Roles of Cathepsins B and L in the Th1/Th2 polarization by dendritic cells

"Die Rolle von Kathepsinen B und L wärend der Th1/Th2 Polarisierung durch Dendritische Zellen"

Doctoral thesis for a Doctoral Degree at the Section Infection Immunology, Julius-Maximilians Universität Würzburg, Graduate School of Life Sciences

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Würzburg 2014

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Ab	antibody
AEP	asparagine endopeptidase
Akt	protein kinase B
AMC	4-methyl-coumarin-7-amide
APC	antigen presenting cells
APS	Ammonium persulfate
BCA	bicinchoninic acid
BMDC	bone marrow-derived dendritic cells
BMM	bone marrow-derived macrophages
CA074	L-trans-Epoxysuccinyl-Ile-Pro-OH propylamide
CA074Me	L-trans-Epoxysuccinyl-Ile-Pro-OMe propylamide
cDC	conventional DC
CPI	cysteine protease inhibitors
CPS	counts per second
cTEC	cortical thymic epithelial cells
DALYs	disability-adjusted life years
DC	dendritic cells
DMEM	Dulbecco's Modified Eagles Medium
DMSO	dimethyl sulfoxide
DTT	Dithiothreitol

E-64	L-trans-Epoxysuccinyl-5Leu-4-guanidinobutylamide
E-64d	L-trans-Epoxysuccinyl-Leu-3-methylbutylamide-ethyl ester
EF-1	elongation factor-1
eGFP	enhanced-green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
ERK	extra-cellular signal-regulated kinase
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
HEL	egg white lysozyme
HRP	horseradish peroxidase
IFN	interferon
Ig	immunoglobulin
Ii	invariant chain
IL	interleukin
iNOS	inducible nitric oxide synthase
JAK	Janus kinase
JNK	Jun N-terminal kinase
LACK	*Leishmania* homolog of receptors for activated kinase
LmAg	*L. major* soluble antigen
LPG	lipophosphogylcan
LPS	lipopolysaccharide
Luc-tg	luciferase-transgenic
M6P	mannose-6-phosphate
M6P/IGF2R	mannose-6-phosphate/insulin-like growth factor II receptor
MAPK	mitogen activated protein kinase
MARCKS	myristoylated alanine-rich C kinase substrate
MENT	myeloid and erythroid nuclear termination stage-specific protein

MHC	major histocompatibility complex
MIF	migration inhibition factor
NADPH	nicotinamide adenine dinucleotide phosphatase
NFkB	nuclear factor-κB
NK	natural killer cells
NO	nitric oxide
OVA	ovalbumin
PAMP	pathogen-associated molecular patterns
PBS	phosphate-buffered saline
pDC	plasmacytoid DC
PE	Phycoerythrin
PECy7	phycoerythrin-cyanine 7
PFA	Paraformaldehyd
PI3K	phosphoinositol-3-kinase
PKC	protein kinase C
PKDL	post-kala azar dermal leishmaniasis
PMSF	Phenylmethylsulfonyl fluorid
PRR	pattern recognition receptors
PTP-1	protein-tyrosine-phosphatase-1
PV	parasitophorous vacuoles
PVDF	polyvinylidene difluoride
RA	retinoic acid
ROS	reactive oxygen species
RT	room temperature
SDS	Sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHP-1	Src homology region 2 domain-containing phosphatase-1
STAT	signal transducer and activator of transcription
TCR	T cell receptor

TGF	transforming growth factor
Th	T helper
TLR	Toll-like receptor
TNF	tumor necrosis factor
TTCF	tetanus toxin antigen
WB	Western Blot
WHO	World Health Organization

Abstract

Leishmaniasis is a neglected tropical disease that can be manifested through different clinical forms, ranging from cutaneous to visceral. The host response against *Leishmania* spp. is greatly dependent on T cell-mediated immunity, in which T helper 1 responses are associated with macrophage activation and elimination of the parasite, while regulatory T cells and T helper 2 responses are correlated with parasite survival and persistence of infection. *Leishmania* uses different virulence factors as strategies for evading the immune response of the host. One of them are cathepsin-like cysteine proteases, which are currently under extensive investigation as targets for drug development. Previous studies with inhibitors of cathepsins B and L *in vivo* revealed an outstanding modulation of the host T helper cell response. However, the mechanisms behind these observations were not further investigated. Given the urgent need for better treatments against leishmaniasis, the aim of this study was to investigate the effects that the lack of cathepsin B and L activity have on the signals that dendritic cells use to instruct T helper cell polarization in response to infection with *Leishmania major*.

The cathepsin inhibitors tested showed low or no cytotoxicity in bone marrow-derived dendritic cells, and dendritic cells and macrophages could be generated from cathepsin B and cathepsin L-deficient mice without apparent alterations in their phenotype in comparison to wild-type controls. Furthermore, lack of cathepsin B and L activity showed no impact in the rate of promastigote processing by dendritic cells. Cathepsin B and cathepsin L-deficient macrophages showed no differences in parasite proliferation and capacity to produce nitric oxide in comparison to wild-type macrophages. In response to the parasite, dendritic cells treated with a cathepsin B inhibitor and dendritic cells from cathepsin B-deficient mice showed higher levels of expression of major histocompatibility complex (MHC) class II molecules than dimethyl sulfoxide (DMSO) or wild-type controls, but it was not accompanied by changes in the expression of costimulatory molecules. Wild-type dendritic cells and macrophages are not able to express the pro-inflammatory cytokine interleukin (IL)-12 in response to promastigotes. However, cells treated with a cathepsin B inhibitor or cells deficient for cathepsin B were able to express IL-12, while the expression of other cytokines -including IL-6 and tumor necrosis factor (TNF)-α- remained unchanged. These characteristics point towards a more "pro-Th1" profile of dendritic cells in the absence of cathepsin B.

This data is the first report on IL-12 regulation depending on cathepsin B. The IL-12 up-regulation observed was already present at the transcriptional level. Furthermore, it was also present in macrophages and dendritic cells in response to LPS, and the latter had

a higher capacity to induce T cell helper 1 polarization *in vitro* than wild-type dendritic cells. The activation of different signaling pathways was analyzed, but the up-regulation of IL-12 could not be attributed to modulation of nuclear factor-κB (NFκB), p38 mitogen activated protein kinase (MAPK) and extra-cellular signal-regulated kinase (ERK)1/2 pathways. Thus, the mechanism behind IL-12 regulation by cathepsin B remains to be elucidated, and the impact of these effects is yet to be confirmed *in vivo*. Altogether it is tempting to speculate that cathepsin B, in addition to its role in processing endocytosed material, is involved in the modulation of the pro-inflammatory cytokine IL-12.

Zusammenfassung

Leishmaniose ist eine hauptsächlich in den Tropen vorkommende Infektionskrankheit, die sich in verschiedenen klinischen Formen, von kutan bis viszeral, manifestieren kann. Die Reaktion des Wirtes gegen *Leishmania* spp. hängt stark von der T-Zell-vermittelten Immunantwort ab, wobei die Antwort der T1-Helferzellen assoziiert ist mit der Aktivierung von Makrophagen und der Beseitigung des Parasiten, während die regulatorischen T-Zellen und T2-Helferzellen mit dem Überleben der Parasiten und der Fortdauer der Infektion in Verbindung stehen. *Leishmania* verwendet verschiedene Virulenzfaktoren als Strategie zur Umgehung der Immunantwort des Wirtes. Darunter zählen Cathepsin-ähnliche Cysteinproteasen, die derzeit Gegenstand umfangreicher Untersuchungen sind mit dem Ziel, für die Arzneimittelentwicklung eingesetzt werden zu können. Frühere Studien mit Inhibitoren von Cathepsin B und L *in vivo* zeigten eine hervorragende Modulation der Wirt-T-Helferzellantwort. Jedoch wurden die Mechanismen, die diesen Beobachtungen zu Grunde liegen, nicht weiter untersucht. Angesichts der dringenden Notwendigkeit einer besseren Behandlung gegen Leishmaniose war das Ziel dieser Studie die Auswirkungen zu untersuchen, die das Fehlen von Cathepsin B und L-Aktivität auf die Signale hat, welche die dendritischen Zellen verwenden, um die Reaktion der T-Helferzellen auf eine Infektion mit *Leishmania major* zu beeinflussen.

Die getesteten Cathepsin-Inhibitoren zeigten geringe oder keine Cytotoxizität in den aus dem Knochenmark präparierten dendritischen Zellen. Dendritische Zellen und Makrophagen von Cathepsin B- und Cathepsin L-defizienten Mäusen zeigten keine offensichtlichen Veränderungen ihres Phänotyps im Vergleich zu Wildtypkontrollen. Weiterhin zeigte das Fehlen von Cathepsin B- und L-Aktivität keine Auswirkung auf die Prozessierung der Promastigoten durch dendritische Zellen. Auch zeigten Cathepsin B- und Cathepsin L-defiziente Makrophagen keine Unterschiede in der Parasitenproliferation und der Fähigkeit Stickoxid zu produzieren im Vergleich zu Wildtyp-Makrophagen. In Reaktion auf den Parasiten war bei mit einem Cathepsin-B-Inhibitor behandelten dendritischen Zellen und dendritischen Zellen von Cathepsin-B-defizienten Mäusen eine höhere Expression von MHC-Klasse-II-Molekülen ersichtlich im Vergleich zu DMSO oder Wildtyp-Kontrollen, aber es wurden keine Veränderungen in der Expression von costimulatorischen Molekülen festgestellt. Dendritische Zellen und Makrophagen von Wildtyp-Mäusen sind nicht in der Lage das pro-inflammatorische Zytokin IL-12 als Reaktion auf die Promastigoten zu exprimieren. Jedoch konnten dendritische Zellen, die mit einem Cathepsin-B-Inhibitor behandelt waren oder Cathepsin-B-defiziente Zellen, IL-12 exprimieren, während die Expression von anderen Zytokinen - einschließlich IL-6 und

TNF-α - unverändert blieb. Diese Eigenschaften weisen in die Richtung einer "pro-Th1"-Antwort der dendritischen Zellen in Abwesenheit von Cathepsin B.

Diese Daten sind der erste Bericht über die IL-12-Regulierung in Abhängigkeit von Cathepsin B. Die Hochregulation von IL-12 war bereits auf der Transkriptionsebene zu beobachten. Weiterhin war sie in Makrophagen und dendritischen Zellen auch als Reaktion auf LPS vorhanden. Dendritische Zellen von Cathepsin B-defizienten Mäusen hatten eine höhere Kapazität zur Induktion einer eine T1-Helferzell-Polarisierung *in vitro* als dendritische Zellen von Wildtyp-Mäusen. Die Aktivierung von verschiedenen Signalwegen wurde untersucht, jedoch konnte die Hochregulierung von IL-12 nicht auf die Modulation von NFκB, p38 MAPK und ERK1/2-Signalwege zurückgeführt werden. Damit ist der für die IL-12-Regulierung durch Cathepsin B verantwortliche Mechanismus noch nicht geklärt; auch die Auswirkungen dieser Effekte *in vivo* müssen noch bestätigt werden. Insgesamt lässt die vorliegende Studie vermuten, dass Cathepsin B nicht nur an der Prozessierung von endozytiertem Material sondern auch an der Regulierung des pro-inflammatorischen Zytokins IL-12 beteiligt ist.

CHAPTER 1

Introduction: Leishmaniasis

1.1 General aspects of leishmaniasis

Leishmaniasis is considered by the World Health Organization (WHO) as a neglected tropical disease that affects "the poorest of the poor".[1,2] It affects more than 10 million people worldwide, with 350 million people considered to be at risk. It is caused by protozoan parasites of the *Leishmania* genus, with more than 20 species identified, and transmitted by the bite of *Phlebotomous* spp., in the Old World, and *Lutzomya* spp. sandflies in the New World. Leishmaniasis comprises a wide variety of clinical manifestations, from cutaneous to visceral, which can be lethal if left untreated. There is not yet an available human vaccine against leishmaniasis, and the commonly used drugs for treatment pose the risk of considerable side effects or are associated with great costs for the patients.

Leishmaniasis is a disease known from longtime. Figures from prehispanic cultures in the Americas already depicted skin lesions and facial deformities typical of cutaneous and mucocutaneous leishmaniasis. Fig. 1.1 summarizes a historic time line from the discovery of *Leishmania* parasites as the agent causative of leishmaniasis in the early 20th century, to the introduction of different therapeutics, from trivalent and pentavalent antimonials (1904-1920s), to liposomal amphotericin B (1990s), and the registration of miltefosine and paramomycin in the 2000s as the most recent therapeutics against the disease. Importantly, the first reports of HIV-*Leishmania* coinfection and of antimonial resistance in the 1980s have added to the need of novel therapeutic and preventive approaches to control the disease.

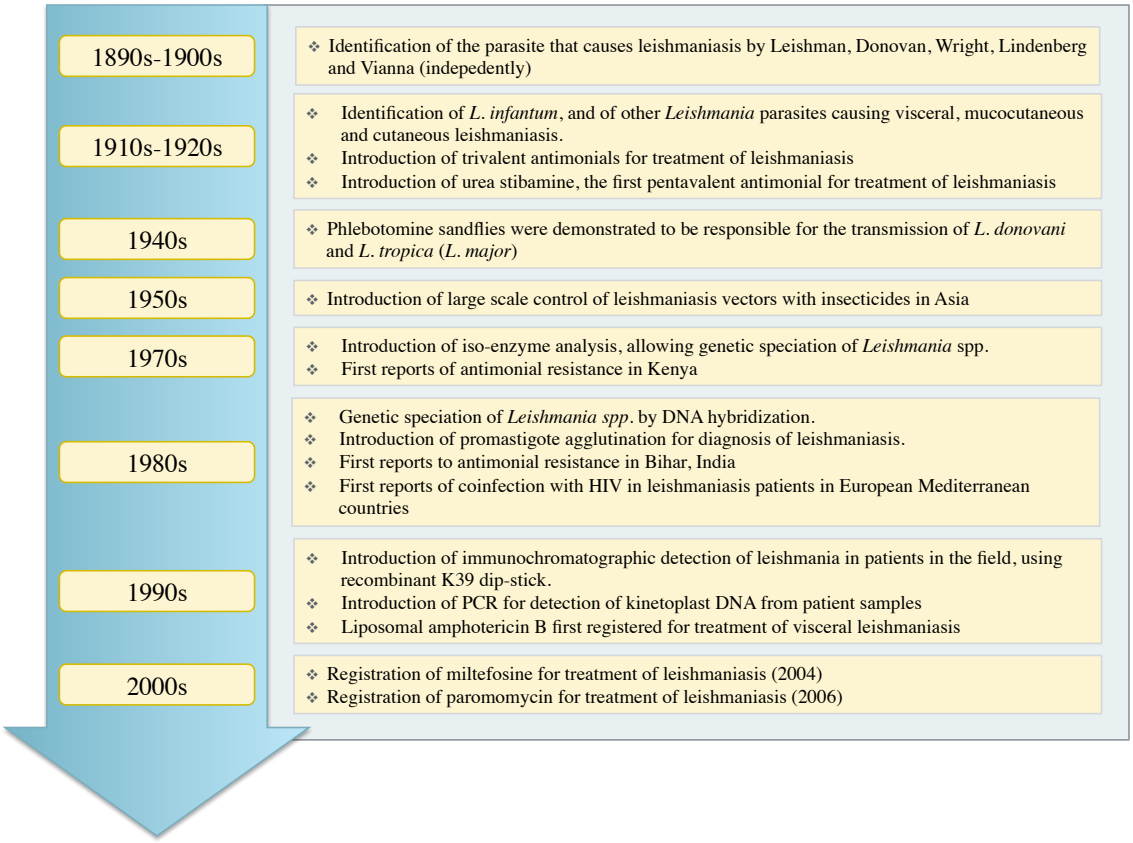

Figure 1.1. **Time-line of main events in treatment and control of leishmaniasis, according to the WHO** [1]

This chapter describes the main aspects of clinical and experimental leishmaniasis. The first sections focus on the biology of the parasites, the distribution of the disease, and the pathologies observed in the different forms of leishmaniasis observed in the clinics. Next, the current status of chemotherapy and immunotherapy against leishmaniasis is reviewed, and the need for novel approaches against the disease is highlighted. Finally, the experimental model of cutaneous leishmaniasis is reviewed, with emphasis on the knowledge gained in how the immune system of the host interacts with the parasite, and ultimately determines the outcome of the disease.

1.1.1 The parasite

Leishmania spp. are intracellular trypanoplastid, protozoan parasites that belong to the order of Kinetoplastids. Kinetoplastids are characterized by a unique mitochondrial DNA structure, the kinetoplast, which contains approximately 30% of total cell DNA.[3] *Leishmania* parasites have a digenetic life cycle, which requires a susceptible vertebrate host and a permissive insect vector. The life cycle of *Leishmania* parasites is illustrated in Fig. 1.2. The extracellular form of the parasites is the promastigote, which is flagellated and motile, and colonizes the digestive tract of *Phlebotomous* spp. and *Lutzomia* spp.

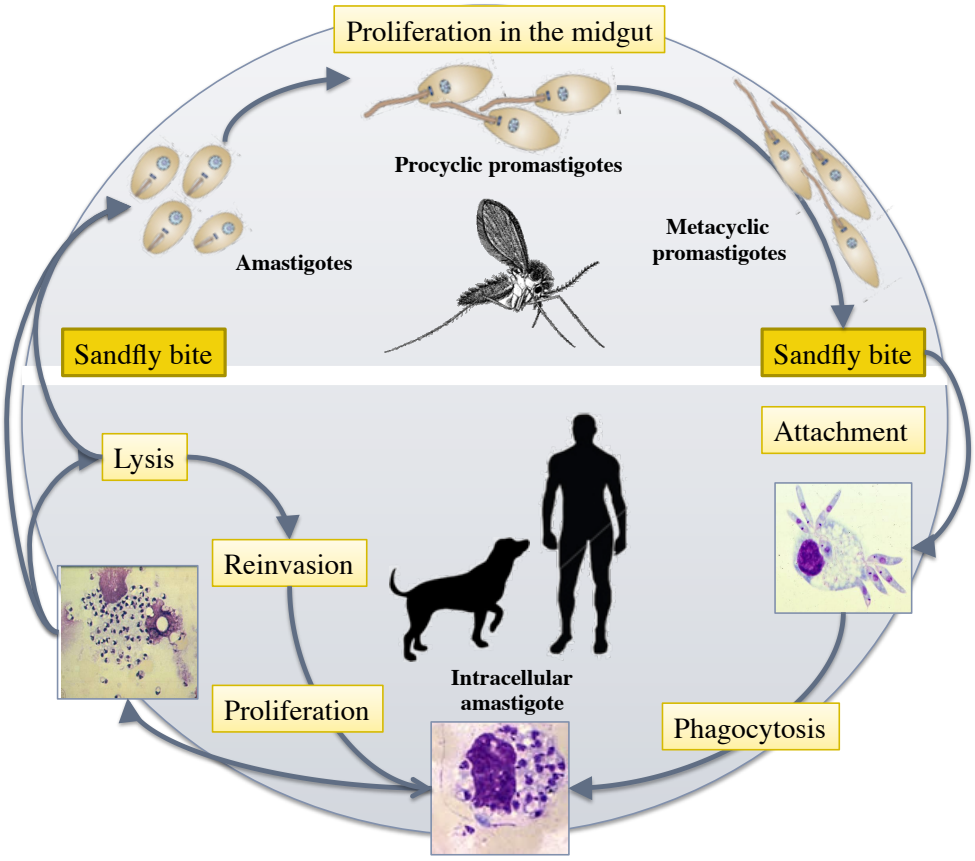

Figure 1.2. **Life cycle of *Leishmania* spp.** Life cycle of *Leishmania* spp. *Leishmania* metacyclic promastigotes reside in the midgut of an infected sandlfy, and are inoculated to the host when the sandfly takes a blood meal. It is rapidly taken up by cells of the innate immune system, macrophages being their preferred host. The promastigotes first attach to the membrane of the macrophages and are taken up by receptor-mediated phagocytosis. Within the macrophage, the promastigotes survive inside a PV, where they differentiate to amastigotes. Amastigotes are responsible for the clinical manifestation of the disease, as they proliferate in the macrophages and invade uninfected cells in the vicinity. When a sandfly takes a blood meal from an infected host, the cycle is completed, as amastigotes are ingested either in free form, or contained within the cells taken up. Inside the sandfly, the amastigotes will transform back to promastigotes, first in a highly replicative form (procyclic promastigotes) and then to a metacyclic form, concomitant with the up-regulation of virulence factors for an upcoming invasion of a new host.

sandflies.[4] During the insect blood feeding, metacyclic promastigotes are inoculated into the host dermis, together with saliva and parasitic phosphoglycans are also introduced into the host, the latter playing an important role in the establishment of the infection and modulation of the host response. Once inside the host, the promastigotes are quickly taken up by mono- and polymorphonuclear cells. The preferred host are macrophages, which take up the promastigotes by receptor-mediated phagocytosis.[5] Inside the macrophages, the promastigotes reside in phagolysosomes, where they transform into the non-flagellated amastigote form. Amastigotes are obligate intracellular, and account for the clinical manifestation of the disease, as they replicate and infect additional macrophages.

When the sandfly takes a blood meal from a *Leishmania*-infected host, the cycle is completed, as it ingests free amastigotes or infected cells. The amastigotes then are

transformed into proliferative, procyclic promastigotes, which can eventually become metacyclic promastigotes in a process known as metacyclogenesis. Metacyclogenesis is characterized by the modification of surface proteins and glycoconjugates, which confers virulence to the parasites should they be inoculated in a new host.[6,7] The changes that promastigotes undergo in this process can be mimicked in liquid culture of promastigotes, as logarithmically growing promastigotes resemble to procyclic promastigotes, and stationary phase promastigotes, to metacyclic promastigotes.[5]

The genus *Leishmania* is divided into two subgenera -*Leishmania* and *Viannia*- based on the place within the alimentary tract of the sandfly vectors where they develop. Species of the subgenus *Leishmania* present suprapylarian development, as their growth is restricted to the section anterior to the pylorus at the junction of midgut and hindgut. *Viannia* species, on the other hand, show peripylarian development, as they can grow in both the midgut and the hindgut.[1,8] The subgenera *Leishmania* is present in both the Old and the New Worlds, while *Viannia* is restricted to the New World.[1] The clinical form of leishmaniasis developed by a patient varies greatly depending on the parasite species. Identification of *Leishmania* spp. can be performed by culturing parasites from lesion biopsies, and PCR-based methods, particularly with multiplex PCR assays, or isoenzyme electrophoresis. However, the use of these methods is limited to reference laboratories, since they require significant infrastructure and technical expertise.[1] Other approaches can be used for diagnosis of the parasite in the field, including rK39 antigen-based immunochromatographic test, and agglutination tests[1] but they do not allow to differentiate between different species of *Leishmania*.

Leishmania parasites use different evasion mechanisms to avoid the attack of the host immune response. As most of them act at the level of silencing the effector functions of macrophages, the interaction of *Leishmania* virulence factors with these cells are reviewed in Section 1.2.1.4. Cysteine proteases are virulence factors from the parasites of great importance for this study, and are described in more detail in Section 2.8.

1.1.2 Epidemiology

The leishmaniases can be classified according to the reservoir hosts that are source of human infection: zoonotic leishmaniases, in which the reservoir hosts are wild, peridomestic or domestic animals, and anthroponoic leishmaniases, in which the reservoir host is human.[1] The transmission cycles of leishmaniasis can furthermore be classified into primary and secondary transmission cycles for zoonotic leishmaniasis, and into tertiary cycles for anthroponoic leishmaniases,[9] as illustrated in Fig.1.3. The great gerbil *(Rhombomys opimus)* and the sand rat *(Psammomys obesus)* are the primary reservoir hosts

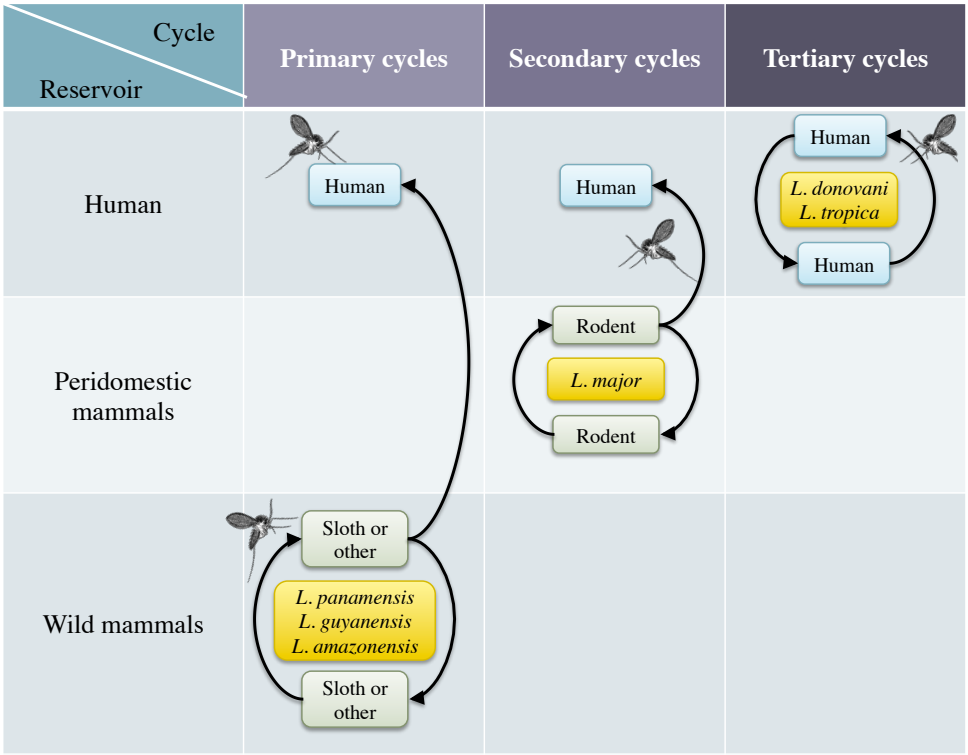

Figure 1.3. **Types of transmission cycles found in leishmaniasis.**. Adapted from P. Buffet.[9]

for *L. major* in Central Asia, and in West Asia and North Africa, respectively.[1] For *L. infantum*, dogs are considered the principal reservoir hosts, and it is estimated that more than 50% of all infected dogs are asymptomatic carriers.[1] Anthroponoic transmission posses the risk of increase in drug resistance.[9] Human beings are considered to be the principal reservoir host of *L. donovani*, which causes visceral leishmaniasis, and of *L. tropica*, which causes cutaneous leishmaniasis.[1] HIV and *Leishmania* co-infection was first reported in the mid-1980s, and it is accounted to be present in one third of the endemic countries. Patients co-infected with *Leishmania* and HIV are furthermore known to be highly infectious to sandflies.[1] Other forms of leishmaniasis transmission included the sharing of syringes among intravenous drug users, blood transfusion, and congenitally, although these are considerably rarer than vector-borne transmission.[1]

Cutaneous leishmaniasis in the New World is predominantly zoonotic, and in the past it was considered mostly an occupational disease, related to activities in forests areas. One example is the chiclero ulcer in southern Mexico, found in workers who tap rubber trees.[10] However, widespread deforestation and the extension of human settlements into formerly uninhabited lands has resulted in a sharp increase in peridomestic, periurban, and in some instances, urban transmission of leishmaniasis.[1,11] In the Old World, cutaneous leishmaniasis is also predominantly zoonotic, attributed in most cases to *L. major*

and *L. aethiopica*. Increased risk of infection is attributed not only to man-made ecological changes and immigration of non-immune population, but also to practices such as sleeping outdoors without a bednet.[1] Epidemics of cutaneous and visceral leishmaniasis in the Old World and the New World are associated with population movements, and the introduction of non-immune people to areas were the disease is present.[1] Examples of this case are the kala-azar epidemic in war refugees from southern Sudan in the 1990s, resulting in the death of 100,000 people[12] and recent reports of cutaneous leishmaniasis in Syrian refugees.[13]

Poverty plays a key role in the risk of transmission of leishmaniasis. Poor housing and sanitary conditions contribute to the increase of sandfly breeding sites. Furthermore, the crowding of a large number of people into a small space -i.e. the *favelas* in Brazil's periurban areas or war refugee camps in the Middle East- attracts sandflies and provides a large biomass for blood meals.[1] Additionally, malnutrition and consequent anemia contributes to the severity of the disease, particularly in cases of visceral leishmaniasis and mucocutaneous leishmaniasis.[1,11] Furthermore, treatment of leishmaiasis is expensive for the patients. In India, it has been estimated that the median total cost for visceral leishmaniasis treatment per patient is equivalent to 1.2-1.4 times the annual income per capita.[1] The burden of the disease, expressed in disability-adjusted life years (DALYs) is estimated at 2,357,000.[14]

Endemic leishmaniasis transmission has been reported in 98 countries and 3 territories.[2] Out of these 98 countries, 72 are developing nations, with 13 of them corresponding to the least developed countries, and cases of HIV-*Leishmania* co-infection have been reported in 35 countries.[15] It has been recently estimated that approximately 0.7 to 1.2 million cases of cutaneous leishmaniasis, and 0.2 to 0.4 million cases of visceral leishmaniasis occur each year.[2] Furthermore, more than 90% of the cases of visceral leishmaniasis in the world are concentrated in six countries: Bangladesh, Brazil, Ethiopia, India, South Sudan and Sudan. Furthermore, 70% to 75% of all cases of cutaneous leishmaniasis occur in ten countries: Afghanistan, Algeria, Brazil, Colombia, Costa Rica, Ethiopia, Iran, North Sudan, Peru, and Syria. It is estimated that leishmaniasis causes 20,000 to 40,000 deaths per year, a 10% case-fatality rate.[2] The overall prevalence of leishmaniasis is 12 million cases, with 350 million people estimated to be at risk.[16] Fig. 1.4 depicts the geographical distribution of cutaneous leishmaniasis in the Old World, particularly the areas affected by *L. major*. Since it has been reported that changes in the environment have a strong influence on the epidemiology of leishmaniasis, it has been suggested that the distribution of leishmaniasis might be affected by climate changes resulting from global warming.[1]

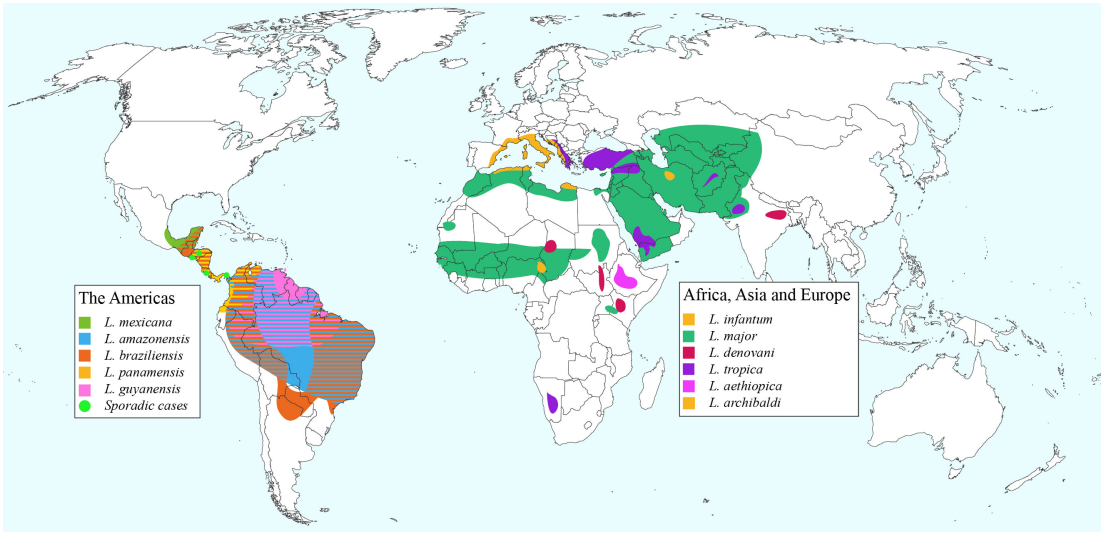

Figure 1.4. **Geographical distribution of cutaneous leishmaniasis**. Adapted from P. Buffet.[9]

1.1.3 Pathology

Depending on the parasite species, genetic factors and the immune status of the host, leishmaniasis can manifest in three main clinical forms: cutaneous leishmaniasis, visceral leishmaniasis, and mucocutaneous leishmaniasis. Fig. 1.5 depicts examples of *Leishmania* spp. classified according to the clinical form of leishmaniasis that they cause in human patients. The control of *Leishmania* parasites within the host is mediated by innate and adaptive immune responses, and this interplay between parasite and host immune response is ultimately reflected in the clinical outcome of the infection.[1] While important variations characterize the different clinical forms of leishmaniasis, some of the common features are:

- Neutrophils are the first cells to encounter *Leishmania* parasites at the site of infection, followed by skin dendritic cells
- Macrophages are the preferred host cell for *Leishmania*
- While in murine models disease resistance or susceptibility is marked by a strict dichotomy of T helper (Th)1 and Th2 immune responses, respectively, while in human disease a rather mixed Th1/Th2 response is observed.[1] The Th cell immunity corresponding to each clinical form of leishmaniasis is further reviewed in Section 1.2.3.2
- Clinical cure confers lifelong immunity, but it is not sterile. Small amounts of parasite persist, and can be reactivated in conditions of immunosuppression.[17,18]

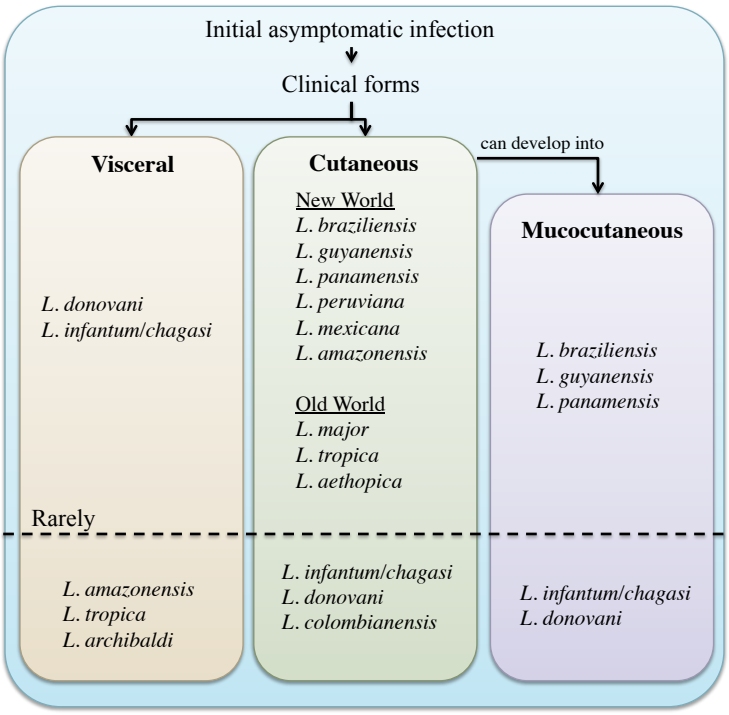

Figure 1.5. **Clinical forms of leishmaniasis and the *Leishmania* spp. that cause them.** Several factors influence the clinical form of leishmaniasis that a patient develops, and the parasite species is of the most important. [9]

Treatment recommendations are formulated based on the clinical form of leishmaniasis present, after considering the cost-benefit for the patient. The use of different chemotherapeutic and immunotherapeutic strategies against *Leishmania* is discussed in section 1.1.4.

1.1.3.1 Visceral leishmaniasis

Visceral leishmaniasis is comprised by a broad variety of disease severity and manifestations. The incubation period of the disease varies from weeks, months, or sometimes even years. Patients infected with *L. donovani* or *L. infantum* develop a reticuloendothelial hyperplasia, affecting the spleen, liver, small intestine mucosa, bone marrow, lymph nodes and other lymphoid tissues. Other symptoms include prolonged fever and cachexia, as malnutrition is both a risk factor and also a sequel of the disease. [19] Furthermore, anemia, leukopenia and granulocytopenia are developed, as a result of a reduction of the lifespan of leukocytes and erythrocytes. In later stages, prothrombin production is decreased, and in combination with thrombocytopenia, severe mucosal hemorrhage might be present. Hyperglobulinaemia is also present, in most of the cases characterized by polyclonal immunoglobulin (Ig) G, as a result of B cell inactivation. [1] The three classical symptoms hepatosplenomegaly, fever, and pancytopenia are however absent in more than 10% of immunocompetent patients, and in 50% of immunosuppressed patients. [9] The high antibody response observed in the patients is not protective, but it is useful for

diagnosis. Spontaneous healing is rare. If left untreated, concomitant infections frequently take place, mainly pneumonia, dysentery and tuberculosis, which are common causes of death.[1,20]

The term *kala-azar* (black fever in Hindi) is used to refer to an advanced, life threatening visceral leishmaniasis form in the Indian subcontinent, which, in some cases, presents darkening of the skin.[19] Post-kala azar dermal leishmaniasis (PKDL) is a form of cutaneous leishmaniasis that takes place in some patients during or after an apparently successful treatment of visceral leishmaniasis. It is triggered by the immune response of the host. The skin lesions can occur as macules, papules, nodules or patches, and appear most prominently on the face. In some cases, PKDL may heal spontaneously, but in others, further treatment might be required.[19]

1.1.3.2 Cutaneous leishmaniasis

The typical incubation time for cutaneous leishmaniasis ranges from weeks to months. The lesions usually progress from papules to plaques, which might develop central ulceration. The lesions might be presented as papulonodular, nodular, and noduloulcerative. Multiple primary lesions or satellite lesions might also appear. Other symptoms include regional adenopathy, nodular lymphangitis, thick hemorrhagic crusts, and in occasions might become infected with bacteria.[19] In early forms of the disease or in cases of patients with low levels of antibodies, there are large numbers of parasite-carrying macrophages. As the lesion evolves, lymphocytes and plasma cells infiltrate, and the destruction of host infected macrophages usually leads to elimination of the parasites. Generally, the cutaneous lesions are painless, but require months for healing and leave permanent scars, which might be disfiguring.

Depending on the strength of the cellular response developed by the patient and the parasitic burden found in the lesions, three main manifestations of cutaneous leishmaniasis have been described: localized, recidivans, and diffuse.[1,9] A schematic representation of these forms can be found in Fig. 1.6. In Leishmaniasis recidivans, the lesions present a heavy lymphocyte infiltrate, while presenting few or not visible parasites. In contrast, diffuse cutaneous leishmaniasis is characterized by low or absent cell-mediated immunity; instead of lymphocyte infiltrates in the lesions, dermal infiltration is composed of vacuolated, parasite-carrying macrophages. Only after treatment, signs of acquired cellular immunity are present.[1] The lesions of localized cutaneous leishmaniasis can appear as self-healing ulcers, or as dermal granulomas, that require several months or years to heal,[21] and in some cases, can become chronic.[22] Cutaneous leishmaniasis from the New

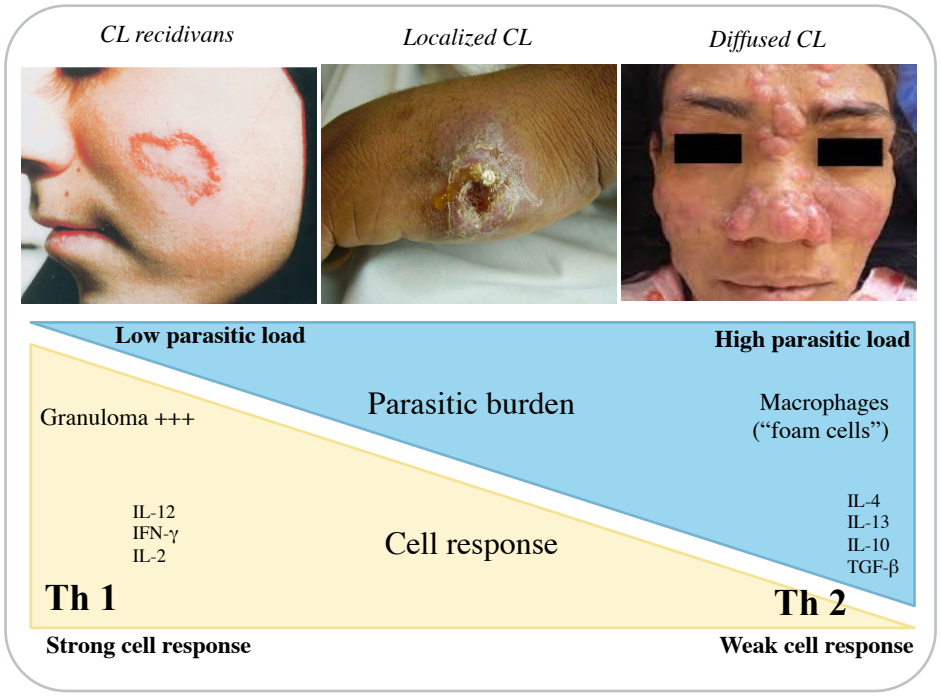

Figure 1.6. **Clinical manifestations of cutaneous leishmaniasis** Cutaneous leishmaniasis (CL) can be presented in patients in three main forms, which are characterized by distinctive cell response and parasitic burden: CL recidivans, localized CL, and diffuse CL. Adapted from P. Buffet,[9] with photographs (from left to right) from: I. Esfandiarpour,[25] A. Bari,[26] and N. Mehrolhasani[27]

World present a relatively more rapid pathological development, wetter and larger ulcers, longer ulcerative phase and more involvement of the connective tissue in comparison with cutaneous leishmaniasis of the Old World.[23,24]

1.1.3.3 Mucocutaneous leishmaniasis

The term mucocutaneous leishmaniasis (also known as mucosal leishmaniasis or *espundia*) has been traditionally used to refer to a disfiguring form of sequel of New World cutaneous leishmaniasis. Mucocutaneous leishmaniasis is characterized by dissemination of the parasites from the skin to the naso-oropharyngeal mucosa.[19] Mucosal and cutaneous lesions can occur at the same time, or appear years apart. Typically, the mucosal lesions appear several years after the resolution of the initial cutaneous lesions that were not treated, or were treated suboptimally.[19]

The initial manifestations of the disease include epistaxis and stuffiness in the nose, with erythema and edema of the nasal mucosa.[19] Minor necrotic and granulomatous reactions appear, associated to the infiltration of lymphocytes, macrophages and plasmocytes. As the disease evolves, acute vasculitis and necrosis of the walls of the small blood vessels appear,[1] and a progressive and ulcerative destruction of the naso-oropharyngeal mucosa and surrounding tissues takes place.[19]

Table 1.1. Summary of therapeutic approaches implemented in comparative studies for leishmaniasis treatment. [9]

Mode of administration	Antibiotics	Antineoplastics and immunomodulators	Antiparasitics	Antifungals
Parenteral	Paromomycin	Destroyed promastigotes + BCG	Pentavalent antimonials	Amphotericin B
		IFN-γ	Sodium stibulconate	Liposomal amphotericin B
		GM-CSF	Meglumine antimoniate	Lipidic complex amphotericin B
			Pentamidine	
Oral	Allopurinol	Miltefosine	Sitamaquine (WR6026)	Ketoconazole
	Azithromycin		Dapsone	Itraconazole
				Fluconazole
		Physical methods		
Local	Topic paromomycin	Cryotherapy		
		CO_2 laser		
		Thermotheraphy		
	Imiquimod	Dynamic phototherapy		
		Intralesional zinc sulfate		
		Intralesional pentavalent antimony		

1.1.4 Current treatments against leishmaniasis

1.1.4.1 Chemotherapy

In the absence of an available effective human vaccine against leishmaniasis, the control of the disease relies on chemotherapy and control of the transmission vector. [28,29] Table 1.1 summarizes the different chemotherapeutic agents against leishmaniasis currently in use. The choice of a chemotherapeutic will depend greatly on the form of leishmaniasis present (cutaneous, mucocutaneous, or visceral), the species of *Leishmania* suspected, and the data available on success rates in different geographical populations. In addition, the potential side effects of these chemotherapeutics are considered. For example, the most serious side effects of the current antimonials available -meglumine antimoniate and sodium stibogluconate- include pancreatitis, cardial and renal toxicity. [28,30] Miltefosine, originally developed as an anticancer drug, is the first effective oral drug against leishmaniasis. However, a limitation for its use is its reported teratogenicity, and therefore it is not suitable for use during pregnancy, and it is ruled out for treatment in women of childbearing age for whom adequate contraception cannot be assured. [31] Amphotericin B also presents nephrotoxicity as a common side effect. However, it is highly effective against antimonial resistant *L. donovani*, and newer formulations, including liposomal amphotericin B reduce the associated toxicity. [32] The use of pentamidine isenthionate is limited, due to the severity of its reported side effects: diabetes mellitus, severe hypoglycemia, shock myocarditis and renal toxicity. [1]

Table 1.2. Treatments for Old World (*) and New World (**) cutaneous leishmaniasis. [9]

Parasite species	First line treatment	Second line treatment	Observations from ongoing research
*L. major**	Pentavalent antimonials (intralesional) and superficial criotherapy Alternatively, fluconazole, local paromomycin, miltefosine	Pentavalent antimonials (parenteral)	Topical paromomycin/-gentamycin: good efficacy Parenteral antimonials: mixed efficacy in children Itraconazole: low or nule efficacy
*L. tropica**	Pentavalent antimonials (intralesional) and superficial criotherapy Thermotherapy	Pentavalent antimonials (parenteral)	
*L. infantum**	Pentavalent antimonials (intralesional) and superficial criotherapy	Pentavalent antimonials (parenteral)	
*L. aethiopica**	Pentavalent antimonials (parenteral) Miltefosine		
*L. guyanensis***	Pentamidine isethionate (i.v. with better results than i.m.)	Miltefosine Pentavalent antimonials (parenteral)	
*L. panamensis***	Pentamidine isethionate (i.v. with better results than i.m.) Miltefosine Pentavalent antimonials (parenteral)		Topical paromomycin/-gentamycin: good efficacy Imiquimod: moderate adjuvant effect associated to pentavalent antimonials
*L. braziliensis***	Pentavalent antimonials (parenteral)	Amphotericin B deoxycolate Amphotericin B liposomal	Pentamidine: no efficacy Miltefosine: no efficacy Local paromomycin: good efficacy
*L. mexicana***	Ketoconazole Thermotherapy	Fluconazole	Miltefosine: no efficacy Pentavalent antimonials: low efficacy

Table 1.2 summarizes the recommended lines for treatment of cutaneous leishmaniasis.[9] Depending on the status of the patient, the size of the lesions and their location. In general, local treatment is recommended for cutaneous leishmaniasis caused by *L. major*, because the lesions are associated with a self-cure rate of about 50% at 6 months, and because of the side effects associated with systemic treatment using the currently available antileishmanial drugs.[1] However, if the lesions are disfiguring or disabling, bigger than 5 cm in diameter or numerous (more than 4), or if the patient is immunocompromised, systemic treatment is recommended.[1]

Treatment of leishmaniasis is quite challenging in the poorest and most remote regions, not only in terms of medicament supply, but also because of the health infrastructure and

personnel required for their administration. Pentavalent antimonials need to be administered intramuscularly or intravenously, and according to the WHO treatment guidelines, the patients should be monitored by serum chemistry, complete blood counts and electrocardiography.[1] Amphotericin B is administered by intravenous infusion, and due to its toxicity, continuous monitoring in a hospital is recommended.[2] For cutaneous leishmaniasis, topical administration of paromomycin ointments have shown promising results, and is expected to reach the clinics within the next years.[9] Ultimately, the therapeutic decisions are based on the benefit-risk ratio for each patient, the health service setting and their availability of such medicines.

A last, but not least, concern about the currently available drugs against leishmaniasis, is the emergency of drug resistant parasites. Antimonials have been one of the first chemotherapeutics used against *Leishmania*, and for more than half a century they have been the first line of treatment in several countries for leishmaniasis.[1] However, antimonials are obsolete for use in the Indian subcontinent, due to the emergence of resistance in Bihar, India.[33] In this region, antimonials show up to 65% failure rate . Therefore, other chemotherapeutics are used, among them miltefosine. Recent reports from India,[34] Bangladesh,[35] and Nepal[36] on miltefosine treatment efficacy indicate the emergency of miltefosine resistance.[28] The risk of selection of drug resistant parasites is higher in anthroponotic leishmaniases, because of the higher exposure of the parasites to antileishmanial drugs.[1,9]

Altogether, there is a need for novel antileishmanial drugs, with fewer side effects as the currently available options, and ideally with easier modes of administration, i.e. oral or topical.

1.1.4.2 Immunotherapy

Immunotherapy consists in the use of agents for modulating the immune response of a patient for prophylactic or therapeutic purposes.[37] The strategy of action of immunotherapeutic agents may be to (1) augment the host natural defenses, (2) restore effector functions, or (3) decrease host excessive response.[37,38] The aim of immunotherapeutic approaches against leishmaniasis is to obtain a therapeutic cure by modulating and activating the immune response of the host,[37] overcoming the control strategies that the parasite uses to escape the immune system.

Vaccines and immunotherapeutics

The first hints for the possibility to develop a vaccine against *Leishmania* spp. came from a practice used in endemic areas for centuries, consisting in the inoculation of live

and virulent *Leishmania* parasites obtained from cutaneous lesions. This "leishmanization" resulted in lesion development, but once it healed, conferred subsequent immunity to infection.[39] This approach was tested in vaccination trials, but due to serious safety concerns, its use was no longer recommended.[40] Although several studies have investigated the use of virulence-attenuated parasites or parasites with a so called "suicidal cassette",[41,42] due to the risks associated to the handling of live vaccines a different strategy was explored: the use of killed parasites.

