pp. 2364-81, 2011, doi: 10.1100/2011/741046.
264. D. Li, Z. Ouyang, and X. Ma, "Mass Spectrometry Imaging for Single-Cell or Subcellular Lipidomics: A Review of Recent Advancements and Future Development," *Molecules*, vol. 28, no. 6, p. 2712, 2023. [Online]. Available: <https://www.mdpi.com/1420-3049/28/6/2712>.
265. J. A. Herrera *et al.*, "Laser capture microdissection coupled mass spectrometry (LCM-MS) for spatially resolved analysis of formalin-fixed and stained human lung tissues," *Clinical Proteomics*, vol. 17, no. 1, p. 24, 2020/06/17 2020, doi: 10.1186/s12014-020-09287-6.

266. P. Lönnbro, P. Nordenfelt, and H. Tapper, "Isolation of bacteria-containing phagosomes by magnetic selection," *BMC Cell Biology*, vol. 9, no. 1, p. 35, 2008/06/27 2008, doi: 10.1186/1471-2121-9-35.
267. J.-A. Herweg *et al.*, "Purification and proteomics of pathogen-modified vacuoles and membranes," (in English), *Frontiers in Cellular and Infection Microbiology*, Review vol. 5, 2015-June-02 2015, doi: 10.3389/fcimb.2015.00048.
268. P. R. Wheeler, "Catabolic pathways for glucose, glycerol and 6-phosphogluconate in *Mycobacterium leprae* grown in armadillo tissues," (in eng), *J Gen Microbiol*, vol. 129, no. 5, pp. 1481-95, May 1983, doi: 10.1099/00221287-129-5-1481.
269. D. E. Matthews, "An overview of phenylalanine and tyrosine kinetics in humans," (in eng), *J Nutr*, vol. 137, no. 6 Suppl 1, pp. 1549S-1555S; discussion 1573S-1575S, Jun 2007, doi: 10.1093/jn/137.6.1549S.
270. H. Akashi and T. Gojobori, "Metabolic efficiency and amino acid composition in the proteomes of *Escherichia coli* and *Bacillus subtilis*," *Proceedings of the National Academy of Sciences*, vol. 99, no. 6, pp. 3695-3700, 2002, doi: doi:10.1073/pnas.062526999.
271. G. Stadthagen *et al.*, "p-Hydroxybenzoic Acid Synthesis in *Mycobacterium tuberculosis*," *Journal of Biological Chemistry*, vol. 280, no. 49, pp. 40699-40706, 2005/12/09/ 2005, doi: <https://doi.org/10.1074/jbc.M508332200>.
272. E. Pérez, P. Constant, A. Lemassu, F. Laval, M. Daffé, and C. Guilhot, "Characterization of Three Glycosyltransferases Involved in the Biosynthesis of the Phenolic Glycolipid Antigens from the *Mycobacterium tuberculosis* Complex*," *Journal of Biological Chemistry*, vol. 279, no. 41, pp. 42574-42583, 2004/10/08/ 2004, doi: <https://doi.org/10.1074/jbc.M406246200>.
273. S. Samanta, A. Singh, P. Biswas, A. Bhatt, and S. S. Visweswariah, "Mycobacterial phenolic glycolipid synthesis is regulated by cAMP-dependent lysine acylation of FadD22," *Microbiology*, vol. 163, no. 3, pp. 373-382, 2017, doi: <https://doi.org/10.1099/mic.0.000440>.
274. J. A. Ferreras *et al.*, "Mycobacterial Phenolic Glycolipid Virulence Factor Biosynthesis: Mechanism and Small-Molecule Inhibition of Polyketide Chain Initiation," *Chemistry & Biology*, vol. 15, no. 1, pp. 51-61, 2008, doi: 10.1016/j.chembiol.2007.11.010.
275. M. B. Reed *et al.*, "A glycolipid of hypervirulent tuberculosis strains that inhibits the innate immune response," *Nature*, vol. 431, no. 7004, pp. 84-87, 2004/09/01 2004, doi: 10.1038/nature02837.
276. P. Constant *et al.*, "Role of the *pks15/I* Gene in the Biosynthesis of Phenolglycolipids in the *Mycobacterium tuberculosis* Complex: EVIDENCE THAT ALL STRAINS SYNTHESIZE GLYCOSYLATED *p*-HYDROXYBENZOIC METHYL ESTERS AND THAT STRAINS DEVOID OF PHENOLGLYCOLIPIDS HARBOR A FRAMESHIFT MUTATION IN THE *pks15/I* GENE *," *Journal of Biological Chemistry*, vol. 277, no. 41, pp. 38148-38158, 2002, doi: 10.1074/jbc.M206538200
277. P. J. Brennan and W. W. Barrow, "Evidence for species-specific lipid antigens in *Mycobacterium leprae*," (in eng), *Int J Lepr Other Mycobact Dis*, vol. 48, no. 4, pp. 382-7, Dec 1980.
278. J. Hessel M. van Dijk, G. A. van der Marel, and J. D. C. Codée, "Developments in the Synthesis of Mycobacterial Phenolic Glycolipids," *The Chemical Record*, vol. 21, no. 11, pp. 3295-3312, 2021, doi: <https://doi.org/10.1002/tcr.202100200>.

