ACIBADEM MEHMET ALI AYDINLAR UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

ESTABLISHMENT OF INTRACELLULAR INFECTION AND ASSESSMENT OF THE DEVELOPING IMMUNE RESPONSE IN CELL CULTURES WITH DIFFERENT LEISHMANIA SPECIES OBTAINED FROM CLINICAL SAMPLES IN TURKEY AND PROTEOMIC CHARACTERIZATION OF LEISHMANIA SURFACE PROTEINS

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PH.D. THESIS

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Department:	Medical Biotechnology
Program:	Medical Biotechnology Ph.D. Program
Thesis Title:	Establishment of intracellular infection and assessment of the developing immune response in cell cultures with different *Leishmania* species obtained from clinical samples in Turkey and proteomic characterization of *Leishmania* surface proteins.
Student's name and Surname:	Nesteren MANSUR ÖZEN
Date of Defense:	12/04/2022

This is to certify that I have examined this copy of Ph.D. thesis. I have found that she prepared after fulfilling specified requirements in the associated legislations before the final examining committee whose signatures are below.

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DECLARATION

I declare that this thesis work is my own work, I had no unethical behavior at any stages from the planning to the writing of the thesis, I obtained all the information in this thesis in accordance with academic and ethical rules, I cited all the information and comments that were not obtained with this thesis work, and I provided resources in the list of references. I also declare that there was no violation of any patents and copyrights during the study and writing of this thesis.

10/05/2022

Nesteren MANSUR ÖZEN

PREFACE AND ACKNOWLEDGEMENT

I would like to thank to my thesis supervisor Prof. Dr. Özgür KURT for guiding me into the fascinating world of parasites and providing an opportunity to carry out this thesis with his advices.

I also want to thank Prof. Dr. Tanıl KOCAGÖZ who directed me to the work with surface proteins and shared his opinions with me. I also appreciate Prof. Dr. Ahmet ÖZBİLGİN for his help on *Leishmania* strains and related techniques that allowed me to carry out the thesis. I also want to thank to Orhan ÖZCAN for his suggestions on proteome experiments.

I have a special thanks to my dearest friend and colleague Assist. Prof. Sinem ÖKTEM OKULLU for her invaluable support and guidance during my thesis study. I owe an appreciation to my PhD partner Sümeyye AKÇELİK DEVECİ for her kind friendship and contributions to my life in this graduate study. It was very valuable to spend six years with these two lovely people.

I would also like to thank to Selma ÇAT and Görkem GÜN for patiently helping under any circumstances and answering every questions. I would like to thank to Elif KILIÇ for help with the experiments, and Yaren BÜYÜKÇOLAK for her friendship. I also appreciate to meet and spend time with Gözde Ervin KÖLE, Simge ŞENAY, Ayşegül EKMEKÇİOĞLU and other graduate researchers. They made me happy during my PhD life and I had fun and unforgettable times with them.

Finally, I gratefully thank to my husband Mehmet Ali ÖZEN for his endless support, motivation, and encouragement in all these years. I would like to thank my other family members for their love not only during this study, but also in my life.

I would like to thank to ABAPKO for the financial support for the project (Project number: ABAPKO 2019/01/04).

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LIST OF ABBREVIATIONS

ABC	: ATP-Binding Cassette
ACTB	: β-actin
APS	: Ammonium persulfate
ATCC	: American Type Culture Collection
BCA	: Bicinchoninic acid
BSA	: Bovine serum albumin
CBA	: Cytometric bead array
CCL1	: C-C Motif Chemokine Ligand 1
cDNA	: Complementary DNA
CL	: Cutaneous leishmaniasis
CO_2	: Carbon dioxide
CXCL8	: CXC motif chemokine ligand 8
DB	: Database
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribonucleic acid
dsRNA	: Double strand RNA
DTT	: Dithiothreitol
EDTA	: Ethylenediaminetetraacetic acid
ER	: Endoplasmic reticulum
FBS	: Fetal bovine serum
FDA	: Food and Drug Administration
GO	: Gene ontology
Gp63	: Glycoprotein 63
GPI	: Glycosylphosphatidylinositol
HASPs	: Hydrophilic acylated surface proteins
HPLC	: High-performance liquid chromatography
ID	: Identity
IL-8	: Interleukin-8
IFN-γ	: Interferon-gamma
kDa	: Kilodalton

KMP-11	: Kinetoplastid Membrane Protein-11
LC-MS	: Liquid chromatography–mass spectrometry
LRV	: *Leishmania* RNA Virus
MCL	: Mucocutaneous leishmaniasis
MDM	: Monocyte-derived macrphages
miRNA	: Micro RNA
mM	: Milimolar
mRNA	: Messenger RNA
NCBI	: National Center for Biotechnology Information
NET	: Neutrophil extracellular traps
NO	: Nitric oxide
NTD	: Neglected tropical disease
OD	: Optical density
ON	: Overnight
PBMC	: Polymorphonuclear cells
PBS	: Phosphate Buffered Saline
PCR	: Polymerase Chain Reaction
PMA	: Phorbol 12-myristate-13-acetate
PRP1	: Pentamidine resistance protein 1
RNA	: Ribonucleic acid
ROS	: Reactive oxygen species
RPMI	: Roswell Park Memorial Institute
RT-PCR	: Real-time PCR
SDS-PAGE	: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SNP	: Single nucleotide polymorphism
SP	: Signal peptide
TEMED	: Tetramethylethylenediamine
TLR	: Toll-like Receptor
TMH	: Transmembrane Helix
TNF-α	: Tumor Necrosis Factor-alpha
VL	: Visceral leishmaniasis
WHO	: World Health Organization

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ÖZET

Türkiye'de Klinik Örneklerden İzole Edilen Farklı *Leishmania* Türleri ile Hücre Kültüründe Hücre İçi Enfeksiyonların Oluşturulması, Gelişen İmmün Yanıtın Ölçümü ve *Leishmania* Yüzey Proteinlerinin Proteomik Karakterizasyonu

Leishmaniasis, *Leishmania* cinsi kamçılı protozoonlarca oluşan, klinikte kutanöz, viseral veya mukokutanöz tutuluma yol açabilen vektör kökenli bir enfeksiyondur. Bazı *Leishmania* suşlarının sadece deride kalırken bazılarınınsa iç organlara göç edebilmeleri birçok araştırmanın merak konusudur. Dış çevre ile etkileşimde olan yüzey proteinlerinin bu süreçteki rolü henüz bilinmemektedir. Parazitlerin konağa girişi sonrası vücutta, suşların virülansıyla paralel bağışıklık yanıt oluştuğu bilinmektedir. *Leishmania* RNA virüsü (LRV) içeren suşlarda virüsün bağışık yanıta etkisi güncel araştırmalardandır. Çalışmamda, *L. donovani*, *L. infantum* ve *L. major* yüzey proteinlerinin tripsinle tıraşlanıp tanımlanması ile makrofajlarda LRV+ *L. major* izolat ile enfeksiyon oluşturulması ve bağışık yanıtın ölçümü hedeflenmiştir. Biyoinformatik analizler *L. donovani*, *L. infantum* ve *L. major*'de hücre zarında yerleşik sırasıyla 86, 85 ve 37 protein tanımlamıştır. Proteinlerden, biyolojik süreçleri varsayımsal olanlar yüksek oranda bulunmuştur. Viseral ve kutanöz tutulum yapan suşlar karşılaştırılmış, daha antijenik proteinlerin B-hücre epitopları incelenmiştir. Viseral suşlar ABC-tipi taşıma proteinleri açısından zengin bulunmuştur. Ayrıca, LRV+ *L. major* izolatı, THP-1'den dönüşen makrofajlardaki kemokin ve sitokinler açısından değerlendirilmiştir. Elde edilen bulgular, *L. major* klinik izolatların CCL1, CCL2 ve CCL5 ekpresyonunu baskıladığı ve bu baskılanmanın LRV varlığıyla CCL5 kemokininde anlamlı düzeyde arttığı gözlenmiştir. IL-6, IL-8, IFN-β ve TNF-α sitokinlerinden özellikle IL-8'in klinik izolatlarda anlamlı düzeyde arttığı, ancak bu artışın LRV^+ suşta gecikmeli olduğu gözlenmiştir. Çalışmada *Leishmania*'ların yüzey proteinlerinin tanımlanmasında tripsin tıraşlama yöntemi ilk kez kullanılmış, viseralizasyonda önemli proteinlerle birlikte olası aşı adayı yüzey proteinleri hakkında özgün bilgiler toplanmıştır. Makrofajlarda enfeksiyon modeli çalışması içinse LRV ile ilişkili olabilecek, ileri

çalışmalar gerektiren yeni hedefler gösterilmiştir.

Anahtar Sözcükler: *Leishmania*, Yüzey proteinleri, B-hücre epitopları, *Leishmania* enfeksiyonu, Doğal bağışıklık cevabı

ABSTRACT

Establishment of Intracellular Infection and Assessment of The Developing Immune Response in Cell Cultures with Different *Leishmania* Species Obtained from Clinical Samples in Turkey and Proteomic Characterization of *Leishmania* Surface Proteins

Leishmaniasis is a vector-borne disease caused by flagellate protozoa of genus *Leishmania*, which can cause cutaneous, visceral or mucocutaneous clinic forms. Some *Leishmania* strains remain only in the skin, while others can migrate to the internal organs and the role of surface proteins in this process is unknown. The entry of parasites into host induces the immune response dependent on the virulence of strains. Effect of *Leishmania* RNA virus (LRV) having strains on host immunity is under investigation. In thesis study, I aimed to identify trypsin-shaved *L. donovani*, *L. infantum* and *L. major* surface proteins and assess the infection of LRV^{+} *L. major* isolate in macrophages. Bioinformatic analyzes identified 86, 85, and 37 proteins localized in cell membrane for *L. donovani*, *L. infantum*, and *L. major*, respectively. Biological process analysis revealed mostly hypothetical proteins. Visceral and cutaneous strains were compared, and antigenic proteins were examined for B-cell epitopes. Visceral strains were found having more ABC-type transport proteins. Additionally, infected THP-1-derived macrophages with LRV^{+} *L. major* was evaluated for chemokines and cytokines. *L. major* isolates suppressed CCL1, CCL2 and CCL5 expression, and this suppression increased significantly with the presence of LRV. Moreover, IL-6, IL-8, IFN-β and TNF-α secretion was measured, and IL-8 increased significantly in isolates with delayed response in LRV^{+} strain. Consequently, trypsin-shaving method was used for the first time to detect *Leishmania* surface proteins, and possible surface vaccine candidates and important proteins in visceralization were indicated. Assessment of LRV presence in *Leishmania* infection showed new targets for further studies.

Keywords: *Leishmania*, Surface proteins, B-cell epitopes, *Leishmania* infection, Innate immune response

1 INTRODUCTION

There is an ongoing balance between microorganisms and humans (with other hosts) in the world for years. Some infectious diseases are better known around the world and have been investigated in detail by many studies until today. Examples to these are acquired immunodeficiency syndrome, malaria, tuberculosis and influenza. However, some disease-causing microbiological agents are less studied than the others and their impacts are more restricted to the endemic areas that we call neglected tropical diseases (NTD). Leishmaniasis is one of the NTD that is seen in various parts of the world and has an increasing risk in Turkey.

First findings of *Leishmania* are dated back centuries with reading of tablets and texts indicating the symptoms seen in the patients, or later the evaluation of ancient DNAs. Years after, it was possible to identify, classify and name New and Old World parasites in different regions with the gained information on disease diagnosis and prognosis (1). *Leishmania* species can cause different clinical symptoms and two of them are important for our country: cutaneous leishmaniasis (CL), which is seen as skin lesions and ulcers, visceral leishmaniasis (VL), which is spreading to visceral organs and can be fatal.

The manifestation of the disease in the clinical outcome depends on many factors such as the type of parasite, the virulence factors of the parasite, and the host characteristics. Risk factors can increase the spreading of disease and increase the number of cases. Because it is a vector-borne disease, environmental changes such as variations in temperature and water storage, decline in forest area, climate changes are important factors for vectoral transmission. Additionally, host-parasite related circumstances like suppression of the immune system, drug resistance or other situations like travel to endemic regions, wars and migrations, low level of socio-economic conditions are also effective in the spread of the diseases. Turkey has drawn more attention to leishmaniasis due to the migration from Syria and climate changes in recent years.

The disease has not any approved vaccine for humans and drug resistance is increasing in parasites. Therefore, novel targets for diagnostics, treatment and preventive medicine are highly required. Visceralization properties of certain type of strains are under investigation to find out potential genes and proteins in this mechanism. One of the candidate genes was vaccine-target stress-response A2 gene family members that having a pseudogene in cutaneous agents, and a functional protein in visceral strains. However, studies were indicated that possible other factors are required for migration of strains.

The primary goal of our study is to increase the knowledge about leishmaniasis and to strengthen the data on *Leishmania* parasites and their infection mechanisms. The plasma membrane proteins are the good candidates of vaccine studies such as glycoprotein 63, hydrophilic acylated surface protein B or kinetoplastid membrane protein-11 which are exposed to extracellular milieu and host cells. The identification of novel antigenic membrane proteins that expressed differentially in various strains has been performed with several strategies. However, surface-shaving of extending membrane proteins could be a potential method to detect antigenic proteins of *Leishmania*, which has not been applied, yet.

Other than type of strains, some virulence factors can regulate outcomes of the disease. *Leishmania* RNA virus (LRV) is a double-stranded RNA virus was detected in some *Leishmania* parasites. It has been associated with the disease exacerbation or drug failure in the studies of New World leishmaniasis. However, the effect of the LRV in the immune response or drug failure to Old World leishmaniasis is still unclear.

1.1 Aim of the Study

The presented thesis study constitutes from two independent parts to increase knowledge about leishmaniasis. In the initial part, the first aim of the study was to optimize surface shaving of live *Leishmania* species with trypsin enzyme and identification of *L. major*, *L. donovani* and *L. infantum* surfacome (surface proteome) with LC-MS/MS. From the data, comparison of visceral strains of *L. donovani* and *L. infantum* to cutaneous *L. major* according to spectral counts and sequence properties was the other subject to be enlightened. Last but not least, antigenic properties and B-cell epitope sites were evaluated to find out potential vaccine targets for further studies.

Second part relies on the in vitro assessment of the immune response against to LRV infected *L. major* clinical isolate in THP-1 derived macrophages. Selected inflammatory chemokines and cytokines were examined as a final goal.

2 BACKGROUND

2.1 Leishmaniasis

Leishmaniasis, is a vector-borne and neglected tropical disease caused by more than 20 species of the genus protozoan parasite *Leishmania*. This disease can be classified as Old World or New World leishmaniasis according to the geographical areas where it occurs: Old World leishmaniasis is mostly seen in Eastern Hemisphere (Asia, Africa, and Southern Europe) and New World leishmaniasis is mostly seen in Western Hemisphere (Central and South America). Australia, Antarctica and Pacific islands are the regions where are known to be free of the leishmaniasis (2). The disease transmission occurs with the bite of a female sand fly, mainly species of *Phlebotomus* (Old World) and *Lutzomyia* (New World). Clinical outcomes of the disease may vary according to infecting parasites, transmitting vector and host characteristics especially immune response to parasites (3). Regional division of the disease leads to the existence of subgenus types of *Leishmania* and several *Leishmania* strains under various clinical types of leishmaniasis (Figure 2.1) (4).

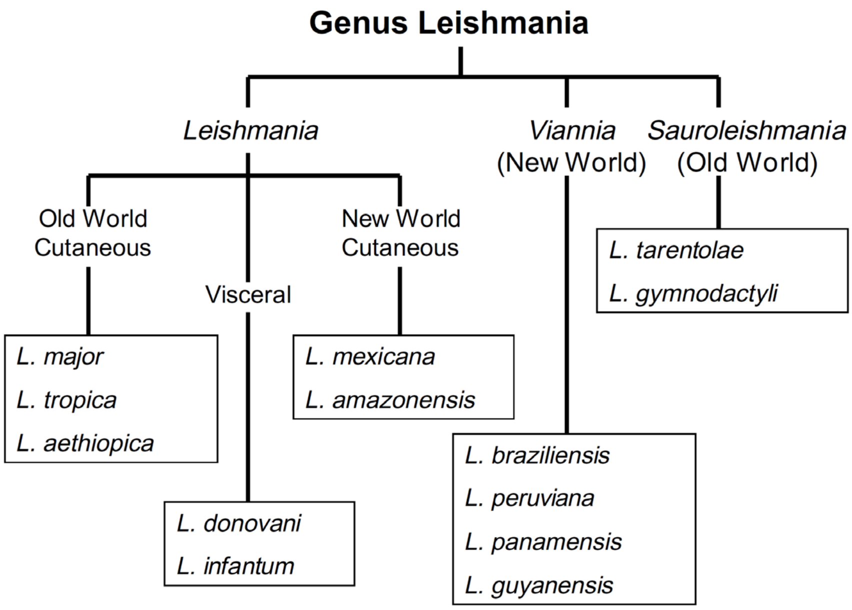


Figure 2.1 *Leishmania* classification for subgenera. Reproduced from (4) under the terms of the Creative Commons CC-BY license, which permits unrestricted use.

In Old World leishmaniasis, *L. major* and *L. tropica* are classified in cutaneous type of disease, whereas *L. donovani* and *L. infantum* are major visceral agents. *L. mexicana* and *L. amazonensis* are New World cutaneous leishmaniasis agents. *L. braziliensis*. *L panamensis* and *L. guyanensis* are mainly seen in mucocutaneous leishmanisis (4).

2.1.1 Clinical forms of the disease

The disease can be seen in a variety of clinical manifestations but mainly there are three distinct forms: cutaneous leishmaniasis (CL), visceral leishmaniasis (VL) and mucocutaneous leishmaniasis (MCL). CL is the most common form of the leishmaniasis and can appear days or months after sandfly bites. It may be mild as self-limiting small nodules to skin ulcers (5). The lesion size may be greater than >5cm and appearance may be different in cases. It may cause pain and healing may need longer time if any secondary infections occur (6). The other form of the disease is visceral leishmaniasis which is almost fatal if it is not treated. VL symptoms can appear after weeks to months of parasite transmission by a vector. VL affects internal organs by the migration of parasites and causes hepatomegaly, splenomegaly, persistent fever, weight loss and anemia. Some of the visceral leishmaniasis patients develop skin lesions years after treatment of the disease and the situation is called as post-kala-azar-dermal leishmaniasis (5,7). The other form is mucocutaneous leishmaniasis (MCL) which is caused by some of the species of New World leishmaniasis parasites. This form progresses after cutaneous infection of skin with the inflammation of mucous membranes such as the nasal, pharyngeal or laryngeal mucosa (5).

2.1.2 Epidemiology

Leishmaniasis is endemic in almost 100 countries around worldwide. The number of new cases changes yearly however it is estimated that almost 0.7-1.2 million new cases are reported for leishmaniasis annually. Around 0.7 million to 1 million are count for cutaneous leishmaniasis and less than 100.000 cases for visceral leishmaniasis recently which was up to 400.000 cases per year in the previous decade (3,8). Estimated total infected cases are currently 12 million and 350 million people are at risk for this neglected tropical disease (9). In total, about 20,000–30,000 deaths are reported per year. World Health Organization (WHO) data in 2020 revealed that most of the total cases of visceral leishmaniasis in endemic areas were reported from Brazil, Eritrea, Ethiopia, India, Kenya, South Sudan and Sudan. In addition, Afghanistan, Algeria, Brazil, Colombia, Iraq, Iran (high in previous years, no data), Libya, Morocco, Nicaragua, Pakistan, Peru, Syria (more than 50.000>cases per year), Tunisia, Yemen have high burden of CL compare to other endemic areas (10).

Turkey is one of the endemic countries for leishmaniasis. According to WHO leishmaniasis data, nearly 1800-2000 CL cases and 25-30 VL cases per year are reported from Turkey (Table 2.1) (10). CL is seen in the especially Southeastern Anatolia Region and Mediterranean, Central Anatolia and Aegean Regions of the country whereas VL is seen in the Aegean, Mediterranean and Central Anatolian Regions (11). Leishmaniasis has become a growing concern in Turkey due to the migration of Syrians to the country.

Table 2.1 Reported CL and VL cases from Turkey in WHO database for Leishmaniasis between 2010-2020 years (10)

	2010	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**	**2017**	**2018**	**2019**	**2020**
CL	2237	1803	1898	2268	1678	1986	1474	N.D.	1554	N.D.	N.D.
VL	36	30	13	33	22	23	23	N.D.	N.D.	N.D.	N.D.

Risk factors can increase the spreading of disease and increase the number of cases. Because it is a vector-borne disease, environmental changes such as variations in temperature and water storage, decline in forest area, climate changes are important factors for vectoral transmission. Additionally, host-parasite related circumstances like suppression of the immune system, drug resistance or other situations like travel to endemic regions, wars and migrations, low level of socio-economic conditions are also effective in the spread of the disease (12).

2.1.3 Diagnosis and treatment of leishmaniasis

Leishmaniasis is diagnosed by several techniques from DNA from infected areas for CL or bone marrow samples for VL patients. Specially stained samples can be visualized under microscope. To further identify the strains, molecular and biochemical methods are used. Serological test can be preferred for visceral leishmaniasis detection (13).

Treatment of leishmaniasis is depended on strain and disease type. Food and Drug Administration (FDA) approved drugs are liposomal amphotericin B (AmBisome®) for treatment of VL; miltefosine for CL, MCL and VL. Other treatment agents amphotericin B deoxycholate, pentamidine isethionate, azoles (ketoconazole, itraconazole, and fluconazole) are also used however drugs are FDA-approved but not for leishmanaiasis. In addition, cutaneous cases might be treated with cryotherapy, thermotherapy or intratopical pentavalent antimonial or topical paromomycin (13).

2.1.4 Vaccine studies

Leishmania vaccine trials have been categorized as first, second and third generation vaccines. In first generation, killed parasites are one of the choices such as Leishvaccine which was applied in Phase I and II but failed in Phase III in dogs (14). *Leishmania* antigenic fragments are the other targets to be used in immunization. Leishmune® and CaniLeish® are licensed vaccines for dog immunization (15). Live-attenuated vaccines were used in some Old World species and gentamicin attenuated *L. major* immunization in Iran was subjected (14,16).

In second generation vaccine trials use recombinant proteins of different *Leishmania* strains. LEISH-F1 was one of the vaccine candidates for combinatorial proteins from the parasites which was found efficient for treatment and protective for leishmaniasis. It reached to Phase II in clinical trials for CL and MCL (17). Similarly, LEISH-F2 which was used in the Phase II of clinical trial and LEISH-F3 was also formulated from recombinant DNA technology for *Leishmania* proteins (18,19). Leish-Tec® is a recombinant vaccine used in dogs from A2 antigen of *L. infantum* which is known to be a visceral determinant (20).

In third generation vaccines, first clinical trial in Phase I was with the usage of ChAd63-KH, adenovirus expressing two membrane protein Kinetoplastid membrane protein-11 (KMP-11) and Hydrophilic acylated surface protein B (HASPB) of *Leishmania* (21).

2.2 *Leishmania* Parasites

Leishmania belongs to the Trypanosomatidae family under Kinetoplastida order which characterized by presence of a unique mitochondrial DNA: kinetoplast (22). More than 30 species of *Leishmania* are identified so far however around 12 of them are medically important. Under subgenus *Leishmania*, there are *L. donovani* complex (*L. donovani*, *L. infantum*), *L. mexicana* complex (*L. amazonensis, L. mexicana*, *L. venezuelensis*), *L. major L. tropica* and *L. aethiopica*; and under the subgenus Viannia there are *L. braziliensis*, *L. guyanensis*, *L. panamensis*, and *L. peruviana* (23). *Leishmania* can be hosted by several vertebrates other than humans such as dogs, forest rodents, foxes etc. Exceptionally, *L. tropica* and *L. donovani* are transmitted between human to human -anthroponotic, but the others can be transmitted from animals to humans-zoonotic with specific insect vectors (4,22).

2.2.1 Life cycle

Leishmania has digenetic life cycle similar to other protozoans. Promastigote form of the parasites is the extracellular stage and found in the midgut of insect vector, amastigote form is the intracellular stage and found in the mammalian host. Briefly, female sandfly transmits infective promastigotes during blood feeding to host. Phagocytic cells such as macrophages, dendritic cells take parasites inside and development of amastigotes occur in phagolysosome of infected cells. Amastigotes start to replicate themselves and burst out of surrounding cells. In the another blood feeding, amastigotes are transferred to insects and transformed into promastigotes form again (24,25).

The amastigote form of the parasite is oval shaped, non-flagellated and non-motile form with a 2 to 4 µm in length. The promastigote form is elongated, motile, flagellated form with a changing size from 5 to 14 µm in length and 1,5 to 3,5 µm in width (22). Unlike amastigotes, there are several developmental stages in promastigote form in the sandfly vector. After blood meal, amastigotes are taken from infected host, and start to transform into slowly moving, replicating procyclic promastigotes. After a while, promastigotes move to the anterior part in the midgut by the differentiation of procyclic form into the highly motile and elongated nectomonad promastigotes. When the second stage promastigotes reach to the proper region where they interact, they start to differentiate into leptomonad promastigotes that are shortened and replicating form of parasites. Some of them are transformed into metacyclic promastigotes which is the infective form of parasites. They resist complement mediated lysis of host immune response better than procyclic form (Figure 2.2) (4).

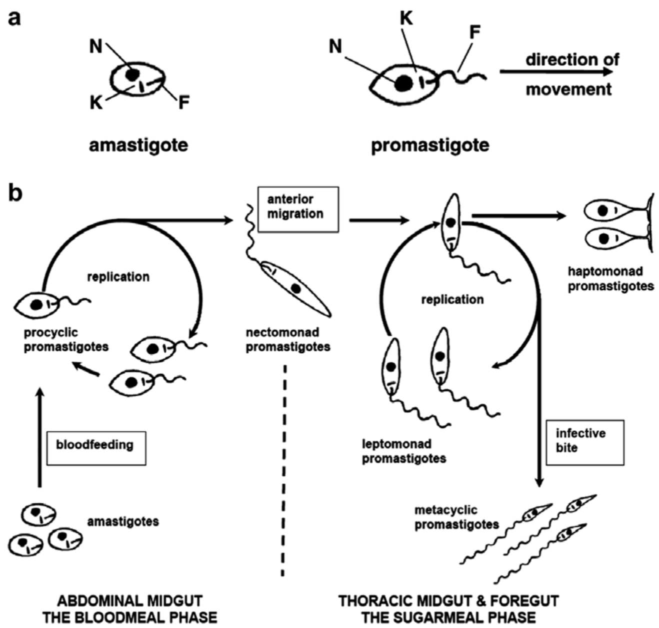


Figure 2.2 Development of *Leishmania* in the sandfly vector (4). Figure reproduced here under the terms of the Creative Commons Attribution License.

2.2.2 Virulence factors

Leishmania parasites undergo various alterations in both amastigote and promastigote stages to sustain infection in host cells, maintain nutritional balance, adapt to stress conditions, counteract immune response of host and be transmitted. In order to manage infectivity and survival, virulence factors are the main factors and of great importance for parasites (26). Several groups of surface, cytosolic or secreted molecules such as some proteins (e.g. proteinases, nutrient transporters) (27,28), glycosylphosphatidylinositol (GPI)- anchored molecules (e.g. gp63, lipophosphoglycan) (29,30), heat-shock proteins (31) or kinetoplastid membrane protein-11 (KMP-11) (32) are present in the dynamic structure of parasites and these molecules may change expressional patterns and shift during stages.

2.2.3 Visceralization-associated determinants

Identification of molecular differences between visceral and cutaneous strains that may cause migration of parasites into internal organs will provide an important insight to understand the spread of the disease. Parasites that metastasize to visceral organs after skin infection can survive here and make this disease fatal, if it is not treated. One point of view is that these parasites may be more heat tolerable to resist internal temperatures than the skin resident parasites. In addition, the flow of immune cells raises into the infection site with the increase in fever, and it resulted with the oxidative stress on parasites. *L. major* is more susceptible to nitric oxide and hydrogen peroxide compare to *L. donovani* (33) Besides, mammalian host and sandfly determinants may also be effective in the spread of parasites in internal organs. Parasite determinants are the most influential factors for the fate of disease progression. Strain-specific genes, pseudogenes, polymorphic differences and level of gene expression may effect disease outcome (reviewed in (34)).

Most prominent candidate of visceralization determinant is A2 gene family members. A2 gene was shown to express as amastigote-specific endoplasmic reticulum (ER) stress response protein (35) in species *L. donovani*, *L. infantum* and

L. mexicana species but not present in *L. major, L. tropica* or *L. braziliensis* (36,37). This family encodes 40 to 100kDa proteins with repetitively aligned ten amino acid sequence (38,39). To elucidate the role of A2 in visceral strains, depletion of A2 with antisense RNA or gene partial knock-out, decreased the amount of parasites in the infected liver (40,41). On the other hand, inserting A2 gene into *L. major* increased migration of parasites out of skin in internal organs (42) and similarly, expressing this gene in non-A2 expressing parasite *L. tarantolae* (lizard parasite) has increased parasites infectivity and visceralization (43). One of the study showed the immunization of mice with a recombinant expressed A2 protein (44), which is used as a commercial vaccine Leish-Tec® (Hertape Calier Animal Health®, Minas Gerais, Brazil) for canine leishmaniasis in Brazil (45,46).

Visceralization factors were further studied with comparing *L. infantum* genome with *L. major* and *L. braziliensis*. Some of the species-specific genes from *L. donovani* ortholog are cloned into *L. major* and functional analysis pointed out three candidate genes LinJ.28.0340, cytosolic unknown protein; LinJ.15.0900, a nucleotide sugar transporter of Golgi apparatus; and LinJ.36.2480, a cytosolic glyceraldehyde 3-phosphate dehydrogenase enhanced *L. major* survival in visceral organs. Also, deletion of LinJ.28.0340 ortholog in *L. donovani* decreased virulence of parasites in mice (47). Similarly, introducing *L. donovani* ortholog of nucleotide sugar transporter (LinJ.15.0900) into *L. major* increased survival of parasites in mice internal organs, however the deletion of gene from *L. donovani* did not affect parasite virulence (48).

Other than strain-specific genes for visceral leishmaniasis, possible single nucleotide polymorphisms (SNPs) and gene copy numbers were evaluated with attenuated cutaneous form of clinical isolate and virulent visceral clinical isolate of *L. donovani*. A2 gene copy number was found to a lesser extent and ras-like RagC GTPase (49) and RagC GTPase related protein Raptor and additional 14 coding genes (50) were mutated.

Overall experiments indicate that there has been uncovered genes and proteins significant in visceralization of some strains of parasites with potential effects, despite none of them are completely responsible for restoring cutaneous strains into visceral forms. Other factors such as gene expression levels or post-transcriptional modifications might be counted as additional factors beyond genomic findings.

2.3 *Leishmania* Surface Proteins

The surface determinants of pathogenic microbes are important subjects of studies due to the interaction and communication with environment and other cells, having virulent characteristics or continuity of microorganisms' survival on the other hand being recognized by host's immune systems. There are several membrane embedded, membrane-associated and extracellularly exposed proteins in many important tasks that have become important vaccine target proteins (51).

In *Leishmania* species, surface proteins are critical players in the determination of life cycle of parasites and survival in the sandfly vector or infected hosts. Expression and role of GPI-anchored glycoproteins, type-1 membrane proteins, transmembrane spanning proteins or peripheral membrane proteins have been extensively studied in parasite virulence (Figure 2.3) (52).

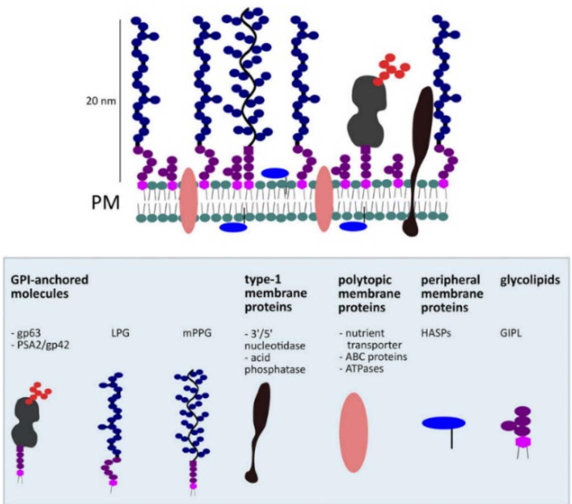


Figure 2.3 Schematic representation of the promastigote-specific *Leishmania* plasma membrane components (53) permission was requested to copy and redistribute the material from Copyright Clearance Center.

2.3.1 KMP-11

Kinetoplastid membrane protein 11 (KMP-11) is a 11-kDa membrane glycoprotein of all *Leishmania* spp. It is expressed in both amastigote and promastigote stages and it has 95% homology when the KMP-11 sequence data were compared with Old World *L. donovani* and *L. infantum* to New World *L. panamensis* (54). It has been seen that peripheral blood mononuclear cells (PBMC) cultures from *L. braziliensis* patients, KMP-11 increased the level of production of Interleukin-10 (IL-10) cytokine and also it is found as Interferon-gamma (IFN- γ) production was repressed in these cells depending on IL-10 (55). In a study with *L. infantum* parasites, KMP-11 gene expression was shown to be down-regulated during infective promastigote transition from log to stationary phase and in amastigotes (56). However, KMP-11 was found to be expressed higher in intracellular amastigotes form of *L. amazonensis* promastigotes compare to promastigotes and its expression was increased in stationary phase progression and metacyclogenesis (57). It has been proposed with its virulent features for promastigotes, KMP-11 is a possible vaccine candidate for leishmaniasis (32).