Several developments of a vaccine for cutaneous leishmaniasis using killed parasites or parasite lysate have been reported, and are considered to be the first-generation candidate vaccines. Convit and colleagues found a 94% of cure rate in patients in Venezuela, using three injections of a vaccine composed of *L. mexicana* lysate with Bacillus Calmette–Guérin (BCG; formulation referred as the "Convit vaccine").[43] In another study using 5,341 patients with mucocutaneous and chronic cutaneous leishmaniasis, treatment with this vaccine resulted in cure rate between 91.2% and 98.7%.[37,44] Mayrink and colleagues evaluated vaccination with a mixture of lysates from 5 *Leishmania* strains using BCG as adjuvant (the "Mayrink vaccine"), and observed a cure rate of 76% in patients with cutaneous leishmaniasis.[37,45,46] In a later clinical trial with 542 patients, the authors found that repeated daily doses of killed *L. amazonensis* and BCG resulted in a cure rate of 98.1%.[46] This vaccine showed comparable results as the use of the drug antimoniate N-methyglucamine (Glucantime ®) and as a combined chemotherapeutic treatment, discussed in the next section. Another candidate was produced in Iran (the "Razi Institute vaccine") for *L. major* given in combination with BCG. While these results have been encouraging for therapeutic indications, they have been inconclusive or negative for prophylaxis.[1]

The second generation vaccines consist of recombinant proteins and DNA vaccines.[1] Up to date, the only commercially available vaccine for leishmaniasis is formulated for dogs. Canine visceral leishmaniasis is challenging to treat, because under the WHO guidelines human chemotherapeutics are not recommended to be used, in order to prevent the development of drug resistance.[37,47] The vaccine Leishmune ® was developed in Brazil, and it is composed by the fucose-mannose-ligand (FML) antigen of *L. donovani*.[48] Currently only one defined vaccine, Leish-111f+MPL-SE, has reached clinical trials in Sudan, Peru and India for its use in humans.[49] Leish-111f is a polyprotein, composed of *L. major* homolog of eukaryotic thiol specific antioxidant (TSA), *L. major* stress-inducible protein-1 (LmSTI-1) and *L. braziliensis* elongation and initiation factor (LeIF).[50,51] The adjuvant, monophosphoryl lipid A-stable emulsion (MPL-SE) stimulates Toll-like receptor (TLR)-4, and is the only TLR agonist in approved human vaccines.[49] A version of this vaccine lacking the His-tag, for manufactory and regulatory purposes, is currently

under clinical trials in India.[50]

Immunochemotherapy

Immunochemotherapy involves the combination of immunotherapy with chemotherapeutic drugs, with the aim to create a synergy between the activation of the immune system, and direct action of drugs against an infectious agent.[37] The benefit of this type of strategy is to reduce the amount of drug required, the duration of treatment or both. By this mean, the toxic side effects associated to leishmanicidal drugs could in turn be reduced.

An approach used in Venezuela is the administration of three injections of the "Convit vaccine" to patients of cutaneous leishmaniasis; if the patient does not respond, then chemotherapy treatment is started.[1] Mayrink and colleagues reported the evaluation of an immunochemotherapy protocol, consisting on a combination of the "Mayrink vaccine" with the pentavalent antimonial Glucantime®. This protocol was associated with a 17.9% reduction of the volume of drug required, and a 31.6% (about 1 month) reduction of the treatment duration time.[46] Currently, the use of repeated daily doses of the "Mayrink vaccine" with low dose of antimonial is applied in the clinics in Brazil, and the "Mayrink vaccine" is registered as an adjuvant for low-dose chemotherapy.[1] Furthermore, a study in Sudan performed by Musa and colleagues in patients with persistent PKDL, the effects of immunochemotherapy with sodium stibogluconate and a mixture of killed *L. major* parasites with alum and BCG were analyzed. The treatment was administered four times at weekly intervals. The cure rate of patients treated with this immunotherapeutic approach presented a 87% of cure rate, while for sodium stibogluconate alone was of only 53%.[52]

Another immunochemotherapeutic strategy is the combination of leishmanicidal drugs with cytokines. Interferon (IFN)-γ is key for the activation of macrophages to kill *Leishmania* amastigotes, and it is reported to be clinically well-tolerated.[37,53] Different studies have evaluated the use of pentavalent antimonials in combination with IFN-γ.[37,54–58] Overall, these results indicate that IFN-γ enhances the efficacy of the pentavalent antimonials, reflected in higher cure rates and faster parasitological control in comparison with pentavalent antimonials alone. Granulocyte-macrophage colony-stimulating factor (GM-CSF) has also been studied in combination with pentavalent antimonials, either by intralesional injection or applied topically,[59,60] and reported to accelerate the healing of lesions, even in patients that have previously not responded successfully to antimonial therapy.[61]

All together, the use of immunotherapeutic approaches, alone or in combination with

chemotherapeutics, have demonstrated promising results, and further emphasize the importance on the immune response of the host for control of the disease.

1.2 The murine model of cutaneous leishmaniasis

Several mouse models are available for studying cutaneous leishmaniasis, based on different sites of infection (i.e., the footpad, the ears, the rump) and parasite dose. One of the most widely used involves the subcutaneous injection of *L. major* promastigotes in the footpad of the mice. Depending on the mouse strain, the infection may proceed as lesions that grow and are self-healing over the course of 6 to 8 weeks -as in the case of C57BL/6, CBA/N, DBA2, C3H/He, Sv129/Ev mice-, or in non-healing lesions, as observed in BALB/c mice. BALB/c mice not only fail to contain the development of the skin lesions, but also present dissemination of the parasites to internal organs, and therefore must be euthanized.[62,63] In resistant mice, healing of the lesions is followed by long-lasting immunity against infection. As observed in humans, this immunity is not sterile, and one year after cure viable parasites can still be detected.[64] The strong difference in outcome of infection indicated a genetically determined predisposition for susceptibility or resistance, and led to intensive investigations on the mechanisms behind this phenomenon. It should be noticed that other experimental setups reflect better the clinical outcome of cutaneous leishmaniasis observed in humans, particularly the intradermal injection of parasites in the ears of mice.[65,66]

Susceptibility and resistance in the murine model of cutaneous leishmaniasis is controlled by cell-mediated immunity, but not by humoral immunity. Mice deficient in antibody (Ab) response are reported to be still resistant to *L. major* infection,[67] and transfer of Ab from immune mice showed no protection to infection in BALB/c mice.[68] In contrast, T-cell deficient mice -athymic nude mice- are susceptible to *L. major* infection, despite being in a C57BL/6 or CBA background.[63,69] After syngeneic transfer of T cells, the ability to resist infection was restored in these mice.[63] Later on, it was shown that the syngeneic transfer of purified $CD4^+$T cells from mice that have been cured from a primary infection or from immunized mice to naïve mice conferred protective immunity.[63,70] While these results strongly indicated the role of protective $CD4^+$T cells, studies in BALB/c mice showed that blocking the functions of $CD4^+$T cells by injection of anti-CD4 Ab or cyclosporin A[71,72] resulted in protection against the parasite. Therefore, $CD4^+$T cells in this model where responsible for the protection and susceptibility of the different mouse strains.

In 1986, Mosmann and colleagues discovered that $CD4^+$T cells can be divided in two subsets, based on the cytokines they produce. The first group, Th1 cells, responds to antigens by secreting the cytokines interleukin (IL)-2, interferon (IFN)-γ and tumor necrosis factor (TNF). The second group, Th2 cells, produce IL-4, IL-5 and IL-10,[73–75] as well as the later discovered IL-13. Resistant mouse strains against *Leishmania* develop a Th1 immune response, while susceptible BALB/c mice develop a Th2 immune response. The importance of Th1 response for the control of leishmaniasis relies greatly upon the activating function of IFN-γ to activate macrophages for the killing of the parasites residing within them. IFN-γ-deficient mice are unable to cure infection,[76] while IL-4-deficient BALB/c mice were reported to cure.[77,78] These observations resulted in the formulation of what is known as the "Th1/Th2 paradigm" of resistance/susceptibility to *L. major* infection. While some of the main principles of this paradigm still hold true, more recent investigations indicate a more complex system with the discovery of other Th cell populations. This aspect is explored in more detail in Section 1.10.

The integration of different components from the innate and the adaptive immune system of the host ultimately result in control or susceptibility to the disease. This section summarizes the contribution of cells and chemical signals from the innate and adaptive immune responses against leishmaniasis.

1.2.1 The role of the innate immune system in cutaneous leishmaniasis

The defense against a pathogen is mediated by two main systems. The first system, innate immunity, is set up in place in steady state, ready to act rapidly upon infection. This system reacts upon detection of pathogen-associated molecular patterns (PAMP), which are structures highly conserved in a wide variety of microorganisms during evolution, but are not expressed on the body's own cells. PAMP are recognized by cells of the innate immune system by using pattern recognition receptors (PRR), which activates the effector mechanisms aimed to eliminate or control the pathogens. The response orchestrated by the innate immune system in turns provides signals that initiate the second system, adaptive immunity, to develop a pathogen-adapted response to eliminate the microbe, and to that generate long-lasting memory to rapidly respond in case of a reinfection. Furthermore, the immune response against a pathogen can be classified into a cell-mediated response, and a humoral immune response. The latter corresponds to mechanisms of defense based on proteins circulating in the blood, including the Ab produced by adaptive immunity, or complement proteins corresponding to the innate immune system.[79–81]

Although the murine model of cutaneous leishmaniasis has demonstrated a paramount role of the adaptive cell-mediated immunity in the susceptibility or resistance to leishmaniasis, the innate immune system in its own contributes in different levels to the outcome of the disease. The innate immune system is comprised by physical barriers (i.e. the skin and skin-resident effector cells), circulating effector cells (including neutrophils, macrophages, and natural killer cells), circulating effector proteins (including the complement system), and cytokines and chemokines.[79] The innate immune response of the host contributes to resistance against the infection by controlling the parasite growth during the early stages, by directing the recruitment of cells from other compartments of the immune system, and by contributing to the cytokine microenvironment in which parasite-specific T cells are primed.[37,82,83] The roles of the different compartments of the innate immune system are described next.

1.2.1.1 The skin

The skin is the first barrier against infection. It contains a network of immune cells, either as patrols or residents, whose purpose is to sense pathogens and control infection.[84] The immune cells comprised in this network include Langerhans cells -a specialized type of dendritic cells (DC)-, tissue residents T cells, mast cells, monocytes, and dermal DC.[23] Furthermore, neutrophils continuously circulating through dermal vessels extravasate quickly upon signs of inflammation. The skin is the site of primary infection, and while the mechanisms of Th cell differentiation have been proposed to primarily occur in the lymph node,[85] there is an increased amount of evidence indicating that the skin also plays a role influencing the direction of the immune response. For example, one of the differences observed in the skin of BALB/c mice in comparison to C57BL/6 mice is that the first present a higher percentage of infiltrating granulocytes, -particularly neutrophils[86] and macrophages[87]-, and depletion of neutrophils in BALB/c mice resulted in a Th1 response.[86] Furthermore, it has been proposed that the infected tissue generates danger signals within the first hours after infection, which could be integrated by DC and transferred to the lymph nodes, were they instruct the polarization of naïve Th cells (Th0).[88,89]

Keratinocytes constitute more than 90% of epidermal cells.[84] They express different PRR, as well as MHC class II molecules that are up-regulated upon activation for the presentation of antigens.[90] IL-6 production from keratinocytes has been reported to play an important role for the innate immune response against *Leishmania*, and for the development of a Th1 immune response.[91,92] Ehrchen and colleagues reported the expression of chemokines and cytokines in the skin within the first hours at the site of infection.[91] The skin of C57BL/6 mice presented higher gene expression levels of the cytokines IL-1β, IL-4, IL-12p40, IL-12p35, and TNF-α, and osteoponin, as well as

of the chemokines CXCL2, CXCL10, CCL2, CCL4 in comparison to BALB/c mice. At the secretion level, the authors found higher amounts of secreted CCL2, osteoponin, IL-6, and TNF-α. The authors attributed the expression of these cytokines and chemokines to keratinocytes. They found that chimeric mice in a C57BL/6 background laking IL-6 expression in keratinocytes but not in immune cells showed a Th2 immune response.[91]

The roles of different immune cells that also infiltrate the skin lesions are discussed in the next pages.

1.2.1.2 Neutrophils

Following parasite inoculation into the dermis, neutrophils rapidly infiltrate the wounded skin, and immediately take up *Leishmania* promastigotes. Neutrophils are thus the first cells to encounter the parasites.[93,94] After an initial acute inflammation, the infection proceeds "silently", as the parasite proliferates without any visible pathology in the patient, and the peak in parasite load is found when the skin lesions start to become apparent.[65,95] In lesions from localized cutaneous leishmaniasis, neutrophils have been reported in the vicinity of the keratinocyte layer of the skin, and are presumably involved in the destruction of keratinocytes, resulting in ulceration.[23,96] As neutrophils are found in necrotic and perinecrotic areas from lesions of human cutaneous leishmaniasis, it is suggested that neutrophils are involved in the protection, but also in the pathology of the disease.[23,97,98]

Laskay and colleagues have proposed that neutrophils could act as "Trojan horses" for *Leishmania* infection, by allowing the parasites to invade macrophages silently.[99,100] They observed *in vitro* that neutrophils rapidly take up *L. major* promastigotes, but the parasites were able to survive as promastigotes within them. Furthermore, the parasites induced the neutrophils to undergo apoptosis, which in turn made them a target for macrophages as these cells routinely clear up apoptotic cells from the system. By this mechanism, the parasites able to enter silently to the macrophages together with the dead neutrophils, and ultimately to find a niche for replication within the macrophage phagosomes.[100] Furthermore, infected neutrophils released MIP1-β, a monocyte-attracting chemotactic factor.

Two-photon intravital microscopy showed *in vivo* that neutrophils are indeed rapidly recruited to the site of infection. 40 min. after exposure to sandflies, neutrophils could be already detected into the skin, localized around the site of apparent bite sites.[94] The parasites were readily taken up by the neutrophils, but remained viable, and were proposed to contribute with the establishment of infection. Moreover, they results indicated

that macrophages were also recruited to the site of infection, and suggested the release of viable parasites from apoptotic neutrophils in the vicinity of surrounding macrophages. They found however no evidence *in vivo* for the uptake of apoptotic neutrophils harboring living parasites. Nevertheless, the importance of neutrophils in the infection outcome was confirmed in this study, as they also found that neutrophil depletion resulted in reduced disease.

A different feature of neutrophils, is that upon recruitment to the site activation they can be activated to die and release "nets" of DNA and granular contents, but there is currently no consensus yet on the fate of the entrapped parasites.[101] Recently, Ribeiro-Gomes and colleagues reported the uptake of apoptotic neutrophils harboring *L. major* by DC, which prevented the activation of infected DC in the skin.[102] Furthermore, exposure to *L. major* was shown to induce the secretion of CCL3 by neutrophils from C57BL/6 mice, in levels considerably higher than in neutrophils from BALB/c mice.[103] The authors of this study also showed that CCL3 acted as a strong DC-attracting chemokine, and that a higher number of Langerhans, dermal and monocyte-derived DC were recruited to the infection site of C57BL/6 mice in comparison to BALB/c mice. Therefore, it has been proposed that the crosstalk between neutrophils and DC may play an important role to the outcome of *L. major* infection.[104]

1.2.1.3 Natural killer cells

Natural killer cells (NK) are members of the innate immune system, whose function is to eliminate host cells harboring intracellular pathogens, or cells that present that have undergone malignant transformation, or cells presenting a strong stress reaction.[66] NK cells are activated in response to an up-regulation of ligands for NK-cell activating receptors, or the down-regulation of NK cell-inhibitory receptors. *Leishmania* infection has been reported to trigger a rapid but transient activation of NK cells. They have been found accumulated at the site of *Leishmania* inoculation 24 h after infection of C57BL/6 mice[66,105] and in the lymph nodes.[106] The cytotoxic activity of NK cells in *Leishmania* infection is dispensable, as myeloid cells infected with *Leishmania* are reported to resist NK-mediated lysis.[66] Nevertheless, mice depleted of NK cells show an aggravated course of *L. major* infection, whereas transfer or activation of NK cells is reported to have an ameliorating effect.[66,107–109]

In addition to their cytotoxic role, NK cells are also known to produce the cytokines IFN-γ and TNF.[66,110,111] IFN-γ plays a crucial role in the activation of macrophages for the killing of the *Leishmania* parasites they harbor, The cytotoxic activity of NK cells and

IFN-γ production has been detected as early as 1 day post infection in resistant mouse strains.[66,106,108,112] It has also been observed that BALB/c mice present a significantly weaker NK cell activity in response to *L. major* than C57BL/6 mice.[66,108,113,114] The activation of NK cells has been attributed to different factors, including: cytokines (IL-2, IL-12, IFN-α/β), IFN-α/β-induced inducible nitric oxide synthase (iNOS), the receptor tyrosine kinase Tyk2, and the transcription factor IRF-2.[66] It has been proposed that the activation of NK cells, in experimental mouse models and in human infection with *Leishmania*, is mediated by the interaction with myeloid cells, as the production of NK cell-stimulatory cytokines is required.[66] In addition, the activity of NK cells has been reported to be transient, and it has been demonstrated that *L. major* inhibits IFN-γ production, proliferation and cytotoxic activity of human NK cells, as shown with promastigotes, parasite lysate, and the 63 kDA glycoprotein GP63 of *L. major*.[66,115]

While there is a consensus on NK cells exerting a protective function in the murine model of cutaneous leishmaniasis, they are non-essential for the resolution of the infection,[66] as a Th1 immune response against the parasite can still be induced in the absence of NK cells- derived IFN-γ.[66,116] Nevertheless, the activation of NK cells has been proposed as a useful strategy to improve the course of infection by immunotherapies or immunochemotherapies.[66]

1.2.1.4 Macrophages

Macrophages are cells of the immune system with great phagocytic capacity that are located in various tissues. They serve different important functions; they are able not only to engulf and degrade microbial pathogens, but also to produce cytokines and chemokines in response to detection of PAMP, which in turn promote the recruitment of other immune cells and the triggering of an immune response. Furthermore, they are able to present antigens from the degraded pathogens to T cells, participating in the promotion or effector phase of adaptive immunity.[117] In addition, they are the main cell type responsible for engulfing and eliminating apoptotic cells, or efferocytosis.[118] Macrophages take up microbial pathogens by phagocytosis, a process in which the cell forms actin protrusions around the microbe that fuse and result in its engulfment. The microbe is then inside the cell within an intracellular vesicle, called the phagosome. The phagosome then fuses with lysosomes, forming a phagolysosome that has an acidic pH and contents hydrolytic and catabolic enzymes. This environment is enough to destroy some microorganisms.[118,119] Activated macrophages kill the phagocytosed pathogens by the action of microbicidal molecules in the phagolysosomes.

One mechanism for the elimination of pathogens in the phagolysosomes of macrophages is the conversion of molecular oxygen into reactive oxygen species (ROS), also known as respiratory burst. This process is mediated by the nicotinamide adenine dinucleotide phosphatase (NADPH) oxidase complex, which is assembled in the phagosolysosome membrane.[120] The oxidase complex is induced and activated by different stimuli, including the recognition of PAMP by TLR and IFN-γ. Moreover, in response to these stimuli macrophages produce reactive nitrogen intermediaries, in particular nitric oxide (NO), by the enzymatic action of iNOS. iNOS catalyzes the conversion of arginine to citrulline and NO, which can acts in synergy with ROS.[79]

Macrophages form the second wave of cells infiltrating the site of infection. While *Leishmania* parasites can infect phagocytic cells and some non phagocytic cells (i.e. fibroblasts), macrophages are widely recognized as the preferred host cell for *Leishmania* parasites.[99,121] Upon entering the host, *Leishmania* promastigotes are exposed to the antimicrobial properties of serum components. Serum-opsonized promastigotes are taken up by macrophages in a process mediated by the complement receptor 3, depending on the GTPase RhoA.[120,122] C3b has been documented to bind to leishmanial surface lipophosphogylcan (LPG) and metalloprotease GP63.[5,123,124] In *in vitro* systems, macrophages in culture synthesize small amounts of proteins, including C3 for the opsonization of nearby particles or cells.[5] C3 has been detected in the surface of *L. donovani* amastigotes and promastigotes incubated with murine peritoneal macrophages without serum pretreatment.[125] Other receptors associated with the phagocytosis of *Leishmania* parasites by macrophages include mannose receptors, complement 1 receptor, fibronectin receptors, and Fc gamma receptors.[5]

Although most of the microbial pathogens that proliferate in macrophages do so by escaping the phagocytic pathway, a characteristic feature of *Leishmania* parasites is their ability to survive and proliferate within the mature phagolysosomes of macrophages.[99,126] Promastigotes taken up by macrophages are delivered to the phagolysosome -also referred in the literature as parasitophorous vacuoles (PV)[127]- where they differentiate into amastigotes. The PV in which amastigotes reside contain lysosomal hydrolases, and their membranes are enriched with late endosomal/lysosomal proteins, as well as an acidic pH (4.7-5.2).[120] Unlike promastigotes, whose metabolic activities are optimal at neutral pH, amastigotes are greatly adapted to the acidic pH of the phagolysosome environment. A pH between 4.0 and 5.5 optimal for their metabolism.[120,128]

Leishmania parasites have been shown to actively manipulate the PV where they reside. They are reported to: alter the composition of the lipid bilayer of this compartment membrane, prevent its fusion with lysosomes, and to remodel the surrounding cytoskeletal

network.[118,120,129] The arrest on phagosome maturation is specific for promastigotes, but not for amastigotes, and it is characterized by periphagosomal F-actin accumulation.[120] Furthermore, amastigotes are able to avoid the generation of ROS from the macrophages by degrading and preventing the assembly of the NADPH oxidase assembly.[120,130,131] NO-dependent *Leishmania* elimination is crucial in macrophages, as treatment with inhibitors of iNOS result in a drastic reduction of parasite elimination *in vitro* and *in vivo*.[132–134] Furthermore, mice lacking iNOS have been shown to be highly susceptible to *L. major* despite the development of a Th1 immune response.[135] Pro-inflammatory cytokines characteristic of a Th1 immune response are strong inducers of iNOS, particularly IFN-γ and TNF-α.[136,137] Conversely, Th2 typical cytokines, including IL-4, IL-10, IL-13 and transforming growth factor (TGF)-β have been reported to inhibit macrophage activation and NO-mediated elimination of the parasites.[138–141]

In addition, to survive within the macrophage intracellular amastigotes must satisfy their metabolic requirements by scavenging nutrients from their host. It is predicted that amastigotes must obtain all their purine requirements, several vitamins and about ten essential amino acids from the phagolysosome.[126] The uptake of nutrients has been attributed to different membrane transporters, including amino acid permease 3, biopterin transporter 1, inositol transporter 1, folate transporter 1, glucose transporters 1 to 3, purine transporters 1 to 4, and polyamide transporter 1.[142] Furthermore, it has been suggested that the amastigotes might obtain essential amino acids and heme from the proteolysis of host phagolysosome proteins.[143]

As previously mentioned, besides phagocytosis macrophages play a key role in inflammation, due to their capacity to produce cytokines in response to detection of PAMP. Examples of inflammatory cytokines typically secreted by activated macrophages include IL-1, IL-6, IL-12, TNF-α, among others.[144] *L. major* promastigotes have been documented to attach and enter into bone marrow-derived macrophages (BMM) in a silent manner. Particularly, the production of IL-12 has been shown to be actively impaired in infected BMM,[145] as well as in granuloma macrophages,[146] and human monocytes.[147] This effect has been confirmed in BMM from susceptible and resistant mouse strains,[145] and it was speculated that it might explain the delayed onset of parasite killing observed even in resistant mice.[146] The suppression of IL-12 expression in macrophages is maintained even after further stimulation with lipopolysaccharide (LPS),[146,148,149] a strong inducer of proinflammatory cytokine expression. The observed regulation of IL-12 synthesis has been attributed to different virulence factor from the parasite, including *Leishmania* phosphoglycans[148] and cysteine proteases.[150] The effect of cysteine proteases of *Leishmania* in the macrophage is discussed in detail in Section 2.8.

At the molecular level, it has been shown that *Leishmania* parasites efficiently silence different signaling pathways important for the immune response of macrophages. One strategy is the interference with TLR signaling. *L. major*, *L. mexicana* and *L. donovani* have been reported to exploit the macrophage Src homology region 2 domain-containing phosphatase-1 (SHP-1) to inactivate kinases necessary for proper TLR signaling.[4,151] SHP-1 is modulated by GP63, one of the major virulence factors of *Leishmania*, and it is also proposed to be up-regulated by *Leishmania* elongation factor-1 (EF-1)β.[152] Fig. 1.7 summarizes the effect of GP63 in different signaling pathways of the infected macrophage. *Leishmania*-mediated modulation of SHP-1 results in an inhibition of Janus kinase (JAK)/signal transducer and activator of transcription (STAT), of mitogen activated protein kinase (MAPK) -including extra-cellular signal-regulated kinase (ERK)1/2, Jun N-terminal kinase (JNK), and p38 MAPK-, as well as nuclear factor-κB (NFkB) signaling pathways. Another important effector activated by *Leishmania*-induced SHP-1 is protein-tyrosine-phosphatase-1 (PTP-1), which dephosphorylates intermediaries of the Janus kinase (JAK)/STAT signaling pathway.[152] lipophosphogylcan (LPG) is known to interact with TLR2, and it has been recently reported that TLR9 expression is decreased in response to this interaction.[153] Furthermore, LPG has been shown to block protein kinase C (PKC) signaling, which is important for the NO and oxidative burst processes. In addition, the down-regulating effect of cysteine proteases from *Leishmania* in macrophages is reported to be dependent on NF-κB signaling pathway.[150] Furthermore, *L. major* infection has been documented to down-regulate IL-12 production by alteration of CD40-mediated signaling.[154] It has also been proposed that *Leishmania* might down-regulate IL-12 expression by modulating the phosphoinositol-3-kinase (PI3K)/protein kinase B (Akt) signaling pathway.[4,155]

Altogether, macrophages are the preferred host cell for *Leishmania* parasites, which have evolved different strategies for invasion and survival. These strategies include adaptation and active modulation of the phagolysosomal environment, mechanisms for uptake of the nutrients available within the host, and silencing of the host defense pathways. Macrophages thus are left dependent on exogenous IFN-γ for their activation, in order to be able to successfully eliminate the intracellular parasites. As discussed in the next pages, Th1 polarized cells are a crucial source of IFN-γ, playing a key role in the control of the disease.

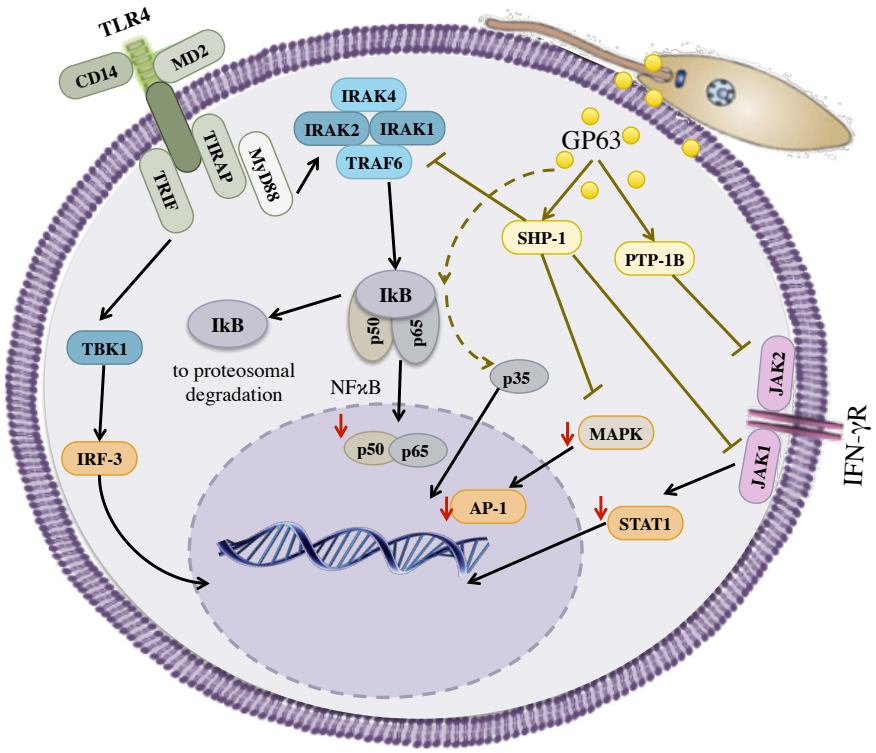

Figure 1.7. ***Leishmania* interactions with host macrophages**. *Leishmania* parasites use different strategies in order to silence their host macrophage. One of the most important virulence factors is the metalloprotease GP63, which directly activates the host phosphatases SHP-1 and PTP-1B in order to block multiple signaling pathways including: JAK/STAT, MAPK, and NF-κB. Furthermore, GP63 is also able to cleave the p65 subunit of NF-κB (represented by the dashed lines). The red arrows indicate down-regulation. Adapted from Shio and colleagues.[152]

1.2.2 Dendritic cells: the bridge between innate and adaptive response to *L. major*

Innate immunity alone is not enough for the control of *Leishmania* infection, since a Th1-mediated macrophage activation is required for the intracellular killing of the parasites. DC play a key role in inducing a cell-mediated immune response against pathogens, serving as a bridge between innate and adaptive immune systems. DC were first reported by Steinman and Cohn in 1973, describing them as cells in lymphoid organs with a dendritic appearance.[156] DC differentiate from haematopoietic progenitor cells, and can be organized in three main stages of differentiation: DC precursors, immature DC, and mature DC.[157] Fully differentiated DC exist as a heterogeneous mixture of populations.[158] They have been subdivided into two major different populations, based on their developmental origin, expression of surface antigens, cytokine production and functional capacity.[157] The first population are conventional DC (cDC), and include Langerhans cells found in the skin and interstitial DC.[157,159] The second population are plasmacytoid DC (pDC), which secrete high amounts of type 1 interferons and are therefore strong inducers of cytotoxic immunity.[157,160] Fig.1.8 depicts the origins and development of different DC

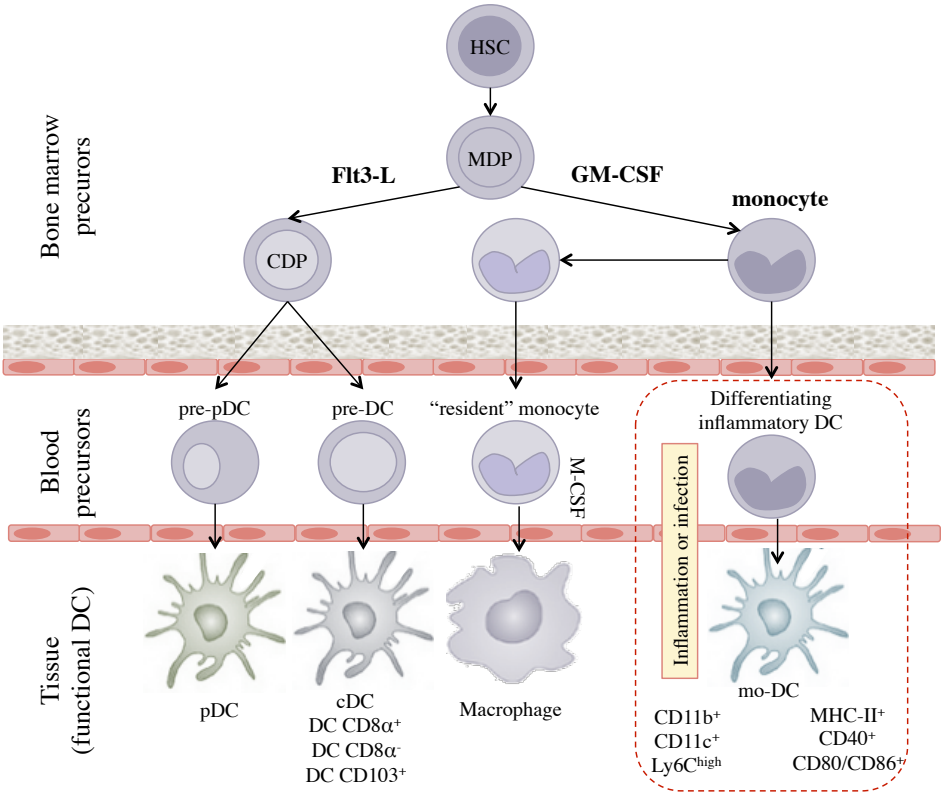

Figure 1.8. **Origin of different DC subsets**. All DC are generated from hematopoietic stem cells (HSC), which differentiate in the bone marrow to macrophage/DC progenitors (MDP). In the presence of Flt-3 ligand, MDP give rise to common DC progenitors (CDP). CPD can be further differentiated into plasmacytoid and DC precursors (pre-pDC and pre-DC, respectively), that travel through the blood to lymphoid and nonlymphoid tissues in steady-state and inflammatory conditions. MDP can also differentiate in the presence of GM-CSF into monocytes that can be further differentiated into macrophages, or in the case of inflammation/infection, into inflammatory DC (mo-DC). Adapted from C. Hespel and M. Moser.[161]

populations. It should be noticed that this is rather a simplistic classification, as also spleen precursors have been identified, and emerging data from the field indicate that DC precursors have a multilineage potential, with the possibility of multiple alternative routes for DC differentiation.[158]

DC are known as "professional" antigen presenting cells (APC), due to their capacity to internalize pathogens and process them into antigens, and further migration to the draining lymph nodes to present them to Th0 cells. They are considered to be the most potent APC known.[162] In steady-state conditions, immature DC reside in peripheral tissues, sampling and processing the antigens present in their microenvironment in the search for danger signals. Immature DC have a high endocytic capacity, and continuously sample their environment by macropinocytosis, receptor-mediated endocytosis, and phagocytosis.[163] Danger signals for infection include host-derived inflammatory cytokines (including TNF-α, IL-1, IL-6, and IFN-α), CD40 ligand (CD40L), molecules released by damaged host tissues, and microbial products.[164] The latter are detected by PRR in the surface, including TLR.

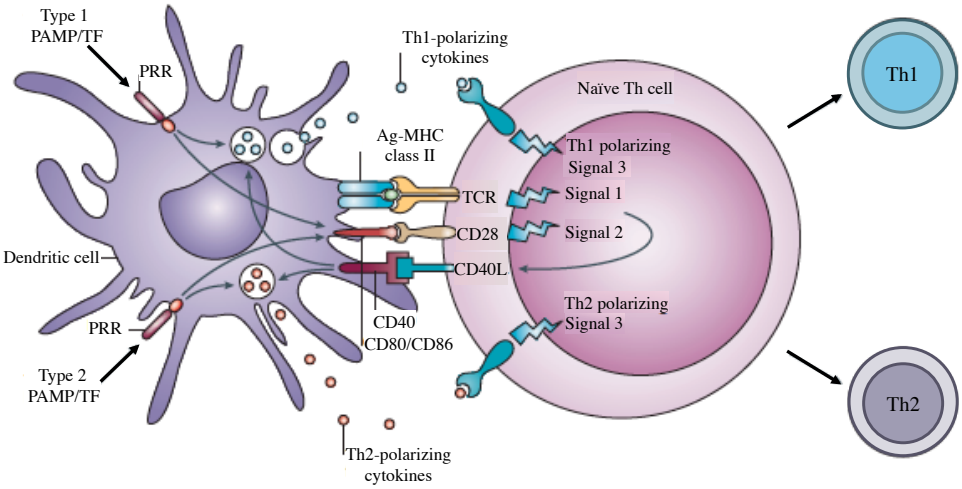

Figure 1.9. **Polarization of naïve Th0 cells by DC**. Th0 cells require three main signals for polarization: (1) the recognition of antigen, by means of their TCR, which is presented in MHC class II molecules on the surface of DC. (2) The recognition of costimulatory molecules expressed by the DC (CD80/CD86, and CD40) by the receptors CD28 and CD40, respectively, and (3) the recognition of cytokines expressed by DC. DC express these signals in response to activation by PAMP. Some stimuli, like LPS, are associated with strong DC maturation and the polarization of Th0 cells to Th1 cells; other signals, including TNF-α induce only a semi-maturation of the DC, and the polarization of Th0 cells to Th2 cells. Adapted from M. Kapsenberg [89]

These stimuli trigger the maturation of DC, characterized by a reduced phagocytic uptake, the acquisition of cellular motility and migration to lymphoid tissues, and the expression of signals that mediate the polarization of Th0 cells. [157] DC use three main signals for instructing Th cell polarization: (1) the presentation of antigen in MHC class II molecules, (2) the expression of costimulatory molecules, and (3) the secretion of cytokines (Fig. 1.9). Several authors proposed in the late 90s that specific DC populations were predetermined into promoting Th responses. [165–167] However, it was later demonstrated that each DC subset has the capacity to induce different forms of Th cell polarization [157,168,169] , and that qualitative and quantitative differences in the signals they present determine the polarization fate of Th0 cells. [170] Presentation of antigen in the absence of costimulation by CD80 and CD86 results in T cell anergy. [171] Polarization by semi-mature DC (as in DC not producing IL-12, [172] or presenting low amounts of antigen [173] is associated with the development of Th2. [170] In contrast, strongly matured DC -as observed by stimulating them with LPS- typically express high levels of MHC class II and costimulatory molecules, and of proinflammatory cytokines, including IL-12. The sum of this signals result in Th1 responses. [174] IL-12 is a key cytokine for the development of Th1 polarization, and it is crucial for protection against leishmaniasis.

DC are key players in the cell-mediated immune response against *Leishmania*, and their contributions to the control of the disease have been extensively documented. [175]

DC are able to carry parasite antigens for an extended period of time, and these DC are able to stimulate T cells in the lymph nodes.[176] Several subsets of DC have been documented to capture *Leishmania* parasites and induce Th cell polarization, including DC residing in the skin -such as Langerhans cells[177] and dermal DC[178]-, lymph node resident DC, and monocyte-derived DC.[179,180] Lesions of human cutaneous leishmaniasis show strong tissue infiltration of inflammatory, monocyte-derived DC, where it has been suggested that they may stimulate T cells in an antigen-dependent manner.[181,182] It has been proposed that at early stage of infection, DC residing in the skin would be the first to capture *Leishmania* parasites,[183] while at a later stage of infection, monocyte-derived DC would differentiate in the inflamed skin, migrating then to the draining lymph nodes.[179]

Monocyte-derived DC have also the capacity to produce NO. STAT6-deficient BALB/c, which present defective IL-4 and IL-13-mediated signaling, display higher recruitment of monocyte-derived DC producing NO.[184] Leon and colleagues reported two de novo formed DC subsets in popliteal lymph nodes of mice infected with *L. major*. These two subsets were infected by the parasites, and were proposed to be involved in the Th cell polarization. The first DC subset was derived from monocytes directly recruited to the lymph nodes. The second subset consisted of DC derived from monocytes that were first recruited to the dermis, and then migrated to the lymph nodes. The authors reported that only the second subset was essential for the induction of Th1 responses.[161,179]

The entry of *L. major* to DC is not a passive event, as it has been reported that DC actively extend capture the parasite, by elaborating motile pseudopods.[183] It has been reported that DC are able to uptake amastigotes by Fcγ receptor (FcγR)I and FcγRIII. Mice lacking functional FcγR have been shown to present decreased numbers of infected lesional DC.[185] This feature has been correlated with the capacity of DC to cross-present *Leishmania* antigens in MHC class I to $CD8^+$ T cells.[175] The C-type lectin receptor DC-SIGN has also been reported to participate in the uptake of some *Leishmania* spp., including *L. infantum* and *L. pifanoi*, but not for *L. major*.[186]

In terms of the signals used by DC to instruct Th cell polarization, IL-12 is a key cytokine for inducing a Th1 response. Even in resistant mouse strains, neutralization of IL-12 by Ab results in susceptibility to *L. major* infection.[187,188] In contrast, treatment of BALB/c mice with IL-12 develop a Th1 response.[189] Von Stebut and colleagues have reported that DC are the primary source of IL-12 in lymphoid tissues,[190] although it IL-12 production was found to vary depending on the DC subset, maturation status, and life stage of the parasites. Furthermore, other members of the IL-12 family, including IL-27 and IL-23, as well as the proinflammatory cytokine IL-1, have been shown to contribute to the establishment of Th1 responses.[175,191–193] However, DC are also affected by the

evasion mechanisms of *Leishmania*. It has been reported that *Leishmania* parasites are also able to inhibit the production of IL-12 in DC.[194] In addition, it has been documented that *Leishmania* has a detrimental influence in the expression of MHC class I and MHC class II molecules in macrophages[195] and a similar effect has been observed in DC during *L. major* infection.[196]

The key role played by DC during *L. major* infection has been experimentally exploited by means of DC vaccination. This concept was first explored by Inaba and colleagues, showing that DC pulsed with antigen *ex vivo* and injected to mice would sensitize them to the protein antigen.[197] In the model of cutaneous leishmaniasis, DC activated *ex vivo* with TLR agonists have been reported to be potent inducers of host resistance in otherwise susceptible BALB/c mice.[198,199] As previously described, antigen persistence has been shown to be important for the maintenance of T cell memory, and IL-10 has been shown to play an important role in this phenomenon. Long-term infected mice have been shown to present persisting parasites, and it has been therefore proposed that DC contributes to the maintenance of memory responses.[176]

1.2.3 Cell-mediated immunity and the Th1/Th2 paradigm

An adaptive immune response involves the selection and amplification of clones of lymphocytes carrying receptors that recognize a foreign antigen. It is initiated when the mechanisms of defense from the innate immune system fail to eliminate a new infection, and aims to eliminate pathogens via humoral immunity, cell-mediated immunity or both.[200] T cells perform two main functions within the adaptive immune system: (1) to orchestrate cell mediated immunity, and (2) to regulate B cell responses to most antigens.[200] Furthermore, T cells can be classified into two main classes, based on their activity. The first class, are cytotoxic T cells, which control infection by killing directly the infected cell to prevent further pathogen replication. They express the co-receptor CD8, and recognize antigens bound to MHC class I molecules. The second class are T helper cells, which interact with B cells for the production of Ab, and can be further organized into different subsets depending on the effector functions they perform upon differentiation. They express the co-receptor CD4, and recognize antigens bound to MHC class II molecules.

T cells have a hematopoietic origin, and differentiate and develop in the thymus, where they undergo a stringent process of selection. The final competent T cells emigrate from the thymus to the bloodstream, from where they are able to home towards lymphoid organs.[200] Differentiated $CD4^+$T cells are able to recognize antigen, but will remain

"naïve" (Th0) and do not perform any effector functions unless activated or "polarized" by the signals provided by APC, particularly DC. These signals include the presentation of antigen in MHC class II molecules -which is recognized by their T cell receptor (TCR)-, the expression of cytokines, and the ligation of costimulatory molecules.

1.2.3.1 The Th1/Th2 model in cutaneous leishmaniasis

The murine model of cutaneous leishmaniasis allowed the confirmation *in vivo* of the presence of two different subsets of $CD4^+$T cells that differed in their cytokine profile, Th1 and Th2, and documented their importance for the resistance and susceptibility to infection, respectively.[73,201] This model grounded the basis for the Th1/Th2 paradigm, in which susceptibility to intracellular infection was originated by an IL-4 driven Th2 response. This Th2 response counteracts with a protective Th1 response, thus resulting in susceptibility.[201] Although the basic premise of this model is still valid, the use of different transgenic mice and the discovery of more subsets of $CD4^+$T cells -namely Th17, Th9, follicular helper cells, and regulatory T cells- have questioned its apparent simplicity.[75]

Fig.1.10 represents a model proposed by Alexander and Brombacher, integrating the involvement of all these new players with the previous Th1/Th2 paradigm.[75] An important feature is that protective immune response depends on Th1 immunity, and this applies to infections with *Leishmania* spp. causing cutaneous leishmaniasis and visceral leishmaniasis.[75,142] Furthermore, it is still recognized that IL-12 is the key cytokine driving the differentiation and proliferation of Th1 cells, and it is produced by APC. Th1 responses can be further enhanced by other inflammatory cytokines, including IL-1β, IL-6, IL-18, and IL-17,[75,202–205] and particularly, the IFN-γ-mediated killing of amastigotes by macrophages can be further augmented by TNF-α and migration inhibition factor (MIF).[75,206,207]

An infection model with a virulent *L. major* strain that causes non-curing infection in C57BL/6 has been reported. These mice surprisingly presented IFN-γ production, but the Th1 population responsible for this IFN-γ expression produced as well considerable levels of IL-10.[75,208,209] These cells are represented in Fig.1.10 as Type-1 regulatory cells (Tr1; $CD4^+CD25^+FoxP3^-$).[208] Th17 cells have also been proposed to contribute with increased pathologies, as it has been observed that they cause an influx into lesions.[75] In contrast, the cytokines produced from Th2 and Th9 populations induce alternative macrophage activation, which is characterized by arginase expression and the proliferation

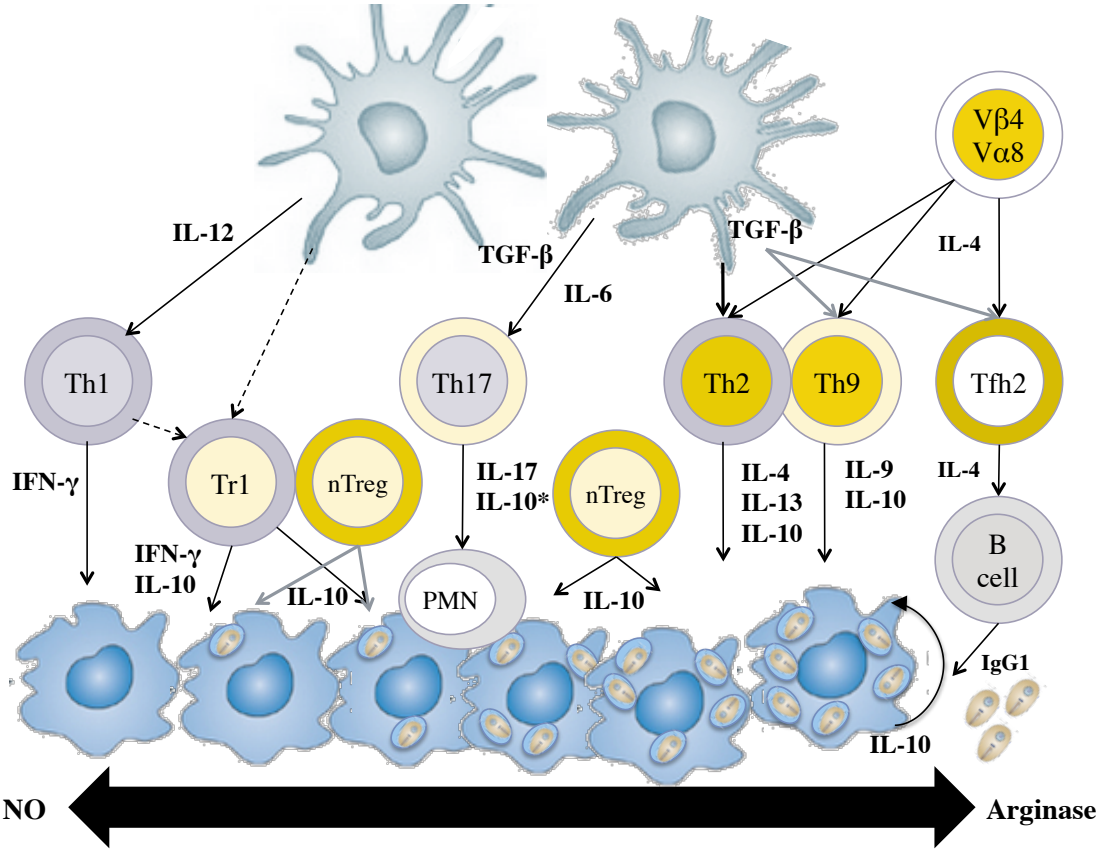

Figure 1.10. **Impact of different T cell populations on the outcome of cutaneous leishmaniasis**. In the *L. major* infection model, Th1 polarization mediated by IL-12 leads to the production of IFN-γ, which results in macrophage activation, production of NO, and parasite elimination. In the case of latent infection, or use of more virulent strains, the persistence of infection is associated with a type-1 regulatory T cells (Tr1), which produce IFN-γ and IL-10. If IL-4 is absent at early time points, but IL-10 is present, the polarization of Th0 cells to other populations except Th1 is observed. TGF-β and IL-6 induce the polarization of Th17 cells producing IL-17 and (*) IL-10, which cause a neutrophil influx associated with pathology. The production of IL-4 by a Vβ4Vα8CD4$^+$T cell population that recognizes LACK antigen from the parasites is associated with Th2 and Th9 polarization. The cytokines produced by Th2, Th9, and natural regulatory T cells (nTregs) result in alternative macrophage activation, arginase expression, and parasite proliferation. It also induces B cell expansion mediated by follicular Th2 cells (Tfh2), and production of IgG1. The uptake of amastigotes opsonized with IgG1 leads to the production of IL-10 in macrophages, and further promotes the progression of the disease. Adapted from J. Alexander and F. Brombacher.[75]

of the parasites.[210]

Launois and colleagues demonstrated in BALB/c mice that the IL-4 produced by the Th2 response developed could be traced back to CD4$^+$T cells expressing the Vβ4 and the Vα8 TCR chains which recognized the antigen *Leishmania* homolog of receptors for activated kinase (LACK).[75,211] Treatment of BALB/c with neutralizing anti-IL-4 Ab during the early stage of infection was reported to direct the immune response towards Th1.[212] It was initially thought that Th2 cells depended on IL-4 for their polarization, as IL-4 activates STAT6, which in turn induces GATA 3, known as the master regulator of Th2 differentiation.[75] However, later studies showed that GATA3 could be also be induced independently of STAT6 activation.[75,213–215] Furthermore, an early IL-4 response has

been detected as well in mice resistant to *L. major* infection, although it was observed to be transient.[65,216–218] Studies using IL-4-deficient and IL-4Rα-deficient mice have put into question the role of IL-4/IL-13, with contradictory results. While IL-4-deficient BALB/c mice were reported to cure from infection,[77,78] other studies have reported that IL-4 and IL-13 in certain experimental conditions were actually able to facilitate a Th1 response by promoting IL-12 production in DC and macrophages.[75,219–222] It has been proposed that an important factor for this apparent contradicting roles of IL-4 and IL-13 is the variation of the expression of IL-4R (which also signals in response to IL-13) in different target cells of the immune system.[75]

In contrast to IL-4 and IL-13, the importance of IL-10 in parasite persistence has been clearly defined. Strong evidence indicating that IL-10 plays a key role in the persistence of infection, since sterile cure has been observed in BALB/c mice treated with anti-IL-10 receptor Ab,[223] which was also confirmed in IL-10 deficient BALB/c mice.[224] Surprisingly, after sterile cure these mice are no longer immune to *L. major* reinfection. Macrophages have been observed to produce IL-10 in response to uptake of *L. major* amastigotes opsonized with IgG1 Ab via Fcγ receptors.[224] The induction of IgG1 production by B cells has been vinculated to follicular Th cells.[75] Furthermore, different $CD4^+$T cell populations have been found to produce IL-10, including: $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs),[225]and the $CD4^+CD25^+FoxP3^-$ Th1 population described above.[208]

The susceptibility of BALB/c mice to *L. major* has been related to deficient CXCR3 up-regulation in their Th1 cells, which prevents them to reach the site of infection,[226] a similar effect also observed in their $CD8^+$ T cells. The role of $CD8^+$T cells in cutaneous leishmaniasis has not been as extensively characterized as for $CD4^+$T cells.[227] Initial studies reported that $CD8^+$T were dispensable for control of a primary infection, although they contributed in resistance to a reinfection.[228] However, a different experimental infection setup, using low doses of parasites, indicated that $CD8^+$T cells were required for control of a primary infection.[229] In contrast, infection models of *L. braziliensis* and *L. donovani*, suggest that $CD8^+$T cells are involved in lesion progression and chronicity of infection.[227]

1.2.3.2 Th1 and Th2 responses in human patients of leishmaniasis

In terms of the adaptive immune response elicited, human visceral leishmaniasis is associated with mixed Th1 and Th2 responses. In *L. infantum* cases, absence of lymphocyte proliferation and production of IFN-γ is associated with progression of the infection. Cure following treatment is concomitant to increased levels of IFN-γ and IL-12, and to decreased levels of IL-10 and TGF-β. High serum concentrations of IL-10 during visceral

leishmaniasis is correlated with PKDL.[1] In the case of PKDL, considerable infiltration of macrophages and DC is present. IL-10 is the predominant cytokine present in the lesions.[1] On the other hand, a wide profile of different Th1 and Th2 cytokines are found in localized cutaneous leishmaniasis lesions. The majority of infiltrating cells are macrophages, B cells, and $CD4^+$ and $CD8^+$ lymphocytes, which produce IFN-γ and TNF-α. IL-10 and IL-13 are associated to chronic lesions.[1] Similarly, mucocutaneous leishmaniasis is characterized by a huge proliferation of lymphocytes, and mixed Th1 and Th2 cytokine responses. IFN-γ producing $CD4^+$ and $CD8^+$T cells can be found in biopsy samples of mucosal regions, as well as a reduced expression of IL-10 and IL-10 receptor in comparison to cutaneous leishmaniasis.[1] Th1 polarization is not necessarily enough for elimination of the infection.This phenomenon has been attributed to the activation of regulatory T cells.[209,223,230,231]

Altogether, Th1 and Th2 immune responses are essential for the resistance and susceptibility of mice to cutaneous leishmaniasis, respectively. The original paradigm of Th1/Th2 polarization in *L. major* has been expanded, incorporating studies on the involvement of newly described Th cell subsets and the cytokines they produce. The main principles of this model include the need of IFN-γ mediated activation of macrophages, which is characteristic of a Th1 response, and the roles of Th2 cytokines on parasite persistence. Although the immune responses observed in humans is not as clear-cut as observed in the BALB/c *versus* C57BL/6 *L. major* infection model, it is still a useful tool that has allowed to investigate the contribution of different components of T cell mediated immunity, and a better insight to prospective immunotherapeutic approaches.

