



Efficacy of the Imiquiline analog EAPB0503 against Cutaneous Leishmaniasis: A promising new treatment paradigm

Rana El Hajj

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THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

En Microbiologie, Maladies transmissibles et hygiène

École doctorale Sciences chimiques et biologiques pour la santé (CBS2)
Unité de recherche Institut des Biomolécules Max Mousseron IBMM UMR 5247, Université de Montpellier

*EFFICACITE D'UN ANALOGUE D'IMIQUALINES, L'EAPB0503: UN
NOUVEAU TRAITEMENT PROMETTEUR CONTRE LA LEISHMANIOSE
CUTANEE*

**Présentée par Rana EL HAJJ
Le 22 juin 2018**

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DE MONTPELLIER**

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LIST OF ABBREVIATIONS

ACL	Anthroponotic Leishmaniasis	Cutaneous	GPI	Glycosylphosphatidyl-inositol
AP	Alternative Pathway		GP63	Glycoprotein 63
C2	Complement Component 2		GSPL	Glyco-sphingophospholipid
C3	Complement Component 3		H&E	Hematoxylin and eosin
C3b	Cleaved Product of C3		HRP	Horseradish peroxidase
C4	Complement Component 4		HTLV-I	human T-lymphotropic virus type I
C5	Complement Component 5		iC3b	The proteolytically Inactive product of the Complement Cleavage fragment C3b
C5b-C9	The assembly of complement plasma glycoproteins C5b, C6, C7, C8 and polymeric C9 as a group on biological membranes		IFN γ	Interferon gamma
°C	Celsius Degrees		IKK	IKb Kinase
CD	Cluster of differentiation		IC50	Inhibitory Concentration 50
CL	Cutaneous Leishmaniasis		IL	Interleukin
CLRs	C-type lectin like receptors		iNOS	inducible Nitric Oxide Synthase
CR	Cure rate		IRB	Institutional Review Board
Ct	Threshold cycle		ITS1	Internal transcribed spacer-1
DALY	Disability-adjusted life year		kDNA	Kinetoplast DNA
DC	Dendritic cells		L.	<i>Leishmania</i>
DMSO	Dimethylsulfoxide		LD50	Lethal dose 50
DNA	Deoxyribonucleic acid		LPG	Lipophosphoglycans
DsRNA	Double stranded RNA		LPS	Lipopolysaccharide
ECG	Electrocardiography		MAC	Membrane Attack Complex
ELISA	Enzyme-linked immunosorbent assay		MASPs	MBL-associated serine proteases

FBS	Fetal Bovine Serum	MBL	Mannose Binding Lectin
FDA	Food and Drugs Administration	MCP-1	Monocyte Chemoattractant Protein-1
FFPE	Formalin-Fixed Paraffin Embedded	MIP	Macrophage Inflammatory Proteins
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	MyD88	Myeloid Differentiation primary response 88
GIPLs	glycoinositol phospholipids	NHS	Normal Human Serum
NK	Natural Killer	TLRs	Toll-like receptors
NLRP3	NOD-Like-Receptors Protein 3	TNF	Tumor necrosis factor
NO	Nitric oxide	Ub	Ubiquitylation
NWCL	New World Cutaneous Leishmaniasis	WHO	World Health Organization
O.D	Optic density	WT	Wild Type
OWCL	Old World Cutaneous Leishmaniasis	ZCL	Zoonotic Cutaneous Leishmaniasis
P	Phosphorylation	Th-2	T- helper 2
PBS	Phosphate Buffered saline	Th-17	T-helper 17
PCR	Polymerase Chain Reaction	PRRs	Pattern Recognition Receptors
P8GLC	Proteoglycolipid complex	qRT-PCR	Quantitative Real time PCR
PMA	phorbol 12-myristate 13-acetate	RANK	Receptor Activator of NF- κ B
PPG	Proteophosphoglycan	rDNA	Ribosomal DNA
RNA	Ribonucleic acid	RFLP	Restriction-Fragment-Length Polymorphism
ROI	Reactive Oxygen Intermediates	ROS	Reactive Oxygen Species
ssRNA	Single stranded RNA	SAP	Secretary Acids Phosphatases
TGF- β	Tumor Growth Factor β	SD	Standard Deviation
Th-1	T- helper 1		

Résumé

La leishmaniose cutanée (LC) est une infection parasitaire classifiée par l'Organisation de Santé Mondiale (WHO) comme étant une des maladies tropicales négligées non-contrôlées. Dans la région du Moyen Orient, la LC est généralement endémique en Syrie et elle est causée principalement par *Leishmania tropica* et *Leishmania major*. La LC a été récemment introduite à des pays non endémiques, suite au déplacement intense des réfugiés Syriens échappant à la crise. Les interventions thérapeutiques contre la LC incluent des traitements locaux, systémiques et physiques. En revanche, le risque élevé de sélection et de résistance des parasites aux traitements actuels suscitent une quête sérieuse, pour trouver de nouvelles approches thérapeutiques. L'Imiquimod est un composé immunomodulateur approuvé pour utilisation clinique, et présente une efficacité vis-à-vis de certaines espèces de *Leishmania*. Dans cette étude, notre intérêt s'est focalisé sur l'efficacité d'un analogue de l'Imiquimod, l'EAPB0503, contre les stades promastigotes et amastigotes de *L.tropica* et *L.major*.

Nos résultats montrent que l'Imiquimod et particulièrement l'EAPB0503 affectent les deux espèces. L'Imiquimod affecte majoritairement la motilité des promastigotes des deux espèces, alors que l'EAPB0503 affecte la motilité des promastigotes de *L.major* mais surtout l'invasion des promastigotes de *L.tropica* dans les macrophages. Les deux composés réduisent la réplication des amastigotes, avec un effet plus prominent de l'EAPB0503. Cet effet est médié par l'augmentation de l'expression du récepteur toll-Like-7 (TLR7), particulièrement pour l'Imiquimod et d'une manière moins importante pour l'EAPB0503. Les deux composés induisent l'activation de la voie de signalisation canonique de NF- κ B. Ceci conduit à une production des cytokines pro-inflammatoires, et une diminution des cytokines anti-inflammatoires expliquant l'activité leishmanicide des deux composés. L'EAPB0503 semble agir *via* un autre TLR que l'imiquimod,

comme il induit une expression plus élevée des transcrits TLR8 et TLR9, conférant une protection contre l'infection.

Collectivement, nos résultats montrent l'effet de l'Imiquimod contre l'espèce la plus agressive, *L. tropica*, et souligne l'activité plus puissante de l'EAPB0503 contre les deux espèces. De plus, cette étude montre le mécanisme d'action de ces deux composés, qui vraisemblablement activent des TLRs différents, mais finissent par induire la voie NF- κ B et la réponse immunitaire correspondante. Ces résultats soulignent l'importance des drogues immuno-modulatrices contre la LC et ouvrent des perspectives sur des études précliniques puis cliniques de ces composés.

Mots clés

Leishmaniose cutanée, *Leishmania tropica*, *Leishmania major*, Imiquimod, EAPB0503, Moyen Orient.

ABSTRACT

Cutaneous Leishmaniasis (CL) is a parasitic infection classified by the WHO as one of the most uncontrolled spreading neglected diseases. In the Middle East Region, CL is mostly endemic in Syria where it is mainly caused by *Leishmania tropica* and *Leishmania major*. CL has been lately introduced to under endemic countries, following the large-scale displacement of refugees from Syria fleeing the crisis. Therapeutic interventions against CL include local, systemic and physical treatments. However, the high risk for selection and spread of drug-resistant parasites is high; consequently, new therapeutic approaches are still needed. Imiquimod is an FDA approved immunomodulatory compound with a tested efficacy against some *leishmania* species. In this study, our interest was to investigate the efficacy of an Imiquimod analog, EAPB0503 in comparison to the original compound, against promastigote and amastigote stages of *L.tropica* and *L.major*.

We showed that Imiquimod affects the motility of promastigotes of both strains. EAPB0503 affected *L. major* promastigotes' motility and impaired the invasion of *L.tropica* promastigotes into macrophages. Both drugs reduced amastigote replication, with a higher potency of EAPB0503. This activity is due to the upregulation of Toll-Like Receptor-7 (TLR7), mainly by Imiquimod, and to a lesser extent by EAPB0503. Importantly, both drugs activated the NF- κ B canonical pathway leading to production of pro-inflammatory cytokines and upregulation of i-NOS levels. A decrease of anti-inflammatory cytokines secretion was obtained, explaining the leishmanicidal activity of both drugs. Importantly, EAPB0503 led to a prominent increase in TLR8 and TLR9 transcripts, presumably conferring protection against the infection.

Collectively, our findings show the effect of Imiquimod against both strains especially, the aggressive *L.tropica* strain. We also show that EAPB0503 displays a more potent *in vitro*

leishmanicidal activity than Imiquimod. These drugs seemingly activate different TLRs, but both activate the canonical NF- κ B pathway and its subsequent mediated immune response. These results highlight the promising effect of immunomodulatory drugs against CL and warrant an in depth *in vivo* preclinical then clinical studies of these compounds.

Keywords:

Cutaneous leishmaniasis, *Leishmania tropica*, *Leishmania major*, Imiquimod, EAPB0503, Middle East.

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Chapter 1

Introduction

1.1- Leishmaniasis

1.1.1- Definition

Leishmaniasis is a vector-born parasitic disease, caused by a flagellated protozoan parasite of the genus *Leishmania* [1]. It is transmitted to humans following the bite of an infected female *Phlebotomus* sandfly. This infection is considered as a significant cause of morbidity and mortality in several countries [2], and it is categorized as one of the Neglected Tropical Diseases (NTD) distributed worldwide [3]. Many of the current disability-adjusted life year (DALYs) for neglected tropical diseases is underestimated because they do not consider the long-term chronic disabilities. However, leishmaniasis is still one of the major leading-to-death parasitic diseases (Table 1).

Table 1: Estimates of death and disability-adjusted life year (DALYs) for the neglected tropical diseases [3].

Variable	Deaths	DALYs ^a
Schistosomiasis	280,000 ^b	4.5 million ^c
Hookworm infection	65,000 ^c	22.1 million ^{c,d}
Ascariasis	60,000 ^c	10.5 million ^{c,d}
Leishmaniasis	51,000 ^e	2.1 million ^f
Trypanosomiasis	48,000 ^c	1.5 million ^f
Chagas disease	14,000 ^c	0.7 million ^f
Trichuriasis	10,000 ^b	6.4 million ^{c,d}
Leprosy	6,000 ^c	0.2 million ^f
Lymphatic filariasis	0 ^c	5.8 million ^f
Trachoma	0 ^c	2.3 million ^f
Onchocerciasis	0 ^c	0.5 million ^f
Dracunculiasis	ND	< 0.1 million ^f
Buruli ulcer	ND	ND
Taeniasis and cysticercosis	ND	ND
Food-borne trematodiasis	ND	ND
Total	534,000	56.6 million

There are over than 20 *leishmania* species causing leishmaniasis [4]. These species are responsible of three forms of the disease [4]: the visceral form, also known as Kala-Azar, the cutaneous form, a chronic but not life-threatening disease, and the mucocutaneous form, which begins by cutaneous lesions that alter later mucosal cavities [5].

Visceral leishmaniasis (VL) is the most aggressive form of the disease. The geographical distribution of the strains causing this infection is variable and distributed in the tropics and subtropics of Asia and Africa, as well as in the drier parts of Latin America and the Mediterranean climate regions [6, 7]. VL occurs mostly in children but it is also highly reported in adults with a prevalence of 500 000 death every year [8, 9, 10]. In this form of the disease, the parasite migrates to the internal organs of the patient, causes hepatosplenomegaly and ascites release in the peritoneal cavity. In addition to these symptoms, patients mainly present weight loss, chronic fever, bleeding and swollen lymph nodes (Figure 1A) [11]. Furthermore, immunocompromised patients present with the most aggressive complications when affected with this infection [12]. In most cases, VL is lethal without a proper treatment.

The second form of the infection is Cutaneous Leishmaniasis (CL). Skin lesions that appear on the bite site characterize this form. These lesions evolve to erythematous papules, may persist and enlarge into a nodule-plaque over a period of 4–12 weeks. The incubation period varies from 1 week to several months (average 1 week–3 months). Most CL lesions heal spontaneously (Figure 1B) [13]. Approximately, one million cases of CL have been reported in the last 5 years and 310 million people are at risk [14].

Mucocutaneous leishmaniasis (MCL) is the third form of the disease, and it is mainly found in Latin America [15]. MCL is characterized by the destruction of oral–nasal and pharyngeal cavities. The infection begins by skins lesions with the capability of the parasite to migrate to the

mucosal cavities if left untreated (Figure 1C). The initial symptoms are mild, with nasal inflammation and stuffiness, but ulceration and perforation of the septum may also occur. The lesion may extend to the face, soft palate, pharynx or larynx [16].



Figure 1. Different forms of Leishmaniasis: visceral leishmaniasis (A), cutaneous leishmaniasis (B), and mucocutaneous leishmaniasis (C).

1.1.2- Epidemiology of leishmaniasis and different *Leishmania* species

Leishmaniasis is categorized as the third most parasitic disease spread worldwide, after malaria and filariasis [17]. It is endemic in more than 98 countries distributed over the 5 continents (Figure 2). The distribution of leishmaniasis is directly linked to poverty, malnutrition, mass migration, lack of sanitation and control in the endemic regions [18]. Its prevalence reaches 12 million infected people with 367 million persons at risk [14]. It is mostly prevalent in Latin America, Africa, Asia, the Middle East and the Mediterranean basin [19]. Moreover, 1.5 to 2 million new cases of leishmaniasis are reported every year [20].



Figure 2: Geographical distribution and incidence of leishmaniasis worldwide [21].

Among many classifications of the disease, the concept of the geographical distribution of species causing the infection remains one of the most common and efficient ways of classifying its epidemiology. Leishmaniasis is classified into the Old World and the New World distributed disease (Table 2). Old World leishmaniasis refers to its endemicity in the old continents regrouping Asia, Europe and Africa, whereas New World Leishmaniasis refers to its distribution in the new continent of America [22, 23]. *L. donovani*, *L. chagasi* (New World) and *L. infantum* (Old World) are known to cause VL (Table 2) that leads to death if untreated [24]. *L. major*, *L. tropica* cause CL in the Old World, especially in Syria, Iran, Morocco, Yemen and Algeria [25, 26]. *L. mexicana*, *L. amazonensis*, *L. guyanensis*, *L. panamensis* and *L. braziliensis* cause CL in the New World especially in Central and South America (Table 2) [27].

The Mucocutaneous form of the disease is only found in the New World, more precisely in South Latin America and is mainly caused by *L. braziliensis* and less frequently by *L. guyanensis* and *L. panamensis* (Table 2) [27].

Table 2: Different species causing leishmaniasis, types of leishmaniasis and their geographical distribution.

<i>Leishmania</i> species	Disease	Epidemiology	Endemic countries	References
<i>L. tropica</i> <i>L. major</i> <i>L. infantum</i>	OWCL	Middle East, Mediterranean basin, Africa, America	Syria, Saudi Arabia, Iraq, Afghanistan, Iran, Morocco	[25], [26]
<i>L. aethiopica</i>	OWCL	Africa	Ethiopia	[28]
<i>L. mexicana</i> <i>L. braziliensis</i> <i>L. peruviana</i> <i>L. guyanensis</i> <i>L. amazonensis</i> <i>L. panamensis</i> <i>L. venezuelensis</i>	NWCL	North, South and Central America	Rio de Janeiro, Brazil, Mexico,	[29], [30]
<i>L. infantum</i> <i>L. chagasi</i> <i>L. donovani</i>	VL	Mediterranean, South America	Bangladesh, Ethiopia, India, Nepal, Sudan Brazil	[31], [32]
<i>L. braziliensis</i> <i>L. panamensis</i> <i>L. guyanensis</i>	MCL	South, Latin America	Paraguay, Ecuador, Peru, Colombia, Venezuela	[33]

1.1.3- *Leishmania* Taxonomy

Phylogenetic analysis for *Leishmania* reveals that all species of this parasite have the same taxonomy and parenteral relationship [34].

Kingdom	Protista
Subkingdom	Protozoa
Phylum	Sarcomastigophora
Subphylum	Mastigophora
Class	Zoomastigophora
Order	Kinetoplastida

Family	Trypanosomatidae
Section	Salivaria
Genus	<i>Leishmania</i>
Species	<i>Donovani, tropica, mexicana, braziliensis...</i>

1.1.4- Life cycle of *Leishmania*

Despite the difference in the geographical distribution caused by different *Leishmania* spp, all species share the same life cycle occurring between an infected female *Phlebotoma* sandfly and a mammal. Different factors depending on the parasite, and on the host, determine the spectrum and the outcome of the disease.

The cycle begins when infected female *Phlebotoma* sandflies take a blood meal and inject the promastigote infective stage from their proboscis. Motile flagellated promastigotes released in the blood get phagocytosed by reticulo-endothelial cells, mostly macrophages and other types of mononuclear cells such as dendritic cells. Once internalized, promastigotes lose their flagellum and transform into non-motile intracellular amastigotes. These stages replicate by binary fission, eventually rupturing the macrophage and spreading to uninfected cells [35, 36].

Sandflies become infected by ingesting amastigote infected reticulo-endothelial cells upon a blood meal [36]. In sandflies, amastigotes transform back into procyclic promastigotes in the midgut, and then migrate to the proboscis, where they transform into infective metacyclic promastigotes (Figure 3). The decrease in temperature and increase in pH triggers the development of the parasite stages within the vector [37, 38].

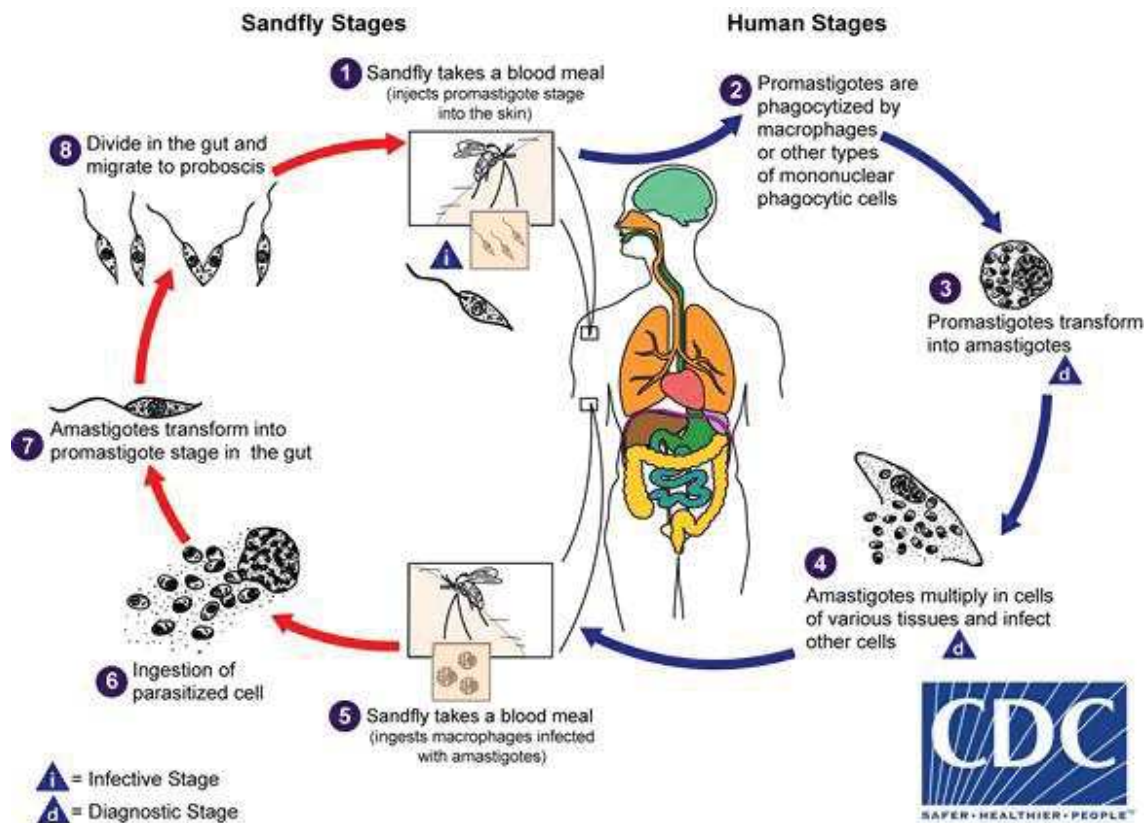


Figure 3: *Leishmania* spp life cycle: *Leishmania* spp present a digenetic life cycle, composed of a mammal and a phlebotomine sandfly. 1-The life cycle begins when an infected sandfly injects the flagellated promastigotes in the skin of the human. 2-Promastigotes are then phagocytized by macrophages. 3- They will be transformed into non flagellated amastigotes. 4- Amastigotes will divide and infect other cells. 5- A second phlebotoma takes a blood meal from the infected host. 6-The sandfly ingests macrophages containing amastigotes. 7- Amastigotes transform back into procyclic promastigotes in the sandfly gut. 8- They will divide in the gut and migrate to proboscis, where they will become infective metacyclic promastigotes ready to be injected during a second blood meal into a new host [26, 27].

1.1.5- *Leishmania* morphology

Leishmania species are generally differentiated based on geographical, biological and clinical features. All species share a similar morphology in both their promastigote and amastigote stages. They are composed of a flagellum, kinetoplast, a mitochondrion, Golgi, nucleus surrounded by a cytoskeleton.

Promastigotes represent the flagellar stage of the parasite. They are spindle shaped, and they measure 15-20 micrometers in length, 1-2 micrometers in width with a small nucleus and a free flagellum. Their kinetoplast is located in front of the nucleus near the anterior end of the body. A small ring of vacuolated cytoplasm (Figure 4A, C) surrounds the nucleus and the anterior kinetoplast.

Amastigotes measure 2-3 micrometers, have a round or oval shape, with a large nucleus and kinetoplast. These forms are the non-flagellated and non-motile stages of the parasite [39] with the base of the flagellum still present. The kinetoplast is usually detectable as a darkly staining body near the nucleus. This form is the intracellular stage (Figure 4B, C) replicating within immune cells, mainly macrophages and monocytes.

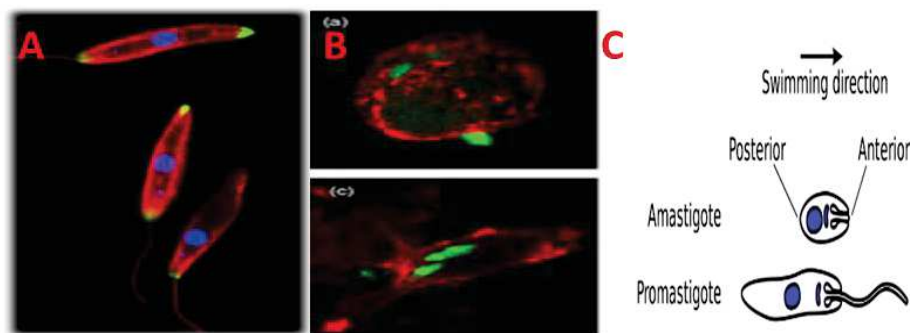


Figure 4: (A) Promastigote stage of *Leishmania spp* having the cytoplasm stained in red, nucleus in blue. (B) Amastigote stage of *Leishmania spp* stained in green inside macrophages. (C) Morphological structure of *Leishmania* promastigotes and amastigotes.

1.1.6- Kinetoplast

Leishmania species belong to the order of kinetoplastidae. The members of this order exhibit several morphological differences characterized by the position of the kinetoplast. The kinetoplast is defined as a network of concatenated circular DNA molecules and their associated structural proteins along with DNA and RNA polymerases, in a specific portion of the protozoan mitochondria [40]. The size of the kinetoplast is variable according to the species. It is constituted

of two types of circular DNA: minicircles and maxicircles. There are thousands of minicircles whose size ranges between 0.5 to 2.5 kilobases among species. They present high heterogeneity, but have conserved regions as replication origin sites. Maxicircles are few dozen, with a size from 20 to 40 kb, and are functionally and structurally analogous to mitochondrial DNA. *Leishmania* kinetoplast has been used as a marker to detect amastigotes in clinical specimens but also in basic research [41].

1.2- Cutaneous leishmaniasis

1.2.1- Definition

Cutaneous leishmaniasis is also known as oriental sore, tropical sore, or Aleppo boil. According to the World Health Organization (WHO), it is defined as “the most common form of leishmaniasis. It usually produces ulcers on the exposed parts of the body, such as the face, arms and legs, where a large number of lesions – sometimes up to 200 –can cause serious disability [42]. When the ulcers heal, they invariably leave permanent scars, which are often the cause of serious social prejudice”. Skin infections commonly associate with secondary infections [43] and CL is endemic in many developing countries [44].

1.2.2- Epidemiology of *Leishmania* species causing CL

A wide range of *Leishmania* species spread in both the Old and New Worlds (Table 2) causes CL. OWCL is present in many endemic areas in North Africa, the Mediterranean Basin, the Middle East, the Indian subcontinent and Central Asia [45]. OWCL is mostly caused by *L.major* and *L.tropica* (Table 2), which will be the focus of our study. Nonetheless, *L.infantum*

and *L.donovani* can also cause localized CL and can be observed less frequently in the Mediterranean areas [30]. *L.aethiopica* can also cause CL in a diffuse manifestation, characterized by diffusion of lesions, great abundance of parasites. This form is very uncommon and is seen in Africa [46] (Table 2). In contrast, NWCL is mainly found in Central and North America and is mainly caused by *L.mexicana*, *L.brazilensis* (Table 2) [47].

1.2.3- Types and transmission of OWCL

Sandflies are the main vectors transmitting leishmaniasis. Similar to the difference of *Leishmania* species in the New and the Old Worlds, different species of sandflies are reported. For instance, in the Old World, Sandflies from the genus *Phlebotoma* are the exclusive vectors of *Leishmania* whereas, sandflies from the genus *Lutzomya* are the vector for CL in the New World (figure 5) [48]. In these vectors, *Leishmania* express various "virulence factors" which may facilitate transmission to and infection of the mammalian host [49].

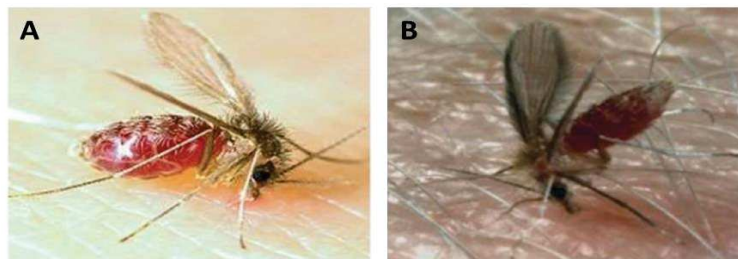


Figure 5. CL transmission vector: (A) *Phlebotoma* sandfly transmitting the OWCL and (B) *Lutzomya* transmitting the NWCL [48].

Since OWCL will be our focus, we will expand in the following section on its transmission. Although the main vector of OWCL is the *Phlebotoma* sandfly, it has been fully documented that the disease can be transmitted from animals to humans defining the zoonotic cutaneous leishmaniasis (ZCL) or from a person to another person defining the anthroponotic cutaneous leishmaniasis (ACL).

- Zoonotic cutaneous leishmaniasis (ZCL): occurs when the parasite is transmitted from a range of animals to humans. It is seen in rural areas and is geographically distributed in the Middle East, Northwestern China and North Africa. ZCL is mostly caused by *L.major* and often heals spontaneously in about two to four months, although in some cases, it may persist for as long as five years [50] (Table 3).

- Anthroponotic cutaneous leishmaniasis (ACL): occurs when the parasite is transmitted from a person to person. It is an urban disease and is geographically distributed in the Middle East, the Indian Subcontinent, and Western Asia. ACL is mostly caused by *L.tropica*. Lesions may persist for 6 to 15 months before healing with significant scarring [51] (Table 3).

Table 3: Types of Old World Cutaneous Leishmaniasis according to species and transmission

Types of CL	Transmitted by	Parasite	Geographic distribution	Healing period
Zoonotic	Animals to humans	<i>L.major</i>	Middle East, Northwestern China, North Africa	2 to 4 months
Anthroponotic	Humans to Humans	<i>L.tropica</i>	Middle East, Indian Subcontinent, Western Asia	6 to 15 months

1.2.4- Factors favoring CL transmission

Climate and other environmental changes have the potential to expand the geographic range of the sandfly vectors thus the areas of incidence of CL [52, 53]. The transmission of this infection is mainly favored by disrupted insecticide control, poor water and sanitation services, which create a ripe breeding ground for sandflies. Poor health systems make the treatment difficult to reach. Moreover, war and political instability as well as refugees fleeing conflict take the disease into non-endemic areas [54, 55, 56].

1.2.5- Cutaneous leishmaniasis in the Middle East Region

In the Middle East area, Syria is characterized by the highest estimated case counts of CL [57]. In the North of Syria, especially in Aleppo, CL belongs to the anthroponotic type and is mainly caused by *L.tropica*. In the suburbs of the capital Damascus, CL belongs to the zoonotic type and is due to *L.major* [58]. Moreover, some reports showed that *L.infantum* was associated with CL in the Syrian Mediterranean area [57, 59, 60].

After the war, Syrian Ministry of Health revealed shocking statistics of CL outbreaks in Syria and its neighborhood. According to the Leishmaniasis Center in Aleppo, 22 365 cases were reported in Syria before the politic crisis, and infections reported to the Ministry of Health doubled from 23 000 before the war to 41 000 in 2013 [61]. In 2012, a CL outbreak was observed among 948 Syrian refugees (Figure 6). The mostly isolated strains were *L.tropica* and *L.major*, occurring in 85% and 15% of patients, respectively [62]. This significant increase in the disease burden among refugees is mostly attributed to inadequate sanitation, poor hygiene and housing, malnutrition, and absence of clean water [63]. Thus, CL remains an alarming category of emerging and uncontrolled disease. Intensified research programs to improve vector control, diagnosis, and therapeutics are therefore badly needed.

Furthermore, neighboring countries which were non-endemic for CL and which have received millions of Syrian refugees also reported large numbers of cases [62, 64]. For instance, Turkey and Jordan have reported hundreds of cases [65, 66]. A similar situation was reported in Eastern Libya with evidence supporting the spread of the disease in refugee camps to neighboring Tunisia [67].

In Yemen, 10,000 new cases are roughly reported every year [25]. In this country, the number may be significantly underestimated because surveillance systems are badly affected or even absent due to the current conflict.

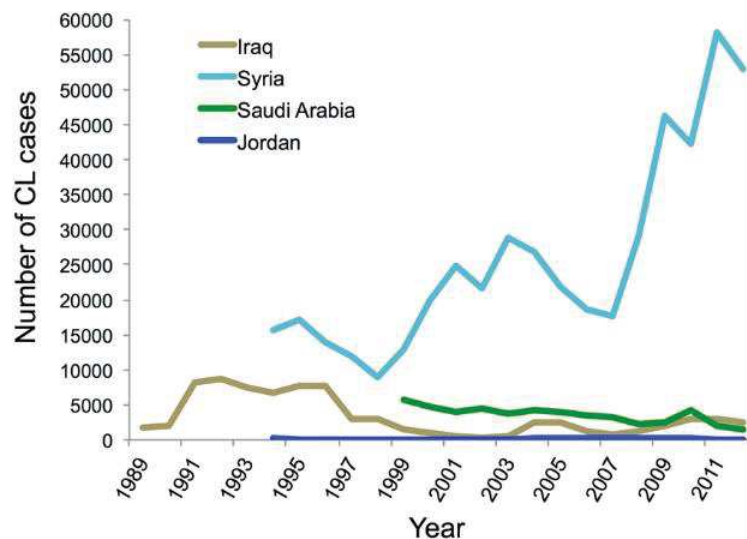


Figure 6: Year-wise trend of CL cases reported in Middle East [63]

Similarly, in Saudi Arabia where CL is present and affects males and females equally [68], witnessed an increase in CL number following the Syrian war. The species causing the disease in Saudi Arabia are *L.major* in central and eastern provinces and *L.tropica* in west and southwest provinces.

1.2.6- Clinical manifestations of CL

Cutaneous Leishmaniasis is categorized into three different clinical forms:

1.2.6.1- Localized

In the localized form, the parasite is confined to the skin (Figure 7). After an incubation period of 1 to 12 weeks, a papule or bump develops at the site of the insect bite. The papule grows and turns into an ulcer. Most people with CL have one or two lesions varying in size from 0.5 to

3 cm in diameter, usually on exposed parts of the body such as the face, arms or legs. Scarring of Leishmaniasis is typical with a depigmented center and a pigmented border [69].



Figure 7: Lesion form a patient with localized cutaneous leishmaniasis [70].

1.2.6.2- Recidivans

This form appears in around 5% of CL patients infected with *L.tropica* and is characterized by microsatellite and confluent lesions that relapse and finally ulcerate in the border of previous scars [71] (Figure 8). Leishmaniasis recidivans may occur decades after the resolution and healing of the primary lesion [72]. This stage of CL infection requires treatment often with two modalities of therapy [73].



Figure 8: Lesions form a patient with recidivans cutaneous leishmaniasis [74].

1.2.6.3- Diffuse

It affects only the skin but with generalized skin lesions (Figure 9). It is seen mainly in Africa and is caused by *L.aethiopica* [75]. Post kala-azar dermal leishmaniasis is a form of diffuse CL and a sequel of visceral leishmaniasis that may appear in affected individuals up to 20 years after being partially treated, untreated or in those considered adequately treated [76].



Figure 9: Lesions from a patient with diffuse cutaneous leishmaniasis [70].

Some lesions of CL heal within a few months, leaving a scar; others are more persistent and need a systemic or intralesional treatment. The natural history depends primarily on the species of *Leishmania* [77].

1.2.7- Diagnosis of CL

Clinical manifestations of CL depend on the species, the genetic, immunological and cultural background of the patient. Diagnosis of CL can be made using the traditional diagnostic techniques of smear, parasite culture and histological analysis of skin biopsies. Thus, modern molecular diagnostic techniques, mainly the polymerase chain reaction test (PCR), appear to be

the most sensitive single diagnostic test for species identification in skin samples. The diagnosis is often made based on a clinically typical lesion in conjunction with an appropriate history of exposure. It is achieved through an association of clinical, epidemiological and laboratory characteristics. Several methods have been used for the diagnosis of leishmaniasis including direct investigation and serological tests [78].

1.2.7.1- Smear

Parasite isolation is performed on material obtained from scratches from the lesion margins, using a sterile surgical blade. Smears are then bleached using May-Grünwald-Giemsa stain (Figure 10) to identify amastigotes forms by means of optical microscopy with sensitivity rate ranging from 64% to 80% depending on technique quality [79]. The specificity of the dermal smear is excellent (100%).

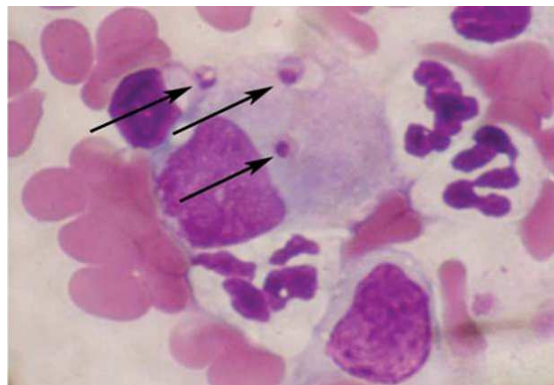


Figure 10: smear showing extracellular and intracellular amastigotes from a lesion aspirate from a feline infected with leishmanial [80].

1.2.7.2- Culture

The parasite can be isolated in appropriate media and incubated at 28°C from a tissue fragment removed from the border of an active lesion. Positivity of the culture varies depending on the presence of amastigotes in the smear. Culture methods generate promastigote stage of

Leishmania. These promastigotes can be grown in blood agar, Schneider's medium or RPMI [81]. Specificity is about 100%, but sensitivity rate only reaches 84% [82].

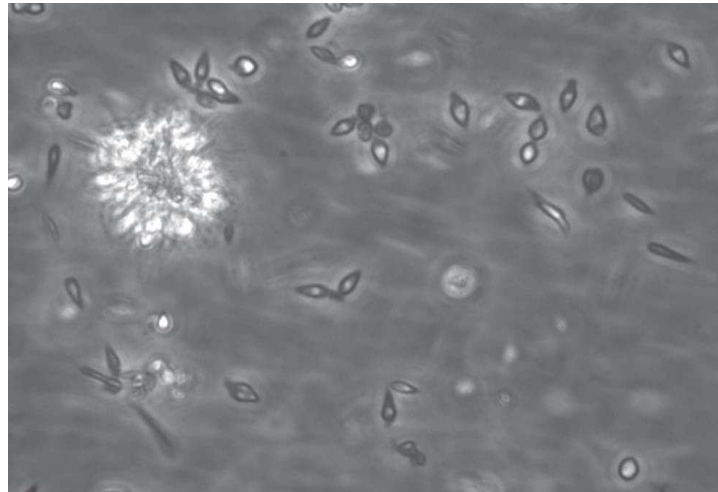


Figure 11: Promastigotes of *Leishmania tropica* after culture in RPMI media (photo captured in our lab)

1.2.7.3- Polymerase Chain Reaction (PCR) and Restriction-fragment-length polymorphism (RFLP)

Although a standardized, commercially available PCR assay for *Leishmania* diagnosis is still unavailable, it is well accepted that PCR can be applied to different clinical samples to obtain higher sensitivity than conventional methods for the diagnosis of CL [83]. Indeed, the characterization of *Leishmania* species is based on biochemical criteria (electrophoresis of isoenzymes) or genetic criteria using various molecular methods including PCR and monoclonal antibody techniques. Those techniques are only used in sophisticated centers because of their high cost.

Based on distinct loci of kinetoplast (kDNA), or ribosomal (rDNA), and others, species identification and parasites quantification have been successful. For instance, in an Iranian study,

PCR-RFLP (Restriction Fragment Length Polymorphism) assay with materials punctured from CL patients using *HaeIII* enzyme was useful for the rapid identification of *Leishmania* [84].

In addition to the regular PCR, Real-time PCR was also used for *Leishmania* detection and it allows the consistent quantification of parasite DNA with an analytical sensitivity of around one femto-gram, which corresponds to 0.01 parasites per reaction [65]. Using quantification by the real-time PCR of 7SL RNA gene expression levels, Romero et al. provided proof that parasites are present in extralesional sites (including normal skin, tonsils, and blood monocytes) of patients with dermal leishmaniasis [85].

More recently, in our lab, a diagnostic molecular method was implemented for speciation and differentiation in the context of CL. This method reliably yielded high quality and quantity of parasitic DNA used for the identification of infection in less than 72h from formalin-fixed paraffin-embedded (FFPE) skin biopsies using the ribosomal Internal transcribed spacer-1 (ITS1)-PCR on DNA extracted from FFPE punch biopsies (Figure 12) [86].

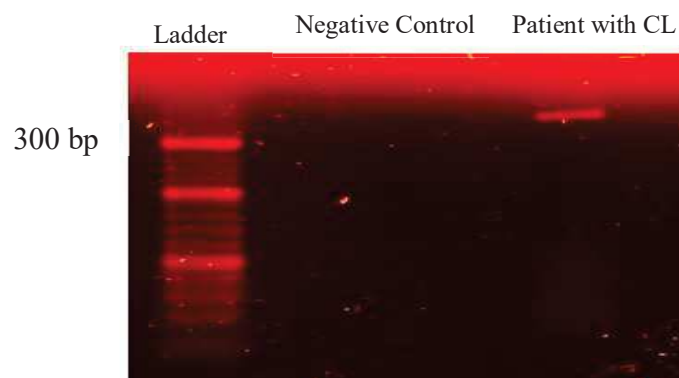


Figure 12: Ribosomal Internal transcribed spacer-1 (ITS1)-PCR for a CL patient showing a band of 300 bp indicating positivity to the infection (PCR performed in our lab).

1.2.7.4- Histology

The histopathological presentation of CL shows a great variability, but a predominant pattern is characterized by the presence of unorganized granuloma without necrosis. CL displays

a wide spectrum of microscopic manifestations and *Leishmania* organisms are typically intensely blue with Giemsa stain (Figure 13).

According to Ridley's classification, CL microscopic manifestations of 147 patients from four geographical areas, Africa/Asia, Ethiopia, Central America and Brazil were studied. These patients were categorized according to the host tolerance of the parasite, the lysis of macrophages and the damage to connective tissue into five groups as I-unreactive, II-reactive, III-infiltrative, IV-tuberculoid and V-hypersensitive (Table 4). In Groups I and II, the histological designations labeled only the changes in the connective tissue of the dermis, since no significant cellular response was demonstrated. In Groups III, IV and V, these changes were present to a different degree in and around the cellular lesion. They consist of fibrinoid change or necrosis with altered staining reaction, edema of collagen, fibrocytosis or fibroblast proliferation, often associated with some cellular infiltration of the epidermis [87].

Table 4: Ridley's classification for mucocutaneous and cutaneous leishmaniasis.

Group I	Group II	Group III	Group IV	Group V
Approximately normal dermis	Necrosis in the dermis	Dermis infiltration with plasma cells, eosinophils and macrophages	Abundance of lymphocytes and some plasma cells	Granuloma with developed epithelioid cells
Patches of collagen degeneration	Fibrocytosis	Presence of giant cells with a predomination of plasma cells	Scattered laghans giant cells.	Globally no necrosis and fibrocytosis
Absence of inflammation and granuloma	Chronic but no large inflammatory cells	Vasculitis in the superficial epidermis, no granuloma	Necrosis in the central area of the granuloma with primitive epithelioid granuloma	Heavy fibrosis

In the Middle East Region, Dr Ibrahim Khalifeh and his team established a registry for CL patients from different countries including Iran, Pakistan, Saudi Arabia, and Iraq. The histopathologic features of these patients were classified based on Ridley's Pattern, but modified further for a better classification (Table 5) [88].

Table 5: Modified Ridley's pattern classification [88].

Group	Histopathologic Response
Group I	Normal Appearing skin biopsy with patches of collagen degeneration
Group II	Predominant severe necrotizing process in the dermis
Group II	Dermis involved by a diffuse and heavy mixed inflammatory infiltrate
Group IV	Scattered Langhans giant cells and primitive epithelioid histiocytes
Group V	Well-Formed granulomas and well-developed epithelioid histiocytes

Another classification was also suggested according to a prospective study conducted in Saudi Arabia in 2005. Four distinct groups were individualized. Type A, where macrophages are heavily parasitized and vacuolated with few lymphocytes, correlates with an early immune response or an anergic diffuse CL. Types B and C consist of a mixed inflammatory response with or without necrosis and present the most common types in Old World CL. Finally, type D, characterized by a tuberculoid granuloma with absent or low parasite load, correlates with chronic forms such as lupoid leishmaniasis or the end stage of spontaneous healing [45].

