2024-09-24

Defining the role of the pore-forming apolipoprotein L APOL7C in a Leishmania infection

Cedeno, Eymi

UNIVERSITY OF CALGARY

Defining the role of the pore-forming apolipoprotein L APOL7C in a *Leishmania* infection

by

Eymi Cedeno

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

GRADUATE PROGRAM IN IMMUNOLOGY

CALGARY, ALBERTA

SEPTEMBER, 2024

Abstract

Leishmaniasis, is a neglected tropical disease caused by infection with the intracellular parasite *Leishmania*. The parasite targets phagocytic cells, including dendritic cells (DCs), where it proliferates and spreads, leading to various forms of the disease, from cutaneous lesions to visceral organ damage. Despite the clinical significance, the molecular interactions between *Leishmania* and DCs remain largely uncharacterized. Dendritic cells, particularly conventional dendritic cells (cDCs), are pivotal in generating cytotoxic T lymphocyte (CTL) responses crucial for controlling *Leishmania* infections. The cross-presentation ability of cDCs has been identified as a key process in mounting these immune responses, primarily studied in murine models. However, the molecular mechanisms underlying the cross-presentation of *Leishmania* antigens by cDCs are still not fully elucidated.

In this study, we investigated the role of apolipoprotein 7C (APOL7C), a cDC-specific pore-forming protein, in *Leishmania* infection. Our results show that that APOL7C, is upregulated in response to a *Leishmania* infection. In addition, we find that APOL7C is recruited to the parasitophorous vacuole (PV) containing *Leishmania* parasites, particularly in late-stage PVs and that its recruitment creates damage in the membrane of the PV. Furthermore, our data showed that APOL7C recruitment to these compartments is dependent on the NADPH oxidase and that its insertion is voltage dependent. Interestingly, this recruitment of APOL7C appears to diminish the cross-presentation of *Leishmania* derived antigens of our infected cell lines in our in-vitro experiments. However, in our in vivo infection model using an *Apol7c-/-* mice we identified differences in the immune response in mice following inoculation of the ear. Following the course of infection the absence of *Apol7c* $^{-/-}$ mice showed a decrease in ear lesion at week 7 with higher parasite burden in the dermis. These results showed the relevance of APOL7C in

Leishmania infections providing new insights into the molecular mechanism underlying its role .

Acknowledgments

First, I would like to extend my sincere thanks to my academic supervisor, Dr. Johnathan Canton, and my co-supervisor, Dr. Robin Yates, for giving me the opportunity to join their research team and embark on my academic journey. I am especially grateful to Dr. Canton for his mentorship, guidance, and constant support, which allowed me to grow during my graduate studies. I also extend my thanks to my committee members, for their guidance and feedback through my research project.

I wish to express my deep appreciation to all the members of the lab Melanie Moore, Gerone Gonzales, Jen Nguyen, Liam Wilkinson, Cassie Woods, and our lab manager, Neil Mckenna. Their support and warm companionship made my time adjusting to Canada an enjoyable experience. Thanks for all the good times and laughs; I could not have asked for a better environment to pursue my academic goals as a graduate student Additionally, I would like to thank Matheus Carneiro for his assistance with the infection assays and his advice throughout my project.

My sincere thanks also go to my dear friends, Alexa Zhang and Saidili Lopez, for their strong support. Their good morning messages and long video calls were invaluable in helping me through my studies. A special thanks to Farheen Akthar for ensuring I always had a warm cup of Chai and homemade food during the many long days and nights during my studies.Finally, I would like to extend my gratitude to the Panamanian Secretariat for Science, Technology, and Innovation (SENACYT) and the consortium agency CALDO Scholarship for their contribution during my studies.

Dedication

This work is dedicated to my parents, Gloria Moreno and Alberto Cedeño, my sisters, Olga Cedeño and Anabelsis Moreno, and to my source of inspiration, my dear Grandma, Olga Sanchez. Thank you for your unconditional love, hard work, and unwavering support, which allowed me to expand my horizons.

Table of Contents

List of Figures

List of Abbreviations and Nomenclature

AmB	Amphotericin B
APCs	Antigen-presenting cells
APOL	Apolipoproteins L
BATF3	Basic leucine zipper transcriptional factor ATF-like 3
BCRs	B-cell receptors
BGS	Bovine growth serum
BM	Bone marrow
CPRG	Chlorophenol-Red β-D-galactopyranoside
CLIP	Class II-associated invariant chain peptide
cDCs	Classical or conventional dendritic cells
cDC1	Conventional dendritic cells type 1
cDC2	Conventional dendritic cells type 2
CLRs	C-type lectin receptors
CL	Cutaneous Leishmaniasis
DAMPs	Damage-associated molecular patterns
EDTA	Ethylenediaminetetraacetic Acid
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FV1	Friedlin V1
HSCs	Hematopoietic stem cells
IMDM	Iscove's Modified Dulbecco's Medium
IRF-8	Interferon regulatory factor 8 Lysosomal-
LAMP1	associated membrane protein 1 Limiting
LDA	dilution analysis
MAD	Membrane-addressing domain
MHC I	Major histocompatibility complex class I
MHC II	Major histocompatibility complex class II
MCL	Mucocutaneous Leishmaniasis

moDCs	Monocyte-derived dendritic cells
MOI	Multiplicities of infection
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NK	Natural killer cells
NBT	Nitro blue tetrazolium
NW	New World
OW	Old World
SIINFEKL	Ovalbumin epitope
PV	Parasitophorous vacuole
PAMPs	Pathogen-associated molecular patterns
PRRs	Pattern recognition receptors
SbV	Pentavalent antimonial compounds
PBS	Phosphate Buffered Saline
pDCs	Plasmacytoid DCs
PFD	Pore-forming domain
PFA	Paraformaldehyde
RFP	Red fluorescent protein
RLU	Relative luminescence units
RT	Room temperature
RPMI	Roswell Park Memorial Institute
sRBCs	Sheep red blood cells
SP	Signal peptide
TCRs	T cell receptors
Th2	T helper type 2
TLRs	Toll-like receptors
Tregs	T regulatory cells
TGF	Transforming growth factor
TAP	Transporter associated with antigen processing
Th1	Type 1 T helper
VL	Viseral Leishmaniasis

Chapter 1. Introduction

1.1 Epidemiology

Leishmaniasis is a infectious disease caused by an intracellular parasite of the genus *Leishmania*[1]. The parasite is transmitted to humans and other mammalian hosts by the bite of the sand fly[2].

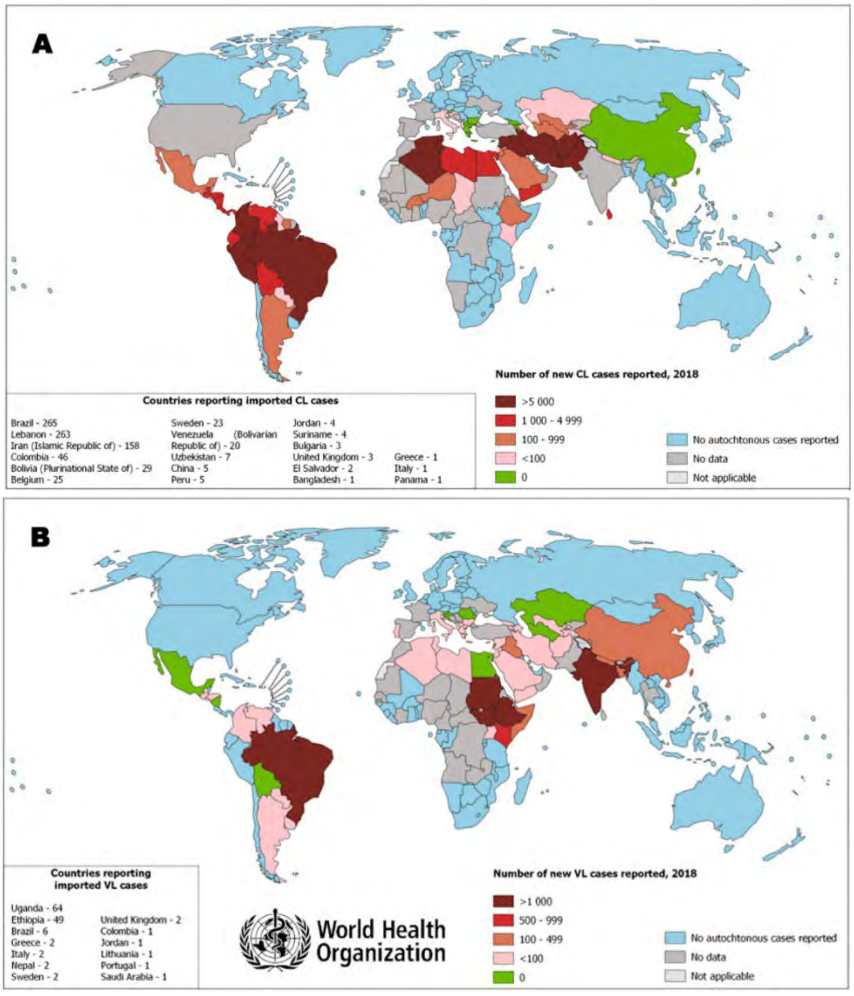

Figure 1. Leishmaniasis world distribution map. Worldwide geographical distribution of leishmaniases in 2018. **A)** The number of new cases of cutaneous and **B)** visceral leishmaniasis are indicated by colour codes. Non-endemic regions are shown in light blue, grey denotes countries with no data available [12].

It affects over 90 different countries throughout Asia, Africa, the Middle East, Central and South America [2,3,4]. The World Health Organization has recognized it as a neglected tropical disease since 2012, with an estimated annual burden of up to 2 million new cases each year and around 12 million people currently infected[1]. According to their geographical distribution, *Leishmania* species can be categorized into Old World (OW), referring to the species endemic to the Eastern Hemisphere, and New World (NW) referring to the species in the Western Hemisphere. Over 50 species of *Leishmania* parasites have been described and characterized, with over 20 species pathogenic to humans that cause the different clinical manifestations of leishmaniasis[3.] Clearly, better control strategies to block the transmission of the parasite are needed.

1.1.1 Clinical Features

Leishmaniasis encompasses a broad spectrum of clinical manifestations that can be classified into three main types: Cutaneous (CL), Mucocutaneous (MCL), and Visceral (VL). The pathology of the different manifestations and the progression of the infection depend on a complex interaction between the species of *Leishmania* that infects and the genetic and immunological condition of the host.

1.1.2 Cutaneous

Cutaneous leishmaniasis (CL) is the most common form of clinical manifestation, with a high disease burden and morbidity among all tropical neglected diseases. CL is described as a localized skin lesion known as a papule at the site of parasite inoculation that usually develops on exposed body sites, such as the face and extremities[4]. The papule slowly enlarges through infection and evolves into a nodule that progressively ulcerates. The ulcer is characteristic of CL and can self-heal over 3 to 18 months, depending on the species that infected the host. However, more than 8% of CL cases are estimated to show progression and become chronic, leading to

severe tissue destruction, disfigurement, and permanent scar formation resulting in a decrease in the quality of life for patients[5,6]. CL is mainly caused by *Leishmania* species such as*: L. tropica, L. aethiopica*, and *L. major* in the OW. However, in the NW, CL is caused by multiple species of both Leishmania subgenera: Leishmania (L. amazonensis, L. infantum, and L. mexicana) and Vianna subgenera (*L. braziliensis, L. guyanensis, L. panamensis*, and *L. peruviana*) [7].

1.1.3 Mucocutaneous

Mucocutaneous leishmaniasis (MCL) is a more severe manifestation that can develop as an extension of local cutaneous leishmaniasis (CL) and is characterized by destructive and disfiguring lesions in the nasopharyngeal mucosa, which can appear months or years after the resolution of primary lesions. MCL can occur due to infection by *Leishmania* species of the *viannia* subgenus, including *L. braziliensis, L. amazonensis, L. panamensis*, and *L. guyanensis*, with a prevalence rate of 20% in endemic areas[8]. MCL causes symptoms such as nasal congestion, bleeding, mucosal erosion, inflammation, perforation of the nasal septum, and partial destruction of the tissues in the mouth, nose, and pharynx. Treating MCL is challenging due to the unregulated inflammatory response that leads to tissue destruction[9] .

1.1.4 Visceral

Visceral leishmaniasis (VL), also known as kala-azar, is the most severe clinical manifestation of leishmaniasis, with 95% of untreated cases leading to death[10]. This manifestation is predominantly caused by species of the *L. donovani* complex (*L. donovani* and *L. infantum*) in Asia and Eastern Africa and by *L. infantum* in Latin America, Central Asia, and the Mediterranean region[11]. It is characterized by a systemic infection that predominantly affects the liver and spleen, with an incubation period of 2-6 months. In the early stages, signs of infection

include fever, weight loss, fatigue, and abdominal pain, evolving into more severe manifestations such as hepatosplenomegaly, pancytopenia, and hypergammaglobulinemia[12]. If left untreated VL is fatal, primarily due to severe cachexia, secondary infections, and hemorrhage. A notable characteristic of clinical VL is the apparent cellular anergy to parasite antigens. This anergy may result from the improper presentation of antigens by T-cells and the induction of cytokines that inactivate macrophages [13,14]. In cases where VL is treated, documented causes of death in clinical studies include severe intractable diarrhea and antimonial toxicity [15].

1.2 Life Cycle

The life cycle of *Leishmania* alternates between the phlebotomine sandfly vector and its final mammalian host. *Leishmania* presents two main morphological stages through its life cycle: the motile promastigotes in the sandfly and intracellular amastigotes in host cells. Inside their sandfly vector, *Leishmania* is known to exist in its promastigote form, characterized by its large elongated ovoid cell body and its flagellum[16]. This motile form allows the parasite to move through the intestines of its vector. The sandflies from the *Phlebotomus* or *Lutzomyia* genera ingest and acquire *Leishmania* parasites when they take a blood meal from their mammalian reservoir (humans, rodents, or canids), this rapid shift in temperature and pH due to vector uptake causes the immotile amastigotes within the bloodmeal to transform into procyclic promastigotes[17] .

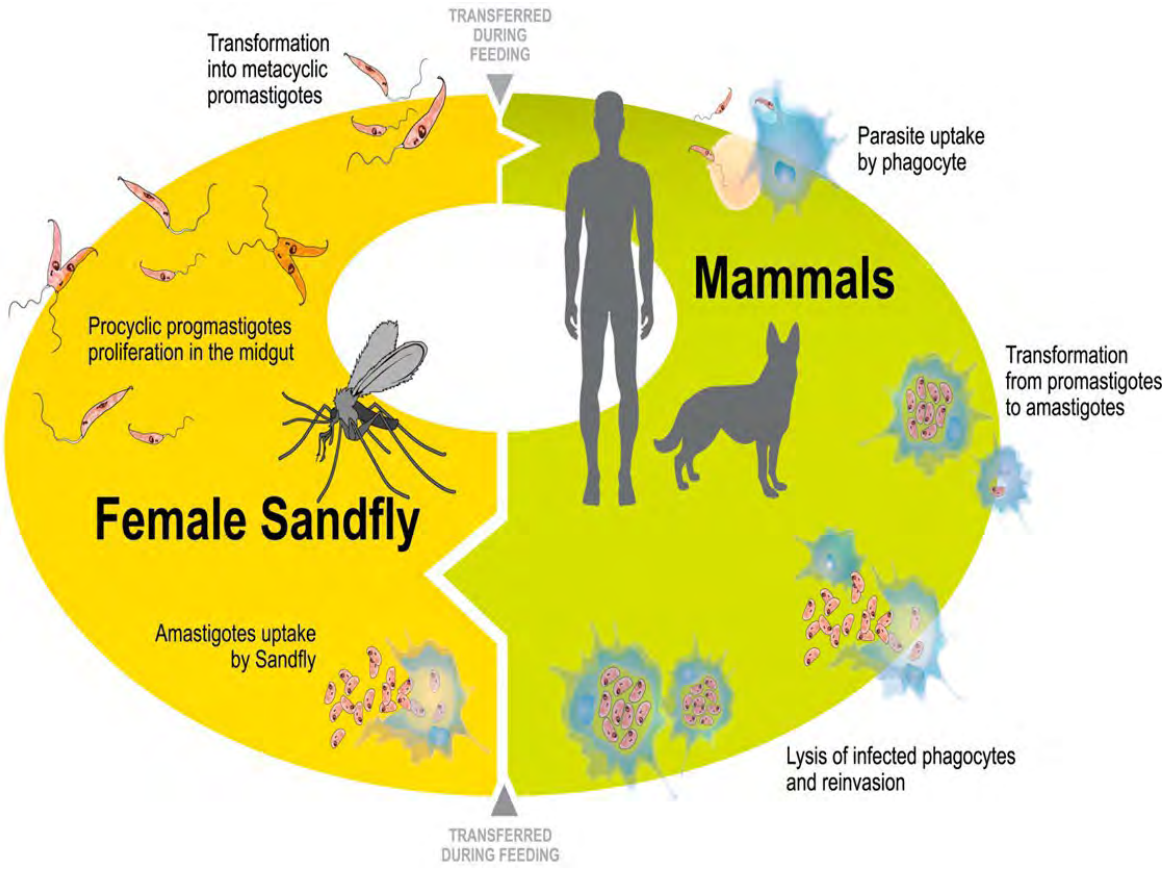

Figure 2. *Leishmania* life cycle. As the female sandfly takes a blood meal, it introduces metacyclic promastigotes into the host's skin. These promastigotes are subsequently engulfed by phagocytic cells at the bite site. Within these cells, the promastigotes differentiate into amastigotes, the intracellular replicative form of the parasite. The proliferation of amastigotes eventually leads to the lysis of the host cell, facilitating the dissemination of the parasites and the subsequent infection of adjacent phagocytic cells. When another sandfly feeds on the infected host, it ingests these amastigote-laden cells, which then differentiate into procyclic promastigotes within the sandfly's midgut. These procyclic promastigotes further develop into metacyclic promastigotes, thereby completing the transmission cycle [20].

Over several days, the promastigotes then migrate throughout the digestive tract of the sandfly and attach to the epithelium of the midgut or the hindgut to transform into their metacyclic and infective stage[19.]

Upon their next blood meal, sandflies inoculate infective and motile metacyclic promastigotes into the mammalian host's dermis. After inoculation, multiple types of phagocytes are recruited to the site, which internalizes the parasites via phagocytosis. After phagocytosis, *Leishmania* promastigotes reside in a membrane-bound phagosome, which undergoes acidification following

fusion with lysosomes to develop a phagolysosome-derived structure known as parasitophorous vesicles (PV)[20,21]. The PV is an acidic compartment where the ingested parasites delay the cell's microbicidal mechanisms to allow for their differentiation into the intracellular amastigote form. In the PV, amastigotes replicate and establish infection in the mammalian host [22].

