Studying MHC I-dependent $CD8^+$ T cell responses in the model of murine cutaneous leishmaniasis

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Date of oral exam 20.08.2010

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1. Summary

Healing of *Leishmania major* infections is based on IFN-γ secretion of both $CD4^+$ Th1 and $CD8^+$ Tc1 cells.

Since only a single epitope for effective $CD4^+$ T cell-mediated immune responses has been identified from the LACK protein so far [1], the aim of this work was to gain more insight in MHC class I-dependent CD8 T cell responses.

For this approach, we first analysed the vaccination effect of the LACK protein fused to the protein transduction domain of the HIV-1 (TAT), which directly translocates proteins into the cytosol and renders processed peptides from these translocated proteins able to be presented to $CD8^+$ T cells. We confirmed the role of $CD8^+$ T cells after *in vivo* protein vaccination of self-healing C57BL/6 mice with TAT-LACK in depletion experiments.

Processing of proteins prior to effective presentation of immunogenic peptides for T cells is absolutely required. Thus, processing of *L. major* proteins and presentation of peptides in the context of MHC class I pathway was analysed by investigating the role of the IFN-γ-inducible immunoproteasome for priming $CD8^+$ T cells *in vivo* and *in vitro*. Within this work, we showed that shaping of immunogenic $CD8^+$ T cell epitopes is mediated in an immunoproteasome-independent pathway.

Additionally, fractions of soluble *Leishmania* antigen (SLA) from either the promastigote or the amastigote life form of the parasite were generated by size exclusion chromatography. In ongoing experiments, these fractions will be analysed for potential immungenic contents.

Finally, epitope prediction of possible $CD8^+$ T cell peptides was performed of life form-dependend protein expression based on computer algorithms. 300 of these peptides have been synthesized and will be further analysed for their immunogenic properties

In conclusion, this work aimed at contributing to the knowledge about antigen processing and the identification of possible $CD8^+$ T cell epitopes. A detailed understanding of the pathway utilized for presentation of *L. major* derived CTL-epitopes and the characterisation of these epitopes will be helpful for the development of new vaccine candidates against this important human pathogen.

Zusammenfassung

Der Ausheilung von Infektionen mit *Leishmania major* liegt die Sekretion von IFN-γ von sowohl $CD4^+$ als auch $CD8^+$ T Zellen zugrunde.

Aktuell konnte in der Literatur nur ein Epitop aus dem parasitären LACK Protein für eine effektive $CD4^+$ T Zell-vermittelte Immunantwort beschrieben werden. Das Ziel der vorliegenden Arbeit bestand daher darin, mögliche MHC I abhängige $CD8^+$ T Zell Antworten zu untersuchen.

Für diesen Ansatz wurde als erstes der Effekt einer Vakzinierung mit LACK Protein fusioniert an die Protein-Transduktionsdomäne des HIV-1 (TAT) analysiert. Die Effektivität von TAT-LACK gegenüber $CD8^+$ T Zellen wurde mittels *in vivo* Protein-Vakzinierung von resistenten C57BL/6 Mäusen in Depletions-Experimenten gezeigt.

Die Prozessierung von Proteinen vor der Präsentation immunogener Peptide gegenüber T Zellen ist unbedingt erforderlich. Daher wurde in dieser Arbeit die Rolle des IFN-γ-induzierbaren Immunoproteasoms bei der Prozessierung von parasitären Proteinen und Präsentation von Peptiden gebunden an MHC I Moleküle durch *in vivo* und *in vitro* Experimente untersucht. Es konnte in dieser Arbeit eine Immunoproteasom-unabhängige Prozessierung aufgezeigt werden.

Weiterhin wurde Parasitenlysat (SLA) von sowohl Promastigoten als auch Amastigoten fraktioniert. In weiterführenden Experimenten können diese Fraktionen auf immunodominante Proteine/Peptide hin untersucht werden.

Letztlich wurden Epitop-Vorhersagen für $CD8^+$ T Zellen mittels computergestützer Software von beiden parasitären Lebensformen durchgeführt. 300 dieser Epitope wurden synthetisiert und werden in weiterführenden Experimenten zur Charakterisierung immunogener Eigenschaften weiter verwendet.

In ihrer Gesamtheit trägt die vorliegende Arbeit wesentlich zum Verständnis über die komplexen Mechanismen der Prozessierung und letztendlich zur Identifikation von möglichen $CD8^+$ T Zell Epitopen bei. Ein detailiertes Verständnis der Prozessierung von $CD8^+$ T Zell Epitopen von *Leishmania major* über den MHC Klasse I Weg ist von höchster Bedeutung. Die Charakterisierung sowie die Identifikation dieser Peptide wird einen maßgeblichen Einfluss auf die weiteren Entwicklungen von Vakzinen gegen diesen bedeutenden human-pathogenen Parasiten mit sich bringen.

2. Introduction

2.1 Leishmaniasis

Although leishmaniasis is not a household name like malaria, the disease caused by infection with *Leishmania spp*. continues to have a major impact on mankind worldwide. Leishmaniasis currently threatens 350 million men, women and children in 88 countries around the world (mostly in the world´s inter-tropic and temperate regions, Fig. 1), affecting 12 million people with 2 million new cases each year (World Health Organization, Leishmaniasis Control home page: www.who.int/leishmaniasis/disease_epidemiology/en/index.html).

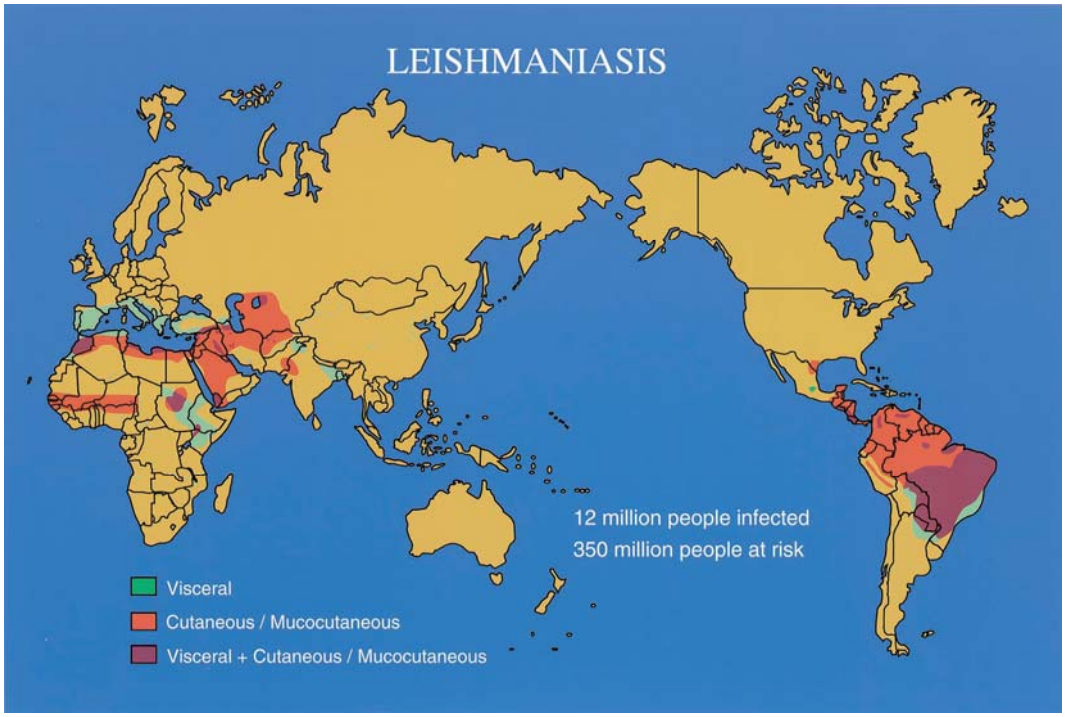

Fig. 1: **Distribution of leishmaniasis**
World map highlighting areas where the three different forms of leishmaniasis, cutaneous (bright red), visceral (green) and mucocutaneous (dark red) leishmaniasis are endemic. 12 million people are currently infected with the disease and 350 million people are at risk of infection. Graph taken from [2].

The disease is transmitted to the host by the bite of an infected female sandfly of the genus *Phlebotomus* or *Lutzomyia* during the blood meal. Leishmaniasis is considered a zoonosis, involving domestic or wild animals as reservoir hosts, or an anthroponosis, with humans as hosts. The animal reservoir shows geographic variations and includes rodents, dogs and other mammals, all of which plays an important role in the

distribution of the disease [3]. Leishmaniasis can be caused by 20 different parasite species with a wide range of clinical symptoms [4].

Parasites of *Leishmania spp.* cause human diseases with different pathological responses (Table 1). The manifold clinical manifestations of leishmaniasis are a result of the interaction between the diverse genetic variability of both the parasites and the hosts. In human infections, the host population is highly heterogeneous and the parasites are not clonal. This makes it difficult to assess the relative contibution of the parasite and the host to disease outcome. However, the common feature to all forms of leishmaniasis is the chronic manifestation of disease.

Cutaneous form

90% of all *leishmania* cases manifest as the localized cutaneous form (CL) of the disease caused by different species of the parasite, including mild or severe ulcers depending on the immune status of the patient, i.e. HIV-*leishmania* co-infections [5]. In general, this form of the disease is non-lethal and skin lesions are spontaneously self-healing with scarring or ulcerated plaques or nodules on exposed sites. Usually, the cutaneous form is restricted to the skin, which is dependent on the immune response of the infected host. Species inducing solely cutaneous disease may also promote more severe forms of the disease [6].

