Processing of *Leishmania dononvani* by human dendritic cells

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Declaration of Authorship

I hereby declare that I have written the present dissertation with the topic:

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Independently, using no other aids then those I have cited. I have clearly mentioned the source of the passages that are taken word for word or paraphrased from other works.

The presented thesis has not been submitted in this or any other form to another Faculty or Examination Institution.

Ibrahim M. I. Azzouz

Berlin, January 2020

List of Abbreviations

WHO	World Health Organization
INFɣ	Interferon gamma
TNF α	Tumor Necrosis Factor Alpha
p	Protein
IL- 12	interleukin-12
IL-4	Interleukin-4
IL-10	Interleukin-10
DCs	Dendritic Cells
MΦs	Macrophages
hDCs	human dendritic cells
pDCs	plasmacytoid Dendritic Cells
mDCs	myeloid Dendritic Cells
hMDDCs	human Monocyte Differentiated Dendritic cells
ihMDDCs	immature human Monocyte Differentiated Dendritic cells
mhMDDCs	mature human Monocyte differentiated Dendritic cells
hMDMs	human Monocyte Differentiated Macrophages
CD	Cluster of Differentiation
CLR	C-type Lectin Receptors
PRRs	Pattern Recognition Receptors
TLRs	Toll Like Receptors
TLRLs	Toll Like Receptors Ligands
Th1	T helper cell
CTL	Cytotoxic T lymphocyte

APCs	Antigen Presenting Cells
L.donovani	*Leishmania. donovani,*
L.major	*Leishmania Major*
CMI	Cell-Mediated adaptive Immunity
MDDCs	Monocyte Derived Dendritic Cells
MDMs	Monocyte Derived Macrophages
PBMCs	Peripheral Blood Mononuclear Cells
MHC-I	Major Histocompatibility Complex molecule class I
MHC-II	Major Histocompatibility Complex molecule class II
HLA-DM	Human Leukocyte Antigen DM
CLRs	C- Type Lectin Receptors
PRRs	pattern recognition receptors
NO	Nitric Oxide
iNOS	inducible NO synthase
TGF-β	Transforming Growth Factor- beta
Granzyme	Granule-secreted enzymes
GrB	Granzyme B
PEF	Perforin
GNLY	Granulysin
NK	Natural Killer cells
NKT	Natural Killer T cell
TCR	T cell Receptor
TRAIL	Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand
ICAD	Inhibitor of Caspase Activated DNAse

YFP	Yalow Fluorescent Protein
PBS	Phosphate buffer solution
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
FACS	Flow cytometry
YFP	Yellow Fluorescent Protein
APC	Allophycocyanin
FCS	Fetal Calf Serum
mAB	monoclonal antibody
PMA	phorbol myristate acetate
IFN-γ mRNA	Interferon Gamma messenger RiboNucleic Acid
JIA	Juvenile Idiopathic Arthritis
PFA	paraformaldehyde
NaOH	Sodium Hydroxide
HCl	Hydrochloric acid

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1. A. Abstract

Leishmania parasites are intracellular protiststhat cause various human diseases ranging from self-healing cutaneous to fatal visceral leishmaniasis. The host cells are phagocytes, primarily neutrophils and macrophages, where the parasites neutralize innate immune defenses, proliferate and finally infect other cells. Despite that *Leishmania* induce vigorous T cell responses, which require antigen presentation and stimulation by phagocytes, importantly dendritic cells. So far it is not clear how to align the blockade of phagocyte functions with the efficient immune stimulation. We found that, in contrast to other phagocytes, human dendritic cells digest the parasites through an apoptotic process, granzyme B and maybe granulysin manage this killing, and digestion of parasites is delayed in granzyme B inhibited cells. The digested parasites co-localise with components of the MHC class I and II antigen processing pathways. Furthermore, the infection leads to enhanced activation of dendritic cells triggered by inflammatory cytokines.

The data presented herein emphasize the need to address the DCs when developing anti-*Leishmania* vaccines or immunotherapies in order to induce efficient CD4+ helper and CD8 effector T cell responses. They may explain why leishmanization, i.e. immunization with life parasites, is efficient whereas subunit vaccines are not. However, leishmanization induces immunity through deliberate infection with subsequent disease, which may come with severe adverse effects. Alternative strategies may consider TLR agonists or inflammatory cytokines for *in situ* vaccination and immunotherapy to activate parasite-bearing DCs and thereby induce parasitocidal CD8 effector T cell and innate immune reaction.

1. B. Zusammenfassung

Leishmania-Parasiten sind intrazelluläre Protisten, die verschiedene humane Krankheiten verursachen, die von selbstheilenden kutanen Leishmaniosen bis hin zu tödlichen viszeralen Leishmaniosen reichen. Die Wirtszellen sind Phagozyten, hauptsächlich Neutrophile und Makrophagen, in denen sich die Parasiten der angeborenen Immunabwehr entziehen, sich vermehren und anschließend andere Zellen infizieren können. Trotzdem induzieren Leishmanien heftige T-Zell-Reaktionen, die eine Antigenpräsentation und Stimulierung durch Phagozyten erfordern, vor allem durch dendritische Zellen. Bisher ist jedoch nicht klar, wie die Blockierung der Phagozytenfunktionen mit der effizienten Immunstimulation in Einklang gebracht werden kann. Wir fanden heraus, dass im Gegensatz zu anderen Phagozyten humane dendritische Zellen die Parasiten durch einen apoptotischen Prozess verdauen können, wobei Granzym B und Granulysin essentiell fürdiese Abtötung sind und die Verdauung der Parasiten in Granzym B-inhibierten Zellen verzögert ist. Die verdauten Parasiten kolokalisieren mit Komponenten der MHC-Klasse I- und II-Antigen-Prozessierungswege. Darüber hinaus führt die Infektion zu einer verstärkten Aktivierung von dendritischen Zellen, die durch entzündliche Zytokine ausgelöst werden.

Die hier präsentierten Daten unterstreichen die Notwendigkeit bei der Entwicklung von anti-Leishmania-Impfstoffe oder Immuntherapien, dendritische Zellen miteinzubeziehen, um so eine effiziente CD4+ Helfer- und CD8+ Effektor-T-Zell-Antwort zu induzieren. Dies könnte erklären, warum die Leishmanisierung, eine Immunisierung mit lebenden Parasiten, effizient ist, während das bei Untereinheitenimpfstoffe nicht der Fall ist. Allerdings kann die Leishmanisierung, die eine Immunität durch das Auslösen einer Infektion mit anschließender Erkrankung herbeiführt, schwerwiegende nachteilige Auswirkungen haben. Alternative Strategien könnten TLR-Agonisten oder inflammatorische Zytokine für eine in-situ-Impfung und einer Immuntherapie sein, um parasitentragende DCs zu aktivieren und dadurch parasitozidal CD8+ Effektor-T-Zellen und Reaktionen des angeborenen Immunsystems zu induzieren.

2. Introduction

2.1. Background of leishmaniasis

Leishmaniasis is a set of infectious diseases caused by single cellular protest parasites of the genus *Leishmania*. These parasites are transmitted to the vertebrate host as a result of the bites by infected sand flies. A variety of mammalian are permissive hosts for these parasites including dogs, rats, hamsters, gerbils and humans as well as other animal species. Of note, more than 70 animal species have been identified as natural reservoir hosts of *Leishmania* parasites [1]. Basically, the outcome of the infections depends on the species of both parasites and hosts. Human leishmaniasis has been split into three type: cutaneous leishmaniasis (CL), muco-cutaneous leishmaniasis (ML; also known as espundia), and visceral leishmaniasis (VL; also known as kala-azar). Moreover, in cutaneous leishmaniasis, the patients present with either dry or wet ulcers(s), or papules on the skin.

Leishmaniasis is classified as disease causing among the highest rate of morbidity globally despite extensive investigations into the disease and preventive and therapeutic measures, and efforts to decrease the number of sandflies in recent years to control the spread of infection. However, it was not possible to reduce the epidemic level, on the contrary, in many endemic regions the numbers of cases are increasing. Statistically, VL is the form of leishmaniasis leading to the highest morbidity rate [2].

VL has a complication known as post-kala-azar dermal leishmaniasis (PKDL), which is a skin manifestation in individuals cured from VL. PKDL is believed to be reservoir for anthroponotic transmission of VL and difficult to cure, particularly in patients in East Africa who suffer from a severe form of PKDL [3, 4]. The time between treated VL and PKDL is 0–6 months in Sudan and 6 months to 3 years in India. Nodular lesions of PKDL contain high numbers of parasites and lead to highly transmittable infection [5].

Leishmaniasis is globally distributed in tropical, subtropical and adjacent regions. Even though it was estimated to cause the ninth largest disease burden among infectious diseases [6], leishmaniasis is largely ignored in discussions of tropical disease priorities. In addition, to date, more than 147 million people living in the South Asian region alone are at risk of being infected. The disease prevails among poor people in marginalized communities. According to WHO reports, unstable increase and decrease of the numbers of VL cases in South Asia show that the

infection rate of this disease is difficult manage (**Figure 1**) [7]. According to various recent studies between 98 and 102 countries and 3 territories on 5 continents have been designated endemic regions for *Leishmania* transmission. The formal case counts totals annually more than 58,000 new VL cases and 220,000 new CL cases [2]. However, the underreporting rate has been estimated to be up to four-fold due to lack of access to medical services and misdiagnosis.

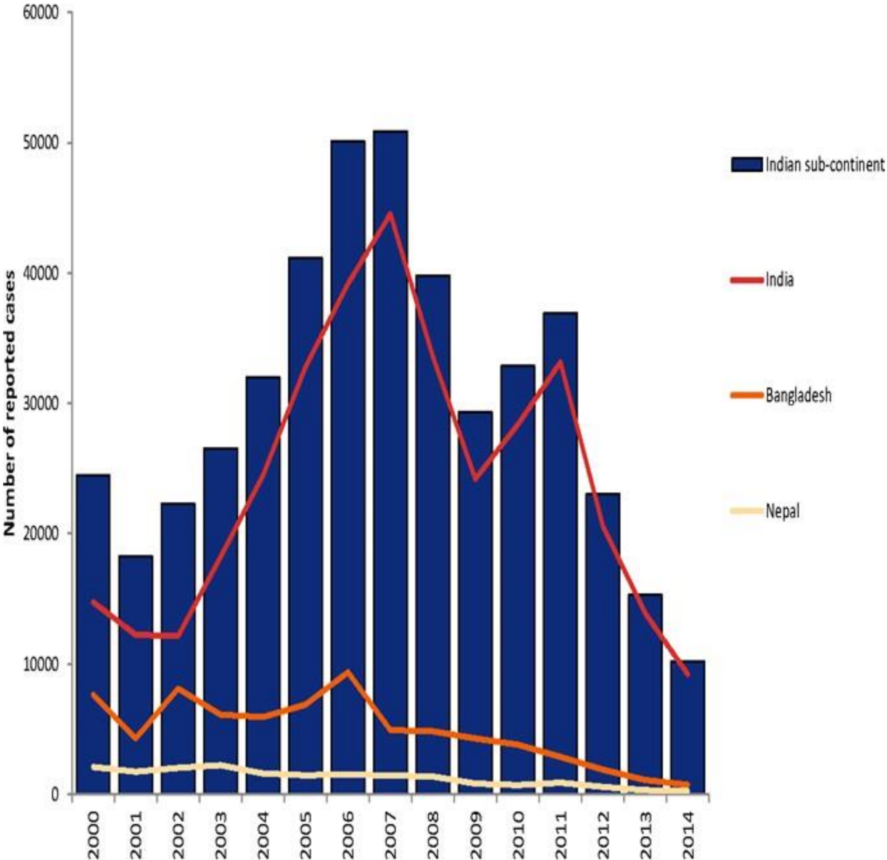


Figure1: The numbers of visceral leishmaniasis cases in the WHO South Asian region 2000−2014 [7].

2.1.1. The clinical presentations of VL

After an incubation time of between 2 and 6 months, VL patients develop a variety of symptoms including fever, fatigue, weakness, loss of appetite and weight loss. These manifestations are accompanied by enlarged lymph nodes, spleen and liver, which results from the parasitic invasion of blood and the reticulo-endothelial system, also known as phagocytic system, and infiltration of white blood cells, mainly granulocytes, into the lymphatic organs and liver. Fatigue and weakness are aggravated by anemia caused by the persistent inflammatory state [8]

There are differences in the clinical manifestation of VL in different endemic areas. A good illustration here is that enlarged lymph nodes are infrequently in Indian VL patients but are frequent in Sudanese VL patients [9, 10]. Hyperpigmentation, which led to the name kala-azar (black fever), is common in India but not in Sudan. As the disease progresses, abdominal pain can occur and worsen along with splenomegaly and hepatomegaly. In addition, accompanying complications may result from bacterial co-infections such as diarrhea, pneumonia or tuberculosis, or *Pseudomonas aeruginosa* sepsis and can confuse the clinical picture at the time of initial diagnosis. The VL symptoms may persist for several months before the patients receive medical care or die from bacterial complications, massive bleeding or severe anemia.

2.1.2. Life cycle of Leishmania parasites

In the phlebotomine sandfly, the *Leishmania* parasite lives and propagates in the gut as flagellated promastigote form. Upon digest and excretion of the undigested remainders of the blood meal, the sandfly switches to sugar meals, which triggers the transformation of the *Leishmania* parasites to the highly mobile and highly infectious metacyclic promastigote form. This form is transmitted to the vertebrate host by the bite of female sandflies during a subsequent blood meal, which the sandfly requires for egg production. In the skin of the infected vertebrate host parasites are taken up by phagocytes (neutrophils, macrophages and dendritic cells) into phagolysosomes where they lose their flagella to transform to an amastigote form (**Figure 2**) [11]. Their survival and then propagation in phagolysosomes goes along with the transformation of the host organelle into parasitophorous vacuoles [12, 13]. The parasites spreads with its phagocytic host cells through the body of the patient via the lymphatic and vascular systems and infect other immune cells in the reticulo-endothelial system, resulting in infiltration of the bone marrow, spleen, liver and lymph nodes (lymphadenopathy). The life cycle is concluded by the bite of another sandlfy.

2.2. Immunology of leishmaniasis

Frequently, *Leishmania* infections remain asymptomatic as no clinical symptoms are presented. Asymptomatic *Leishmania* infection may relate to a previous leishmaniasis and indicates immunity to the disease without prevention of the infection. Understanding the roles of *Leishmania* parasites in terms of its ability to evade the immune system on cellular and systemic levels, as well as the mechanisms of developing immunity of the host, is crucial for the development of prophylactic measures and immune-therapeutic approaches such as vaccines. Besides disease-induced immunity there might also be other factors that predispose some individuals to develop the disease or to control the infection such as genetics or nutritional status.

The vector-born transmission and the intracellular existence and propagation of the parasites render antibody-based immune responses ineffective. Immunity against leishmaniasis is based on T cell-mediated immune responses leading to killing of the parasite inside the phagocytic host cells resulting in cure and cell-mediated immunity (CMI) to subsequent infections. In VL patients, the inability to control *Leishmania donovani* (*L. donovani*) infection is associated with a profound T cell unresponsiveness to *L. donovani* antigens [14].

In addition, the crucial role of CMI has been recognized by the increasing the risk of developing clinical illness in cases of malnutrition or concomitant immunosuppressive diseases such as HIV infection [15, 16]. Other risk factors implicated in progressing clinical illness have been identified to be reduction of IFN-ɣ production [17] and polymorphisms in the promoter of the TNF-α gene [18] as well as young age [19-21].

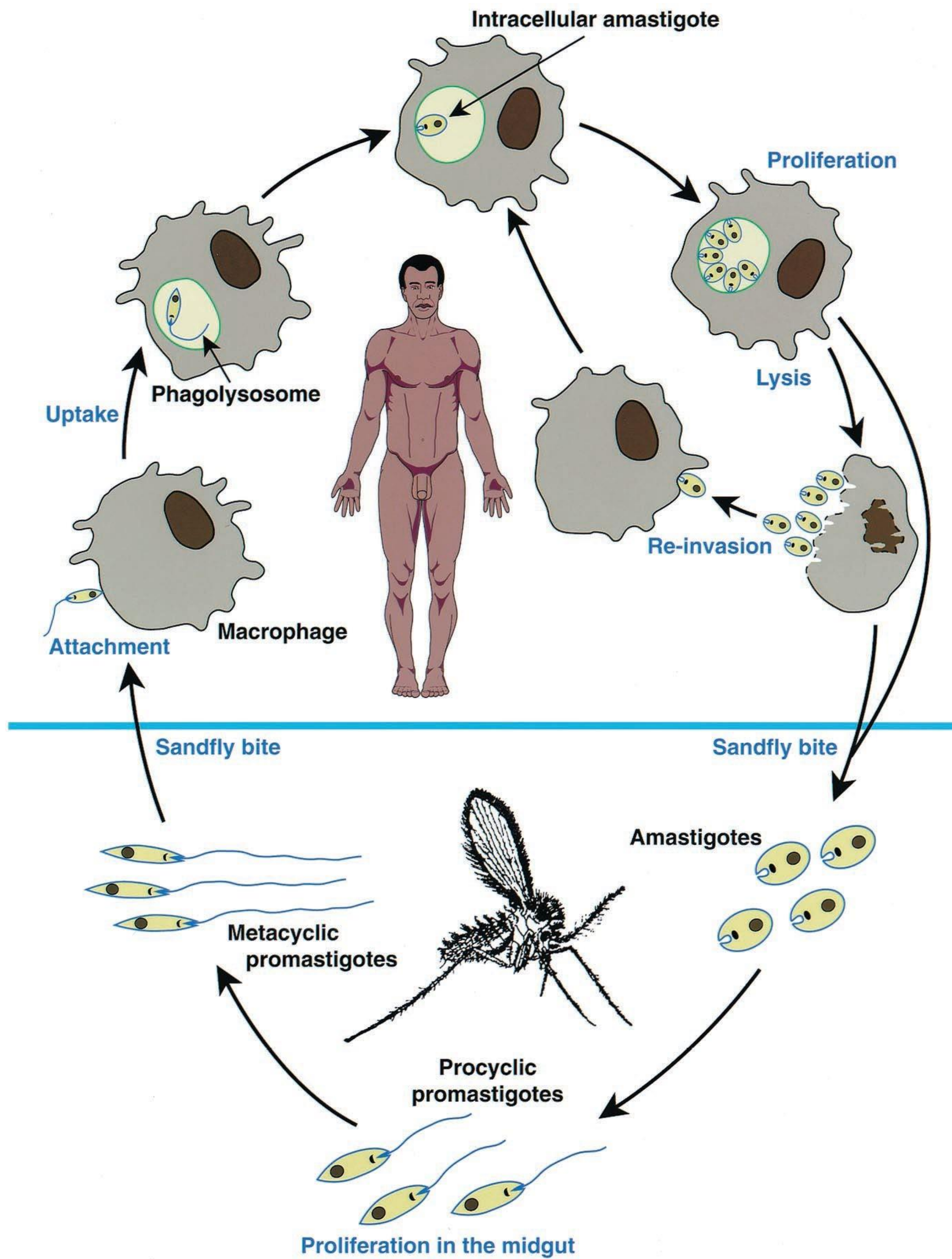


Figure 2: Life cycle of *Leishmania* parasite [11].

The host immune system can be tricked or exploited by *Leishmania* parasites. Immune evasion, immune suppression or a failure to mount immune response could be the cause of why people get leishmaniasis once they injected with *Leishmania* parasites. A thorough understanding of these mechanisms could lead to better strategies for effective management of *Leishmania* infections.

The immunology and immunopathology in humans, dogs, and experimental rodent models in visceral infections have been extensively studied, with many points characterized and others still to be elucidated [22]. A consensus is that, despite differences in the course of infection in the different species, the outcome of the disease is critically influenced by the host immune response. Several studies have shown that susceptibility to human VL is related to an elevated titer of circulating antibodies and a reduction of type-1 T cell-mediated immunity, mainly with decreased production of interferon gamma (IFNɣ) and interleukin 12 (IL-12), plus a marked up-regulation of IL-10 and IL-4 cytokines [23, 24].

The innate immune response contributes to VL resistance, acting to control parasite growth during the early stages of infection. Macrophages, neutrophils and dendritic cells have a central role for host resistance or susceptibility to *Leishmania* infection [25]. As intracellular parasites, *Leishmania* parasites have developed many of sophisticated mechanisms to block leishmanicidal activities of macrophages and overcome the host innate immunity. Certainly, *Leishmania* parasites inhibit, upon infection, antigen presentation and other defense activities required for induction of efficient T cell responses. [26].

2.2.1. Neutrophils in leishmaniasis

Studies of mouse models show that neutrophils travel to the site of infection soon after the sand fly bite, and are the early tissue infiltrating cells to phagocyte *Leishmania major* [27-29]. The interactions mediating this migration between endothelial cells involve adhesion molecules expressed on neutrophil surface membrane, which allow for binding and “rolling” prior to extravasation from vasculature [30] . Other papers show that neutrophils may influence adaptive immune responses by expressing chemokines which recruit other immune cell types that in turn participate in the response to infection [31, 32]. A partial list of neutrophil microbicidal responses includes assembly of the multi-protein NADPH oxidase complex with resultant

production of reactive oxygen species, release of granule contents into intracellular microbial compartments, and release of defensins [30, 33].

The role of neutrophils in *Leishmania spp*. infection has been extensively investigated in murine models, and the outcomes have varied depending on either the resistant or susceptible genetic background of the mouse and the species of *Leishmania* used [34-36]. There are indications that a subset of *L. donovani* survive intracellularly in murine neutrophils [37]. Furthermore, neutrophils are also found to eliminate parasites as reported for an experimental model of *Leishmania braziliensis* infection, in which the infection induces activation of the neutrophils with increased ROS production leading to parasite clearance [38, 39].

Previous studies on human innate immune cells have suggested that these neutrophils could play an important role in human leishmaniasis. Neutrophils from healthy donors infected with *Leishmania major* produce a strong oxidative response that eliminates internalized parasites [40]. Infection with *Leishmania amazonensis* promotes neutrophils activation, degranulation and production of leukotriene B4 which promotes the killing of parasite [41]. Moreover, interactions between *Leishmania*-infected macrophages and healthy human neutrophils modulate the intracellular replication of both *Leishmania amazonensis* [42] and *Leishmania braziliensis* [43].

Latest works show that circulating peripheral blood neutrophils from patients with CL were more activated, they expressed more elevated levels of reactive oxidants, and they produced more elevated amounts of the proinflammatory chemokines CXCL8 and CXCL9 than neutrophils from healthy subjects [44].

Many results suggest that the failure to respond to *Leishmania* antigen stimulation observed in VL patients is not due to a defect in the ability to mount protective Th1 responses per se but rather to induction of suppressive factors, e.g. IL-10, resulting in unresponsiveness of infected macrophages to activation signals [45].

2.2.2. Macrophages in leishmaniasis

Macrophages (MΦs) alongside neutrophils are the main host cells for the intracellular *Leishmania* parasite [46]. These cells have a vital role for parasite proliferation and disease consequences as well. However, the immunologic part of MΦ in human *leishmaniasis* is still largely elusive. It is well documented that the secretion of cytokines such as TNF-α and IL-1β, and chemokines by these cells in response to

Leishmania parasite infection reflects their importance in inducing immune responses in line with the function of phagocytosis and presentation of antigens to T cells [47].

Some studies have revealed high expression of CCL2, CXCL9 and CXCL10 in lesions from CL patients. CCL2 and CCL3 are known to enhance the leishmanicidal capacity of human MΦ to the same level as IFN-ɣ [48]. On the other hand, expression of some molecules such as CXCL9, CXCL10 and TNF-α at high level in the tissue of CL and ML patients can lead to tissue damage and chronic inflammatory reactions[49, 50]. However, the mechanisms by which human MΦs are able to digest *Leishmania* parasite are still ambiguous. It was confirmed that once *Leishmania* parasites enter host MΦs and become intercellular; an oxidative burst occurs characterized by an increase in reactive oxygen species (ROS) and reactive nitrogen intermediates such as (NO). The production of NO participates in immune response against *Leishmania* parasites eliminating the pathogens particles in mice, yet the role of NO in humans is still not clear [51, 52]. Interestingly, due to the importance of MΦs in leishmaniasis further investigations are required to get deep insight into the function of MΦs during the invasion of humans *Leishmania* parasites

The present study aims at exploring the fate of *leishmania* parasites when infecting human MΦ and dendritic cells (DCs) thus to bring new light on the interaction of macrophages and *Leishmania* parasite **(Figure 3)** [53]**.**

2.2.3. Dendritic cells in leishmaniasis

The ability of DC's prime and trigger adaptive immune responses to foreign antigens is indisputable, and its role in the induction of tolerance to self-antigens is becoming ever more evident.

Dendritic cells are the master cells for antigen presentation and have the dominant role in T cells priming [54]. They exist in all peripheral tissues in an immature state but are efficient in antigen uptake and processing. They evolve from hematopoietic bone marrow progenitor cells and are classified according to origin, function and site of resident in tissues.

Three DC subpopulations have been identified in the human blood: (pDC) and two subsets of (mDC) expressing CD1c (BDCA1) and CD141 (BDCA3), respectively. pDCs are characterised by their specific expression of CD123, BDCA2, BDCA4 and ILT7 [55, 56]. After interaction with microorganisms or substances associated with infection or inflammation, DCs undergo a process of maturation and migrate to the T

cell areas of lymphoid organs. Subsequently, they present antigens to immature T cells and regulate their responses [57]. The maturation phase is associated with high expression of MHC and costimulatory molecules, such as CD40, CD80, CD86, and CD54 and enhanced cytokine secretion. At the same time antigen capture and phagocytic capacity are down-regulated and different patterns of chemokine receptors and chemokines are produced that permit DC migration and recruitment of multiple cell types [57, 58].

Antigens can be up taken by DCs via different groups of receptor families, such as Fc receptors, C- Type Lectin Receptors (CLRs), pattern recognition receptors (PRRs), and Toll-like receptors (TLRs) [59]. The engagement of the receptors by their cognate ligands enhances the capability of DCs to recognize a wide range of microbial stimuli [60]. The polarization of naïve CD4 T cells toward the Th1 subset and subsequent IFN-γ production depends on the production of IL-12 by the APCs. *Leishmania*-infected DCs secrete IL-12p70 which should push CD4 T cell development in that direction [61, 62].

More recently, the interaction of DCs and *Leishmania*-parasites has been investigated to address the roles of DCs in *Leishmania* parasite infection and leishmaniasis. Previous studies had demonstrated that MΦs and DCs are critical during leishmaniasis as they trigger the adaptive immune reactions [63]. Moreover and important for the development of an immunogenic vaccine against leishmaniasis, recent reports have led to the conclusion that highly potent DCs primed with *Leishmania* antigens can induce effector CTL activity against infected cells and the parasites [64]. As a part of this present study, the handling of *L. donovani* by DCs was studied as shown in the subsequent chapters.

Upon contact, both MΦs and DCs engulf and phagocytose *Leishmania* parasites however with different functional outcomes. Infected DCs produce IL-12 [61], which is critical for the development of IFN-γ-producing CD4Th1 cells [65]. IFN-γ acts on the activation of MΦs (conventional activation) to up regulation of iNOS with production of nitric oxides and free oxygen radicals that are important for intracellular parasite killing and on CD8 T cells to induce their differentiation to effector cytotoxic cell [66]. In contrast, the production of IL- 4 by other cell types (including keratinocytes and CD4 T cells) supports CD4 Th2 cell development. Th2 cells produce IL-4 and IL-13, which leads to upregulation of arginase, alternative MΦ activation and the

production polyamines that promote intracellular parasite proliferation [67]. Furthermore, the infected MΦs also produce a variety of immune-regulatory cytokines including IL-10 and TGF-β, which further deactivate the killing function of infected cells towards intracellular parasite, thereby supporting parasite survival [68, 69].

Recently, DCs were found to express the serine protease granzyme B (GrB), previously only known as the apoptosis-inducing effector protease of natural killer cells and cytotoxic T lymphocytes [70].

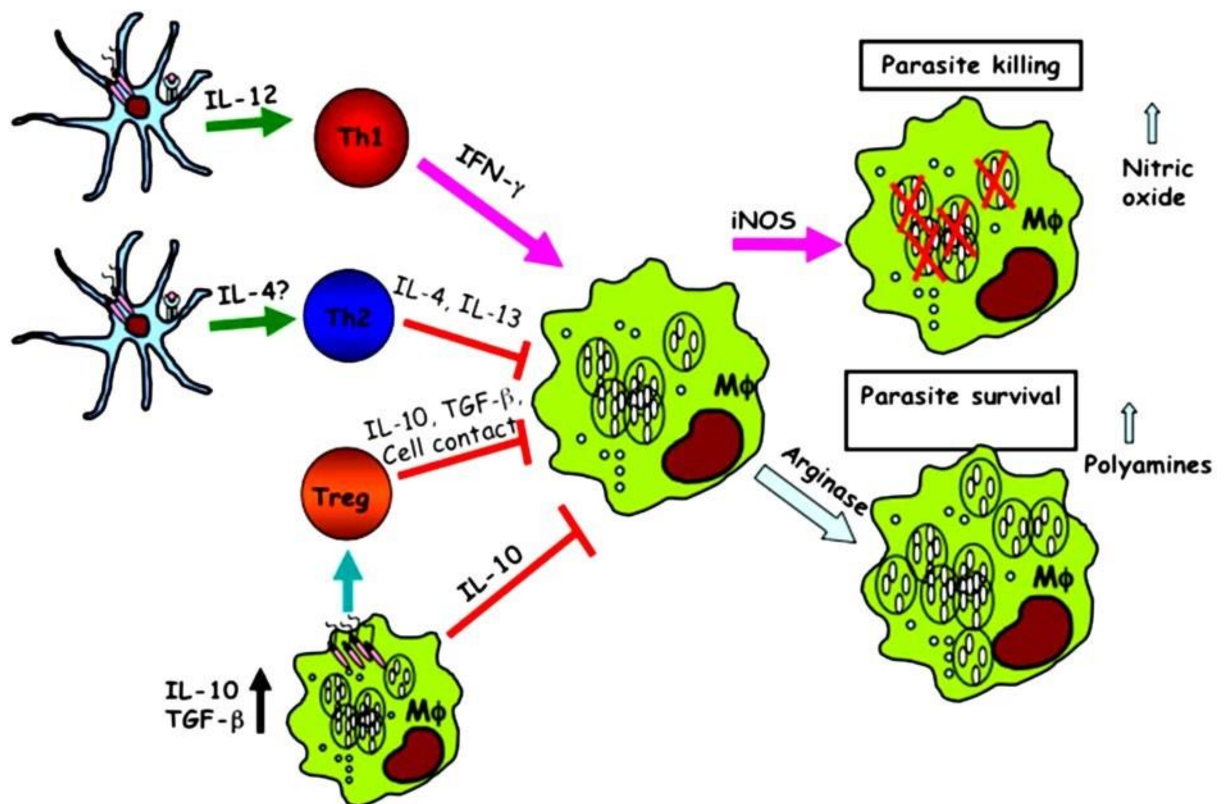


Figure 3: Macrophages and dendritic cells control the outcome of *Leishmania* infection.

The early interaction of *Leishmania* parasite with macrophages and dendritic cells and its influence on the host immune response adapted by [53].

2.3. Antigen processing and presentation

DC and MΦs are utilizing three major endocytotic cellular processes to internalize exogenous antigens as described in (**Figure 4**). These processes are phagocytosis, marcopinocytosis and pinocytosis and [71].

Phagocytosis has defined as the clathrin-independent manner by which cells internalize extensive particulate material such as apoptotic bodies, cellular debris or bacteria, which are typically anticipated to be degraded by lysosomal enzymes [72]. The phagosome is a membrane-bound organelle formed when a phagocytic cell ingested particulate material [73]. There are three stages of the phagocytic process: attachment of the particle to the outer cell surface facilitated by surface receptors; engulfment, characterized by the closure of the plasma membrane around the particle; and creation of the phagosome, which finally evolves into phagolysosome by fusion with lysosomes. Macropinocytosis is a manner whereby large vacuoles, named macropinosomes, generate at the plasma membrane that nonspecifically trap large capacities of the extracellular media. Macropinosomes are typically 200–500 nm in diameter and are thought to form at locations of membrane ruffling [74]. During pinocytosis, soluble antigen is taken up at the same time as extracellular fluid in areas around of budding endosome. Uptake of these vesicles can be both clathrin dependent and independent.