2.3.2 Gp63

Glycoprotein 63 or leishmanolysin is a surface zinc metalloproteinase of *Leishmania* parasites. It is found that Gp63 is mostly expressed in promastigotes compare to amastigotes (58). In the study conducted with *L. amazonensis* and *L. major*, Gp63 was shown to act on complement system of host immune response by converting C3b to iC3b and resist to lysis through this mechanism (59). In the initial infection with promastigote stage of parasite, Gp63 was thought to enhance adherence to macrophage receptors such as fibronectin receptor of host macrophages (60) and cleave extracellular matrix proteins to be able to survive in mammalian host (61). In host macrophages, Gp63 mediates degradation of host molecules such as the myristoylated alanine-rich C kinase substrate (MARKS) and MARKS related proteins (MRPs) and interfere their signaling pathways (62). In addition, it was shown that Gp63 regulates protein tyrosine phosphatases (PTP) which induce inactivation of several JAK kinases (JAK1, JAK2) related to IFN-gamma mediated immune responses and so increase parasite persistence in host (63).

2.3.3 Transporters

Leishmania has several transport proteins to scavenge essential nutrients or ions from the host environment that parasites are present and additionally known to resist drugs. Some of them are required for carbohydrate mechanisms, nucleotide mechanism, cofactor mechanism, polyamine mechanism and some are responsible for amino acid or ion uptake. In digenetic lifecycle of parasites, stage-specific transport dynamics may be present because of the milieu differences in the midgut of sandfly and macrophage phagolysosome vesicles (27).

Amino acid transporters

There are several types of transporters for salvaging of amino acids from the host milieu such as arginine, lysine, or proline transporters. Amino acids are mostly utilized by promastigotes than amastigotes, but some are still needed for parasite growth (64). Amino acid permease 3 (AAP3) is permissive transporter for arginine which is one of the essential amino acids for *Leishmania* survival by polyamine production and nitric oxide (NO) synthesis. It may also regulate host component mediate activation (65). AAP7 has affinity to lysine and AAP7 deleted *Leishmania* studies show that it is an indispensable transporter for parasite viability (66).

Hexose transporters

Leishmania transporters obtain glucose with higher extend but it may also permit fructose, mannose, or galactose (67). Promastigotes are prone to uptake glucose 10-fold higher than amastigotes regarding to environmental pH condition (68). In *Leishmania* strains, a few glucose transporters have been identified such as GT1, GT2 and GT3 in *L. Mexicana* with distinct functional properties (69).

Nucleoside transporters

Leishmania parasites are incapable of purine ring synthesis and so purines must be obtained from the extracellular space. With this point of view, it may be targeted for the drug design (reviewed in 60). Transport of nucleosides are carried out with the family of equilibrative nucleoside transporter (ENT) which consists of four members NT1, NT2, NT3 and NT4 (71).

Folate/Biopterin transporters

Leishmania is needed to obtain folate and pterin cofactors from the host environment. The antifolate methotrexate (MTX) was used as a drug possibility to leishmaniasis (72).

Iron/heme transporters

Leishmania is iron auxotroph and needed to acquire it. It has been found that *Leishmania* iron transporter 1 (LIT1) is able to uptake of ferrous iron but also cationic manganese, cadmium, and zinc (73). Later, it is reported that *Leishmania* heme response-1 (LHR1) transports heme and studies performed with the deletion of LHR1 alleles showing that LHR1 is necessary for *Leishmania* virulence and survival (74).

Ion transporters

Several ATP-driven H+ , K+ , and Na+ proton pumps are present in *Leishmania* and have a role in ion balance of parasites.

ATP-Binding Casette (ABC) Transporters

ATP-binding cassette (ABC) family is one of the largest transmembrane proteins family that present in both eukaryotes and prokaryotes. They export several molecules through the membranes with nucleotide binding domain hydrolases ATP and they have a direct relation with drug-resistance in *Leishmania* (29, reviewed in 30). *Leishmania* genomes has 42 and 43 ABC-transport genes including subfamilies from ABCA to ABCH for *L. major* and *L. infantum*, respectively (77). ABCB, ABCC and ABCG subfamilies were found to related to drug-resistance and among them, ACG4 and ABCG6 localized in plasma membrane were shown to resist miltefosine drug (78,79).

2.3.4 Hydrophilic Acylated Surface Proteins (HASPs)

Hydrophilic acylated surface proteins (HASPs) are highly immunogenic and acidic proteins which are encoded by the cDNA16 gene family. They are inserted to membrane non-classical way with dual acylation at the N-terminal site of the proteins (80). *L. donovani* HASPB protein was shown to lead long term resistance in VL mice model (81).

2.4 Proteomic Approaches for Analysis of *Leishmania* Surface Proteins

Leishmania surface proteins attract attention for many purposes such as vaccine strategies, stage differences or functional studies. The fact that these proteins are mostly hydrophobic and that makes their extraction more difficult. Few studies have extracted and identified *Leishmania* surface proteins using different methods, and later analyzed by Liquid Chromatography Mass Spectrometry (LC-MS/MS) and compared with related database. Advanced bioinformatics analyzes were also performed with the various tools. Previous studies in the literature support and complement each others in terms of findings.

L. chagasi surface protein differences of logarithmic-avirulent parasites and metacyclic-virulent parasites were obtained either by separating these proteins with streptavidin affinity after labeling with biotinylation or by ultracentrifuge using a detergent called octyl glucoside. Biotinylation of proteins has shown that it is an efficient method to distinguish protein content differences between stages (82)

Cutaneous *L. mexicana* and visceral *L. infantum* strains were compared on amastigote and promastigote stages by extracting membrane proteins after parasites are dissected by nitrogen cavitation and surface proteins are obtained by density gradient and sucrose separation. Accordingly, differences were observed between promastigotes and amastigotes of the strains, especially in translation and metabolic pathways. Comparative studies were indicated some common proteins and expressional differences (83).

Comparison of the visceral leishmaniasis agent *L. infantum* plasma membrane and cutaneous leishmaniasis agents *L. amazonensis* plasma membrane proteins were performed with extraction of membrane fraction sodium carbonate lysis together with sonication and ultracentrifugation of membrane proteins. Around 7.5-15.15% and 7.8-17.4% of identified proteins were found to be plasma membrane proteins for *L. infantum* and *L. amazonensis*, respectively. Strain-specific or commonly expressed proteins evaluated for their functions (84)

In another study, to evaluate the membrane protein differences between *L. infantum* promastigote and axenic amastigotes, the parasites were fragmented by sonication. Membrane fractions were obtained by measuring densities in a plate system called free-flow zone electrophoresis (ZE-FFE) after serial centrifugation. After all, possible GPI-anchor proteins and stage-specific protein differences with transmembrane domains in both stages were determined (85).

Focusing on a single strain can also be used for surface membrane analysis. Clinically isolated sodium antimonate sensitive *L. donovani* strain log-phase promastigotes were used to fractionate membrane part with sodium carbonate solution after freeze-thaw and ultrasonication disruption. About 50% of enriched proteins were identified as membrane proteins (86).

Membrane vesicles that can display membrane protein profiles have been studied for the ATP-driven As(III)-glutathione transport system and pentostam drug resistance, which is an important first-line drug used in leishmaniasis. For this purpose, parasites were fragmented with glass beads and used to obtain enriched of plasma membrane vesicles by differential centrifugation (87).

2.5 Trypsin Shaving Method for the Analysis of Surface Proteome

Surface proteins are the one of the best targets for vaccine studies because of the interaction of these proteins with the environment. These proteins can possess several functions such as transport of nutritional molecules, binding host to initiate infection or inhibition of host immune responses for pathogens. The surface shaving method is used to obtain surface-exposed proteins from live organisms with protease enzyme digestion and referred as surfome or surfaceome (reviewed in (51)). Trypsin is the most used enzyme which cleaves from lysine or arginine residues of proteins (88). The method has been applied for several organisms up to date: gram-positive (89–91), gram-negative bacteria (92–94), fungus (95–97) or parasites (98,99).

There are some advantages and disadvantages of the applied method which can be useful to find out novel drug or vaccine targets in a fast way however the presence of cytoplasmic proteins can make this approach challenging. There are three possible hypothesis for non-surface proteins: it might be contaminated through the lysis of cells in enzyme incubation; these proteins might be released into incubation buffer with non-canonical pathway or outer membrane vesicle with cytoplasmic content might be collected with surface-shaved proteins (51).

2.6 *Leishmania* and Immune Response

Leishmania inoculation into vertebrate host by the bite of sandfly vector initiates several complex mechanisms regarding infective parasite and host factors that makes unpredictable to estimate disease outcome. Innate immune response cells are the first triggered immune players recruited to the infection sites despite this recruitment is the way to establish a proper infection for *Leishmania* in the host. Adaptive immune cells are indispensable to resolve the disease and be able to have a memory for further infections. In the following parts, innate immunity against *Leishmani*a parasites is summarized.

2.6.1 Innate immune response to *Leishmania* infection

Leishmania deposition into skin together with sandfly saliva which consists of several immunomodulatory and chemoattractant molecules initiates the first respond to parasites to activate and recruit other immune cells (100). Keratinocytes are found to interact with pathogens and induce inflammatory response with sensing to microbial agents with surface or endosomal receptors such as Toll-like receptors (TLR)(101). *L. major* induce TLR2 expressing keratinocytes which produce neutrophil chemoattractant molecules (102). In addition, *L. infantum* induces pro-inflammatory immune modulators IL-6, IL-8, tumor necrosis factor (TNF-α) and IL-1β from human keratinocytes, *L. major* is unable to do this inducement, but IL-4 has been released (103). Keratinocytes obtained from *L. major* resistant mice express higher levels of immune mediators compare to resistant mice to promote T helper 1 (Th1) cell polarization (104).

In innate immunity against leishmaniasis, macrophages are the main host cells to eliminate pathogens however neutrophils are the cells that primarily recruited to the infection area in hours by secreted pro-inflammatory molecules from skin resident cells (105,106). Complement cascade through *Leishmania* infection also induces activation of neutrophils with complement factor C3 which help to opsonize parasites for phagocytosis in *L. major* infected BALB/c mice (107). Neutrophils are classified in polymorphonuclear leukocytes and have a role in the first line defense against microbial agents to eliminate them. They may use phagocytosis, release reactive oxygen species (ROS) or do degranulation. They may constitute externalized chromatin formation called neutrophil extracellular traps (NETs) to kill pathogens such as *L. amazonensis* infection in human neutrophil cells (108). These cells may also alter immune response of other immune players following interaction with them (106). On the other hand, parasite replication in neutrophils have been reported after *L. mexicana* infection in chronic phase but *L. major* amastigotes were not able to replicate in neutrophils which might be the result of parasitophorous vesicle structure and surface composition of the different species (110,111).

2.6.1.1 Macrophages and inflammatory response to *Leishmania* parasites

Macrophages are important players of *Leishmania* infection, who allows either the multiplication and persistence of parasites or responsible for their destruction and clearance. Activation, and transition to subtypes of macrophages are key determinants for disease resolution, and the tendency of infection is dependent on the communication between the host macrophages and the infecting parasite (112). Macrophages are phagocytic cells that are important in the innate immune system for the pathogen response and for dead tissue clearance. It recognizes the self or harmed cells with pattern recognition receptors such as TLRs. They also introduce processed antigens to adaptive immune cells via major histocompatibility complex (MHC) class II (113). All tissue macrophages develop after terminal differentiation of circulating monocytes into tissue-specific macrophages (114).

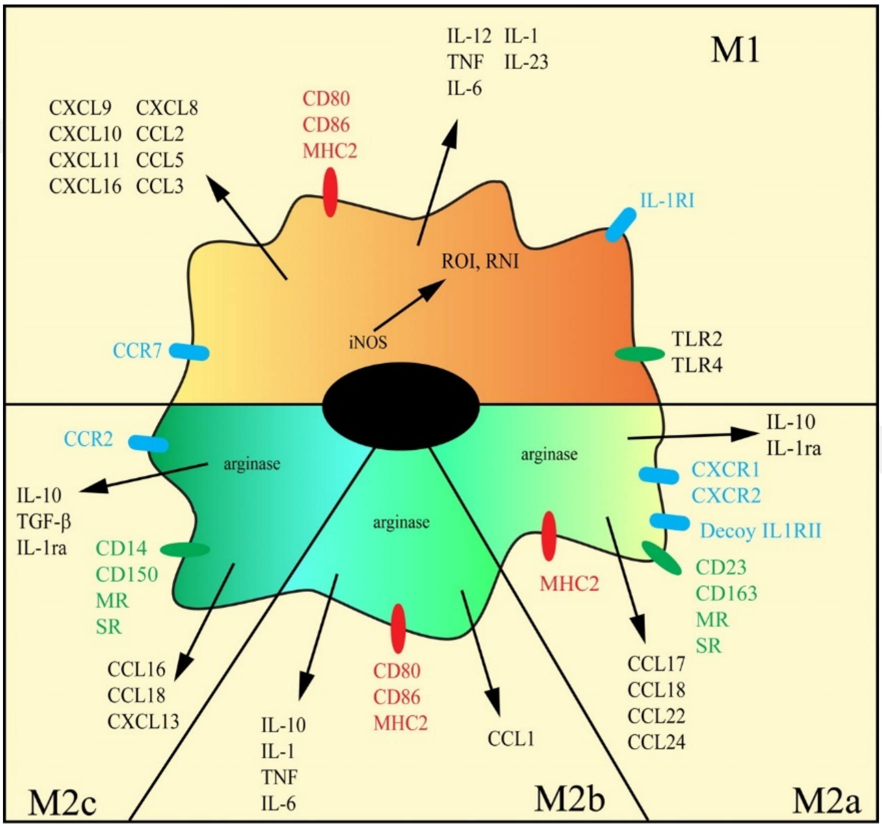


Figure 2.4 General concepts and properties of polarized macrophages. Figure reproduced with permission from (115)

Polarization of non-activated, naïve macrophages (M0) into classically activated M1 and alternatively activated M2 phenotype (M2a, M2b, M2c and M2d) was depended on environmental signals that trigger pro- or anti-inflammatory response, respectively (112,114). Each sub-type secretes specialized cytokine and chemokine profile that are resultant from the induced sub-type (115)(Figure 2.4). M1 subtype of macrophages produces TNF-α, IL-1β, IL-6, IL-12, or type I IFN such cytokines and reactive oxygen and nitrogen species upon activation. They can also express CCL2, CCL3,CCL5, CXCL10 as chemokine response. On the other hand, M2 type releases mainly IL-10 and express IL-1Ra and increases phagocytosis but chemokine response depends on subtypes which might be CCL18, CCL22 for M2a, CCL1 for M2b and CCL16, CXCL13 for M2c (112,114).

Leishmania infects the first neutrophils, but macrophages are the main route for amastigote transition. In the feeding of sandfly, Promastigote Secretory Gel (PSG) with high proteophosphoglycan content produced by the parasite is transferred to the host which provides activation for macrophage uptake. In fact, this fluid increases arginase activity in macrophages and provides nutritional support for the parasite (116). Interaction of promastigote flagella with macrophages triggers phagocytosis (117). Different strains of *Leishmania* may interact with certain macrophage receptors such as complement receptors (CRs), mannose receptors, fibronectin receptors and Fcγ receptors (FcγRs) (118), and caveolin-dependent internalization of *L. donovani* parasites by host cells was lately indicated (119). After phagocytosis of parasites, they manipulate host cells to persist and multiply within structures called as *Leishmania* parasitophorous vacuoles (LPVs) (120).

Leishmania can manipulate infected host cells to favor parasite persistence by altering several signaling pathway (Figure 2.5). *L. amazonensis* infected mouse macrophages were shown to suppress pro-inflammatory cytokines IL-12, IL-17 and IL-6 in lipopolysaccharide (LPS)-induced cells and increased pro-inflammatory cytokines IL-1α, TNF, CCL3 (MIP-1α) and CCL2 (MCP-1) and suppressive IL-10 (121). Macrophages produce reactive oxygen species (ROS) or nitric oxide (NO) to overcome parasites' infection. Interferon-γ (IFN-γ) and tumour necrosis factor (TNF) have a role on nitric oxide species production from host cells to kill *Leishmania* parasites (122,123). Cutaneous leishmaniasis patients have increased arginase-1, tumor growth factor-β (TGF-β) and prostaglandin E2 with a alternatively activated macrophage phenotype which was supported with *L. major* and *L. amazonensis* growth in vitro studies with M2 macrophages (124,125). Infected macrophages can express peroxisome proliferator-activated receptors (PPARs) which are either shown as important for the activity of M2 macrophages in *L. major* model (120) or murine macrophage cell line were induced to M1 macrophages with PPAR to produce pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 against *L. mexicana* (126,127).

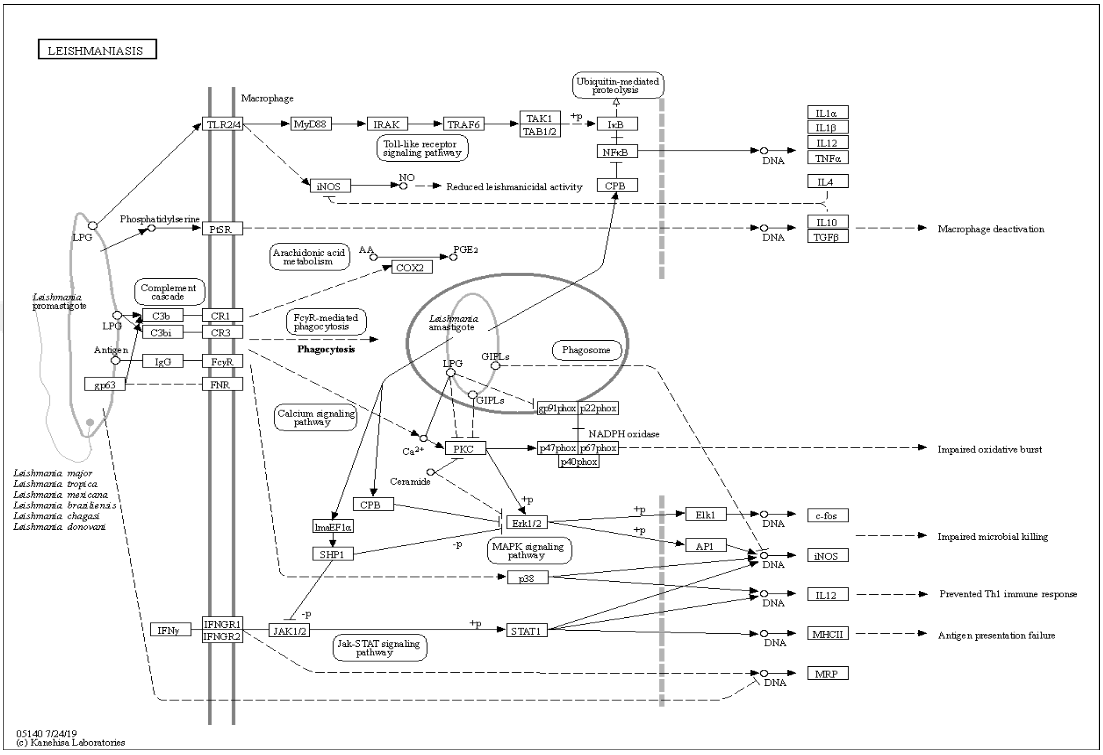


Figure 2.5 Leishmaniasis pathway. Retrieved from KEGG DB (128).

M1/M2 polarization is a balanced system to favor parasite elimination with antimicrobial activity of M1 in initial response and regulatory role of M2 in afterwards (112).

2.7 *Leishmania* RNA virus (LRV) of *Leishmania* Parasites

Leishmania RNA virus (LRV) is an endobiont virus present in Old World and New World *Leishmania* species. The viral properties and effect of the virus on the pathogenesis of parasites in cutaneous, mucocutaneous and visceral leishmaniasis will be presented.

2.7.1 *Leishmania* RNA Virus (LRV)

The *Leishmania* virus - LRV is a member of *Totrividae* family viruses. The virus is about 40nm and carrying ~4-8kb double strand RNA (dsRNA) which encodes major capsid protein and RNA-dependent RNA polymerase (RDRP) (129). LRV can be divided into two groups as LRV-1 and LRV-2. New World Leishmaniasis strains *L. guyanensis* and *L. braziliensis* has been shown to be infected by LRV-1 (129,130), whereas Old World Leishmaniasis strains *L. major* (131), *L. aethiopica* (132), *L. tropica* (133) , *L. infantum* (134) was shown to be infected by LRV-2.

2.7.2 Effect of LRV on inflammatory response against leishmaniasis

The pathogenicity of the LRV and severity of the leishmaniasis has been studied in different types of disease with several strains that it is hypothesized to be a virulence factor for worsening of the disease progression, or not (135–137). It was also investigated whether there is a relationship between drug resistance and LRV presence in infecting *Leishmania* strains. However, there is a lack of agreement which some studies were shown to have no relation with therapy failure with especially LRV-2 (134,138), conversely, LRV-1 was found correlated with first-line treatment failure in tegumentary leishmaniasis (136,139)

The association of inflammatory response by LRV was assessed in the view of metastatic *L. guyanensis* parasites which was shown to be bearing LRV-1 dsRNA. In vitro infection of murine macrophages with these parasites increased the expression of CCL5, CXCL10 chemokines and TNF-α, and IL-6 cytokines. Moreover, induction of macrophages with purified LRV-1 dsRNA was also resulted in expression of pro-inflammatory molecules in a TLR3 dependent manner (140). Human patient data and mouse studies were shown that after TLR3 activation, autophagy was induced by activation of type I interferons which interfere with the inhibition of *Leishmania* survival (141) .

Presence of LRV-1+ *L. guyanensis* decreased IFN-γ expression in infected patients of cutaneous leishmaniasis but IL-17A levels were significantly higher compared to LRV-1- *L. guyanensis*. In murine infection model, IL-17A was shown to contribute parasite persistence in LRV-1 mediated pathology in the absence of IFN-γ (142).

Induction of RNA interference (RNAi) pathway *in L. guyanensis* and *L. braziliensis* which is a defense mechanism to protect organisms from their RNA viruses eliminated LRV-1 in these strains. Infection of bone marrow derived macrophages from mouse *in vitro* with LRV-depleted *L. guyanensis* and *L. braziliensis* could not induce Toll-like receptor-3 (TLR3) dependent inflammatory response and production of TNF-α and IL-6 compared to LRV-1 positive ones (143).

Anti-viral target screening to LRV-1 with nucleoside analogs was revealed a potential drug which inhibited LRV-1 in *L. guyansesis*. Bone marrow derived macrophages or BALB/c mice were infected with drug-treated parasites and decrased inflammatory response against LRV-1^{-} *L. guyanensis* was observed (144).

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 *Leishmania* strains

Leishmania strains used in the surface proteomic analysis were the reference strains of *Leishmania donovani* (MHOM/IN/1980/DD8), *Leishmania infantum* (MHOM/TN/1980/IP-1) and *Leishmania major* (MHOM/IL/67/JERICHO II). In the macrophage infection and immune response experiments, two patient-derived isolates positive for *Leishmania* RNA Virus (LRV) or lacking virus (LRV+ or LRV- *L. major,* respectively) were used in addition to *L. major* reference. LRV+ *L. major* strain was isolated from 15-year-old schoolgirl from Manisa province located in Western Anatolia. She had 6-month-old, 4 cm long, ulcerated skin lesion near her left ear. Initially, the patient was treated with intralesional 20 mg/kg glucantime treatment, three times a week for 3 weeks. This treatment protocol was needed to be repeated two more times due to treatment failures. All the indicated strains were obtained from Prof. Ahmet Özbilgin at Parasitology Laboratory in Manisa Celal Bayar University.

3.1.2 *Leishmania* culture

Ingredients of liquid culture of *Leishmania* strains were RPMI (GIBCO® with L-glutamine) supplemented with 10% fetal bovine serum (FBS, GIBCO®), 1% Penicillin-Streptomycin (Penicillin, 10,000 units/ml - Streptomycin, 10 mg/ml, GMBiolab®, Taiwan) and 0.2% Gentamicin (Gentamicin, 50 mg/ml, GMBiolab®, Taiwan). The volume of media was prepared freshly depending on needs of experimental design.

The contents of the solution used in the freezing of *Leishmania* strains are a mixture of RPMI (35%), FBS (50%) and DMSO (15%). Upon preparation, freezing media stored at -20˚C for further use.

3.1.3 Cell line

A human monocytic cell line, THP-1 cells (ATCC: TIB-202™) were kindly provided by Assoc. Prof. Ayça Sayı Yazgan, Istanbul Technical University, Istanbul, Turkey.

3.1.4 Cell culture

THP-1 cells were maintained in a complete RPMI media similar to Leishmania culture media except Gentamicin is not added. In macrophage differentiation experiments, PMA is used as an inducer with different concentrations. The solutions used during cell culture experiments are shown in Table 3.1. Freezing of cells were performed with a freezing media like used in Leishmania parasite freezing except 10% DMSO is used instead of 15%.

Table 3.1 Solution used in cell culture experiments

Chemicals/Medium	**Supplier Company**
Roswell Park Memorial Institute (RPMI) Medium with L-Glutamine	GIBCO
Fetal Bovine Serum (FBS)	GIBCO
Penicillin/Streptomycin	GIBCO
Trypan Blue	Sigme
DMSO (Dimethyl sulfoxide)	Sigma
PMA (Phorbol-12-Myristate-13-Acetate)	Sigma

3.1.5 Primers

Primers' sequences designed for this study using National Center for Biotechnology Information (NCBI) primer blast for the indicated mRNA sequences. Sequences of primers used in the whole study and expected product lengths are given in Table 3.2.

Table 3.2 Primers used in the infection experiments

Primers	Forward primer 5'->3'	Reverse primer 5'->3'	Product length
Leishmania primers			
KMP-11	AGATGCAGGAACAGAACGCC	TGCTTGAAGTGCTCCGAGTG	160bp
LRV-2	ATGCTGATAACTTGAAACAGGAG	CATCATTGCCTGTAAGTGAGTAG	954bp
Human primers			
hCD11b	GCCAATGTGACCAGTGAGAACA	CTGAGGCCGTGAAGTTGAGAT	139bp
hCD36	GACGCTGAGGACAACACAGTC	GCCACAGCCAGATTGAGAACTG	133bp
hACTB	GAGCATCCCCCAAAGTTCACA	GTGGGGTGGCTTTTAGGATGG	127bp
hCCL1	CTTGGACACAGTTGGATGGGT	AGCCATGTGGTTTCCAGAGC	111bp
hCCL2	ACAAGCAAACCCAAACTCCG	AAACAGGGTGTCTGGGGAAAG	99bp
hCCL5	CCGAAAGAACCGCCAAGTGT	AGCTAGGACAAGAGCAAGCAGA	144bp
hCCL22	TTACGTCCGTTACCGTCTGC	CCCTGAAGGTTAGCAACACCA	98bp
hCXCL13	CCGTGGGAATGGTTGTCCA	CCATTCAGCTTGAGGGTCCA	85bp

3.1.6 Proteomics analysis

Chemicals that used in the protein studies and their suppliers were indicated in Table 3.3.

Table 3.3 Chemicals used in protein studies

Chemicals	Suppliers
Trypsin, MS Grade, porcine pancreas **(Cat. No: 37286)**	SERVA
Ammonium bicarbonate	SIGMA
Sucrose	Merck
DTT (1,4-Dithiothreitol)	GeneDirex
Urea	Merck
Thiourea	Merck

3.1.7 Equipments

General labware equipments, machines and their accessories are shown on Table 3.4 with their supplier companies.

Table 3.4 General equipments used in the studies

Equipments	Supplier Company
Biosafety Class II Cabinet	Thermo Scientific
Thermo Cycler, PCR	BIO-RAD, T100
CFX-96^{TM} Real time PCR LightCycler® Nano	Bio-Rad Roche
Water purification system	Merck Millipore, Milli-Q® Advantage A10
FACS Verse Flow Cytometer	Becton Dickinson, ABD
Microplate Spectrophotometer	BioTek, PowerWave XS2
NanoDrop	Thermo Scientific One^{C}
Light microscope	Leica DM500
Fluorescence microscope	ZEISS, AXIO
Imaging System	BIO-RAD, ChemiDoc^{TM} MP
pH meter	Thermo Scientific, ORION STAR A211
Electrophoresis system	MS Measure Science, BIO-RAD, Mini Protean
Power supply	BIO-RAD, Power^{TM} Pac Basic
Mini-Puroteain® Glass Plates	BIORAD, 1mM spacers
Mini-Puroteain® Short Plates	BIO-RAD
Microcentrifuges	Thermo Scientific, MICROCL 21R Thermo Scientific, MICROCL 17
Centrifuges	Beckman Coulter^{TM} Allegra 64R, Thermo Scientific, SL16R
Vortex	bioSan Combi-Spin FVL-2400N
Incubator	Thermo Scientific, HERATHERM
Shaker	Witeg
Magnetic shaker with heat	Thermo Scientific, CIMAREC
Autoclave	Witeg,Nüve steamArt
Dry heat sterilizator	Nüve FN120
Pipettes	CappAid Thermo Scientific
Syringe Filters 0.45µm-0.22 µm	Sartorius
Pipette Tips	RatioLab®

Table 3.4 (cont'd) General equipments used in the studies

Equipments	Supplier Company
Serological Pipette Tips	Greiner bio-one
Pipet Controller	Sarstedt
Water Bath	EMCO, ESM-3710
Dry Block Heating Thermostat	BIOSAN, Bio TDB-100
Microwave	SAMSUNG T.D.S
Weighing Machine	UNIBLOC, SHIMADZU UW620H
Fridges	Arctiko/ Kirsch

3.1.8 General chemicals

General chemicals and their supplier companies were indicated in Table 3.5.

Table 3.5 General chemicals that used in the studies

Chemicals	Supplier Company
Hydrocloric Acid Fuming 37%	Merck
Sodium Hydroxide	Merck
Ethanol 100%	Merck
Methanol 100%	Merck
2-Propanol	Merck
Acetic Acid ≥99%	SIGMA-ALDRICH
Acrylamide/Bis-acrylamide 30% solution	SIGMA Life Science
Ammonium Per Sulfate (APS)	BioFroxx
TEMED	BioFroxx
PBS 10X	GeneMark
Trizma® Base	SIGMA Life Science
Sodium Dodecycle Sulfate	SIGMA Life Science
4X Laemmli Sample Buffer	BIO-RAD
Fixation solution	Biolegend

3.1.9 Bioinformatics tools

Bioinformatics tools and web servers used in the identification of properties of proteins are given in Table 3.6.

Table 3.6 Bioinformatics tools and web servers used in the proteomics data analysis studies

Tools	Predictions		Reference
DeepLoc	Eukaryotic Protein Subcellular Localization	https://services.healthtech.dtu.dk/service.php?DeepLoc-1.0	(145)
TMMHM-2.0	Transmembrane Helices in Proteins	https://services.healthtech.dtu.dk/service.php?TMHMM-2.0	(146,147)
SignalP -5.0	Signal Peptide and Cleavage Sites	https://services.healthtech.dtu.dk/service.php?SignalP-5.0	(148)
IEDB (The Immune Epitope Database) Analysis Resource	BepiPred Linear Epitope Prediction- B Cell Epitope Site Prediction	http://tools.iedb.org/bcell/	(149)
VaxiJen 2.0	Prediction of Protective Antigens and Subunit Vaccines	http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html	(150)
TriTrypDB	Kinetoplastid Informatic Resources	https://tritrypdb.org/tritrypdb/app/	(151)
KEGG	KEGG: Kyoto Encyclopedia of Genes and Genomes	https://www.genome.jp/kegg/	(152)
Leish-ESP	Leish-ESP: Leishmania Expression and Sequencing Projects	http://leish-esp.cbm.uam.es/	
InterPro	Classification of Protein Families	http://www.ebi.ac.uk/interpro/	(153)
BlastP	Basic Local Alignment Search Tool for Proteins	https://blast.ncbi.nlm.nih.gov/blast/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome	
Pfam	Protein Families Database	http://pfam.xfam.org/	(154)

3.1.10 Other statistics, analysis and graphics tools

GraphPad Prism 6 Software for all the graphs and statistical analysis, ImageJ for band intensity analysis, Unipro UGENE for blasted sequences alignments and Excel for tables and standard curve preparation were other softwares or programs used in the thesis.

3.2 Methods

3.2.1 Cultivation of *Leishmania*

Leishmania strains were grown in liquid complete media including RPMI with 25mM L-glutamine, 10% FBS (fetal bovine serum, GIBCO), 1% Penicillin-Streptomycin (Penicillin, 10,000 units / ml- Streptomycin, 10 mg / ml, GIBCO) and 0.2% Gentamicin (Gentamicin) to obtain a high amount of promastigote. Some strains were first thawed in complete media including 15% FBS to induce survival.

3.2.2 Growth curve of parasites

Growth curve of *Leishmania* parasites were prepared for *L. major*, *L. infantum* and *L. donovani* to determine culture stages. Cells were counted and seeded as $5x10^5$/ml or 10^6/ml in complete RPMI medium supplemented with 10% FBS, 1% Pen-Strep and 0.2% Gentamicin. Number of parasites were counted daily, and line graphs were drawn using GraphPad program.