Mucocutaneous form

The mucocutaneous form (MCL) of the disease is a more severe form of CL with parasites of the *L. braziliensis* complex disseminating towards mucous membranes. After an initial skin lesion, that slowly but spontaneously heals, chronic ulcers appear after months or years on the skin, mouth and nose, with destruction of underlying tissue (nasal cartilage, for example). Tissue destruction with disfigurement can be very severe. The pathogenicity of this form is strongly dependent on additionally occuring infections of the patient.

Visceral form

Some *Leishmania* species cause the visceral form (VL) of infection characterized by spreading of parasites to and invasion of inner organs like liver, spleen, bone marrow and lymph nodes. Untreated, this form of the disease is lethal in almost 100% of cases. Medication with antimonial drugs or liposomal amphotericin B is not highly efficient and includes severe side effects [7]. A promising new drug and by the first to be orally applicated is miltefosin (hexadecylphosphocholine), which is currently being tested in phase IV trials [8].

To date, there are no effective vaccines against leishmaniasis.

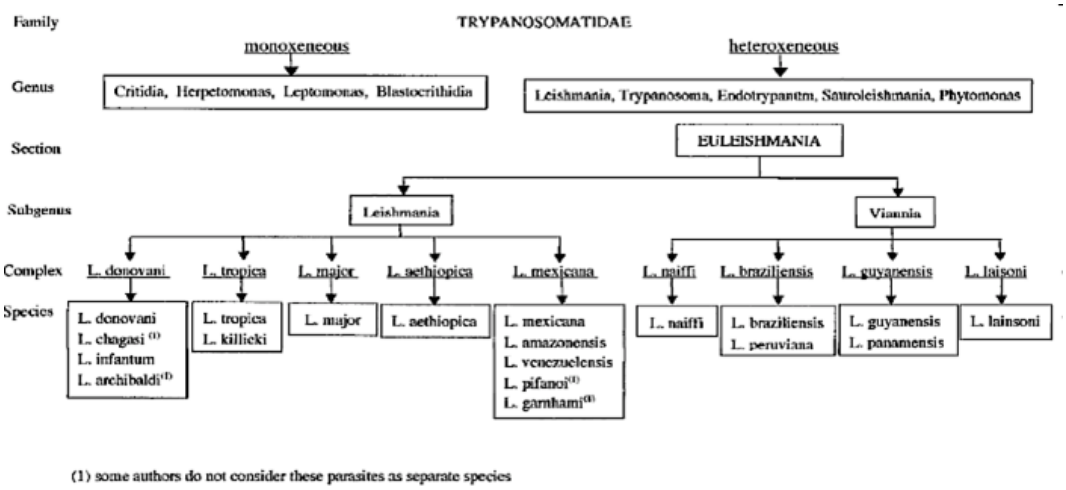

Table 1 **Classification of *Leishmania* species.** Graph taken from: http://awmf.org

2.2 The life cycle of *Leishmania major*

Leishmania major exist in two different life forms, the extracellular promastigote form (Fig. 2A) and the intracellular amastigote form (Fig. 2B). Promastigotes are elongate, slender and measure about 10-12 µm in length. They have a large central nucleus and a kinetoplast located near the anterior end. A flagellum arises at the anterior end, that may be longer than the rest of the promastigote. Amastigotes of *Leishmania* are spherical to ovoid and have a length of approximately 1-5 µm and a width of 1-2 µm. They possess a large nucleus and a prominent kinetoplast.

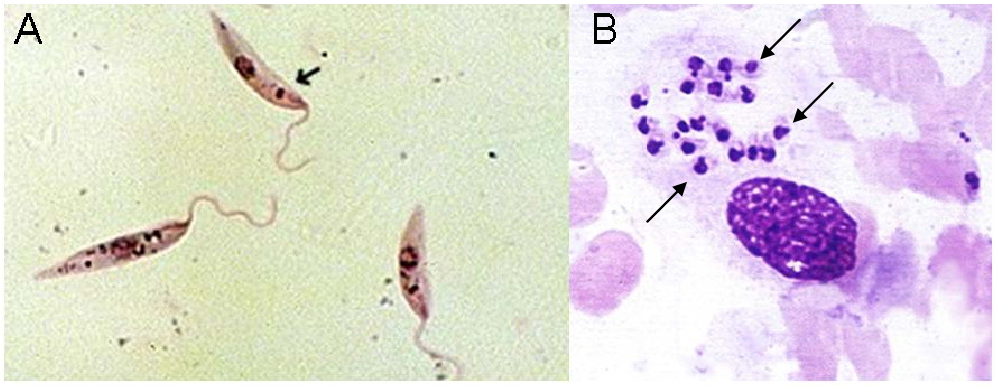

Fig. 2 **Promastigote and amastigote life form of *Leishmania spp*.**
The extracellular promastigote life form of *Leishmania spp*. (A) is about 10-12 µm in length, with a central nucleus, an anterior located kinetoplast and a flagellum. The intracellular amastigote life form (B, arrows) remains within phagolysosomes of phagocytes. Amastigotes are of ovoid shape with 1-5 µm in length and 1-2 µm in width.

The life cycle is initiated when female sandflies feed from infected tissue or blood that contains macrophages infected with the amastigote life form of the parasite. The infected bloodmeal passes from the sandfly abdominal midgut towards the foregut (Fig. 3). Different parasite stages were characterized by morphological and functional changes taking place to survive in the fly [9]. First, amastigotes differentiate into small procyclic promastigotes with short flagella commencing to the first multiplication cycle in the fly [10]. Finally, in the infective stage of the parasite, the metacyclic promastigotes are accumulated in the stomodeal valve multiplying in a series of replicative cycles. This form of the parasite is highly adapted for successful transmission to the upper dermis of the skin of mamalian hosts [11].

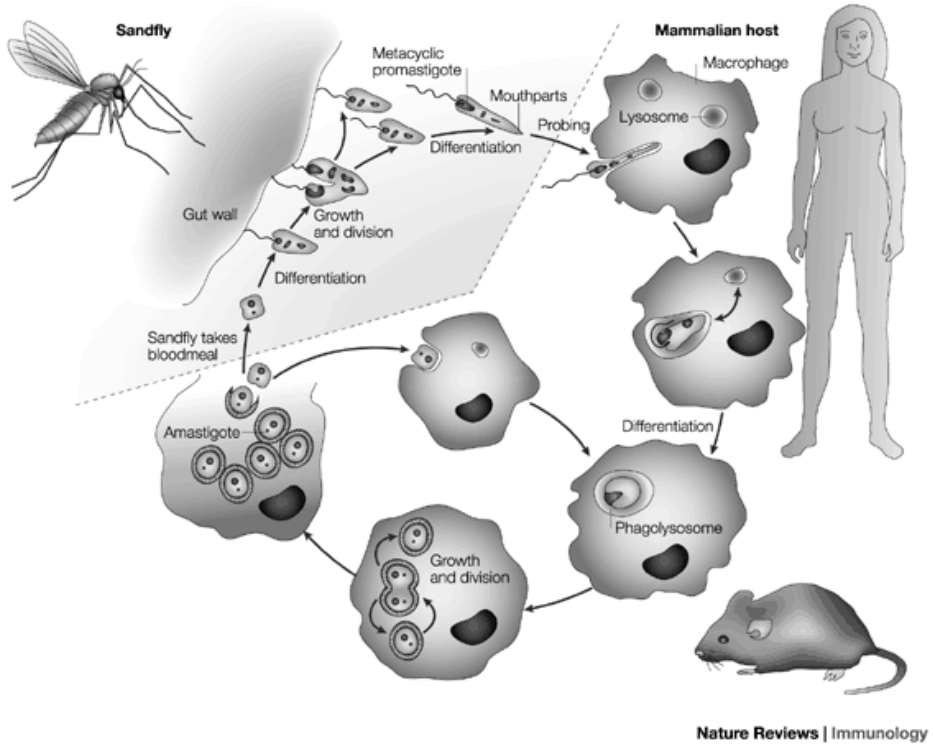

Fig. 3 **Life cycle of *Leishmania major***
Released amastigotes or amastigote-infected MΦ are taken up by the sand fly during blood feeding. In the midgut of the fly, amastigotes transform into rapidly dividing, non-infectious stage promastigotes. These forms undergo a process of attachment to the midgut wall, release and anterior migration that is accompanied by their differentiation to non-dividing, metacyclic promastigotes which can be transmitted when the sand fly takes another blood meal. 10-1,000 infectious stage promastigotes are inoculated into the upper dermis of the skin of the host. Parasites are opsonized efficiently by serum components and taken up by complement receptor (CR) 3 of MΦ. Within MΦ, promastigotes reside in

phagolysosomes and transform into replicating amastigotes. Graph taken from [12].

Upon feeding, 10-1,000 promastigotes are inoculated into the skin and are rapidly phagocytosed by macrophages (MΦ). Phagocytosis serves as an important defense mechanism against pathogens, however, *Leishmania* parasites have adapted to take advantage of this cellular uptake, since they are obligate intracellular protozoan microorganisms. *Leishmania* promastigotes are resistant to the lytic complex of complement [13] and the main receptor responsible for parasite entry into MΦ is complement receptor (CR) 3 [14]. Once within the MΦ, the infectious stage promastigotes transform into the amastigote life form of the parasite capable of surviving inside of phagolysosomes. Amastigotes replicate within the phagolysosomes and rupture of the MΦ leads to the release of high parasite numbers into the adjacent tissue leading to further infection. The life cycle of *Leishmania major* is completed when a sand fly feeds from infected tissues or blood.