CHAPTER 2

Introduction: Cysteine cathepsins

Cathepsin B (Ctsb; EC 3.4.22.1) and cathepsin L (Ctsl; EC 3.4.22.15) are proteases belonging to the class CA of cysteine peptidases, of the C1 family of enzymes similar to papain. Cysteine proteases are expressed by all organisms, from bacteria to mammals.[232,233] The name "cathepsin" is used to refer to intracellular proteases, which are active at weakly acid pH values.[233] Thus, most of them are located in the lysosomes, where they play a key role in protein degradation. This chapter summarizes the biochemical properties of Ctsb and Ctsl, as well as the role they play in numerous physiological processes, including immune responses. Furthermore, their regulation from transcription to activity is discussed, as well as the relevance of cathepsin inhibitors in cutaneous leishmaniasis.

2.1 Activity and structure of cysteine cathepsins

The term cysteine protease comes from the presence of cysteine residues in their active site. In Ctsb, the cysteine residue C29 interacts with the histidine residue H199 to catalyze peptide bond cleavage, which is mediated by nucleophilic attack by S- from C29 on the carbonyl carbon atom, and proton donation from H199.[234] Most cysteine cathepsins present predominantly endopeptidase activity.[235] Ctsb is able to exert endopeptidase and exopeptidase activity. This particular dual activity results from the presence of an occluding loop.[236] Ctsl, in contrast, lacks exopeptidase activity.[233] In environments with an acidic pH, the active site of Ctsb is partially blocked by the occluding loop, which allows access to small substrates or the carboxy terminus of proteins, and thus Ctsb presents exopeptidase activity. When the pH is increased to neutral levels, the occluding loop is displaced, and the active site can now accommodate larger substrates.[237] Thus,

at neutral pH, Ctsb can function as an endopeptidase.

A total of 11 human cysteine cathepsins have been described: B, C, F, H, L, K, O, S, V, X, and W.[238] In human tissues, cathepsins B, H, L, C, X, F, and O appear expressed ubiquitously, while cathepsins K, S, V and W present predominance for specific tissues or cell types. Cathepsin S (Ctss) is predominantly expressed in APC.[239,240] Cathepsin V (Ctsv) is highly homologous to Ctsl, but its expression is restricted to the thymus and testis.[241] Out of these 11 cathepsins, only the 3-D structure of cathepsins O and W are still unknown. Mature Ctsb can be found as a glycoprotein in two forms: a single-chain form of 29 kDa, and a processed two-chain form consisting of a 25 kDa and 4 kDa chains.[242] It is described as a bilobal protein, in which the active site and substrate-binding cleft are located between the two lobes.[243]

Similarly, Ctsl is composed of two domains (L-domain and R-domain), which form an interface opened at the top, where the active-site cleft is located. The reactive Cys and His residues are located at the center of the active-site cleft, each one of them coming from a different domain. All together, the residues from four loops contained on both domains integrate the active site, where the substrate binds in an extended conformation.[235] 3-D based sequence alignment of the mature forms of cathepsins B, C, F, H, L, K, S, V, and X exhibits conservation of the active site residues and of the residues interacting with the main chain of the bound substrate.[235] A common feature of the 3-D structures of most cysteine cathepsins is the presence of three defined substrate-binding sites, named S2, S1, and S1'. The S2 site forms a pocket, while S1 and S1' represent side-chain interactions.[235] A schematic representation of Ctsb and Ctsl structure can be found in Fig. 2.1. Ctsb and Ctsl are synthesized as an inactive precursor protein, and require removal of their N-terminal prodomain for activation. Different studies have documented the inhibitory activity of this propeptide against mature cathepsins.[244,245] The prodomain serves also for stabilization. In procathepsin B, the propeptide interacts with the occluding loop region, which is lifted away from the enzyme surface. This conformation provides structural support, and shields the active site.[246]

The following sections summarize the known substrates of Ctsb and Ctsl. Furthermore, a roadmap from *ctsb* and *ctsl* genes to the synthesis, processing and trafficking of mature active Ctsb and Ctsl to different cellular locations is presented. Finally, the regulation of cathepsin activity by endogenous and synthetic inhibitors is discussed.

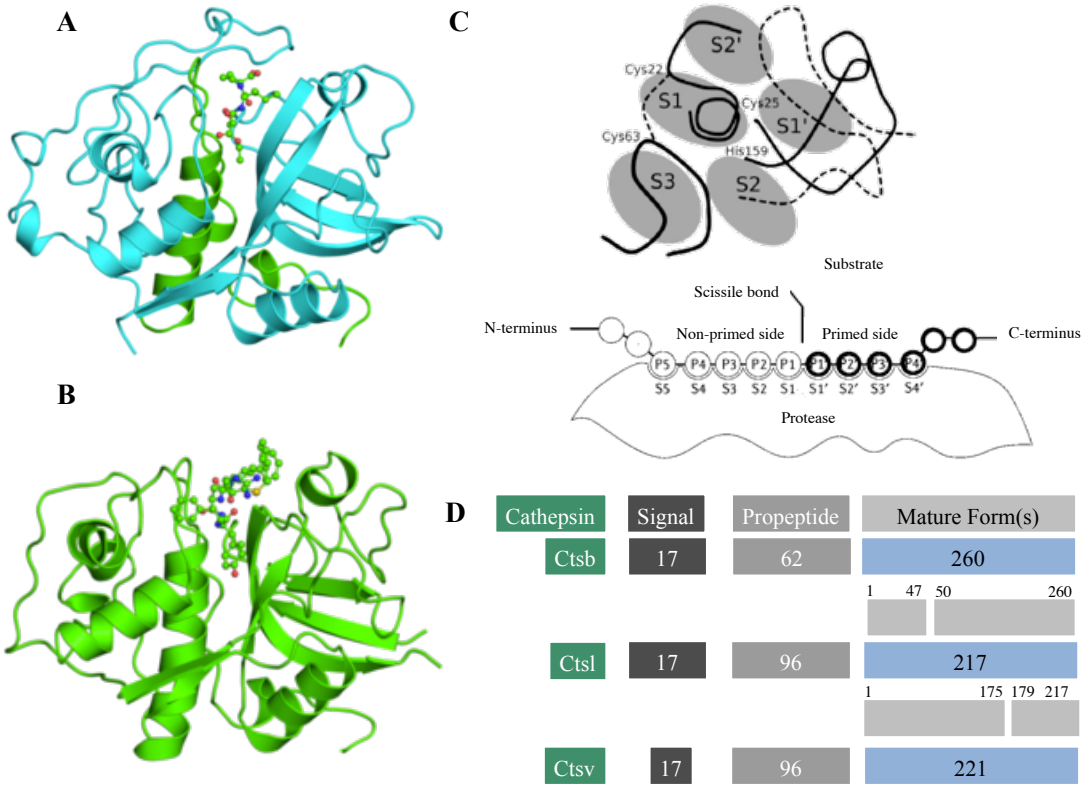

Figure 2.1. **Structure of cysteine cathepsins**. A) 3-D model of the quaternary structure of human Ctsb, consisting of light and heavy chain, in complex with the inhibitor CA030. B) Quaternary structure of human Ctsl, consisting of light and heavy chain, in complex with an inhibitor. C) Schematic representation of S3, S2, S1, S1', and S2´ sites in cysteine proteases, with reference to the 3-D structure of the cathepsin itself (1; adapted from Turk and colleagues [235]), or with reference to the substrate (2; adapted from Otto and Schirmeister [233]). D) Schematic representation of pre-procathepsins. Pre-procathepsins are an immature of cathepsins, containing still a signal peptide (17 amino acids for Ctsb) to target them to Golgi for additional modifications, a propeptide, that conveys protection and stabilizes the structure until arrival to the lysosomes, and the sequence corresponding to the mature forms (adapted from Aggarwal and colleagues [243])

2.2 Cathepsin gene expression

Qian and colleagues first cloned and characterized the *ctsb* gene and its flanking regions. They postulated that the lysosomal cysteine protease genes are evolutionary ancient, and that intron shifting had occurred as they diverted from a common ancestral form. Furthermore, they described the presence of several potential specificity protein 1 (Sp1) binding sites, and, all together, concluded that the characteristics from this region resembled those present in "house-keeping genes", [247] which would explain the constitutive expression of this protease. Currently it is known that the 5' flanking region of *ctsb* gene contains six Sp1-binding sites, four Ets-binding sites, and one enhancer box (E-box) for transcriptional regulation. [243] Up-stream stimulatory factor 1 (USF1) and USF2 have been shown to bind to the E-box of ctsb gene, thus enhancing its expression, while the alternatively spliced form of USF2, USF2c acts as a repressor. [248] Studies of Ctsb from *Sarcophraga peregrina* by Yano and colleagues revealed that the 3'-untranslated region (3'-UTR) of the ctsb gene was necessary for repression of its translation, and identified a

ctsb 3'-UTR-binding protein (CBBP) as responsible for this repression. Furthermore, they documented that purified CBBP represses *ctsb* mRNA translation in rabbit reticulocyte lysate.[249]

Although pre-procathepsin B is synthesized from a single gene, there is more than one mRNA species for Ctsb. These transcripts vary in size depending on the length of the untranslated regions[234] and constitute a form of Ctsb regulation at the post-transcriptional level. Evidence of different transcripts for Ctsb can be found in reports from Moin and colleagues, who analyzed the relative amounts of mRNA for Ctsb in murine tumors, namely hepatoma and B16 melanoma. They found higher levels of Ctsb transcripts, and these higher levels were reflected in higher Ctsb activity.[250] Qian and colleagues confirmed the up-regulation of Ctsb at the transcriptional level in B16a melanoma cells, and described the up-regulation of three different transcripts.[251] They analyzed the cDNA sequences corresponding to these three different transcripts, and found that while the three contain identical coding sequences for normal pre-procathepsin B, two of them presented unusually long extended 3´ untranslated regions. Later studies performed by Gong and colleagues, comparing Ctsb mRNAs from human kidney and human cancer tissues, showed that theses transcripts correspond to alternate splicing from a single gene.[252] In rheumatoid synovial tissue, Lemaire and colleagues found alternative splicing of the 5'region of ctsb pre-messenger RNA.[253] Up to date, 35 splice variants and 27 protein products have been identified.[243]

Differentiation of human promonocytes (cell line U937) by 12,0,tetradecanoyl-phorbol-13-acetate (TPA) or GM-CSF was reported to be accompanied by an increase of Ctsb activity.[254] This observation was later supported by the study performed by Krause and colleagues, who screened genetic markers of human monocyte to macrophage differentiation, and found an up-regulation of *ctsb*, among other genes.[255] Multiple stimuli have been reported to influence the expression of Ctsb and Ctsl. Table 2.1 enlists different stimuli shown to up- or down-regulate the expression, activity or both, of these cathepsins.

2.3 Cathepsin synthesis and maturation

2.3.1 Cathepsin synthesis

Ctsb is synthesized at the rough endoplasmic reticulum as a pre-proenzyme consisting of 339 amino acids, out of which 17 are a signal peptide that directs it to the lumen. Once in the lumen, the signal peptide is removed, resulting in a 43-46 kDa precursor form or procathepsin B.[243] This proform is transported to the Golgi apparatus where it is

Table 2.1. Stimuli influencing Ctsb and Ctsl expression and activity; n.a., data non available; —, no effect detected.

Stimulus	Level at which regulation was observed			Cell type or tissue	Reference
	Gene expression	Mature protein	Activity		
Estradiol-17β	n.a.	n.a.	increase	rat preputial gland	[256]
Interferon-α	n.a.	n.a.	decrease	human blood monocytes	[257]
Interferon-β	n.a.	n.a.	decrease	cell line U937	[257]
Interferon-γ	n.a.	n.a.	decrease	rabbit articular chondrocytes	[257]
	increase*	n.a.	increase*	THP-1 cells	[258] [259] [260]
	increase	increase	increase	U937 and macrophages	[261]
LPS	increase	n.a.	increase	THP-1 cells	[259]
D-glucose	—	increase	increase	fibroblasts kBALB	[262]
IL-1 β	—	—	—	human lung epithelial cell line	[263]
IL-10	—	—	—		
HGF	—	—	—		
IL-6	increase	increase	increase		
TGF-β	decrease	decrease	—		

(*) The cells used for this study were previously primed with phorbol 12-myristate 13-acetate (PMA)

glycosylated with oligosaccharides containing mannose residues, which are important for the trafficking of cathepsins to endosomes.

The first studies on the kinetics of Ctsb synthesis used pulse-chasing experiments in culture rat hepatocytes in the presence of [^{35}S] methionine. It was found that after short time of labeling, a glycosylated 39 kDa pro-cathepsin B, which was then converted to a 29-kDa form by limited proteolysis. A latent form of pro-cathepsin B was also found by immunoblotting in microsomal lumen.[242] Since it was found that the conversion of pro-cathepsin B to the mature form was blocked by pepstatin, the authors postulated that cathepsin D would act as a processing protease for pro-cathepsin B, and that this processing would take place within lysosomes. This last hypothesis was tested by labeling living rats with [^{35}S] methionine *in vivo*, and it was found that processing of a 39 kDa pro-cathepsin B to a 29 kDa mature cathepsin B indeed took place at the lysosomes. A two-chain form consisting of a 25 kDa subunit and a 4 kDa subunit was also detected. The authors described the kinetics for cathepsin B synthesis and processing as follows: Within 10 min after injection of [^{35}S] methionine, a newly synthesized pro-cathepsin B (39 kDa) was detected in rough microsomal fraction, and almost simultaneously in the smooth microsomal fraction. This form was sensitive to endoglycosidase H treatment, which indicated that the proenzyme is glycosylated. 30 min after injection, pro-cathepsin B was detected in Golgi subfractions. 60 min. after injection, a mature single form of 29 kDa appeared in lysosomal fractions, and a two chain form of 25 kDa.[242]

Similar synthesis kinetics were found in rat peritoneal macrophages by Kominami and

colleagues.[264] In this study, the authors used a similar pulse and chase approach with [^{35} S] methionine, in which they immunoprecipitated cathepsins B, L, and H from lysate preparation and medium. The kinetics for synthesis of a 39 kDa pro-cathepsin B precursor and subsequent processing to a single-chain form of 29 kDa followed comparable kinetics as described by Nishimura and colleagues,[264] and the appearance of the two chain form took place over a period of 21 h. Moreover, after 30 min of chase the pro-cathepsin B form was also found in the culture medium, and the authors estimated that about 30% of the labeled Ctsb was secreted by the macrophages. The authors found similar kinetics for the synthesis of the pro-cathepsin L, although the single-chain form of Ctsl was only found transiently. Moreover, approximately 30% of the labeled Ctsl was found released to the culture medium.

2.3.2 Cathepsin trafficking

The targeting of newly synthesized lysosomal enzymes in mammalian cells depends on the presence of mannose-6-phosphate (M6P) residues in their oligosaccharide chains, and their recognition by M6P receptors. The phosphorylated pro-cathepsin B and pro-cathepsin L bind to M6P receptors, which mediate their transport to lysosomes by means of transport vesicles. Evidence for M6P receptor-dependent cathepsin trafficking was found in a study with retinoic acid. Retinoic acid (RA) was known to bind to the mannose-6-phosphate/insulin-like growth factor II receptor (M6P/IGF2R), and it was found that RA induced a shift of proteins, including Ctsb, from the perinuclear area by cytoplasmic vesicles. This translocation of Ctsb was not observed in M6P/IGF2R-deficient cells.[265] A later study by Takana and colleagues suggested that about half of the newly synthesized Ctsb was targeted to lysosomes in an M6P-independent mechanism;[266] however, this study was performed in hepatocytes, a cell type described to present a M6P-independent pathway for lysosomal sorting.[267]

2.3.3 Cathepsins in endosomes and lysosomes

Pro-cathepsins B and L are inactive, as their propeptide functions as an inhibitor. Different studies have documented the inhibitory activity of this propeptide against mature cathepsins.[244,245] Once they reach the acidic environment of lysosomes, the low pH triggers the proteolytic removal of the propeptide. This proteolysis occurs as an autocatalytic process.[268,269] In some circumstances, Ctsb can alternatively be activated by the aspartic protease cathepsin D, by cathepsin G, urokinase-type plasminogen activator (uPAR), tissue-type plasminogen activator, and elastase.[270–272]

Claus and colleagues characterized the steady state presence of cathepsins in J774 macrophage early endosomes, late endosomes and lysosomes enclosing latex beads. They documented higher levels of Ctsb activity in lysosomes than in early and late endosomes. In contrast, Ctsh was highly enriched in early endosomes, and Ctss activity levels were higher in late endosomes than in lysosomes and early endosomes.[273] Furthermore, they found in the same study that treatment with chloroquine or bafilomycin A1 led to complete loss of Ctsb and Ctsl activity,[273] due to the capacity of chloroquine and bafilomycin A1 to increase lysosomal pH.[274,275] Later experiments from Muno and colleagues suggested that Ctsb and Ctsl were located in different lysosomal subpopulations, defined by their density in a Percoll gradient.[276] Although the majority of cathepsin activity in resting DC has been found restricted to lysosomes, Lautwein and colleagues have shown that cathepsins selectively accumulate in late endosomes after LPS-induced stimulation.[277] Similarly, stimulation with IFN-γ has also been reported to induce a shift in the pattern of cathepsin activity in endosomes vs. lysosomes.[278]

Within the endosomes and lysosomes, cathepsins are responsible for different important functions. One of them is the proteolytic processing of endocytosed material, which can be in turn used to generate peptides to be presented to induce an immune response in APC. Furthermore, they contribute to process the MHC class II molecules-invariant chain complexes, to allow the presentation of antigens. In addition, they also play a key role in the processing of endosome-associated Toll-like receptor (TLR), including TLR3, TLR7 and TLR9. The contributions of cysteine cathepsins in these processes is discussed in detail in Section 2.6.

2.4 Beyond the lysosome

2.4.1 Cathepsins in the nucleus and association with histones

Szengo and colleagues documented evidence for translocation of minute amounts of Ctsb in rat preputial gland to the nucleus in response to hormonal stimulation. The authors used fluorometric assays with different synthetic substrates and a combination of the inhibitors leupeptin, antipain and pepstatin to identify a purified protease from lysosomal preparations of rat preputial gland as Ctsb. Surprisingly, Ctsb activity was detected not only in lysosomes, but also in ultrapurified nuclei.[256] Ctsb activity was later reported in nuclear preparations from ovaries, from healthy donors and patients with stage III and IV ovarian cancer,[279] although this activity was smaller as the levels detected in mitochondrial, plasma membrane and lysosomal fractions. Furthermore, Tedelind and colleagues documented active variants of Ctsb and Ctsv in the thyroid carcinoma cell

lines KTC-1, HTh7, and HTh74.[280] The authors later tested the trafficking of Ctsb and an active-site mutant counterpart as chimeric proteins fused to the enhanced enhanced-green fluorescent protein (eGFP), and concluded that the trafficking of both chimeric proteins was altered in thyroid carcinoma cells, in comparison with normal, differentiated thyrocytes.[281] A substrate for cysteine cathepsins identified in the cell nucleus is the transcription factor YY1, which regulates cell differentiation and has been reported to be processed by Ctsb.[282]

Active Ctsl variants in the nucleus have also been documented. Goulet and colleagues reported a Ctsl variant lacking the signal peptide, that was able to translocate to the nucleus and proteolytically process the transcription factor CDP/Cux,[283] which is involved in the regulation of cell cycle progression.[284] Later work from Duncan and colleagues showed that nuclear Ctsl proteolytically processes histone H3 during embryonic stem cell differentiation in mice.[285] Furthermore, recent data indicates that the endogenous cysteine protease inhibitor stefin B is also localized in the nucleus, and interacts with nuclear Ctsl,[286] which suggests that stefin B plays a regulatory role in the proteolytic activity of nuclear Ctsl. Similarly, myeloid and erythroid nuclear termination stage-specific protein (MENT), is another serpin shown to interact with chromatin for its condensation. It has been reported that MENT exerts strong inhibition against Ctsv, but not Ctsl, in DNA-rich environments,[287] and that MENT interaction with nuclear papain-like cystene proteases resulted in a block of cell proliferation.[288]

2.4.2 Cell membrane-bound cathepsins

Ctsb has also been found bound to the membrane, as demonstrated in epithelial cells transformed with the ras oncogene,[289] in human breast carcinoma lines,[290] and in B16 melanoma cells.[291] The authors of the later study proposed that this membrane association of Ctsb could play a role in the degradation of the extracellular matrix. Evidence supporting this hypothesis was found in a study using human prostate tumors, in which the mature form of Ctsb was found in cancer cells and in the subjacent extracellular matrix.[292] It has been reported that Ctsb is able to associate with tumor cell surface by binding to the annexin II heterotetramer, which localizes to the caveolae in the cell membrane.[293]

2.4.3 Extracellular functions of cathepsins

The pH for optimal activity of cysteine cathepsins is slightly acidic, and they are mostly unstable at neutral and basic pH. Among them, Ctsl is the most unstable at neutral

pH, although association with a substrate conferred some protection.[294] An exception to this is Ctss, which is able to retain most of its activity at neutral or slightly basic pH. The extracellular environment of tumors is slightly acidic,[295] thus allowing Ctsb to retain its optimal proteolytic capacity.[296] Since the extracellular localization of cysteine cathepsins is concomitant to increased expression or activity, or both, it has been proposed that pH might not the only factor responsible for their activity in the extracellular environment.[235] For example, Herve-Grepinet and colleagues have reported the protective action of extracellular catalases against cathepsin inactivation by hydrogen peroxide.[297]

2.5 Inhibitors of cysteine cathepsins

A crucial element for control of cathepsin activity is the presence of inhibitors. In this section, the different types of endogenous cysteine protease inhibitors are summarized. Next, small molecule inhibitors of cysteine cathepsins are described, with emphasis on those used throughout the present study.

2.5.1 Endogenous cysteine protease inhibitors

Endogenous Cysteine protease inhibitors (CPI) play an important role in the regulation of cathepsin activity. Based on their physiological role, endogenous protease inhibitors can be described as either emergency or regulatory inhibitors.[298] Emergency inhibitors are characterized by a large excess concentration of the inhibitor with respect to their target protease, rapid binding kinetics, and by being separated from their targets. They are meant to target proteases from pathogens, or proteases escaping their usual compartments. Regulatory inhibitors, on the other hand, are co-localized with their target proteases, and can be further divided into threshold-type, buffer-type, and delay-type.[298] Threshold inhibitors are present in low concentration and show rapid binding kinetics. Buffer-type inhibitors bind to their target protease or proteases in a weak manner, and can be easily displaced. An example of buffer-type inhibitors are the propeptides of cathepsins. Delay-type inhibitors are often irreversible, although they present slow kinetics of binding.

Endogenous CPI can be further classified by their structure into the cystatin protein superfamily, which is integrated by the following families: stefins (type 1 cystatins), cystatins (type 2 cystatins) and kininogens (type 3 cystatins).[299] A common feature among CPI is their stability at high temperatures, extreme pH, and their specificity against cysteine proteases.[233] Cystatins and stefins have low molecular weights (11-13kDa) and, with the exception of cystatin C, have no carbohydrate residues.[233] In contrast, kininogens have higher molecular weights, ranging from 50-80 kDa (low molecular weight

Table 2.2. Inhibitory activity of different CPI

Family	Inhibitor	K_i (nM)			
		Ctsb	Ctsl	Cruzipain	Papain
Stefins (Type I cystatin)	Stefin A	8.2	1.3	0.0072	0.019
	Stefin B	73	0.23	0.06	0.12
Cystatins (Type II cystatines)	Cystatin C	0.27	<0.005	0.014	0.00001
	Cystatin D	>1000	18	n.d.	1.2
	Cystatin E/M	32	n.d.	n.d.	0.39
	Cystatin F	>1000	0.31	n.d.	1.1
	Cystatin S	n.d.	n.d.	n.d.	108
	Cystatin SA	n.d.	n.d.	n.d.	0.32
	Cystatin SN	19	n.d.	n.d.	0.016
	Chicken cystatin	1.7	0.019	0.001	0.005
Kininogens	L-kininogen	600	0.017	0.041	0.015
Thyropins	p41 fragment	>1000	0.002	0.058	1.4

kininogens), to circa 120 kDa (high molecular weight kininogens).[233] Stefins are synthesized without signal peptide, and lack disulphide bonds. They are present mainly as intracellular proteins, although they can be also be found in body fluids.[235] Cystatins, on the other hand, contain a signal peptide for their secretion to the extracellular milieu and two highly conserved intra-molecular disulphide bonds.[235] Kininogens possess also characteristics typical of extracellular proteins: they contain a signal peptide, disulfide bridges, and carbohydrate residues.[233] Furthermore, they are precursors of the kinins found predominantly in blood plasma.[235]

A fourth group of CPI has recently been proposed, based on two findings. First, the inhibition of Ctsl by a fragment of the p41 invariant chain (Ii) associated with MHC class II molecules. Second, the discovery of equistatin, a protein from *Actinia equina*,that strongly inhibits Ctsl and papain. Both equistatin and p41 fragment show no homology to cystatins, but do present significant homology to thyroglobulin type-1 domain. Thus, the name thyropins has been proposed for this new group of CPI.[235] Moreover, some serpins, known as inhibitors of serine proteases, can also present inhibitory activity against cystein proteases.[235] Table 5.3 enlists the interactions between selected human CPI against representative cysteine proteases.

Cystatin C is reported to be the most potent inhibitor of Ctsb, Ctsh, Ctsl, and Ctss.[300] It is ubiquitously expressed, although its expression levels vary between different tissue types.[301] Huh and colleagues reported that cystatin C-deficient mice present reduced growth of melanoma lung metastases in comparison to wild-type mice.[302] During the development of DC, it has been reported that the intracellular levels of cystatin C increase during the differentiation of monocytes to immature DC,[303] and decrease upon DC maturation.[304] Furthermore, stimulation of DC with TNF-α induces secretion of

cystatin C.[303] It has been proposed that cystatin C participates in the regulation of antigen presentation in MHC class II molecules in DC, although there is still controversy. On one hand, work from El Sukkari and colleagues with cystatin C-deficient mice showed that the expression levels of MHC class II molecules in DC from these mice was not altered, and neither was its subcellular distribution, nor the formation of peptide-loaded MHC class II complexes in different DC types.[305] Furthermore, the efficiency of antigen presentation in cystatin C-deficient mice was comparable to wild-type mice.[305] However, later work from Kitamura and colleagues reported that overexpression of cystatin C in DC suppressed IL-6-dependent increase of Ctss activity, and in a reduction of MHC class II α-β dimer, Ii, and H2-DM levels in DC.[306] It has been also proposed that cystatin C may play a role in antigen presentation on MHC class I molecules by $CD8^+$ DC,[299] but this hypothesis has not been confirmed experimentally.

Stefin B, also known as cystatin B, has been shown *in vitro* to bind tightly to Ctsh, Ctsl, and Ctss, and to a lesser extent, to Ctsb.[307] While it has been shown that stefin B is up-regulated in the differentiation of macrophages from monocytes,[308] no co-localization or interaction with Ctsb, Ctsl or Ctss could be found.[299] A serial analysis of gene expression (SAGE) study performed by Suzuki and colleagues revealed an up-regulation of stefin B in human monocytes stimulated with LPS.[309] Stefin B-deficient mice have been recently shown to be more sensitive to lethal LPS-induced sepsis, secreting higher amounts of the pro-inflammatory cytokines IL-1β and IL-18.[310] This effect was associated by increased gene expression of caspase-11, and of better activation of caspases 1 and 11.[310] Surprisingly, the authors found that pre-treatment of macrophages with E-64d did not affected IL-1β secretion, and therefore suggested that the inflammasome activation in stefin B-deficient mice was not due to an increase in cathepsin activity.[310] Stefin B has been reported to act as a regulator of cathepsin activity in the cell nucleus, binding to cathepsin F in hepatic stellar cells[311] and to Ctsl in TG98G human glioblastoma cell line.[286]

Some CPI have also been reported to modulate nitric oxide (NO) production in macrophages. Verdot and colleagues documented that the NO production in macrophages stimulated with IFN-γ was further enhanced with the use of chicken cystatin, cystatin C, and stefin B.[312] Treatment with E-64 did not cause any increase of NO production, suggesting that this effect was no dependent on cathepsin activity.[313] In addition to increase NO production, IFN-γ activated macrophages treated with chicken cystatin were shown to release TNF-α and IL-10.[312]

Treatment with recombinant IFN-γ (rIFN-γ) has been proved safe in other models

such as leprosy, cancer and AIDS, but rIFN-γ alone is not enough to induce a Th1 response in BALB/c mice. Since NO is a key effector molecule for antileishmanial activity in macrophages, the effect of cystatins has been tested in infection models with *L. donovani*. Kar and colleagues found *in vivo* that even at subtreshold concentrations of IFN-γ, cystatin could induce Th2 to Th1 phosphorylation levels of JAK1, JAK2, and IRF-1.[314] In agreement with early observations by Verdot and colleagues, the therapeutic effect of cystatin was suggested to be independent from its cathepsin inhibitory capacity. By using synthetic overlapping peptides from cystatin, Mukherjee and colleagues found that the NO-regulatory activity of cystatin was confined to a 10-mer sequence, which corresponds to a different region than the region responsible for cathepsin inhibition.[315]

Cytotoxic T-lymphocyte antigen-2 (CTLA-2) is a Ctsl inhibitor recently identified and characterized.[316,317] The protein sequence of CTLA-2 has been reported to be homologous to the pro-region of mouse Ctsl.[317] The expression of CTLA-2 has been related to immuno-priviledged organs -uterus, brain and retina- and its role in immune response is currently under investigation.[318]

2.5.2 Small-molecule inhibitors

The discovery in 1978 of the epoxyscuccinyl-based inhibitor L-trans-Epoxysuccinyl-5Leu-4-guanidinobutylamide (E-64) by Hanada and colleagues was a crucial milestone in the field of cysteine cathepsins.[319] E-64 is a non-selective inhibitor of cysteine cathepsins and calpain, isolated from culture extract of *Aspergillus japonicus*. Its inhibitory activity results from selectively alkylating the cysteine residue at the active site, and remaining covalently bond to the enzyme.[235] The scaffold of E-64 was used to develop the first specific inhibitors of Ctsb, CA030 and CA074.[320,321] These inhibitors bind to the S1' and S2' of the active-site cleft. Furthermore, derivatives of these inhibitors with increased cell permeability were developed, namely E-64d and CA074Me,[322] as well as the Ctsl inhibitor Katunuma (CLIK)-series of inhibitors for Ctsl.[323] These inhibitors have been extensively used to study the roles of cysteine cathepsins and of Ctsb in different disease models. The mode of inhibition by the epoxysuccinyl group has been a useful concept for the development of cathepsin inhibitors and activity-based probes.[324]

Based on their reactive groups, in addition to epoxysuccinyl-based inhibitors other types of cathepsin inhibitors developed include: aldehydes, semicarbazoles, methyl ketones and trifluoromethyl ketones, α-keto acides, α-keto esters, α-keto amides and diketones, nitriles, halomethyl ketones, diazomethanes, acyloxymethyl ketones, methylsulfonium salts, disulfides, azapeptides, azobenzenes, aziridines, o-acylhydroxamates, azepanone-based,

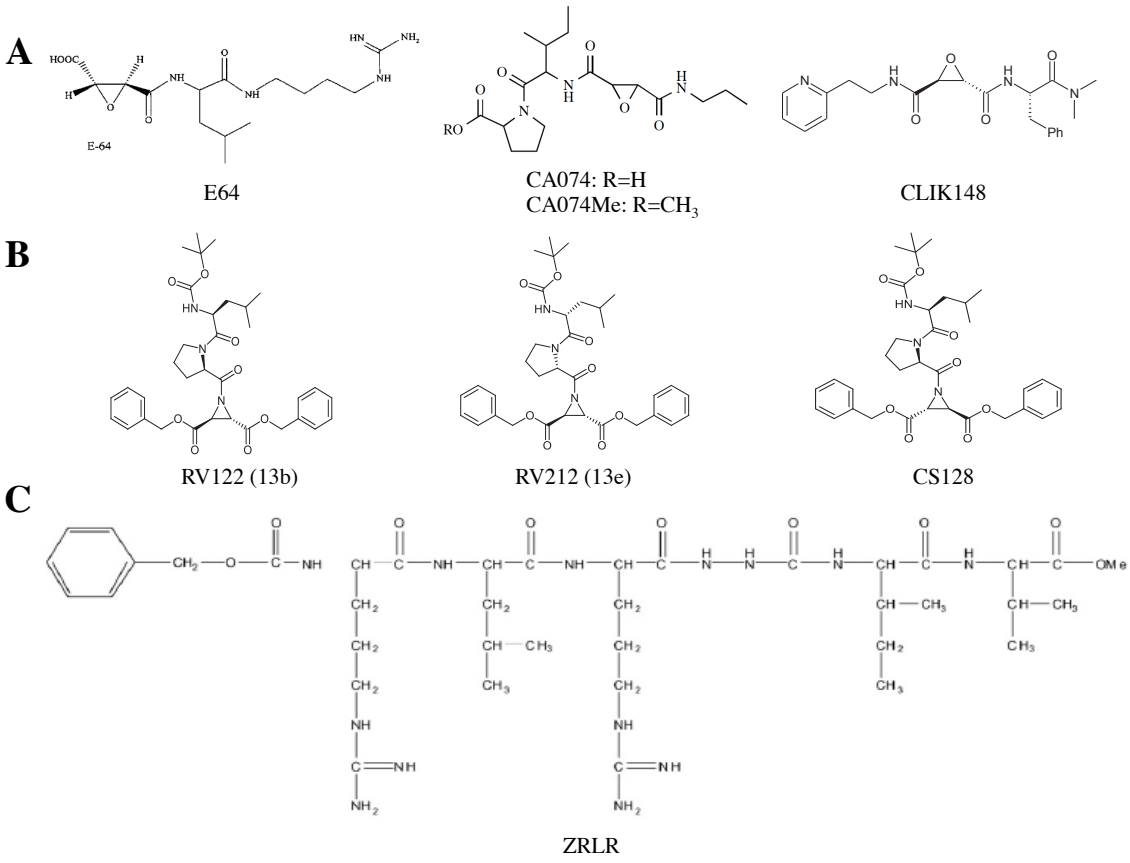

Figure 2.2. **Structure of cathepsin inhibitors**. Schematic representation of the structures of A) the expoxysuccinyl-based inhibitors E-64, CA074, CA074Me, and CLIK148. B) the aziridine-based inhibitors RV122C, RV212C, and CS128; C) and the azapeptide ZRLR.

vinyl sulfones and allylsulfones.[233,235] A common characteristic of most of these inhibitors is that they comprise a peptide segment for recognition by cathepsins, based on the sequence of a good substrate, and that this peptide segment is bound to a group suitable to nucleophilic attack or substitution, to react with the cysteine residue of the active site.[233] An example of a cathepsin inhibitor with anti-parasitic activity is K11777. K11777 is a vinyl sulfone inhibitor of cathepsin L-like proteases, that has been shown to cause reduction in worm burden and parasite egg output in mice infected with *Schistosoma mansoni*.[325] Furthermore, this inhibitor also presents potent activity against *Entamoeba histolytica*,[326] *Trypanosoma cruzi*,[327] *T. brucei*[328] and *L. tropica*.[329]

The inhibitors used in the present study include epoxysuccinyl-based inhibitors, aziridine-based inhibitors and one azapeptide inhibitor. The structures of these inhibitors can be found in Fig. 2.2. The following sections will summarize their main biochemical characteristics and mode of action, as well as their used in parasitic disease models.

2.5.2.1 Azapeptides

One strategy for the design of cathepsin inhibitors has been the synthesis of peptidyl derivatives whose structure is based in on the inhibitory sites of cystatins.[330,331] Unfortunately, they have shown to present poor bioavaiblity due to proteolytic degradation and rapid secretion.[331,332] To circumvent these problems, peptidomimetic approaches have been used, leading to the development of azapeptides. Azapaptides are peptide analogs, characterized by the replacement of one or more of the amino acid residues by a semicarbazide.[333] An α-carbon is replaced by nitrogen,[233] and this results in conformational restrictions that cause the peptide to bend from a linear geometry,[333] as well as a loss in chirality and reduction of flexibility of the linear peptide.[334] This aza-substitution in azapeptides is reported to result in enhanced activity and selectivity, as well as aid to prolong their stability in a biological context[333] as they are less susceptible to breakdown by proteases.[334] Wieczerzak and colleagues have reported an azapeptide with a great inhibitory capacity towards Ctsb (K_i = 480 pM): Z-Arg-Leu-Arg-alpha-aza-glycyl-Ile-Val-OMe (also known as ZRLR).[331] In addition to its high inhibitory capacity, ZRLR was shown to be cell-permeable and to have a higher specificity to Ctsb than the inhibitor CA074Me.[335] Furthermore, it has been reported that inhibition of Ctsb by ZRLR resulted in enhanced presentation of tetanus toxin C-fragment (TTC) to T cells by different APC.[335] These results suggest that ZRLR is a useful inhibitor for study of Ctsb functions.

2.5.2.2 Epoxysyccinyl-based inhibitors

After the discovery of E-64 in *A. japonicus*, systematic studies were performed to determine the role of its different structural components in cathepsin inhibition. As a result, it was determined that the reactive group essential for inhibition was trans-L-(S,S)-epoxysuccinic acid.[233] Analysis of E-64-papain and E-64c-papain complexes revealed that the epoxide residue interacts with the S1 site while the leucil-residue binds to the S2 site.[233,336,337] The inhibition of Ctsb by epoxysuccinyl-(iso)leucyl-proline derivatives -including CA074- demonstrate a different pattern for inhibitor binding, by interacting with the S' sites.[233] A schematic representation of cathepsin inhibition by E-64 and CA074 can be found in Fig. 2.3 . Out of different epoxysuccinyl-(iso)leucyl-proline derivatives synthesized, using different ester and amide substitutes in the epoxide ring, CA074 has been shown to be the most selective inhibitor for Ctsb *in vitro*.[233]

Both E-64 and CA074 present poor cell permeability. Therefore, chemical modifications were developed to improve the permeability of the inhibitors, in order to allow the study of cysteine cathepsins in living cells. One of the first derivatives of E-64 was E-64c, in which the agmatine residue is replaced by an isoamylamide residue.[233] E-64d

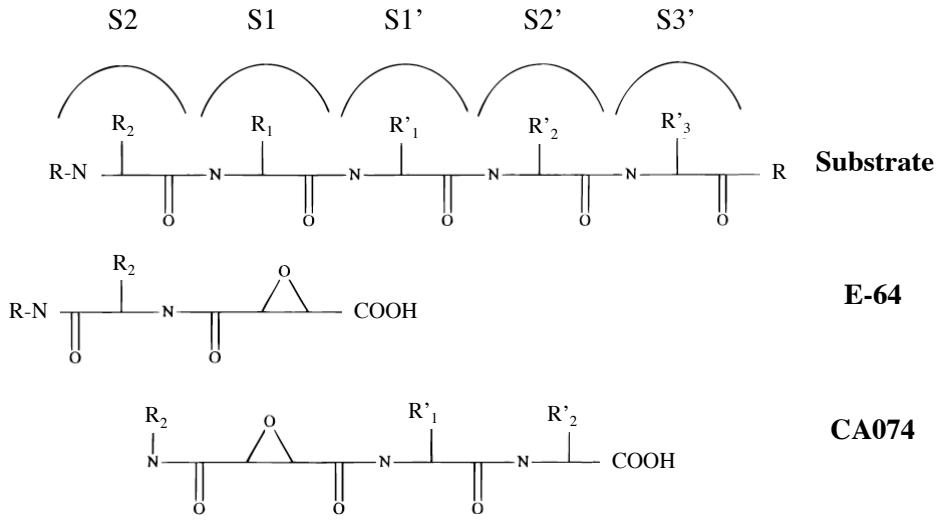

Figure 2.3. **Interaction of substrates and inhibitors with cysteine cathepsins**. Schematic representation of the mode of the sites of binding of substrate or the inhibitors E-64 and CA074 to the active site of cathepsins (adapted from Otto and Schirmeister[233]).

is the ethyl ester of E-64c, and is nowadays widely used due to its great cell permeability. CA074Me is the methyl ester modification of CA074.[322] Upon uptake by the cell, CA074Me is hydrolyzed to CA074 by intracellular esterases.[322] Incomplete hydrolysis causes unspecific inhibition of other cysteine cathepsins.[338] Another important group of inhibitors developed based in the structure of E-64 are the series of Ctsl inhibitor Katunuma (CLIK)-I. The essential common structure from these inhibitors necessary for Ctsl inhibition is N-(L-transcarbamoyloxyrane-2-carbonyl)-phenylalaninde-dimethylamide).[323] Among these inhibitors, CLIK148 showed strong inhibition of Ctsl, and strong resistance to enzymatic degradation.[323] Therefore, it was later used for studies *in vivo*.

CA074 and CLIK148 have been used in the infection model of cutaneous leishmaniasis, showing marked effects in the regulation of the immune response developed by the host. Fig.2.4 summarizes the effects of CA074 and CLIK148 treatment of mice infected with *L. major*. Treatment of BALB/c mice infected with *L. major* with CA074 resulted in control of the parasite infection by inducing a protective Th1 immune response.[339,340] Moreover, treatment of resistant DBA/2 mice and C57BL/6 mice with CA074 showed no alteration in the onset of a Th1 response against the parasite.[341] In contrast, treatment of *L. major*-infected BALB/c mice with CLIK148 resulted in the development of a stronger Th2 response than control mice, which resulted in higher parasitic burden.[342] Similarly, *L. major*-resistant mouse strains also developed a Th2 immune response when treated with CLIK148.[341] Furthermore, in these studies neither CA074 nor CLIK148 showed a direct effect on the proliferation of the parasite *in vitro*, and the authors showed that the host cathepsins were inhibited. It was therefore proposed that the effects of CA074 and CLIK148 observed were due to modulation of the host's Ctsb and Ctsl, respectively, and

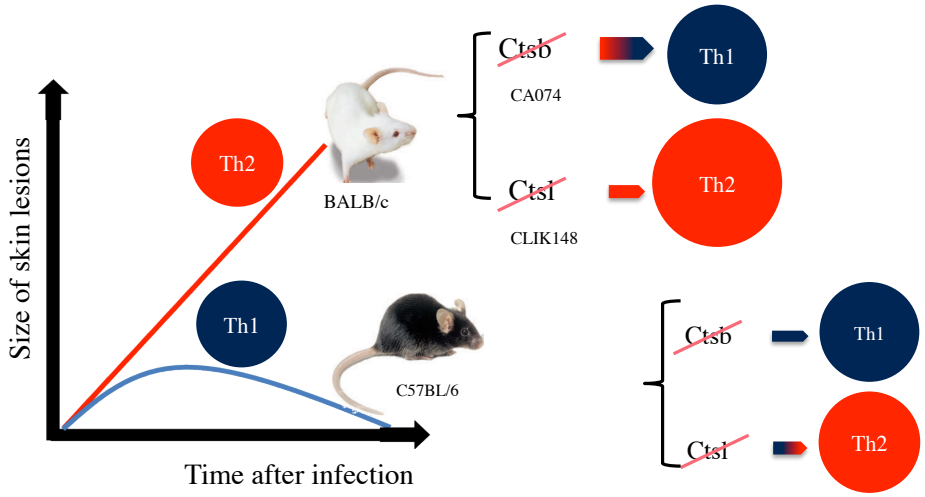

Figure 2.4. **Effect of cathepsin inhibitors in *L. major* infection**. Treatment of mice infected with L. major and treated with the inhibitors CA074 and CLIK148 is associated with a shift in Th polarization. Susceptible BALB/c and resistant DBA2 and C57BL/6 mice develop a Th1 response when treated with the inhibitor of Ctsb CA074. In contrast, these mouse strains are reported to develop a Th2 response when treated with the inhibitor CLIK148.

the authors hypothesized that the lack of Ctsb or Ctsl activity would lead to different patterns for proteolytic processing of the parasite into antigens, thus altering the polarization of naïve Th cells. This hypothesis however was not further investigated, and the roles of Ctsb and Ctsl in Th polarization remained unclear.

2.5.2.3 Aziridine-based inhibitors

(S)-Aziridine-2-carboxylic acid is an irreversible inhibitor of papain.[233,343] Therefore aziridine bound peptides are another strategy for the development of cysteine protease inhibitors.[233] Aziridine-2,3-dicarboxylates are analogous to epoxide derivatives, and irreversible cysteine protease inhibitors. Vicik and colleagues reported the development of different aziridine-2,3-dicarboxylate inhibitors, with the aim to target Ctsl.[344] The authors also tested the inhibitory capacity of these compounds against rhodesain, the main cysteine protease from *Trypanosoma brucei rhosesiense*, and found that some of them presented trypanocidal activities.[345]

These inhibitors were then also tested *in vitro* for leishmanicidal activity against *L. major*. Out of them, two in particular -named 13b (also called RV122C) and 13e (also called RV212C)- caused impaired promastigote growth at mid-micromolar concentrations, while showing no toxicity against fibroblasts, macrophages and dendritic cells. Furthermore both compounds decreased the infection rate of peritoneal macrophages,[346] and in the case of compound 13b, this effect correlated with increased production of NO. Moreover, treatment of peritoneal macrophages with 13b and 13e resulted in higher expression

of IL-12 and TNF-α upon *L. major* infection.[346] When the mechanisms of parasite killing were investigated, it was found that the cell death of *L. major* promastigotes upon treatment with the inhibitors was characterized by cell shrinkage, reduction of mitochondrial transmembrane potential, and increased DNA-fragmentation.[347] Furthermore, undigested debris in lysosome-like organelles was detected, which appeared to participate in authophagy-related processes, differing from typical mammalian apoptosis.[347]

Based on these results, the compound RV122C was tested *in vivo* in BALB/c mice. Despite the leishmanicidal activity of this compound *in vitro*, the tested dosis of RV122C (0.25 mg per day for 6 weeks, intra peritoneal) was not enough to convey protection from *L. major* infection. Furthermore, re-stimulation of splenocytes from infected mice with *L. major* lysate revealed higher levels of IL-4 expression in splenocytes from RV122C-treated mice, in comparison with non-treated controls. These results indicate the induction of a stronger Th2 response, possibly by modulation of the host cathepsin activity.[348] This prompted to the development of a new generation of aziridine-based inhibitors by Schirmeister and colleagues, aiming to higher specificity towards parasitic cathepsins. One of this new generation inhibitors is CS128, which is used for the present study.