3.2.3 Trypsin shaving experiments

3.2.3.1 Optimization of shaving conditions

Trypsin shaving of parasites were conducted in a suitable buffer by changing the type of ingredients, their quantities, incubation time and enzyme amount. *Leishmania major* reference strain was used to optimize several conditions before final treatment.

i) First, enzyme activity was checked for shaving experiments by incubating the parasites with trypsin enzyme for several hours and taking samples at various time points. Briefly, *Leishmania* promastigotes were cultured in RPMI complete medium and approximately 100 x 10^6 cells were harvested. Cells were pelleted and washed three times with 1x PBS. In the last washing step, cells were divided into 8 tubes and centrifuged to obtain a cell pellet. 60µl of 50mM ammonium bicarbonate solution containing 0.8µg trypsin was added to the cells and mixed gently. Supernatants of samples incubated at 37°C for 1 minute, 3, 5, 15, 30, 60 minutes and overnight were obtained after centrifugation of the tubes at 13.000rpm from the indicated time points. (Ammonium bicarbonate is a more commonly used buffer in trypsin enzyme digestion assays (155)).

ii) To determine the effect of centrifuge speed in the last step, incubation time and presence or absence of sucrose (as used in (92,156) for shaving experiments) on the release of proteins from promastigotes, parasites were prepared as done before. Then, cells were incubated with 50mM ammonium bicarbonate buffer with or without 0.5M sucrose at 37°C for 5 or 60min and centrifuged at 1000g, 4000g, 13.000rpm. Supernatants were collected and protein profiles were evaluated in SDS-PAGE.

iii) Concentration of ammonium bicarbonate was also optimized to compare protein release from cells without addition of trypsin enzyme. Promastigotes were counted and prepared as previous experiments. Incubation of cells were made in 25mM or 50mM ammonium bicarbonate with 0.5M sucrose for 5min or 45min. Supernatants were obtained after 1000g centrifugation.

iv) Following performing optimization experiments, the effect of ammonium bicarbonate or PBS on trypsin enzyme digestion activity was evaluated. Promastigotes were incubated with 25mM ammonium bicarbonate buffer containing 0.5M sucrose or 1x PBS containing 0.5M sucrose for 15min or 45min with 1µg trypsin in 60µl total volume. Cells were centrifuged at 1000g, and supernatants were transferred to a new tube.

v) Trypsin shaving was repeated by increasing the amount of enzyme, addition of reducing agent DTT (as applied in the study (157)). Parasites were collected at 2500g for 8 minutes and washed with 1x PBS three times. Shaving of parasite surface was performed using 5 µg Trypsin enzyme. Digestion buffer, containing 25mM ammonium bicarbonate, 0.5M sucrose and with an addition of 5mM DTT was mixed gently with cells and the incubation of samples was carried out at 37°C for 45 minutes. Afterwards, the tubes were centrifuged at 3000 g for 7 minutes. The supernatant was collected and stored at -20°C.

3.2.3.2 Trypsin shaving of *Leishmania* strains

L. major, L. infantum and *L. donovani* parasites were used to shave surface proteins with trypsin enzyme. Parasites were counted and 100×10^6 parasites were used in the experiment following 3.2.3.1 part (v). In addition to applied optimized conditions, surface trypsin digestion was followed by overnight trypsin digestion based on the work of Benachour and his colleagues performed (92). After last centrifugation of parasites, supernatants were taken into new eppendorf tubes and 600 µl of 25mM ammonium bicarbonate buffer was added on samples. Each solution was passed through a 0.2µM filter. To complete the trypsin enzyme cleavage, the solution was brought back to 37°C and left overnight. Enzyme activity was stopped by addition of acid and prepared for LC-MS/MS analysis. SDS-PAGE gel was run and gel was stained with silver solution. The physical appearance of the parasites after digestion was examined under the microscope

3.2.3.3 Total protein extraction

To control trypsin digested proteins, total protein isolates were obtained from the cells and protein profiles were compared. Approximately 10×10^6 cells were pelleted and lysed with the solution containing 6M urea, 2M thiourea, 2% SDS and 2mM EDTA. The cells were incubated for 1 hour at room temperature with 500 µl of this solution. Tubes were centrifuged at 16.000g for 10 min. and the supernatant was taken. Proteins were stored at -80 °C.

3.2.4 Detection of proteins

3.2.4.1 SDS-PAGE

As a protein separation method, SDS-PAGE was performed to visualize peptides/proteins in the optimization steps of the trypsin shaving method and to detect the obtained cell surface-shaved peptides/proteins. For this purpose, a 12% separation gel with stacking gel were prepared. Ingredients of the gel and required volumes of the solutions are indicated in Table 3.7. After preparation, gels were covered in a wet tissue and stored at 4°C until further use.

Table 3.7 SDS-PAGE gel contents for separating and stacking gels

Components	**Volume**
Separating Gel (10ml)	
Distilled water	3.2 ml
30% Acrylamide Bis-acrylamide	4 ml
1.5 M Tris HCl pH 8.8	2.6 ml
10% SDS	100 µl
10% APS	100 µl
TEMED	10 µl
Stacking gel (5ml)	
Distilled water	2.975 ml
30% Acrylamide Bis-acrylamide	0.67 ml
0.5 M Tris HCl pH 6.8	1.25 ml
10% SDS	50 µl
10% APS	50 µl
TEMED	5µl

Since the protein concentrations obtained after trypsin shaving were not very high, the highest volume of protein was loaded on the SDS gels. 4X Laemmli Buffer (Biorad) prepared with β-mercaptoethanol (9:1 Laemmli Buffer: β-mercaptoethanol) solution was added to proteins as to be 1X working concentration and the mixture was incubated at 95°C for 5 minutes. Total protein concentrations, on the other hand, were prepared with a lower amount since they were having higher concentrations. All samples were loaded on the gels with a pre-stained protein ladder (GeneMark) and they were run at 60V 30 minutes, 100V 30 minutes and 150V until the dye reached to the end.

3.2.4.2 Silver staining

Protein detection on polyacrylamide gels was perfomed with Silver Stain Plus™ (Bio-Rad) kit. Staining procedure was carried out according to manufacturer's instructions. Briefly, the gel after running was incubated in the fixative enhancer solution specified in Table 3.8 for a minimum of 20 minutes at room temperature. It is then washed twice with deionized distilled water for 10 min. Afterwards, the staining solution was prepared by adding in an order of water, silver complex solution, reduction moderator solution, image development reagent and development accelerator solution as indicated volumes in Table 3.9. The gel was taken into this solution and stained on a shaking platform until bands were observed. Gels were then incubated with 5% acetic acid solution for 15 minutes to stop silver staining. The gel was then washed with deionized water and visualized on the ChemiDoc (Bio-Rad).

Table 3.8 Silver staining fixation solution content

Content	**% (V/V)**	**Volume (ml)**
Methanol	50	10
Acetic acid	10	2
Fixation Enhancer Concentrate	10	2
Deionized distilled water	30	6

Table 3.9 Silver staining solution content

Content	**Volume (ml)**
Deionized distilled water	3.5
Silver complex solution	0.5
Reduction moderator solution	0.5
Image development reagent	0.5
Development accelerator solution*	5

** 0.25 g of development accelerator chemical is dissolved in 5 ml of water.*

3.2.4.3 BCA Assay

Bicinchoninic acid (BCA) assay kit was used to quantitate of protein concentration. The proteins are known to reduce Cu^{+2} to Cu^{+1} in alkaline medium.

BCA and reduced Cu ion reaction gives purple color which can be measured at 562 nm absorbance (158). In this assay, bovine serum albumin (BSA) standards were prepared from given 2000µg/ml stock concentration. The diluted BSA standard concentrations range between 20µg/ml-2000µg/ml. Reagent A and reagent B was mixed 50:1 ratio by calculating required total amount and the mixture was distributed to a 96-well plate well as 200µl per sample. Extracted proteins and standard samples were added on wells in 10µl volume. The plate was incubated at 37°C for 30min. The absorbance at 562nm was measured with microplate spectrophotometer (BioTek, PowerWave XS2) and obtained values were used to plot a line graph for concentration versus optical density (OD). With the equation, samples' concentration was calculated from their OD values.

3.2.5 LC-MS/MS analysis

LC-MS/MS analysis was carried out with C18 nanoflow reversed-phase HPLC (Dionex Ultimate 3000,3500 RSLC nano, Thermo Scientific) integrated with Orbitrap Mass Spectrometer (Q Exactive Orbitrap, Thermo Scientific) at Koç University Translational Medicine Research Center Omics Laboratory with the protocol indicated in Table 3.10.

Table 3.10 LC-MS/MS protocol for *Leishmania* shaved samples

LC Gradient	4-25%, 25-45%, 45-95% acetonitrile in 0.1% formic acid witl 300 nl/min flow, 90 min
MS Method:	MS1: 60 000 resolution; AGC 3e6; Max IT 60 ms; Scan range 400-1500 m/z
	MS2: 15 000 resolution; AGC:5e4; Max IT:60 ms; Top 15; Isolation window:2.2 m/z; NCE: 26
Analysis	Thermo Scientific Proteome Discoverer 2.3
Search engine	Sequest
Fasta file	Leishmania donovani_uniprot-proteome_UP000318447_20210518 Leishmania infartum_uniprot-proteome_UP000008153_20210518 Leishmania major_uniprot-proteome_UP000000542_20210518
Filters	Protein FDR confidence, at least High; Peptide Seguence Lenght beween 7-25; Peptide confidence, at least High

3.2.6 Bioinformatics analysis

After instrument analysis, raw peptide sequences data were blasted with proteome of *Leishmania donovani*_uniprot-proteome_UP000318447_20210518; *Leishmania infantum*_uniprot-proteome_UP000008153_20210518; *Leishmania major*_uniprot-proteome_UP000000542_20210518 in Swiss-Prot and TrEMBL database. At least 1000 proteins were listed from 3 database for each *Leishmania* species.

Unique peptides count lower than two was distracted from total list. Multifasta formats of protein sequences were prepared in Excel. To filter cell membrane proteins, Deeploc-1.0 was used with Blosum63 protein encoding for maximum 500 sequences for each run. Protein length greater than 6000 aa were excluded from analysis (threshold for tool). Transmembrane helix domains were predicted with TMHMM-2.0 for all proteins. Similarly, signal peptide sequence predictions were performed with SignalP-5.0 web server. Ortholog protein or gene findings of *Leishmania* strains were assessed in TriTrypDB, KEGG DB or Leish-ESP from Uniprot IDs or gene IDs. Additional analysis of proteins families, domains and functional sites were done in Interpro or Pfam databases from Uniprot IDs of each targeted sequence. Multi alignments of ortholog proteins were prepared in first NCBI Blast, then applied to Unipro UGENE software. Vaxijen-2.0 was used to score antigenic properties of indicated proteins sequences by choosing parasite as an organism which accepts 0.5 for threshold for antigenicity. B-Cell epitope predictions of targeted sequences were examined with BepiPred-2.0 and shown in multiple alignment images.

3.2.7 Leishmania RNA virus (LRV) Detection

3.2.7.1 RNA isolation

Leishmania LRV+ *L. major* isolate, LRV- *L. major* isolate and reference strains were cultured as mentioned previously. Promastigotes were centrifuged at 1000g for

8 min and pellets were used for RNA extraction. Total RNA isolation was performed with Total RNA Miniprep Purification Kit® (GMBiolab, Taiwan) according to manufacturer's instructions. Briefly, cells were lysed with 350 µl β-mercaptoethanol containing RNA lysis buffer and mixed with equal volume of 70% ethanol. Mixture was transferred to spin column filters and centrifuged at 12.000 x g for 1 min. Filter was washed with 500 µl Wash I Buffer and DNase I treatment was performed on filters for 15 min at room temperature. 600 µl Wash II was added on columns and centrifuged at 12.000 x g for 1 min and washing repeated again. Elution was performed with 35 µl nuclease-free water. Eluted RNA concentration was measured with NanoDrop (Thermo Scientific) and RNA integrity was check with agarose gel electrophoresis. RNA samples were stored at -80˚C until further use.

3.2.7.2 cDNA synthesis

Conversion of RNA to single-strand cDNA was performed with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™) according to manufacturer's instructions. Briefly, volume of RNA up to 2 µg was calculated for each sample depending on the concentration of the samples and mixed with nuclease-free water. Reaction mixture was prepared in total and distributed equally into each tube. Amounts of ingredients used in the total volume of 20 µl reverse transcription reaction were used as 2 µL 10X RT Buffer, 0.8 µL 25X dNTP mix, 2 µL 10X RT Random Primers, 1 µL Ribolock RNase Inhibitor, 1 µL Reverse Transcriptase mixture added on 13.2 µL RNA (up to 2 µg) and ddH_20 mixture. Reaction steps and their incubation times were followed as: 10 min at 25°C, 120 min at 37°C and 5 min at 85°C.

3.2.7.3 Conventional LRV-2 PCR

LRV-2 presence in two *L. major* isolates and reference strain was evaluated with specific detection primers that targeting the viral capsid antigen of LRV using conventional PCR. As an internal control, *L. major s*pecific KMP-11 PCR was run simultaneously. PCR reactions were performed with PCR Hot Start Master Mix II

(GMBiolab®) and reaction mixture was 1µl of cDNA and 7.5 pmol from each LRV-2 primer pairs in a total of 25 µl reaction mixture. Thermal cycler conditions are specified as 3 min at 95°C for initial denaturation; followed by 35 cycles of 30 sec at 95°C for denaturation, 30 sec at 53˚C for annealing, 2 min at 72 ˚C for extension; 7min at 72˚C for final extension. Amplification of targeted sequences were checked with agarose gel electrophoresis. Two percent agarose gel was prepared, and PCR products were loaded on gels with Sybr Gold (TIBO) and Loading Buffer (6X) (GMBiolab®). 100 bp DNA marker was used (GMBiolab®). Results were visualized with ChemiDoc (BioRad).

3.2.7.4 Sequencing

The LRV-2 PCR product was sequenced in GATC Biotech laboratories in Germany, and the data was blasted to LRV-2 nucleotide sequence in NCBI.

3.2.8 THP-1 cells and differentiation into macrophages

3.2.8.1 THP-1 culture

Human monocytic cell line, THP-1 (ATCC® TIB-202 ™) was grown at RPMI-1640 with L-Glutamine (GIBCO) medium containing 10% FBS (GIBCO) and 1% Penicillin-Streptomycin (GIBCO) in a 37˚C incubator with 5% CO_2. Passages were performed every two to three days by monitoring cells and counting them to not exceed 10^6 cells/ml. Culture volume was depended on required experimental procedure.

3.2.8.2 PMA induction

Monocyte derived macrophages (MDMs) were obtained from THP-1 monocytes with PMA induction. PMA is a widely used chemical agent to induce monocytes resemble like macrophage profile (159). To perform the induction, THP-1 cells were seeded as 500.000cells/well in 6-well plates with 25nM or 80nM PMA or without addition PMA. Cells morphology was monitored at 24h, 48h and 72h and cells were collected for RNA extraction with Trypsin-EDTA (GIBCO) treatment. Total RNA isolation procedure was same as the previous ones mentioned before in Section 3.2.7.1 and converted to cDNA as explained in Section 3.2.7.2.

3.2.8.3 Microscopy analysis

THP-1 monocyte cells morphology change into macrophages was examined with fluorescent microscopy (ZEISS, AXIO) using bright field and photographed daily at 400X magnification for PMA induced or non-induced conditions.

3.2.8.4 Macrophage marker expression

Differentiation of THP-1 monocytic cells to macrophage-like profile was also assessed by comparing expression of two known macrophage related surface markers: CD36 (160) and CD11b (161) for each case. Conventional PCR was used to observe expression profile with 25 cycles of denaturation-annealing-extension stages before reach to the plateau of PCR curve. PCR products were run 2% agarose gel with Sybr Gold (TIBO) using 100-bp marker (GMBiolab) and visualized with ChemiDoc (BioRad). PCR conditions were given as initial denaturation at 95°C for 3 min, and 25 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 1 min, and final extension at 72°C for 5 min. Intensity of bands was calculated semi-quantitatively using ImageJ program and bar graphs were drawn using GraphPad program.

3.2.9 *Leishmania* infection experiments

THP-1 monocytic cells were collected and centrifuged at 500g for 5min. Supernatant was discarded, and pellet was counted with Improved Neubauer counting chamber. Cells were seeded as 330.000cells/well for 24-well plates in 500µl or 170.000cells/well for 48-well plates in 300µl with 25nM PMA in complete RPMI media and cultured for 3 days. That day, culture supernatant was replaced with fresh

RPMI media without PMA and infection was done one day after. LRV+ *L. major* isolate, LRV- *L. major* isolate and reference strain were grown in liquid RPMI complete media mentioned before and kept at 25˚C. Instead of 10% FBS, in some steps 15% FBS was used to increase growth rate.

Leishmania promastigotes were collected at day 5-7. Cells were centrifuged and supernatant discarded. Pellet was dissolved with fresh RPMI media. After, they were counted with Thoma counting chamber and macrophages were infected with *Leishmania* strains with 1:4 ratio (macrophages:promastigotes). Macrophages were kept together with parasites for 24 hours or 48 hours at 37˚C incubator with 5% CO_2.

For all experiments, supernatants were collected to Eppendorf tubes, centrifuged at 5000g for 5min, transferred to new tubes and stored at -80˚C until further use. Infected cells were directly lysed with lysis buffer of Total RNA isolation kit (GMBiolab) on plates and the extraction protocol was continued as written before in Section 3.2.7.1. RNA concentrations were measured with NanoDrop and single strand cDNA synthesis was performed as mentioned before in Section 3.2.7.2 using 0.5 or 1 µg RNA depended on the concentration of experimental sets. The experiment sets were repeated at least 3 times to.

3.2.10 Giemsa staining

Control THP-1 derived macrophages and *L. major* isolates and reference strain infected macrophages were collected and air-dried on microcopy slides. Samples were fixed with methanol and stained with 1:20 diluted Giemsa dye for 10 min. Slides were rinsed with distilled water. Dried samples were observed under light microscope.

3.2.11 Relative expression levels of chemokines

To determine relative expression levels of chemokines in infected macrophages, CCL1, CCL2, CCL5, CCL22 were analyzed with real time PCR (RT-PCR). Optimal annealing temperatures of primers were determined, and experimental work was done for proper cDNA concentration.

Samples were diluted 1:6 for chemokine expressions and 1:12 for ACTB endogenous control. Sybr Green I (GMBiolab) was used to detect PCR products and experiment was conducted in CFX96 (BioRad) and LC Nano (Roche). PCR conditions were as following: initial denaturation at 95°C for 2 min, 40 cycles for denaturation at 95°C for 20 sec, annealing at 60°C for 30 sec and extension at 72°C for 45 sec. Cycle threshold (Ct) values obtained after run were analyzed with $2^{-\Delta Ct}$ method. Bar graphs and statistical analysis was performed in GraphPad program.

3.2.12 Human inflammatory cytometric bead array (CBA)

Production of selected cytokines were evaluated with a specialized Human inflammatory cytometric bead array (CBA). The kit is used for cell-free samples and detects IL-8, IL-1β, IL-6, IL-10, TNF, and IL-12p70 cytokines with a bead-based system. Beads had distinct CBA-Red fluorescence intensities and antibody spesific for one of the cytokine which can be analyzed with a proper flow cytometer (162). Standard samples were prepared from a top standard 5000 pg/mL with a 1:2 serial dilution until 1:256 with assay diluent. The mixture of bead populations was prepared as combining 10µl of each cytokine specific bead into one tube and mixing 50 µl of it with 50 µl of each standard, negative sample with only assay diluent or experimented sample supernatants. After addition of 50 µl phycoerythrin (PE)-conjugated cytokine antibody mixture into tubes, samples were incubated for 3 hours at room temperature at dark. One ml of Wash Buffer was added on samples and centrifuged at 200g for 5 minutes. Beads were dissolved with 300 µl wash buffer and mixed with Fixation Buffer (Biolegend), then solutions were transferred to flow tubes. Detection of cytokines were done with FACS Verse flow cytometer (BD). Instrument setup was performed for the detection of CBA-Red signal. Data were acquired with CBA-Red to PE (FL-2 channel) plot.

4 RESULTS

4.1 Surface Protein Analysis of Cutaneous and Visceral Strains

4.1.1 Growth curve of *Leishmania* strains

Comparative surface protein analysis of parasites was studied in the promastigote form of parasites. Unlike amastigotes, this parasitic form has several infectivity stages. To assess the proliferation dynamics of parasites, visceral leishmaniasis associated strains *L. donovani* and *L. infantum* strains and cutaneous leishmaniasis associated *L. major* strain were subjected to growth curve analysis. Briefly, 5 x 10^5 /ml or 10^6 / ml cells were cultured at day 0 and parasite concentrations were obtained by counting cells with a counting chamber every day. Growth curves of *L. donovani*, *L. infantum* and *L. major* were given in Figure 4.1.

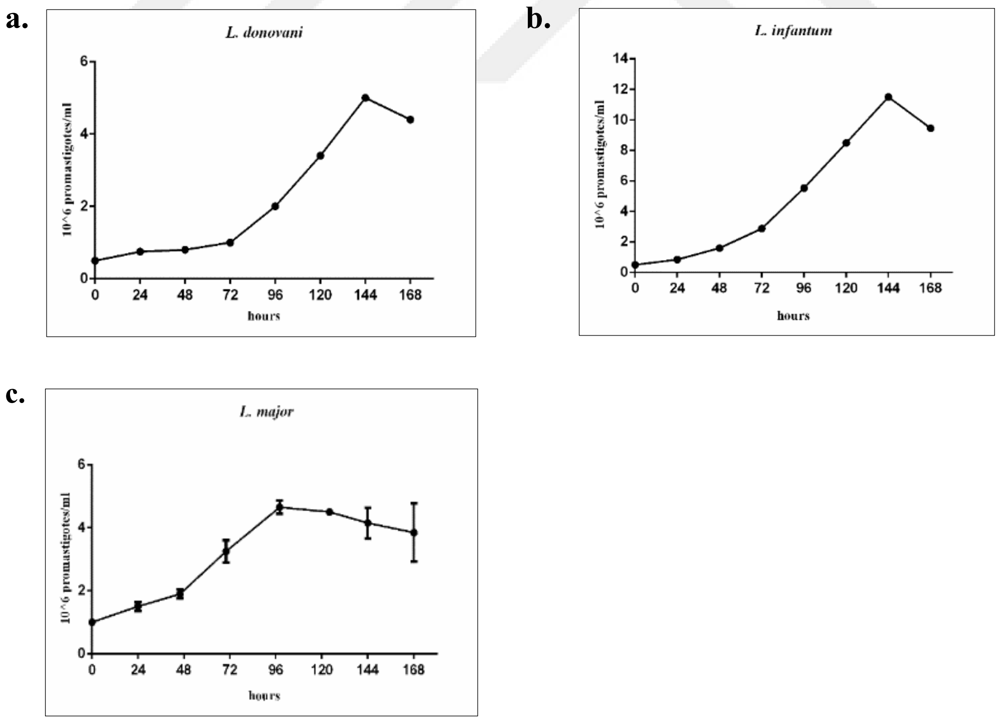


Figure 4.1 Growth curve graphs a. *L. donovani*, b. *L. infantum* and c. *L. major*. Line graphs were drawn in GraphPad Prism (cell number (x10^6) vs time (0-168h))

Logaritmic phase of the growth curve consists of dividing, motile, non-infective procyclic promastigotes whereas stationary phase parasites are more infective and non-dividing metacyclic promastigotes (163). *L. infantum* and *L. donovani* reference strains were started with 5 x 10^5/ml concentration and reached to logarithmic phase after day 3 and stationary phase after day 6. *L. major* reference strain was cultured with 10^6 / ml concentration and reached to logarithmic phase after day 2 and stationary phase after day 5. Experiments were performed between days 5-7 according to estimated phases to be able to increase metacyclic promastigote percentage.

4.1.2 Selection of conditions for trypsin shaving experiments

To find out proper enzyme activity which depends on temperature, pH and type of the solution, or concentration, several conditions were tested on live parasites. To shave surface proteins, enzyme should work properly, and incubation buffer should not induce cell lysis or cytoplasmic content release. First of all, activity and digestion capability of the trypsin enzyme (MS approved, from porcine pancreas, SERVA) were observed with a time course experiment on *Leishmania* promastigotes. SDS-PAGE results were shown in Figure 4.2.

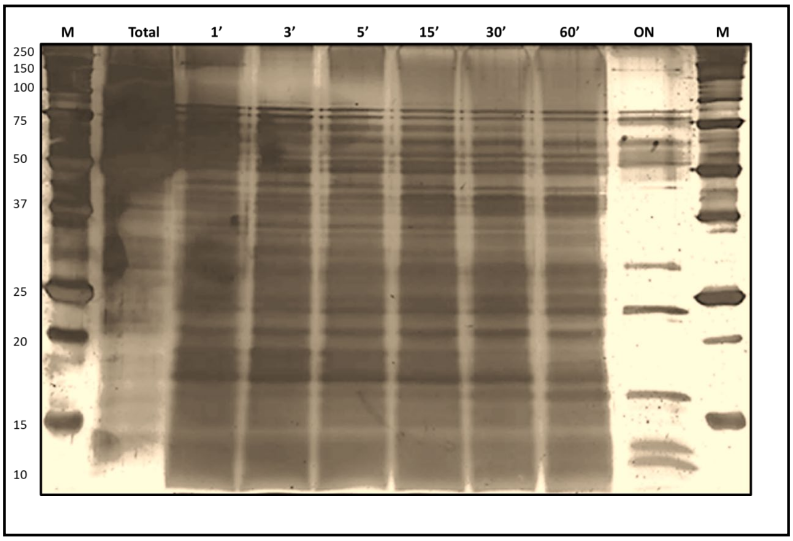


Figure 4.2 Efficiency analysis of trypsin enzyme and digested proteins of live promastigotes in time-dependent manner. Promastigotes were split into tubes and

Figure 4.2 (cont'd) treated with 0.8μg trypsin enzyme in 50mM ammonium bicarbonate for 1, 3, 5, 15, 30, 60 minutes or overnight (ON). SDS-PAGE shows total protein content (Total), supernatant of trypsin-treated samples for each time (labels 1'-60' or ON) and protein marker (M).

Trypsin was efficiently digested proteins in the environment of promastigotes in the increasing time points (Figure 4.2). Overnight incubation showed total digestion of proteins that present in the incubation buffer. However, presence of several proteins even in the first minute of incubation led to optimize conditions for experiments to decrease cell content contamination.

Various factors can be effective on protein release from cells. Therefore, protective role of additive sucrose in incubation buffer and/or centrifuge speed effect on parasites cells break down in different time points were compared without adding Trypsin enzyme. Parasites were incubated with 50mM ammonium bicarbonate with or without 0.5M sucrose for 5 or 60 minutes and centrifuged at 1000g, 4000g, 13.000rpm for removal of cells. Supernatant were run on SDS-PAGE and silver-stained gel results were indicated in Figure 4.3.

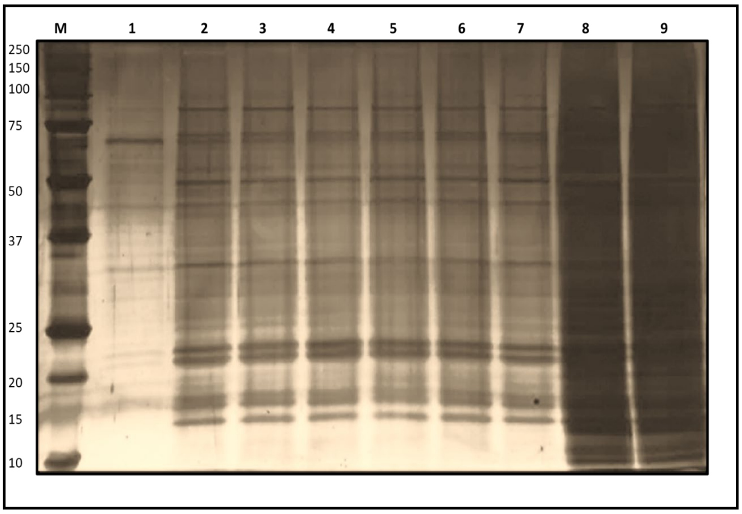


Figure 4.3 Effect of sucrose addition, centrifuge speed and incubation time on protein release from parasites. Silver stained SDS-PAGE results for supernatants for promastigotes treated with 50mM ammonium bicarbonate buffer only with sucrose (labels 2-4 for 5 min, 5-7 for 60 min) or without sucrose (labels 8-9 for 60 min). Centrifuge speed was used as 1000g (labels 2, 5, 8), 4000g (labels 3, 6, 9) or 13.000rpm (labels 4, 7). M is protein marker and label (1) refers to last PBS wash before incubation.

Supernatant of promastigote cells after incubation led to compare conditions for protein release before trypsin enzyme addition. To prevent cell lysis, sucrose was found as protective when added 0.5M into 50mM ammonium bicarbonate buffer (labels 2-7 compared to 8-9). Longer incubation time increased protein bands very slightly when compared to shorter one. However, centrifuge speed does not have a significant effect on this releasement.

Ammonium bicarbonate concentration was another tested factor to minimize cellular lysis from before trypsin treatment of *Leishmania* parasites. For this reason, 0.5M sucrose added 50mM and 25mM ammonium bicarbonate were compared for two incubation times 5 minutes or 45 minutes with similar procedures. Figure 4.4 shows the SDS-PAGE results of equal volumes of released supernatant proteins. Lowering the concentration of ammonium bicarbonate from 50mM to 25mM decreased detected bands on SDS-PAGE after 45 minutes so experiments were carried out with 25mM concentration with 0.5M sucrose.

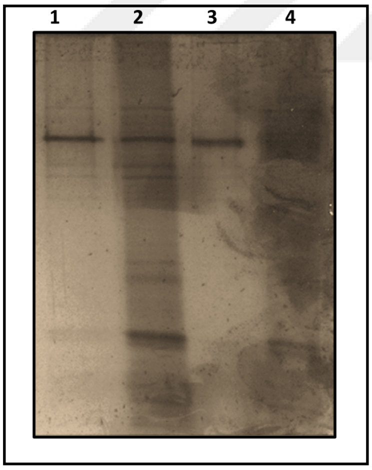


Figure 4.4 Effect of ammonium bicarbonate concentration on protein release from promastigotes. Silver stained SDS-PAGE for supernatant samples of parasites which were incubated in 50mM ammonium bicarbonate for 5 (label 1) or 45 min (label 2) or 25mM ammonium bicarbonate buffer for 5 (label 3) or 45 min (label 4).

After most of the conditions were optimized, promastigotes' surface proteins were digested with 1 μg trypsin in the PBS or 25mM ammonium bicarbonate with or without 0.5M sucrose for 15 or 45 minutes. Silver stained SDS-PAGE results were compared according to bands profile (Figure 4.5).

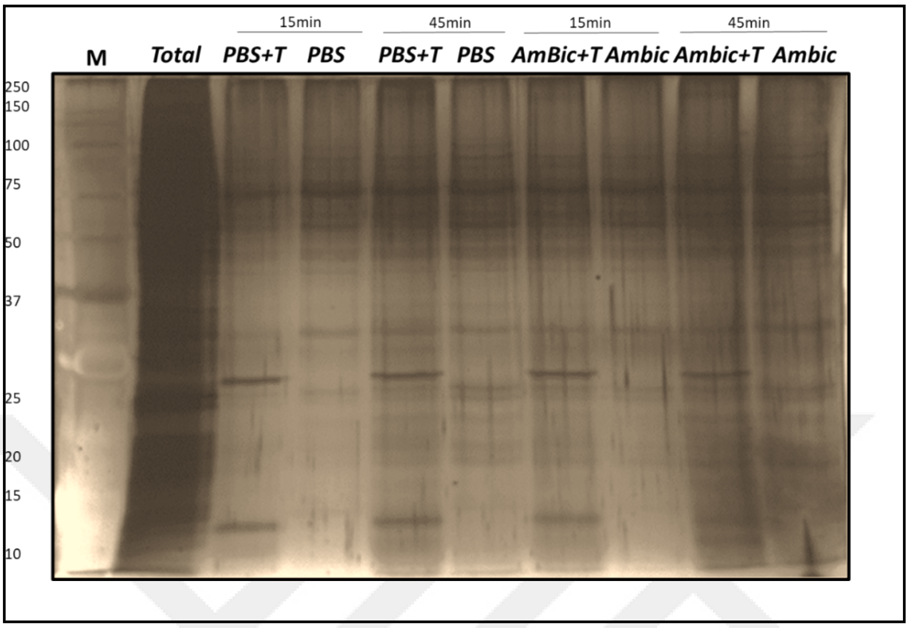


Figure 4.5 Comparison of phosphate buffered saline (PBS) and ammonium bicarbonate (AmBic) incubation buffer on trypsin enzyme digestion. Parasites were treated with 1µg trypsin in 1x PBS or 25mM ammonium bicarbonate for 15 or 45 minutes. Total protein extract (lane 2), samples with PBS (lanes 3-6) or AmBic (lanes 7-10) buffers with or without trypsin addition, and M protein marker (lane 1) are indicated in Silver-stained SDS-PAGE image.

Trypsin enzyme is around 24 kDa. SDS-PAGE bands near 25kDa in protein ladder in trypsin added samples shows enzyme itself. Comparing 25mM ammonium bicarbonate with PBS in shaved samples, detected protein bands especially in lower kDa proteins were higher in trypsin enzyme incubated in ammonium bicarbonate buffer for 45 minutes. Experiments were repeated for this condition by increasing promastigote amount, trypsin concentration, centrifuge speed (to eliminate lighter or smaller cell contamination) and addition of DTT.

4.1.2.1 Shaving of cutaneous and visceral strains with trypsin enzyme

To obtain trypsin-shaved surface proteins for LC-MS/MS analysis with comparable results, conditions were applied for all three Leishmania species: *L.*

major, *L. infantum* and *L. donovani* simultaneously. After last centrifuge, supernatant may include lysed but not degraded cells which these cells may contaminate surface digested proteins with cellular proteins. Therefore, the supernatant samples were filtered with 0.2µm filter and trypsin cleavage was extended until next day to complete protein digestion into peptides. Trypsin shaved proteins and overnight digested proteins were separated with SDS-PAGE (Figure 4.6). Digested peptides were sequenced for protein determinations in LC-MS/MS analysis.