2.3 Experimental murine cutaneous leishmaniasis

2.3.1 Th1/Th2 immune responses

The first direct demonstration of the relevance of the T helper (Th) 1/Th 2 balance for regulation of disease outcome *in vivo* arose from mouse studies in the *Leishmania major* model [13;15;16]. Certain mouse strains like BALB/c mice fail to control infection and develop progressive lesions and systemic disease. The genetic predisposition for susceptibility or resistance to *L. major* infection in mice correlates with the dominance of an interleukin-4 (IL-4)-driven Th2 response that eventually causes disease progression and death (BALB/c mice) or an IL-12-driven, interferon-γ (IFN-γ)-dominated Th1 response that promotes healing and parasite clearance (C57BL/6 mice). It was already been shown in the late 80´s that $CD4^+$ Th1 cells induce resistance to *Leishmania* infections [15;17]. Both, *Leishmania*-specific $CD4^+$ Th1 cells and $CD8^+$ Tc1 cells [18] were primed by infected dendritic cells (DC) after uptake of released amastigotes of *L. major* (aLm; Fig. 4).

Interestingly, and in contrast to MΦ, DC have been shown to phagocytose only the amastigote life form, whereas infectious stage promastigotes (pLm) were phagocytosed to a lesser extent [19-23]. Infected DC migrate to the draining lymph nodes and activate $CD4^+$ Th1 cells and $CD8^+$ Tc1 cells in an IL-12 dependent manner [24]. T cells, in turn, migrate back to the side of infection and secrete the pro-inflammatory cytokine IFN-γ, which induces autocrine NO^{2-} secretion in MΦ. NO^{2-} induces intracellular parasite elimination [4]. Thus, healing of murine

Leishmania infection requires both, $CD4^+$ Th1 and $CD8^+$ Tc1 immune responses with secretion of IFN-γ.

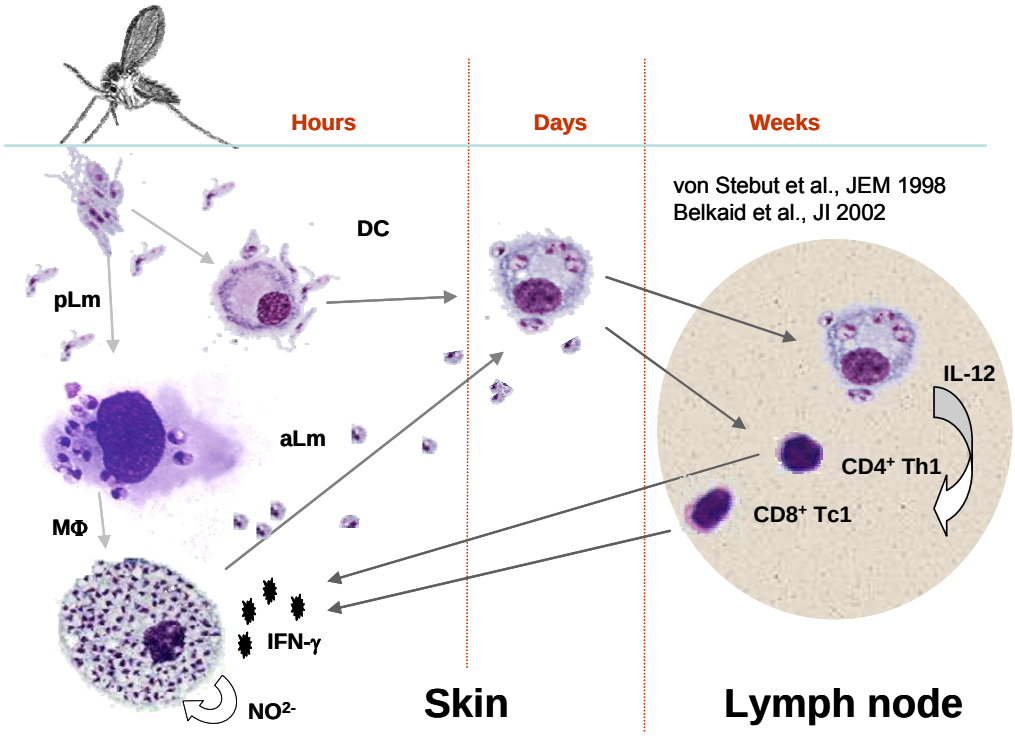

Fig. 4 **The *Leishmania* paradigm**
After inoculation of metacyclic promastigotes of *Leishmania major* (pLm) into the skin, parasites invade macrophage (MΦ) host cells within hours post infection but not dendritic cells (DC) and – within phagolysosomes – they transform into obligate intracellular amastigotes (aLm). MΦ infection does not lead to activation of the adaptive immune system. Release of amastigotes into the tissue of rupted MΦ results in their phagocytosis by DC within days post infection, which activates DC and enables them to secrete IL-12. DC, in turn, effectively induce activation of $CD4^+$ Th1 and $CD8^+$ Tc1 cells. Both subsets of effector T cells secrete IFN-γ, which plays a critical role for protection by inducing NO^{2-} in MΦ. NO^{2-} renders these cells able to kill the intracellular form of *L. major*.

Infection of *Leishmania major* in resistant C57BL/6 mice is used as a surrogate model of the cutaneous from of the disease in humans, in contrast to infection of susceptible BALB/c mice, which resembles the visceral form of leishmaniasis in humans induced by *L. donovani* or other *Leishmania spp*.

2.3.2 Experimental infections of mice

Unfortunately, there is no standardized experimental infection model of mice with *L. major* and various approaches are described in the literature. It cannot be ruled out that varying conditions

might have influenced the outcome of infection. The most frequently used model is the subcutaneously inoculation of a high dose (10^4 – 10^7) of stationary-phase promastigotes of the parasite into the hind footpad. However, sometimes different developmental stages of the parasite were used [25]. Alternative routes of infection such as intravenous [26], intradermal [27] or intranasal [28] administration as well as the delivery of parasites using the sandfly vector [29] have also been described. Additionally, different *L. major* substrains can impact disease outcome as well [30].

All experiments within this work were performed with the *L. major* substrain clone VI (MHOM/IL/80/Friedlin), which was inoculated intradermally in either "high dose" or "low dose" numbers of metacyclic promastigotes. In the high dose inoculation, mice were infected with $2x10^5$ metacyclic promastigotes. The advantage of this model is the rapid development of lesions within a few weeks post infection. In the low dose model, mice were infected with 1,000 metacyclic promastigotes. The low dose model mimics the natural transmission by sand fly bites and thus has a much higher physiological relevance [26]. A clear disadvantage of the low dose model is the slower development of lesions, which first appear at week three post infection. The infection is performed intradermally into the ear of anasthetized mice and the developing granuloma can be measured in three dimensions, which is in contrast to the subcutaneous inoculation of the hind foot pads allowing only two dimensional quantification. Further, the migration of inflammatory cells to the infected ear dermis is methodically more reliable [31] and harvest of submandibulary draining lymph nodes is easier to perform [32].

2.3.3 Leishmaniasis in resistant C57BL/6 mice

The infection of resistant C57BL/6 mice with low dose inocula of *Leishmania major* in experimental designs can be separated into three distinct phases [26].

Silent (first) phase

After the inoculation of promastigotes, parasites are taken up by MΦ and fibroblasts [33] and transformed into the amastigote life form. The uptake of the promastogote life form of the parasite at this time point is mainly mediated by CR3-dependent phagocytosis by MΦ. The phagocytes that engulf parasites in infected tissue are kept in a "silent state", unable to initiate T cell-mediated immune responses. The presentation of antigen [34-36], the expression of co-stimulatory molecules [37-39], the production of IL-12 [40-43] for efficacious Th1/Tc1 immune responses and expression of iNOS (inducible nitric oxide synthase) of these cells is inhibited efficiently [44]. The amastigote life form of *L. major* can survive in the phagolysosomes of inactivated MΦ and can amplify until

infected cells are ruptured. This 4-5 weeks lasting process is called “silent phase”, because of the lack of visible clinical symptoms during this phase.

Second phase

First clinical symptoms such as redness and swelling of the skin at the site of infection are visible in the second phase of the disease. Neutrophils, eosinophils and MΦ migrate towards the infected tissue [26], a process, which is partly mediated by complement [45] and cutaneous mast cells [46].

Third phase

DC as efficiacious T cell activators are found in infected tissue 6 weeks post infection [26;47]. The migration of DC towards the side of infection might be mediated by mast cell-derived mediators [46], IgG-mediated mechanisms [47] and cytokine and chemokine release [48]. It has already been reported [26] that the occurance of DC correlates with the migration of *Leishmania*-specific T cells (both, Th1 and Tc1) towards and parasite elimination at the side of infection. Visible symptoms like ulceration of infected tissue can be observed at this time point.

2.3.4 Dendritic cells and macrophages in leishmaniasis

Both, DC that migrate towards the site of infection several weeks post infection, as well as skin residental MΦ contribute to anti-*Leishmania* immunity. MΦ are among the first cells of the innate immune system that take up inoculated promastigotes as well as liberated amastigotes from prior lysed MΦ [49], parasite phagocytosis by these cells is a “silent” process. MΦ are not activated and no protective T cell-mediated immune response is initiated by these cells. This might be explained by CR3-mediated phagocytosis of parasites by MΦ [50]. In contrast, uptake of amastigotes by DC is mediated more frequently by Fcγ receptor (FcγR) I and FcγRIII [47] that resulted in activation of DC. Activation of DC is solely achieved by engulfment of the amastigote life form of the parasite. Additionally, the induction of both, $CD4^+$ and $CD8^+$ T cell-mediated immunity is restricted to activated DC [4;18]. It has been reported that MΦ are unable to prime naïve T cells against *Leishmania* antigen [51] and cannot re-stimulate primed $CD8^+$ T cells [26]. Thus, different recognition and engulfment processes might contribute to different priming of T cells [47].