Although both MΦs and DCs are phagocytes, there are differences in the evolution of the phagosomes. In the course of maturation, the phagosomes go through a cascade of gradually acidified membrane-bound states. In MΦs, acidification occurs comparatively early after phagocytosis, after which the cargo is degraded robustly [75]. In DCs, the phagosomal lumen alkalinizes the initial few hours after phagocytosis [76]. As effect, degradation of the endocytosed material occurs slowly, promoting the formation of antigenic peptides. Over time, the pH level declines with increasing lysosomal fusions resulting in cleavage of the MHC-II invariant chain (Ii) by cathepsins, producing a small peptide, named class II-associated Ii peptides (CLIP) that remains in the MHC-II peptide-binding groove. CLIP is then replaced by antigenic peptides, a process mediated by acidic pH and the chaperone HLA-DM. The MHC-II molecules loaded with antigenic peptides are then transported to the cell surface and presented to T cells of the adaptive immune system [77, 78].

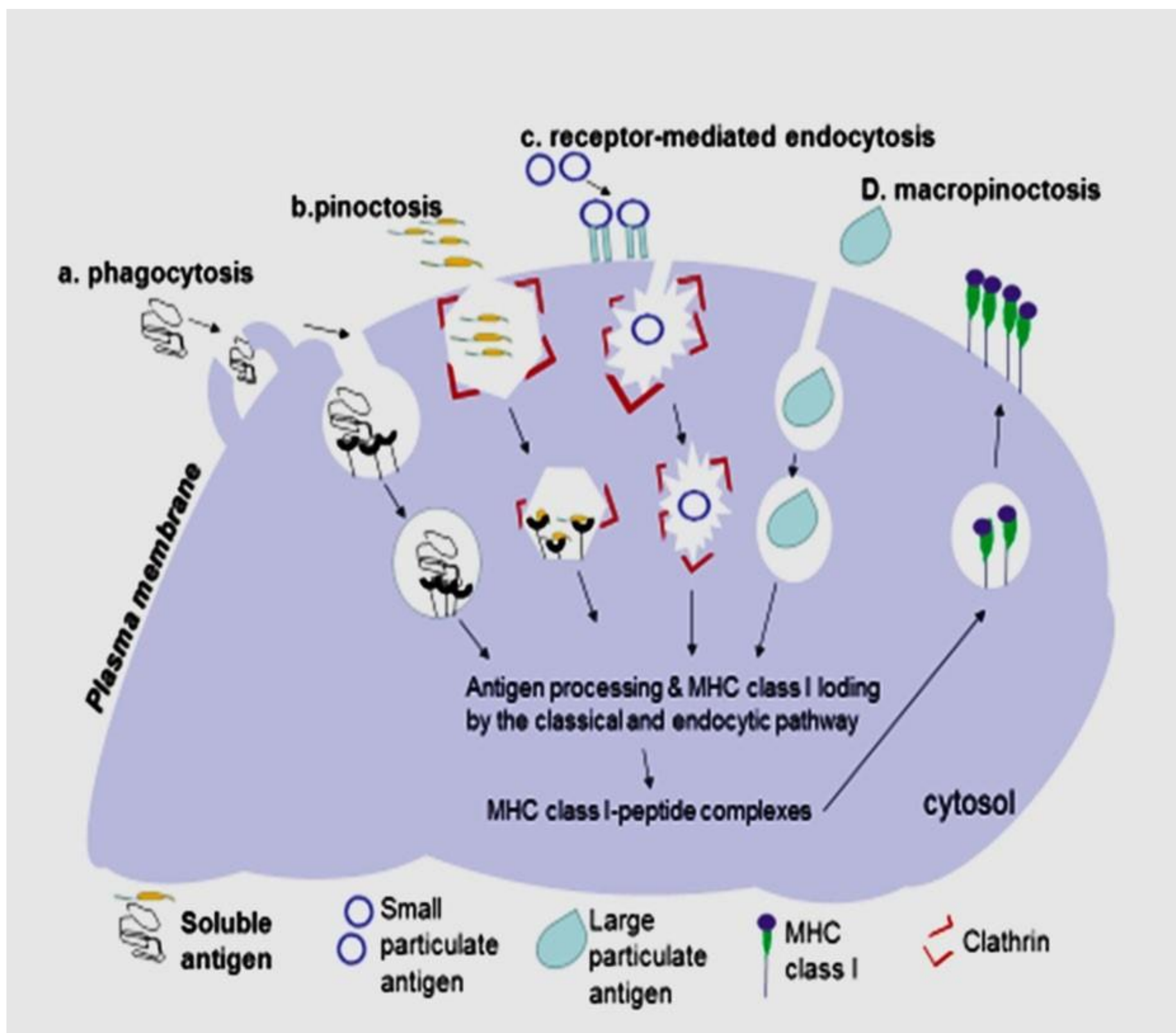


Figure 4: General pathways for the internalization of exogenous antigens by professional antigen presenting cells (pAPC).

DC obtains exogenous antigens through four major paths. The nature of the antigen determines which internalization mode is involved. a) Large particulate antigens such as biologically inert particles, apoptotic cells and opsonized ⁄complement fixed bacteria, are internalized by phagocytosis. b) Pinocytosis defines the uptake of soluble antigens as portion of the extracellular fluid in the surrounding area of budding endosome. c) Small particulate antigens move in the cell by receptor-mediated endocytosis. d) Large fluid volumes are internalized by marcopinocytosis. Antigens from endocytic vesicles are either translocated into the cytosol for processing and presentation by MHC class I molecules and CD8 T cell stimulation or peptides generated inside the endosomes are loaded onto MHC class II molecules for presentation to and stimulation CD4 T cells. Adapted from Mellman I. Endocytosis and molecular sorting [71].

2.4. Human Leukocyte Antigen System

The major histocompatibility complex (MHC) genes code for related proteins identified as the human leukocyte antigens (HLA). In mammalians, the MHC comprises of 200 genes that in humans are situated on chromosome 6. These genes are categorized functionally into MHC class I, MHC class II and MHC class III genes, and encode for cell surface molecules dedicated to present antigenic peptides to T cells for recognition by the T-cell receptor (TCR) [79, 80]. The MHC class I, class II and class III molecules differ in their protein structures and membrane anchoring and cell type that express them. MHC class I and class II genes are highly polymorphic with, to date, more than 15,000 alleles identified for MHC class I and more than 5,000 for MHC class II. This polymorphism predominantly translates to amino acids in the peptide-binding groove and affects the peptide-binding specificity of the MHC molecules.

2.4.1. MHC Class I molecules

The MHC Class I are transmembrane glycoproteins at the cell surface of all but a few immune-privileged nucleated cells. Intact MHC class I molecules consist of the heavy alpha chain non-covalently associated with β-2 microglobulin [81]. The α chain consists of α1, α2, and α3 domains, the transmembrane region and a short intracellular sequence rich in positively charge amino acids. The α1 and α2 domains form the peptide-binding groove that is closed at the ends and can bind peptides of predominantly nine amino acids. There are three loci coding for the heavy chains of MHC class I molecules: HLA-A, HLA-B, and HLA-C. They present antigenic peptides to T cells that express CD8 cell surface molecules that also bind HLA class I molecules to form the TCR signal transduction complex together with the TCR, CD3 and the transmembrane proteins on T cells [82]. Activated CD8 T cells have a cytolytic function and are able to specifically recognize infected or otherwise aberrant cells like tumor cells and kill them [83]. Every nucleated cell expressing MHC class I molecules canact as antigen-presenting cells for CD8 T cells [84].

MHC class III molecules or HLA-III in humans are structurally similar to HLA I but bind different ligands like main chain-acetylated peptides or lipids, and may have different function such as HLA-G, which or HLA-E, which presents specific peptides to their cognate receptors on natural killer [NK] cells [85].

2.4.2. MHC Class II molecules

The MCH class II molecules are expressed only at the surface of specialised antigen-presenting cells such as DCs, B cells, Langerhans cells, MΦs as well as thymic epithelium and in humans on some activated T cells. Some other nucleated cells can be triggered to express MHC class II molecules by specific cytokines such as interferon (IFN)-gamma [86]. Structurally, the MHC class II molecules consist of 2 polypeptides, alpha (α) and beta chains (β), each with two extracellular domains, of which the outer domains of both chains form the peptide-binding superdomain. Both chains are membrane anchored with a single strand transmembrane section and a short cytoplasmic tail [87]. The polypeptide α and β chains are encoded by genes located on chromosome 6 at the HLA-DR, -DQ and -DP loci [88]. In contrast to the MHC I molecule, the peptide binding groove of MHC II molecules are open at the ends so that longer peptides with eleven and above amino acids can bind. MHC class II molecules present antigenic peptides to CD4 T cells.

2.4.3. Association of leishmaniasis and HLA

Like most disease-HLA associations, the association of the HLA genetics with the capacity of patients to cope with *Leishmania* infection is still controversial and needs to be elaborated. Previous studies have reported correlations of specific HLA alleles or haplotypes with susceptibility for or resistance to leishmaniasis. However, due to the complex parasite-host relationship, and influence of various other genetic factors such as cytokine gene polymorphism or of non-genetic parameters such as infection history and general constitutional or nutritional states, the statistics of such correlations is poor [89]. A few studies have looked in monozygotic twins with segregation explorations for a possible impact of HLA genetics on the degree of susceptibility to leishmaniasis and found some indications for an association [89-91]. For instance, HLA-Cw7 was reported to be associated with cutaneous leishmaniasis and was suggested as marker for susceptibility [92]. HLA-A26 was correlated with kala-azar in Iran [93]. From a Brazilian study, HLA-DQw3 was reported to be related with susceptibility, whereas HLA-DR2 is associated with greater resistance [94]. HLA-A11, -B5 and -B7 were suggested to be related to leishmaniasis in Egypt [95, 96]. One study in India using transcriptome array analyses has related abundant expression of HLA-DR with the initiation of effective T cell immune responses against the disease without breaking it down to specific HLA alleles or haplotypes [96].

Other investigations have shown that infection of MΦs with *L. donovani* parasites leads to reduced MHCII expression or, in again another study, to reduced capacity of infected MΦs to stimulate CD4 T cells while not affecting expression levels, which was related to depletion of cholesterol from and higher fluidity of the cell membrane. This defect could be overcome by incubating the cells with liposomal cholesterol. These reports show thatthe parasites have developed a range of mechanism to interfere with effective T cell responses against infected host cells [97]. A recent study in our lab has shown that the infection of MΦs with *L. donovani* has profound effects on the self-peptide repertoire expressed by MHC class I molecules, which in parts can be explained with modifications in antigen processing including the composition of the proteasomes, and altered protein expression and turn-over in different cellular compartments [98].

2.5. **Role of adaptive immunity in response to *L. donovani***

In human, CD4 T cells and CD8 T cells interact in response to and in the depletion of *Leishmania* parasites, CD4 T cells as helper cells for CD8 T cell differentiation to effector CTL and for proliferation of the CD8 T cells. Effector CD8 T cells can kill the infected host cells and the parasites within whereby the killing of the parasites is faster than the induced lysis of the host. That way the parasites are killed before the host cells disintegrate. When stimulated, both T cell types will produce IFNγ acts on infected MΦs to triggers oxidative bursts, which contributes to the elimination of intracellular *Leishmania* parasites. In mouse models, the later mechanism, killing the parasites by triggering infected MΦs, is the main mode of action to eliminate the infection. The T-cellular immune responses can be very vigorous and cause tissue damage at the infection sites. In fact, much of the pathology in leishmaniasis is immune pathology as effects of strong anti-*Leishmania* immune responses. Recently, it was reported that granzyme expression by CD8 T cells is higher in lesions of CL patients than in patients in early phases of CL, and that the frequency with which CD8 T cells express granzyme is directly related to the intensity of the inflammatory reaction observed in CL lesions [99, 100].

Generally, individuals who had recovered from leishmaniasishave a strong immunity against the disease across different *Leishmania* species [101]. They will not get the same disease again and after VL some individuals may present with the much milder PKDL but nether with VL again. This is different from other parasitic infections

such as malaria. This observation strongly advocates the development of anti-leishmanial vaccines that could induce a long lasting immunity similar to that acquired naturally in healed individuals.

A key immunologic feature of VL is the inability of the adaptive immune system to mount timely effective curative antigen specific immune responses [102, 103]. There appears to be no inherent defect in antigen-induced Th1 responsiveness. Cured individuals are resistant to reinfection, are leishmanin skin test positive, and their PBMC readily mount *Leishmania* antigen-specific IFN-γ responses *ex vivo* [104, 105]. Furthermore, even during the acute phase of the disease, elevated levels of IFN-γ mRNA have been found in lesional tissue, such as the spleen and bone marrow [106, 107]. There are ample evidence for high anti-*Leishmania* immune reactions during the VL infection in acute and chronic phases of the disease.

Extensive studies with experimental mouse models of leishmaniasis have shown that the outcome of the infection is critically dependent on the activation of one of the two subsets of CD4T cells, namely Th1 and Th2 cells. IFN-γ, secreted by Th1 cells leads to host resistance to infection with *Leishmania* parasites [108], IL-4 secreted by Th2 cells is associated with the down-modulation of IFN-γ-mediated macrophage activation [109]. However, the Th1/Th2 paradigm could not be translated to human leishmaniasis where both Th1 and Th2 cytokine profiles mix in all stages of the disease.

2.6. Granzymes, perforin and granulysin

2.6.1. The Granzymes and perforin

Granzymes are a family of serin proteases that have first been described present in the cytolytic granules of CTL [110]. They are cytolytic molecules that cleave members of the caspase cascades, in particular effector caspases, and trigger apoptosis of the affected cell. There are five human granzymes with a range of substrate specificities: granzyme A (GrA-tryptase), granzyme B (GrB-aspase), granzyme H (GrH-chymase), granzyme K (GrK-tryptase), and granzyme M (GrM-metase) [111]. Granzyme B and granzyme A are the most abundant and most often expressed granzymes and are predominantly involved in immune-mediated killing of transformed, allogeneic, and/or pathogen-infected cells through a mechanism involving the membrane perforating molecule, perforin, that enables granzyme entry into the target cell leading to induction of cell death [112].

Granzyme B is expressed mostly by NK, NKT cells and activated memory CD8 CTL and some memory CD4 T cells during inflammations, anti-tumor immune responses and in infections. Other noncytotoxic leukocytes such as B cells, dendritic cells, macrophages, and mast cells can express granzyme B but rarely and mostly at lower levels [113-115]. Emerging evidence reported the role of granzyme B in mediating cellular apoptosis as well as acting as an extracellular protease. In memory and effector CD4 T cells, Treg, Th1, and Th17 cells, granzyme B is induced after TCR activation and by common γ-chain cytokines including IL2 and IL15, and by TLRLs [116, 117]. Generally, granzyme B is upregulated in CD8 T cells after CD3/TCR-triggered activation plus the same cytokines. Importantly, memory CD4 T cells kill virally-infected or tumor cells via granzyme B similarly to effector CD8 T cells [118, 119].

One study has compared the expression and bioactivity of granzyme B in CD4 and CD8 T cells, and CD8 T cells express more intracellular granzyme B. A comparison of extracellular granzyme B between CD4 and CD8T cells was not examined [120]. Other groups compared directly human memory CD4 and memory CD8 T cells by flow cytometry and they found that resting and activated memory CD8 T cells store significantly more granzyme B, whereas resting and activated memory CD4 T cells store little to no granzyme B intracellularly [121]. In a mouse model of LCMV infection, direct comparison of antigen-specific CD4 and CD8 CTLs by flow cytometry showed that CD8 T cells express more granzyme B and CD107a, a membrane protein of the inner leaflet of cytolytic granuals. However, *in vivo* CTL killing measurements in mice showed that CD4 T cells can eliminate target cells with comparable efficiency and magnitude as CD8 T cells [122, 123]. Hence, CD4 and CD8 T cells differ in granzyme B synthesis, storage and secretion. Other interesting work demonstrated that activated human pDCs express high levels of granzyme B that surpass the expression level in CTL [70].

They found in patients with juvenile idiopathic arthritis (JIA)not only significantly elevated amounts of granzyme B in fresh synovial fluid specimens but also high granzyme B expression in the corresponding synovia-derived pDCs compared with normal healthy controls. They investigated in healthy subjects how granzyme B production and secretion in pDCs is conducted by cytokines, TLR ligands, and

costimulatory signals. Moreover, they provided novel insights into how pDCs may regulate antiviral, autoimmune, and antitumor immune responses [70].

Perforin (PFN), a pore forming cytolytic protein also present in the lytic granules of very specific lymphoid cells, (NK) cells, (CTL), B-lymphocytes [117] and some other non-lymphoid cells which can secret granzyme B and with or without PFN [124]. Emerging evidence suggests that the human MΦs are not these protease-secreted cells. However, upon degranulation, perforin binds to the target cell's plasma membrane, and oligomerises in a Ca2+ dependent manner to develop pores on the target cell. The pore formed allows for the passive diffusion of a family of granzymes into the target cell [118]. There are different subtypes of DCs, which vary in the expression of these proteases. Peritumoral mDCs stained positive for perforin and granzyme B in patients with carcinoma after in vivo stimulation, whereas infiltrating pDCs expressed (TRAIL) and release granzyme B alone [124]. In addition, the essential role of both human granzyme B and PFN-secreting DCs in killing of *leishmania* parasite infected human cells has not addressed yet. Moreover, whether TCL contribute in *leishmania* parasite deletion or not still need more realization.

2.6.2. The role of perforin and granzyme B in killing parasite infected cells

Cytotoxic T lymphocytes as well as other perforin and granzymes producing cells have the ability to eliminate such target cells by interaction between FasL/Fas and extrinsic pathway interaction is the standard manner of CTL-induced apoptosis[125]. Conversely, these cells can apply their cytotoxic effects on virus-infected cells and tumour cells also by excretion of the transmembrane pore-forming molecule perforin with secretion of cytoplasmic granules through the pore and inside the target cell (Figure 5).

Granzyme B can cleave proteins at aspartate residues followed by the activation of pro-caspase-10 cleaving many factors like (ICAD) to facilitate cell death [126]. Further evidence also supports that granzyme B can induce cell death by either activation of caspase 3 directly or by cleavage of Bid to tBid and that leads to activate the mitochondrial pathway [127, 128]. However, caspase-3 can be activated via granzyme B directly. Using this approach, the upstream signalling pathways are side stepped and execution phase of apoptosis will be activated directly. It is proposed that both direct activation of caspase-3 and intrinsic mitochondrial pathways are fundamental for granzyme B induced cell death [129] (**Figure 5**).

However, there are many indications that granzyme B can also activate other pathways of cell death (particularly in the mitochondrion) [130].

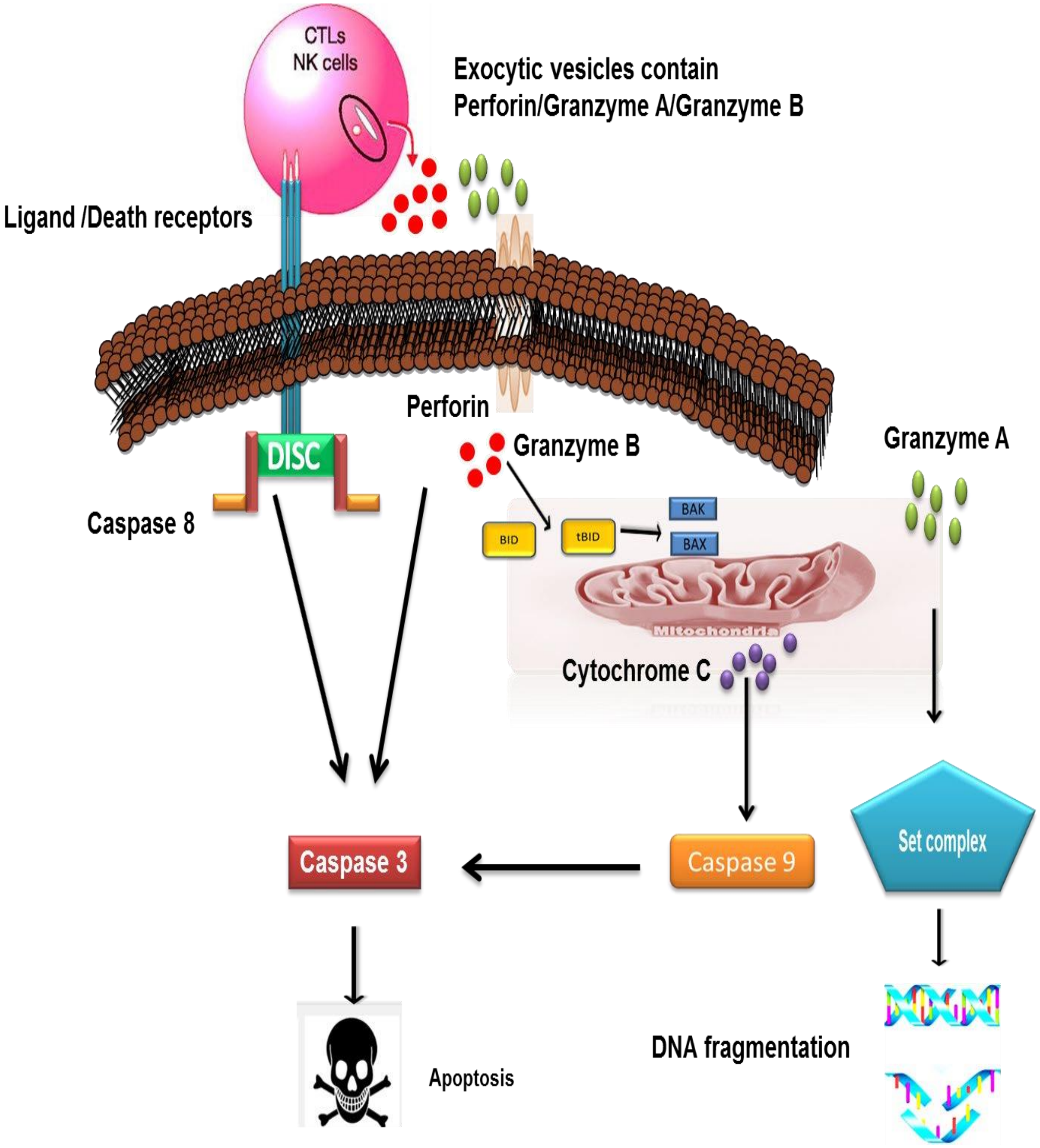


Figure 5: The perforin/granzyme pathway of apoptosis cell death

T-cell mediated cytotoxicity and perforin-granzyme dependent killing of the cell. The perforin/granzyme pathway can prompt apoptosis via either granzyme B or granzyme A. The extrinsic, intrinsic, and granzyme B pathways converge on the same terminal, or execution pathway. This pathway is initiated by the cleavage of caspase-3 and results degradation of cytoskeletal and nuclear proteins and DNA fragmentation that lead eventually to form apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells. The granzyme A pathway activates a parallel, caspase-independent cell death pathway via single stranded DNA damage. Adapted from [131]

2.6.3. The cytolytic granulysin and its role during the infection

Cytolytic granules in CTLs and NK cells of humans and some other mammals but not rodents , contain granulysin, a membrane destabilising protein of the saposin familiy that preferentially attacks cholesterol-low bacterial, fungal and parasite membranes [132]. Granulysin is a cytolytic and proinflammatory molecule expressed by NK cells and by CTLs 3–5 days after activation [133]. It is synthesized as a 15-kDa molecule that is cleaved at both the amino and the carboxyl termini to a 9-kDa form [134]. Perforin and granzymes do not kill intracellular parasites in intact host cells but in presence of granulysin granzymes does so [135].Incubation of extracellular bacteria with granulysin is cytolytic but only at high concentrations in the micromolar range or in highly hypotonic or acidic buffers [136]. It was therefore suggested granulysin acts against bacteria within acidic phagolysosomes or may together with other agents. Granzymes, especially granzyme B, and the granulysin are upregulated when T cells are incubated with bacteria [137]. Granulysin has been implicated in a range of human diseases, and expression of granulysin correlates with good clinical outcomes in cancer and infections [138-140]. Transcriptome analyses have shown that in addition to CTL and NK cells also DCs express granzyme B and granulysin [141]

As to date, it is well established that the cytolytic effector molecules granzyme, perforin and granulysin in cytolytic granules combine to kill intracellular protozoan parasites that cause human disease. Perforin form pores in the membrane of the host cells, which allow granzyme and granulysin to enter the cell wheregranulysin disruptsthe parasite membranes and granzyme enters the intracellular parasites where it cleaves a large series of proteins including members of the respiratory chain to generate ROS and wreck the oxidative defenses of the parasite (**figure 7**) [142].

As reported previously, [143], NK cells lacking either granzyme B or perforin are defective in their ability to kill target cells. NK cells expressing granulysin but lacking perforin are unable to cytolysis target cells. NK cells that express granulysin but lack granzyme B are similarly efficient in killing target cells as wild-type (WT) NK cells. Recombinant 9-kDa granulysin can cause mitochondrial damage and activate the intrinsic apoptosis pathway leading to caspase-3 and/or caspase-9 activation, whereas granulysin delivered by NK cells does not activate these pathways. Also, granulysin delivered by NK cells triggered cellular processes that lead to activation of caspase-7 and endoplasmic reticulum (ER) stress in the target cells, whereas recombinant 9-kDa

granulysin does not. Thus, recombinant and NK cell-delivered granulysin induce target cell death through separate mechanisms [144].

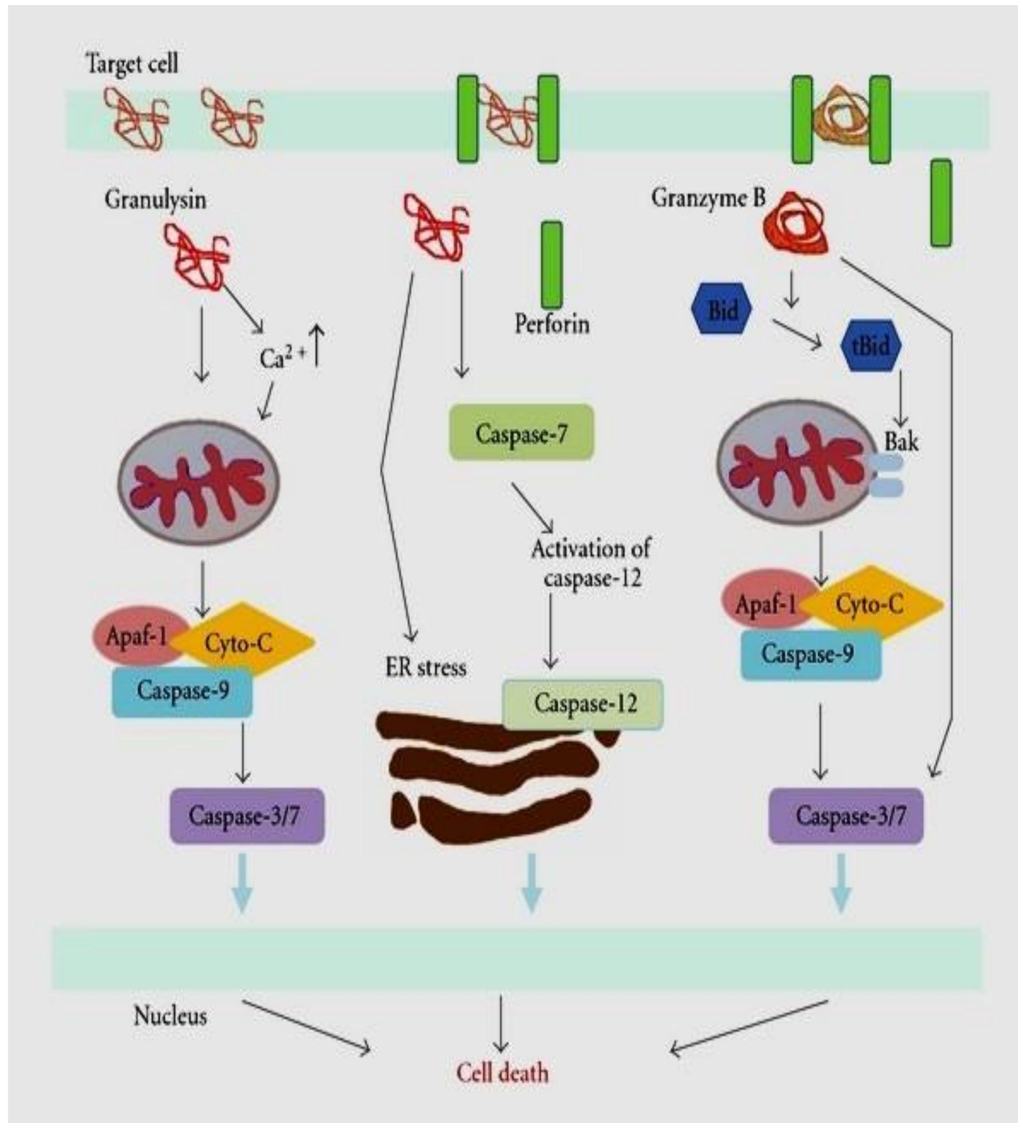


Figure 6: Model of apoptosis induced by granulysin and granzyme B. Recombinant 9-kDa granulysin (left panel), cytotoxic cell-delivered granulysin (middle panel), and cytotoxic cell-delivered granzyme B (right panel) activating different apoptotic pathways. (Ref. R. V. Saini, C. Wilson, M. W. Finn, T. Wang, A. M. Krensky, and C. Clayberger, "Granulysin delivered by cytotoxic cells damages endoplasmic reticulum and activates caspase-7 in target cells. Adapted by [144]

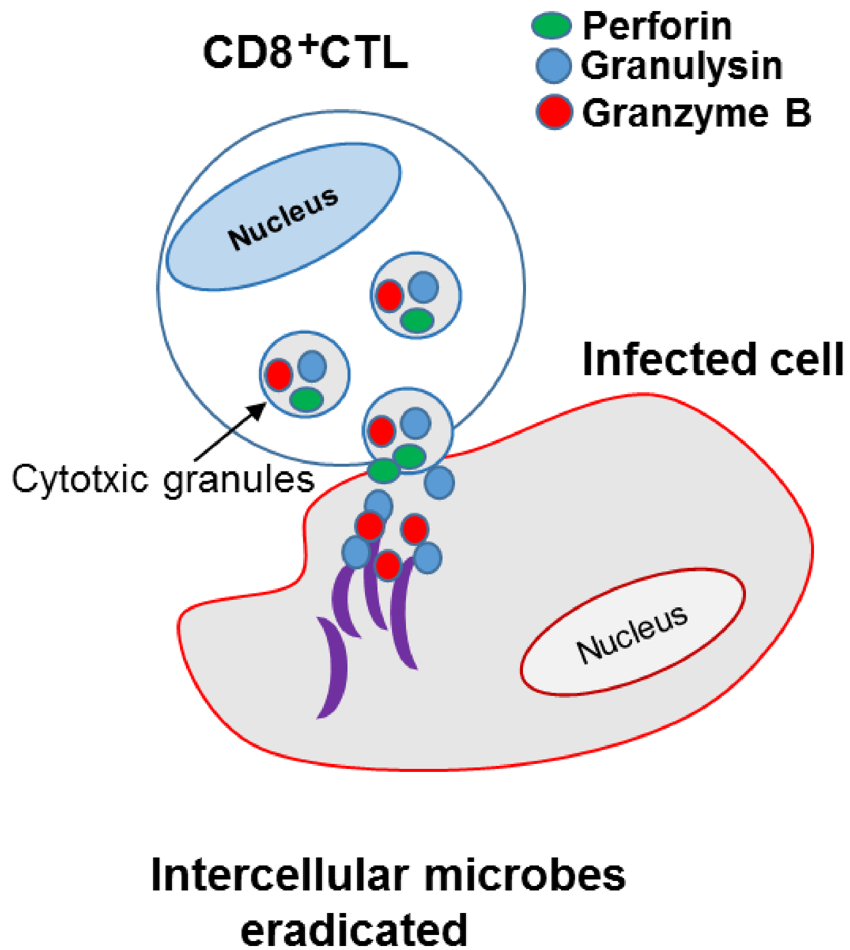


Figure 7: Model of combination of granulysin, perforin, and granzyme B to kill intercellular microbes in most mammalian cells.