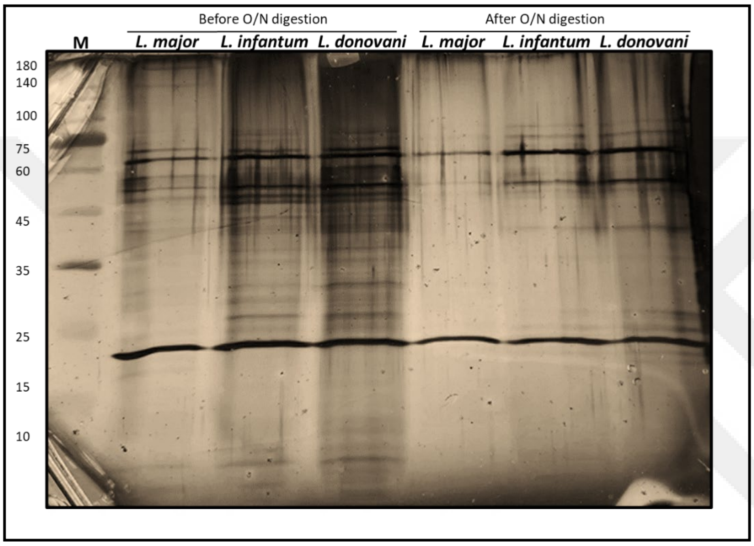


Figure 4.6 Trypsin digestion of *L. major*, *L. infantum* and *L. donovani* promastigotes before and after overnight incubation. All three strains were digested with 5µg trypsin enzyme at 37°C and supernatants of *L. major*, *L. infantum* and *L. donovani* were collected, filtered and run on SDS-PAGE, respectively (lanes 2-4). Digestion was completed with overnight incubation of pre-cut proteins that are shown silver stained SDS gels (lanes 5-7).

4.1.3 Analysis of trypsin-shaved proteins of *Leishmania* strains

4.1.3.1 Protein database search for *Leishmania*

Leishmania species were shaved *in vivo* with trypsin enzyme and obtained peptide sequencing data after LC-MS/MS were analyzed with bioinformatics approaches. Listed peptides were searched in Sequest search engine for Uniprot databases: Leishmania donovani_uniprot-proteome_UP000318447_20210518; Leishmania infantum_uniprot-proteome_UP000008153_20210518; Leishmania major_uniprot-proteome_UP000000542_20210518. Proteins were primarily sorted for unique peptide numbers (#/Unique peptides) and unique peptide number equal and greater than 2 were taken into further analysis. After threshold, remaining protein counts were 1795 for *L. donovani*, 1519 for *L. infantum* and 969 for *L. major*. Uniprot IDs were used to retrieve FASTA sequences of indicated proteins and collected as multi-FASTA format.

4.1.3.2 Evaluation of plasma membrane proteins and their biological processes within species

To elaborate properties of plasma membrane proteins in cumulative data, protein subcellular localizations were predicted with DeepLoc 1.0. The model predicts eukaryotic protein localizations with a deep neural networks based on only protein sequence data with 92% accuracy for membrane-bound proteins (145). Algorithm was used with prepared multi-FASTA data for scoring possible placement of proteins in nucleus, cytoplasm, extracellular, mitochondrion, cell membrane, endoplasmic reticulum (ER), plastid, golgi apparatus, lysosome/vacuole or peroxisome. Highest valued places were chosen for each protein regarding cell membrane. Among 1795 proteins, 86 proteins were predicted as plasma membrane for *L. donovani*. Around 85 of 1519 proteins for *L. infantum* and 37 of 969 proteins for *L. major* were classified in plasma membrane.

Furthermore, presence of transmembrane helix (TMH) topology and number of these helices were predicted with TMHMM-2.0 web server for integral membrane proteins and signal peptide (SP) predictions were done with SignalP-5.0 for proteins having cleavable membrane insertion sequences. In total, 229, 209 and 104 transmembrane helix domain residing proteins and 59, 57, 36 signal peptide sequence inserted proteins were predicted for *L. donovani*, *L. infantum* and *L. major*, respectively. All cell membrane proteins sub-listed with TMH and SP domains were presented for all three strains (Table 8.1 for *L. donovani*, Table 8.2 for *L. infantum* and Table 8.3 for *L. major*, Appendix 1).

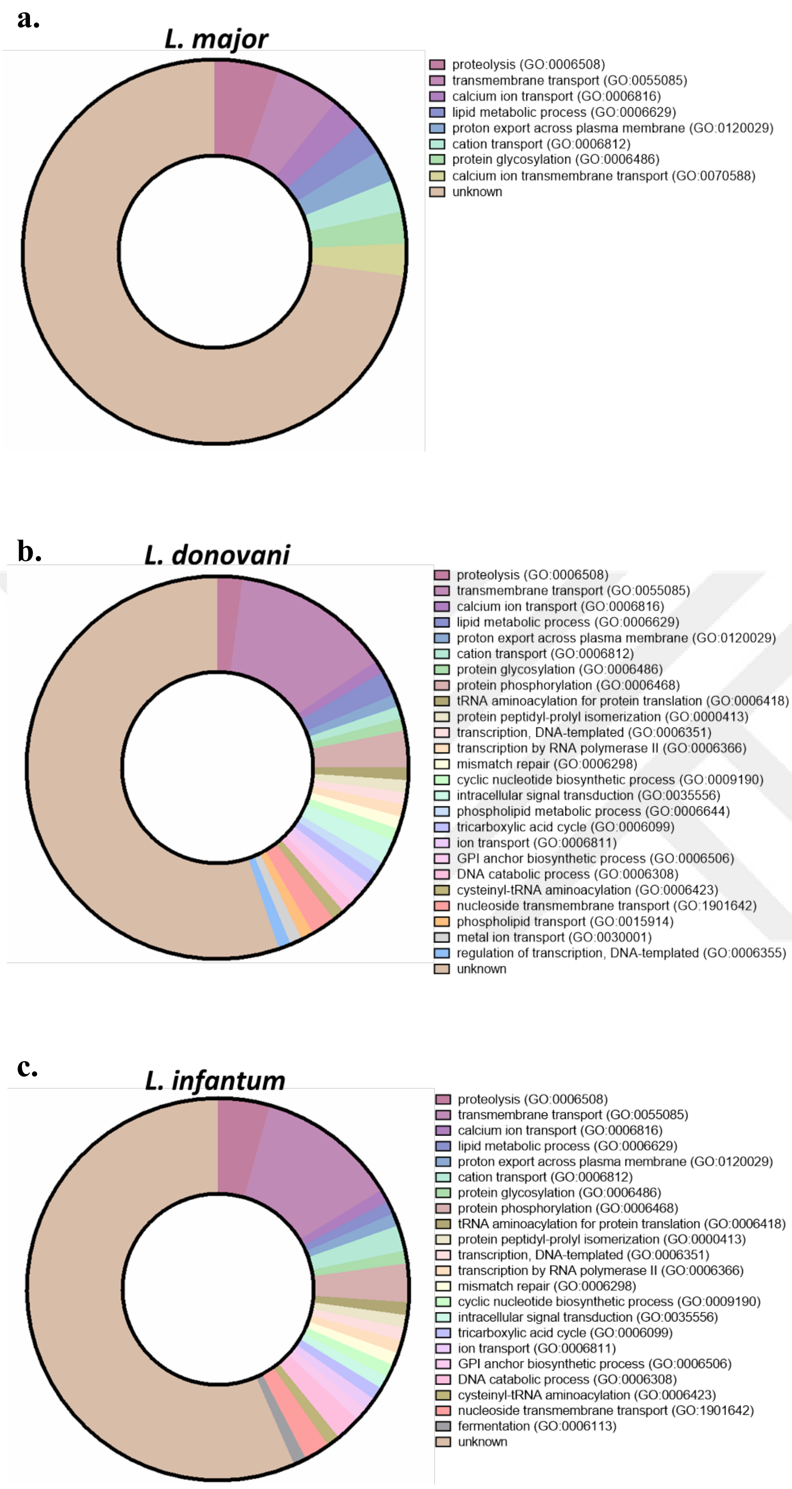


Figure 4.7 Distribution of biological process of *L. major*, *L. donovani* and *L. infantum* plasma membrane proteins. Data obtained from InterPro server for was drawn as pie charts in GraphPad prism for a. *L, major,* b. *L. donovani* and c. *L infantum.* GO:Biological Process numbers given in brackets for each cluster annotation with same color code. Unknown represents proteins with any known biological function information.

Plasma membrane localized proteins were further evaluated for gene ontology (GO) terms protein classes in InterPro analysis tool. It predicts protein families and domains and related annotations using several member databases with protein signature models (153). Functional properties of proteins are classified with GO term enrichment method regarding biological process, molecular function, and cellular component. Each of the proteins were submitted to database and biological processes of identified proteins were collected with their specific GO term codes and classes. Pie chart graphs of plasma membrane proteins for all three species were made for each GO:Biological Process category for *L. major*, *L. donovani* and *L. infantum* (Figure 4.7a, b, c, respectively).

Cell membrane properties of all species for identified proteins were subjected to GO annotation. Most of the proteins were found unknown which means without any pre-defined biological processes. Proteins with specified GO classifications were mainly involved in the transport mechanism. *L. infantum* and *L. donovani* showed similar protein profiles and classification compared to *L. major*. Membrane transport proteins constituted most of the identified proteins yet again. However, number and varieties of proteins presented in *L. donovani* and *L. infantum* are more than *L. major*. ATP-binding cassette (ABC) family proteins and ABC-related transport proteins are abundant in this sub-group of visceral strains plasma membrane transporters (Appendix 1).

4.1.3.3 Comparison of *L. donovani* and *L. infantum* plasma membrane proteins with *L. major* orthologs

Cell membrane localized proteins of *L. donovani*, *L. infantum* visceral strains were collated against *L. major* proteins to identify possible visceral enriched proteins. Proteins that commonly expressed in both *L. donovani* and *L. infantum* were listed and compared with identified proteins of *L. major* counterparts. Ortholog genes were fished out from TriTrypDB, KEGG database and Leish-ESP database for each mutual protein. Spectral peptides count equal and greater than 2 for *L. donovani* & *L. infantum* versus *L. major* was accepted as differentially expressed (Table 4.1).

Table 4.1 Differentially expressed proteins of *L. donovani* and *L. infantum* compared to *L. major* orthologs (variation of #Peptides ≥ 2).

Uniprot ID Identified	Protein name	Length	#Peptides (Unique Peptides)		Uniprot ID	Protein names	# Peptides (Unique Peptides)	Gene ID *Lm* ortholog	Length
	L. donovani & L. infantum		*Ld*[1]	*Li*[2]		*L. major*	*Lm*[3]		
A0A504X6J6 Q4Q6L9	Calpain cysteine protease family protein Putative cytoskeleton-associated protein CAP5.5	724 723	18 (18) 0(0)	19 (15) 5(1)	Q4Q6L9 A0A504X6J6	Putative cytoskeleton-associated protein CAP5.5 Calpain cysteine protease family protein	22 (18) 6(2)	LMJF_31_0440 CGC21_12120	723 724
A0A504XF03	Uncharacterized protein	1502	13 (13)	8 (8)	E9ADG8	Uncharacterized protein	2 (2)	LMJF_27_1895	1177
A0A504X9G9	HAD ATPase, P-type, IC family protein	1196	10 (10)	8 (8)	E9AF31	Putative calcium motive P-type ATPase (EC 3.6.3.-)	2 (2)	LMJF_35_2080	1109
A0A504XE14	Protein kinase domain family protein	752	10 (10)	6 (6)	E9ACB1 E9AEZ3	Putative casein kinase II, alpha chain- Putative cyclophilin 14	- -	LMJF_02_0360 LMJF_35_1720	381 444
A0A3S7X8Z9	Thioredoxin family protein	559	10 (10)	9 (9)	Q9GRP8	Uncharacterized protein L7845.03	3 (3)	L7845.03 LMJF_35_1330	562
A4HXD4	Putative P-type ATPase (Vacuolar-type_Ca2+-ATPase_-_putative)	1134	7 (7)	5 (5)	Q4QED4	Putative P-type ATPase	-	LMJF_17_0600	1051
A4I0N4	Hypothetical predicted transmembrane protein (Protein_of_uncharacterized_function_(DUF3184)_-_p utative)	1375	7 (7)	7 (7)	Q4QAT7	Hypothetical predicted transmembrane protein	3 (3)	LMJF_24_0440	1369
A0A504XVP5	5-histidylcysteine sulfoxide synthase	1280	7 (7)	5 (5)	E9ADV2 E9ADV3	Uncharacterized protein Uncharacterized proteins	3 (3) -	LMJF_29_0940 LMJF_29_0950	757 494
A4I9N7	ATP-binding cassette protein subfamily C, member 8 (ATP-binding_cassette_protein_subfamily_C_-_member_8_-_putative)	2089	7 (7)	4 (4)	Q4Q3E0	Putative ATP-binding cassette protein subfamily C,member 8	2 (2)	ABCC8 LMJF_34_0670	2087
A4I9R3	ATP-binding_cassette_protein_subfamily_B_-_member_2_-_putative (p-glycoprotein	1341	7 (7)	8 (8)	Q4Q3A6	P-glycoprotein	-	LMJF_34_0990	1341
A0A504XNW5	Papain cysteine protease family protein	4209	7 (6)	5 (5)	Q4QI61- Q4QI68	Cathepsin L-like protease Cathepsin L-like protease	-	LMJF_08_1080- LMJF_08_1010	443-348
A4I065	ABC-thiol transporter (Pentamidine resistance_protein_1)	1569	7 (6)	6 (3)	Q4QBE9	ABC-thiol transporter	-	LMJF_23_0250	1568
A0A504Y207	ABC transporter family protein	1971	6 (6)	3 (3)	Q4QJ71 Q4QJ70 Q4QJ68 Q4QF95	Putative ATP-binding cassette protein subfamily G, member 1-4	- - - -	LMJF_06_0080 LMJF_06_0090 LMJF_06_0110 LMJF_15_0890	657 663 601 741
A4I078	ATP-binding cassette protein subfamily G, member 5 (ATP-binding_cassette_protein_subfamily_G_-_member_5)	1246	5 (5)	6 (6)	Q4QBD6	Putative ATP-binding cassette protein subfamily	3 (3)	ABCG5 LMJF_23_0380	1241
A4HRH2	Long-chain-fatty-acid-CoA_ligase_-_putative	698	5(3)	6(4)	E9AC40	Putative long-chain-fatty-acid-CoA ligase (EC 6.2.1.3)	3(3)	LMJF_01_0490	698
A0A504Y3U6	Protein kinase domain family protein	5959	5 (5)	2 (2)	Q4QJ16 Q4QJ15 Q4QJ14	Uncharacterized protein	- - -	LMJF_06_0620 LMJF_06_0630 LMJF_06_0640	1010 1775 3142
A0A504X927	ABC transporter family protein	2912	4 (4)	5 (5)	Q4Q6C0 Q4Q6B9	Uncharacterized protein Pentamidine resistance protein 1	- -	LMJF_31_1420 LMJF_31_1430	1157 1807
A0A504XN37	DHHC palmitoyltransferase family protein	1106	4 (4)	4 (4)	Q4QB09 Q4QB08	Palmitoyltransferase	- -	LMJF_23_1420 LMJF_23_1430	321 450
A4I0D4	Na/H antiporter-like protein (Na/H_antiporter-like_protein)	1500	4 (4)	3 (3)	Q4QB80	Na/H antiporter-like protein	-	LMJF_23_0830	1500
E9AG27	Glycerol-3-phosphate acyl transferase (EC 2.3.1.15) (Glycerol-3-phosphate_acyl_transferase)	628	4 (4)	4 (4)	E9ACF5	Glycerol-3-phosphate acyl transferase	-	LMJF_03_0080	628
A0A504XZ59	MutS domain V family protein	1179	4 (4)	6 (6)	Q4Q4J6	Putative DNA mismatch repair protein	-	LMJF_33_0410	939
A0A504XQH8	Histidine phosphatase (Branch 2) family protein	571	3 (3)	4 (4)	Q4Q1G2	Putative membrane-bound acid phosphatase 2	-	LMJF_36_2590	571
A4HX85	ATP pyrophosphate-lyase (Adenylyl cyclase)	1399	6 (3)	6 (6)	Q4QEI2	Putative receptor-type adenylate cyclase	-	LMJF_17_0190	1414
A0A504XLH3	Uncharacterized protein	861	3 (3)	2 (2)	Q4QBP9	Uncharacterized protein	-	LMJF_22_0980	706
A4HYG7	Phosphatidic acid phosphatase protein-like protein (Phosphatidic_acid_phosphatase_protein-like_protein)	400	3 (3)	2 (2)	Q4QD76	Phosphatidic acid phosphatase protein-like protein	-	LMJF_19_1350	400
A4HUE7	Putative folate/biopterin transporter	700	3 (3)	3 (3)	Q4QHI0	Putative folate/biopterin transporter	-	LMJF_10_0380	691
A4I6Q3	Pentamidine_resistance_protein_1 (p-glycoprotein e)	1679	3 (3)	3 (3)	Q4Q6D6	P-glycoprotein e	-	LMJF_31_1270	1677
A4HSH8	Hypothetical_protein_-_conserved	419	3 (3)	4 (4)	Q4QJE6	Uncharacterized protein	-	LMJF_05_0560	418
A0A504XAH5	Nucleoside transporter family protein	501	2 (2)	2 (2)	Q4QG33	Nucleobase transporter	-	LMJF_13_1210	501
A4I0F1	Hypothetical_protein_-_conserved	131	2 (2)	2 (2)	Q4QB61	Uncharacterized protein	-	LMJF_23_1020	150
A0A504XQZ9	RSN1_7TM domain-containing protein	1400	2 (2)	2 (2)	Q4QEP8	RSN1_7TM domain-containing protein	-	LMJF_16_1190	1441

[1]*Ld* refers to *L. donovani*

[2]*Li* refers to *L. infantum*

[3]*Lm* refers to *L. major*

Comparisons of *L. donovani* and *L. infantum* plasma membrane proteins with *L. major* data assigned 31 proteins into differentially expressed group. Nine of 31 proteins were expressed in three strains and only putative cytoskeleton-associated protein CAP5.5 (LMJF_31_0440 gene) has slightly more spectral count for *L. major*. Other 8 proteins were majorly expressed in *L. donovani* and *L. infantum*. Out of 31 proteins, 22 proteins were not identified in *L. major* plasma membrane proteins (unidentified or unique peptide count was 1, thus excluded from the final analysis table) but shared by other two strains.

Some of the *L. major* orthologs were not listed in cell membrane localization analysis which *L. donovani* plasma proteins were clustered because of any sequence differences. *L. donovani* A0A504XF03 Uncharacterized protein (LdBPK_271800.1 gene) was blasted to *L. major* ortholog E9ADG8 Uncharacterized protein (LMJF_27_1895 gene) in NCBI blastP and aligned. *L. donovani* has an extra around 360aa region at the beginning which encloses transmembrane domain with 5 helix region and eukaryotic domain of unknown function 846 (DUF846) (Figure 4.8-a). In a similar way, WD repeat containing domain family protein *L. donovani* A0A504XLH3 Uncharacterized protein (LdBPK_220800.1 gene) was localized in plasma membrane however *L. major* ortholog Q4QBP9 Uncharacterized protein (LMJF_22_0980 gene) was found as a soluble cytoplasmic protein that missing signal peptide and 1 transmembrane helix domain in around missing 150aa region (Figure 4.8-b). Another analyzed protein was *L. donovani* A0A504XE14 Protein kinase domain family protein (LdBPK_020330.1, LdBPK_351710.1 genes) which the subcellular localization prediction placed it into cell membrane. *L. major* orthologs, E9ACB1 Putative casein kinase II, alpha chain (LMJF_02_0360 gene) and E9AEZ3 Putative cyclophilin 14 (LMJF_35_1720 gene) were found localized in inner part of the cell although they have complementary profile with similar domains with *L. donovani* protein (Figure 4.8-c).

Identified cell membrane protein A0A504XVP5 5-histidylcysteine sulfoxide synthase encompasses two genes (LdBPK_291030.1 and LdBPK_291040.1 genes) and their *L. major* orthologs E9ADV2 and E9ADV3 Uncharacterized proteins

(LMJF_29_0940 and LMJF_29_0950) are cytoplasmic and endoplasmic reticulum membrane localized, respectively. After further analysis, it seems that all the peptides sequenced for this protein matched with LdBPK_291030.1 gene product which does not predicted as cell membrane member. Since DeepLoc predicted DNA-related A0A504XZ59 MutS domain V family protein (LdBPK_330420.1) and the protein and its *L. major* ortholog Q4Q4J6 Putative DNA mismatch repair protein (LMJF_33_0410) or ER-related A4HRH2 Long-chain-fatty-acid-CoA_ligase – putative (LINJ_01_0510) and its *L. major* ortholog E9AC40 Putative long-chain-fatty-acid-CoA ligase (LMJF_01_0490) in the target group, these proteins were also re-analyzed with a more accurate prediction to place into nucleus or ER instead of cell membrane, respectively.

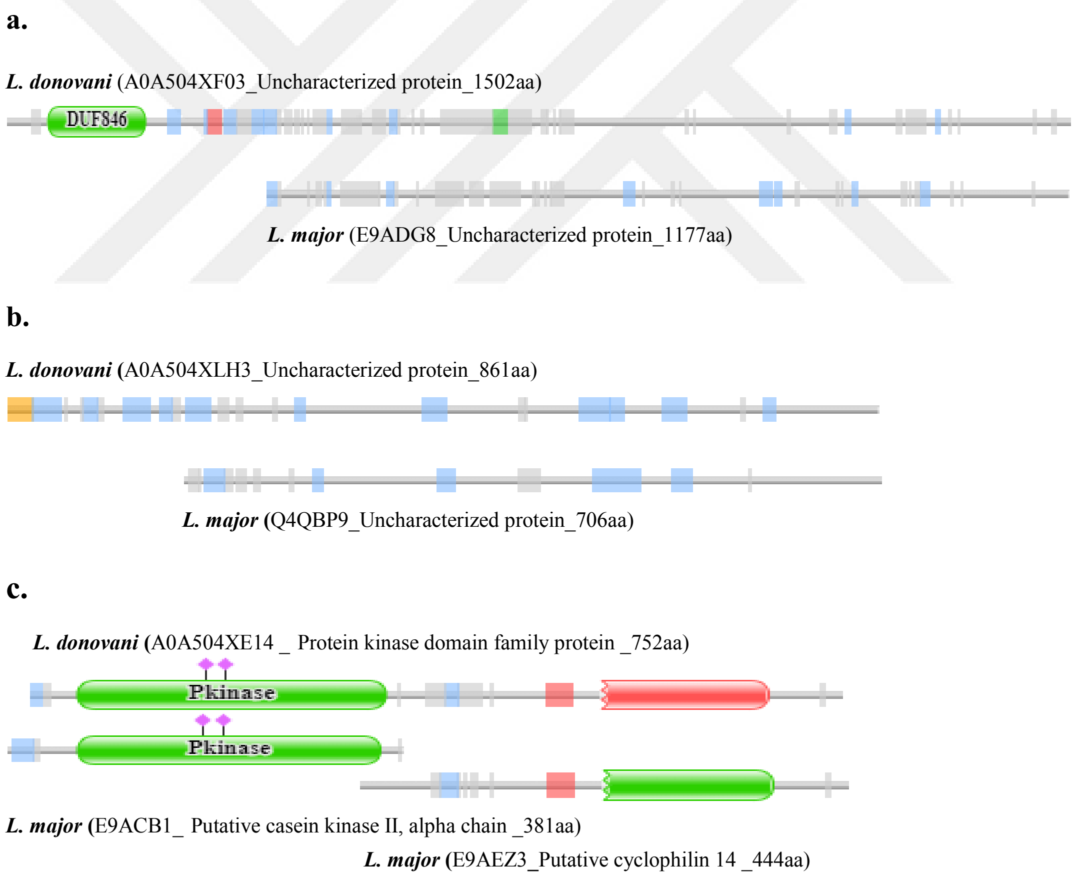


Figure 4.8 Pfam analysis of differentially localized protein orthologs of *L. donovani* plasma membrane proteins shows domain varieties. a. *L. donovani* A0A504XF03 Uncharacterized protein and *L. major* E9ADG8 Uncharacterized protein; b. *L. donovani* A0A504XLH3 Uncharacterized protein and *L. major* Q4QBP9 Uncharacterized protein; c. *L. donovani* A0A504XE14 Protein kinase

Figure 4.8 (cont'd) domain family protein and *L. major* E9ACB1 Putative casein kinase II, alpha chain & *L. major* E9AEZ3_Putative cyclophilin 14. Red boxes indicate transmembrane helix, yellow box indicates signal peptide, rod-shaped parts represent Pfam domains and purple flags are the active site of the domain.

4.1.3.4 Comparison of *L. major* plasma membrane proteins with *L. donovani* and *L. infantum* orthologs

To analyze identified plasma membrane properties of *L. major* strain, similar approach was used in the evaluation of *L. major* cell membrane proteins comparison to *L. donovani* & *L. infantum* protein data. Mutually but differentially expressed *L. donovani* & *L. infantum* cell membrane localized proteins against to *L. major* cell membrane proteins were listed before (Table 4.1). In here, proteins from only *L. major* expressed in identified protein list or differentially expressed (at least 2 peptide difference) from other two reference strains were presented with peptide counts (Table 4.2). Sequence differences of ortholog genes might cluster target proteins in different subcellular localizations which led to the further analysis.

Table 4.2 *L. major* cell membrane proteins that expressed alone or differentially expressed proteins compared to *L. donovani* or *L. infantum* orthologs (variation of #Peptides ≥ 2).

Uniprot ID Identified	Protein name	Length	#Peptides (Unique Peptides)		Uniprot ID	Protein names	# Peptides (Unique Peptides)	Gene ID *Lm* ortholog	Length
	L. donovani & L. infantum		*Ld*[1]	*Li*[2]		*L. major*	*Lm*[3]		
A0A6L0WQM5	Calcium-transporting ATPase	1119	-	-	Q4QIM6	Calcium-transporting ATPase (EC 7.2.2.10)	7(7)	LMJF_07_0650	1119
A4I005	I/6 autoantigen-like protein	207	-	-	Q4QBK1	I/6 autoantigen-like protein	7(5)	LMJF_22_1460	206
A0A3Q8IIN0	Eukaryotic translation initiation factor 3 subunit L	544	9(9)	9(9)	Q4Q253	Eukaryotic translation initiation factor 3 subunit L	5(5)	LMJF_36_0250	633
A4I7K1	Protein SEY1 homolog (EC 3.6.5.-)	895	5(5)	8(8)	Q4Q5P8	Protein SEY1 homolog (EC 3.6.5.-)	3(3)	LMJF_32_0370	891
A4HRC3	Protein_of_uncharacterized_function_(DUF2946)	333	-	-	E9ABZ0	Uncharacterized protein	3(3)	LMJF_01_0010	332
A4I4L4	Hypothetical_protein_conserved	711	-	-	E9AE12	Uncharacterized protein	3(3)	LMJF_29_1530	708
A0A3S5H7A6	MRH domain-containing protein	1186	-	-	Q4QBV4	MRH domain-containing protein	3(3)	LMJF_22_0410	1185
A4HTD0	Cation-transporting_ATPase_-_putative	1244	11(11)	8(8)	Q4QII2	Putative cation-transporting ATPase	3(3)	LMJF_07_1050	1244
E9AGG2	Putative surface antigen protein 2	664	-	-	Q4QGL8	Putative surface antigen protein	3(2)	LMJF_12_0990	610
A0A3S7X408	Intergrin alpha chain protein, putative	785	-	-	Q4Q6Q3	Putative intergrin alpha chain protein	2(2)	LMJF_31_0110	785
A4I0F2	Hydrophilic acylated surface protein a	80	-	-	P90552	HASPA1 (Hydrophilic acylated surface protein a) (Hydrophilic surface protein 2)	2(2)	haspa2 HASPA1 HASPA2 LMJF_23_1040 LMJF_23_1082 LMJF_23_1088	73

[1]*Ld* refers to *L. donovani*

[2]*Li* refers to *L. infantum*

[3]*Lm* refers to *L. major*

Assessing listed 11 proteins was revealed that *L. major* had 8 proteins were uniquely expressed by itself. In *L. major* identified proteins, Q4QIM6 Calcium -transporting ATPase (LMJF_07_0650 gene) and Q4QBK1 I/6 autoantigen-like protein (LMJF_22_1460) have highest peptide counts in the list. Although E9AE12 Uncharacterized protein (LMJF_29_1530) has almost similar sequence length, one of the TMH domains was seemed to lack regarding its ortholog (Figure 4.9).

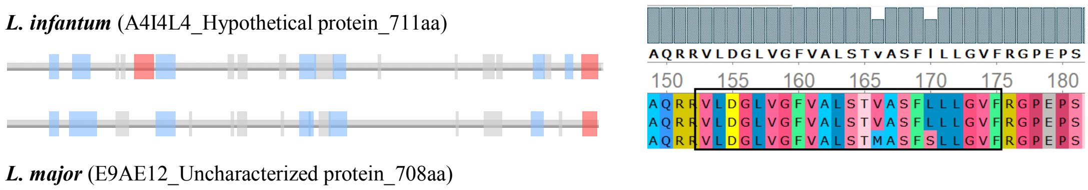


Figure 4.9 Pfam analysis of *L. major* E9AE12 Uncharacterized protein shows transmembrane helix domain difference compared to its *L. infantum* ortholog. Red boxes indicate transmembrane helices.

Only 3 proteins were shared with other strains with higher peptide counts in *L. donovani* and *L. infantum* strains. However, these 3 proteins were further analyzed whether localization differences are from analysis accuracy and found out to be predicted in inside cellular compartments. Proteins: Q4Q253 Eukaryotic translation initiation factor 3 subunit L (LMJF_36_0250 gene) and its *L. donovani* ortholog A0A3Q8IIN0 Eukaryotic translation initiation factor 3 subunit L (LdBPK_360270.1 gene) in cytoplasm/nucleus; Q4Q5P8Protein SEY1 homolog (LMJF_32_0370 gene) and its *L. infantum* ortholog A4I7K1 ProteinSEY1 homolog (LINF_320008800 gene) in endoplasmic reticulum; A4HTD0 Cation -transporting ATPase – putative (LMJF_07_1050 gene) and *L. infantum* ortholog Q4QII2 Putative cation -transporting ATPase (LINF_070017000 gene) in ER.

4.1.3.5 Mutually expressed cell membrane proteins with similar peptide counts for *L. donovani, L. infantum and L. major*

Cell membrane localized proteins with shared by all three strains were listed for almost equally identified peptide counts. These proteins might be important players in the complexity of *Leishmania* infection apart from any type of disease (Table 4.3).