In addition, activated DC are more potent in expressing inflammatory cytokines and the upregulation of MHC as well as co-stimulatory molecules [52-56]. IL-12 as a key mediator of the induction of Th1/Tc1 immune responses [57-59] has already been reported to be secreted mainly by activated DC in lymphoid tissue [21;60]. In conclusion,

activation of DC by engulfment of amastigotes is absolutely required for efficacious priming of $CD4^+$ and $CD8^+$ T cells by DC rather than by MΦ.

2.3.5 $CD8^+$ T cells in leishmaniasis

Despite the fact that *Leishmania major* is an obligate intracellular parasite, the relevance of $CD8^+$ T cells in addition to $CD4^+$ T cells in healing *Leishmania* infections was neglected for a long time, only gaining appreciation in the last decade. It was assumed in the past that the main role of $CD8^+$ T cells was to provoke immunity against re-infection with the same pathogen and to maintain memory responses [61-63]. Resistant C57BL/6 mice showed higher frequencies of *Leishmania*-specific $CD8^+$ T cells compared to susceptible BALB/c mice [64]. After depletion of these cells in resistant mice, animals elicited an exacerbated disease outcome, but recovered from the disease [65]. Findings in line with results from CD8 depletion were obtained with mice deficient for CD8 or β_2-globulin [66-68]. In all these studies, high dose infection of parasites was implemented, which were injected subcutaneously into the foot pad.

In contrast to these studies, it has been shown in the physiologically more relevant low dose infection model [18] that $CD8^+$ T cells are required for primary immunity for C57BL/6 mice. CD8-depleted or -deficient C57BL/6 mice did not recover from infection following intradermal challenge with *Leishmania major*. Further, T cell-deficient $RAG^{-/-}$ mice were resistant to *Leishmania major* after adoptive transfer of $CD8^+$ T cells from C57BL/6 wild type mice. It appears that IFN-γ secretion of both $CD4^+$ and $CD8^+$ T cells plays a pivotal role for healing, whereas it is uncertain if the cytotoxic activity contributes to parasite elimination [69-71]. Additionally, $CD8^+$ T cells regulated the expression of IFN-γ by $CD4^+$ T cells, indicating that cross-talk between $CD4^+$ and $CD8^+$ T cells is important in primary *L major* infections [72]. Thus, in addition to $CD4^+$ T cells, $CD8^+$ T cells are absolutely required for primary immunity in murine leishmaniasis.

2.4 $CD8^+$ T cell priming

2.4.1 The MHC class I processing pathway

Efficacious T cell-mediated anti-*Leishmania* immune responses require initial activation and priming of naïve $CD4^+$ and $CD8^+$ T cells. In general, priming of $CD8^+$ T cells is induced by presentation of peptides bound to MHC class I molecules [73]. MHC class I associated peptides are 8-11 residues in length, typically nine residues, and most of these peptides are generated by an abundant and ubiquitious protease, the proteasome [74] (Fig. 5). Under normal conditions, proteasomes freely diffuse in the cytoplasm and nucleus and, therefore, might find substrates

by simple collision [75]. As most peptides have limited capacity to diffuse across membranes, transport from the cytoplasm to the endoplasmatic reticulum (ER) requires transporter for antigen processing (TAP) – an ER-resident, heterodimeric peptide transporter [76]. TAP binds cytosolic peptides in an ATP-dependent manner and expells the bound peptide into the lumen of the ER [77].

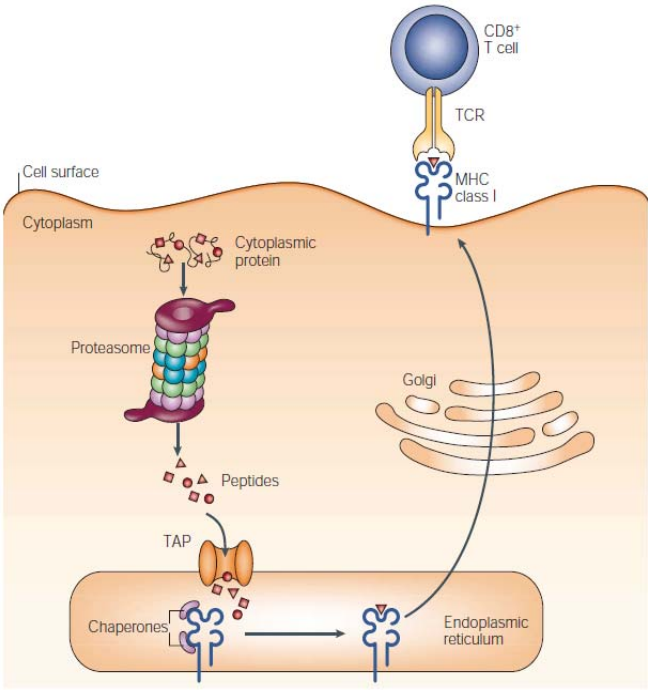

Fig. 5 **MHC class I antigen presentation: the basics** Cytosolic and nuclear proteins are degraded by the proteasome into peptides. The transporter for antigen processing (TAP) then translocates peptides into the lumen of the endoplasmic reticulum (ER) while consuming ATP. MHC class I heterodimers wait in the ER for the third subunit, a peptide. Peptide binding is required for correct folding of MHC class I molecules and release from the ER and transport to the plasma membrane, where the peptide is presented to the immune system. TCR, T-cell receptor. Graph taken from [78].

TAP is one subunit of the MHC class I loading complex, which increases the efficiency of peptide loading by clustering the relevant molecules that are involved in MHC class I loading in a single location. The loading complex includes 3 or 4 copies of the dedicated molecule chaperone tapasin, each bound to a peptide-receptive MHC class I molecule, and one copy each of the general purpose molecular chaperones calreticulin and ERp60 [79]. Tapasin interacts directly with MHC class I molecules and is required for optimal peptide loading [79;80]. Tapasin simultaneously interacts with ERp60, which in turns interacts with calreticulin. ERp60 is a thioreductase that supports the formation of a disulphide bridge that connects the walls of the MHC class I peptide-binding groove with its base [81]. Successful peptide binding releases the MHC class I molecule from the MHC class I loading complex for delivery to the cell surface through the standard secretory pathway. $CD8^+$ T cells recognize the complex of peptide and MHC class I molecule and, in turn, can be activated.

2.4.2 The (immuno-) proteasome

The 26S proteasome degrades polyubiquitinated cellular protein substrates, making it the key enzyme forming $CD8^+$ T cell epitopes. The proteasome is composed of the catalytic 20S core complex and two 19S regulator complexes that confer binding and unfolding of ubiquitinated substrates (Fig. 6) [82]. The 20S core complex is composed of 14 nonidentical subunits, which form 4 stacked rings of seven different subunits each. The hydrolysing activity is conferred by three of these seven β subunits in the two inner rings β1, β2 and β5 [83].

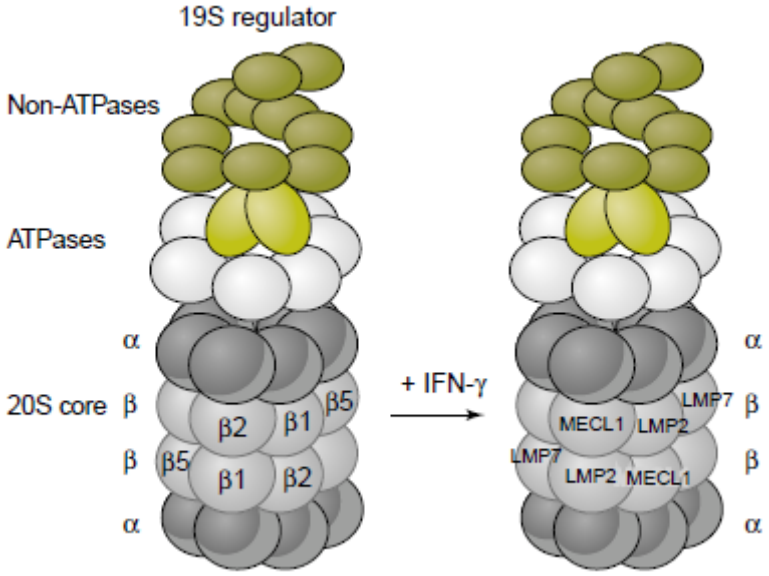

Fig. 6 **The proteasome and conformational changes in the IFN-γ-induced immunoproteasome**
The 20S proteasome consists of 28 (14 different) subunits, 21–31 kDa in molecular mass, which are arranged as four heptameric staggered rings. The outer rings contain the a subunits, the inner rings the β subunits. Three of the seven β subunits in each β ring harbour the active sites (β1, β2 and β5). In total there are six active sites within one 20S core complex. The 19S regulator, which attaches to both sides of the 20S core comprises the base, composed of six ATPase subunits and two non-ATPase subunits, and the lid, which contains 8–10 non-ATPase subunits. Upon IFN-γ induction, the synthesis of three β subunits, all with active sites, is induced. These so called immunosubunits, LMP2, MECL1 and LMP7, are incorporated into the complex upon de novo synthesis of the 20S core and form, together with the other subunits, the newly assembled immunoproteasome. Graph taken from [84].

There are two different types of proteasomes: the constitutive form, present in every cell, and the IFN-γ-inducible immunoproteasome. IFN-γ induces the synthesis of immunosubunits β1i (also named low molecular weight protein 2; LMP2), β2i (multicatalytic endopeptidase complex-like 1;

MECL-1) and β5i (LMP7) all of which replace the constitutive β1, β2 and β5 subunits in the 20S core proteasome [85-88]. The β5i/LMP7 subunit is essential for an accelerated up-regulation of the functional immunoproteasome catalytic core complex [89]. Constitutive proteasomes and immunoproteasomes have distinct proteolytic activities [90-93] and thus it is not surprising that the immunoproteasome can enhance the presentation of some epitopes [94-97], but abrogates the presentation of others [98].
In conclusion, efficacious priming of $CD8^+$ T cells required most frequently a proteasomal-dependent protein degradation pathway [99].