2.7. Persistence of Parasites

Mostly, regulatory agencies do not accept leishmanization because it is actually inducing disease and can be accompanied by complications and is problematic in immunocompromised recipients. In mice however, injection of live parasites together with CpG oligodeoxynucleotides as antigen-independent immune stimulator debilitates the severity of the following leishmanization [145]. Vaccination with live pathogens transgenic with a suicide cassettes making them susceptible to treatment certain prodrugs was tested successfully in mice as a safe live challenge. However, it has not been pursued further into clinical trials [146]. Leishmanization has been tested against cutaneous leishmaniasis using life *Leishmania major* or more recently with *Leishmania tarentolae* as vaccine. In analyses with human sera and cells, and in tests in mice, leishmanisation was found to be crossprotective between *Leishmania* species. Epidemiological investigation have shown that individuals from Sudan with a history of CL have lower incidence of VL [147]. Okwor and Uzonna argued that vaccination with life-parasite-based vaccines for CL would induce central memory and effector cells. The challenge will be to achieve attenuation of life parasites without losing efficacy. Observations in animal models indicate that antigen persistence may be as important as the specific protein or parasite component employed in a vaccine [148]. In the work presented herein, human innate immune cells have been incubated with both life and dead *Leishmania* parasite to test their suitability as vaccines.

3. Aims and hypothesis

The aims of this present work was to figure out the different out comes from the *Leishmania* parasite infection on human dendritic cells comparing with the *Leishmania* human host cell (macrophage), to assess the role of granzyme B and granulysin during *leishmania* infection and to give more contributing scientific understanding for the strategies of Leishmaniazation. Vaccination for the *Leishmania* still need more optimistic future because the high potentiality of occurrence.

Therefore, an increased understanding of host pathogen interactions is required for which have the hypothesis:

“Processing of *L. donovani* by human dendritic cells is granzyme B and granulysin dependent, although both human macrophages and dendritic cells are monocyte-differentiated cells; they have different aspects during Leishmaniasis.

4. Materials and methods

4.1. Subjects

This study included 30 healthy donors. They are all from Berlin, Deutschland. All consent forms have been prepared and obtained by the Zentrum für Transfusionsmedizin und Zelltherapie Berlin.

The filtered blood bags collected in appropriate conditions and send to our labs as fresh (within 2 hours) as possible.

4.2. Parasites

The wild-type *Leishmania donovani* MHOM/IN/02/BHU5 (BHU5) was isolated and established as a line from splenic aspirates from a patient with VL in Muzaffarpur, Bihar, India [149]. Fluorescent *L. donovani* were developed after transfection with integration plasmids containing yellow fluorescent protein (YFP). After transfection, the wild-type and transfected parasites were grown and maintained at 25°C in M199 culture medium supplemented with 20% heat-inactivated fetal bovine serum. For the experiments, the parasites were harvested from end-log-phase cultures, centrifuged at 2800 rpm, suspended in RPMI 1640 GlutaMax culture medium, counted in a Neubauer chamber after fixation with 4% paraformaldehyde, and adjusted to the required parasite densities for addition to the DC cultures at the ratio of 10:1 (parasites : DCs ratio). To kill the parasites in some experiments, they were killed by incubation in 4% paraformaldehyde for 15 min followed by extensive washing with PBS before they used.

4.3. Materials

Table 1. Cell culture requirements

Product	Source
Fetal Calf serum (FCS)	Biochrom AG, Germany
RPMI 1640 GlutaMax culture medium	Invitrogen, Carlsbad, CA, USA
Phosphate-Buffered Saline x10 (PBS)	Thermo Fisher Scientific Inc, Germany
Biocoll separating solution	Biochrom GmbH, Berlin, Germany
recombinant human Granulocyte Macrophage-Colony Stimulating Factor (rGM-CSF)	Genzyme, Cambridge, MA, USA
Interleukin-4 (IL-4)	PromoCell GmbH, Germany
monoclonal antibody mAB (OKT3)	Produced in house from culture supernatant of OKT3 hybridomas
Rabbit complement MA	CEDARLANE®.Ontario, Canada
M199 culture medium	Gibco Invitrogen, Germany
Ionomycine	Sigma-Aldrich Chemie GmbH, Germany
Phorbol 12-Myristate 13-Acetate (PMA)	Sigma-Aldrich Chemie GmbH, Germany
Ac-IETD-CHO caspase-8/granzyme B inhibitor	ENZO LIFE SCIENCES GmbH, NY, USA
Z-IETD-AFC caspase-8/granzyme B substrate	Biomol GmbH, Hamburg, Germany
Brefeldin A	Sigma-Aldrich Chemie GmbH, Germany

Table 2. The fluorescent dyes and antibodies

Product	Source
Anti-human CD11c PerCP/Cy5.5	Ebioscince, Germany
Anti-human CD11c Allophycocyanin (APC)	Becton Dickinson,CA, USA
Anti-human CD11b APC	Thermo Fisher Scientific Inc, Germany
Alexa Fluor® 647 Mouse Anti-Human Perforin	Becton Dickinson,CA, USA
Alexa Fluor® 647 Mouse Anti-Human Granzyme B	Becton Dickinson,CA, USA
Alexa Fluor® 647 Mouse Anti-Human Granulysin	Becton Dickinson,CA, USA
FITC Mouse Anti-Human CD3	Becton Dickinson,CA, USA
PE Mouse Anti-Human CD8	Becton Dickinson,CA, USA
DAPI(4',6-Diamidino-2-Phenylindole, Dihydrochloride)	Thermo Fisher Scientific. MA, USA
Calcein-AM	Thermo Fisher Scientific Inc, Germany
LysoTracker Red DND-99	Thermo Fisher Scientific Inc, Germany

Table 3. Instruments

The BD FACSCalibur™	Becton Dickinson, San Jose, CA, USA
The centrifuge	Beckman Coulter GmbH, Krefeld, Germany
Heracell™ 240i CO2- Incubators	Thermo Fisher Scientific Inc, Germany
Single-& two-photon confocal microscopy	Leica Microsystems, Germany
Perkin Elmer LS-50B Luminescence Spectrophotometer	Perkin Elmer Ltd, UK

UV/VIS spectrophotometer Lambada2	Perkin Elmer Ltd, UK
Cytospin 2	Shandon, UK
PTC-200 Peltier Thermal Cycler	BIO-RAD, München, Germany
Vilber Lourmat Super Bright transilluminators	France

Table 4. Software

CellQuest software	Becton Dickinson, Heidelberg, Germany
WinMDi 2.9	Purdue University, USA
FLOWJO v10 software	Becton, Dickinson & Company

Table 5. Reagents and other requirements

Fix/Perm Diluent	Ebioscince, Germany
Fix/Perm concentrate	Ebioscince, Germany
10x Permeabilization Buffer	Ebioscince, Germany
paraformaldehyde powder	Sigma-Aldrich Chemie GmbH, Germany
Sodium Hydroxide 1N	Sigma-Aldrich Chemie GmbH, Germany
Hydrochloric acid	Sigma-Aldrich Chemie GmbH, Germany
superscript III reverse transcriptase kit	Invitrogen, CA, USA

Nucleospin RNA II Purification Kit	Macherey-Nagel, Duren, Germany
Dream Taq DNA polymerase	Thermoscientific, Darmstadt, Germany
Dream Taq green buffer	Thermoscientific, Darmstadt, Germany
ethidium bromide	Roth, Karlsruhe, Germany.
Agarose SERVA 500g	SERVA Electrophoresis GmbH, Heidelberg, Germany
Cell culture flasks 25 cm^2, 75 cm^2	TPP Techno Plastic Products AG, Switzerland
Cell culture plate 12-well	TPP Techno Plastic Products AG, Switzerland
Cell Scrapers	Thermo Fisher Scientific Inc, Germany
Falcon Round-Bottom Polystyrene Tubes, 5 mL	Thermo Fisher Scientific Inc, Germany
Falcon™ 50mL and 15mL Conical Centrifuge Tubes	Thermo Fisher Scientific Inc, Germany
Pipette 2, 5, 10, 25, 50 ml	Thermo Fisher Scientific Inc, Germany
BD FACSFlow™ Sheath Fluid	Thermo Fisher Scientific Inc, Germany
Neubauer chamber	BLAUBRAND, Germany
Coverslips	Thermo Fisher Scientific Inc, Germany

4.4. Methods

4.4.1. Cell culture

In purpose to work under sterile conditions, cell culture work was performed in a class II laminar airflow workbench under endotoxin free conditions. Human cells were cultivated in humidified incubators.

4.4.2. Methods in parasitology

4.4.2.1. Culturing *Leishmania* promastigotes

The wild-type and YFP-transfected *Leishmania donovani* parasite strain BHU5 (MHOM/IN/02/BHU5) [149] promastigotes were grown and maintained at 25°C in M199 culture medium with 20% heat-inactivated fetal bovine serum. For the experiments, the parasites were harvested from end-log-phase cultures, centrifuged at 2800rpm, and then suspended in RPMI 1640 GlutaMax culture medium supplemented with 10% FCS. After undergoes throw extensive wash process 2 times with 1xPBS and last time with RPMI 1640 medium, the parasites counted in a Neubauer Chamber after fixation with 4% paraformaldehyde, and adjusted to the required parasite densities for addition to the human dendritic cells and macrophages cultures at a multiplicity of infection (MOI) of 1:10.

In purpose to kill the parasite to use as positive control for the digesting and none digesting cells, they were killed by incubation in 4% paraformaldehyde for 15 min followed by extensive washing with PBS.

4.4.3. Methods regarding human cells

4.4.3.1. Isolation of monocyte from peripheral blood mononuclear cells (PBMCs) and preparation of human monocyte differentiated dendritic cells (hMDDCs) and human monocyte differentiated macrophages (hMDMs)

PBMCs were isolated from heparinized filtered blood using Biocoll separating solution at 2000rpm for 25min. Which then were collected in RPMI 1640 GlutaMax culture medium supplemented with 2% FCS and incubated for 1-2 hours at 37°C and 8% CO2 (monocyte adherent step), in this time of incubation the monocyte adhere to the bottom of cell culture flask. Consecutively, the monocyte differentiation protocol will be onset. Non-adherent PBMCs were lifted and cultured in 75 cm2 cell culture flasks in RPMI 1640 GlutaMax culture medium supplemented with 10% FCS for other experiments, then the adhered cells washed gently 3 times with warmed 1xPBS to decontaminated the most of lymphocytes. These adhered cells then were cultured in RPMI 1640 GlutaMax culture medium supplemented with 10% FCS for different incubated times and treated with different cytokines depending on the cells of interest. The culture medium was supplemented on day 0 and 4 of culture with 50 µg/mL recombinant human GM-CSF and 50 µg/mL IL-4 to get an immature hMDDCs (ihMDDCs) on the day 5. The flask for hMDMs was supplemented with only 50 µg/mL recombinant human GM-CSF at day 0 and 4 and continue cultured for 7-8 days, the culture medium refreshed 2 times and every time the cytokine treatment was refreshed too. All flasks were maintained during all this time at 37°C in humanized atmosphere with 8% CO2.

On the 5th day of differentiation, the ihMDDCs were washed then re-suspended in fresh medium. For the hMDMs they were washed 3-4 times with PBS in aim to discard most of lymphocytes and then the adherent cells were lifted with cell scraper in fresh medium. Then all human cells were used as describe later.

4.4.3.2. Incubation of hMDDCs and hMDMs with *L. donovani* parasites

In our experiments, we infected human cells separately with dead or live YFP-transfected *L. donovani* parasites or wild type non-transfected parasites, the cells were washed and re-suspended in the culture medium at $1x10^6$ cell/ml. The parasites washed 2 times with 1x PBS and then washed with the RPMI 1640 GlutaMax culture medium supplemented with 10% FCS, then suspended in this culture medium. The

human cells then infected with parasite at MOI 1:10 (cell: parasites). The flasks keep during the infection time points at 37°C in humanized atmosphere with 8% CO_2.

4.4.3.3. In vitro activation of human CD8 T lymphocytes

To address the positive and negative control for the CTL activation. None-adhered autologous T lymphocytes which lifted with the suspension were cultured in cell culture flask with RPMI 1640 GlutaMax culture medium supplemented with 10% FCS at 37°C in 8% CO_2. The activation assay of lymphocytes was carried out by treatment with Ionomysin (500ng/ml) and PMA (5ng/ml) for 30min at RT, and for 30min at 37°C in a cell culture incubator with 8 % CO_2. The cells then incubated for more 5h with Brefeldin A at final concentration of 10µg/ml. The cells then were washed with cell culture medium and prepared for cell surface and intercellular staining. Other autologous lymphocytes have been none-activated as a negative control.

4.4.3.4. Depletion of human T lymphocyte with OKT3 monoclonal antibodies and rabbit complement

To check the expression of granzyme B and granulysin genes in pure populations of ihMDDCs, mhMDDCs, and hMDMs, T lymphocytes depleted through a reaction of the complement with anti CD3 OKT3 monoclonal antibodies. Briefly, the cell suspension has been obtained of both hMDDCs and hMDMs, the suspension centrifuged and the cell pellets re-suspended with 2 ml fresh culture medium in sterile 5ml falcon tube. This suspension has been incubated with OKT3 Anti-CD3 for 1h at 4°C and shaken every 15min, the tubes then centrifuged and refreshed the cells pellets with culture medium and incubated with reconstituted working solution of Rabbit Complement at 1:4 dilution for 1h at 37°C and shaken every 15min. The tubes then centrifuged and the cells pellets were extensively washed with PBS X 3 times, the cells then re-suspended in 1-2ml of PBS and frozen for 24h at – 20°C. The purification of hMDDCs and MDMs population was carried out by flow cytometry.

In the other experiments, the cells pellet re-suspended with fresh culture medium and incubated at 37°C and with 8 % CO_2.

4.4.4. Flow cytometry

To assess several aspects, such as surface and transcription factor expression on human cells, proliferation of PBMCs, phenotypical characterization of cells, lysosomal acidification, etc. flow cytometry analysis was performed. For experiments a FACSCalibur was used and data analysis was carried out with FlowJoTM software.

4.4.5. Preparation of human cells for cell surface and intracellular staining

For measurement of perforin, granzyme B and granulysin produced by either hMDDCs or MDMs before and after they infected, the intracellular staining for these cells has performed and normalized properly. Briefly, cells pellets were harvested and suspended in PBS, the cells were stained with different CD markers for phenotyping and after incubation; they washed 1-3 times with 1xPBS. The cells pellets were fixed for 30min in dark at RT with a Fixation Solution (Fix/Perm Diluent + Fix/Perm Concentrate), the 1x Permeabilization Solution was added. This suspension has been gently shaken and centrifuged. The cells have re-suspended in 1x Permeabilization Solution and were incubated for 45min with antibodies for intracellular staining at RT in dark. Ultimately, the cells were washed with Permeabilization Solution one time then washed with 1xPBS for 2 times and suspended with FACS buffer and the measurement was done with FACScalipur.

4.4.6. Laser Scanning Confocal Microscopy (LSCM)

The uptake and routing of *L. donovani* parasites a in hMDDCs were monitored by LSCM. To investigate the merge of YPF-transfected intercellular phagocytized parasites with intercellular proteins of hMDDCs, and combine of the fluoresced parasites with the intercellular granzyme B, cells were harvested at different time points depending on the experiment. The cells were stained for CD marker with anti CD11c, for hMDDCs and CD11b for MΦs, they then have been washed with 1xPBS and the cells pellets fixed with Fix/Perm buffer for 30 min at RT in dark, 1x permeabilization buffer solution was added and gently shaken, the suspension then centrifuged for 5 min at 1200 rpm and re-suspended in 1x permeabilization buffer solution and then incubated the pellet with Alexa Fluor 647-labeled anti- granzyme B Ab for another 30-45min. the pellets then incubated for 10min with 4% PFA, the suspension then

centrifuged with cytospin at 80rpm for 8min. Then the spots mounted before covered with cover slip. The slide then kept in dark at 4-6C°.

The slides for intracellular localization and routing of intercellular parasites in hMDDCs were analyzed with laser scanning confocal microscopy (Leica TCS SP2). The images were processed with the Leica Confocal Software Version 2.5 Build 1227.

4.4.7. Evaluation of granzyme B activity

To investigate the activity of granzyme B in none-infected hMDDCs. The isolated hMDDCs were incubated with/without granzyme B inhibitor for 20min before infected with *L. donovani* parasites as previous described in this chapter and then at determined times of infection 24h, 48h, and 96h, the suspensions were incubated with granzyme B substrate for one hour then the samples measured with LS-50B Luminescence Spectrophotometer at recommended wavelength. For detection the granzyme B activity in samples on microscopic slides with LSCM, the samples were prepared as previously described above.

4.4.8. Preparation of 4% paraformaldehyde

We added 400 mL of 1X PBS to a glass beaker on a stir plate in a ventilated hood. Heat while stirring to approximately 60°C. 20 g of paraformaldehyde powder was added to the heated PBS solution. In aim to dissolve the powder. The pH was slowly raised by added 1 N NaOH dropwise from a pipette until the solution was cleared. Once the paraformaldehyde was dissolved, the solution was cooled and filtered. We adjusted the volume of the solution to 500 mL with 1X PBS. The pH was adjusted with small amounts of dilute HCl to approximately 6.9. At the end, the solution stored at 2- 8 °C.

4.4.9. Total RNA isolation and cDNA synthesis

Cells obtained from siRNA knockdown experiments were harvested. Total RNA was extracted using Nucleospin RNA II Purification Kit as the manufacturer's instructions. Briefly, $1x10^6$ cells were put in 2ml eppendorf tubes and lysed with 350µl lysis buffer. Samples were centrifuged and the supernatant was mixed with 350µl (70%) ethanol and centrifuged through a nuceospin RNA column, to bind the RNA to the silica gel membrane. Traces of DNA were removed by DNAse treatment. DNAse

and any contaminant were washed away with wash buffer and RNA was eluted in RNase-free water. RNA concentration was measured at room temperature with a UV/VIS spectrophotometer according to manufacturer's instruction with 1µl of the RNA sample diluted 50 times with RNase-free water. cDNAs were synthesized from 500 ng each of the DNase-treated total RNA using superscript III reverse transcriptase kit as the manufacturer's instructions. cDNA-3' Primer (AAG CTG TGG TAA CAA CGC AGA GTC GAC TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT VN) was used in a cDNA synthesis mixture containing (20µl) 5x First strand Buffer, (4 µl) 0.1M DTT, 200U superscript III reverse transcriptase enzyme, 20 pmol cDNA-3' primer, 10mM dNTP mix and 500ng RNA. The cycling condition comprised of denaturation at 65°C for 5 minutes, annealing and cDNA synthesis at 50°C for 60 minutes and termination at 72°C for 15 minutes.

The samples were put on ice for further use or frozen at -80°C for a later use. For measuring RNA concentrations (duplicates), a NanoDrop2000c was used. RNA was verified to be DNA-free by a test-PCR.

4.4.10. GrB and GNLY gene expression in hMDDCs and hMDMs

The expression of GrB and GNLY gene by hMDDCs and hMDMs was determined by semi-quantitative RT-PCR as described previously [98]. Briefly, RT-PCR was carried out with 500ng of hiMDDCs, hmMDDCs, and hMDM of each cDNA using the following constitutive (GrB and GNLY, and β-actin -sequence specific forward and reverse primers). GrB: CTTCTGCTGCCTTCCTCC, GACTTGGCTCCAGAGAAGGT (799 bp; 004131.6, NM_001346011.2); GNLY: AGGGTGTGAAGGCATCTCA, AAGGACTACACAGCTCACCC (725 bp; 006433.5, NM_ 001302758.2); β-actin CTTGATGTCACGGACGATTT, CACGGCATTGTCACCAACT (500bp; NM_002046.2, NM_017008.2). Each RT PCR setup contained (5µl) 10x Dream Taq green buffer, (2µl) 2.5 mM dNTP mix, (0.5 µl) of each 30 pmol/µl β-actin -sequence specific forward and reverse primers, (1µl) of each 100 pmol/µl constitutive or immunoproteasome subunits sequence specific forward and reverse primer, 500ng cDNA,15,875µl PCR grade water and 0.125 µl Dream Taq DNA polymerase. PTC-200 Peltier Thermal Cycler was used and thermo-cycling conditions were denaturation at 96°C for 2 min, 35 cycles of denaturation at 95°C for 40 sec, primer annealing at 55°C to 68°C for 1 min, primer extension at 72 °C for 40

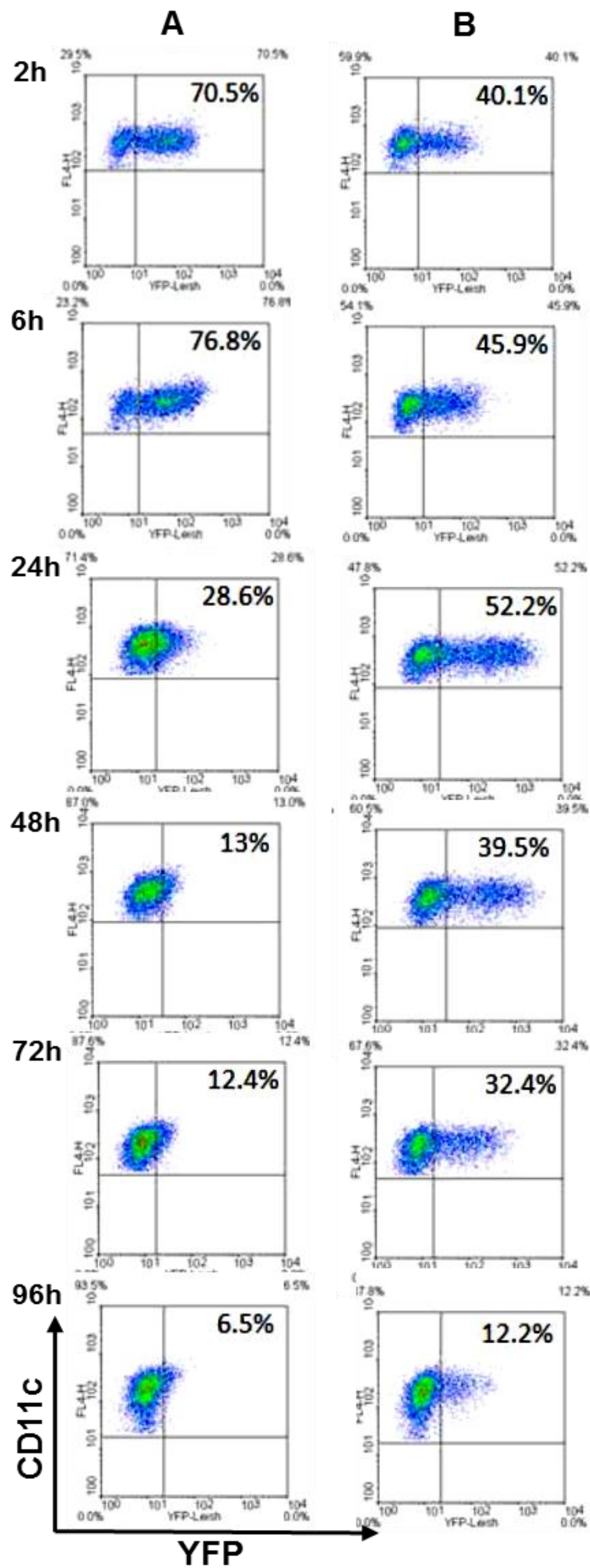
sec and a final cycle of extension at 72°C for 10 min. then the sample tubes keep in 4°C. The amplified DNA fragments were analyzed by electrophoresis using 1% agarose gels in 1x TBE buffer with 0,006 % ethidium bromide. The gel read was done with Vilber Lourmat Super Bright transilluminators.

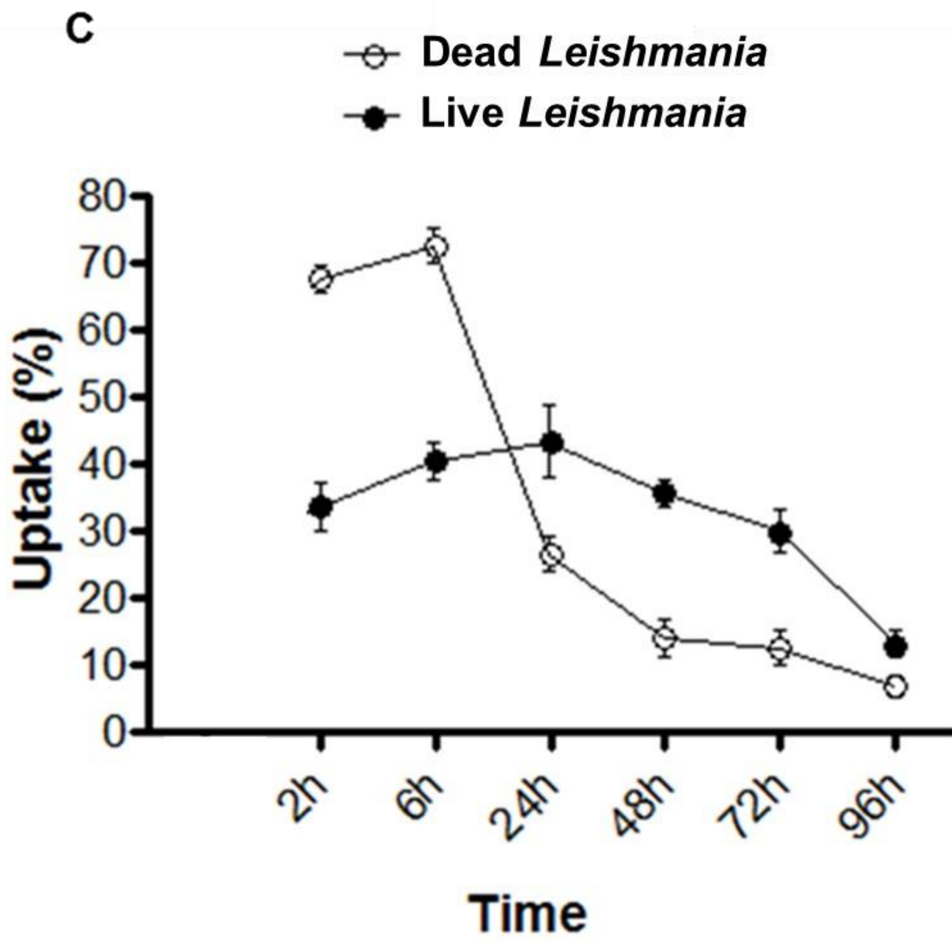
5. Results

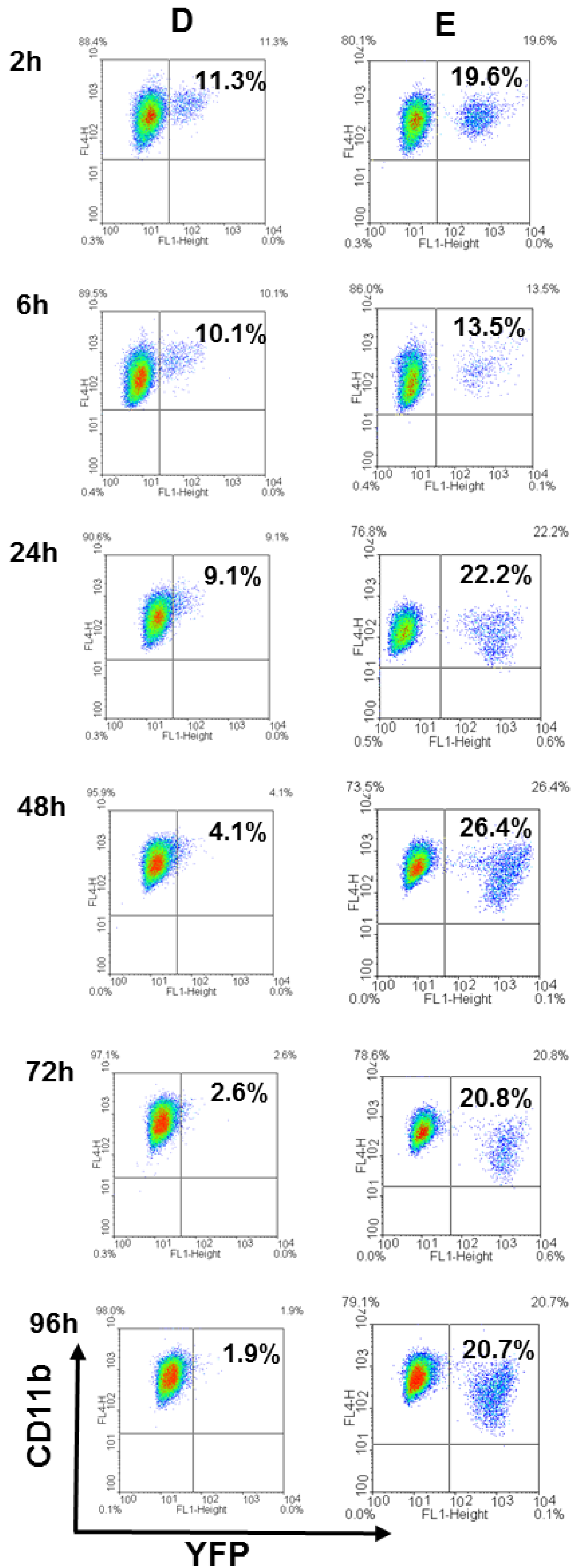
5.1. Uptake of *L. donovani* by hMDDCs and hMDMs

The impact of the first interaction of innate immune cells with the protozoa parasites have a large impact on the infection progression [64] . In the *Leishmania* endemic areas, the infection during the rainy season can be managed, not all exposed people got the infection [150]. In order to figure out the phagocytic capabilities and weather the intercellular phagocytized parasites are proliferated or digested in the phagocyte cells; therefore, the experimental work was accomplished to estimate the proliferation and the killing rate via the expression of the fluorescent signal amplification. To achieve this, the uptake of *L. donovani* promastigotes by both hMDDCs and hMDMs was analyzed by infecting the cells with YFP-transfected parasites. The differentiated ihMDDCs of adhered isolated monocytes were prepared as previously described in the chapter 4. On the day 5 of differentiation, the human cells were either infected with dead YFP-transfected *L. donovani* parasites (**Figure 8A),** or with live YFP-transfected *L. donovani* parasites (**Figure 8B**) at MOI of 1:10. For the hMDMs, the cells were detached with cells scraper at the day 8 of differentiation from the PBMCs as described previously in the chapter 4, they are infected as well with either dead YFP-transfected *L. donovania* parasites (**figure 8D**) or with live YFP-transfected *L. donovania* parasites (**figure 8E**) at MOI of 1:10. The cell suspensions for both cells were collected at different time points 2, 6, 24, 48, 72, and 96 hours (h) as detailed in (**Figure 8**). Subsequently, the cells were washed one-time with 1x10 PBS and stained for 25min with Allophycocyanin (APC) labeled anti-human CD11c for the DCs, and APC labeled anti-human CD11b for the MΦs. The flow cytometry was measured with FACSCalibur. The uptake percentages were measured by overlapping the fluorescent signal expression on FL-1 and FL-4 for both hMDDCs and hMDMs, and this population indicates to either YFP positive hMDDCs or YFP positive hMDMs. The acquired data was analyzed using FLOWJO v10 software. The results showed that, the live parasites were gradually taken up by hMDDCs, and responded on the time points manner, and that was manifestly through the population of hMDDC positive YFP up to 24h of the incubation then the ratios have descended, thus indicating the YFP signal was decreased due to the destruction of parasites. The dead parasites were taken up very fast by both hMDDC and MDM, and the YFP positive population in both

hMDDC and hMDM was drooping gaily with the time, while the ratios of uptake of viable parasites by hMDM started with increasing the YFP positive population ratios, these ratios showed unstable up take capability as they were decreased and increased in response to the determined time points were preformed (**figure 8**)**.**


A
B
2h
70.5%
40.1%
6h
76.8%
45.9%
24h
28.6%
52.2%
48h
13%
39.5%
72h
12.4%
32.4%
96h
6.5%
12.2%
CD11c
YFP


C
Dead Leishmania
Live Leishmania
Uptake (%)
80
70
60
50
40
30
20
10
0
2h
6h
24h
48h
72h
96h
Time


D
E
2h
11.3%
19.6%
6h
10.1%
13.5%
24h
9.1%
22.2%
48h
4.1%
26.4%
72h
2.6%
20.8%
96h
1.9%
20.7%
CD11b
YFP