Table 4.3 Identified proteins with similar unique peptide numbers for all strains (variation of #Peptide <2)

Uniprot ID Identified	**Protein name**	**Length**	**# Peptides** (Unique Peptides)**		**Uniprot ID Identified**	**Protein names**	**# Peptides* (Unique Peptides)**	**Gene names**	**Length**
	L. donovani & L. infantum		***Ld*[1]**	***Li*[2]**		***L. major***	***Lm*[3]**		
A4I060	**ATP-binding cassette protein subfamily C, member 2 (Pentamidine_resistance_protein _1)**	**1562**	**10 (9)**	**12 (9)**	**Q4QBF3**	**Putative ATP-binding cassette protein subfamily C, member 2**	**11 (11)**	**ABCC2 LMJF_23_0220**	**1570**
A0A504XVU0	**AAA domain (Cdc48 subfamily) protein**	**2588**	**6 (6)**	**7 (5)**	**A0A504XVU0**	**AAA domain (Cdc48 subfamily) protein**	**5 (5)**	**CGC21_28190**	**2588**
A4HTX5	**VTC domain family protein**	**813**	**6(6)**	**5(5)**	**Q4QI04**	**VTC domain-containing protein**	**7(7)**	**LMJF_09_0220**	**813**
A4HRZ6	**Calcium-transporting ATPase**	**1023**	**9 (4)**	**7 (7)**	**Q95Z93**	**Calcium-transporting ATPase (EC 7.2.2.10)**	**6 (6)**	**LMJF_04_0010**	**1023**
A4I809	**Metalloprotease_M41_FtsH_-_putative**	**716**	**7(7)**	**8(8)**	**Q4Q5D1**	**Putative ATP-dependent zinc metallopeptidase (EC 3.4.24.-)**	**7(7)**	**LMJF_32_1500**	**718**
A4I274	**Hypothetical_protein_-_conserved**	**548**	**4 (4)**	**2 (2)**	Q4Q906	Uncharacterized protein	1 (1)	LMJF_26_1860	550
A0A504X4R7	**Cysteinyl-tRNA synthetase**	**1707**	**4 (4)**	**3 (3)**	**Q4QGR8**	**Cysteinyl-tRNA synthetase**	**4 (4)**	**LMJF_12_0250**	**784**
A0A504XL74	**Amastin surface glycofamily protein**	**300**	**4 (4)**	**2 (2)**	**Q4QIA5**	**Uncharacterized protein**	**2 (2)**	**LMJF_08_0640**	**299**
A4I0B5	**Hypothetical_protein_-_conserved**	**556**	**4 (4)**	**6 (6)**	**Q9U0V4**	**Uncharacterized protein L7836.08**	**6 (6)**	**L7836.08 LMJF_23_0640**	**555**
A0A504XEW7	**Diacylglycerol acyltransferase family protein**	**1863**	**4 (4)**	**3 (3)**	**E9ADD4 E9ADD5 E9ADD8**	**DNA-directed RNA polymerase II-like protein Putative diacylglycerol acyltransferase Acyltransferase**	**2 (2) - -**	**LMJF_27_1550 LMJF_27_1560 LMJF_27_1590**	**134 1556 330**
A0A504XRS0	**DnaJ domain family protein**	**3914**	**4 (4)**	**5 (2)**	A0A504XRS0	DnaJ domain family protein	4 (1)	CGC21_30690	3914
A0A504XVX4	**Major Facilitator Superfamily protein**	**1329**	**4 (4)**	**3 (3)**	**E9AE01 E9AE11 E9AE12**	**Uncharacterized protein**	**- - 3 (3)**	**LMJF_29_1420 LMJF_29_1520 LMJF_29_1530**	**655 648 708**
A4HVJ3	**Palmitoyltransferase (EC 2.3.1.225)**	**607**	**4 (4)**	**2 (2)**	Q4QG89	Palmitoyltransferase	1 (1)	LMJF_13_0650	607
A4IBK2	**Hypothetical_protein_-_conserved**	**602**	**3 (3)**	**4 (4)**	**E9AFD5**	**Uncharacterized protein**	**2 (2)**	**LMJF_35_3110**	**602**
A4IAC3	**Hypothetical_protein_-_conserved**	**940**	**3 (3)**	**2 (2)**	Q4Q2I7	Uncharacterized protein	1 (1)	LMJF_34_3570	941
A0A504XNP1	**P-type H(+)-exporting transporter (EC 7.1.2.1)**	**2018**	**3 (3)**	**6 (6)**	**Q4QDN9 Q4QDN8 Q4QDN7**	**Uncharacterized protein Plasma membrane ATPase**	**- - 6(6)**	**LMJF_18_1500 LMJF_18_1510 LMJF_18_1520**	**1046 974 974**
A0A504XYQ7	**N-acetylglucosaminyl transferase component (Gpi1) family protein**	**2310**	**3 (3)**	**3 (3)**	**E9AE65 E9AE66**	**N-acetylglucosaminyl transferase component Gpi1, putative**	**- 3 (3)**	**LMJF_29_2030 LMJF_29_2040**	**888 1280**
A4HU10	**Phospholipid_transporting_ATPase-like_protein_-__putative (Putative phospholipid transporting ATPase-like protein)**	**2528**	**3 (3)**	**2 (2)**	Q4QHT5	Putative phospholipid transporting ATP	1 (1)	LMJF_09_0890	2525
A0A504XB68	**Dolichyl-diphosphooligosaccharide--protein glycotransferase (EC 2.4.99.18)**	**837**	**4 (2)**	**3 (2)**	E9AET8	Dolichyl-diphosphooligosaccharide--protein glycotransferase	2 (1)	LMJF_35_1150	790
A0A3Q8IHB3	**Uncharacterized protein**	**321**	**2(2)**	**4(4)**	**Q4QH88**	**Uncharacterized protein**	**3(3)**	**LMJF_10_1240**	**321**
A0A3S7XAD8	**Hypothetical_protein_conserved**	**152**	**2 (2)**	**2 (2)**	Q4Q276	Hypothetical_protein_conserved	1 (1)	LMJF_36_0030	152
A4I3R5	**Palmitoyltransferase (EC 2.3.1.225)**	**457**	**2 (2)**	**2 (2)**	A4I3R5	Palmitoyltransferase	1 (1)	LINF_280025200 LINJ_28_2010	457
D1GJ51	**Putative surface antigen protein 2 (Soluble promastigote surface antigen PSA-50S) (Surface_antigen_protein_2_-_putative)**	**463**	**2 (2)**	**4 (3)**	**Q4QGJ9**	**Putative surface antigen protein 2**	**5 (3)**	**LMJF_12_0760**	**587**
A0A504Y863	**S1/P1 Nuclease family protein**	**378**	**2 (2)**	**2 (2)**	Q4Q630	Putative 3'-nucleotidase/nuclease	1 (1)	LMJF_31_2310	378
A4HSV7	**Hypothetical_protein_-_conserved**	**679**	**2 (2)**	**4 (4)**	Q4QJ12	Uncharacterized protein	1 (1)	LMJF_06_0660	679
E9ACP0	**Uncharacterized protein**	**635**	**2 (2)**	**2 (2)**	E9ACP0	Uncharacterized protein	1 (1)	LMJF_03_0950	635
A4HYX4	Putative calpain-like cysteine peptidase (Small myristoylated protein 1)	131	1(1)	**3(3)**	**Q5SDH5**	**Putative calpain-like cysteine peptidase (Small myristoylated protein 1)**	**3(3)**	**SMP-1 LMJF_20_1310**	**131**
A4I283	Hypothetical_protein_-_conserved	655	1(1)	1(1)	**Q4Q945**	**Uncharacterized protein**	**2(2)**	**LMJF_26_1490**	**655**
A4HS14	**Surface antigen-like protein**	**709**	1(1)	**2(2)**	**Q9XZX9**	**Surface antigen-like protein**	**2(2)**	**LMJF_04_0190**	**709**

*Proteins written in bold letters are included in the actual analysis, proteins written in not bold excluded from analysis but only presented here.

[1]*Ld* refers to *L. donovani*

[2]*Li* refers to *L. infantum*

[3]*Lm* refers to *L. major*

To determine plasma membrane proteins having comparable peptide counts, cell membrane localized proteins of *L. donovani* and *L. infantum* strains were compared with *L. major* orthologs for number of peptides were lower than 2 for at least one of two strains. Results indicated 29 proteins were sub-listed for this analysis. Proteins had unique peptide counts 1 or 0 were excluded in the experiment but only presented here to not to miss similarities for less counted peptides. Among them, ATP-binding cassette protein subfamily C, member 2 (Pentamidine_resistance_protein_1) in *L. donovani* and *L. infantum*, its *L. major* ortholog Putative ATP-binding cassette protein subfamily C, member 2 were the most counted protein in this mutual sub-list. It is followed by AAA domain (Cdc48 subfamily) protein, VTC domain-containing protein, Calcium-transporting ATPase and Putative ATP-dependent zinc metallopeptidase. Nine of 29 proteins were found as uncharacterized proteins which should be further analyzed to predict their importance.

4.1.3.6 Antigenicity and B cell epitope predictions of selected proteins

To determine antigenic sites that might be recognized by B cells, identified cell membrane proteins from each subjected group according to their peptide counts were further investigated. Antigenicity predictions of proteins listed in Table 4.1, Table 4.2 and Table 4.3 were performed with Vaxijen-2.0 tool for parasites targets with threshold value 0.5 for probable antigens (complete lists are shown in Appendix 2, Table 8.1-Table 8.4). The proteins over 0.6 antigenic value were further examined to predict B-cell epitope sequences in BepiPred Linear Epitope Prediction 2.0 which uses Random Forest algorithm improved by training with epitopic or non-epitopic amino acids derived from crystal structures (149). Selected proteins' FASTA files for *L. donovani*, *L. infantum* and *L. major* strains were combined and aligned in UGene Software. Alignments were validated from NCBI blast tool. Comparison of highly antigenic proteins' epitopic regions from differentially counted peptides for *L. donovani* and *L. infantum* from Table 4.1 indicated in Figure 4.10 (a-f); from differentially counted peptides for *L. major* from Table 4.2 indicated in Figure 4.11 (a-d) and from mutual and similar peptides counted proteins for all strains from Table 4.3 indicated in Figure 4.12 (a-f).

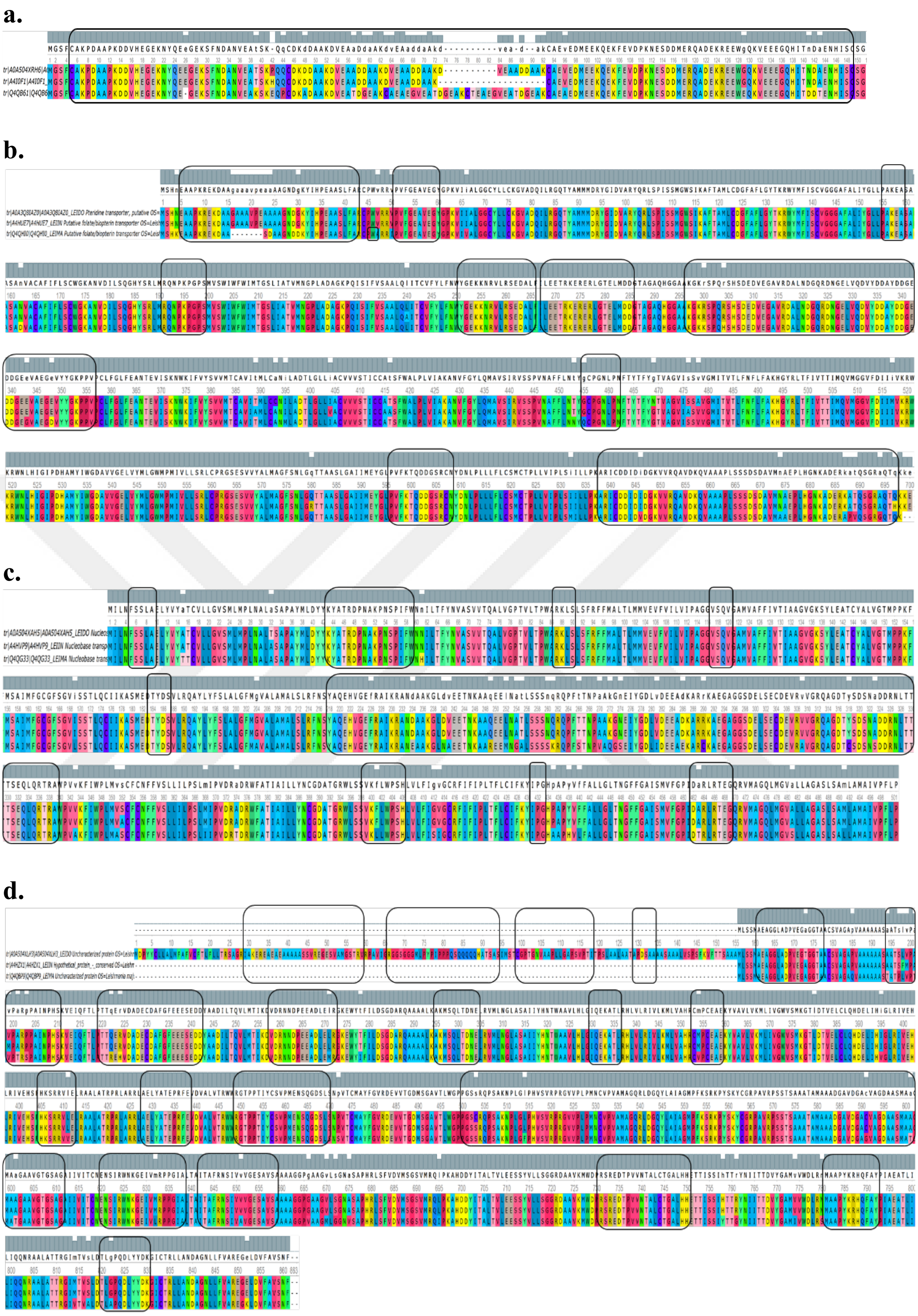
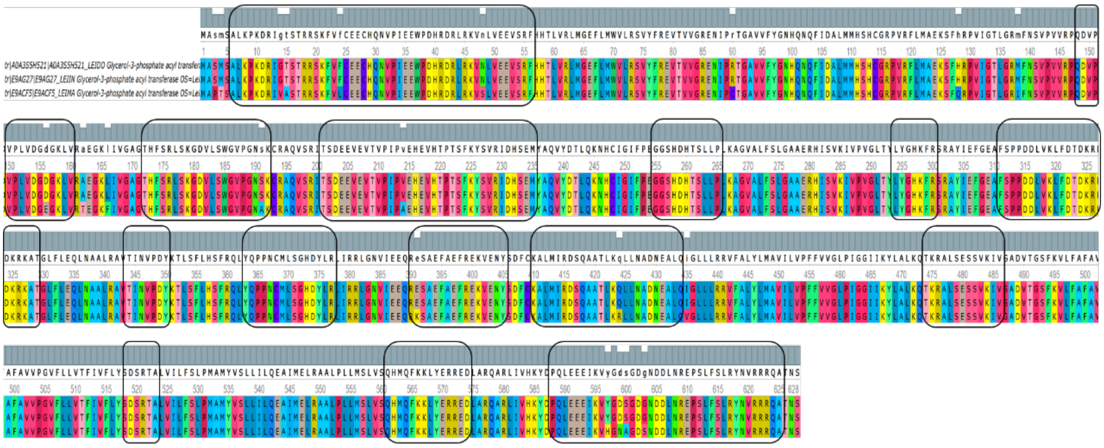


Figure 4.10 B cell epitope regions and highly antigenic plasma proteins exclusively detected in *L. donovani* and *L. infantum* and sequence alignments with *L. major* orthologs.

e.



f.

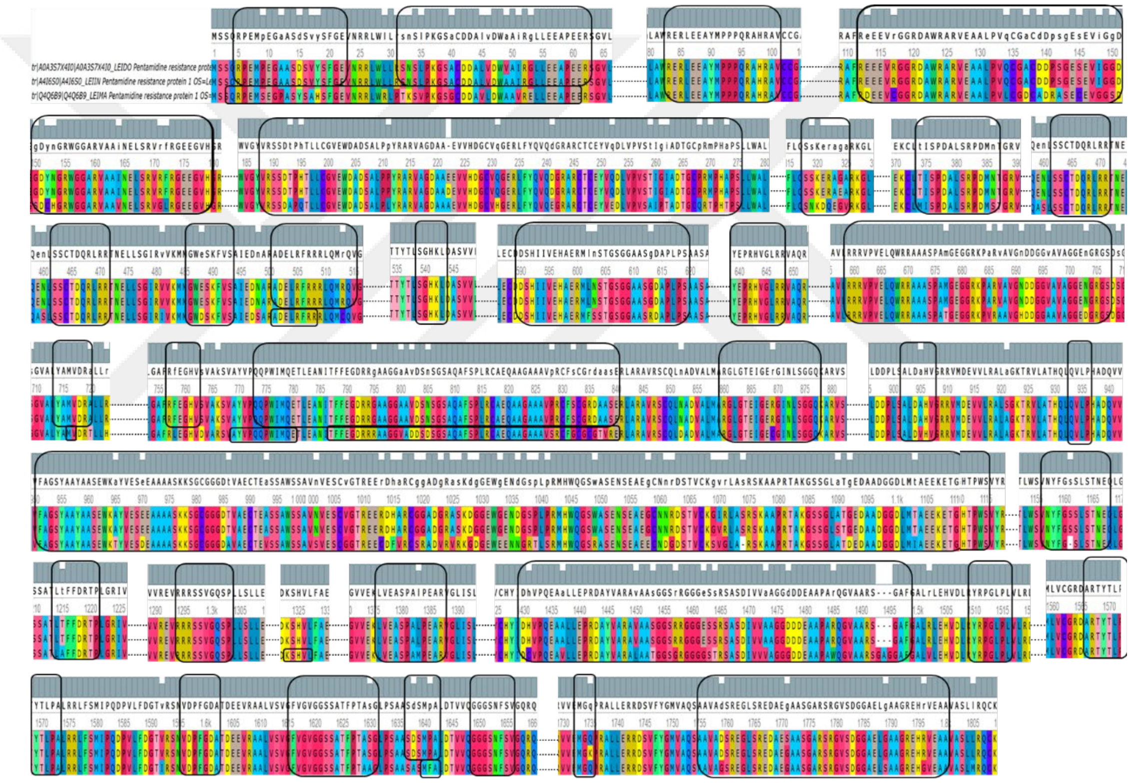


Figure 4.10 (cont'd) B cell epitope regions and highly antigenic plasma proteins exclusively detected in *L. donovani* and *L. infantum* and sequence alignments with *L. major* orthologs. Predicted proteins a. Hypothetical protein – conserved (A0A504XRH6/A4I0F1); b. Putative folate/biopterin transporter (A0A3Q8IAZ0 /A4HUE7); c. Nucleoside transporter family protein (A0A504XAH5/A4HVP9); d. Uncharacterized protein (A0A504XLH3/A4HZX1); e. Glycerol-3-phosphate acyl transferase (A0A3S5H521/E9AG27) and f. ABC transporter family protein (A0A504X927/A4I6S0) were aligned in order of *L. donovani*, *L. infantum* and *L. major* and epitopic regions were picked up from BepiPred-2.0 Tool analysis. Sequences were colored for each amino acids, black boxes were B-cell epitope regions of indicated sequences.

Antigenic prediction of 31 proteins detected mainly or only in *L. donovani* and *L. infantum* proteins resulted with those 22 proteins are probable antigens (score > 0.5). In this group, B-cell epitope analysis were applied to more antigenic (score > 0.6) proteins groups which are A0A504XRH6/A4I0F1 Hypothetical protein – conserved (score= 0.9240), A0A3Q8IAZ0/A4HUE7 Putative folate/biopterin transporter (score= 0.7128); A0A504XAH5/A4HVP9 Nucleoside transporter family protein (score= 0.6606); A0A504XLH3/A4HZX1 Uncharacterized protein (score 0.6452); A0A3S5H521/E9AG27 Glycerol-3-phosphate acyl transferase (score 0.6221) and A0A504X927/A4I6S0 ABC transporter family protein (score=0.6167).

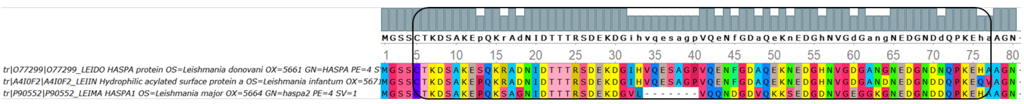
Epitope regions of orthologous proteins were compared for *L. donovani*, *L. infantum* and *L. major* strains. Although there are some conserved epitope regions, all the proteins have some extent mismatches or multiple sequence differences between species especially in *L. major* strain compare two visceral agents. Some proteins have missing gap regions especially seen in the epitope sites of A0A504XRH6/A4I0F1 Hypothetical protein – conserved which antigenicity score was increased from 0.9240 for *L. infantum* to 0.9893 for *L. major* (Figure 4.10-a). Moreover, *L. donovani* A0A504XLH3 Uncharacterized protein was identified in both *L. donovani* and *L. infantum* which having an extra 155 aa region compared to *L. infantum* and *L. major* orthologs with several epitope sites in the N-terminal of the protein (Figure 4.10-d). This protein starts with a signal peptide and is predicted to be localized in cell membrane unlikely to its orthologs. Interestingly, identified unique peptides for *L. donovani* and *L. infantum* were EREAEAEAAAAASSVR and EAEAEAAAAASSVR which are found in the epitope region of flanking N-terminal site and might be an important target for further studies. Additionally, sequence differences or similarities might be important in the terms of conserved, non-conserved or semi-conserved regions between strains.

In identified proteins, some mismatch residues are classified in same amino acid groups such as non-polar or polar however there are some amino acid group variances in epitope regions. A0A504X927/A4I6S0 ABC transporter family protein gene consists of an Uncharacterized protein and a A0A3S7X4I0/ A4I6S0

Pentamidine resistance protein (PRP1) which epitope analysis was performed for this membrane localized region. The PRP1 protein has a role in drug resistance (164). From the sequence alignment, 1485. hydrophilic and positively charged arginine residue (R) found in epitope region in *L. donovani* and *L. infantum* sequences were replaced with Tryptophan (W) residue in *L. major* ortholog (Figure 4.10-f). Tryptophan is highly unique aromatic and hydrophobic amino acids present mainly in membrane proteins (165) which this sequence disconcordance might be one of the significant point worth studying.

The other plasma membrane group of uniquely identified *L. major* proteins were also subjected to antigenicity scoring analysis with Vaxijen-2.0 and B-cell epitope analysis with BEpiPred-2.0. Out of 8 proteins, 7 of them were found to be a probable antigen with a value over 0.5. Multiple sequence alignments for *L. donovani*, *L. infantum* and *L. major* ortholog groups were prepared. B-cell epitope predictions were assessed in a similar way for the proteins P90552 Hydrophilic acylated surface protein a (HASPA1) (score= 1.3841), E9ABZ0 Uncharacterized protein (score= 0.6385), Q4Q6Q3 Putative intergrin alpha chain protein (score= 0.6285) and E9AE12 Uncharacterized protein (score= 0.6271) in more antigenic protein listed groups (score > 0.6).

a.



b.

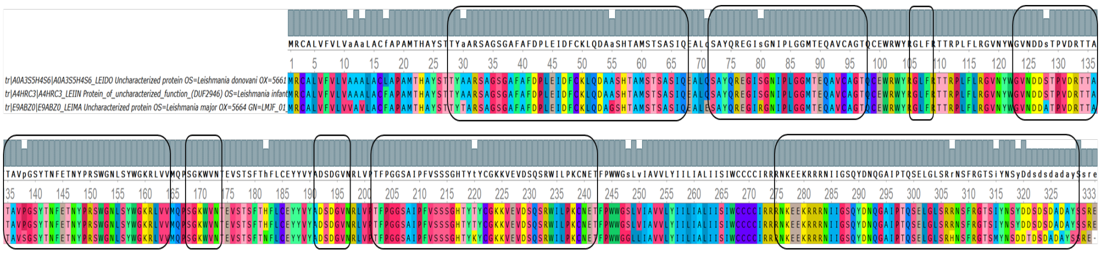
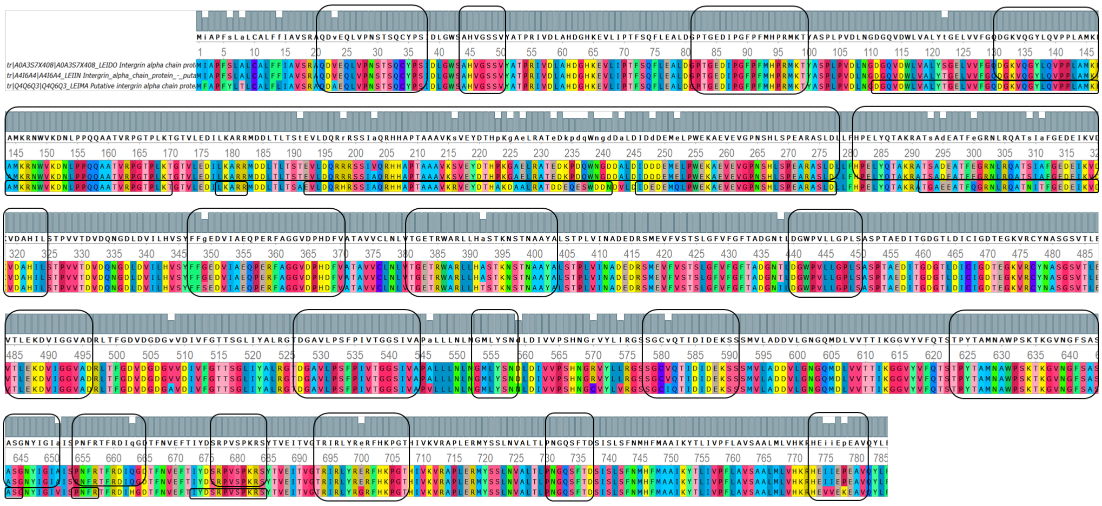


Figure 4.11 B cell epitope regions and highly antigenic plasma proteins exclusively detected in *major* and sequence alignments with *L. donovani* and *L. infantum* orthologs.

c.



d.

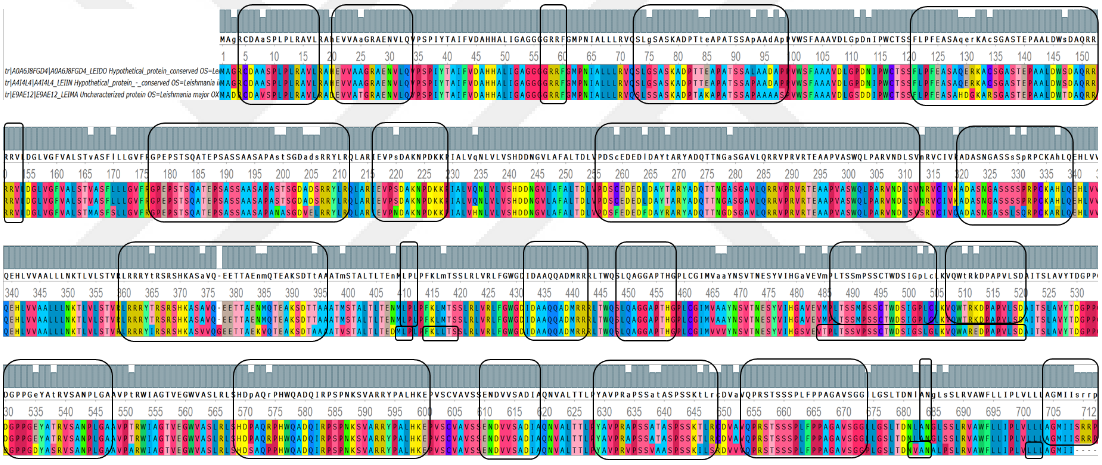
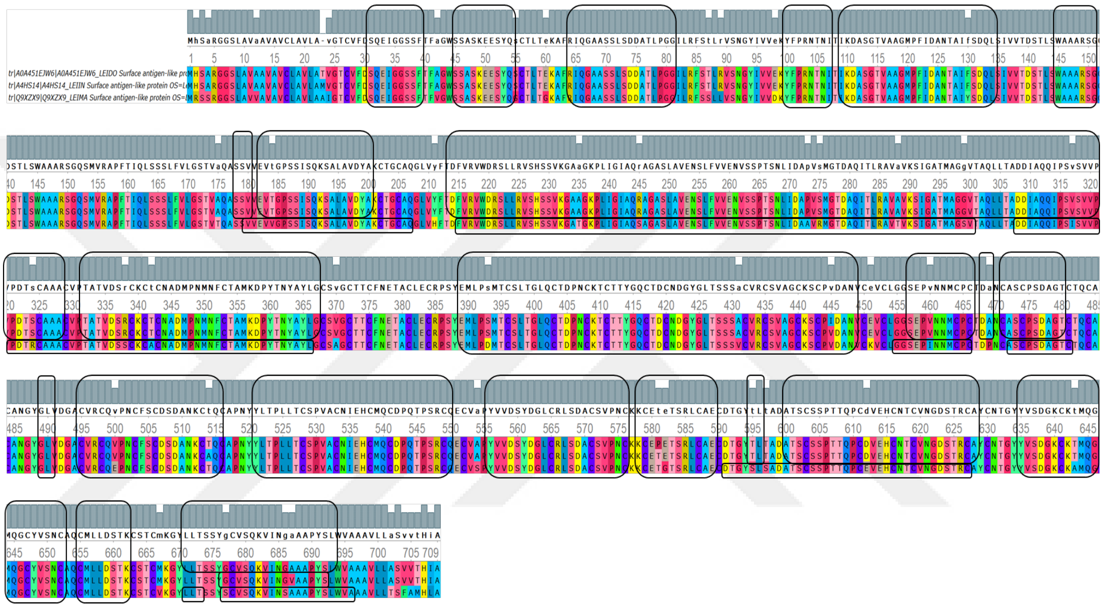


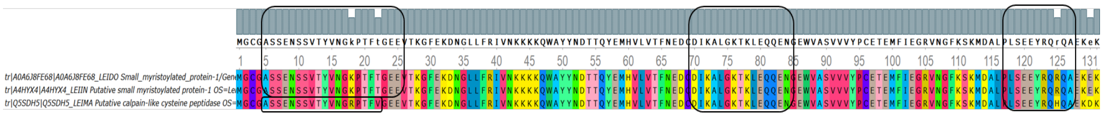
Figure 4.11 (cont'd) B cell epitope regions and highly antigenic plasma proteins exclusively detected in *L. major* and sequence alignments with *L. donovani* and *L. infantum* orthologs. Predictions were applied to proteins a. Hydrophilic acylated surface protein a (HASPA1) (P90552); b. Uncharacterized protein (E9ABZ0); c. Putative intergrin alpha chain protein (Q4Q6Q3) and d. Uncharacterized protein (E9AE12) aligned in order of *L. donovani*, *L. infantum* and *L. major* sequences. Epitopic regions were selected from BepiPred-2.0 Tool analysis. Sequences were colored for each amino acids, black boxes were B-cell epitope regions of indicated sequences.

Results were analyzed for epitopic regions for differential sequence properties. The most antigenic protein P90552 Hydrophilic acylated surface protein a (HASPA1) has a gap region between 33-39 residues in aligned sequence compared to *L. donovani* and *L. infantum* sequences and there are some mismatch residues (Figure 4.11-a). It is realized that these sequence varieties led to a slightly lower Vaxijen antigenicity scores in *L. infantum* (1.1572) and *L. donovani* (1.1667) ortholog proteins compared to *L. major* (1.3841). Though, there are several conserved B-cell epitope regions, the single aa changes were observed in these sites might be a concern in the antigenicity of proteins.

a.



b.



c.

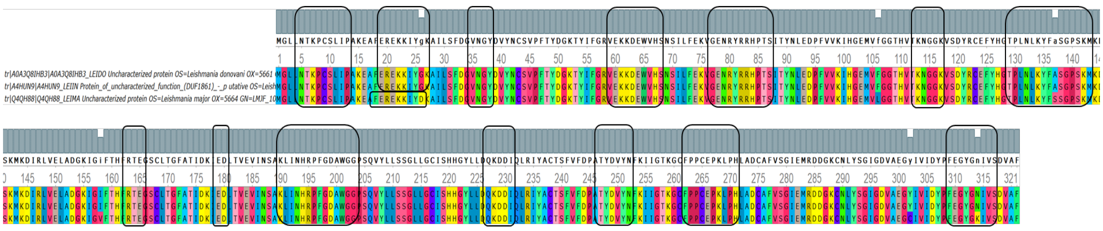
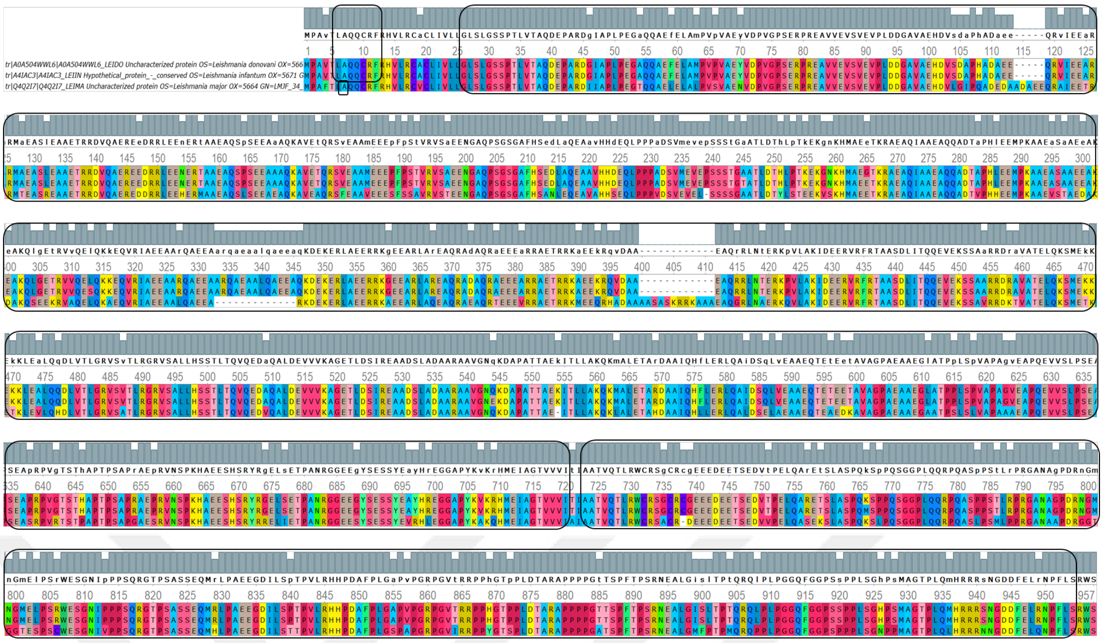
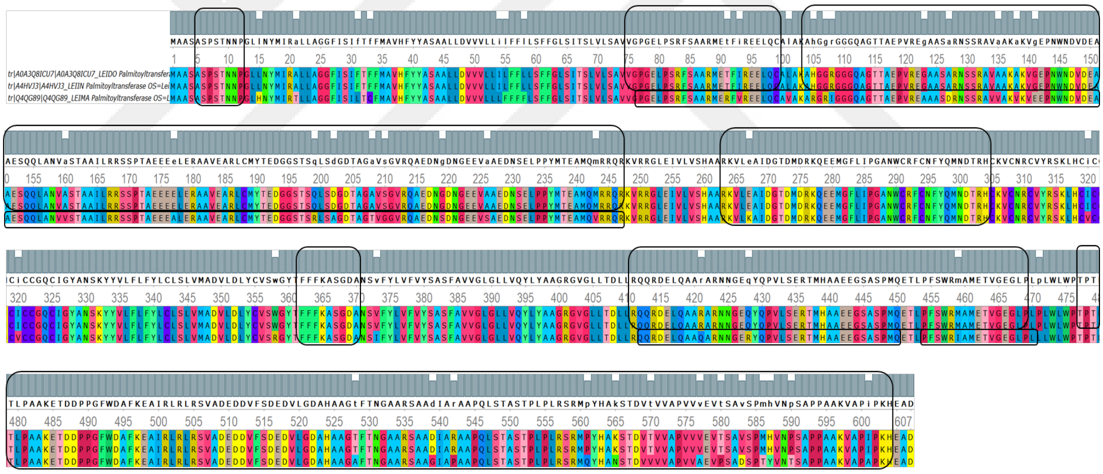


Figure 4.12 B cell epitope regions and highly antigenic plasma proteins commonly detected *L. donovani*, *L. infantum* and *L. major*.

d.



e.



f.