279. G. Tabouret *et al.*, "Mycobacterium leprae Phenolglycolipid-1 Expressed by Engineered M. bovis BCG Modulates Early Interaction with Human Phagocytes," *PLoS Pathogens*, vol. 6, no. 10, p. e1001159, 2010, doi: 10.1371/journal.ppat.1001159.
280. L. S. Schlesinger and M. A. Horwitz, "Phenolic glycolipid-1 of Mycobacterium leprae binds complement component C3 in serum and mediates phagocytosis by human monocytes," *Journal of Experimental Medicine*, vol. 174, no. 5, pp. 1031-1038, 1991, doi: 10.1084/jem.174.5.1031.
281. G. Stadthagen *et al.*, "Comparative investigation of the pathogenicity of three Mycobacterium tuberculosis mutants defective in the synthesis of p-hydroxybenzoic acid derivatives," *Microbes and Infection*, vol. 8, no. 8, pp. 2245-2253, 2006/07/01/ 2006, doi: <https://doi.org/10.1016/j.micinf.2006.04.008>.
282. F. Pierrel, "Impact of Chemical Analogs of 4-Hydroxybenzoic Acid on Coenzyme Q Biosynthesis: From Inhibition to Bypass of Coenzyme Q Deficiency," (in eng), *Front Physiol*, vol. 8, p. 436, 2017, doi: 10.3389/fphys.2017.00436.
283. T. Parish and N. G. Stoker, "The common aromatic amino acid biosynthesis pathway is essential in Mycobacterium tuberculosis," *Microbiology*, vol. 148, no. 10, pp. 3069-3077, 2002, doi: <https://doi.org/10.1099/00221287-148-10-3069>.
284. J. J. Fournie, E. Adams, R. J. Mullins, and A. Basten, "Inhibition of human lymphoproliferative responses by mycobacterial phenolic glycolipids," *Infection and Immunity*, vol. 57, no. 11, pp. 3653-3659, 1989, doi: doi:10.1128/iai.57.11.3653-3659.1989.
285. M. A. Neill and S. J. Klebanoff, "The effect of phenolic glycolipid-1 from Mycobacterium leprae on the antimicrobial activity of human macrophages," *Journal of Experimental Medicine*, vol. 167, no. 1, pp. 30-42, 1988, doi: 10.1084/jem.167.1.30.
286. K. Green and S. Garneau-Tsodikova, "Resistance in tuberculosis: what do we know and where can we go?," (in English), *Frontiers in Microbiology*, Mini Review vol. 4, 2013-July-23 2013, doi: 10.3389/fmicb.2013.00208.
287. J. E. S. Nunes *et al.*, "Mycobacterium tuberculosis Shikimate Pathway Enzymes as Targets for the Rational Design of Anti-Tuberculosis Drugs," (in eng), *Molecules*, vol. 25, no. 6, Mar 11 2020, doi: 10.3390/molecules25061259.