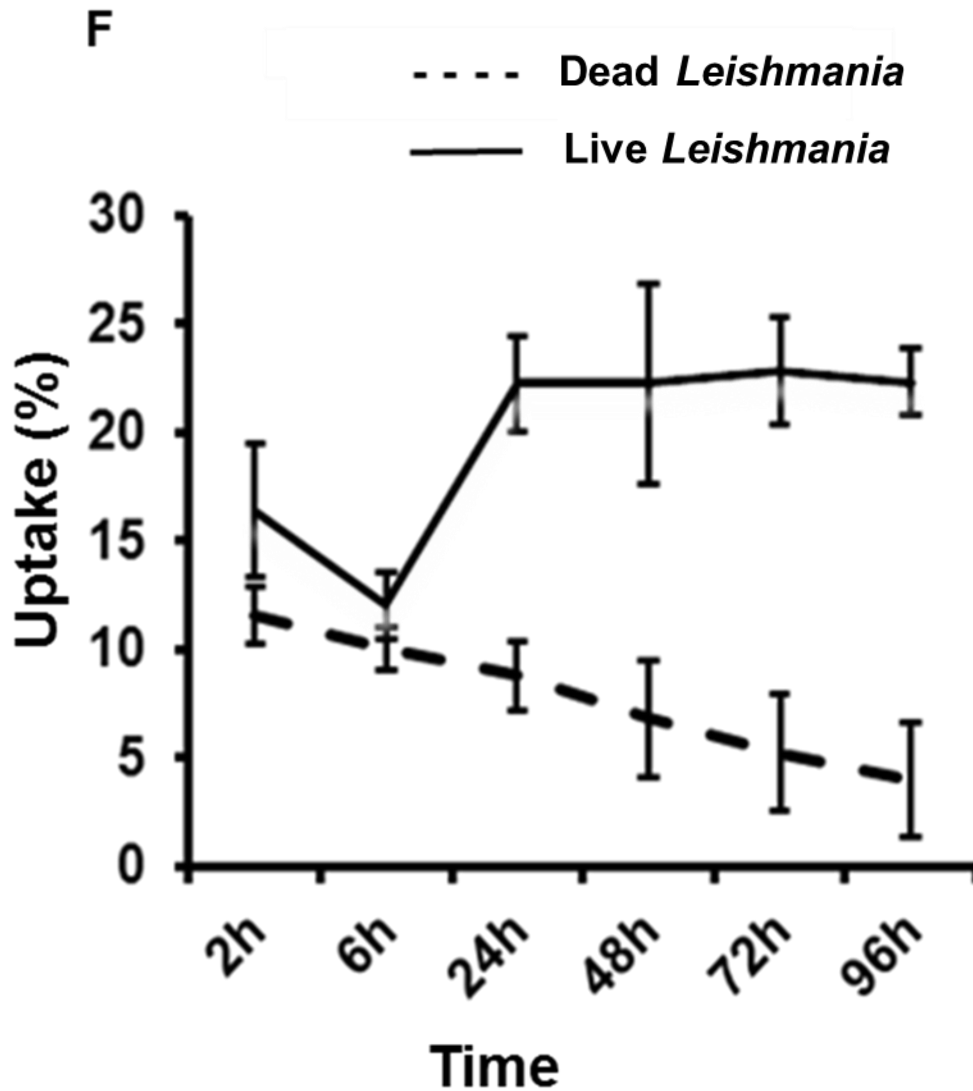
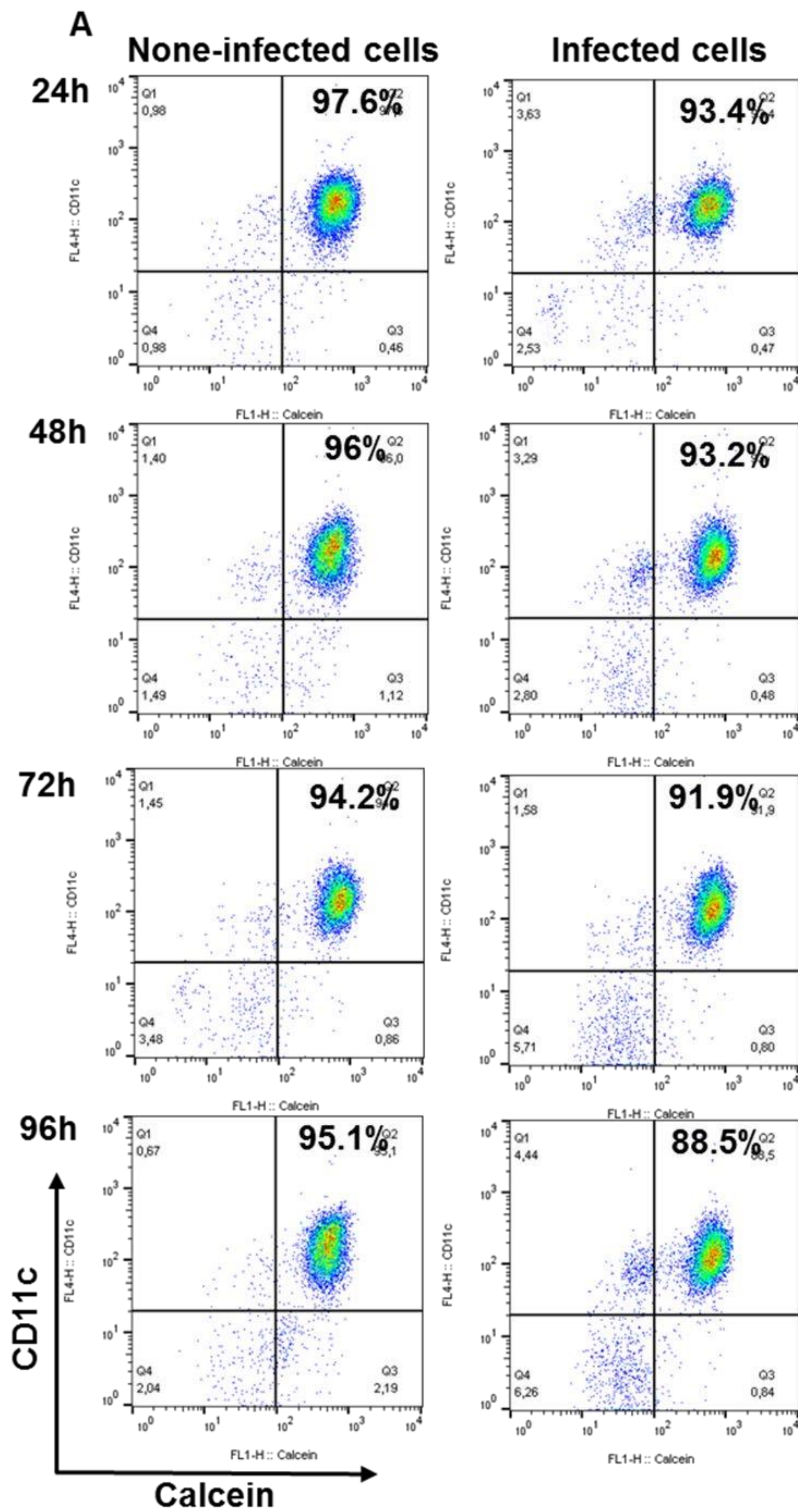


Figure 8: The uptake of dead and live *L. donovani* parasites by hMDDCs and hMDMs

On the 5th Day, ihMDDCs were incubated with either dead or live YFP- *L. donovani* parasites, harvested at the indicated time points, stained with fluorochrome-labeled anti-CD11c. on the day 8, detached hMDMs were infected with either dead and live YFP- *L. donovani* parasites, harvested at the same indicated time points of infected hMDDCs, the infected cells then stained with the fluorochrome-labeled anti-CD11b. both infected hMDDCs and hMDMs then analyzed by flow cytometry. Data displayed in (A, B, D and E) are representative of three independent experiments performed with cells from three different donors. The cells after stained were measured with FACSCalibur. The data were processed with the CellQuest software and analyzed with FLOWJO v10. A) The infection of hMDDCs with dead parasites. B) Infected hMDDCs with the live parasites. C) As depicted, the hMDDCs positive YFP fluorescent percentage of both dead and live parasites were plotted against the time of detection. D) The infection of hMDMs with dead parasites. E) The infection of hMDMs with live parasites. F) hMDMs positive YFP fluorescent percentage of both dead and live parasites were plotted against the time of detection. The determined incubation time points were shown in the lift side of graphs. The number of the independent samples was 3 in all experiments.

5.2. Impact of *L. donovani* infection on viability of hMDDCs and hMDMs

During the *Leishmania* infection, the parasites attempt to evade the killer immune cells, they distinguished by their antigens, which enhance the taken up them by phagocytes [151] . To complete their live cycle, the parasites start to differentiate and proliferate inside their host cells, which cause at the end rapture the membrane of host cells, thus losing cell membrane integrity. In this current study, we aimed to evaluate the viability of the hMDDCs and hMDMs upon infection with wild type *L. donovani* parasites at different time points 24, 48, 72, and 96h, as indicated in (**Figure 9 A, B**). The cells were harvested at determined time points in flow cytometry tubes, washed tow times with x1 PBS and stained for max. 25min with none-fluorescent cell-permeable Calcein-AM, which is metabolized in the cytoplasm of live cells into the green fluorescent calcein, and APC labeled anti-CD11c for hMDDCs, and anti-CD11b for hMDMs. After staining, the cells were washed one time with x1 PBS, and then the samples were reconstituted in FACSFlow Sheath Fluid. The sample were measured with FACSCalibur and the data were acquired using the CellQuest software and analyzed with the FLOWJO v10. The graphs showed that no significant effect of infection was detected on the viability of hMDDCs and MDMs compared with non-infected cells (**Figure 9**).


A
None-infected cells
Infected cells
24h
48h
72h
96h
97.6%
93.4%
96%
93.2%
94.2%
91.9%
95.1%
88.5%
CD11c
Calcein
FL1-H :: Calcein
FL4-H :: CD11c
Q1
0,98
Q4
0,98
Q3
0,46
Q1
3,63
Q4
2,53
Q3
0,47
Q1
1,40
Q4
1,49
Q3
1,12
Q1
3,29
Q4
2,80
Q3
0,48
Q1
1,45
Q4
3,48
Q3
0,86
Q1
1,58
Q4
5,71
Q3
0,80
Q1
0,67
Q4
2,04
Q3
2,19
Q1
4,44
Q4
6,26
Q3
0,84

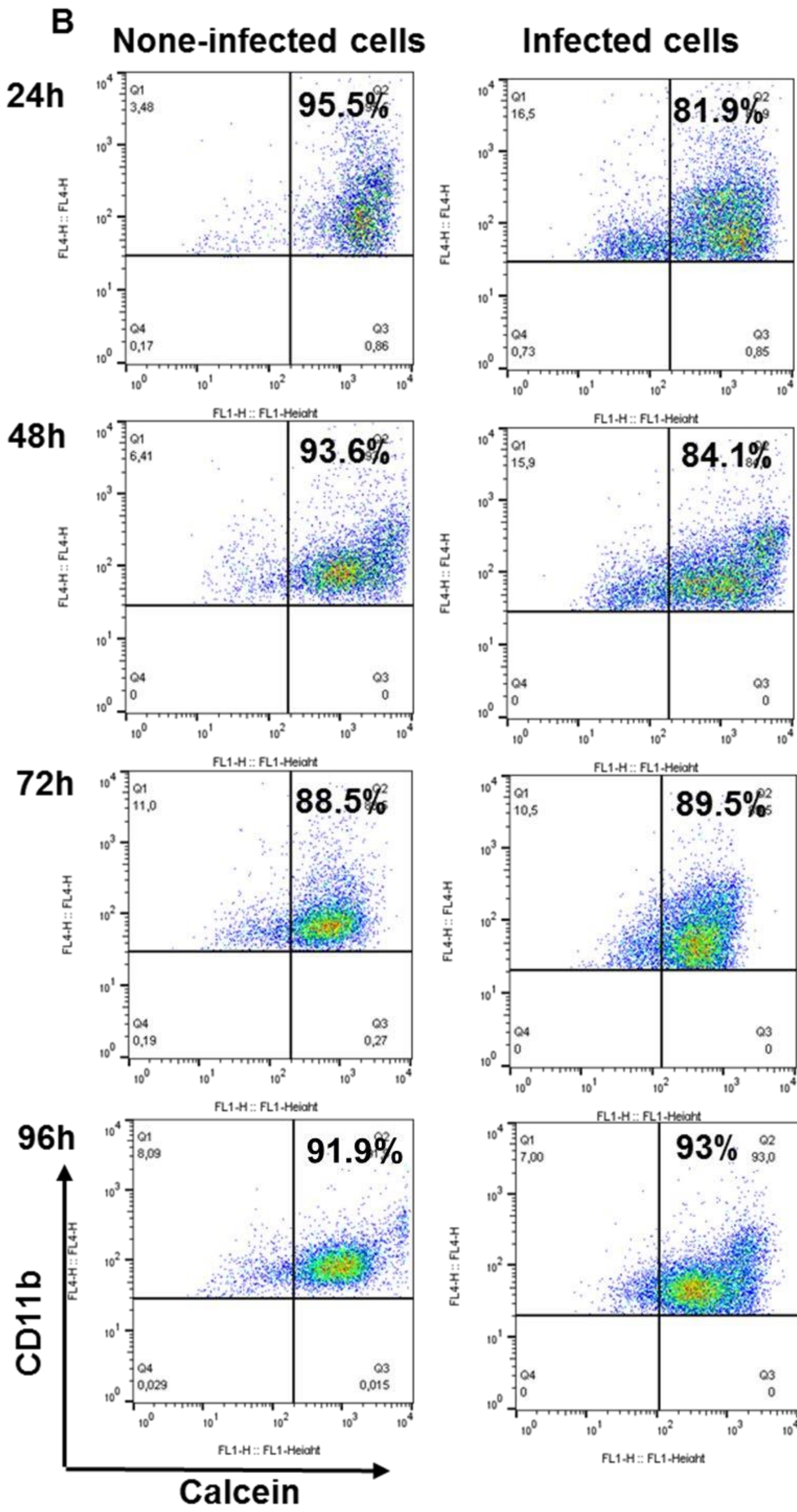

B
None-infected cells
Infected cells
24h
48h
72h
96h
95.5%
81.9%
93.6%
84.1%
88.5%
89.5%
91.9%
93%
Q1
Q2
Q3
Q4
3,48
0,17
0,86
16,5
0,73
0,85
6,41
15,9
11,0
0,19
0,27
10,5
8,09
0,029
0,015
7,00
93,0
FL4-H :: FL4-H
FL1-H :: FL1-Height
CD11b
Calcein

Figure 9: The effect of *L. donovani* infection on viability of hMDDCs and hMDMs

ihMDDCs and hMDMs were infected with wild-type *L. donovani* for different time points as indicated. Consequently, the cells were harvested, washed and stained with both Calcein-AM and fluorochrome-labeled monoclonal antibodies against-CD11c and anti-CD11b for both hMDDCs and hMDMs, respectively. After they were washed, the samples were measured using flow cytometry. A) Represent non-infected hMDDCs left and infected hMDDCs represented on right. B) Shown non-infected hMDMs, lift and represent infected hMDMs, right. These results illustrative one of 3 independent experiments.

5.3. Intracellular routing of the intercellular parasites in hMDDCs

It has been widely reported that utmost of investigations on the *Leishmania* infection were involved animal model [161], *in vivo* studies the phagocytic capacity and the faith of the taken up parasites by different human immune cells needs to be more elaborated. This experiment aimed to detect the subcellular localisation of intracellular parasite with the antigen processing compartments, the hMDDCs incubated for different time points 2h and 24h with live/dead YFP-transfected *L. donovani* parasites at MOI 1:10, and the infection process was achieved in culture plate 12-well and incubated as mentioned above. After the incubation time, the cells were harvested, washed and then stained intracellularly with fluorochrome-labeled antibodies against different intercellular proteins. Including HLA class I and class II, the antigen presenting molecules for CD8 and CD4 T lymphocytes, respectively. The cells stained also with the invariant chain CD74, which is associated with newly biosynthesized, but not degraded. HLA class II to stabilize the molecule and protect the peptide-binding groove, and calnexin, a membrane protein in the ER that acts as chaperon for nascent HLA class I. Already 2h after addition to the cultures the dead parasites were found in these compartments; in the case of the live parasites, the translocation into these compartments appears to be slower. After 24h of incubation, the live parasite intercellular infected cells appear aggregated with the different intercellular compartments of interest (**Figure 10**).

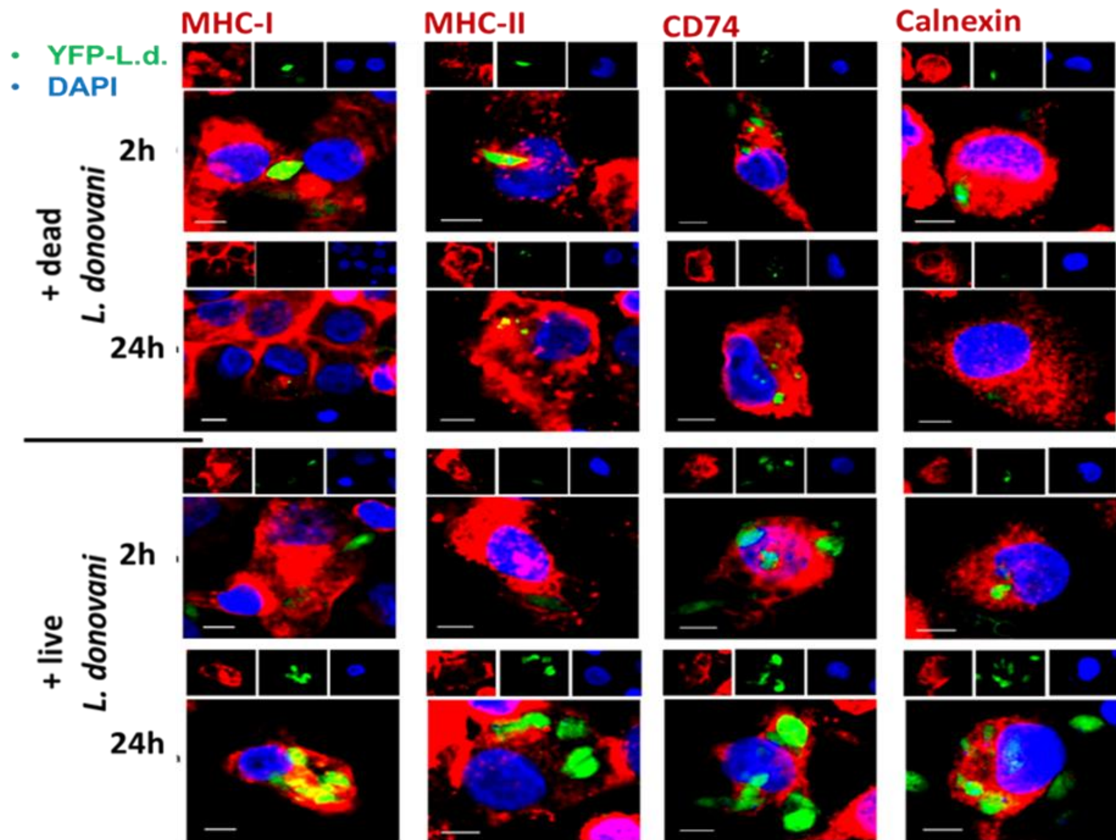


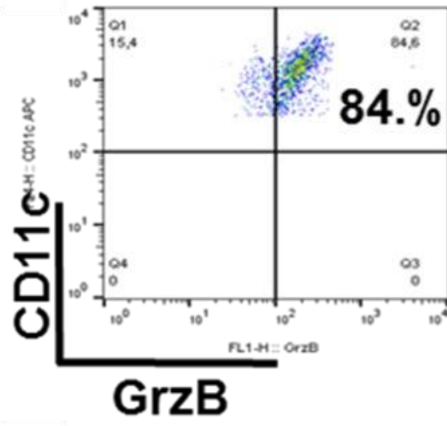
Figure 10: The intracellular localization of the phagocytized parasites in hMDDCs

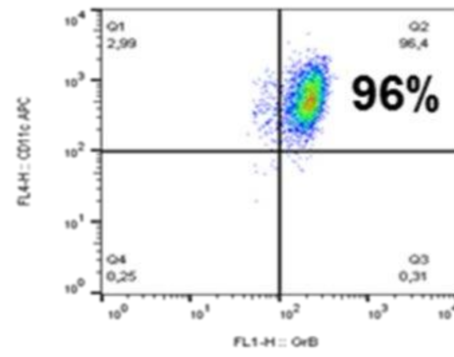
On the 5th Day ihMDDCs were incubated with dead or live YFP- *L. donovani* parasites (green fluorescence), harvested at the indicated time points, fixed, permeabilized and stained for MHC class I, MHC class II, CD74 and calnexin (red fluorescence), and counterstained with DAPI (blue fluorescence). After staining the cells were imaged by confocal microscopy. Images were deconvolved using ImageJ software Scale bar= 5µm.

5.4. Determination of granzyme B in *L. donovani* infected and none-infected hMDDCs and hMDMs

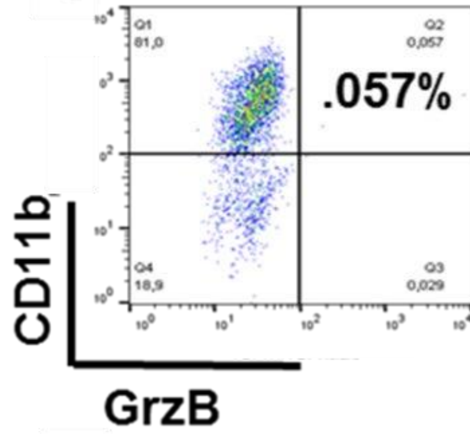
To evaluate the expression of granzyme B in hMDMs and hMDDCs in response to infection with *L. donovani*. The ihMDDCs at day 5 of differentiation were incubated with wild type *L. donovani* in 12 well cell culture plate at MOI 1:10 for 48 h at 37 °C and 8% CO_2. The cells then washed with PBS and stained for cell surface with APC labeled anti-CD11c, fixed, permeabilized and incubated with Fluorescein (FITC) labeled anti-human granzyme B. To determine the expression of these proteins in hMDMs, the cells were incubated for 48h at a MOI 1:10 as described previously, the cells then washed, stained with FITC labeled anti-CD11b, fixed, permeabilized and incubated with Alexa flour 647 labeled anti-human granzyme B for 30-40min in dark. The samples were splitted, and then measured with FACSCalibur. Other samples were incubated for 10min with 4% PFA in dark and then were centrifuged on microscope slide, fixed, mounted, and covered with coverslip. The images of these samples on microscope slides got by confocal microscopy. Depending on the mean MFI, the expression of granzyme B was high in none infected hiMDDCs, this expression up-regulated after 48hr of infection with the parasite, in contrast, the expression of granzyme B was very low in none infected hMDMs and no significant change after infection (**Figure 11**).

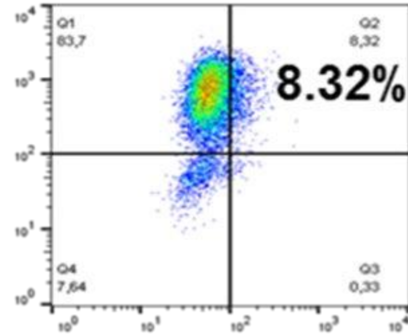
A



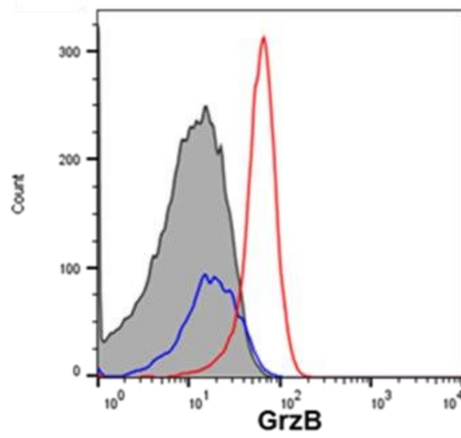


B

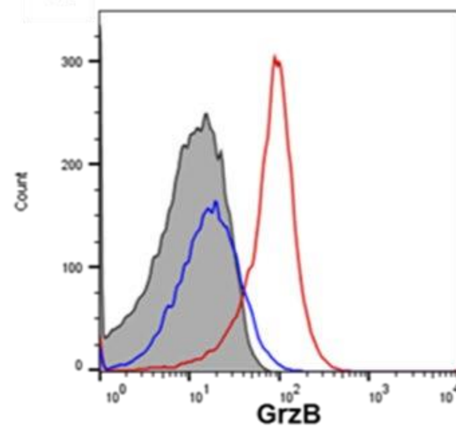


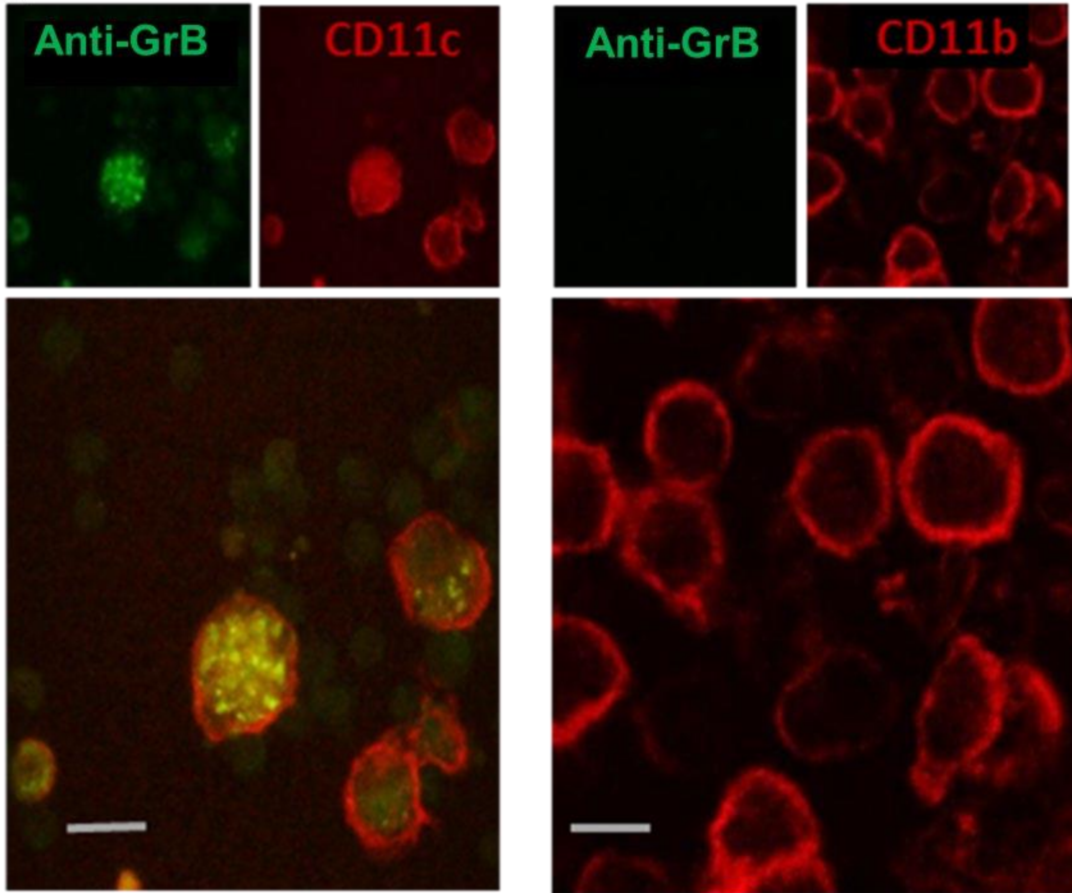


C



D




E
Anti-GrB
CD11c
Anti-GrB
CD11b

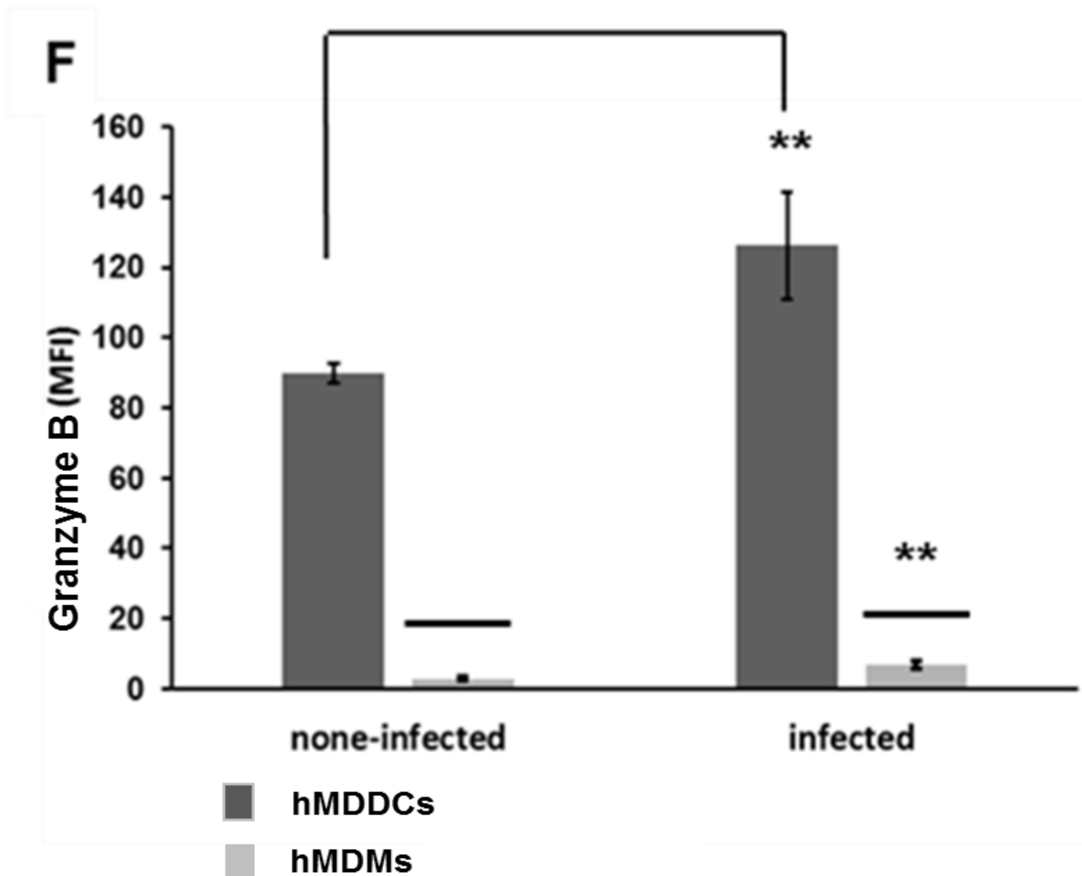


Figure 11: Granzyme B expression in *L. donovani* infected and none-infected hMDDCs and hMDMs.

The hMDDCs and the MDMs incubated with/without wild type *L. donovani* at MOI 1:10 for 48h. The cells then washed and stained with APC anti CD11c for the hMDDCs (red fluorescent) and for the hMDMs stained with APC anti CD11b (red fluorescent), after that the cells incubated with fixation buffer for 30min before they permeabilized with permabilization buffer and combined with FITC anti human granzyme B (green fluorescent) for 30-40min for both hMDDCs and hMDMs. A) The granzyme B expression in none- infected hMDDcs left, and infected hMDDCs right. B) The granzyme B expression in none infected hMDMs, left, and infected hMDMs, right. C) Show the mean MFI of granzyme B expression in none infected both DCs, red line and hMDMs, blue line. D) Show the mean MFI of granzyme B expression in infected both hMDDCs, red line and hMDM, blue line. E) Show the localization of granzyme B inside the infected hMDDCs, left and infected hMDM, right. F) Show the data analysis, these results got from 3 different samples, ** = p<0.01; comparing the mean of MFI of granzyme B expression obtained from none infected and infected cells.

Obtained and processed images was done with the Leica Confocal Software Version 2.5 Build 1227. Scale bar= 5µm.

5.5. Detection of granulysin in *L. donovani* infected and none-infected hMDDCs and hMDMs

To evaluate the granulysin expression in *L. donovani* infected and none-infected both hMDMs and hMDDCs. The non-infected and infected cells were processed as previously prescribed but stained to detect CD markers precisely; PerCP/Cy5.5 anti-human CD11c and CD11b for hMDDC and MDM, respectively. Then the cells were stained intracellularly with Alexa flour 647 labeled anti-human granulysin. The expression was low in aforementioned cells as exhibited in (**Figure12**). hMDDCs showed no effect of infection on the granulysin expression, but the granulysin expression was significantly increased in hMDMs after became infected.