2.5 Vaccination against leishmaniasis

2.5.1 Treatment of leishmaniasis

Therapeutic options to cure infections with *Leishmania spp*. range from topical treatment of simple cutaneous leishmaniasis to systemic therapy, which is needed for more complex cases of cutaneous as well as mucocutaneous and visceral disease [100]. The efficacy of each therapy is dependent on its species- and stage-specific effect on *Leishmania* growth. In addition, most therapeutics can have severe side effects and are relatively cost intensive making treatment in endemic regions problematic and difficult. Due to the development of life-long immunity after resolution of infection, the development of a vaccine should be possible [101]. Despite several attempts, this has proven to be difficult and to date, there is no effective vaccine against *Leishmania*.

2.5.2 Examples of *Leishmania* vaccines

Historically, cutaneous leishmaniasis has been the focus of various vaccination attempts, probably because it has been recognized since antiquity that individuals, whose skin lesions healed, were protected from further infections. Large-scale vaccination trials (controlled infections) using live promastigotes were carried out [102;103], but the use of live vaccines has many problems, including the development of large uncontrolled skin lesions and other skin diseases. In addition to their missing potency and durability, vaccines based on parasites lysates showed low reproducibility, due to versatile variations among different parasites preparations. A successful vaccine against leishmaniasis should induce both, $CD4^+$ and $CD8^+$ T cells [12] in an IL-12-dependent manner [105] as well as long-lasting memory immune responses. Therefore, well-defined, recombinant proteins may be promising antigens for a successful vaccination strategy. Several *Leishmania*-derived proteins have already been identified as possible vaccine candidates (Table 2). These include the *Leishmania* surface glycoprotein 63 (gp63) and the histone H1, which

have been tested in mice [106-109]. Immunization with the promastigote surface antigen-2 (PSA-2) resulted in complete protection against infectious challenge with *L. major* in resistant mice, but mediated only partial protection in susceptible mice [110]. Three classes of cathepsin L-like cystein proteinases (CP) have been described, which induced partial protection so far [111;112], but one of the most frequently used *Leishmania*-antigens appears to be the *Leishmania* homologue of receptors for activated C kinase (p36/LACK).

Protein used for vaccination	Protein mixture of wild type parasites
Promastigote lysate	—
Heat killed parasites	—
Leishmania surface glycoprotein (gp) 63	—
Histone (H)1	—
Promastigote surface antigen (PSA)-2	—
Cathepsin L-like cysteine proteinase (CP)	—
Leishmania homologue of receptors for activated C kinase (p36/LACK)	—
L. major homologue of the eucaryotic thiol-specific antioxidant (TSA)	Leish-111f (tri-fusion protein)
L. major homologue of the eucaryotic stress-inducible protein-1 (LmSTI1)	—
Leishmania elongation and initiation factor (LeIF)	—

Table 2 **List of antigens used for vaccines** (taken from [104]; for references see text)

2.5.3 *Leishmania* homologue of receptors for activated C kinase (LACK)

LACK is a well-conserved, highly immunogenic 36 kDa protein [113], expressed in both life forms of all *Leishmania spp*. [1] responsible for the rapid expansion of Vβ4-Vα8$^+$ CD4$^+$ T cells [114]. Furthermore, deletion experiments with *L. major* revealed that LACK is required for parasite viability as well as pathogenesis [115].

Consequently, LACK was utilized as target antigen in several vaccine strategies. Immunization of mice with rLACK protein in combination with rIL-12 resulted in partial protection of BALB/c mice against infectious challenge with *L. major* [1;105;116]. Notably, analysis of possible immunogenic peptides of the LACK protein revealed only a single CD4$^+$ T cell activating peptide [1], but to date no counterparts for CD8$^+$ T cell activating peptides were identified.

2.5.4 Vaccination with TAT-LACK

It has been shown that exogenous TAT protein from human immundeficiency virus (HIV)-1 is taken up by cells when added to culture medium [117;118]. The 86 aa TAT protein is a strong transactivator and

essentiell for replication of the virus [119;120], as well as crossing cell membranes and translocation directly into the cytosol of target cells. This process is referred to as protein transduction, which has been observed in many cells treated with TAT protein [121]. This macropinocytosis-mediated process [122] is mediated by a 11 aa protein domain (YGRKKRRQRRR), which is referred to as a protein transduction domain (PTD) [123;124]. Within the last decade many TAT-fusion proteins have been generated for cytosolic transduction of these proteins [125-132] and their biological activity within cells has been verified *in vitro* [123;125]. TAT-fusion proteins were used for translocation of antigens directly into the cytosol of antigen presenting cells (APC) like DC for effective presentation of MHC class I antigens to $CD8^+$ T cells. Such a TAT-fusion protein-mediated activation of $CD8^+$ T cells has already been effectively utilized in tumor models [133-135] and the general mechanism is depicted for *Leishmania* antigen vaccinations *vs*. natural infections in Fig. 7. A successful vaccine against leishmaniasis should induce both *Leishmania*-specific $CD4^+$ and $CD8^+$ T cells [6], sustained IL-12 production [105] as well as long-lasting memory immune responses. Non-live, protein-based vaccines, which are capable of expanding memory T cells that can survive independently of life, persisting parasites and that are capable of producing both IL-2 and IFN-γ might therefore also be efficacious and become an alternative solution to live vaccines.

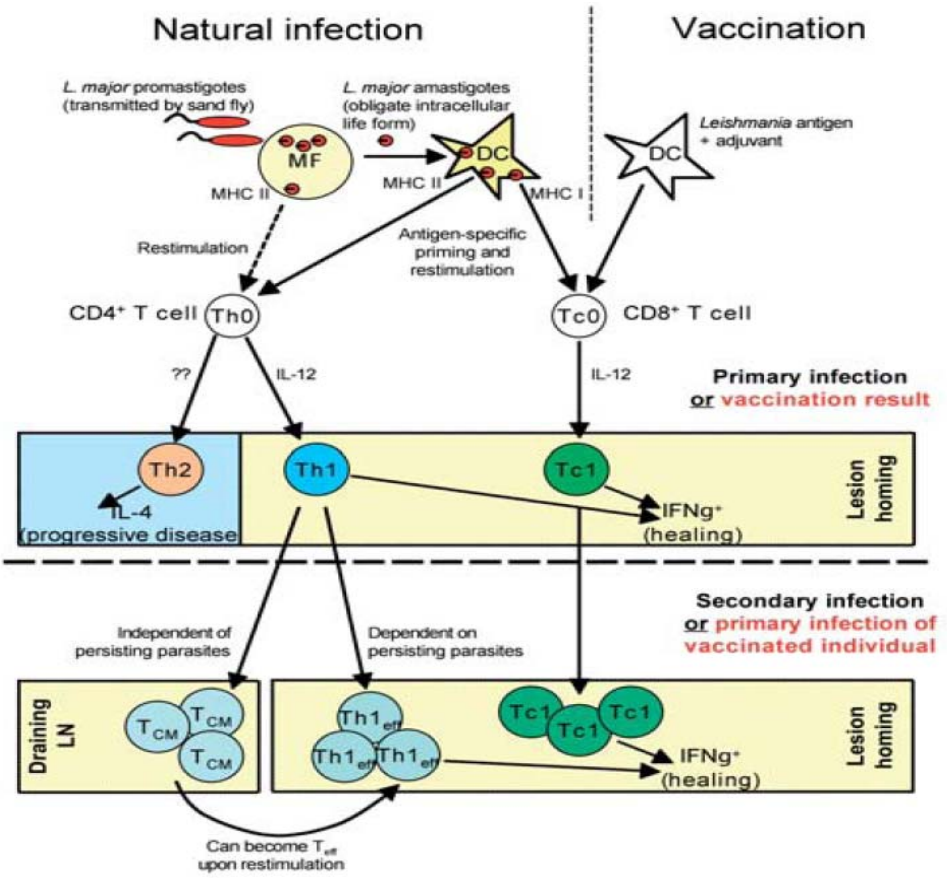

Fig. 7: **Upon infection with *L. major* or, optimally, vaccination, protective Th1/Tc1-dependent immunity develops.**
Alternatively to natural infection, targeting DC with antigen in the presence of adjuvant leads to induction of Th1/Tc1 immunity directed against *Leishmania* in a vaccination approach. Healing and lesion resolution in leishmaniasis is dependent on the release of IFN-γ from *Leishmania*-specific T cells (Th1/Tc1) homing to the lesion. Upon restimulation of antigen-specific T cells in an immune host (due to a resolved primary infection or a successful vaccination), IFN-γ is rapidly released from antigen-specific effector T cells and infection is prevented. The maintenance of Th1 cells is dependent on a few life parasites persisting in the host. In addition, central memory T cells capable of becoming effector T cells after proliferation in the draining lymph node upon re-challenge can also maintain protective immunity. Central memory T cell development is independent of persisting parasites and can be induced by recombinant vaccines. Graph taken from [104].