APPENDIX A: SUPPLEMENTARY TABLES

Supplementary Table 1A: LC-MS MRM Specifications

Compound	Formula	Monoisotopic mass (g/mol)	Precursor ion [M+H] ⁺ (m/z)	Product ion (m/z)		Collision energy (V)	Fragmentation energy (V)	Retention time (min)	LOD (Abundance)	LOQ (Abundance; ng/mL)	ISTD Compound
				Quantifier	Qualifier						
1-methyltryptophan	C12 H14 N2 O2	218.1055	219.1	202.1		8.0	82.0	6.395	683.9159	2581; 2.4414	Tryptophan-d ₅
					157.0	30.0					
					115.1	57.0					
2-Picolinic acid	C6 H5 N O2	123.032	124.0	78.1		21.0	69.0	1.920	316.9155	795; 0.6104	Picolinic acid-d ₄
					51.2	8.0					
2-Picolinic acid-d ₄	C6 H D4 N O2	127.0571	128.1	82.2		25.0	69.0	1.912			
					54.2	45.0					
3-hydroxyanthranilic acid	C7 H7 N O3	153.0426	154.0	136.0		64.0	64.0	4.864	24.8026	661; 0.6104	3-hydroxykynurenine- ¹³ C ₃ (¹⁵ N)
					80.2	29.0					
3-hydroxykynurenine- ¹³ C ₃ (¹⁵ N)	C7 [13C]3 H12 N [15N] O4	228.0868	229.1	211.0		4.0	74.0	3.043			
					110.1	17.0					
3-hydroxykynurenine	C10 H12 N2 O4	224.0797	225.1	208.0		6.0	73.0	3.035	6.7607	45; 0.1526	3-hydroxykynurenine- ¹³ C ₃ (¹⁵ N)
					110.1	14.0					
5-hydroxyindoleacetic acid	C10 H9 N O3	191.0582	192.1	146.0		13.0	83.0	6.087	23.2547	110; 1.2207	5-hydroxyindoleacetic acid-d ₆
					51.2	73.0					
5-hydroxyindoleacetic acid-d ₆	C10 H3 D6 N O3	197.0959	198.1	151.1		17.0	83.0	6.045			
					54.1	81.0					
5-hydroxytryptophan	C11 H12 N2 O3	220.0848	221.1	204.0		6.0	73.0	4.058	9.0371	251; 0.6104	5-hydroxytryptophan-d ₃
					162.0	18.0					
		224.1099	224.1	206.0		9.0	74.0	4.041			

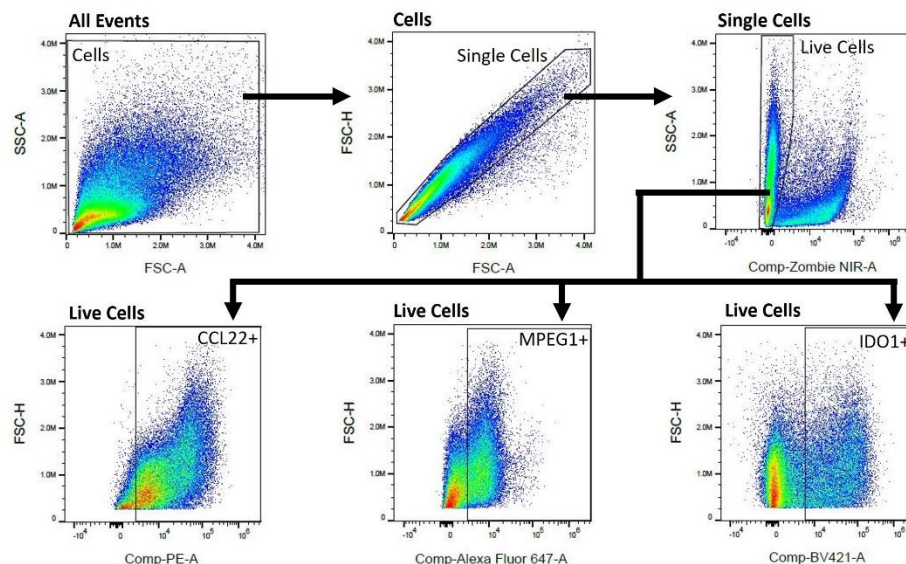
5-hydroxytryptophan-d ₃	C11 H8 D4 N2 O3				164.0	17.0					
Anthranilic acid	C7 H7 N O2	137.0477	138.1	120.0		10.0	64.0	7.268	149.2989	2016; 1.2207	Anthranilic acid- ¹³ C ₆
					65.2	34.0					
Anthranilic acid- ¹³ C ₆	C [13C]6 H7 N O2	143.0678	144.1	126.1		9.0	64.0	7.268			
					70.2	33.0					
Indoleacetic acid	C10 H9 N O2	175.0633	176.1	130.1		18.0	73.0	8.432	19.7033	94; 1.2207	None
					77.1	50.0					
Indolelactic acid	C11 H11 N O3	205.0739	206.1	118.1		22.0	81.0	7.775	4.5224	54; 2.4414	None
					130.1	34.0					
Kynurenic acid- ¹³ C ₆	C4 [13C]6 H7 N O3	195.0627	196.1	150.1		21.0	83.0	5.545			
					95.1	49.0					
Kynurenic acid	C10 H7 N O3	189.0426	190.0	144.0		18.0	82.0	5.547	38.0600	925; 1.2207	Kynurenic acid- ¹³ C ₆
					89.1	46.0					
Kynurenine- ¹³ C ₁₀	[13C]1 H14 N2 O7 S	316.22	219.1	202.1		4.0	74.0	4.462			
					100.1	13.0					
Kynurenine	[13C]10 H12 N2 O3	218.1183	209.1	192.0		6.0	73.0	4.472	65.1571	198; 0.6104	Kynurenine- ¹³ C ₁₀
					94.1	14.0					
Melatonin	C13 H16 N2 O2	232.1212	233.1	174.0		10.	87.0	8.029	126.5866	14628; 12207	Tryptamine-d ₄
					130.0	54.0					
N-acetylserotonin	C12 H14 N2 O2	218.1055	219.1	160.0		14.0	82.0	6.041	16.7533	330; 0.1526	None
					159.0	38.0					
					115.1	38.0					
N-formylkynurenine	C11 H12 N2 O4	236.0797	237.1	146.0		25.0	78.0	4.519	18.0338	213; 1.2207	3-hydroxykynurenine- ¹³ C ₃ (¹⁵ N)
					91.1	53.0					
Quinolinic acid	C7 H5 N O4	167.0219	168.0	150.0		6.0	63.0	1.969	244.2641	907; 9.7656	Quinolinic acid-d ₃
					78.1	22.0					
Quinolinic acid-d ₃	C7 H2 D3 N O4	170.0407	171.0	153.0		8.0	69.0	1.968			
					81.2	41.0					
Serotonin	C10 H12 N2 O	176.095	177.1	160.0		6.0	63.0	4.081	18.2686	3550; 0.6104	Serotonin-d ₄
					115.1	30.0					
Serotonin-d ₄	C10 H8 D4 N2 O	180.1201	181.1	164.1		6.0	68.0	4.064			
					118.1	34.0					