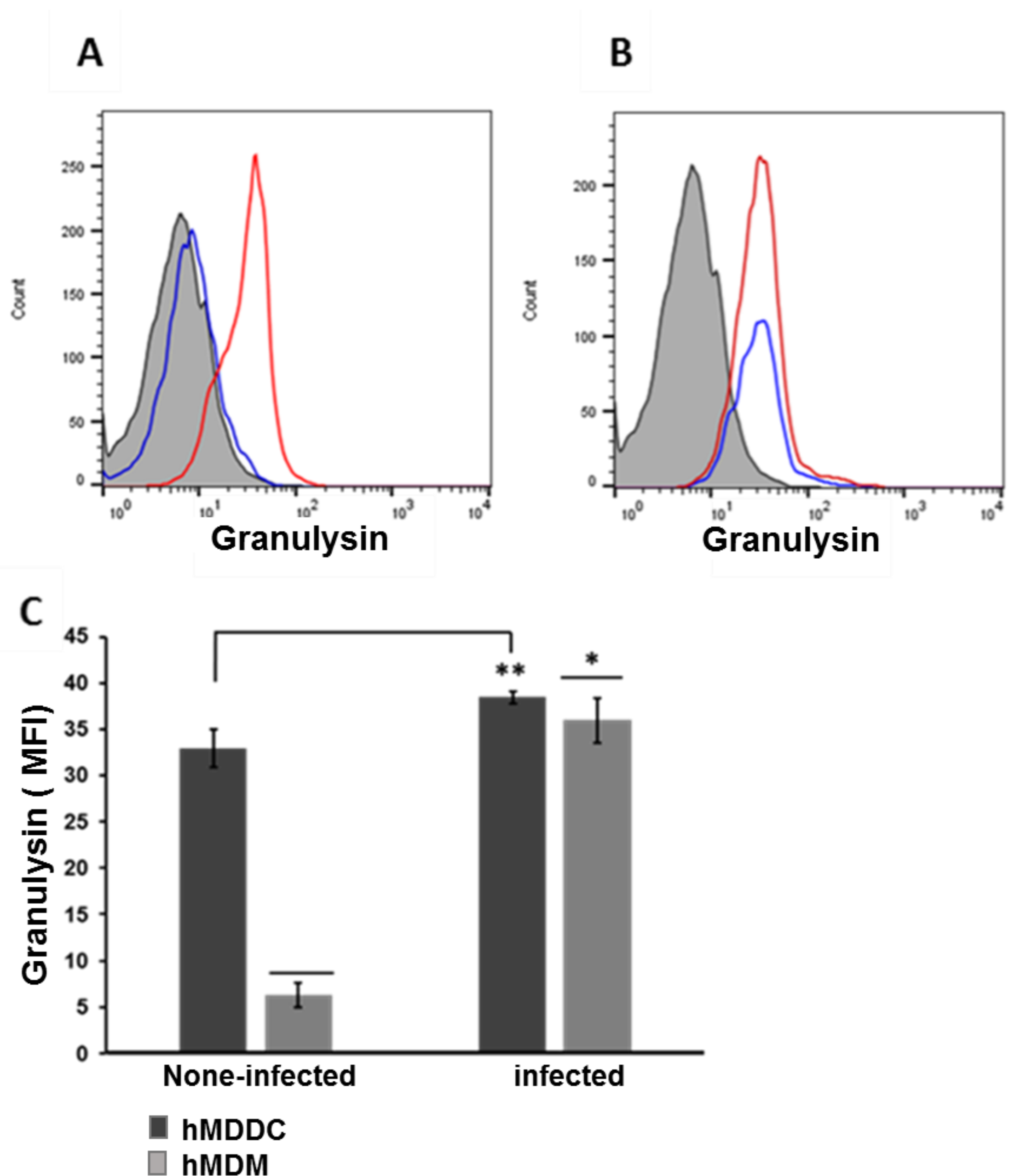


Figure 12: The expression of granulysin in *L. donovani* infected and none-infected hMDDCs and hMDM.

The ihMDDCs and the hMDM incubated with/without wild type *L. donovani* at 1:10 cell to parasites ratio for 48h, the cells then washed and stained with PerCp/Cy5.5 anti CD11c and CD11b for hMDDCs and MDMs, respectively. After that, the cells incubated with fixation buffer for 30min before they permeabilized with permabilization buffer and combined with Alexa flour anti-human granulysin for 30-40min for both hMDDCs and MDM, the cells then measured with FACSCalibor. A) Show the MFI of granulysin expression in both none infected hMDDCs, red line and hMDM, blue line. B) Show the MFI of granulysin expression in both infected hMDDCs, red line and hMDM, blue line. C) Represent the analysis of data of 3 different samples, * = $p<0.05$; ** = $p<0.01$; comparing the mean of MFI of granulysin expression obtained from none infected and infected cells.

5.6. Detection of GrB and GNLY mRNA in ihMDDCs, mhMDDCs, and hMDMs

Following assessment of the granzym B and granulysin expression, the expression of granzyme B and granulysin has been shown by fluorescent intercellular staining only in hMDDCs, while granulysin only was detected also in hMDMs. The detection of the proteins expression level in these cells was aimed to provide a better sympathetic into the role of granzyme B and or granulysin-secreting hMDDCs and hMDMs, and the importance of their action on the immune response during leishmaniasis. Thus, to confirm our flow cytometry data, the gene expression of GrB and GNLY in ihMDDCs, mhMDDCs, and hMDMs, was detected by semi-quantitative RT-PCR, and was carried out as detailed in section 4.4.9**.** For this present experiment, ihMDDCs, mhMDDCs and hMDMs were obtained after the T Lymphocytes were depleted, then the cells were washed extensively with PBS and the purification of cell populations were determined with flow cytometry. Immature and mature MDDCs expressed both GrB and GNLY genes (**Figure 13 A, B**). The Findings showed that both gene bands expression was thicker after maturation, whereas, hMDMs were expressed GNLY gene only (**Figure 13 C**). As a positive control for genes expression of GrB and GNLY, the expression was detected in cDNA obtained from PHA activated PBMCs (**Figure 13 D**).

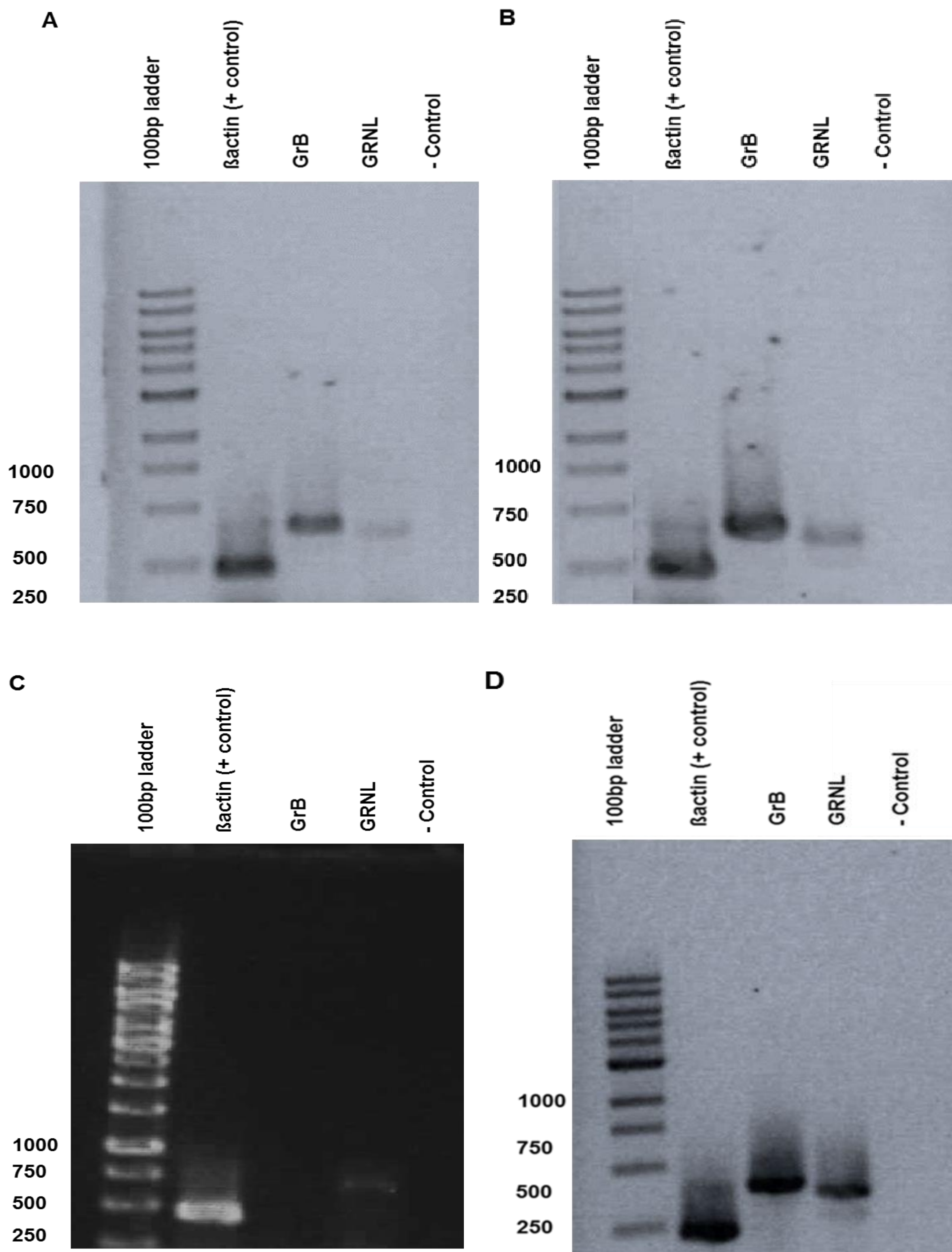
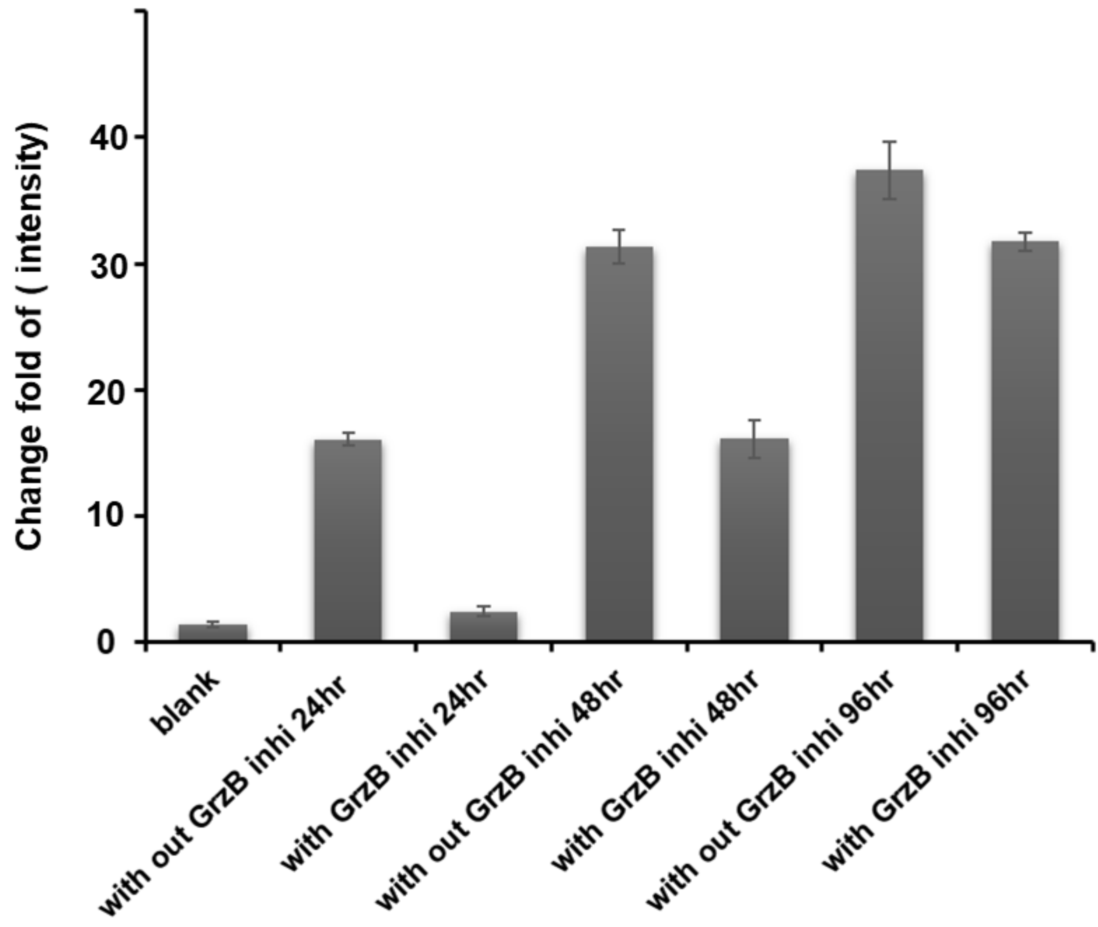


Figure 13: GrB and GNLY mRNA expression in ihMDDCs, mhMDDCs, and hMDMs.

A) Agarose gel electrophoresis of RT-PCR of the GrB (799bp)and GNLY (725bp) expressed by ihMDDCs B) Agarose gel electrophoresis of RT-PCR of the GrB (399bp)and GNLY (558bp) expressed by mhMDDCs C) Agarose gel electrophoresis of RT-PCR of the GrB (399bp)and GNLY (558bp) expressed by hMDMs. D) Gene expression of GrB and GNLY in PHA activated PBMCs sample.

5.7. Evaluation of granzyme B activity in *L. donovani* infected hMDDCs

Granzyme B is an apoptosis-inducing protease their expression by hMDDCs was determined and confirmed in this present study. The activity of granzyme B inside infected phagocytic cells still needs to be evaluated. In this experiment, we aimed to evaluate the granzyme B activity and test the effectiveness of granzyme B inhibitor for the coming experiment, hiMDDcs were treated with/without granzyme B inhibitor 20min before they were incubated with wild type *L. donovani* and at different time points as indicated. The supernatants were collected and incubated with the fluorogenic granzyme B substrate for one hour, and then the samples were measured with LS-50B Luminescence Spectrophotometer. The results showed that the granzyme B was in active form after 24h of infection and its activity was increased 16-fold, the effect of granzyme B inhibitor was evident, granzyme B activity was decreased to the same non-stained sample. After 96h, the activity of granzyme B inhibitor was significantly decreased (**Figure 14 A**). In addition, to show the activity of granzyme B inside the *Leishmnaia* infected hMDDCs and beside that co-localized with the intercellular parasites. Therefore, the ihMDDCs were incubated with YFP-transfected *L. donovani* combined with/without the fluorogenic granzyme B substrate, then, at 24h, the infected cells were washed, and stained with APC anti-human CD11c. after 25min, the cells fixed and mounted on a slide to examine by confocal microscopy. The pictures show the activity of granzyme B inside and outside the infected cells in comparison with a negative control, and the active granzyme B combined with intercellular parasites (**Figure 14 B, C**).


A
Change fold of (intensity)
40
30
20
10
0
blank
with out GrzB inhi 24hr
with GrzB inhi 24hr
with out GrzB inhi 48hr
with GrzB inhi 48hr
with out GrzB inhi 96hr
with GrzB inhi 96hr

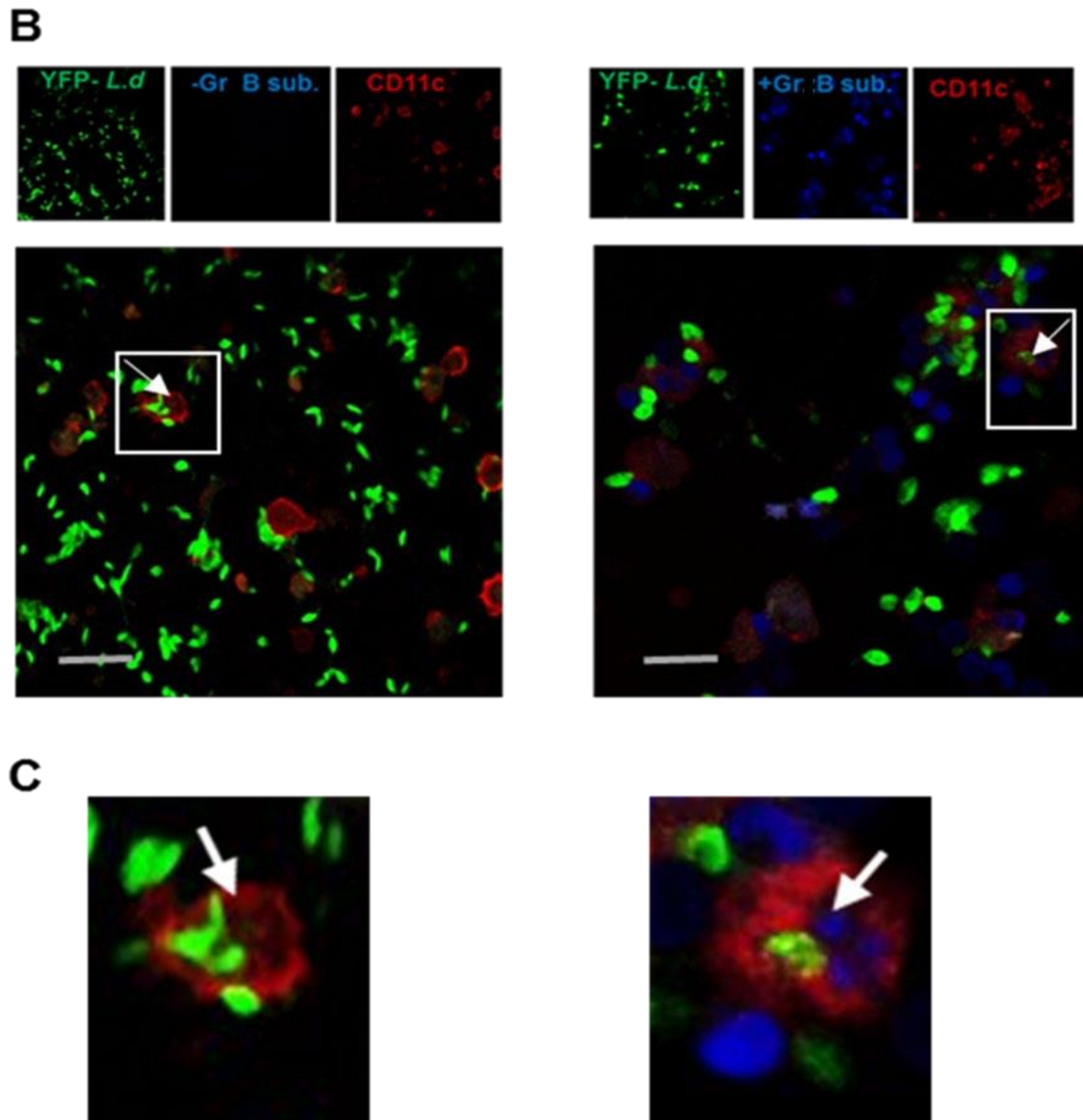


Figure 14: Granzyme B activity in *L. donovani* infected ihMDDCs.

On the 5th Day, ihMDCs cells were treated with/without granzyme B inhibitor for 20min before they incubated with wild type *L. donovani* for different time points, as indicated, the supernatants were collected at that determined time points and incubated with the fluorogenic granzyme B substrate. The granzyme B substrate signal was detected using Perkin Elmer LS-50B Luminescence Spectrophotometer. In addition, to show the activity and localization of active granzyme B protein inside the infected cells, the ihMDDCs incubated at the same time with YFP- transfected. *L. donovani* (green fluorescent) and the fluorogenic granzyme B substrate (blue fluorescent) for 24h before they stained with APC anti-human CD11c (red fluorescent). The cells then were analysed using confocal microscopy. Images were deconvolved using ImageJ software. A) Presents the intensity of granzyme B activity with time points of infection, the data analysed from 3 different samples. B) The infected cells incubated without granzyme B substrate, lift, the infected cells incubated with granzyme B substrate, right. C) Shown is a sequence of detailed images, the blue fluorescent express the active granzyme B co-localized with the green fluorescent from the parasite (indicated by white arrow) inside the red fluorescent infected cell, right, while the none-treated sample with granzyme B substrate presented without the blue fluorescent (pointed out by white arrow) as a negative control, left. The laser scanning confocal microscopy was operated to examine the slides. In addition, the images processed with the Leica Confocal Software Version 2.5 Build 1227. Scale Bar=5µm.

5.8. Detection the Co-localisation of granzyme B with digested *L. donovani* inside hMDDCs

Granzyme B has the most robust apoptotic activity of all granzymes, as a result of its caspase-like ability to cleave substrates at key aspartic acid residues [118]. To get our data together, we design this experiment to detect the granzyme B protein integrated with the intercellular parasite at a determined time point of infection. In this purpose, ihMDDCs were incubated with YFP-transfected *L. donovani* for 48h in flask, and then the cells lifted, washed, and fixed with the fixation buffer for 30min and after that, incubated with permeabilized with permeabilization buffer combined with Alex flour 647 anti-humans granzyme B for 30-40min before they washed, fixed and centrifuged on the slide to detect with confocal microscopy. Images were deconvolved using ImageJ software. The pictures show visibly the overlap of the green fluorescent from the parasite with the granzyme B (red fluorescent) inside the infected cells. The yellow fluorescent shown the parasite digested inside the hMDDC (**Figure 15**).

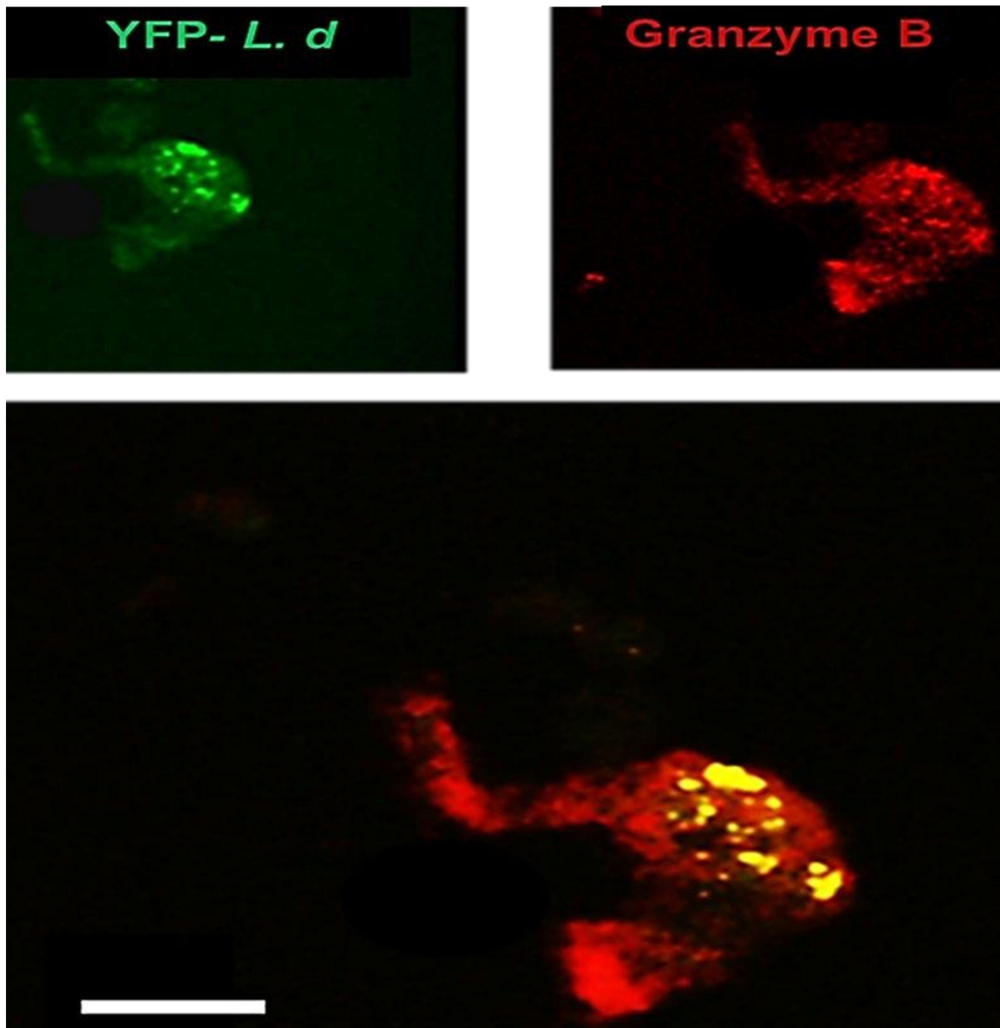
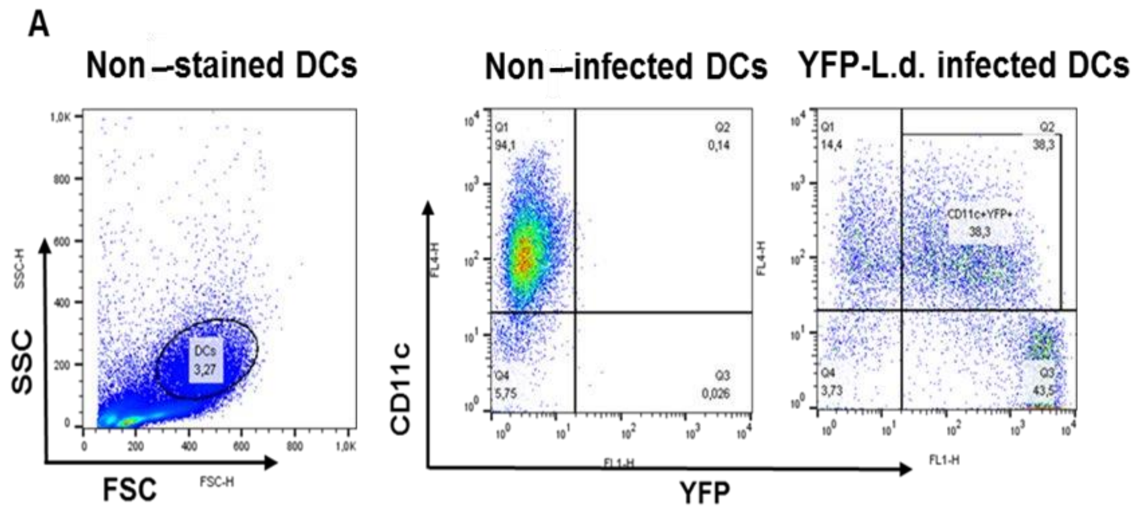


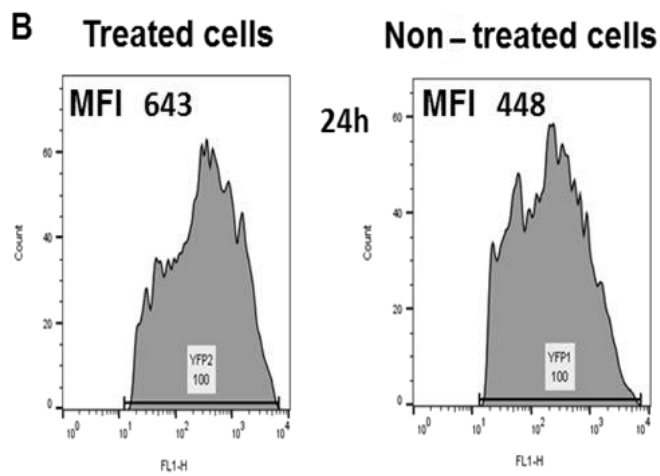
Figure 15: Co-localization of granzyme B with the digested *L. donovani* inside hMDDCs

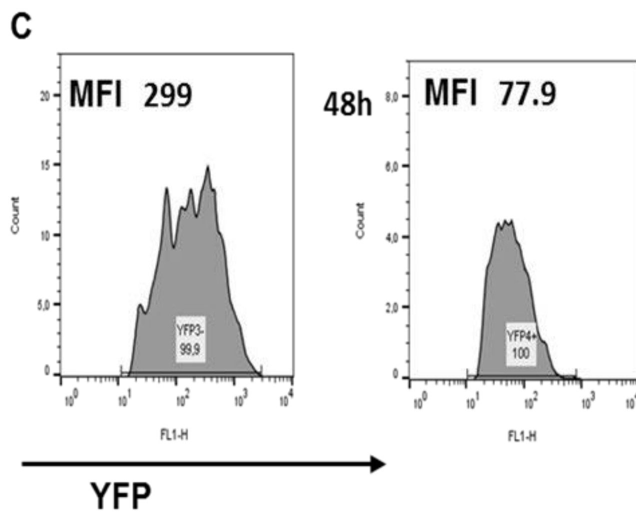
ihMDDCs were incubated at day 5 of differentiation with YFP- transfected *L. donovani* for 48h, before they washed and stained intercellular for the produced granzyme B with Alexa flour 647 anti-human granzyme B. The cells then fixed and centrifuged on slid and detected under the confocal microscopy, the pictures shown clearly the overlap, which is displayed in (yellow fluorescent) of degraded parasites (green fluorescent) inside the DCs with the secreted granzyme B protein (red fluorescent). The images processed with the Leica Confocal Software Version 2.5 Build 1227. Scale bar= 5µm.

5.9. Evaluation of granzyme B role in the digestion process of *Leishmania* parasite inside *Leishmania* infected hMDDCs

To define the role of granzyme B produced in hDCs, the ihMDDCs differentiated at day 5, they incubated with granzyme B inhibitor at the concentration of 100µM/ml for 20min before they incubated with YFP transfected *L. donovani* for 24h and 48h, the cells then washed and stained with anti-human CD11c. The cells were prepared for flow cytometry for detecting the signal of hMDDCs positive YFP, this fluorescent signal rate shows the killing of the intercellular parasite, the cells were measured with FACSCalibur, and the result analyzed with FLOWJO single cells analysis software v10. The data analyzed with Excel 2016. The cells during the measurement were gated on the positive CD11c in combination with the positive YFP and evaluated the histogram of YFP in this gated population. The YFP signal in the granzyme B inhibitor-treated infected hMDDCs showed slightly more mean fluorescent intensity MFI after 24h comparing with none treated infected hMDDCs. However, after 48h of infection, which is that time point of processing of intercellular pathogens as we showed before, the granzyme B inhibitor-treated infected hMDDCs had a more than two folds YFP signal comparing with none treated cells, they displayed highly decreased in the YFP signal (**Figure 16**).