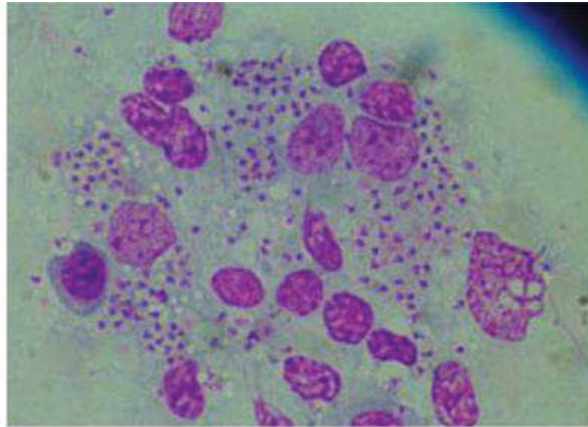


Figure 13: Amastigotes forms of *leishmania spp* by Giemsa stain [89].

1.2.8- *Leishmania* Immunology

The Successful elimination of *Leishmania* depends on the coordinated action of different players of the host immune system. From promastigote entry, into the host bloodstream, to the final amastigote stage, inside mammalian cells, the battle between disease establishment and parasite eradication is partly decided by the ability of *Leishmania* to evade host immunity. Components of the host defense playing a role in the eradication of *Leishmania* include both elements of the innate and adaptive immune system. Therefore, there is an unmet need of a cell-mediated response to resist against this pathogen [90].

1.2.8.1- *Leishmania* Antigenicity

Leishmania parasite presents several surface glycoconjugates that are involved in the survival of the microorganism. Among these molecules, the proteophosphoglycan (PPG), the secretory acids phosphatases (SAPs), the lipophosphoglycan and glycosylphosphatidyl-inositol (GPI) were described [91]. These components may have a major role in the parasite survival process and the progression of the disease within the vertebrate host [92].

Furthermore, the surface of this parasite is covered with molecules that vary depending on its development stage. The procyclic promastigotes are covered with a 7-nm-thick glycocalyx, whereas the infective metacyclic promastigotes are covered with a glycocalyx layer that is at least 17 nm thick. In contrast, this covering is almost absent from the amastigotes, suggesting that the promastigote cell coat is a stage-specific structure that may be lost after phagocytosis and after intracellular transformation [93]. The glycocalyx consists of glycoproteins and related products. The latter components are composed of members of a family of glycoinositol phospholipids (GIPLs) and lipophosphoglycans (LPG) [94, 95]. The whole structure is species specific and constitutes the first antigens to encounter the host's immune system. The two major virulence factors of *Leishmania* are the metalloprotease leishmanolysin GP63 and the glycocalyx component lipophosphoglycan (LPG) [96].

1.2.8.1.1- Glycoprotein 63 (GP63)

Leishmanolysin or GP63 is a glycosylphosphatidyl-inositol (GPI) - anchored glycoprotein expressed on the surface of *Leishmania* parasite [97]. This component plays an important role in the parasite binding process to the host macrophages [98]. GP63 is thought to be able to promote the migration of the parasite and the progression of the disease within tissues by destroying fibronectin and type IV collagen [99]. In addition, this molecule can affect the complement system by transforming C3b into iC3b or by cleaving C3 allowing promastigotes to avoid a complement-mediated degradation [100]. *L. major* parasites lacking these glycocalyx receptors are highly susceptible to complement killing [101]. Genetic deletion of GP63 in *L. major* parasite does not affect its growth in sandflies or culture; however, these promastigotes are highly susceptible to

complement killing and the disease induced by these Gp63^{-/-} *L. major* are delayed in comparison to WT *L. major* [102].

1.2.8.1.2- Lipophosphoglycan (LPG)

Lipophosphoglycan molecules are abundantly present on the surface of all *Leishmania* promastigotes species. They are important for the parasite survival [103]. LPG provides the parasite with the ability to evade the complement system and the digestion by enzymes within the macrophages phagolysosomes [104]. Furthermore, this component impairs the secretion of IL-12 [92].

1.2.8.2- Innate Immunity against CL

Innate immune response is critical in the rapid clearance of invading pathogens and further shaping the adaptive immune responses against them. Upon entry into the mammalian hosts, *Leishmania* parasites first encounter the complement system [105].

1.2.8.2.1- The Complement Cascade

The complement system is constituted of more than 30 soluble proteins in the blood plasma; these are involved in the rapid clearance of invading pathogens from the body. There are three major pathways that can activate the complement system: Classical, Lectin and Alternative pathway (Figure 14). Antibodies binding to the pathogen initiate the classical pathway. However, the binding of Mannose Binding Lectin (MBL) and ficolins activates lectin pathway. In contrast, alternative pathway is directly activated by the pathogens [106].

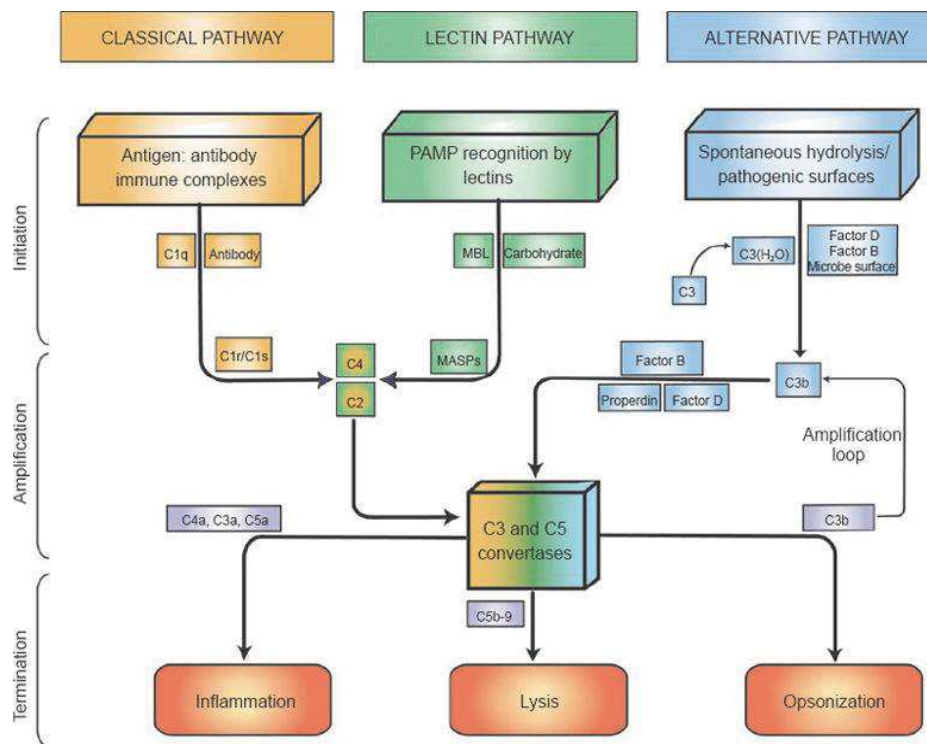


Figure 14: The complement pathway following a pathogen invasion. The Complement pathway can be activated through three pathways: classical, lectin, and alternative. The classical pathway is activated when C1q binds to antibody attached to antigen, activating C1r and C1s, which cleave C4 and C2. The lectin pathway is activated when mannose-binding lectin (MBL) encounters conserved pathogenic carbohydrate motifs, activating the MBL-associated serine proteases (MASPs) and again cleaving C4 and C2. C4 and C2 cleavage products form the classical and lectin pathway C3 convertase, C4bC2a, which cleaves C3 into C3b and C3a. A second molecule of C3b can associate with C4bC2a to form the C5 convertase of the classical and lectin pathways, C4bC2aC3b. The alternative pathway (AP) is activated when C3 undergoes spontaneous hydrolysis and forms the initial AP C3 convertase, C3 (H₂O) Bb, in the presence of Factors B and D, leading to additional C3 cleavage and eventual formation of the AP C3 convertase (C3bBb) and AP C5 convertase (C3bBbC3b). Properdin facilitates AP activation by stabilizing AP convertases. All three pathways culminate in the formation of the convertases, which in turn generate the major effectors of the complement system: anaphylatoxins (C4a/C3a/C5a), the membrane attack complex (MAC), and opsonins (e.g., C3b). Anaphylatoxins are potent proinflammatory molecules derived from the cleavage of C4, C3, and C5. The MAC is a terminal assembly of complement components C5b through C9, which can directly lyse targeted surfaces. C3b induces phagocytosis of opsonized targets and serves to amplify complement activation through the AP [106].

As an obligate intracellular parasite, *Leishmania* needs an intracellular niche to survive and propagate. The complement system is one of the first barriers facing this parasite. Once the complement pathway is activated, C3b facilitates C5b-C9 membrane attack complex (MAC) on

the *Leishmania* surface and lyses the parasite. Conversely, *Leishmania* virulence factor LPG inhibits MAC complex formation on the surface, while GP63 inactivates C3b (iC3b) and inhibits MAC complex. Opsonization of *Leishmania* by C3b and iC3b facilitates its uptake by CD11b and FcγR receptor on neutrophils and macrophages [106] (Figure 15). These pathways result in common activation of C3 convertase that cleaves C3 to generate C3b. Deposition of C3b on the pathogen surface will induce the deposition of C5b-C6-C7-C8-C9 complex that promotes lysis of the target pathogen. In addition, C3b promotes phagocytosis of the pathogen by neutrophils and macrophages.

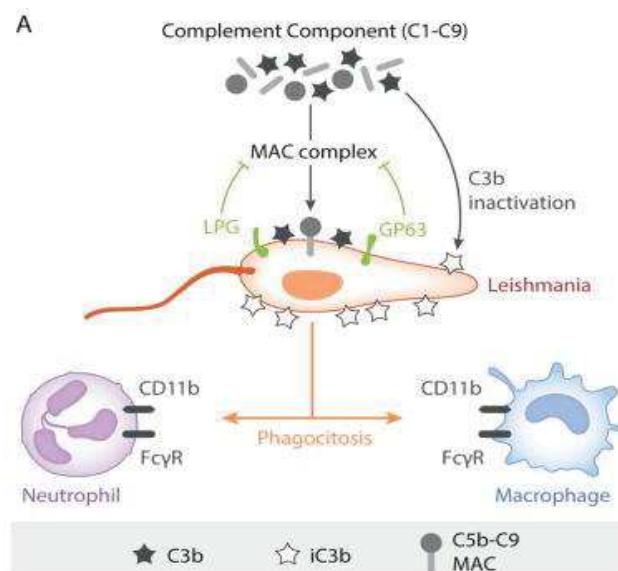


Figure 15: The innate immunity against *Leishmania*. Role of the complement cascade [105]. Upon entry into the mammalian hosts, *Leishmania* parasites first meet the complement system. Once the complement pathway is activated, C3b facilitates C5b-C9 membrane attack complex (MAC) on the surface of the parasite and leads to its lysis. Conversely, *Leishmania* virulence factor LPG inhibits MAC complex formation on the surface, while GP63 inactivates C3b (iC3b) and inhibits MAC complex. Opsonization of *Leishmania* by C3b and iC3b facilitates its uptake by CD11b and FcγR receptor on neutrophils and macrophages.

Complement systems showed a prominent efficacy in clearing *Leishmania*. Studies have shown that a concentration of 1/120 of normal human serum (NHS) is cytotoxic for *L. major* parasites [105]. Moreover, there is an evidence for the involvement of the three cascades in the

clearance of *Leishmania*. Alternative complement pathway is widely accepted as the major pathway involved in *Leishmania* clearance [107, 108, 109]. However, IgM anti-*Leishmania* antibodies induced activation of classical pathway has also been shown to be important and specific for *Leishmania* promastigotes agglutination and killing [110]. The Alternative pathway kills *Leishmania* amastigotes [111]. Finally, serum MBL binds *L. major* and *L. mexicana* promastigotes suggesting a possible contribution of the Lectin pathway in killing *Leishmania* [111].

1.2.8.2.2- Pattern Recognition Receptors

Following *Leishmania* infection, neutrophils and macrophages are the major innate immune cells that respond and attack the parasite. These cells are equipped with several germline encoded pattern recognition receptors (PRRs), helping them to sample the host environment from invading pathogens. In addition, the membrane bound toll-like receptors (TLRs) and C-type lectin like receptors (CLRs) recognizes and responds to *Leishmania* infection [105].

1.2.8.2.3- Toll-like receptors (TLRs)

1.2.8.2.3.1- Different types of TLRs

The TLR signaling pathway is one of the first defensive systems against invasive microorganisms. TLRs are transmembrane proteins that confer specificity to the innate immunity cells by recognition of every known category of pathogen that may cause a human disease. The TLR family consists of 11 members (TLR1 to TLR11) differing in their specificity for different pathogens and their capability to induce different cytokines production (Figure 16) [112]. TLRs are located on either the plasma membrane or internal membranes of macrophages, DCs, and NK

cells. T and B-lymphocytes also express TLRs. Among the TLRs located on internal membranes, such as lysosomes, TLR3, TLR7, TLR8, and TLR9 are the receptors that have been described so far. TLR1, TLR2, TLR4, TLR5, and TLR6 are located on plasma membranes. It is noteworthy that TLR1 and TLR6 are anchored to the TLR2 using the same signaling pathway despite the fact that they have a different ligand-recognizing extracellular domain [113, 114, 115].

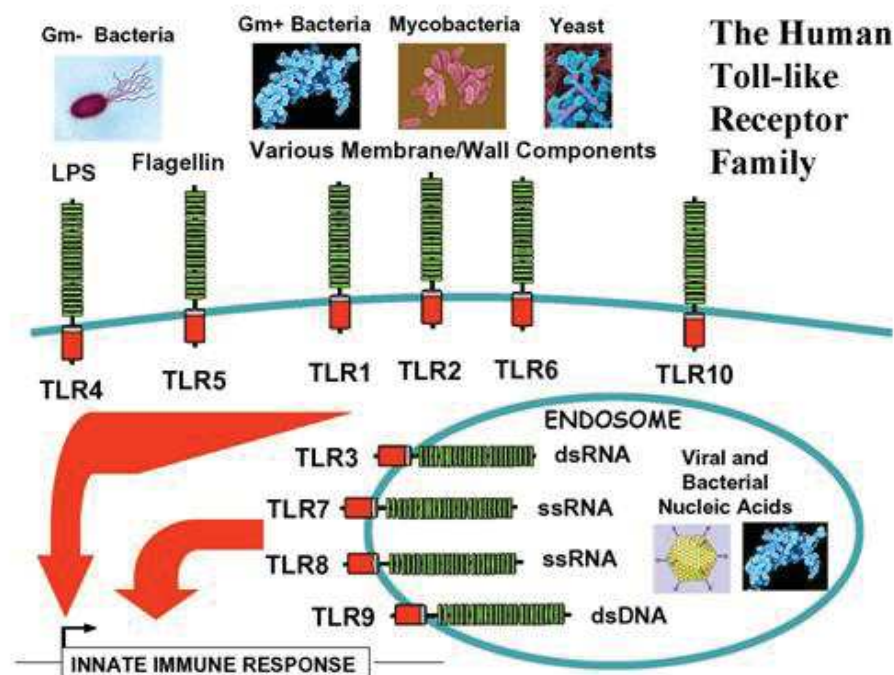


Figure 16: Localization of Toll-like receptors [116]. TLR1, 2, 3, 4, 5, 6 and 10 are located on the cellular membrane and are involved in the recognition of Gram negative (TLR 4, 5), Gram-positive bacteria (TLR1), mycobacteria (TLR2), and yeast components (TLR6). TLR3, 7, 8 and 9 are located on the endosomal membrane and are involved in the recognition of viral and bacterial nucleic acids. These TLRs bind double stranded RNA (TLR3), single stranded RNA (TLR7, 8), and double stranded DNA (TLR9).

After recognition of specific pathogen antigens, TLRs trigger the NF- κ B transcription factor, which then translocate to the nucleus and promotes the transcription and synthesis of pro-inflammatory cytokines [117]. This TLR signaling induces the phosphorylation of I κ -B α , which targets this protein for degradation, leading to the release of NF- κ B dimers and their translocation to the nucleus (Figure 17) [118, 119]. The NF- κ B family is composed of five related transcription

factors: p50, p52, RelA (p65), c-Rel and RelB [37]. In unstimulated cells, NF- κ B is found as an inactive cytosolic form, associated with an inhibitory subunit known as I κ B (I κ B- α , I κ B- β or I κ B- ϵ). Upon cell stimulation by binding of ligand to a cell surface receptor like Toll-like receptor superfamily, the canonical pathway is activated *via* activation of I κ B-bound NF- κ B species (such as RelA/p50 or cRel/p50). This activation may occur *via* either the canonical and non-canonical pathway. The canonical NF- κ B activation mainly occurs through activation of an upstream multimeric IKK complex, formed by IKK α , IKK β , and IKK γ or NEMO. This activation results in the phosphorylation (P) of I κ B α , its ubiquitylation (Ub) and subsequent degradation by the 26S proteasome. Release of the NF- κ B complex allows it to relocate to the nucleus, bind specific promoters, and activate gene transcription implicated in many functions including the immune and inflammatory response [215]. On the other hand, the non-canonical activation of NF- κ B occurs *via* binding of RelB complex to p100, leading to the translocation of RelB/p52 to the nucleus and the transcription of target genes [120].

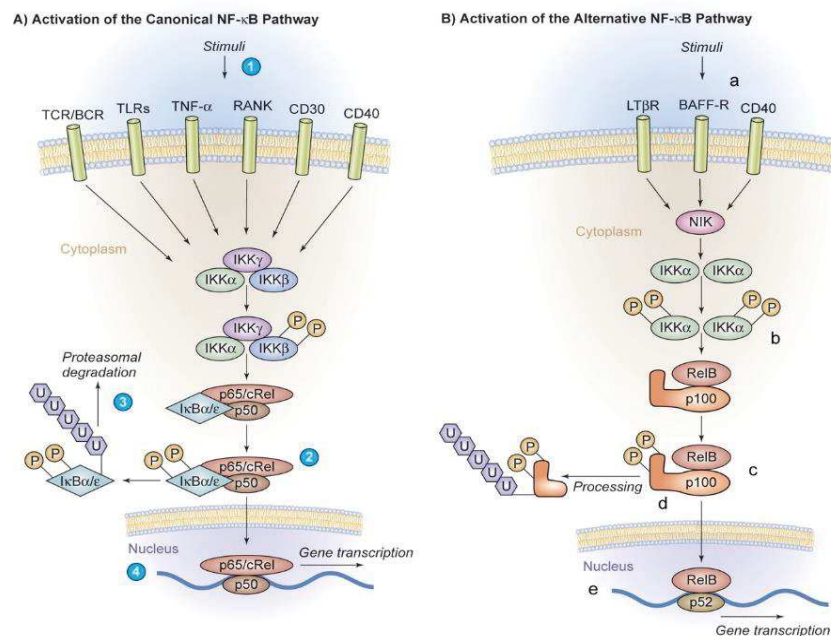


Figure 17: Canonical and alternative pathway of NF- κ B activation [120]. Activation of the canonical NF- κ B pathway. A series of stimuli activate the canonical pathway of NF- κ B

activation, including proinflammatory cytokines such as IL-1, TNF- α , or pathogen-associated molecular patterns that bind to TLRs, the antigen receptors TCR/BCR, or lymphocyte co-receptors such as CD40, CD30, or receptor activator of NF- κ B (RANK) (1). Activated IKK phosphorylates I κ B proteins on 2 conserved serine residues and induces I κ B polyubiquitinylation (2), which in turn induces their recognition by the proteasome and causes successive proteolytic degradation (3). Following the I κ B degradation, the cytoplasmic NF- κ B dimers are released and translocate into the nucleus, where gene transcription is activated (4). (B) Activation of the alternative NF- κ B pathway. The alternative pathway of NF- κ B activation is engaged by a restricted set of cell-surface receptors that belong to the TNF receptor superfamily, including CD40, the lymphotoxin β receptor, and the BAFF receptor (a). This pathway culminates in the activation of IKK α (b), which can directly phosphorylate NF- κ B2/p100 (c), inducing partial proteolysis of p100 to p52 by the proteasome (d). The p52 protein lacks the inhibitory ankyrin repeats and preferentially dimerizes with RelB to translocate into the nucleus (e).

1.2.8.2.3.2- TLRs in context of Leishmaniasis

We have described the importance of LPG and GP63 in the immune response. Adaptor molecules mediate TLR activation. MyD88 is the most common adaptor molecule for the activation of NF- κ B and is present in most TLRs [105].

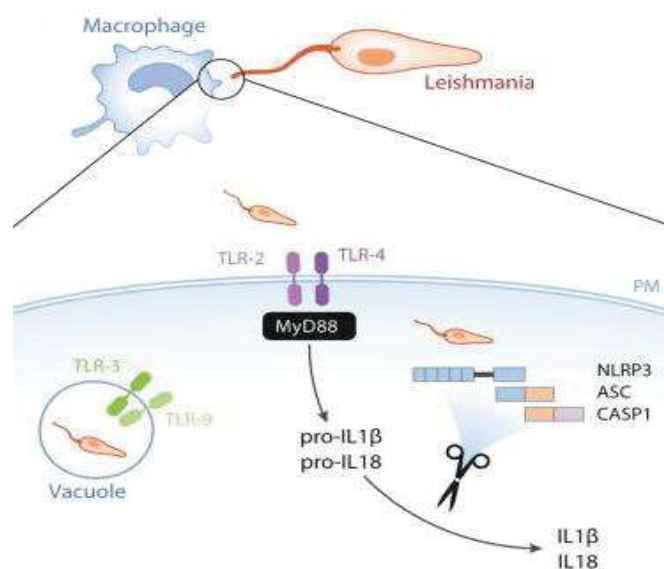


Figure 18: Mediation of TLRs by MyD88 upon *Leishmania* infection [105]. TLR2 and TLR4 on macrophage recognize *Leishmania* outside, whereas TLR3 and TLR9 recognize *Leishmania* in the vacuole. Activation of TLRs activates the NF κ B and MAP kinase signaling through MyD88, which is important for *Leishmania* clearance. In the cytoplasm, NLRP3 inflammasome senses *Leishmania* to produce IL-1 β and IL-18, which can have diverse cellular functions depending on the genetic background of the host [106].

MyD88 activation is mediated by the binding of Leishmanial LPG, GPIL and TLR2 [101]. Macrophage expressed TLR2 and TLR4 recognize the extracellular *Leishmania*, whereas TLR3 and TLR9 recognize internalized *Leishmania* within the vacuole (Figure 18) [105]. Activation of TLRs activates the NF- κ B and MAP kinase signaling through MyD88 and leads to *Leishmania* clearance. In the cytoplasm, NLRP3 inflammasome senses *Leishmania* to produce IL-1 β and IL-18, which can have diverse cellular functions depending on the genetic background of the host (Figure 18). NF- κ B activation by LPG is mediated by TLR2. However, other leishmanial components can also activate other TLRs [121]. Furthermore, NK cells infected with promastigotes of *L.major in vitro* revealed that three molecules of LPG aggregate with one molecule of TLR2, thus corroborating the theory of interaction between *Leishmania* parasite and TLRs [122,123]. These data invalidate the concept of unresponsiveness of TLR2 and TLR4 in leishmaniasis [124]. Beyond the studies that have demonstrated the importance of TLR2, studies on TLR4 have also yielded promising results. Some studies have demonstrated that LPG and GPIL have no effect on TLR4 [122, 124]. A later study demonstrated that TLR4 was required for efficient parasite control, probably due to the activity of inducible Nitric Oxide Synthase (iNOS) [125]. The activation of iNOS leads to NO synthesis and *Leishmania* death. In the absence of TLR4, the more intense activity of arginase increases the formation of urea and reduces the formation of NO [126]. In addition, the leishmanicidal activity of TLR4 was evaluated by pancreatic elastase in studying the activation of systemic inflammation. This enzyme produced by neutrophils induces the leishmanicidal activity of macrophages through TLR4 activation [127]. The last studies confirmed the benefits of TLR4 activation in terms of *Leishmania* death, a concept that has been demonstrated for other parasites and bacteria [128, 129, 130]. Moreover, mice with a mutation in the *TLR4* gene were unable to heal from CL lesions [131, 132]. The same group,

using an experimental model that was responsive to the pro-inflammatory cytokine IL-12 [125], later confirmed these results. In view of this, Li et al. demonstrated the production of pro-inflammatory cytokines, especially IL-12, *via* TLR9 in *L. major* infected mice [133]. In humans, TLR9 is present only in plasmacytoid DCs and B lymphocytes. The importance of this receptor in the acute phase of the disease has not been well established yet. Nevertheless, recent studies have demonstrated that it may enhance the effect of vaccination against *Leishmania* [134]. Recently, some evidence has shown that TLR3 can also contribute to the recognition of *Leishmania* [135]. This receptor, like TLR7, TLR8, and TLR9, is located in intracellular endosomal membranes, recognizes double-stranded RNA, and triggers NF- κ B and the production of IFN- γ [136]. Besides the localization of TLR3, it induces cytokine production by means of a signaling pathway similar to that of the TLR from the plasma membrane. Thus, MyD88 is an adaptor protein that is shared by all the known TLRs [137]. However, TLR3 also uses a MyD88-independent pathway to NF- κ B and production of IFN- γ [138]. The MyD88-dependent TLR pathway is involved in the induction of DC maturation, which was demonstrated previously with *L. major* and *L. donovani* [139, 140].

1.2.8.2.4- Nitric Oxide production by iNOS

Nitric Oxide synthases (NOSs) are a family of enzymes catalyzing the production of Nitric Oxide (NO) from L-arginine. The inducible isoform, iNOS, is involved in immune responses, binds calmodulin at physiologically relevant concentrations [141]. It is the proximate cause of septic shock and may function in autoimmune diseases [141]. NO is generated by at least three different isoforms of Nitric Oxide Synthases. Inducible iNOS (iNOS/NOS2) is one of these isoforms and is a Ca^{2+} independent defense mechanism initiated by many cell types, particularly

macrophages [141]. It is expressed in response to pro-inflammatory cytokines (e.g. IFN- γ , IL-1 β , TNF- α) [142]. Macrophage derived NO acts as both a highly cytotoxic molecule, generated in response to microbial stimuli, pro-inflammatory cytokines, and as a regulatory molecule, that controls T lymphocyte proliferation and cytokine secretion during adaptive immune responses [141].

In leishmanial infection, the action of iNOS in the regulation of innate immunity has been further identified in the protective response of mice to *Leishmania major* (Figure 19). In this experimental model, small quantities of iNOS-derived NO, generated by activated macrophages, activate natural killer (NK) cells to respond to IL-12 and IFN- α/β , which allows them to become cytotoxic and to release IFN- γ , thus controlling the spread of the parasite. This initial response is critical for the containment of the infection and hence for subsequent T cell-dependent clinical resolution of the disease, despite the fact that in the late phase of infection iNOS is further up regulated by IFN- γ producing CD4⁺ T lymphocytes and the antimicrobial activity of cytotoxic cells predominates [143].

1.2.8.3- Adaptive Immunity

When the innate strategies fail to kill *Leishmania* parasites, T helper-mediated immune responses are induced in order to regulate the disease progression. Resistance to leishmaniasis requires the activation of Type 1 helper cells (Th-1) and the production of IFN- γ (Figure 19) [144].

Th1-mediated response is enhanced by IFN- γ -induced secretion of nitric oxide (NO), which leads to parasite destruction. In contrast, Th2-mediated response activation resulting in IL-4, IL-5, IL-10 and IL-13 production, leads to a humoral immune reaction and then to disease progression (Figure 19)[145].

Of note, it is important to mention that the outcome of the infection is mainly determined by interactions between Th1 and Th2 cytokines. Furthermore, many studies revealed that Th2-mediated response leads to susceptibility to *L. major* in animal models, therefore, a Th1 response is needed for the cure of the animal (Figure 19) [146, 147].

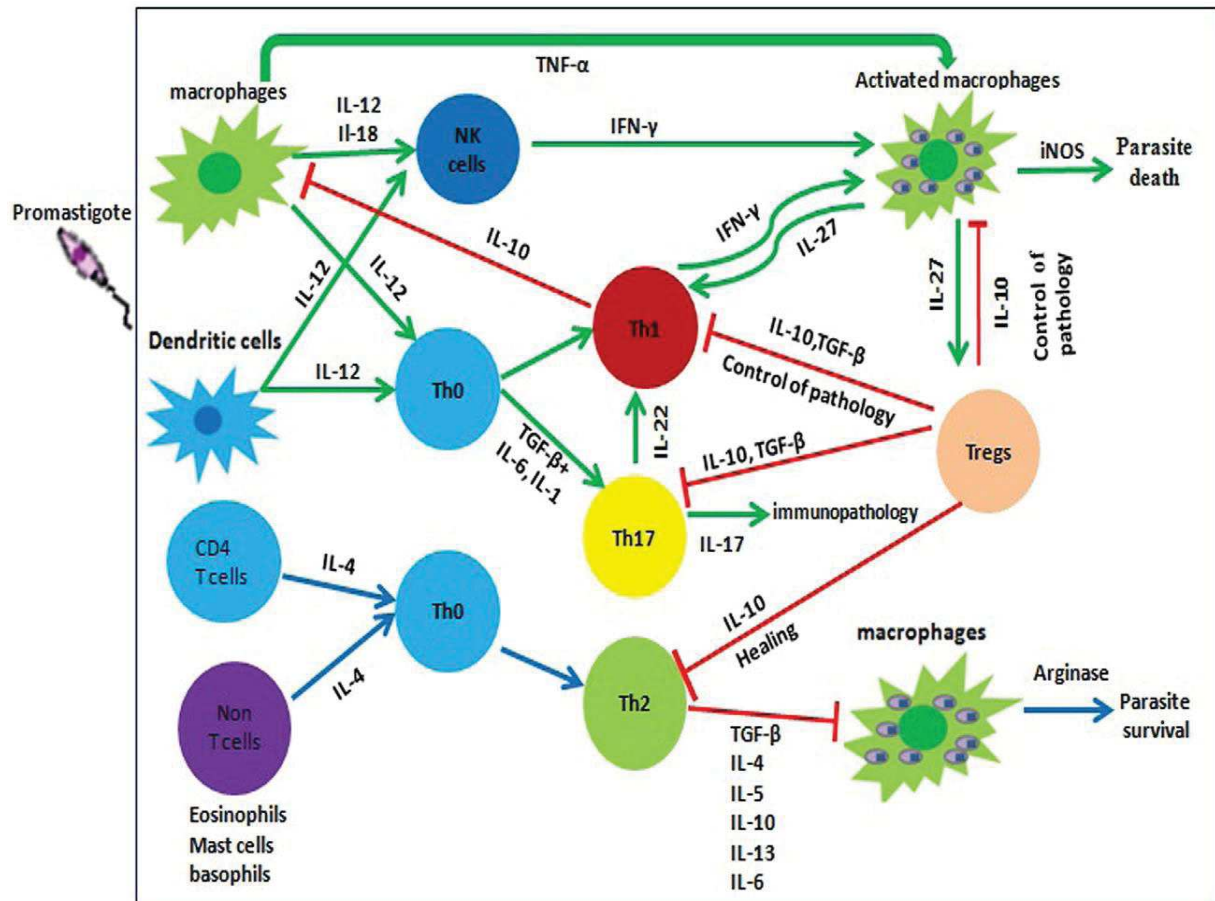


Figure 19: Immune response following infection with *Leishmania*. Different immunological pathways in CL all depending on the differentiation of CD4⁺ T cell subsets into Th1, Th2, T_{reg}, and Th17. Following parasite entry, APCs (macrophages and dendritic cells) are stimulated to produce pro-inflammatory cytokines such as IL-12. These cytokines promote Th1 differentiation and IFN-γ production lead to activation of macrophages and parasite killing by NO production. Conversely, anti-inflammatory cytokines promote differentiation of Th0 toward Th2 that inhibit macrophage activity and lead to parasite survival. Overproduction of inflammatory cytokines results in severe immunopathology and non-healing infection. TGF-β and IL-27 cytokines secreted by macrophages or DCs stimulate T_{reg} cells to produce IL-10 that act back on the macrophages and DCs to reduce the release of inflammatory mediators, forming a negative feedback loop and the balance of pro- and anti-inflammatory cytokines controls pathology and tissue destruction [148].

1.2.8.4- Survival mechanisms of the parasite

The innate immune mechanism is considered as the first-line defense implicated in eliminating pathogens [149]. However, one of the major problems with *Leishmania* infections is the ability of these parasites to evade and subvert host immune responses. These qualities allow the parasites to persist in the host and establish chronic infections. Tremendous selective pressure to survive the complement pathway and his capacity to kill the parasite, has resulted in the evolution of several immune evasion strategies employed by *Leishmania*.

Leishmania parasites can prevent the complement system mediated lysis [150]. Intracellular amastigotes are capable of modifying their membrane configuration leading to the failure of C5b-C9 complex to be inserted into the parasites' membrane [151, 152]. Furthermore, several kinases produced by the parasites can deactivate many complement system elements (C3, C5 and C9) by a phosphorylation process. Most importantly, the previously mentioned parasite membrane components, GP63 and LPG, can convert C3b into an inactive form and inhibit the complement system [151].

1.2.8.4.1- Oxidative burst: reactive oxygen intermediates production

Oxidative burst is one of the most important mechanisms involved in a macrophage-mediated phagocytosis. At the site of infection, reactive oxygen intermediates (ROI) such as Hydroxyl radicals, highly reactive superoxide and hydrogen peroxide are abundantly found and are capable of killing parasites by affecting their membrane [153,154]. However, many studies revealed that LPG could neutralize these intermediates and protect parasites [155].

1.2.8.4.2- ROS production

In order to establish a successful infection, *Leishmania* parasites must counter the immune responses evoked by macrophages. One of the primary microbicidal molecules in macrophages efficient against *Leishmania* are the reactive oxygen species (ROS). Successful survival of *Leishmania* within the host macrophages depends on the impairment of host immune responses. Inhibition of ROS generation is a crucial adaptive strategy through which *Leishmania* can survive within the hostile environment of macrophages [156].

1.2.8.4.3- Acidification and digestion: Macrophages high acidity

Although studies have shown that the high acidity within the macrophages phagolysosomes provides them with an important anti-leishmanial activity and that low pH makes proteins, carbohydrates, RNA and DNA very sensitive to acid hydrolases-mediated degradation [90], many reports suggest several strategies that allow the parasite to prevent macrophages killing mechanisms. In an acidic environment, the membrane molecule Gp63 reaches its maximal activity in lysing proteins. Thus, this molecule is able to degrade different enzymes found in phagolysosomes [157]. Moreover, the negative charge of LPG may contribute in protecting the parasite from lysosomal enzymes [154].

1.2.9- Susceptibility versus resistance to CL

A huge number of reports talking about BALB/c and C57bl/6 mice infected by *L.major* indicated two extremely distinct outcomes of infection [158]. However, this remarkable difference between the two cases is mainly due to the different strains of mice [159]. In BALB/c mice, the multiplication of parasites and the progression of the disease are not controlled. Therefore,

susceptibility is due to Th2-mediated response induction characterized by a considerable secretion of IL-4, IL-10, IL-13 and IgE [160]. In contrast, IFN- γ secretion induced by Th1 response activation in C57bl/6 mice is the major reason behind resistance and self-healing capacity shown in these models [161].

Several immunological studies have increased our understanding of the adaptive response in leishmaniasis. Thus, this disease has been used as a model of Th1 and Th2 responses. Unfortunately, there are still several aspects of the initial steps of *Leishmania* infection in humans that are largely unknown. Most importantly, the dose of parasites, the cytokine milieu and the genetic background of the host are three main factors, which determine the course and the outcome of infection.

1.2.10- Treatment of CL

There are different factors that influence CL treatment. Drug's efficacy is dependent on lesion size, number and appearance. In addition, disease duration prior to treatment, re-infection, immunosuppression, and co-infections make CL treatment inefficient [162]. Moreover, the location of the lesion (e.g. face or joints), age of the patient (e.g. adults or children) and gender are also factors that often determine the choice of treatment [163].

Other factors affecting CL treatment are intrinsic and related to the different *Leishmania* species. An effective treatment in one geographical area for a given organism may not work in different geographical areas or for different organisms in the same location [163, 164]. In these cases, efficacy depends not only on the *Leishmania* species but also on the patient's response and health status. Another main disadvantage and paradox is the lack of availability of most of these drugs in rural and poor areas where the majority of leishmaniasis cases is encountered [165].

In this study, we focused on *L.tropica* and *L.major* strains, being the endemic strains causing CL in the Middle East Region, and whose dissemination to the neighborhood countries was increased due to the Syrian conflict.

Therapies used against CL regroup physical, oral, topical and parenteral therapies (Summarized in Table 6 at the end of this section).

1.2.10.1-Physical therapies

Physical therapies against CL include thermotherapy, CO2 laser, photodynamic therapy, and cryotherapy regimens.

1.2.10.1.1- Thermotherapy

Thermotherapy is the application of heat to directly destroy the parasite. Many studies showed that this method achieved the same efficacy as intralesional antimonials and in some cases, proved its superiority on them. However, thermotherapy has some disadvantages related to cost and availability, thus it should not be applied near mucosal areas [166, 167, 168].

1.2.10.1.2- CO2 laser

This is another physical method that can barely damage the healthy tissue with a good efficacy on CL. This treatment is capable of thermolysis on damaged tissues, and had showed better efficacy than cryotherapy combined to intralesional antimonials [169, 170].

1.2.10.1.3- Photodynamic Therapy

Photodynamic Therapy is a Light-mediated cytolysis of *Leishmania* parasites that is proposed as a safe, rapid and highly effective choice for CL [171, 172].

1.2.10.1.4- Cryotherapy

Cryotherapy by using liquid nitrogen was demonstrated to reach a high efficiency rate against CL. In addition, even, the combination of cryotherapy and intralesional pentavalent antimonials has been shown to induce 100 % CR rates against *L. major* and *L. tropica* CL [170, 173,174].

1.2.10.2- Topical therapies

1.2.10.2.1- Paromomycin

Several clinical trials undertaken since the 1990s have already described the possible efficacy of topical paromomycin for the treatment of CL [175, 176]. However, no greater efficacy was observed in patients after long term follow up and this was due to the short duration of treatment [176]. Topical paromomycin versus intralesional administration of antimonials comparison was tested for both *L.major* and *L.tropica* infections. Both treatments achieved low response rates [170, 175].

1.2.10.2.2- Intralesional Pentavalent Antimonials

The efficacy of intralesional pentavalent antimonials has been observed mainly for *L.major* and *L.tropica* infections in Asia and the Mediterranean basin area [177, 178]. A randomized

clinical trial undertaken in Syria for *L.tropica* infections administered intralesional antimonials once weekly for 5 consecutive weeks. The results showed a significantly higher response when compared to treatment with recombinant Interferon gamma (IFN- γ) [177, 178]. In Saudi Arabia, a randomized clinical trial compared parenteral therapy with antimonials and intralesional therapy with antimonials. The response rates were found to be similar for systemic and local therapy [177,178]. On the other hand, there are various studies about the benefits of the combination of intralesional antimonials with other therapeutic options. A study undertaken in Iran showed that the efficacy of the combination of intralesional antimonials with parenteral pentavalent or intramuscular antimonials or also cryotherapy was higher than its administration as monotherapy [177,178].

1.2.10.2.3-Topical Imidazole drugs

Formulated as an ointment, topical applications of clotrimazole and miconazole for one month have been tested in Saudi Arabia without any cure of the patients. Topical ketoconazole was also tested in Afghanistan, but it did not significantly change the course of the lesions [177,178].

1.2.10.3-Oral Drug Therapy

1.2.10.3.1-Azole drugs

Fluconazole was tested in Saudi Arabia against *L.major* and proved its efficacy and tolerance when a dose of 200 mg/day was administered for 6 weeks [177, 178, 179]. A better complete remission (CR) was achieved in Iran against *L.major* obtained when the dose of fluconazole was doubled per day for 6 weeks [176, 180, 181]. Several randomized clinical trials

have compared itraconazole with placebo. A very high cure rate was obtained in Iran against *L. major*. Nonetheless, itraconazole in Iran against *L. major* presented lowered cure rates by half [176, 180, 181].

1.2.10.3.2- Miltefosine drugs

Miltefosine has been mainly used against *L. major* infections, and response rates vary between 87 and 100%. The only clinical trial performed in Iran against *L. major* infection concluded that oral miltefosine is as effective as the intralesional antimonials [176, 180, 181].

1.2.10.4- Parenteral Therapy

1.2.10.4.1- Pentavalent Antimonials

Intramuscular or intravenous pentavalent antimonials were tested in Eastern Africa, the Middle East and the Mediterranean countries and have proven effectiveness against CL. However, different response rates were obtained between species, with higher CRs for *L. major* than for *L. tropica* [165, 175, 176, 180, 182]. The combination of topical 5% imiquimod cream three times per week with parenteral pentavalent antimonials for 14 days did not result in additional benefits [183].

1.2.10.4.2- Liposomal Amphotericin B

Experience with Liposomal Amphotericin B for the treatment of OWCL is very limited. The majority of cases that required systemic parenteral treatment received pentavalent antimonials. However, the high toxicity observed with these later, encouraged the increasing choice and use of liposomal Amphotericin B by more specialists [184]. An American study collected therapeutic

response at doses of 3 mg/kg/day of Amphotericin B up to ten doses, given within over 3 weeks, in ten travelers. At the end of the treatment, the general response rate was 84%. However, when another cycle was given, no response was observed and all attempts with Liposomal Amphotericin B failed [176, 180, 185].

Table 6: Different CL treatment and their disadvantages.

Treatment	Types	Problems
Physical therapy	-thermotherapy	-size, number and appearance of the lesions.
	-CO ₂ laser	-the duration of the disease prior to treatment
	-photodynamic therapy	-frequency and time to self-healing
	-cryotherapy regimens	-re-infection
Topical therapy	-paromomycin -intralesional pentavalent antimonials -topical imidazole drugs	-lack of availability of most of these drugs in rural and poorer areas where the majority of leishmaniasis are encountered
Oral therapy	-azole drugs -miltefosine	-mucosal or diffuse involvement -immunosuppression
Parenteral therapy	-pentavalent antimonial -liposomal Amphotericin B	- location of the lesion

1.2.10.5- Anti-cancer drugs

1.2.10.5.1- Tamoxifen

With all the above-mentioned trials, some clinicians have tested some anti-cancer drugs such as Tamoxifen. This anti-estrogen has been extensively used in the treatment and prevention of breast cancer for decades [186]. Some studies have demonstrated its anti-leishmanial activity, either alone or combined to amphotericin B, *in vitro* and in murine studies, in which there was a significant reduction in both lesion size and parasite burden [187, 177].