Tryptamine	C10 H12 N2	160.1	161.1	144.1		9.0	59.0	5.917	45.8119	8021; 0.6104	Tryptamine-d ₄
					115.1	41.0					
Tryptamine-d ₄	C10 H8 D4 N2	164.1252	165.1	148.1		9.0	64.0	5.900			
					118.1	45.0					
Tryptophan	C11 H12 N2 O2	204.0899	205.1	188.0	146.0	6.0	68.0	5.412	152.1974	1003; 1.2207	Tryptophan-d ₅
						18.0					
Tryptophan-d ₅	C11 H7 D5 N2 O2	209.1213	210.1	192.1		6.0	73.0	5.377			
					150.1	18.0					
Xanthurenic acid	C10 H7 N O4	205.0375	206.0	160.0		18.0	91.0	5.164	161.0387	568; 1.2207	Tryptophan-d ₅
					132.0	30.0					

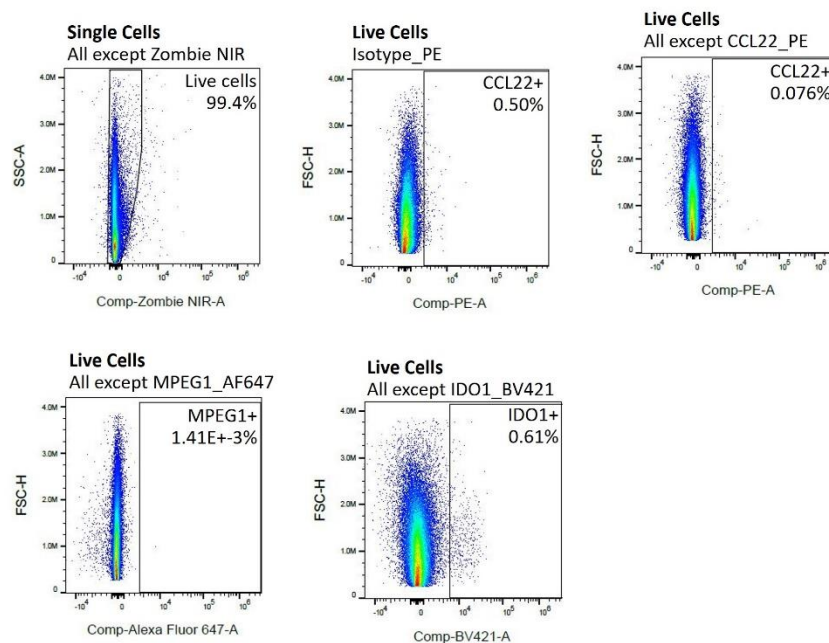
Transitions and Compound Specific MS Parameters, Liquid Chromatography Retention Time, Limit of Detection (LOD) and Limit of Quantification (LOQ)