2.6 Substrates of cysteine cathepsins

While various proteins have been demonstrated *in vitro* to be degraded by cathepsins, up to date there is still limited data about intracellular physiological substrates of cathepsins.[235] A common feature derived from *in vitro* studies, is that cathepsins present broad specificity, and that they cleave their substrates preferentially after basic or hydrophobic residues.[235,349] While cysteine cathepsins were first believed to be involved only in protein degradation during necrotic and autophagic cell death[235] and intracellular protein turnover,[298] it is now well documented that cystein cathepsins play important role in different physiological processes. Seminal work by Davie and Ratnoff, and by MacFarland and colleagues, resulted in the concept of protein activation by limited proteolysis,[298] and thereby the current concept of protease signaling.[298,350] By definition, protease signaling is irreversible. Moreover, in order to understand the role of a given protease, is necessary to know not only their immediate physiological substrates -the protease degradome[351]-, but also the downstream effects of the proteolysis of those substrates.[298] This section summarizes investigations on substrates of physiological relevance of Ctsb and Ctsl.

The proapoptotic Bcl-2 homolog protein Bid was one of the first intracellular proteins shown to be a substrate of cathepsins. Cathepsin-mediated processing of Bid into a proapoptotic form leads to the release of cytochrome c from mitochondria. Bid has

been found to be efficiently processed by cathepsins B, K, L, and H.[352] Furthermore, cathepsins also degrade several antiapoptotic proteins, including Bcl-2, Bcl-xL, XIAP, and Mcl-1[353] which, in combination with the processed Bid, synergize to drive the cell into apoptosis. Another important target of cysteine proteases is myristoylated alanine-rich C kinase substrate (MARCKS),[354] which is a PKC. MARCKS concentrations within the cell can be regulated by gene transcription, and by proteolytic cleavage by cathepsins and calpains. Increased MARCKS cleavage was observed by Kopitar-Jerala and Turk in brain and macrophages from stefin B-deficient mice, in comparison with wild-type mice.[354] Since the processing of cathepsin B was not altered, the authors concluded that the increased cleavage could be attributed to the lack of cysteine cathepsin inhibition by stefin B.[354]

Another role of cysteine cathepsins is the processing of TLR. One of them is TLR9, which signals in response to DNA. Ewald and colleagues demonstrated recently that the proteolytic processing of TLR9 is a multistep process, first requiring the participation of asparagine endopeptidase (AEP), followed by cathepsin-mediated trimming.[355] Furthermore, the authors showed that TLR7 and TLR3 are processed in a similar manner.[355] Improper proteolytic processing of this receptor would thus result in impaired signaling in response to pathogens.

The thyroid epithelium is one of the few locations where a tissue-specific natural substrate of cysteine cathepsins has been identified.[280] Thyroglobulin is synthesized as a pro-hormone, and requires proteolytic processing by Ctsb, Ctsk, and Ctsl.[356,357] Furthermore, this processing takes place sequentially in different compartments within the thymocyte: the extracellular follicle lumen, and endo-lysosomes. The secretion of mature Ctsb in the follicular lumen is regulated by thyroid stimulating hormone (TSH).[358]

Ctsb-deficient mice present a normal phenotype in comparison to wild-type littermates.[359] In contrast, lack of Ctsl in mice results in a phenotype including defects in the positive selection of T cells in the thymus, epidermal thickening, periodical loss of hair and develop a progressive dilated cardiomyopathy. Ctsl-deficient cardiomyocytes present impaired collagen turnover,[360] as well as an increased number and altered morphology of acidic compartments. These defects have been reported to ultimately result in loss of cytoskeletal proteins and mitochondrial impairment.[361] This phenotype is associated with deficient termination of macroauthophagy process.[362] The periodic hair loss and epidermal thickening of these mice has been attributed to involvement of Ctsl in termination of growth factor signaling in keratinocytes in endosome and lysosomal compartments.[363]

The activity of cathepsins outside endosomes and lysosomes can be found discussed in detail in Section 2.4.

2.7 Cathepsins and antigen presentation

Cysteine proteases contribute greatly to the proteolytic degradation of proteins taken up by endocytosis into antigens, and to process the Ii in MHC class II complexes for antigen presentation. Elucidation of a specific role for each cathepsin has been challenging due to different constrains. On one hand, cysteine cathepsins present high redundancy in the type of substrates they can process. On the other hand, early studies relied on the use of inhibitors, with the disadvantage of potential unspecific inhibition of more than only one cysteine cathepsin. Furthermore, the expression of different cysteine cathepsins is not homogenous in all cell populations.[364–372] Moreover, the absence of one cathepsin may affect the activity of other proteases.[270,373] This section summarizes the relevant studies investigating the participation of different cathepsins in antigen presentation.

Lang and Antoine first reported the localization of MHC class II molecules in murine BMM, in compartments rich in proteolytic activity. They detected MHC class II molecules in the plasma membrane of BMM stimulated with recombinant IFN-γ, as well as on the limiting membrane and internal structures of vesicular acidic compartments. These compartments were identified as early and late endosomes.[374] Furthermore, the authors found that MHC class II molecules within these endosomes co-localized with Ctsb and the aspartic protease Ctsd.[374] The same year, Roche and Cresswell showed that treatment of purified HLA-DR α-β dimers in complex with the Ii with Ctsb resulted in the proteolytic degradation of the HLA-DR-associated-Ii.[375] These results were confirmed by Reyes and colleagues, who showed that Ctsb at pH 5.0 cleaved and released Ii from MHC class II α- and β- chains. This proteolytic release was dose-dependent, and resulted in a different fragmentation pattern than treatment with Ctsd.[376] The involvement of Ctsb and Ctsd in the processing and release of Ii was also supported by experimental data from Daibata and colleagues.[377] By using charge-loss mutations in three putative sites for Ctsb cleavage in Ii, Xu and colleagues proposed a staged cleavage by Ctsb.[378]

Several more studies at the time analyzed the capacity of proteolytic processing of different proteins by Ctsb to generate antigens recognized by T-cells. Van Noort and colleagues analyzed the cleavage of sperm-whale myoglobin within the endocytic pathway of macrophages, and found that Ctsd accounted for most of the initial cleavage of myoglobin, while Ctsb appeared to be involved in the subsequent trimming at the C-terminal of these products. Furthermore, this proteolytic processing resulted in products that contained all major T cell epitopes for myoglobin known at the time.[379] In a different

model, Bushell and colleagues presented evidence for the generation of T cell epitopes from recombinant human growth hormone after proteolytic processing with Ctsb.[380] In addition, the insertion of a Ctsb cleavage sites between determinants recognized by B and T cells in a peptide was shown to enhance its immunogenicity.[381]

Later studies incorporated the use of cathepsin inhibitors. For example, Matsunaga and colleagues used E-64 and CA074 to investigate the involvement of Ctsb in the processing of vaccines for hepatitis type B (HBsAg) and rabies. They found that CA074 suppressed the primary antibody response to HBsAg *in vivo*, as well as an inhibitory effect on the priming of mice and rechallenge *in vitro* with rabies vaccine or the antigenic peptides. Treatment with the Ctsb inhibitor Cbz-Phe-Ala-CHN2 was shown to block the presentation of conalbumin and ovalbumin (OVA), while having no effect in the presentation of an OVA peptide.[382] The presentation of insulin, however, was enhanced when this inhibitor was used.[382]

Mitzuochi and colleagues investigated the capacity of antigen presenting cells to process OVA in the presence of CA074 or the Ctsd inhibitor pepstatin A, and to present it to T helper cell clones. Both inhibitors impaired the IL-2 production using OVA, but no alterations in the presentation of an OVA peptide $_{(327-339)}$, and therefore concluded that both Ctsb and Ctsd are necessary for the processing of OVA.[383] In contrast, in another study using a different OVA-specific Th clone, digestion of OVA with Ctsd produced the antigenic OVA epitope 322-336, while Ctsb and Ctsl failed to generate this epitope and caused its destruction.[384] *In vitro* studies on the proteolytic generation of antigens from egg white lysozyme (HEL) by van Noort and Jacobs suggested that reduced HEL was cleaved by Ctsd, but not by Ctsb; Ctsb was rather active in trimming HEL peptides after their processing by Ctsd.[385]

Later research revealed the essential role of Ctss in the processing of MHC class II-associated Ii. Riese and colleagues showed that inhibition of Ctss with LHVS (morpholinurea-leucine-homphenylalanine-vinylsulfone phenyl) in lymphoblastoid cells prevented complete proteolysis of the Ii, and as a result observed an accumulation of 13 kDa Ii fragment.[386] The authors observed that purified Ctss could digest Ii from α-β Ii trimers to generate α-β-CLIP complexes, while purified Ctsb, Ctsh and Ctsd failed to do so. Moreover, they reported that administration *in vivo* of LHVS caused a diminished immune response to OVA.[387] In contrast, Morton and colleagues showed in B-lymphoblastoid cells that inhibition of cysteine proteases, particularly of Ctsb, resulted in the accumulation of incompletely processed MHC class II-Ii complexes within lysosomal compartments.[388]

A more refined approach became available with the use of APC from cathepsin-deficient mice. Deussing and colleagues showed that the formation of α-β dimers of MHC class II molecules was not altered by the absence of Ctsd or Ctsb.[359] The authors concluded that multiple proteases could probably generate the same antigenic determinants.[359] A year later, Driessen and colleagues reported the control of trafficking and maturation of MHC class II molecules in DC from Ctss-deficient mice.[389] Their results provided more evidence for proteolytic processing of the Ii by Ctss, and documented that lack of Ctss resulted in a drastic reduction of the flow of MHC class II molecules to the cell surface, related to the retention of MHC class II molecules in late endocytic compartments. The same year, Shi and colleagues reported that APC from Ctss-deficient mice failed to process the Ii beyond a 10 kDa peptide fragment. This resulted in turn in delayed peptide loading, and also an accumulation of MHC class II-Ii (10 kDa) complexes in the cell surface.[240] In agreement with this data, Nakagawa and colleagues documented that Ctss-deficient mice presented a marked inhibition of degradation of the Ii in APC, and a diminished susceptibility to collagen-induced arthritis.[390]

A later report from Zhang and colleagues still emphasized the participation of Ctsd in the degradation of Ii.[391] In this study, the authors tested *in vitro* and *in vivo* the processing and presentation of OVA in the presence of the inhibitors pepstatin A and CA074. While a Th1-type immune response was reported with the inhibitor CA074, treatment of mice with pepstatin A resulted in a suppression of OVA-specific lymphocytes, and the development of both Th1 and Th2 responses.[391] Cathepsin E (Ctse), an aspartic protease, was also reported to participate in the processing of exogenous antigen in primary cultured murine microglia.[392]

More studies confirmed the key role of Ctss in antigen presentation. Fiebiger and colleagues showed that TNF-α and IL-1β cause an increase in Ctss and Ctsb activity in human DC, and higher levels of formation of MHC class II dimers in a Ctss-dependent manner.[369] IL-10, in contrast, was reported to impair the capacity of DC to up-regulate Ctss and Ctsb activity, and this suppressed activity coincided with delayed formation of MHC class-II dimers and degradation of antigens.[369] In a different model, Driessen and colleagues analyzed the degradation of an immune complex (Ig-125I-labeled F(ab')2) delivered via FcγR-uptake in APC from mice lacking Ctsb, Ctsd, Ctsl, and Ctss. They found that Ctsd and Ctsl were dispensable for the degradation of this immune complex, while Ctsb and Ctss mediated the major part of its proteolytic processing.[393] In contrast, Plüger and colleagues showed *in vitro* that Ctsb, Ctsd, Ctsl, and Ctss digested HEL with considerable redundancy, although there was evidence for preferential cleavage patterns.[394] Therefore, the authors concluded that while the proteolytic processing presented high redundancy among the different cathepsins, Ctss played in this system an

important and specific role in antigen presentation.

Studies on antigen presentation in Ctsl-deficient mice revealed that while Ctsl was dispensable for degradation of the Ii in bone marrow-derived APC, it was necessary for its degradation in cortical thymic epithelial cells (cTEC). Furthermore, the authors found that the positive selection of $CD4^+$T cells *in vivo* was reduced.[364] As a result, these mice have a markedly reduced number of $CD4^+$ T cells than their wild-type counterparts. Ctsv is a cysteine cathepsin highly homologous to Ctsl, expressed exclusively in human tymus and testis. In cTEC, Ctsv is the dominant cysteine protease expressed, while Ctss and Ctsl is expressed mainly in DC and macrophages.[395] Tolosa and colleagues found that recombinant Ctsv is capable of processing the Ii into CLIP efficiently, and proposed that Ctsv - in analogy to Ctsl in mice- was the protease responsible for the generation of α-β CLIP complexes.[395]

Another protease extensively investigated in the context of antigen processing and presentation is asparagine AEP. Studies from Manoury and colleagues reported that using a peptide inhibitor of AEP resulting in an inhibition of *in vitro* processing of tetanus toxin antigen (TTCF). Moreover, they observed *in vivo* that this inhibitor slowed TTCF presentation to T cells, while preprocessing of TTCF with AEP accelerated its presentation.[396] Furthermore, it was later suggested that AEP can initiate the removal of MHC class II Ii.[397] It was proposed that AEP would have an "unlocking function", necessary for the further processing by AEP itself or other cysteine proteases.[398] However, it was later shown that AEP-deficient mice presented no differences in comparison to wild-type mice in processing of the Ii, in maturation of MHC class II molecules, and in the presentation of antigen to T-cells.[399]

Finally, cathepsin G (Ctsg), a serine protease, has also been recently investigated in the context of antigen presentation, as it is found in primary human B cells, DC, and mouse microglia, but not in B cell lines or monocyte-derived DC.[400] While it has been shown that Ctsg plays no relevant role in the degradation of the Ii, it has been associated to the processing of antigens, notably of autoantigens, including myelin basic protein, myelin oligodendrocyte glycoprotein, and pro-insulin.[401–403] However, important variations in substrate specificity between mouse and human Ctsg have been reported, and therefore Ctsg-deficient mice are not suitable for drawing any conclusions on human antigen processing.[401]

In summary, Ctss plays an important role in the proteolytic processing of Ii, as confirmed by different approaches over the past years, while Ctsl - or Ctsv in humans- are key to the processing of antigens for presentation in cTECs, thus allowing positive selection

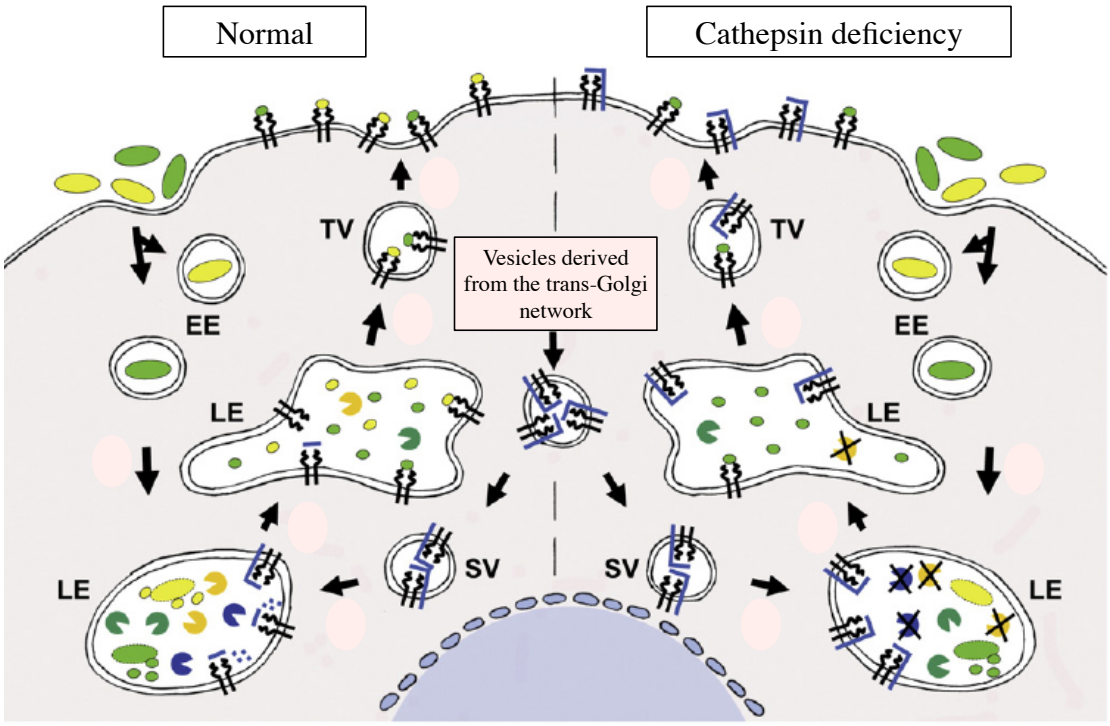

Figure 2.5. **Cysteine cathepsins and antigen processing**. Schematic representation of the processing of endocytosed material under normal conditions and cathepsin-deficiency. EE: early endosomes; LE: late endosomes; SV: sorting vesicles, TV: transport vesicles. The endocytosed material (i.e. a pathogen) is first located within EE, which transport them to the LE compartment. Within LE, the material is proteolyticaly processed into antigens for loading onto MHC class II molecules. The antigen peptide-MHC class II molecule complex is transported to the surface for antigen presentation by means of TV. If Ctss is missing, the invariant chain bound to newly synthetized MHC class II molecules cannot be processed for removal, and thus antigen cannot be presented. Furthermore, missing of one cathepsin could potentially reduce the repertoire of antigens generated for presentation. Adapted from Müller and colleagues. [362]

of T cells. The participation of other cathepsins remains still controversial, and it is likely that rather than each of them having a specific role, the balance in the proteolytic activity of the sum of all of them ultimately determines the relative levels of antigenic peptides generated for presentation. Fig.2.5 represents a model proposed by Müller and colleagues on the participation of cathepsins in antigen presentation. [362]

2.8 Cathepsin-like cysteine proteases from *Leishmania*

Cysteine proteases have been reported as virulence factors of *Leishmania* parasites. Three cysteine proteases of the papain family have been reported in *Leishmania* spp.: two cathepsin L-like, CPA and CPB, and one cathepsin B-like, named CPC. Among them, numerous studies point out CPB as the most critical cysteine protease for parasite survival and virulence. CPB-deficient promatigotes have been shown to be less infective to macrophages in comparison to wild-type promastigotes, as well as presenting a reduced virulence in BALB/c mice, being able to form only small and slow-growing lesions. [404,405]

Studies from Alexander and colleagues reported that mice infected with CPB-deficient *L. mexicana* developed a protective Th1 immune response.[406] The *cpb* genes are multicopy, with 19 copies arranged in a tandem repeat within a single locus.[404,407] In order to completely rescue the virulence of CPB-deficient *L. mexicana*, Denise and colleagues reported that the expression of multiple *cpb* genes was necessary, in contrast to insertion of individual *cpb* genes.[408]

CPB has been shown to regulate IL-4 production. A lack of IL-4 expression was reported in BALB/c mice infected with CPB-deficient *L. mexicana*, leading to the onset of a Th1 response. In contrast, reinsertion of multiple *cpb* genes into the CPB-deficient parasites restored the parasite capacity to induce IL-4.[408] Furthermore, injection of recombinant CPB alone in BALB/c mice paws was shown to cause an increase in IL-4 and IL-5 expression, and in the levels of IgE.[409] In contrast, overexpression of endogenous cysteine protease inhibitors from *Leishmania* resulted in reduced levels of Ab and IL-4 production as well as higher levels of IFN-γ in infected mice than infection with wild-type parasites.[410]

Besides affecting IL-4 expression, *Leishmania* CPB has been also reported to alter IL-12 production in macrophages and DC.[150,411,412] CPB-deficient *L. mexicana* amastigotes were less efficient to inhibit IL-12 in response to LPS than wild-type amastigotes; in concordance with this observation, inhibition of *L. mexicana* CPB also altered the ability of amastigotes to inhibit IL-12 expression in infected macrophages.[150] This data suggested that CPB might affect transcription factors relevant for IL-12 expression in the host. Indeed, Cameron and colleagues reported that the observed regulation of IL-12 expression was related to cleavage of the p65 subunit of NF-κB, which could still translocate to the nucleus, but failed to correctly bind to DNA. Moreover, infection with wild-type amastigotes resulted in cleavage of IκB-α and IκB-β, which was not observed in infection with CPB-deficient amastigotes.[150] Furthermore, infection with wild-type amastigotes resulted in cleavage of JNK and ERK, while pre-incubation with a CPB inhibitor prevented it.[150] In addition CPB from *L. mexicana* has been shown to affect the transcription factors STAT-1 and AP-1 by impeding their translocation to the nucleus.[413]

Similar as in *L. mexicana*, CPB has also been reported to contribute to the virulence of other *Leishmania* species. *L. major* parasites transfected with *cpb*-containing cosmids were shown to cause stronger infection in C3He/FeJ mice than their wild-type counterparts, which was characterized by higher parasitic loads and reduced expression of IFN-γ.[414] Furthermore *L. amazonensis* amastigotes have been shown to internalize and cleave MHC class II proteins inside the parasitophorous vacuoles (PV) of infected host cells[415] and CPB is one of the parasite proteases accounted for degradation of

internalized MHC class II molecules.[416] In addition to the proteolytic activity of CPB, its COOH-terminal extension (CTE) is known to play a role in the modulation of the host immune system. CTE is a polypeptide hydrolyzed during the processing of CPB to its mature form, and the use of a synthetic peptide based on its structure in *L. amazonensis* was reported to cause an increase in lesion growth in BALB/c mice.[417]

In contrast to the observations with CPB-deficient *Leishmania*, CPC-deficient *L. mexicana* promastigotes presented reduced infectivity to macrophages *in vitro*, but showed to induce comparable rate of lesion development as in mice infected with wild-type parasites.[418] The authors found that re-expression of the *cpc* gene restored infectivity *in vitro*, and suggested that although CPC played a role in the interaction of the parasite with its host macrophage, it was not crucial overall for virulence *in vivo*.[418] Similarly, although CPA has been reported to be relevant for host-parasite interaction of *L. infantum* infection, it was not shown to be essential for parasite replication.[419] Studies of infection of BALB/c mice with different CPA-, CPB- or CPC-deficient *L. mexicana*, lack of CPA or CPC affected less the virulence of the parasites than CPB deficiency.[406] Nevertheless, studies of *L. mexicana* parasites lacking both CPA and CPB suggest a synergistic role of these proteases, as they displayed a greater impairment in infectivity in comparison with parasites lacking only CPA or CPB.[420] *L. mexicana* parasites lacking CPA and CPB present an impairment in authophagy, which is believed to be an important step for cell differentiation as these parasites are unable to transform into amastigotes.[421]

In order to modulate its host, *Leishmania* cysteine proteases must be delivered. Brooks and colleagues showed that the major route of trafficking of *Leishmania* cysteine proteases to lysosomes is via the flagellar pocket, and that glycosylation was not required for trafficking.[422] Temperature shift, as occurring between the transfer of promastigotes from the sandfly to the host, has been reported in *L. mexicana* to induce the secretion of CPB, among other immunomodulatory molecules, in exosomes.[423] Moreover, the authors found that the release of these exosomes was taking place already within 4 h of temperature shift, suggesting that this might be a strategy for immune modulation within the early moments of interaction of the parasites with their host.[423] The delivery of immunomodulatory cargo to the host via exosomes has also been documented for *L. donovani*, which presented a direct suppressive effect in human monocyte-derived dendritic cells.[424] Fig. 2.6 presents a model of action of cysteine proteases of *Leishmania* in the modulation of the host's immune response, proposed by Mottram and colleagues.[420]

Altogether, cysteine proteases from *Leishmania* have been shown to play an important role in the parasite survival and virulence. Therefore, they have been extensively

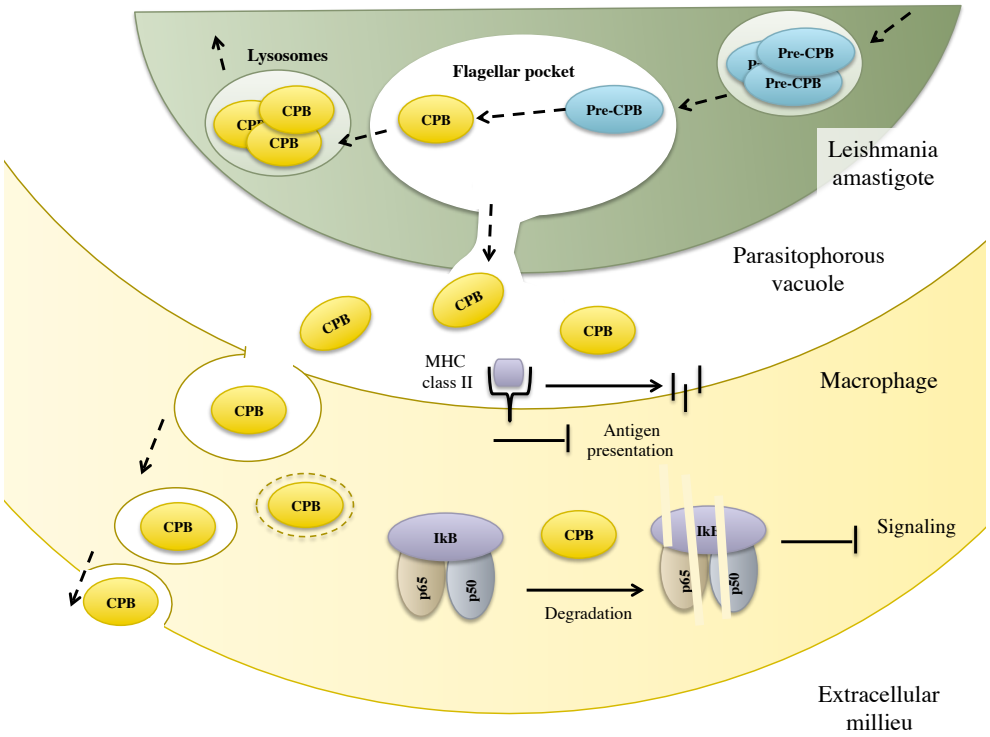

Figure 2.6. **Cysteine protease B-like (CPB) as a virulence factor from *Leishmania*.** Model proposed by Mottram and colleagues, in which the immature form of CPB is trafficked to the flagellar pocket, where it is processed to its mature form. From there, it is proposed to be either sorted to the lysosomes, or secreted towards the PV. In this space, CPB interacts with MHC class II molecules of macrophages, in order to impair the presentation of antigens. It is also proposed that CPB within the parasitophorous vacuole could also reach the macrophage cytoplasm, where it is able to cleave important transcription factors for the immune response of the host, including NF-κB. Finally, the authors propose that CPB could also reach the extracellular space, where it can interact with other components of the host's immune system. Adapted from Mottram and colleagues.[420]

studied as potential targets for drug development. For example, the inhibitor N-Pip-F-hF-VS Phenyl was reported to cause arrest of *L. tropica* promastigote pathogenicity and growth. *L. major* showed to be sensitive to cysteine protease inhibitors in both the promastigote and amastigote stages[425] while *L. mexicana* promastigotes appeared to be resistant to the loss of cysteine protease activity.[329,404] A more detailed description of cysteine protease inhibitors with leishmanicidal activity can be found in section 2.5.

CHAPTER 3

Aims of the study

Leishmania parasites use different strategies to manipulate the immune response of their host. One of them is the use of cysteine proteases. Therefore, cathepsin inhibitors have been investigated as potential drug candidates. Reports of their use *in vivo* suggested that treatment with cathepsin inhibitors resulted in a drastic change in the immune response orchestrated by the mice, either towards Th1 and increased resistance, or towards Th2 and enhanced susceptibility, depending on the cathepsin targeted. Mammals also express cysteine cathepsins, which have been related to different functions, including the processing of endocytosed material and protein turnover. It was demonstrated in those studies that the inhibitors had no leishmanicidal activity *per se*, and that the cathepsins of the mice were also being inhibited.

Considering that in APC cysteine cathepsins are known to participate in the process of antigen presentation, it was proposed that the effects on the immune system observed were due to changes in the pattern of processing of *Leishmania* antigens. This hypothesis, however, remained to be tested. The urge for newer therapeutics to treat leishmaniasis has led to the development of new cysteine protease inhibitors. With them comes the need to understand the effects that they exert not only to the parasites, but also to the host.

This project aims to understand the effect that the lack of Ctsb and Ctsl activity has in the immune response of the host against *L. major*. Given the critical role that DC play in the polarization of $CD4^+$ Th cells into Th1 or Th2 subsets, and thus the control of infection, the aims of this project are:

- To determine the effect that the absence of cathepsin B and cathepsin L activity have on the signals that these cells use to instruct Th cell differentiation during *L.*

major infection: signal 1 (antigen presentation via MHC-class II molecules), signal 2 (expression of co-stimulatory molecules), and signal 3 (cytokine production).

- To investigate the mechanisms behind these effects

In order to address the aforementioned objectives, two approaches for this study were used: cathepsin inhibitors, and APC derived from cathepsin-deficient mice.

CHAPTER 4

Materials and methods

4.1 Mice

The following mice strains were used to generate BMM and bone marrow-derived dendritic cells (BMDC): BALB/c, C57BL/6, C57BL/6 *Ctsb* $^{-/-}$ and C57BL/6 *Ctsl* $^{-/-}$. The *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ mice were kindly provided by Prof. Thomas Reinheckel (University of Freiburg, Germany) together with wild-type littermates, and their generation has been previously described.[359,426,427]

In addition, for *in vitro* co-culture experiments, OT-II mice were used to isolate $CD4^+$ T cells from lymph nodes and spleen. These mice were kindly provided by Prof. Manfred Lutz. All mice were 6 to 12 weeks old, and were kept under conventional conditions in accordance with the guidelines of the local authorities.

4.2 Parasites

4.2.1 Culture of wild-type *L. major*

The *L. major* isolate MHOM/IL/81/FE/BNI was maintained by continuous passage in female BALB/c mice, from whose lesions the parasites were isolated, and grown *in vitro* in blood-agar cultures as described previously[137] at 27 °C, 5% CO_2 and 95% humidity. Only promastigotes passaged 5 to 8 times were used for *in vitro* infection experiments, in order to preserve optimal infectivity levels. This *L. major* isolate was used to generate two different transgenic (tg) strains, used in some of the experiments for the present study. One of these strains expresses luciferase, while the other expresses eGFP. The generation and culture of both strains is described below.

4.2.2 Generation and culture of Luc-tg *L. major*

The generation of the luciferase-transgenic (Luc-tg) strain was previously described.[428] Briefly, the luciferase (Luc) coding region was cut from pGL4.13 (Promega, Mannheim, Germany) by NcoI-XbaI, and the resulting fragment was cloned into the NcoI-NheI-restricted *Leishmania* expression vector pLEXSY-hyg2 (Jena Bioscience, Jena, Germany), which contains a marker gene for selection with hygromycin (HYG). The resulting plasmids were linearized by SwaI (New England Biolabs, Frankfurt, Germany), and the parasites were transfected by electroporation. The genes for Luc and HYG were integrated into the 18S rRNA locus of the parasites by homologous recombination. As described for the wild-type (WT) strain, the virulence of both the Luc-tg and eGFP-tg strains was maintained by passage of the parasites in female BALB/c mice. Luc-tg promastigotes were grown in blood-agar cultures supplemented with 50 μg/ml hygromycin (Sigma-Aldrich, Taufkirchen, Germany), and only promastigotes at *in vitro* passages 5 to 8 were used for the experiments here reported.

4.2.3 Generation and culture of eGFP-tg *L. major*

The generation of the eGFP-tg *L. major* strain here reported was performed by Angela and Tobias Schwarz. Briefly, the eGFP-coding region from EGFP-N1 (Clontech, Saint-Germain-en-Laye, France) by BamHI-NotI (Promega) was cut, and cloned into the Bglll-NotI-restricted *Leishmania* expression vector pLEXSY-hyg2 (Jena Bioscience,). This vector contains a marker gene for selection with hygromycin. The resulting plasmids were linearized by SwaI (New England Biolabs), and the parasites were transfected by electroporation. The genes for eGFP and hygromycin were integrated by homologous recombination into the 18S rRNA locus of the parasites. As described above, the transgenic promastigotes were grown in blood-agar cultures with 50 μg/ml hygromycin, and only *in vitro* passages 5 to 8 were used for experiments. The stability of the integrated eGFP was assessed *in vitro* and *in vivo* by flow cytometry.

4.2.4 Preparation of *L. major* lysate and heat-killed parasites

For preparation of *L. major* soluble antigen (LmAg), stationary-phase WT promastigotes were washed three times in cold phosphate-buffered saline (PBS), counted, resuspended at a concentration of 1×10^9 parasites/ml in PBS, and subjected to three cycles of freezing in liquid nitrogen and thawing in a warm bath for no longer than 1 min. The aliquots were stored at -80 °C, and each aliquot was thawed not more than twice. For preparation of heat-killed parasites, WT promastigotes were harvested, washed three times with in complete RMPI medium, counted, and adjusted to a concentration of 1×10^9 parasites/ml. Next, the parasites were incubated for 30 min at 80 °C, and used for stimulation of BMDC in a ratio equivalent of 5 parasites per BMDC.

4.3 Buffers

All the buffers used in the present study can be found enlisted in Table A.1. The final concentrations of each reagent are included, and those that were added freshly to the buffers prior to each experiment are marked with the symbol (*).

4.4 Culture media and cells

4.4.1 Culture media

RPMI 1640 medium (Invitrogen, Darmstadt, Germany) was used, either containing phenol red or phenol red-free, as indicated for each experiment. This medium was supplemented with heat-inactivatedfetal calf serum (FCS) (10% v/v; PAA Laboratories, Pasching, Austria), L-glutamine (final concentration 2 mM; Biochrom AG, Berlin, Germany), HEPES (pH 7.2, 0.01 M; Invitrogen, Darmstadt, Germany), penicillin G (0.2 U/ml; Sigma-Aldrich), gentamicin (0.05 mg/ml; Sigma-Aldrich), and 2-mercaptoethanol (0.05 mM; Sigma-Aldrich), and hereby will be referred as "complete RPMI medium". For the generation of BMM, a conditioned medium was used containing Dulbecco's Modified Eagles Medium (DMEM) from Invitrogen, heat-inactivated FCS (10% v/v; PAA Laboratories), heat-inactivated horse serum (0.5%; Invitrogen), 2-mercaptoethanol (0.05 mM; Sigma-Aldrich), nonessential amino acids (Invitrogen), HEPES (0.01 M; Invitrogen), L-glutamine (4 mM; Biochrom) and L929 supernatant (15% v/v). *L. major* promastigotes were cultured in a biphasic medium consisting of a solid base of rabbit-blood agar (Elocin-lab, Gladbeck, Germany) and a liquid phase of phenol red-free RPMI medium.

4.4.2 Generation of BMDC

BMDC were generated from bone marrow progenitors following the protocol from Lutz *et al.*[429] Briefly, total bone marrow cells were flushed from femurs and tibiae with a syringe containing complete RPMI medium. The cell suspension was washed and resuspended in fresh RPMI medium, and the cell number was determined by trypan blue staining. The cell suspension was adjusted to a concentration of 0.2×10^6 cells/ml, and seeded in bacteriological petri dishes in a final volume of 10 ml/dish, in the presence of recombinant murine granulocyte-macrophage colony-stimulating factor (GM-CSF, 0.04 μg/ml; Invitrogen). The cultures were incubated at 37 °C, 5% CO_2. On days 3 and 6, 5 ml of complete RPMI medium supplemented with GM-CSF were added to each dish. At day 8, the non-adherent cells were collected, washed with complete RPMI medium and resuspended at 2×10^6 cells/ml in complete RPMI medium.

4.4.3 Generation of BMM

BMM were generated by culturing 0.67×10^6 cells/ml of total bone marrow progenitors as described for BMDC, but using conditioned DMEM instead, at 37 °C and 5% CO_2. On day 6, the culture medium was removed carefully and replaced with cold RPMI complete medium, and the petri dishes were kept on ice for 10 min. Thereafter, the macrophages were removed with a cell scrapper, washed with fresh complete medium without phenol red, and resuspended at 2×10^6 cells/ml. As quality control, the morphology of the obtained BMDC and BMM was analyzed. Part of the cells was used for cytospin preparations stained with Diff-Quik II dye (Medion Diagnostics, Düdingen, Switzerland) according to the manufacturer's instructions, and observed under the light microscope (see section 4.6.1). Furthermore, the expression of the phenotypic markers CD11c in DC and F4/80 in macrophages was assessed by flow cytometry (see section 4.8.2)

4.4.4 Isolation of T cells for polarization assays

Lymph nodes and spleens were removed from OVA-specific TCR-transgenic OT-II mice, and kept in ice-cold complete RPMI medium in 60 mm × 15 mm petri dishes. Lymphocytes were isolated by mechanical dissociation using the sterile plunger of a 5 ml syringe and a cell strainer (70 μm, BD Falcon, Durham, USA), and the obtained cell suspension was kept on ice. Similarly, splenocytes were isolated by mechanical dissociation in a cell strainer. The obtained cell suspension was centrifuged at 300 × *g*, 4 °C for 5 min, and the pellet was incubated with red blood cell lysis buffer for 5 min at 37 °C. The cells were washed with fresh cold medium afterwards. Non-$CD4^+$ cells were depleted using the $CD4^+$T cell enrichment kit (StemCell Technologies, Grenoble, France). $CD25^+$ cells were further depleted, using anti-CD25-Phycoerythrin (PE) and anti-PE magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany). The obtained cell population was used for T cell differentiation assays as described in Section 4.11.1, and its purity was assessed by flow cytometry.

4.4.5 Preparation of cell lysates

For preparation of total cell lysates from BMDC to be used for activity assays, active site labeling, and Western Blot (WB) of cysteine cathepsins, the cells were harvested either directly from the petri dishes where they were generated, or after treatment with different stimuli including: LPS (1 μg/ml, Sigma-Aldrich), CpG ODN 1668 (5´-TCCATGACGTTCCTGATGCT-3´, 25 μg/ml, Qiagen Operon, Cologne, Germany), TNF-α (500 U/ml, Peprotech, Rocky Hill, NJ, USA), or infection 1:5 with *L. major* promastigotes. The cells were washed twice in PBS, and centrifuged at 300 × *g* for 5 min at room temperature (RT) (Heraeus Multifuge X1R, Thermo Scientific). After the last washing step, the cells were resuspended in 5 ml PBS and counted with trypan blue

staining. The samples were centrifuged again, and the cell pellet was resuspended in sodium acetate buffer (see composition on A.1), using 1 ml of buffer per 1×10^7 cells. The samples were then immediately frozen in liquid nitrogen, and thawed at 37 °C in a water bath for 1 min. This freeze-and-thaw process was repeated 3 times. The samples were then centrifuged at 900 × *g* for 10 min at 4 °C (Heraeus Fresco21, Thermo Scientific), and the supernatant was aliquoted and stored at -20 °C for later use. Each aliquot was used no more than three times.

For experiments in which cathepsin expression was assessed, lysosome-enriched lysates were prepared using a Lysosome isolation kit (Sigma-Aldrich), following the manufacturer's instructions. Briefly, the stimulated cells were harvested after 24 h, washed twice in cold PBS, and resuspended in extraction buffer. The cells were lysed using a Dounce homogenizer, and after every 5 strokes a small sample was observed under the light microscope using trypan blue staining to ascertain the level of breakage, until achieving between 80% to 85% of lysed cells. The samples were then centrifuged at 1000 × g for 10 min, and the supernatant was further centrifuged at 20,000 × *g* for 20 min. For further enrichment, a multi-step Optiprep gradient, provided within the kit, was prepared, and the samples were centrifuged for 12 h at 45,000 × *g* Optima L-80XP Ultracentrifuge (Beckman Coulter, Fullerton, CA, USA). After centrifugation, the tubes showed multiple bands floating in the gradient, with a volume of approximately 0.5 ml-0.7 ml. Each fraction was collected with glass Pasteur pipettes starting from the top of the gradient and stored for analysis. The protein concentration was determined using Bradford reagent (Sigma-Aldrich), and lysosome-enriched fractions were identified by measuring their acid phosphatase activity with the Acid phosphatase activity kit (Sigma-Aldrich) according to the manufacturer's instructions.

BMDC lysates for WB of intermediaries of different signaling pathways where prepared as follows. 4×10^6 cells were incubated in 50-ml tubes with a loosen lid at 37 °C, with or without *L. major* promastigotes at a 1:5 cell-to-parasites infection ratio. Incubation in 50-ml tubes allows to minimize the amount of cells lost (in comparison with culture plates) due to adherence as a result of activation of the cells. After different time points, 15 ml of pre-warmed PBS was added, and the cells were washed as described previously to remove any remaining parasite. After the last washing step, the cell pellet was resuspended in 150 μl of ice-cold Triton X-100 Lysis Buffer, transferred to 1.5-ml tubes, and left on ice for 30 min for lysis. The lysates are centrifuged at 18,000 × *g* for 15 min at 4 °C (Heraeus Fresco21, Thermo Scientific), and the supernatants were aliquoted and stored at -20 °C for later use. Each aliquot was used no more than three times. For preparation of lysates from BMM with this protocol, 4×10^{10} cells per well were plated in a final volume of 2 ml, and the cells were allowed to adhere for 4 h. After this time, the culture medium was carefully removed, and replaced by either 2 ml of fresh medium, or 2 ml of medium containing *L. major* promastigotes in a 1:15 cell-to-parasites

infection ratio, and incubated at 37 °C, 5% CO2. At different time points, the medium was carefully removed, and the wells were washed three times with warm PBS. After the last washing step, 150 μl of ice-cold Triton X-100 Lysis Buffer were added to each well, and the plate was incubated in ice for 30 min. The lysed cells were collected in 1.5-ml tubes, and centrifuged at 18,000 $\times$ *g* for 15 min at 4°C (Heraeus Fresco21, Thermo Scientific). The supernatants were aliquoted and stored as described above.

4.4.6 Preparation of cytoplasmic and nuclear extracts

In order to prepare cytoplasmic and nuclear extracts, 5×10^6 BMDC and BMM were seeded in 50-ml falcon tubes or cell culture plates, respectively, and the cells were allowed to rest for 90 min at 37 °C, 5% CO_2. The cells were thereafter stimulated with *L. major* promastigotes using the same infection ratios as described above, and further incubated at 37 °C. Two different buffers were prepared: cell fractionation buffer A, and cell fractionation buffer B. Both were supplemented with DTT (final concentration 0. 5 mM), protease inhibitor cocktail (1:100 dilution, Sigma-Aldrich), and Na3VO4 (final concentration 1 mM). At different time points, the stimulated cells were washed twice with cold PBS and centrifuged (300 $\times$ *g*, 4°C, 5 min), and resuspended in 90 μl of ice-cold cell fractionation buffer A. 10 μl of 1% Triton X-100 in cell fractionation buffer A were added to the samples, and they were further incubated for 5 min in ice. The samples were then centrifuged at 2000 $\times$ *g* for 5 min at 4 °C, and the supernatants were collected as cytoplasmic fraction, and stored at -20 °C. The pellets were then washed with 100 μl of cell fractionation buffer A, and the samples were centrifuged again as described above. The supernatants were discarded, and the pellets were resuspended in 60 μl of cell fractionation buffer B (see composition of cell fractionation buffers A and B in table A.1). The samples were further incubated for 30 min in ice. Then, they were sonicated in ice (Sonoplus, Bandelin, Berlin, Germany) using two cycles of 20 s each, with 40% of amplitude. The resulting suspensions were collected as nuclear fraction, and were stored at -20°C. MEK1/2 and lamnin A/C were used as protein markers for cytoplasm and nucleus, respectively, and their presence in the obtained fractions was determined by WB (see section 4.12).

4.5 Cytotoxicity assays

4.5.1 Alamar Blue assay

An Alamar Blue assay was used to measure the cytotoxic activity of cathepsin inhibitors against *L. major* promastigotes, following the protocol described by Ponte-Sucre et al.[346] The inhibitors included for these assays were: CA074Me (Bachem, Bubendorf, Switzerland), E64-d (Bachem), CLIK148 (kindly provided by Prof. Tanja Schirmeister),

and CS128 (provided by Prof. Tanja Schirmeister), and all were dissolved in dimethyl sulfoxide (DMSO, Applichem). Briefly, 200 μl of a 1×10^7 parasites/ml suspension in complete RPMI medium without phenol red were seeded into 96-well plates, in the presence or absence of different concentrations of the inhibitors, in a range from 100 μM to 0.8 μM. The plates were then incubated for 24 h at 27 °C, 5% CO_2 and 95% humidity, and 20 μl of Alamar Blue (Trinova Biochem, Giessen, Germany) were added per well. The plates were incubated again, and after 24 h and 48 h the optical densities (OD) of the wells were measured at a test wavelength of 540 nm and a reference wavelength of 630 nm, using a Multiskan Ascent microplate reader (Thermo Electronic Corporation). OD values at 48 h were used to calculate the concentration that inhibits 50% cell proliferation (IC_{50}) by linear interpolation. Each plate included growth controls in which the parasites were incubated without any inhibitor, in medium with an equivalent volume of DMSO as used in the inhibitor-treated wells, which never exceeded 1% (vol/vol). In addition, amphotericin B was used as positive control. The same protocol was followed and adapted to measure the cytotoxic activity of cathepsin inhibitors in BMM, using 200 μl of a 2×10^5 cell/ml cell suspension per well. For each experiment, each inhibitor concentration was tested in duplicates.

4.5.2 Amastigote Assay

In the present study, this assay was used to determine (1) the toxicity of different cathepsin inhibitors against *L. major*, and (2) the differences in the proliferation of *L. major* in cathepsin-deficient BMM, in comparison with WT BMM. This assay was previously described by Bringmann et al.[428]

BMM from BALB/c, *Ctsb* $^{-/-}$, *Ctsl* $^{-/-}$, and their WT C57BL/6 counterparts were harvested as previously indicated, and adjusted to a concentration of 2×10^5 cells/ml in phenol red-free complete RPMI medium. This medium was used in all the following steps of the assay. The cells were seeded into white 96-well plates with clear bottom (Greiner Bio-One, Frickenhausen, Germany) in a final volume of 200 μl, and were incubated for 4 h at 37°C, 5% CO_2 to allow cell adhesion. For the assays in which the toxicity of chemical compounds against amastigotes was tested, blanks were set by using empty wells containing medium with an equivalent volume of the solvent used for each compound, water or DMSO. Similarly, growth control wells were included, using medium with an equivalent volume of the solvent used, and different concentrations of amphotericin B were used as positive controls. After the aforementioned incubation time, the culture medium was carefully removed, and replaced by 200 μl of a 3×10^6 parasites/ml suspension of Luc-tg. *L. major* promastigotes, to obtain an infection ratio of 1:15. The plates were then incubated for 24 h at 37 °C, 5% CO_2. Any remaining extracellular parasites were eliminated by washing the plate wells 3 times with pre-warmed medium, and 200 μl of

fresh medium were added per well. For toxicity assays, instead of only fresh medium, different concentrations of compounds (from 100 μM to 0.4 μM) were used. The plates were further incubated for 24 h at 37 °C, 5% CO_2. For measurement of the luciferase activity from the intracellular amastigotes, 50 μl of the luciferin-containing lysis buffer Britelite Plus (PerkinElmer, Waltham, USA) were added to each well, and the plate was incubated in the dark for 5 min at RT. The resulting luminescence was measured as counts per second (CPS) with a Victor X Light 2030 luminometer (PerkinElmer).

4.6 Microscopy

4.6.1 Diff-Quik staining of BMDC and BMM

The morphology of BMDC and BMM generated from WT, *Ctsb* $^{-/-}$, *Ctsl* $^{-/-}$ mice was compared by Diff-Quik II dye (Medion Diagnostics, Düdingen, Switzerland) staining, following the manufacturer's instructions. Briefly, cytospin preparations of 2×10^5 cells were prepared, and the glass slides were allowed to air-dry overnight at RT. The slides were then dipped 5 times in the Diff-Quik fixative solution, which contains Fast Green (0.002 g/l) in methanol, followed by five times dipping into Diff-Quik stain solution I, which contains eosin Y (1.22 g/l), and five times dipping into Diff-Quik stain solution II, containing thiazine dye (1.1 g/l). The slides were afterwards rinsed with distilled water, air-dried overnight at RT, and mounted with Aquatex (Merck, Darmstadt, Germany) for observation under the light microscope (Axiolab, Carl Zeiss, Oberkochen, Germany). Infected macrophages were also stained following this method, in order to determine the infection rate.

4.6.2 Transmission electron microscopy of BMDC and BMM

The morphology of BMDC and BMM from WT, *Ctsb* $^{-/-}$, *Ctsl* $^{-/-}$ mice was additionally compared by transmission electron microscopy (TEM). Samples of the obtained cells were prepared for TEM using an adapted version of the protocol previously described by Schurigt et al.[347] Briefly, 2×10^6 cells were harvested, washed in PBS, centrifuged, and the resulting cell pellet was resuspended in a fixation solution containing 2.5% glutaraldehyde (Sigma-Aldrich) in 0.2 M sodium cacodylate (Sigma-Aldrich) buffer,and incubated for 2 h at 4 °C. The fixed cells were afterwards washed with 0.5 M cacodylate buffer, and incubated with 2% OsO4 in 0.5 M cacodylate buffer overnight at 4 °C for lipid staining. The samples were afterwards washed with distilled water, and contrasted with aqueous 0.5% uranyl acetate overnight at 4 °C. The samples were centrifuged, and thereafter subjected to a series of incubation steps with 50%, 70%, 90%, 96%, and three times in 100% ethanol, each of 30 min at 4 °C, for dehydration. The samples were then incubated in 100% propylene oxide (Sigma-Aldrich), for 30 min, followed by centrifugation, addition of

a 1:1 solution of Epon 812 (Sigma-Aldrich) and propylene oxide, and overnight incubation at RT. The samples were finally embedded by replacing this mixture with pure Epon 812, incubating overnight at RT, and polymerized at 60 °C for 3 days. Ultrathin sections were prepared and mounted at the Central Department for Electron Microscopy, University of Würzburg, and analyzed with an EM 10 transmission electron microscope (Carl Zeiss). The levels of vacuolization of at least 100 cells per sample were analyzed by using the following scoring system: 0 if no vacuoles were visible; 1 if the area of the sum of all the vacuoles corresponded to aprox. 1 to 20% of the cell surface; 2 if said area was of aprox. 21% to 40%; 3 if it was of 41% to 60%; 4 for 61% to 80%; and 5 if it was over 80%. Furthermore, an extra score point was added if the vacuoles presented myeloid-like structures and a double membrane, which are characteristic of autophagosomes.