1.3 Mechanism of evasion

Leishmania, similar to other intracellular parasites such as *Toxoplasma gondii* and *Trypanosoma cruzi*, has developed mechanisms to evade immune responses, ensuring its survival within the mammalian host[23] . Once successfully infecting the host cells, the parasite navigates the hostile environment by disrupting various host cell signals. *Leishmania* parasites modify molecular pathways in the immune response, including the production of microbicidal factors, pro-inflammatory cytokines, and antigen presentation, which allows the parasite to evade the host's immune defenses[24,25]. The parasites manipulate or inhibit the effective type 1/Th1 immune response to ensure their survival. Research has demonstrated that *Leishmania* reduces T cell priming by downregulating the expression of toll-like receptors (TLRs) and IL-12 production in macrophages [26.] Additionally, *Leishmania* parasites enhance the activity of T helper type 2 (Th2) and T regulatory cells (Tregs), increasing the secretion of anti-inflammatory cytokines such as transforming growth factor (TGF)-β, IL-4 and IL-10, promoting the parasite's survival and contributes to chronic infection [27.]

1.4 Treatment

Currently, the first line of treatment for the different manifestations of leishmaniasis for over 50 years has relied on the intravenous or intramuscular application of pentavalent antimonial

compounds (SbV). The most common formulations for treatment are antimoniate (Glucantime) and sodium stibogluconate (Pentosam), due to their high clinical effectiveness[28,29]. However, the administration of these compounds requires prolonged treatment regimens of up to 30 days and is associated with a range of side effects, from mild symptoms like headache, nausea, dizziness, abdominal pain, and weakness to severe adverse effects such as nephrotoxicity, pancytopenia, cardiopathy, and peripheral neuropathy[30]. As an alternative to these outcomes, several drugs, including amphotericin B (AmB), azoles, paromomycin, antifungal azoles, and miltefosine, have been approved as second-line treatments[31].In recent years, the World Health Organization has included liposomal amphotericin B in its recommended treatments for leishmaniasis. This formulation, which utilizes a novel controlled-release system, has achieved an 80% efficacy rate and has been associated with reduced side effects compared to conventional formulations. [32]. However, its high cost, transportation, and storage requirements limit its use in developing regions. Additionally, in the last decade, multiple studies have shown an emerging and increasing drug resistance to first-line treatments in endemic regions[28]. Furthermore, challenges such as variability in parasite species, treatment duration, host immune response, and treatment accessibility emphasize the need for new therapeutic targets and effective treatments for leishmaniasis. As a result, numerous studies have focused on the development and experimental testing of vaccines and immunotherapy candidates for leishmaniasis, such as immunomodulators, TLR agonists, and cytokines[33,34]. Currently, both first- and second-generation therapeutic vaccines are undergoing clinical trials aimed at enhancing the host immune response, improving parasite clearance, minimizing side effects, and overcoming drug resistance.

1.5 Immunity

The immune system is a complex and versatile network of molecular and cellular mechanisms that have evolved to recognize and eliminate exogenous foreign particles. In vertebrates, the immune system is traditionally categorized into two main branches: the innate and the adaptive immune systems.

1.5.1 Innate Immunity

Innate immunity is characterized by physical barriers like the skin and mucosal surfaces, as well as immunological cells such as monocytes, macrophages, natural killer (NK) cells, and dendritic cells [35]. These components constitute the first line of defense against pathogenic invasion. The innate immune system functions through pattern recognition receptors (PRRs) that detect conserved molecular patterns shared among pathogens, apoptotic host cells, and damaged cells. These patterns are divided into pathogen-associated molecular patterns (PAMPs), which indicate the presence of pathogens, and damage-associated molecular patterns (DAMPs), which signal tissue damage[36]. The recognition of PAMPs and DAMPs by PRRs triggers a cascade of proinflammatory signals that includes the release of cytokines and antimicrobial peptides, initiating an early defensive response. Although the innate immune response provides a rapid reaction within minutes to hours, its non-specific receptor repertoire limits its ability to identify specific pathogens[33.]

1.5.2 Adaptive Immunity

In contrast to the innate immune response, adaptive immunity is characterized by a slow but highly specific response that develops over days to weeks upon first exposure to a new pathogen[38]. This immune response relies on the recognition functions of B and T cell lymphocytes. Both B and T cells generate receptor diversity and specificity through a process of

gene rearrangement involving variable (V), diversity (D), and joining (J) gene segments, which undergo splicing, somatic recombination, and random nucleotide addition at VD or DJ junctions(39). T cells recognize antigens using T cell receptors (TCRs) on their surface, and antigen-presenting cells (APCs) such as dendritic cells and macrophages present these antigens on major histocompatibility complex (MHC) molecules to T cells, providing co-stimulatory signals that drive T cell activation and proliferation[40]. B lymphocytes, on the other hand, recognize antigens directly through B-cell receptors (BCRs). Although B cells serve as APCs, another function of these cells is to differentiate into plasma cells that secrete antibodies. These antibodies circulate throughout the body, facilitating pathogen clearance[41,42]. In addition, B cells also generate memory cells that persist long after an infection has been cleared, allowing for a rapid and effective response to future encounters with the same pathogen[43].

1.6 Dendritic Cells

Dendritic cells (DCs) serve as professional antigen-presenting cells (APCs) that reside in various lymphoid and non-lymphoid tissues. Derived from hematopoietic stem cells (HSCs) in the bone marrow (BM), DCs are pivotal in immune responses by acting as a bridge between the innate and adaptive immune systems through the detection of danger signals[44]. In a steady-state environment, dendritic cells (DCs) function as regulators of immune tolerance by presenting self-antigens to T cells. However, in response to infection or inflammation, immature DCs located in peripheral tissues act as vigilant sentinels, constantly sampling their environment to detect exogenous “danger” signals. Upon recognizing these danger signals, DCs undergo activation and maturation, which is characterized by the upregulation of specific chemokine receptors like CCR7 and CD62L[45]. These receptors guide the DCs' migration to secondary lymphoid organs where they play a vital role in the activation of naïve T cells and the subsequent

modulation of immune responses[46]. Based on their functional localization and phenotype, DCs can be classified into four different types: classical or conventional (cDCs), Plasmacytoid DCs (pDCs), monocyte-derived DCs (moDCs), and Langerhans cells[47,48]. cDCs can be further subdivided based on their specialization into conventional dendritic cells type 1 (cDC1) and conventional dendritic cells type 2 (cDC2). The cDC1 subset requires interferon regulatory factor 8 (IRF-8) and the basic leucine zipper transcriptional factor ATF-like 3 (BATF3) for their development, are identified by the expression of CD8α in mice and CD141 (BDCA-3) in humans. These cells are essential for the cross-presentation of exogenous antigens through major histocompatibility complex (MHC) class I molecules, which is crucial for the activation of $CD8^+$ cytotoxic T lymphocytes[49.] In contrast, conventional dendritic cells type 2 (cDC2s) are a specialized subset of dendritic cells distinguished by their expression of CD11b and their role in antigen presentation through MHC class II molecules[50.]

To drive adaptive immune responses, DCs digest internalized antigens into peptides, which subsequently associate with the major histocompatibility complex class I (MHCI) and class II (MHC II) molecules. These peptide-MHC complexes are then transported to the cell surface, where they can activate T cells. The MHC class I molecule is expressed on the cell surface of all nucleated cells and is composed of a heavy and invariant light chain known as β2-microglobulin [46]. In this pathway, the peptide fragments of endogenous antigens from intracellular events such as viral infections, cellular or intracellular bacteria are assembled onto MHCI in the endoplasmic reticulum (ER) and transported to the cell surface[51]. This is referred to as the endogenous pathway of MHCI loading.

In the case of exogenous antigens, DCs present them through their MHCII surface molecules. In contrast with MHCI, MHCII molecules are composed of an α and a β chain that is stabilized by

an invariant chain in the ER. From the Golgi, both the invariant chain and MHCII complex are transported to a late endosome. After getting into endocytic organelles, cathepsin S and L digest the invariant chain and produce an MHCII-associated invariant chain peptide (CLIP). Peptides generated in endocytic organelles such as lysosomes and phagolysosomes replace CLIP and can then be presented on the cell surface to $CD4^{+}$T cells [52,53]. This is referred to as the exogenous pathway of MHCII presentation.

1.7 Cross-presentation

Antigen-presenting cells (APCs) have the ability to uptake and present exogenous antigens on MHCI[54]. This process, referred to as cross-presentation, departs from both the endogenous pathway for loading MHCI and the pathway for loading exogenous antigens onto MHCII.

Previous studies have defined cross-presentation as a function that is efficiently carried by DCs,

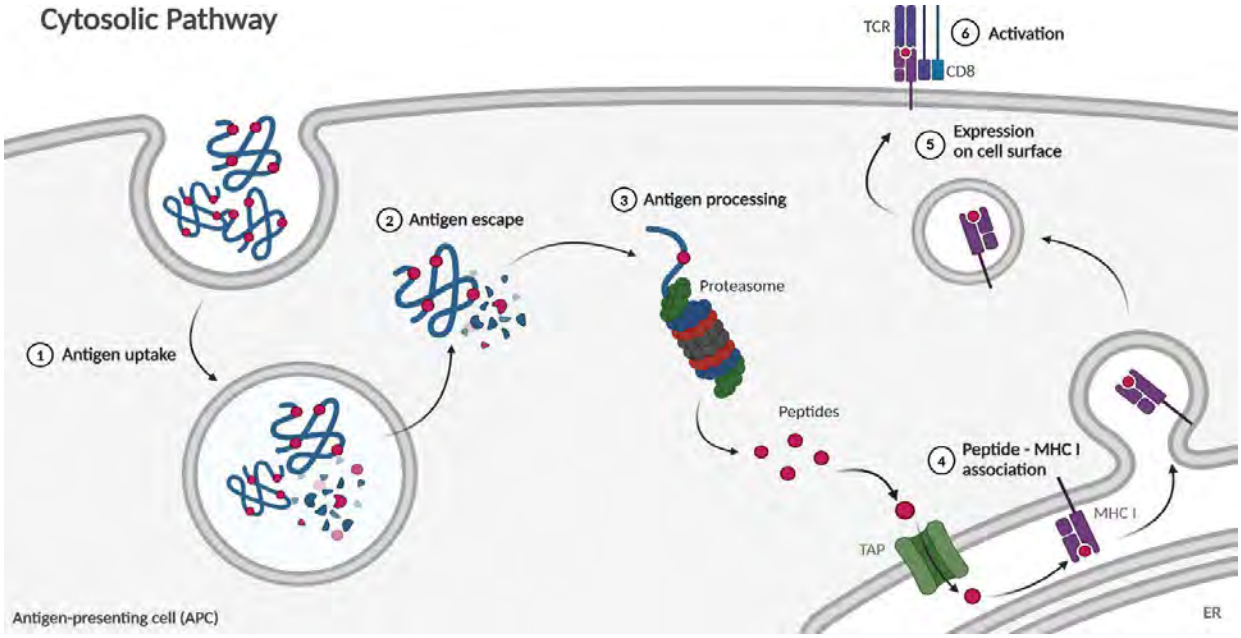

Figure 3. Schematic overview of the cytosolic pathway of cross-presentation. After the phagocytosis of exogenous antigens by APCs into endosomal compartments, the antigens gain access to the cytosol. In the cytosol, the antigens are further degraded by the proteasome, and the resulting antigen-derived peptides are then transported into the ER. Within the ER, these peptides are loaded onto MHC I molecules, which are then transported to the cell surface to activate $CD8^{+}$ T cells. Diagram created with biorender

specifically the cDC1 subset[51,56].Although the specific molecular mechanism of cross-presentation has been studied over the last decade, two main pathways have been proposed: the cytosolic pathway and the vacuolar pathway.

In the cytosolic pathway (**Figure 3**), after phagocytosis, exogenous antigens in the endosomal compartment gain access to the cytosol, where the proteasome degrades them into peptides that can be loaded by the transporter associated with antigen processing (TAP) in the MHCI in the endoplasmic reticulum.[57,58]

By contrast, in the vacuolar pathway, the peptide degradation of exogenous proteins is performed by lysosomal enzymes within the endocytic compartments (phagosomes or endosomes), and peptides are loaded onto MHCI molecules in endocytic organelles. In this mechanism, cathepsin S has been described as the main protease that generates antigenic peptides[55] and is characterized by not being dependent on the proteasome machinery or the TAP protein[59.] The potential of cDC1s to cross-present antigens has initiated research aimed at finding strategies to improve tumor- and viral-specific $CD8^+$ T cell responses as an immunotherapy strategy for the treatment of cancer or infectious diseases.

In the case of *Leishmania* antigen cross-presentation, it has been suggested that this process may not strictly follow the cytosolic pathway, as observed in other intracellular microorganisms such as *Escherichia coli* (1), *Mycobacterium tuberculosis* (2), and *Listeria monocytogenes* (3), where TAP dependency is well established . A 2006 study demonstrated that dendritic cells (DCs) infected with *Leishmania major* can prime CD8+ T cells in a TAP-deficient mouse model, indicating that *Leishmania* antigens may bypass the classical TAP-dependent pathway (4). The authors proposed that *Leishmania* antigens could be degraded within endosomal compartments, bypassing cytosolic translocation and TAP-mediated transport to the endoplasmicreticulum (ER) (5).

However, intracellular pathogens like *Leishmania* may employ multiple cross-presentation pathways.

For example, a study showed that cross-presentation of OVA by *Toxoplasma gondii*-infected DCs was strictly TAP- and proteasome-dependent (6). These findings suggest that *Leishmania* antigens might evade the more efficient TAP-dependent cross-presentation machinery by remaining Sequestered within their parasitophorous vacuoles (PVs). The cross-presentation of *Leishmania* antigens thus presents a significant contradiction between vacuolar and cytosolic pathways, raising important questions about the relative contributions of each mechanism. Resolving this contradiction remains an ongoing area of research, aimed at identifying and characterizing the molecular players involved in *Leishmania* antigen processing

1.8 DCs and *Leishmania*

The outcome of Leishmania infection is influenced not only by the species of the parasite but also by the host's immune response. Although most studies on immune cells involved in *Leishmania* infections have focused primarily on macrophage cell lines, evidence shows that DCs also internalize *Leishmania* parasites and play a crucial role in regulating the infection. During the early stages of *Leishmania* infection, DCs help manage parasite proliferation by shaping the cytokine microenvironment and fostering a protective type 1 T helper (Th1) immune response. After phagocytosing *Leishmania* parasites, DCs migrate to primary lymphoid organs

where they process and present antigens to naïve T cells, thus initiating an adaptive immune response. The Th1 response is vital for disease resolution, as it involves the production of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-12, and IL-18, which collectively promote the clearance of *Leishmania* infection[60].

DCs recognize and internalize antigens through various surface receptors, including pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) [61,62] . Upon phagocytosis, *Leishmania* parasites are internalized into a phagosome, which later develops into a parasitophorous vacuole (PV). Although information on PVs harboring *Leishmania* parasites is derived almost exclusively from studies with macrophages and macrophage-like cell lines, several studies have shown that DCs also internalize and form *Leishmania*-harboring PVs[63,64]. Recent advancements in the study of DC subsets have deepened our understanding of DC-*Leishmania* interactions, suggesting that DCs recognize and ingest the parasites through receptors such as TLR-2, TLR-4, and TLR-9[65].In addition, the role of cross-presentation by cDC1 in *Leishmania* has been described using mice that lack the BATF3 transcriptional factor. During infection with *L. major*, *Batf3*-/- mice, which lack cDC1s, showed a strong defect in the capacity to generate a Th1 immunity, and this resulted in a high parasite load and non-healing lesions[56,66]. In another study with *L. infantum,* BATF3-dependent DCs also played a protective role during *L. infant* infection, as higher numbers of parasites were observed in the liver, but not spleen or bone marrow, around 7 weeks post-infection in *Batf3*$^{-/-}$ versus wild-type mice [12].

However, the specific role played by the cDC1 subset during *Leishmania* infection is still unclear, and the molecular mechanism by which these cells cross-present *Leishmania* antigens remains elusive.

1.9 The apolipoprotein L family

The apolipoproteins L (APOL) gene family consist of six genes in human, and thirteen genes in mice[64].

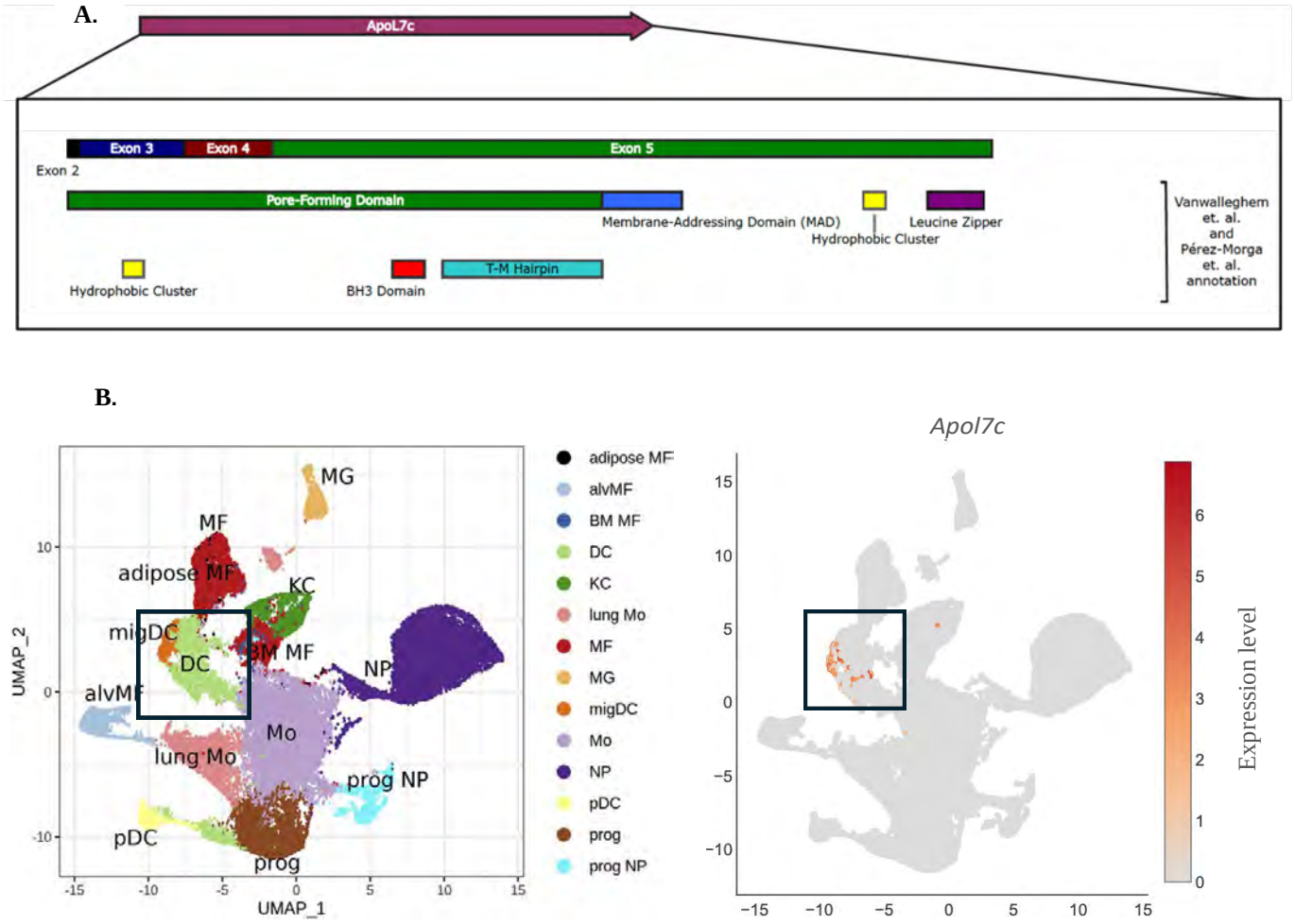

Figure 4. APOL7C is expressed exclusively by cDCs. A) Domain structure of APOL7C as characterized by Vanwalleghem et al. and Perez-Morga et al.[67] **B)** ImmGen open-source single-cell RNA-seq profiling of myeloid cells tabula Muris Senis (mTMS) separated by cell type showing DCs as expressors of APOL7C. alvMF, alveolar macrophage; bone marrow macrophage, BM MF; DC, dendritic cell; KC, Kupffer cell; MF, macrophage; MG, microglia; migDC, migratory dendritic cell; Mo, monocyte; NP, neutrophil; pDC, plasmacytoid dendritic cell; prog, progenitor;pDC.