A
Non-stained DCs
Non-infected DCs
YFP-L.d. infected DCs
SSC
FSC
DCs
3,27
CD11c
YFP
Q1
94,1
Q2
0,14
Q4
5,75
Q3
0,026
Q1
14,4
Q2
38,3
CD11c+YFP+
38,3
Q4
3,73
Q3
43,5


B
Treated cells
Non-treated cells
MFI 643
24h
MFI 448
YFP2
100
YFP1
100


C
MFI 299
48h
MFI 77.9
YFP3-
99,9
YFP4+
100
YFP

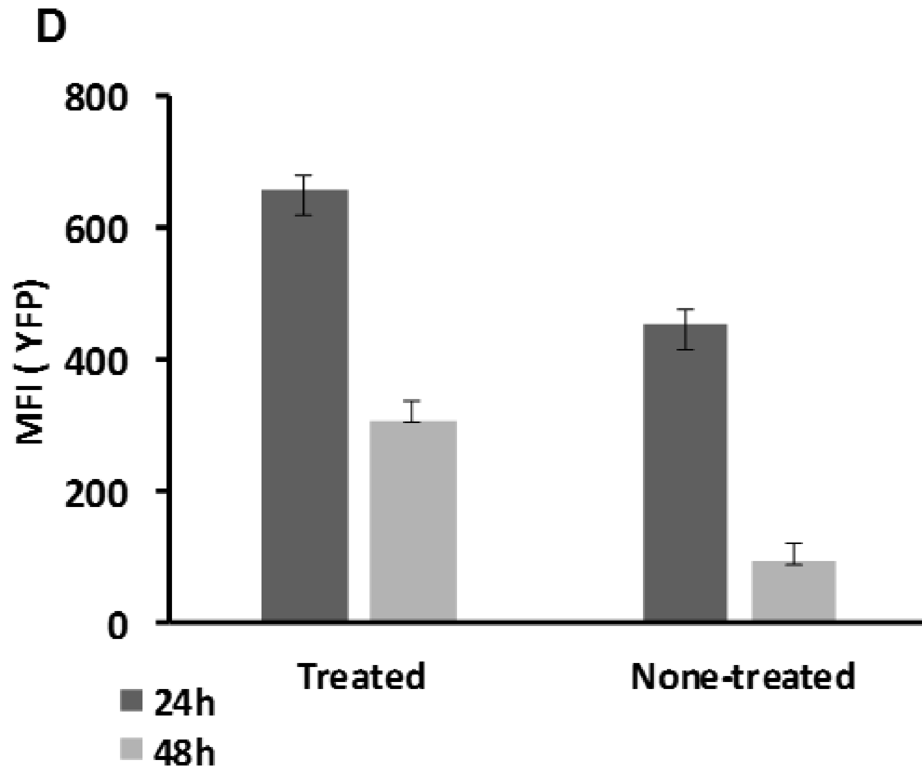
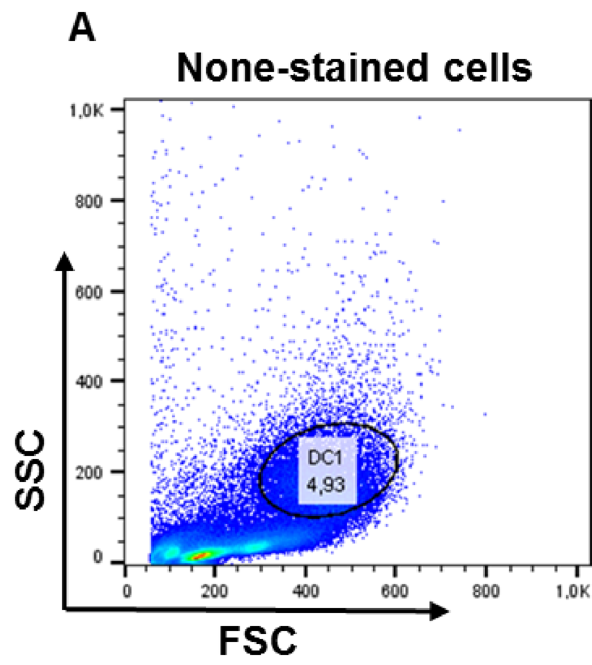


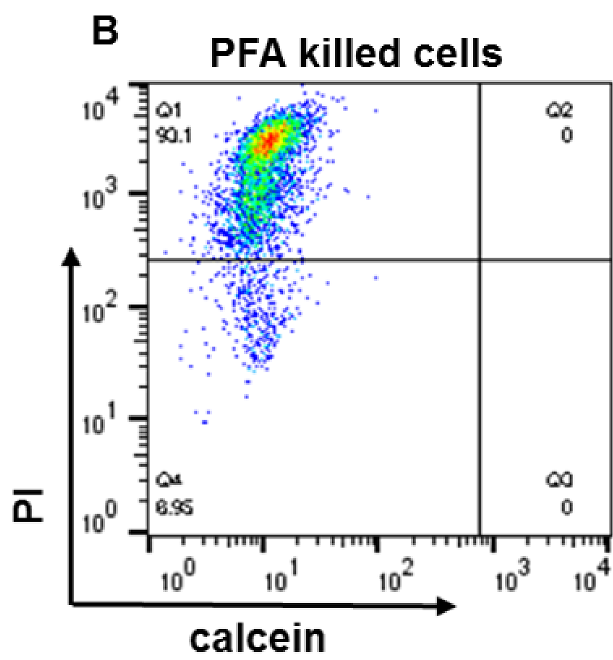
Figure 16: The role of granzyme B in digestion of phagocytized parasite in hMDDCs

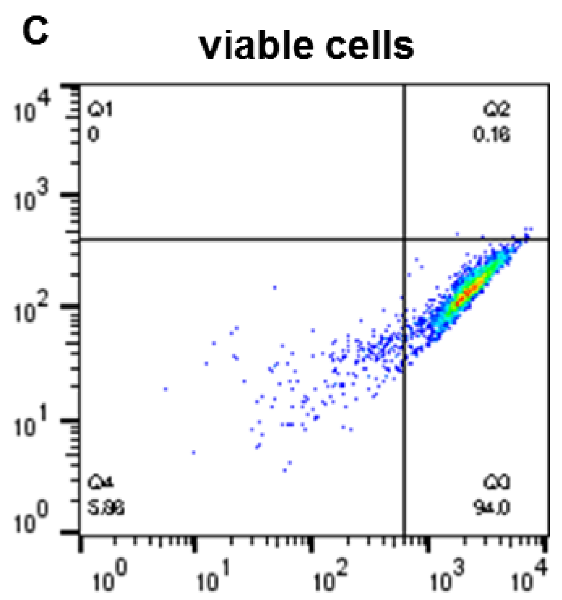
The hMDDCs were differentiated for 5 days from adhered monocytes, they were treated with/ without granzyme B inhibitor before the infected for 24h and 48h with YFP-transfected *L. dononvani*. The mean fluorescent intensity (MFI) was measured by using the FLOWJO single cells analysis software v10. The none-stained cells for events gating (A) left, the gated events none stained cells. Gated stained infected cells with APC anti- CD11c, middle, the infected cells gated for + CD11c and +YFP (A) right. (B) The flow cytometry histograms show the MFI of YFP signal of infected cells for 24h treated cells, left, and of none-treated cells, right. The MFI signal after 48h of the cells infection showed in (C) treated cells, left, and the none-treated cells, right. (D) Histograms and data, presented as mean ± SEM, are representative for 3 independent experiments (**: $p < 0.01$).

5.10. The impact of granzyme B inhibitor on hMDDCs viability

In order to confirm the outcome of treatment of hMDDCs with the human granzyme B inhibitor, the ihMDDCs were obtained as previously described, and they incubated with/ without human granzyme B inhibitor (100µM/ml) for 24h. The cells were then harvested, washed one time with PBS and stained with the nonfluorescent cell-permeable calcein-AM, which is metabolised in the cytoplasm of live cells into the green fluorescent calcein, and propidium iodide (PI), which only enters cells with perforated cell membranes and binds nucleic acids, staining the cells red. After staining, the cells were measured with FACSCalibur, and the data were analysed with FLOWJO single cells analysis software v10. To prepare the dead and live cells as a positive control for both, the cells incubated with/ without paraformaldehyde (PFA) for 15min and stained separately with (PI) and Calcein-AM, respectively. The graphs showed there was no significant effect of the treatment with the granzyme B inhibitor with a recommended concentration on the viability of hMDDCs (**Figure 17**).


A
None-stained cells
DC1
4,93
SSC
FSC


B
PFA killed cells
Q1
93.1
Q2
0
Q4
6.95
Q3
0
PI
calcein


C
viable cells
Q1
0
Q2
0.16
Q4
5.88
Q3
94.0

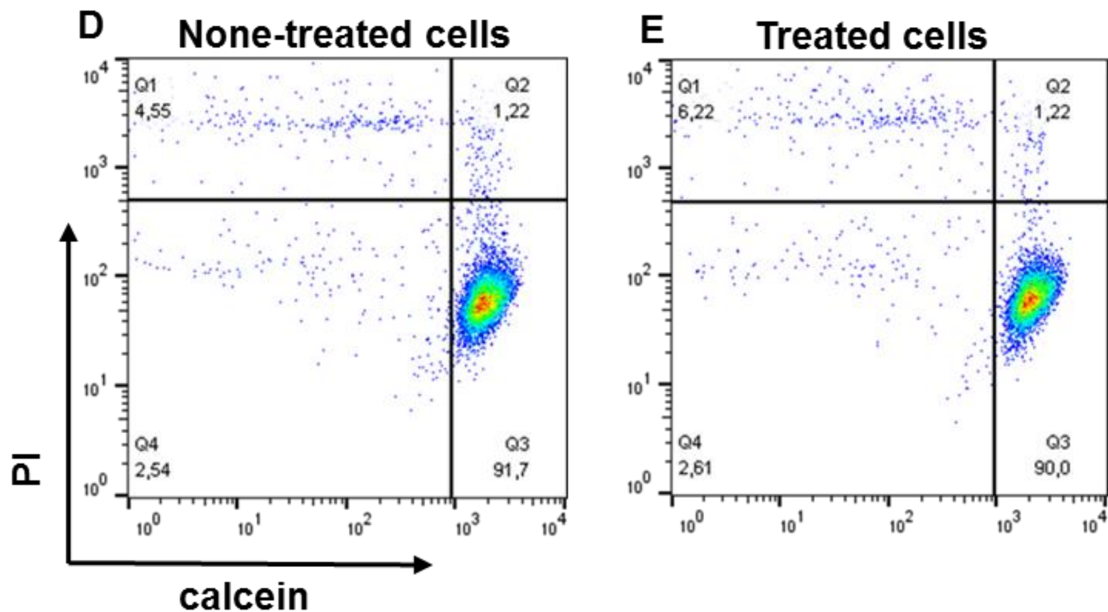


Figure 17: Impact of granzyme B inhibitor on hMDDCs viability

The hMDDCs incubated for 24h with/without granzyme B inhibitor incubated (100µM/ml), the cells then washed and stained with the nonfluorescent cell-permeable calcein-AM, which is metabolized in the cytoplasm of live cells into the green fluorescent calcein, and propidium iodide (PI), which only enters cells with perforated cell membranes and binds nucleic acids, staining the cells red. The histograms showed no effect of the granzyme B inhibitor on the viability of DCs. A) The none-stained cells. B) Positive control for dead cells. C) Positive control for viable cells. D) The cells incubated without granzyme B inhibitor and stained for both (PI) and Calcein-AM. E) The 24h incubated cells with granzyme B inhibitor.

5.11. Impact of *L. donovani* infection on hMDDCs activation

In the immune response against different infections, including parasitic infections, DCs comprise a complex array of cell populations that play a leading role. In an immature state, they can sense and phagocyte the antigens. Due to up taking antigens, they become activated, mature and prime naïve T lymphocytes within lymph nodes. It is essential to evaluate the activation of DCs during *Leishmania* infection. hMDDCs were obtained from filtered blood samples from healthy donors, ihMDDCs at day 5 of differentiation were infected with wild-type *L. donovani* promastigotes for 48h in the presence or absence of a cocktail of cytokines IL-1β (10 ng/mL), IL-6 (25 ng/mL) and TNF-α (10 ng/mL), the cells incubated with these cocktail 2h after the infection and then continued for 48h. In addition, as a positive control, none infected hMDDCs were incubated with LPS for 48h. The cells then lifted, washed and incubated for 25min with fluorescent labeled specific activation markers for the hMDDCs, they included anti-CD1a, anti-CD40, anti-CD58, anti-CD83, anti CD80, anti-CD86, anti-HLA-DR and anti-

HLA-ABS. The stained cells were washed and the fluorescent signals determined with FACSCalibor, and the results were analyzed with the WinMDi 2.9. The data showed a slightly up-regulation of most of the activation markers; this up-regulation was significant in those infected cells and treated with the cocktail of cytokines. The positive control of activated DCs showed downregulation of CD1a; this is also was decreased in the infected cells and treated with the cocktail of activating cytokines (**Figure 18**).

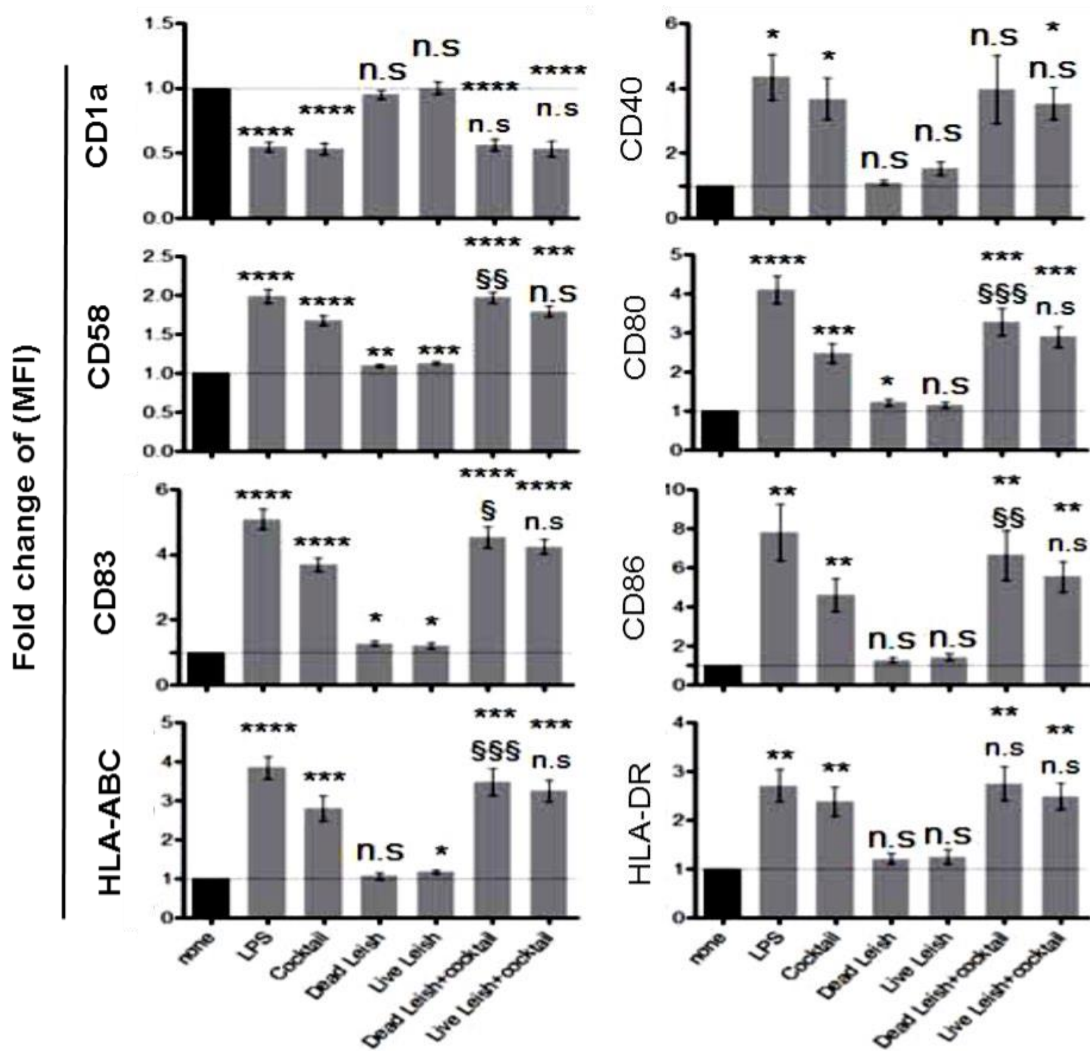
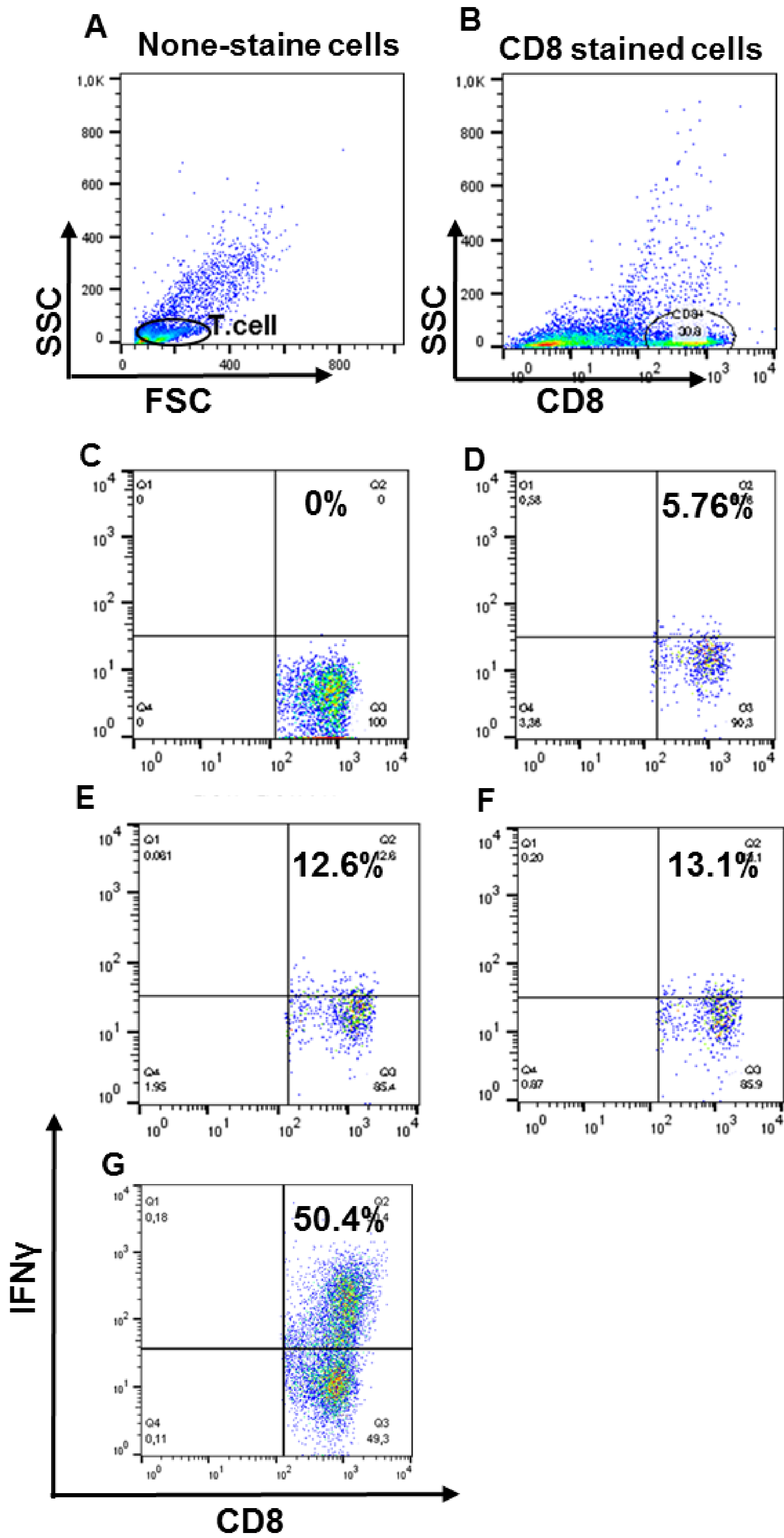


Figure 18: The impact of *L. donovani* infection on the activation of hMDDCs

hMDDCs were infected *with L. donovani* parasites for 48h with/without treated with a cocktail of cytokines, which added after 2h of infection. The positive control were cells treated with even LPS or a cocktail of activation cytokines for 48h. The samples washed, stained, and measured with FACSCalibur. The data obtained from 11 independent experiments done with different samples and analyzed with WinMDi 2.9.

5.12. Determination of granzyme B role in priming autologous cytotoxic T lymphocyte by *L. donovani* infected hMDDCs

Not all granzyme B-expressing cells are co-express perforin, providing further support to the notion that granzyme B also exerts functions that are independent of its cytotoxic effects. The role of granzyme B expressed in hMDDCs in activation of naïve T lymphocytes need to be addressed. In this present experiment, the PBMCs were isolated as described previously, the PBMCs were incubated for 2hours at 37°C in 8% CO_2, the nonstick lymphocytes were lifted with the suspension were culture in refreshed cell culture medium in cell culture flask and maintained at 37°C in 8% CO_2, the medium was refreshed 2-3 days. The adhered monocytes differentiated to DCs as previously described, the ihMDDCs differentiated at day 5, they were counted and incubated with/without granzyme B inhibitor at the concentration of 100µM/ml for 20min before they incubated with/without wild type *L. donovani* for 48h. The cells then were co-cultured then with the autologous lymphocytes in an effector: target ratio of 10:1 in the presence of Brefeldin A for 6hours at 37°C in 8% CO_2. As a positive control for the activation, the cytotoxic T cells were incubated with PMA and Ionomycin for 6hous in the presence of Brefeldin A. All the samples then were stained intracellularly with anti-human IFN-γ Antibody after they were stained with anti-human CD8 Antibody and fixed for 30min, as a negative control, the cells stained only with human CD8 Antibody. The samples were then washed and measured with FACSCalibur the data were analyzed with FLOWJO single cells analysis software v10.


A
None-staine cells
T.cell
SSC
FSC
B
CD8 stained cells
SSC
CD8
C
0%
D
5.76%
E
12.6%
F
13.1%
G
50.4%
IFNγ
CD8

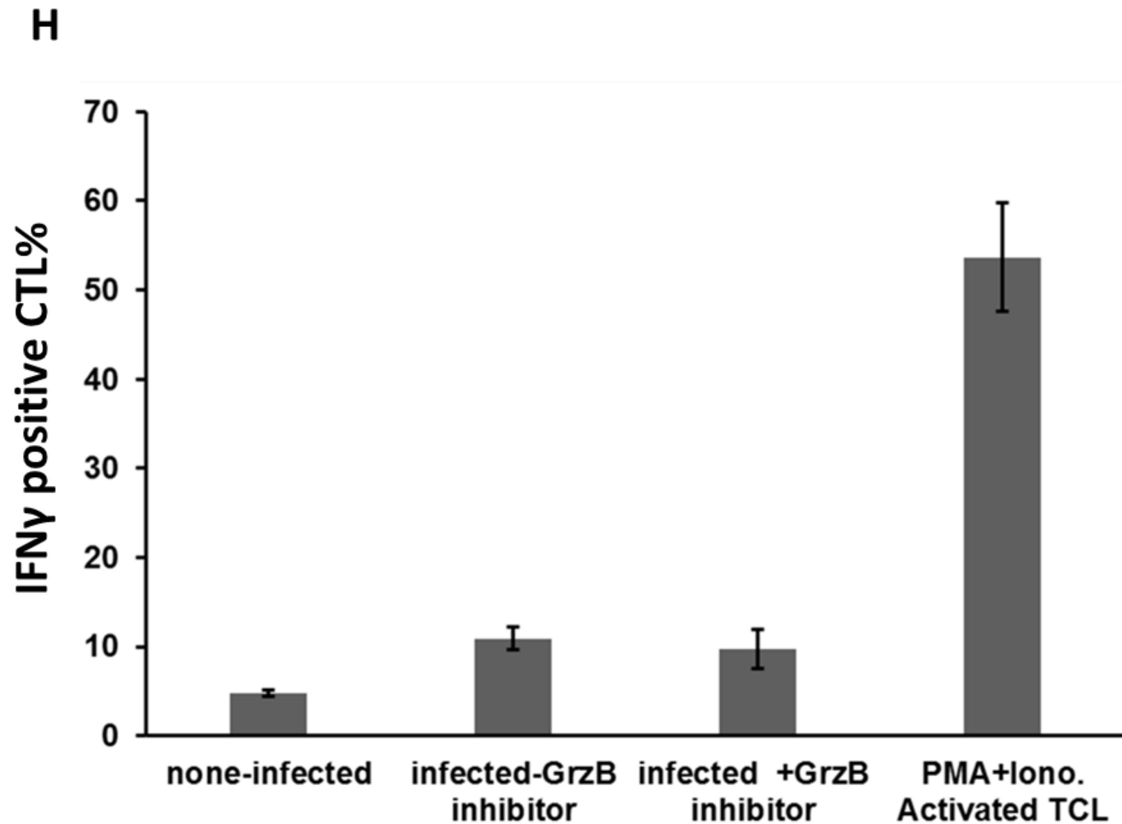


Figure 19: The role of granzyme B on priming of T lymphocytes by hMDDCs during leishmanaiasis

The autologous T lymphocytes were cultivated in RPMI 1640 GlutaMax culture medium supplemented with 10% FCS at 37°C in 8% CO_2 and refresh twice, the autologous hMDDCs and hMDMs were infected as mentioned before and then co-cultured with their autologous T lymphocytes for 6h in the presence of Brefeldin A. The cells then washed and stained for the surface CD markers with anti-CD8 AB, and intercellular with anti-INF gamma AB. The samples were measured with FACSCalibur. The results show no significant effects of inhibited granzyme B protein on the activation of T lymphocytes, the positive control of T lymphocyte activation was carried out with treated cell with a combination of Ionomysin and PMA for 6h in the presence of Brefeldin A. A) None stained lymphocytes, B) lymphocytes stained only with anti-CD8, C) Gated on CD8+ cells without Anti-IFNγ, D) Co-culture of none-infected DCs with autologous CD8 T lymphocytes, E) *Leishmania* infected hMDDCs without granzyme B inhibitor co-cultured with autologous lymphocytes, F) *Leishmania* infected hMDDCs with granzyme B inhibitor co-cultured with autologous lymphocytes, G) The positive control of CTL activation, H) diagram show the percentages of CTL positive IFNγ of 3 independent samples.

6. Discussion

6.1. Intercellular *Leishmania* parasites are digested inside the hMDDCs but not hMDMs

During life, our body is exposed to various threats including infectious agents like bacteria, viruses or protozoal parasites. For protection against such agents, several mechanisms of immune defence have evolved. By gaining more insight into host-pathogen interactions, a better understanding arose of how infections occur and what protective and curative immune responses are that led to new prophylactic and intervention strategies.

In the presented project, we focused on the interaction of human innate immune cells with *L. donovani*. As widely known, the monocyte-derived macrophage are host cells for *Leishmania* parasite. We tested the different interactions between human monocyte-derived dendritic cells as antigen presenting cells and human monocyte-derived macrophages with the *L. donovani* with special attention to *Leishmania*-dendritic cell interaction. We hypothesised that that processing of *Leishmania* parasites by human dendritic cells is dependent on the cytolytic molecules granzyme B and granulysin, and that both proteins have an essential role in the antigen processing mechanism for induction of *Leishmania*-specific T cell responses.

Upon infection of hMDM and hMDDCs with *Leishmania* parasites, we observed macrophages to be permissive for intracellular *Leishmania* parasite survival and propagation whereas dendritic cells digest the intracellular parasites. Furthermore, killing of the parasites induce hMDDCs suitable for priming *Leishmania*-specific T cell lymphocytes. This ability increased upon maturation of the *Leishmania* infected DCs.

The hMDDCs had been identified before as granzyme B expressing cells **[70, 117, 118]**. We established expression of both granzyme B and granulysin by hMDDCs and hMDMs. In addition, we established the essential role of granzyme B and maybe also granulysin in the process of parasite digestion by hMDDCs. As the expression of granzyme B was only found in hMDDCs but not in human macrophages, our data suggest that this expression difference could explain why hMDDCs in contrast to hMDMs can kill the intercellular parasites.

Most of the leishmaniasis studies in humans were done with macrophages as they are their main host cells **[152-155].** Also in murine models of leishmaniasis, macrophages were the focus of the investigations **[156-165]**. Only few studies were done on the response of human dendritic cells to *Leishmania* infection. Although *Leishmania* parasites infect and interact with a variety of phagocytic host immune cell types, macrophages and dendritic cells (DCs) are the most important ones for the outcome of the infection. After promastigotes are taken up by macrophages into the phagosomes, subsequent fusion with lysosomes occurs **[166, 167]** . Although this is the most deadly environment for most pathogens, *Leishmania* are among the few that can survive and proliferate in such condition **[167, 168].** To control and eliminate the parasites efficiently, the macrophages must be activated, which is usually induces by cytokines such as INF-γ or TNF-α being produced by DC-primed helper CD4 and cytolytic CD8 T, and NK cells **[169]**. In our study, human macrophages were infected with live promastigotes of YFP-transfected *L. donovani* parasites that were compared to dead parasites as a positive control for phagocytosis and digestion. The destruction of phagocytised parasites was monitored by the YFP fluorescence. We saw that the parasites proliferate inside hMDMs even when treated with GM-CSF. Live parasites are taken up at a slower rate than dead parasites, and our data showed no decrease of YFP fluorescence in live parasite-infected hMDMs. In contrast, in case of dead parasites, the YFP vanished over time. These findings confirm that the hMDMs cannot digest the *L. donovani* parasites, nor could GM-CSF activated hMDMs. *Leishmania* parasites may be destroyed in macrophages by a granulysin/granzyme B-dependent mechanism delivered by CD8 cytotoxic T cells. However, these effector cells have to be induced first, which requires prior processing and presentation of leishmanial antigens by antigen presenting cells, most importantly DCs **[170].** Handling of the parasites was found to be different in dendritic cells. Considering the pivotal role of DCs in the induction of adaptive immune response and their capacity to take up and destroy *Leishmania* parasites **[53]**, they must express special killing mechanisms. In concordance it has been demonstrated that granulysin/granzyme B-dependent processes are essential for clearance of the parasites in human leishmaniasis, for cure from the disease and for immunity against subsequent infections **[170, 171]**. This mechanism cannot play a role in mice as mice do not have granulysin **[172]**, which makes it difficult to draw conclusions from murine models of leishmaniasis.

6.2. Both hMDDCs and hMDMs do not lose viability after *Leishmania* infection

The proliferation of amastigotes inside their infected host cells, together with their evading immune cell-killing mechanisms, and their regular infection of other cells augments the infection and, thus, the severity of the disease **[172, 173].** The mechanism that intercellular amastigotes use to leave macrophages to infect neighbouring cells is unknown. It has been postulated, usually based on static images only, that unrestricted proliferation of the amastigotes causes host cell rupture **[174,175]** or that amastigotes are released by exocytosis with membrane shrivelling but without cell rupture **[176]**. In addition, there is a major role of *Leishmania*-specific cytotoxic T lymphocytes in destroying the infected target cells. Cross-presentation of *Leishmania* antigens via MHC class I during in vivo infections may result from several internalisation pathways, such as direct infection, receptor-mediated uptake **[177]**, or the internalisation of apoptotic vesicles of infected cells **[178]**. After activation, antigen-specific CD8 T cells differentiate into activated effector cells and acquire the capacity to kill target cells, and produce several cytokines and chemokines **[179]**. The precise mechanism underlying CTL killing of microbes is still under investigation. For viability assay in our work, we stained the infected and none-infected cells with non-fluorescent calcein-AM dye, which passes easily through cell membranes into live cells and is hydrolysed by cellular esterases to give calcein, which is fluorescent and retained in the cytoplasm. The intensity of calcein dye measured with a fluorimeter is directly proportional to the activity of cellular esterases, which in turn is proportional to viable cells. Here, we found that both hMDDCs and hMDMs were not dead after *in vitro* infection with *L. donvania* parasites. We have checked the viability of infected and compared to none-infected cells. The time points of infection and MOI were the same used for uptake and processing experiment before. These factors could affect the viability of the infected cells. We found that the viability of *L. donovani* infected cells declined with higher number of infecting parasites. The data are not shown in this work.

6.3. The phagocytised parasites inside hMDDCs co-localise with components of antigen processing and presentation

The plasticity in both type and magnitude of the immune responses is a basic feature of the immune reactions induced by various microbial infections **[180]**. The type and magnitude of this response is determined by the interaction between phenotypically and functionally heterogeneous antigen-presenting DCs and naïve or previously primed T cells in spleen and lymph nodes. In murine models of leishmaniasis, this interaction determines the disease manifestations depending on whether it leads to differentiation of a host-protective Th1 and CTL or a disease-exacerbating Th2 type of T lymphocyte cells. Some studies found that the parasites are localised in intracellular compartments containing MHC class II, Lamp1, and cystatin C, which supports the notion that the infected DCs should be able to present parasite antigens to T cells **[181, 182]**. In our present work, the cells were stained with anti-MHC-II, these glycoproteins are synthesised in the endoplasmic reticulum (ER) where they associate with MHC-II-associated invariant-chain (Ii or CD74), a chaperone that forms a nonameric complex. Our data showed that the phagocytised YFP-transfected *L. donovani* parasites co-localise with MHC class II and CD74. These stained compartments overlayed with the YFP fluorescence in dead *Leishmania*-infected hMDDCs at 24h of infection when the YFP fluorescence of dead parasites was disappearing as by flow cytometric results. While the co-localisation was clear in live parasite infected hMDDCs, the merge was weak generally at the time point of 2h, which suggests that at this time point of infection, the process of phagocytosis was still on going and the intercellular parasites were being transported within vacuoles. Interestingly these data suggest that the *Leishmania* infection generates signals leading to activation of CD4 T cell lymphocytes.

Cross-presentation by MHC-I is a distinctive process in which antigens from phagocytosed particles or soluble proteins internalised by other routes are assembled with MHC-I. This process is largely restricted to specific DC subsets *in vivo* **[105, 183]**. In addition, transfer of the trimmed peptides from TAP onto folding MHC-I is promoted by a loading complex composed of dedicated chaperones, tapasin and a series of generic membrane-bound and soluble chaperones including calnexin, ERP57 and calreticulin **[184]**. We stained the *Leishmania*-infected cells for both MCH-I and calnexin. Interestingly, here we found at 24h of infection an overlay of life YFP-

transfected *L. donovani* inside hMDDCs with intercellular MHC-I and calnexin. This data suggests a role of DCs in priming CTL during leishmaniasis, which will contribute significantly to the development of vaccines against leishmaniasis.