2.6 Epitope prediction

The development of vaccines or methods for monitoring $CD8^+$ T cell responses depends on the identification of suitable epitopes from immunologically relevant antigens. The prediction of such epitopes can also be obtained by algorithms that are currently available based on structure, bindings motifs, matrices or artificial neural networks (ANNs). In

motif-based algorithms, the occurrence of certain residues at specific positions in a peptide sequence is used to predict MHC ligands. This approach yields a low prediction accuracy of 60-70% because not all binding peptides contain the exact motif. The limitation of this approach is overcome by the use of matrices, which are essentially refined motifs covering all of the amino acids in a MHC class I ligand. An interesting example is the SYFPEITHI database (www.syfpeithi.de) [136], which provides a matrix-based MHC-binding prediction. In contrast to other prediction algorithms, however, only naturally occuring MHC ligands were used to generate the matrices in the SYFPEITHI database. In ANN-based methods, like NetMHC (www.cbs.dtu.dk/services/NetMHC), complex networks are used to predict binding peptides in an antigenic sequence.

2.7 Aim of the work

The aim of the present work was to study MHC class I-dependent CD8 T cell responses in murine cutaneous leishmaniasis. For this approach the role of $CD8^+$ T cells in TAT-LACK fusion protein-vaccinated C57BL/6 mice was analysed by depletion experiments. Further, contribution of LMP7 as an essential domain of the immunoproteasome for effective activation of $CD8^+$ T cells was analysed in this model. Next, fractions of parasite lysate were generated for further analysis of immunogenic contents and stimulation of T cells. Additionally, the entire proteom as well as the life form-specific protein expression of *L. major* was analysed for $CD8^+$ T cell epitopes by computer-based algorithms.

3. Results

3.1 Immune cells in murine leishmaniasis

3.1.1 Dendritic cells in murine leishmaniasis

Effective T cell-mediated immunity in murine leishmaniasis is mediated by pathogen phagocytosis of DC prior to processing and presenting antigen to T cells. Activated DC are the only cells capable of processing *Leishmania* antigen in both the MHC class I and class II pathways [26] and thus can prime $CD4^+$ and $CD8^+$ T cells efficiently [4;18]. Infected DC upregulate MHC class I and II molecules as well as co-stimulatory molecules like CD54 and CD86.

To confirm these findings in the experimental procedures used within this work, we initially analysed the expression of surface molecules on *L. major* amastigote-infected DC compared to immature or LPS-stimulated DC (Fig. 8). DC were generated from bone marrow of C57BL/6 mice and immature DC were harvested on day 6. Cells were either infected with *L. major* amastigotes (DC:parasites 1:10) or incubated with 100 ng/ml LPS for maximum expression of surface markers for additional 18 hrs. For analysis of surface molecules, cells were co-stained with αCD11c antibody as a specific marker for DC and additionally with αMHC class I and class II or αCD54 and αCD86 antibodies.

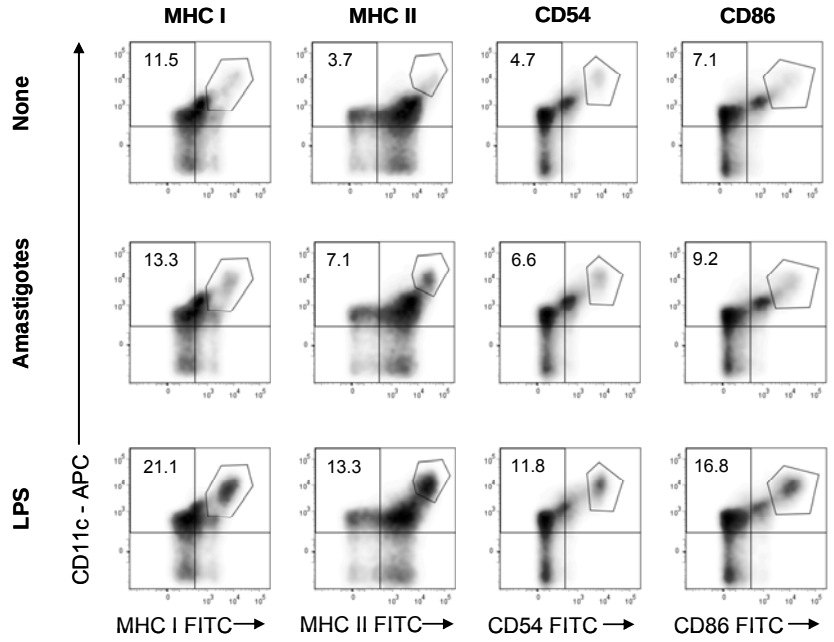

Fig. 8: **Surface markers on BMDC**
Bone marrow of C57BL/6 mice was isolated and cultured in the presence of IL-4 and GM-CSF for 6 days. Immature DC were harvested on day 6 and cultured

with either *L. major* amastigotes (DC: parasites 1:10) or 100 ng/ml LPS for 18 hrs at 37°C. Expression of surface markers of matured DC (amastigotes & LPS) was compared to immature DC (non) by co-staining with αCD11c-APC and either MHC I-FITC, MHC II-FITC, CD54-FITC or CD86-FITC. Analysis of DC only was obtained by gating on high expression of each surface marker in addition to $CD11c^{high}$. Percentages of double-positive cells were depicted in gates of each dotblot. One representative experiment out of two is shown.

Analysis of both, MHC class I and class II molecules as well as co-stimulatory molecules CD54 and CD86 revealed slightly increased expression of these surface molecules on DC infected with *L. major* amastigotes compared to unstimulated controls. We detected an increase of MHC class I molecule expression. Maximum MHC class I expression was observed in LPS-stimulated DC. In parallel, MHC class II expression was increased. Co-stimulatory molecule expression was increased with regard to CD54 and CD86 expression.

DC secrete a variety of proinflammatory cytokines including IL-12 [52-56], which is the key cytokine for induction of Th1/Tc1 responses [57-59]. To confirm the capability of infected DC to secrete IL-12p40, supernatants of DC were harvested after 18 hrs of culture with *L. major* amastigotes and IL-12p40 levels were determined by ELISA (Fig. 9).
IL-12p40 levels of DC infected with *L. major* amastigotes were ~3-fold increased compared to unstimulated controls. Cytokine secretion can even be enhanced by stimulating DC with LPS, which resulted in ~5-fold increased IL-12p40 levels of these cells compared to unstimulated controls.

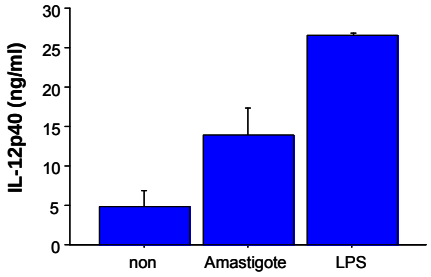

Fig. 9: **IL-12p40 secretion of BMDC**
Bone marrow of C57BL/6 mice was isolated and cultured in the presence of IL-4 and GM-CSF for 6 days. Immature DC were harvested on day 6 and were cultured with either *L. major* amastigotes (DC: parasites 1:10) or 100 ng/ml LPS for 18 hrs at 37°C. IL-12p40 secretion was determined in supernatants by ELISA and expressed as mean±SEM. Pooled data from two independent experiments are shown.

3.1.2 Cytokine secretion of CD4$^+$ and CD8$^+$ T cells

To further investigate the functional role of T cell subsets in *L. major* infected C57BL/6 mice, we analysed the cytokine expression of isolated CD4$^+$ and CD8$^+$ T cells (Fig. 10). Draining lymph nodes of C57BL/6 mice were harvested 6 weeks post infection with low doses of *L. major* metacyclic promastigotes. T cell subsets were enriched using magnetic micro beads, 2x10^5 cells/ 200 µl were plated with immature DC and were antigen-specifically restimulated with SLA. Cytokine secretion was determined after 48 hrs by ELISA.

CD4$^+$ T cells secreted most of the detected IFN-γ, but CD8$^+$ T cells secreted high amounts of IFN-γ as well. Only low levels of IL-4 as a key cytokine for Th2 immune responses were found, and marginal amounts of IL-10 were secreted by T cells.

In summary, the major source of important T cell-released cytokines were secreted from activated CD4$^+$ T cells, but CD8$^+$ T cells contributed to effective T cell-mediated immune responses by secreting protective IFN-γ as well.

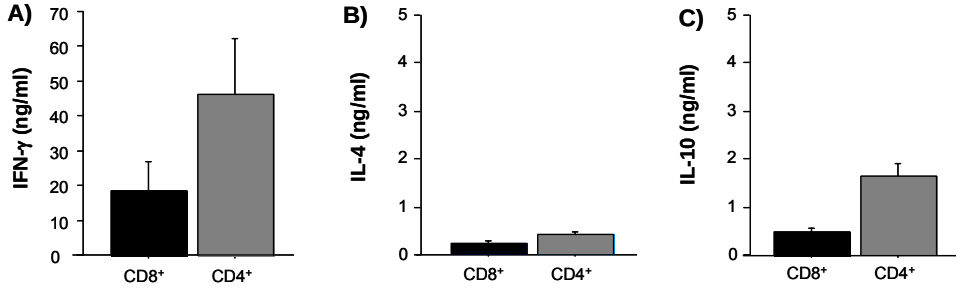

Fig. 10: **Cytokine secretion of T cell subsets from draining lymph nodes**
Draining lymph nodes of low dose *L. major*-infected C57BL/6 mice were isolated 6 weeks post infection and T cell subsets were positively isolated using magnetic micro beads. Isolated cell fractions were cultured with DC (T cell/DC ratio: 10:1) and restimulated antigen-specifically with SLA. Secretion of cytokines **A)** IFN-γ, **B)** IL-4 and **C)** IL-10 were measured by ELISA and expressed as mean±SEM. Pooled data from two independent experiments are shown.