4.6.3 Fluorescence microscopy

BMM from WT, *Ctsb* $^{-/-}$, and *Ctsl* $^{-/-}$ mice were harvested, counted, and adjusted to a concentration of 5×10^5 cells/ml in complete RPMI medium without phenol red. 250 μl were seeded in duplicates into chambered cover glasses (Nunc Thermo Scientific), and incubated for 4 h at 37 °C, 5% CO_2 to promote cell adhesion. Meanwhile, eGFP-tg *L. major* promastigotes were harvested, washed 3 times with warm PBS, counted, and adjusted to a concentration of 3×10^6 parasites/ml in complete medium without phenol red. After the 4 h incubation time of BMM was completed, the culture medium was carefully removed, and replaced by 250 μl of the eGFP-tg parasite suspension, to obtain an infection ratio of 1:15. The samples were further incubated for 24 h at 37 °C, 5% CO_2. The cells were then washed 3 times with warm PBS to remove any free parasites. The samples corresponding to the time point of 24 h were incubated with Hoechst solution 0.5% v/v (Immunochemistry Technologies, Bloomington, USA) for 15 min at 37°C protected from the light, followed by washing 3 times with warm PBS and addition of 250 μl of complete medium. Then, they were observed under a fluorescence microscope (Leica Microsystems). The rest of the samples corresponded to the time point of 48 h, and were incubated in fresh medium for further 24 h, stained and observed under the fluorescence microscope as described above. The amount of cells and *L. major* bodies were quantified with the Cell Counter plug-in from the ImageJ software.[430]

4.7 Quantification of cathepsin expression and activity

4.7.1 Measurement of total protein by BCA

The protein concentration of lysates to be used for WB analysis was determined using a microplate bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockfort, IL, USA), following the manufacturer's instructions. This method is based on the reduction of

cupric ions to cuprous ions by cysteine, cystine, tyrosine, and tryptophan residues within proteins, and the quelation of these resulting cuprous ions with BCA, to form a purple complex. For this assay, dilutions of the lysates (1:5 to 1:25) were prepared using the same buffer in which the samples were lysed, and 25 μl of each dilution were pipetted in duplicates into transparent U-bottomed 96-well plates provided with the kit. As standard, 25 μl/well of serial dilutions of BSA in lysis buffer were included, using a concentration range of 0.05 to 0.4 μg/μl. Next, reagents A and B were mixed (1:50), and 200 μl of this solution were added per well. Reagent A contains BCA, while reagent B is composed of 4% cupric sulfate. The plate was incubated for 30 min at 37 °C. The absorbance per well was measured with a Multiskan Ascent microplate reader, using a test wavelength of 562 nm.

4.7.2 Cathepsin B and cathepsin L activity assay

The proteolytic activity of cathepsins B and L was measured from cell lysates prepared in sodium acetate buffer (pH 5.5) described in section 4.4.5. The protein concentration for those lysates was measured using the BCA protein Assay protocol described in the previous section, and the concentrations of the obtained lysates were in the range of 0.5 μg/μl to 1.0 μg/μl. The cathepsin activity assay used in the present study was previously described by Schurigt et al,[347] and is based on the proteolytic cleavage of the fluoropeptide z-Phe-Arg-4-methyl-coumarin-7-amide (AMC) (Z-Phe-Arg-AMC, Bachem), which is a substrate for cysteine cathepsins B and L. Briefly, sodium acetate buffer was supplemented with 0.5 mM DTT, and used as reaction buffer. 5 μg of total protein from each sample were pipetted in triplicates in black flat-bottom 96-well plates (Nunc, Thermo Scientific). For the experiments in which the activity of cathepsin inhibitors was tested against murine lysates, 1 μl DMSO, or 1 μl of the inhibitors CA074 (10 μM Bachem) or E64 (10 μM Bachem) were added for each sample, followed by addition of sodium acetate reaction buffer to complete a volume of 94 μl/well, and a 15 min incubation step at 37 °C. After this incubation, 1 μl of DMSO, CA074, E64, or the inhibitors CS128, CLIK148, and RV212C were added per sample, and the plate was incubated again for 15 min at 37 °C. In the experiments in which the lysates were generated from cells already pre-incubated with cathepsin inhibitors during culture, the samples were incubated only with 1 μl of DMSO, CA074 or E64 for 30 min at 37 °C. Standard curves were prepared with serial dilutions of the fluorochrome 7-amino-4-methyl-coumarin (AMC; Bachem). Finally, the proteolytic reaction was initiated by addition of 5 μl of 500 μM Z-Phe-Arg-AMC. The proteolytic release of AMC was continuously monitored for 45 min by fluorescence spectroscopy, using an excitation wavelength of 355 nm, and an emission wavelength of 460 nm with a Fluoroskan Ascent fluorescence reader (Thermo Electron Corporation, Langenselbold,

Germany). The proteolytic activity of each sample was calculated using the linear range of the reaction curves.

4.7.3 Cathepsin S activity assay

The inhibitory capacity of some of the cathepsin inhibitors used in the present study against Ctss was assessed using a Cathepsin S drug discovery kit (Enzo Life Sciences, Plymouth Meeting, PA, USA). This assay is based on the proteolytic cleavage of a fluorogenic substrate, z-Val-Val-Arg-AMC, by recombinant human Ctss, which results in the release of AMC. The inhibitors were tested following the manufacture's instructions. Briefly, all the components of the kit were allowed to warm up to 37°C, including assay buffer (50 mM potassium phosphate, pH 6.5), recombinant Ctss, DMSO, cathepsin inhibitors, the substrate z-Val-Val-Arg-AMC, as well as AMC calibration standard. First, assay buffer was pipetted to a 1/2 volume white 96-well plate, followed by addition of Ctss (0.06 mU/well). Next, cathepsin inhibitors were added: CS128, CA074Me, and 13b, in a range of concentrations from 0.1 to 10 μM. In addition, for some wells 1 μM E64 was used as control. Finally, AMC standard was added, and the plate was incubated for 30 min at 37°C. The reaction was started by addition of z-Val-Val-AMC substrate (40 μM final concentration), and the plate was continuously measured for 20 min, at intervals of 30 s each, using an excitation wavelength of 355 nm, and an emission wavelength of 460 nm with a Fluoroskan Ascent fluorescence reader. The remaining proteolytic activity of each sample was calculated using the linear range of the reaction curves.

4.7.4 Determination of cathepsin B and L expression by WB

The expression of Ctsb and Ctsl in BMDC in response to different stimuli was analyzed by Western Blot (WB). The composition of all the buffers described in this section can be found on Table A.1. 10 μg of total protein from each sample were mixed with 5× PAGE-Sample Buffer, and boiled for 5 min at 95 °C. The samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using a 4% polyacrylamide collecting gel, and a 12% separating gel. The samples were run through the collecting gel at 80 V, and at 110 V at the separating gel, using SDS Transfer Buffer I (1×

). After electrophoresis, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (PALL, Dreieich, Germany) at 140 V, 250 mA for 1 h using Transfer Buffer I. The protein transfer was assessed by incubating the membranes for 5 min at RT in Ponceau S solution (0.05% Ponceau S, 3% acetic acid), followed by continuous washing with distilled water. The membranes were then blocked overnight at 4°C with 10% non-fat, blotting grade milk (Biorad, Hercules, CA, USA) in PBS-Tween buffer. For detection of Ctsb and Ctsl, the membranes were first incubated for 1 h at RT with the

following biotinylated Ab: anti-mCathepsin-B (0.5 μg/ml, R&D Systems, Minneapolis, MN, USA) or anti-mCathepsin-L (0.1 μg/ml, R&D Systems), followed by incubation with horseradish peroxidase (HRP)-streptavidin (0.1 μg/ml in 0.5% milk, Thermo Scientific). The membranes were developed using chemiluminescent HRP substrate (Millipore, Billerica, MA, USA), and visualized using an ImageQuant LAS400 luminescent image analyzer (GE Healthcare Life Sciences, Uppsala, Sweden). As loading control, the membranes were stripped by incubation with Stripping buffer, freshly supplemented with 0.8% 2-mercaptoethanol for 20 min at 70 °C. The membranes were then washed with PBS-Tween buffer, and blocked overnight at 4 °C with 10% non-fat, blotting grade milk. Next, the membranes were incubated with anti-m-β-Actin (0.5 μg/ml, Sigma-Aldrich) for 1 h at RT, followed by incubation with biotinylated goat anti-mIgG1 (0.1 μg/ml, Abcam,Cambridge, UK) for 1 h at RT, and HRP-streptavidin (0.1 μg/ml in 0.5% milk) for 1 h at RT. The membranes were developed and visualized as described above. The images were analyzed using the software ImageJ.

4.7.5 Active site-labelling of cathepsins

The biotinylated probe DCG-04 was kindly provided by Prof. Matthew Bogyo (University of California, San Francisco, CA, USA). This probe was engineered to track the activity of cysteine proteases, using the structure of E64 as a scaffold. DCG-04 was used in the present study to label simultaneously the cysteine cathepsins present in lysates prepared from freshly generated BMDC from WT, *Ctsb* $^{-/-}$, *Ctsl* $^{-/-}$ mice. 7 μg of total protein were incubated with 100 μM DCG-04 in citrate reaction buffer (see Table A.1) for 1 h at RT, followed by addition of 5X PAGE sample buffer and boiling for 5 min at 95 °C. The samples were then resolved in 12% SDS-PAGE, and blotted to a PVDF membrane, as described previously in section 4.7.4. The membranes were then incubated for 1 h at RT in HRP-Streptavidin (0.1 μg/ml in PBS-T), followed by addition of chemiluminescent HRP substrate, and imaging. The images were analyzed using the software ImageJ.

4.8 Flow cytometry

4.8.1 Measurement of uptake and processing of eGFP-*L. major* by BMDC

1×10^6 BMDC/ml were harvested at day 7 of culture, plated in 6-well plates and incubated overnight at 37°C, 5% CO_2. For some experiments, BALB/c BMDC were pre-incubated with cathepsin inhibitors for 4 h prior to infection. eGFP-*L. major* promastigotes were harvested, washed 3 times in warm PBS, added to the BMDC at a 1:5 infection ratio, and further incubated at 37°C. After 2 h of exposure of the BMDC to the parasites, the cells were washed with warm PBS, and resuspended in fresh medium at a concentration

of 1×10^6 cells/ml. Part of the cells was fixed in paraformaldehyde fixation buffer (PFA, 4%; Applichem, Darmstadt, Germany). The remaining cells were incubated for a total of 4 h or 24 h post infection, fixed, and the amount of infected cells at the different time points was determined by flow cytometry, together with the expression of maturation markers as described next.

4.8.2 Measurement of extracellular markers by flow cytometry

BMDC infected with e-GFP *L. major*, stimulated with LmAg (30 μl LmAg/ml, equivalent to 30 parasites per BMDC), LPS (1 μg/ml) or TNF-α (500 U/ml) were fixed with 4% PFA and resuspended in FACS buffer containing the following Ab: phycoerythrin-cyanine 7 (PECy7)-conjugated anti-CD11c (BD Biosciences, Heidelberg, Germany), PE-conjugated anti-CD86 (BD Biosciences), allophycocyanin-conjugated anti-MHC class II (Miltenyi, Bergisch Gladbach, Germany). For some assays, BMDC were infected with WT *L. major* promastigotes instead, and fluorescein isothiocyanate (FITC)-conjugated anti-CD40 (Biolegend, San Diego, USA) and FITC-conjugated anti-CD80 (eBioscience, San Diego, USA) Ab were used. Data was obtained using the MACSQuant flow cytometer (Miltenyi) and analyzed using FlowJo (Tree Star Inc., CA, USA). The expression of F4/80 in BMM was determined using FITC-conjugated anti-F4/80 Ab (Biolegend).

4.8.3 Measurement of intracellular cytokines by flow cytometry

The expression of intracellular IL-12 and IL-6 was analyzed in BMDC after 24 h stimulation with LPS (1 μg/ml) at 37°C, 5% CO_2, in the presence or absence of different cathepsin inhibitors, and brefeldin A (3 μg/ml, eBioscience). The cells were then incubated for 20 min in 4% PFA fixation buffer, permeabilized for 20 min at 4 °C using permeabilization buffer (see table A.1), and incubated for 1 h with (PECy7)-conjugated anti-CD11c, PE-conjugated anti-IL-12(p40/p70, BD Biosciences), and APC-conjugated anti IL-6 (Biolegend). Data was obtained using the MACSQuant flow cytometer. Furthermore, cells from polarization assays described in section 4.11.1 were fixed with 2% formaldehyde for 20 min at 4°C, permeabilized for 20 min at 4°C using permeabilization buffer, and stained with the following Ab diluted in permeabilization buffer: Pacific Blue-conjugated anti-CD4 (Biolegend), FITC-conjugated anti-IFN-γ (BD Biosciences), PE-conjugated anti-CD25 (BD Biosciences), and allophycocyani-conjugated anti-IL10 (Biolegend). Data was obtained using a LSR-II flow cytometer (BD Biosciences, San Jose, USA). All results were analyzed using the software FlowJo.

4.9 Measurement of cytokine expression

4.9.1 Measurement of cytokines in supernatants by ELISA

1×10^6 BMDC were seeded in a final volume of 1 ml in 24-well plates, and were stimulated with 5×10^6 *L. major* WT promastigotes (infection ratio 1:5), LmAg (30 μl/ml), LPS (1 μg/ml), or CpG ODN (25 μg/ml). The cells were further incubated for 24 or 48 h, and the supernatants were collected. The concentration of the cytokines in the supernatants was determined by sandwich enzyme-linked immunosorbent assay (ELISA), using capture-detection Ab pairs purchased from BD Biosciences for IL-12p40, IL-6 and TNF-α, and R&D Systems for IL-10 (Wiesbaden, Germany) following the suppliers' instructions.

Briefly, 96-well plates were coated with the corresponding capture Ab diluted in coating buffer (IL-12p40: 3.0 μg/ml; IL-6: 1.67 μg/ml; TNF-α: 4 μg/ml), or in PBS (IL-10: 2 μg/ml), followed by overnight incubation at 4 °C. The plates were then washed 3 times in PBS-T, and blocked overnight at 4 °C with 10% FCS. Next, the plates were washed 3 times in PBS-T, and supernatants or standards were added, and the plates were further incubated overnight at 4 °C. The plates were afterwards washed, incubated for 1 h at RT with the corresponding biotinylated secondary Ab (IL-12p40: 2.5 μg/ml; IL-6: 1 μg/ml; TNF-α: 1 μg/ml, IL-10: 0.2 μg/ml). Next, the plates were washed again, and incubated for 45 min at RT with a 1:1000 dilution of alkaline phosphatase-streptavidin complex (BD Biosciences) in blocking solution. To detect the cytokines, alkaline phosphatase substrate (1 mg/ml in diethanolamin buffer) was added to each well, and the developing color in the wells was measured at a test wavelength of 405 nm, and a reference wavelength of 490 nm in a Multiskan Ascent microplate reader. In addition, IL-12p70 was measured by using the IL-12p70 ELISA Ready-SET-Go kit from eBioscience according to the manufacturer's instructions. This system uses as detection enzyme avidin-HRP, and includes a tetramethylbenzidine substrate solution. To analyze the cytokine production in BMM, 1×10^6 cells were seeded in 500 μl into 24-well plates, together with 15×0^6 *L. major* WT promastigotes (infection ratio 1:15), in the presence or absence of LPS (1 μg/ml). The cells were incubated for 24 and 48 h, and the supernatants were collected. Cytokine measurements by ELISA were performed as described above.

4.9.2 Measurement of cytokine expression by RT-PCR

Total RNA from 2×10^6 BMDC or BMM, stimulated as described above, was isolated using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA synthesis was performed using the iScript cDNA synthesis kit (BioRad, Munich, Germany) and the resulting cDNA was used at a 1:8 dilution to assess the expression of IL-12a(p35) by real-time PCR. The real-time PCR was performed in a final volume of 25 μl per well using Maxima SYBR Green/Fluorescein qPCR Master

Mix (Thermo Scientific, Schwerte, Germany) and run with a CFX96 Touch real-time PCR detection system (BioRad) for 40 cycles. The primer pairs used were: *Il12p35* forward: TGGCTACTAGAGAGACTTCTTCCACAA, *Il12p35* reverse: GCACAGGGTCATCATCAAAGAC; *Il12p40* forward: CGTGCTCATGGCTGGTGCAAA, *Il12p40* reverse: ACGCCATTCCACATGTCACTGCC. The housekeeping gene β-actin was used for normalization of the samples: *β-actin* forward: CATTGCTGACAGGATGCAGA, *β-actin* reverse: TTGCTGATCCACATCTGCTG. Relative gene expression values were calculated with the $2^{-\Delta\Delta CT}$ method

4.10 Quantification of nitric oxide production

BMM from WT and cathepsin-deficient mice were seeded and infected as described above, followed by incubation at 37°C, 5% CO_2 for 24 h. The cells were washed afterwards with phenol-free complete RPMI medium to eliminate any extracellular parasites and incubated for further 48 h in the absence or presence of 1 μg/ml LPS. The supernatants were collected, and the concentration of nitrite (NO_2^-) was determined by addition of 100 μl of culture supernatant to 100 μl of Griess reagent (Sigma-Aldrich) and incubation for 15 min at RT. The resulting absorbance at 540 nm was measured with the Multiskan Ascent ELISA reader (Thermo Electronic Corporation). The nitrite concentrations were determined using sodium nitrite (NaNO) as a standard, and reflect the nitrite oxide levels released by macrophages.

4.11 Polarization assays

4.11.1 Th1 polarization assay

In order to evaluate the effect of different cathepsin inhibitors on the Th1 polarization by BMDC, a Th1 polarization assay was adapted from the protocol described by Pletinckx et al,[431] and performed at the laboratory of Prof. Manfred B. Lutz (University of Würzburg, Würzburg, Germany).For this assay, 1×10^4 BMDC were co-cultured with 5×10^4 purified $CD4^+CD25^-$ T cells from OT-II mice (see section 4.4.4), in the presence of OVA (1 mg/ml, Hyglos, Bernried, Germany)or $OVA_(327-339)$ peptide (100 ng/ml, Activotec, Cambridge, UK), and LPS (0.1 μg/ml), in U-bottom 96-well plates, with a final volume of 200 μl/well for 5 days at 37°C, 5% CO2. The cells were then harvested, counted, and adjusted to a concentration of $1x10^6$ cell/ml for restimulation with PMA (10 ng/ml, Sigma-Aldrich) and ionomycin (1 μg/ml, Sigma-Aldrich) at 37 °C, 5% CO_2. After 1 h incubation, Brefeldin A was added (3 μg/ml), and the plates were further incubated for 4 h more. The cells were then washed, fixed in 2% formaldehyde, incubated for 20 min in

permeabilization buffer, and the expression of Th1 cytokines was assessed by staining of intracellular cytokines, and flow cytometry (see 4.8.3).

4.12 Analysis of intermediates of signaling pathways

Differences in expression levels and activation status of intermediates of signaling pathways relevant to immune response against leishmaniasis were analyzed by WB, all performed at the lab of PD Dr. Heike Hermanns (University of Würzburg, Würzburg, Germany). Lysates from WT and cathepsin-deficient BMDC and BMM were prepared as described in section 4.4.5, after different time points of infection with *L. major* promastigotes (1:5 infection ratio for BMDC, and 1:15 for BMM). The samples were mixed with 4× Lämli buffer, and boiled for 5 min at 95°C, and their proteins were separated by SDS-PAGE (10% acrylamide gels). The proteins were then transferred to PVDF membranes using a semi-dry electro-blotter (Peqlab, Erlangen, Germany), which consists of a 3-buffer system (see table A.1 for reference of anode buffer I, another buffer II, and cathode buffer). The membranes were then blocked for 20 min with 10% bovine serum albumin (BSA, Applichem) at RT, followed by overnight incubation at 4°C with the following antibodies against murine proteins, diluted in 5% BSA in Tris-buffered saline (TBS)-Tween, all of them from Cell Signaling (Danvers, MA, USA): anti-p65, anti-IκB, anti-phosphorylated p38, anti-p38, anti-phosphorylated ERK1/2, anti-ERK1/2, anti-phosphorylated MAPK, and anti-MAPK The membranes were then washed, incubated for 1 h at RT with HRP-conjugated anti-rabbit IgG or anti-mouse IgG1, respectively (both from DAKO, Hamburg, Germany), and developed using a chemiluminescence kit (GE Healthcare, Munich, Germany). The membranes were then visualized using a FluorChem Q imager (Biozym Scientific, Oldendorf, Germany). For loading control, the membranes were stripped as previously described in section 4.7.4, followed by incubation with anti-GAPDH as primary antibody, followed by incubation with HRP-conjugated anti-rabbit IgG1. The images were analyzed using the software ImageJ.

4.13 Statistical analysis

Values are provided as mean ± standard deviations from at least 2 independent experiments. Statistical significance was determined by the unpaired 2-tail Student's t test (Microsoft Excel Software). In experiments using cathepsin-deficient BMDC or BMM, this statistical test was used to compare, for each treatment, the results from *Ctsb* $^{-/-}$ or *Ctsl* $^{-/-}$ cells with their WT counterparts, and in experiments were different cathepsin inhibitors were tested, inhibitor-treated cells were tested against DMSO controls.

CHAPTER 5

Results

5.1 Effects of cathepsin deficiency and inhibition on BMDC generation and survival

The first part of this section focuses on the cytotoxicity of the different cathepsin inhibitors used for the present study. Next, BMDC and BMM from *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ mice were generated and characterized in terms of their morphology, yield of cells generated and expression of typical markers.

5.1.1 The cathepsin inhibitors used in the present study showed no cytotoxicity in BMDC

The cytotoxic activity of different cathepsin inhibitors was evaluated in BMDC, using an Alamar Blue assay. The active ingredient of Alamar Blue reagent is resazurin, a blue cell-permeable compound with almost no fluorescence. When resazurin enters a viable cell, the reducing environment within the cytosol results in the reduction of resazurin to resorufin, which has a red color and is highly fluorescent, resulting in a change in the color and increase in the fluorescence in the medium surrounding the viable cells. In this assay, different cathepsin inhibitors were tested in BMDC, in a concentration range of 0.8 μM to 100 μM, and their IC_{50} values were calculated after 48 h.

Table 5.1 summarizes the IC_{50} values found. Almost all the cathepsin inhibitors tested had very low cytotoxicity, since their IC_{50} values were superior to 100 μM, with the exception of CA074Me, which had an IC_{50} value of 55.35 $\pm$ 6.76 μM. Therefore, the

Table 5.1. Cytotoxicity of cathepsin inhibitors in BMDC

Compund	IC_{50} value
RV212C	>100 μM
CS128	>100 μM
CA074Me	55.35 μM $\pm$ 6.76
CLIK148	>100 μM
E64d	>100 μM
CA074	>100 μM
ZRLR	>100 μM

viability of BMDC after 48 h incubation with different concentrations of the inhibitors was assessed by Trypan Blue staining (see Appendix, Fig. A.1). Concentrations up to 20 μM showed no significant effect in BMDC viability. Additionally, different concentrations of these inhibitors were titrated in BMDC, by pre-treating them for 1 h with the inhibitors and preparing lysates. The remaining proteolytic activity corresponding to cathepsins in these lysates was measured (see Appendix, Fig. A.2). All together, a concentration of 10 μM was chosen for pre-treatment of BMDC, and unless stated otherwise, was used throughout the rest of this study.

5.1.2 BMDC and BMM from cathepsin B- and cathepsin L-deficient mice present comparable phenotypes as their wild-type counterparts

BMDC and BMM from cathepsin-deficient mice were generated from stem cell progenitors. The morphology of the obtained cells was analyzed by light microscopy, and TEM. BMDC from cathepsin-deficient mice displayed a typical immature DC phenotype, similar as BMDC from WT mice (Fig. 5.1 A). Comparably, no morphological differences were found in BMM from WT and cathepsin-deficient mice.

CD11c and F4/80 are commonly used as characteristic markers for BMDC and BMM, respectively, and their expression in the generated cells was evaluated by flow cytometry at day 8 in BMDC, and day 6 in BMM. The expression of these markers was comparable between wild-type and cathepsin-deficient BMDC and BMM, and no significant differences were found in the yields of $CD11c^+$ cells and $F4/80^+$ cells obtained per plate at the end of the generation culture (Fig. 5.1 B and C). In addition, the harvested BMDC from WT and cathepsin-deficient mice presented comparable levels of MHC class II molecules and CD86 expression (Fig. 5.1 D and E).

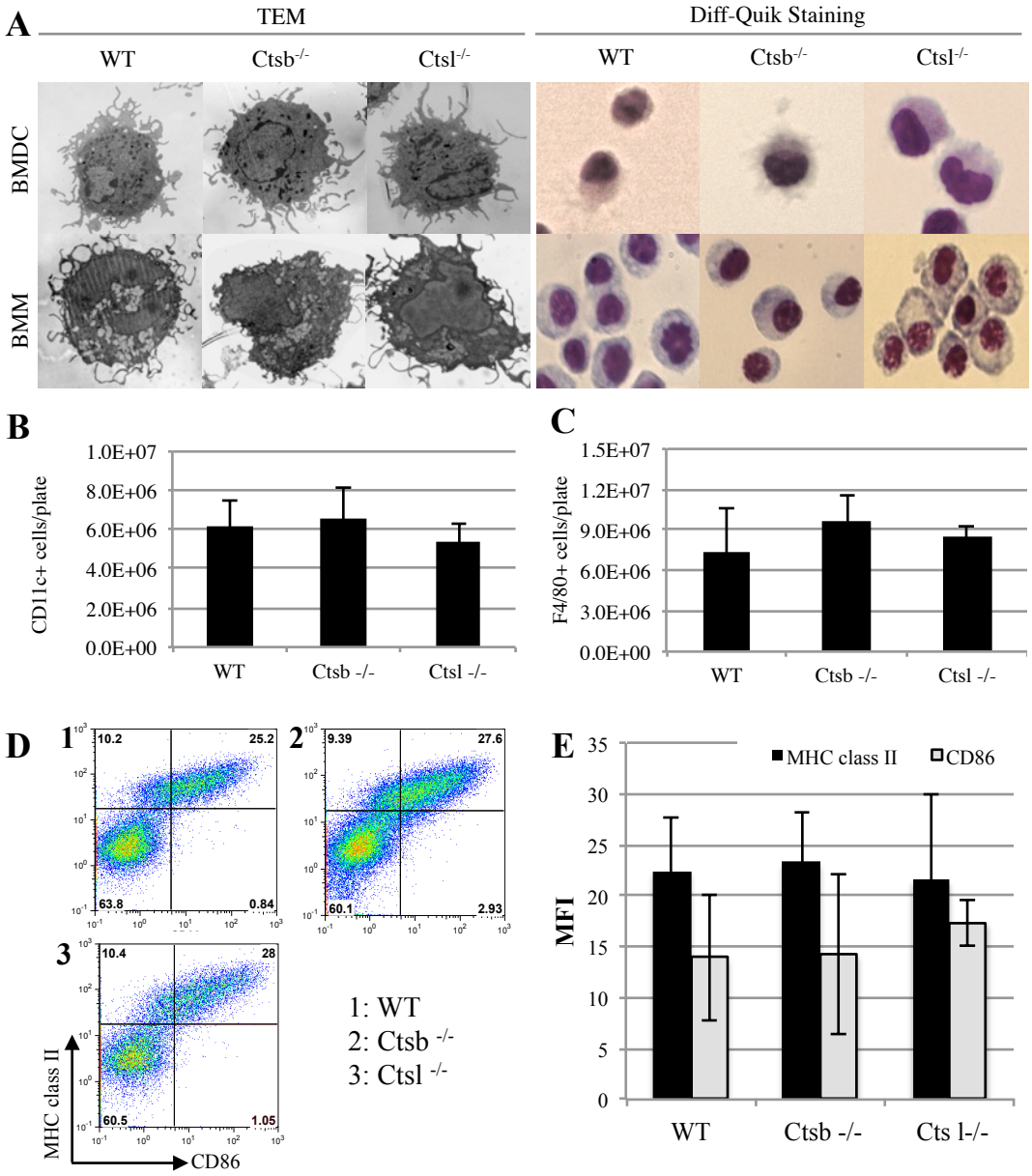

Figure 5.1. **Morphology and phenotype of bone marrow derived macrophages and dendritic cells from wild-type and cathepsin-deficient mice.** (A) The generated cells present comparable morphologies, as illustrated here in representative TEM pictures (4400x magnification) and light microscopy pictures (Diff-Quik staining, 40x magnification) of BMDC and BMM from wild-type, *Ctsb* $^{-/-}$ and *Ctsb* $^{-/-}$ mice. (B) Comparable yields of CD11c^{+} cells per plate were obtained in cultures of BMDC from wild-type and cathepsin-deficient mice. (C) Yields of F4/80^{+} cells per plate obtained in cultures of BMM from wild-type and cathepsin-deficient mice. (D) Representative density plots comparing the expression of MHC class II molecules and CD86 in BMDC from wild-type and cathepsin-deficient mice. (E) Expression levels of MHC class II and CD86 in BMDC from wild-type and cathepsin-deficient mice, represented by their medium fluorescence intensity (MFI). The results in (B), (C), and (E) are expressed as mean ± SD from cells generated out of 3 different animals per genotype. The results in (A), (B) and (C) are reported in [432]

Therefore, BMDC and BMM can be generated in the absence of Ctsb and Ctsl, and these cells present comparable morphology and expression of characteristic markers as cells generated from WT mice.

Table 5.2. Toxicity of cathepsin inhibitors in *L. major* promastigotes

Compund	IC_{50} value	% of inhibition
RV212C	59.94 ± 8.82 μM	82.13%
CS128	51.28 ± 2.41 μM	89.7%
CA074Me	>100 μM	0%
CLIK148	>100 μM	5.52%
E64d	>100 μM	19.21%
CA074	>100 μM	0%
ZRLR	>100 μM	0%

5.2 Toxicity of cathepsin inhibitors in *L. major*

In this section, the toxicity of cathepsin inhibitors used for the present study was assessed against *L. major* parasites.

5.2.1 Toxicity against promastigotes

The cytotoxicity of different cathepsin inhibitors was tested in *L. major* promastigotes using the Alamar Blue Assay previously described. The results of these assays can be found in Table 5.2. CS128 had an IC_{50} value of 51.28 ± 2.41 μM, showing similar leishmanicidal activity in comparison with RV212C, which had an IC_{50} value of 59.94 ± 8.82 μM. Moreover, CS128 at a concentration of 100 μM resulted in 89.7% inhibition of parasite growth, while RV212C at 100 μM resulted in 82.13% of parasite growth.

The rest of the cathepsin inhibitors tested showed little or no leishmanicidal activity in concentrations up to 100 μM, including CA074, CA074Me, CLIK148, E64d, and ZRLR.

5.2.2 Toxicity against amastigotes

After successfully infecting a host cell, *L. major* promastigotes differentiate into amastigotes within parasitophorous vacuoles. To reach the amastigote, a leishmanicidal compound should permeate through three physical barriers: the cell membrane of the host, the membrane of the PV where the amastigote is contained, and the parasite cell membrane. Therefore, cell permeability is a critical factor to consider when evaluating a potential leishmanicidal compound.

The leishmanicidal activity of CS128 was further examined using an amastigote assay in which BMM are infected with Luc-tg *L. major* promastigotes. After 24 h, the parasites would have transformed into amastigotes, and at this time point they are challenged with different concentrations of CS128 or amphotericin B for another 48 h. With the addition of D-luciferin in the presence of ATP and oxygen, the luciferase expressed by viable Luc-tg

L. major amastigotes catalyzes its conversion to oxyluciferin and the generation of light. CS128 presented an IC_{50} value of 1.34 ± 0.12μM, in comparison with amphotericin B, which had an IC_{50} value of 14.87 ± 0.82 nM.

5.3 Expression of cathepsins in BMDC

The first part of this section concentrates on the expression of Ctsb and Ctsl in BMDC in response to multiple stimuli, including infection with *L. major* promastigotes. Further on, the expression of different cysteine cathepsins was compared in lysates generated from *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ BMDC.

5.3.1 Expression of cathepsins B and L in BMDC during infection with *L. major* promastigotes

The expression levels of Ctsb and Ctsl in BMDC during infection with *L. major* promastigotes was examined by WB. BMDC were incubated with the parasites using an infection ratio of 5 parasites per BMDC, and whole cell lysates were prepared at the following time points after infection: 0 h, 1 h, 4 h, 10 h, and 24 h. Figure 5.2 summarizes the expression levels found. Ctsb is slowly up-regulated in BMDC upon infection with *L. major*, as no increase is detected at 10 h p.i., but until a later time point, at 24 h p.i. Ctsb is also slightly up-regulated in non treated BMDC, although their levels are considerably lower as in infected cells. Ctsl is also up-regulated as a result of infection with *L. major*.

5.3.2 Expression of cathepsins B and L in BMDC in response to Th1- and Th2- inducing stimuli

The expression of Ctsb and Ctsl was analyzed in BMDC in response to different stimuli, including CpG, lipopolysaccharide (LPS), and TNF-α. First, lysates of freshly generated BMDC from BALB/c and C57BL/6 mice were compared. Some differences in the basal expression levels of Ctsb and Ctsl were observed between BALB/c and C57BL/6 BMDC. However, the up-regulation of Ctsl and Ctsb as a response to the different maturation stimuli was comparable (Fig. 5.3). Moreover, TNF-α induced higher expression levels of Ctsb in comparison to CpG, and LPS.

5.3.3 Expression of cysteine proteases in *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ BMDC

As a control, the expression of other cathepsins in *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ BMDC was analyzed. Lysates from these cells were prepared and incubated with DCG-04, a biotinylated substrate that targets the same broad set of for cysteine proteases as E64, and resolved by SDS-PAGE. The resulting blots allowed assessing the expression of multiple cysteine

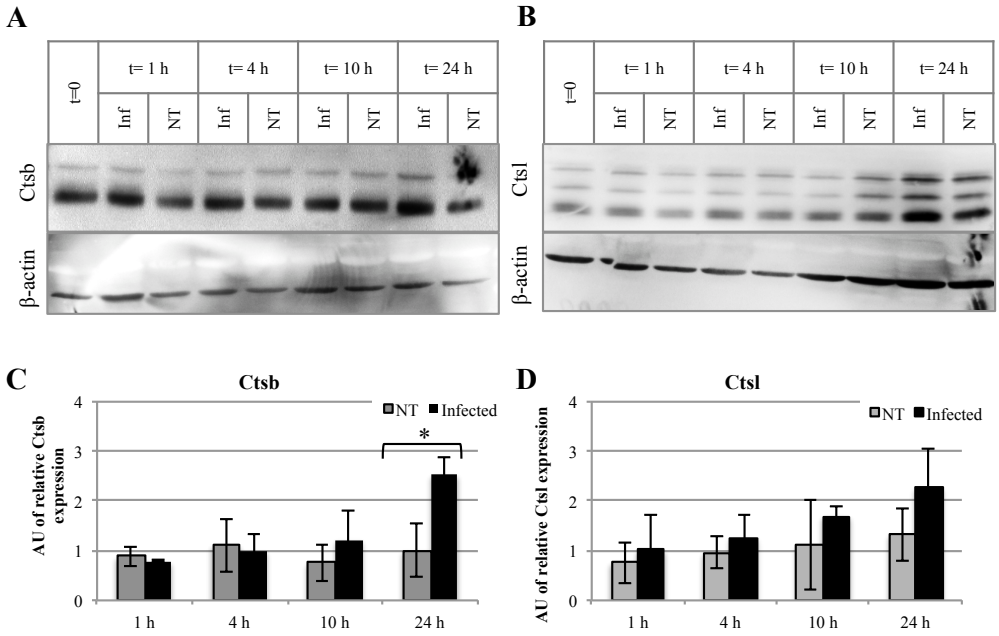

Figure 5.2. **Expression of Ctsb and Ctsl in BMDC in response to *L. major* infection.** BALB/c BMDC were infected with *L. major*, and lysates from infected (inf.) and non-treated BMDC (NT) were prepared at 1 h, 4 h, 10 h, and 24 h post-infection (p.i.). The expression of Ctsb and Ctsl was then analyzed by WB. (A) Immunoblots for Ctsb from one representative experiment. (B) Immunoblots for Ctsl from one representative experiment. (C) Summary of expression levels of Ctsb, described in arbitrary units (AU) relative to NT BMDC at 1 h. (D) As in (C), expression of Ctsl. The results are shown as mean ± SD from 3 independent experiments, *p<0.05.

proteases, identified by their apparent molecular weight (Fig.5.4 A). Among them were Ctsb, Ctsl, cathepsin S (Ctss), and cathepsin X (Ctsx). There were no significant differences in the expression of Ctss and Ctsx between WT, *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ BMDC (Fig. 5.4 B). Moreover, *Ctsb* $^{-/-}$ BMDC expressed comparable levels of Ctsl as WT BMDC, and *Ctsl* $^{-/-}$ BMDC expressed similar levels of Ctsb as WT BMDC. Therefore, Ctsb or Ctsl deficiency had no effect in the expression levels of Ctss and Ctsx.

5.4 Impact of cathepsin deficiency in uptake and processing of *L. major* promastigotes by BMDC

5.4.1 Processing of *L. major* promastigotes in BMDC in the presence of cathepsin inhibitors

EGFP-tg *L. major* promastigotes were used to monitor the amount of living *L. major* in BMDC and BMM. When BMDC were first analyzed by fluorescence microscopy, it was found that although an infection ratio equivalent to 5 parasites per BMDC was used, after 24h few cells bearing at least one parasite could be detected. The infection was then monitored by flow cytometry during different time points (Figure 5.5 A). While 2

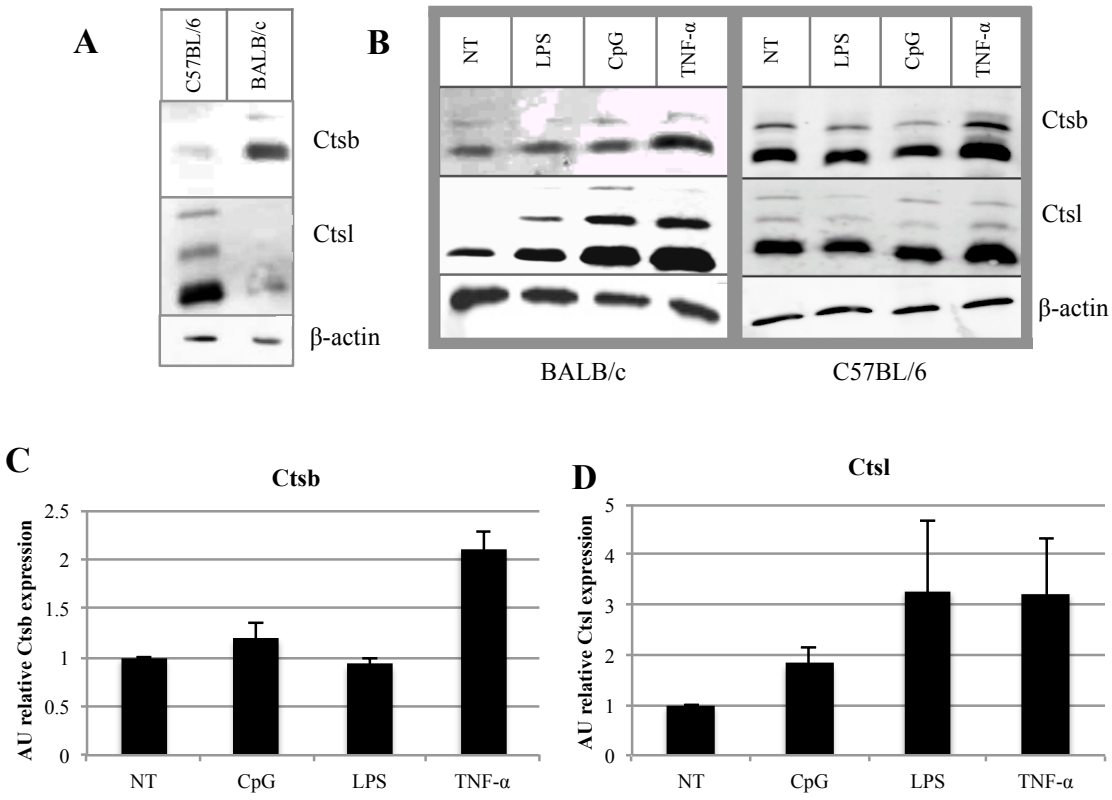

Figure 5.3. **Expression of Ctsb and Ctsl in BMDC in response to LPS, CpG, and TNF-α** (A) Expression levels of Ctsb and Ctsl in lysates from BALB/c and C57BL/6 BMDC, freshly harvested. (B) Immunoblots for Ctsb and Ctsl in lysates of BMDC from BALB/c and C57BL/6 mice, 24 h after stimulation with LPS, CpG or TNF-α. Non-treated cells (NT) were used as negative control. (C) Summary of expression levels of Ctsb in BALB/c BMDC lysates, described in arbitrary units (AU) relative to NT BMDC. (D) As in (C), expression of Ctsl. The results are shown as mean ± SD from cells generated out of 3 independent experiments.

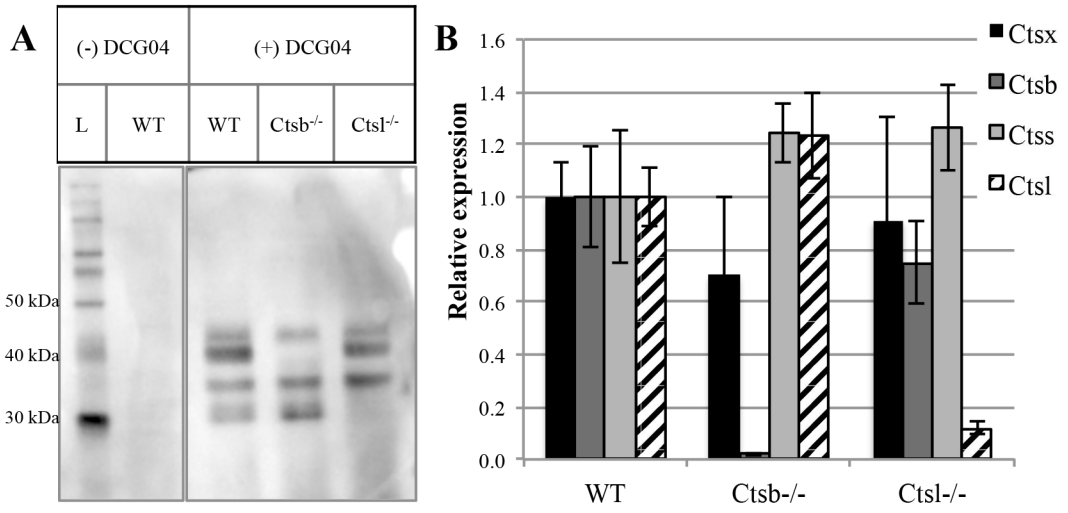

Figure 5.4. **Expression of cysteine proteases in *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ BMDC**. (A) Active-site labeling of multiple cysteine cathepsins using biotinylated DCG04 in lysates of wild-type (WT), *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ BMDC. The image corresponds to one representative experiment out of 3. (B) Determination of relative expression levels of cathepsins X, B, S, and L in lysates from WT, *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ BMDC. The results are shown as mean ± SD from lysates prepared from 3 different animals per genotype

h p.i. up to 65% BMDC were positive for infection, this proportion gradually decreases over time, and 24 h later, less than 15% of the BMDC were still positive for infection. This suggests that *L. major* promastigotes do not replicate inside BMDC, but are rather processed during this timeframe.

Next, the effect of different cathepsin inhibitors in the aforementioned processing of *L. major* was evaluated. BMDC from C57BL/6 and BALB/c mice were pre-incubated with 10 μM of CA074Me, CS128, or RV212C, followed by infection with enhanced-green fluorescent protein (eGFP)-tg promastigotes, and the fraction of $CD11c^{+}$, $eGFP^{+}$ BMDC was used as a readout for infected cells. In BMDC from both mouse lines, the decrease in the percentage of infected cells over the course of 24 h was comparable, and no significant differences were found in BMDC pre-incubated with the cathepsin inhibitors tested (Fig.5.5 B and C). Therefore, the processing of *L. major* promastigotes in BMDC was not altered by the use of cathepsin inhibitors. Moreover, this experiment was repeated using the peptide-based cathepsin B inhibitor ZRLR. As observed with the epoxide- and aziridine-based inhibitors, ZRLR showed no effect in the processing of *L. major* promastigotes (Fig.5.5 D).

5.4.2 *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ BMDC present similar uptake and processing rate of promastigotes as WT BMDC

The uptake and processing of *L. major* promastigotes was analyzed in *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ BMDC, using the same infection setup as described above. As expected from the results in section 5.4.1, while up to 77% of WT BMDC are infected after 2 h, this proportion decreases over time, and by 24 h p.i. less than 15% of BMDC are still infected (Fig. 5.6 A). In agreement with the experiments using cathepsin inhibitors, *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$BMDC presented a similar decrease in the percentage of infected cells over the course of 24 h, with no significant difference with respect to WT BMDC (Fig. 5.6 B).

5.5 Parasite survival in *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ *macrophages*

5.5.1 *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ present smilar parasite survival as WT BMM

The cathepsin inhibitors used in the present study target murine cathepsins as well, and some of them, like CS128 and RV212C, also showed leishmanicidal activity. Therefore it was necessary to evaluate if host Ctsb and Ctsl could have a beneficial or detrimental effect on the survival of *L. major* in infected BMM. With this aim, the proliferation of *L. major* in BMM was analyzed using two different approaches: (1) infection with eGFP-tg promastigotes, followed by monitoring using fluorescence microscopy, and (2) infection

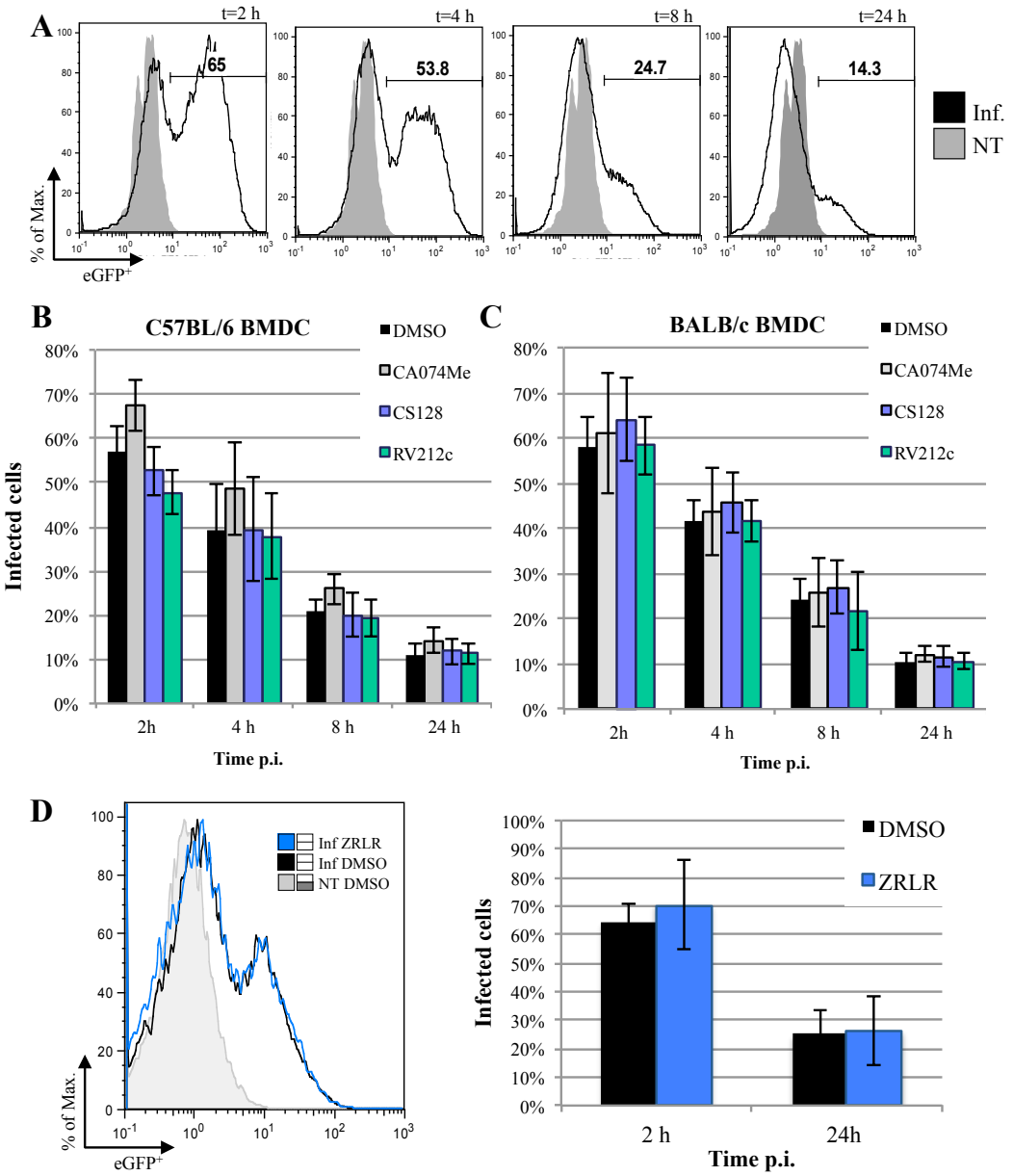

Figure 5.5. **Processing of *L. major* promastigotes in BMDC in the presence of cathepsin inhibitors** (A) Representative histograms from one experiment with BALB/c BMDC, infected with eGFP-tg promastigotes in the presence of DMSO (0.1% v/v) at different time points. (B) Processing of eGFP-tg promastigotes in the presence of different cathepsin inhibitors by C57BL/6 BMDC and (C) BALB/c BMDC. The results are expressed as mean ± SD of 3 independent experiments. (D) Representative histogram of infected BALB/c BMDC in the presence or absence of the cathepsin B inhibitor ZRLR, and summary from 3 independent experiments.

with Luc-tg promastigotes, having luciferase activity as an indicator for parasite viability and proliferation as described in section 5.2.2.