1.2.10.5.2- Imidazoquinolines family

1.2.10.5.2.1- Imiquimod

Imiquimod (Figure 20), (S-26308, R-837) (1-(2-methylpropyl)-1H-imidazo [4, 5-c] quinolin-4-amine), is the first member of the imidazoquinolone family, and belongs to the class of medications called immune response modifiers.

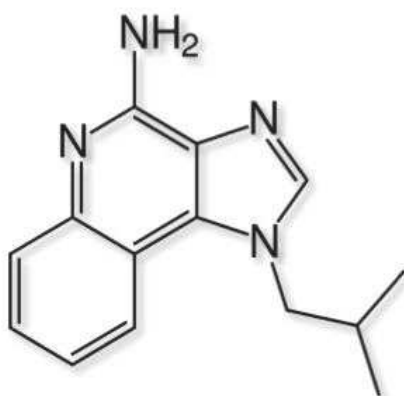


Figure 20: Chemical structure of Imiquimod

This low molecular weight derivative of the Imidazoquinoline family was the first immune response modifier used for the treatment of infectious skin conditions and shown great anti-viral and anti-tumor activities *in vivo* [188]. The United States Food and Drugs Administration (FDA) approved this agent in 1997 for the topical treatment of external perianal warts because of its action on increasing the activity of the body's immune system. This drug was proven also efficacious as a topical therapy for certain types of skin cancers: basal cell carcinoma, Bowen's disease, superficial squamous cell carcinoma, some superficial malignant melanomas, and actinic keratosis [189].

In pre-clinical studies, Imiquimod inhibits melanogenesis and proliferation of human melanocytes. Its therapeutic spectrum was also extended to cutaneous B-cell lymphomas [189]. The exact mechanism of action in which Imiquimod and its analogs activate the immune system

is not known yet. Nevertheless, it is known that Imiquimod activates immune cells by ligating the Toll-like receptor 7 (TLR-7), commonly involved in pathogen recognition, on the cell surface [190]. Cells activated by imiquimod *via* TLR-7 secrete cytokines (primarily IFN- α , IL-6, and TNF- α). There is evidence that imiquimod, when applied to skin, can lead to the activation of Langerhans cells, which subsequently migrate to local lymph nodes to activate the adaptive immune system [190]. Other cell types activated by Imiquimod include natural killer cells, macrophages, and B-lymphocytes. There are case reports and preliminary studies suggesting Imiquimod effectiveness in the treatment of CL [191].

However, the effects of Imiquimod on innate immune responses, *via* TLRs, suggest a potential anti-leishmanial activity that was demonstrated by inducing the release of nitric oxide. Less than 1% of the drug is recovered in urine after topical application. Topical Imiquimod (5% cream) is only mildly irritating and does not lead to systemic toxic effects. Imiquimod has been used in combination with a systemic antimonial in the treatment of CL and presented a cure rate of 90% in patients with refractory CL to pentavalent antimonial treatment [190]. It has been also shown that it is also more effective in the initial treatment of CL [192]. Another clinical trial in Peru, Miranda-Verastegui et al. showed that this combination was better than placebo plus pentavalent antimony [193].

1.2.10.5.2.2- Imiquimod analogs

1.2.10.5.2.2.1- EAPB0203

Since several years, the group of Bonnet and Masquefa in Montpellier, France, has developed analogs of Imiquimod. Three chemical series have been synthesized: 1) the imidazo[1,2-a]quinoxalines, 2) the imidazo[1,5-a]quinoxalines, and 3) the pyrazolo[1,5-a]qui-

noxalines [174]. Among the imidazo[1,2-a]quinoxaline series, one of these compounds (EAPB0203) exhibits an important *in vitro* cytotoxic activity and is 50 times more potent than imiquimod against human melanoma cell lines [194]. Studies have shown that EAPB0203 exhibits inhibition of cell proliferation G2/M cell cycle arrest [195]. In a mouse model, EAPB0203 treatment schedules caused a significant decrease in tumor size compared to vehicle control and fotemustine treatment. EAPB0203 has also been evaluated in T-cell lymphomas and human T-lymphotropic virus type I (HTLV-I) associated adult T-cell leukemia/lymphoma [188].

1.2.10.5.2.2- EAPB0503

This compound also has a potential therapeutic role in the treatment of malignant melanoma. EAPB0503, has 7–9 times higher cytotoxic activity against the human melanoma cell line (A375) than EAPB0203 [174]. Thus, EAPB0503 has emerged as very promising drug. Since these analogs of Imiquimod have shown a better potency than their original compound used in *Leishmania* treatment, we investigated in this thesis work their anti-leishmanial activity. EAPB0503 (1-(3-methoxyphenyl)-N-methylimidazo [1, 2-a] quinoxalin-4-amine) was synthesized as described by Deleuze- Masquefa et al. The synthesis of EAPB0503 was subsequently optimized by microwave-assisted chemistry (Figure 21) [195]. Briefly, carbonyl-imidazole dimer 1 (95%) that resulted from the bimolecular condensation of 2-imidazole carboxylic acid was coupled with ortho-fluoroaniline to yield the intermediate 2 (90%), which is then cyclized to yield compound 3 (80%). Compound 3 was treated with phosphorus oxychloride and N,N-diethylaniline in a sealed tube at 130°C for 15 min using a Biotage initiator microwave synthesizer (Biotage, Uppsala, Sweden), leading to compound 4 (90%) after column chromatography on silica gel purification. Compound 4 was coupled with methylamine in a

microwave (150°C, 20 min) to yield compound 5 (95%). The bromination of compound 5 by N-bromosuccinimide in a microwave (100°C, 6 min) led to compound 6 (85%). EAPB0503 was obtained by Suzuki coupling of compound 6 with 3-methoxyphenylboronic acid in the presence of a palladium catalyst, basic conditions, and under microwave assistance (140°C, 20 min), and was purified by column chromatography on a silica gel, leading to the pure product (90%) [196,197].

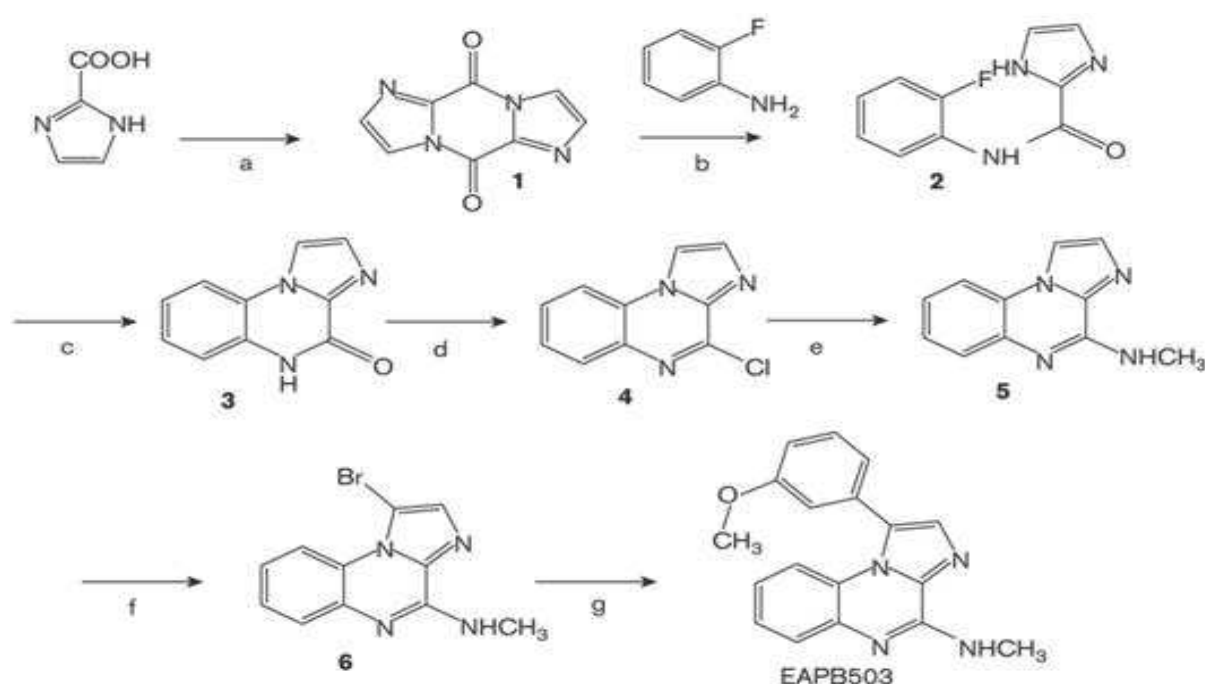


Figure 21: Synthesis reaction steps to obtain EAPB0503

1.2.11- Disadvantages of current treatment of CL

All the CL therapies mentioned earlier are expensive, associated with various adverse and toxic effects and require careful monitoring. Most importantly, organic antimonials considered as the most efficient available treatment, have a very complicated administration route and their use is frequently associated with cardiac toxicity. Antimonials increase the cardiac calcium current leading to serious ECG (electrocardiography) changes [198]. Thus, an inexpensive and safe

systemic treatment for *Leishmania* is urgently needed [199]. The current safest treatment for CL is Glucantime. However, it is not practical since the patient must take painful and multiple injections on a weekly basis for up to 6 months, with the need for intramuscular injections, that are limited by drug toxicity and side effects.

1.2.12- Problematic and Hypothesis

A huge CL outbreak has disseminated in the Middle East Region after the Syrian crisis. All available treatments against CL are associated with high rates of toxicity, high cost, elevated risks of relapse and resistance to drugs; the drug of choice is still out of reach and new strategies must be set. The lack of a gold standard prompted us to seek for novel therapeutic approaches.

Since EAPB0503 has proven a higher potency than imiquimod in many models, our main aim in this work was to address its potency against CL. We have tested its efficacy on both promastigote and amastigote stages of *L. tropica* and *L. major* parasites, being the most endemic strains in our region. The mechanism of action as well as the elicited immune response was also investigated. Our results will give a better insight about the effect of Immunomodulatory drugs derivatives on CL, and will open horizons for new and promising treatment paradigm.

Chapter 2

Materials and Methods

2.1- Parasites Culture and Maintenance

Leishmania major (MHOM/MA/81/LEM265 and MMER/MA/81/LEM309) and *Leishmania tropica* parasites (MHOM/LB/76/LEM61, MRAT/IQ/72/ADHANIS1) were purchased under MTA agreement from the CRHU “Parasitologie-Mycologie, CHU Montpellier; UMR MIVEGEC; Centre National de Référence des Leishmanioses. These parasites were maintained in a standard medium at a temperature ranging from 22 to 25°C. The medium consists of RPMI (Lonza) supplemented with 10% Fetal Bovine Serum (FBS), 100IU/ml streptomycin and 100IU/ml penicillin (Sigma). These parasites were subcultured on weekly basis.

2.2- Drugs

Imiquimod was purchased from Molekula (Wessex House, Shaftesbury, Dorset, UK) and EAPB0503 was synthesized as described by Deleuze-Masquefa et al. [200]. The synthesis of EAPB0503 was performed using microwave-assisted chemistry (as described above). These drugs were prepared as a 0.1 M stock in dimethylsulfoxide (DMSO) and stored at -80°C. Working solutions of 0.1 µM were freshly prepared in culture media. Treatment was performed and tested for 2, 6, 10 and 24h. The most important effect was obtained 10 and 24h post-treatment, and these time points were adopted for the remainder of the study.

2.3- Culture and treatment

Human THP-1 cells (American Type Culture Collection (ATCC TIB-202), Manassas, VA) were grown in RPMI medium with L-Glutamine, 25 mM Hepes (Lonza), supplemented with 10% FBS, 1% penicillin-streptomycin, 1% kanamycin and 1% glutamine (Invitrogen). The differentiation of THP-1 cells was performed using 50 ng/mL of phorbol 12-myristate 13-acetate (PMA, Sigma) overnight. Following their adherence, these differentiated macrophages were then activated with 1 µg/mL of Lippopolysaccharide (LPS) for 4h.

For the amastigote experiments, 1 million of THP-1 cells were seeded in 6-well-plate, differentiated onto macrophages. Activated macrophages were then infected with *L.major* or *L.tropica* at the ratio of 5 parasites/one macrophage, and incubated for 24h at 37 °C. Non-internalized promastigotes were removed by two gentle washes with PBS.

2.4- Efficacy of Imiquimod and EAPB0503 on *L.tropica* and *L.major* strains

2.4.1- *In vitro* anti-promastigote activity

Promastigotes of *L.major* and *L.tropica* were seeded into 96-well flat-bottomed microtiter plates at the density of 1×10^5 cells per well, in a final volume of 100 µl medium, and were incubated with ascending concentrations of Imiquimod and EAPB0503 (0.1, 0.5, 1, and 10 µM). Parasites were incubated at 25°C for 24, 48 and 72 h. Surviving promastigotes were selected according to their mobility. A blinded count was performed and all experiments were done at least 3 times, with triplicates in each experiment.

2.4.2- Effect of Imiquimod and EAPB0503 on the infectivity of *L.major* and *L.tropica*

Parasites of both strains *L.major* or *L.tropica* were treated with 0.1 μ M of Imiquimod and EAPB0503 for 72h. Promastigotes were then incubated with differentiated macrophages derived from THP1 cells, and incubated for 24h at 37 °C. Treated infected cells were washed twice with PBS, scrapped and prepared for RNA extraction.

2.4.3- *In vitro* anti-amastigote activity against *L.major* and *L.tropica*

2.4.3.1- RNA preparation

Infected THP1 with *L.major* or *L.tropica* strains were treated with different concentrations of Imiquimod and EAPB0503 (0.01 μ M, 0.05 μ M, 0.1 μ M, 0.5 μ M and 1 μ M). Treated infected cells were then incubated for 24h. Cells were then washed with PBS, scrapped and total RNA was extracted using Trizol extraction method. Experiments were performed starting from 2 μ g of RNA in a total of 20 μ l. Briefly, cells were resuspended in 1 mL of TRIzol. Next, 200 μ l of chloroform were added, mixture was vortexed for 1 min, and then incubated for 1 min at room temperature. Mixture was then centrifuged at 15000g for 10min at 4°C. After obtaining a clear layer separation, the upper phase containing the total RNA was gently taken, transferred into a new Eppendorf tube and precipitated by adding 0.7V isopropanol. After centrifugation at 15000g for 10 min at 4°C, RNA pellet was allowed to air-dry for few minutes to remove any excess of isopropanol, and then was resuspended in 30 μ l of RNase/DNase Free water. RNA concentration was then quantified using the nanodrop machine (ND-1000 Spectrophotometer).

2.4.3.2- cDNA preparation

For cDNA synthesis, components were added in the same order indicated in Table 7. The Revert Aid First cDNA synthesis Kit (Thermo Scientific) was used. The mixture was gently mixed and incubated for 5 min at 25°C followed by 60 min at 42°C, then for 5 min at 70°C and the reaction was stopped at 4°C.

Table 7: Components of cDNA Preparation process and volumes

RNase/DNase free water containing 5 µg of template RNA	11 µl
random primers	1 µl
5X Reaction Buffer	4µl
Ribolock RNase Inhibitor(20U/µl)	1µl
10 Mm dNTP Mix	2µl
RevertAid M-MuLV RT (200U/µl)	1µl
Total Volume	20µl

2.4.3.3- Quantitative Real Time PCR qRT PCR

Syber green qRT PCR was performed using the BIORAD machine (CFX96 Optics Module, Serial No. 785BR04788). Primers for the housekeeping gene are directed against the host cell Glyceraldehyde-3-Phosphate dehydrogenase GAPDH. Primers for amastigotes detection chosen in this study recognize an amplicon of 116-bp fragment of the minicircle kinetoplast DNA (kDNA). Their sequences are presented in Table 8. In qRT-PCR, individual reactions were prepared with 0.25 µM of each primer, 150 ng of cDNA and SYBR Green PCR Master Mix to a final volume of 10 µl. PCR reaction consisted of a DNA denaturation step at 95°C for 3min, followed by 40 cycles (denaturation at 95°C for 15 sec, annealing at 56°C for 60 sec, extension at 72°C for 30 sec). For each experiment, reactions were performed in duplicates and expression of individual genes was normalized to GAPDH Ct values. The Threshold cycle (Ct) corresponds to the cycle at which there is a significant detectable increase in fluorescence. So, comparing the CT values of a target gene (amastigotes in this case) to that of an endogenous reference gene

(GAPDH), allows the gene expression level of the target gene to be normalized to the amount of input RNA or cDNA. This is mainly done by calculating ΔCt ($Ct_{(Kinetoplast)} - Ct_{(GAPDH)}$). Thereafter, $\Delta\Delta Ct$ is calculated according to the Livak method: $2^{-\Delta\Delta Ct}$ to obtain the percentage of expression. This method is widely used for relative gene expression analysis [201].

Table 8: Forward and reverse primers used in this study.

Primer	Sequence	Annealing temperature	References
GAPDH Forward Primer	5'- CATggCCTTCCgTgTTCCTA-3'	58.9 °C	[202]
GAPDH Reverse Primer	5'- CCTgCTTCACCACCTTCTTgAT-3'	59.2°C	[202]
HHLM-1 Forward Primer	5'- CCTATTTTACACCAACCCCCAGT-3'	59.6°C	[86]
HHLM-2 Reverse Primer	5'- GGGTAGGGGCGTTCTGCGAAA-3'	65.9°C	[86]

2.4.3.4- Assessment of the transcription level of INOS in untreated or treated THP1 infected with *L.major* or *L.tropica*

Infected THP1 with *L.tropica* and *L.major* were treated with 0.1 μ M of Imiquimod and EAPB0503 for 10 and 24h. Cells were then scrapped and RNA extraction was performed (as described section 4.3.1), cDNA synthesis (as described section 4.3.2) and Syber-green RT-PCR was performed (as described section 4.3.3) using specific INOS primers (Table 9).

Table 9: INOS forward and reverse primers used in this study

Primer name	Sequence	Annealing temperature	References
INOS Forward primer	5'-GGGAGCCAGAGCAGTACAAG-3'	61.9°C	[203]
INOS Reverse primer	5'-GGCTGGACTTCTCACTCTGC-3'	59.7°C	[203]

2.4.3.5- Assessment of the transcription level of different TLRs in the context of *L.tropica* infection and then upon treatment with 0.1 μ M of Imiquimod or EAPB0503

Infected THP1 with *L.tropica* and *L.major* were treated with 0.1 μ M of Imiquimod and EAPB0503 for 10 and 24h. Cells were then scrapped and RNA extraction was performed (as described section 4.3.1), cDNA synthesis (as described section 4.3.2) and Syber-green RT-PCR was performed (as described section 4.3.3) using specific TLR primers (Table 10). The expression level of TLRs was normalized to the reference gene GAPDH.

Table 10: Forward and reverse primers of different tested TLRs

Primer	Sequence	Annealing temperature	Reference
TLR1 Forward primer	5'-TCTTCGGCACGTTAGCACTG-3'	60.5°C	[206]
TLR1 Reverse primer	5'-CCAAACCGATCGTAGTGCTGA-3'	61.3°C	[206]
TLR2 Forward primer	5'-GGGGCTTCACTTCTCTGCTT-3'	61.9°C	[206]
TLR2 Reverse primer	5'-AGCATCCTCTGAGATTTGACG-3'	59.7°C	[206]
TLR4 Forward primer	5'-GGACTCTGATCATGGTAGGT-3'	59.4°C	[206]
TLR4 Reverse primer	5'-CTGATCCATGCATTGGTAGGT-3'	56.4°C	[206]
TLR6 Forward primer	5'-GGTACCGTCAGTGCTGGAA-3'	58.4°C	[207]
TLR6 Reverse primer	5'-GGGTTTTCTGTCTTGGCTCA-3'	59.4°C	[207]
TLR7 Forward primer	5'-GATCCTGGCCTATCTCTGACTC-3'	64°C	[208]
TLR7 Reverse primer	5'-CGTGTCCACATCGAAAACAC-3'	58.4°C	[208]
TLR8 Forward primer	5'-CAAACGTTTTACCTTCCTTTGTC-3'	59.3°C	[209]
TLR8 Reverse primer	5'-CGGTAACGTCAATGAGCAAA-3'	58.4°C	[209]
TLR9 Forward primer	5'-GAATCCTCCATCTCCCAACAT-3'	59.4°C	[206]
TLR9 Reverse primer	5'-CCAGAGTCTCAGCCAGCACT-3'	62.5°C	[206]

2.4.3.6- Enzyme-linked immunosorbent assay (ELISA)

Supernatants of infected and treated THP1 were collected 10h and 24h after treatment, and ELISA was performed using Multi-Analyte ELISArray Kit (Qiagen) according to the manufacturer's instructions. Briefly, supernatants of *L.tropica* infected THP1 and treated with 0.1 μ M of Imiquimod or EAPB0503 were collected. Supernatants were spun for 10 min at 1000g and transferred to new Eppendorf tubes, and diluted using a specific cocktail of antigens (IL-12, IL-1 β , IL-6, and TNF- α , MIP-1 α , MIP-1 β , MCP-1, IL-10 and IL-4) provided by the kit. Samples were then loaded in the coated ELISA plaque, and were incubated for 2 hours. Then, 3 washes were performed, and the detection antibody was added and incubated for 2 hours. After that, Avidin-HRP was added for 30 min, and 4 washes were performed. Development solution was then added in dark and kept for 15 min, before addition of the stop solution. The secreted levels of the following cytokines and chemokines: IL-12, IL-1 β , IL-6, and TNF- α , MIP-1 α , MIP-1 β , MCP-1, IL-10 and IL-4 were then assessed. The optic density (O.D) was determined at 450 and 570 nm and calculated according to the standard values of a positive control provided by the kit.

2.4.3.7- Immunofluorescence and confocal microscopy

For Immunofluorescence assay, p6 well plates were seeded with THP1 cells and activated as described under section 3. Macrophages were infected with *L.tropica* (5p/c) for 24h and treated with Imiquimod or EAPB0503 for 10 or 24h. At these time points, coverslips were fixed in 4% paraformaldehyde for 20 minutes. Permeabilization was performed in Triton (0.2%) for 10 minutes. Following one PBS wash, blocking for 30 min with PBS-10% FBS was performed. Primary antibody directed against the NF- κ B p65 subunit (Santa Cruz, Sc-8008) was used at the dilution of 1:50. For *Leishmania* parasite staining inside macrophages, an anti-Gp63 (LifeSpan

BioSciences, LS-C58984) was used at the dilution 1:50. Anti-mouse secondary antibodies (Abcam, ab150116) were used at the concentration of 1:100. Staining of nuclei was performed using 1 µg/mL of Hoechst 33342, trihydrochloride trihydrate solution (Invitrogen, H33342) for 5 min and then coverslips were mounted on slides using a Prolong Anti-fade kit (Invitrogen, P36930). Observations were performed on a confocal microscope (Carl Zeiss) and all images were acquired and analyzed using Zeiss LSM 710 software.

2.4.3.8- H&E staining and microscopic visualization of amastigotes upon treatment with Imiquimod or EAPB0503

THP-1 plating, activation, infection and treatment were performed as described under sections 2 and 3. H&E staining was performed as described by Grosset et al., 2017 [210]. Briefly, hematoxylin (Fisher Scientific, Canada) was added on cells, and a counterstaining for 30 seconds was performed followed by a water rinse for 5 minutes. Slides were then dipped in 50% (vol/vol) alcoholic eosin Y solution (Leica Microsystems, Canada) then rinsed in ethanol before slide mounting.

2.4.3.9- Western blot analysis

THP-1 plating, activation, infection and treatment were performed as described under sections 2 and 3. Cells were scrapped, washed with PBS, and pellets were re-suspended in 1x Laemmli buffer. Following denaturation, samples were run on 10% polyacrylamide gels. Proteins were then transferred to nitrocellulose membranes (BIO RAD Cat# 162-0112) at 30V overnight using a BioRad transfer unit. To verify the protein transfer, nitrocellulose membranes were stained with Ponceau Red. Blocking was performed for 1h in 5% of Bovine Albumin serum (BSA) in wash buffer (15 mM Tris-HCL pH8, 150 mM NaCL and 0.05 Tween 20) and probed with specific

primary antibodies against TLR7 (sc- 57463 Santa Cruz Biotechnology, CA, 1:100), NF- κ B p65(sc-8008; Santa Cruz Biotechnology, Inc., Santa Cruz ,CA, 1:250), or p52 (sc-7386, Santa Cruz Biotechnology, CA, 1:250). Equal loading was tested following probing with the anti- GAPDH antibody (MAB5476; abnova, 1:20 000). Nitrocellulose membranes were then washed three times with wash buffer for 5 minutes each, before incubation with the appropriate anti-mouse secondary antibody conjugated to Horseradish peroxidase (HRP) conjugated secondary antibodies (m-IgGk BP-HRP, Santa Cruz, sc-516102, 1:5000). Bands were visualized by autoradiography, following incubation with luminol chemiluminescent substrate (Bio-Rad, Cat# 170-5061).

2.5- Isolation of promastigotes of *L.tropica* from biopsies of CL Patients

Punch biopsy from cutaneous lesion of one CL positive patient (3 or 4 mm of diameter) was performed and incubated in a sterile tube containing 2 mL sterile physiological serum, supplemented with Penicilline G (50 or 100 000 UI/mL). The specimens were cut to small sections and each section was incubated in a semi-solid culture media (consisting of 10 g of agar with 3 g of sodium chloride NaCl, and dissolved in 500 mL of de-ionized water, and autoclaved for 30 min). Parasites were cultured for 2 to 3 weeks. Growing promastigotes were then transferred to a liquid medium (RPMI, Lonza), and split on a weekly basis. Freshly isolated promastigotes were then assessed for their responsiveness to Imiquimod and EAPB0503. Cell and parasite culture, infection, treatment and molecular analysis such as cDNA synthesis and real time PCR were performed as previously described under sections 1, 3, 4.3.1, 4.3.2.and 4.3.3.

2.6- Statistical analysis

Continuous variables were analyzed by the unpaired Student's *t* test. *P* value was determined and values for $p < 0.05$ were considered as significant.

2.7- IRB approval

These samples were collected after approval by the Institutional Review Board of the American University of Beirut and after patients provided informed consent in accordance with the Declaration of Helsinki. The data was collected from de-identified tissue.

Chapter 3

Results

Dr Bonnet's lab has been synthesizing Imiquimod derivatives for more than one decade and validated the potency of certain derivatives, particularly EAPB0503, against many diseases, including melanomas and leukemias [188, 194, 197]. Imiquimod has been introduced by the WHO to the guidelines of treatment of CL after improving the disease progression, when compared to available treatment strategies [183]. To the best of our knowledge, there is no study assessing the effect of Imiquimod against the aggressive *L. tropica* strain. Moreover, no studies have been conducted to elucidate the mechanism of action dictating its anti-parasitic activity. We have investigated this efficacy against promastigotes and amastigotes of *L. tropica* and demonstrated the mechanism of action against these strains. More importantly, one of its analogs, EAPB0503, showed higher anti-tumor activity in many cancer models. We extrapolated this efficiency and tested the effect of EAPB0503 on CL.

Since Imiquimod is known to act *via* activation of TLR7, and since TLRs play major roles in the context of CL, we investigated whether EAPB0503 displays a similar mode of action.

The results section of this work will be divided into two parts:

I-Effect of Imiquimod and its analog EAPB0503 on promastigote and amastigote stages of *L. major* and *L. tropica*

II-Screening of TLRs in the context of CL and effect of Imiquimod and EAPB0503 on these TLRs

3.1-Effect of Imiquimod and its analog EAPB0503 on promastigote and amastigote stages of *L. major* and *L. tropica*

All available treatments against CL are associated with high toxicity rates, high cost, elevated risks of relapse and resistance to drugs; the drug of choice is still out of reach and new strategies must be set. Imiquimod proved potent against *L. major* caused CL. We investigated its effect against the aggressive *L. tropica* strain and characterized its mode of action. Furthermore, and since EAPB0503 has proven a higher anti-tumor potency than Imiquimod in many models, our main aim was to address its potency against CL.

3.1.1- Imiquimod and EAPB0503 affect *L. major* and *L. tropica* promastigotes with a higher potency of EAPB0503 on the aggressive *L.tropica* strain

We have started by testing the efficacy of Imiquimod and its analog EAPB0503, on promastigotes of *L.major* or *L.tropica*. We have treated these motile stages with increasing concentrations of 0.1, 0.5, 1 and 10 μ M of either drugs for up to 3 days. Blinded count based on promastigote motility was performed on a daily basis. Results were expressed as percentage of untreated control (\pm) SD. The percentage of parasites mobility of both strains decreased in a concentration dependent manner upon treatment with both drugs (Figure 22A, B, C, D). In more details, for imiquimod drug, the motility decreased by 50% upon treatment with 10 μ M after 24h, and with 1 μ M at 48h for *L. major* (Figure 22A). Furthermore, Imiquimod was only capable of decreasing the motility of around 60% of promastigotes after 3 days of treatment (Figure 22A). Longer treatment time points for up to five days didn't lead to less motility (data not shown). In case of *L. tropica*, Imiquimod showed better results whereby motility decreased by 50% at 0.1 μ M and 48h post-treatment (Figure 22C).

We then addressed the effect of EAPB0503 on promastigotes' motility of both strains. Our results indicate a higher potency of this analog on *L.major* promastigotes, with a 50% inhibition of motility at 0.1 μM obtained at 48h post-treatment (10 folds lower than imiquimod at the same time point) (Figure 22B). This effect was less prominent when EAPB0503 was tested on *L. tropica* promastigotes and whereby the mobility decreased by 50% at 1 μM after 48h of treatment (10 folds higher than imiquimod at the same time point) (Figure 22D).

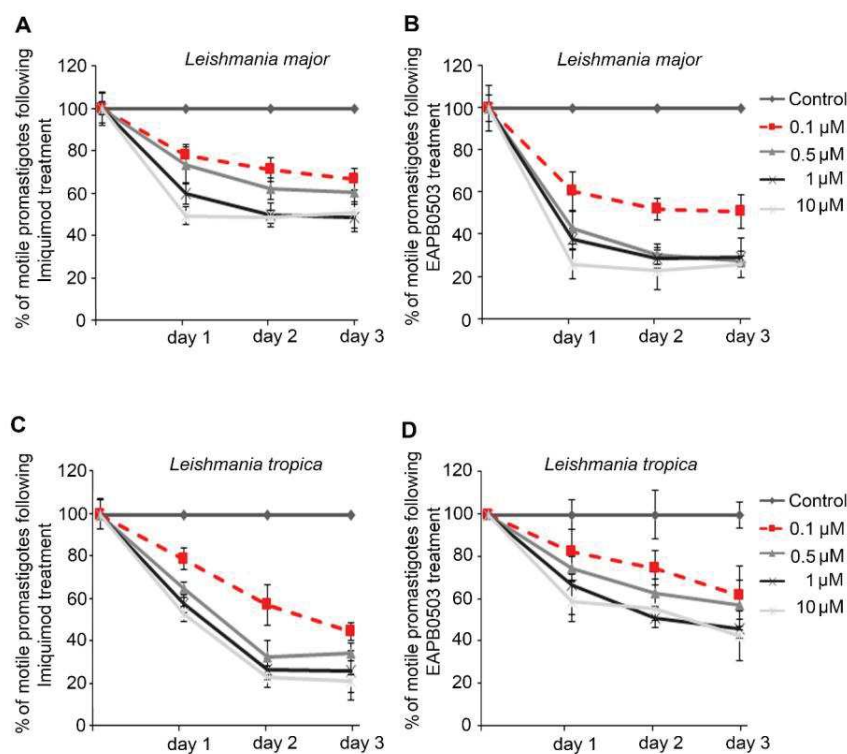


Figure 22: Imiquimod and EAPB0503 affect *L.major* and *L.tropica* promastigotes' motility. Effect of different concentrations of Imiquimod (A, C) and EAPB0503 (B, D) on promastigotes of *L.major* and *L.tropica* respectively. Blind count of the parasites based on their motility was performed on a daily basis. Results are expressed as percentage of untreated control (\pm SD).

The question that followed was to rule out whether the observed results with either drugs was due to a static or to a leishmanicidal effect. For that purpose, we have assessed the capacity of infection of macrophages by treated promastigotes with 0.1 μM of either drugs, being the lowest

dose obtained with one of both drugs, and showing an effect in our previous assay. Briefly, *L. major* or *L. tropica* promastigotes were treated for 72h with 0.1 μ M of Imiquimod or EAPB0503. Then, treated promastigotes of both strains were put on activated macrophages for 24h. Cells were washed thoroughly and the infection capacity of treated promastigotes was assessed by their capacity to transform into intracellular amastigote stages. Then amastigotes were quantified using primers addressed against the kinetoplast marker by quantitative RT-PCR. Interestingly, our results showed that *L. major* treated promastigotes with either Imiquimod or EAPB0503 lose their capacity of infecting macrophages only by 50%, seemingly showing that there is a combination of both static and leishmanicidal role of these drugs on *L. major* promastigotes (Figure 23A). Strikingly, the most prominent effect on the infection capacity of *L. tropica* promastigotes was obtained upon treatment with EAPB0503 (Figure 23B) whereby only 20% of infection was observed as compared to 60% infection with Imiquimod treated promastigotes (Figure 23A). This result is not in line with the motility assay where Imiquimod showed a better effect, but strongly suggests a leishmanicidal effect of EAPB0503 on *L. tropica* promastigotes.

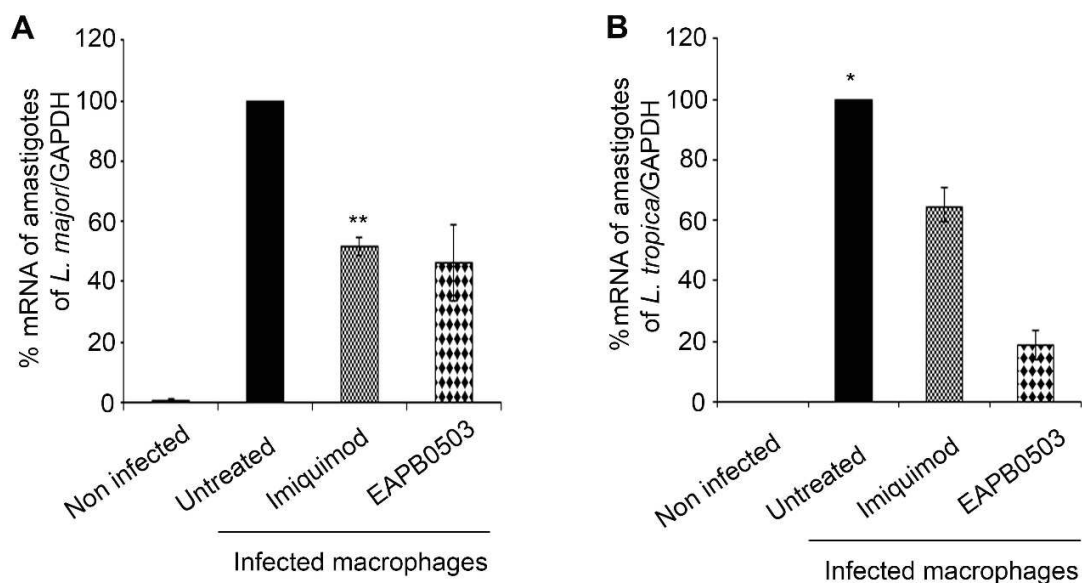


Figure 23: Imiquimod and more importantly EAPB0503 affect macrophage invasion by promastigotes of *L.tropica* strain. (A, B) Capacity of the treated of *L.major* and *L.tropica* promastigotes respectively to infect macrophages. To investigate the capacity of treated promastigotes to infect macrophages, the IC₅₀ of 0.1μM of EAPB0503, obtained on the more susceptible strain *L. major* was adopted. 10⁶ Promastigotes were treated with 0.1 μM of Imiquimod or EAPB0503 for 3 days, and their ability to infect macrophages was evaluated by Syber green RT-PCR using kinetoplast specific primers. Percentage of expression of amastigotes was normalized to GAPDH. Results are expressed as percentage of untreated control (±) SD and are representative of three independent experiments. The t-test was performed to validate significance. *, ** and *** indicate p values ≤ 0.05; 0.01 and 0.001, respectively. P-values less than 0.05 were considered significant.

3.1.2- EAPB0503 exhibits a higher potency than Imiquimod on amastigotes replication:

3.1.2.1- EAPB0503 displays a similar activity than Imiquimod against *L. major* amastigotes replication

To compare the effect of EAPB0503 and Imiquimod on *L. major* amastigotes, THP-1 derived macrophages were infected at the ratio of 5 parasites: 1 cell. Treatment was performed with ascending concentrations (0.01, 0.05, 0.1, 0.5, and 1 μM) of either Imiquimod or its analog EAPB0503 for 24 hours. Amastigotes were then quantified by real time quantitative PCR using kinetoplast specific primers.

Our results demonstrated that both drugs exhibit a similar and prominent effect on amastigote replication at the concentration of 1μM (Figure 24A and B) against *L.major* strain (Figure 24A and B).

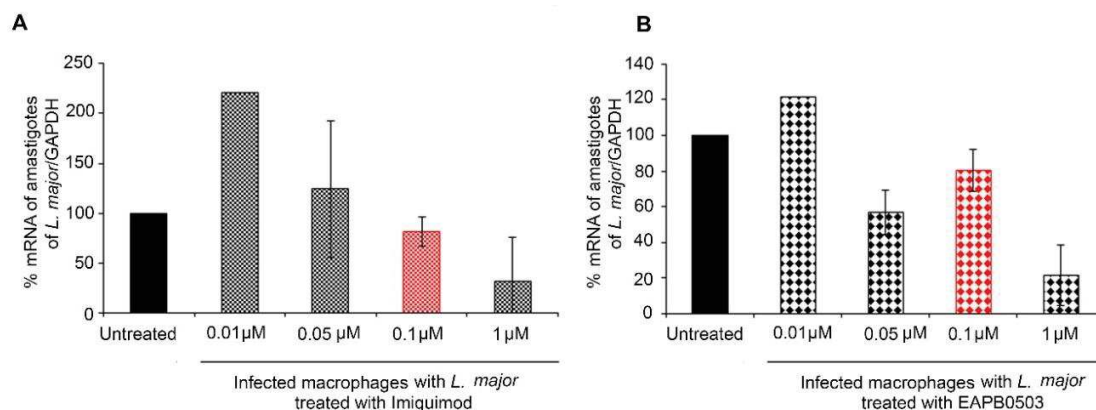


Figure 24: EAPB0503 displays a similar effect on amastigote replication against *L. major* strain as compared to Imiquimod. Real-time quantitative PCR detection of infected macrophages with *L. major* amastigotes treated with different concentrations of Imiquimod (A) or EAPB0503 (B). Briefly, differentiated THP-1 into macrophages were infected with *L. major* or *L. tropica* at the ratio of 5 parasites/cell for 24h. Treatment with 0.1, 0.5, 1 or 10 μM of Imiquimod or its analog EAPB0503 was performed for 24h. The results are shown as percentage of untreated infected macrophages. Amastigote transcripts were evaluated by Syber green RT-PCR using kinetoplast specific primers and their percentage of expression was normalized to GAPDH. Results are expressed as percentage of untreated control (±) SD and are representative of at least three independent experiments.

3.1.2.2- EAPB0503 displays a more potent activity than that of Imiquimod against the most aggressive *L. tropica* amastigotes

L. tropica is the most aggressive and most endemic strain in the Middle East area [20]. It has been associated with 85% of CL cases in this region [62]. To compare the effect of EAPB0503 and Imiquimod on *L. tropica* amastigotes, we followed the similar approach performed for *L. major* strain. THP-1 derived macrophages were infected at the ratio of 5 parasites: 1 cell. Treatment was performed with ascending concentrations (0.01, 0.05, 0.1, 0.5, and 1 μM) of either Imiquimod or its analog EAPB0503 for 24 hours. Amastigotes were then quantified by real time quantitative PCR using kinetoplast specific primers. Our results demonstrated that both drugs exert an anti-amastigote activity in a concentration dependent manner. The doses of 0.01 and 0.05 were not efficient against the infection. Strikingly, an IC₅₀ of 0.1 μM was obtained upon treatment with EAPB0503 as compared to that of Imiquimod obtained at 1 μM (10 folds higher than EAPB0503)

(Figure 25A and B). This significant decrease obtained with EAPB0503 was more prominent at 0.5 and 1 μ M, and was more prominent than the same doses of Imiquimod (Figure 25A and B). **This promising data clearly shows a different response of leishmanial strains to treatment with a better response obtained upon treatment of the most aggressive *L. tropica* strain with EAPB0503.** The IC₅₀ of 0.1 μ M was adopted for the remainder of the study.

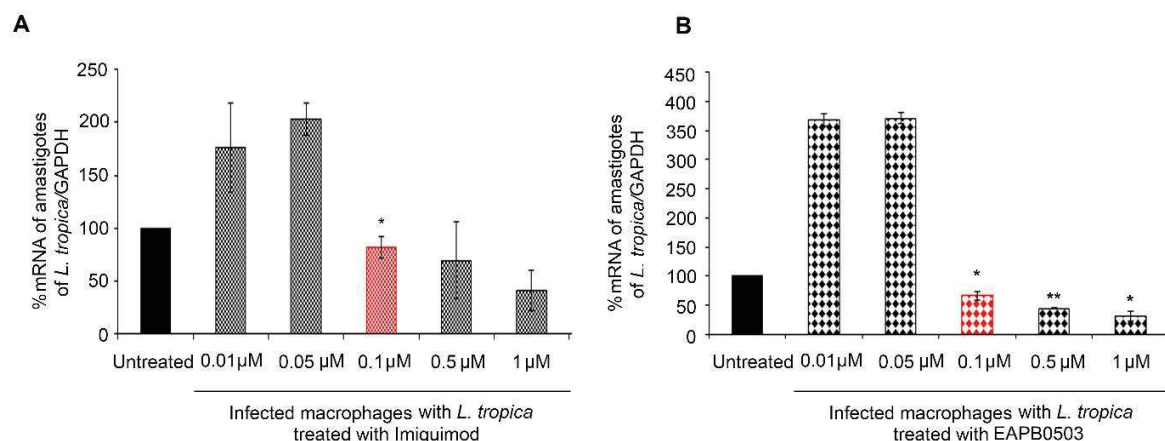


Figure 25: EAPB0503 displays a better effect on *L.tropica* amastigote replication than Imiquimod, and in a concentration and time dependent manner. Real-time quantitative PCR detection of infected macrophages with *L.tropica* amastigotes treated with different concentrations of Imiquimod (A) or EAPB0503 (B). Briefly, differentiated THP-1 into macrophages were infected with *L.major* or *L.tropica* at the ratio of 5 parasites/cell for 24h. Treatment with 0.1, 0.5, 1 or 10 μ M of Imiquimod or its analog EAPB0503 was performed for 24h (A-B). The results are shown as percentage of untreated infected macrophages. Amastigote transcripts were evaluated by Syber green RT-PCR using kinetoplast specific primers and their percentage of expression was normalized to GAPDH. Results are expressed as percentage of untreated control (\pm) SD and are representative of at least three independent experiments. The t-test was performed to validate significance. *, ** and *** indicate p values \leq 0.05; 0.01 and 0.001, respectively. P-values less than 0.05 were considered significant.