3.2 Role of CD8$^+$ T cells in TAT-LACK vaccinated mice

3.2.1 Lesion development in TAT-fusion protein vaccinated C57BL/6 mice

Both, CD8$^+$ T cells and CD4$^+$ T cells are required for primary immunity against murine leishmaniasis and DC play a crucial role in activating these T cells. This concept was confirmed in the *L. major* infection model by using protein-pulsed DC vaccination strategies in

BALB/c and C57BL/6 mice (PhD-Thesis K. Kronenberg; 2006). In this work, the vaccination efficacy of the leishmanial protein LACK fused to TAT was analysed. TAT is the protein transduction domain of HIV-1 that transloctes proteins into the cytosol of infected cells and renders these proteins able for the processing by the MHC class I pathway. Vaccination with DC pulsed with LACK induced comparable CD4 priming as did DC pulsed with TAT-LACK, whereas CD8 priming was weaker in mice vaccinated with DC pulsed with LACK protein alone.

Though vaccination with protein-pulsed DC has shown to have a high potency in mice, they are hardly applicable in *Leishmania*-endemic areas. DC-based vaccinations can only serve as proof-of-principle experiments for the development of directly inoculated protein-based vaccines since DC generation from humans is laborious and an expensive procedure. Thus, other methods for developing vaccines might be more suitable.

It has already been shown (MD-Thesis F. Butsch, 2009) that direct vaccination of susceptible BALB/c mice with TAT-LACK + CpG resulted in decreased progression of disease.

To enhance vaccination effects and to modify the immune response towards protective, long-lasting Th1/Tc1 immunity, immunostimulatoric CpG oligonucleotides (CpG) were administered 24 hrs after the second vaccine inoculation (10 µg/ear). CpG contains microbial DNA with its characteristic non-methylated cytidine-guanosine-dinucleotides, which activate the immune system via TLR9 [137;138].

Two weeks later, all mice were infected with ~1,000 metacyclic promastigotes of *L. major* in the contralateral ear and lesion development was assessed weekly.

Based on these results in BALB/c mice, we now vaccinated C57BL/6 mice directly with TAT-LACK, since C57BL/6 mice are discussed to be a mouse strain with higher clinical relevance for human *L. major* infections [6]. To address antigen presentation to $CD8^+$ T cells, we investigated the effect of a 2-times intradermal vaccination with 10 µg TAT-LACK into ear skin of C57BL/6 mice. As a control, we vaccinated mice with 10 µg LACK, which lacks the protein transduction domain TAT. To rule out antigen-unspecific immunological effects of the TAT domain due to vaccination with fusion proteins, we additionally vaccinated mice twice with 10 µg TAT-ovalbumin (TAT-OVA) and, as a control for effects of vaccination performed by needle inoculation, mice were also administered sterile PBS.

All groups developed lesions starting on week 3 post infection and after 13 weeks, all mice showed no measurable lesions any more (Fig. 11). Mice vaccinated with TAT-LACK developed smaller lesions compared to all control groups, and only in TAT-LACK-vaccinated mice we observed significantly decreased lesion sizes at week 5-7 and week 9 post infection compared to PBS-treated mice. Significant differences were also observed at week 7 when comparing groups vaccinated with TAT-LACK and. TAT-

OVA. Significant differences were not obvious between TAT-LACK-vaccinated mice and LACK-vaccinated mice at any time point. Thus, vaccination of C57BL/6 mice with TAT-LACK resulted in more effective immune responses and smaller lesion sizes. Vaccination with LACK is not sufficient for reducing lesion development compared to controls.

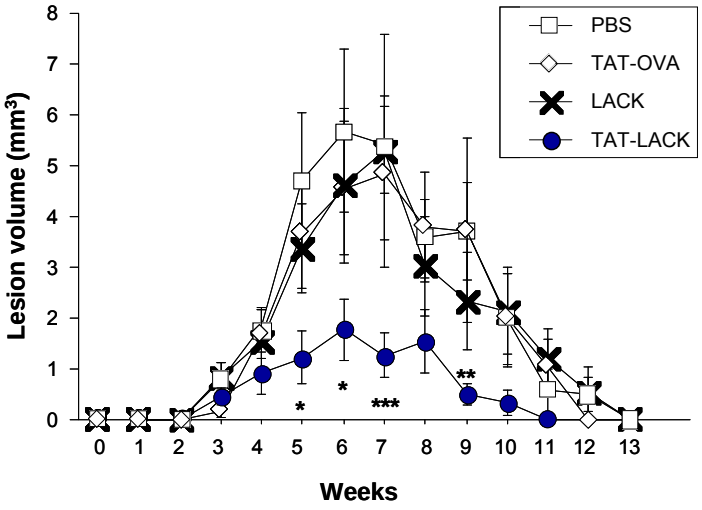

Figure 11: **Vaccination of C57BL/6 mice with fusion proteins.**
C57BL/6 mice (5 mice/group) were vaccinated twice intradermally with 10 µg TAT-OVA, LACK or TAT-LACK into ear skin of one ear. CpG oligonucleotide (ODN) 1826 was administered the following day (10 µg/ear). Two weeks later, mice were infected in the contralateral ear with 1,000 metacyclic promastigotes of *L. major*. Lesion development was monitored weekly in 3 dimensions and expressed as mean ± SEM (pooled data from 2 independent experiments are shown, n≥10, statistical differences to unvaccinated groups are shown, *p≤0.05, **p≤0.005 and ***p≤0.002).

3.2.2 Depletion of CD8$^+$ T cells in TAT-LACK vaccinated mice

Based on the results of vaccination with different proteins, we next investigated the role of CD8$^+$ T cells *in vivo* in the context of TAT-LACK-vaccinated C57BL/6 mice after intradermal, low dose infection with metacyclic promastigotes of *L. major*. CD4$^+$ or CD8$^+$ T cells were depleted by intraperitoneal application of anti-CD4 (125 µg/mouse) or anti-CD8 antibody (50 µg/mouse) 2 days prior to vaccination, respectively.

The efficiency of T cell depletion was determined 3 days post depletion by analysing CD4$^+$ and CD8$^+$ T cell subsets in the peripheral blood by flow cytometry (Fig. 12 A). A small amount of blood was collected from the tail vein of two randomly chosen mice (out of five mice per group) each treated with either anti-CD4- or anti-CD8-depleting antibody, respectively, or isotype control antibody.

No CD4$^+$ T cells were detected in both mice treated with anti-CD4-depleting antibody (0.0 and 0.1%), whereas the CD8$^+$ T cell number was

not decreased as shown by normal numbers in these animals (17.5 and 15.7%) compared to isotype control-treated mouse (12.4%). In line, we detected only marginal numbers of $CD8^+$ T cells (1.5 and 0.3%) in anti-CD8-depleting antibody-treated mice. $CD4^+$ T cell numbers were not affected (22.7 and 31.5%) in these mice compared to isotype controls (22.0%). Thus, at the time point of vaccination with TAT-LACK, no $CD4^+$ T cells in the blood of CD4-depleted mice and only a little $CD8^+$ T cells in the blood of CD8-depleted mice were found.

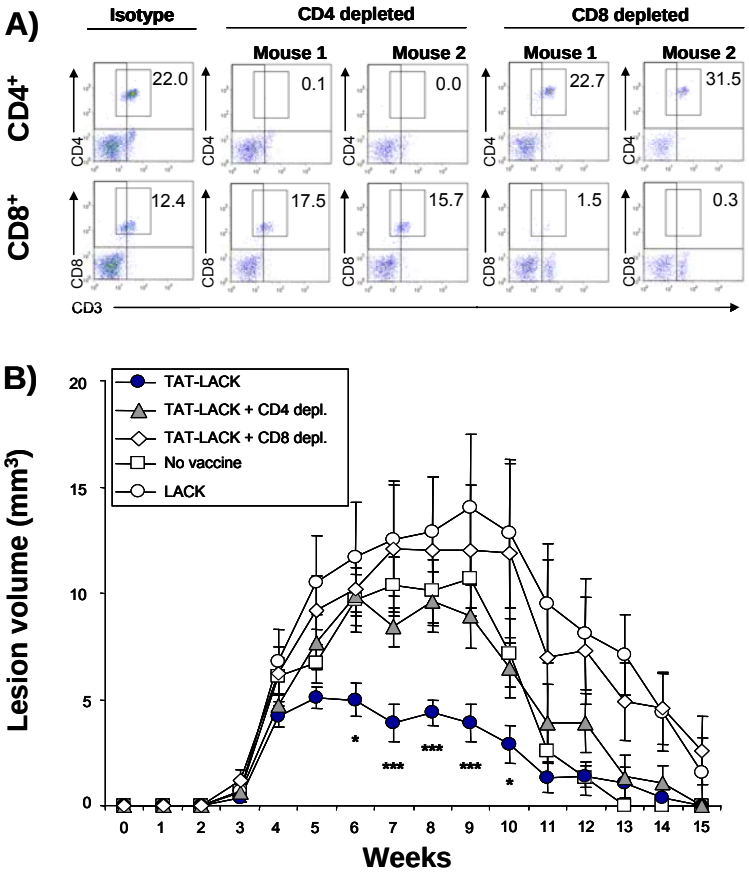

Figure 12: **Vaccination with TAT-LACK fusion protein directly *in vivo* depends on both $CD4^+$ and $CD8^+$ T cells**
C57BL/6 mice (5 mice/group) were injected with anti-CD4 (125 µg/mouse), anti-CD8 (50 µg/mouse) or control mAb (i.p.) and vaccinated twice two days later with 10 µg TAT-LACK or LACK intradermally into ear skin. CpG oligonucleotide (ODN) 1826 was administered the following day (10 µg/ear). **A)** 3 days post depletion, the presence of the T cell subsets was analysed. Peripheral blood was collected from the tail vein of two randomly chosen mice of each group, stained for CD3 and either CD4 or CD8 and the T cell frequency was determined by using flow cytometry. **B)** Two weeks later mice were infected with 1,000 metacyclic promastigotes of *L. major*. Lesion development was monitored weekly in 3 dimensions and expressed as mean ± SEM (pooled data from 3 independent

experiments are shown, n≥9 mice/group, statistical differences to unvaccinated group are shown, *p≤0.05 and ***p≤0.002).