6.4. *Leishmania*-infected hMDDCs but not hMDMs express granzyme B, but both granulysin

The basic components of the cytotoxic granules of NK cells and CTLs are granzymes, closely related serine proteases. In general, our understanding of the functions of granzymes is still limited, perhaps with the exception of granzyme B, which is the most extensively studied member of the family. Granzyme B is expressed not only by cytotoxic cells such as CTLs and NK cells but recently many studies have shown the expression of granzyme B by a variety of normally non-cytotoxic cell types including $CD34^+$ hematopoietic stem cells **[185]**, pDCs **[70]**, B cells **[186, 187]**, basophils **[188]**, mast cells **[189]** and neutrophils **[190]**.

Interestingly, EBV enhanced granzyme B production and secretion by pDCs **[70]**. Concordantly to our data that show that *Leishmania* infection boosts high expression of granzyme B in infected hMDDCs. The expression of granzyme B was already detected in ihMDDCs.

The other important cytolytic protein, granulysin, is a saposin-like pore-forming protein. Granulysin preferentially disrupts cholesterol-poor bacterial, fungal and parasitic membranes **[144]**. The expression of granulysin was very low in the none-infected hMDDCs and slightly up-regulated after *Leishmania* infection. We confirmed our flow cytometry findings with the results of confocal microscopy and detection of the RNA coding for these products via PCR. Our study showed the expression of granzyme B and granulysin by ihMDDCs and mhMDDCs and the increase of the expression levels upon *Leishmania* infection.

On the other hand, we found that the expression of granulysin in hMDM was at the same level as in hMDDCs, but no granzyme B expression was detected. These findings were confirmed by the PCR. Our findings showed for the first time the expression of granzyme B in human myeloid DCs inside intracellular vesicles, also in *L. donovani* infected hMDDCs. In addition, this for the first time visualised the expression of granzyme B inside hMDDCs.

These findings open new insights into a novel role of hMDDCs during *Leishmania* infection.

Because of the low-level expression of granulysin in both hMDDCs and hMDMs, we could not see this expression by confocal microscopy.

Translating these findings into our model for the differential handling of intracellular *Leishmania* parasites, we can hypothesize that granzyme B in hMDDCs might have a role in processing of phagocytised intercellular parasites. The role of granulysin in both hMDDCs and hMDMs was not clear and is the focus of our following work.

6.5. Granzyme B is up-regulated in hMDDCs upon *L. donovani* infection

It is commonly accepted that during cytolysis of target cells by CTL and NK cells, granzyme B, following entry into the cytosol of the target cell, induces apoptosis by activating caspases, prominently effector caspases like caspase 3 **[191, 192]**. In addition, granzyme B has been found to cleave key caspase substrates **[193]** such as the BH3-only protein Bid **[194, 195]** and ICAD (inhibitor of the caspase-activated DNAse) **[196, 197]**. Due to its cytotoxic nature, it is expressed as an inactive prepro-enzyme in the expressing effector cells. Granzyme B becomes functional by the removal of the pro-peptide dipeptide Gly-Glu from its N-terminus by lysosomal dipeptidyl peptidase I/cathepsin C **[198]**. It is not clear yet if granzyme B expressed by dendritic cells is active or not. After we demonstrated the expression of granzyme B in hMDDCs before and after infection with *L. donovani* we aimed to test whether it is proteolytically active. To this end, we incubated *L. donovani*-infected hMDDCs with a selective granzyme B inhibitor and a fluorogenic granzyme B substrate. Our respective data suggest that the granzyme B expressed by *Leishmania*-infected and non-infected hMDDCs is proteolytically active. During infection, the activity of granzyme B increased. The granzyme B inhibitor showed maximal effect on the activity of the enzyme on *Leishmania* parasites after around 24h of infection/incubation. At this time point, we had observed before the onset of the process of parasite digestion inside the infected hMDDCs. These data also showed for the first time enhanced activity of granzyme B with an extended time of infection; the granzyme B inhibitor we used lost

its activity when it was incubating for longer time than recommended by the manufactories. Confocal microscopy images showed for the first time active granzyme B co-localised with digested *L. donovani* parasites inside hMDDCs. Moreover, our confocal microscopy analyses showed for the first time the co-localisation of YFP fluorescence from digested *L. donovani* parasites with fluorescent granzyme B enzyme product. These findings suggest that granzyme B contributes to apoptosis of intercellular *Leishmania* parasite.

6.6. The inhibition of granzyme B suppresses digestion of intracellular *L. donovani* parasite by hMDDCs

Generally, granzymes act on different primary substrates and are able to cleave various cellular protein substrates to induce apoptosis **[199]**. *In vitro* studies have suggested that granzyme B induces target cell death through two essential pathways, one triggering outer mitochondrial membrane permeabilisation via cleavage of the proapoptotic protein BH3-interaction domain death agonist (Bid), and the other involving direct proteolytic activation of caspases ultimately leading to DNA damage **[200]**. The absence of granzyme B during apoptosis of most, but not all, cell types delayed target cell DNA fragmentation **[201, 202]**. However, gene deletion mice deficient in granzyme B show diminish levels of CD8 T cell-mediated cytotoxicity and have increased susceptibility to some viral infections. Despite the residual ability of CD8 T cells from granzyme $B^{-/-}$ mice to kill target cells, they were unable to induce DNA fragmentation **[203]**. There are several ways to delete the GrB gene in mice cells, which is impractical in primary human cells. For this reason, the cells in our work were incubated with granzyme B inhibitor. In accordance with the results from other works in animal model, we found that the inhibition of granzyme B delayed destruction of intracellular phagosytised *L. donovani* by hMDDCs, the YFP fluorescence signal in infected hMDDCs increased compared with non-treated infected MDDCs. Depending on the suggested killing time point after 24h of infection, we detected the effect of granzyme B inhibitor between 24h to 48h. A greater effect occurred at 48h; the killing process was reduced by around 60%, up from 30% at 24h. The finding that in the presence of granzyme B inhibitor still some degree of destruction was observed suggests that other intercellular elements could contribute to the process of

Leishmania parasite digestion by hMDDCs. Besides cathepsins, endosomal proteases, granulysin, which we detected in DCs, could contribute to the processing.

6.7. Effect of *L. donovani* infection on hMDDCs activation

Since the destruction by DCs follows an initial intracellular proliferation of the parasites, it appears that the parasitocidal capacity is induced by live *Leishmania* parasites; not so by dead parasites, which may be processed like other particulate antigens. While this implies some degree of activation of the DCs, this activation does not extend to the well-established indicators of DC activation, CD83, the co-stimulatory molecules CD40, CD80 and CD86, and the cytokine IL-12. This, again, is surprising as components of *Leishmania* parasites have been reported to address innate pathogen-associated molecular pattern (PAMP) recognition receptors (PRRs), such as Toll-like receptors (TLRs), which does result in the induction or up-regulation of said activation markers **[204]**. It may be conjectured that immunostimulatory leishmanial factors do not get access to the respective PRRs or that these receptors are not expressed by iDCs. However, in the presence of proinflammatory cytokines, activation of *Leishmania*-bearing DCs is strongly enhanced beyond what can be observed with the cytokines alone, which is reminiscent of recently published observations with TLR agonists **[205]**. Some reports indicated that *Leishmania* alter DC maturation but the data are controversial as some studies show the induction of maturation by some species of *Leishmania* while others show impairment of DC maturation **[206, 207]**. However, in the experiments that showed impairment of DC function, far higher MOI than in our experiments were used. Obviously, when comparing different studies on the immunology of leishmaniasis, careful attention needs to be paid to the detailed design of the different studies. In particular, it needs to be acknowledged that there are significant differences between different *Leishmania* species and the immune constitution of different host species.

Antigen presentation by DCs is critical for the generation of memory and effector T cells from primary naïve T cells *in vivo*. Activated DCs express costimulatory receptors and the cytokines necessary for the initiation of functional and memory T cell responses. The responses of T cells induce and regulate T cell expansion, the generation of effector functions and T cell survival. The exact contribution of each component alone and in combination in mediating these processes is still not fully

clear. Some studies on MDDCs in murine models of leishmaniasis have been published. It has been concluded that *Leishmania*-infected DCs upregulate the levels of costimulatory molecules such as CD40, CD80, and CD86 as well as of MHC II, and of the adhesion molecule CD54. Such activated and matured DC are able to trigger the activation of T lymphocytes **[208]**. Other authors have reported that during the course of chronic infection of C57BL/6 mice with *L. major* the inflammatory DC are the main producers of iNOS and are recruited in a CCR2-dependent manner. The induction of iNOS depends on the development of a local Th1-dominated microenvironment and could contribute to the clearance of the parasites **[209]**. According to another work, infection of hDCs with *L. major* promastigote does not inhibit the process of maturation. Production of high amounts of IL-12 is reported to require CD40-CD40L interaction, although infected DC are able to produce some low level IL-12 without that. **[210]**. However, other authors found that *L. major* parasites in a mouse model produce and secrete a soluble factor that binds to the macrophage inducible Ca^{2+}-dependent lectin receptor (Mincle), a C-type lectin, of DCs, which inhibits their maturation. They report that Mincle deficiency leads to stronger DC activation represented by a higher expression of costimulatory molecules, migration to draining lymph nodes (dLNs) and priming of Th1 responses. Mice deficient in Mincle are capable of controlling parasite replication and have smaller lesions **[211]**. To translate these conclusions to human leishmaniasis, the different human dendritic cell (hDC) types and their differentiation, functional and activation states need to be characterised in leishmaniasis patients. Such information will help understand how the whole macrophage/DC system works in the disease situation and may open new opportunities for therapeutic immune interventions **[212]**.

From this PhD work we can conclude that the hDCs should address when developing anti-*Leishmania* vaccines or immunotherapies in order to induce efficient CD4+ helper and CD8+ effector T cell responses. They may explain why leishmanization is efficient whereas subunit vaccines are not. However, leishmanization induces immunity through deliberate infection with subsequent disease, which may come with severe adverse effects. Alternative strategies may consider TLR agonists or inflammatory cytokines for *in situ* vaccination and immunotherapy to activate parasite-bearing DCs and thereby induce parasitocidal CD8+ effectorT cell and innate immune reaction.

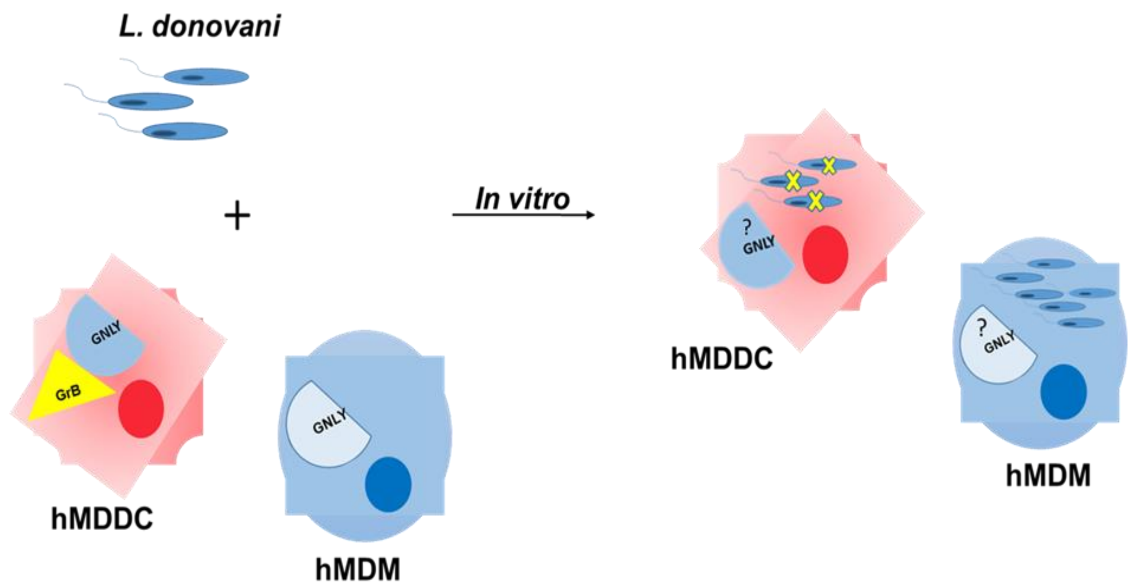


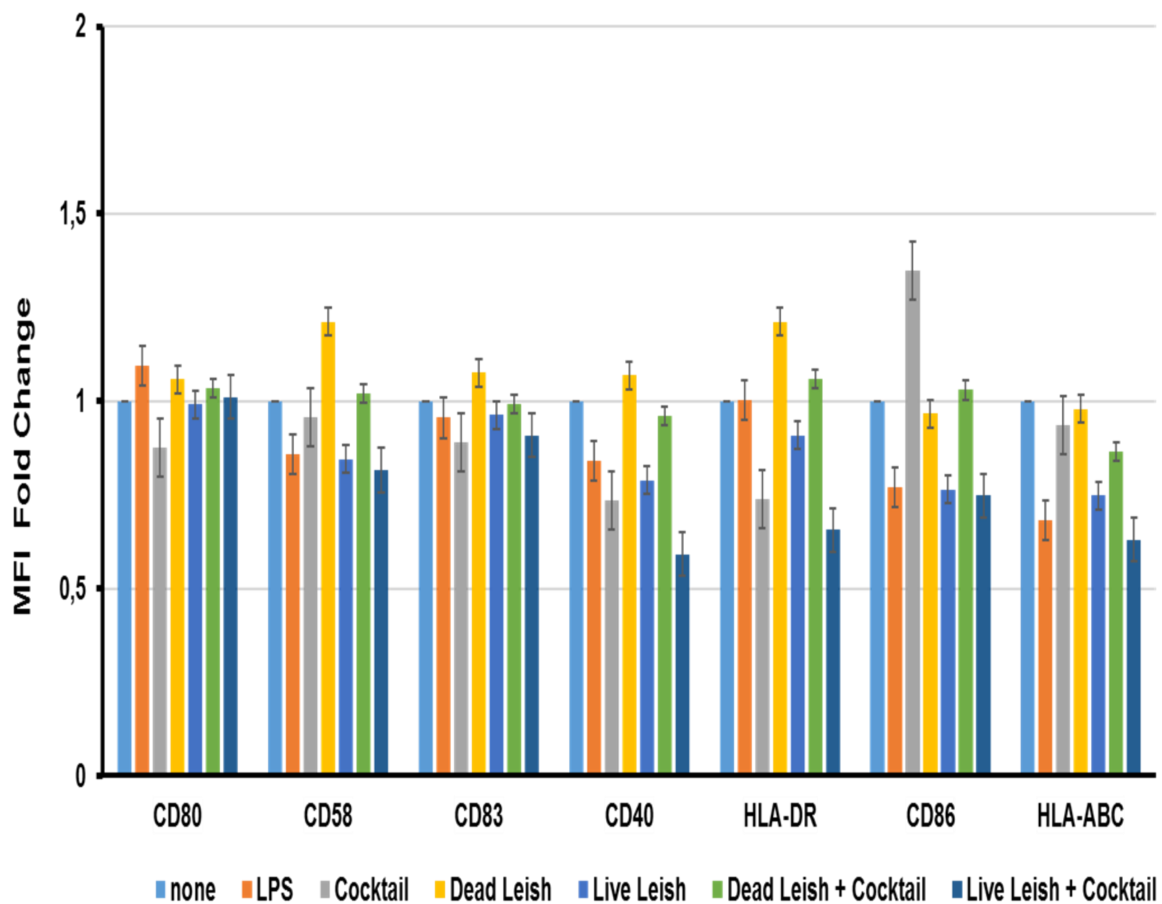
Figure 20: Parasite transformation occurs more efficiently in hMDDCs compared to hMDMs.

During *in vitro* infection with *L. donovani*, the human myeloid cells, hMDDCs and hMDMs differently treated their intracellular phagocytised parasites. The hMDMs couldn't digest these parasites, while the hMDDCs can process them. The role of granzyme B expressed in hDCs was completely figured out in the hMDDCs, although the role of granulysin still not clear in both myeloid cells.

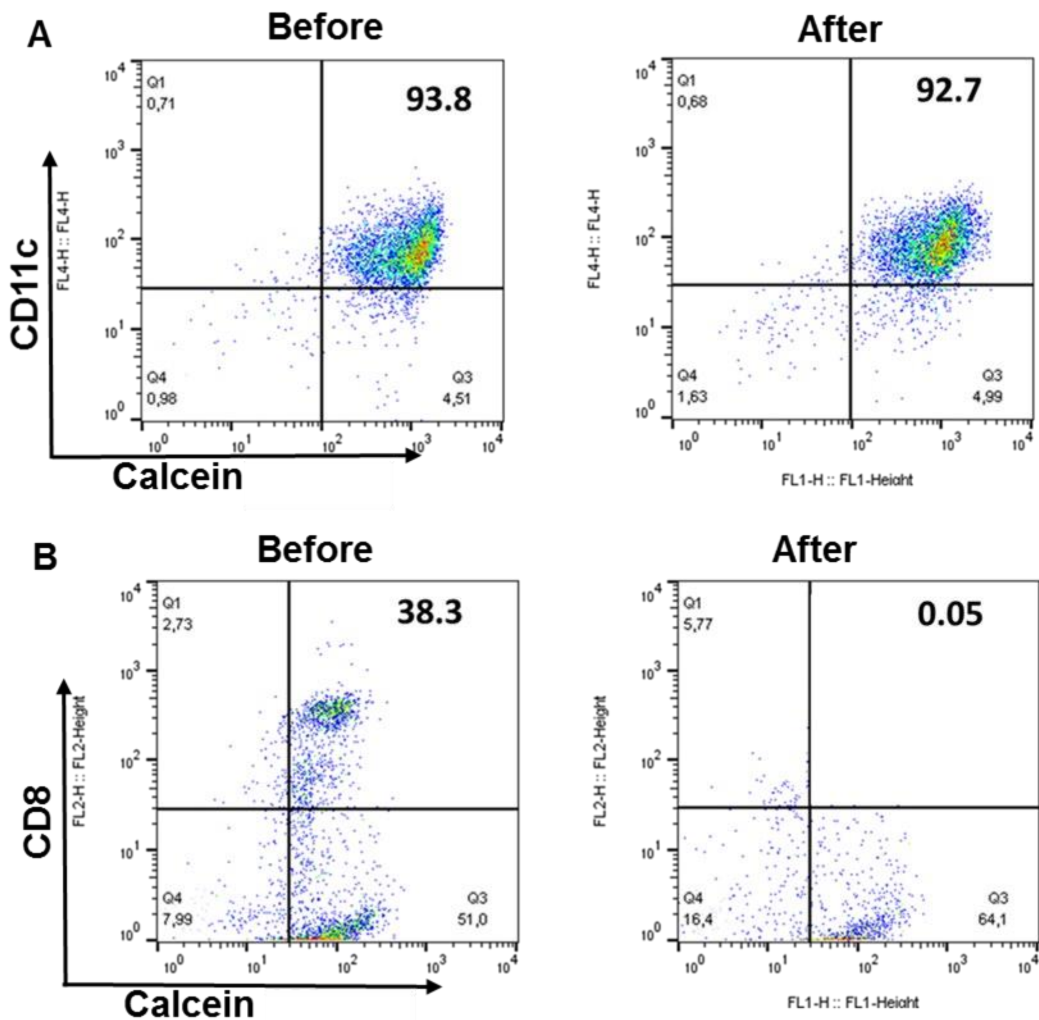
7. Appendix

7.1. Supplementary figure:

hMDMs activation during *Leishmania* infection, hMDM infected with wild-type *L. donovani* for 48h and as a positive control of maturation and activation, the cells were incubated with LPS for 48h. After incubation time the cells were washed and incubated for 25min with fluorescent antibodies against CD11b, CD40, CD58, CD83, CD86, CD80, HLA-DR and HLA-ABC. During flow cytometry is measured, we gated on population of CD11b+ cells and determine the expression of mentioned markers.



7.2 Supplementary figure: T Lymphocytes depletion from PBMCS, hMDDCs contaminated T lymphocyte were incubated with OKT3 Anti-DC3 and Rabbit Complement, the cells were washed and stained for the viability. They incubated for 25min with Calcein AM and fluorescent antibodies against CD8, CD11b and CD11c compared with none-treated cells. The cells stained with specific anti-CD11c and Calcein AM for viability. A) Show the effects of T cell depletion assay on hMDDCs viability. B) Show the of depletion percentage T lymphocytes before and after treatment. C) Show the impact of treatment with the OKT3 and the rabbit complement on the hMDMs viability.



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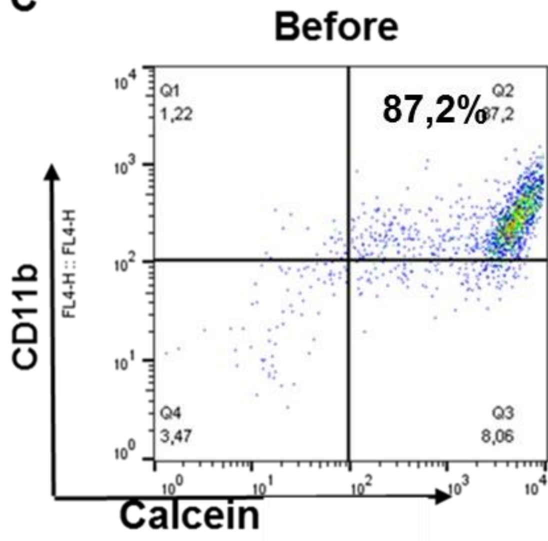

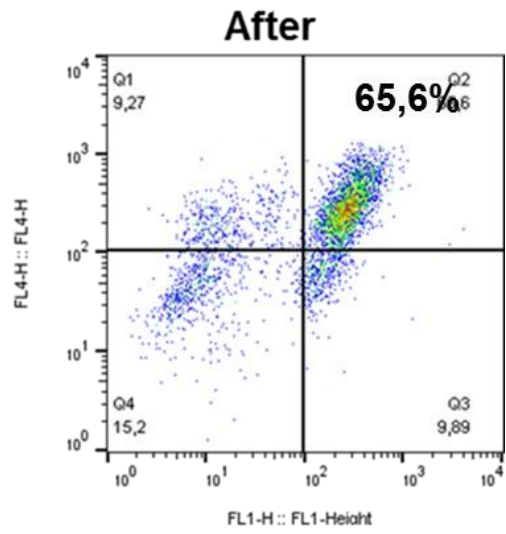

7.3. List of Publications and ongoing projects

1. AA Aljabali, A., A Bakshi, H., L Hakkim, F., A Haggag, Y., M Al-Batanyeh, K., Azzouz IM, M.,…& Pabreja, K. (2020). Albumin Nano-Encapsulation of Piceatannol Enhances Its Anticancer Potential in Colon Cancer Via Downregulation of Nuclear p65 and HIF-1α. *Cancers*. *Cancers* 2020, *12*(1), 113; https://doi.org/10.3390/cancers12010113
(Contribution ≥ 20%)

2. Azzouz IM, Nyambura, L.W., Baleeiro, R.B., Walden, P. Clearance and processing of *Leishmania* parasites by infected human dendritic cells with efficient induction of T cell responses. Ongoing to be publish soon. (Contribution ≥ 70%)

3. Azzouz IM, Nyambura, L.W., Baleeiro, R.B., Walden, P. Interaction of *Leishmania donovani* with human DCs, MΦs, THP1-derived MΦs and MUTZ3-derived DCs. Ongoing to be publish soon
(Contribution ≥ 70%)

8. References

1. World Health, O., *Control of the leishmaniases: report of a meeting of the WHO Expert Commitee on the Control of Leishmaniases, Geneva, 22-26 March 2010*: World Health Organization.

2. Alvar, J., et al., *Leishmaniasis worldwide and global estimates of its incidence.* PloS one. **7**(5): p. e35671.

3. Hotez, P.J., et al., *Combating tropical infectious diseases: report of the Disease Control Priorities in Developing Countries Project.* Clinical infectious diseases, 2004. **38**(6): p. 871-878.

4. Desjeux, P., et al., *Report of the post kala-azar dermal leishmaniasis (PKDL) consortium meeting, New Delhi, India, 27-29 June 2012*, BioMed Central.

5. Seaman, J., A.J. Mercer, and E. Sondorp, *The epidemic of visceral leishmaniasis in western Upper Nile, southern Sudan: course and impact from 1984 to 1994.* International journal of epidemiology, 1996. **25**(4): p. 862-871.

6. Bern, C., J.H. Maguire, and J. Alvar, *Complexities of assessing the disease burden attributable to leishmaniasis.* PLoS neglected tropical diseases, 2008. **2**(10): p. e313.

7. WHO, *South-East Asia poised to defeat visceral leishmaniasis (kala-azar)*. 2015, WHO: Geneva

8. Naeem, A.T., et al., *Clinical features and laboratory findings of visceral leishmaniasis in children referred to Children Medical Center Hospital, Tehran, Iran during 2004-2011.* Iranian journal of parasitology. **9**(1): p. 1.

9. Zijlstra, E.E., et al., *Kala-azar in displaced people from southern Sudan: epidemiological, clinical and therapeutic findings.* Transactions of the Royal Society of Tropical Medicine and Hygiene, 1991. **85**(3): p. 365-369.

10. Siddig, M., et al., *Visceral leishmaniasis in Sudan. Clinical features.* Tropical and geographical medicine, 1990. **42**(2): p. 107-112.

11. Handman, E., *Leishmaniasis: current status of vaccine development.* Clinical microbiology reviews, 2001. **14**(2): p. 229-243.

12. Lodge, R., T.O. Diallo, and A. Descoteaux, *Leishmania donovani lipophosphoglycan blocks NADPH oxidase assembly at the phagosome membrane.* Cellular microbiology, 2006. **8**(12): p. 1922-1931.

13. RITTIG, M. G.; BOGDAN, C. Leishmania–host-cell interaction: complexities and alternative views. *Parasitology Today*, 2000, 16. Jg., Nr. 7, S. 292-297.

14. Sacks, D.L., et al., *An analysis of T cell responsiveness in Indian kala-azar.* The Journal of Immunology, 1987. **138**(3): p. 908-913.

15. Schaefer, K.U., et al., *A prospective sero-epidemiological study of visceral leishmaniasis in Baringo District, Rift Valley Province, Kenya.* Transactions of the Royal Society of Tropical Medicine and Hygiene, 1995. **89**(5): p. 471-475.

16. Cerf, B.J., et al., *Malnutrition as a risk factor for severe visceral leishmaniasis.* The Journal of infectious diseases, 1987. **156**(6): p. 1030-1033.

17. Carvalho, E.M., et al., *Immunologic markers of clinical evolution in children recently infected with Leishmania donovani chagasi.* Journal of Infectious Diseases, 1992. **165**(3): p. 535-540.

18. Blackwell, J.M., H.S. Mohamed, and M.E. Ibrahim, *Genetics and visceral leishmaniasis in the Sudan: seeking a link.* Trends in parasitology, 2004. **20**(6): p. 268-274.

19. Davies, C.R. and A.S.M. Gavgani, *Age, acquired immunity and the risk of visceral leishmaniasis: a prospective study in Iran.* Parasitology, 1999. **119**(3): p. 247-257.

20. Zijlstra, E.E., et al., *Endemic kala-azar in eastern Sudan: a longitudinal study on the incidence of clinical and subclinical infection and post-kala-azar dermal leishmaniasis.* The American journal of tropical medicine and hygiene, 1994. **51**(6): p. 826-836.

21. Evans, T.G., et al., *Epidemiology of visceral leishmaniasis in northeast Brazil.* Journal of Infectious Diseases, 1992. **166**(5): p. 1124-1132.

22. Kaye, P.M., et al., *The immunopathology of experimental visceral leishmaniasis.* Immunological reviews, 2004. **201**(1): p. 239-253.

23. Murray, H.W., *Endogenous interleukin-12 regulates acquired resistance in experimental visceral leishmaniasis.* The Journal of infectious diseases, 1997. **175**(6): p. 1477-1479.

24. Manna, P.P., G. Chakrabarti, and S. Bandyopadhyay, *Innate immune defense in visceral leishmaniasis: cytokine mediated protective role by allogeneic effector cell.* Vaccine. **28**(3): p. 803-810.

25. Moreno, I., et al., *Kinetic analysis of ex vivo human blood infection by Leishmania.* PLoS neglected tropical diseases. **4**(7): p. e743.

26. Reiner, N.E., W. Ng, and W.R. McMaster, *Parasite-accessory cell interactions in murine leishmaniasis. II. Leishmania donovani suppresses macrophage expression of class I and class II major histocompatibility complex gene products.* The Journal of Immunology, 1987. **138**(6): p. 1926-1932.

27. Beil, W.J., et al., *Differences in the onset of the inflammatory response to cutaneous leishmaniasis in resistant and susceptible mice.* Journal of Leukocyte Biology, 1992. **52**(2): p. 135-142.

28. Peters, N.C., et al., *In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies.* Science, 2008. **321**(5891): p. 970-974.

29. MuÈller, K., Zandbergen, G., Hansen, B., Laufs, H., Jahnke, N., Solbach, W., & Laskay, T. (2001). Chemokines, natural killer cells and granulocytes in the early course of Leishmania major infection in mice. *Medical microbiology and immunology*, *190*(1-2), 73-76.

30. Amulic, B., et al., *Neutrophil function: from mechanisms to disease.* Annual review of immunology. **30**: p. 459-489.

31. Charmoy, M.I., et al., *Neutrophil-derived CCL3 is essential for the rapid recruitment of dendritic cells to the site of Leishmania major inoculation in resistant mice.* PLoS pathogens. **6**(2): p. e1000755.

32. Hilda, J.N., M. Narasimhan, and S.D. Das, *Neutrophils from pulmonary tuberculosis patients show augmented levels of chemokines MIP-1Î±, IL-8 and MCP-1 which further increase upon in vitro infection with mycobacterial strains.* Human immunology. **75**(8): p. 914-922.

33. Pham, C.T.N., *Neutrophil serine proteases: specific regulators of inflammation.* Nature Reviews Immunology, 2006. **6**(7): p. 541.

34. Charmoy, M.I., et al., *Leishmania major induces distinct neutrophil phenotypes in mice that are resistant or susceptible to infection.* Journal of Leukocyte Biology, 2007. **82**(2): p. 288-299.

35. Tacchini-Cottier, F., et al., *An immunomodulatory function for neutrophils during the induction of a CD4+ Th2 response in BALB/c mice infected with Leishmania major.* The Journal of Immunology, 2000. **165**(5): p. 2628-2636.

36. Sousa, L.M.A., et al., *Neutrophils have a protective role during early stages of L eishmania amazonensis infection in BALB/c mice.* Parasite immunology. **36**(1): p. 13-31.

37. Gueirard, P., Laplante, A., Rondeau, C., Milon, G., & Desjardins, M. (2008). Trafficking of Leishmania donovani promastigotes in non-lytic compartments in neutrophils enables the subsequent transfer of parasites to macrophages. *Cellular microbiology*, *10*(1), 100-111.

38. Carlsen, E.D., et al., *Interactions between neutrophils and Leishmania braziliensis amastigotes facilitate cell activation and parasite clearance.* Journal of innate immunity. **7**(4): p. 354-363.

39. Falcao, Sarah AC, et al. Exposure to Leishmania braziliensis triggers neutrophil activation and apoptosis. *PLoS neglected tropical diseases*, 2015, 9. Jg., Nr. 3.

40. Laufs, H., et al., *Intracellular survival of Leishmania major in neutrophil granulocytes after uptake in the absence of heat-labile serum factors.* Infection and immunity, 2002. **70**(2): p. 826-835.

41. Tavares, N.M., et al., *Understanding the mechanisms controlling Leishmania amazonensis infection in vitro: the role of LTB4 derived from human neutrophils.* The Journal of infectious diseases. **210**(4): p. 656-666.

42. Afonso, L., et al., *Interactions with apoptotic but not with necrotic neutrophils increase parasite burden in human macrophages infected with Leishmania amazonensis.* Journal of Leukocyte Biology, 2008. **84**(2): p. 389-396.

43. Novais, F.O., et al., *Neutrophils and macrophages cooperate in host resistance against Leishmania braziliensis infection.* The Journal of Immunology, 2009. **183**(12): p. 8088-8098.

44. Conceicao, Jacilara, et al. Characterization of neutrophil function in human cutaneous leishmaniasis caused by Leishmania braziliensis. *PLoS neglected tropical diseases*, 2016, 10. Jg., Nr. 5.