3.1.2.3- EAB0503 affects amastigote replication as early as 10h post-treatment

After testing different concentrations of Imiquimod or EAPB0503 on amastigotes of *L.major* and *L.tropica* strains at 24h, we wanted to examine the effect of these compounds at an earlier time point of 10h post-treatment. We chose the concentration of 0.1 μ M, showing a prominent effect against the most aggressive *L. tropica* strain. Our results showed that amastigotes

transcription levels decreased in a time-dependent manner upon treatment of infected macrophages by either strains. Imiquimod induced a decrease in *L. major* amastigotes expression by 50% at 10h post-treatment, and by 65% at 24h post-treatment (Figure 26A). More interestingly, EAPB0503 showed a more prominent decrease of amastigotes expression at 10h or 24h post-treatment, whereby only 10% of amastigote transcripts were detected by RT-PCR (5 folds less than what we obtained with Imiquimod) (Figure 26A).

In case of *L.tropica*, Imiquimod was able to reduce amastigotes transcription level to 60% at 10h post-treatment and to around 20% at 24h post-treatment, as compared to infected and untreated macrophages (Figure 26B). Strikingly, EAPB0503 showed a better effect than Imiquimod with a reduced amastigotes transcript levels to 30% (almost 3 folds less than Imiquimod effect at the same time-point) at 10h post-treatment and to 10% (around 2 folds less than Imiquimod effect at this same time-point) at 24h post-treatment (Figure 26B). **Altogether, these data show a very promising potency of the EAPB0503 against amastigote replication, at the low dose of 0.1 μ M and as early as 10h, when compared to Imiquimod.**

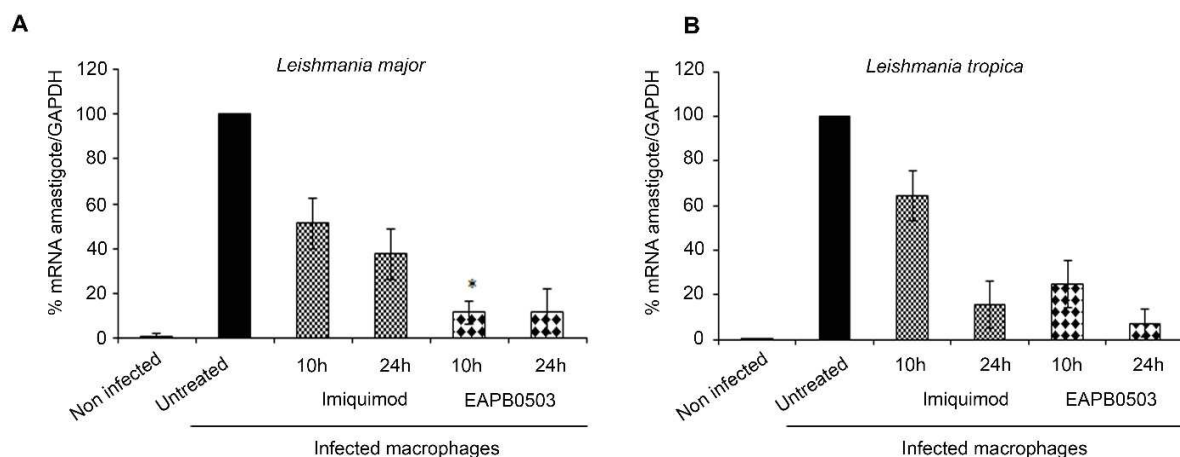


Figure 26: EAPB0503 affects *L.major* and *L.tropica* amastigote replication in a time dependent manner and as early as 10h post-treatment. Real-time quantitative PCR detection of infected macrophages with *L. major* (A) or *L.tropica* (B) amastigotes treated with 0.1 μ M of

Imiquimod or EAPB0503 for 10 and 24h. Briefly, differentiated THP-1 into macrophages were infected with *L. major* or *L. tropica* at the ratio of 5 parasites/cell for 24h. Treatment with 0.1 μ M of Imiquimod or EAPB0503 was performed for 10 and 24h (A, B). The results are shown as percentage of untreated infected macrophages. Amastigote transcripts were evaluated by Syber green RT-PCR using kinetoplast specific primers and their percentage of expression was normalized to GAPDH. Results are expressed as percentage of untreated control (\pm) SD and are representative of at least three independent experiments. The t-test was performed to validate significance. *, ** and *** indicate p values \leq 0.05; 0.01 and 0.001, respectively. P-values less than 0.05 were considered significant.

3.1.2.4- The anti-amastigote activity of Imiquimod and EAPB0503 is mediated by the canonical NF- κ B pathway activation following an increase in TLR-7 expression and triggering an anti-leishmanial immune response:

3.1.2.4.1- Imiquimod and EAPB0503 lead to an increase of TLR7 expression in *L. tropica* infected macrophages:

In this section, we narrowed our analysis to one *Leishmania* strain, *L. tropica*, being the most aggressive in the Middle East region [20]. We adopted the concentration of 0.1 μ M and both time points 10 and 24h post-treatment (as optimized and described in the above section).

Imiquimod belongs to the class of Toll-like receptor (TLR) agonists with high affinity to TLR7/8 and probably 6 [191, 211, 212, 213]. It has been documented in many studies that Imiquimod can exert its biological activity *via* modulation of variable signaling pathways [38], one main of which, is the activation of the canonical NF- κ B pathway [214]. In more details, It is known that imiquimod activates immune cells by ligating the Toll-like receptor 7 (TLR7), commonly involved in pathogen recognition, on the cell surface [190]. Cells activated by imiquimod *via* TLR7, secrete cytokines (primarily IFN- α , IL-6, and TNF- α) [190]. We wanted to decorticate the molecular mechanisms underlying the potency of Imiquimod and more importantly its analog EAPB0503 against *L. tropica* amastigotes. We started by assessing the expression of TLR7 in the context of the treatment by either drugs. Our results showed that TLR7 protein level increased after treatment with either drugs, in comparison to uninfected or untreated infected

macrophages (Figure 27). The highest level of upregulation was obtained upon treatment with Imiquimod for 10h. TLR7 protein expression decreased 24h post-treatment but remained higher than in non-treated *L.tropica* infected macrophages (Figure 27). Similar results were obtained upon treatment with EAPB0503, with a higher protein expression of TLR7 at 10h and a decrease in its expression to similar levels of untreated infected macrophages. **Taking into account that the highest obtained TLR7 expression level was upon 10h post-treatment with Imiquimod, our results confirm the mechanism of action of this drug *via* this receptor, but in the context of CL. The lower expression level obtained for EAPB0503, seemingly shows that these drugs may have different modes of action, or may act through different TLRs.**

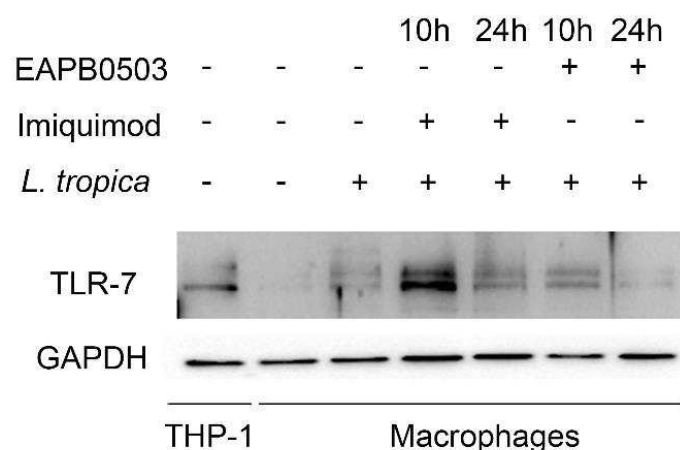


Figure 27: The anti-amastigote effect of Imiquimod and to a lesser extent EAPB0503 induces an increase in TLR7 expression. Western blot analysis for TLR7 in *L.tropica* infected macrophages treated with 0.1 μ M of Imiquimod or EAPB0503 for 10 and 24h. The results depict one representative experiment among three independent ones.

3.1.2.4.2- The anti-amastigote effect of Imiquimod and EAPB0503 is mediated by the canonical NF- κ B pathway activation

It has been widely documented that following recognition of specific pathogen antigens, TLRs trigger the activation of the NF- κ B pathway [117]. This TLR signaling induces the phosphorylation of I κ B α triggering its degradation, and leading to the release and translocation of

NF- κ B dimers to the nucleus (Figure 17) [118, 119]. This translocation activates gene transcription implicated in many functions including the immune and inflammatory response [215].

Our results have shown that Imiquimod and to a lesser extent, EAPB0503, triggers a higher expression of TLR7 in *L.tropica* infected macrophage (Figure 27). This led us to investigate whether treatment with either drugs acts through the activation of NF- κ B pathway. We have tested different subunits of this complex. Western blot analysis clearly showed an activation of an upstream multimeric IKK complex (IKK α /IKK β) (Figure 28A) and an induction of the phosphorylated form of the I κ B α , presumably leading to its degradation (Figure 28B), upon treatment for 10 or 24h with Imiquimod or EAPB0503. We then examined whether the activation involves the canonical or non-canonical NF- κ B pathway. This led us to test the p50 and p65 subunits known to belong to the canonical pathway. Our results demonstrate that the p50 subunit was upregulated especially upon 24h post-treatment with either drugs (Figure 28B).

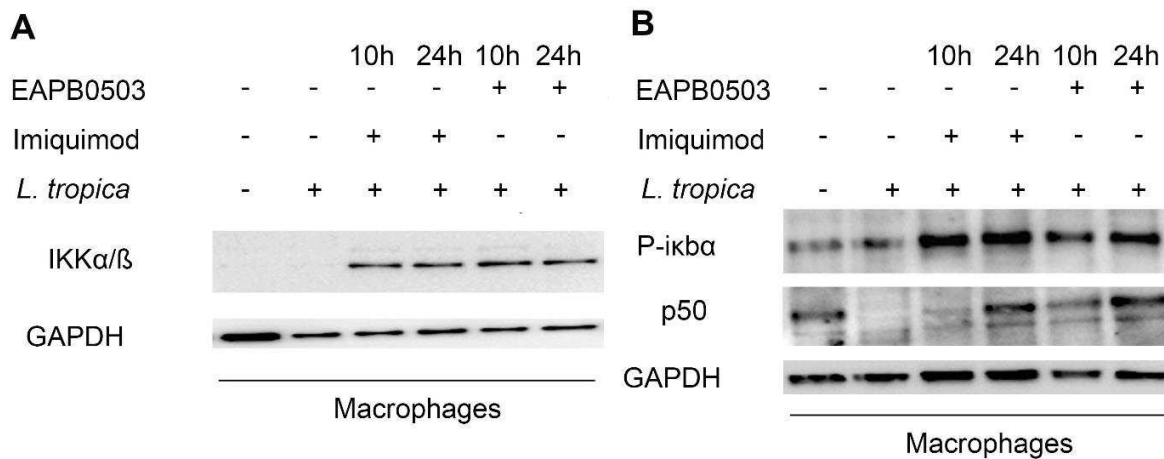


Figure 28: The anti-amastigote effect of Imiquimod and EAPB0503 is mediated by the canonical NF- κ B pathway activation, following an increase in TLR7 expression. Western blot analysis for IKK α / β (A), P-I κ B α and P50 (B) in *L.tropica* infected macrophages treated with 0.1 μ M of Imiquimod or EAPB0503 for 10 and 24h. The results depict one representative experiment among three independent ones.

We further investigated the effect of Imiquimod and EAPB0503 on the nuclear translocation of p65 which represents the active NF- κ B subunit by confocal microscopy. In comparison to non-infected or non-treated but infected macrophages, Imiquimod and EAPB0503 induced a nuclear translocation of p65 at both 10 and at 24h time points (Figure 29).

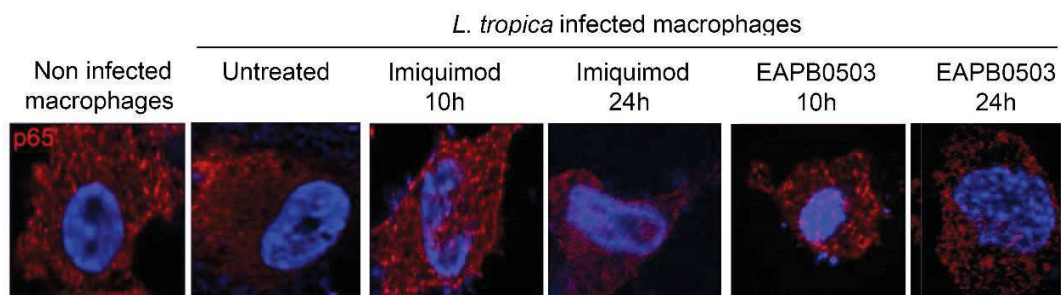


Figure 29: The anti-amastigote effect of Imiquimod and EAPB0503 is mediated by the canonical NF- κ B pathway activation, following an increase in TLR7 expression. Confocal microscopy on *L.tropica* infected macrophages treated with 0.1 μ M of Imiquimod or EAPB0503 for 10 and 24h. The NF- κ B p65 subunit was stained with an anti-p65 antibody (red), and nuclei were stained with DAPI (blue). The results depict one representative experiment among three independent ones.

3.1.2.4.3- Imiquimod and EAPB0503 trigger an anti-leishmanial immune response following activation of the canonical NF- κ B pathway and i-NOS production

It has been well documented that after recognition of specific pathogen antigens, TLRs trigger the activation of the NF- κ B pathway leading promotes the transcription and synthesis to pro-inflammatory cytokines production [117].

Our data has so far demonstrated TLR7 upregulation, and canonical NF- κ B activation upon treatment of *L.tropica* infected macrophages with Imiquimod or EAPB0503. This led us to investigate the resulting secretion levels of pro-inflammatory and anti-inflammatory cytokines in the context of the treatment. Our results clearly indicate an up regulation of the Macrophage Inflammatory Proteins (MIP-1 α and β) upon treatment with Imiquimod for 10h. These proteins

are known to be produced by macrophages, to recruit immune responses to the site of infection (e.i. pro-inflammatory cytokines such as IL-6, IL-1 β and Tumor necrosis factor TNF- α) [216]. This increase in both proteins was significant upon 10h treatment with EAPB0503 (Figure 30A and B). Furthermore, this increased secreted level was sustained at 24h treatment with either drug.

We then examined the Monocyte Chemoattractant Protein (MCP-1) levels, known to be secreted by monocytes/macrophages and crucial for the regulation of their migration and infiltration [217]. Our results indicate that MCP-1 levels increase upon at 10h treatment with Imiquimod and further increase after 24h of treatment (Figure 30C). More importantly, EAPB0503 induced higher levels of secreted MCP-1, when compared to Imiquimod, at both 10h and 24h post-treatment with a significantly sustained increase at 24h post-treatment (Figure 30C).

We then measured the secretion levels of depicted pro-inflammatory cytokines known to be important in the context of clearance of CL, namely IL-12, IL-1 β , TNF- α and IL-6 [148]. In CL, production of IL-12 by macrophages and dendritic cells (DC) is linked to the resistance against Leishmaniasis. Our results showed a gradual increase in IL-12 secretion upon 10 then 24h of treatment with Imiquimod (Figure 30D). Strikingly, upon treatment with EAPB0503, IL-12 increased significantly to reach its highest levels upon 24h post-treatment, as compared to untreated infected macrophages or Imiquimod treated macrophages. We then looked at the IL-1 β and TNF- α secretion levels. Our results indicated an increase of both pro-inflammatory cytokines secretion levels upon 10h treatment with either drugs; this increase was sustained at 24h post-treatment and was higher upon treatment with EAPB0503 (Figure 30E and F).

We then examined IL-6 secretion levels and showed that this cytokine displayed a different pattern than the previously discussed pro-inflammatory cytokines. IL-6 secretion levels increased at 10h post treatment with either Imiquimod or EAPB0503. These levels dropped at 24h to reach

undetectable levels upon treatment with EAPB0503 (Figure 30G). **Altogether, our data clearly demonstrate an increase of pro-inflammatory cytokines in response to treatment with Imiquimod or its analog EAPB0503, presumably indicating a role in the clearance of the infection.**

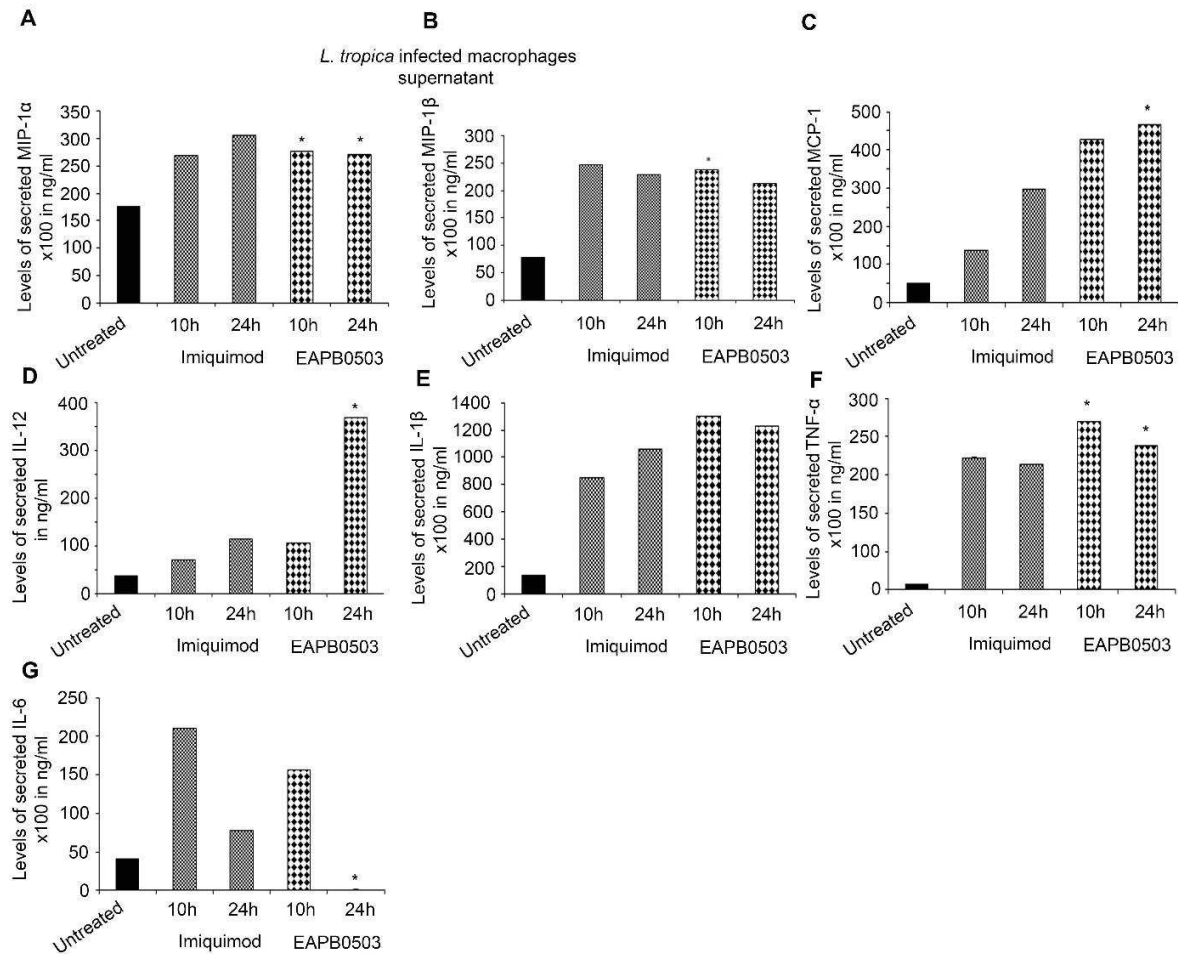


Figure 30: The anti-amastigote effect of Imiquimod and EAPB0503 is mediated by the canonical NF- κ B pathway activation resulting in a higher pro-inflammatory cytokine immune response. ELISA showing the secretion level of the pro-inflammatory cytokines MIP-1 α (A), MIP-1 β (B), MCP-1 (C), IL-12 (D), IL-1 β (E), TNF- α (F), and IL-6 (G) upon treatment of *L.tropica* infected macrophages with 0.1 μ M of Imiquimod or EAPB0503 for 10 and 24h. Results are expressed as percentage of untreated control (\pm) SD and are representative of at least three independent experiments. The t-test was performed to validate significance. *, ** and *** indicate p values \leq 0.05; 0.01 and 0.001, respectively. P-values less than 0.05 were considered significant.

It has been well documented that Macrophage derived NO acts as both a highly cytotoxic molecule, generated in response to microbial stimuli, pro-inflammatory cytokines, and as a regulatory molecule that controls T lymphocyte proliferation and cytokine secretion during adaptive immune responses [141]. Inducible iNOS is a Ca^{2+} independent defense mechanism initiated by many cell types, particularly macrophages to produce NO [141]. It is expressed in response to pro-inflammatory cytokines (e.g. IFN- γ , IL-1 β , TNF- α) [142]. In leishmanial infection, the action of iNOS in the regulation of innate immunity has been identified in the protective response of mice to *Leishmania major*. This initial response is critical for the containment of the infection [143].

With the increased secreted levels of pro-inflammatory cytokines we obtained upon treatment of *L.tropica* infected macrophages with Imiquimod or EAPB0503, it was worth investigating whether this induces NO production by macrophages. This led us to assess iNOS production by measuring the transcript levels upon treatment with either drugs. **Our data demonstrated that both drugs led to iNOS increased transcripts at both time points (10 and 24h) with a very prominent effect at 10h post treatment with EAPB0503** (Figure 31).

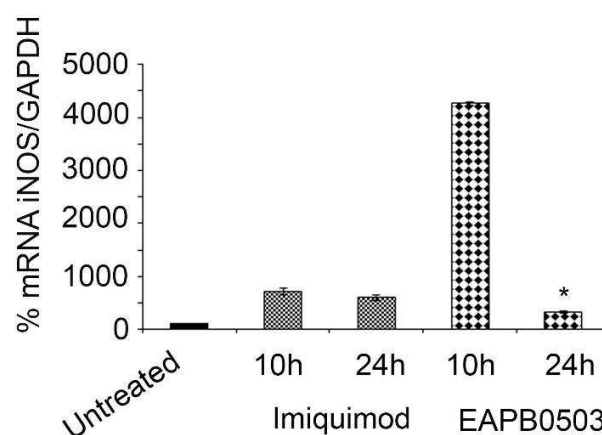


Figure 31: Imiquimod and EAPB0503 induce higher iNOS transcript levels explaining their leishmanicidal activity. Real-time quantitative PCR detection of iNOS in infected macrophages with *L.tropica* amastigotes treated with 0.1 μM of Imiquimod or EAPB0503 for 10 and 24h. iNOS

percentage of expression was normalized to GAPDH. Results are expressed as percentage of untreated control (\pm) SD. The t-test was performed to validate significance. *, ** and *** indicate p values ≤ 0.05 ; 0.01 and 0.001, respectively. P-values less than 0.05 were considered significant.

In CL, pro-inflammatory cytokine production is linked to the resistance against leishmaniasis; whereas production of anti-inflammatory cytokines, such as IL-4, IL-10 and TGF- β , has been related with parasite replication, disease susceptibility and progression [151, 218, 219, 220]. After showing an increase in the pro-inflammatory cytokine production in response to treatment with EAPB0503, we examined the secretion levels of two depicted anti-inflammatory cytokines, IL-10 and IL-4. In comparison to non-treated *L.tropica* infected macrophages, secretion levels of IL-10 decreased by 4 folds, 10h after treatment with either drugs and this decrease was sustained by 24h post-treatment (Figure 32A). Similarly, secretion levels of IL-4 decreased by 3.75 folds and 5 folds than non-treated macrophages, at 10h and 24h post-treatment with Imiquimod respectively. More interestingly, treatment with EAPB0503 showed a significant decrease by 15 folds at 10h post-treatment as compared to non-treated infected macrophages. This decrease was sustained at 24h post-treatment (Figure 32B). **Collectively, our results show that NF- κ B activation by Imiquimod and EAPB0503 induces an upregulation of pro-inflammatory cytokines. This leads to i-NOS upregulation, presumably leading to NO production and leishmanicidal activity. In addition, and concomitantly with the upregulation of pro-inflammatory cytokines, a decrease in the anti-inflammatory cytokines is obtained, diminishing macrophage susceptibility to *L.tropica* infection, and dictating the leishmanicidal effect of the tested drugs.**

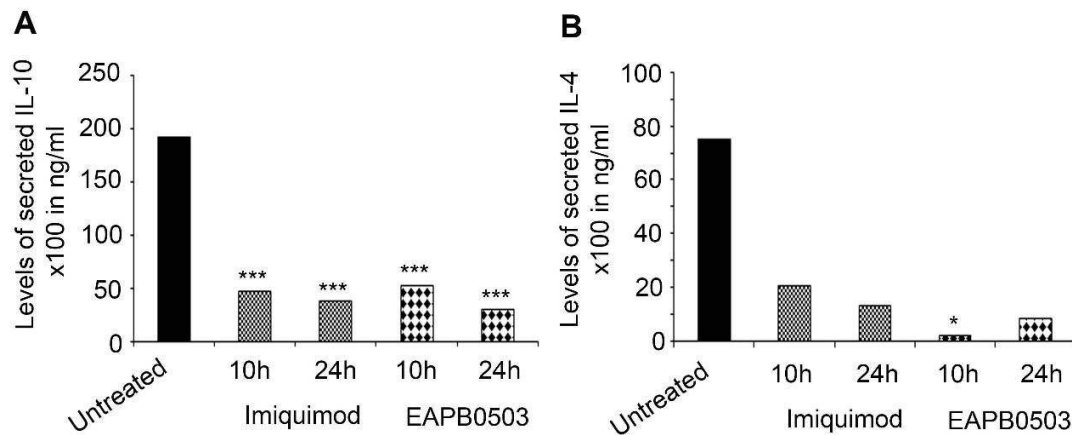


Figure 32: Imiquimod and EAPB0503 trigger a decrease in the anti-inflammatory cytokine response. ELISA showing the secretion level of the anti-inflammatory cytokines (IL-10 and IL-4) (A, B respectively) upon treatment of *L.tropica* infected macrophages with 0.1 μ M of Imiquimod or EAPB0503 for 10 and 24h. Results are expressed as percentage of untreated control (\pm) SD and are representative of at least three independent experiments. The t-test was performed to validate significance. *, ** and *** indicate p values ≤ 0.05 ; 0.01 and 0.001, respectively. P-values less than 0.05 were considered significant.

3.1.2.5- EAPB0503 potentially affects freshly isolated *L.tropica* from patients' biopsies when compared to Imiquimod

To eliminate any discrepancy on the susceptibility of cultured *L. tropica* and *L. major* strains to our tested treatments due to a variation in genetic drift (e.i less virulent strains after culture for a long time), we have investigated the efficacy of Imiquimod and its analog EAPB0503 on freshly isolated parasites from untreated patients' biopsies. For that purpose, we used an optimized protocol for extraction of *Leishmania* amastigotes from biopsies of untreated patients (developed at CRHU, Montpellier; unpublished data). Briefly, punch biopsy from cutaneous lesions (3 or 4 mm of diameter) of 4 CL patients was performed and cut to small sections. Each section was incubated in a semi-solid culture media, consisting of agar and blood isolated from rabbit. Parasites were cultured for 2 to 3 weeks and the freshly isolated promastigotes were then be assessed for their responsiveness to Imiquimod and EAPB0503.

3.1.2.5.1- Imiquimod and EAPB0503 display a prominent effect against *L. tropica* promastigotes freshly isolated from patients' biopsies

We have tested the efficacy of Imiquimod and its analog EAPB0503, on freshly isolated promastigotes of *L. tropica* from four patients' biopsies. We have tested increasing concentrations of 0.1, 0.5, 1 and 10 μM of Imiquimod or EAPB0503 for up to 3 days. Blinded count based on promastigote motility was performed on a daily basis. Results were expressed as percentage of untreated control (\pm) SD. The percentage of parasites motility from all patients' biopsies decreased in a concentration dependent manner upon treatment with both drugs (Figure 33A, B). The concentration that led to 50% of inhibition of motility was obtained at 0.5 μM of Imiquimod drug and at 0.1 μM of EAPB0503 (five folds less than imiquimod) after 48h of treatment (Figure 33A and B).

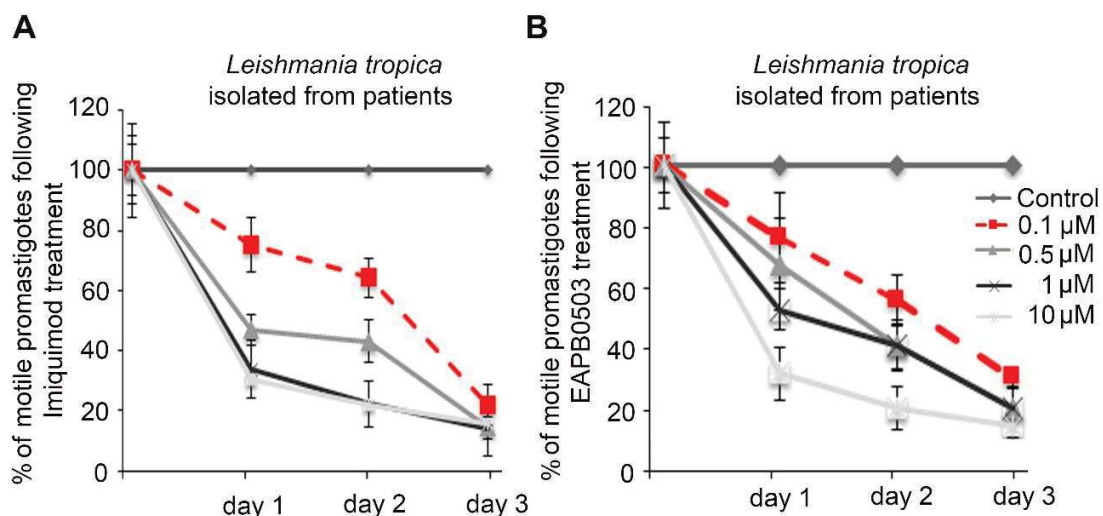


Figure 33: EAPB0503 displays a prominent inhibition of motility against freshly isolated *L. tropica* from patients' biopsies when compared to Imiquimod. Effect of different concentrations of Imiquimod or EAPB0503 on promastigotes of *L. tropica* freshly isolated from three patients' biopsies. Results are expressed as percentage of untreated control (\pm) SD and are representative of three independent experiments.

3.1.2.5.2- Imiquimod and EAPB0503 exhibit a prominent effect on the replication of *L. tropica* amastigotes freshly isolated from patients' biopsies

We then compared the effect of EAPB0503 and Imiquimod on *L. tropica* amastigotes derived from promastigotes freshly obtained from patients' biopsies. THP-1 derived macrophages were infected at the ratio of 5 parasites: 1 cell. Treatment was performed at 0.1 μ M, for 10 and 24h with either Imiquimod or EAPB0503. Amastigotes were then quantified by real time quantitative PCR using kinetoplast specific primers. Our results demonstrated that both drugs affect the replication of amastigotes in a time dependent manner. The transcription level of amastigotes in infected macrophages treated with Imiquimod decreased by 30 and 55% at 10 and 24h post-treatment respectively, when compared to non-treated infected macrophages (Figure 34). More importantly, treatment with EAPB0503 induced a more prominent effect whereby, at 10h post-treatment, the transcription level of *L.tropica* amastigotes decreased by 70 and 90% at 10 and 24h post-treatment (Figure 34). **This promising data clearly shows that EAPB0503 is more potent than Imiquimod on *L.tropica* amastigotes derived from freshly isolated promastigotes of patients' biopsies.**

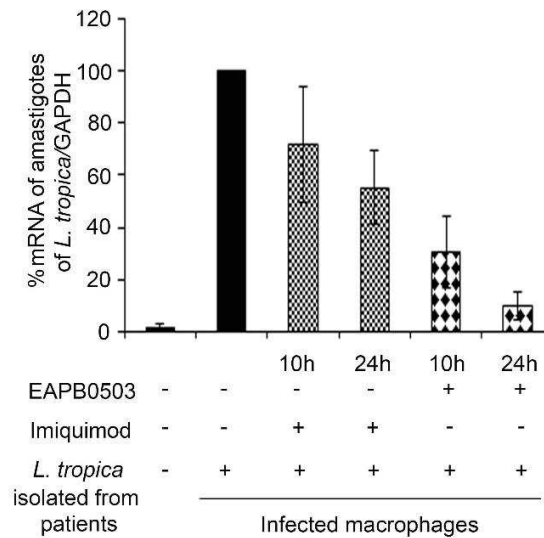


Figure 34: EAPB0503 potentially affects the replication of amastigotes derived from freshly isolated *L.tropica* from patients' biopsies when compared to Imiquimod. Real-time quantitative PCR detection of infected macrophages with patients' isolated *L.tropica* amastigotes treated with 0.1 μ M of Imiquimod or EAPB0503 for 10 or 24h. Percentage of expression of amastigotes was normalized to GAPDH. Results are expressed as percentage of untreated control (\pm) SD and are representative of three independent experiments.

3.1.2.5.3- Histological analysis confirms the high potency of Imiquimod and more importantly EAPB0503, on the replication of fresh amastigotes

In order to confirm the anti-amastigote effect of Imiquimod and EAPB0503 on the freshly virulent *L.tropica* strain isolated from patients, amastigotes inside macrophages were stained by H&E. Our results were very consistent with the real-time PCR data. Following treatment with Imiquimod or EAPB0503, at 10 and 24h, infected macrophages presented less amastigotes, marked as purple dots, in comparison with the non-treated infected macrophages (Figure 35).

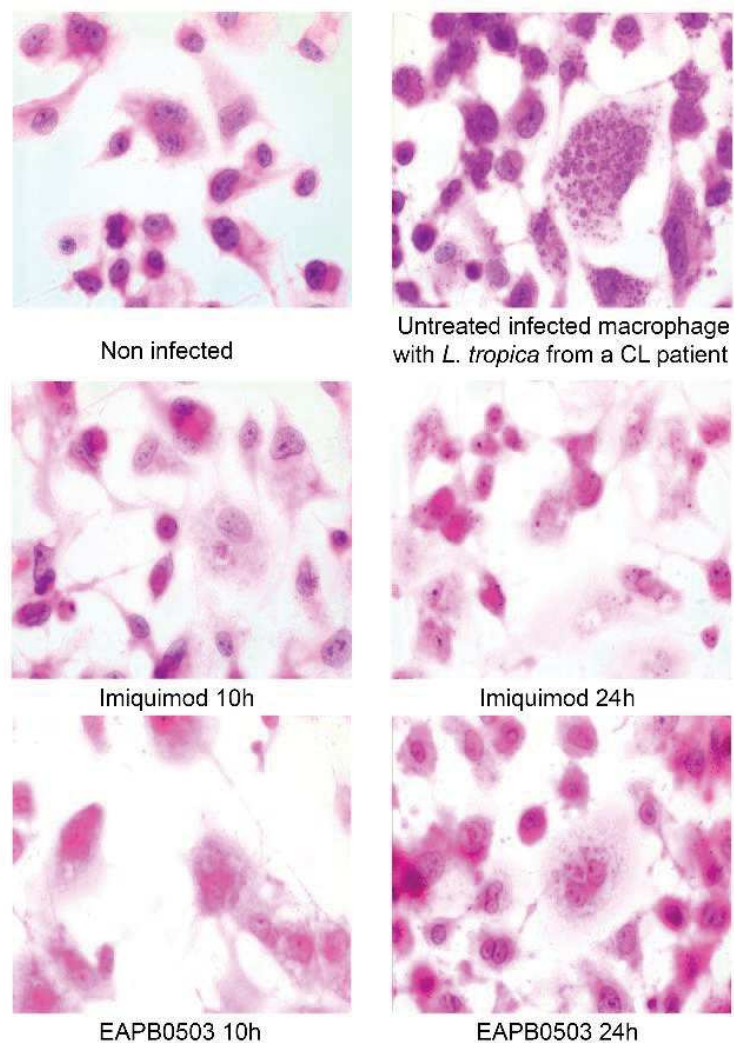


Figure 35: H&E staining on untreated or treated macrophages infected with patients' isolated *L.tropica* amastigotes with 0.1 μ M of Imiquimod or EAPB0503 for 10 or 24h. The results depict one representative patient. Similar results were obtained on the remaining three patients.

The leishmanicidal effect of Imiquimod and EAPB0503 on amastigotes of patient-derived *L.tropica* was further confirmed by confocal microscopy. We have used the Glycoprotein Gp63 marker, which is known to contribute in parasite binding on macrophages [97, 98]. In one representative field, and in comparison to non-treated *L.tropica* amastigotes, Imiquimod treatment for 10h led to a decrease in amastigotes percentage represented by Gp63 inside macrophages (Figures 36A and B). In more details, this decrease reached 61% at 10h and 35% at 24h post-

treatment. More interestingly, EAPB0503 induced a more important decrease in amastigotes number, to 39% 10h following treatment, and further decreased to 24% 24h post-treatment. Consistently with the transcript data, EAPB0503 was able to induce a more prominent decrease in amastigotes upon treatment of 10h or 24h as compared to untreated infected macrophages with *L.tropica* as well as Imiquimod (Figures 36A and B).

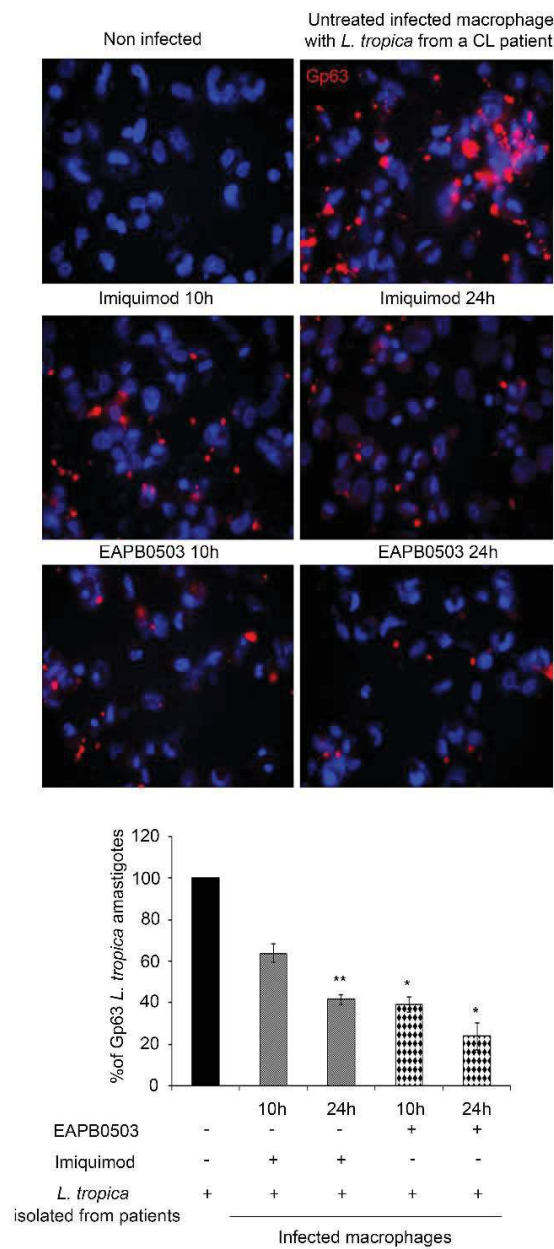


Figure 36: Imiquimod and EAPB0503 trigger an anti-amastigote activity against *L.tropica*. A. Confocal microscopy on patients' derived *L.tropica* infected macrophages treated with 0.1 μ M of Imiquimod or EAPB0503 for 10 and 24h. B. Blind count of Gp63 (reflecting amastigotes) in *L.tropica* infected macrophages treated with 0.1 μ M of Imiquimod or EAPB0503 for 10 and 24h. Results are expressed as percentage of amastigotes in non-treated *L.tropica* infected macrophages. The amastigote marker GP63 was stained with an anti-GP63 antibody (red), and nuclei were stained with DAPI (blue). The results depict one representative experiment among three independent ones.

Altogether, these data show a very promising potency of the EAPB0503 at the low dose of 0.1 μ M and as early as 10h on patients' derived *L.tropica* stages, confirming the *in vitro* results obtained on cultured strains.

3.1.2.5.4- Transcription level of i-NOS in patients' derived *L.tropica* infected macrophages treated with Imiquimod and EAPB0503

To rule out whether the same mechanism of action of either drugs is obtained on fresh stages of *L. tropica*, we tested i-NOS levels at 10 and 24h post-treatment of infected macrophages. Both drugs induced an increase in i-NOS transcript levels at both time points (Figure 37). More importantly, 10h post-treatment with EAPB0503, transcription levels of i-NOS increased tremendously to 40 folds higher than the non-treated infected macrophages. These levels decreased then by 24h post-treatment.

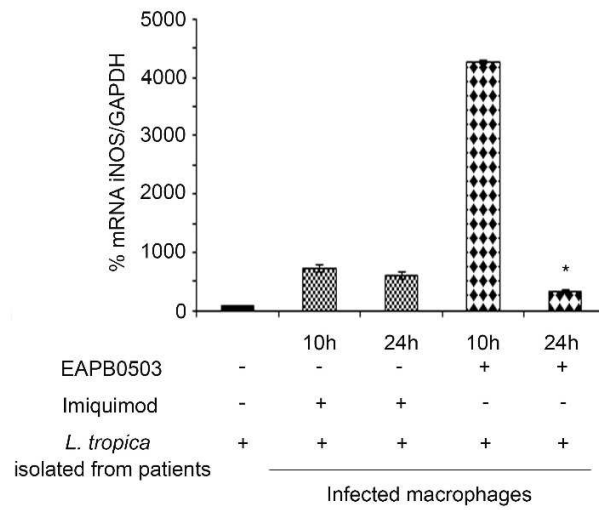


Figure 37: Real-time quantitative PCR detection of iNOS in infected macrophages with patients' isolated *L.tropica* amastigotes upon treatment with 0.1 μ M of Imiquimod or EAPB0503 for 10 and 24h. Percentage of expression of amastigotes was normalized to GAPDH. Results are expressed as percentage of untreated control (\pm) SD and are representative of three independent experiments. The t-test was performed to validate significance. *, ** and *** indicate p values ≤ 0.05 ; 0.01 and 0.001, respectively. P-values less than 0.05 were considered significant.