These proteins can be upregulated in response to inflammatory molecules such as IFN-α, IFN-β, and TNF-α [65]. Due to the high sequence homology among APOL family members, they share three main functional domains: a pore-forming domain (PFD), a membrane-addressing domain (MAD), and a SRA-interacting-protein-like domain (SID), which are conserved across different isoforms [66, 67].

Recent studies have highlighted that several human APOL proteins, including APOL1, APOL2, APOL3, and APOL6, exhibit antiviral activity against RNA viruses [68]. Among these, APOL1 has been the most extensively studied due to its unique ability to be secreted extracellularly via an N-terminal signal peptide (SP), which enables it to form colicin-like pores capable of lysing *Trypanosoma brucei* parasites [69, 70]. Although the PFD's pore-forming function is believed to be conserved across APOL isoforms due to their sequence similarities, structural and functional knowledge for most members of the APOL family remains limited, with current research primarily focused on APOL1 [71].

The overall goal of my thesis is to better understand the molecular mechanism of the novel APOL7C protein in a Leishmania major infection using a combination of both in-vitro and in-vivo mouse approaches.

1.10 T-Cell Immune Response in Leishmaniasis

In *Leishmania* infections, the immune response plays a critical role in determining disease outcomes. The immune system's ability to recognize and mount an effective response relies on the activation and coordination of T lymphocytes, which are the key drivers of adaptive immunity. T cells originate in the bone marrow and mature in the thymus, where they differentiate into two classes: CD4+ helper T cells or CD8+ cytotoxic T cells (CTLs), based on their interaction

with major histocompatibility complex (MHC) molecules. CD4+ T cells recognize antigens presented on MHC class II molecules, typically by professional antigen-presenting cells (APCs), while CD8+ T cells respond to antigens presented on MHC class I molecules, which are expressed by most nucleated cells[78]. Once matured, T cells circulate through peripheral lymphoid tissues, primed by antigens presented by APCs, such as dendritic cells and macrophages. Naive CD8+ T cells emerging from the thymus are inherently programmed to become CTLs, although they do not yet express the functional characteristics of activated effector cells. In contrast, the fate of naive CD4+ T cells is more complex . Upon activation, naive CD4+ T cells can differentiate into distinct subsets, primarily Th1, Th2, Th17 , or regulatory T cells (Tregs), each of which plays a unique role in the immune response. This differentiation process, known as clonal expansion, is triggered upon the first antigen encounter and is guided by the cytokine environment and signals provided by APCs, which ultimately shape the immune response and determine its effectiveness in pathogen clearance[79].

The development of a Th1 response is driven primarily by key cytokines such as interleukin-12 (IL-12) and interferon-gamma (IFN-γ) in the surrounding microenvironment. APCs, particularly dendritic cells and macrophages, secrete IL-12 in response to pathogen-associated molecular patterns (PAMPs) that are recognized by Toll-like receptors (TLRs) on their surface. IL-12 stimulates the activation of signal transducer and activator of transcription 4 (STAT4) in naive CD4+ T cells, which directs their differentiation into Th1 cells [80]. Once differentiated, Th1 cells secrete large amounts of IFN-γ, which amplifies the Th1 response and induces macrophage activation . IFN-γ is critical in driving macrophages toward the M1 phenotype, also known as classically activated macrophages. These M1 macrophages are highly effective in killing intracellular pathogens such as *Leishmania,* as they upregulate inducible nitric oxide synthase (iNOS), resulting

311 in the production of nitric oxide (NO), a reactive nitrogen species essential for the destruction
312 of intracellular parasites. Additionally, M1 macrophages produce reactive oxygen species (ROS),
313 pro-inflammatory cytokines, and chemokines that recruit additional immune cells to the site of infection
314 [81]. The M1 phenotype, characterized by enhanced antimicrobial activity, is critical for controlling
315 *Leishmania* infections in genetically resistant hosts such as C57BL/6 mice.
316 In contrast, the development of a Th2 response is promoted by the cytokine interleukin-4 (IL-4),
317 , which can be produced by various immune cells, including innate lymphoid cells, basophils,
318 or even CD4+ T cells themselves. IL-4 activates signal transducer and activator of transcription 6
319 (STAT6) in naive CD4+ T cells, leading to their differentiation into Th2 cells (5). Th2 cells
320 secrete cytokines such as IL-4, IL-5, and IL-13, which promote humoral immunity and polarize
321 macrophages into the M2 phenotype. M2 macrophages, also known as alternatively activated
322 macrophages are primarily involved in tissue repair and immune regulation rather than microbial
323 killing. They exhibit reduced microbicidal activity due to the suppression of iNOS and NO
324 production. Instead, M2 macrophages facilitate anti-inflammatory responses and tissue remodeling
325 by secreting growth factors, anti-inflammatory cytokines such as IL-10 and TGF-β, and enzymes
326 involved in extracellular matrix repair [82]. In *Leishmania* infections, the polarization of macrophages
327 into the M2 phenotype by Th2 cytokines supports parasite persistence, as M2 macrophages are
328 ineffective at clearing intracellular pathogens. This Th2/M2 response is typically seen in susceptible
329 mouse strains, such as BALB/c mice, where parasites persist due to impaired macrophage killing
330 capacity.
331 Th17 cells play a role in the immune response by secreting pro-inflammatory cytokines,
332 such as interleukin-17 (IL-17) and interleukin-6 (IL-6), which promote the recruitment of neutrophils
333 and other immune cells to the site of infection. While Th17 cells enhance the immune system's ability t

o control *Leishmania* through their inflammatory effects, excessive or unregulated Th17 activity can result in tissue damage and exacerbate disease pathology. Therefore, Th17 cell responses require careful regulation to prevent excessive inflammation and tissue injury during *Leishmania* infections (82).

Regulatory T cells (Tregs), defined by the expression of the transcription factor Foxp3 and the production of immunosuppressive cytokines such as IL-10 and transforming growth factor-beta (TGF-β), are essential modulators of immune responses. While Tregs help prevent excessive inflammation in the context of chronic *Leishmania* infections, Tregs can promote parasite survival by suppressing the protective Th1 response in a *Leishmania* infection[84] . Tregs accumulate at the infection site, where they limit the ability of the host immune system to eradicate the parasite, leading to persistent infection. Similarly, secretion of IL-10 produced by Tregs has been demonstrated to downregulate macrophage activation, reducing the effectiveness of parasite clearance[85]. Elevated Treg activity is often linked to the persistence of *Leishmania* infection, where the immune system fails to eliminate the parasite, resulting in chronic disease. Although it has been shown that depletion of Tregs in experimental models can enhance the immune response, allowing for improved parasite clearance, however, it also may lead to excessive tissue damage[86]. The balance between Th1-driven protective immunity and Th2/Treg-mediated immune suppression is thus crucial in determining the outcome of infection. A dominant Th1 response facilitates effective parasite control and lesion healing, while a skewed Th2/Treg response contributes to chronic infection, promoting sustained parasite persistence[87]. This complex interplay between immune regulation and pathogen persistence underscores the challenge of developing treatments that can modulate immune responses without exacerbating tissue in Leishmania infections.

1.9.1 Hypothesis

The putative pore-forming protein APOL7C is recruited to *Leishmania*-containing PVs. Once there, APOL7C drives the formation of large, non-selective pores that allow for the escape of *Leishmania* antigen to the cytosol for cross-presentation by DCs.

My project has two specific aims:

1.9.2 Specific aims:

Aim 1: Characterize APOL7C recruitment to PVs harboring *L. major* parasites. Previous work in the Canton lab has characterized APOL7C recruitment to DC phagosomes challenged with experimental particles such as silica beads. Here, I wish to extend these findings to the intra-phagosomal pathogen *Leishmania*.

Aim 2: Establish the role of APOL7C for cross-presentation during *L. major* infection. Previous studies in DCs have addressed the importance of cDC1s for the control of *Leishmania* infection. In these studies, the role of cross-presentation was addressed using parasites that express the model antigen ovalbumin (OVA). Several OVA-expressing *Leishmania* parasites have been generated. Notably, only parasites that secrete OVA via their flagellar pocket generate robust anti-CD8 T cell responses. This is informative to the pathology of *Leishmania* infections as it suggests that secreted proteins are likely to serve as the primary source of antigen for generating anti-*Leishmania* CD8 T cell responses. Indeed, *Leishmania* parasites are known to secrete virulence factors during infection via their flagellar pocket, many of which gain access to the host cell cytosol by an unknown mechanism. To date, studies on cross-presentation usually implemented BATF3-dependent cDCs; however, they have not used parasites that secrete OVA

to assess the cross-presentation of *Leishmania* derived antigens by cDCs. I will, therefore, optimize cross-presentation assays to assess the role of APOL7C in the cross-presentation of secreted antigens.

Chapter 2. Materials and methods

2.1 Culture and isolation of *Leishmania* parasites

Infection assays were carried out by using three lines of *Leishmania* promastigotes; the *L. major* NIH Friedlin V1 (FV1) from the JordanValley (MHOM/IL/80/FN), a stable transfected line of *L. major* FV1 promastigotes expressing a red fluorescent protein (RFP) RFP-L. major [67] and, *L. major* stable line that expresses a portion of the OVA gene encoding amino acids 232 to 288 containing the MHC class I-restricted OVA257-264 (SIINFEKL) epitope OVA-*L. major* [68] (kindly provided by Dr. Nathan Peter's laboratory). Promastigotes were cultured in and maintained in a logarithmic phase with a routine passage every 2-3 days in medium 199, supplemented with heat-inactivated fetal bovine serum (FBS), penicillin, streptomycin, L-glutamine, HEPES, adenine (in 50mM HEPES), hemin, and biotin, in an incubator at 26°C. For experiments, active, fast-swimming infective-stage metacyclic promastigotes were isolated from the stationary culture of 4 days old by a ficoll gradient [69]. After the infective parasites were isolated, the pellet was resuspended in a non-supplemented RPMI 1640 medium, and the parasites were quantified using a hemocytometer.

2.2 Cell culture Maintenance

RawKb.APOL7C::mCherry, RawKb.APOL7C::GFP, $p22^{phox-/-}$RawKb.APOL7C::mCherry, RawKb.APOL7C$_{EE}$::mCherry, and RawKb.APOL7C$_{EEE}$::mCherry cell lines were cultured in 10

mm tissue-culture-treated polystyrene plates using complete RPMI supplemented with bovine growth serum (BGS), penicillin, streptomycin, glutamate, sodium bicarbonate, and HEPES. When cell confluency of 75-85% was reached, the cells detached with a cell scraper, diluted (1:40) and seeded into new plates. The cell culture were splitted every 2-3 days and maintained in an incubator at 37°C and 5% CO_2.

MutuDCs were cultured in 10 mm tissue-culture-treated polystyrene dishes using complete IMDM supplemented with heat-inactivated fetal bovine serum (FBS), penicillin, streptomycin, glutamate, sodium bicarbonate, and HEPES. When cell confluency of 75-85% was reached, the MutusDCs were lifted by adding 5 ml of 10 mM EDTA in PBS for 10min at room temperature (RT). After the time was reached, cells were pelleted at 1200 rpm for 5 minutes, diluted (1:10 or 1:5), and finally resuspended in complete IMDM into new plates. The cells were splitted every 2-3 days and were maintained in an incubator at 37°C and 5% CO_2.

2.3 Mice

C57BL/6J mice were obtained from Jackson laboratories and *Apol7c*$^{-/-}$ mice were generated by GemPharmatech llc by microinjecting precomplexed recombinant Cas9 and sgRNAs that targeted sequences flanking exons 4 and 5 of *Apol7c* into fertilized eggs of C57Bl/6J mice. Fertilized eggs were transplanted to obtain positive F0 mice which were confirmed by PCR. Stable F1 generation mice were generated by breeding the F0 mice with C57BL/6J mice. All mice were bred at the University of Calgary Foothills Campus mouse facility under specific pathogen-free conditions. All mouse assays were performed in accordance with national with institutional guidelines for animal care under specific protocols.

2.4 Generation of a RawKb.APOL7C::GFP cell line

The synthetic construct expressing APOL7C::GFP was cloned into a pSBtet-blasticidin vector using a standard Gibson cloning method. Next, the vector was transfected along with the SB100X transpose using the FugeneHD transfection reagent to the Raw 264.7 cells to generate a stable cell line. Then, the transfected cells were plated into 6-well plates containing RPMI medium. Twenty-four hours post-transfection, the selection antibiotic was added to the medium at the concentration (1 µg/mL) Cells were grown for 5-7 days for selection based on blasticidin sensitivity. The generated cell line RawKb.APOL7C::GFP is a gain-of-function system that facilitates the visualization of APOL7C.

2.5 APOL7C recruitment with different phagocytic particles

RawKb.APOL7C::mCherry cell lines were plated at of $2X10^5$ cells per well onto an Ibidi 8-well µ-dish, treated with doxycycline overnight to induce the expression of the APOL7C, and left to adhere overnight. Then, the cells were challenged with the different phagocytic targets *Staphylococcus aureus*, zymosan, and sheep red blood cells (sRBCs) for 3 hours and immediately fixed with 4% of paraformaldehyde (PFA) in PBS. PFA was then quenched with 50 mM NH_4Cl in PBS for 5 minutes followed by 3X washes with PBS. Next, the cells were permeabilized with 0.4% Triton X 100 in PBS for 10 minutes followed by 3X washes with PBS. Next, the cells were blocked for 30 minutes with 2% low-fat milk in PBS and then incubated with the primary antibodies for 1-2 hours at room temperature (RT). Finally, the plates were imaged on a Leica SP8 confocal microscope (equipped with an HC PL APO 63´/1.40 Oil CS2) to visualize the localization of APOL7C.

2.6 Quantitative reverse transcription polymerase chain reaction (q-RT PCR)

q-RT PCR was performed using the Arum Total RNA Mini kit (BIO-RAD) to isolate the mRNA following the manufacturer's instructions. The infected and non-infected cells were lysed with lysis the lysis solution for 1 minute, followed by supplementation with 100% ethanol.

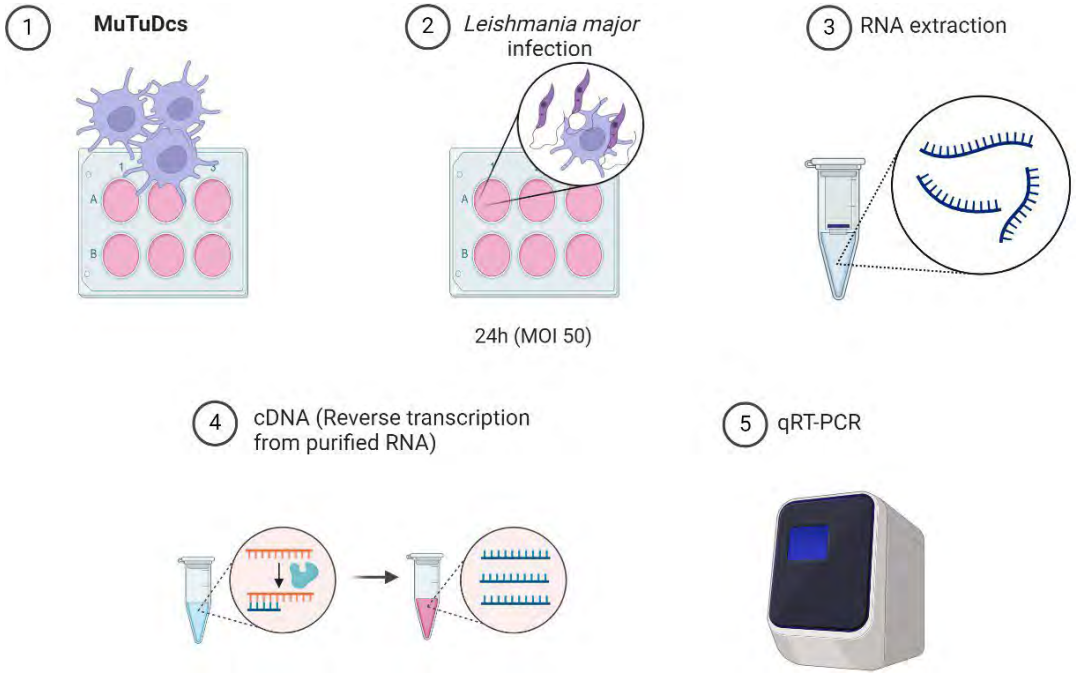

Figure 5. Graphical summary of q-RT PCR protocol. Diagram created with BioRender.com

The mixture was then transferred into a purification column at room temperature and spun down at 8,000 rpm for 1 minute at 4°C and the supernatant was discarded. The column was washed with wash buffers A and B before being transferred into a new collection tube. Finally, 100 µl of nuclease-free water was added to the purification column, and it was spun down at 12,000 rpm for 1 minute to collect the flow-through. The cDNA was prepared using the QuantaBio qScript cDNA synthesis kit according to the manufacturer's instructions. To summarize, 0.1 µg of

mRNA was combined with 1 µl of reverse transcriptase, 4 µl of reaction mix, and nuclease-free water was added to reach a final volume of 20 µl. The PCR reaction was run on a BioRad iQ5 thermocycler using iQ SYBR Green Supermix according to the manufacturer's instructions.