The percentage of infected cells at 24 h and 48 h p.i. was comparable among WT, *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ BMM (Fig. 5.7 A). The number of parasites per infected cell increased between 24 h and 48 h p.i., indicating parasite proliferation (Fig. 5.7 B and C). No significant differences were found between WT, and *Ctsb* $^{-/-}$ or *Ctsl* $^{-/-}$ BMM. Similarly, it

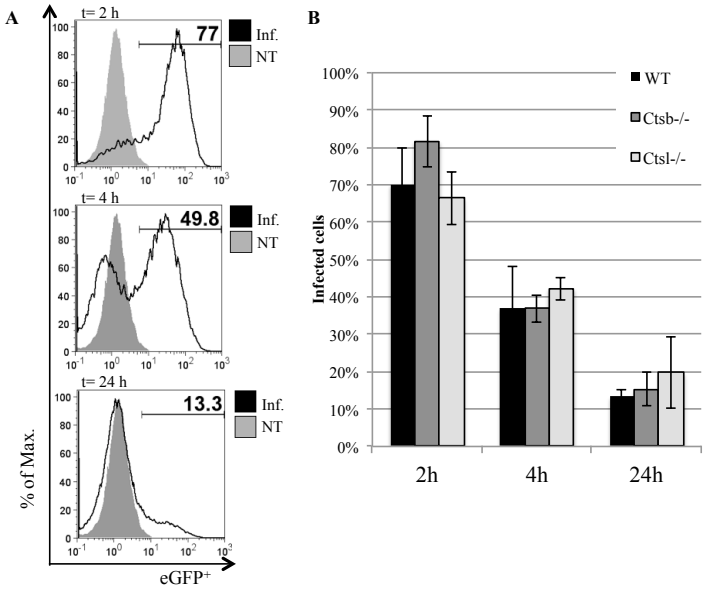

Figure 5.6. ***Ctsb*** $^{-/-}$ **and** ***Ctsl*** $^{-/-}$ **BMDC present similar uptake and processing rate of promastigotes as WT BMDC.** BMDC were infected for 2 h with eGFP-tg *L. major* promastigotes, and the percentage of infected cells was monitored over the course of 24 h. (A) Representative histogram from one experiment with WT BMDC. (B) No significant differences between BMDC from WT and cathepsin-deficient mice were found in the uptake and processing of eGFP-tg promastigotes at 2 h, 4 h, and 24 h. The results are expressed as mean ± SD of 3 independent experiments and can be found reported in [432]

was found that the proliferation of Luc-tg *L. major* 48 h p.i. was comparable between WT, and *Ctsb* $^{-/-}$ or *Ctsl* $^{-/-}$ BMM (Fig. 5.7 D).

Additionally, the morphology of infected BMM was analyzed by TEM. For each infected cell detected, a score representing its level of vacuolization was assigned, in order to evaluate if the BMM presented signs that would indicate autophagy. No significant differences were found in the vacuolization scores estimated for WT BMM, in comparison to *Ctsb* $^{-/-}$ or *Ctsl* $^{-/-}$ BMM (Fig. 5.8).

5.5.2 *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ BMM produce comparable levels of nitric oxide in response to *L. major* and to LPS

BMM use NO as an effector to eliminate intracellular pathogens. Since *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ BMM presented comparable levels of survival and proliferation of intracellular amastigotes, it would be expected that the levels of NO expressed in response to infection would be comparable to those of WT BMM. Therefore, the levels of NO produce by BMM were measured in supernatants after infection with *L. major*, stimulation with LPS, or infection with *L. major* followed by LPS stimulation. No significant differences in the levels of NO were detected between WT, *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ BMM, with neither of the stimuli used (Fig. 5.9), indicating that Ctsb and Ctsl deficiency have no effect in the

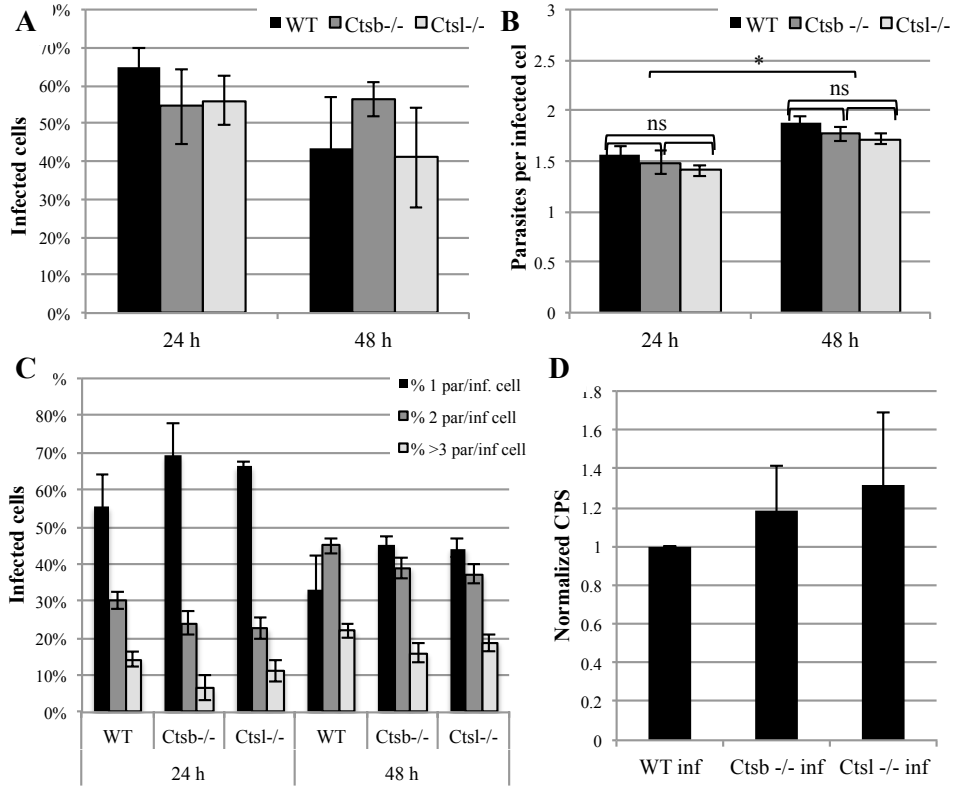

Figure 5.7. ***Ctsb*** $^{-/-}$ **and** ***Ctsl*** $^{-/-}$ **BMM present similar parasite survival as WT BMM.** WT, *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ BMM where infected with eGFP-tg *L. major* promastigotes, and the percentage of infected cells was determined by fluorescence microscopy at 24 h. and 48 h. post-infection (p.i.). (A) Percentage of infected cells, determined by fluorescence microscopy. (B) As in (A), the number of parasites per infected cells was determined by fluorescence microscopy. No significant differences in parasite numbers were found among WT, *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ BMM, although the average number of parasites per infected cell increased from 24 h. p.i. and 48 h p.i.(C) As in (B), percentage of infected cells harboring 1, 2, or 3 or more parasites. (D) BMM where infected with Luc-tg *L. major*, and the luminescence detected was used as a reference for parasite survival within the infected cell at 48 h. p.i., here reported as counts per second (CPS). In all the graphs, the results are expressed as mean ± SD of 3 independent experiments (*p$<$0.05) and can be found reported in [432]

activity of inducible iNOS.

5.6 Effect of cathepsin inhibitors on BMDC maturation

The recognition of a potential pathogen triggers the maturation of BMDC, characterized by an increase in the expression of antigen-presenting MHC class II molecules and co-stimulatory molecules in their surface. In this section, BMDC are treated with different cathepsin inhibitors in order to evaluate their effect in BMDC maturation. The first part of this section concentrates on the maturation of BMDC in response to *L. major* promastigotes and soluble antigen (LmAg). The second part focuses on the maturation of BMDC stimulated with LPS or TNF-α in the presence of cathepsin inhibitors.

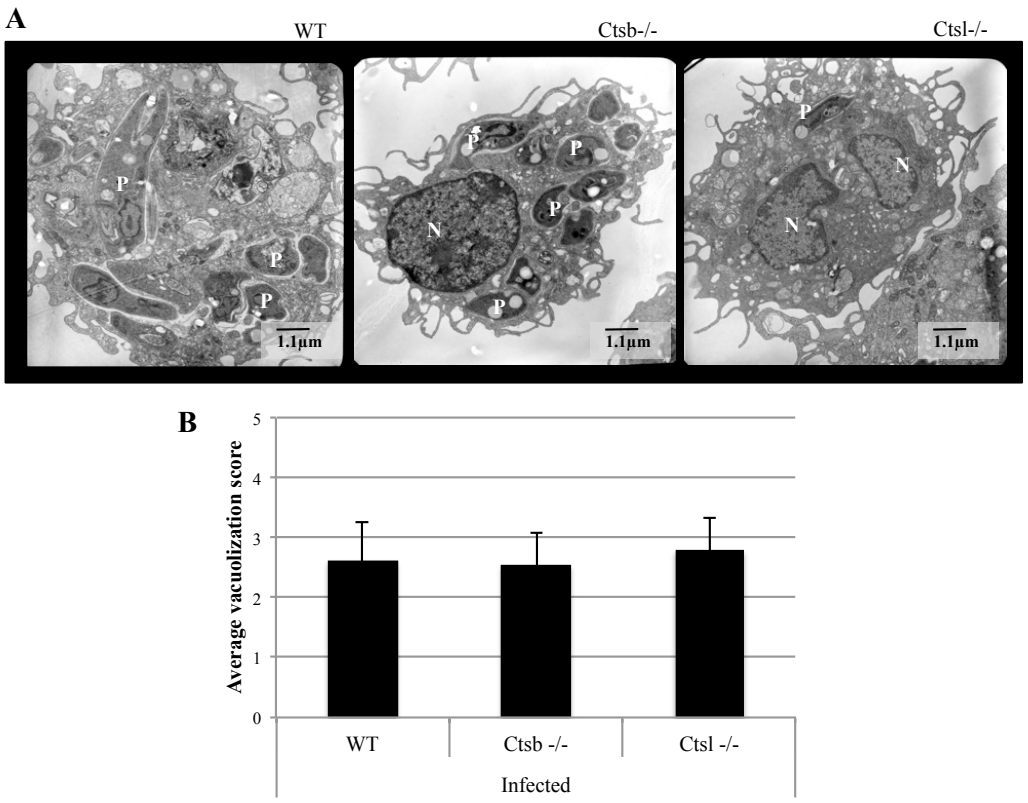

Figure 5.8. **Infected *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ BMM showed comparable levels of vacuolization as WT BMM.** Samples from infected BMM where analyzed by TEM, and vacuolization scores from 0 to 5 where assigned depending on the approximate percentage of cell surface occupied by vacuoles. (A) Representative pictures from WT, *Ctsb* $^{-/-}$, and *Ctsl* $^{-/-}$ BMM 24 h. p.i. with *L. major* promastigotes. N: cell nucleus, P= parasite. (B) Summary of vacuolization scores from 100 BMM from each mouse line.

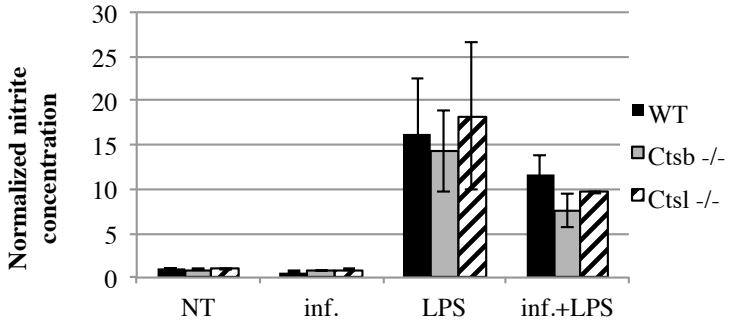

Figure 5.9. ***Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ BMM produce comparable levels of nitrite oxide in response to *L. major* and to LPS as WT BMM.** Nitric oxide (NO) production in supernatants from BMM, 48 h. p.i. with *L. major* or stimulation with LPS. The results are reported as mean ± SD of 3 independent experiments, and can be found reported in [432]

5.6.1 BMDC pre-treated with the cathepsin B inhibitor CA074Me up-regulate MHC class II molecule expression in response to *L. major* promastigotes

BMDC use three key signals to induce the polarization of naïve Th cells: (1) the presentation of antigen in MHC class II molecules, (2) the expression of co-stimulatory

molecules, and (3) the production of cytokines. In order to investigate the effect of cathepsin inhibitors in the first two signals, BMDC were pre-incubated with 10 μM of different cathepsin inhibitors, infected with *L. major* as described in the previous sections, and the expression levels of MHC class II molecules and co-stimulatory molecules were analyzed by flow cytometry.

First, the inhibitors CA074Me and CLIK148 were analyzed, as their effects on *L. major* infection have been previously documented *in vivo*. These inhibitors had no significant effect in the expression levels of MHC classs II molecules and CD86 in non-infected BMDC. However, infected BMDC pre-treated with CA074Me expressed higher levels of MHC class II molecules, but not of CD86, in comparison with BMDC pre-treated with CLIK148 and DMSO (Fig. 5.10). The inhibitors CS128 and RV212C showed no effect in the expression of MHC class II, and CD86 (Fig. 5.11). Similarly, CA074Me enhanced the expression of MHC class II, but not of co-stimulatory molecules in BMDC stimulated with LmAg (Fig.5.12). On the other hand, the inhibitors CLIK148, CS128, and RV212C showed no effect in the expression neither of MHC class II molecules, nor of co-stimulatory molecules in BMDC stimulated with LmAg.

5.6.2 Effects of cathepsin inhibition in the response to LPS and TNF-α by BMDC

LPS is a TLR4 agonist and strong maturation inducer in BMDC, which in turn provide signals for Th1 maturation. BMDC stimulated with TNF-α, on the other hand, have been reported as inducers for Th2 maturation. Being these stimuli characteristic "pro-Th1" or "pro-Th2" inducers, the effects of cathepsin inhibitors in BMDC maturation to LPS and TNF-α were thus evaluated.

Except for CA074, all the inhibitors tested using LPS as a stimulus showed a detrimental effect in the expression of MHC class II molecules. However, only CA074Me and RV212C resulted in a decrease of the expression of CD86, but not of CD80 or CD40 (Fig. 5.13). When TNF-α was used as a stimulus, CA074Me pre-treatment resulted rather in an enhancement of the expression of MHC class II molecules. CA074Me showed no significant effect in the expression of CD86 and CD40, and only a slight decrease in expression of CD80. The inhibitors RV212C and CS128 showed no significant effect neither in the expression of MHC class II molecules, nor of co-stimulatory molecules (Fig. 5.14).

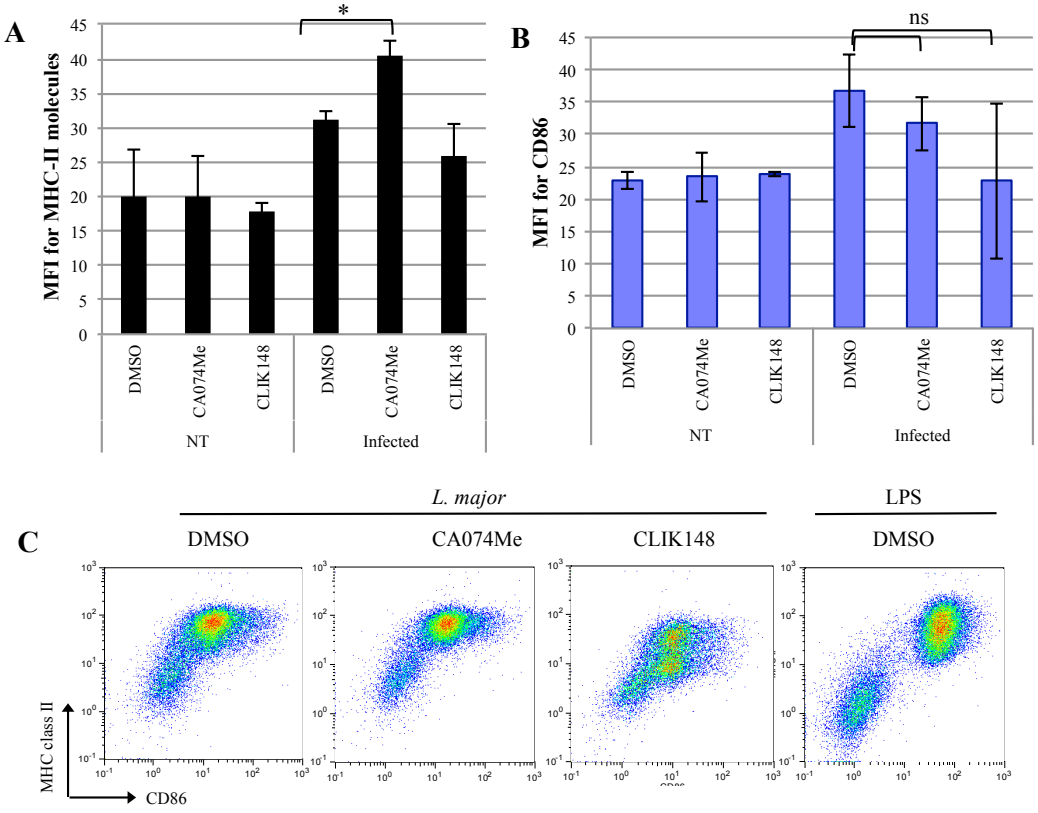

Figure 5.10. **BMDC pre-treated with the cathepsin B inhibitor CA074Me up-regulate the expression of MHC class II molecules in response to *L. major* promastigotes.** BALB/c BMDC where pre-incubated with 10 μM CA074Me, 10 μM CLIK148, or an equivalent volume of DMSO (0.01% v/v), and infected with *L. major* promastigotes. (A) Expression of MHC class II molecules 24 h p.i. (B) Expression of CD86 24 h p.i. The chart bars represent the mean $\pm$ SD of 3 independent experiments, *p$<$0.05. (C) Representative density dot plots depicting the maturation of infected BMDC, treated with DMSO, CA074Me, or CLIK148. LPS stimulation is also shown as a positive control.

A summary of the effects of cathepsin inhibitors in BMDC subjected to infection and the aforementioned stimuli can be found in Table 5.3. The responses observed would suggest that the effects can be grouped in two categories: semi-maturation stimuli (*L. major* infection, LmAg, and TNF-α), and strong maturation stimulus (LPS). When a semi-maturation stimulus is used, only CA074Me has an impact in MHC class II molecule expression, which is enhanced, but there is no dramatic effect in the expression of co-stimulatory molecules. Moreover, CLIK148, RV212C, and CS128 had no impact in the maturation of BMDC. The use of cathepsin inhibitors in a strong maturation stimulus, such as LPS, results rather in a detrimental effect on maturation.

5.7 Maturation of *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ deficient BMDC

The effects of cathepsin inhibition in BMDC maturation were next compared to the maturation of *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ BMDC in response to *L. major* infection, as well as

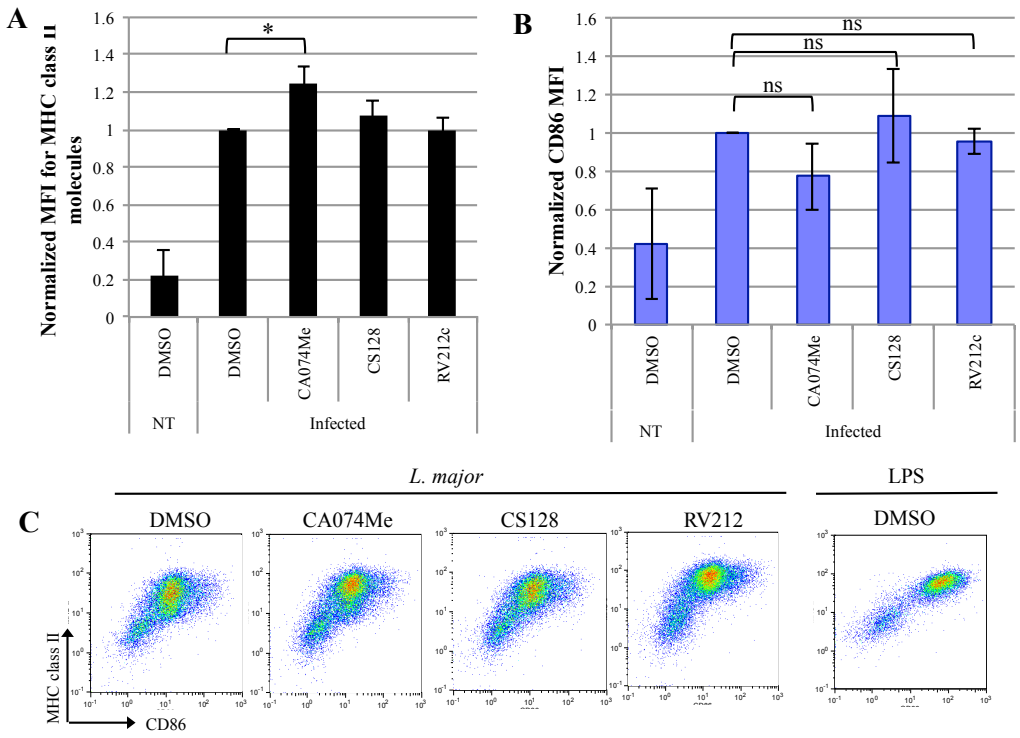

Figure 5.11. **The aziridine-based cathepsin inhibitors CS128 and RV212C have no effect in the maturation of BMDC in response to *L. major* promastigotes.**(A) Effect of different cathepsin inhibitors on the expression of MHC class II molecules and (B) CD86, as determined by their mean fluorescence intensity (MFI) normalized to the values obtained for BALB/c BMDC infected and treated with DMSO. All inhibitors were tested at a 10 μM concentration. The chart bars represent the mean $\pm$ SD of 3 independent experiments, *p$<$0.05. (C) Representative density dot plots depicting the maturation of infected BMDC, treated with DMSO, CA074Me, CS128, or RV212C. LPS stimulation is also shown as a positive control.

different pro-Th1 and pro-Th2 stimuli.

5.7.1 *Ctsb*$^{-/-}$ BMDC express higher levels of MHC class II molecules in response to *L. major* than WT and *Ctsl*$^{-/-}$deficient BMDC

BMDC from WT, *Ctsb*$^{-/-}$ and *Ctsl*$^{-/-}$ mice were infected with *L. major* promastigotes as described in the previous sections, and the expression of MHC class II molecules was determined by flow cytometry. Similarly as observed with the cathepsin B inhibitor CA074Me in section 5.6.1, *Ctsb*$^{-/-}$ expressed higher levels of MHC class II molecules. Although the *Ctsl*$^{-/-}$ inhibitor CLIK148 had no effect on the expression of MHC class II molecules, *Ctsl*$^{-/-}$ also expressed higher levels of MHC class II molecules than WT BMDC, although slightly lower than observed in *Ctsb*$^{-/-}$ BMDC (Fig. 5.15).

The expression levels of MHC class II molecules in response to LmAg were lower than observed in infected BMDC. While CA074Me pre-treated BMDC expressed more MHC class II molecules than those pre-treated with DMSO (see section 5.6.1), *Ctsb*$^{-/-}$ expressed

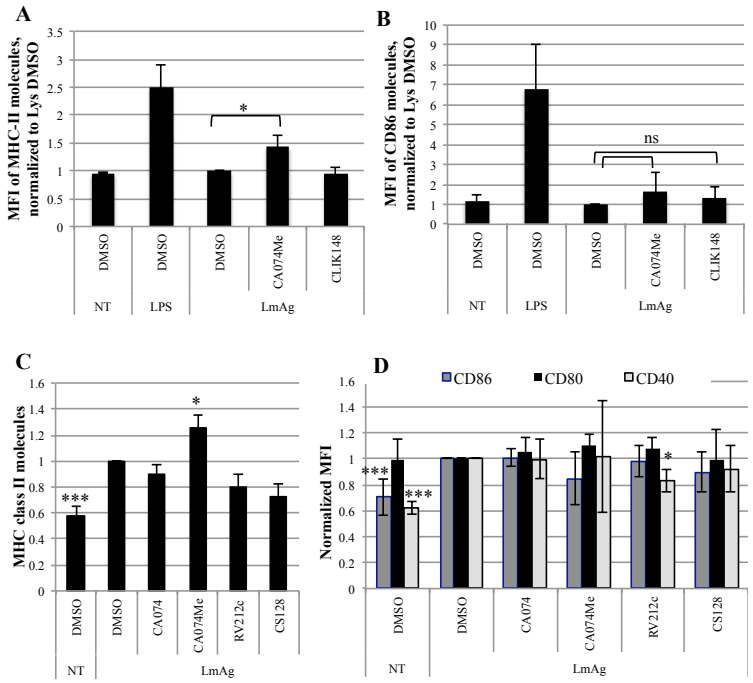

Figure 5.12. **CA074Me, but not CS128 and RV212C, enhances the up-regulation of MHC class II molecules in BMDC in response to *L. major* soluble antigen (LmAg).** BALB/c BMDC were pre-incubated with DMSO, CA074Me or CLIK148, and stimulated with LmAg for 24 h. (A) Expression of MHC class II molecules, expressed as the MFI normalized to the values of DMSO+LmAg treated cells. (B) As in (A), expression of CD86. (C) Normalized MFI for MHC class II molecules in BMDC pre-incubated with CA074, CA074Me, RV212C, and CS128, and stimulated with LmAg. (D) As in (C), normalized MFI for CD40, CD80, and CD86 expression. In all the graphs, the results are expressed as mean ± SD of 3 independent experiments, and the significance was estimated for every treatment *versus* DMSO + LmAg treated cells; *$p<0.05$, ***$p<0.005$.

comparable levels of MHC class II molecules as their WT and *Ctsl* $^{-/-}$ counterparts. In response to heat-killed parasites, BMDC expressed more MHC class II molecules than those in response to LmAg. However, no significant differences were found among WT, *Ctsb* $^{-/-}$, and *Ctsl* $^{-/-}$ BMDC. Moreover, in the absence of stimulation, non-treated WT BMDC expressed similar levels of MHC class II molecules as *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ BMDC (Fig. 5.15).

5.7.2 Cathepsin B and L deficiency have no effect on the expression of co-stimulatory molecules in BMDC in response to *L. major*

In section 5.6, it was shown that the different cathepsin inhibitors used in the present study showed no significant effect in the expression of co-stimulatory molecules by BMDC in response to infection with *L. major*. Similarly, neither *Ctsb* $^{-/-}$ nor *Ctsl* $^{-/-}$ presented differences in their expression of CD86, CD80, and CD40 in response to promastigotes (Fig. 5.16). Upon use of LmAg or heat-killed parasites as stimuli, *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ BMDC expressed comparable levels of co-stimulatory molecules as their WT counterparts (Fig. 5.16).

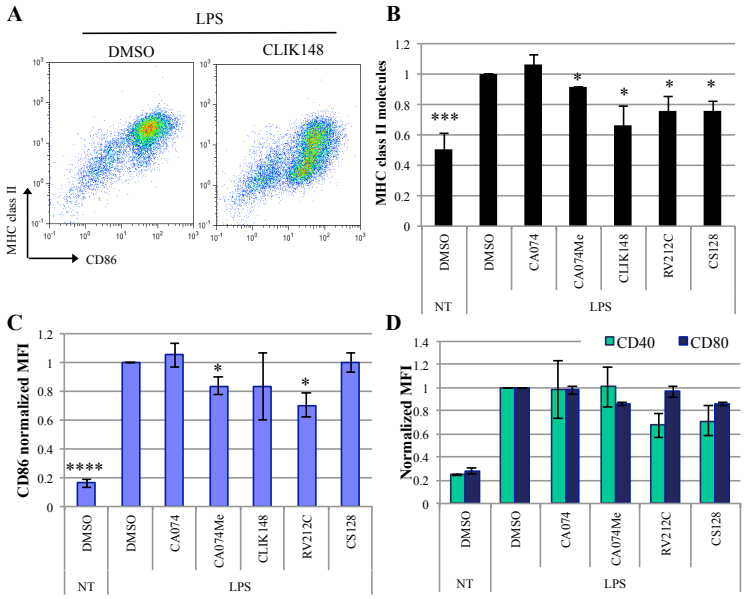

Figure 5.13. **Effect of different cathepsin inhibitors in the maturation of BMDC in response to LPS.** BMDC were pre-incubated with 10 μM of different cathepsin inhibitors or DMSO, and further stimulated with LPS for 24 h. (A) Representative density plots of LPS stimulated BMDC in the presence of DMSO or CLIK148. (B) MFI of MHC class II molecules, normalized to DMSO + LPS-treated cells. (C) As in (B), normalized MFI of CD86. (D) As in (C), normalized MFI of CD40 and CD80. The bars represent the mean ± SD of 3 independent experiments, and the significance was estimated for every treatment *versus* DMSO + LPS treated cells; *p<0.05, ***p<0.005, ****p<0.001.

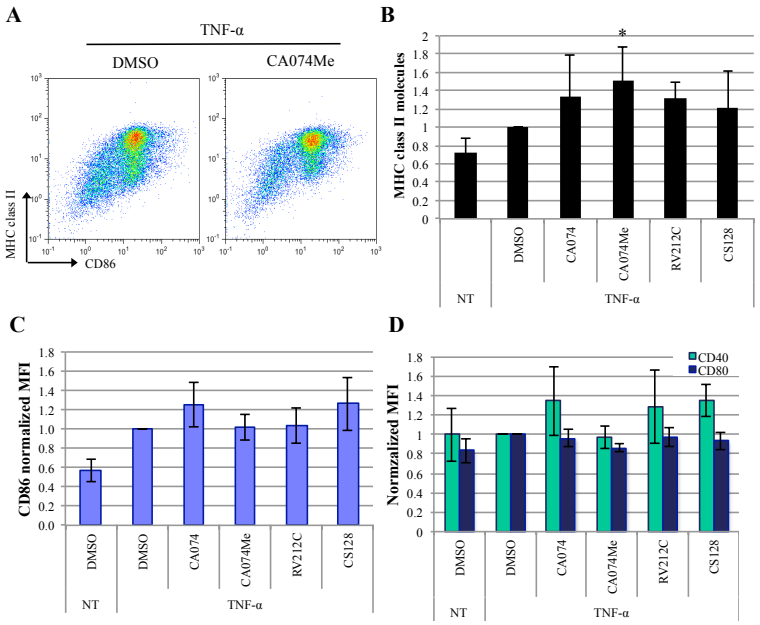

Figure 5.14. **Effect of different cathepsin inhibitors in the maturation of BMDC in response to TNF-α.** BMDC were pre-incubated with 10 μM of different cathepsin inhibitors or DMSO, and further stimulated with TNF-α for 24 h. A) Representative dot plots of TNF-α-stimulated BMDC in the presence of DMSO or CLIK148. (B) MFI of MHC class II molecules, normalized to DMSO + TNF-α-treated cells. (C) As in (B), normalized MFI of CD86. (D) As in (C), normalized MFI of CD40 and CD80. The bars represent the mean ± SD of 3 independent experiments, and the significance was estimated for every treatment *versus* DMSO + TNF-α-treated cells; *p<0.05.

Table 5.3. **Overview of the effects of cathepsin inhibitors on BMDC maturation in response to different stimuli.** (—) Indicates that no statistically significant effect was detected in 3 independent experiments.

Stimulus	Inhibitor	Effect			
		MHC Class II	CD86	CD80	CD40
Non-treated	CA074	—	—	—	—
	CA074Me	—	—	—	—
	CLIK148	—	—	—	—
	RV212C	—	—	—	—
	CS128	—	—	—	—
L. major	CA074	—	—	—	—
	CA074Me	Increase	—	—	—
	CLIK148	—	—	—	—
	RV212C	—	—	—	—
	CS128	—	—	—	—
LmAg	CA074	—	—	—	—
	CA074Me	Increase	—	—	—
	CLIK148	—	—	—	—
	RV212C	—	—	—	Decrease
	CS128	—	—	—	—
LPS	CA074	—	—	—	—
	CA074Me	Decrease	Decrease	Decrease	—
	CLIK148	Decrease	—	—	—
	RV212C	Decrease	Decrease	—	Decrease
	CS128	Decrease	—	Decrease	Decrease
TNF-α	CA074	—	—	—	—
	CA074Me	Increase	—	Decrease	—
	CLIK148	—	—	—	—
	RV212c	—	—	—	—
	CS128	—	—	—	—

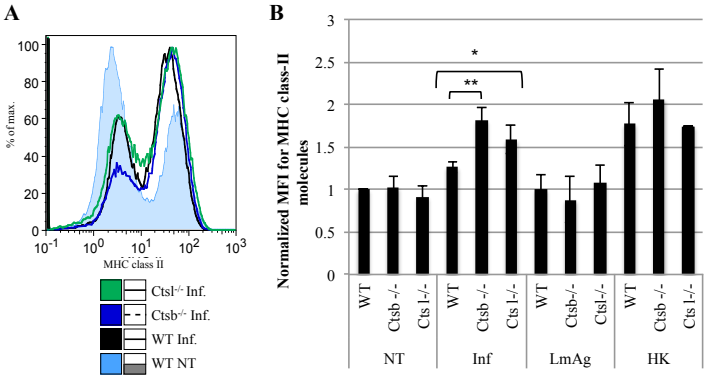

Figure 5.15. **textitCtsb $^{-/-}$ BMDC express higher levels of MHC class II molecules in response to L. major than wild-type, and *Ctsl* $^{-/-}$ BMDC.** (A) Representative histogram for MHC class II molecule expression in BMDC 24 h p.i. with *L. major* promastigotes. (B) Normalized MFI for MHC class II molecules 24 h after stimulation with LmAg, heat-killed parasites (HK), or infection with promastigotes. The obtained MFI values were normalized to WT, non-treated (NT) BMDC. The bars represent the mean ± SD of 3 independent experiments, and the significance in every treatment was estimated between WT and *Ctsb* $^{-/-}$ BMDC, and between WT and *Ctsl* $^{-/-}$BMDC; * p<0.05, **p<0.01.

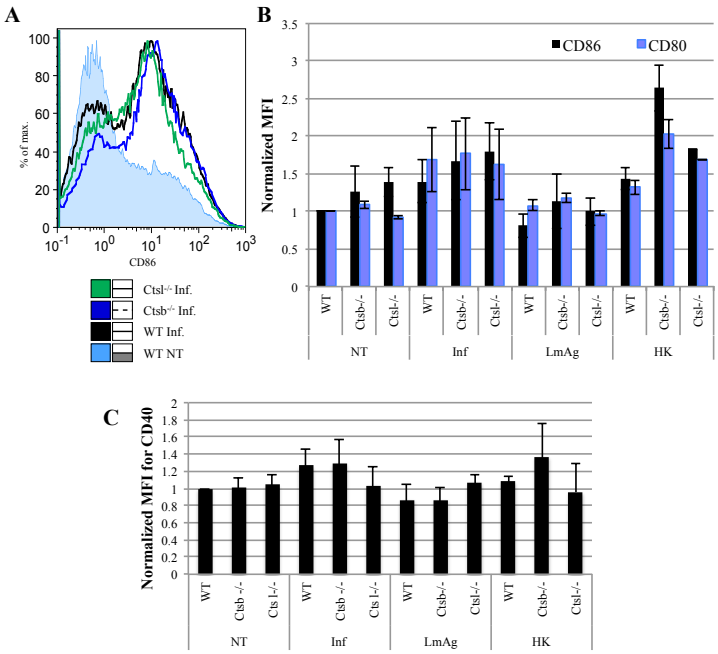

Figure 5.16. **Wild-type and cathepsin-deficient BMDC express comparable levels of costimulatory molecules in response to *L. major*.** (A) Representative histogram for CD86 expression in BMDC 24 h p.i. with *L. major* promastigotes. (B) Normalized MFI for CD80, and CD86 24 h after stimulation with LmAg, heat-killed parasites (HK), or infection with promastigotes. (C) As in (B), normalized MFI of CD40. The obtained MFI values were normalized to WT, non-treated (NT) BMDC. The bars represent the mean ± SD of 3 independent experiments

5.7.3 Cathepsin B and L deficiency have no effect on the maturation of BMDC in response to LPS

The effects of Ctsb and Ctsl deficiency were next evaluated using LPS as a stimulus. Unlike the results observed using cathepsin inhibitors, which showed in general a detrimental effect on the maturation of BMDC, neither *Ctsb* $^{-/-}$ nor *Ctsl* $^{-/-}$ BMDC expressed reduced levels of MHC class II molecules (Fig. 5.17 A), and of co-stimulatory molecules (Fig. 5.17 and C).

5.8 Effect of cathepsin inhibitors on cytokine expression

Besides the presentation of antigen in MHC class II molecules and the expression of co-stimulatory molecules, a key signal BMDC use for Th cell polarization is the expression of different cytokines. The following sections -from 5.8 to 5.12- summarize the effect of cathepsin inhibitors in the expression of cytokines relevant for Th1 or Th2 polarization, and the cytokines expressed by cathepsin-deficient BMDC.

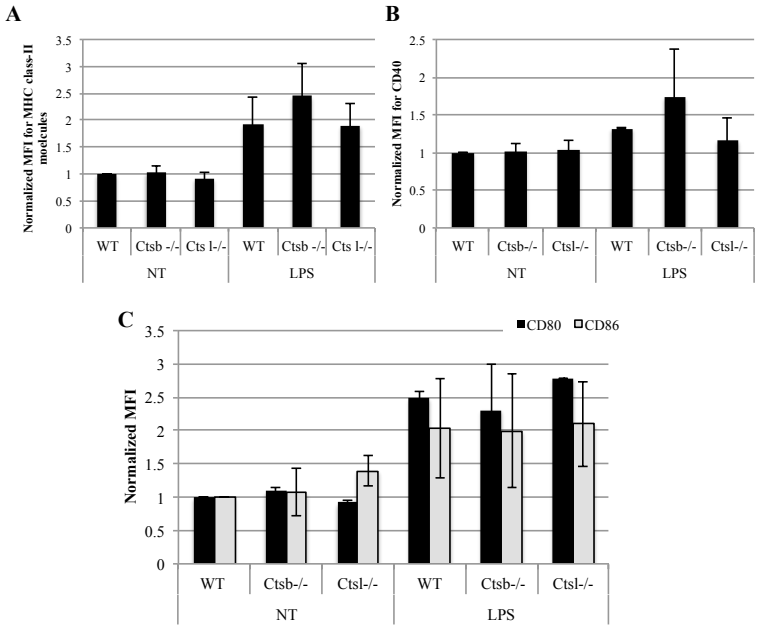

Figure 5.17. **Cathepsin-deficient BMDC express levels of MHC class II molecules and co-stimulatory molecules in response to LPS comparable to wild-type BMDC.** (A) Normalized MFI of MHC class II molecules in BMDC, 24 h after stimulation with LPS. (B) As in (A), normalized MFI of CD40, and (C) CD80, and CD86. All MFI values were normalized to WT NT BMDC. The bars represent the mean ± SD of 3 independent experiments.

5.8.1 The tested cathepsin inhibitors have a negative effect on IL-12 expression

IL-12 is a key inducer of Th1 polarization, and the response to LPS by BMDC is characterized by a high expression of IL-12. In order to analyze the effect of cathepsin inhibitors in BMDC stimulated with LPS, two approaches were used: (1) intracellular staining and analysis by flow cytometry, and (2) measurement of IL-12 in supernatants from stimulated cells.

Figure 5.18 summarizes the results obtained by intracellular staining of IL-12. With exception of CA074, all the cathepsin inhibitors tested resulted in a reduction of IL-12 expression. In particular, BMDC treated with CA074Me showed lower levels of IL-12 already at a concentration of 5 μM. The antibody used for intracellular staining recognizes both the p40 and p70 forms of IL-12. Being IL-12p70 the bioactive form relevant for inducing Th1 polarization, the levels of IL-12p70 in supernatants of LSP-stimulated BMDC were measured by ELISA. In agreement with the results found by intracellular staining, the cathepsin inhibitors tested –with the exception of CA074- resulted in lower levels of IL-12p70 BMDC treated with DMSO (Figure 5.19). In contrast, most of the cathepsin inhibitors that showed a down-regulation of IL-12p70 expression had no effect on IL-10 expression. CA074Me, however, also induced a dose-dependent decrease in IL-10 expression.

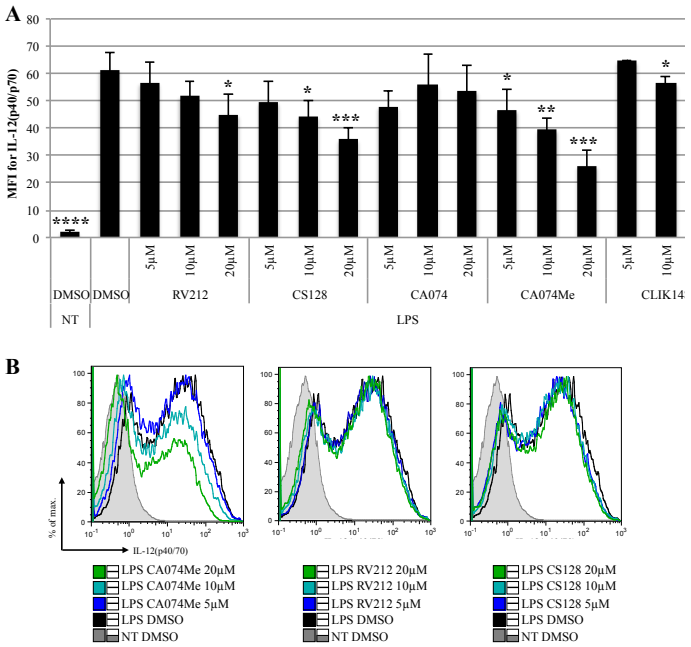

Figure 5.18. **The tested cathepsin inhibitors have a negative effect on the expression of IL-12 by BMDC in response to LPS.** (A) MFI of IL-12(p40/p70), determined by intracellular staining of BMDC in response to LPS after 24 h. The bars represent the mean ± SD of 3 independent experiments, and the significance in every treatment was estimated against DMSO+LPS treated BMDC; *p<0.05, **p<0.01, ***p<0.005. (B) Representative histograms of IL-12(p40/p70), detected by intracellular staining.

The negative effect of CA074Me in IL-12 expression was unexpected. IL-12 is key for induction of Th1 polarization; therefore, a decrease of IL-12 in BMDC treated with CA074Me would contradict the results reported in the literature vivo with cathepsin inhibitors.[340] Therefore, it was suspected that CA074Me at the concentrations used in our experiments could be also inhibiting other cysteine cathepsins besides Ctsb. The inhibitory capacity of CA074Me, RV212, and CS128 against Ctss was tested, and it was confirmed that indeed, all three inhibitors present some degree of unspecific inhibition of Ctss (see Appendix, Fig. A.3).

Furthermore, the effect in IL-12 expression of these cathepsin inhibitors was evaluated in BMDC infected with *L. major*. In preliminary experiments, no IL-12 could be detected 48 h p.i. by ELISA and intracellular staining of infected BMDC. Similarly, neither of the inhibitors tested –CLIK148, RV212, CA074, CA074Me, and CS128– induced detectable levels of IL-12 (data not shown).

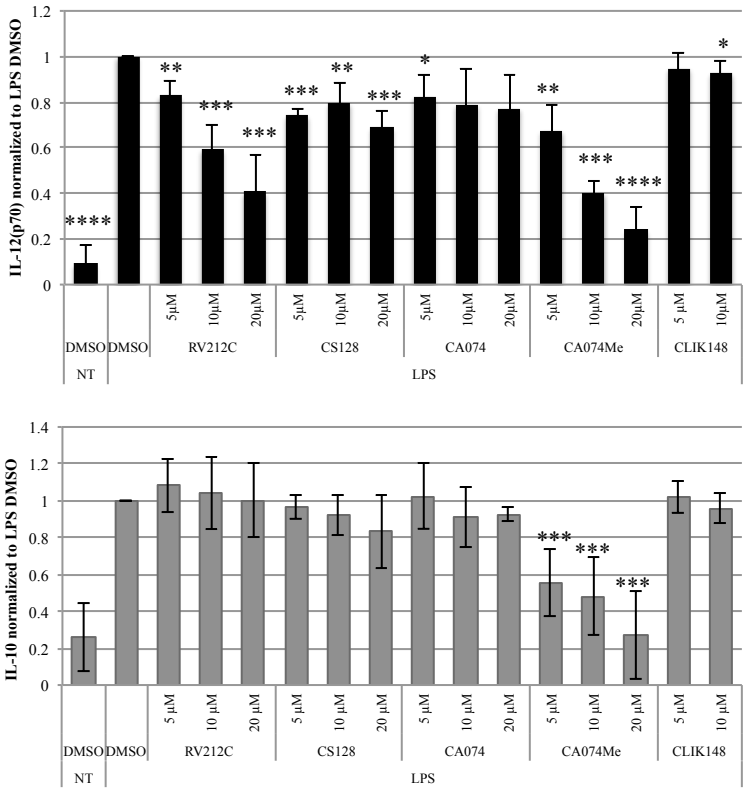

Figure 5.19. **Negative effect of cathepsin inhibitors in IL-12p70 expression in response to LPS.** (A) IL-12(p70) was measured by ELISA in supernatants from BMDC pre-treated with different cathepsin inhibitors, and stimulated for 48 h. with LPS. The obtained measurements were normalized to DMSO+LPS-treated BMDC. (B) As in (A), IL-10 measurements of BMDC in response to LPS, normalized to DMSO+LPS-treated BMDC. The results are presented as mean ± SD of 3 independent experiments, and the significance in every treatment was estimated against DMSO+LPS treated BMDC; *p<0.05, **p<0.01, ***p<0.005, ****p<0.001.

5.9 Effect of cathepsin inhibitors on *in vitro* Th1 polarization

A Th1 polarization assay was optimized, in order to determine if the cumulative effects of cathepsin inhibitors in BMDC could play a role in their ability to induce *in vitro* the polarization of naïve Th cells to Th1 cells. The standard protocols reported for this kind of assay require previous stimulation of BMDC with LPS, and to supplement the co-cultured BMDC and $CD4^{+}CD25^{-}$ T cells with IL-12. Because the cathepsin inhibitors tested had only a significant effect in IL-12 expression, but not in expression of MHC class II molecules and of co-stimulatory molecules, a protocol was adapted to require no addition of IL-12 (see section 4.11.1).

The results of these polarization assays are summarized in Fig. 5.20. While RV212 showed a slightly lower amounts of Th1 polarized cells in comparison to DMSO controls, treatment with CA074Me resulted in a marked decrease in Th1 polarization. On the

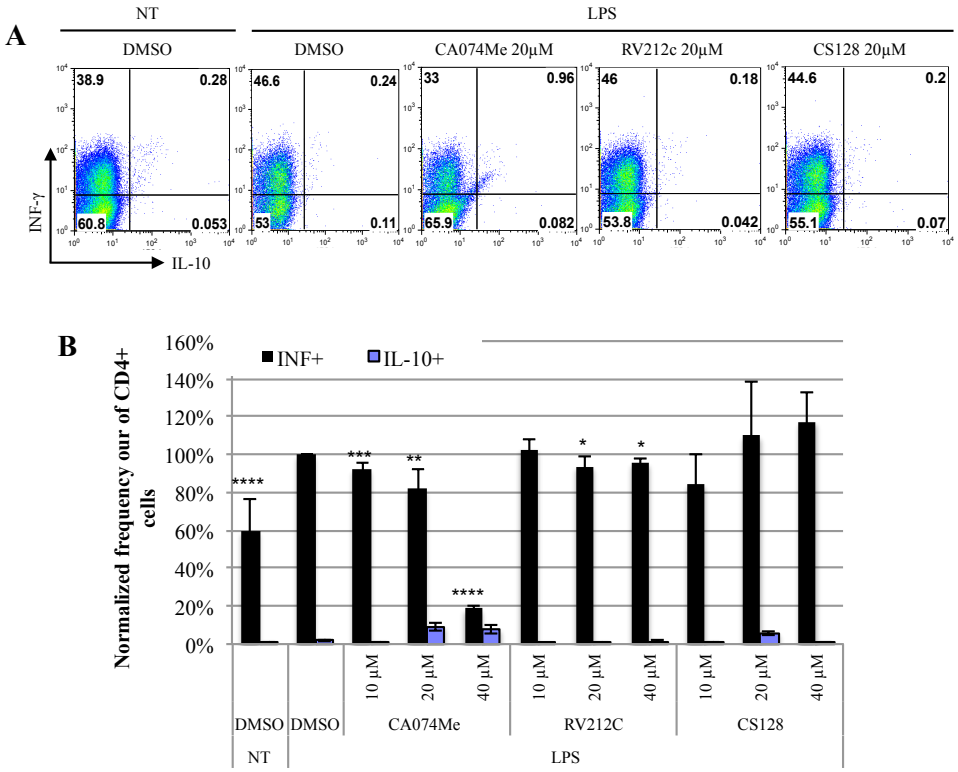

Figure 5.20. **Effect of cathepsin inhibitors on Th1 polarization *in vitro*.** . $CD4^+CD25^-$ T-cells were isolated from OT-II mice, and co-cultured with BMDC in the presence of ovalbumin, LPS, and different cathepsin inhibitors or DMSO for 5 days. (A) Density plots of $CD4^+$ gated cells at the end of the co-culture, stained intracellularly for IFN-γ and IL-10. (B) Frequency of IFN-γ^+ and IL-10^+ $CD4^+$ T cells, normalized to samples with LPS stimulation in the presence of DMSO. The results are presented as mean ± SD of 3 independent experiments, and the significance in every treatment was estimated against DMSO+LPS treated samples; *$p<0.05$,**$p<0.01$, ***$p<0.005$, ****$p<0.001$.

other hand, CS128 had no significant effect in the amount of Th1-polarized cells.

5.10 Cytokine expression in cathepsin-deficient BMDC and BMM

Analogous to the experiments using cathepsin inhibitors, BMDC from WT, *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ were used to assess the effects of Ctsb and Ctsl deficiency in cytokine expression.