APPENDIX B: SUPPLEMENTARY FIGURES

Gating Strategy



Gating Controls

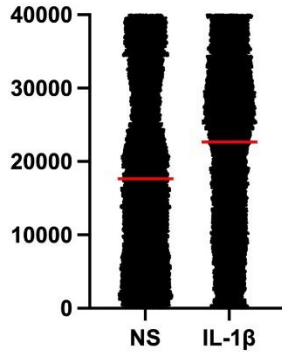


Supplementary Figure 1B: Flow Cytometry Gating Strategy

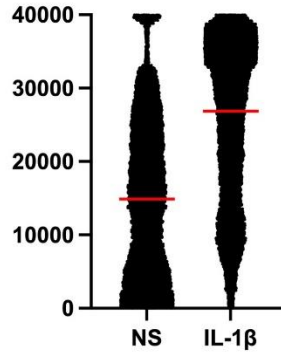
The acquisition threshold was set to 250,000 eliminating the acquisition of debris. Therefore, all the events within the plot were gated as cells. Next the single cells gated and from the FMO the live cells (viability dye negative) were gated. These live cells were used for Figures 6B, E, H. The protein-positive cells (CCL22+, MPEG1+, or IDO1+) were gated next based off the fluorescence-minus-one (FMO) and isotype gating controls. These protein-positive cells were used in Figures 6C, F, I.

Single Live Cells

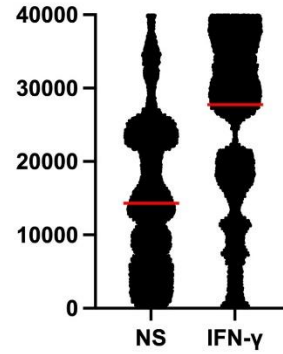
CCL22 NS vs IL-1 β
Mann-Whitney t-test Ranks



MPEG1 NS vs IL-1 β
Mann-Whitney t-test Ranks

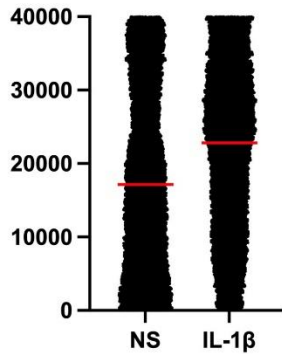


IDO1 NS vs IFN- γ
Mann-Whitney t-test Ranks

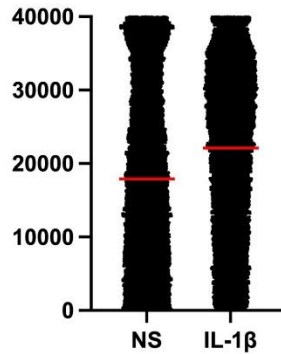


Protein Positive Cells

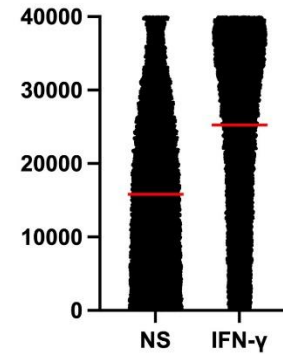
CCL22 NS vs IL-1 β
Mann-Whitney t-test Ranks