2.7 Infection Assay

RawKb.APOL7C::GFP or RawKb.APOL7C::mCherry cell lines were plated onto Ibidi 8-well µ-dishes at $2x10^5$ cells per well ; the cells were treated with doxycycline overnight to induce the expression of the APOL7C and left to adhere overnight. The next day, cells were challenged with the RFP-*L. major* or *L. major* metacyclic promastigotes previously isolated by a ficoll gradient at a multiplicity of infection (MOI) of 40 parasites to each cell (40:1) for different times

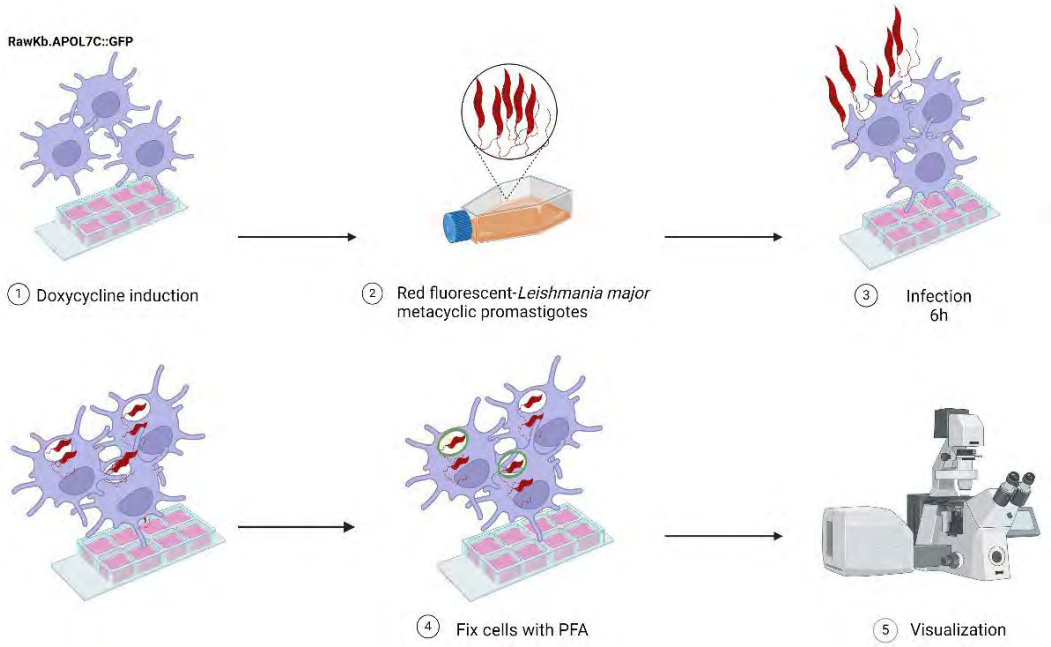

Figure 6. Graphical summary of infection protocol. Diagram created with BioRender.com

points or a final time of 6h at 34°C.After the time point was reached, 4% PFA diluted in PBS was added for 20 minutes of incubation the fixated cells were visualized in the microscope

2.8 Immunofluorescence Assay (IF)

For immunofluorescence assays after the infection protocol, the RawKb.APOL7C::GFP permeabilized cells were blocked for 30 minutes with 2% low-fat milk in PBS. Then, the cells were further probed with one or a combination of specific antibodies such as: anti-mCherry, anti-LAMP1,anti-Galectin-3 for 1h at 4°C RT. Next, cells were washed three times with 2% low-fat milk in PBS and incubated with the fluorescent-labeled secondary antibody of choice for 1h at RT, followed by a final 3X PBS wash. Images were then acquired by confocal microscopy, and the number of PVs positive for APOL7C::mCherry,APOL7C::GFP, LAMP1, and Galectin-3 were quantified.

2.9 Luminol Assay

RawKb.APOL7C::GFP cells were lifted, resuspended, and plated at a 5×10^6 cell per well in a 96-well white-sided microwell plates on RPMI complete medium and left for an hour incubation at 37°C.

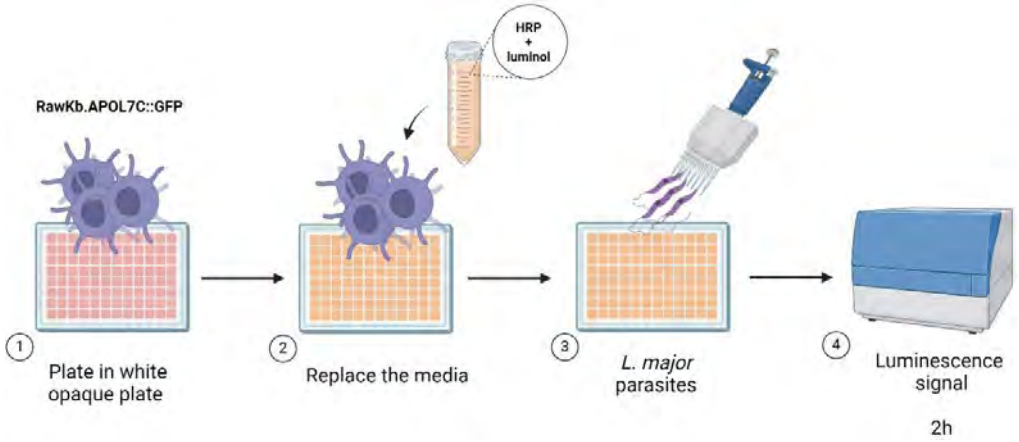

Figure 7. Graphical summary of a luminol assay protocol. Diagram created with BioRender.com

Next, the media was replaced with a 50 μM luminol diluted in PBS, and the cells were further challenged with the different (MOI) of *L. major* parasites 10:1, 20:1, 40:1 and 60:1. Finally, the production of Reactive oxygen species (ROS) was monitored by analyzing chemiluminescence every 2 min for a total of 120 min at 34°C. For statistical analysis, 3 biological replicates were measured for each treatment. ROS levels were represented as relative luminescence units (RLU) at each time point.

2.10 Nitro blue tetrazolium (NBT) reduction assay

RawKb.APOL7C::mCherry cells were plated onto Ibidi 8-well μ-dishes at $2x10^5$ cells per well and incubated for 1h to let the cells adhere to the plate at 37°C. The next day, the cells were treated with 50 μl of a 1mg/mL stock solution of nitroblue tetrazolium (NBT) diluted in PBS and further challenged with the *L. major* metacyclic promastigotes previously isolated by a ficoll gradient at an MOI of 40 parasites to each cell (40:1). After the required time point was reached cells were immediately visualized on the microscope, and the images were taken.

2.11 Cross-presentation Assay

RawKb.APOL7C::mCherry cells were plated in RPMI complete media with or without doxycycline treatment overnight at 37°C. The next day, the cells treated with doxycycline were washed multiple times with a fresh RPMI medium to remove any doxycycline left that could harm *Leishmani*a parasites.

The cells were lifted, pelleted and plated in a 96-well U bottom plate at of $5X10^4$ cells per well. Then cells were challenged with different MOI (20:1,40:1,80:1) of the *L.major*-OVA metacyclic promastigotes. The cells were incubated with the parasites for 6h at 34°C to allow for phagocytosis, processing, and presentation of the ovalbumin-derived peptide SIINFEKL peptide

409 on the cell surface.

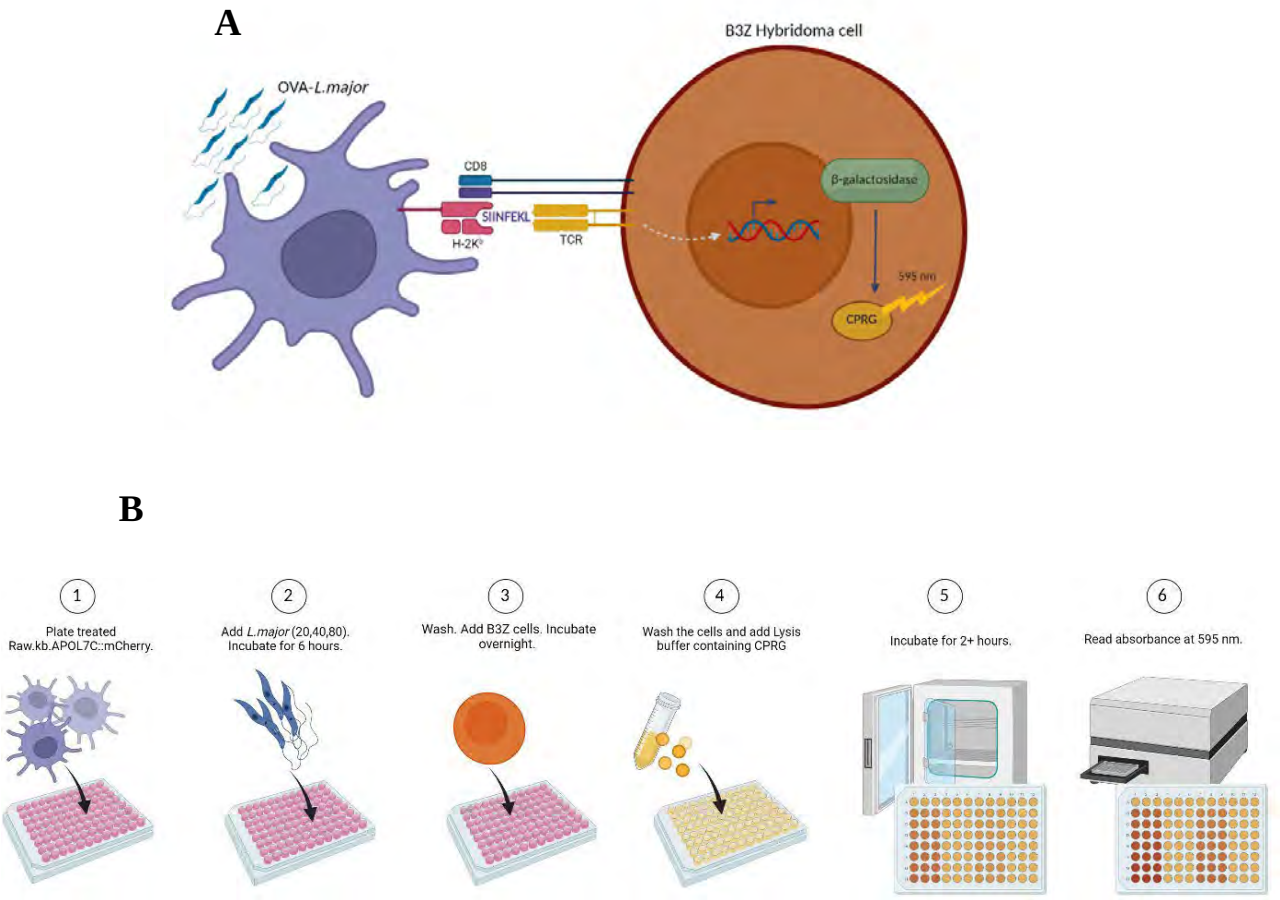

Figure 8. **A) Representative schematic demonstrating the cross-presentation mechanism of OVA-*L. major* antigens**. RawKb.APOL7C cells phagocytose the OVA-*L. major* parasites and load SIINFEKL peptide onto MHCI. When SIINFEKL is presented to B3Zs, they are activated and induce β-galactosidase synthesis. The assay is performed in CPRG 488 substrate, which turns red in the presence of β-galactosidase. Absorbance is then measured to determine cross-presentation efficiency. **B)** Schematic summary of the cross-presentation assay protocol. Diagram created with BioRender.com

After the time point was reached, the non-internalized promastigotes were removed by a 3X wash of fresh serum-free RPMI. For the next step, B3Z cells were counted and resuspended in fresh R10 medium at $1X10^6$ cells/mL and incubated with the infected RawKb.APOL7C::mCherry cells for 18-24h. The B3Z cell line specifically recognizes OVA peptides presented on an MHC class I complex to their TCR and, upon activation, will induce the synthesis of β -galactosidase. The cells were washed twice with 1X PBS and lysed with chlorophenol-Red β-D-galactopyranoside (CPRG) solution. Then, the plate was incubated for 1-3 h at 37 °C. Cross-presentation of antigens secreted by the parasite was assessed by measuring absorbance at 595nm on a plate reader.

2.12 *In vivo* Infection Experiments

C57BL/6 and *Apol7c*$^{-/-}$ mice were infected using an intradermal injection of $1X10^4$ *L. major* metacyclic promastigotes in a 10 -µl volume into the ear dermis[70]. During the 18-week period, the progress of infection was monitored by measuring weekly the diameter of the ear lesion. At 7 weeks post-infection, ear tissue and the spleen from the mice in each group were taken for analysis to determine the parasite burden by limiting-dilution analysis.

2.13 Tissue processing

Briefly, for ears, ventral and dorsal sheets were separated, then submerged in 0.5-1mL DMEM containing 16µg/mL of Liberase and incubated at 37°C for 90–120 minutes. Following Liberase treatment, ear tissue was homogenized for 3.5 minutes in a Medicon using a Medimachine (Becton Dickson).

Tissue homogenates were then flushed from the Medicon with 8mL DMEM with 0.05% DNase I and filtered using a 50µm-pore-size cell strainer. Spleens were removed and homogenized with a

1mL syringe plunger on a 70μm cell strainer. Red blood cells (RBCs) in the spleen were eliminated using ACK lysing buffer.

2.14 Parasites and parasite quantification

Parasite loads were determined by limiting dilution analysis (LDA). Briefly, two-fold serial dilutions in 96-well flat-bottom plates were performed by administering 100μL of diluted tissue suspension to 100μL M199 complete medium. The number of viable parasites in the ears and spleens was determined from the highest dilution at which parasite growth was observed after 7-10 days of incubation at 26°C, and results were expressed as the mean values of the negative logarithm of the titer.

Chapter 3. Results

3.1 APOL7C recruitment to phagosomes

As there is limited knowledge of the functions of the APOL family, our lab has begun exploring one specific member, APOL7C. Previous studies in the Canton lab had found that APOL7C is uniquely expressed in DCs and recruited to phagosomes containing inert silica beads[71.] To investigate whether the APOL7C can be recruited to phagosomes harboring different phagocytic targets, we challenged the RawKb.APOL7C::mCherry cells with various targets, including live *Staphylococcus aureus*, zymosan, and sheep red blood cells (sRBCs).

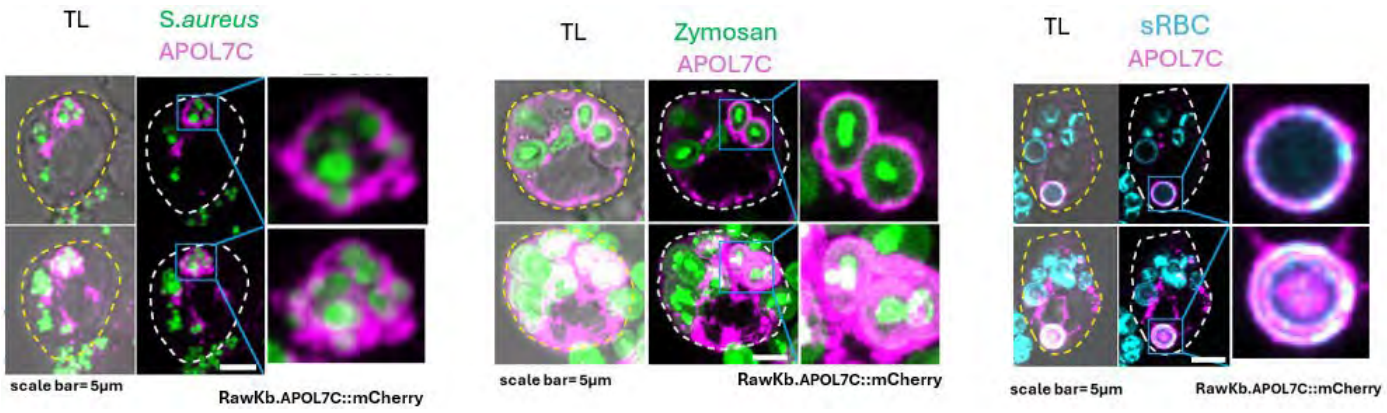

Figure 9. Visualization of APOL7C recruitment to phagosomes containing different phagocytic targets. APOL7C recruitment was not limited to OVA-bead-containing phagosomes, as it was recruited to the phagosomes of different particles including *S.aureus*, zymosan, and sheep red blood cells (sRBC)

After 3 hours of incubation, our results revealed that APOL7C, seen in the bright magenta rings is similarly recruited to phagosomes containing *S. aureus*, zymosan, and sRBCs (**Figure 9**). These findings suggest that APOL7C recruitment is not limited to inert particles but extends to more complex and biologically relevant targets. This consistent recruitment pattern enhances our interest of further characterize APOL7C function in immune responses.

3.2 APOL7C is recruited to *Leishmania* parasitophorous vesicles (PVs)

Building on our initial findings, we next investigated whether APOL7C is recruited during *Leishmania* infection. By implementing our RawKb.APOL7C::GFP cell line and infecting them with RFP-*L. major* promastigotes, we visualized APOL7C recruitment using confocal microscopy. Our data showed that APOL7C localized to PVs harboring RFP-*L. major* parasites 6 hrs post-infection (**Figure 10**).

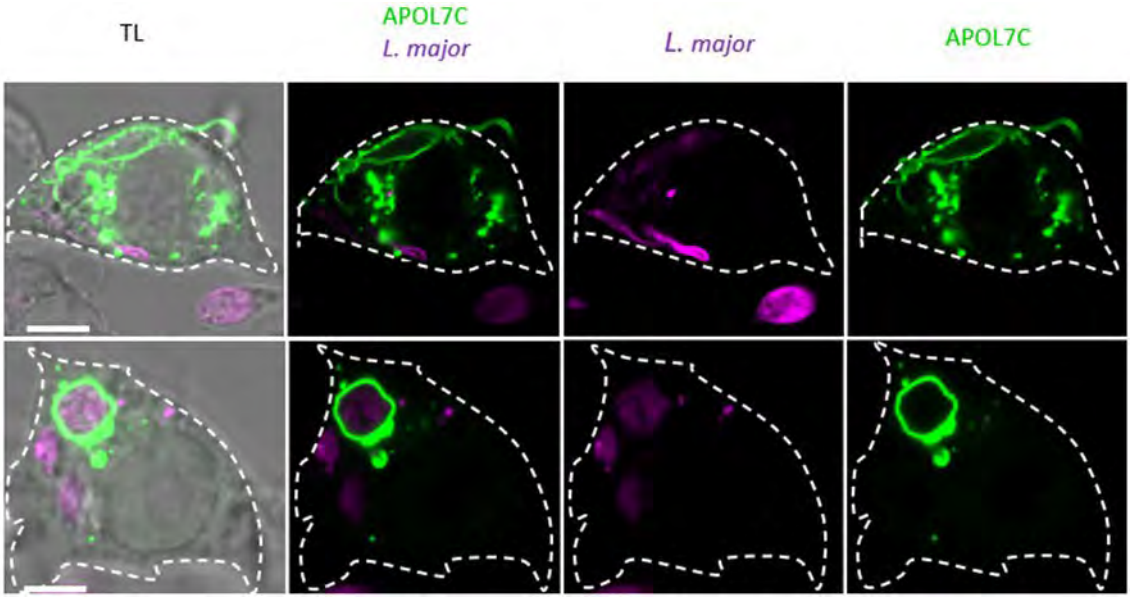

Figure 10. APOL7C recruitment on *L. major* PVs. RawKb.APOL7C::GFP cells were infected with MOI of 50:1 RFP-*L. major* parasites. Recruitment of APOL7C to the phagosomes containing *L. major* parasites was visualized using confocal microscopy. Scale bar = 5 μm.

The visualization of APOL7C recruitment of to *Leishmania* PVs may suggests a potential role for this protein in the host-pathogen interaction, particularly in the modulation of the PV environment.

3.3 *Apol7c* increased expression during *Leishmania* infection

To determine whether *Leishmania* infection can modulate *Apol7c* expression, we performed qRT-PCR on infected DCs at an MOI of 50:1, 24 hrs post-infection. Our results showed a significant upregulation of APOL7C mRNA expression, with a fold increase of over 50-fold compared to non-infected controls (**Figure 11**). Gene expression was normalized to 18S rRNA and calculated using the ΔΔCq method, with experiments repeated three times with biological replicates. This upregulation suggests that APOL7C is likely involved in the host response to *Leishmania* infection, possibly playing a role in immune modulation or pathogen clearance.