Collectively, our results show a more potent effect of EAPB0503 on both stages of *L.tropica* freshly isolated from patients' biopsies.

3.2-Screening of TLRs in the context of CL and effect of Imiquimod and its analog EAPB0503 on these TLRs

The TLR signaling pathway is one of the first defensive systems against invasive microorganisms. TLRs are transmembrane proteins, consisting of 11 members (TLR1 to TLR11), which have different specificity for different pathogens and lead to the production of different cytokines [221]. In case of CL, a number of studies reported the importance of TLRs in the disease progression and/or control. Early studies concluded that TLR2, TLR4, and TLR9, are involved in the recognition of *L. major* [222, 223]. TLR2 ligand was shown to induce the generation of protective immunity against leishmaniasis by NF- κ B activation [224], and induction of TNF- α , IL-12, Reactive Oxygen species, and Nitrogen Oxide [225]. Additionally, TLR2 and TLR4 agonists were shown to play a protective role in CL [223, 226, 227]. In contrast, it was reported that TLR-2 is actually involved in parasite survival in macrophages upon activation by lipophosphoglycan (LPG) and subsequent decrease of TLR9 expression [222] and TLR4 signaling prevention [228], thus the reduction of anti-leishmanial responses. Similar controversies were reported *in vivo*. For instance, C57BL/6 mice deficient in either TLR2, 4, or 9, showed that only TLR9-deficient mice are more susceptible to *L. major* infection, indicating that the reported role of TLR2 and TLR4 in immunity to CL requires re-evaluation [229]. On the contrary, TLR4^{-/-} mice showed a role of TLR-4 in the control of *L. major* infection [137].

Due to the controversial role of TLRs in the context of CL, we started by screening the TLR expression in the context of *L. tropica* infection.

3.2.1-Downregulation in TLR4, TLR7, TLR8 and TLR9 upon infection of macrophages with patients' derived *L.tropica*

Since reports about TLR levels are controversial in the context of CL [223], we started by screening for levels of TLR4, TLR7, TLR8 and TLR9 in patients' derived *L.tropica*-infected macrophages in comparison to non-infected macrophages. Transcription level of TLR4 decreased by 60% in *L.tropica* infected macrophages in comparison to non-infected cells (Figure 38A). Similarly, TLR7 expression was also reduced in infected macrophages by 40% when compared to non-infected macrophages (Figure 38B). The same pattern was observed when we checked for TLR8 transcription level, which was strongly reduced, by 90% in comparison to non-infected macrophages (Figure 38C). As for TLR9, infection by *L.tropica* strain induced a tight decrease in TLR9 transcription, by 20%, in comparison to non-infected macrophages (Figure 38D).

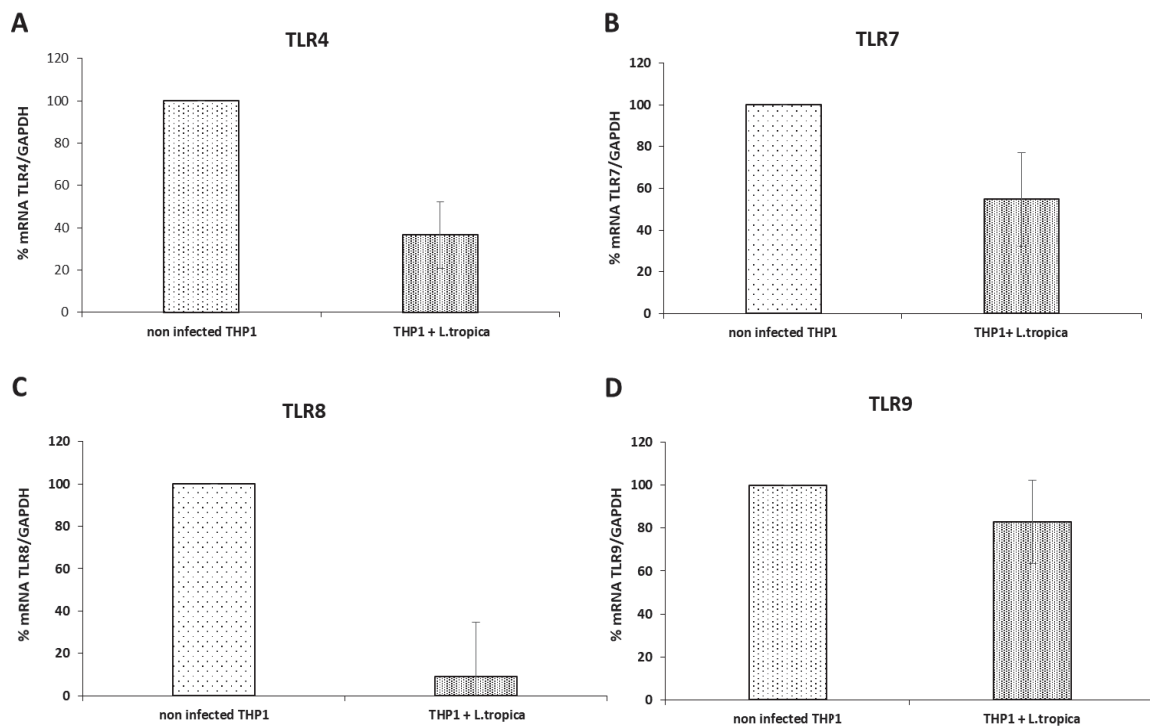


Figure 38: Real-time quantitative PCR detection of TLR4 (A), TLR7 (B), TLR8 (C) and TLR9 (D) in *L.tropica* infected macrophages in comparison to non-infected macrophages. Percentage of expression of amastigotes was normalized to GAPDH. Results are expressed as percentage of untreated control (\pm) SD and are representative of three independent experiments.

3.2.2- Upregulation in TLR4, TLR7, TLR8 and TLR9 in *L.tropica* infected macrophages upon treatment with 0.1 μ M of Imiquimod and EAPB0503

Most studies of TLRs in leishmaniasis have shown that expression of TLR2, TLR4, TLR7, TLR8 and TLR9 have been related to disease outcome, together with other contributing factors such as *Leishmania* species and genetic background [223]. Yet data on the role of TLRs obtained from experimental murine leishmaniasis remain controversial: on one hand, enhanced the expression of these TLRs have been related to protection mediated by cytokine production, whereas their absence has been associated with a Th2 response and elevated *Leishmania* numbers [213, 229, 230].

We investigated transcription levels of different TLRs in patients' derived *L.tropica* infected macrophages upon treatment with Imiquimod or EAPB0503. Our results demonstrated that TLR4, TLR8 and TLR9 transcription level were not increased after Imiquimod treatment for 10h and were close to the transcription level of non-treated *L.tropica* infected macrophages. However, an increase was observed after 24h of treatment (Figure 39A, B, C and D). Importantly and consistently with the known literature [190, 191], and our western blot data (Figure 27), TLR7 transcription level increased at 10h (Figure 39B), and the increase was more prominent at 24h post-treatment (Figure 39B).

Importantly, EAPB0503 induced an upregulation of the four TLRs as early as 10h post-treatment with 2 folds higher in TLR4, 5 folds in TLR7, 10 folds in TLR8 and 70 folds in TLR9 when compared to untreated infected macrophages. Moreover, transcription levels of TLR4, TLR8

and TLR9 further increased 24h post-treatment, with 6, 50 and 80 folds respectively (Figure 39A, C and D). Contrarily to Imiquimod, TLR7 expression decreased at 24h post- treatment.

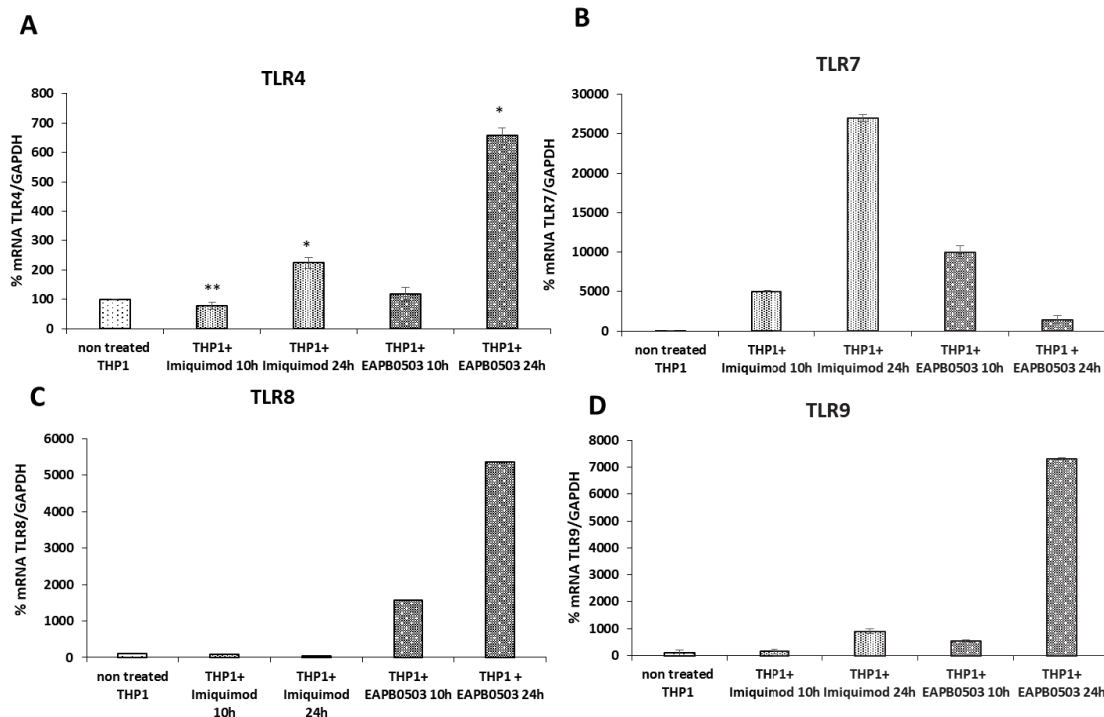


Figure 39: Decrease in the transcription levels of TLR4 (A), TLR7 (B), TLR8 (C) and TLR9 (D) upon treatment with Imiquimod and EAPB0503 in *L.tropica* infected macrophages. Real-time quantitative PCR detection of TLR4, 7, 8 and 9 in infected macrophages with *L.tropica* amastigotes treated with 0.1 μ M of Imiquimod or EAPB0503 for 10 and 24h. TLR percentage of expression was normalized to GAPDH. Results are expressed as percentage of untreated control (\pm) SD. The t-test was performed to validate significance. *, ** and *** indicate p values \leq 0.05; 0.01 and 0.001, respectively. P-values less than 0.05 were considered significant.

Our results indicate that both Imiquimod and EAPB0503 act through TLR activation, however, their mechanism of action may differ in triggering different TLRs and their downstream signaling. While Imiquimod acts through TLR7, the transcript data show that EAPB0503 seemingly act through the upregulation of TLR4/8/9.

3.2.3- Slight upregulation of TLR1, TLR2, and TLR6 upon infection of macrophages with patients' derived *L.tropica*

It is well known that TLR1 interacts with TLR2 [231]. Although TLR2 role in CL is controversial, it is clear that it plays a crucial role in this infection, whether by promoting disease or protecting against it [222,230]. As for TLR1, it was shown to be associated with reduced cytokine production such as IFN- γ and TNF- α in CL [232]. Moreover, TLR1, TLR2 along with TLR6 have the same signaling pathway [233].

Infection of differentiated macrophages with virulent *L.tropica* led to an increase in the transcription level of TLR1 to 175% (Figure 40A). Similarly, TLR2 transcription level was more increased after infection, in comparison to non-infected macrophages, reaching 157% (Figure 40B). The same pattern was observed for the transcription level of TLR6 which also increases following *L.tropica* infection (Figure 40C).

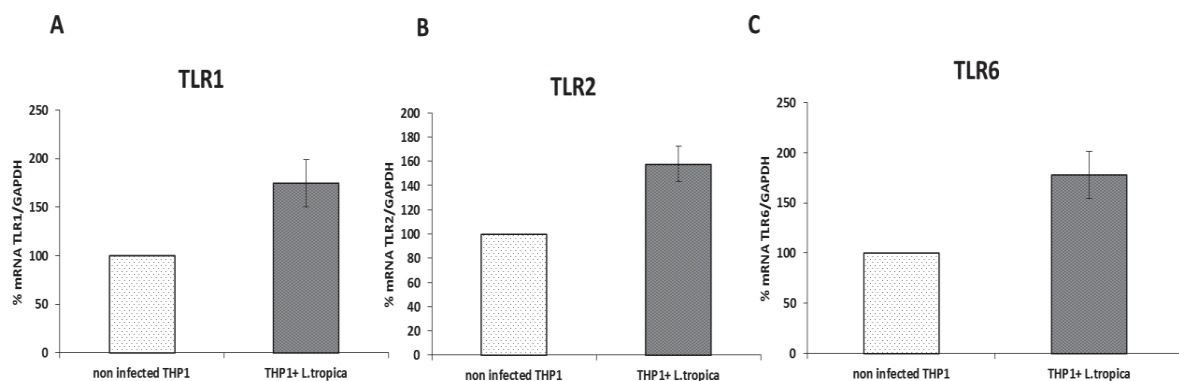


Figure 40: Real-time quantitative PCR detection of TLR1 (A), TLR2 (B) and TLR6 (C) in *L.tropica* infected macrophages in comparison to non-infected macrophages. Percentage of expression of amastigotes was normalized to GAPDH. Results are expressed as percentage of untreated control (\pm) SD and are representative of three independent experiments.

3.2.4- Imiquimod and EAPB0503 trigger a downregulation in TLR1, TLR2 and TLR6 in *L.tropica* infected macrophages

We then assessed TLR1, 2 and 6 levels upon treatment of patients' derived *L.tropica* with Imiquimod or EAPB0503. Our results demonstrate that treatment with 0.1 μ M of Imiquimod for 10h induced a decrease in TLR1 and TLR6 transcription levels to 90% and 20% respectively whereas TLR2 transcription levels at this time point were similar to those of untreated infected macrophages. Interestingly, and following treatment with Imiquimod for 24h, transcription levels of TLR1, TLR2 and TLR6 decreased to 10%, 30% and 45% respectively (Figure 41A, B and C). When we examined the effect of EAPB0503, we noticed a nice decrease of 38%, 25% and 30% for TLR1, TLR2 and TLR6 respectively, and as early as 10h (Figure 41 A, B and C). Transcription levels of TLR1 and TLR2 further decreased to 10 and 18% respectively at 24h post-treatment, whereas TLR6 levels were sustained at around 40% (Figure 41A, B and C).

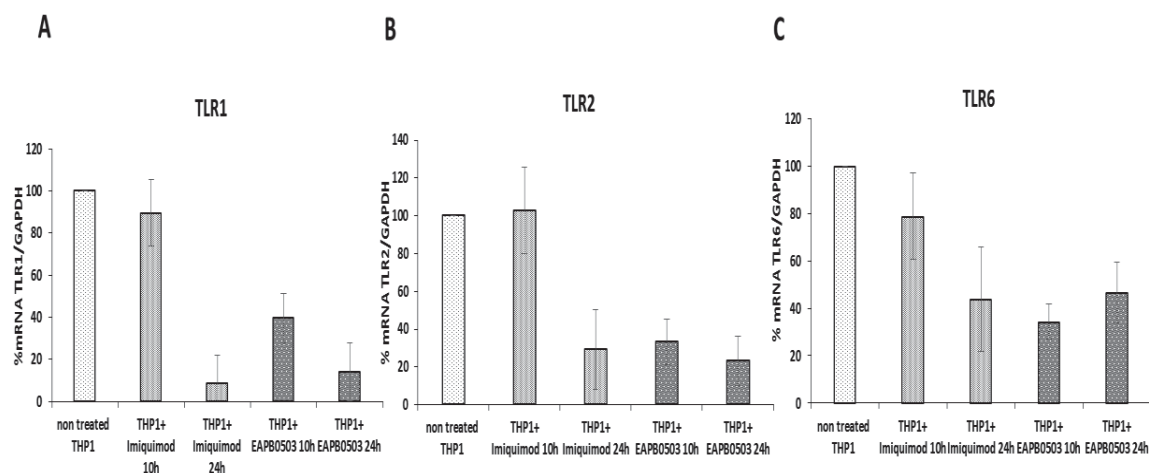


Figure 41: Decrease in the transcription levels of TLR1 (A), TLR2 (B) and TLR6 (C) after treatment with Imiquimod and EAPB0503 in *L.tropica* infected THP1

Our results, showing a slight increase in TLR1, TLR2 and TLR6 following *L.tropica* infection suggest that these TLRs may help in parasite invasion and persistence inside

macrophages. Interestingly, treatment with Imiquimod and particularly EAPB0503 induced a decrease in their level, revealing mechanism of action is linked to their level.

Chapter 4

Discussion

Cutaneous leishmaniasis is one of the most common neglected tropical diseases distributed worldwide. Globally, the annual incidence of CL is estimated to be 0.7 to 1.2 million new cases per year. This infection is still endemic in scattered foci of many countries where poverty and malnutrition exist [20, 52, 56]. In the Middle East Region, Syria remains the most endemic country with the highest number of CL cases [68]. Five years ago, the onset of the Syrian conflict has conducted to a huge outbreak of refugees to the surrounding areas in the Middle East and North Africa. A study performed on infected Syrian refugees diagnosed with CL in Lebanon revealed that 85% of patients were infected with *L.tropica* and 15% were infected with *L.major* [62].

Pentavalent antimony compounds remain the first-line treatment choice for CL treatment. However, these compounds raise many concerns pertaining to cost, availability, poor compliance, and mainly systemic toxicity, and drug resistance [234]. Therefore, there is a high need for alternative treatment modalities. In this study, we focused on testing novel drug's efficacy on *L.tropica* and *L.major* being the causative agents of CL in the Middle East region.

Imiquimod is an immunomodulator known to activate a number of immune cells, including macrophages [190]. These macrophages are known to be the main host cells where *Leishmania* species replicate. In the context of CL, Imiquimod efficacy was tested against some species causing leishmaniasis *in vitro* and *in vivo*. For instance, this compound's efficacy was tested in BALBc mice infected with *L.major* and it increased the protection against CL infection by stimulating a protective immune response [235]. In addition, treatment of *L.donovani* infected macrophages with Imiquimod induced intracellular amastigotes killing by NO production.

Moreover, studies involving CL patients showed that combined treatment of Imiquimod with meglumine antimoniate induced a high healing rate for CL patients who failed to heal when treated with meglumine antimonite alone [183, 184, 193]. Based on these and other clinical trials, the WHO updated the CL treatment guidelines by adding Imiquimod to pentavalent antimony as a second-line therapy of CL [191].

This study is the first to describe the anti-promastigote activity of Imiquimod against *L. major* and *L. tropica* strains. Interestingly, our results showed that *L. major* treated promastigotes with either Imiquimod or EAPB0503 lose their capacity of infecting macrophages only by 50%, seemingly showing that there is a combination of both static and leishmanicidal role of these drugs on *L. major* promastigotes. Strikingly, the most prominent effect on the infection capacity of *L. tropica* promastigotes was obtained upon treatment with EAPB0503 whereby only 20% of infection was observed as compared to 60% infection with Imiquimod treated promastigotes. This result is not in line with the motility assay where Imiquimod showed a better effect, but strongly suggests a leishmanicidal effect of EAPB0503 on *L. tropica* promastigotes. This difference between both assays may reflect a specific effect on invasion of macrophages by treated *L. tropica* promastigotes, leading to a reduction in amastigotes production inside macrophages.

Previous studies assessed the activity of Imiquimod *L. major* at the concentration of 0.15 and 0.3 μM [237]. Moreover, Imiquimod analog, EAPB0503, proved at the concentration of 1 μM a good anti-tumor activity against many types of cancers including melanoma, and leukemias [236]. We assessed the anti-amastigote activity of Imiquimod and its analog EAPB0503 against *L. tropica* and *L. major* strains. We showed at the concentration of 0.1 μM an anti-amastigote efficacy. In more details, the effect of Imiquimod against *L. major* amastigotes was more potent than that of EAPB0503. In contrast, for *L. tropica* strain, the effect of EAPB0503 was more potent.

This result is very promising specially that *L.tropica* is the most aggressive and the most endemic in the Middle East Region (diagnosed in 85% of cases) [62, 238]. We then pursued our study by investigating the mechanism of action of Imiquimod and EAPB0503 against *L.tropica* strain. Previous studies demonstrated that Imiquimod acts *via* binding TLR7, leading to the activation of the NF- κ B signaling pathway [190]. Moreover, Imiquimod induced a protective anti-leishmanial effect on BALBc mice infected with *L.major*, by mediating a Th1 response providing protective immunity against this strain [213]. This protective effect is coupled with the induction of NO synthesis helping in parasite destruction [239]. In this study, and consistently with the published data, we showed that Imiquimod increased the expression of TLR7 in *L.tropica* infected macrophages. However, it will be important to test its activity by for example, silencing this receptor, and check whether this silencing abrogates the efficacy of Imiquimod. For EAPB0503, our results showed that TLR7 expression was higher than that of infected non-treated macrophages with *L.tropica*. However, TLR7 protein levels upon EAPB0503 treatment were lower than those obtained upon treatment with Imiquimod. This finding suggest that EAPB0503 may partially act *via* TLR7, but also may have other protective mechanisms of action. We then aimed at investigating the effect of Imiquimod and EAPB0503 on the activation of the NF- κ B pathway. Our results showed that both drugs activate the canonical NF- κ B, through phosphorylation of p-I κ B α , and nuclear translocation of the active p65 subunit after treatment. Afterwards, we assessed the secretion levels of pro-inflammatory cytokines in *L.tropica* infected macrophages treated with either drugs. We measured secretion levels of MIP-1 α (CCL3) and MIP-1 β (CCL4). These chemokines are known to be involved in the development of Th1 cells, promoting resistance against infections [241]. In addition, MIP-1 α and MIP-1 β play a role in recruiting other cytokines such as TNF- α and IL-6 [242]. In our study, MIP-1 α and MIP-1 β levels were highly increased

after treatment with Imiquimod and EAPB0503. This finding along with the anti-amastigote activity of Imiquimod and EAPB0503 confirm the protective role of these chemokines against CL infection. In addition, we assessed the secretion levels of MCP-1 after treatment with Imiquimod and EAPB0503. Previous studies have shown that MCP1 is highly expressed in lesions of patients with self-healing localized cutaneous leishmaniasis (LCL) whereas it is scarce in those of chronic diffuse cutaneous leishmaniasis (DCL) [243]. This finding indicated that MCP-1 may contribute to the healing process. Results showed that MCP-1 directly stimulates the elimination of intracellular *Leishmania* parasites by human monocytes, a potential that correlates with the induction of reactive oxygen intermediates. Our results showed an increase of MCP-1 levels upon treatment with either drugs, however, the increase was more prominent upon treatment with EAPB0503 and at 24h post treatment.

IL-12 is produced primarily by monocytes, macrophages, and other antigen-presenting cells; it is essential for fighting infectious diseases and cancer. IL-12 is a heterodimeric cytokine crucial to fight against multiple bacterial infections [244]. This cytokine promotes cell-mediated immunity *via* stimulation of Th1 cells. A study conducted on an experimental leishmanial model, it has been shown that daily intraperitoneal administration of *L.major* challenge of either 0.33 micrograms IL-12 or 1.0 micrograms IL-12 per mouse caused a reduction in parasite burden by 75% at the site of infection, in highly susceptible BALB/c mice [245].

Moreover, TNF- α is a glycoprotein initially known for its ability to induce necrosis in certain tumors [246]. It stimulates the acute phase of the immune response. This potent pyrogenic cytokine is one of the first cytokines to be released in response to a pathogen, and is able to exert its effects in many organs [247]. In CL, TNF- α secretion helps in the protection against the infection. Injection of CBA mice with rabbit anti-TNF α induced development of larger lesions

[248]. We showed that secretion levels of TNF- α after treatment with 0.1 μ M of Imiquimod were highly increased in comparison to non-treated infected macrophages. Moreover, EAPB0503 induced a more important significant increase in TNF- α level, supporting the leishmanicidal efficacy of either drugs against infection with *L.tropica* amastigotes and the higher potency of EAPB0503 when compared to Imiquimod.

Similarly to TNF- α , IL-1 β is among the pro-inflammatory cytokines that are primarily secreted after an infection or a stress. It is an endogenous pyrogen released at early stages of infection and involved in the protection against the infection [249]. In our study, we showed that IL-1 β secretion level was elevated after treatment with Imiquimod and EAPB0503, reflecting a potential role of these drugs in the protection against CL. We also measured IL-6 levels, after treatment. IL-6 is a pleiotropic cytokine that has both pro-and anti-inflammatory roles [251]. In CL, most studies describe a role of IL-6 in the induction of a susceptibility to the infection [252]. According to Moskowitz *et al.*, IL-6-deficient C57BL/6 mice infected with *L. major* exhibit a Th1 response controlling the infection [252]. Other studies revealed that pre-treatment of macrophages with IL-6 suppressed IFN- γ and TNF- α production *in vitro* against *L. amazonensis* [253]. Taken together, these studies showed that IL-6 confers susceptibility to CL. We showed that IL-6 secretion was increased following treatment with Imiquimod particularly after 10h, to decrease 24h post-treatment. Similarly, upon EAPB0503 treatment, IL-6 levels were elevated 10h post-treatment, and then strongly and significantly decreased 24h following EAPB0503 treatment. The elevated IL-6 levels obtained at both time points with Imiquimod and at 10h with EAPB0503 are not consistent with the literature that reveals a role of IL-6 in susceptibility to CL. In contrast, only treatment with EAPB0503 for 24h reduced IL-6 levels, presumably suggesting that longer exposure to the drug is needed to decrease IL-6 secretion levels, and to confer resistance to the

infection. Our study did not provide a clear conclusion about IL-6 role in CL, and probably for this cytokine, longer exposures to the drugs particularly to Imiquimod may be needed to elucidate the levels of this cytokine.

We also succeeded to test the efficacy of Imiquimod and EAPB0503 on freshly isolated *L. tropica* promastigotes from skin lesions of CL patients. We confirmed the results obtained on cultured strains, thus eliminating any potential doubt about a response to either drugs due a lower virulence and a genetic drift obtained from long term cultures. We also investigated the anti-amastigote activity of Imiquimod and its analog on macrophages infected with patients' derived *L. tropica* amastigotes. The anti-amastigote activity was consistent with that of the cultured strains and was concentration, time-dependent, and more prominent after EAPB0503 treatment. This time-dependent anti-amastigote activity was confirmed not only by real-time PCR, but also by H&E staining of *L. tropica* inside macrophages, underlying the promising potency of EAPB0503 for CL treatment. In an attempt to better understand the mechanism by which Imiquimod and EAPB0503 act, we checked for the transcription level of i-NOS in treated infected macrophages. This is driven by the idea that NO is produced following production of i-NOS by activated macrophages, and is known to play a major role in fighting against microbicidal infections [254], including *Leishmania* [255]. For instance, inhibition of i-NOS reduced *L. infantum* burden in human macrophages [256]. In addition, the increase of iNOS and NO generation in response to IFN- γ and TNF- α is crucial to control CL [257]. We assessed i-NOS transcription level in *L. tropica* infected macrophages treated with 0.1 μ M of Imiquimod and EAPB0503. Results showed that Imiquimod induced an important increase in i-NOS transcription level 10 and 24h post-treatment. More importantly, EAPB0503 induced a much more important increase in i-NOS levels presumably explaining the higher leishmanicidal potency of this compound.

In the second part of this work, we were interested in screening for TLRs in the context of *L. tropica* infection and following treatment with Imiquimod and EAPB0503. TLRs are important pattern recognition receptors expressed abundantly on macrophages, DCs, and NK cells. TLRs are involved in self/non-self-discrimination by the innate immunity and initiating and directing adaptive immunity [258]. Upon recognition of respective pathogen associated molecular patterns (PAMPs), TLRs recruit adaptor molecules, such as MyD88 and TRIF, and initiate a series of downstream signaling events that lead to the production of pro-inflammatory cytokines, chemokines, and antimicrobial peptides [259]. Early studies concluded that three distinct TLRs, TLR2, TLR4, and TLR9, are involved in the recognition of *L. major*. A study conducted by De Veer in 2003 demonstrated that LPG is a TLR2 ligand, and is responsible for the generation of protective immunity against Leishmaniasis by NF- κ B activation [260], moreover the induction of TNF alpha, IL-12, Reactive Oxygen species, and Nitrogen Oxide [261]. However, more recent studies on C57BL/6 mice deficient in either TLR2, 4, or 9, showed that only TLR9-deficient mice are more susceptible to *L. major* infection, indicating that the reported role of TLR2 and TLR4 in immunity to murine leishmaniasis requires re-evaluation [229].

Moreover, and in contrast to the previous results by De Veer et al., a study conducted in 2013 showed that TLR-2 is actually involved in parasite survival in macrophages upon activation by lipophosphoglycan (LPG) and subsequent decrease of TLR9 expression [222] and TLR4 signaling prevention [228] thus the reduction of anti-leishmanial responses. Similarly, TLR2 was found involved in disease promotion after the lack of TLR2 was found to increase resistance to *L. braziliensis* and *L. amazonensis* infections, and decreased lesion formation and parasite burdens [230, 262]. TLR4 plays a critical role during Leishmania infection, which was affirmed by studies on TLR4^{-/-} mice that demonstrated the role of TLR4 in the control of *L. major* and *L. pifanoi*

infections. Glyco-sphingophospholipid (GSPL) and proteoglycolipid complex (P8GLC) from *Leishmania* induce TLR4, thus promoting a strong anti-parasitic immune response *via* TNF- α . Clearance was also obtained using GSPL treatment in *L. donovani* infection [261]. In this study, we showed that anti-leishmanial activity of Imiquimod and EAPB0503 could be mediated by the increase in TLR4 and TLR9, thus confirming the theory that the high level of these TLRs promote protection against the infection. On the other hand, TLR4, TLR7, TLR8 as well as TLR9 are shown to be linked to resistance against the infection. This was confirmed by the decrease in these TLRs level in *L. tropica* infected macrophages, and their increase following treatment. This finding is consistent with the studies reporting the role of TLR4 and TLR9 in the protection against CL [127, 133, 229]. Moreover, our results were consistent with the literature, showing that the activity of Imiquimod is mediated by TLR7. To a lesser extent, activity of this compound is associated with TLR8 increase. Interestingly, we showed for the first time that the activity of EAPB0503 is associated with an increase in TLR8 expression. In addition, TLR8 and TLR9 levels were more increased after EAPB0503 treatment in comparison to Imiquimod. Moreover, this compound induced a higher decrease in TLR1, TLR2 and TLR6 than Imiquimod. In our study, these TLRs showed to be involved in parasite persistence. Altogether, these findings indicate that the better anti-amastigote activity of EAPB0503 could be associated to the trigger of a series of TLRs helping in parasite clearance, thus decreasing other TLRs levels, helping in parasite persistence. Yet, our results were only obtained at the transcript levels and should be confirmed at the protein levels. Moreover, the activation of downstream cascades following these TLRs activation should be investigated.

TLR1 and TLR6 are suggested to have the same signaling pathway of TLR2. TLR1 expression was not highly evaluated in CL context. In this study, we showed that TLR1 and TLR6

expression decreased after treatment with Imiquimod and EAPB0503. These findings are consistent with TLR2 level that also decreased after treatment.

As for TLR7 expression, and consistently with the known literature, we confirmed that Imiquimod displays its anti-amastigote activity by increasing TLR7 levels in infected macrophages, leading to NF- κ B activation and pro-inflammatory cytokine production. It is worth noting that pro-inflammatory cytokines are known to promote resistance to the infection. When we assessed TLR8 expression, we noticed that its expression is higher following treatment with EAPB0503. Collectively, these results seemingly show that EAPB0503 and Imiquimod may increase the expression of different TLRs and may display their leishmanicidal activity through different signaling pathways.

Chapter 5

Conclusion and perspectives

In this study, we focused on *L.major* and *L.tropica*, the causative agents of CL in the Middle East Region. We demonstrated that Imiquimod exhibits an *in vitro* anti-leishmanial activity against these two strains, by targeting promastigotes growth as well as amastigotes expression inside macrophages.

More interestingly, EAPB0503, the analog of Imiquimod showed a more prominent activity against promastigotes of *L.major* and *L.tropica*. In more details, Imiquimod had mostly a static effect on promastigotes of both strains whereas EAPB0503 displayed mostly a static effect on *L. major* promastigotes and impaired the invasion of *L.tropica* promastigotes inside macrophages. These results strongly suggest a better leishmanicidal effect of EAPB0503 on *L.tropica* promastigotes, which could be linked to an effect on their invasion inside macrophages. In addition, both drugs displayed an anti-amastigote activity with a more prominent effect of EAPB0503.

Investigating the mechanism of action of Imiquimod and its analog revealed that this anti-amastigote activity is due to the upregulation of Toll Like Receptor-7 (TLR7), mainly mediated by Imiquimod, and to a lesser extent by EAPB0503, resulting in the activation of the NF- κ B canonical pathway. NF- κ B activation led to a consequent production of pro-inflammatory cytokines, and a down-regulation of anti-inflammatory cytokines mediating the leishmanicidal activity of the drugs.

Furthermore, Imiquimod and particularly EAPB0503 display a promising leishmanicidal activity against virulent *L.tropica* strain. This activity could be linked to an increased i-NOS level

which contributes in parasite destruction. The potency of Imiquimod and EAPB0503 need further studies *in vivo* mice models, to study their effect on lesions as well as on the immune microenvironment and the recruitment of other immune cells to the site of infection. Last but not least, it is of high interest to suggest a topical use of EAPB0503 and test its efficacy on LC skin lesions.

Our results showed EAPB0503 led to a prominent increase in TLR8 and TLR9 transcripts, presumably showing a role of these TLRs in conferring a protection against the infection. Other TLRs seem also to be affected by EAPB0503, such as elevation of the transcript levels TLR4 which promote protection against the infection. EAPB0503 seems also to slightly downregulate TLR1, TLR2 and TLR6 which seem to be helping in parasite infection inside macrophages. These preliminary results should be confirmed at the protein level and their downstream signaling should be tested.

Our study opened more perspectives towards screening different TLRs in CL patients with different clinical features (parasitic index, chronicity...), and investigate their involvement/relationship with the aggressivity of the infection. This will help in providing a clear conclusion about the contribution of these TLRs in the context of CL, and test different immunomodulatory drugs acting *via* these TLRs. Thereafter, it is of high interest to check the downstream signaling of these TLRs, to better understand their potential role in the infection process and in the response to treatment. This may be understood by silencing these receptors (Knock-down, crispr-cas9...) to clearly identify their role.

Our results give insights on the effect of Immunomodulatory drugs derivatives on CL, and opens horizons for new and promising treatment paradigm.

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Résumé français de la thèse

1- Généralités sur la leishmaniose:

La leishmaniose est une infection parasitaire causée par le protozoaire flagellé du genre *Leishmania*. Elle est transmise suite à la pique d'une mouche de sable du genre *Phlébotome*. Cette infection est classée parmi les maladies tropicales négligées les plus répandues dans le monde et est responsable d'un taux de mortalité et de morbidité assez élevé.

Il existe environ 20 espèces différentes de *Leishmania*; elles sont responsables de trois formes de leishmaniose:

- **La leishmaniose viscérale (LV):** c'est la forme la plus sévère de l'infection. Elle est généralement causée par *Leishmania donovani* et *Leishmania infantum*. Le parasite migre vers le foie et la rate de l'hôte y causant une hépatosplénomégalie. Cette maladie est fatale en l'absence du traitement. Elle est répandue dans les pays tropiques et sous-tropiques, ainsi que les régions sèches de l'Amérique Latine et du bassin Méditerranéen. Cette infection se présente avec une prévalence mondiale de 500 000 cas par an.
- **La leishmaniose cutanée (LC):** c'est la forme la plus endémique de la leishmaniose. elle est causée majoritairement par *L.major* et *L.tropica*. Elle est caractérisée par des lésions cutanées qui apparaissent sur le site de pique, et qui peuvent évoluer en des papules puis des nodules puis des lésions sévères et des ulcères au niveau de la peau en une période de 4 à 8 semaines. Cette forme de leishmaniose est principalement causée par les espèces *Leishmania major* et *Leishmania tropica*. La majorité des lésions de la LC disparaissent sans traitement, mais laissent une cicatrice défigurante. Pendant les cinq dernières années, 1 million de cas de LC environ ont été reportés et 310 millions de personnes sont à risques

d'infection. Cette maladie est en forte expansion au Maghreb et au Moyen Orient et menace l'Europe du Sud.

- **La leishmaniose mucocutanée (LMC):** cette forme est endémique en Amérique Latine. Elle est caractérisée par la destruction des cavités naso-pharyngiale et orale. Elle débute par des lésions cutanées qui évoluent et affectent les cavités muqueuses, en absence de traitement. Cette forme est généralement causée par *L. braziliensis*, et moins fréquemment par *L.guyanensis* et *L.panamensis*.

2- Classification et transmission de la leishmaniose:

Toutes les espèces du genre *Leishmania* partagent la même classification taxonomique, appartenant au règne des Protistes, sous-règne des Protozoaires, ordre des kinetoplastidés, famille des trypanosomes, genre *Leishmania*, et différentes espèces (*major*, *tropica*, *donovani*...).

Ce parasite présente un cycle dixène, impliquant une mouche de sable de genre Phlébotome et un mammifère. Une femelle de Phlébotome infectée transmet les promastigotes flagellés, lors d'un repas sanguin à un mammifère. Les promastigotes infectent les macrophages, y perdent leurs flagelles et s'y transforment en amastigotes non flagellés. Le cycle recommence lors de la pique d'une nouvelle mouche de sable ingérant des macrophages infectés par des amastigotes, qui vont se retransformer dans son intestin en promastigotes; ces derniers migreront vers les glandes salivaires de la mouche et un nouveau cycle recommence.

3- La leishmaniose cutanée et les facteurs favorisant sa transmission :

La leishmaniose cutanée est la forme la plus fréquente de la leishmaniose, distribuée au Maghreb et au Moyen Orient surtout la Syrie et menace l'Europe du Sud. Les changements

climatiques et environnementaux favorisent majoritairement l'expansion de la leishmaniose cutanée et de son vecteur. De plus, la pauvreté, le manque d'hygiène, les conflits politiques et les guerres représentent une cause majeure de la dissémination de la LC.

Récemment, la crise politique qui a eu lieu en Syrie a provoqué une migration intense des réfugiés Syriens vers les pays voisins. Les conditions dramatiques de vie sur les camps des réfugiés (manque d'hygiène, ...), ont favorisé la dissémination de la LC dans des pays non endémiques pour cette maladie, y compris le Liban. Ceci représente un grave problème et requiert des interventions sérieuses et efficaces pour contrôler l'expansion de cette infection. Pour ces raisons, notre étude s'est focalisée sur la leishmaniose cutanée causée par *L.major* et *L.tropica*, étant les espèces les plus répandues en Syrie (représentant 15 et 85% des cas respectivement en Syrie).

4- Traitements actuels de la Leishmaniose cutanée:

Le traitement de la LC dépend de plusieurs facteurs et varie selon le nombre de lésions, le site de ces lésions, le système immunitaire et l'âge du patient... De plus, des facteurs intrinsèques comme l'espèce du parasite responsable de l'infection, et des facteurs extrinsèques comme le manque de disponibilité du traitement dans les régions rurales peuvent affecter le traitement.

Différents traitements sont utilisés contre la LC, regroupant des thérapies physiques, orales, topiques ou parentérales. Parmi ces différentes modalités de traitement, la thérapie parentérale notamment le Glucantime demeure le traitement le plus efficace et le plus répandu de la leishmaniose cutanée. En revanche, ce traitement présente beaucoup de désavantages limitant son efficacité, notamment son injection répétitive hebdomadaire dans les lésions et sur un intervalle de 6 semaines. Ce mode d'injection est très douloureux et non pratique, surtout quand les lésions

se présentent sur le visage, ou à proximité des yeux. Dans ces derniers cas, les injections se font par voie intramusculaire, mais présentent une toxicité hépatique et cardiaque.

L'absence de traitement standard de la LC, et l'inefficacité/la toxicité des traitements actuellement disponibles rendent particulièrement importante la recherche de nouveaux traitements plus efficaces. L'Imiquimod, un immunomodulateur, approuvé par la FDA (Food and Drug Administration) et l'EMA (European Medicines Agency) pour le traitement des infections génitales ainsi que dans certains cancers de peau, a été récemment testée contre la LC et a montré des résultats très encourageants. Ceci a poussé l'Organisation de Santé Mondiale (WHO) à mettre à jour le guide standard de traitement de la LC et inclure l'Imiquimod parmi les traitements efficaces contre cette infection parasitaire.

5- Objectifs, résultats et conclusion :

Nous nous sommes focalisés sur deux espèces responsables de la LC dans la région du Moyen Orient, *L.major* et *L.tropica*. L'équipe des Drs Bonnet et Masquéfa à Montpellier a réussi à synthétiser des analogues de l'Imiquimod, dont l'EAPB0503. Ce composé a été testé dans certains types de cancer et a montré une efficacité prometteuse excédant celle de l'Imiquimod.

L'objectif de cette thèse est de tester l'efficacité de l'EAPB0503 sur les stades promastigotes et amastigotes des deux souches *L.major* et *L.tropica*.

En premier lieu, les promastigotes de ces deux souches ont été traités par différentes concentrations (0.1, 0.5, 1 et 10 μ M) de ces deux composés pendant 72 heures. D'abord, les promastigotes ont été dénombrés en se basant sur leur motilité, puis leur capacité à infecter les macrophages après traitement a été évaluée. Les résultats montrent que pour la souche *L.major*, l'EAPB0503 possède une activité anti-promastigote plus importante en réduisant le taux de croissance des parasites,

alors que pour la souche *L.tropica*, c'est l'Imiquimod qui induit un effet plus important. Concernant l'effet sur l'infection des promastigotes dans les macrophages, les résultats montrent que pour *L.major*, les deux composés affectent à peu près similairement l'infection des parasites, alors que pour la souche *L.tropica*, l'EAPB0503 induit une diminution d'infection notable des promastigotes. Ces résultats montrent une activité potentielle statique de l'Imiquimod contre la souche *L.major*, alors que l'EAPB0503 pourra avoir un effet leishmanicide contre la souche la plus virulente *L.tropica*.