MPEG1 NS vs IL-1 β
Mann-Whitney t-test Ranks

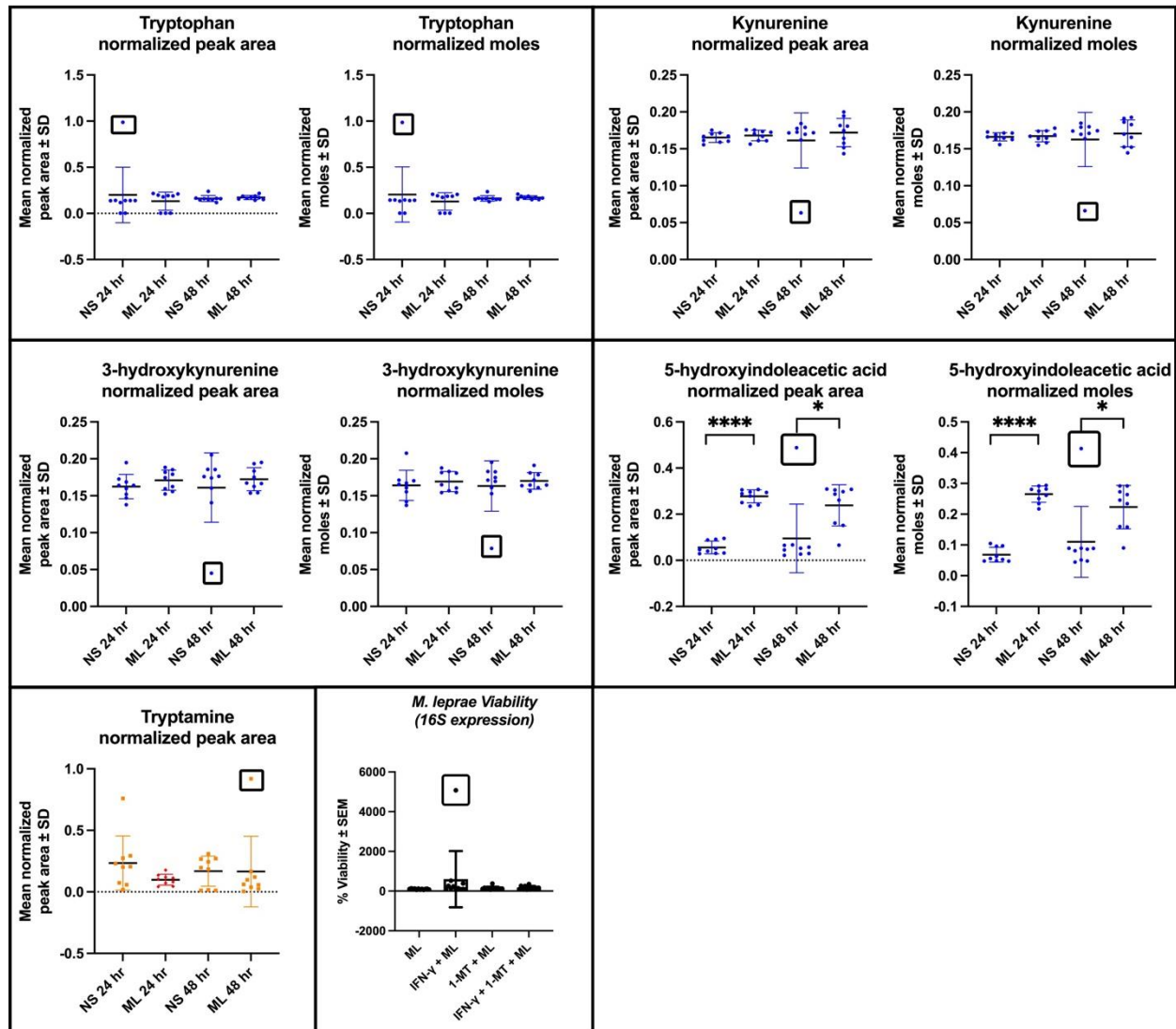


IDO1 NS vs IFN- γ
Mann-Whitney t-test Ranks



Supplementary Figure 2B: Mann-Whitney T-Test Rank Plots

The Mann-Whitney t-test rank plots for the single cell analysis done for Figure 6.



Supplementary Figure 3B: Removed Data Points

Select data points were removed because of the immense value difference compared to the other values intra- and inter-experiment. For Figure 16A, with the value present, there was no statistically significant difference between NS and ML at 24 hours, but there was after the value was removed. Despite this change being concerning, the removed value was ~675% greater than the next greatest value for tryptophan peak area and moles. For Figures 16B, C, and E, removing the value did not change the statistical significance between NS and ML. Removing the value reduced the difference between these conditions. For Figure D, removing the value increased the significance between the NS and ML, but the conditions significantly differed before the value was removed. The values in Figures A-E were removed before the normalization for the data presented in Figure 10. Removing the value in Figure 16F also did not affect the statistical significance between conditions, though it did decrease the differences.