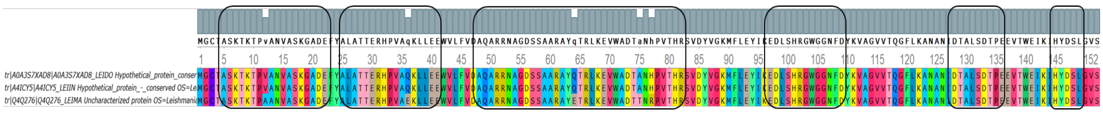


Figure 4.12 (cont'd) B cell epitope regions and highly antigenic plasma proteins commonly detected *L. donovani*, *L. infantum* and *L. major*. Antigenic predictions in Vaxijen were performed with proteins identified in *L. donovani* strain. a. Surface antigen-like protein (A4HS14); b. Putative calpain-like cysteine peptidase (Small myristoylated protein 1) (Q5SDH5); c. Uncharacterized protein (A0A3Q8IHB3); d. Hypothetical protein - conserved (A4IAC3); e. Palmitoyltransferase (A4HVJ3) and f. Hypothetical protein - conserved (A0A3S7XAD8).

Figure 4.12 (cont'd) Sequences from ortholog proteins were aligned in order of *L. donovani*, *L. infantum* and *L. major*. Epitopic regions were selected from BepiPred-2.0 Tool analysis. Sequences were colored for each amino acids, black boxes were B-cell epitope regions of indicated sequences.

To elucidate antigenicity and B-cell epitope region of commonly identified peptides and proteins, sequences of 29 proteins identified in *L. donovani* strains were examined with Vaxijen-2.0. All the proteins except A4I060 ATP-binding cassette protein subfamily C, member 2 (Pentamidine_resistance_protein_1) (score= 0.4608) were found as a probable antigen (score > 0.5). B-cell epitope analysis performed for proteins identified in *L. donovani* with highly scored proteins are A4HS14 Surface antigen-like protein (score= 0.7), Q5SDH5 Putative calpain-like cysteine peptidase (Small myristoylated protein 1) (score= 0.6976), A0A3Q8IHB3 Uncharacterized protein (score= 0.6904), A4IAC3 Hypothetical protein - conserved (score= 0.6591); A4HVJ3 Palmitoyltransferase (score= 0.6285) and A0A3S7XAD8 Hypothetical protein - conserved (score= 0.6221) (Figure 4.12 a-f).

Orthologous proteins of *L. donovani*, *L, infantum* and *L. major* for commonly identified peptides and proteins have similar and dissimilar epitope regions in some sites like other groups of analyzed proteins. The most antigenic protein in identified list is A0A451EJW6/A4HS14/Q9XZX9 (*Ld*/*Li*/*Lm*) Surface antigen-like protein (Figure 4.12-a) with similar Vaxijen score 0.7022/0.7/0.7162. Their sequences are quite similar in epitope regions with a few mismatches. On the other hand, most sequence variety were found in A0A504WWL6/A4IAC3/Q4Q2I7 (*Ld*/*Li*/*Lm*) Hypothetical protein – conserved (Figure 12-d) with Vaxijen score 0.6464/0.6591/0.6375. The protein has very long epitope sites (residues 26-720 and 723-954 in aligned sequences) and mismatches and gaps especially in the groups *L. donovani* and *L. infantum* compared to *L. major*. Whether these differences affect B-cell epitope binding is unknown, but antigenicity of proteins is seemed to be closed to each other.

Taken together, differentially or commonly expressed proteins with high antigenic scores possess several similar or dissimilar B-cell epitope sites. Visceral agents of *L. donovani* and *L. infantum* sequences which are mainly matching with each other but have several dissimilarities with *L. major* proteins.

4.2 *In vitro* Infection of THP-1 Derived Macrophages with LRV - Bearing *L. major* Strains

Leishmania resident an endosymbiont virus *Leishmania* RNA Virus (LRV) are under investigation in many years whether it has an impact on disease progression and severity of leishmaniasis. In our previous studies, an autochthonous *L. major* isolate from Turkey was reported for the first time because of having a subtype LRV: LRV-2 (166). In the present study, this *L. major* isolate contains LRV-2 were examined for innate immune response in monocyte-derived macrophages comparing with another *L. major* isolate without LRV and reference *L. major*.

4.2.1 THP-1 monocyte derived macrophages

Leishmania infection experiments were widely conducted in monocyte derived macrophages *in vitro*. In the study, THP-1, an acute monocytic leukemia-derived cell line was chosen as a host cell for *Leishmania* promastigotes and transformed into macrophages. As an inducing agent, phorbol 12-myristate 13-acetate (PMA) was preferred. There are several treatment conditions for this cellular conversion which can affect gene expression and further immune response experiments. Using higher PMA concentrations tend to interfere M1/M2 polarization (159). PMA induction conditions affecting morphology of cells and macrophage marker expressions were tested for the present study.

4.2.1.1 Differentiation of THP-1 cell morphology with PMA

To decide on required dose of PMA concentration and incubation time on macrophage differentiation, THP-1 cells were incubated with 25nM or 80nM PMA for 1, 2 or 3 days or left untreated. THP-1 cells are morphologically round shaped, suspension cells, whereas THP-1 derived macrophages are directed to more elongated and attached form. Focusing on the physical appearance of cells and adherence capability, microscopic images of cultures were photographed for each condition and each time point (Figure 4.13).

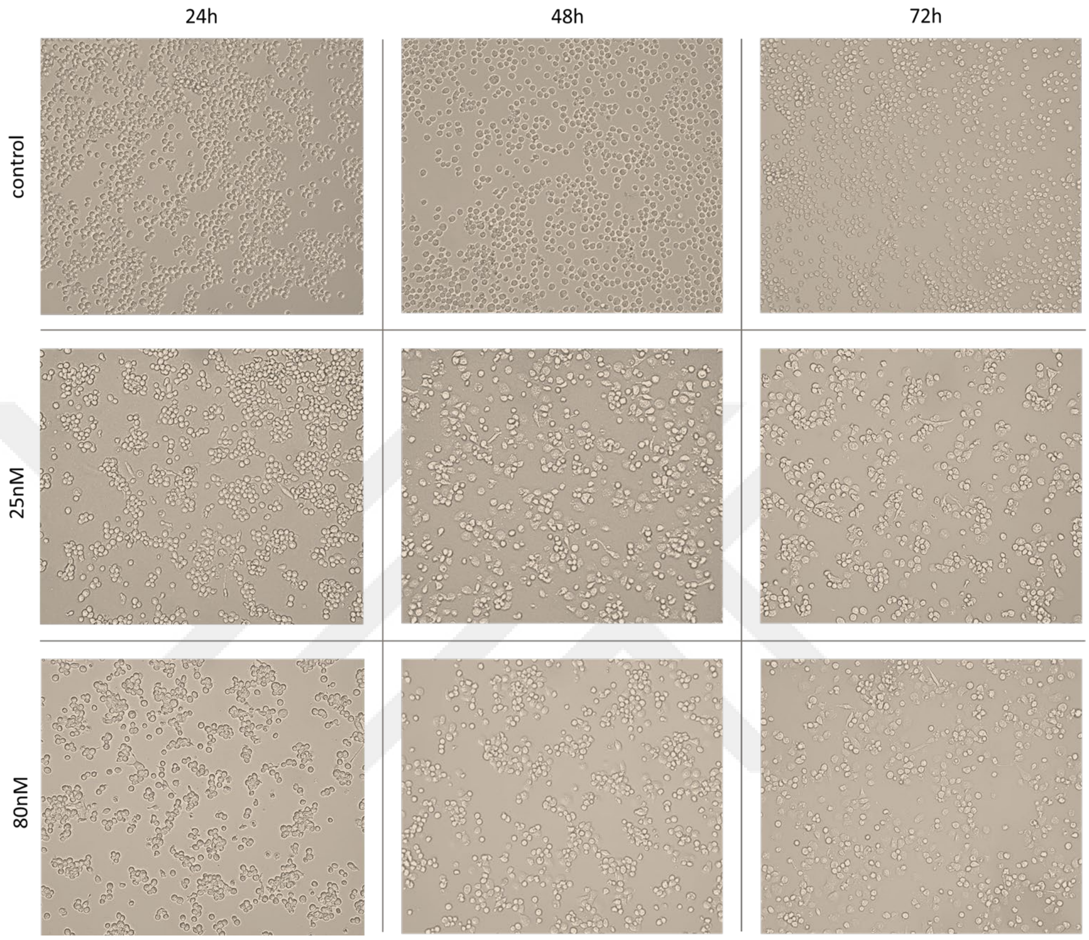


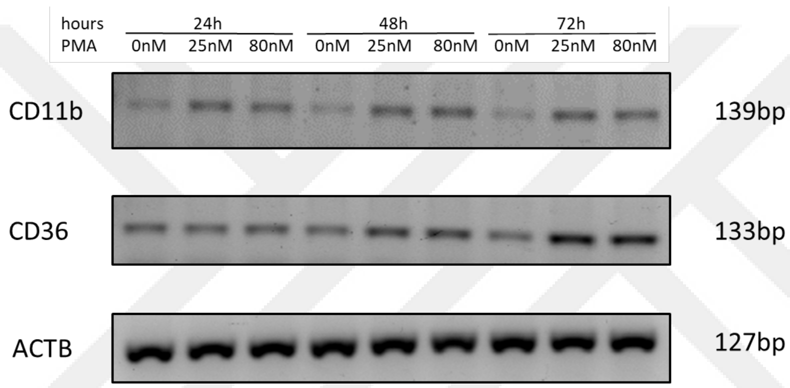
Figure 4.13 Microscopic examination of THP-1 macrophage differentiation and attachment with 25nM, 80nM compared to suspension control THP-1 macrophages lacking PMA. Images were taken under bright field on ZEISS Axio Vert A1 microscopy with 400Xmagnification.

It was observed that using 25nM and 80nM PMA induced cell to be flattened more and adhere to the plate from the 24h. Elongated cells numbers were increased towards to the 72h. Untreated cells were stayed as suspension cells and rounded.

4.2.1.2 CD11b and CD36 expression of PMA induced THP-1 cells

Transformation of PMA induced THP-1 cells into macrophages was assessed in molecular level emphasizing surface molecule CD36 and CD11b expressions. These markers are known to present in macrophage cells (160,161). For this reason, THP-1 cells were treated with 25nM and 80nM PMA or left untreated for 1, 2 or 3 days. Cells were collected from plates in indicated times and RNA isolation was performed. Following cDNA synthesis, equal µg of cDNA was subjected to conventional PCR for CD11b, CD36 and housekeeping ACTB applying lower cycle number (25 cycles) to avoid reaching saturation. PCR gel results for all treatment condition for indicated time points are presented in Figure 4.14-a and band intensity analyses conducted in ImageJ program were indicated in Figure 4.14-b.

a.



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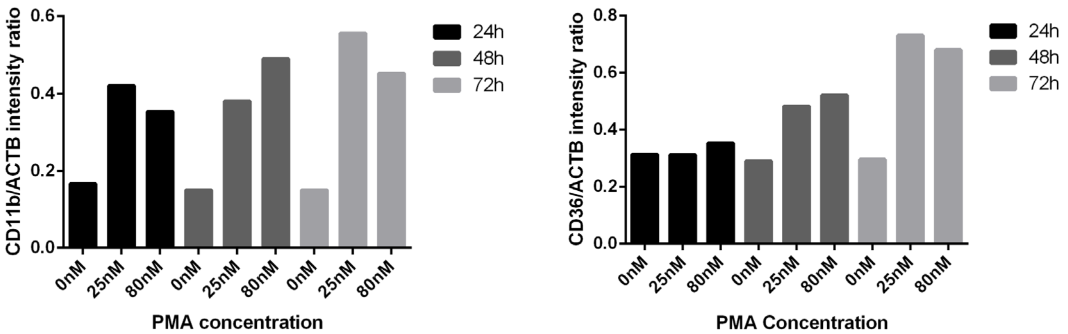

Figure 4.14 CD11b and CD36 expression of THP-1 monocytes increased in the presence of PMA. a. THP-1 cells were incubated with 25nM and 80nM PMA for 3 days or left untreated. CD11b and CD36 surface markers expressions were checked with conventional PCR at early multiplication cycles. ACTB was used as endogenous control. PCR products were run on 2% agarose gel with Sybr Gold. b. Band intensities were measured with ImageJ and bar graphs were drawn with GraphPad Prism.

Conventional PCR gel results came into view as PMA induction increases surface markers expressions regardless of PMA concentration amount. However, band intensities were evaluated to analyze semi-quantitively. Values obtained from CD11b or CD36 lanes were divided with corresponding ACTB values of samples. Ratios were used to draw bar graphs in GraphPad Prism. According to bar graph results, CD11b increased from the 24h PMA treatment with both concentration whereas CD36 expression difference were detectable after 48h. They reach their maximum at day 3. Control THP-1 monocytes (without PMA) were having stable CD11b and CD36 expressions in all 3 days.

Overall experiments shown that, PMA induced macrophages differentiation as expected. However, treatment conditions were chosen for our experiments. To have low PMA concentration, 25nM PMA was preferred with 3 days induction and additionally 1 day rest without PMA which have proper macrophage-like cell profile (Figure 4.10) and relatively higher CD11b and CD36 expression compared to other incubation times and 80nM PMA concentration (Figure 4.11).

4.2.2 Infection of THP-1 derived macrophages with *L. major* LRV+ parasites

To elucidate chemokine and cytokine response of infected macrophages against LRV presence, THP-1 monocytes were prepared as in optimized conditions. In parallel, LRV+, LRV- isolates and reference *L. major* strains were grown in liquid culture until 5-7th days of culture. Since stationary phase parasites indicated as more infective (163), *L. major* parasites undergo stationary phase on those days according to growth curve analysis in this study (Figure 4.1). After macrophage transformation, cells were infected with reference strain and two isolate strains of *L. major* with a macrophage:parasite ratio of 1:4 or left uninfected for 24 or 48 hours. Experiment was repeated 3 three times and cell pellets were used in the chemokine expression studies whereas cell culture supernatants were used in inflammatory cytokine production experiments.

Macrophages infected with *Leishmania* strains was collected and stained with

Giemsa dye after 24h or 48h. Samples were visualized under bright field with 1000X magnification. Representative image was presented in Figure 4.15. However, the desired infection images for all samples could not be obtained after staining. This may be due to the failure of the staining despite the optimized conditions and/or the inability to be observed due to the low level of infection.

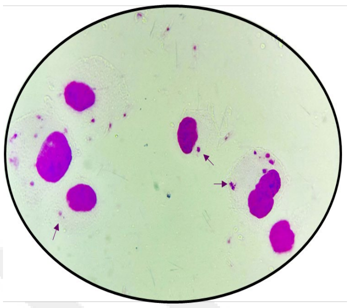

Figure 4.15 LRV- *L. major* infected macrophage cells. Giemsa-stained cell nucleus were colored purple. Amastigotes were shown with black arrows, 1000X magnification.

4.2.3 LRV+ *L. major* infected macrophages and chemokines expression analysis for CCL1, CCL2, CCL5, CXCL13 and CCL22

Chemoattractant chemokines release from macrophages to induce and activate other immune cells. *Leishmania* LRV effect on chemokine expression of infected macrophage cells were examined for parasites harboring or lacking double stranded LRV. LRV+, LRV- *L. major* isolates and reference strain infected macrophages or untreated control cells were harvested after 24h or 48h and total RNA extracted from directly lysing cells on plates. Extracted RNAs were subjected to first strand cDNA conversion procedure.

CCL1, CCL2, CCL5 and CCL22 expressions were assessed by qPCR analysis to understand whether LRV+ *L. major* parasites have a differential effect on this chemokine inducement compared to LRV- *L. major* parasite in mRNA level. ACTB was used as an endogenous control. Gene expression analysis were shown for each chemokine and infection experiment for 24h and 48h in bar graphs or cumulative results for all three experiments were presented (Figure 4.16 a-d).

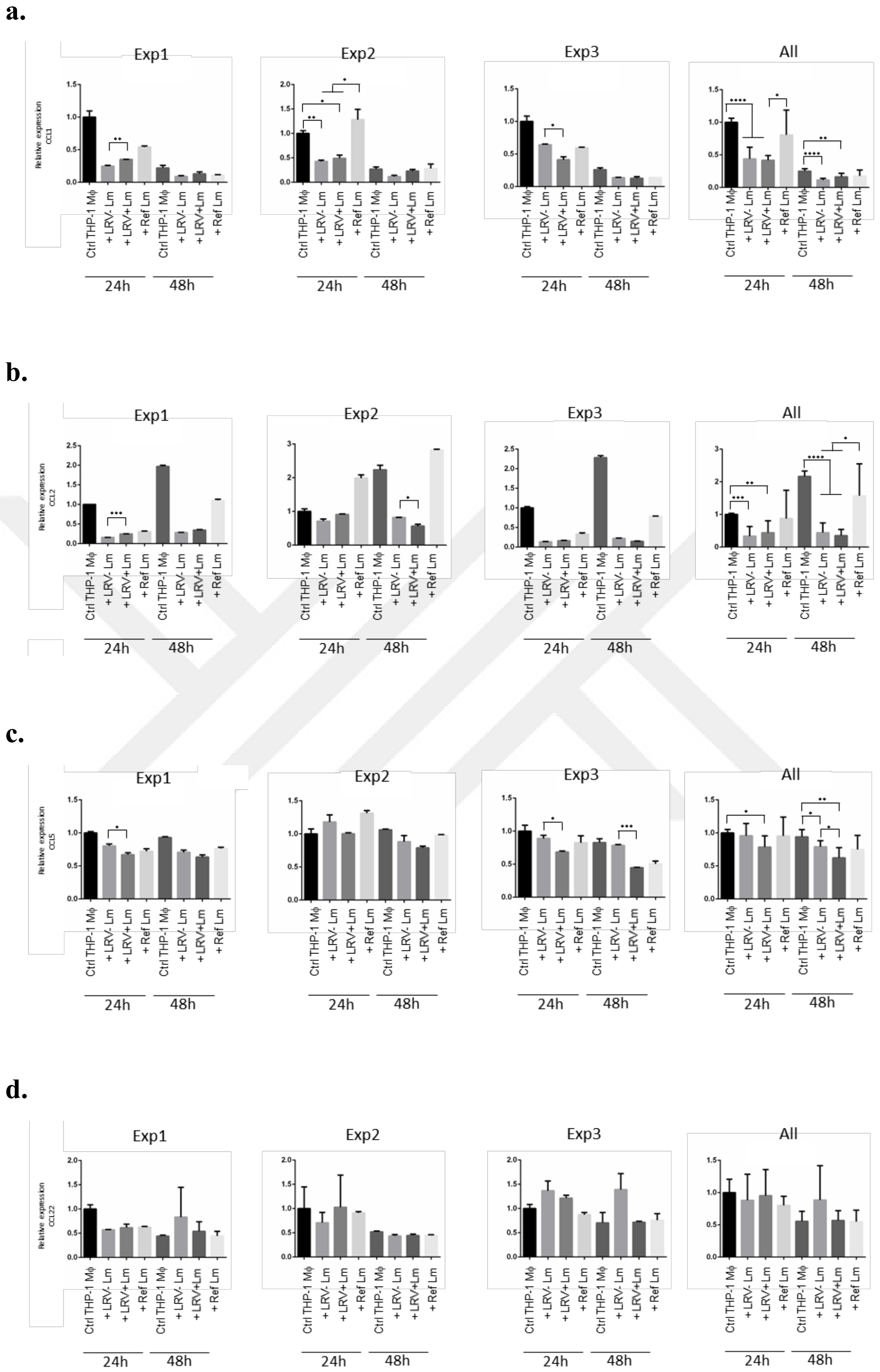

Figure 4.16 CCL1, CCL2, CCL5 and CCL22 gene expression analysis of LRV+ and LRV- *L. major* infected macrophages show variable responses. Macrophages infected or left uninfected and were used to analyze relative expression level of a. CCL1, b. CCL2, c. CCL5 and d. CCL22 by qPCR. Chemokine data were calculated

Figure 4.16 (cont'd) with $2^{-\Delta Ct}$ method and normalized to control THP-1 macrophages. Bar graphs were prepared with GraphPad Prism and shows each of the three experiments (Exp1,2,3) or average of three independent experiments (all). Statistical analysis was performed with unpaired t-test (* $p< 0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p < 0.0001$).

Results showed that LRV+ and LRV- isolates mainly reduced the expression of chemokines compared to reference strain especially in CCL1 for 24h and CCL2 for both 24h and 48h after macrophage infection. Any significant difference was observed for CCL22 expression in infected or uninfected macrophages. Only significant variance was seen in CCL5 expression that it is significantly lower in LRV+ *L. major* infected cells (normalized average value is 0.62357) compared to LRV- *L. major* infected ones (normalized average value is 0.79097) after 48h. *L. major* reference strains is also bearing LRV virus but did not induce this reduction and there was not a comparable inequality between ratios of reference strains and LRV- *L. major* strain infected macrophages except experiment 3. CCL1 is mainly produced in M2b type cells whereas CCL2 and CCL5 is produced from M1 type inflammatory cell. CCL22 is a M2a type releasing chemokines. It is expected to be differentially expressed chemokine profile in cells infected with variable infectious agents (115).

4.2.4 Cytokine production from infected macrophages

Macrophages release to and are induced by several cytokines to respond environmental changes. Presence of LRV in *Leishmania* parasites might regulate this response in cytokine manner. IL-8, IL-1β, IL-6, IL-10, TNF-α and IL-12p70 production from LRV+ *L. major* infected macrophages were analyzed with flow cytometry based Human Inflammatory Cytokine Cytometric Bead Array (CBA) kit and compared with LRV- *L. major* and reference strain infected macrophages data and uninfected control group.

After 24h or 48h of infection with 1:4 macrophage:parasite ratio, culture medium was collected and centrifuged to avoid cellular contamination. Assay was performed with standard samples and experimented supernatant samples to analyze 6 different cytokines in a single tube. Secreted cytokines were sequestered by CBA Red labeled beads and PE-conjugated antibody mixture. Prepared beads were assayed in flow cytometry and aligned from highest values to bottom as IL-8, IL-1β, IL-6, IL-10, TNF-α and IL-12p70 in CBA-Red (FL-3 channel) (Figure 8.3, Appendix 5). Concentrations of each cytokine were calculated from standard samples PE-median value and bar graphs were prepared for three independent experiments (Figure 4.17). IL-12p70 and IL-10 values were under detection level, so results were excluded from final analysis.

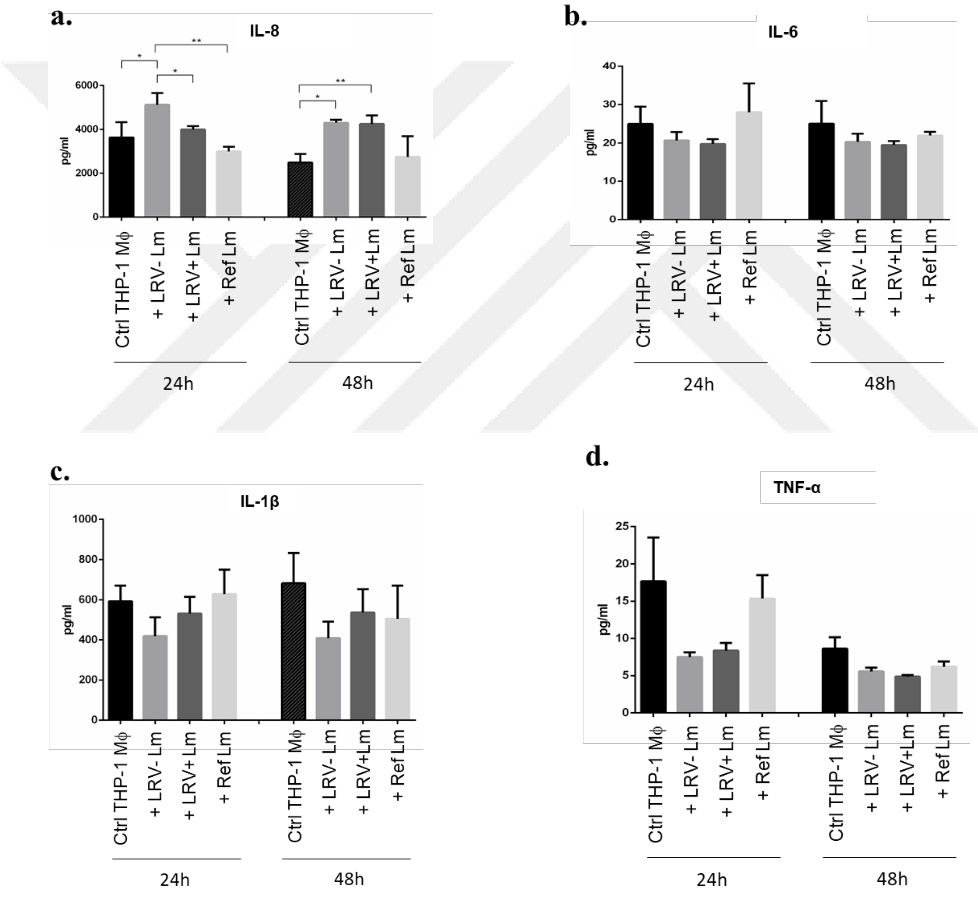

Figure 4.17 Cytokine production from THP-1 cells infected with LRV+, LRV- and reference *L. major* strains or left uninfected. Macrophage supernatants were collected after 24h or 48h and subjected to Human Inflammatory Cytokine CBA assay to measure concentration (pg/ml) of a. IL-8, b. IL-6, c. IL-1β, d. TNF-α. PE-Median intensities of prepared standard samples were used to draw standard curves to calculate produced cytokine amounts. Bar graphs were drawn from data of three independent experiments in GraphPad Prism, and statistical analysis was performed with unpaired t-test (* p< 0.05, ** p<0.01, *** p<0.001 and **** p < 0.0001).

Production of inflammatory cytokines from infected cells were shown for IL-8, IL-6, IL-1β and TNF-α. Among them, IL-8 production was the only target molecule that LRV- *L. major* infected macrophages produced significantly higher cytokine amount (average of 5127,83 pg/ml) compared to LRV+ *L. major* infected cells (average of 3998,08 pg/ml) and uninfected control group (3632,06 pg/ml) after 24h. However, it is noteworthy to indicate that IL-8 production from LRV+ *L. major* infected macrophages were reached the LRV- *L. major* infected macrophage IL-8 level after 48h with a delayed response.

Other cytokines produced slightly lower levels of IL-6, IL-1β and TNF-α in isolates infected macrophage groups compared to uninfected and reference strain treated cells. However, this reduction was not noteworthy because it was not found significant between these groups. In general, *L. major* reference strain was not induced host cells to produce inflammatory cytokines like isolate strains do.

5 DISCUSSION

Leishmaniasis is defined as a neglected tropical disease and is of major concern for public health in some countries. However, incidence of the disease can change as people become more intertwined with the vectors, migrate to/from endemic areas, and have unfavored environmental and socio-economic conditions which all are major factors in the spread of the disease. Presence of virulence factors, transmission of genes within the same species - in fact the hybrid species - should be of interest which may lead to the emergence of new species.

The clinical outcomes of leishmaniasis depends on many factors, such as the species of the parasite, virulence factors, and the host characteristics. Basically, leishmaniasis is seen in three different forms: cutaneous, visceral and mucocutaneous leishmaniasis. Cutaneous leishmaniasis is endemic in our country while the Old World species that causes visceral leishmaniasis are also seen. In the last two decades, leishmaniasis is an emerging infection in Turkey, mainly due to global warming that increased the lifetime and distribution of the vectors, the sandflies, as well as the influx of Syrian refuges due to civil war.

5.1 Identification of Surface Proteins of Old World *Leishmania* Species

Cutaneous leishmaniasis is a disease that manifests itself with small ulcers and papules which can be treated with local or systemic treatment methods or sometimes heals spontaneously without the need for treatment. On the other hand, visceral leishmaniasis is a type of disease that can be treated with drugs such as antimony compounds and miltefosine, but can be fatal if it is left untreated. Although it seems that the strains of the same kind of parasites may cause different clinical forms in diseases (167), generally certain species are of great importance in this distinction. In this context, the differences between functional genes have been noticed, and the best example of this is the A2 gene family. This gene encodes the amastigote-specific ER stress response protein and was previously described in *L. donovani* and *L. infantum*, whereas it is found as a pseudogene in cutaneous strains such as *L. major* and *L.*

tropica knock-in and knock-out studies have shown the importance of this gene in visceralization (31–34).

In addition, three additional candidates for visceral determinants were also indicated which are LinJ.28.0340, cytosolic unknown protein; LinJ.15.0900, a nucleotide sugar transporter of Golgi apparatus; and LinJ.36.2480, a cytosolic glyceraldehyde 3-phosphate dehydrogenase (36). Some strain-specific genes were found to be mutated or have differential copy numbers (38-39). However, any of the proteins or genes identified did not fully restore the infectivity of cutaneous strains into visceral strains. The question arises whether the level of specific protein expressions can contribute to visceralization. In this work, I aimed to understand the cell surface proteins and their varieties in *L. donovani*, *L. infantum* and *L. major* strains.

Trypsin shaving as an identification method for surface proteins

Surface proteome and plasma membrane detected proteins of *Leishmania* strains was the main subject of the first part of the study. Surface proteins interact with the host environment, provide protection against the immune response, are active in the uptake of nutrients and required for the parasite to survive, or are functional in critical situations such as drug resistance. They are good candidates for vaccine development. For instance, *Leishmania* surface proteins Glycoprotein 63, KMP-11, and Hydrophilic acylated surface proteins (HASPs) are some of the target molecules used in vaccine studies. (32,168,169).

Yet, for any of the commercial vaccine registered for humans, it is important to focus on to find out antigenic targets that are effective on visceral strains, which may cause a life-threatening disease if left untreated. There are only a few studies in which specifically enriched membrane proteins of *Leishmania* were identified with different methodologies. Some of these methods rely on the chemical disruption of cells and extraction of membrane proteins by ultracentrifugation (82,84), biotinylation of cell surface and pull-down with streptavidin affinity (82), disruption by nitrogen cavity and sucrose gradient (83) and some other techniques (85–87).

Trypsin shaving of surface proteins is based on the cutting of lysine and arginine residue from accessible cell surface proteins of living cells and it is noteworthy to mention that it has not been used for *Leishmania* species before. Previous studies on gram-positive, gram-negative bacteria, unicellular eukaryotes or some parasites have been conducted with this method. Membrane structure differences of cells and using alive cells make this technique challenging and which may require modifications in the process. Therefore, optimization experiments on different factors such as ingredient of incubation buffer, incubation time, and concentration of enzyme were required. It is also aimed to increase the exposed cut sites with addition of reducing agent DTT without destroying the cell intact structure. Collected peptides were prepared to LC-MS/MS analysis and obtained peptide sequences were blasted to *L. donovani*, *L. infantum*, and *L. major* Uniprot Swiss-Prot and TreEMBL databases. Listed proteins for each strain was filtered to exclude proteins having only 1 unique peptide counts to increase specificity.

After the first filtered-out, 1795, 1519, 969 proteins for *L. donovani*, *L. infantum* and *L. major* were counted, respectively. Most of the protein sequences of *Leishmania* strains are derived from unreviewed data. Bioinformatics approaches are required for further analysis. To focus on my target group, cell membrane localizations, membrane-spanning transmembrane helix (TMH) structures and signal peptide (SP) sequences were predicted for all proteins. Subcellular localization of protein with DeepLoc tool revealed 86, 85 and 37 *L. donovani, L. infantum* and *L. major* cell membrane localized proteins. Of these proteins, 71 of them have TMH and 18 have SP in visceral strains whereas 26 of them have TMH and 9 have SP in *L. major.* However, Blosum62 protein encoding type used in Deeploc-1.0 analyzes, which allows numerous protein analysis rapidly, put some proteins into cell membrane from ER e.g. *L. infantum* Metalloprotease M41 FtsH - putative or nucleus e.g. *L. donovani* MutS domain V family protein. Possible reasons for the detection of intracellular proteins can be summarized as cell lysis and releasing of cellular content while performing the method; unconventional insertion of intracellular membrane proteins into cell membrane or because I collected cell supernatant, possible detection of secreted or extracellular vesicle proteins (51).

Biological processes of cell membrane localized proteins indicated that 53, 52 and 27 of *L. donovani*, *L, infantum* and *L. major* are classified as ‘unknown’, respectively. Rest of them are having a function in ATP binding, transport activity, ATP hydrolysis activity and nucleotide binding. Some specific transport proteins, which are very vital for parasites and allow exchange of substances with the external environment have been identified. ATP Binding Cassette (ABC)-type transporters are found as predominant for the characterized proteins 8/33 (24%) in *L. donovani* and *L. infantum* and 2/10 (20%) *L. major*. It is reviewed that, 47 of 247 transport proteins of *L. major* were shown as ABC-type transport proteins and some subtypes were indicated in drug resistance (84,170).