5.10.1 *Ctsb* $^{-/-}$ BMDC and BMM express higher levels of IL-12 than WT and *Ctsl* $^{-/-}$BMDC and BMM in response to *L. major*

Pre-treatment of epoxide-based and aziridin-based cathepsin inhibitors did not result in an increase in IL-12 expression (see section 8.8.1). Therefore, it was expected that *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$BMDC would express similar levels of IL-12 as WT BMDC in response to *L.*

major. Surprisingly, *Ctsb* $^{-/-}$ BMDC expressed significantly higher levels of IL-12p70 and IL-12p40 than their WT and *Ctsl* $^{-/-}$ counterparts (Fig. 5.21 A and B). This response, however, was exclusive to living *L. major* promastigotes, since no up-regulation of IL-12 was observed in BMDC stimulated with LmAg or heat-killed parasites. Similarly, an increase in IL-10 expression was also observed in *Ctsb* $^{-/-}$ BMDC infected with *L. major* promastigotes, but not in *Ctsb* $^{-/-}$ BMDC stimulated with LmAg or heat-killed parasites (Fig. 5.21 C).

In the light of these results, the cytokine production of *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ BMM in response to *L. major* was measured. Similarly as BMDC, *Ctsb* $^{-/-}$ BMM expressed higher amounts of IL-12p70, IL-12p40, and IL-10 than WT and *Ctsl* $^{-/-}$ BMM (Fig. 5.21 D to F). Moreover, the expression of other cytokines, such as IL-6 and TNF-α in response to *L. major* was comparable among WT, *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ BMDC and BMM (Fig. 5.22).

The striking difference between the results observed using the Ctsb inhibitor CA074Me, and *Ctsb* $^{-/-}$ BMDC and BMM in response to *L. major* urged us to consider the use of a Ctsb inhibitor with a reported higher specificity for Ctsb than CA074Me. Therefore, the effect of the peptide-based inhibitor ZRLR in BMDC infected with *L. major* was tested. Pre-treatment with this inhibitor resulted in IL-12p70 expression levels in infected BMDC comparable as those observed *Ctsb* $^{-/-}$ BMDC, regardless of the mouse line from which the cells were generated. Moreover, pre-treatment of *Ctsb* $^{-/-}$ BMDC with this inhibitor had no further effect in their levels of IL-12p70 expression (Fig. 5.23).

5.10.2 *Ctsb* $^{-/-}$ BMDC express higher levels of IL-12 than WT BMDC in response to LPS

Epoxide- and aziridine-based inhibitors decreased the expression of IL-12 by BMDC in response to LPS (see section 4.8.1). Therefore, it would be expected that *Ctsb* $^{-/-}$ BMDC or *Ctsl* $^{-/-}$ BMDC, or both could also produce less IL-12 in response to LPS than WT BMDC. Contrary to this, *Ctsb* $^{-/-}$ BMDC expressed higher levels of IL-12 than WT and *Ctsl* $^{-/-}$ BMDC, and the later showed no significant impairment in IL-12 expression (Fig. 5.24 A). Moreover, there were no significant differences in the production of IL-6 (Fig. 5.24 B), although *Ctsl* $^{-/-}$ expressed slightly higher amounts of IL-10 than WT and *Ctsb* $^{-/-}$ BMDC (Fig. 5.24 C). Furthermore, *Ctsb* $^{-/-}$ BMDC produced considerably lower levels of TNF-α than WT and *Ctsl* $^{-/-}$ BMDC.

As described in the previous section, the effects were compared to BMDC pre-treated with the Ctsb inhibitor ZRLR. IL-12 production was higher in BMDC treated with ZRLR

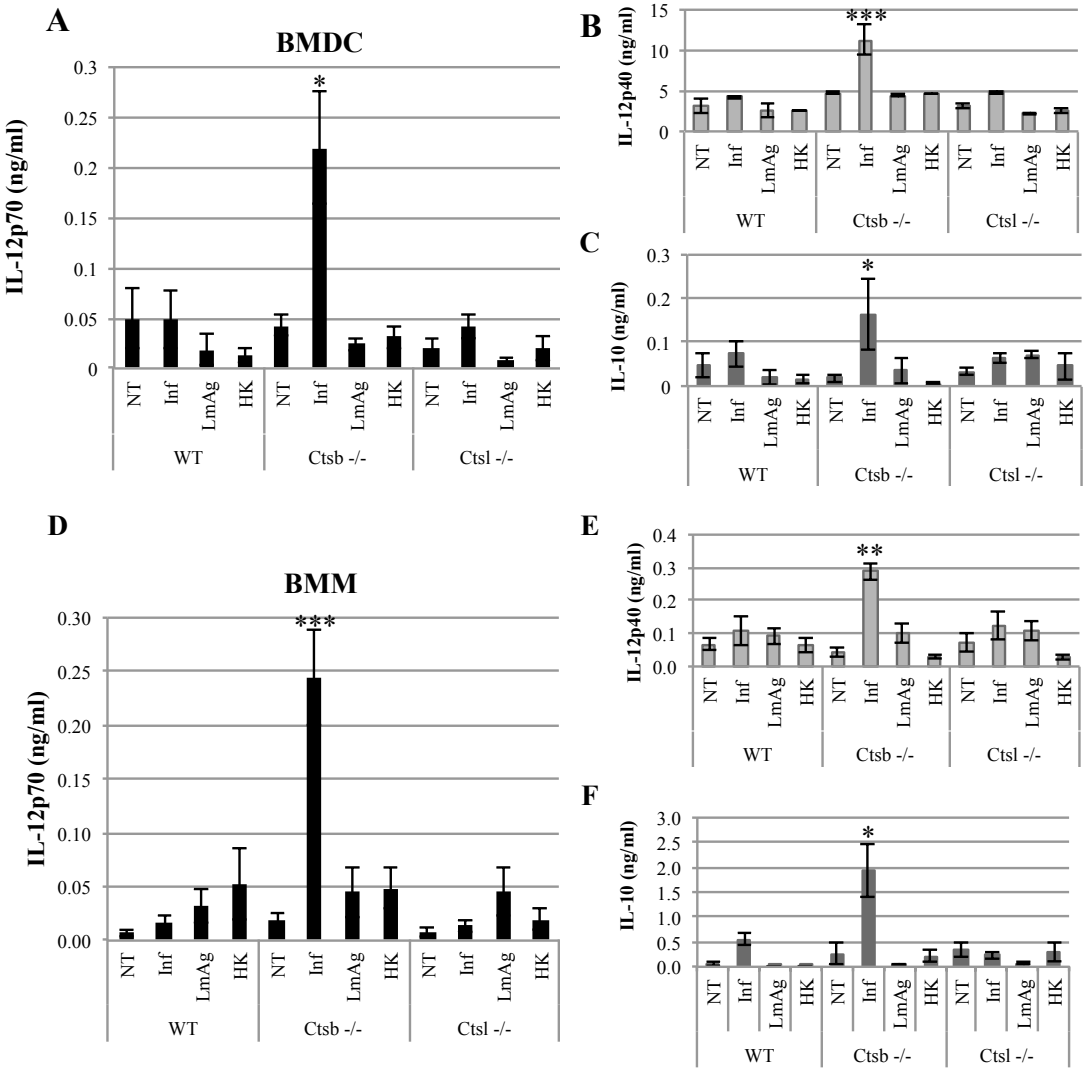

Figure 5.21. ***Ctsb*** $^{-/-}$ **BMDC and BMM express higher levels of IL-12 than** ***Ctsl*** $^{-/-}$ **and WT BMDC and BMM in response to** ***L. major*****.** (A) IL-12p70 in supernatants from non-treated BMDC (NT), BMDC infected (Inf) with *L. major* promastigotes at 48 hours p.i., BMDC stimulated with parasite lysate (LmAg), or with heat-killed parasites (HK), for 48 h. (B) IL-12p40 and (C) IL-10 concentration in supernatants of BMDC at 48 h p.i., or stimulation with LmAg or HK parasites. (D) IL-12p70 production in supernatants from non-treated BMM (NT), BMM infected (Inf) with *L. major* promastigotes at 48 h p.i., and BMM stimulated for 48 hours with LmAg or HK parasites. (E) IL-12p40 and (F) IL-10 concentration in supernatants of BMM at 48 hours p.i., or stimulation with either LmAg or HK parasites. The results are expressed as mean ± SD of 5 independent experiments, and can be found reported in ref. [432] . For each experimental group (NT, Inf, LmAg and HK), statistical significance was estimated between WT and *Ctsb* $^{-/-}$ cells, and between WT and Ctsl-/- cells, * $p<0.05$, ** $p<0.01$, *** $p<0.005$.

in comparison to the DMSO controls, independently of the mouse background from which the cells were generated (Fig. 5.25).

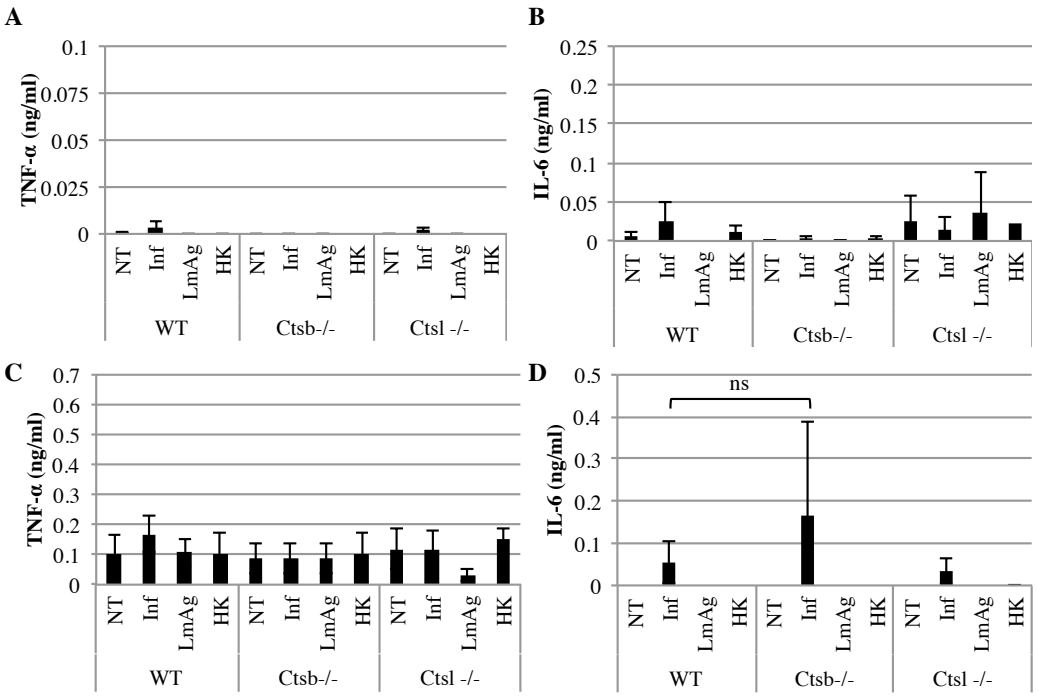

Figure 5.22. **Ctsb and Ctsl deficiency showed no effect in IL-6 and TNF-α expression in response to *L. major*.** A) TNF-α in supernatants from non-treated BMDC (NT), BMDC infected (Inf) with *L. major* promastigotes at 48 h p.i. and BMDC stimulated with parasite lysate (LmAg) or heat-killed parasites (HK) for 48 h. (B) IL-6 concentration in supernatants of BMDC at 48 h p.i. (C) TNF-α in supernatants from non-treated BMM (NT), BMM infected (Inf) with *L. major* promastigotes at 48 h p.i. and BMM stimulated with LmAg or HK for 48 hours. (D) IL-6 concentration in supernatants of BMM at 48 h p.i. The results are expressed as mean ± SD of 3 independent experiments. For each treatment (NT, Inf, LmAg, and HK), statistical significance was assessed between WT and *Ctsb* $^{-/-}$ cells, and between WT and *Ctsl* $^{-/-}$ cells, and in all cases no statistical significance was found ($p>0.05$). The results can be found reported in ref. [432] .

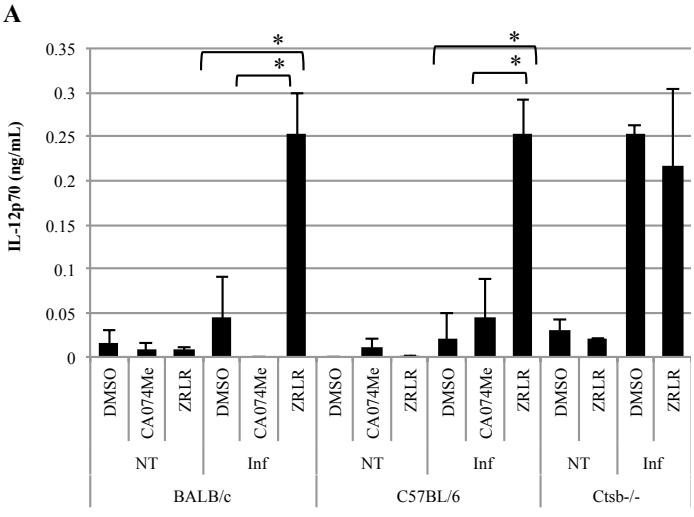

Figure 5.23. **RLR pre-treatment of BMDC results in higher levels of IL-12 expression in response to *L. major*.** Effect of CA074Me (10 *mu*M) and ZRLR (10 *mu*M) in IL-12(p70) expression by BMDC from BALB/c, C57BL/6 or *Ctsb* $^{-/-}$ mice in response to *L. major* promastigotes

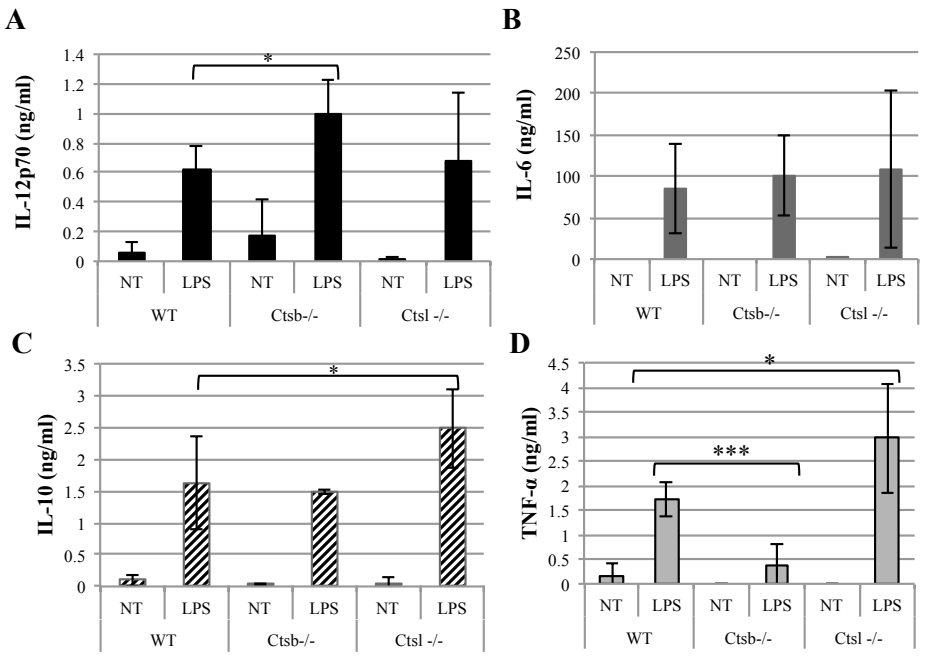

Figure 5.24. ***Ctsb*** $^{-/-}$ **BMDC express higher levels of IL-12 than** ***Ctsl*** $^{-/-}$ **and WT BMDC and BMM in response to LPS.**Concentration of different cytokines in supernatants from non-treated BMDC (NT) or LPS-stimulated BMDC (LPS, 1 μg/ml) after 24 hours: (A) IL-12p70, (B) IL-6, (C) IL-10, and (D) TNF-α. The results are expressed as mean $\pm$ SD of 5 independent experiments. The statistical significance in each treatment was assessed between WT and *Ctsb* $^{-/-}$ BMDC, and between WT and *Ctsl* $^{-/-}$ BMDC. * $p<0.05$, **$p<0.01$, *** $p<0.005$. The results can be found reported in ref.[432] .

5.10.3 IL-12 expression is impaired in cathepsin B-deficient BMDC upon CpG stimulation

CpG is another stimulus known to induce IL-12 expression in BMDC, and thus Th1 polarization. BMDC from *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ BMDC mice were stimulated with CpG, and their capacity to produce IL-12 was examined by ELISA. While *Ctsl* $^{-/-}$ BMDC showed similar expression of IL-12 as WT BMDC, *Ctsb* $^{-/-}$ BMDC presented a significant impairment in IL-12 production (Fig. 5.26).

5.11 The observed up-regulation in IL-12 in response to LPS in Ctsb-/- BMDC is enough to induce higher levels of Th1-polarized cells

Since *Ctsb* $^{-/-}$ BMDC expressed higher levels of IL-12 than WT and *Ctsl* $^{-/-}$BMDC, it was hypothesized that they might thus induce higher levels of Th1 polarization. Therefore, BMDC from WT and *Ctsb* $^{-/-}$ BMDC were co-cultured with $CD4^{+}CD25^{-}$ T-cells as described in section 8.9. *Ctsb* $^{-/-}$ BMDC having LPS as a maturation stimulus resulted in higher frequencies of IFN-γ^{+} T cells, but not of IL-4^{+} T cells than when WT BMDC were used, indicating a Th1 polarization (Fig. 5.27). This effect was found not only when

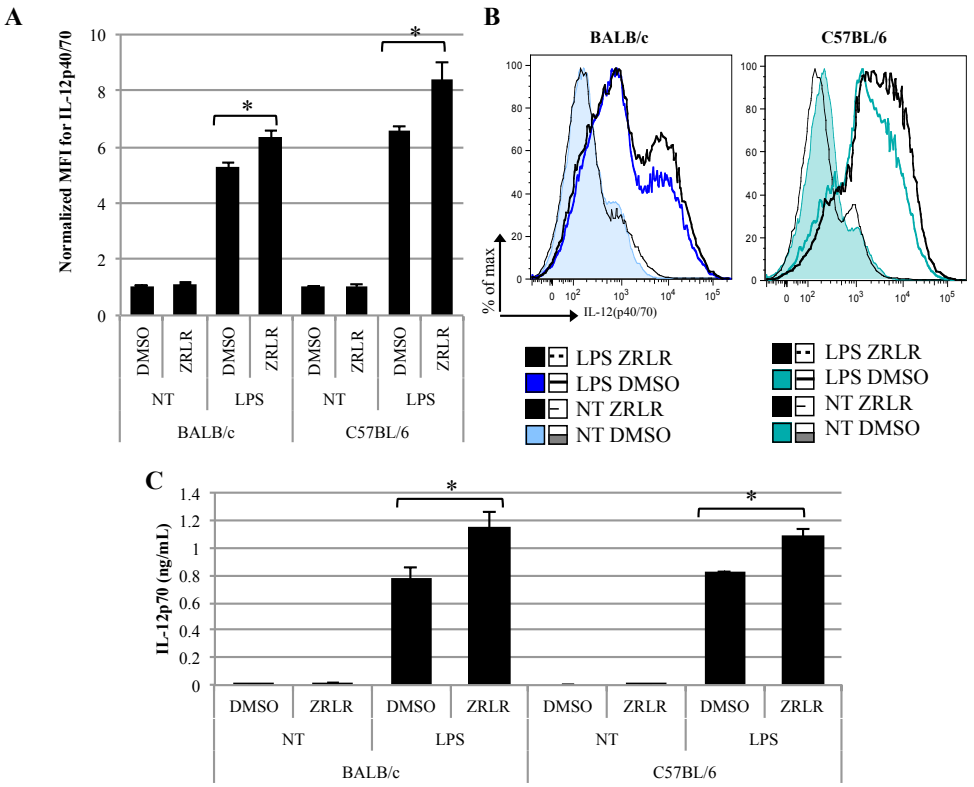

Figure 5.25. **ZRLR enhances the up-regulation of IL-12 production by BMDC in response to LPS.** (A) Normalized MFI for IL-12(p40/70) from BMDC pre-treated with ZRLR in response to LPS. (B) Histograms from a representative experiment, showing the expression of IL-12(p40/p70) by BMDC in response to LPS. (C) Effect of ZRLR in IL-12(p70) expression by BMDC from BALB/c and C57BL/6 mice in response to LPS. Results are presented as mean ± SD of 3 independent experiments, *p<0.05.

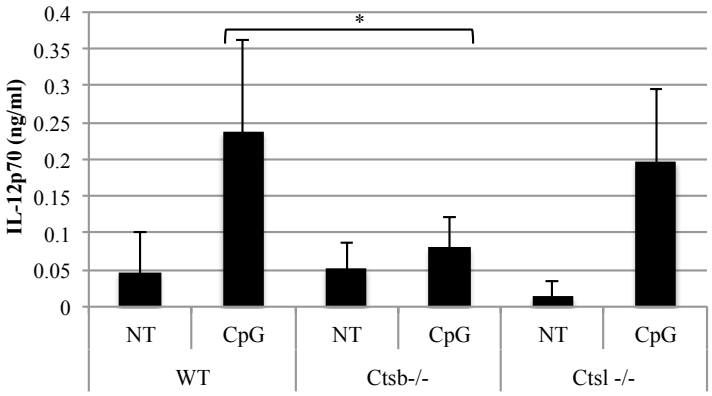

Figure 5.26. **IL-12 expression is impaired in *Ctsb* $^{-/-}$ BMDC upon stimulation with CpG.** Concentration of IL-12 in supernatants of BMDC from WT, *Ctsb* $^{-/-}$ or *Ctsl* $^{-/-}$ mice, either non-treated (NT) or stimulated with CpG for 24 hours. The results are expressed as mean ± SD of 3 independent experiments.

full ovalbumin (OVA) was used as antigen, but also with the OVA peptide 327-339.

Moreover, as described in section 8.10.2, BMDC pre-treated with the Ctsb inhibitor

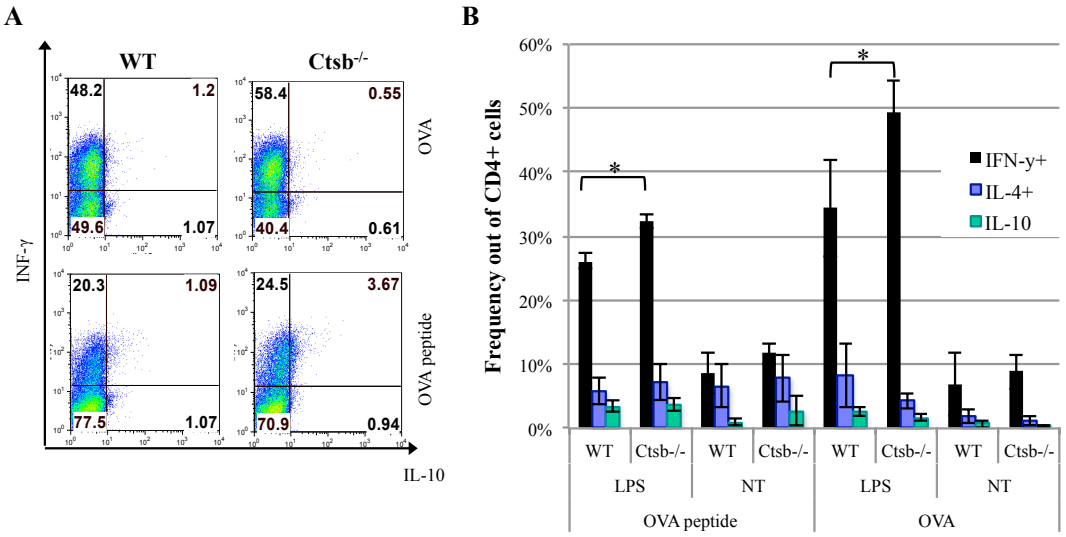

Figure 5.27. **Th1 polarization of OT-II CD4$^+$ naïve T cells by BMDC from WT C57BL/6 and *Ctsb* $^{-/-}$ mice.** Isolated CD4$^+$CD25$^-$ T cells from OT-II mice were co-cultured with BMDC generated from WT C57BL/6 and *Ctsb* $^{-/-}$ mice in the presence of LPS as a stimulus and OVA peptide (327-339) or ovalbumin (OVA) as antigens. A) Density plots from one representative experiment. B) Average percentages of IFN-γ, IL-4, or IL-10$^+$ CD4$^+$ T cells from 3 independent experiments ± SD. * $p < 0.05$. These results can be also found reported in [432] .

ZRLR produced higher levels of IL-12 than BMDC pre-treated only with DMSO. Therefore, it would be expected that ZRLR would also contribute to higher levels of Th1-polarized cells than co-cultures without the inhibitor. In agreement with the observations with *Ctsb* $^{-/-}$ BMDC, treatment with ZRLR increased the frequency of Th1-polarized cells in comparison with DMSO controls (Fig. 5.28). Altogether, these results indicate that Ctsb in BMDC regulates their capacity to induce the polarization of naïve Th cells into Th1 *in vitro*.

5.12 The observed up-regulation of IL-12 by *Ctsb* $^{-/-}$ BMDC in response to *L. major* and LPS takes place at the transcriptional level

In the previous sections, it was shown that *Ctsb* $^{-/-}$ BMDC and BMM expressed higher levels of IL-12 in response to *L. major* promastigotes than WT and *Ctsl* $^{-/-}$ BMDC and BMM. Moreover, *Ctsb* $^{-/-}$ BMDC produced more IL-12 in response to LPS, and therefore induced higher levels of Th1-polarization *in vitro*. IL-12 production was measured by ELISA or intracellular staining. In order to learn more about the possible mechanisms by which Ctsb could regulate IL-12 expression, it was necessary to determine next if the observed up-regulation of IL-12 was present at the transcriptional level.

Effect of ZRLR on in vitro Th1-polarization

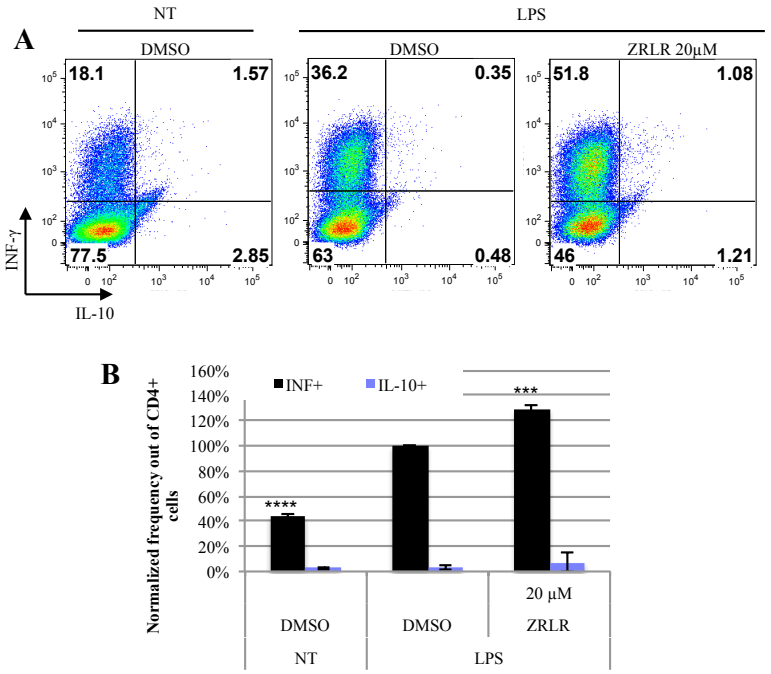

Figure 5.28. **ZRLR enhances *in vitro* Th1-polarization.** $CD4^+CD25^-$ T-cells were isolated from OT-II mice, and co-cultured with BMDC in the presence of ovalbumin, LPS, ZRLR (20 μM) or DMSO for 5 days. (A) Density plots of $CD4^+$ gated cells at the end of the co-culture, stained intracellularly for IFN-γ and IL-10. (B) Frequency of IFN-γ^+ and IL-10^+ $CD4^+$ T cells, normalized to samples with LPS stimulation in the presence of DMSO. The results are presented as mean $\pm$ SD of 3 independent experiments, and the significance in every treatment was estimated against DMSO+LPS treated samples; ***$p<0.005$, ****$p<0.001$.

Total RNA was isolated from WT, *Ctsb* $^{-/-}$, and *Ctsl* $^{-/-}$ BMM infected with *L. major* promastigotes, at 6 h and 24 h p.i. Non-treated BMM, and LPS treated BMM were used as negative and positive controls. The expression levels of the *Il12p35* and *Il12p40* subunits were then determined by real time PCR, and can be found summarized in Fig. 5.29. For both time points, *Ctsb* $^{-/-}$ BMM expressed higher levels of *Il12p35* in response to infection and LPS than WT, and *Ctsl* $^{-/-}$ BMM, while their transcription levels without any stimulation were comparable. Similarly, *Ctsb* $^{-/-}$ BMM expressed higher levels of *Il12p40* in response to infection and LPS than WT, and *Ctsl* $^{-/-}$ BMM.

5.13 Approaches to determine mechanisms of IL-12 up-regulation in cathepsin B-deficient BMDC and BMM

In the previous sections, it was shown that Ctsb plays a role in the expression of IL-12 in response to *L. major* and LPS. Furthermore, this regulation takes place in BMDC and

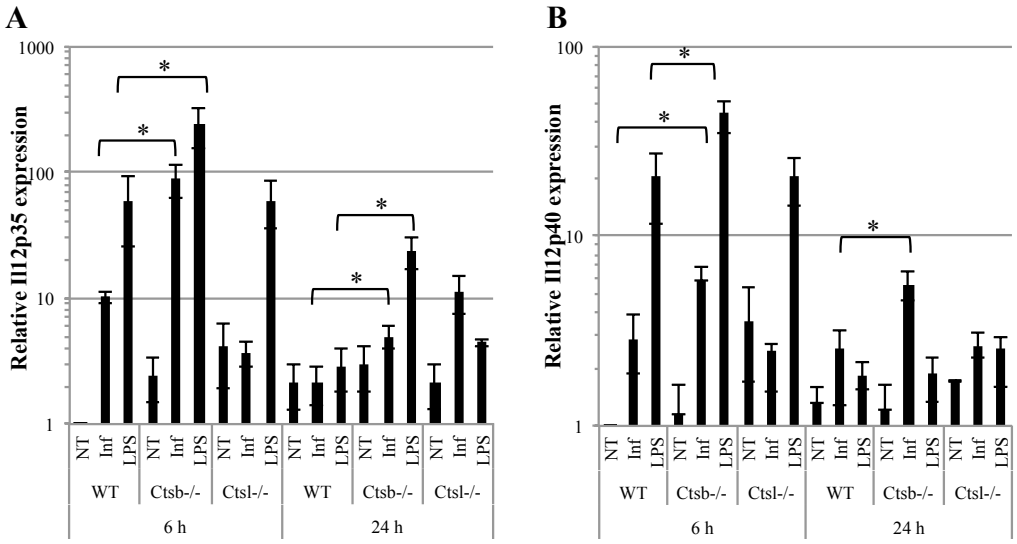

Figure 5.29. **The observed up-regulation of IL-12 in response to *L. major* and LPS takes place at the transcriptional level.** Relative expression levels of (A) *IL12p40* and (B) *IL12p35* transcripts in mRNA from BMM at 6 h or 24 h p.i. with *L. major* promastigotes or LPS stimulation. Non-treated (NT) BMM from each mouse line were used as negative controls. The expression levels were estimated using the $2^{-\Delta\Delta CT}$ method, using WT NT BMM at 6 h as a reference. The results are shown as mean ± SD of 4 independent experiments, (* $p<0.05$), and can be found reported in ref. [432] .

BMM, and the up-regulation of IL-12 in *Ctsb* $^{-/-}$ BMM is present already at the transcriptional level. *Leishmania* parasites are known to silence their host by manipulating key signaling pathways, but somehow, in *Ctsb* $^{-/-}$ BMDC and BMM this silencing appeared to be incomplete, as they were able to express IL-12. Therefore, different signaling pathways relevant to IL-12 expression and immune response to *L. major* were analyzed by WB. The first part of this section focuses on NF-κB signaling pathway. The second part, concentrates in analysis of ERK and p38-MAP kinase pathways.

5.13.1 Analysis of activation of NF-κB signaling pathway

One of the main signaling pathways involved in the expression of IL-12 is the NF-κB pathway. Therefore, it was first investigated if the observed up-regulation of IL-12 was dependent on the NF-κB signaling pathway by assessing the translocation of the p65 subunit from the cytoplasm to the nucleus. Two different monoclonal antibodies were tested separately, to detect the NF-κB p65 subunit by WB. The protein bands with the expected molecular weight (65 kDa) were used for analysis.

Figures 5.30 and 5.31 summarize the results from these experiments. It was found that the different levels of p65 for all the treatments in nuclear fractions from WT and *Ctsb* $^{-/-}$ had no statistical significance. It should be pointed out that we found with both

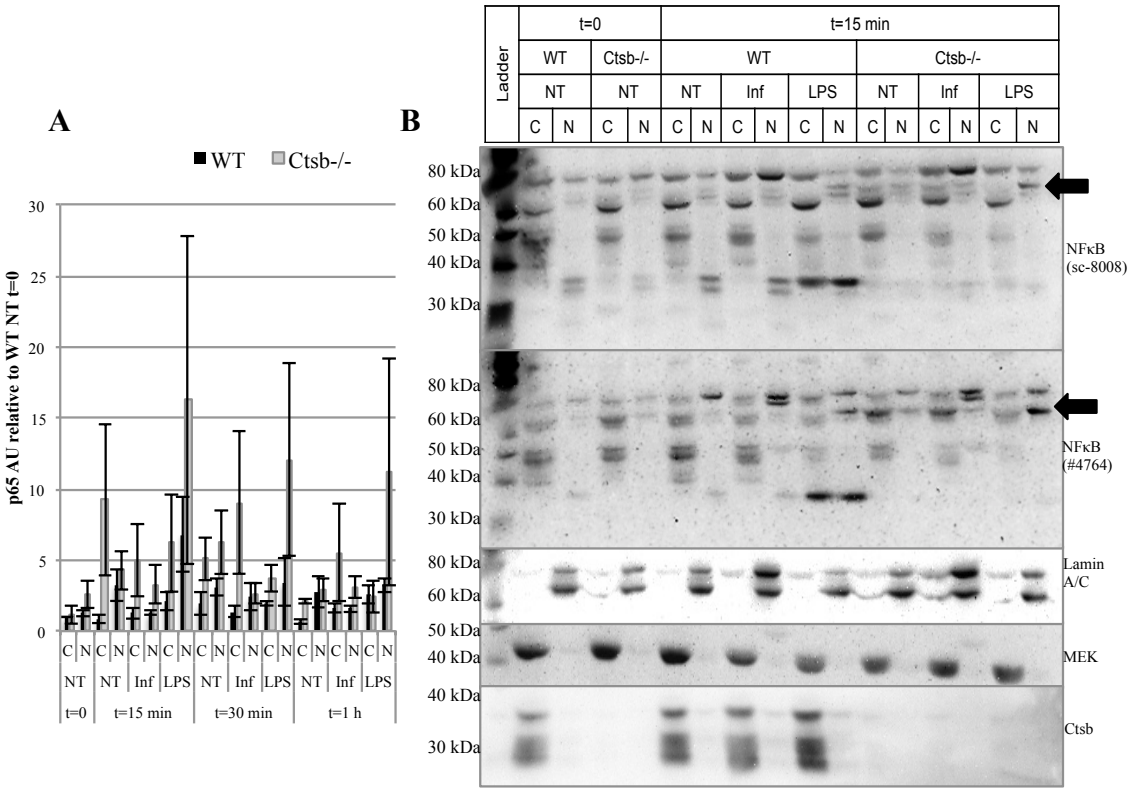

Figure 5.30. **Detection of NF-κB (p65 subunit) in nuclear and cytoplasmic extracts by WB.** Nuclear (N) and cytoplasmic (C) extracts were prepared from WT and *Ctsb* $^{-/-}$ BMM at different time points after infection with *L. major* promastigotes or stimulation with LPS. (A) Quantification of NF-κB (p65 subunit) by WB, represented as arbitrary units (AU) relative to the measurements in WT BMM NT at 0 min. The bars represent the average result from 3 independent experiments $\pm$ SD. For each treatment, no statistical significance was found between samples from WT and *Ctsb* $^{-/-}$ BMM. B) Representative immunoblots from one experiment including samples at 0 and 15 min. Multiple bands were detected independently using two different antibodies against NF-κB (p65 subunit): sc-8008, from Santa Cruz, and #4764, from Cell Signaling. Only those with an apparent molecular weight of 65 kDa (red arrows) were considered for the analysis in (A). The expression levels of MEK and Lamin A/C were used as loading controls for cytoplasmic and nuclear extracts, respectively. The results can be found reported in ref. [432] .

antibodies multiple protein bands with molecular weights other than 65 kDa. In particular, a non-identified protein with a molecular size around 30 kDa was observed in WT LPS-stimulated BMM but not in LPS-stimulated *Ctsb* $^{-/-}$ BMM. While these results suggest that NF-κB is not responsible of the Ctsb-mediated regulation of IL-12, more experiments with different approaches would be needed to confirm this observation.

5.13.2 Analysis of activation of ERK and p38 MAP kinase signaling pathways

The activation of ERK and p38 MAP kinases plays key roles in the immune response against *Leishmania* parasites. Therefore, lysates of WT and *Ctsb* $^{-/-}$ BMM were prepared at different time points of infection, and the phosphorylation of ERK 1/2 and p38 MAP

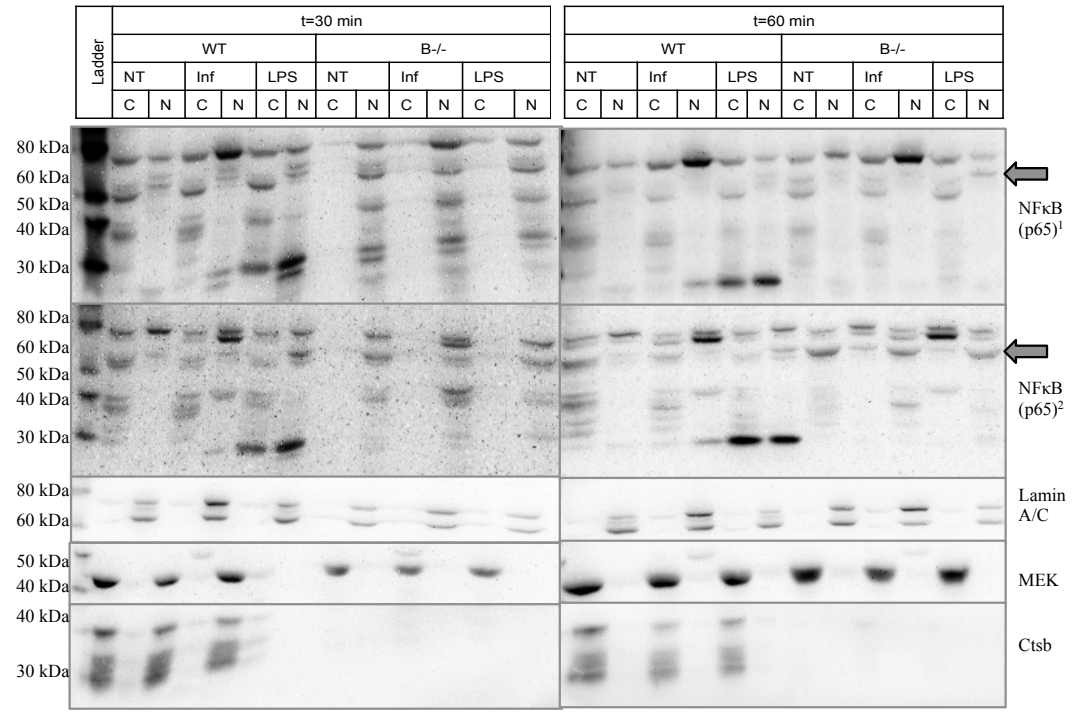

Figure 5.31. **Measurement of NF-κB (p65 subunit) in nuclear and cytoplasmic extracts by WB (Continuation from 5.30).** Representative immunoblots from one experiment, same as shown in Figure 5.30 B, including samples at 30 min and 60 min.

kinase was analyzed by WB. Non-treated BMM were used as negative control, and LPS-stimulated BMM were used as positive control.

Figures 5.32 and 5.33 summarize the results from these experiments. Both pathways appeared silenced in lysates from infected WT and *Ctsb* $^{-/-}$ BMM, as indicated by the low signal detected in phosphorylated p38 and ERK. As shown in the previous sections, Ctsb deficiency resulted in higher levels of IL-12 expression in response to LPS. However, the differences in activation of p38 MAPK and ERK pathways detected in LPS-stimulated BMM were not statistically significant. Therefore, the regulation of IL-12 expression by Ctsb is not by means of these signaling pathways.

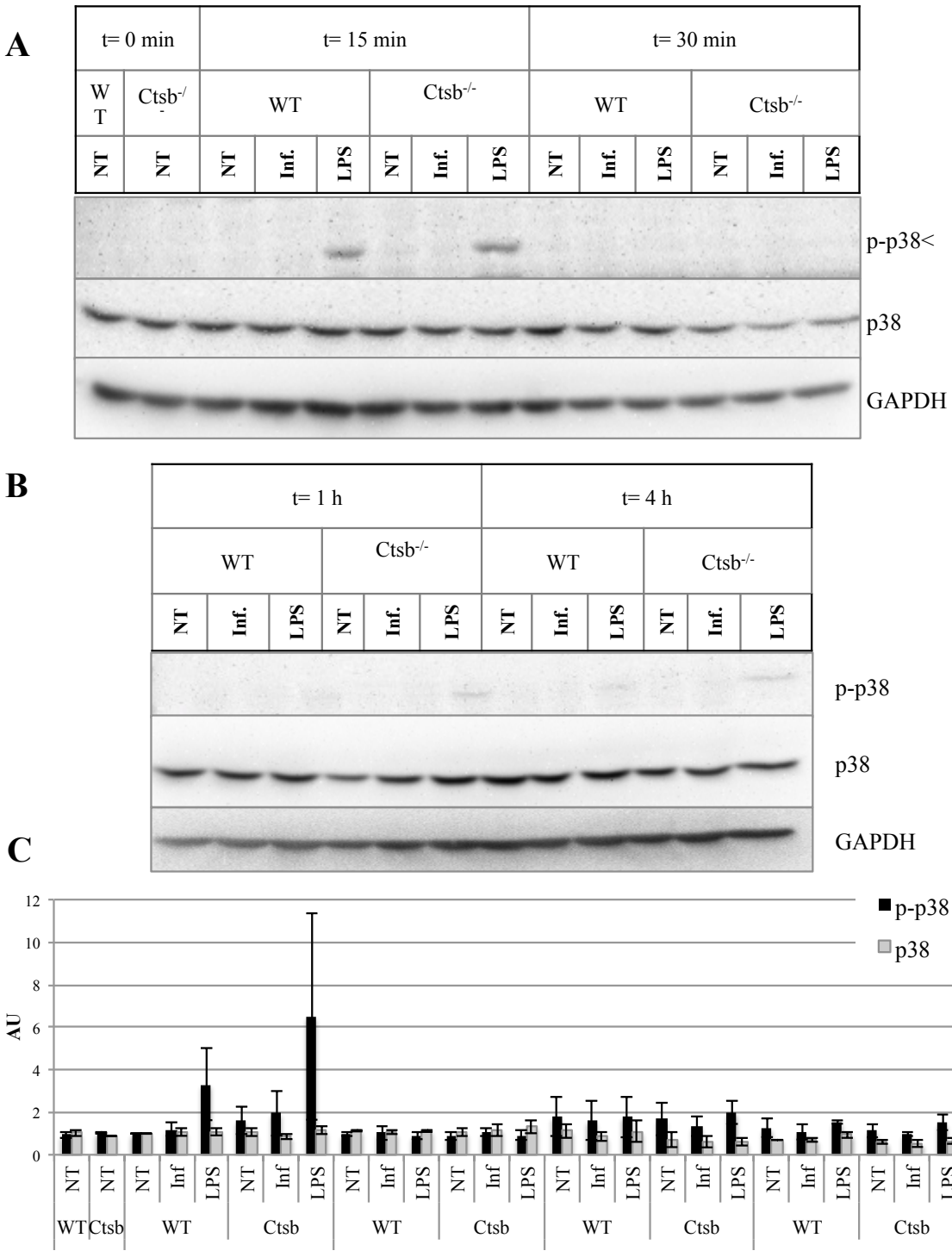

Figure 5.32. **Analysis of p38 MAP kinase signaling pathway activation by WB.**Lysates from WT and *Ctsb* $^{-/-}$ BMM were prepared at different points after infection with *L. major* promastigotes or stimulation with LPS. Immunoblots were prepared to detect phosphorylated p38 MAP kinase (p-p38), total p38 MAPK, and GAPDH as a loading control. (A) Representative immunoblot from one experiment, including samples at 0 min, 15 min, and 30 min. (B) As in (A), representative immunoblot from one experiment, including samples at 1 h and 4 h.(C) Quantification of p-p38 MAPK and p38 MAPK, summarized from 3 independent experiments ± SD. Although some variations were observed in LPS-stimulated BMM, no statistical significance was found.

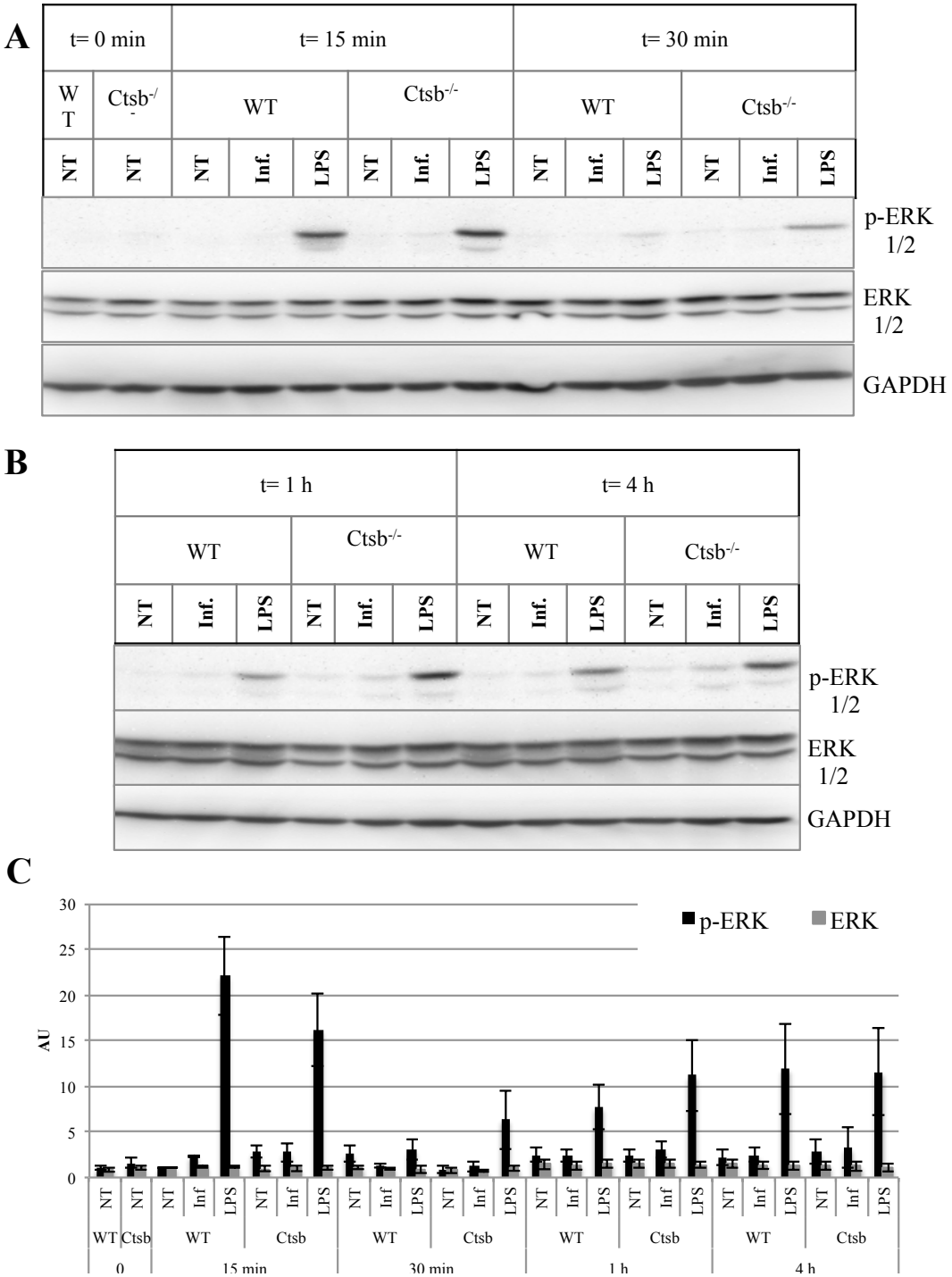

Figure 5.33. **Analysis of ERK signaling pathway activation by WB.** Lysates from WT and *Ctsb* $^{-/-}$ BMM were prepared at different points after infection with *L. major* promastigotes or stimulation with LPS. Immunoblots were prepared to detect phosphorylated ERK 1/2 (p-ERK), total ERK 1/2, and GAPDH as a loading control. (A) Representative immunoblot from one experiment, including samples at 0 min, 15 min, and 30 min. (B) As in (A), representative immunoblot from one experiment, including samples at 1 h and 4 h. (C) Quantification of p-ERK 1/2 and ERK 1/2, summarized from 3 independent experiments ± SD. Although some variations were observed in LPS-stimulated BMM, no statistical significance was found.

CHAPTER 6

Discussion

The drugs currently available for the treatment of leishmaniasis present several disadvantages, including strong side effects, difficulty of administration, and the emergence of resistance. Hence, the need to develop novel therapeutics. The studies on immunochemotherapeutic approaches indicate that a synergy between parasite killing and stimulating the immune system results in shorter duration of treatment and a reduced amount of drug required. Cathepsin B-like and cathepsin L-like cysteine proteases from *Leishmania* have been shown to be relevant for the virulence of the parasite, and some cathepsin inhibitors -including aziridine-based and K11777-have shown to have leishmanicidal properties. The test of two epoxysuccinyl-based cathepsin inhibitors by Katunuma and colleagues [339–341] indicated that these inhibitors had immunomodulatory properties: CA074, the Ctsb inhibitor, promoted a Th1 immune response; in contrast CLIK148, the Ctsl inhibitor, induced a Th2 immune response. These results would suggest that CA074 could be a potential immunochemotherapeutic agent for treatment of leishmaniasis. The mechanisms behind the observed modulation of Th1/Th2 polarization were not further investigated, and thus the aim of this study was to analyze the involvement of Ctsb and Ctsl in this process. Given the importance of DC for instructing the polarization of Th0 cells, this study focused on analyzing the effect of Ctsb and Ctsl inhibition and deficiency in the signals that DC provide to Th0 for this process: the presentation of antigens in MHC class II molecules, the expression of costimulatory molecules, and of cytokines.