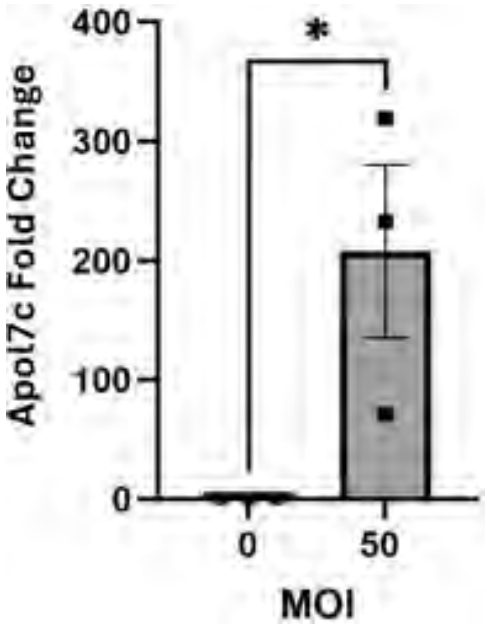

Figure 11. *Apol7c* expression is trigger by *L. major* infection. MutuDCs cells were infected with metacyclic promastigotes of *L. major* for 24hrs. Each dot represents the mean ± s.d. from 3 independent experiments (n = 3). Significance determined using student's t test. *$p \leq 0.05$

3.4 Kinetics of APOL7C recruitment to PVs

To further understand the kinetics of APOL7C recruitment to *Leishmania* PVs, we monitored RawKb.APOL7C::GFP cells infected with RFP-*L. major* at different time points: 2h, 4h, and 6h post-infection (**Figure 12A**). Notably, APOL7C recruitment was significantly higher at 6h post-infection, compared to earlier time points **(Figure 12B**). These findings contrast with the recruitment observed with other phagocytic targets, such as beads and zymosan, where recruitment occurs earlier. This delayed recruitment to *Leishmania* PVs may reflect the parasite's ability to modulate host cellular pathways to its advantage, potentially delaying the host's defensive responses.

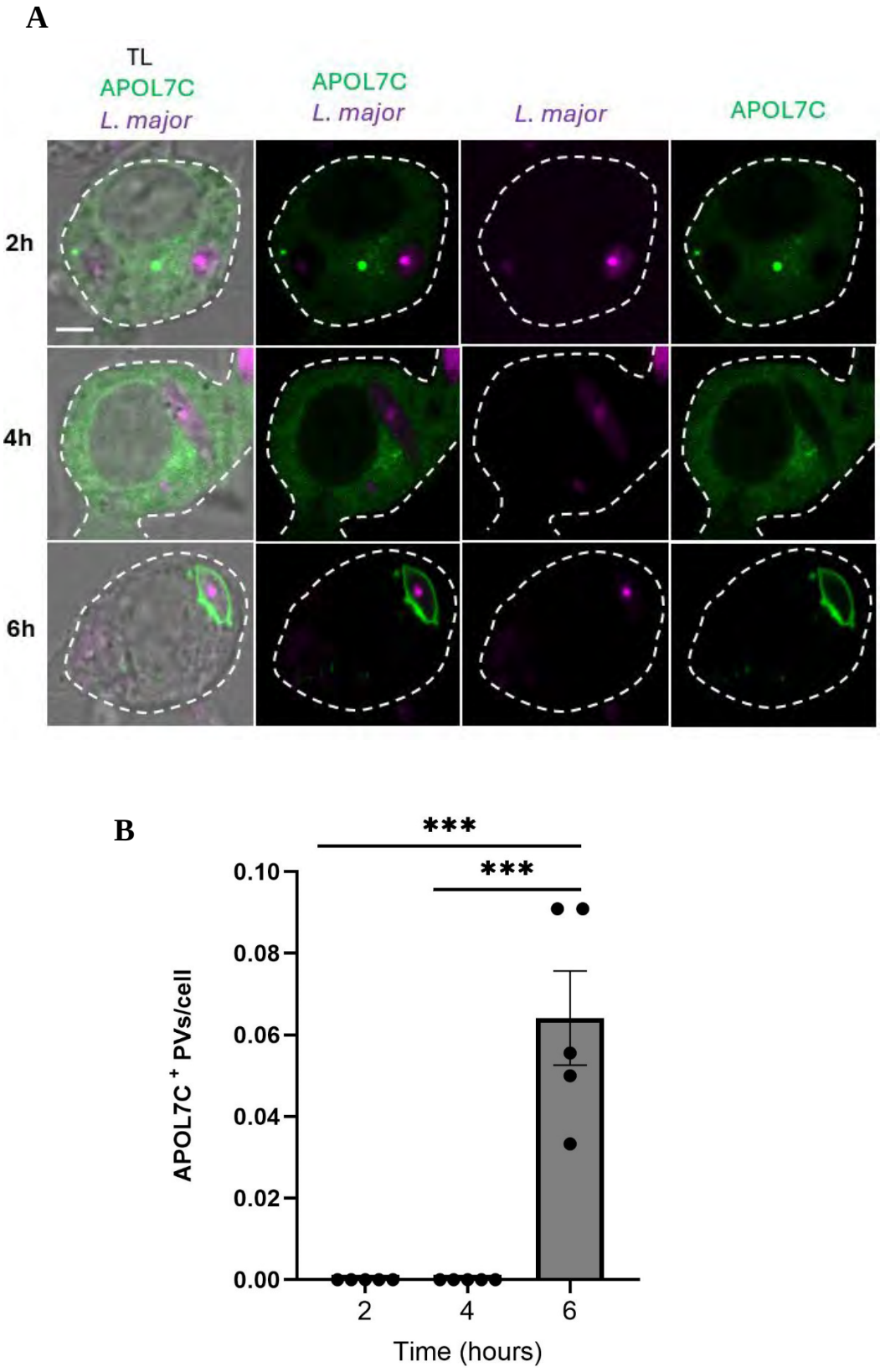

Figure 12. Kinetics of APOL7C recruitment to *Leishmania* PV. A) Visualization of APOL7C recruitment to *Leishmania* PVs. Scale bar=5 μm. **B)** Quantification of APOL7C-positive PVs at different time points of infection 2h, 4h, and 6h. Data are plotted as mean ± s.d. of the number of APOL7C+ PV/cell. Each dot represents a field of view, compiled from 3 independent experiments (n = 3). Significance determined using student's t-test.

3.5 APOL7C recruitment to late-stage PVs

To further characterize the nature of APOL7C-positive *Leishmania* PVs, we performed an immunofluorescence assay targeting the lysosomal-associated membrane protein LAMP1.

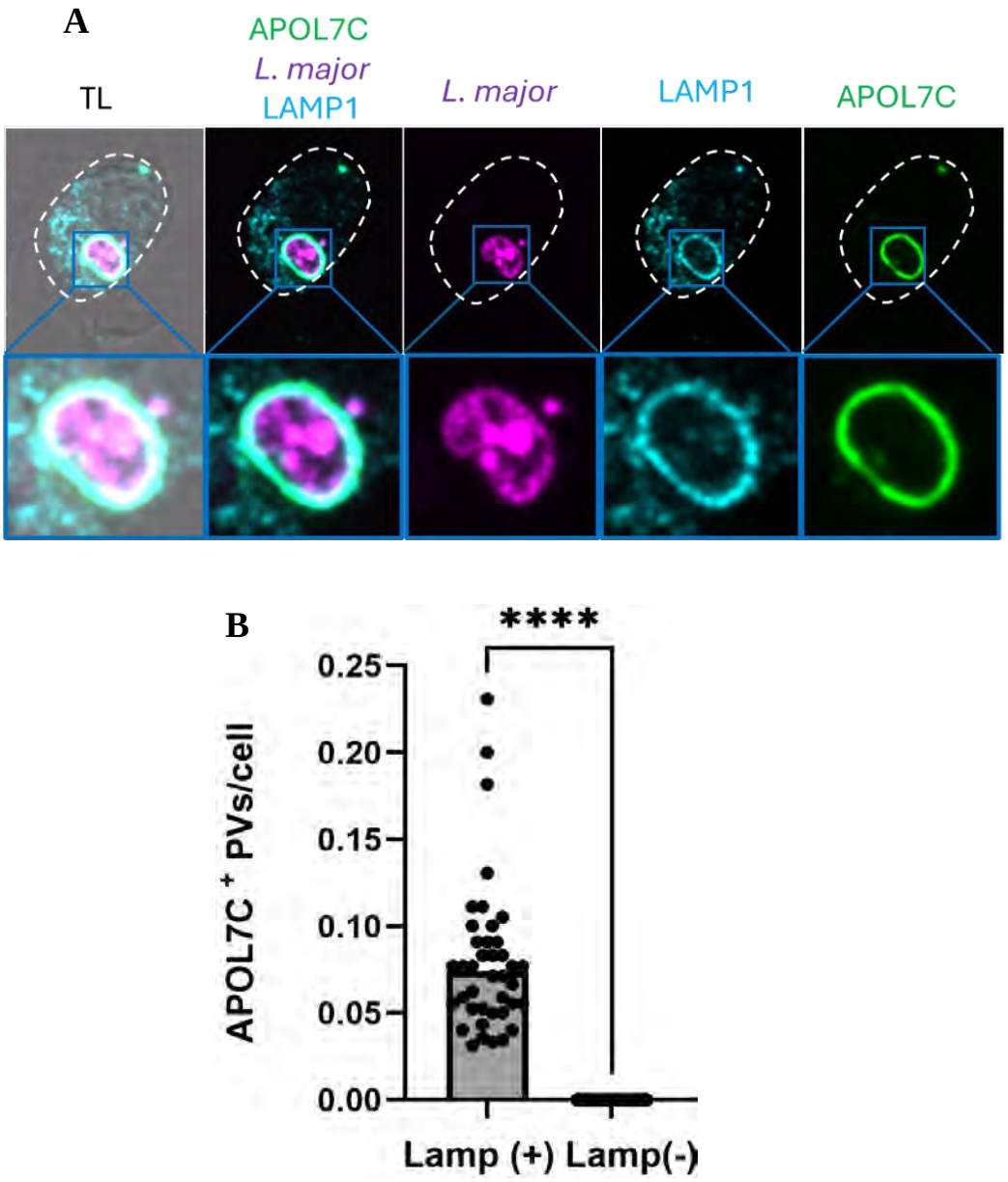

Figure 13. APOL7C recruitment co-localize with LAMP1 on *Leishmania* PVs. A) Doxycycline Inducible RawKb.APOL7C::GFP cells were infected for 6 hours and later stained for LAMP1. Scale bar= 5 µm. **B**) Number of APOL7C::GFP and Lamp1+ PVs. Each dot represents a field of view, compiled from 3 independent experiments (n = 3). Significance was determined using a student t-test. ****$P \leq$ 0.0001.

Following infection with RFP-*L. major* at an MOI of 40:1, we observed that APOL7C-positive PVs colocalized with LAMP1 at 6 hours post-infection (**Figure 13A**). This colocalization indicates that APOL7C is recruited to late-stage LAMP1-positive PVs, consistent with previous studies on silica bead-containing phagosomes. The recruitment of APOL7C to late-stage PVs suggests a role in the maturation and potential disruption of the parasite-containing compartments, aligning with its predicted pore-forming function (**Figure 13B**)

3.6 APOL7C recruitment causes membrane disruption PVs

Given the presence of a putative pore-forming domain in APOL7C, we next investigated whether APOL7C could induce membrane damage in *Leishmania* PVs. RawKb.APOL7C::GFP cells were infected with RFP-*L. major*, and immunofluorescence assays for the damage-associated biomarker Galectin-3 were performed 6 hrs post-infection (**Figure 14A**). Analysis showed that Galectin-3 localized mainly on APOL7C-positive PVs harboring RFP-*L. major* (**Figure 14B**). This finding suggests that APOL7C disrupts the membrane integrity of Leishmania PVs, potentially exposing the parasite to cytosolic immune defenses. This membrane disruption could play a critical role in the host's strategy to combat intracellular pathogens

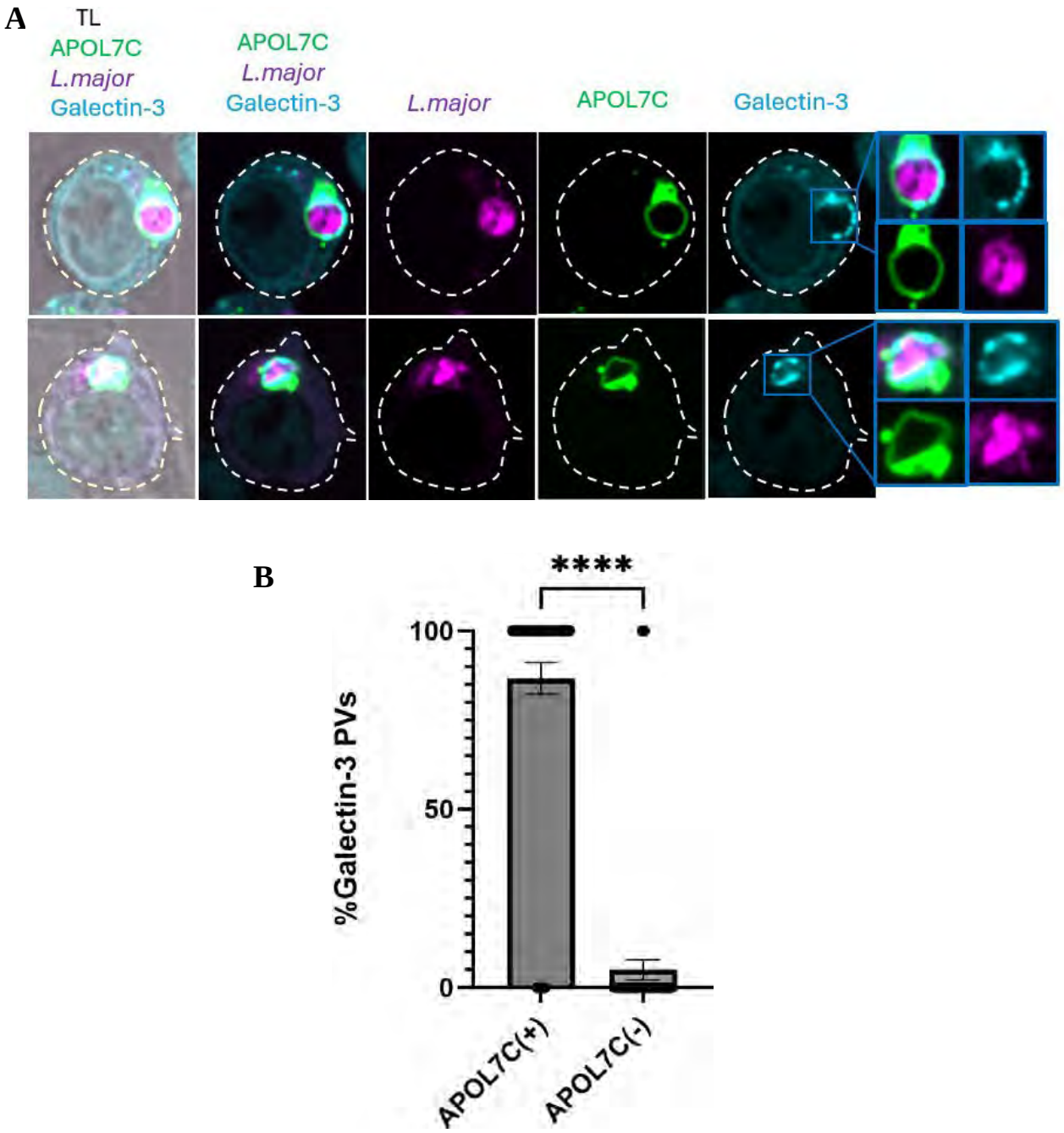

Figure14. APOL7C Co-localizes with damage marker Galectin-3 on PVs. A) Doxycycline Inducible RawKb.APOL7C::GFP cells were infected for 6 hours and later stained for Galectin-3. Scale bar 5 μm. **B)** Percentage of APOL7C^{+} orAPOL7C^{-} PVs that were positive for Galectin 3. Each dot represents a field of view, compiled from 3 independent experiments (n = 3). ****$P \leq 0.0001$.

500

3.7 Role of NADPH Oxidase Activity in APOL7C Recruitment

Previous research from our lab has shown that APOL7C recruitment is dependent on NADPH oxidase activity. To investigate this in our *Leishmania* infection model, we first assessed NADPH oxidase activity using a nitro blue tetrazolium (NBT) reduction assay.

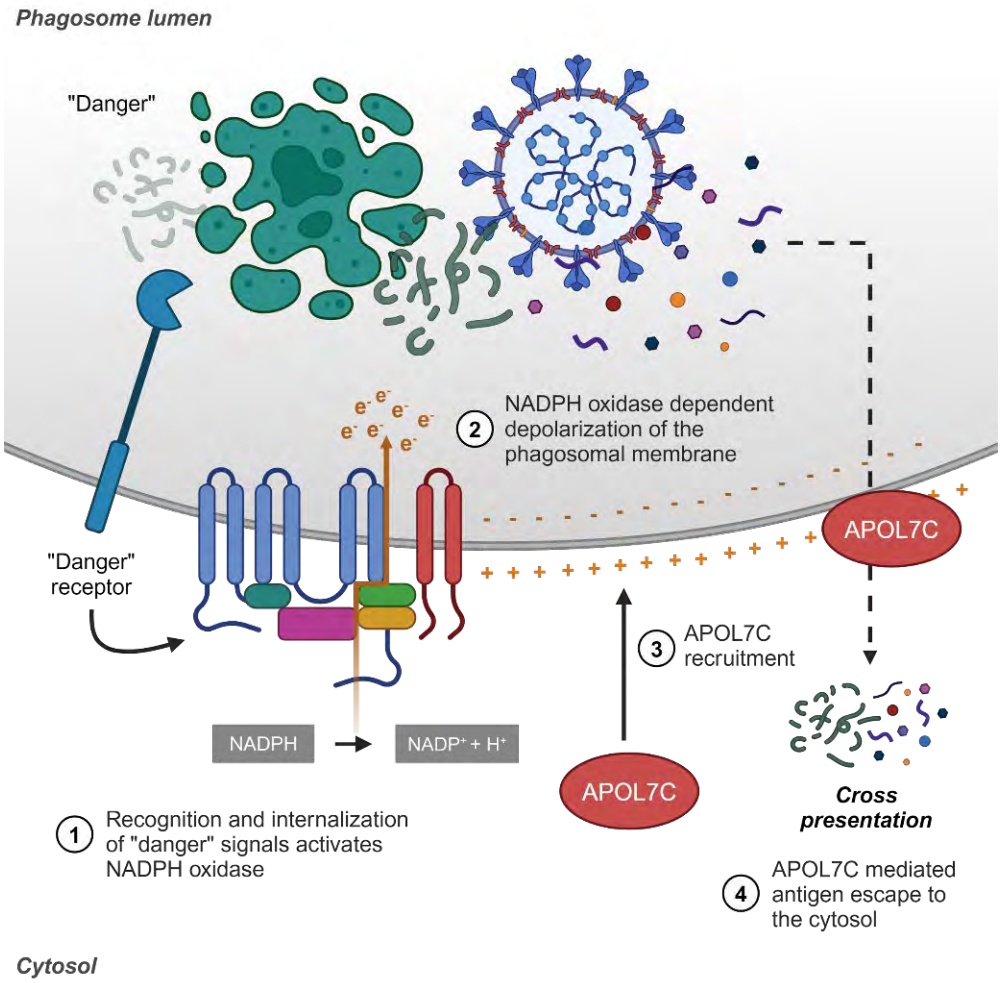

Figure 15. Mechanism of APOL7C recruitment mediated by the NADPH oxidase activity. The recognition and internalization of exogenous particles lead to the activation of the NADPH oxidase; once activated, it depolarizes the phagosomal membrane. This depolarization leads to the recruitment of APOL7C to the phagosome, where the protein forms a pore, causing membrane disruption. Diagram created by Gerone Gonzales with BioRender.com.