Parasites’ surface proteins in the way of visceralization

Initially, common proteins of visceral strains, *L. donovani* and *L. infantum* were compared with designated ortholog proteins of the cutaneous *L. major*. Proteins having two or more peptide differences between two groups were listed for protein priorly aligned with *L. donovani*. In total, 31 proteins were identified and, of which 22 were detected exclusively in *L. donovani* and *L. infantum.* Putative cytoskeleton-associated protein CAP5.5 (Q4Q6L9) was the only protein having slightly higher peptide count in *L. major* in 9 common proteins, and it was previously identified in metacyclic *L. chagasi* membrane proteins (82). Most of the detected ABC transporters were classified in this group. Defined ABC-transporter B, C and G superfamily members ABCB4 (A4I9R3), ABCC3 (A4I065), ABCC4 (A4I6Q3), ABCC7 (A4I6S0), ABCG1-4 (A0A504Y207) and ABCG5 (A4I078) except ABCC8 (A4I9N7) were identified so far in membrane enrichment studies of *L. infantum* (84–86), although some of them were shown to be localized in intracellular membranes or vesicles. Na/H antiporter-like protein (A4I0D4) was represented in membrane fraction of *L. amazonensis* (84), and Putative folate/biopterin transporter (A4HTX5) were shown in logarithmic promastigotes of *L. chagasi* (51) which was intended to be enriched metacyclic promastigotes in the present study.

Proteins were further examined in antigenic scoring and B-cell epitope prediction analyzes to underline potential immunogenic proteins significant for visceral strains. These *in silico* proteomic approaches can be used for studies targeting vaccine candidates (171). Therefore, six highly antigenic proteins were chosen for B-cell epitope predictions for *L. donovani* and *L. infantum* enriched-proteins. Highest scored A0A504XRH6/A4I0F1 Hypothetical protein was shown to be identified in plasma membrane proteins in logarithmic and metacyclic form of *L. chagasi* (82) and membrane fraction of *L. infantum* and *L. Mexicana* (83) that contains a gap in the single epitope region of protein orthologs in visceral strains. The other noticeable protein was A0A504XLH3/A4HZX1 Uncharacterized protein. It has a potentially significant 155 aa flank region in the identified A0A504XLH3 ortholog protein but not in *L. infantum* and *L. major* counterparts. A signal peptide in 155 aa N-terminal site is not present in these orthologs and lack of SP localizes the proteins intracellularly. This extra part includes several epitope regions for B-cell recognition that identified peptides from both visceral strains were matched with one of them but any peptides were detected in *L. major*. A0A504X927/A4I6S0 ABC transporter family protein has the A0A3S7X4I0/A4I6S0 Pentamidine resistance protein (PRP1 / ABCC7). Brotherton and her colleagues were identified PRP1 in membrane extraction of *L. infantum* promastigotes but not in amastigotes (85). The protein was shown in resistance to a second-line drug pentamidine used in leishmaniasis in overexpressed *L. major* (172) and *L. infantum* (164). Apart from unmatching residues which might dislocate B-cell epitope sites, one tryptophan (W) residue in *L. major* was of interest. Tryptophan is a unique a hydrophobic amino acid encoded with a single codon which mainly present in the lipid bilayer interface of membrane proteins. All of tryptophan residues might have a specific function in folding and stability of membrane (173). PRP1 (Q4Q6B9) has a tryptophan at 1482. residue in *L. major* and when is aligned with *L. donovani*, *L. infantum*, it matches with positively charged arginine (R). Interestingly, ortholog proteins from New World *L. mexicana* (Diffuse CL agent) (174) has a tryptophan residue, *L*. guyanensis, *L. braziliensis*, *L. panamensis* (MCL agents) (174) have arginine residue at that site in the alignment of same PRP1 proteins (Appendix 4, Figure 8.4). Given that single mutations in ABC-transporter proteins have a large impact on function (175,176) it is a mystery whether this residue has any effect on disease progression.

L. major plasma membrane enriched proteins revealed 11 proteins where eight of them were listed only in *L major*. Four proteins were classified in highly antigenic group and P90552 Hydrophilic acylated surface protein a (HASPA1) has the highest score. These HASPs are present in the surface of metacyclic promastigotes and amastigotes and HASPB is used as a vaccine candidate in visceral leishmaniasis (21). The epitope region of the protein has a gap in *L. major* ortholog which makes the protein more antigenic (1.3841) compared to its visceral orthologs *L. donovani* (1.1667) and *L. infantum* (1.1572). Most of them were not detected before in membrane protein studies.

L. donovani. *L. infantum* and *L. major* shared 29 proteins that were detected with similar spectral counts. It can be inferred that these proteins might be required for all these three strains in normal conditions. The highest spectral count was owned by A4I060 ABC subfamily C member 2 (ABCC2) with around 10-12 unique peptides. The localization of proteins were shown as in the network of intracellular membranes (77) but listed in plasma membrane sorted proteins. A4HTX5 VTC domain family protein and A4I0B5 Hypothetical proteins was previously shown in the extracted membrane fraction of *L. chagasi* (82). A4HS14 Surface antigen-like protein (score= 0.7) was the most ranked protein for antigenicity scoring and its B-cell epitope sites were compatible with each other. Putative calpain-like cysteine e peptidase (Small myristoylated protein 1 / SMP-1) (Q5SDH5) was indicated to possess a regular *Leishmania* flagella protein which has a role in flagellum elongation during amastigote to promastigote differentiation (177). Thus, it is expected that the protein commonly expressed in all strains besides SMP-1 was demonstrated in membrane enrichments of *L. infantum*, *L. Mexicana* (83) and *L. chagasi* (82). In mutually detected proteins, two Palmitoyltransferase (A4HVJ3 and A4I3R5) were identified and these group of enzymes have an impact on flagellar structural changes which partially inhibition was shown to decrease motility of metacyclic promastigotes and macrophage invasion (178).

Trypsin shaving method can be accepted as a complementary method for the detection of plasma membrane proteins in addition to other studies. However, there are a few restrictions in the purity of protein content regarding the source of surface proteins. The nature of technique collecting live parasites' surface-shaved proteins in the cell-free supernatants however released vesicles and secreted proteins are also released to this environment. Filtering of samples might eliminate vesicles greater than 200nm while the others might be subjected to surface shaving. There might be other optimization steps for increasing the trypsin enzyme activity to decrease incubation time to lower released proteins and it is a must to evaluate undigested parasite supernatants to see the effect of trypsin alone .

5.2 Inflammatory Response Evaluation to LRV Carrying *L. major*

Presence of double-strand *Leishmania* RNA virus (LRV) in *Leishmania* species has previously been detected in different parasite isolates. It has been investigated in patient data, mouse models or in vitro studies whether presence of LRV may increase the complexity of the disease and affect the inflammatory response or drug treatment failure (135–142). Recently, our group was reported the first LRV-positive *L. major* clinical isolate from an drug-resistant autochthonous patients which later the virus was classified in LRV-2 with sequence analysis as expected in Old World strains (166). In the present study, I hypothesized that LRV carrying *L. major parasites* can modulate immune response of THP-1 derived macrophages differentially.

LRV-bearing *L. major* was used to evaluate the inflammatory response of macrophages with selected chemokines and cytokines. An acute monocytic leukemia-derived cell line, THP-1 cells were differentiated into macrophages with PMA and infected with LRV carrying *L. major* isolate, reference strain and a clinical isolate of *L. major* lacking LRV-2. Macrophages can be induced with several pathogens to polarize their subsets and produce several different cytokines and chemokines according to targeted function. *L. major* infected macrophages were investigated for the expression of C–C motif chemokine ligand 1 (CCL1), CCL2, CCL5 and CCL22 chemokines in mRNA level after 24h or 48h. The results showed that CCL1, CCL2 and CCL5 but not CCL22 was significantly down-regulated in clinically isolated *L. major* strains after 24h and 48h with the exception of LRV- *L. major* strain infection after 24h did not induced CCL5 suppression. However, reference strain was neither induced nor suppressed chemokines' expression. This might be explained with the infectivity and virulence of clinical isolates and reference strains might vary in several aspect that evolved with copy number increase of the genes or polymorphisms during transmission to other hosts (179). Only expressional difference was observed in the CCL5 chemokine that was significantly lower in LRV+ *L. major* infected macrophages compare to LRV- *L. major* ones. CCL2, CCL3, CCL4, CCL5, CXCL8 and CXCL10 are associated with viral infections (136), but especially CCL5 involvement was indicated in several RNA viruses such as influenza, respiratory syncytial virus (RSV), and hepatitis C virus (HCV) via dsRNA recognizing TLR3 (180,181).

The study conducted with ATL patients and THP-1-derived macrophages infected *L. braziliensis* revelaed a noteworthy mRNA regulating miRNA (miR-548d-3p) that upregulated in self-healed patients' serum and infected macrophages. Inhibition of this miRNA resulted in increased CCL2, CCL5 and IL-6 production in *L. braziliensis* infection which was predicted to suppress CCL-2 and CCL5 to control inflammation but raised parasite growth (182). This phenomenon was supported with the *L. major* infected macrophages derived from human PBMC monocytes which were up-regulated IL-8, CXCL2, CXCL3 but down-regulated CCR2, CCL5, CCL17, CXCL9 and CXCL10 chemokine expressions (183) or similarly down-regulated the expression of CCL2, CCL5, CXCL10, CXCL11 and CXCL12 mRNA levels after 24h with a negative correlation of selected miRNAs up-regulation (184). However, increased secretion of CCL5, CCL2, IL-8, TNF-α was observed after *L. major* infection of lymphoblastoid cell line but CXCL10 products were found to be dramatically decreased in protein level via gp63 cleavage (140).

Influence of LRV on inflammatory cytokines response was measured for IL-8, IL-1β, IL6 and TNF-α. In cells treated with LRV- and LRV+ *L. major* isolates, a slight but neglectable decrease was observed in IL-6, IL-1β and TNF-α production. On the other hand, elevated levels of IL-8 production were observed in cells infected with LRV- *L. major* after 24h; this elevation in IL-8 levels was observed after 48h in cells infected with LRV+ *L. major*.

The effect of the LRV-1 status of *L. braziliensis* and *L. panamensis* on inflammatory response from monocytic cell line U937 derived macrophages was shown for clinical isolates and *L. braziliensis* LRV+ strains was shown to respond with lower levels of IL-1β, CXCL10, and TNF-α and IL-6 unlike LRV- ones. A minimal but insignificant decrease in CCL5 secretion was observed in LRV-1+ isolates of *L. panamensis* after 48h (185). De Carvalho and his collegues indicated the inhibition of inflammasome activation which was required to control parasite replication through TLR activation because of LRV+ *L. guyanensis* strain and clinical isolates and decreased levels of IL-1β but increased level of IL-6 and TNF- α was observed (141). On the other hand, in an infection model of clinically isolated *L. guyanensis* LRV+ parasites comparison to LRV- strains showed increased level of CCL5, CXCL10, TNF-a, and IL-6 (140). IL-8 and IL-1β was shown to be some of the CCL5 inducible cytokines (186). However, in the present study only IL-8 cytokine which is a chemo-attractant for neutrophils was upregulated although CCL5 was down-regulated. It can be referred that there might be some additional mechanisms for this activation and delayed response in the presence of LRV.

In future studies, possible role of LRV in CCL5 suppression might be further investigated to find out underlined mechanisms. However, isogenic mutants that only differ for LRV presence might be the better choice to observe inflammatory responses. The methods generally based on the elimination of viral content from LRV-carrying parasites. In general, *Leishmania* strains use several mechanisms to evade host inflammatory response. Production of IL-8 and suppression CCL5 were shown with some other studies also seen in these experiments. Increased CCL5 down-regulation with LRV-bearing parasites might increase parasite persistence and delayed pro-inflammatory IL-8 response of LRV can contribute this.

6 CONCLUSION

In the first part of the study, the examination of the surface proteins of *L. donovani*, *L. infantum* and *L. major* was performed by trypsin-shaving, which the method was hitherto not used in *Leishmania.* Other part was focused on the macrophage immune response to LRV-carrying *L. major* clinical isolate.

Proteolytic digestion of surface proteins with trypsin enzyme revealed some surface proteins that were not mentioned before in literature although several unrelated proteins were also detected. *Leishmania* has only a group of reviewed proteins, so the usage of prediction programs was pivotal for the categorization of proteins to classify into cell membrane. Some intracellular membrane proteins are localized in this group, so the unusual cell surface localization of these proteins may be the next target to investigate. Our findings indicate that several proteins were identified in visceral strains of *L. donovani* and *L. infantum,* and these enriched proteins were highly abundant in ABC-type transporters which some of them are associated with drug-resistance. However, the role of these proteins in visceralization is still unknown. Additionally, several proteins were listed in common list for all strains, and some are shown in the classical mechanisms of *Leishmania* infectivity. Informatic approach for B-cell epitope predictions of highly enriched antigenic proteins were highlighted some proteins such as Hypothetical protein, Hydrophilic acylated surface protein a, Surface antigen-like protein which can be targeted for vaccine studies. It is important to mention that most of the proteins indicated in all strains were hypothetical proteins with unknown function. Further studies should be carried out to clarify their role in the *Leishmania* biology and could be good candidates for prophylactic effects. There is still a need for an efficient vaccine to prevent leishmaniasis in humans.

To point out the effect of LRV in the macrophage immune response for chemokine and cytokine expression, a clinical isolate of *L. major* was used which was recently reported having LRV-2 for the first time in our country. It was shown that clinical isolates of *L. major* were suppressed CCL1, CCL2 and CCL5 expression of THP-1 derived macrophages independent from LRV. However, additional down-regulation was observed in CCL5 expression in LRV-carrying strain after 48h. Moreover, clinical isolates induced IL-8 production significantly but not IL-1β, IL-6 and TNF-α and this induction was seen in LRV- strain after 24h, but in LRV+ strain after 48h with a delayed response. Next arised questions related to LRV are how to regulate CCL5 expression and to impede IL-8 production.

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8 APPENDIX

APPENDIX 1

PLASMA MEMBRANE PROTEIN LISTS

Table 8.1 Plasma membrane predicted proteins for *L. donovani* and their properties

Uniprot ID	Protein names	Gene names	Coverage [%]	# Peptides	# Unique Peptides	Length	Deeploc	SP	TMH	TMH No	Biological Process
A0A504X6J6	Calpain cysteine protease family protein	CGC21_12120	37	18	18	724	x				proteolysis (GO:0006508)
A0A504XF03	Uncharacterized protein	CGC21_30515	16	13	13	1502	x		x	5	unknown
A0A504X9G9	HAD ATPase, P-type, IC family protein	CGC21_6555	16	10	10	1196	x		x	8	unknown
A0A504XE14	Protein kinase domain family protein	CGC21_6365	21	10	10	752	x		x	1	protein phosphorylation (GO:0006468) protein peptidyl-prolyl isomerization (GO:0000413)
A0A3S7X8Z9	Thioredoxin family protein (Thioredoxin, putative) (Thioredoxin_putative/Pfam:PF00085)	CGC21_6205 LdCL_350018300 LDHU3_35.1770	20	10	10	559	x	x	x	2	unknown
A4I060	ATP-binding cassette protein subfamily C, member 2 (Pentamidine_resistance_protein_1)	putative ABCC2 LINF_230007300 LINJ_23_0240	8	10	9	1562	x		x	8	transmembrane transport (GO:0055085)
A4HXD4	Putative P-type ATPase (Vacuolar-type_Ca2+-ATPase_-_putative)	LINF_170012300 LINJ_17_0660	9	7	7	1134	x		x	6	unknown
A4I0N4	Hypothetical predicted transmembrane protein (Protein_of_uncharacterized_function_(DUF3184)_-_p utative)	LINF_240009200 LINJ_24_0440	10	7	7	1375	x		x	2	unknown
A0A504XVP5	5-histidylcysteine sulfoxide synthase	CGC21_27990	7	7	7	1280	x		x	1	unknown
A4I9N7	ATP-binding cassette protein subfamily C, member 8 (ATP-binding_cassette_protein_subfamily_C_-_member_8_-_putative)	putative ABCC8 LINF_340012300 LINJ_34_0690	6	7	7	2089	x		x	11	transmembrane transport (GO:0055085)
A4I9R3	ATP-binding_cassette_protein_subfamily_B_-_member_2_-_putative (p-glycoprotein) (EC 3.6.3.44)	MDR1 LINF_340016200 LINJ_34_1060	9	7	7	1341	x		x	9	transmembrane transport (GO:0055085)
A0A504XNW5	Papain cysteine protease family protein	CGC21_0105	4	7	6	4209	x	x	x	1	proteolysis (GO:0006508)
A0A3S7X1W4	Histidine phosphatase (Branch 2) family protein (Membrane-bound acid phosphatase, putative)	CGC21_22820 LdCL_280033600	16	6	6	548	x	x	x	1	unknown
A0A504XVU0	AAA domain (Cdc48 subfamily) protein	CGC21_28190	4	6	6	2588	x		x	8	lipid metabolic process (GO:0006629)
A4I065	ABC-thiol transporter (EC 3.6.3.44) (Pentamidine_resistance_protein_1)	MRPA LINF_230007800 LINJ_23_0290	6	7	6	1569	x		x	8	transmembrane transport (GO:0055085)
A0A504Y207	ABC transporter family protein	CGC21_24500	5	6	6	1971	x		x	8	unknown
A4I078	ATP-binding cassette protein subfamily G, member 5 (ATP-binding_cassette_protein_subfamily_G_-_member_5)	putative ABCG5 LINF_230009200 LINJ_23_0430	7	5	5	1246	x		x	10	unknown
A0A504X2Q8	Glycosyl transferases group 1 family protein	CGC21_11290	9	5	5	902	x				unknown
A0A504Y3U6	Protein kinase domain family protein	CGC21_24260	1	5	5	5959	x		x	4	protein phosphorylation (GO:0006468)
A4HRZ6	Calcium-transporting ATPase (EC 7.2.2.10)	LINF_040005000 LINJ_04_0010	12	9	4	1023	x		x	7	calcium ion transport (GO:0006816)
A4I274	Hypothetical_protein_-_conserved	LINF_260023900 LINJ_26_1860	12	4	4	548	x		x	1	unknown
A4HSI0	Hypothetical_protein_-_conserved	LINF_050010900 LINJ_05_0580	11	4	4	779	x		x	5	unknown
A0A504X4R7	Cysteinyl-tRNA synthetase (EC 6.1.1.16)	CGC21_9935	4	4	4	1707	x		x	1	cysteinyl-tRNA aminoacylation (GO:0006423) tRNA aminoacylation for protein translation (GO:0006418)
A0A504XL74	Amastin surface glycofamily protein	CGC21_0335	11	4	4	300	x		x	4	unknown
A0A504X927	ABC transporter family protein	CGC21_11525	2	4	4	2912	x		x	8	transmembrane transport (GO:0055085)
A0A504XN37	DHHC palmitoyltransferase family protein	CGC21_16895	5	4	4	1106	x		x	8	tricarboxylic acid cycle (GO:0006099)
A4I0B5	Hypothetical_protein_-_conserved	LINF_230014100 LINJ_23_0810	11	4	4	556	x	x	x	4	unknown
A4I0D4	Na/H antiporter-like protein (Na/H_antiporter-like_protein)	LINF_230016100 LINJ_23_1000	5	4	4	1500	x		x	10	cation transport (GO:0006812) transmembrane transport (GO:0055085)
A0A504XEW7	Diacylglycerol acyltransferase family protein	CGC21_30690	2	4	4	1863	x		x	24	transcription, DNA-templated (GO:0006351) transcription by RNA polymerase II (GO:0006366)
A0A504XRS0	DnaJ domain family protein	CGC21_3905	2	4	4	3914	x		x	6	unknown
E9AG27	Glycerol-3-phosphate acyl transferase (EC 2.3.1.15) (Glycerol-3-phosphate_acyl_transferase)	GAT LINF_030005600 LINJ_03_0070	10	4	4	628	x		x	3	unknown
A0A504XVX4	Major Facilitator Superfamily protein	CGC21_28340	5	4	4	1329	x		x	14	transmembrane transport (GO:0055085)
A4HRN9	Hypothetical_protein_-_conserved	LINF_020009200 LINJ_02_0390	6	4	4	1019	x		x	8	unknown
A0A504XZ59	MutS domain V family protein	CGC21_38370	5	4	4	1179	x				mismatch repair (GO:0006298)
A4HVJ3	Palmitoyltransferase (EC 2.3.1.225)	LINF_130011400 LINJ_13_0540	11	4	4	607	x		x	4	unknown
A0A504XQH8	Histidine phosphatase (Branch 2) family protein	CGC21_13650	10	3	3	571	x	x	x	2	unknown
A4HX85	ATP pyrophosphate-lyase (Adenylyl cyclase)	RAC-B2 LINJ_17_0140	7	6	3	1399	x		x	1	cyclic nucleotide biosynthetic process (GO:0009190) intracellular signal transduction (GO:0035556)
A4HRH2	Long-chain-fatty-acid-CoA_ligase_-_putative (Putative long-chain-fatty-acid-CoA ligase) (EC 6.2.1.3)	LINF_010010000 LINJ_01_0510	9	5	3	698	x				unknown
A4I676	Putative calcium-binding protein	LINJ_30_1300	9	3	3	528	x		x	1	unknown
A4IBK2	Hypothetical_protein_-_conserved	LINF_350036400 LINJ_35_3160	8	3	3	602	x		x	2	unknown
A0A504XLH3	Uncharacterized protein	CGC21_2250	4	3	3	861	x		x	1	unknown
A0A504X6M4	Uncharacterized protein	CGC21_12270	6	3	3	939	x	x			unknown
A4IAC3	Hypothetical_protein_-_conserved	LINF_340043100 LINJ_34_3350	5	3	3	940	x	x			unknown

Table 8.1 (cont'd) Plasma membrane predicted proteins for *L. donovani* and their properties

Uniprot ID	Protein names	Gene names	Coverage [%]	# Peptides	# Unique Peptides	Length	Deeploc	SP	TMH	TMH No	Biological Process
A4HYG7	Phosphatidic acid phosphatase protein-like protein (Phosphatidic_acid_phosphatase_protein-like_protein)	LINF_190019800 LINJ_19_1380	10	3	3	400	x		x	4	unknown
A0A3S7X4L0	Protein kinase domain family protein (Protein kinase, putative)	CGC21_11325 LdCL_310026200	4	3	3	1359	x		x	3	phospholipid metabolic process (GO:0006644)
A4HUE7	Putative folate/biopterin transporter	LINJ_10_0390	5	3	3	700	x		x	12	unknown
A0A504XNP1	P-type H(+)-exporting transporter (EC 7.1.2.1)	CGC21_5410	2	3	3	2018	x		x	7	proton export across plasma membrane (GO:0120029)
A4I6Q3	Pentamidine_resistance_protein_1 (p-glycoprotein e)	ABCC4 LINF_310019400 LINJ_31_1290	3	3	3	1679	x		x	10	transmembrane transport (GO:0055085)
A4HSH8	Hypothetical_protein_-_conserved	LINF_050010700 LINJ_05_0560	12	3	3	419	x		x	1	unknown
A0A504XWL9	Oxidoreductase FAD-binding domain family protein	CGC21_1075	4	3	3	1287	x		x	3	unknown
A4I0Z4	Hypothetical_protein_-_conserved	LINF_240024800 LINJ_24_1940	4	3	3	1286	x				unknown
A0A504WVT1	Ion transport family protein	CGC21_25585	2	3	3	2823	x		x	19	ion transport (GO:0006811) transmembrane transport (GO:0055085)
A0A504WYB7	Uncharacterized protein	CGC21_36930	5	3	3	1247	x		x	13	unknown
A0A504X595	Glycosyltransferase (GlcNAc) family protein	CGC21_29930	2	3	3	2263	x		x	2	unknown
A0A504XYQ7	N-acetylglucosaminyl transferase component (Gpi1) family protein	CGC21_28570	2	3	3	2310	x		x	4	GPI anchor biosynthetic process (GO:0006506)
A4HU10	Phospholipid_transporting_ATPase-like_protein_-__putative (Putative phospholipid transporting ATPase-like protein)	LINF_090015100 LINJ_09_0940	2	3	3	2528	x		x	8	unknown
A0A504XB68	Dolichyl-diphosphooligosaccharide--protein glycotransferase (EC 2.4.99.18)	CGC21_6110	8	4	2	837	x		x	12	protein glycosylation (GO:0006486)
A0A504XIG7	Nucleoside transporter family protein	CGC21_13315	4	2	2	573	x		x	10	nucleoside transmembrane transport (GO:1901642)
A0A504XAH5	Nucleoside transporter family protein	CGC21_33475	7	2	2	501	x		x	11	nucleoside transmembrane transport (GO:1901642)
A4I3S1	Divalent_cation_transporter_-_putative	LINF_280025900 LINJ_28_2070	8	3	2	678	x		x	2	metal ion transport (GO:0030001) transmembrane transport (GO:0055085)
A4I4L2	Nodulin-like domain-containing protein	LINJ_29_1600	6	2	2	672	x		x	14	unknown
A4I5Q4	Phospholipid-transporting ATPase (EC 7.6.2.1)	LINF_300028000 LINJ_30_2270	2	2	2	1279	x		x	4	phospholipid transport (GO:0015914)
A0A3S7X6J5	Hypothetical_protein_conserved	CGC20_10895 CGC21_38065 LdCL_330017000 LDHU3_33.1690	28	2	2	177	x				unknown
A0A3S7XAD8	Hypothetical_protein_conserved	CGC20_29900 CGC21_12345 LdCL_360005100 LDHU3_36.0060	22	2	2	152	x				unknown
A4I0F1	Hypothetical_protein_-_conserved	LINF_230018500 LINJ_23_1190	31	2	2	131	x				unknown
A4I594	Phosphoinositide-specific_phospholipase_C_-_efhand-like/Phosphatidylinositol-specific_phospholipase_C_-_X_domain/PDZ_domain_(Also_known_as_DHR_or_GLGF)/PDZ_domain_containing_protein_-_putative	LINF_300011700 LINJ_30_0680	5	2	2	730	x				lipid metabolic process (GO:0006629) intracellular signal transduction (GO:0035556)
A0A3Q8IKI2	Hypothetical_protein_conserved	CGC20_29020 CGC21_13220 LdCL_360023800 LDHU3_36.2390	5	2	2	544	x	x	x	1	unknown
A0A504WWX8	Protein kinase domain family protein	CGC21_32165	2	2	2	1188	x		x	2	regulation of transcription, DNA-templated (GO:0006355) protein phosphorylation (GO:0006468)
A0A504XF88	C3H1-type domain-containing protein	CGC21_31340	9	2	2	372	x		x	1	unknown
A4I3R5	Palmitoyltransferase (EC 2.3.1.225)	LINF_280025200 LINJ_28_2010	6	2	2	457	x		x	4	unknown
A0A3S5H4S6	Uncharacterized protein	CGC20_4190 CGC21_18085 LdCL_010005100 LDHU3_01.0030	10	2	2	333	x	x	x	2	unknown
A0A3S7WP69	Surface antigen-like protein (Surface_antigen-like_protein/GeneDB:LmjF.05.0900)	CGC20_24180 CGC21_9670 LdCL_050014100 LDHU3_05.0990	12	2	2	286	x	x	x	1	unknown
A0A504X3S0	Major intrinsic family protein	CGC21_20800	3	2	2	596	x		x	5	transmembrane transport (GO:0055085)
A4HWY7	EF-hand_domain_containing_protein_-_putative (Putative flagellar calcium-binding protein)	LINF_160014800 LINJ_16_0930	19	2	2	203	x				unknown
E9AC40	Putative long-chain-fatty-acid-CoA ligase (EC 6.2.1.3)	LMJF_01_0490	2	2	2	698	x				unknown
D1GJ51	Putative surface antigen protein 2 (Soluble promastigote surface antigen PSA-50S) (Surface_antigen_protein_2_-_putative)	PSA-50S LINF_120014700 LINJ.12.0665	5	2	2	463	x	x	x	2	unknown
A4HUE2	Hypothetical_protein_-_conserved	LINF_100008600 LINJ_10_0340	1	2	2	1957	x		x	1	unknown
A0A504XQZ9	RSN1_7TM domain-containing protein	CGC21_24790	2	2	2	1400	x		x	8	unknown
A4I2P8	ATP-binding cassette protein subfamily A, member 8 (ATP-binding_cassette_subfamily_A_-_member_1_-_putative)	putative ABCA8 LINF_270016000 LINJ_27_0830	3	2	2	1843	x		x	12	transmembrane transport (GO:0055085)
A4HYK6	Domain_of_uncharacterized_function_(DUF1736)_-_pu tative	LINF_200006500 LINJ_20_0170	3	2	2	908	x		x	10	unknown
A0A3Q8IHB3	Uncharacterized protein	CGC20_35470 CGC21_23235 LdCL_100021000 LDHU3_10.1760	9	2	2	321	x				unknown
A0A504Y863	S1/P1 Nuclease family protein	CGC20_9640 CGC21_11085	7	2	2	378	x	x	x	2	DNA catabolic process (GO:0006308)
A4HSV7	Hypothetical_protein_-_conserved	LINF_060012200 LINJ_06_0680	4	2	2	679	x				unknown
A4I9X9	Regulator_of_chromosome_condensation_(RCC1)_repeat_-_putative	LINF_340025200 LINJ_34_1770	4	2	2	629	x				unknown
E9ACP0	Uncharacterized protein	LMJF_03_0950	4	2	2	635	x	x	x	1	unknown
E9AG80	Hypothetical_protein_-_conserved	LINF_080009700 LINJ_08_0470	16	2	2	291	x	x	x	2	unknown

Table 8.2 Plasma membrane predicted proteins for *L. infantum* and their properties

Uniprot ID	Protein names	Gene names	Coverage [%]	# Peptides	# Unique Peptides	Length	Deeploc	SP	TMH	TMH No	Biological Process
A0A504X6J6	Calpain cysteine protease family protein	CGC21_12120	36	19	15	724	x				proteolysis (GO:0006508)
A4I809	Metalloprotease_M41_FtsH_-_putative (Putative ATP-dependent zinc metallopeptidase) (EC 3.4.24.-)	LINF_320020800 LINJ_32_1570	18	9	9	716	x		x	1	proteolysis (GO:0006508)
A0A3S7X8Z9	Thioredoxin family protein (Thioredoxin, putative) (Thioredoxin_putative/Pfam:PF00085)	CGC21_6205 LdCL_350018300 LDHU3_35.1770	21	9	9	559	x	x	x	2	unknown
A4I060	ATP-binding cassette protein subfamily C, member 2 (Pentamidine_resistance_protein_1)	putative ABCC2 LINF_230007300 LINJ_23_0240	10	12	9	1562	x		x	8	transmembrane transport (GO:0055085)
A0A504X9G9	HAD ATPase, P-type, IC family protein	CGC21_6555	12	8	8	1196	x		x	8	unknown
A4I9R3	ATP-binding_cassette_protein_subfamily_B_-_member_2_-_putative (p-glycoprotein) (EC 3.6.3.44)	MDR1 LINF_340016200 LINJ_34_1060	10	8	8	1341	x		x	9	transmembrane transport (GO:0055085)
A0A504XF03	Uncharacterized protein	CGC21_30515	9	8	8	1502	x		x	5	unknown
A4I0N4	Hypothetical predicted transmembrane protein (Protein_of_uncharacterized_function_(DUF3184)_-_p utative)	LINF_240009200 LINJ_24_0440	9	7	7	1375	x		x	2	unknown
A4HRZ6	Calcium-transporting ATPase (EC 7.2.2.10)	LINF_040005000 LINJ_04_0010	10	7	7	1023	x		x	7	calcium ion transport (GO:0006816)
A0A504XNP1	P-type H(+)-exporting transporter (EC 7.1.2.1)	CGC21_5410	4	6	6	2018	x		x	7	proton export across plasma membrane (GO:0120029)
A4I0B5	Hypothetical_protein_-_conserved	LINF_230014100 LINJ_23_0810	9	6	6	556	x	x	x	4	unknown
A0A504XE14	Protein kinase domain family protein	CGC21_6365	11	6	6	752	x		x	1	protein phosphorylation (GO:0006468) protein peptidyl-prolyl isomerization (GO:0000413)
A4HX85	ATP pyrophosphate-lyase (Adenylyl cyclase)	RAC-B2 LINJ_17_0140	7	6	6	1399	x		x	1	cyclic nucleotide biosynthetic process (GO:0009190) intracellular signal transduction (GO:0035556)
A0A504XZ59	MutS domain V family protein	CGC21_38370	9	6	6	1179	x				mismatch repair (GO:0006298)
A4I078	ATP-binding cassette protein subfamily G, member 5 (ATP-binding_cassette_protein_subfamily_G_-_member_5)	putative ABCG5 LINF_230009200 LINJ_23_0430	9	6	6	1246	x		x	10	unknown
A4HXD4	Putative P-type ATPase (Vacuolar-type_Ca2+-ATPase_-_putative)	LINF_170012300 LINJ_17_0660	6	5	5	1134	x		x	6	unknown
A0A504XVP5	5-histidylcysteine sulfoxide synthase	CGC21_27990	5	5	5	1280	x		x	1	unknown
A0A504XVU0	AAA domain (Cdc48 subfamily) protein	CGC21_28190	4	7	5	2588	x		x	8	lipid metabolic process (GO:0006629)
A0A504XNW5	Papain cysteine protease family protein	CGC21_0105	3	5	5	4209	x	x	x	1	proteolysis (GO:0006508)
A0A504X927	ABC transporter family protein	CGC21_11525	3	5	5	2912	x		x	8	transmembrane transport (GO:0055085)
A4HRH2	Long-chain-fatty-acid-CoA_ligase_-_putative (Putative long-chain-fatty-acid-CoA ligase) (EC 6.2.1.3)	LINF_010010000 LINJ_01_0510	11	6	4	698	x				unknown
E9AHP6	Uncharacterized protein	LINJ_33_2400	48	4	4	540	x				unknown
A4IBK2	Hypothetical_protein_-_conserved	LINF_350036400 LINJ_35_3160	13	4	4	602	x		x	2	unknown
E9ADR4	Putative ATP-binding cassette protein subfamily A,member 10	ABCA10 LMJF_29_0620	3	4	4	1866	x		x	16	transmembrane transport (GO:0055085)
A0A504XN37	DHHC palmitoyltransferase family protein	CGC21_16895	5	4	4	1106	x		x	8	tricarboxylic acid cycle (GO:0006099)
A4I9N7	ATP-binding cassette protein subfamily C, member 8 (ATP-binding_cassette_protein_subfamily_C_-_member_8_-_putative)	putative ABCC8 LINF_340012300 LINJ_34_0690	3	4	4	2089	x		x	11	transmembrane transport (GO:0055085)
A0A3Q8IHB3	Uncharacterized protein	CGC20_35470 CGC21_23235 LdCL_100021000 LDHU3_10.1760	14	4	4	321	x				unknown
E9AG27	Glycerol-3-phosphate acyl transferase (EC 2.3.1.15) (Glycerol-3-phosphate_acyl_transferase)	GAT LINF_030005600 LINJ_03_0070	10	4	4	628	x		x	3	unknown
A4HSH8	Hypothetical_protein_-_conserved	LINF_050010700 LINJ_05_0560	17	4	4	419	x		x	1	unknown
A4HSV7	Hypothetical_protein_-_conserved	LINF_060012200 LINJ_06_0680	8	4	4	679	x				unknown
A0A504XQH8	Histidine phosphatase (Branch 2) family protein	CGC21_13650	8	4	4	571	x	x	x	2	unknown
A4HUE7	Putative folate/biopterin transporter	LINJ_10_0390	5	3	3	700	x		x	12	unknown
A0A504Y8Q0	Uncharacterized protein	CGC21_27430	2	3	3	1140	x	x	x	1	unknown
A4I0D4	Na/H antiporter-like protein (Na/H_antiporter-like_protein)	LINF_230016100 LINJ_23_1000	4	3	3	1500	x		x	10	cation transport (GO:0006812) transmembrane transport (GO:0055085)
A0A504XEW7	Diacylglycerol acyltransferase family protein	CGC21_30690	2	3	3	1863	x		x		transcription, DNA-templated (GO:0006351) transcription by RNA polymerase II (GO:0006366)
A4I065	ABC-thiol transporter (EC 3.6.3.44) (Pentamidine_resistance_protein_1)	MRPA LINF_230007800 LINJ_23_0290	6	6	3	1569	x		x	8	transmembrane transport (GO:0055085)
A0A504X4R7	Cysteinyl-tRNA synthetase (EC 6.1.1.16)	CGC21_9935	3	3	3	1707	x		x	1	cysteinyl-tRNA aminoacylation (GO:0006423) tRNA aminoacylation for protein translation (GO:0006418)
A0A504XVX4	Major Facilitator Superfamily protein	CGC21_28340	4	3	3	1329	x		x	14	transmembrane transport (GO:0055085)
A4I0D6	Hypothetical_protein_-_conserved	LINF_230016400 LINJ_23_1020	6	3	3	527	x	x	x	2	unknown
D1GJ51	Putative surface antigen protein 2 (Soluble promastigote surface antigen PSA-50S) (Surface_antigen_protein_2_-_putative)	PSA-50S LINF_120014700 LINJ.12.0665	9	4	3	463	x	x	x	2	unknown
A0A504Y207	ABC transporter family protein	CGC21_24500	2	3	3	1971	x		x	8	unknown
A4I6Q3	Pentamidine_resistance_protein_1 (p-glycoprotein e)	ABCC4 LINF_310019400 LINJ_31_1290	3	3	3	1679	x		x	10	transmembrane transport (GO:0055085)
A4I9L8	Calcium_channel_protein_-_putative (Putative calcium channel protein)	LINF_340010000 LINJ_34_0500	2	3	3	2556	x		x	21	ion transport (GO:0006811) transmembrane transport (GO:0055085)