Dans la deuxième partie de notre étude, nous avons étudié l'effet de l'Imiquimod et de l'EAPB0503 sur les amastigotes des deux souches *L.major* et *L.tropica*. Les macrophages humains ont été infectés par un taux de 5 parasites/cellule/souche pendant 24h. Ensuite, les macrophages infectés ont été traités par différentes concentrations d'Imiquimod ou de l'EAPB0503 et l'effet des traitements sur les amastigotes ont été évalué par PCR en temps réel, utilisant des amorces spécifiques pour le kinétoplaste du parasite. Nos résultats montrent que les deux composés induisent une réduction du taux d'expression des amastigotes, avec un effet plus prononcé suite au traitement par l'EAPB0503. Afin de comprendre le mécanisme d'action de ces composés, nous avons choisi la souche la plus agressive et la plus répandue dans la région du Moyen Orient, *L.tropica*. Nos résultats sont consistants avec la littérature montrant que l'Imiquimod agit via l'activation de TLR7, et montrent que les macrophages infectés et traités par l'Imiquimod expriment un niveau plus élevé de ce récepteur. En revanche, le niveau d'expression de TLR7 après traitement par l'EAPB0503 était inférieur à celui induit par l'Imiquimod. De plus, nos résultats montrent une activation de la voie canonique de signalisation NF- κ B (phosphorylation de l'unité I κ B α , translocation nucléaire de la sous-unité p65...). L'activation de cette voie de

signalisation, suite au traitement par les deux composés, induit l'élévation du taux de sécrétion de cytokines pro-inflammatoires (MIP-1 α , MIP-1 β , MCP-1, IL-12, IL-1 β , IL-6 et TNF- α), et une diminution du taux de sécrétion de cytokines anti-inflammatoires (IL-10 et IL-4). L'élévation du taux des cytokines pro-inflammatoires induit à son tour une élévation des transcrits i-NOS, vraisemblablement conduisant à la production du NO, connu pour son effet leishmanicide.

Pour éliminer les doutes qui pourraient être évoqués concernant l'utilisation pour ces études de souches maintenues en culture depuis longtemps, ce qui fait qu'elles pourraient être devenues moins virulentes donc plus susceptibles à l'effet des drogues, nous avons testé l'effet leishmanicide de ces drogues sur des parasites fraîchement isolés à partir des patients infectés par la LC. Nos résultats se sont révélés conformes avec les résultats obtenus avec les souches de culture et indiquent que l'Imiquimod et plus particulièrement l'EAPB0503 possèdent une activité leishmanicide contre les promastigotes et les amastigotes (résultats obtenus par PCR en temps réel, microscope confocale et marquage à l'Hématoxyline et l'Eosine) de *L.tropica* dérivé des patients. De plus, il a été montré une augmentation du taux d'i-NOS après traitement par l'Imiquimod et plus particulièrement par l'EAPB0503, suggérant une production de NO, et expliquant l'effet leishmanicide contre ce parasite.

Comme l'Imiquimod agit via TLR-7/8, et comme les cellules hôtes sont des macrophages qui sont connus par l'expression de différentes classes de TLRs suite à l'infection par un pathogène, la deuxième partie de notre travail s'est focalisée sur l'expression des TLRs dans le contexte de la LC et de l'effet de l'Imiquimod et de son analogue EAPB0503 sur leur expression. Nous avons d'abord vérifié l'hypothèse de variation de l'expression des TLRs en fonction de l'infection par *L.tropica*. Nos résultats montrent que *L.tropica* induit une diminution d'expression de TLR2 connu pour avoir un effet favorisant l'invasion du parasite dans l'hôte. L'expression des récepteurs TLR1

et TLR6, qui possèdent la même voie de signalisation que TLR2, est aussi augmentée après infection de même que ceux des récepteurs TLR4, TLR7, TLR8 et TLR9. Ces résultats confirment des hypothèses validées dans la littérature suggérant que ces récepteurs sont impliqués dans la protection contre l'infection, ce qui explique leur diminution après infection par *L.tropica*.

En second lieu, nous avons déterminé l'effet de l'Imiquimod et de son analogue sur les TLRs exprimés par les macrophages infectés par *L.tropica*. On a démontré que le taux d'expression de TLR7 augmente d'une manière significative après traitement par l'Imiquimod et d'une manière moins prononcée suite au traitement par l'EAPB0503. Cependant, l'analyse du taux d'expression des TLRs montre une augmentation remarquable des taux de TLR8 et de TLR9 après traitement par l'EAPB0503, qui est supérieure aux niveaux induits par l'Imiquimod. En revanche, les taux d'expression des TLR1, TLR2, TLR6 sont inférieurs après traitement par cet analogue. Ces résultats montrent que l'effet supérieur induit par l'EAPB0503 contre les macrophages infectés par *L.tropica* pourra être expliqué par un effet potentiel sur les récepteurs TLR8 et TLR9 conférant une résistance à l'infection, ainsi qu'une diminution de l'expression des TLR1 et TLR2, TLR6 qui jouent un rôle dans la susceptibilité à l'infection. Nos résultats montrent que l'Imiquimod et son analogue EAPB0503 agissent sur différents TLRs suggérant des mécanismes d'action différents pouvant expliquer l'effet plus prometteur de l'EAPB0503. Ces résultats devraient être confirmés au niveau protéique et les voies de signalisation en cause investiguées.

Collectivement, nos résultats montrent un effet puissant et prometteur de l'EAPB0503, supérieur à l'effet de l'Imiquimod, dans le traitement de la leishmaniose cutanée. Ces résultats nécessitent d'être confirmés *in vivo* dans des modèles murins. Une fois ces résultats obtenus et ces modèles établis, il serait d'un grand intérêt de développer des traitements sous forme topique, et tester leur efficacité.

Published Research papers

Fatal Visceral Leishmaniasis Caused by *Leishmania infantum*, Lebanon

Rana El Hajj, Hiba El Hajj,¹ Ibrahim Khalifeh¹

Visceral leishmaniasis, a fatal disease if not treated, is caused by *Leishmania* parasites. This disease might be overlooked in the Middle East because of limited awareness and low incidence. We report 5 patients who died of visceral leishmaniasis in Lebanon and make recommendations to improve faster diagnosis and treatment.

Leishmaniasis is a parasitic disease characterized by different clinical manifestations depending on patient immune response and causative species (1). Visceral leishmaniasis, the most severe form, is fatal if untreated (2). This disease is caused by *Leishmania donovani*, which is endemic to Africa and Asia, causes anthroponotic visceral leishmaniasis, and is associated with high mortality rates (3). However, *L. infantum*, which undergoes zoonotic transmission, is associated with fewer deaths and is endemic to Latin America and the Middle East (4). This species shows a mortality rate of 6% for children (5).

In the Middle East, cutaneous leishmaniasis is the most common endemic form of leishmaniasis and is caused mainly by *L. tropica* and *L. major* (6). *L. infantum* is reported to cause cutaneous leishmaniasis and visceral leishmaniasis in Syria, but only cutaneous leishmaniasis in Lebanon (7,8). During 1958–2014, visceral leishmaniasis showed a low incidence in Syria; 17 cases were reported in 2008 and 36 cases in 2014 (9,10). However, no molecular or biochemical typing was performed to identify the causative species and strains (11). Moreover, visceral leishmaniasis caused by *L. infantum* has not been reported in Lebanon (12).

Recently, displacement of refugees during the ongoing crisis in Syria resulted in a massive population migration and spread of communicable diseases, including cutaneous leishmaniasis (6,11). In Lebanon, 2,420 families from Syria were given a diagnosis of cutaneous leishmaniasis. As of April 2017, a total of 2,057 (85%) of these families were infected with *L. tropica* and 363 (15%) with *L. major* (6). These infections indicate the need for early diagnosis of visceral leishmaniasis and prevention of deaths in the Middle East. We report visceral leishmaniasis in refugees from Syria in Lebanon who acquired *L. infantum* in Syria.

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The Study

Five refugee children from Syria (age range 2–11 years) died of visceral leishmaniasis during 2014–2017 because of a late diagnosis and lack of awareness of this disease in Lebanon. All 5 children had migrated from the northern coast of Syria to Lebanon and had resided in Beirut for an average of 9 months (range 7–11 months). Visceral leishmaniasis developed 4–6 months after they left Syria. Further investigations showed that the siblings of 3 of these patients were infected while staying in Syria.

All patients visited medical institutions in Lebanon and had fever, abdominal distension, ascites, hepatosplenomegaly, and pancytopenia (Table). Three patients were given incorrect diagnoses of leukemia and were given steroids and blood transfusions. The remaining 2 patients were given incorrect diagnoses of a hemophagocytic syndrome with an idiopathic etiology and treated accordingly.

These 5 patients were then reevaluated at the American University of Beirut Medical Center (Beirut, Lebanon) after an average of 4.8 months. Microscopic examination of bone marrow aspirates and smear specimens showed a few scattered amastigotes within macrophages for 2 patients (Figure).

A diagnosis of visceral leishmaniasis caused by *L. infantum* was confirmed by PCR amplification of the internal transcribed spacer 1 region of the parasite (13), followed by restriction fragment length polymorphism analysis of the internal transcribed spacer 1 region amplicon (14). This analysis specifically distinguishes *L. infantum* from other *Leishmania* species. After confirmation of visceral leishmaniasis, the patients were treated with Abelcet (amphotericin B lipid complex) (Teva Pharma BV, Harlow, UK) according to the manufacturer's guidelines. However, the delay in diagnosis led to an advanced disease stage and lack of response to treatment, followed by death.

Conclusions

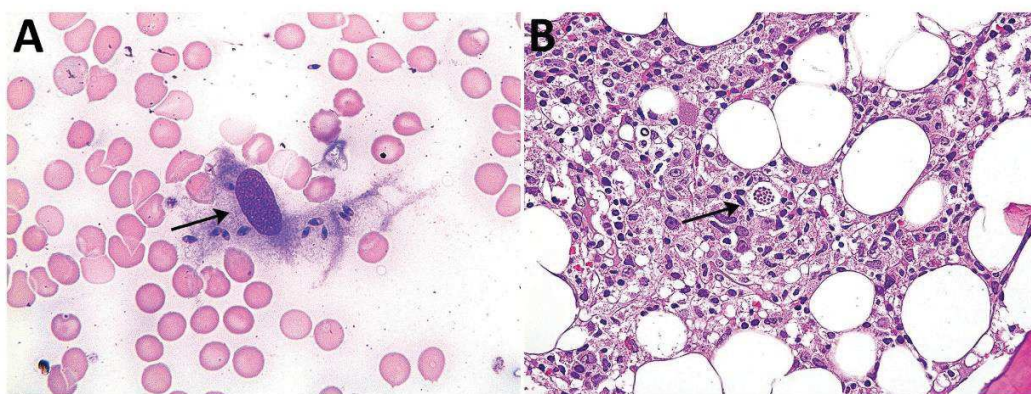
We report 5 children among refugees from Syria in Lebanon who died of visceral leishmaniasis caused by *L. infantum*. Our report provides insightful baseline information about knowledge, practices, and control regarding this disease. The combination of hepatosplenomegaly, fever, and pancytopenia should raise the suspicion for visceral leishmaniasis and result in prompt intervention. These interventions might prevent misdiagnosis and enable appropriate treatment strategies at early stages of the disease to reduce the number of deaths.

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Table. Clinical characteristics of 5 patients with visceral leishmaniasis, Lebanon, 2014–2017*

Patient no.	Age, y/sex	Abdominal distension	Hepatosplenomegaly	Fever	Ascites	Blood	Lung infiltrates	Insect bite	Delay in diagnosis, mo
1	11/F	+	+	+	+	Pancytopenia	+	+	4
2	7/M	+	+	+	+	Pancytopenia	+	—	5
2	5/M	+	+	+	+	Pancytopenia	+	—	6
4	4/M	+	+	+	+	Pancytopenia	+	—	5
5	2/F	+	+	+	+	Pancytopenia	+	—	4

*, negative; +, positive.

**Figure.** Bone marrow smear sample (A) and bone marrow aspirate (B) for patient 2 with visceral leishmaniasis caused by *Leishmania infantum*, Lebanon. Arrows show amastigotes within macrophages. Panel A, Wright Giemsa stain, original magnification x400; panel B, hematoxylin and eosin stain, original magnification x200.**About the Author**

Rana El Hajj is a doctoral student at the American University of Beirut Medical Center, Beirut, Lebanon. Her primary research interest is investigation of specific therapies for leishmaniasis.

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RESEARCH ARTICLE

Toxoplasma gondii autophagy-related protein ATG9 is crucial for the survival of parasites in their host

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Abstract

Autophagy is a conserved, life-promoting, catabolic process involved in the recycling of nonessential cellular components in response to stress. The parasite *Toxoplasma gondii* is an early-diverging eukaryote in which part of the autophagy machinery is not exclusively involved in a catabolic process but instead has been repurposed for an original function in organelle inheritance during cell division. This function, depending essentially on protein TgATG8 and its membrane conjugation system, is crucial for parasite survival and prevented an in depth study of autophagy in the mutants generated so far in *Toxoplasma*. Thus, in order to decipher the primary function of canonical autophagy in the parasites, we generated a cell line deficient for TgATG9, a protein thought to be involved in the early steps of the autophagy process. Although the protein proved to be dispensable for the development of these obligate intracellular parasites *in vitro*, the absence of TgATG9 led to a reduced ability to sustain prolonged extracellular stress. Importantly, depletion of the protein significantly reduced parasites survival in macrophages and markedly attenuated their virulence in mice. Altogether, this shows TgATG9 is important for the fate of *Toxoplasma* in immune cells and contributes to the overall virulence of the parasite, possibly through an involvement in a canonical autophagy pathway.

KEYWORDS

ATG9, autophagy, stress response, toxoplasma, virulence

1 | INTRODUCTION

Macroautophagy (simply referred to as "autophagy" thereafter) is a self-degradation process conserved among eukaryotes. It allows cells to eliminate undesired cytosolic materials such as misfolded proteins or damaged organelles under normal growth conditions and recycle their own components under stress conditions such as starvation. During the autophagy process, cytosolic components are sequestered within a double membrane vesicle, called the autophagosome, and then delivered into a lysosomal compartment for degradation (Shibutani & Yoshimori, 2014). Autophagosome formation requires autophagy-related (ATG) proteins; many of which were originally identified in the yeast *Saccharomyces cerevisiae* (Devenish & Klionsky, 2012). One of the primary functions of autophagy is participating in the maintenance of basal cellular homeostasis in normal growth conditions. Usually, lack of basal autophagy leads to no apparent

abnormalities, like for yeast cells growing in nutrient-rich medium (Tsukada and Ohsumi, 1993), although this housekeeping function is indispensable for some mammalian cell types such as neural cells (Hara et al., 2006). Starvation-induced autophagy, on the other hand, is essential for stress management in almost all eukaryotes. The lack of oxygen or nutrients such as amino acids or glucose, are known to trigger autophagy, which plays a key part in mobilizing diverse cellular energy and nutrient stores (Kaur & Debnath, 2015; Russell, Yuan, & Guan, 2014) to maintain normal functions of the cells. Autophagy can also be induced by other stress, reactive oxygen species and reactive nitrogen species in particular, which are harmful for cellular components, as they generate damaged proteins and DNA (Filomeni, De Zio, & Cecconi, 2015).

The protist *Toxoplasma gondii* (*T. gondii*) is an obligate intracellular parasite whose survival is based on scavenging essential nutrients from its host cell (Blader & Koshy, 2014). Infection can range from asymptomatic to severe, depending on the virulence of the parasite cell lines and the immune status of the host (Hunter & Sibley, 2012). The

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majority of infection cases in humans are asymptomatic, as the parasite is controlled by the immune system and remains in a dormant state in the brain and other tissues. However, *T. gondii* can provoke severe diseases that may be fatal for immunocompromised patients and for developing fetuses of primary-infected pregnant women (Montoya & Liesenfeld, 2004). Tachyzoites, the invasive and fast-replicating forms of the parasite, which are responsible for the symptoms of the acute form of the disease, undergo multiple rounds of division within their host cells, leading to significant tissue damage. Immunological studies have shown that the elimination of the parasite involves interferon- γ (IFN- γ)-dependent mechanisms, implicating both innate and adaptive immune responses (Sturge & Yarovsky, 2014).

T. gondii possesses an apparently reduced autophagy machinery but is nevertheless able to generate autophagosome-like structures under acute starvation condition or following drug treatment (Besteiro, Brooks, Striepen, & Dubremetz, 2011; Ghosh, Walton, Roepe, & Sinai, 2012; Lavine & Arrizabalaga, 2012). These vesicles resemble bona fide autophagosomes: they appear as double-membrane compartments containing more or less intact cytosolic materials and are decorated with protein ATG8, which is the most widely used autophagosomal marker (Slobodkin & Elazar, 2013). However, there is no clear evidence so far that these vesicles, once generated, are being degraded and their content is recycled as expected in a canonical autophagy pathway. In a very unusual fashion though, during normal intracellular growth, TgATG8 also localizes at the outermost membrane of the apicoplast, a relict plastid found in apicomplexan parasites (Kong-Hap et al., 2013), where it plays a noncanonical role in organelle inheritance during cell division (Lévêque et al., 2015). The apicoplast is involved in several key metabolic pathways, so it is essential for the survival of the parasites (Sheiner, Vaidya, & McFadden, 2013). Accordingly, interfering with the function of TgATG8, or of proteins regulating its membrane association such as TgATG3 and TgATG4, is lethal for the parasites (Besteiro et al., 2011; Kong-Hap et al., 2013; Lévêque et al., 2015). These mutants are thus essentially unsuitable for functional studies of the canonical autophagy pathway.

There is still a great deal of uncertainty concerning the origin of the membranes required for autophagosome formation. They seem to originate from multiple compartments, such as the endoplasmic reticulum, Golgi, mitochondria, plasma membrane, and early and recycling endosomes (Lamb, Yoshimori, & Tooze, 2013). ATG9 is a transmembrane ATG protein whose precise function is elusive but is thought to deliver membranes to the preautophagosomal structures and autophagosomes (Mari et al., 2010). It is an essential component of the autophagy initiation machinery, which has been shown to be necessary for optimal autophagy in mammalian cells and in budding yeast (Noda et al., 2000; Young et al., 2006). With the aim of interfering only with autophagy-related functions in the parasites, we thus sought to study and to disrupt the function of the single *T. gondii* ATG9 homolog.

In contrast with TgATG8 and its related machinery, in *Toxoplasma* ATG9 depletion had no effect on apicoplast homeostasis. *In vitro*, TgATG9-depleted parasites only displayed a mild phenotype, when experiencing stress due to long extracellular incubation. They also showed a reduced proteolytic capacity, suggestive of an impaired autophagy pathway, although direct implication of the protein in the

biogenesis of autophagosomes could not be demonstrated. Importantly, however, further characterization of the mutant *in vivo* revealed that TgATG9 is crucial for the virulence of the parasites in mice. Thus, our study highlights that there might be important roles for canonical autophagy in the context of the host organism.

2 | RESULTS

2.1 | *T. gondii* contains a single putative ATG9 homolog which partially localizes to the trans-Golgi network and endolysosomal compartments

ATG9 is the only known multipass transmembrane autophagy protein among about 40 ATG proteins identified to date. It has six conserved transmembrane domains (He et al., 2006; Noda et al., 2000; Young et al., 2006). We performed homology searches in the *T. gondii* genomic database (www.toxodb.org) and identified a single putative homolog for ATG9 (TGME49_260640). TgATG9 is predicted to be a 1655 amino acids (aa)-long protein, with a generally well-conserved "ATG9 domain" (accession number: PF04109 in the Pfam database <http://pfam.xfam.org/>) extending from aa 370 to aa 870 (Figure 1a, Figure S1a,c). However, other parts of the proteins have less overall homology. This is quite common in ATG9 from other eukaryotes: for instance, the cytosolic N- and C-terminal domains of mammalian ATG9 have no significant homology to the respective yeast domains (Webber & Tooze, 2010).

Another characteristic of ATG9 is the presence of six transmembrane domains (Figure S1c). We generated a C-terminally-HA-tagged version of the native protein (TgATG9-HA) by endogenous locus recombination (Figure 1b), and found after gel electrophoresis and immunoblot analysis that TgATG9 migrates close to its predicted molecular mass of 184 kDa (Figure 1c). After parasite fractionation, the protein was found to be exclusively associated with the membrane fraction (Figure 1d). Moreover, we found this association of TgATG9 with the insoluble fraction is resistant to chaotropic agents such as urea or high salt concentrations, as expected for a transmembrane protein (Figure 1e). We also analyzed the exposure of the C-terminal HA tag, by proteinase K digestion assays and found it is exposed to the cytosolic face of the membranes (Figure 1f). Thus, combining bioinformatic predictions of the transmembrane domains, together with biochemical analysis, we found the overall topological model of TgATG9 (Figure 1a) to be similar to its counterparts in budding yeast (He et al., 2006) and mammals (Young et al., 2006) (Figure S1c).

We then assessed the subcellular localization of TgATG9 by immunofluorescence assay (IFA) using an anti-HA antibody. The protein was found to localize to vesicular structures, usually in the apical part of the parasites (Figure 2a). Contrarily to TgATG8, TgATG9 does not seem to be associated with the apicoplast (Figure 2a). We tested for colocalization with a number of organelles and found no particular association of TgATG9 with the rhoptries, micronemes and mitochondrial network either (Figure S2). On the other hand, we could detect partial colocalization with several markers, which are, from the highest to the lowest correlation coefficient, the vacuolar compartment (costained with the TgCRT marker [Warring, Dou, Carruthers,

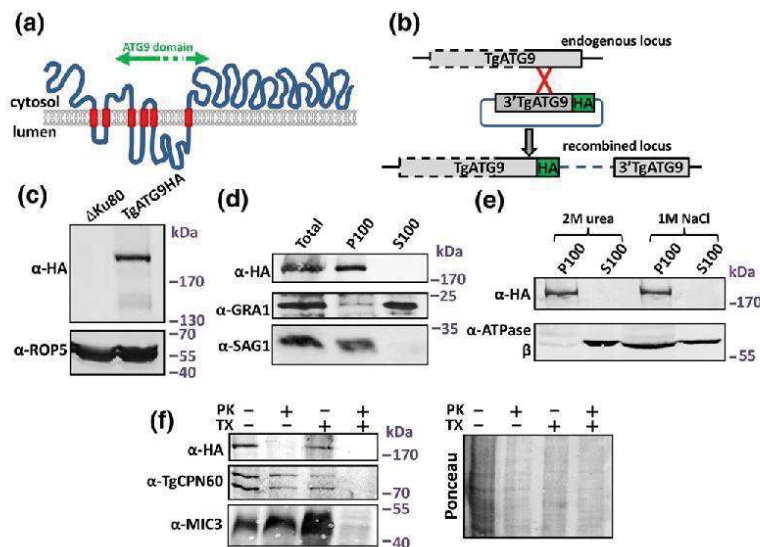


FIGURE 1 TgATG9 is a membrane-associated protein. (a) Topological model for TgATG9 based on bioinformatics predictions and biochemical analyses displayed in (e). The conserved ATG9 domain (Pfam PF04109) is delineated in green. (b) Strategy for adding a C-terminal triple hemagglutinin (HA) tag to TgATG9 by single homologous recombination at the endogenous locus of the corresponding gene. (c) Detection by immunoblot of the HA-tagged TgATG9-HA protein in parasite extracts. ROP5 has been used as a loading control. (d) Immunoblot analysis of parasite lysates after fractionation shows TgATG9-HA is associated with membrane fractions (P100 and S100 are the pellet and soluble fractions, respectively, after a 100,000 g centrifugation). GRA1 is a soluble protein control and SAG1 is a membrane-associated protein control. (e) Immunoblot analysis shows TgATG9-HA present in the P100 fraction described in (d) is resistant to high salt or urea extraction, in contrast with the β subunit of the F₁ ATPase, which was used as a peripheral membrane protein control. (f). Proteinase K digestion assay, in the presence or absence of detergent, shows that the C-terminus of TgATG9-HA is exposed on the cytoplasmic side. CPN60 and MIC3, which are proteins contained within the lumen of membrane-bound organelles (the apicoplast and micronemes, respectively) are protected from Proteinase K digestion in the absence of detergent

McFadden, & van Dooren, 2014)), the Golgi apparatus (costained with the GRASP marker [Lorestani et al., 2010]), as well as vesicles of the endolysosomal pathway (early endosomes were costained with the pro-M2AP marker [Harper et al., 2006]; Figure 2a,b). We also evaluated the localization of the protein in autophagy-inducing conditions and observed an occasional colocalization of the TgATG9 signal with some TgATG8-positive vesicles, which are distinct from the apicoplast or Golgi apparatus signals, and thus likely represent autophagic vesicles (Figure 2c). ATG9 is an early marker of autophagosome biogenesis and is only transiently associated with these vesicles (Orsi et al., 2012); it is thus unsurprising to see only a partial colocalization of the two markers. Overall, these localization data are consistent with previous studies on other eukaryotic ATG9: in mammalian cells, for instance, ATG9 is found on vesicles trafficking between the Golgi, diverse endocytic vesicles, and autophagosomes (Longatti et al., 2012; Orsi et al., 2012; Takahashi et al., 2011).

2.2 | Intracellular growth of ATG9-depleted parasites is unaffected in nutrient-rich conditions in vitro

In order to functionally characterize TgATG9, we sought to generate a TgATG9-depleted cell line. All autophagy-related genes previously characterized in our laboratory (TgATG3, TgATG4 and TgATG8) are

indispensable for intracellular growth of the parasite, likely due to their role in apicoplast homeostasis. In case TgATG9 would also be essential, we generated an inducible knock-down TgATG9 (KDTgATG9) cell line (Figure 3a). The endogenous promoter of TgATG9 was replaced by an inducible TetO7Sag4 promoter, which can be reversibly turned on or off thanks to a tetracycline-based transactivator system (Meissner, Brecht, Bujard, & Soldati, 2001). Replacement of the endogenous promoter by the inducible promoter system in KDTgATG9 was confirmed by polymerase chain reaction (PCR; Figure 3b). The promoter replacement was done in the TgATG9-HA background cell line and did not affect extensively TgATG9 expression levels (Figure S3a), or its localization (Figure S3b). Also, this way we could monitor the down-regulation of the protein by immunoblot using an anti-HA antibody. TgATG9 was no longer detectable after 2 days of anhydrotetracycline (ATc) treatment (Figure 3c, Figure S3b). However, in contrast with the apicoplast-related TgATG mutants, ATc-driven depletion of TgATG9 in this cell line did not affect the parasite lytic cycle in vitro (Figure S4a). Consistent with this, we found that conditional depletion of TgATG9 had no particular impact on the apicoplast or on the mitochondrial network (Figure S5a). Loss of TgATG9 had also no apparent effect on TgATG8 localization at the apicoplast either (Figure S5b).

Overall, this suggested that TgATG9 is a dispensable gene, but to rule out an absence of phenotype that could be due to some residual

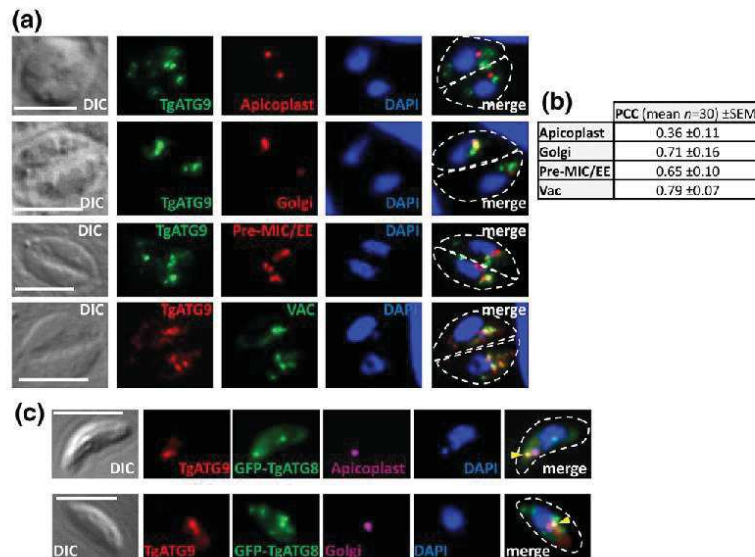


FIGURE 2 TgATG9 localizes to multiple membrane-bound subcellular compartments. (a) Co-labeling, in intracellular parasites, of TgATG9-HA with markers from the apicoplast (TgATR1), Golgi apparatus (GRASP-RFP), pre-micronemal or early endosomal compartment ("Pre-MIC/EE," proTgM2AP), and vacuolar compartment ("VAC," TgCRT-GFP). (b) Pearson's correlation coefficient values for costainings between TgATG9 and indicated markers; values are mean \pm standard error calculated from $n = 30$ cells. (c) Colabeling, in starved extracellular parasites, of TgATG9-HA, with GFP-TgATG8-decorated vesicles (yellow arrowhead) distinct from the apicoplast (labelled with TgATR1) or the Golgi apparatus (stained for the GRASP marker), and thus likely representing autophagic vesicles. Differential interference contrast images are shown on the left. DNA is stained in blue with DAPI. Scale bar = 5 μ m.

proteins, we went on to completely delete TgATG9. A TgATG9 knock-out cell line (Δ TgATG9) was generated by double homologous recombination (Figure 3d). Two independent clones were selected and were found to be similar in subsequent phenotypic analyses (not shown). The replacement of the entire TgATG9 coding region by a chloramphenicol acyltransferase (CAT) selection cassette was confirmed by PCR (Figure 3e). Accordingly, semiquantitative RT-PCR analyses using TgATG9-specific primers showed that there was no more TgATG9 transcript in the Δ TgATG9 cell line (Figure 3f). Similar to the inducible TgATG9 knock-down cell line, Δ TgATG9 parasites showed no intracellular growth defect in vitro (Figure S4b). Real-time quantitative PCR analysis of parasite growth in human foreskin fibroblasts (HFF) confirmed the mutant parasites were not impaired in growth, even suggesting a slightly increased growth rate (Figure S4c).

The absence of an apicoplast-related phenotype and a preserved viability in nutrient-rich conditions for TgATG9-depleted parasites make this protein a suitable candidate to assess its putative involvement in canonical autophagy in *T. gondii*.

2.3 | Assessing alterations of the autophagy pathway due to the loss of TgATG9

T. gondii tachyzoites spend most of their lytic cycle inside their intracellular replicative niche. Hence, the extracellular milieu is very likely for them a source of a significant environmental stress. In particular, the ability to respond to nutrient stress is important for the fitness of

extracellular parasites (Konrad, Wek, & Sullivan, 2011; Konrad, Wek, & Sullivan, 2014). Our previous studies have shown that extracellular parasites, even incubated in Dulbecco's modified eagle medium (DMEM) medium with a full complement of amino acids, already display a basal level of TgATG8-decorated autophagosomes significantly higher than intracellular parasites (Besteiro et al., 2011). Autophagosomes numbers are further increased when extracellular parasites are incubated in an amino acids-depleted medium such as host-based security system (HBSS; Besteiro et al., 2011; Kong-Hap et al., 2013). To investigate the involvement of TgATG9 in extracellular survival and its possible role in autophagy, we first used extracellular Δ TgATG9 parasites transiently expressing the GFP-TgATG8 autophagosomal marker to quantify the formation of autophagosome-like structures. Surprisingly, the Δ TgATG9 cell line was still able to generate multiple GFP-TgATG8-decorated vesicles (Figure 4a). Quantification of the percentage of puncta-bearing parasites, or the number of puncta per parasite, showed no significant difference between extracellular parasites of the Δ TgATG9 and parental cell line, both in amino acids starvation (HBSS) and complete (DMEM) media (Figure 4b,c). As shown in previous studies in other eukaryotic models, depletion of ATG9 does not lead to a complete inhibition of GFP-ATG8 puncta appearance following starvation (Bader, Shandala, Ng, Johnson, & Brooks, 2015; Young et al., 2006). It was suggested in the original description of the yeast ATG9 mutant that, instead of being needed for the initiation of autophagy vesicle formation, the protein is more likely needed for the completion of the sequestering

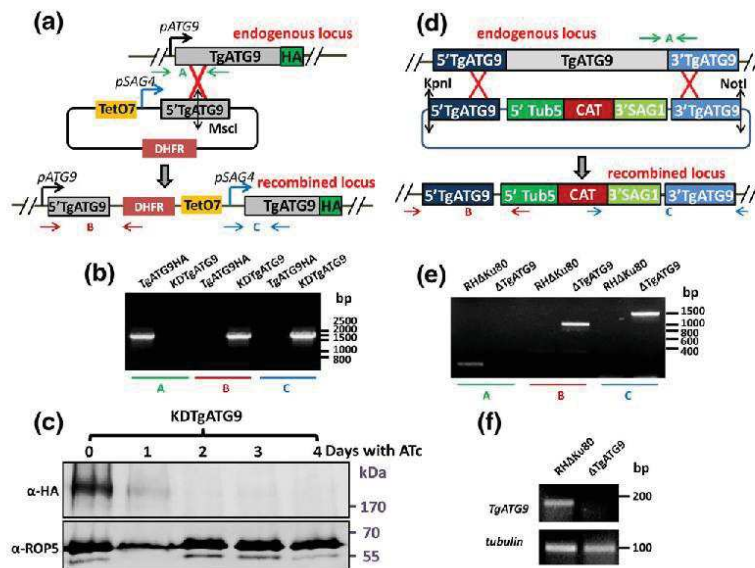


FIGURE 3 Two parallel strategies were implemented to generate TgATG9-depleted parasites. (a) A conditional knock-down cell line was generated in the TgATG9-HA background by replacing the endogenous promoter with an ATc-regulated promoter. Clones were obtained after pyrimethamine selection. Arrows represent primers used to verify integration by the PCR shown in (b). TetO7 = tet operator, DHFR = dihydrofolate reductase selection marker, pSAG4 = SAG4 minimal promoter. (b) Genomic DNA regions from TgATG9-HA and KDTgATG9 cell lines were amplified with primers couples depicted in (a) for PCR detections of the endogenous and recombined loci. (c) Immunoblot assessment of TgATG9-HA depletion in protein lysates from parasites incubated in absence or presence of ATc for up to 4 days. ROP5 was used as a loading control. (d) A TgATG9 knock-out cell line was generated in the RHΔKu80 background by double homologous recombination. Clones were obtained after chloramphenicol selection. Arrows represent primers used to verify integration by the polymerase chain reaction (PCR) shown in (e). CAT = chloramphenicol acetyl transferase; 5'Tub5 and 3'SAG1: untranslated regions from the *tubulin* and *SAG1* genes, respectively, for driving chloramphenicol acetyl transferase expression. (e) Genomic DNA regions from RHΔKu80 and ΔTgATG9 cell lines were amplified with primers couples depicted in (d) for PCR detections of the endogenous and recombined loci. (f) Semi-quantitative RT-PCR analysis of TgATG9 expression in the ΔTgATG9 mutant and parental cell lines. Specific β -tubulin primers were used as controls

membrane of the autophagosome (Noda et al., 2000). Of course, because of the limit of optical microscopy, we could not distinguish between functional autophagosomes or incomplete GFP-TgATG8-positive structures. We tried to assess morphological differences by electron microscopy, but these structures are particularly difficult to identify, and we could not obtain quantitative data (not shown).

One major hindrance to a more complete analysis of the mutant was the lack of functional assay available for measuring the autophagic flux in *T. gondii*. One way to monitor the autophagic flux is to measure the turnover rate of long-lived proteins, which are normally degraded by autophagy, while short-lived proteins are thought to be degraded by the proteasome (Bauvy, Meijer, & Codogno, 2009). We thus sought to adapt to *Toxoplasma* a protocol using a "clickable" methionine analog (L-azidohomoalanine or AHA) for global protein labeling, followed by quantification by flow cytometry after autophagy-inducing conditions for measuring autophagy-dependent proteolysis (Zhang, Wang, Ng, Lin, & Shen, 2014). After trying different concentrations and incubation times, we found conditions where the incorporation of AHA is suitable for protein labeling without affecting parasite viability (Figure 5a,b). To avoid labeling of short-lived proteins (whose turnover depends on the proteasome),

extracellular parasites were submitted to a 1 hr chase in methionine-rich DMEM after AHA incorporation, and they were subsequently incubated for 4 hrs in either starvation (HBSS) or complete (DMEM) media. A viability dye was also added to the end of autophagy-inducing treatment with the aim of labeling dead parasites, which would be subsequently excluded from our flow cytometry analysis (Figure 5c). After incubation in HBSS, the majority of the parasite population was dead and this rendered the quantification difficult (not shown). Although extracellular parasites kept in complete medium are likely experiencing a significant nutrient stress (Besteiro et al., 2011; Konrad et al., 2011; Konrad et al., 2014), they remained essentially viable (Figure 5c), and thus much more suitable to measure proteolysis and the involvement of autophagy. Interestingly, after 4 hrs incubation in complete DMEM, flow cytometry analyses showed a moderate, but statistically significant, reduction of proteolysis in ΔTgATG9 mutant compared to the parental cell line (Figure 5d). As a control, we used a cathepsin L mutant cell line (ΔTgCPL), deficient for a major vacuolar protease (Parussini, Coppens, Shah, Diamond, & Carruthers, 2010), thus likely impaired in its overall proteolytic capacity, and it showed the same trend as the ΔTgATG9 mutant (Figure 5d).

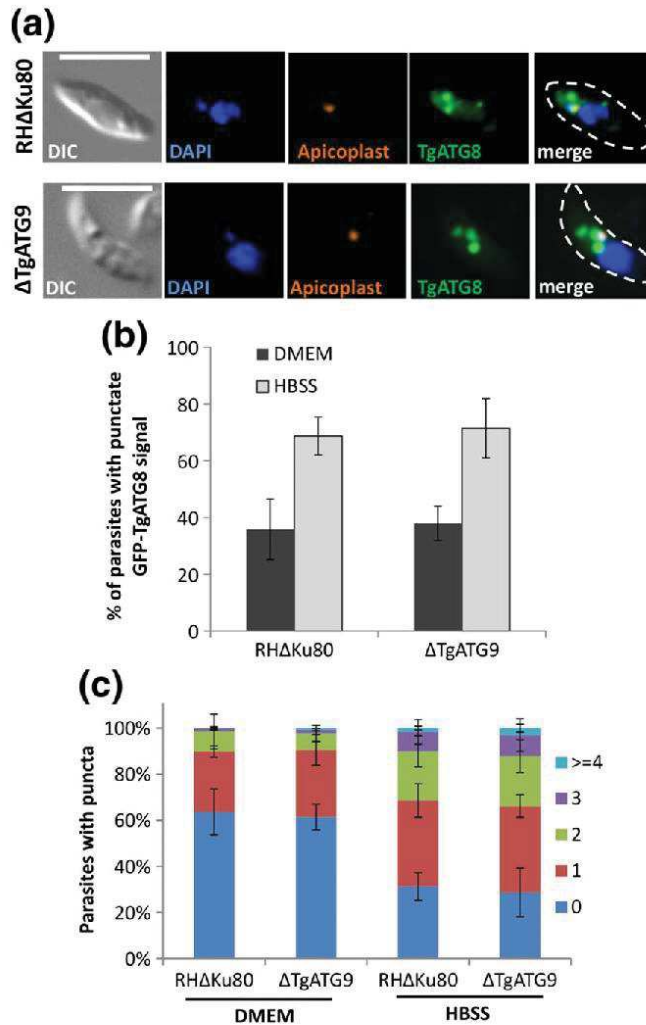


FIGURE 4 The formation of TgATG8-decorated vesicles is unaffected in TgATG9-depleted parasites. (a) Examples of extracellular RHΔKu80 and ΔTgATG9 parasites transiently expressing GFP-TgATG8 that were starved for 6 hrs in HBSS and found to display autophagosome-like punctate signal (distinct from the apicoplast, which was stained with TgATRX1). DIC = differential interference contrast. DNA is stained in blue with DAPI. Scale bar = 5 μm. (b) Quantification of ΔTgATG9 parasites transiently expressing GFP-TgATG8 that displayed a punctate GFP-TgATG8 signal (other than the apicoplast) following a 6-hr starvation in HBSS or incubation in DMEM as a control. Mean values from three independent experiments were represented ± SEM. (c) Quantification of GFP-TgATG8 puncta (other than the apicoplast) in ΔTgATG9 parasites transiently expressing GFP-TgATG8 starved for 6 hrs in HBSS or kept in DMEM. Mean values from three independent experiments were represented ± SEM

2.4 | Depletion of TgATG9 reduces extracellular survival of *T. gondii*

To assess precisely whether the viability of extracellular parasites was differently affected in the mutant, we incubated freshly egressed parasites in amino acid-free or in complete media. The use of a membrane impermeable fluorescent viability dye, coupled with quantification by microscopy, allowed us to measure the mortality rate at any given time during the incubation period (Figure 6a, Figure S6a). In the absence of amino acids, both the parental and the TgATG9 mutant cell lines showed a rapidly increasing mortality rate that began to plateau (or even decrease, possibly due to fluorescence loss after parasite degradation), after 2 hrs of treatment (Figure S6a). Parasites incubated in complete DMEM, however, displayed generally a much more reduced mortality rate. Interestingly though, ΔTgATG9 mutant parasites

displayed a systematically higher mortality rate than the control, and it increased drastically after 16 hrs of treatment (Figure 6a). To assess the overall viability of these parasites kept in extracellular conditions, we used their ability to invade and establish a parasitophorous vacuole inside host cells as a readout. This confirmed that, for both cell lines, an incubation in HBSS for several hours dramatically decreased parasite viability (Figure S6b). As for the proteolysis assay described above, the high mortality rate due to these particularly harsh conditions was not suitable to observe any significant difference between the two cell lines. However, ΔTgATG9 parasites kept in DMEM for 16 hrs showed a reduced viability in comparison to the parental cell line kept in the same conditions (Figure 6b). This is in accordance with the increased death rate observed for the mutant parasites in these conditions, and this suggests TgATG9 is important for viability in the extracellular state.