The formation of dark blue formazan deposits indicates an oxidative burst in infected cells after 1 hour (**Figure 16A**), confirming ROS production. Further, we performed a luminol-based assay to measure intraphagosomal ROS production, with luminescence detected at 2-minute intervals over 120 minutes. Peak ROS activity was observed at 22 minutes post-infection for MOIs of 10:1, 20:1, and 40:1, before declining (**Figure 16B**). Our results showed a consistent ROS production across most of the different MOI suggesting that *Leishmania* infection triggers a NADPH oxidase response.

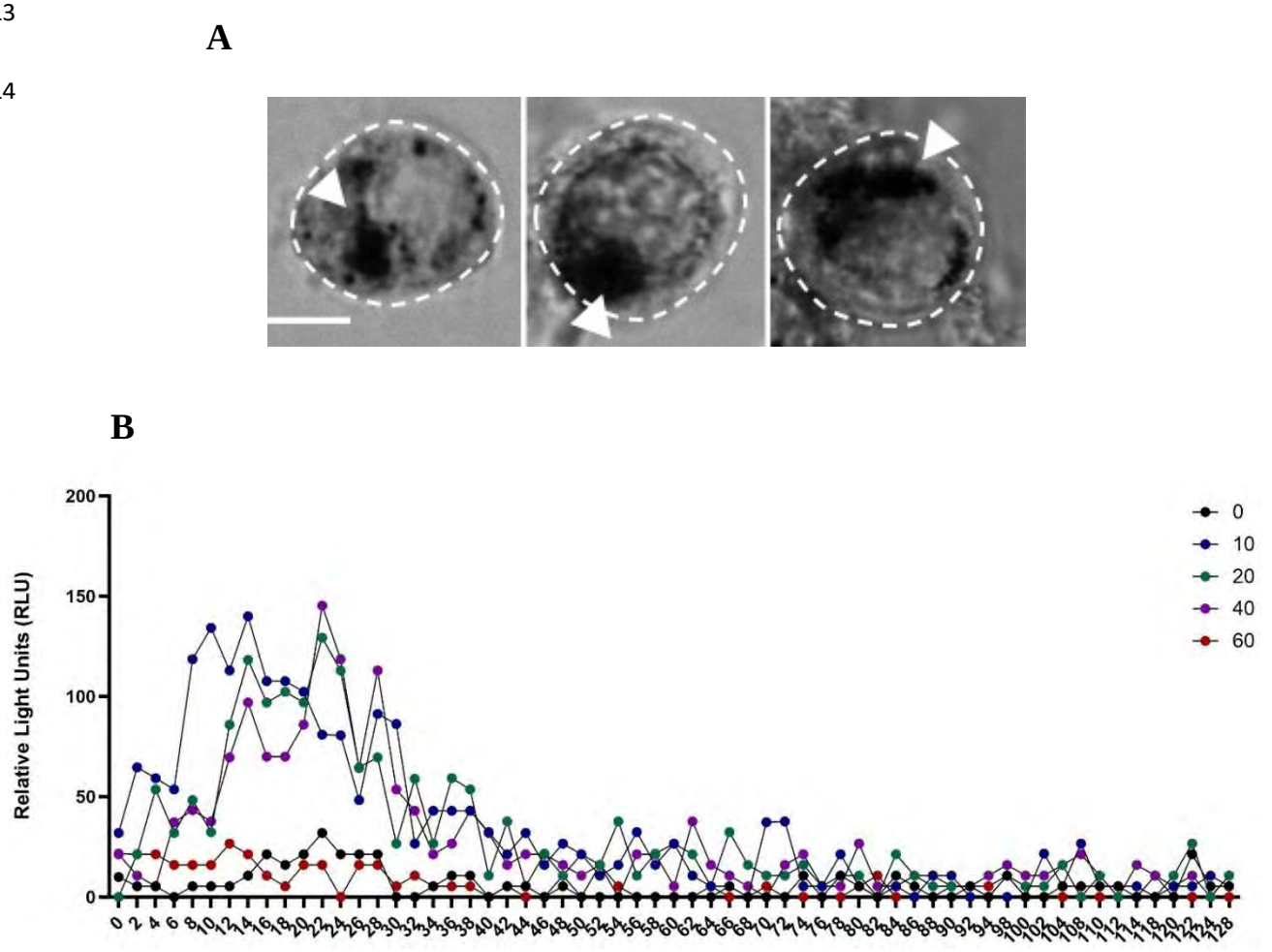

Figure 16. Activation of NADPH activity upon *L. major* infection. A) RawKb.APOL7C::mCherry were challenged with *L. major* for 1h in the presence of NBT at 1 μg/ml, as indicated. Cells were then washed three times with PBS, and imaged. Dark formazan deposits indicate local production of ROS. Scale bar =5 μm**. B)** RawKb.APOL7C::GFP were challenged with *L. major* parasites the presence of 8 U/ml HRP. Luminescence was measured for a total of 120 min.

To validate the role of NADPH oxidase in APOL7C recruitment, we used $p22^{phox\ -/-}$ RawKb.APOL7C::mCherry cells, which lack functional NADPH oxidase. After performing an infection and immunofluorescence assays, our results indicates that $p22^{phox\ -/-}$cells did not recruit APOL7C to *Leishmania* PVs after 6 hours of infection, emphasizing the essential role of NADPH oxidase in this recruitment process (**Figure 17**). This finding aligns with the previous observations that APOL7C insertion into phagosomal membranes, influenced by NADPH oxidase activity.

As previous studies have showned, the membrane insertion of APOL family members depends on three key amino acid residues in their MID. By mutating these critical residues in our RawKb.APOL7C::mCherry cell line, we created two mutant cell lines where APOL7C insertion is insensitive to voltage. We then investigated APOL7C insertion into PVs using these voltage-insensitive mutants ($APOL7C_{EE}$::mCherry and $APOL7C_{EEE}$::mCherry) in an infection assay. After infection, the analysis with confocal microscopy showed that both of these mutants failed to recruit APOL7C to PVs (**Figure 17 and Figure 18**), this results suggest APOL7C inserts to the PVs in a voltage dependent manner and further supports the importance of the NADPH oxidase changes for APOL7C in our *Leishmania* infection model.

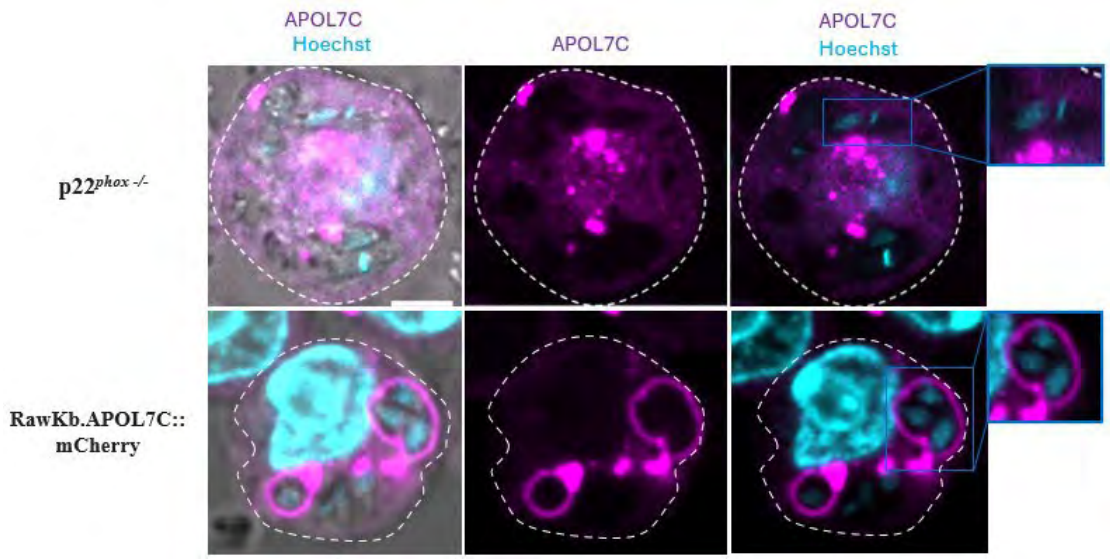

Figure 17. APOL7C recruitment in p22$^{phox-/-}$RawKb.APOL7C::mCherry and RawKb.APOL7C::mCherry cells. Both cell lines were incubated with doxycycline overnight and further infected with *L. major* parasites for 6 hours, followed by an immunofluorescence assay before imaging. Our results show that p22$^{phox-/-}$RawKb.APOL7C:mCherry cells could not recruit APOL7C to the PVs in contrast with our control cell line RawKb.APOL7C::mCherry. Scale bar= 5 μm.

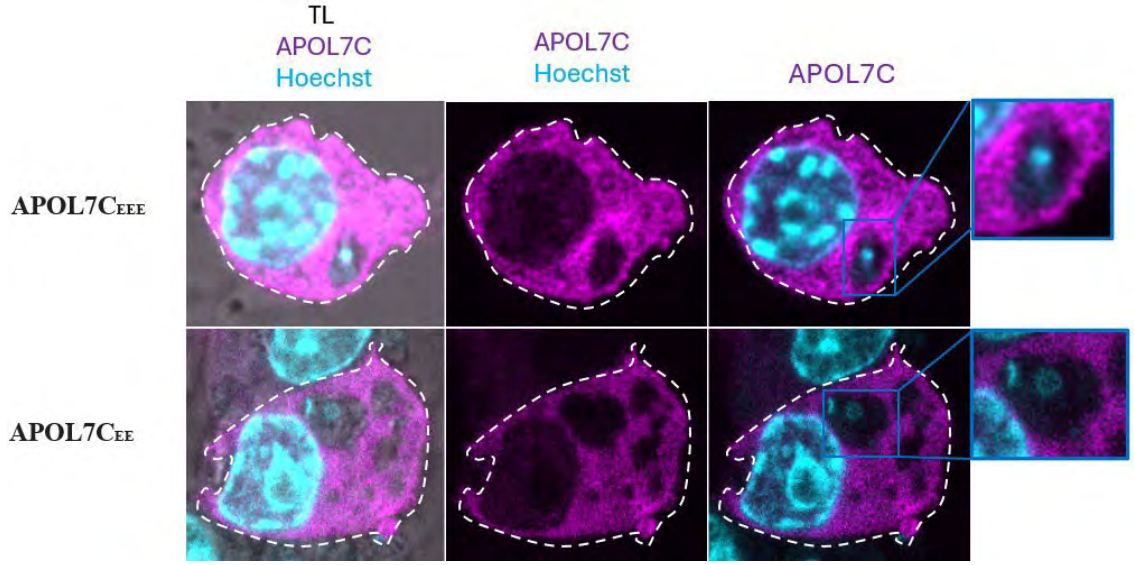

Figure 18. APOL7C recruitment in voltage insensitive APOL7C mutant cell lines. Both cell lines were incubated with doxycycline overnight and further infected with *L. major* parasites for 6 hours, followed by an immunofluorescence assay before imaging. Our results show that RawKb.APOL7C$_{EE}$ and **B)**RawKb.APOL7C$_{EEE}$ and cells could not recruit APOL7C to the PVs. Scale bar =5 μm.

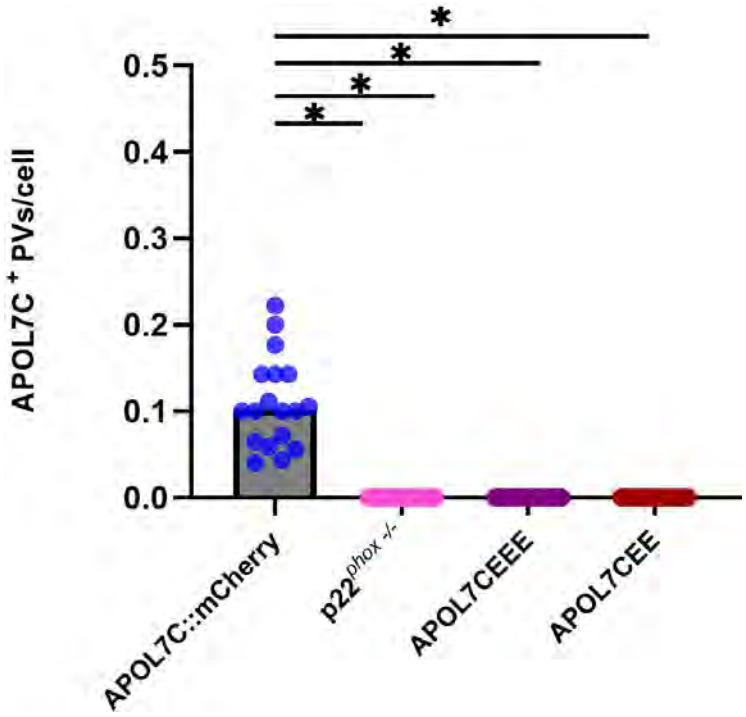

Figure 19. Quantification of APOL7C$^+$ PVs in all the cell lines infected. The **p22$^{phox-/-}$**and voltage insensitive mutant cell lines showed no recruitment of APOL7C. Number of APOL7C::mCherry PVs is shown. Each dot represents a field of view, compiled from 3 independent experiments (n = 3). Significance was determined using a student t-test. *$P \leq 0.05$.

.

3.8 APOL7C diminishes cross-presentation during *Lesihmania* infection

Previous studies in DCs have addressed the importance of cDC1s for the control of *Leishmania* infection. For this experiment, we obtained an ovalbumin-expressing *Leishmania major* from Dr. Nathan Peters and established the optimum conditions for assessing cross-presentation *in vitro* using the B3Z hybridoma T cell line. First, we treated the RawKb.APOL7C::mCherry cells with doxycycline to induce the expression of APOL7C. After overnight incubation, the cells were washed multiple times with fresh medium to remove any doxycycline left that can be harmful to *Leishmania* parasites, and then cells were challenged with different MOI (20:1,40:1,80:1) of the

543 *L.major*-OVA metacyclic promastigotes. The cells were incubated for 6h at 34°C with the cells
544 to allow for phagocytosis, processing, and presentation of the ovalbumin-derived SIINFEKL
545 peptide on the cell surface. For the next step, the cells were further co-cultured overnight with
546 B3Z hybridoma cells. β-galactosidase production was measured the next day by transferring the
547 B3Z cells into a new plate and then lysing them with a CPRG solution and measuring the
548 absorbance. Our results, contrary to previous experiments with ovalbumin-coated beads (OVA-
549 beads), showed a decrease in cross-presentation when APOL7C expression was induced during
550 infection (**Figure 20**). Although APOL7C had previously enhanced cross-presentation by
551 facilitating the escape of antigen from OVA-bead-containing phagosomes, we may consider that
552 the function of APOL7C during *Leishmania* infections may differ from previous observations.

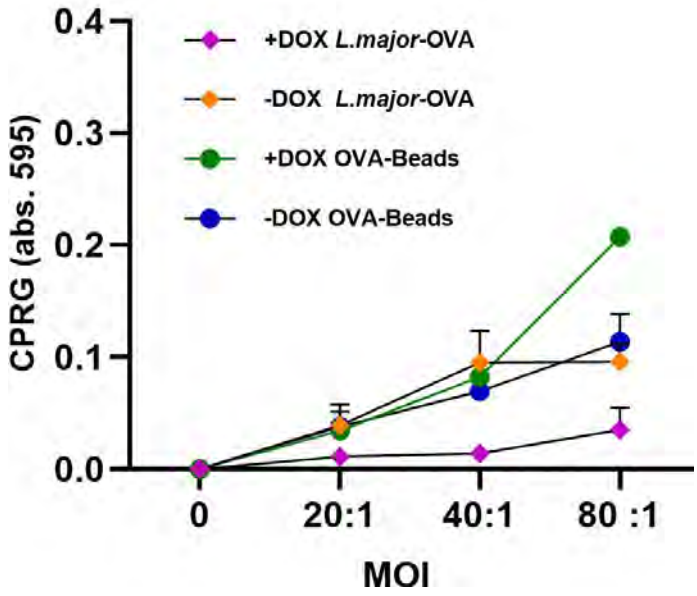

Figure 20. APOL7C expression diminished cross-presentation of *L. major*-OVA antigens. RawKb.APOL7C::mCherry were infected with different concentrations of *L. major*-OVA parasites for 6 hrs. Next, the cells were co-cultured overnight with B3Z cells. B3Z cells were lysed in a CPRG-containing buffer, triggering the production of β-galactosidase. Cross presentation efficiency was determined by the measurement of CPRG absorbance after 1-2hr incubation. Plotted as mean ± s.d. of experimental triplicates and is representative of 3 independent experiments (n = 3). Significance determined using students t test.

For instance, it is conceivable that APOL7C disrupts the replicative niche of *Leishmania,* thereby reducing available antigens for cross-presentation; however, further research is needed in order to fully comprehends its role.

3.9 APOL7C modulates lesion size and parasite burden

Upon infection, both *Apol7c*$^{-/-}$ and C57BL/6 mice exhibited a progressive increase in lesion size. However, by week 8 post-infection, *Apol7c*$^{-/-}$ mice demonstrated a significant reduction in lesion size compared to C57BL/6 mice strain (**Figure 21**). This reduction in lesion size was particularly evident at the later time points of infection. Analysis of parasite burden in the spleen revealed the presence of parasites in both *Apol7c*$^{-/-}$ and C57BL/6 mice across all time points examined.