The inhibitors tested presented no cytotoxicity at the working concentrations (5 μM to 20 μM). Higher concentrations were avoided, as they are associated with unspecific inhibition of other cysteine cathepsins. Furthermore, it was possible to generate functional BMDC and BMM from *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ mice, with no apparent differences in their

morphology and expression of characteristic markers (CD11c and F4/80, respectively). Some of the inhibitors -RV212C and CS128- showed leishmanicidal activity *in vitro* . In contrast, the survival and proliferation of *L. major* parasites was not altered in infected *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ BMM. This comparable survival and proliferation of the parasites in Ctsb/- and *Ctsl* $^{-/-}$ BMM was correlated with similar levels of NO expression in comparison to WT BMM, and at the time point analyzed, no apparent differences in the presence of autophagosome-like structures were detected. In response to *L. major* infection, both Ctsb and Ctsl are up-regulated in BMDC. Other stimuli, including LPS, CpG and TNF-α were also found to induce an up-regulation of Ctsb and Ctsl, and this pattern of expression appeared to be different depending on the genetic background of the mice (BALB/c or C57BL/6). Lack of Ctsb and Ctsl showed no effect in the expression of other cysteine cathepsins, including Ctss and Ctsx. The capacity of BMDC of uptake and processing of *L. major* promastigotes was not altered neither by inhibition of cathepsins, nor by *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ deficiency. However, some differences in the maturation of BMDC were detected.

The inhibitor CA074Me enhanced the expression of MHC class II molecules in BMDC in response to *L. major* promastigotes and of LmAg. This effect was not detected with other cathepsin inhibitors, including CLIK148, RV212C, and CS128. Similarly, *Ctsb* $^{-/-}$ BMDC showed higher expression of MHC class II molecules in response to *L. major* promastigotes. Furthermore, none of the inhibitors showed an effect on the expression of costimulatory molecules -CD40, CD80 and CD86- and a comparable observation was made with BMDC from *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ mice. In contrast, BMDC stimulated with LPS showed a small down-regulation of the expression of MHC class II molecules, and in some cases, of CD86. However, these effects were not observed in *Ctsb* $^{-/-}$ or *Ctsl* $^{-/-}$ BMDC. When analyzing the cytokines expressed by BMDC, *L. major* promastigotes did not induce significant expression of cytokines in BMDC.

In response to LPS, BMDC express high concentrations of pro-inflammatory cytokines and IL-10. Most of the tested inhibitors showed a detrimental effect in the expression of IL-12 in LPS-stimulated BMDC. When co-culturing these cells with naïve CD4^{+} T cells from OT-II mice, it was found that this was correlated with a lower frequency of Th1-polarized cells. In contrast, when analyzing the cytokine expression of *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ BMDC, it was found that *Ctsb* $^{-/-}$ BMDC are able to express IL-12 in response to *L. major*. Furthermore, in response to LPS *Ctsb* $^{-/-}$ express higher levels of IL-12, and in co-culture assays, *Ctsb* $^{-/-}$ BMDC induced higher frequencies of Th1 polarized cells than when WT BMDC were used. Thus, lack of Ctsb was found to result in the up-regulation of two of the signals used by BMDC to induce Th cell polarization, particularly of Th1. The observed up-regulation of IL-12 was found to occur at the transcriptional level, but it could not be

attributed to differences in the activation of the signaling pathways NF-κB, p38 MAPK, and ERK 1/2. The next pages contrast these observations against reports in the literature.

Lysosomal cathepsins have been described as effector molecules in the lysosomal pathway of apoptosis.[433] In this pathway, lysosomal proteases are released into the cytosol in response to different stress factors, including oxidative stress. Lysosomal cathepsins -particularly Ctsb- have been reported to process the pro-apoptotic protein Bid into its active form, and to proteolytically inactivate anti-apoptotic proteins of the Bcl-2 family, which results in apoptosis induction via the mitochondrial release of cytochrome c.[433] Therefore, it would be tempting to speculate that inhibition of Ctsb rather than having a cytotoxic effect would actually prevent the activation of apoptosis. However, the Ctsb inhibitor Z-Phe-Gly-NHO-Bz has been reported to induce apoptotic cell death in cancer cells,[434] and the Ctsb inhibitor z-FA-FMK was shown to have a detrimental effect on T cell proliferation by blocking NF-κB activation and cell cycle progression.[435] Therefore the cytotoxicity of all the inhibitors in this study was determined for BMDC. The cathepsin inhibitors used showed no cytotoxicity in BMDC in the range of concentration used for this study. Most of them presented an IC_{50} value no lower than 100 μM, with the exception of CA074Me, which showed some cytotoxicity at concentrations higher than 50 μM. Furthermore, BMDC and BMM could be generated from *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ mice, with no differences in terms of morphology, yields of cells, and the expression of the characteristic markers CD11c and F4/80 in comparison with those from WT mice. All these cells showed a similar capacity for the uptake of *L. major* parasites regardless of their genetic background. In addition, the generated immature BMDC showed comparable levels of expression of MHC class II molecules and of the costimulatory molecule CD86 as the WT BMDC.

As mentioned in Table 2.1, several reports in the literature indicated an up-regulation of cysteine cathepsins in response to inflammatory stimuli (including the cytokines IFN-γ[436] and IL-6[263]), and LPS.[437] BMDC were found to up-regulate the expression of Ctsb, and to a lesser extent of Ctsl in response to *L. major*. The expression of Ctsb and Ctsl was then analyzed in BMDC in response to other stimuli: LPS and CpG, known to induce the maturation of BMDC to induce Th1 immune responses, and TNF-α, which causes a semi-maturation of BMDC and subsequent induction of Th2 responses.[170,431]

The expression of Ctsb and Ctsl was different in BMDC freshly harvested depending on their genetic background: BMDC from BALB/c mice appeared to express higher levels of Ctsb and lower levels of Ctsl than their C57BL/6 counterparts. However, the regulation of Ctsb expression in response to different stimuli was found to be similar. As opposed to the report form Li and colleagues, showing an up-regulation of Ctsb in

response to LPS in THP-1 cells,[437] BMDC stimulated with LPS did not show significant changes in the expression of Ctsb regardless of their genetic background, and similar results were found in BMDC stimulated with CpG. However, the expression of Ctsb was up-regulated in BMDC stimulated with TNF-α in BMDC from both BALB/c and C57BL/6 mice. In contrast, the expression of Ctsl was found to vary depending on the genetic background of the BMDC. In C57BL/6, Ctsl followed a similar expression pattern as Ctsb, with a significant up-regulation only in response to TNF-α. BALB/c BMDC on the other hand, up-regulated Ctsl in response to LPS, CpG, and TNF-α. Therefore, it was not possible to conclude from these results a pattern of Ctsb and Ctsl expression characteristic for strong maturation (or pro-Th1) or semi-maturation (pro-Th2) of BMDC.

It is possible that the deletion of one cysteine cathepsin could impact the expression of other cathepsins. For example, Honey and colleagues described that Ctss had the capacity to control the turnover of other enzymes in the endosomes, including Ctsl.[373] As a control, the expression of other cysteine cathepsins in *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ BMDC was evaluated by labeling the active site with biotinylated probe -DCG04- which was developed using the E64 scaffold. With this approach, the other cathepsins detected corresponded in size to Ctsx, and Ctss. Both were expressed at comparable levels in WT and cathepsin-deficient BMDC. Furthermore, no significant differences in Ctsl expression were found between WT and *Ctsb* $^{-/-}$ BMDC. Similarly, no differences in Ctsb expression were found between WT and *Ctsl* $^{-/-}$ BMDC. Therefore, any effects found in this study would likely not be due to impaired expression or overexpression of other cysteine cathepsins.

Following their uptake by macrophages, *Leishmania* parasites transform into amastigotes that survive and proliferate within their host cell. The process of differentiation of promastigotes to amastigotes has been characterized *in vitro* by Tsigankov and colleagues in four main stages: first, signal perception (0 to 5 h); second, movement cessation and aggregation (5 to 10 h); third, amastigote morphogenesis (10 to 24 h) and maturation (24 to 120 h).[438] Therefore, the time course analyzed for parasite survival and proliferation in macrophages would correspond to fully formed amastigotes, undergoing maturation. During this stage, the parasites not only survived, but the average number of parasites per infected cell increased, indicating proliferation. The parasite survival and proliferation was found comparable between cathepsin-deficient BMM and WT BMM, indicating that the macrophage Ctsb and Ctsl play no role in controlling the parasite survival. When the production of NO was analyzed, negligible levels of NO were detected in infected BMM from WT and cathepsin-deficient mice. Furthermore, no differences in NO expression were found between WT and cathepsin-deficient BMM upon LPS stimulation. Chicken cystatin -an inhibitor of cysteine cathepsins- has been reported to stimulate NO release

in IFN-γ-activated peritoneal macrophages.[312] Furthermore, in a mouse model of visceral leishmaniasis, the combination of chicken cystatin with IFN-γ resulted in complete elimination of parasite burden, which was attributed to an enhanced production of NO in cystatin-treated animals.[439] However, the up-regulation of NO associated to cystatin was not dependent on cathepsin inhibition: the NO-regulatory of cystatin was associated with a 10-mer sequence not corresponding with the site for interaction with cathepsins, and this peptide alone was enough to result in enhanced NO production.[315] These results suggest that the differential cytokine expression detected in *Ctsb* $^{-/-}$ BMM was not correlated with differences in the parasite load and NO production in comparison to WT BMM.

Macrophages infected *in vitro* with *L. amazonensis* have been reported to develop autophagy, and this phenomenon was also reported in skin lesions of BALB/c mice,[440] and in a model of *L. donovani* infection.[441] Furthermore, treatment with an autophagy inhibitor resulted in this model in the reduction of the infection index, and it was proposed that autophagy would be a medium for the parasite for nutritive support.[440] Autophagy involves the proteolytic degradation of cellular material in lysosomes, mediated by the activity of different proteases. The initial steps rely on the proteolytic processing of Atg8 by the cysteine protease Atg4.[442] Lysosome cysteine proteases proteolytically process the contents of the autophagosome, disposing the autophagic flux.[442] Besides degrading autophagosome contents, Ctsl is also known for degrading the membrane proteins GABARAP-II and LC3-II.[442] It has been reported that *Ctsl* $^{-/-}$ mouse embryonic fibroblasts present no apparent defects in the initiation of autophagy, the formation of autophagosomes or the fusion of autophagosomes with lysosomes. However, it was at the last stage, the degradation of autophagolysosomal content that some impairment was observed.[443] Similarly, *Ctsb* $^{-/-}$ cells have been reported to present no defects in the fusion of LC3-containing autophagic vacuoles, but a delay in the autophagic flux was observed.[442,444] At the time point in which infected BMM were observed by TEM (48 h p.i.) the presence of some autophagic-like vacuoles was identified, although no significant differences were detected. It is possible that at later time points, the defects on degradation of autophagolysosomal content could have been reflected. However, it is at this and earlier time points that the differences in cytokine expression in BMM were observed.

Inhibitors of Ctsb, including CA074Me and ZRLR, showed no cytotoxic activity against *L. major*. However, the inhibitors RV212C and CS128 did show a cytotoxic activity against promastigotes, in the range of IC_{50} values of 50 to 60 μM. In comparison, the cathepsin L-like inhibitor K11777 has been reported about 30% of growth inhibition of *L. major* promastigotes at a concentration of 50 μM. These inhibitors were designed against the cathepsin L-like CPB. Interestingly, neither the Ctsl inhibitor CLIK148 nor the broad cathepsin inhibitor E64d presented cytotoxic activity against *L. major* promastigotes.

Similarly, it was reported that CA074 and CLIK148 had no effect on the proliferation *in vitro* of *L. major* promastigotes.[340,341] While it might be possible that CLIK148 has a better affinity to mammal Ctsl than to *Leishmania* Ctsl-like, it is surprising that E64d did not show comparable IC_{50} values as theRV212C and CS128 inhibitors. It has been reported that amastigotes and promastigotes of CPB-deficient *Leishmania* can be cultured *in vitro*, although they present reduced virulence.[404] In *L. mexicana*, the cathepsin L-like CPB and CPA have been described as crucial for autophagy, which greatly contributes to the organelle remodeling required for the transformation of promastigotes into amastigotes.[445] In *L. major*, however, the cell death observed was associated with the accumulation of debris in autophagy-related vacuoles.[347] The discrepancies in the effects of the different CPB inhibitors used in this study might be an indication of involvement with other targets besides CPB.

The lack of Ctsb and Ctsl has not been shown to impair the presentation of antigens. However, it could be possible that the absence of one cysteine cathepsin causes a "slower" antigen presentation. A slower antigen processing is reported to be beneficial antigen presentation. Delamarre and colleagues reported that DC and B lymphocytes express lower levels of proteases than macrophages, and this was associated with a capacity to retain antigens for extended periods and favor antigen presentation.[367] Therefore, the processing of *L. major* parasites was analyzed by infecting BMDC with eGPF-tg promastigotes, and following the rates of infected cells over the course of 24 h. 2 h after infection no differences in parasite uptake were detected in BMDC upon treatment with different cathepsin inhibitors. As the time progressed, the amount of BMDC in which the $eGFP^+$ parasites could be detected was progressively reduced, which was interpreted as a result of parasite destruction and processing, and it was similar in BMDC from BALB/c and C57BL/6 background. Furthermore, none of the inhibitors tested showed an effect on the kinetics of parasite processing. Similarly, BMDC from *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ mice presented comparable kinetics of parasite processing as WT BMDC.

The maturation of DC in response to *Leishmania* infection has been addressed by numerous studies. There is a great variation in the reported levels of DC maturation in response to *Leishmania* found in the literature, which varies depending on the *Leishmania* species and strain, the developmental stage of the parasite, the DC studied, if the parasites were opsonized.[194] DC have been reported to present incomplete maturation after uptake of *L. major* promastigotes[199] and of *L. amazonensis* amastigotes.[446] The impaired activation of DC observed upon infection with *L. amazonensis* has been associated with interference of the JAK/STAT, NF-κB, IRF pathways[447] and ERK.[448] Incomplete DC maturation, as observed after stimulation with *Trypanosoma brucei* antigens or TNF-α,

has been shown to induce activation of genes correlated with the induction of Th2 polarization.[431] Upon recognition of PAMP or danger signals, immature DC become activated and mature, in a process characterized by the up-regulation of the surface expression of MHC class II molecules for the presentation of antigens, of costimulatory molecules, and of cytokines. These in turn are used as signals for instructing the polarization of Th0 cells in the lymph nodes.

Studies using the Ctsb inhibitor CA074 and the Ctsl inhibitor CLIK148 reported drastic effects on the Th response elicited, and it was hypothesized that the observed change in Th polarization could be attributed to different patterns of antigen processing. The process of antigen presentation requires multiple serial steps. On one hand, the processing of proteins into antigenic peptides that fit into the peptide-binding cleft of MHC class-II molecules. On the other hand, the processing and eventual removal of the invariant chain (Ii) from MHC class-II molecules in order to load the processed antigen. A more recent model for the presentation of some antigens in MHC class II molecules has been described as "bind first trim later", in which large fragments of antigen bind to adjacent MHC class II molecules in the endosome membrane plane, and once captured, are trimmed by further proteolytic processing.[449]

The individual contributions of cysteine cathepsins to antigen presentation have been extensively investigated. Up to date, the only cysteine cathepsins with a confirmed essential role for this process are Ctss for the degradation of the Ii in APC[240,386,387] and of Ctsl for the processing of antigens specific of cTECs.[450] In contrast, other cathepsins including Ctsb, Ctsd, and Ctsl contribute to processing the bulk of proteins contained in endolysosomal compartments. Because of their broad substrate specificity,[349] the combined proteolytic activity of the cathepsins present could compensate the absence of a single one. In general, the antigen processing requirements for the MHC class II presentation pathway appear to be minimal and quite redundant.[451] Indeed while Ctsb has been described as the most abundant cysteine cathepsin in the lysosome,[359,452] Ctsb has been reported to be dispensable for the maturation of MHC class II molecules.[359]

In the studies using the inhibitors CA074 and CLIK148, the authors incubated *Leishmania* lysate together with a lysosomal crude extract and observed different patterns of processing, reflected as the generation of peptides with diverse molecular weights that varied depending on the inhibitor used.[340–342] Studies evaluating the presentation of antigens to T-cell hybridomas reported that splenocytic APC from *Ctsb* $^{-/-}$ mice were more efficient to present certain antigens,[359] and similar results were found with the use of inhibitors in murine splenocytes[453] and primary human DC.[391] The latter is a report by Reich and colleagues, in which it was observed that the azapeptide inhibitor of

Ctsb, ZRLR, enhanced the presentation of TTCF.

In the present study, *Ctsb* $^{-/-}$ BMDC expressed higher levels of MHC class II molecules than WT and *Ctsl* $^{-/-}$ BMDC in response to *L. major* promastigotes. Similar results were found with the Ctsb inhibitor CA074Me. However, no effect was detected in the expression of the costimulatory molecules CD40, CD80 and CD86. As previously discussed, the kinetics of parasite processing were similar between WT and cathepsin-deficient BMDC, and between BMDC treated with cathepsin inhibitors and DMSO controls. These observations suggest that the different levels of MHC class II molecules expressed are not explained by faster or slower parasite processing. Deussing and colleagues suggested that some antigenic determinants may present different degrees of susceptibility to proteolytic degradation by cathepsins before being able to bind to MHC class II molecules.[359] It is possible therefore, that in the absence of Ctsb antigens may have a prolonged survival to proteolytic destruction. Higher levels of antigen presented have been associated with the induction of Th1 responses.[173] While in this study the immunogenicity of the antigens generated by *Ctsb* $^{-/-}$ BMDC, data from Delamarre and colleagues suggest that protein antigens resistant to proteolytic processing were better immunogens compared with antigens more susceptible to proteolytic attack.[454]

It should be noted that no differences in the expression of MHC class II molecules were found when stimulating WT and cathepsin-deficient BMDC with LmAg. This observation might be related to the differences in uptake mechanism and subsequent processing between intact promastigotes and lysates.[453,455] On the other hand, while *Ctsb* $^{-/-}$ BMDC tended to express higher levels of MHC class II molecules in response to heat-killed parasites than WT BMDC, the difference was not statistically significant, which could reflect the interaction of the living parasite with the host cell. For example, *L. mexicana* parasites have the capacity to internalize and cleave MHC class II molecules in the infected macrophage, in a process in which CPB is believed to play an important role.[416] Furthermore, for the preparation of LmAg, the parasites were killed by repeated cycles of freeze and thaw, whereas heat-killed parasites and both procedures could result in different forms of cell death (i.e. necrosis and apoptosis). Different modes of cell death are associated with more or less immunogenicity of tumor lysates for DC vaccination,[456] and the detection of posphatidylserine in the membrane of apoptotic parasites is associated with a silent invasion of the remnant viable parasites.[457] Indeed, BMDC stimulated with heat-killed parasites expressed higher levels of MHC class II molecules than BMDC stimulated with living *L. major* promastigotes and LmAg.

DC have different capacity to express IL-12 in response to *Leishmania*, depending on the DC subset and life stage of the parasite (i.e., whether the infection was carried out

using amastigotes or promastigotes).[458] The semi-maturation profile observed in BMDC in response to *L. major* promastigotes is accompanied by poor cytokine expression.[412] In agreement with these observations, in this study WT BMDC were unable to produce IL-12 in response to promastigotes, regardless of their genetic background (BALB/c or C57BL/6). In contrast, *Ctsb* $^{-/-}$ BMDC were able to express higher levels of IL-12 (both p70 and p40 forms) than WT and *Ctsl* $^{-/-}$ BMDC. No significant differences were detected in the expression of other pro-inflammatory cytokines, including IL-6 and TNF-α, which could indicate that this effect was not a generalized up-regulation of cytokine expression, but rather a more specific mechanism. IL-10 was also up-regulated in *Ctsb* $^{-/-}$ BMDC. IL-10 is an anti-inflammatory cytokine expressed by BMDC also in response to stimuli inducing Th1 responses, such as LPS. In terms of kinetics, the pro-inflammatory cytokines IL-12 and IL-6 are expressed first, and hours later, the expression of IL-10 starts.[172] At the time point in which the supernatants were collected (48 h), it is therefore expected to find both IL-12 and IL-10.

BMDC and BMM have been reported to be modulated differently by *Leishmania* parasites.[458] However, *Leishmania*-infected macrophages do not secrete IL-12,[190] and produce poor expression of cytokines in general,[145,149] similarly as observed with BMDC in response to promastigotes. In order to address if the observed up-regulation of IL-12 was specific for BMDC, the cytokine expression of BMM from WT, *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ mice in response to promastigotes was analyzed. Similar as observed in *Ctsb* $^{-/-}$ BMDC, *Ctsb* $^{-/-}$ BMM were able to produce IL-12, with similar level of IL-12p70 as *Ctsb* $^{-/-}$ BMDC, although the up-regulation of IL-12p40 was not as high. A similar observation has been reported by Pompei and colleagues in response to *Mycobacterium tuberculosis*, and the authors suggested that this effect was dependent upon the level of engagement of different TLR, particularly TLR9 in BMDC.[459] It has been suggested that TLR2 -which is engaged by LPG-, TLR3, 4, 7 and 9 are involved in the response to *Leishmania* infection. However, the engagement of these TLR by promastigotes has been described as relatively weak, and it is associated to the weak activation of DC.[194]

Therefore, the response of *Ctsb* $^{-/-}$ BMDC to stimulation of specific TLR was analyzed. LPS is recognized by TLR4 and it has been recently reported that the inhibition of IL-12 production in macrophages infected by *L. mexicana* occurs in a TLR4-dependent manner.[460] In response to LPS, *Ctsb* $^{-/-}$ BMDC present comparable maturation as WT and *Ctsl* $^{-/-}$ BMDC. However, they did produce higher levels of IL-12. When the expression of other cytokines was analyzed, it was found that *Ctsb* $^{-/-}$ BMDC expressed significantly lower levels of TNF-α. Ha and colleagues have also reported that BMM lacking Ctsb secrete significantly less TNF-α in response to LPS, due to an accumulation of TNF-α-containing vesicles that could not reach the plasma membrane.[461] Next, the

response of *Ctsb* $^{-/-}$ BMDC to stimulation with CpG was analyzed, and it was found that they produced considerably lower levels of IL-12 than WT and *Ctsl* $^{-/-}$ BMDC. CpG is recognized by TLR9, which is known to require processing by endosomal cathepsins to initiate signaling.[355,462] Therefore, these results agree with a previous report from Matsumoto and colleagues,[462] and suggest that the up-regulation of IL-12 expression observed in *Ctsb* $^{-/-}$ BMDC and BMM was independent from TLR9 signaling.

These results using cathepsin-deficient BMDC can be contrasted to BMDC treated with different cathepsin inhibitors. A striking difference found, was that the BMDC treated with the Ctsb inhibitor CA074Me did not expressed IL-12 as observed in *Ctsb* $^{-/-}$ BMDC. Furthermore, except from CA074 -which has poor cell permeability- all of the tested inhibitors presented a negative, dose-dependent effect on IL-12 expression in response to LPS. In particular, CA074Me appeared to be the most detrimental inhibitor for IL-12 expression. A previous report from Schotte and colleagues showed that the Ctsb inhibitor z-FA.FMK, a fluoromethylketone[463] down-regulated cytokine expression in macrophages in response to LPS. However, a common problem of the use of cathepsin inhibitors is the potential of unspecific targeting other cathepsins. As described in Section 2.5.2.2, the methyl ester modification of CA074, CA074Me, allows the inhibitor to be cell permeable, but if it is not completely processed intracellularly by esterases to CA074, it is reported to inhibit other cysteine proteases besides Ctsb.[323]

Therefore the inhibitors were tested for activity against Ctss. Indeed, the inhibitors CA074Me, CS128, and RV212C showed a dose-dependent inhibition of Ctss, in the concentration range used in the present study. Ctss has been reported to have an immunosuppressive activity, and inhibitors against this cathepsin have been studied in models of autoimmune diseases.[464] Thus, the azapeptide Ctsb inhibitor ZRLR was also incorporated to the present study, as it has been reported to be highly selective towards Ctsb.[335] Pre-treatment of BMDC with ZRLR resulted in IL-12 expression levels comparable to those observed with *Ctsb* $^{-/-}$ BMDC. The *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ mice used in this study were in a C57BL/6 background. However, the up-regulation of IL-12 expression by ZRLR was observed in BMDC from BALB/c and C57BL/6, indicating that this effect was mediated by Ctsb deficiency -or lack of activity- rather than by the BALB/c or C57BL/6 background of the mice. Furthermore, *Ctsb* $^{-/-}$ BMDC showed no significant differences in their expression of IL-12 upon treatment with ZRLR, indicating that the up-regulation of IL-12 observed with this inhibitor was dependent only on abrogation of Ctsb activity.

In contrast to *Ctsb* $^{-/-}$ BMDC and BMM, their *Cstl* $^{-/-}$ counterparts did not present significant differences in cytokine production in comparison with WT cells in response to *L. major* promastigotes or to CpG. When LPS was used as a stimulus, they produced

higher levels of IL-10 and TNF-α, but no significant differences were found in IL-12 expression. When the effects of CLIK148 were analyzed in BMDC, no significant effects were detected in the expression of MHC class II molecules, costimulatory molecules, and IL-12 expression in response to *L. major* promastigotes. In response to LPS, CLIK148 resulted in a modest down-regulation of MHC class II molecules, and a slight down-regulation of IL-12 expression, which was not as marked as observed with the other inhibitors tested. Therefore, these results alone would not explain the observations made by Onishi and colleagues, who observed that the Ctsl inhibitor CLIK148 caused a Th2-like immune response to *L. major* in otherwise resistant mice.[341] However, CLIK148 is also able to inhibit other cathepsins, including Ctsc, Ctsk, and Ctss,[323] and the authors of this study did not analyze if the dose administered *in vivo* of CLIK148 was enough to inhibit them.

Altogether, in response to *L. major* promastigotes, *Ctsb* $^{-/-}$ BMDC present higher levels of two signals for the polarization of Th0 towards Th1: (1) the expression of costimulatory molecules, and (2) the expression of IL-12. The latter is also up-regulated in response to LPS. Higher secretion of IL-12 would be expected to result in higher levels of Th1 polarized cells. Therefore, the capacity of *Ctsb* $^{-/-}$ BMDC to polarize Th0 cells towards Th1 was tested *in vitro* using $CD4^{+}CD25^{-}$ T cells from OT-II mice, using full OVA and $OVA_{(327-339)}$ peptide as antigens and LPS as a maturation stimulus. With both antigens, co-culture of *Ctsb* $^{-/-}$ BMDC resulted in higher levels of polarized Th1 cells than WT BMDC. Similarly, BMDC treated with ZRLR were able to induce higher levels of Th1 polarization than DMSO controls. As expected from their effects on IL-12 production in BMDC, CA074Me and RV212C resulted in lower levels of Th1 polarized cells. It should be noted that in contrast to other co-culture protocols for Th1 polarization, no exogenous cytokines were added to the culture. Although this approach results in lower levels of Th1 polarized cells it allows to directly appreciating the effects of the cytokines directly produced by the stimulated BMDC. The results obtained indicate that the observed regulation of IL-12 expression in BMDC from *Ctsb* $^{-/-}$ mice, or upon Ctsb inhibition with ZRLR, can have a direct impact in the polarization of Th cells towards Th1. Of note, although the inhibitor CS128 did not showed a clear negative effect in Th1 polarization *in vitro*, during the course of this study it was independently tested *in vivo*, and it was found that it did not protected BALB/c mice from *Leishmania* infection. It was found that CS128 also targeted Ctss, and it is thus possible that the negative effect observed *in vivo* was mediated by Ctss inhibition.

In order to determine if the observed up-regulation in *Ctsb* $^{-/-}$ BMM and BMDC was taking place at the transcriptional level, the genetic expression of the two IL-12 subunits was analyzed. Indeed, at the time points analyzed, the genes composing the two subunits of IL-12p70 were up-regulated in response to LPS and *L. major*. If lack of Ctsb activity

results in an up-regulation of IL-12 expression, how is it that Ctsb influences cytokine production in BMDC and BMM?

From the model of cutaneous leishmaniasis, it has been reported that *L. major* parasites induce tolerance in macrophages-characterized by poor or absent cytokine production- by a process involving the MAPK and NF-κB pathways of the host.[465,466] Although these pathways are initially activated by contact with the parasite, but once the infection is firmly established, they become silenced and render the cell unresponsive even to further stimulation with LPS.[465] This silencing has been attributed to different virulence factors from *Leishmania*, including surface phosphoglycans,[148,467] GP63,[468] and cysteine proteases.[150] Despite these strategies for silencing, somehow the infected BMM and BMDC were able to produce IL-12. Therefore a next point to address was how Ctsb may influence cytokine production, and the approach taken was to analyze the activation status of different signaling pathways that are reported to be silenced by *Leishmania*. The first signaling pathway chosen for analysis was NF-κB, as it has been described that upon translocation to the nucleus, the p65 subunit interacts with the upstream promoter region of the *Il12p40* gene.[469]

Upon analyzing the nuclear translocation of the p65 subunit of NF-κB, it was not possible to conclude that IL-12 expression was up-regulated directly by a higher activation of this pathway. On the other hand, IL-6 expression was not found to be up-regulated in *Ctsb* $^{-/-}$ BMDC and BMM, and IL-6 transcription has been shown to be also dependent on NF-κB signaling pathway. Therefore, it is likely that the molecular mechanism behind the involvement of Ctsb in the expression of IL-12 would not be shared by IL-6. These results contrast with the observations of Lawrence and colleagues, who found that the Ctsb inhibitor z-FAM-FMK blocked NF-κB activation, inhibiting T cell blast formation, and preventing the cells from entering and leaving the cell cycle.[435] Therefore, this inhibitor showed an immunosuppressive activity.

Thus, the activation of two other signaling pathways was analyzed, p38 MAPK and ERK 1/2. The p38 MAPK appeared phosphorylated transiently during the first time point of LPS stimulation, but no activation was detected with *L. major* promastigotes. In contrast, ERK1/2 remained phosphorylated upon stimulation, and the levels of activation were more strongly activated when LPS was used as a stimulus than with *L. major* promastigotes. In both signaling pathways, no significant differences in activation were detected, and therefore could not be accounted for the up-regulation of *Ctsb* $^{-/-}$ observed. Therefore, the molecular mechanism behind the regulation of IL-12 expression by Ctsb remains still to be elucidated. Some directions for further investigation would be to analyze the activation of JAK/STAT, mTOR and PI3K signaling pathways,[470] and

the intracellular location of Ctsb during *L. major* infection, as it might be possible that Ctsb participates in the proteolytic processing of signaling intermediates in the cytoplasm, or of transcription factors in the nuclear space, as described in thyroid carcinoma cells.[280]

Altogether, while previous studies hypothesized that the absence of Ctsb or Ctsl activities during *L. major* infection would lead to different Th polarization due to alterations in antigen presentation,[340,341,391] the results found in this study indicate that *Ctsb* $^{-/-}$ BMDC up-regulate two of the three types of signals for instructing T cell polarization: the expression of higher levels MHC class II molecules for antigen presentation than their WT counterparts, and expression of IL-12. Thus, these cells exhibit a "more mature" or "pro-Th1"-like profile. Moreover, a similar trend was observed upon LPS stimulation, and co-culture of purified naïve CD4+ T cells with *Ctsb* $^{-/-}$ BMDC resulted in a higher frequency of polarized T cells than using WT BMDC.

The results from this study were the first to document a Ctsb-mediated regulation of IL-12 expression, and the mechanisms responsible for this effect remain yet to be answered. Furthermore, although the results obtained strongly indicate a profile more favorable for a Th1 response to *L. major* infection, a definitive confirmation would still require *in vivo* experiments. At the time of this study, only *Ctsb* $^{-/-}$ and *Ctsl* $^{-/-}$ mice on a C57BL/6 background were available. While assays with the Ctsb inhibitor ZRLR confirmed the observations with *Ctsb* $^{-/-}$ BMDC in BMDC derived from C57BL/6 and BALB/c mice, it would be necessary to backcross the *Ctsb* $^{-/-}$ C57BL/6 mice to the *Leishmania*-susceptible BALB/c background in order to evaluate if they are protected against infection with the parasite. Furthermore, transfer experiments of *Ctsb* $^{-/-}$ BMDC would be necessary in order to determine if Ctsb deficiency in these cells is enough to result in Th1 polarization during infection.

The concept of "protease signaling" described by Turk and colleagues has gained increasing attention in different research fields,[350] especially in the context of therapeutic applications. However, the interplay between proteolytic networks and other signaling pathways is still to be addressed in different disease models. Based on the results presented in this study, a novel role for Ctsb during *L. major* infection is proposed: in addition to its involvement in antigen presentation, it is also a regulator of cytokine expression. Thus, pharmacological inhibition of Ctsb might have the potential to improve the Th1-mediated clearance of *L. major*.

Appendix A

A.1 Buffers and solutions

Table A.1. Buffers.

Buffer name	Compound	Concentration	Supplier	Location
10X PBS	Sodium Chloride	1.37 M	Sigma-Aldrich	St. Louis, MO, USA
(pH=7.4)	Potassium Chloride	26.83 mM	Roth	Karlsruhe, Germany
	di-Sodium hydrogen phosphate	101.44 mM	Roth	
	Potassium dihydrogen phosphate	17.64 mM	AppliChem	Darmstadt, Germany
10X TBS-T	Sodium Chloride	1.37 M	Sigma-Aldrich	
(pH=7.6)	Tris	200 mM	Sigma-Aldrich	
	Tween-20	1%	AppliChem	
1X PBS-T	10X PBS	10%	See 10X PBS	
	Tween-20	0.05%	AppliChem	
Sodium acetate	Sodium acetate	200 mM	Sigma-Aldrich	
buffer	EDTA	1 mM	Roth	
(pH=5.5)	Brij35	0.05%	Thermo Scientific	Rockfort, IL, USA
Citrate reaction	Citric acid	50 mM	Sigma-Aldrich	St. Louis, MO, USA
buffer	DTT *	50 mM	Sigma-Aldrich	Oakville, Canada
(pH=5.5)				
Fixation buffer	PFA	4%	AppliChem	
4% PFA	10X PBS	10%	See 10X PBS	
FACS Buffer	Sodium Azide	0.1%	Sigma-Aldrich	St. Louis, MO, USA
	FCS	2.5%	PAA Laboratories	Pasching, Austria
	10X PBS	10%	See 10X PBS	
Permeabilization	FCS	1%	PAA Laboratories	
buffer	Sodium Azide	0.10%	Sigma-Aldrich	
(pH=7.6)	Saponin	0.10%	AppliChem	
	10X PBS	10%	See 10X PBS	
Coating buffer	Sodium hydrogen carbonate	0.1 M	Roth	
(pH= 8.3)				

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Table A.1 – continued from previous page

Buffer name	Compound	Concentration	Supplier	Location
Diethanolamin buffer (pH=9.8)	Magnesium chloride hexahydrate	0.393 mM	Roth	
	Diethanolamin	9.70%	Merck	Darmstadt, Germany
Triton X-100 lysis buffer (pH=7.5)	Tris	20 mM	Sigma-Aldrich	
	Sodium Chloride	150 mM	Sigma-Aldrich	
	Triton X-100	1%		
	Sodium fluoride*	10 mM	AppliChem	
	Sodium ortho-vanadate*	1 mM	AppliChem	
	PMSF*	1 mM	AppliChem	
	Aprotinin*	5 μg/ml	Sigma-Aldrich	Taufkirchen, Germany
	Leupeptin*	5 μg/ml	MP Biomedical Inc.	Illkirch, France
5X PAGE-Sample Buffer	Tris.HCl (pH=6.8)	250 mM	Applichem	
	Glycerol	50%	Roth	
	SDS	5%	Roth	
	Bromphenolblue	0.05%	Sigma-Aldrich	
	DTT	250 mM	Sigma-Aldrich	
4X Laemmli Buffer (pH=6.8)	Tris	0.25 mM		
	SDS	8%		
	Glycerol	40%		
	2-Mercaptoethanol	20	AppliChem%	
	Bromphenol Blue	0.1%		
10x SDS Running Buffer I	Tris	25 mM	Sigma-Aldrich	
	Glycin	190 mM	Sigma-Aldrich	
	SDS	1%	Roth	
10X SDS Running Buffer II	Tris	0.25 M		
	Glycin	1.92 M		
	SDS	35 mM		
Transfer Buffer I	Tris	25 mM	Sigma-Aldrich	
	Glycin	190 mM		
	Methanol	30%	Honeywell	Seelze, Germany
3-Buffer Transfer System				
Anode Buffer I	Tris	300 mM		
	Methanol	20%		
Anode Buffer II	Tris	25 mM		
	Methanol	20%		
Cathode Buffer	6-Aminocaproic acid	25 mM	AppliChem	
	Methanol	20%		

Continued on next page

Table A.1 – continued from previous page

Buffer name	Compound	Concentration	Supplier	Location
Cell fractionation buffer A	HEPES	10 mM		
	Potassium chloride	10 mM		
	Magnesium chloride hexahydrate	1.5 mM		
	D-sucrose	0.34 M	Sigma-Aldrich	
	Glycerin	10%	Roth	
	DTT	1 mM		
Cell fractionation buffer B	EDTA	3 mM		
	EGTA	0.2 mM	Roth	
	DTT	1 mM		

Reagents marked with (*) were freshly added before use

A.2 Effect of inhibitors in BMDC viability

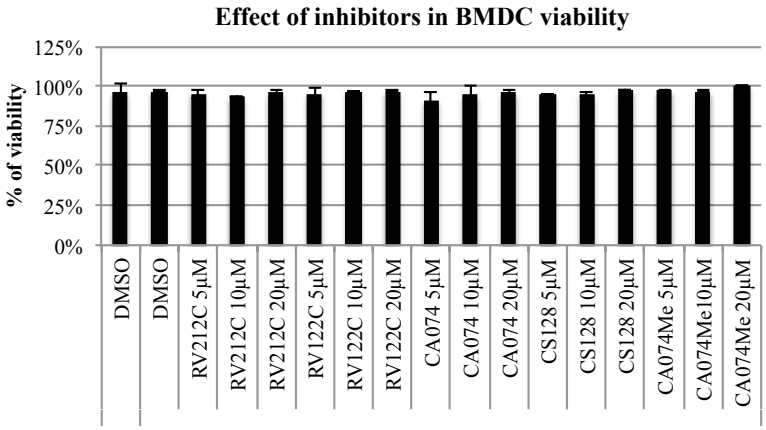

Figure A.1. **Effect of inhibitors in BMDC viability.** BMDC form BALB/c mice were incubated for 48 h with different concentrations of cathepsin inhibitors, and the percentage of viable cells was determined by using Trypan Blue staining.

A.3 Titration of cathepsin inhibitors

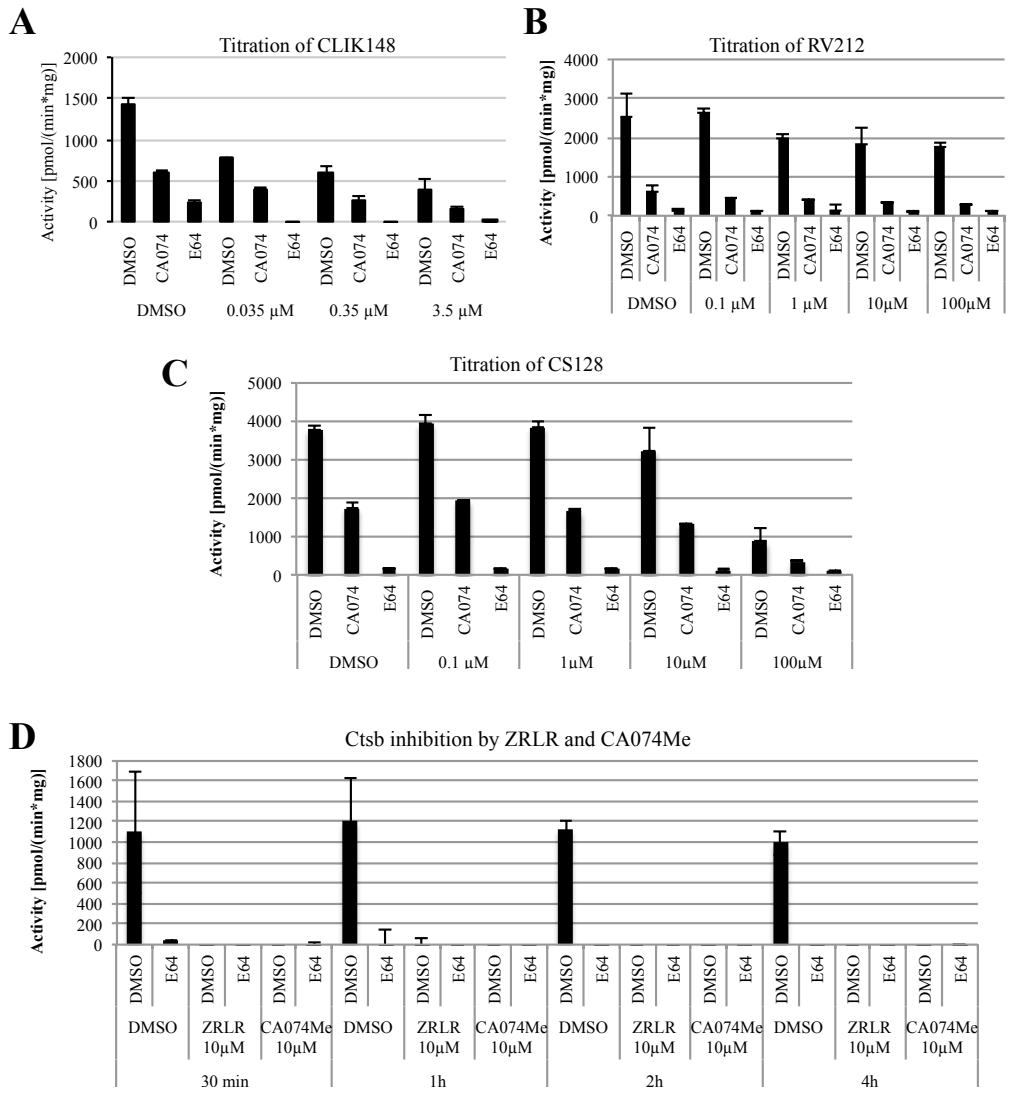

Figure A.2. **Titration of cathepsin inhibitors.** BMDC form BALB/c mice were incubated for 1 h with different concentrations of cathepsin inhibitors, and lysates were prepared from these cells. Incubation of the lysates with CA074 and E64 was used to differentiate between Ctsb and Ctsl activity. The remaining proteolytic activity of the lysates against z-Phe-Arg-AMC was then measured. Titration of (A) CLIK148, (B) RV212C, and (C) CS128. (D) BMDC were incubated for 30 min, 1 h, 2 h, and 4 h with 10 μM of ZRLR or CA074Me, and lysates were prepared. The remaining Ctsb activity in these lysates was measured by using the substrate z-Arg-Arg-AMC.

A.4 Cathepsin S inhibition

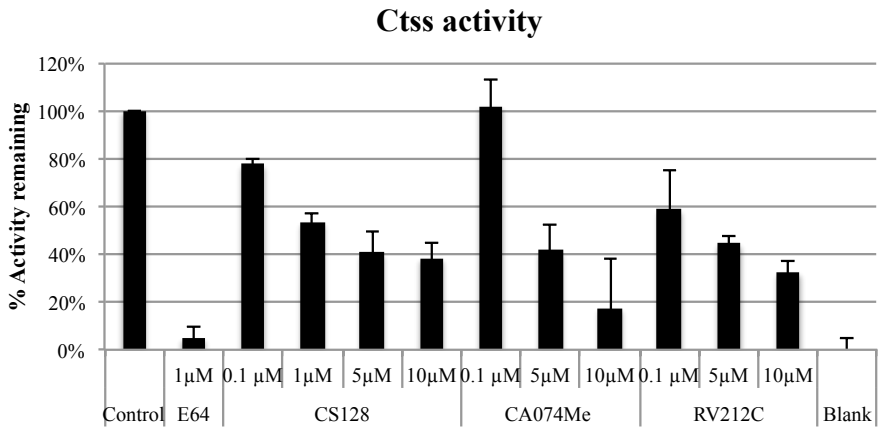

Figure A.3. **Unspecific cathepsin S inhibition.** The unspecific inhibitory activity of CA074Me, CS128, and RV212C was measured against recombinant Ctss, and the remaining proteolytic activity is here reported.

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Acknowledgements

I would like to thank all the members of my thesis committee. To Prof. Heidrun Moll, for her valuable guidance during my thesis, for her trust to allow me to work very independently and for being a mentor for me. To Prof. Manfred B. Lutz for his very helpful suggestions on experiments and very exciting scientific discussions, and for giving me the chance to perform some of the experiments here included in his lab. To Prof. Tanja Schirmeister, for providing me with some the inhibitors used in this study and for her guidance on the biochemistry of cysteine cathepsins.

The work here presented was possible thanks to different funding sources. This work was supported by a grant of the German Excellence Initiative to the Graduate School of Life Sciences, University of Würzburg. Thanks to the SFB 630 for funding some of the materials used for this study, and myself for the period of July-November 2014. Thanks to the Frauenbüro of the University of Würzburg for funding me during the period March-June 2014 with the program "Chancengleichheit für Frauen in Forschung und Lehre".

An important part of my thesis would not have been possible without the contributions of the collaboration partners that I had the privilege to work with. Thanks to Prof. Thomas Reinheckel, from the University of Freiburg, for kindly providing me with the cathepsin-deficient mice, and for the helpful scientific discussions. To PD Dr. Timo Burster, from the University of Ulm, and Dr. Ewa Wieczerzak, from the University of Gdansk, for providing me the cathepsin B inhibitor ZRLR and analyzing lysates from my cells looking for unspecific inhibition. Special thanks to PD Dr. Heike Hermanns, for allowing me to perform WB for intermediates of different signaling pathways in her lab, and for the great scientific discussions.

I would like also to thank for their contributions to all the members of the AG Moll lab: Dr. Uta Schurigt, Dr. Anita Masic, Bianca Röger, Martina Schultheis, Rebuma Fiderssa Fite, and Christina Daumberger. Among them, I want to particularly thank Bianca Röger for assisting me in several experiments included in this study, and to Anita Masic for the helpful scientific discussions. Many thanks also to the members of the AG Lutz lab for helping me with the co-culture experiments performed there, specially to Cinthia Silva-Vilches. In addition, thanks to Sabine Walter from the AG Hermanns, for her help on the WB that I performed in this lab.

Also many thanks to Dr. Alicia Ponte-Sucre and Dr. Robison Ramírez-Pineda, for their tips and fruitful scientific discussions. Thanks to Dr. Garbielle Blum-Oehler, for her support and advice during all the course of my PhD. Special thanks to M.Sc. Pável A. Marichal-Gallardo, for his valuable suggestions on the figures included in this dissertation and his patient help with my questions on LaTeX, and for his great support during the course of my PhD.

To my family, for their great support throughout my life, particularly on my quest to the other side of the Atlantic to become a scientist. To my sister Claudia, and my brother Ray, for being a great motivation for me. *A mi familia, por su gran apoyo durante toda mi vida, y particularmente, en esta aventura al otro lado del Atlántico para convertirme en una científica.*

Affidavit

I hereby confirm that my thesis entitled "Roles of Cathepsins B and L in the Th1/Th2 polarization by dendritic cells" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg, December 8th, 2014 Iris J. Gonzalez Leal

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation "Roles of Cathepsins B and L in the Th1/Th2 polarization by dendritic cells" eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg, December 8th, 2014 Iris J. Gonzalez Leal

List of publications

Publications

Gonzalez-Leal I J, Röger B, Schwarz A, Schirmeister T, Reinheckel T, Lutz M B, Moll H (2014) Cathepsin B in antigen-presenting cells controls mediators of the Th1 immune response during *L. major* infection. *PLoS NTD.* 8 (9) e3194.

Posters and Presentations in International Symposia

14-17.03.2012. Joint Meeting of the German Society for Tropical Medicine and Health and the German Society for Parasitology (DGP). Heidelberg, Germany.
Contribution: Poster "Expression and Activity of lysosomal cysteine cathepsins in dendritic cells from mice that are susceptible and resistant to *Leishmania* infection".

11-14.09.2013. 43rd Annual Meeting of the German Society for Immunology (DGfI). Mainz, Germany.
Contribution: Poster "Cysteine cathepsin inhibitors alter murine dendritic cell maturation and cytokine production".

09-10.10.2013. 8th GSLS International Student Symposium. Würzburg, Germany.
Contribution: Poster "Infection of bone-marrow derived dendritic cells and macrophages from cathepsin B and cathepsin L deficient mice with *Leishmania major*: a tale of susceptibility and resistance".

10-12.10.2013. 27th Annual Meeting oft he Macrophage and Dendritic Cell Society. Erlangen, Germany.
Contribution: Poster "Infection of bone-marrow derived dendritic cells and macrophages from cathepsin B and cathepsin L deficient mice with *Leishmania major*: a tale of susceptibility and resistance".

20-22.11.13. SFB630, International Symposium "Novel Agents Against Infectious Diseases-An Interdisciplinary Approach". Würzburg, Germany.
Contribution: Talk "Cathepsin-deficient dendritic cells present alterations in maturation and cytokine production in response to *L. major* infection"

09-07.05.2014. 3rd Molecular Microbiology Meeting Würzburg (M3W3). Würzburg, Germany.
Contribution: Poster "Enhanced maturation and IL-12 production by cathepsin B deficient dendritic cells and macorphages in response to *L. major*".