45. Vouldoukis, I., et al., *Interleukin-10 and interleukin-4 inhibit intracellular killing of Leishmania infantum and Leishmania major by human macrophages by decreasing nitric oxide generation.* European journal of immunology, 1997. **27**(4): p. 860-865.

46. Ribeiro-Gomes, F.v.L., et al., *Macrophage interactions with neutrophils regulate Leishmania major infection.* The Journal of Immunology, 2004. **172**(7): p. 4454-4462.

47. Arango Duque, G. and A. Descoteaux, *Macrophage cytokines: involvement in immunity and infectious diseases.* Frontiers in immunology. **5**: p. 491.

48. Brandonisio, O., et al., *Macrophage chemotactic protein-1 and macrophage inflammatory protein-1Î± induce nitric oxide release and enhance parasite killing in Leishmania infantum-infected human macrophages.* Clinical and experimental medicine, 2002. **2**(3): p. 125-129.

49. Faria, D.R., et al., *Decreased in situ expression of interleukin-10 receptor is correlated with the exacerbated inflammatory and cytotoxic responses observed in mucosal leishmaniasis.* Infection and immunity, 2005. **73**(12): p. 7853-7859.

50. Ritter, U. and H. Körner, *Divergent expression of inflammatory dermal chemokines in cutaneous leishmaniasis.* Parasite immunology, 2002. **24**(6): p. 295-301.

51. Murray, H.W. and R.F. Teitelbaum, *L-arginine-dependent reactive nitrogen intermediates and the antimicrobial effect of activated human mononuclear phagocytes.* Journal of Infectious Diseases, 1992. **165**(3): p. 513-517.

52. Assreuy, J., et al., *Production of nitric oxide and superoxide by activated macrophages and killing of Leishmania major.* European journal of immunology, 1994. **24**(3): p. 672-676.

53. Liu, D. and J.E. Uzonna, *The early interaction of Leishmania with macrophages and dendritic cells and its influence on the host immune response.* Frontiers in cellular and infection microbiology. **2**: p. 83.

54. Steinman, R.M. and M.D. Witmer, *Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice.* Proceedings of the National Academy of Sciences, 1978. **75**(10): p. 5132-5136.

55. Ziegler-Heitbrock, L., et al., *Nomenclature of monocytes and dendritic cells in blood.* Blood. **116**(16): p. e74-e80.

56. Dzionek, A., et al., *BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood.* The Journal of Immunology, 2000. **165**(11): p. 6037-6046.

57. e Sousa, C.R., *Activation of dendritic cells: translating innate into adaptive immunity.* Current opinion in immunology, 2004. **16**(1): p. 21-25.

58. Sundquist, M., A. Rydström, and M.J. Wick, *Immunity to Salmonella from a dendritic point of view.* Cellular microbiology, 2004. **6**(1): p. 1-11.

59. Moll, H., *Dendritic cells and host resistance to infection.* Cellular microbiology, 2003. **5**(8): p. 493-500.

60. Geijtenbeek, T.B.H., et al., *Self-and nonself-recognition by C-type lectins on dendritic cells.* Annu. Rev. Immunol., 2004. **22**: p. 33-54.

61. Stebut, B.E.V., et al., *Uptake of Leishmania major Amastigotes Results in Skin-derived Dendritic Cells: Implications for the Initiation of Anti-Leishmania Immunity.* The Journal of experimental medicine, 1998. **188**(8).

62. Takeda, K. and S. Akira, *Microbial recognition by Toll-like receptors.* Journal of dermatological science, 2004. **34**(2): p. 73-82.

63. Marovich, M.A., et al., *IL-12p70 production by Leishmania major-harboring human dendritic cells is a CD40/CD40 ligand-dependent process.* The Journal of Immunology, 2000. **164**(11): p. 5858-5865.

64. Crauwels, P., *The interaction of Leishmania major parasites with human myeloid cells and its consequence for adaptive immunity*, in *Biology*. 2014, Der Johannes Gutenberg-Universität: Mainz. p. 153.

65. Kima, P.E., et al., *Leishmania-infected macrophages sequester endogenously synthesized parasite antigens from presentation to CD4+ T cells.* European journal of immunology, 1996. **26**(12): p. 3163-3169.

66. Liew, F.Y., Y. Li, and S. Millott, *Tumour necrosis factor (TNF-alpha) in leishmaniasis. II. TNF-alpha-induced macrophage leishmanicidal activity is mediated by nitric oxide from L-arginine.* Immunology, 1990. **71**(4): p. 556.

67. Gordon, S., *Alternative activation of macrophages.* Nature Reviews Immunology, 2003. **3**(1): p. 23.

68. Kane, M.M. and D.M. Mosser, *Leishmania parasites and their ploys to disrupt macrophage activation.* Current opinion in hematology, 2000. **7**(1): p. 26-31.

69. Kane, M.M. and D.M. Mosser, *The role of IL-10 in promoting disease progression in leishmaniasis.* The Journal of Immunology, 2001. **166**(2): p. 1141-1147.

70. Panitz, V.M., *Regulation of human granzyme B-producing plasmacytoid dendritic cells by viral stimuli*, Universität Ulm.

71. Mellman, I., *Endocytosis and molecular sorting.* Annual review of cell and developmental biology, 1996. **12**(1): p. 575-625.

72. Arandjelovic, S. and K.S. Ravichandran, *Phagocytosis of apoptotic cells in homeostasis.* Nature immunology. **16**(9): p. 907.

73. Blander, J.M. and R. Medzhitov, *On regulation of phagosome maturation and antigen presentation.* Nature immunology, 2006. **7**(10): p. 1029.

74. de Carvalho, T.M.U., E.S. Barrias, and W. de Souza, *Macropinocytosis: a pathway to protozoan infection.* Frontiers in physiology. **6**: p. 106.

75. Russell, B.H., et al., *Potential dissemination of Bacillus anthracis utilizing human lung epithelial cells.* Cellular microbiology, 2008. **10**(4): p. 945-957.

76. Vyas, N., et al., *Nanoscale organization of hedgehog is essential for long-range signaling.* Cell, 2008. **133**(7): p. 1214-1227.

77. Verpelli, C., et al., *Comparative neuronal differentiation of self-renewing neural progenitor cell lines obtained from human induced pluripotent stem cells.* Frontiers in Cellular Neuroscience. **7**: p. 175.

78. Neefjes, J., et al., *Towards a systems understanding of MHC class I and MHC class II antigen presentation.* Nature Reviews Immunology. **11**(12): p. 823.

79. Wu, J., V. Groh, and T. Spies, *T cell antigen receptor engagement and specificity in the recognition of stress-inducible MHC class I-related chains by human epithelial γδ T cells.* The Journal of Immunology, 2002. **169**(3): p. 1236-1240.

80. Beck, S. and J. Trowsdale, *The human major histocompatibility complex: lessons from the DNA sequence.* Annual review of genomics and human genetics, 2000. **1**(1): p. 117-137.

81. Mack, S.J., et al., *Common and well-documented HLA alleles: 2012 update to the CWD catalogue.* Tissue antigens. **81**(4): p. 194-203.

82. Gewurz, B.E., et al., *Antigen presentation subverted: structure of the human cytomegalovirus protein US2 bound to the class I molecule HLA-A2.* Proceedings of the National Academy of Sciences, 2001. **98**(12): p. 6794-6799.

83. Zhang, N. and M.J. Bevan, *CD8+ T cells: foot soldiers of the immune system.* Immunity. **35**(2): p. 161-168.

84. Ploegh, H.L., *Viral strategies of immune evasion.* Science, 1998. **280**(5361): p. 248-253.

85. Borrego, F., et al., *Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis.* Journal of Experimental Medicine, 1998. **187**(5): p. 813-818.

86. Nickoloff, B.J. and L.A. Turka, *Immunological functions of non-professional antigen-presenting cells: new insights from studies of T-cell interactions with keratinocytes.* Immunology today, 1994. **15**(10): p. 464-469.

87. Alberts, B., et al., *Molecular Biology of the Cell, Garland Science; 2002.* Bray D. Cell movements: from molecules to motility. 2nd ed: Garland Science, 2000.

88. Maffei, A. and P.E. Harris, *Peptides bound to major histocompatibility complex molecules.* Peptides, 1998. **19**(1): p. 179-198.

89. Price, P., et al., *The genetic basis for the association of the 8.1 ancestral haplotype (A1, B8, DR3) with multiple immunopathological diseases.* Immunological reviews, 1999. **167**(1): p. 257-274.

90. Meddeb-Garnaoui, A., et al., *Association analysis of HLA-class II and class III gene polymorphisms in the susceptibility to mediterranean visceral leishmaniasis.* Human immunology, 2001. **62**(5): p. 509-517.

91. Peacock, C.S., et al., *Genetic analysis of multicase families of visceral leishmaniasis in northeastern Brazil: no major role for class II or class III regions of HLA.* Genes and immunity, 2002. **3**(6): p. 350.

92. Olivo-Díaz, A.I., et al., *Role of HLA class II alleles in susceptibility to and protection from localized cutaneous leishmaniasis.* Human immunology, 2004. **65**(3): p. 255-261.

93. Faghiri, Z., S.Z. Tabei, and F. Taheri, *Study of the association of HLA class I antigens with kala-azar.* Human heredity, 1995. **45**(5): p. 258-261.

94. Petzl-Erler, M.L., M.P. Belich, and F. Queiroz-Telles, *Association of mucosal leishmaniasis with HLA.* Human immunology, 1991. **32**(4): p. 254-260.

95. Morsy, T.A., et al., *Histocompatibility (HLA) antigens in Egyptians with two parasitic skin diseases (scabies and leishmaniasis).* Journal of the Egyptian Society of Parasitology, 1990. **20**(2): p. 565-572.

96. Lal, S., et al., *Role of HLA-DR antigen on T-cell activation in visceral leishmaniasis.* Indian journal of experimental biology, 1991. **29**(12): p. 1101-1103.

97. Roy, K., et al., *Class II MHC/peptide interaction in Leishmania donovani infection: implications in vaccine design.* The Journal of Immunology. **192**(12): p. 5873-5880.

98. Nyambura, L.W., *Impact of monocyte differentiation and intracellular infection on processing and presentation of autoantigen.*

99. Shen, Z., et al., *Cloned dendritic cells can present exogenous antigens on both MHC class I and class II molecules.* The Journal of Immunology, 1997. **158**(6): p. 2723-2730.

100. Sigal, L.J., et al., *Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen.* Nature, 1999. **398**(6722): p. 77.

101. Schulz, O., et al., *CD36 or αvβ3 and αvβ5 integrins are not essential for MHC class I cross-presentation of cell-associated antigen by CD8α+ murine dendritic cells.* The Journal of Immunology, 2002. **168**(12): p. 6057-6065.

102. Malherbe, L., et al., *Selective activation and expansion of high-affinity CD4+ T cells in resistant mice upon infection with Leishmania major.* Immunity, 2000. **13**(6): p. 771-782.

103. Jung, S., et al., *In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens.* Immunity, 2002. **17**(2): p. 211-220.

104. Heinzel, F.P., et al., *Reciprocal expression of interferon gamma or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets.* Journal of Experimental Medicine, 1989. **169**(1): p. 59-72.

105. Belkaid, Y., et al., *CD8+ T cells are required for primary immunity in C57BL/6 mice following low-dose, intradermal challenge with Leishmania major.* The Journal of Immunology, 2002. **168**(8): p. 3992-4000.

106. Faria, D.R., et al., *Recruitment of CD8+ T cells expressing granzyme A is associated with lesion progression in human cutaneous leishmaniasis.* Parasite immunology, 2009. **31**(8): p. 432-439.

107. Uzonna, J.E., K.L. Joyce, and P. Scott, *Low dose Leishmania major promotes a transient T helper cell type 2 response that is down-regulated by interferon γ–producing CD8+ T cells.* Journal of Experimental Medicine, 2004. **199**(11): p. 1559-1566.

108. da Silva, S.C., et al., *CD8 (+) Granzyme B (+)-Mediated Tissue Injury versus CD4 (+) IFNγ (+)-Mediated Parasite Killing in Human Cutaneous Leishmaniasis.* The Journal of investigative dermatology. **134**(11): p. 2850.

109. Manson-Bahr, P.E.C., *Immunity in Kala-Azar*.* Transactions of the Royal Society of Tropical Medicine and Hygiene, 1961. **55**(6): p. 550-555.

110. Chowdhury, D. and J. Lieberman, *Death by a thousand cuts: granzyme pathways of programmed cell death.* Annu. Rev. Immunol., 2008. **26**: p. 389-420.

111. Haldar, J.P., et al., *Cell-mediated immune response in Indian kala-azar and post-kala-azar dermal leishmaniasis.* Infection and immunity, 1983. **42**(2): p. 702-707.

112. Kim, W.-J., et al., *Macrophages express granzyme B in the lesion areas of atherosclerosis and rheumatoid arthritis.* Immunology letters, 2007. **111**(1): p. 57-65.

113. Garg, R., et al.,*Immunostimulatory cellular responses of cured Leishmania-infected patients and hamsters against the integral membrane proteins and non-membranous soluble proteins of a recent clinical isolate of Leishmania donovani.* Clinical & Experimental Immunology, 2005. **140**(1): p. 149-156.

114. Ho, M., et al., *Immunosuppression in Kenyan visceral leishmaniasis.* Clinical and experimental immunology, 1983. **51**(2): p. 207.

115. Hagn, M., et al., *Human B cells secrete granzyme B when recognizing viral antigens in the context of the acute phase cytokine IL-21.* The Journal of Immunology, 2009. **183**(3): p. 1838-1845.

116. Strik, M.C.M., et al., *Human mast cells produce and release the cytotoxic lymphocyte associated protease granzyme B upon activation.* Molecular immunology, 2007. **44**(14): p. 3462-3472.

117. Karrich, J.J., et al., *IL-21-stimulated human plasmacytoid dendritic cells secrete granzyme B, which impairs their capacity to induce T-cell proliferation.* Blood. **121**(16): p. 3103-3111.

118. Jahrsdörfer, B., et al., *Granzyme B produced by human plasmacytoid dendritic cells suppresses T-cell expansion.* Blood. **115**(6): p. 1156-1165.

119. Tamang, D.L., et al., *Induction of granzyme B and T cell cytotoxic capacity by IL-2 or IL-15 without antigens: multiclonal responses that are extremely lytic if triggered and short-lived after cytokine withdrawal.* Cytokine, 2006. **36**(3-4): p. 148-159.

120. Meyer, T., et al., *poly (I: C) costimulation induces a stronger antiviral chemokine and granzyme B release in human CD4 T cells than CD28 costimulation.* Journal of Leukocyte Biology. **92**(4): p. 765-774.

121. Appay, V., et al., *Characterization of CD4+ CTLs ex vivo.* The Journal of Immunology, 2002. **168**(11): p. 5954-5958.

122. Brown, D.M., *Cytolytic CD4 cells: Direct mediators in infectious disease and malignancy.* Cellular immunology. **262**(2): p. 89-95.

123. Grossman, W.J., et al., *Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells.* Blood, 2004. **104**(9): p. 2840-2848.

124. Niiya, H., et al., *Differential regulation of perforin expression in human CD4+ and CD8+ cytotoxic T lymphocytes.* Experimental hematology, 2005. 33(7): p. 811-818.

125. Cai, S.F., et al., *Differential expression of granzyme B and C in murine cytotoxic lymphocytes.* The Journal of Immunology, 2009. 182(10): p. 6287-6297.

126. Hildemann, S.K., et al., *High efficiency of antiviral CD4+ killer T cells.* PloS one. **8**(4): p. e60420.

127. Swihart, K., et al., *Mice from a genetically resistant background lacking interferon γ receptor are susceptible to infection with Leishmania major but mount a polarized T helper cell 1-type CD4+ T cell response.* Journal of Experimental Medicine, 1995. 181(3): p. 961-971.

128. Sakahira, H., M. Enari, and S. Nagata, *Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis.* Nature, 1998. **391**(6662): p. 96-99.

129. Trapani, J.A. and M.J. Smyth, *Functional significance of the perforin/granzyme cell death pathway.* Nature Reviews Immunology, 2002. **2**(10): p. 735.

130. Kummer, J.A., et al., *Production and characterization of monoclonal antibodies raised against recombinant human granzymes A and B and showing cross reactions with the natural proteins.* Journal of immunological methods, 1993. **163**(1): p. 77-83.

131. Fan, Z., et al., *Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor.* Cell, 2003. **112**(5): p. 659-672.

132. Krensky, A.M. and C. Clayberger, *Biology and clinical relevance of granulysin.* Tissue antigens, 2009. **73**(3): p. 193-198.Stenger, S., et al., An antimicrobial activity of cytolytic T cells mediated by granulysin. Science, 1998. 282(5386): p. 121-125.

133. Goping, S., et al., Granzyme B-induced apoptosis requires both direct caspase activation and relief of caspase inhibition. Immunity, 2003. 18(3): p. 355-365.

134. Barry, M. and R.C. Bleackley, Cytotoxic T lymphocytes: all roads lead to death. Nature Reviews Immunology, 2002. 2(6): p. 401.

135. Jongstra, J., et al., The isolation and sequence of a novel gene from a human functional T cell line. Journal of Experimental Medicine, 1987. 165(3): p. 601-614.

136. Walch, M., et al., Involvement of CD252 (CD134L) and IL-2 in the expression of cytotoxic proteins in bacterial-or viral-activated human T cells. The Journal of Immunology, 2009. 182(12): p. 7569-7579.

137. Peña, S.V., et al., Processing, subcellular localization, and function of 519 (granulysin), a human late T cell activation molecule with homology to small, lytic, granule proteins. The Journal of Immunology, 1997. 158(6): p. 2680-2688.

138. Ochoa, M.-T., et al., T-cell release of granulysin contributes to host defense in leprosy. Nature medicine, 2001. 7(2): p. 174.

139. Su, A.I., et al., A gene atlas of the mouse and human protein-encoding transcriptomes. Proceedings of the National Academy of Sciences, 2004. 101(16): p. 6062-6067.

140. Kishi, A., et al., Differential expression of granulysin and perforin by NK cells in cancer patients and correlation of impaired granulysin expression with progression of cancer. Cancer Immunology, Immunotherapy, 2002. 50(11): p. 604-614.

141. Kägi, D., et al., Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature, 1994. 369(6475): p. 31.

142. Dotiwala, F., et al., Killer lymphocytes use granulysin, perforin and granzymes to kill intracellular parasites. Nature medicine. 22(2): p. 210.

143. Di Liberto, D., et al., Decreased serum granulysin levels in childhood tuberculosis which reverse after therapy. Tuberculosis, 2007. 87(4): p. 322-328.

144. Saini, R.V., et al., Granulysin delivered by cytotoxic cells damages endoplasmic reticulum and activates caspase-7 in target cells. The Journal of Immunology. 186(6): p. 3497-3504.

145. Shresta, S., et al., Natural killer and lymphokine-activated killer cells require granzyme B for the rapid induction of apoptosis in susceptible target cells. Proceedings of the National Academy of Sciences, 1995. 92(12): p. 5679-5683.

146. Rauch, S., et al., New vaccine technologies to combat outbreak situations. Frontiers in immunology. 9.

147. Mendez, S., et al., Coinjection with CpG-containing immunostimulatory oligodeoxynucleotides reduces the pathogenicity of a live vaccine against cutaneous Leishmaniasis but maintains its potency and durability. Infection and immunity, 2003. 71(9): p. 5121-5129.

148. Davoudi, N., et al., Development of a recombinant Leishmania major strain sensitive to ganciclovir and 5-fluorocytosine for use as a live vaccine challenge in clinical trials. Vaccine, 2005. 23(9): p. 1170-1177.

149. Forgber, Michael, et al. "Mapping the antigenicity of the parasites in Leishmania donovani infection by proteome serology." *PloS one* 1.1 (2006): e40.

150. An E-mail interview with Dr. Pradeep Das. *Trop Parasitol.* 2018;8(2):127–131. doi:10.4103/tp.TP_57_18

151. Santos-Mateus, D., et al. "The battle between Leishmania and the host immune system at a glance." *Int Trends Immun* 4.1 (2016): 28-34.

152. Peters, Nathan C., and David L. Sacks. "The impact of vector-mediated neutrophil recruitment on cutaneous leishmaniasis." *Cellular microbiology* 11.9 (2009): 1290-1296.

153. Beattie, Lynette, and Paul M. Kaye. "Leishmania–host interactions: what has imaging taught us?" *Cellular microbiology* 13.11 (2011): 1659-1667.

154. Moll, Heidrun, et al. "Langerhans cells transport Leishmania major from the infected skin to the draining lymph node for presentation to antigen-specific T cells." *European journal of immunology* 23.7 (1993): 1595-1601.

155. Liese, J., Schleicher, U. and Bogdan, C. 2007. TLR9 signaling is essential for the innate NK cell response in murine cutaneous leishmaniasis. *Eur. J. Immunol.* 37:3424.

156. Silveira, F. T., Lainson, R., De Castro Gomes, C. M., Laurenti, M. D. and Corbett, C. E. 2009. Immunopathogenic competences of *Leishmania* (V.) *braziliensis* and *L. (L.) amazonensis* in American cutaneous leishmaniasis. *Parasite Immunol.* 31:423.

157. Vargas-Inchaustegui, D. A., Xin, L. and Soong, L. 2008. *Leishmania braziliensis* infection induces dendritic cell activation, ISG15 transcription, and the generation of protective immune responses. *J. Immunol.* 180:7537.

158. Agallou, M., Margaroni, M. and Karagouni, E. 2011. Cellular vaccination with bone marrow-derived dendritic cells pulsed with a peptide of *Leishmania infantum* KMP-11 and CpG oligonucleotides induces protection in a murine model of visceral leishmaniasis. *Vaccine* 29:5053.

159. Lopez Kostka, S., Dinges, S., Griewank, K., Iwakura, Y., Udey, M. C. and von Stebut, E. 2009. IL-17 promotes progression of cutaneous leishmaniasis in susceptible mice. *J. Immunol.* 182:3039.

160. Hartley, M. A., Bourreau, E., Rossi, M. *et al.* 2016. Leishmania virus dependent metastatic leishmaniasis is prevented by blocking IL-17A. *PLoS Pathog.* 12:e1005852.

161. Belkaid, Y., Piccirillo, C. A., Mendez, S., Shevach, E. M. and Sacks, D. L. 2002. CD4+CD25+ regulatory T cells control *Leishmania major* persistence and immunity. *Nature* 420:502.

162. Uzonna, J. E., Joyce, K. L. and Scott, P. 2004. Low dose *Leishmania major* promotes a transient T helper cell type 2 response that is down-regulated by interferon gamma-producing CD8+ T cells. *J. Exp. Med.* 199:1559.

163. Smelt, S. C., Cotterell, S. E., Engwerda, C. R. and Kaye, P. M. 2000. B cell-deficient mice are highly resistant to *Leishmania donovani* infection, but develop neutrophil-mediated tissue pathology. *J. Immunol.* 164:3681.

164. Calvopina, M., Barroso, P. A., Marco, J. D. *et al.* 2006. Efficacy of vaccination with a combination of *Leishmania* amastigote antigens and the lipid A-analogue

ONO-4007 for immunoprophylaxis and immunotherapy against *Leishmania amazonensis* infection in a murine model of New World cutaneous leishmaniasis. *Vaccine* 24:5645.

165. Gregory, D. J., and M. Olivier. "Subversion of host cell signalling by the protozoan parasite Leishmania." *Parasitology* 130.S1 (2005): S27-S35.

166. Xin, Lijun, Kui Li, and Lynn Soong. "Down-regulation of dendritic cell signaling pathways by Leishmania amazonensis amastigotes." *Molecular immunology* 45.12 (2008): 3371-3382.

167. Ribeiro-Gomes, Flavia L., et al. "Neutrophils activate macrophages for intracellular killing of Leishmania major through recruitment of TLR4 by neutrophil elastase." *The Journal of Immunology* 179.6 (2007): 3988-3994.

168. Seger, Rony, and Edwin G. Krebs. "The MAPK signaling cascade." *The FASEB journal* 9.9 (1995): 726-735.

169. Zijlstra, E.E., et al., Endemic kala-azar in eastern Sudan: a longitudinal study on the incidence of clinical and subclinical infection and post-kala-azar dermal leishmaniasis. The American journal of tropical medicine and hygiene, 1994. 51(6): p. 826-836.

170. Matsui, T., et al., CD2 distinguishes two subsets of human plasmacytoid dendritic cells with distinct phenotype and functions. The Journal of Immunology, 2009. 182(11): p. 6815-6823.

171. Stenger, S., K.R. Niazi, and R.L. Modlin, Down-regulation of CD1 on antigen-presenting cells by infection with Mycobacterium tuberculosis. The Journal of Immunology, 1998. 161(7): p. 3582-3588.

172. Lindner, S., et al., Interleukin 21-induced granzyme B-expressing B cells infiltrate tumors and regulate T cells. Cancer research. 73(8): p. 2468-2479.

173. Eiras, D.P., L.A. Kirkman, and H.W. Murray, Cutaneous leishmaniasis: current treatment practices in the USA for returning travelers. Current treatment options in infectious diseases. 7(1): p. 52-62.

174. Saxena, A., et al., Analysis of the Leishmania donovani transcriptome reveals an ordered progression of transient and permanent changes in gene expression

during differentiation. Molecular and biochemical parasitology, 2007. 152(1): p. 53-65.

175. Laskay, T.s., G. van Zandbergen, and W. Solbach, Neutrophil granulocytes as host cells and transport vehicles for intracellular pathogens: apoptosis as infection-promoting factor. Immunobiology, 2008. 213(3-4): p. 183-191.

176. Wilson, M.E. and R.D. Pearson, Immunology of leishmaniasis. Modern parasite biology: cellular, immunological, and molecular aspects., 1990: p. 200-221.

177. World Health, O., Control of the leishmaniases: report of a meeting of the WHO Expert Commitee on the Control of Leishmaniases, Geneva, 22-26 March 2010: World Health Organization.

178. Lainson, R., et al., The leishmaniases in biology and medicine, vol. 1 (biology and epidemiology). 1987.

179. Liew, F.Y. and C.A. O'Donnell, Immunology of leishmaniasis, in Advances in parasitology. 1993, Elsevier. p. 161-259.

180. Handman, E., Cell biology of Leishmania, in Advances in parasitology. 1999, Elsevier. p. 1-39.

181. Rittig, M.G. and C. Bogdan, Leishmania-host-cell interaction: complexities and alternative views. Parasitology today, 2000. 16(7): p. 292-297.

182. Woelbing, F., et al., Uptake of Leishmania major by dendritic cells is mediated by FcÎ³ receptors and facilitates acquisition of protective immunity. Journal of Experimental Medicine, 2006. 203(1): p. 177-188.

183. Kaech, S.M., et al., Molecular and functional profiling of memory CD8 T cell differentiation. Cell, 2002. 111(6): p. 837-851.

184. Winau, F., et al., Apoptotic vesicles crossprime CD8 T cells and protect against tuberculosis. Immunity, 2006. 24(1): p. 105-117

185. Berthou, C., et al., Granzyme B and perforin lytic proteins are expressed in CD34+ peripheral blood progenitor cells mobilized by chemotherapy and granulocyte colony-stimulating factor. Blood, 1995. 86(9): p. 3500-3506.

186. Hagn, M., et al., Human B cells secrete granzyme B when recognizing viral antigens in the context of the acute phase cytokine IL-21. The Journal of Immunology, 2009. 183(3): p. 1838-1845.

187. Hagn, M., et al., Human B cells differentiate into granzyme B-secreting cytotoxic B lymphocytes upon incomplete T-cell help. Immunology and cell biology. 90(4): p. 457-467.

188. Tschopp, C.M., et al., Granzyme B, a novel mediator of allergic inflammation: its induction and release in blood basophils and human asthma. Blood, 2006. 108(7): p. 2290-2299.

189. Strik, M.C.M., et al., Human mast cells produce and release the cytotoxic lymphocyte associated protease granzyme B upon activation. Molecular immunology, 2007. 44(14): p. 3462-3472.

190. Wagner, C., et al., Granzyme B and perforin: constitutive expression in human polymorphonuclear neutrophils. Blood, 2004. 103(3): p. 1099-1104.

191. Stenger, S. and R.L. Modlin, Cytotoxic T cell responses to intracellular pathogens. Current opinion in immunology, 1998. 10(4): p. 471-477.

192. Martin, S.J., et al., The cytotoxic cell protease granzyme B initiates apoptosis in a cell-free system by proteolytic processing and activation of the ICE/CED-3 family protease, CPP32, via a novel two-step mechanism. The EMBO Journal, 1996. 15(10): p. 2407-2416.

193. Medema, J.P., et al., Cleavage of FLICE (caspase-8) by granzyme B during cytotoxic T lymphocyte-induced apoptosis. European journal of immunology, 1997. 27(12): p. 3492-3498.

194. Andrade, F., et al., Granzyme B directly and efficiently cleaves several downstream caspase substrates: implications for CTL-induced apoptosis. Immunity, 1998. 8(4): p. 451-460.

195. Barry, M., et al., Granzyme B short-circuits the need for caspase 8 activity during granule-mediated cytotoxic T-lymphocyte killing by directly cleaving Bid. Molecular and cellular biology, 2000. 20(11): p. 3781-3794.

196. Waterhouse, N.J., et al., A central role for Bid in granzyme B-induced apoptosis. Journal of Biological Chemistry, 2005. 280(6): p. 4476-4482.

197. Alimonti, J.B., et al., Granzyme B induces BID-mediated cytochrome c release and mitochondrial permeability transition. Journal of Biological Chemistry, 2001. 276(10): p. 6974-6982.

198. Sharif-Askari, E., et al., Direct cleavage of the human DNA fragmentation factor-45 by granzyme B induces caspase-activated DNase release and DNA fragmentation. The EMBO Journal, 2001. 20(12): p. 3101-3113.

199. Thomas, D.A., et al., DFF45/ICAD can be directly processed by granzyme B during the induction of apoptosis. Immunity, 2000. 12(6): p. 621-632.

200. Cremer, C., et al., Engineered versions of granzyme b and angiogenin overcome intrinsic resistance to apoptosis mediated by human cytolytic fusion proteins, in Resistance to Immunotoxins in Cancer Therapy, Springer. p. 185-219.

201. Jenne, D.E. and J.r. Tschopp, Granzymes, a family of serine proteases released from granules of cytolytic T lymphocytes upon T cell receptor stimulation. Immunological reviews, 1988. 103(1): p. 53-71.

202. Lieberman, J., Cell death and immunity: the ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. Nature Reviews Immunology, 2003. 3(5): p. 361.

203. Heusel, J.W., et al., Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells. Cell, 1994. 76(6): p. 977-987.

204. Mayer, M.S., et al., Development of a DNA marker for Fusarium wilt resistance in chickpea. Crop Science, 1997. 37(5): p. 1625-1629.

205. Marovich, M.A., et al., IL-12p70 production by Leishmania major-harboring human dendritic cells is a CD40/CD40 ligand-dependent process. The Journal of Immunology, 2000. 164(11): p. 5858-5865.

206. Anthony, D.A., et al., Functional dissection of the granzyme family: cell death and inflammation. Immunological reviews. 235(1): p. 73-92.

207. Huang, L., M. Hinchman, and S. Mendez, Coinjection with TLR2 agonist Pam3CSK4 reduces the pathology of leishmanization in mice. PLoS neglected tropical diseases. 9(3): p. e0003546.

208. Chandel, H.S., et al., Toll-like receptors and CD 40 modulate each other's expression affecting L eishmania major infection. Clinical & Experimental Immunology. 176(2): p. 283-290.

209. Butler, M., et al., Modulation of dendritic cell phenotype and functionin an in vitro model of the intestinal epithelium. European journal of immunology, 2006. 36(4): p. 864-874.

210. Von Stebut, E., et al., Uptake of Leishmania major amastigotes results in activation and interleukin 12 release from murine skin-derived dendritic cells: implications for the initiation of anti-Leishmania immunity. Journal of Experimental Medicine, 1998. 188(8): p. 1547-1552.

211. De Trez, C., et al., iNOS-producing inflammatory dendritic cells constitute the major infected cell type during the chronic Leishmania major infection phase of C57BL/6 resistant mice. PLoS pathogens, 2009. 5(6): p. e1000494.

212. Iborra, S., et al., Leishmania uses mincle to target an inhibitory ITAM signaling pathway in dendritic cells that dampens adaptive immunity to infection. Immunity. 45(4): p. 788-801.