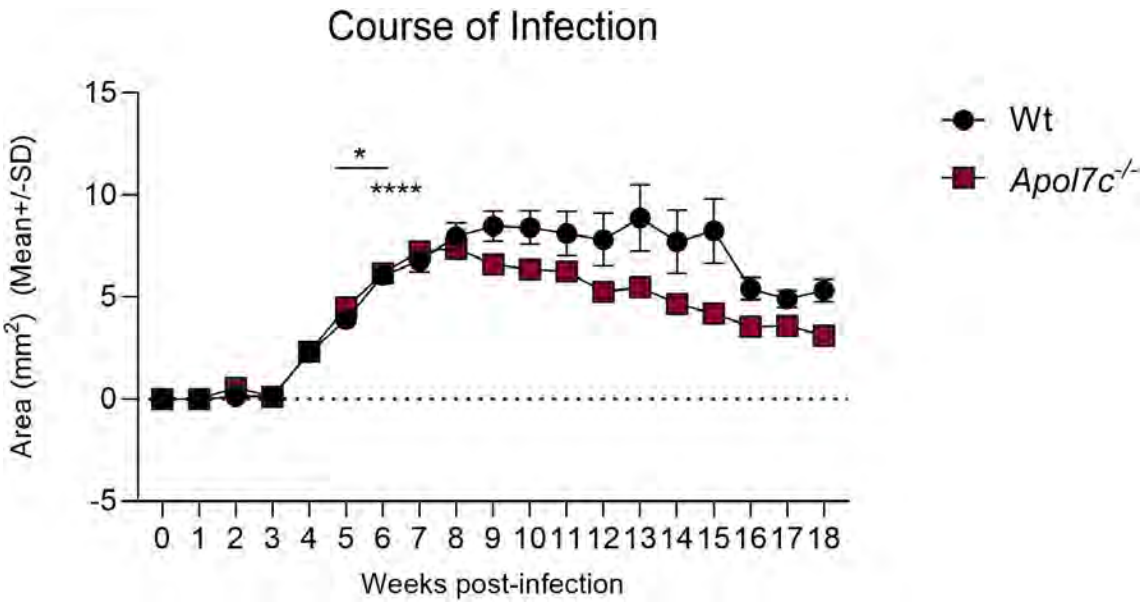

Figure 21. In vivo infection. *Apol7c*$^{-/-}$ mice display faster healing during *L. major* infection. C57BL/6 and *Apol7c*$^{-/-}$ mice were infected with 1× 10^4 *L. major* promastigotes in the ear dermis and monitored for lesion size growth over 18 weeks. Significance was determined using a two-way ANOVA -test. n=3–4 mice per time point per experiment. $^{*}P = 0.005$; $^{***}P \leq 0.0001$;

563 However, no significant differences in parasite load were observed between the two strains over
564 the experimental periods (**Figure 22B**).In contrast, examination of the ear tissue demonstrated a
565 significantly higher parasite load in *Apol7c*-/- mice compared to C57BL/6 mice (**Figure 22A**).

566 This increased parasite burden in the ear tissue of *Apol7c*-/- mice indicates a distinct infection
567 pattern, reminiscent of previously described conditions in *L. major*-infected BALB/c mice.

568

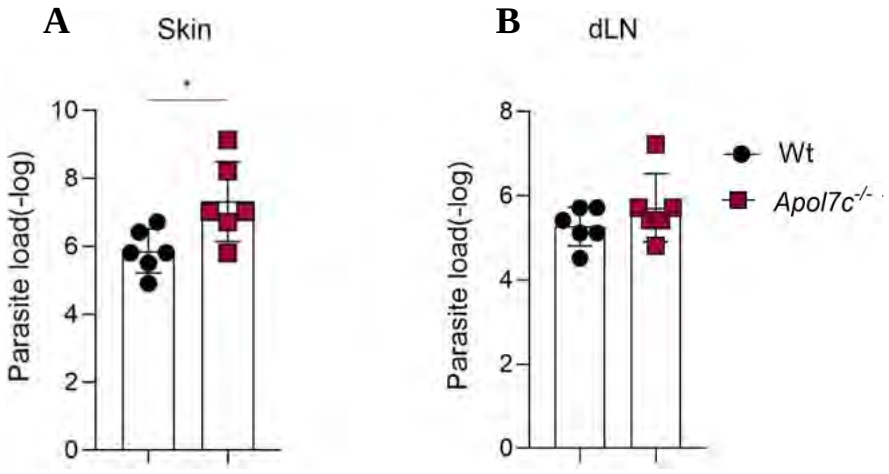

Figure 22. Parasite load at the ear dermis and spleen. After reaching 7 weeks, **A)** ear dermis tissue B**)** spleen were isolated from both C57BL/6 and *Apol7c*$^{-/-}$ infected mice strains. Parasite load was calculated by limiting dilution. Data are from a single experiment with n = 6 per experimental group and are shown as mean ± SD. Significance was determined using a student t-test **P* < 0.005

Chapter 4. Discussion

This study sought to characterize the mechanism and potential role of APOL7C, a pore-forming protein, in a *Leishmania* infection model. First, our findings demonstrated a significant upregulation of *Apol7c* expression in our DCs 24 hours post-infection (**Figure 11**). These observations are corroborated by recent work from our laboratory, which has shown that conventional dendritic cells (cDCs) exhibit a marked upregulation of *Apol7*c upon stimulation with the innate immune stimulus of polyinosine-polycytidylic acid (PolyI:C)[68]. In addition, previous studies reported the upregulation of Apolipoprotein L (APOL) family members in response to various inflammatory stimuli[69,70] with potential antiviral activity[71]. Within the context of *Leishmania* infection, cDCs, particularly the cDC1 subset, are crucial in orchestrating immune responses through antigen presentation and the activation of effective T-cell responses. However, given that the survival and proliferation of *Leishmania* parasites during infection involve multiple phagocytes our results could yield valuable insights into further understanding the host-pathogen interactions in DCs. Our data strongly showed that *Leishmania* infection can modulates the expression of the gene encoding the APOL7C protein in MuTuDC cells. These result support our objective to further elucidate the potential function of APOL7C in *Leishmania* infection.

Building on knowledge from previous studies in our lab, shown that recruitment of APOL7C to phagosomes harboring inert silica beads, we now show recruitment to other, more physiological, phagocytic targets. Initially, I sought to investigate whether APOL7C is recruited more broadly to a range of phagocytic targets such as *S. aureus*, sheep red blood cells (sRBCs) and zymosan. Notably, I found that APOL7C is similarly recruited to sRBC-, zymosan- and *S. aureus*-containing phagosomes (**Figure 9**). This indicates that APOL7C recruitment to phagosomes is

likely not an artifact of silica beads but is more broadly recruited to phagosomes containing distinct cargoes.

We next investigated whether APOL7C is recruited to *Leishmania* PVs, by infecting the RawKb.APOL7C::GFP cell line with RFP-*L. major*. The data presented showed the recruitment of APOL7C to *Leishmania* PVs **(Figure 10**). However, unlike previous finding our results show that in the case of RFP-*L. major* infection, the recruitment of APOL7C could not be visualized before 6 hrs of infection (**Figure 12B**). These results suggest that in contrast to previously studied particles, the recruitment of APOL7C to PVs appeared to be a slightly later event. As an intracellular parasite, it is known that upon phagocytosis in the early stages of infection, *Leishmania* alters multiple signaling pathways involved in the clearance of foreign particles (e.g., ROS production). It is worth considering that the parasite may modulate the recruitment of APOL7C to enhance its survival and successful infection of the host cells.

One of the focus of this study was to further characterize the *Leishmania* PVs that recruit APOL7C. A key element of early *Leishmania* infection in host cells is the formation of the parasitophorous vacuole (PV), an organelle that plays a crucial role in the life cycle of the parasite. The PV provides a protective niche that allows the promastigote form of *Leishmania* to differentiate into the intracellular amastigote form, which then replicates within this compartment. The survival and persistence of intracellular pathogens like *Leishmania* within host cells depend heavily on their ability to modulate the composition and function of the phagosomes they occupy[67]. This modulation involves several critical processes, such as the regulation of vacuolar pH through vacuolar ATPases, the acquisition of substrates via specific membrane transporters and channels, and fusion events with lysosomes, other phagosomes, and various vesicles[72] . The PV originates from the host cell plasma membrane and is formed through a series of coordinated fusion events with vesicles from early and late endocytic pathways,

lysosomes, the ER, and possibly autophagic vesicles[75]. Following the phagocytosis event, the maturation of phagosomes can be monitored by the acquisition of proteins specific to each endocytic stage. Initially, the phagosome is associated with the rapid recycling pathway, as evidenced by the presence of markers such as transferrin, early endosomal antigen 1 (EEA1), and the small GTPase Rab5. The phagosome then transitions into a more mature stage by fusing with late endosomal compartments, characterized by the presence of lysosome-associated membrane proteins LAMP1 and LAMP2[76]. However, in *Leishmania* infections, the PVs can display distinct characteristics reflecting the diversity in their biogenesis, influenced by signals from both the host cell and the parasite itself[15]. While most studies on Leishmania PVs have focused on macrophages, research has shown that DCs also internalize *Leishmania* parasites[61, 77, 78]. Our results showed that APOL7C is recruited to PVs that express a late phagosome marker LAMP1(**Figure 13A**). This outcome is consistent with previous observations of APOL7C recruitment to silica bead-containing phagosomes.

As previously mentioned, APOL7C is a cytosolic apolipoprotein featuring a putative colicin-like pore-forming domain, known for inducing membrane pores. We aimed to evaluate the membrane integrity of *Leishmania* parasitophorous vacuoles (PVs) that recruit APOL7C. Consistent with earlier findings, our data revealed that APOL7C recruitment to the PVs is associated with the localization of Galectin-3, a damage marker that appeared specifically localized to APOL7C-positive PVs **(Figure 14A).** Galectins are proteins that specifically bind to carbohydrates in a reversible manner without chemical modification[79]. Research has shown that Galectin 3, is crucial for lysosomal repair. It recognizes membrane damage by binding to luminal β-galactosides once glycoconjugates on the luminal leaflet are exposed to the cytosol, thereby recruiting membrane repair and autophagic receptors. Previous studies have demonstrated that Galectins are recruited to ruptured endocyti organelles containing bacteria [80, 81, 82].

Our data suggest that APOL7C recruitment results in a loss of membrane integrity in *Leishmania*-harboring PVs. These results are consistent with prior research on APOL1, which indicates that trypanosome lysis is mediated by the uptake of APOL1 into lysosomes, leading to disruption of the lysosomal membrane[83]. Overall, the identification and characterization of the PVs where APOL7C is recruited in infected DCs presents a significant step toward gaining a more comprehensive understanding of leishmaniasis pathology and the role of APOL7C in infection.

When professional phagocytes, which are crucial defenders in our innate immune system, are activated and engulf pathogens, there is a marked increase in their use of molecular oxygen (O_2). This heightened oxygen consumption is harnessed by the NADPH oxidase complex to produce reactive oxygen species (ROS). The process begins with the one-electron reduction of O_2 to form superoxide anion ($O_2^{\bullet-}$), followed by the production of hydrogen peroxide (H_2O_2) through further reduction. The cytotoxic effects of $O_2^{\bullet-}$ and H_2O_2 are not confined to their direct antimicrobial properties but extend to their ability to interact with other microbicidal systems in phagocytes, leading to the generation of additional ROS such as hydroxyl radicals ($OH^{\bullet}$)[73]. However, previous studies in our lab have shown that the activity of NADPH also leads to depolarization of the phagosomal membrane which triggers the recruitment of APOL7C (**Figure 15**).

To understand role of NADPH oxidase in APOL7C recruitment in our infection model, we use the p22 $^{phox-/-}$ RawKb.APOL7C::mCherry cells. The $p22^{phox}$ subunit, is a crucial component of the NADPH oxidase complex as it forms a heterodimeric complex with $gp91^{phox}$, known as flavocytochrome b558 (cyt b558), which is essential for the enzyme's catalytic activity[73] .Our data demonstrated that in these cells, which lack the $p22^{phox}$ subunit, APOL7C was not recruited

to *Leishmania*-containing PVs (**Figure 16**), highlighting the indispensable role of NADPH oxidase in this recruitment process.

Further supporting these findings, similar results were observed with RawKb.APOL7C::mCherry cells expressing voltage-insensitive mutants of APOL7C (**Figure 17 and Figure 18**). This suggests that NADPH oxidase activation and the resultant changes in the PV membrane potential are key triggers for APOL7C insertion into this organelle. These conclusions are consistent with prior research, including unpublished data from our laboratory on silica bead phagosomes, which showed comparable mechanisms of the APOL7C insertion in phagosomes.

In summary, our data characterize the recruitment of APOL7C to *Leishmania* PVs as being dependent on the activity of the NADPH oxidase complex (**Figure 19**). This enzyme's role during the respiratory burst creates the necessary oxidative stress and membrane potential changes that signal the recruitment of APOL7C to PVs. This work not only sheds light on the molecular interactions between host cells and pathogens but also provides valuable insights into the mechanisms driving APOL7C. As previously described in the literature *Leishmania* infections in DCs are integral to the immune response. Previous studies, including those involving *L. major* have highlighted the complexities of this process. Notably, DCs infected with live promastigotes secreting OVA were able to induce robust $CD8^+$ T cell responses, whereas DCs exposed to heat-killed promastigotes were significantly less effective. However, during the initial stages of Leishmania infection in DCs the mechanism of cross-presentation twarrants further investigation. Studies using the *L.m*-NT-OVA system demonstrated that cross-presentation of *L. major*-derived antigens by DCs can be presented by a TAP-independent mechanism and that instead is reliant on an intraphagosomal pathway[74] .

In our study, we conducted *in vitro* cross-presentation assays to study the potential role of

APOL7C in this mechanism. By using a B3Z reporting cell line to asses cross-presentation, we observed a diminished ability of the RawKb.APOL7C::mCherry cell line to present *Leishmania* antigens during infection with *L. major* parasites (**Figure 20**). This suggests that the role of APOL7C in enhancing cross-presentation that was previously described may be influence by different factors such as antigen uptake and processing mechanism in the case of an infection model. Our data suggested that APOL7C may have a distinct function. It is important to take in consideration the cross-presentation of a live parasite could further influence APOL7C's function and its impact on antigen presentation[75]. These results show the impact of APOL7C in the cross-presentation of *Leishmania* antigens unveiling that this protein may modulate cross-presentation in different ways depending on the context.

In addition, our *in vivo* assay in which we usine a novel *Apol7c-/-* mice, have provided further insights into the role of APOL7C in *Leishmania* infection. In the case of *in vivo* studies to investigate *Leishmania* infection, murine models have been extensively utilized to investigate the interactions between the host's immune system and different *Leishmania* species that cause clinical manifestation. These models have provided critical insights into the immune mechanisms involved in CL, particularly regarding the roles of DC and in disease progression and resolution.

Multiple studies, performing an intradermal infection of C57BL/6 mice deficient in the transcription factor BATF3 have revealed critical insights into the role of DCs in the progression of CL. In these *Batf3*-deficient mice, the disease progression was notably exacerbated, as evidenced by unresolved lesions and increased parasitic burdens. This outcome underscores the essential role DCs in managing *Leishmania major* infections. However, despite the information gathered for similar studies using BATF3-dependent DCs in CL, the molecular mechanism that leads to this phenotype implemented by this DC subset in leishmaniasis remains a gap in our knowledge [56,66].

Our results show that in our *Apol7c-/-* mice exhibit a reduced ear lesion size compared to our control C57BL/6 mice, during the 18 weeks of infection (**Figure 21**). Interestingly, the *Apol7c -/-* mice presented high parasite load in the ear dermis. Although, in contrast in the *Apol7c*$^{-/-}$ mice this increase was not found to be significantly different in the case of the spleen (**Figure 22B**). Our data, offers new insights into the role of APOL7C in a *Leishmania* infection highlighting the potential impact of the expression of this protein by DCs on the immune response and disease progression. These findings suggest that APOL7C may significantly influence how DCs responds against infection of *Leishmania* parasites, providing a new information that can be useful to characterize its function.

Chapter 5. Significance

This thesis investigates a novel protein APOL7C and employs advanced molecular techniques, confocal microscopy, and both *in vivo* and *in vitro* methodologies to study its role in a *Leishmania* infection model. Prior studies have elucidated the critical role of cross-presentation of microbial peptides on MHCI molecules by cDC1s in eliciting and modulating a robust $CD8^+$ T cell response against various pathogens, including *Leishmania* parasites. Despite the recognized importance of this process in adaptive immunity, the molecular mechanisms underlying the activation and modulation of this response in DCs, which facilitate the generation of *Leishmania*-specific CD8+ T cell responses, remain poorly understood. This research delineates the potential role of APOL7C, in modulating the cross-presentation of *Leishmania* antigens. The findings presented in this thesis describe that expression and recruitment of APOL7C to PVs can modulate cross-presentation upon interaction with this intracellular parasite, thereby expanding current understanding of the mechanisms employed by DCs against *Leishmania* infections. Additionally, this study offers insights into the pathogenesis of *Leishmania* infection within DCs,

an immune cell type whose role in *Leishmaniasis* is poorly characterized. The absence of an effective vaccine against Leishmaniasis and the limitations of conventional treatments, such as toxicity, cost, and the emergence of drug resistance, highlight the necessity for exploring more immunotherapeutic strategies. Consequently, investigating the role of APOL7C may reveal new avenues for research focused on this protein as a potential immunotherapy approach to enhance control of the disease by implementing DC directed approaches.

5.1 Future Directions

To fully understand the role of APOL7C in DCs during *Leishmania* infection, future research should aim to optimize and refine experimental approaches. While this study utilized RAW 264.7 cells, which are more closely related to the monocyte/macrophage lineage, these cells may not accurately reflect the behavior of APOL7C in cDCs. DCs, particularly the cDC1 subset, are essential for initiating immune responses, including the cross-presentation of antigens to $CD8^+$ T cells. Since cDC1s are known for their superior ability to cross-present antigens, using a bone marrow-derived cDC1 cell line could provide critical insights into the specific behavior of APOL7C within this context.

Future experiments should focus on employing a bone marrow-derived cDC1 cell line to describe the behavior of APOL7C in a *Leishmania* infection model. Additionally, extending the analysis of APOL7C function beyond the early stages of infection to include mid and late stages would further enhance the findings of this project. This expanded timeline would allow for monitoring the role of APOL7C as the infection progresses, particularly to include factors such parasite replication. This approach can further elucidate whether APOL7C plays a role in controlling parasite burden or if its activity diminishes over time as the infection becomes established.

Finally, exploring the therapeutic potential of targeting APOL7C for immunotherapy could present an exciting avenue for future research, particularly in the context of Leishmaniasis. Overall, these proposed research directions could significantly advance our understanding of APOL7C's role in immune defense, with the potential to translate these findings into novel therapeutic strategies.

Chapter 6. Appendices

Appendix A. Primers use in this study for q-RT PCR

Primer name	Primer Sequence
APOL7C qPCR forward	CCATCCAGAATATACAAGGCATCCAAA
APOL7C qPCR reverse	CTGGCATCATTTGCTAGGCGAGTG

Appendix B. Unconjugated primary antibodies used in this study

Target	Stock	Application	Company
mCherry	0.5mg/mL	IF 1:5000	Biolegend
LAMP1	0.5 mg/mL	IF 1:50	Biolegend

Appendix C. Conjugated primary antibody used in this study

Target	Conjugated	stock	Application	Company
Galectine-3	AF 647	0.50 mg/mL	IF 1:500	Biolegend

Appendix D. Conjugated secondary antibodies used in this study

Target	Conjugate	Species	stock	Application	Company
Mouse IgG	AF488	Goat	2 mg/mL	IF 1:1000	Invitrogen
Rat IgG	AF 647	Goat	2 mg/mL	IF 1:1000	Invitrogen

Chapter 7. References

1. Steverding D. The history of leishmaniasis. Parasites & Vectors. 2017 Feb 15;10(1):82.

2. The Weekly Epidemiological Record (WER). https://www.who.int/publications/journals/weekly-epidemiological-record

3. Desjeux P. Leishmaniasis: current situation and new perspectives. Comparative Immunology, Microbiology and Infectious Diseases. 2004 Sep 1;27(5):305–18.