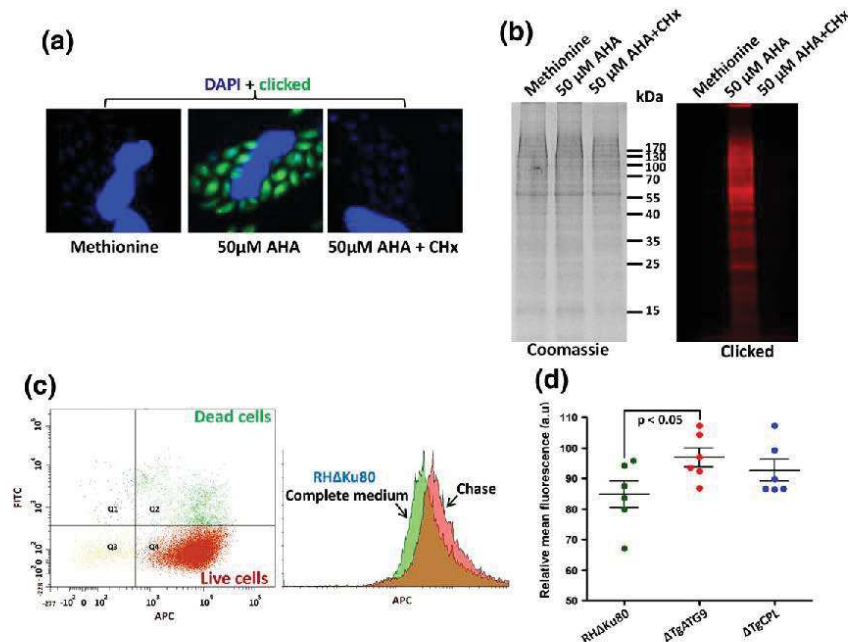


FIGURE 5 Quantitative measurement of the proteolysis from long-lived proteins using AHA metabolic labeling. (a) Fluorescence microscopy after the click reaction shows methionine analog AHA was successfully incorporated into intracellular tachyzoites following a 6-hr incubation. The use of protein synthesis inhibitor cycloheximide (Chx) together with AHA prevented its incorporation. DNA is stained in blue with DAPI. (b) Protein-labeling profile of parasites incubated in the conditions described in (a) was assessed by in gel fluorescence imaging. (c) Example of flow cytometry analysis of AHA-labeled extracellular parasites. Selected parasites were negative for the viability exclusion dye eFluor520 (FITC channel) and positive for clicked AHA labeling (APC channel). Decrease of AHA labeling in these parasites after extracellular incubation in complete medium likely reflects proteolysis (right). (d) Quantification of AHA fluorescence values after incubation of RHΔKu80, ΔTgATG9 and ΔTgCPL extracellular parasites kept in DMEM for 4 hrs suggests that the ΔTgATG9 and ΔTgCPL mutants have a lesser ability to degrade proteins that the RHΔKu80 control cell line. Mean values from six independent experiments were represented \pm SEM. p value was calculated using unpaired Student's T test with two-tailed equal variance

2.5 | Depletion of TgATG9 strongly diminishes parasite virulence in vivo

During acute infection of intermediate hosts, *T. gondii* tachyzoites multiply rapidly following complete cycles of invasion, intracellular replication, and lytic egress. Being obligate intracellular parasites, their survival depends primarily on their ability to invade their host cells, which provide for their nutritional needs, because if they persist in the long term as an extracellular form, they are more prone to external stresses (Khan, Behnke, Dunay, White, & Sibley, 2009). On the other hand, the parasites also have to face a significant pressure from their hosts' immune system and, in particular, activated effector cells (Yarovinsky, 2014). Therefore, we sought to investigate how the loss of TgATG9 would impact parasite virulence in vivo. BALB/c mice were infected by intraperitoneal injection of 100 ΔTgATG9 parasites, and their survival was monitored on a daily basis (Figure 7b). Compared with mice infected with the RHΔKu80 parental cell line control that died after 11 days, most ΔTgATG9-infected mice showed no fatality throughout the whole duration of the experiment. To verify that the lack of virulence was due to the specific absence of TgATG9, we also

used the KDTgATG9 cell line in the presence or absence of ATc (Figure 7b). In the absence of ATc, KDTgATG9 parasites were found to be slightly less efficient in killing mice than the parental cell line, which might be due to a subtle alteration in TgATG9 basal protein levels, or timing of expression during the cell cycle, resulting from the promoter change strategy we used for generating this conditional knock-down cell line (Figure S3a). Also, it should be noted that in the presence of ATc, the survival of mice infected with TATi1-ΔKu80 control parasites was slightly prolonged, which might be attributed to a direct effect of the drug present in the drinking water of mice throughout the experiment. More importantly, mice infected with KDTgATG9 parasites and kept in the presence of ATc survived throughout the whole duration of the experiment. Overall, this shows TgATG9 is crucial for parasite fitness in the host.

Parasite burden in peritoneal exudates was evaluated at day 4 post-infection, using real time quantitative PCR to measure levels of transcripts of the tachyzoite-specific surface antigen SAG1, and showed an apparently reduced number of ΔTgATG9 parasites compared with control (Figure 7c). Moreover, parasites were not detected in spleen extracts (not shown), which is consistent with an early

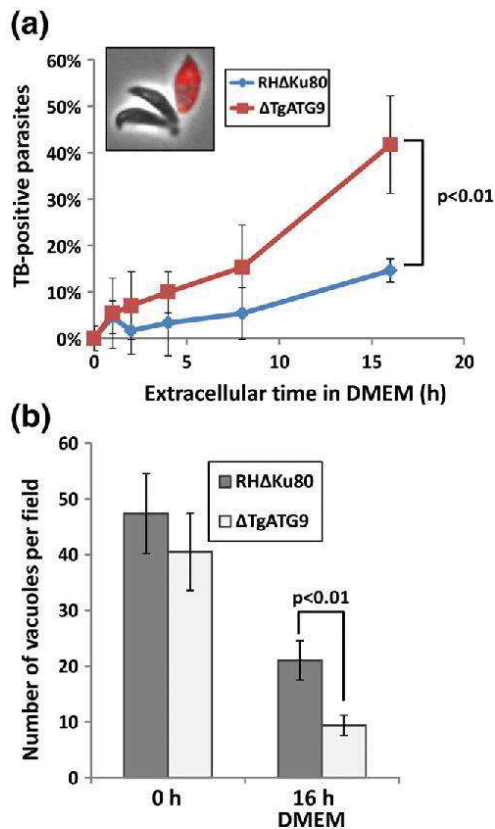


FIGURE 6 TgATG9 mutant parasites are less able to sustain extracellular stress. (a) Mortality rate of RHΔKu80 and ΔTgATG9 extracellular parasites kept in DMEM medium for increased periods of time was assessed by their permeability to trypan blue, which is impermeable to intact membranes and becomes fluorescent when complexed with intracellular proteins (inset). Shown are mean values \pm SEM from three independent experiments. The p value was calculated using unpaired Student's T -test with two-tailed equal variance. (b) Viability of RHΔKu80 and ΔTgATG9 parasites at the end of the experiment described in (a) was assessed by their ability to invade host cells. Shown are the mean values \pm SEM from one representative experiment out of three. The p value was calculated using unpaired Student's T test with two-tailed equal variance

clearance of ΔTgATG9 parasites in the peritoneum. When injected in the peritoneal cavity, the parasite is able to invade macrophages and other cells of innate immunity, which have been suggested to be instrumental in disseminating the parasites in the host (Da Gama, Ribeiro-Gomes, Guimarães, & Arnholdt, 2004). Thus, we next assessed the ability of TgATG9-depleted parasites to survive within macrophages. We isolated thioglycolate-elicited peritoneal macrophages from BALB/c mice, and they were infected with freshly egressed parasites of the ΔTgATG9 or the parental control cell lines. Parasite growth was quantified after 48 hrs by real time quantitative PCR using the SAG1 marker (Figure 7d). In sharp contrast with experiments where

the same technique was used to assess parasite growth in HFFs (Figure S4c), ΔTgATG9 parasites in macrophages were drastically affected two days after invasion. This suggests a reduced growth and/or an increased clearance for TgATG9-depleted parasites, specifically in the context of the macrophages. This is likely the explanation for the absence of parasitemia we found in the spleen and the overall reduced virulence of these mutant parasites for their host.

3 | DISCUSSION

Autophagy is an important mechanism for maintaining cell homeostasis in eukaryotes. This biological process has only recently started to be characterized in apicomplexan parasites, and it appears that these early-branching eukaryotes bear a number of original features compared with classic eukaryotic models. In response to starvation, *Toxoplasma* tachyzoites are able to generate subcellular structures resembling autophagosomes and decorated with autophagosomal marker TgATG8. Electron microscopic studies also brought morphological evidences of degraded cellular material in these autophagic vesicles (Besteiro et al., 2011; Ghosh et al., 2012). However, there has been no clear demonstration yet that the autophagosomes formed under stress conditions and their content are actually recycled within tachyzoites. The main reason for this is the lack quantitative assay for measuring the autophagic flux in the parasites. Moreover, all autophagy-related mutants that were generated so far in *Toxoplasma* (TgATG3, TgATG4, and TgATG8 [Besteiro et al., 2011; Kong-Hap et al., 2013; Lévêque et al., 2015]) are affected for TgATG8 function, and this protein happens to also have a peculiar noncatabolic role at the apicoplast membrane (Lévêque et al., 2015). This role being essential for the survival of the parasites, these mutants are unsuitable for studying canonical autophagy.

In the present study, we describe TgATG9, which is the *Toxoplasma* homolog of a protein known to be important in the early steps of autophagosome formation in other eukaryotes. The predicted topology of TgATG9 and its localization, both during normal growth and in starvation conditions, are very similar to what has been described for its other eukaryotic counterparts (Noda et al., 2000; Orsi et al., 2012), suggesting it might also be involved in canonical autophagy in the parasites. Depletion of TgATG9 did not affect parasite growth and apicoplast homeostasis, confirming that the lethal phenotype of other previously generated TgATG mutants was probably due to the impairment of an apicoplast-related function, and not to a lack of canonical autophagy. It also suggests autophagy is not essential for parasite growth in normal in vitro culture conditions, as observed in several other eukaryotic cell types when grown with a full complement of nutrients. In the yeast *Saccharomyces cerevisiae*, for example, the autophagy machinery is dispensable for vegetative growth in rich medium but essential to survive nitrogen starvation, which was at the basis of the strategy originally used to decipher the molecular components of the machinery in this model eukaryote (Devenish & Klionsky, 2012; Tsukada and Ohsumi, 1993).

When we next investigated the fate of the parasites upon prolonged absence of amino acids, we could observe that these conditions led to an equally detrimental phenotype for mutant and control

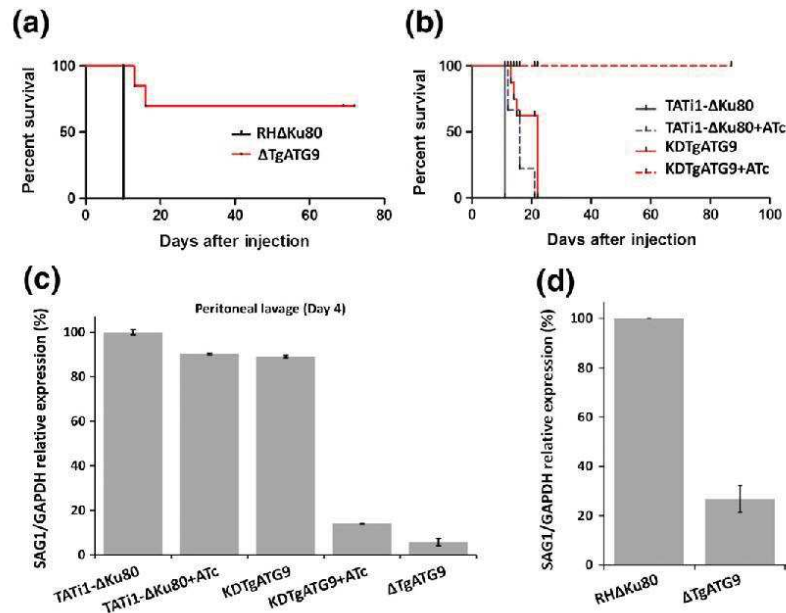


FIGURE 7 TgATG9 is crucial for parasite survival inside macrophages and for virulence in vivo. (a) Survival curve of BALB/c mice ($n = 10$) infected intraperitoneally with 100 parasites from the Δ TgATG9 and RH Δ Ku80 cell lines. Data are representative from three independent experiments. (b) Survival curve of BALB/c mice ($n = 10$) infected intraperitoneally with 100 parasites from the KDTgATG9 and TAT11- Δ Ku80 cell lines, and kept, or not, in the presence of ATc. (c) Real-time quantitative PCR detection of parasites in peritoneal extracts of mice ($n = 5$), four days post-infection with the cell lines described in (a) and (b). *T. gondii* SAG1 and mouse *Glyceraldehyde-3-Phosphate Dehydrogenase* were used to quantify relative abundance of parasite and host messenger RNAs, respectively. Data presented are mean value from triplicate \pm SEM. (d) In vitro parasite growth in peritoneal macrophages was evaluated by real-time quantitative PCR. Peritoneal macrophages isolated from mice were infected at a ratio of 1:1 with parasites, and 48 hrs later relative abundance of parasite and host messenger RNAs was quantified as described in (c). Mean values from three independent experiments were represented \pm SEM

cell lines. Harsh starvation conditions, although useful to induce and observe the appearance of autophagy vesicles (Besteiro et al., 2011; Ghosh et al., 2012), are rapidly inducing death in *Toxoplasma* tachyzoites. It has been previously suggested that autophagy is actually a key actor of this starvation-induced death (Ghosh et al., 2012). However, our study shows that the mortality rate and the overall loss of viability in the population are similar between TgATG9-depleted and control parasites. Not only this would argue against the occurrence of an autophagy-mediated cell death (Ghosh et al., 2012), but it also suggests the inability of the autophagy pathway to efficiently protect extracellular parasites from the detrimental consequences of a prolonged amino acids starvation.

Assessing the formation of TgATG8-decorated vesicles upon starvation in TgATG9-depleted parasites, however, could not give us a definitive answer on the involvement of TgATG9 in autophagosome biogenesis. It has been shown in other eukaryotes that the absence of ATG9 does not completely abolish the formation of autophagosomes (Bader et al., 2015; Young et al., 2006), driving researchers towards alternative methods, like measuring the proteolytic capacity of mutant cells (Young et al., 2006), to show autophagy was impaired. We thus implemented this approach in our model, by using a metabolic labelling technique involving a methionine analog

that can be linked chemically to a fluorophore for quantification (Zhang et al., 2014). Once again, the high mortality in the parasite population following harsh starvation conditions prevented the analysis of the mutant. However, using this technique, we could detect a proteolysis defect in the Δ TgATG9 extracellular parasites incubated in complete DMEM, compared with parental parasites kept in the same conditions. This would suggest TgATG9 is indeed involved in the proteolytic capacity of tachyzoites through its function in the autophagy pathway.

One should note that in spite of these inferred evidences, TgATG9 might also have another function which is beyond canonical autophagy. Descriptions of nonautophagy-related functions for ATG proteins have been increasing in the recent literature (Bestebroer, V'kovski, Mauthe, & Reggiori, 2013; Mauthe et al., 2016; Subramani & Malhotra, 2013). Another possibility is that there might be a fully functional autophagy pathway in *Toxoplasma*, but TgATG9 might not be necessary for it. For instance, it is surprising to see that while ATG9 homologs are clearly present in coccidia (Figure S1a,b), they seem to be absent from other apicomplexan species. This is also true for *Plasmodium*, which has been nevertheless suggested to contain a functional catabolic autophagy pathway on the basis of colocalization studies between autophagosomal vesicles and PfRAB7-endosomes (Tomlins et al., 2013). Whether this suggests canonical autophagy might not,

after all, be functional in these apicomplexan parasites or they have evolved a different way of initiating early steps of autophagosome biogenesis remains to be investigated further.

As they invade, tachyzoites establish themselves inside a parasitophorous vacuole, from where they will get access to nutrient sources from the host (Blader & Koshy, 2014). On the other hand, at the end of the lytic cycle, when searching for a new host cell to reinvade, the parasites are under the stress of the extracellular environment. Extracellular tachyzoites can use their own internal carbon sources to power the ATP demanding gliding motility initially (Lin, Blume, Ahsen, Gross, & Bohne, 2011), but a prolonged extracellular state leads to a significant decrease in viability (Khan et al., 2009 and this study). In these conditions, tachyzoites seem to be able to sense stress and initiate a response, like for instance, triggering conversion into bradyzoites (Yahiaoui et al., 1999; a slow-growing form more resistant to metabolic, immunological, or chemical stress) upon reentry into a host cell. Thus, even in complete DMEM, the extracellular environment probably lacks essential nutrients, or extracellular tachyzoites might not be able to uptake efficiently these nutrients. A conserved eukaryotic translational control pathway in *Toxoplasma*, relying on the phosphorylation of the alpha subunit of the eukaryotic initiation factor 2 (eIF2 α) has been characterized in *Toxoplasma* (Sullivan, Narasimhan, Bhatti, & Wek, 2004) and is important to sustain the viability of extracellular tachyzoites (Joyce, Queener, Wek, & Sullivan, 2010; Konrad et al., 2011; Konrad et al., 2014). Interestingly, the eIF2 α pathway is an upstream regulator of autophagy in response to nutrient deprivation and other stresses in mammalian cells (Kroemer, Mariño, & Levine, 2010). The fact that TgATG9-depleted parasites are less able to survive long extracellular incubations would suggest that autophagy could be an important player of the eIF2 α -controlled integrated stress response in *Toxoplasma*, similarly to what has been observed for other eukaryotic models and certainly deserves further investigations.

The ability to survive in the extracellular environment following lysis of a host cell is the key to drive successfully the invasion of a new host and subsequent propagation of the tachyzoites. However, to what extent this really is important in the *in vivo* context is not known. Once the parasites are established within a tissue, they probably have an easy access to potential host cells and might not remain extracellular for extended periods of time. In sharp contrast with the moderate phenotype we observed *in vitro*, TgATG9-depleted parasites were clearly not able to propagate and to generate a sustained infection following intra-peritoneal injection into BALB/c mice. During infection, macrophages are important regulatory and effector cells, as they can both be infected by *T. gondii* and contribute to its propagation within tissues, or they can limit parasite replication and produce cytokines that contribute to resistance (Da Gama et al., 2004). Interestingly, we demonstrated *in vitro* that the survival of TgATG9-depleted parasites in macrophages is compromised, hinting the protein plays a key role in the interaction between parasites and these immune cells.

Host control of *Toxoplasma* depends on the production of pro-inflammatory cytokines and is largely dependent on IFN- γ for activating effector mechanisms to eliminate intracellular parasites (Hunter & Sibley, 2012). Interestingly, the IFN- γ -dependent response in innate immune cells may include depletion of essential amino acids such as tryptophan, arginine, and generation of nitric oxide (Yarovinsky,

2014). Both amino acids starvation and oxidative stress are potent autophagy inducers (Kroemer et al., 2010), suggesting an involvement of this pathway in surviving these stresses. It should also be noted that response to both types of stress can involve the eIF2 α pathway (Harding et al., 2003). However, populations of *T. gondii* in North America and Europe are dominated by three clonal lineages (known as types I, II, and III) which are differentially able to modulate the host immune response. Present work was performed with parasites of a type I strain, which are normally highly virulent for mice, and are known to be able to disrupt the IFN- γ -dependent response to a significant extent (Hunter & Sibley, 2012). Thus, it would now be particularly interesting to investigate TgATG9 function in type II parasites to assess the full contribution of host IFN- γ -dependent stress on the elimination of TgATG9 mutants, as well as the ability of these parasites to enter the chronic dormant bradyzoite stage.

More work is needed to definitely demonstrate that there is a fully functional catabolic autophagy pathway in *Toxoplasma*. However, our data suggest that autophagy could be part of an integrated stress response pathway in the parasite. This response might be of particular importance for tachyzoites in the context of the host for surviving either as extracellular parasites, or inside the hostile environment of immune cells. This can be put in parallel with studies performed on TgCPL, a protease functioning as a classic degradative enzyme within the parasite endolysosomal system, both for extracellular and intracellular parasites (Dou, McGovern, Di Cristina, & Carruthers, 2014; Parussini et al., 2010). Strikingly, TgCPL-depleted parasites have a relatively mild growth phenotype *in vitro*, while they are attenuated for their virulence in mice (Dou et al., 2014). These data, together with our own results on TgATG9, are thus suggesting a potential implication of components of both the autophagic and proteolytic machineries in the survival of *Toxoplasma* tachyzoites in the context of the host and certainly calls for further investigations.

4 | EXPERIMENTAL PROCEDURES

4.1 | Ethics statement

All murine protocols were approved by the Institutional Animal Care and Utilization Committee (IACUC) of the American University of Beirut (AUB; IACUC Permit Number #15-08-346). All animals were housed in specific pathogen-free facilities. Humane endpoints were used as requested by the AUB IACUC according to AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International) guidelines and guide of animal care use book (Guide, NRC 2011). Mice were sacrificed for any of the following reasons: (a) impaired mobility (the inability to reach food and water), (b) inability to remain upright, (c) clinical dehydration and/or prolonged decreased food intake, (d) weight loss of 15–20%, (e) self-mutilation, (f) lack of grooming behavior or rough or unkempt hair coat for more than 48 hours, (g) significant abdominal distension, and (h) unconsciousness with no response to external stimuli. Animals were deeply anesthetized before cervical dislocation. All animals were housed in approved pathogen-free housing. Eye pricks were done following deep anesthesia with isoflurane.

4.2 | Parasites and cells culture

Tachyzoites of the RH Δ Ku80 (Huynh & Carruthers, 2009a), TATI1- Δ Ku80 (Sheiner et al., 2011) *T. gondii* strains, as well as derived transgenic parasites generated in this study were maintained by serial passage in human foreskin fibroblast (HFF, American Type Culture Collection, CRL 1634) cell monolayer grown in DMEM (Gibco) supplemented with 5% decompemented fetal bovine serum (Gibco), 2 mM L-glutamine (Gibco) and a cocktail of penicillin-streptomycin (Gibco) at 100 μ g/mL.

4.3 | Bioinformatic analyses

Sequence alignments were performed using the multiple sequence comparison by log-expectation algorithm of the Geneious software suite (www.genious.com). The phylogenetic tree was built using the neighbor-joining method included in the same software suite. Topology and TgATG9 transmembrane helices prediction was performed using the TMHMM Server v. 2.0 (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>) and the Phobius prediction software (<http://phobius.sbc.su.se/>). Domain searches were performed in the Pfam database (<http://pfam.xfam.org/>).

4.4 | Generation of an HA-tagged TgATG9 cell line

The ligation independent strategy (Huynh & Carruthers, 2009b) was used for C-terminal HA₃-tagging of TgATG9. A 1.66 kbp fragment corresponding to the 3' end of TgATG9 was amplified by PCR from genomic DNA, with the Phusion polymerase (New England BioLabs) using primers ML1946/ML1947 (primers used in this study are listed on S1Table) and inserted in frame with the sequence coding for a triple HA tag, present in the pLIC-HA₃-CAT plasmid. The resulting vector was linearized with MluI and 40 μ g of DNA were transfected into the TATI1- Δ Ku80 cell line to allow integration by single homologous recombination.

4.5 | Generation of conditional knock-down and knock-out TgATG9 cell lines

The conditional knock-down TgATG9 cell line (KDTgATG9) was generated based on the Tet-off system using the plasmid DHFR-TetO7Sag4-TgATG9. In brief, the 5' region of TgATG9 starting with the initiation codon was amplified from genomic DNA by PCR using Q5 polymerase (New England Biolabs) with ML1892/ML1893 primers containing BglII and NotI restriction sites, respectively. The 1.7 kb fragment was then inserted into BglII/NotI site of the DHFR-TetO7Sag4 plasmid (Morlon-Guyot et al., 2014), downstream of the ATc-inducible TetO7Sag4 promoter, obtaining the DHFR-TetO7Sag4-TgATG9 plasmid. The plasmid was then linearized by MscI digestion and transfected into the TATI1- Δ Ku80 cell line expressing 3 \times HA-tagged TgATG9. Transfected parasites were selected with g at a concentration of 1 ng/mL and cloned by serial limiting dilutions. Correct integration in the positive clones was verified by PCR (Figure 3b) using primers ML2530/ML2531 (a), ML1774/ML2531 (b), and ML1771/ML2530 (c).

The TgATG9 knock-out cell line (Δ TgATG9) was generated by replacing the endogenous TgATG9 locus with CAT cassette using double homologous recombination strategy. Briefly, 0.95 kb and 1.1 kb DNA fragments corresponding to the upstream of the initiation codon and downstream of the stop codon of TgATG9, respectively, were amplified from genomic DNA using ML2072/ML2073 and ML2074/ML2075 primers. These fragments were respectively cloned into KpnI/HindIII and BamHI/NotI site of pTub5/CAT plasmid (Soldati & Boothroyd, 1993), containing in between the CAT coding region under *tubulin promoter*. The resulting pTub5/CAT-TgATG9 plasmid was then digested with KpnI/NotI and transfected into the RH Δ Ku80 cell line. Transfected parasites were selected with chloramphenicol at concentration of 20 ng/mL and cloned by limit dilutions. Correct integration in the positive clones was verified by PCR (Figure 3e) using primers ML1851/ML2223 (a), ML2232/ML2233 (b) and ML388/ML2222 (c). Semi-quantitative rt-PCR was used to check for specific TgATG9 mRNA depletion (see below).

4.6 | TgATG9 mRNA analysis

Total mRNAs of freshly egressed extracellular parasites from the TATI1- Δ Ku80, KDTgATG9 (incubated with or without ATc at 1.5 μ g/mL for 3 days), RH Δ Ku80, and Δ TgATG9 cell lines were extracted using Nucleospin RNA II Kit (Macherey-Nagel). The cDNAs were synthesized with 660 ng of total RNA per RT-PCR reaction using Superscript II first-strand synthesis system (Invitrogen). Specific TgATG9 ML2535/ML2536 primers and, as a control, *Tubulin* β ML841/ML842 primers were used to amplify specific transcripts with the GoTaq DNA polymerase (Promega). PCR was performed with 22 cycles of denaturation (30 s, 95 $^{\circ}$ C), annealing (20 s, 56 $^{\circ}$ C), and elongation (20 s, 72 $^{\circ}$ C).

4.7 | Subcellular fractionation

3.10⁷ TgATG9-HA tachyzoites were solubilized in 1 ml of Tris HCl 50 mM pH 7.5 and sonicated twice for 30 seconds. Cellular debris were removed by centrifugation at 500 g for 10 minutes. The supernatant was submitted to an ultracentrifugation at 100,000 g for 30 minutes to yield a membrane-enriched high speed pellet and high speed supernatant soluble fractions, respectively. The supernatant fraction was TCA-precipitated and extracts were resuspended in SDS-PAGE loading buffer prior to immunoblot analysis.

Alternatively, the high speed pellet was further extracted by 1 M NaCl or 2 M urea for 2 hrs at 4 $^{\circ}$ C and submitted to another round of ultracentrifugation to yield a pellet and supernatant fraction for subsequent immunoblot analysis.

4.8 | Proteinase K digestion assay

Parasites were lysed by sonication in homogenization buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS pH 7.2, 2 mM dithiothreitol) and centrifuged at 1,500 g for 10 minutes to remove intact cells. An organellar fraction was obtained by centrifugation at 15,000 g for 30 minutes. The pellet was resuspended in homogenization buffer. Proteinase K (Sigma) and Triton X-100 were optionally added at

0.1 mg/ml and 0.5% v/v, respectively and incubated for 30 minutes at 4 °C before analysis by SDS-PAGE and immunoblotting.

4.9 | Immunoblot analysis

Protein extracts from 10^7 freshly egressed tachyzoites or from fractionation and the proteinase K digestion assays described above were separated by SDS-PAGE. Rat monoclonal anti-HA (3F10, Roche) was used to detect tagged TgATG9. Mouse anti-SAG1 (Couvreur, Sadak, Fortier, & Dubremetz, 1988), mouse anti-ROP5 (Leriche & Dubremetz, 1991), rabbit anti-TgCPN60 (Agrawal, van Dooren, Beatty, & Striepen, 2009), rabbit anti-MIC3 (Garcia-Réguet et al., 2000), or mouse anti-GRA1 (Lecordier, Mercier, Sibley, & Cesbron-Delauw, 1999) antibodies were used as controls.

4.10 | Plaque assays

Confluent monolayers of HFFs grown in 24-well plates were infected with 2×10^5 freshly egressed tachyzoites and incubated with or without ATc (at 1.5 µg/ml) for 6 days. Infected cell layer was then fixed in cold methanol (for 1 min) and stained with Giemsa (for 1 min). Images were acquired with an Olympus MVX10 macro zoom microscope equipped with an Olympus XC50 camera. Plaque area measurements were done using Zen software (Zeiss).

4.11 | Parasite viability assays

Freshly egressed extracellular tachyzoites of the RHΔKu80 and ΔTgATG9 cell lines were incubated in complete (DMEM) or amino acids starvation (HBSS) media at 37 °C with 5% CO₂. The experiment was performed in batch and at 0 h, 1 h, 2 h, 4 h, 8 h, and 16 h after incubation; live extracellular parasites were collected and stained with Trypan Blue (TB, Sigma) to determine the mortality rate by counting TB-positive parasites under a fluorescence microscope (excitation: 550/25 nm, emission: 605/70 nm); indeed, dead parasites take up TB into the cytoplasm because of loss of their membrane selectivity, and there TB becomes fluorescent as it is complexed to proteins (Harrison, Callebaut, & Vakaet, 1981). An assay for assessing parasite viability was also conducted by evaluating their invasive capacity. A total of 2×10^5 of freshly lysed tachyzoites of RHΔKu80 and ΔTgATG9 cell lines was used to infect confluent monolayer of HFFs grown on coverslips for 18 hrs. The number of parasitophorous vacuoles per field was visualized by IFA using anti-ROP1 antibody, with 100× objective lens.

4.12 | Immunofluorescence microscopy

For immunofluorescence assays IFA, intracellular tachyzoites grown on coverslips containing HFF monolayers were fixed for 20 min with 4% (w/v) paraformaldehyde in PBS, permeabilized for 10 min with 0.3% Triton X-100 in PBS and blocked with 0.1% (w/v) BSA in PBS. Primary antibodies used (at 1/1000, unless specified) for detection of the organelles were as follows: for the apicoplast, anti-ATRX1 (DeRocher et al., 2008) and for the mitochondrial network, anti-F₁ β ATPase (P. Bradley, unpublished); for rhoptries, anti-ROP1 (J.F. Dubremetz, unpublished); for micronemes, anti-MIC3 (Garcia-Réguet et al., 2000);

for early endosomes or pre-micronemal compartment, anti-prom2AP (Harper et al., 2006). Rat monoclonal anti-HA antibody (Roche) was used at 1/500 to detect epitope-tagged TgATG9. For colabeling with autofluorescent protein markers of sub-cellular compartments, constructs allowing the transient expression of vacuolar marker TgCRT-GFP (Warring et al., 2014) and Golgi marker GRASP-RFP (Nishi, Hu, Murray, & Roos, 2008) were transfected in HA-tagged TgATG9-expressing tachyzoites. Staining of DNAs was performed on fixed cells incubated for 5 min in a 1 µg/ml DAPI solution. All images were acquired at the Montpellier RIO imaging facility from a Zeiss AXIO Imager Z2 epifluorescence microscope equipped with a Camera ORCA-flash 4.0 camera (Hamamatsu) and driven by the ZEN software. Adjustments for brightness and contrast were applied uniformly on the entire image. Pearson's correlation coefficient was calculated for costainings on at least 30 individual images of parasites using the "Coloc 2" plugin from the ImageJ/Fiji software (http://imagej.net/Coloc_2).

4.13 | Analysis of autophagosome-like structures

To visualize autophagosome-like structures, *T. gondii* tachyzoites were transfected to express transiently GFP-TgATG8 (Besteiro et al., 2011) and costained with antibodies for the TgATRX1 apicoplast marker (DeRocher et al., 2008) to discard the apicoplast-related signal.

4.14 | Virulence assays in mice

10 week-old female BALB/c mice (Jackson laboratories) were infected by intraperitoneal (i.p.) injection of 20 or 100 tachyzoites freshly harvested from cell culture. In order to monitor an equal viability of tachyzoites from the KDTgATG9, ΔTgATG9, and control cell lines, invasiveness of the parasites was evaluated by simultaneous plaque assay with a similar dose of parasites on HFFs. The immune response of surviving animals was tested following eye pricks performed on day 7 postinfection: sera were tested by immunoblotting against tachyzoites lysates (not shown). For inducible knock-down strains survival experiments, ATc (Sigma-Aldrich) was dissolved in water at a concentration of 0.2 mg/ml. The drinking water bottle was wrapped in aluminum foil to prevent precipitation of ATc due to light and the solution changed every 36 hrs. Survival experiments were done on groups of 10 mice per parasite cell line. Mice survival was checked on a daily basis until their death, endpoint of all experiments. Data were represented as Kaplan and Meier plots using Prism software (Graphpad).

4.15 | Analysis of parasite survival in macrophage

Thioglycolate-elicited macrophages were obtained from peritoneal fluids 3 days after i.p. injection of 2 ml thioglycolate at 3.85% into BALB/c mice, and were cultured in RPMI medium (Invitrogen) supplemented with 10% of fetal calf serum, 1% penicillin-streptomycin, 1% kanamycin, 20 mM Hepes pH 7, and 1% glutamine (Invitrogen).

For the determination of parasite replication *in vivo*, 10–12 week-old female BALB/c mice (groups of 5 mice per condition) were infected intraperitoneally with 100 tachyzoites freshly harvested from cell culture. On day 4 post-infection, mice infected with either TAT11-ΔKu80, KDTgATG9 (incubated with or without ATc), RHΔKu80 and ΔTgATG9

cell lines, were deeply anesthetized, and then sacrificed by cervical dislocation. The peritoneal cavity of each mouse was washed with 10 ml of PBS. The i.p. wash was spun at 1000 rpm for 5 min to pellet cells. Cells were processed to extract total RNA to study the capacity of parasite replication. Liver and spleen were harvested for homogenization followed by total RNA extraction and quantification of parasites by real time PCR.

4.16 | Determination of parasitemia by quantitative PCR

Total RNA from infected HFF cells, primary murine macrophages, or from mouse tissue (peritoneum, spleen) was extracted using TRIzol (Thermo Scientific), and experiments were performed from 5 µg of RNA. cDNA was prepared using the Revert Aid First cDNA synthesis Kit (Thermo Scientific). SYBR green-based quantitative PCR was performed using the CFX96 Real-Time PCR Detection System (BioRad, Serial No. 785BR04788). Individual reactions were prepared with 0.25 µM of each primer (HH-34/HH-35 for human glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), HH-36/HH-37 for mouse GAPDH; HH-21/HH-23 for *TgSAG1*), 150 ng of cDNA and SYBR Green PCR Master Mix to a final volume of 10 µl. PCR reaction consisted of a DNA denaturation step at 95 °C for 3 min, followed by 35 cycles (denaturation at 95 °C for 15 s, annealing at 56 °C for 60 s, extension at 72 °C for 30 s). For each experiment, reactions were performed in triplicates and expression of individual genes was normalized to GAPDH Threshold cycle (Ct) values and the percentage of expression was calculated according to the Livak method (Schmittgen & Livak, 2008).

4.17 | Proteolysis assay using AHA labeling

In order to quantify the degradation of proteins by autophagy, we adapted a proteolysis assay based on an existing method described for mammalian cells (Zhang et al., 2014), using a methionine analog, AHA, that can be chemo-selectively linked to a fluorescent moiety for labeling proteins (Click-iT AHA, ThermoFisher Scientific). Freshly egressed parasites were first incubated in methionine-free DMEM for 30 min to deplete intracellular methionine pools. They were then labeled with AHA in methionine-free DMEM for 6 hours, followed by a 1-hr incubation in DMEM containing an excess of methionine (2 mM) to chase out labeled short-lived proteins. Extracellular parasites were then incubated in full (DMEM) or starvation (HBSS) medium for 4 hrs. To label the dead population of parasites prior to flow cytometry analysis, fixable viability dye eFluor520 (eBioscience) was used following the protocol supplied by the manufacturer. Cells were then fixed in 2% (w/v) PFA and permeabilized in 0.5% Triton X-100. Click reaction was performed as previously described (Zhang et al., 2014) with the appropriate fluorophore. AHA-labeled proteins were then visualized in gel with an Odyssey Fc imaging system (LI-COR Biosciences), or whole parasites were analyzed by flow cytometry on a FACSanto flow cytometer (BD Biosciences) from the MRI platform.

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SUPPORTING INFORMATION

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EAPB0503: an Imiquimod analog with potent activity against cutaneous leishmaniasis

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Abstract

Cutaneous Leishmaniasis (CL) is a parasitic neglected tropical disease. Syria is endemic for *Leishmania tropica* and *Leishmania major*, causing CL in the Eastern Mediterranean. The large-scale displacement of Syrian refugees exacerbated the spread of CL into neighboring countries. The high risk for drug-resistance to current treatments stresses the need for new therapies.

Imiquimod is an immuno-modulatory drug currently used in CL treatment. Herein, we investigated the potency of its analog, EAPB0503, against *L.tropica* and *L.major* and described their mechanism of action. Both drugs reduced amastigote replication. EAPB0503 proved more potent, particularly on the most aggressive *L.tropica* amastigotes. Toll-Like Receptor-7 was upregulated, mainly by Imiquimod, and to a lesser extent by EAPB0503. Both drugs activated the NF- κ B canonical pathway triggering an immune response and i-NOS upregulation in infected macrophages. Our findings establish Imiquimod as a strong candidate for treating *L.tropica* and show the higher potency of its analog EAPB0503 against CL.

Keywords: Immunomodulation, TLR, NF- κ B, *L. tropica*, *L. major*

Introduction:

Cutaneous leishmaniasis (CL) is caused by *Leishmania* parasite and is classified by the World Health Organization (WHO) as one of the most common neglected tropical diseases [1].

During the past decade, an alarming increase in the incidence of CL was documented, ranging from 2.1 million case in 2002, to approximately 4 million cases in 2015 [2]. In the Eastern Mediterranean, *Leishmania tropica* and *Leishmania major* cause CL [3]. In Syria, the prevalence lately doubled due to chronic conflicts [4]. The displacement of Syrian refugees to the neighboring countries, including under-endemic ones like Lebanon, promoted the dissemination of this infection [5].

CL treatment varies among patients [6], and include local, systemic and physical approaches [7]. Glucantime is widely used [8], but yet presents with many disadvantages such as the painful intra-lesional injections to be repeatedly injected in each lesion, on a weekly basis and for up to 8 weeks [9]. An intramuscular injection of Glucantime was proposed to overcome this painful process, however it was associated with high hepatic and cardiac toxicity [10]. Imiquimod is an FDA approved imidazoquinoxaline against skin infections, with great anti-viral/anti-tumor activities [16]. Imiquimod proved potent in CL treatment [11, 12]. It was used in combination with systemic antimonials [13], and presented with cure rates exceeding 90% in refractory patients [14]. Accordingly, it was introduced by the WHO to the guidelines of CL treatment [15].

Among several synthesized Imiquimod analogs [16], EAPB0503 (1-(3-methoxyphenyl)-N-methylimidazo[1,2-a]quinoxalin-4-amine) exhibited higher potency than Imiquimod in several cancer models [17,18,19]. We explored whether EAPB0503 might exhibit a higher anti-*Leishmania* potency. We showed that both drugs affected amastigote replication, with a more prominent effect of EAPB0503. An upregulation of Toll-Like Receptor-7 (TLR7), was preferentially mediated by Imiquimod, and to a lesser extent by EAPB0503. Both drugs activated the NF- κ B canonical pathway leading to an increase in the pro-inflammatory cytokines secretion and consequently, an upregulation of i-NOS synthesis. A decrease in the anti-inflammatory

cytokines secretion was obtained, illustrating the leishmanicidal activity of both drugs. Our findings establish Imiquimod as a strong treatment candidate against the aggressive *L. tropica* strain and show the higher potency of EAPB0503 against CL. These results warrant an in depth preclinical study and highlights the importance on immune-modulatory therapy against CL.

Results

EAPB0503 exhibits a higher efficacy on *L. tropica* amastigotes replication

To compare the effect of Imiquimod and EAPB0503 on *L. major* amastigotes, macrophages were infected at the ratio of 5 parasites per cell. Treatment was performed with different concentrations of either drugs for 24 hours. Amastigotes replication was evaluated by real time PCR, using kinetoplast specific primers. Starting the concentration of 0.1 μ M, *L. major* amastigotes transcription levels decreased in a concentration-dependent manner following treatment with either drugs, and leading to 80% inhibition of replication of the parasites at the concentration of 1 μ M (Figure 1A, 1B).

L. tropica, the most aggressive and most endemic strain in the Middle East area [7], showed that both drugs exert an anti-amastigote activity in a concentration dependent manner. Strikingly, a concentration of 0.1 μ M EAPB0503 exhibited the same effect of 1 μ M of Imiquimod (10 folds higher concentration) (Figure 1C, 1D). This decrease in amastigotes was also more prominent at 0.5 and 1 μ M of EAPB0503, compared to the same doses of Imiquimod (Figure 1C, 1D). This promising data clearly shows a different response of leishmanial strains to treatment with either drugs, and a better response obtained upon treatment of the most aggressive *L. tropica* strain with EAPB0503.

EAB0503 inhibits amastigote replication as early as 10h post-treatment

Based on our concentration screening results, we chose the optimal concentration of 0.1 μ M for further analysis. We examined the effect of this concentration at an earlier time point of 10h. Imiquimod induced a decrease in *L. major* amastigotes replication by 50% at 10h post-treatment, and by 65% at 24h post-treatment (Figure 1E). More interestingly, EAPB0503 showed a more prominent decrease of amastigotes expression at 10h or 24h post-treatment, where only 10% of amastigote transcripts were detected by RT-PCR (5 folds less than Imiquimod) (Figure 1E).

Both drugs showed a concentration dependent anti-amastigote activity, against *L. tropica*, the most aggressive and most endemic strain in the Eastern Mediterranean [7]. Imiquimod reduced amastigotes transcription levels to 60% at 10h post-treatment and to around 20% at 24h post-treatment (Figure 1F). Interestingly, EAPB0503 reduced amastigotes transcript levels to 30% (almost 3 folds less than Imiquimod) at 10h post-treatment and to 10% (around 2 folds less than Imiquimod) at 24h post-treatment (Figure 1F). Altogether, these data show that EAPB0503 acts at the low dose of 0.1 μ M and as early as 10h, when compared to its parental compound Imiquimod.

Imiquimod triggers an increase of TLR-7 expression in *L. tropica* infected macrophages

Imiquimod belongs to the class of Toll-like receptor (TLR) agonists with high affinity to TLR7, commonly involved in pathogen recognition [13, 20]. We investigated the molecular mechanisms underlying the potency of Imiquimod and its analog EAPB0503 against *Leishmania* amastigotes. We focused on *L. tropica*, being the most aggressive species in the Middle East region [7] and adopted the concentration of 0.1 μ M at both time points 10 and 24h post-treatment. Our results showed that TLR7 protein levels increased after treatment with either drugs, in comparison

to uninfected or untreated infected macrophages (Figure 2A). Consistent with published data, the upregulation was maximal upon treatment with Imiquimod for 10h (Figure 2A). EAPB0503 induced a higher protein expression of TLR7. Nonetheless, the highest induction of TLR7 was obtained upon treatment with Imiquimod. Our results confirm the mechanism of action of Imiquimod *via* this receptor in the context of CL. The lower expression of TLR7 upon treatment with EAPB0503, seemingly shows a potential mode of action through a different TLR.