Table 8.2 (cont'd) Plasma membrane predicted proteins for *L. infantum* and their properties

Uniprot ID	Protein names	Gene names	Coverage [%]	# Peptides	# Unique Peptides	Length	Deeploc	SP	TMH	TMH No	Biological Process (GO)
Q5SDH5	Putative calpain-like cysteine peptidase (Small myristoylated protein 1)	SMP-1 LMJF_20_1310	17	3	3	131	x				unknown
A0A504XYQ7	N-acetylglucosaminyl transferase component (Gpi1) family protein	CGC21_28570	3	3	3	2310	x		x	4	GPI anchor biosynthetic process (GO:0006506)
A4I0F1	Hypothetical_protein_-_conserved	LINF_230018500 LINJ_23_1190	31	2	2	131	x				unknown
A0A3Q8IDX3	PQ loop repeat family protein (PQ loop repeat, putative)	CGC21_32315 LdCL_260026000	15	2	2	368	x		x	6	unknown
A0A504XL74	Amastin surface glycofamily protein	CGC21_0335	7	2	2	300	x		x	4	unknown
A4HS14	Surface antigen-like protein (Surface_antigen-like_protein)	LINF_040007000 LINJ_04_0180	5	2	2	709	x	x	x	1	unknown
A0A504XRS0	DnaJ domain family protein	CGC21_3905	2	5	2	3914	x		x	6	unknown
A0A504XAH5	Nucleoside transporter family protein	CGC21_33475	7	2	2	501	x		x	11	nucleoside transmembrane transport (GO:1901642)
A0A3S7WP69	Surface antigen-like protein (Surface_antigen-like_protein/GeneDB:LmjF.05.0900)	CGC20_24180 CGC21_9670 LdCL_050014100 LDHU3_05.0990	12	2	2	286	x	x	x	1	unknown
A0A381M9M8	Hypothetical_protein_-_conserved	LINF_040006600 LINF_040006700 LINF_040006800 LINJ_04_0160	8	2	2	528	x	x			unknown
A0A3S7XAD8	Hypothetical_protein_conserved	CGC20_29900 CGC21_12345 LdCL_360005100 LDHU3_36.0060	22	2	2	152	x				unknown
E9AHU4	Dolichyl-diphosphooligosaccharide--protein glycotransferase (EC 2.4.99.18)	LINF_350016400 LINJ_35_1170	6	3	2	836	x		x	11	protein glycosylation (GO:0006486)
A0A504XLH3	Uncharacterized protein	CGC21_2250	2	2	2	861	x		x	1	unknown
A0A504X8A6	Uncharacterized protein	CGC21_9525	5	2	2	711	x		x	4	unknown
A4IAC3	Hypothetical_protein_-_conserved	LINF_340043100 LINJ_34_3350	3	2	2	940	x	x			unknown
A4I3R5	Palmitoyltransferase (EC 2.3.1.225)	LINF_280025200 LINJ_28_2010	6	2	2	457	x		x	4	unknown
Q4Q906	Uncharacterized protein	LMJF_26_1860	3	2	2	550	x		x	1	unknown
A0A504XS79	Uncharacterized protein	CGC21_2555	10	2	2	354	x	x	x	2	unknown
A0A504WYF7	Cation efflux family protein (Cation_transporter_putative/GeneDB:LmjF.19.1380)	CGC20_8630 CGC21_18900 LDHU3_19.1720	6	2	2	613	x		x	3	cation transport (GO:0006812) transmembrane transport (GO:0055085)
A4IDE0	Mitochondrial inner membrane protease ATP23 (EC 3.4.24.-)	LINF_360023300 LINJ_36_1770	13	2	2	310	x				unknown
A4HU10	Phospholipid_transporting_ATPase-like_protein_-__putative (Putative phospholipid transporting ATPase-like protein)	LINF_090015100 LINJ_09_0940	1	2	2	2528	x		x	8	unknown
A0A504XQZ9	RSN1_7TM domain-containing protein	CGC21_24790	2	2	2	1400	x		x	8	unknown
E9AGH0	Surface antigen protein 2	LINJ.12.0690	4	2	2	377	x	x	x	1	unknown
A0A504Y4E0	Uncharacterized protein	CGC21_16560	3	2	2	1104	x				unknown
A4IDG6	Inosine-guanosine transporter (Inosine-guanosine_transporter)	NT2 LINF_360026000 LINJ_36_2040	8	2	2	499	x		x	10	nucleoside transmembrane transport (GO:1901642)
A0A504Y3U6	Protein kinase domain family protein	CGC21_24260	0	2	2	5959	x		x	4	protein phosphorylation (GO:0006468)
A0A504Y863	S1/P1 Nuclease family protein	CGC20_9640 CGC21_11085	7	2	2	378	x	x	x	2	DNA catabolic process (GO:0006308)
A4HVJ3	Palmitoyltransferase (EC 2.3.1.225)	LINF_130011400 LINJ_13_0540	4	2	2	607	x		x	4	unknown
Q4QD04	Uncharacterized protein	LMJF_20_0370	3	2	2	1213	x		x	8	unknown
A0A504XG97	Calcium-activated chloride channel family protein	CGC21_33255	3	2	2	988	x		x	8	unknown
A4HYG7	Phosphatidic acid phosphatase protein-like protein (Phosphatidic_acid_phosphatase_protein-like_protein)	LINF_190019800 LINJ_19_1380	10	2	2	400	x		x	4	unknown
A0A504Y8T0	Peptidase M3 family protein	CGC21_18525	3	2	2	1196	x		x	1	proteolysis (GO:0006508)
Q4QGQ3	Putative 3'-nucleotidase/nuclease	LMJF_12_0400	7	2	2	381	x	x	x	2	DNA catabolic process (GO:0006308)
A4IBS9	Hypothetical_protein_-_conserved	LINF_350044600 LINJ_35_3960	4	2	2	804	x		x	1	unknown
A0A504Y105	EGF-like domain-containing protein	CGC21_22700	1	2	2	1691	x		x	12	unknown
A4HRX5	NLI_interacting_factor-like_phosphatase_-_putative	LINF_030014000 LINJ_03_0870	4	2	2	739	x				unknown
A0A504Y1T2	UBA/TS-N domain family protein	CGC21_16425	6	2	2	395	x		x	4	unknown
A0A504XCA7	Uncharacterized protein	CGC21_21295	4	2	2	604	x	x	x	1	unknown
A0A504Y2B0	Eukaryotic-type carbonic anhydrase family protein	CGC21_21790	5	2	2	604	x	x	x	2	unknown
E9ACD1	Protein kinase domain-containing protein	LMJF_02_0570	3	2	2	1424	x		x	2	protein phosphorylation (GO:0006468)
A4HWR6	Hypothetical_protein_-_conserved	LINF_160006800 LINJ_16_0180	1	2	2	4440	x		x	2	fermentation (GO:0006113)
E9ACP0	Uncharacterized protein	LMJF_03_0950	4	2	2	635	x	x	x	1	unknown

Table 8.3 Plasma membrane predicted proteins for *L. major* and their properties

Uniprot ID	Protein names	Gene names	Coverage [%]	# Peptides	# Unique Peptides	Length	Deeploc	SP	TMH	TMH No	Biological Process
Q4Q6L9	Putative cytoskeleton-associated protein CAP5.5	LMJF_31_0440	38	22	18	723	x				proteolysis (GO:0006508)
Q4QBF3	Putative ATP-binding cassette protein subfamily C,member 2	ABCC2 LMJF_23_0220	9	11	11	1570	x		x	6	transmembrane transport (GO:0055085)
Q4QIM6	Calcium-transporting ATPase (EC 7.2.2.10)	LMJF_07_0650	9	7	7	1119	x		x	10	calcium ion transmembrane transport (GO:0070588)
Q4QI04	VTC domain-containing protein	LMJF_09_0220	11	7	7	813	x		x	2	unknown
Q9U0V4	Uncharacterized protein L7836.08	L7836.08 LMJF_23_0640	14	6	6	555	x	x	x	4	unknown
Q4Q5D1	Putative ATP-dependent zinc metallopeptidase (EC 3.4.24.-)	LMJF_32_1500	10	6	6	718	x		x	1	proteolysis (GO:0006508)
Q95Z93	Calcium-transporting ATPase (EC 7.2.2.10)	LMJF_04_0010	9	6	6	1023	x		x	7	calcium ion transport (GO:0006816)
Q4QDN7	Plasma membrane ATPase (EC 7.1.2.1)	H1A-2 LMJF_18_1520	8	6	6	974	x		x	8	proton export across plasma membrane (GO:0120029)
Q4QBK1	I/6 autoantigen-like protein	LMJF_22_1460	35	7	5	206	x				unknown
Q4Q253	Eukaryotic translation initiation factor 3 subunit L (eIF3l)	LMJF_36_0250	10	5	5	633	x		x	1	unknown
A0A504XVU0	AAA domain (Cdc48 subfamily) protein	CGC21_28190	3	5	5	2588	x		x	8	lipid metabolic process (GO:0006629)
Q4Q3W0	Uncharacterized protein	LMJF_33_2270	47	3	3	579	x				unknown
Q4Q5P8	Protein SEY1 homolog (EC 3.6.5.-)	LmjF32.0370 LmjF_32_0370	5	3	3	891	x		x	1	unknown
Q4QGJ9	Putative surface antigen protein 2	LMJF_12_0760	7	5	3	587	x	x	x	2	unknown
E9ABZ0	Uncharacterized protein	LMJF_01_0010	18	3	3	332	x	x	x	2	unknown
Q4QH88	Uncharacterized protein	LMJF_10_1240	9	3	3	321	x				unknown
Q4QBD6	Putative ATP-binding cassette protein subfamily	ABCG5 LMJF_23_0380	4	3	3	1241	x	x	x	10	unknown
E9AE12	Uncharacterized protein	LMJF_29_1530	7	3	3	708	x				unknown
Q4QBV4	MRH domain-containing protein	LMJF_22_0410	5	3	3	1185	x	x	x	2	unknown
Q5SDH5	Putative calpain-like cysteine peptidase (Small myristoylated protein 1)	SMP-1 LMJF_20_1310	17	3	3	131	x				unknown
Q4QII2	Putative cation-transporting ATPase (EC 3.6.3.-)	LMJF_07_1050	4	3	3	1244	x		x	10	cation transport (GO:0006812)
E9AC40	Putative long-chain-fatty-acid-CoA ligase (EC 6.2.1.3)	LMJF_01_0490	6	3	3	698	x				unknown
Q4QAT7	Hypothetical predicted transmembrane protein	LMJF_24_0440	3	3	3	1369	x		x	2	unknown
A0A504X6J6	Calpain cysteine protease family protein	CGC21_12120	11	6	2	724	x				proteolysis (GO:0006508)
A0A504X5S7	Dolichyl-diphosphooligosaccharide--protein glycotransferase (EC 2.4.99.18)	CGC21_6095	4	3	2	863	x		x	11	protein glycosylation (GO:0006486)
Q9XZX9	Surface antigen-like protein	LMJF_04_0190	4	2	2	709	x	x	x	1	unknown
Q4QGL8	Putative surface antigen protein	LMJF_12_0990	4	3	2	610	x	x	x	2	unknown
Q4QJE4	Uncharacterized protein	LMJF_05_0580	7	2	2	784	x		x	5	unknown
Q9N856	Uncharacterized protein	LMJF_04_0130	6	2	2	531	x	x			unknown
Q4QIA5	Uncharacterized protein	LMJF_08_0640	10	2	2	299	x		x	4	unknown
Q4QCR9	DUF1935 domain-containing protein	LMJF_20_1270	14	2	2	189	x				unknown
Q4Q3E0	Putative ATP-binding cassette protein subfamily C,member 8	ABCC8 LMJF_34_0670	2	2	2	2087	x		x	9	transmembrane transport (GO:0055085)
A0A504XF03	Uncharacterized protein	CGC21_30515	2	2	2	1502	x		x	5	unknown
Q4Q6Q3	Putative intergrin alpha chain protein	LMJF_31_0110	3	2	2	785	x	x	x	1	unknown
P90552	HASPA1 (Hydrophilic acylated surface protein a) (Hydrophilic surface protein 2)	haspa2 HASPA1 HASPA2 LMJF_23_1040 LMJF_23_1082 LMJF_23_1088	26	2	2	73	x				unknown
E9AF31	Putative calcium motive P-type ATPase (EC 3.6.3.-)	LMJF_35_2080	3	2	2	1109	x		x	8	unknown
Q4Q945	Uncharacterized protein	LMJF_26_1490	3	2	2	655	x		x	2	unknown

APPENDIX 2

VAXIJEN SCORES

Table 8.4 VaxiJen 2.0 antigenic property analysis of *L. donovani* and *L. infantum* proteins which are expressed differentially compared to *L. major* samples (2< or more peptides)

Uniprot ID	Protein name	VaxiJen 2.0 Score* (0.5< Probable Antigen)
A4I0F1	**Hypothetical_protein_-_conserved**	**0.9240**
A4HUE7	**Putative folate/biopterin transporter**	**0.7128**
A0A504XAH5	**Nucleoside transporter family protein**	**0.6606**
A0A504Y3U6	**Protein kinase domain family protein**	**0.6574**
A0A504XLH3	**Uncharacterized protein**	**0.6452**
E9AG27	**Glycerol-3-phosphate acyl transferase (EC 2.3.1.15) (Glycerol-3-phosphate_acyl_transferase)**	**0.6221**
A0A504X927	**ABC transporter family protein**	**0.6167**
A0A504XNW5	**Papain cysteine protease family protein**	**0.5799**
A0A504XF03	**Uncharacterized protein**	**0.5769**
A0A504XQZ9	**RSN1_7TM domain-containing protein**	**0.5765**
A0A504XE14	**Protein kinase domain family protein**	**0.5679**
A4HSH8	**Hypothetical_protein_-_conserved**	**0.5677**
A4HX85	**ATP pyrophosphate-lyase (Adenylyl cyclase)**	**0.5634**
A4HYG7	**Phosphatidic acid phosphatase protein-like protein (Phosphatidic_acid_phosphatase_protein-like_protein)**	**0.5594**
A4I0D4	**Na/H antiporter-like protein (Na/H_antiporter-like_protein)**	**0.5557**
A4HXD4	**Putative P-type ATPase (Vacuolar-type_Ca2+-ATPase_-_putative)**	**0.5541**
A4I9N7	**ATP-binding cassette protein subfamily C, member 8 (ATP-binding_cassette_protein_subfamily_C_-_member_8_-_putative)**	**0.5533**
A0A504X9G9	**HAD ATPase, P-type, IC family protein**	**0.5388**
A4I6Q3	**Pentamidine_resistance_protein_1 (p-glycoprotein e)**	**0.5374**
A4I078	**ATP-binding cassette protein subfamily G, member 5 (ATP-binding_cassette_protein_subfamily_G_-_member_5)**	**0.5160**
A4I9R3	**ATP-binding_cassette_protein_subfamily_B_-_member_2_-_putative (p-glycoprotein) (EC 3.6.3.44)**	**0.5121**
A4I0N4	**Hypothetical predicted transmembrane protein (Protein_of_uncharacterized_function_(DUF3184)_-_putative)**	**0.5047**
A4I065	**ABC-thiol transporter (EC 3.6.3.44) (Pentamidine_resistance_protein_1)**	**0.5018**
A0A504XN37	DHHC palmitoyltransferase family protein	0.4888
A0A504X6J6	Calpain cysteine protease family protein	0.4853
A0A504Y207	ABC transporter family protein	0.4839
A0A504XZ59	MutS domain V family protein	0.4713
A0A3S7X8Z9	Thioredoxin family protein (Thioredoxin, putative) (Thioredoxin_putative/Pfam:PF00085)	0.4659
A0A504XQH8	Histidine phosphatase (Branch 2) family protein	0.4571
A0A504XVP5	5-histidylcysteine sulfoxide synthase	0.4461
A4HRH2	Long-chain-fatty-acid-CoA_ligase_-_putative (Putative long-chain-fatty-acid-CoA ligase) (EC 6.2.1.3)	0.4278

*Antigenic proteins were shown in bold.

Table 8.5 VaxiJen 2.0 antigenic property analysis *L. major* ortholog proteins of *L. donovani* and *L. infantum* differentially expressed proteins

Uniprot ID	Protein name	Vaxijen 2.0 Score* (0.5< Probable Antigen)
Q4QB61	**Uncharacterized protein**	**0.9893**
Q4QJ14	**Uncharacterized protein**	**0.7176**
Q4QHI0	**Putative folate/biopterin transporter**	**0.7021**
Q4QJ15	**Uncharacterized protein**	**0.6871**
E9AEZ3	**Putative cyclophilin 14**	**0.6858**
Q4QG33	**Nucleobase transporter**	**0.6609**
Q4Q6C0	**Uncharacterized protein**	**0.6600**
Q4QJ68	**Putative ATP-binding cassette protein subfamily G, member 1-4**	**0.6563**
Q4QF95	**Putative ATP-binding cassette protein subfamily G, member 1-4**	**0.6537**
Q4QJE6	**Uncharacterized protein**	**0.6299**
E9ACF5	**Glycerol-3-phosphate acyl transferase**	**0.6229**
Q4QBP9	**Uncharacterized protein**	**0.6219**
Q4QD76	**Phosphatidic acid phosphatase protein-like protein**	**0.5998**
Q4QED4	**Putative P-type ATPase**	**0.5926**
Q4QB80	**Na/H antiporter-like protein**	**0.5894**
Q4QI68	**Cathepsin L-like protease**	**0.5827**
Q4Q6B9	**Pentamidine resistance protein 1**	**0.5817**
Q4QEP8	**RSN1_7TM domain-containing protein**	**0.5658**
Q4Q3E0	**Putative ATP-binding cassette protein subfamily C,member 8**	**0.5535**
Q4QDN9	**Uncharacterized protein**	**0.5513**
E9AF31	**Putative calcium motive P-type ATPase (EC 3.6.3.-)**	**0.5487**
Q4Q6D6	**P-glycoprotein e**	**0.5483**
Q4QB08	**Palmitoyltransferase**	**0.5461**
Q4QGB2	**putative acyl-CoA synthetase**	**0.5207**
Q4QB09	**Palmitoyltransferase**	**0.5194**
Q4QAT7	**Hypothetical predicted transmembrane protein**	**0.5097**
Q4Q1G2	**Putative membrane-bound acid phosphatase 2**	**0.5067**
E9ADG8	**Uncharacterized protein**	**0.5062**
Q4QI61	**Cathepsin L-like protease**	**0.5055**
Q4QBE9	**ABC-thiol transporter**	**0.5055**
Q4QJ16	**Uncharacterized protein**	**0.5031**
Q4QEI2	Putative receptor-type adenylate cyclase	0.4999
Q4Q3A6	P-glycoprotein	0.4966
Q4QDN8	Plasma membrane ATPase	0.4919
Q4QBD6	P-glycoprotein e	0.4918
Q4Q6L9	Putative cytoskeleton-associated protein CAP5.5	0.4912
Q4QDN7	Plasma membrane ATPase	0.4878
E9ADV3	Uncharacterized protein	0.4647
Q9GRP8	Uncharacterized protein L7845.03	0.4607
Q4QJ70	Putative ATP-binding cassette protein subfamily G, member 1-4	0.4519
Q4QJ71	Putative ATP-binding cassette protein subfamily G, member 1-4	0.4495
E9ACB1	Putative casein kinase II, alpha chain-	0.4443
Q4Q4J6	Putative DNA mismatch repair protein	0.4227
E9ADV2	Uncharacterized protein	0.4042

*Antigenic proteins were shown in bold.

Table 8.6 VaxiJen 2.0 antigenic property analysis of proteins that identified only in *L. major*

Uniprot ID	Protein name	Vaxijen 2.0 Score* (0.5< Probable Antigen)
P90552	**HASPA1 (Hydrophilic acylated surface protein a) (Hydrophilic surface protein 2) surface protein**	**1.3841**
E9ABZ0	**Uncharacterized protein**	**0.6385**
Q4Q6Q3	**Putative intergrin alpha chain protein**	**0.6285**
E9AE12	**Uncharacterized protein**	**0.6271**
Q4QBV4	**MRH domain-containing protein**	**0.5573**
Q4QGL8	**Putative surface antigen protein**	**0.5471**
Q4QIM6	**Calcium-transporting ATPase (EC 7.2.2.10)**	**0.5458**
Q4QBK1	I/6 autoantigen-like protein	0.3931

*Antigenic proteins were shown in bold.

Table 8.7 VaxiJen 2.0 antigenic property analysis of identified *L. donovani* cell membrane localized proteins that shown in all species with similar peptide counts

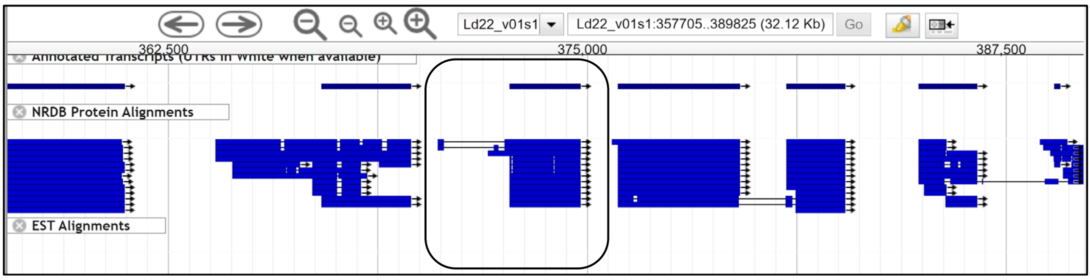
Uniprot ID	Protein name	Vaxijen 2.0 Score* (0.5< Probable Antigen)
A4HS14	**Surface antigen-like protein**	**0.7**
Q5SDH5	**Putative calpain-like cysteine peptidase (Small myristoylated protein 1)**	**0.6976**
A0A3Q8IHB3	**Uncharacterized protein**	**0.6904**
A4IAC3	**Hypothetical_protein_-_conserved**	**0.6591**
A4HU10	**Phospholipid_transporting_ATPase-like_protein_-__putative (Putative phospholipid transporting ATPase-like protein)**	**0.6547**
A4I809	**Metalloprotease_M41_FtsH_-_putative**	**0.6417**
A0A504XYQ7	**N-acetylglucosaminyl transferase component (Gpi1) family protein**	**0.6379**
A4I0B5	**Hypothetical_protein_-_conserved**	**0.6378**
A4HVJ3	**Palmitoyltransferase (EC 2.3.1.225)**	**0.6285**
A0A3S7XAD8	**Hypothetical_protein_conserved**	**0.6221**
A0A504XL74	**Amastin surface glycofamily protein**	**0.5994**
A0A504XVX4	**Major Facilitator Superfamily protein**	**0.5993**
D1GJ51	**Putative surface antigen protein 2 (Soluble promastigote surface antigen PSA-50S) (Surface_antigen_protein_2_-_putative)**	**0.5881**
A0A504Y863	**S1/P1 Nuclease family protein**	**0.5881**
A4I3R5	**Palmitoyltransferase (EC 2.3.1.225)**	**0.5836**
A0A504XVU0	**AAA domain (Cdc48 subfamily) protein**	**0.5814**
A4HSV7	**Hypothetical_protein_-_conserved**	**0.5689**
A0A504XRS0	**DnaJ domain family protein**	**0.5616**
A4IBK2	**Hypothetical_protein_-_conserved**	**0.5614**
A0A504X4R7	**Cysteinyl-tRNA synthetase**	**0.5584**
A4I283	**Hypothetical_protein_-_conserved**	**0.5563**
A4HRZ6	**Calcium-transporting ATPase**	**0.5443**
A0A504XEW7	**Diacylglycerol acyltransferase family protein**	**0.543**
A4HTX5	**VTC domain family protein**	**0.5424**
A0A504XB68	**Dolichyl-diphosphooligosaccharide--protein glycotransferase (EC 2.4.99.18)**	**0.5423**
A0A504XNP1	**P-type H(+)-exporting transporter (EC 7.1.2.1)**	**0.5308**
E9ACP0	**Uncharacterized protein**	**0.5285**
A4I274	**Hypothetical_protein_-_conserved**	**0.5208**
A4I060	ATP-binding cassette protein subfamily C, member 2 (Pentamidine_resistance_protein_1)	0.4608

*Antigenic proteins were shown in bold.

APPENDIX 3

A0A504XLH3 PROTEIN ALIGNMENT

a.



b.

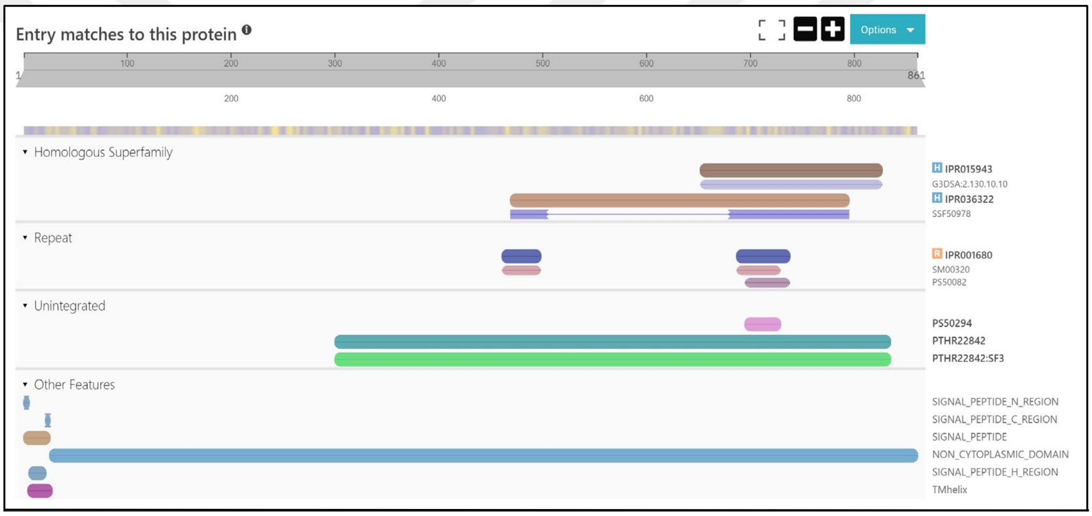


Figure 8.1 *L. donovani* A0A504XLH3_Uncharacterized protein (861aa) sequence alignments with ortholog sequences and protein domain properties. a. Ortholog sequences were aligned in the TriTrypDB (Kinetoplast Informatics Resources). b. Protein domain regions retrieved from Interpro.

APPENDIX 4

PENTAMIDINE RESISTANCE GENE 1

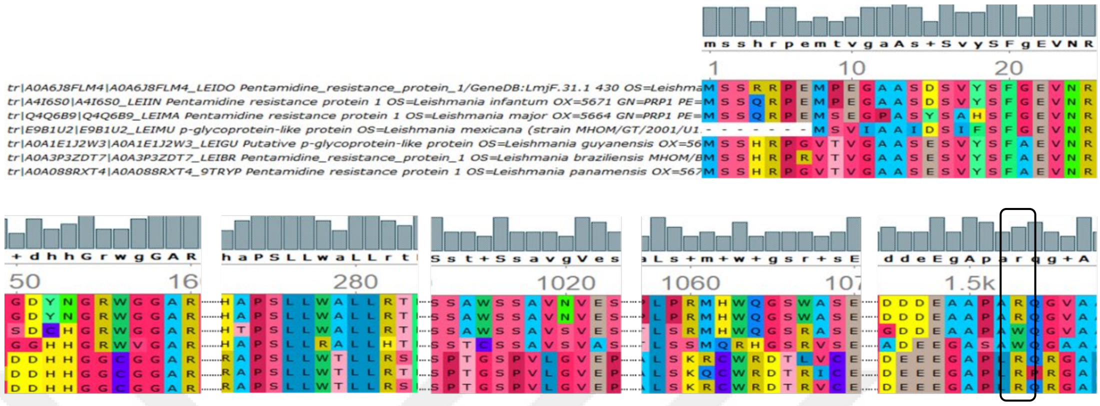


Figure 8.2 Pentamidine resistance gene in *Leishmania* strains

APPENDIX 5

CBA KIT REPRESENTATIVE ANALYSIS

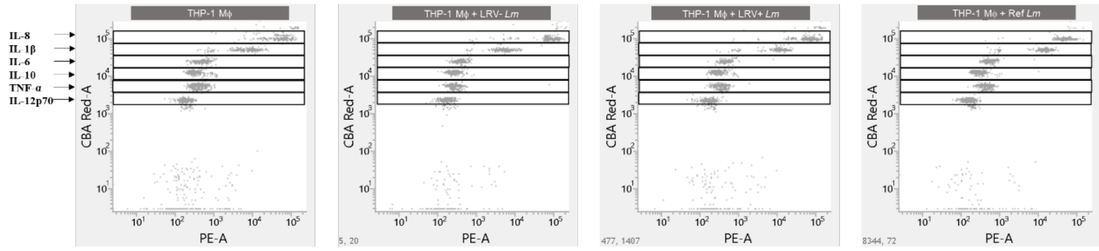


Figure 8.3 Representative image of LRV+, LRV-, reference *L. major* infected or uninfected macrophage cells culture media CBA inflammatory cytokine data.

Table 8.8 PE-Median values of a single experiment derived from CBA inflammatory kit (*Mϕ: Macrophage)

	IL-8	IL-1β	IL-6	IL-10	TNF-α	IL-12p70
THP-1 Mϕ*	68099	9775	543	349	480	205
THP-1 Mϕ + LRV- *Lm*	91573	6106	410	296	295	197
THP-1 Mϕ + LRV- *Lm*	79916	13190	498	310	408	197
THP-1 Mϕ + Ref *Lm*	52827	16547	576	331	491	191

9 CURRICULUM VITAE