4. Burza S, Croft SL, Boelaert M. Leishmaniasis. The Lancet. 2018 Sep;392(10151):951–70.

5. Abadías-Granado I, Diago A, Cerro PA, Palma-Ruiz AM, Gilaberte Y. Cutaneous and Mucocutaneous Leishmaniasis. Actas Dermo-Sifiliográficas (English Edition). 2021 Jul 1;112(7):601–18.

6. Bullón J, Márquez L, Fernández JA, Scorzza C, Scorza JV, Rodríguez J, et al. A Promising Cutaneous Leishmaniasis Treatment with a Nanoemulsion-Based Cream with a Generic Pentavalent Antimony (Ulamina) as the Active Ingredient. Cosmetics. 2021 Dec;8(4):115.

7. de Vries HJC, Schallig HD. Cutaneous Leishmaniasis: A 2022 Updated Narrative Review into Diagnosis and Management Developments. Am J Clin Dermatol. 2022 Nov;23(6):823–40.

8. Gurel MS, Tekin B, Uzun S. Cutaneous leishmaniasis: A great imitator. Clinics in Dermatology. 2020 Mar 1;38(2):140–51.

9. Schwartz E, Hatz C, Blum J. New world cutaneous leishmaniasis in travellers. The Lancet Infectious Diseases. 2006 Jun 1;6(6):342–9.

10. Scorza BM, Carvalho EM, Wilson ME. Cutaneous Manifestations of Human and Murine Leishmaniasis. International Journal of Molecular Sciences. 2017 Jun;18(6):1296.

11. Ready PD. Epidemiology of visceral leishmaniasis. Clin Epidemiol. 2014 May 3;6:147–54.

12. Soto M, Ramírez L, Solana JC, Cook ECL, Hernández-García E, Charro-Zanca S, et al. Resistance to Experimental Visceral Leishmaniasis in Mice Infected With Leishmania infantum Requires Batf3. Front Immunol. 2020 Dec 10;11:590934.

13. Volpedo G, Pacheco-Fernandez T, Bhattacharya P, Oljuskin T, Dey R, Gannavaram S, et al. Determinants of Innate Immunity in Visceral Leishmaniasis and Their Implication in Vaccine Development. Front Immunol. 2021 Oct 12;12:748325.

14. Organ-specific immune responses associated with infectious disease - PubMed. https://pubmed.ncbi.nlm.nih.gov/10652464/

15. Omotade TO, Roy CR. Manipulation of Host Cell Organelles by Intracellular Pathogens. Microbiology Spectrum. 2019 Apr 26;7(2):10.1128/microbiolspec.bai-0022–2019.

16. Zijlstra EE, Musa AM, Khalil E a. G, Hassan IE, El-Hassan AM. Post-kala-azar dermal leishmaniasis. The Lancet Infectious Diseases. 2003 Feb 1;3(2):87–98.

17. Sunter J, Gull K. Shape, form, function and Leishmania pathogenicity: from textbook descriptions to biological understanding. Open Biology. 2017 Sep 13;7(9):170165.

18. Kamhawi S. Phlebotomine sand flies and Leishmania parasites: friends or foes? Trends in Parasitology. 2006 Sep;22(9):439–45.

19. Sand flies: Basic information on the vectors of leishmaniasis and their interactions with Leishmania parasites | Communications Biology

20. Díaz E, Ponte-Sucre A. Leishmaniasis: The Biology of a Parasite. In: Ponte-Sucre A, Padrón-Nieves M, editors. Drug Resistance in Leishmania Parasites: Consequences, Molecular Mechanisms and Possible Treatments. Cham: Springer International Publishing; 2018 https://doi.org/10.1007/978-3-319-74186-4_1

21. Young J, Kima P. The Leishmania Parasitophorous Vacuole Membrane at the Parasite-Host Interface. The Yale journal of biology and medicine. 2019 Sep 20;92:511–21.

22. Real F, Mortara R, Rabinovitch M. Fusion between Leishmania amazonensis and Leishmania major Parasitophorous Vacuoles: Live Imaging of Coinfected Macrophages. PLoS neglected tropical diseases. 2010 Dec 7;4:e905.

23. Chulanetra M, Chaicumpa W. Revisiting the Mechanisms of Immune Evasion Employed by Human Parasites. Front Cell Infect Microbiol. 2021 Jhttps://www.frontiersin.org/journals/cellular-and-infection-microbiology/articles/10.3389/fcimb.2021.702125/full

24. Gupta G, Oghumu S, Satoskar AR. Mechanisms of Immune Evasion in Leishmaniasis. Adv Appl Microbiol. 2013;82:155–84.

25. Walker DM, Oghumu S, Gupta G, McGwire BS, Drew ME, Satoskar AR. Mechanisms of cellular invasion by intracellular parasites. Cell Mol Life Sci. 2014 Apr;71(7):1245–63.

26. Gregory DJ, Olivier M. Subversion of host cell signalling by the protozoan parasite Leishmania. Parasitology. 2005 Mar;130(S1):S27–35.

27. T helper (h)1/Th2 and Leishmania: paradox rather than paradigm - ScienceDirect.. https://www.sciencedirect.com/science/article/pii/S0165247805000143?via%3Dihub

28. Sundar S, Chakravarty J. Liposomal Amphotericin B and Leishmaniasis: Dose and Response. J Glob Infect Dis. 2010;2(2):159–66.

29. Pradhan S, Schwartz RA, Patil A, Grabbe S, Goldust M. Treatment options for leishmaniasis. Clinical and Experimental Dermatology. 2022 Mar 1;47(3):516–21.

30. Torres-Guerrero E, Arenas R. Leishmaniasis. Current therapeutic alternatives. Dermatologia Revista Mexicana. 2018 Sep 1;62:400–9.

31. Musa AM, Mbui J, Mohammed R, Olobo J, Ritmeijer K, Alcoba G, et al. Paromomycin and Miltefosine Combination as an Alternative to Treat Patients With Visceral Leishmaniasis in Eastern Africa: A Randomized, Controlled, Multicountry Trial. Clinical Infectious Diseases. 2023 Feb 1;76(3):e1177–85.

32. Report of a WHO informal consultation on liposomal amphotericin B in the treatment of visceral leishmaniasis. 2013 https://www.who.int/publications/i/item/WHO-CDS-NTD-IDM-2007.4

33. Okwor I, Uzonna JE. Immunotherapy as a Strategy for Treatment of Leishmaniasis: a Review of the Literature. Immunotherapy. 2009 Sep 1;1(5):765–76.

34. Roatt BM, Aguiar-Soares RD de O, Coura-Vital W, Ker HG, Moreira N das D, Vitoriano-Souza J, et al. Immunotherapy and Immunochemotherapy in Visceral Leishmaniasis: Promising Treatments for this Neglected Disease. Front Immunol 2014 https://www.frontiersin.org/journals/immunology/articles/10.3389/fimmu.2014.00272/full

35. Janeway CA, Medzhitov R. Innate immune recognition. Annu Rev Immunol. 2002;20:197–216.

36. Riera Romo M, Pérez-Martínez D, Castillo Ferrer C. Innate immunity in vertebrates: an overview. Immunology. 2016;148(2):125–39.

37. Iwasaki A, Medzhitov R. Control of adaptive immunity by the innate immune system. Nat Immunol. 2015 Apr;16(4):343–53.

38. The Adaptive Immune Response | Biology for Majors II https://courses.lumenlearning.com/suny-wmopen-biology2/chapter/the-adaptive-immune-response

39. Bassing CH, Swat W, Alt FW. The Mechanism and Regulation of Chromosomal V(D)J Recombination. Cell. 2002 Apr;109(2):S45–55.

40. Chi H, Pepper M, Thomas PG. Principles and therapeutic applications of adaptive immunity. Cell. 2024 Apr;187(9):2052–78.

41. B Cell Responses: Cell Interaction Dynamics and Decisions: Cell https://www.cell.com/cell/fulltext/S0092-8674(19)30278-8

42. Nutt SL, Hodgkin PD, Tarlinton DM, Corcoran LM. The generation of antibody-secreting plasma cells. Nat Rev Immunol. 2015 Mar;15(3):160–71.

43. Kurosaki T, Kometani K, Ise W. Memory B cells. Nat Rev Immunol. 2015 Mar;15(3):149–59.

44. Liu K. Dendritic Cells. Encyclopedia of Cell Biology. 2016;741–9.

45. Reis e Sousa C. Activation of dendritic cells: translating innate into adaptive immunity. Current Opinion in Immunology. 2004 Feb 1;16(1):21–5.

46. Mantegazza AR, Magalhaes JG, Amigorena S, Marks MS. Presentation of phagocytosed antigens by MHC class I and II. Traffic. 2013 Feb;14(2):135–52.

47. Hossain MK, Wall KA. Use of Dendritic Cell Receptors as Targets for Enhancing Anti-Cancer Immune Responses. Cancers (Basel). 2019 Mar 24;11(3):418.

48. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. T Cells and MHC Proteins. In: Molecular Biology of the Cell 4th edition. Garland Science; 2002 https://www.ncbi.nlm.nih.gov/books/NBK26926/

49. Hildner K, Edelson BT, Purtha WE, Diamond M, Matsushita H, Kohyama M, et al. Batf3 Deficiency Reveals a Critical Role for CD8α+ Dendritic Cells in Cytotoxic T Cell Immunity. Science. 2008 Nov 14;322(5904):1097–100.

50. Schlitzer A, Zhang W, Song M, Ma X. Recent advances in understanding dendritic cell development, classification, and phenotype. F1000Res. 2018 Sep 26;7:F1000 Faculty Rev-1558.

51. Förster R, Braun A, Worbs T. Lymph node homing of T cells and dendritic cells via afferent lymphatics. Trends in Immunology. 2012 Jun 1;33(6):271–80.

52. Dzopalic T, Rajkovic I, Dragicevic A, Colic M. The response of human dendritic cells to co-ligation of pattern-recognition receptors. Immunol Res. 2012 Apr;52(1–2):20–33.

53. Lindenbergh MFS, Stoorvogel W. Antigen Presentation by Extracellular Vesicles from Professional Antigen-Presenting Cells. Annu Rev Immunol. 2018 Apr 26;36:435–59.

54. Canton J, Blees H, Henry CM, Buck MD, Schulz O, Rogers NC, et al. The receptor DNGR-1 signals for phagosomal rupture to promote cross-presentation of dead-cell-associated antigens. Nat Immunol. 2021 Feb;22(2):140–53.

55. Den Haan JMM, Bevan MJ. Antigen presentation to CD8+ T cells: cross-priming in infectious diseases. Current Opinion in Immunology. 2001 Aug;13(4):437–41.

56. Batf3-dependent CD103+ dendritic cells are major producers of IL-12 that drive local Th1 immunity against Leishmania major infection in mice PMC. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4316187/

57. Shen L, Rock KL. Priming of T cells by exogenous antigen cross-presented on MHC class I molecules. Current Opinion in Immunology. 2006 Feb 1;18(1):85–91.

58. Fehres CM, Unger WWJ, Garcia-Vallejo JJ, van Kooyk Y. Understanding the Biology of Antigen Cross-Presentation for the Design of Vaccines Against Cancer. Frontiers in Immunology. 2014 https://www.frontiersin.org/articles/10.3389/fimmu.2014.00149

59. Chefalo PJ, Grandea AG III, Van Kaer L, Harding CV. Tapasin−/− and TAP1−/− Macrophages Are Deficient in Vacuolar Alternate Class I MHC (MHC-I) Processing due to Decreased MHC-I Stability at Phagolysosomal pH1. The Journal of Immunology. 2003 Jun 15;170(12):5825–33.

60. Tibúrcio R, Nunes S, Nunes I, Rosa Ampuero M, Silva IB, Lima R, et al. Molecular Aspects of Dendritic Cell Activation in Leishmaniasis: An Immunobiological View. Front Immunol. 2019 Feb 22;10:227.

61. Reithinger R, Dujardin JC, Louzir H, Pirmez C, Alexander B, Brooker S. Cutaneous leishmaniasis. The Lancet Infectious Diseases. 2007 Sep 1;7(9):581–96.

62. McDowell MA, Marovich M, Lira R, Braun M, Sacks D. Leishmania Priming of Human Dendritic Cells for CD40 Ligand-Induced Interleukin-12p70 Secretion Is Strain and Species Dependent. Infection and Immunity. 2002 Aug;70(8):3994–4001.

63. Prina E, Abdi SZ, Lebastard M, Perret E, Winter N, Antoine JC. Dendritic cells as host cells for the promastigote and amastigote stages of Leishmania amazonensis: the role of opsonins in parasite uptake and dendritic cell maturation. Journal of Cell Science. 2004 Jan 15;117(2):315–25.

64. Hierarchy of Susceptibility of Dendritic Cell Subsets to Infection by Leishmania major: Inverse Relationship to Interleukin-12 Production | Infection and Immunity. Available from: https://journals.asm.org/doi/full/10.1128/IAI.70.7.3874-3880.2002

65. Kawai T, Akira S. TLR signaling. Semin Immunol. 2007 Feb;19(1):24–32.

66. Ashok D, Schuster S, Ronet C, Rosa M, Mack V, Lavanchy C, et al. Cross-presenting dendritic cells are required for control of Leishmania major infection. European Journal of Immunology. 2014;44(5):1422–32.

67. Fontaine F, Lecordier L, Vanwalleghem G, Uzureau P, Van Reet N, Fontaine M, et al. APOLs with low pH dependence can kill all African trypanosomes. Nat Microbiol. 2017 Nov;2(11):1500–6.

68. Kimblin N, Peters N, Debrabant A, Secundino N, Egen J, Lawyer P, et al. Quantification of the infectious dose of Leishmania major transmitted to the skin by single sand flies. Proc Natl Acad Sci U S A. 2008 Jul 22;105(29):10125–30.

69. Bertholet S, Debrabant A, Afrin F, Caler E, Mendez S, Tabbara KS, et al. Antigen Requirements for Efficient Priming of CD8+ T Cells by Leishmania *major*-Infected Dendritic Cells. Infection and Immunity. 2005 Oct;73(10):6620–8.

70. Späth GF, Beverley SM. A lipophosphoglycan-independent method for isolation of infective Leishmania metacyclic promastigotes by density gradient centrifugation. Exp Parasitol. 2001 Oct;99(2):97–103.

71. Carneiro MB, Lopes ME, Hohman LS, Romano A, David BA, Kratofil R, et al. Th1-Th2 Cross-Regulation Controls Early Leishmania Infection in the Skin by Modulating the Size of the Permissive Monocytic Host Cell Reservoir. Cell Host & Microbe. 2020 May;27(5):752-768.e7.

72. Gonzales GA, Huang S, Rajwani J, Wilkinson L, Nguyen JA, Wood CM, et al. The pore-forming apolipoprotein APOL7C drives phagosomal rupture and antigen cross-presentation by dendritic cells.2023 http://biorxiv.org/lookup/doi/10.1101/2023.08.11.553042

73. Riffaud CM, Rucks EA, Ouellette SP. Persistence of obligate intracellular pathogens: alternative strategies to overcome host-specific stresses. Front Cell Infect Microbiol. 2023 May 22;13:1185571.

74. Konior A, Schramm A, Czesnikiewicz-Guzik M, Guzik TJ. NADPH Oxidases in Vascular Pathology. Antioxid Redox Signal. 2014 Jun 10;20(17):2794–814.

75. Groemping Y, Rittinger K. Activation and assembly of the NADPH oxidase: a structural perspective. Biochem J. 2005 Mar 15;386(Pt 3):401–16.

76. Bertholet S, Goldszmid R, Morrot A, Debrabant A, Afrin F, Collazo-Custodio C, et al. Leishmania Antigens Are Presented to CD8+ T Cells by a Transporter Associated with Antigen Processing-Independent Pathway In Vitro and In Vivo. The Journal of Immunology. 2006 Sep 15;177(6):3525–33.

77. Moll H, Fuchs H, Blank C, Röllinghoff M. Langerhans cells transport Leishmania major from the infected skin to the draining lymph node for presentation to antigen-specific T cells. Eur J Immunol. 1993 Jul;23(7):1595–601.

78. Sacks, D., & Noben-Trauth, N. (2002). The immunology of susceptibility and resistance to *Leishmania* major in mice. *Nature Reviews Immunology*, 2(11), 845-858.
This review highlights the importance of Th1 and Th2 immune responses in mouse models of *Leishmania* infection, and the role of cytokines such as IFN-γ, IL-4, and IL-10.

79. Kaye, P. M., & Scott, P. (2011). Leishmaniasis: complexity at the host-pathogen interface. *Nature Reviews Microbiology*, 9(8), 604-615.
This article covers the interactions between the host immune response and *Leishmania*, particularly the role of T cells and macrophages.

80. Müller, I., Pedrazzini, T., Kropf, P., Louis, J. A., & Milon, G. (1991). Establishment of resistance to *Leishmania* major infection in susceptible BALB/c mice requires parasite-specific CD8+ T cells. *International Immunology*, 3(6), 587-597.
This study focuses on the role of CD8+ T cells in protective immunity during *Leishmania* infections in susceptible mice.

81. Belkaid, Y., Mendez, S., Lira, R., Kadambi, N., Milon, G., & Sacks, D. (2000). A natural model of Leishmania major infection reveals a prolonged "silent" phase of parasite amplification in the skin before the onset of immunity. *Journal of Immunology*, 165(12), 7771-7780.
This paper offers insights into the delayed immune response and the role of T cells in controlling *Leishmania* infection.

82. McMahon-Pratt, D., & Alexander, J. (2004). Does the immune response to *Leishmania* direct the outcome of infection? *Parasitology Today*, 20(7), 342-349.
This article discusses how different T cell subsets, particularly CD4+ and CD8+ T cells, influence the course of *Leishmania* infections.

83. Alexander, J., & Bryson, K. (2005). T helper (h)1/Th2 and *Leishmania*: paradox rather than paradigm. *Immunology Letters*, 99(1), 17-23.
This paper reviews the classical Th1/Th2 paradigm in *Leishmania* infections and the implications for disease resistance and susceptibility.

84. Belkaid Y, Hoffmann KF, Mendez S, Kamhawi S, Udey MC, Wynn TA, et al. The role of interleukin (IL)-10 in the persistence of Leishmania major in the skin after healing and the therapeutic potential of anti-IL-10 receptor antibody for sterile cure. J Exp Med. 2001 Nov 19;194(10):1497–506.

85. Kane MM, Mosser DM. The role of IL-10 in promoting disease progression in leishmaniasis. J Immunol. 2001 Jan 15;166(2):1141–7.

86. Belkaid Y, Piccirillo CA, Mendez S, Shevach EM, Sacks DL. CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity. Nature. 2002 Dec 5;420(6915):502–7.

87. Mendez S, Reckling SK, Piccirillo CA, Sacks D, Belkaid Y. Role for CD4+ CD25+ Regulatory T Cells in Reactivation of Persistent Leishmaniasis and Control of Concomitant Immunity. Journal of Experimental Medicine. 2004 Jul 19;200(2):201–10.

88.