Imiquimod and EAPB0503 induce the canonical NF- κ B pathway activation

Following recognition of pathogens, TLRs trigger the NF- κ B pathway activation [21] inducing immune inflammatory responses [22]. Imiquimod activates the canonical NF- κ B pathway [23]. We explored this pathway in the context of CL. Western blot analysis clearly showed an activation of the multimeric IKK complex (IKK α /IKK β) after 10 or 24h treatment with either drugs (Figure 2B). Furthermore, an induction of the phosphorylated form of the I κ B α at both time points was obtained, presumably leading to its degradation (Figure 2B). We then examined whether this NF- κ B activation involves the canonical pathway. Our results demonstrate that the p50 subunit was upregulated especially upon 24h post-treatment with either drugs (Figure 2B). This led to the nuclear translocation of p65 (Figure 2C and not shown), which represents the active NF- κ B subunit, and known to activate immune response genes. Collectively these results showed that both Imiquimod and EAPB0503 inhibit amastigote replication *via* activation of the canonical NF- κ B pathway.

Imiquimod and EAPB0503 induce NF- κ B mediated macrophage immune response

We investigated the expression of pro- and anti-inflammatory mediators after treatment. Macrophage Inflammatory Proteins (MIP-1 α and β) and Monocyte Chemoattractant Protein

(MCP-1) levels increased upon treatment with both drugs (Figure 2D). The secreted levels of depicted pro-inflammatory cytokines, important in CL clearance [24], namely IL-12, IL-1 β , TNF- α and IL-6, were increased upon treatment with Imiquimod or EAPB0503 (Figure 3A).

Macrophage-derived nitric oxide (NO) is effective against microbes, and synthesized by Nitric Oxide Synthase (i-NOS). i-NOS is induced in response to pro-inflammatory cytokines [25] and in CL-infected macrophages is protective against *L. major* [26]. Both drugs increased i-NOS transcripts, with EAPB0503 inducing a 5-fold higher expression (Figure 3B). This presumably leads to higher NO production, hence enhanced leishmanicidal activity.

In CL, pro-inflammatory cytokines are linked to resistance against leishmaniasis; whereas anti-inflammatory cytokines relate to disease progression [27]. We examined the secretion levels of two depicted anti-inflammatory cytokines, IL-10 and IL-4 after treatment with either drugs. In comparison to non-treated *L. tropica* infected macrophages, secretion levels of IL-10 and IL-4 decreased by around 4 folds after treatment with Imiquimod (Figure 3C). More interestingly, treatment with EAPB0503 showed a significant decrease by 4 and 15 folds of IL-10 and IL-4 respectively and as compared to non-treated infected macrophages (Figure 3C).

Altogether, our results show that NF- κ B activation by Imiquimod and EAPB0503 induces secretion of pro-inflammatory cytokines. This leads to i-NOS upregulation, presumably leading to NO production and leishmanicidal activity. In addition, and concomitantly with the upregulation of pro-inflammatory cytokines, a decrease in the anti-inflammatory cytokines is obtained, probably diminishing macrophage susceptibility to *L. tropica* infection, and triggering the leishmanicidal effect of the tested drugs.

EAPB0503 exhibits a higher efficacy on freshly isolated *L. tropica* from patients' biopsies

To eliminate the potential doubt due to the susceptibility of cultured *L. tropica* and *L. major* strains to our tested treatments (e.g genetic drift and less virulent strains after long term culture), we investigated the efficacy of Imiquimod and its analog EAPB0503 on freshly isolated parasites from untreated patients' biopsies. Our results demonstrated that both drugs inhibit amastigote replication in a time dependent manner. Amastigotes transcription levels decreased by 30 and 55%, at 10 and 24h post-treatment with Imiquimod respectively (Figure 4A). Treatment with EAPB0503 induced a more prominent effect where, *L. tropica* amastigote transcripts decreased by 70 and 90%, at 10 and 24h post-treatment respectively (Figure 4A). We then assessed the effect of both drugs on amastigotes histologically (Figure 4B) and by immunofluorescence confocal microscopy (Figure 4C). Our results were very consistent with the transcript data with less amastigotes detected upon treatment (Figure 4B, 4C). Consistently, and using the *Leishmania* Glycoprotein Gp63 marker for quantification of amastigotes [28], Imiquimod treatment led to a decrease in amastigotes percentage reaching 60% at 10h and 35% at 24h post-treatment (Figure 4C). More interestingly, EAPB0503 induced a more important decrease in amastigotes number, to 40% after 10h of treatment, and to 25% after 24h of treatment (Figure 4C). Altogether, these data show that Imiquimod and mostly EAPB0503 are highly active at the low dose of 0.1 μ M and as early as 10h on patients' derived *L. tropica* stages, confirming the obtained results on *in vitro* cultured strains.

The mechanism of action of either drugs on *L. tropica* obtained from patients' biopsies, was evaluated for TLR7 protein expression and showed an increase after treatment with either drugs, in comparison to uninfected or untreated infected macrophages. Consistently with the cultured strain, Imiquimod induced the highest TLR7 protein levels (Figure 4D). Moreover, i-

NOS transcript levels were increased reaching the highest levels after 10h treatment with EAPB0503 (Figure 4F). These results indicate a similar mode of action of both drugs on freshly isolated parasites from patients' biopsies and confirm the higher potency of EAPB0503 against CL.

Discussion

CL is one of the most common neglected tropical diseases worldwide. Globally, the annual incidence of CL is estimated to be 0.7 to 1.2 million new cases per year. This infection is still endemic in many countries [29]. In the Eastern Mediterranean, Syria presents with the highest number of CL cases [30]. The Syrian conflict exacerbated the spread of the infection to the surrounding areas. In Lebanon, 85% of infected Syrian refugees were diagnosed with *L. tropica* whilst the remaining 15% were infected with *L. major* [5].

Pentavalent antimony compounds remain the treatment choice for CL treatment. However, these compounds associate with high cost, poor availability, drug resistance and systemic toxicity [31]. We focused on testing novel drugs' efficacy on *L. tropica* and *L. major*. Imiquimod activates macrophages [14], the main host cells for *Leishmania* replication. In CL, Imiquimod was mainly tested against *L. major* amastigotes [32]. In Imiquimod treated mice infected with *L. major*, an increased protection was obtained [33]. In CL patients infected with *L. major*, Imiquimod combined to meglumine antimoniate induced a high healing rate in refractory patients [12, 15, 34]. We showed that both drugs affected amastigotes. Imiquimod was more potent than that of EAPB0503, against *L. major* amastigotes, yet it exhibited an effect on *L. tropica* amastigotes. Conversely, EAPB0503 was more potent on the aggressive *L. tropica* strain [4, 5]. Imiquimod acts *via* binding TLR7, leading to the activation of the NF- κ B pathway. Imiquimod protective effect,

on *L. major* infected mice, was coupled with the induction of NO synthesis [26, 35]. Consistent with the published data, but on *L. tropica* strain, we showed that Imiquimod and EAPB0503 upregulated TLR7 expression. Nonetheless, the highest induction was obtained upon Imiquimod treatment. This finding suggests that EAPB0503 may partially act *via* TLR7, or through other TLRs. The canonical NF- κ B pathway was activated by both drugs, leading to increased secretion levels of pro-inflammatory cytokines. MIP-1 α and MIP-1 β , involved in resistance against infections [36] were both secreted at higher levels upon treatment with either drugs. Consistently with the known role of MIP-1 α and MIP-1 β in recruiting other cytokines such as TNF- α and IL-6 [37], levels of secretion for these two cytokines were also increased. TNF- α increased secretion levels were consistent with its protective role against CL [38].

Previous studies have shown that MCP1 is highly expressed in lesions of patients with self-healing localized cutaneous leishmaniasis whereas it is scarce in those of chronic diffuse cutaneous leishmaniasis [39]. This suggests its role in the parasites elimination *via* induction of reactive oxygen intermediates (ROI). Our results showed that MCP-1 levels increased upon treatment with either drugs, but more importantly with EAPB0503, presumably explaining its higher potency. However, the potential involvement of ROIs on the clearance of treated parasites remains to be elucidated.

We also succeeded to test the efficacy of Imiquimod and EAPB0503 on freshly isolated *L. tropica* from skin lesions of CL patients. We confirmed the results obtained on cultured strains, thus eliminating any potential doubt about a lower virulence or a genetic drift obtained from long term cultures. These results highlight the promising potency of EAPB0503 for CL treatment.

Nitric Oxide production by activated macrophages is known to play a major role in fighting against infections [40], including *Leishmania* [41]. Inhibition of i-NOS reduced *L. infantum* burden in human macrophages [42]. In addition, the increase of i-NOS and NO generation in response to IFN- γ and TNF- α is crucial to control CL [43]. We checked for i-NOS transcripts in treated macrophages infected with patients' derived *L. tropica* and showed an important increase with either drugs. Interestingly, the highest levels were obtained upon EAPB0503 treatment, presumably explaining its higher leishmanicidal efficacy.

TLRs are important pattern recognition receptors expressed abundantly on macrophages. Early studies concluded that TLR2, TLR4, and TLR9, are involved in the recognition of *L. major* and that TLR2 ligands play a protective immune role against Leishmaniasis [44]. However, recent studies on C57BL/6 mice deficient in either TLR2, 4, or 9, showed that only TLR9^{-/-} mice are more susceptible to *L. major* infection, indicating TLR2 and TLR4 related immunity to murine leishmaniasis requires re-evaluation [45]. In this study, we confirmed that Imiquimod displays its anti-amastigote activity *via* TLR7 upregulation, leading to NF- κ B activation and pro-inflammatory cytokine production. EAPB0503 effect on TLR7 was less prominent. Whether EAPB0503 acts *via* any of the important TLRs in CL or not, requires further investigation.

Collectively, our results did not only show a promising efficacy of a new compound against CL, but also highlighted the effect of Imiquimod and its analog against the aggressive *L. tropica* strain. We also described the molecular mechanisms of these drugs against amastigotes highlighting the importance of immune-modulatory therapy against CL.

Materials and methods:

Parasite culture

Leishmania major (MHOM/MA/81/LEM265 and MMER/MA/81/LEM309) and *Leishmania tropica* (MHOM/LB/76/LEM61, MRAT/IQ/72/ADHANIS1) were purchased from the CRHU “Montpellier”. Parasites were maintained in RPMI (Lonza) supplemented with 10% Fetal Bovine Serum (FBS), 100IU/ml streptomycin/penicillin (Sigma).

Test agents

Imiquimod was purchased from Molekula (Wessex House) and EAPB0503 was synthesized using microwave-assisted chemistry as described by Khier et al. [46]. Drugs were prepared as a 0.1 M stock in dimethylsulfoxide (DMSO) and stored at -80°C. Working solutions of 0.1 µM were freshly prepared in culture media.

Macrophage culture and treatment

Human monocytic THP-1 cells (ATCC TIB-202), Manassas, VA) were grown in RPMI (Lonza), supplemented with 10% of fetal bovine serum (FBS), 1% penicillin-streptomycin, 1% kanamycin and 1% glutamine (Invitrogen). 1 million THP-1 cells were differentiated into macrophages, using 50 ng/mL of phorbol 12-myristate 13-acetate (PMA, Sigma) overnight. Following their adherence, differentiated macrophages were then activated with 1 µg/mL of LPS for 4h, then infected with *L. major* or *L. tropica* at the ratio of 5 parasites/macrophage, and incubated for 24h at 37 °C. Non-internalized promastigotes were removed by two gentle washes with PBS.

Isolation of fresh *L. tropica* promastigotes from biopsies of CL Patients

Punch biopsies (4 mm of diameter) from three CL patients were performed and incubated in sterile physiological serum, supplemented with Penicilline G (50 000 UI/mL). Specimens were incubated in a semi-solid culture media (10g agar, 3g NaCl, 500 mL water). 3 weeks later, promastigotes were transferred to liquid medium. Sample collection was approved by the Institutional Review Board of the American University of Beirut (PALK.IK.01).

Anti-amastigote activity

Macrophages infected with *L. major*, *L. tropica*, or patients' derived *L. tropica* parasites were treated with Imiquimod and EAPB0503 (0.01 μ M, 0.05 μ M, 0.1 μ M, 0.5 μ M and 1 μ M) for 24h. Total RNA was extracted using Trizol (Qiagen). cDNA synthesis was performed using a Revert Aid First cDNA synthesis Kit (#K1622-Thermo Scientific). Syber green qRT PCR was performed using the BIORAD-CFX96 machine. Primers for the housekeeping Glyceraldehyde-3-Phosphate dehydrogenase GAPDH, and i-NOS are listed in Table. Primers for amastigotes detection target the minicircle kinetoplast DNA (kDNA) (Table). Percentage of expression was calculated according to Livac method [47].

Enzyme-linked immunosorbent assay (ELISA)

Supernatants of infected macrophages in presence or absence of either drugs were collected 10h and 24h after treatment, and ELISA was performed using Multi-Analyte ELISArray Kit (Qiagen) according to the manufacturer's instructions. Briefly, supernatants of *L. tropica* infected macrophages (untreated or treated with 0.1 μ M of Imiquimod or EAPB0503) were collected. Supernatants were spun for 10 min at 1000g, transferred to new Eppendorf tubes, and diluted using

a specific cocktail of antigens (IL-12, IL-1 β , IL-6, and TNF- α , MIP-1 α , MIP-1 β , MCP-1, IL-10 and IL-4) provided by the kit (Qiagen). Samples were then loaded in the coated ELISA plate, and were incubated for 2 hours. 3 washes were performed, and the detection antibody was added and incubated for 2 hours. Then, Avidin-HRP was added for 30 min, and 4 washes were performed. Development solution was then added in dark and kept for 15 min, before addition of the stop solution. The secreted levels of the following cytokines and chemokines were then assessed. The optic density (O.D) was determined at 450 and 570 nm and calculated according to the standard values of a positive control provided by the kit.

Immunofluorescence and confocal microscopy

For Immunofluorescence assay, p6 well plates were seeded with activated macrophages infected with *L. tropica* for 24h and treated with Imiquimod or EAPB0503 for 10 or 24h. At these time points, coverslips were fixed in 4% paraformaldehyde for 20 minutes. Permeabilization was performed in Triton (0.2%) for 10 minutes. Following one PBS wash, blocking for 30 min with PBS-10% FBS was performed. Primary antibody directed against the NF- κ B p65 subunit (Santa Cruz, Sc-8008) was used at the dilution of 1:50. For *Leishmania* parasite staining inside macrophages, an anti-Gp63 (LifeSpan BioSciences, LS-C58984) was used at the dilution 1:50. Anti-mouse secondary antibodies (Abcam, ab150116) were used at the concentration of 1:100. Staining of nuclei was performed using 1 μ g/mL of Hoechst 33342 (Invitrogen, H33342) and coverslips were mounted using a Prolong Anti-fade kit (Invitrogen, P36930). Z-section images were acquired by confocal microscopy using a Zeiss LSM 710 confocal microscope (Zeiss, Germany) and all images were analyzed using Zeiss LSM 710 software.

Hematoxylin and eosin stain

H&E staining was performed as described by Grosset et al., 2017 [48]. Briefly, hematoxylin (Fisher Scientific, Canada) was added on cells, and a counterstaining for 30 seconds was performed followed by a water rinse for 5 minutes. Slides were then dipped in 50% (vol/vol) alcoholic eosin Y solution (Leica Microsystems, Canada) then rinsed in ethanol before slide mounting.

Western blot analysis

Activated macrophages infected with *L. tropica* for 24h were treated with 0.1 μ M of Imiquimod or EAPB0503 for 10 or 24h. Cells were scrapped, washed with PBS, and pellets were re-suspended in 1x Laemmli buffer. Following denaturation, samples were run on 10% polyacrylamide gels. Proteins were then transferred to nitrocellulose membranes (BIO RAD Cat# 162-0112) at 30V overnight using a BioRad transfer unit. Blocking was performed for 1h in 5% of Bovine Albumin serum (BSA) in wash buffer and probed with specific primary antibodies against TLR7 (sc- 57463 Santa Cruz Biotechnology, 1:100), NF- κ B p65 (sc-8008; Santa Cruz Biotechnology, 1:250), or p52 (sc-7386, Santa Cruz Biotechnology, 1:250). Equal loading was tested following probing with the anti- GAPDH antibody (MAB5476; abnova, 1:20 000). Nitrocellulose membranes were then washed, before incubation with the appropriate anti-mouse secondary antibody conjugated to Horseradish peroxidase (HRP) (m-IgGk BP-HRP, Santa Cruz, sc-516102, 1:5000). Bands were visualized by autoradiography, following incubation with luminol chemiluminescent substrate (Bio-Rad, Cat# 170-5061).

Statistical analysis

Continuous variables were analyzed by the unpaired Student's t test. P value was determined and values for $p < 0.05$ were considered as significant.

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Author statement

The authors of this study have consented to its submission. There is no conflict of interest associated with this endeavor.

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Table: List of primers

Primer	Sequence 5'→3'	References
GAPDH Forward Primer	5'-CATggCCTTCCgTgTTCCTA-3'	[49]
GAPDH Reverse Primer	5'-CCTgCTTCACCACCTTCTTgAT-3'	[49]
HHLM-1 Forward Primer	5'-CCTATTTTACACCAACCCCCAGT-3'	[50]
HHLM-2 Reverse Primer	5'- GGGTAGGGGCGTTCTGCGAAA -3'	[50]
i-NOS Forward primer	5'-GGGAGCCAGAGCAGTACAAG-3'	[43]
i-NOS Reverse primer	5'-GGCTGGACTTCTCACTCTGC-3'	[43]

Figure 1

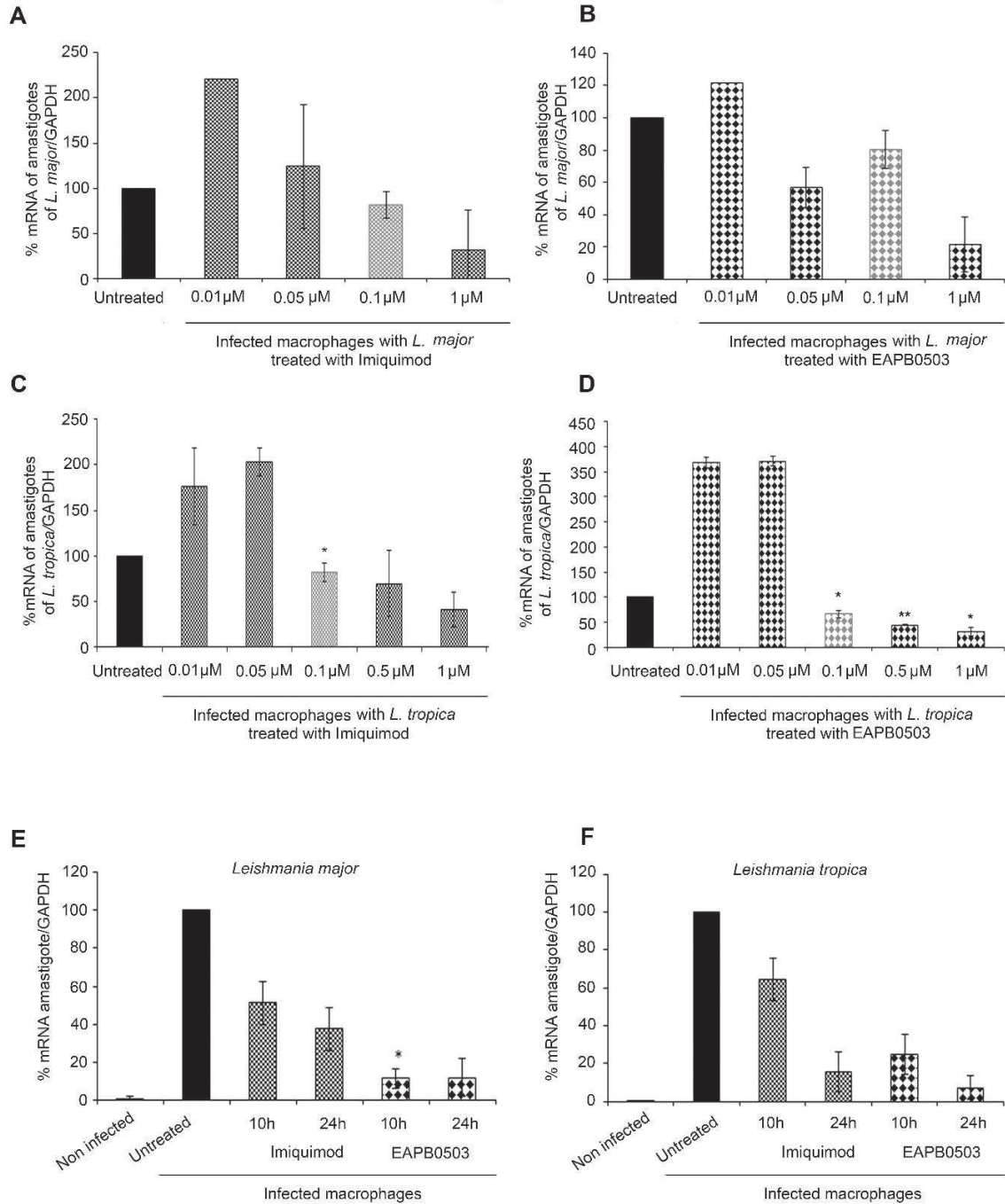


Figure 1: EAPB0503 exhibits a higher efficacy on *L. tropica* amastigotes replication. Real-time quantitative PCR (RT-PCR) detection of infected macrophages with *L. major* or *L. tropica* amastigotes treated with different concentrations of Imiquimod (A, C) or EAPB0503 (B, D). RT-

PCR detection of infected macrophages with *L. major* (E) or *L. tropica* (F) amastigotes treated with 0.1 μ M of Imiquimod or EAPB0503 for 10 and 24h. Briefly, differentiated and activated THP-1 into macrophages were infected with *L. major* or *L. tropica* at the ratio of 5 parasites/cell for 24h. Treatment with 0.1, 0.5, 1 or 10 μ M of Imiquimod or its analog EAPB0503 was performed for 24h (A-D). Treatment with 0.1 μ M of Imiquimod or EAPB0503 was performed for 10 and 24h (E, F). The results are shown as percentage of untreated infected macrophages. Amastigote transcripts were evaluated by Syber green RT-PCR using kinetoplast specific primers and their percentage of expression was normalized to GAPDH. Results are expressed as percentage of untreated control (\pm) SD and are representative of at least three independent experiments. The t-test was performed to validate significance. *, ** and *** indicate P-values \leq 0.05; 0.01 and 0.001, respectively. P-values less than 0.05 were considered significant.

Figure 2

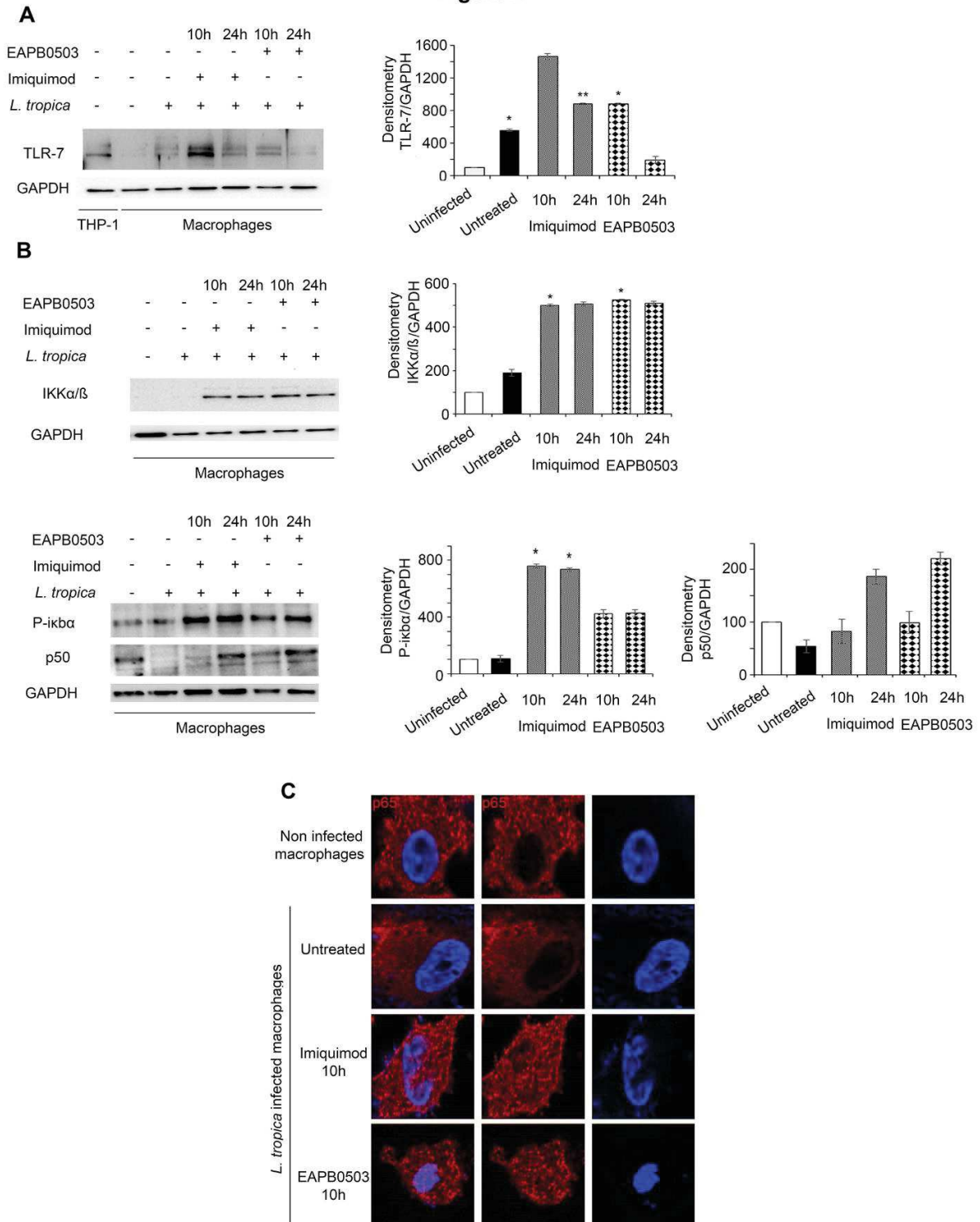


Figure 2: Imiquimod triggers an increase of TLR-7 expression in *L. tropica* infected macrophages leading to the canonical NF- κ B pathway activation. Western blot analysis for TLR-7 (A), IKK α/β , P-I κ B α and P50 (B) in *L. tropica* infected macrophages treated with 0.1 μ M of Imiquimod or EAPB0503 for 10 and 24h. The results depict one representative experiment among three independent ones. Densitometry was performed using Image Lab software (Biorad). Results shown represent the average of quantification of three independent experiments. (C) Confocal microscopy on *L. tropica* infected macrophages treated with 0.1 μ M of Imiquimod or EAPB0503 for 10h. The NF- κ B p65 subunit was stained with an anti-p65 antibody (red), and nuclei were stained with Hoechst 33342 (blue). The results depict one representative experiment among three independent ones.

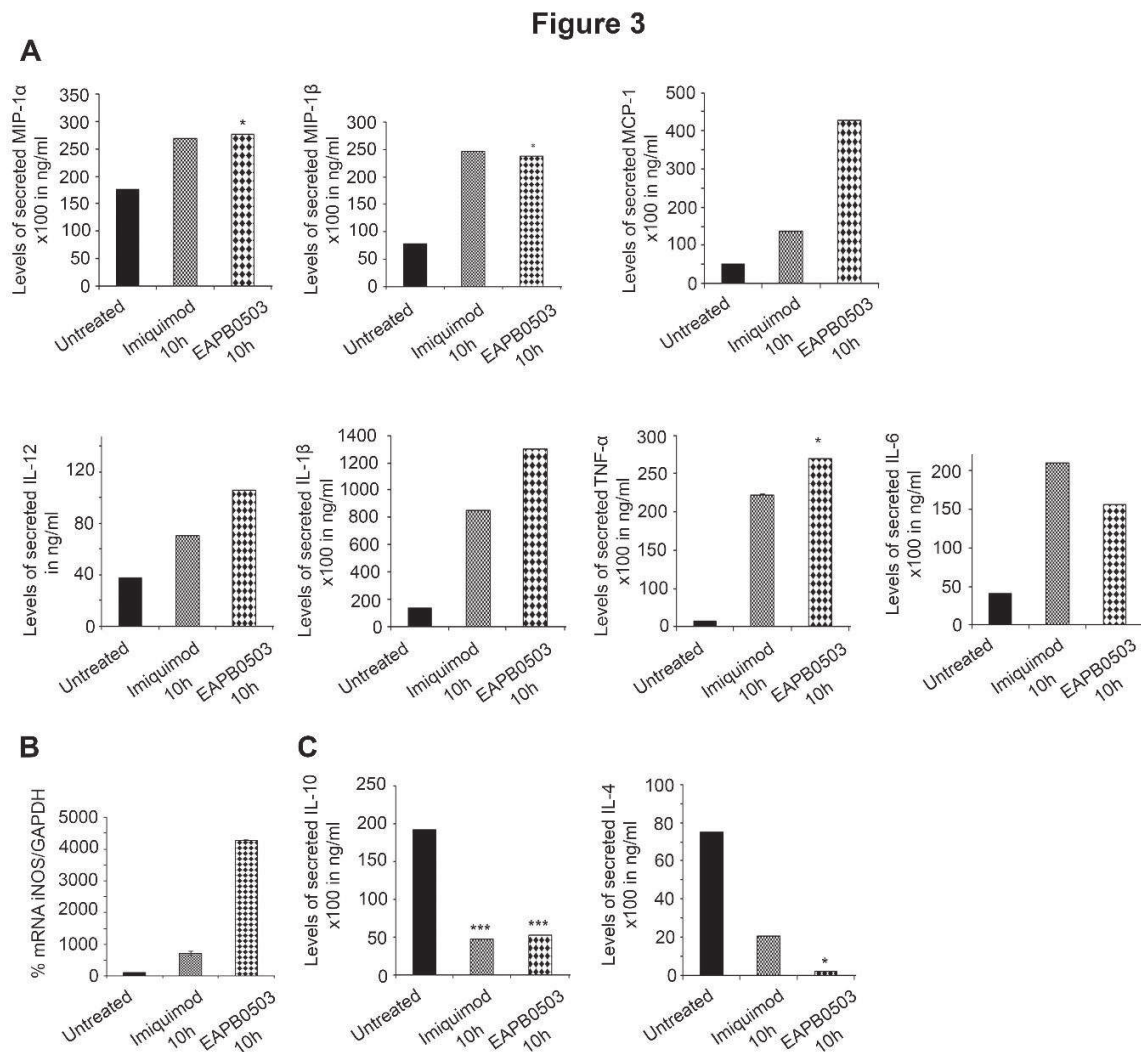


Figure 3: Imiquimod and EAPB0503 induce NF- κ B mediated macrophage immune response. (A) ELISA showing the secretion level of the pro-inflammatory cytokines (MIP-1 α , MIP-1 β , MCP-1, IL-12, IL-1 β , TNF- α , and IL-6) by infected macrophages with *L. tropica* amastigotes treated with 0.1 μ M of Imiquimod or EAPB0503 for 10h. (B) RT-PCR detection of i-NOS in infected macrophages with *L. tropica* amastigotes, treated with 0.1 μ M of Imiquimod or EAPB0503 for 10h. i-NOS percentage of expression was normalized to GAPDH. Results are expressed as percentage of untreated control (\pm) SD. (C) ELISA showing the secretion level of and

anti-inflammatory cytokines (IL-10 and IL-4) upon treatment of *L. tropica* infected macrophages with 0.1 μ M of Imiquimod or EAPB0503 for 10h. Results are expressed as percentage of untreated control (\pm) SD and are representative of at least three independent experiments. The t-test was performed to validate significance. *, ** and *** indicate p values \leq 0.05; 0.01 and 0.001, respectively. P-values less than 0.05 were considered significant.

Figure 4

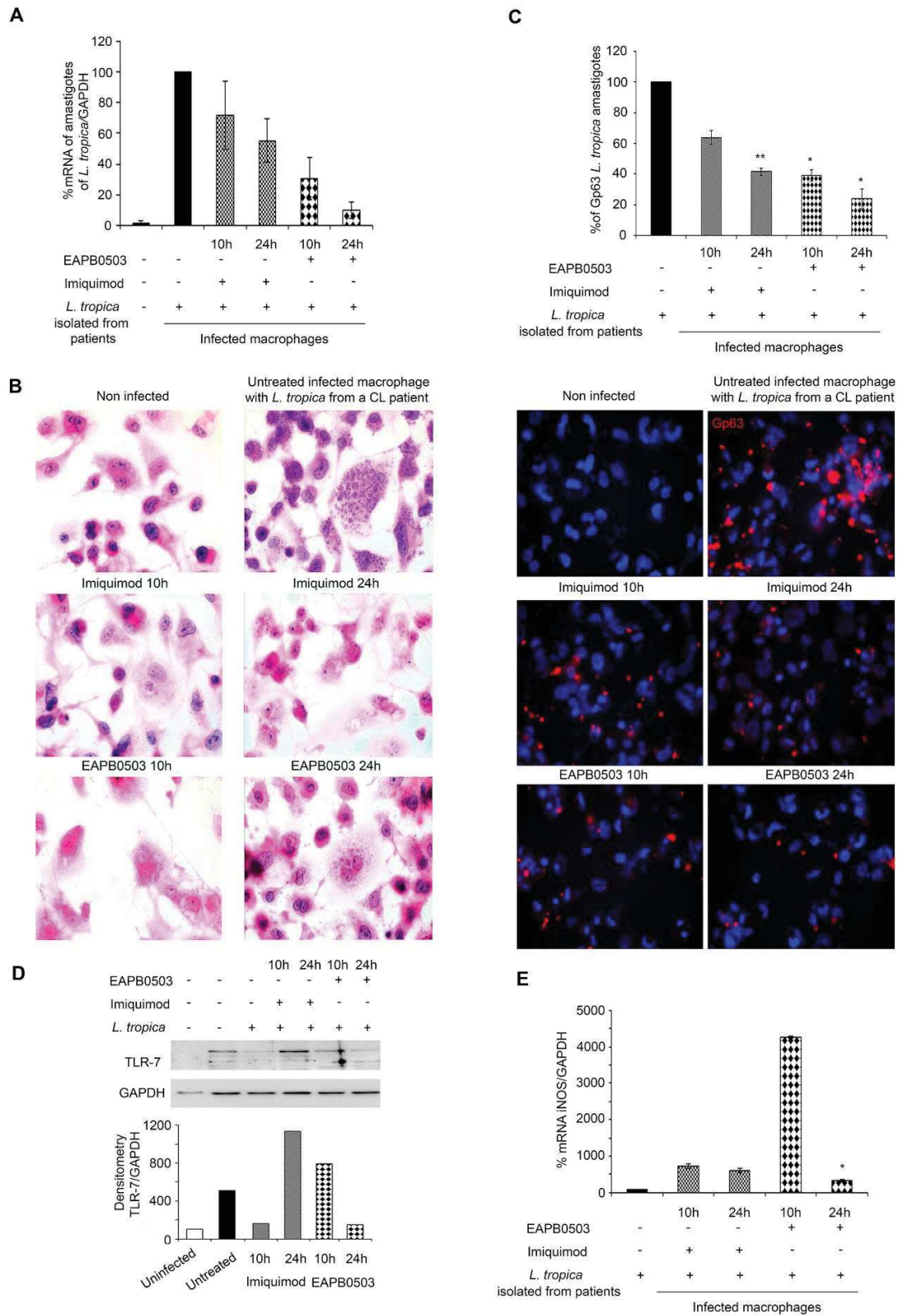


Figure 4: EAPB0503 exhibits a higher efficacy on freshly isolated *L. tropica* from patients' biopsies. (A) RT-PCR detection of infected macrophages with patients' derived *L. tropica* amastigotes treated with 0.1 μ M of Imiquimod or EAPB0503 for 10 or 24h. Percentage of expression of amastigotes was normalized to GAPDH. Results are expressed as percentage of untreated control (\pm) SD and are representative of three independent experiments. (B) H&E staining on untreated or treated macrophages infected with patients' derived *L. tropica* amastigotes with 0.1 μ M of Imiquimod or EAPB0503 for 10 or 24h. The results depict one representative patient. Similar results were obtained on the remaining two patients. (C) Confocal microscopy on patients' derived *L. tropica* infected macrophages treated with 0.1 μ M of Imiquimod or EAPB0503 for 10 or 24h. The Gp63 surface parasite was stained with an anti-Gp63 antibody (red), and nuclei were stained with Hoechst 33342 (blue). Images represent Z sections. Graphs show quantification of Gp63 (Blind count) as averages of one Z section/cell from 50 different cells of 2 independent experiments on two different patients derived amastigotes. (D) Western blot analysis for TLR-7 on patient derived *L. tropica* infected macrophages (from one patient) treated with 0.1 μ M of Imiquimod or EAPB0503 for 10 and 24h. (E) RT-PCR detection of iNOS in infected macrophages with patients' isolated *L. tropica* amastigotes upon treatment with 0.1 μ M of Imiquimod or EAPB0503 for 10 and 24h. Percentage of expression of amastigotes was normalized to GAPDH. Results are expressed as percentage of untreated control (\pm) SD and are representative of three independent experiments. The t-test was performed to validate significance. *, ** and *** indicate p values \leq 0.05; 0.01 and 0.001, respectively. P-values less than 0.05 were considered significant.

Résumé

La leishmaniose cutanée (LC) est une infection parasitaire classifiée par l'Organisation de Santé Mondiale (WHO) comme étant une des maladies tropicales négligées non-contrôlées. Dans la région du Moyen Orient, la LC est généralement endémique en Syrie et elle est causée principalement par *Leishmania tropica* et *Leishmania major*. La LC a été récemment introduite à des pays non endémiques, suite au déplacement intense des réfugiés Syriens échappant à la crise. Les interventions thérapeutiques contre la LC incluent des traitements locaux, systémiques et physiques. En revanche, le risque élevé de sélection et de résistance des parasites aux traitements actuels suscitent une quête sérieuse, pour trouver de nouvelles approches thérapeutiques. L'Imiquimod est un composé immunomodulateur approuvé pour utilisation clinique, et présente une efficacité vis-à-vis de certaines espèces de *Leishmania*. Dans cette étude, notre intérêt s'est focalisé sur l'efficacité d'un analogue de l'Imiquimod, l'EAPB0503, contre les stades promastigotes et amastigotes de *L.tropica* et *L.major*.

Nos résultats montrent que l'Imiquimod et particulièrement l'EAPB0503 affectent les deux espèces. L'Imiquimod affecte majoritairement la motilité des promastigotes des deux espèces, alors que l'EAPB0503 affecte la motilité des promastigotes de *L. major* mais surtout l'invasion des promastigotes de *L. tropica* dans les macrophages. Les deux composés réduisent la réplication des amastigotes, avec un effet plus prominent de l'EAPB0503. Cet effet est médié par l'augmentation de l'expression du récepteur toll-Like-7 (TLR7), particulièrement pour l'Imiquimod et d'une manière moins importante pour l'EAPB0503. Les deux composés induisent l'activation de la voie de signalisation canonique de NF- κ B. Ceci conduit à une production des cytokines pro-inflammatoires, et une diminution des cytokines anti-inflammatoires expliquant l'activité leishmanicide des deux composés. L'EAPB0503 semble agir via un autre TLR que l'imiquimod, comme il induit une expression plus élevée des transcrits TLR8 et TLR9, conférant une protection contre l'infection.

Collectivement, nos résultats montrent l'effet de l'Imiquimod contre l'espèce la plus agressive, *L. tropica*, et souligne l'activité plus puissante de l'EAPB0503 contre les deux espèces. De plus, cette étude montre le mécanisme d'action de ces deux composés, qui vraisemblablement activent des TLRs différents, mais finissent par induire la voie NF- κ B et la réponse immunitaire correspondante. Ces résultats soulignent l'importance des drogues immuno-modulatrices contre la LC et ouvrent des perspectives sur des études précliniques puis cliniques de ces composés.

Mots clés : Leishmaniose cutanée, *Leishmania tropica*, *Leishmania major*, Imiquimod, EAPB0503, Moyen Orient.

Abstract

Cutaneous Leishmaniasis (CL) is a parasitic infection classified by the WHO as one of the most uncontrolled spreading neglected diseases. In the Middle East Region, CL is mostly endemic in Syria where it is mainly caused by *Leishmania tropica* and *Leishmania major*. CL has been lately introduced to under endemic countries, following the large-scale displacement of refugees from Syria fleeing the crisis. Therapeutic interventions against CL include local, systemic and physical treatments. However, the high risk for selection and spread of drug-resistant parasites is high; consequently, new therapeutic approaches are still needed. Imiquimod is an FDA approved immunomodulatory compound with a tested efficacy against some leishmania species. In this study, our interest was to investigate the efficacy of an Imiquimod analog, EAPB0503 in comparison to the original compound, against promastigote and amastigote stages of *L.tropica* and *L.major*.

We showed that Imiquimod affects the motility of promastigotes of both strains. EAPB0503 affected *L. major* promastigotes' motility and impaired the invasion of *L.tropica* promastigotes into macrophages. Both drugs reduced amastigote replication, with a higher potency of EAPB0503. This activity is due to the upregulation of Toll-Like Receptor-7 (TLR7), mainly by Imiquimod, and to a lesser extent by EAPB0503. Importantly, both drugs activated the NF- κ B canonical pathway leading to production of pro-inflammatory cytokines and upregulation of i-NOS levels. A decrease of anti-inflammatory cytokines secretion was obtained, explaining the leishmanicidal activity of both drugs. Importantly, EAPB0503 led to a prominent increase in TLR8 and TLR9 transcripts, presumably conferring protection against the infection.

Collectively, our findings show the effect of Imiquimod against both strains especially, the aggressive *L. tropica* strain. We also show that EAPB0503 displays a more potent in vitro leishmanicidal activity than Imiquimod. These drugs seemingly activate different TLRs, but both activate the canonical NF- κ B pathway and its subsequent mediated immune response. These results highlight the promising effect of immunomodulatory drugs against CL and warrant an in depth in vivo preclinical then clinical studies of these compounds.

Keywords: Cutaneous leishmaniasis, *Leishmania tropica*, *Leishmania major*, Imiquimod, EAPB0503, Middle East.