Mice were vaccinated twice with TAT-LACK or appropriate controls followed by administration of CpG. Two weeks later, mice were infected with *L. major* and lesion development was assessed weekly in 3 dimensions (Fig. 12 B). Again, all mice developed lesions starting at week 3 post infection. Mice treated with isotype control antibody prior to vaccination with TAT-LACK showed significantly smaller lesion size compared to anti-CD4 or anti-CD8 depletion antibody-treated mice from week 5 to week 10 post infection. Control groups were either treated with PBS or vaccinated with LACK. Both controls elicited lesion development similar to CD4 and CD8-depleted, TAT-LACK-vaccinated mice indicating a pivotal role for $CD8^+$ T cells in addition to $CD4^+$ T cells in TAT-LACK vaccination followed by infections with *L. major*.

3.2.3 Vaccination with TAT-fusion protein compared to *Leishmania* lysate

Highly immunodominant effects of soluble *Leishmania* antigen (SLA) upon vaccination in the mouse model have been reported previously [139-141]. Thus, we additionally analysed the role of SLA as a control in vaccination of C57BL/6 mice in comparison to TAT-LACK, LACK and TAT-OVA (Fig. 13). In this experiment, total parasite lysate of physically disrupted metacyclic promastigotes of *L. major* was used as soluble *Leishmania* antigen (SLA).

C57BL/6 mice were vaccinated twice on two consecutive days with 10 µg of proteins or SLA, followed by administration of CpG one day later. After low dose infection in the contralateral ear two weeks later, we observed significantly reduced lesion size in TAT-LACK-vaccinated mice compared to PBS-treated controls in week 5 and week 6 post infection. Mice vaccinated with SLA elicited significantly reduced lesion sizes starting from week 3 post infection compared to all other groups.

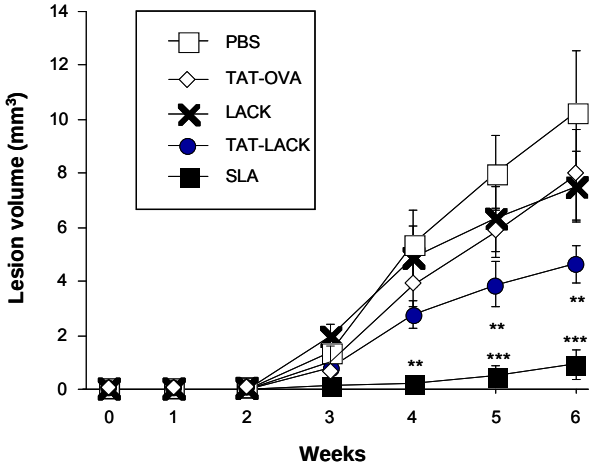

Figure 13: **Vaccination with TAT-LACK fusion proteins vs. SLA *in vivo***
C57BL/6 mice (5 mice/group) were vaccinated intradermally twice with 10 µg TAT-OVA, LACK, TAT-LACK or SLA into ear skin of one ear. CpG oligonucleotide (ODN) 1826 was administered the following day (10 µg/ear). Two weeks later, mice were infected in the contralateral ear with 1,000 metacyclic promastigotes of *L. major*. Lesion development was monitored weekly in 3 dimensions and expressed as mean ± SEM (pooled data from 2 independent experiments are shown, n≥10, statistical differences to unvaccinated groups are shown, **p≤0.005 and ***p≤0.002).

3.2.4 Parasite burdens in infected ears of vaccinated mice

Six weeks post infection, infected ears of fusion-protein- or SLA-vaccinated mice were harvested and the number of parasites was assessed using a limited dilution assay (Fig. 14). We found significantly reduced numbers of parasites in mice vaccinated with TAT-LACK compared to LACK- and PBS-treated mice. Additionally, vaccination with SLA resulted in significantly reduced parasite numbers compared to LACK, PBS and also TAT-LACK. However, no differences in parasite burdens were observed in TAT-OVA-vaccinated mice compared to either TAT-LACK or SLA groups. To investigate the systemic distribution of parasites, the number of parasites in spleens of all groups were analysed. As known for Th1-predominant C57BL/6 mice, we found only low parasite numbers in a single group (PBS-treated) and no parasites were detected in all vaccination groups.

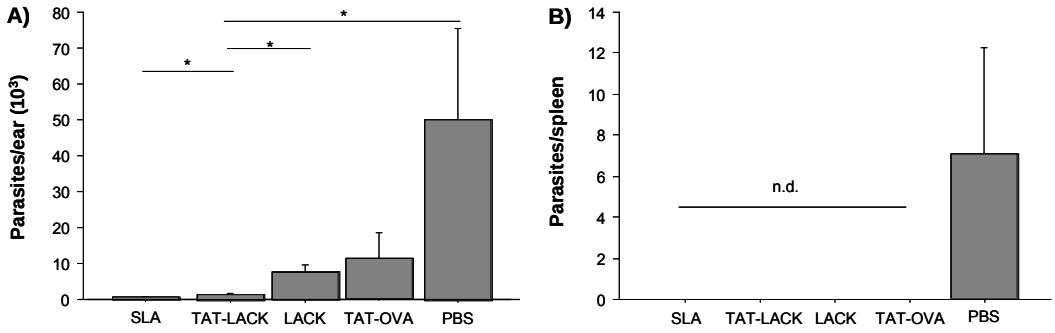

Fig. 14: **Parasite burdens in TAT-LACK- or SLA-vaccinated ears**
C57BL/6 mice (5 mice/group) were vaccinated twice with 10 µg TAT-OVA, LACK, TAT-LACK or SLA intradermally into ear skin of one ear per mouse. CpG oligonucleotide (ODN) 1826 was administered the following day (10 µg/ear). Two weeks later, mice were infected in the contralateral ear with 1,000 metacyclic promastigotes of *L. major*. **A)** Ears and **B)** spleens were harvested 6 weeks post infection and the number of parasites was assessed using a limiting dilution assay. Parasite numbers were expressed as mean ± SEM (pooled data from 2 independent experiments were shown, n≥10 mice/group, statistical differences to unvaccinated groups are shown, *p≤0.05, n.d. = not detected).

3.2.5 Cytokine secretion of antigen-specifically restimulated draining lymph node cells

In addition to parasite burdens in infected ears, we analysed the cytokine secretion of antigen-specifically restimulated draining lymph node cells 6 weeks post infection. Draining lymph nodes were harvested and cell suspensions were plated at $1x10^6$ cells/ 200 µl and restimulated with soluble *Leishmania* antigen (SLA). Cytokine release was determined after 48 hrs by ELISA.

All groups secreted high amounts of IFN-γ ranging from 40 ng released from TAT-LACK- and SLA-vaccinated mice to 65-70 ng released from LACK- or TAT-OVA-vaccinated or PBS-treated mice, respectively (Fig. 15). Interestingly, the levels of secreted IFN-γ, a cytokine for effective immune responses against *L. major*, were diminished in TAT-LACK- and SLA-vaccinated mice, which beared a significantly lower number of parasites in infected ears. However, levels of secreted IFN-γ in control groups (LACK, TAT-OVA and PBS) were higher, albeit the differences to TAT-LACK and SLA groups were not significant. In contrast to secretion of IFN-γ, we detected only marginal levels of IL-4 in antigen-specifically restimulated draining lymph node cells. Moderate levels of IL-10 were observed, with only lymph node cells of LACK-vaccinated mice secreting significantly less IL-10 than the other groups.

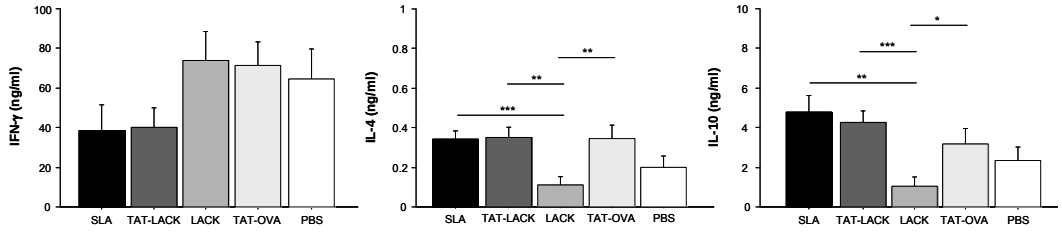

Fig. 15: **Cytokine secretion of draining lymph node cells of TAT-LACK- or SLA-vaccinated mice**
C57BL/6 mice (5 mice/group) were vaccinated intradermally twice with 10 µg TAT-OVA, LACK, TAT-LACK or SLA into ear skin of one ear per mouse. CpG oligonucleotide (ODN) 1826 was administered the following day (10 µg/ear). Two weeks later, mice were infected in the contralateral ear with 1,000 metacyclic promastigotes of *L. major*. Draining lymph nodes were harvested 6 weeks post infection and cytokine secretion was assessed using ELISA. Levels of detected cytokines were expressed as mean ± SEM (pooled data from 2 independent experiments were shown, n≥10, statistical differences between vacciantion groups are shown, *p≤0.05, **p≤0.005 and ***p≤0.002).

3.2.6 Proliferation of antigen-specifically restimulated draining lymph node cells

To investigate the responsiveness of distinct T cell subsets in vaccinated mice to antigen-specific restimulation, we vaccinated C57BL/6 mice twice with TAT-LACK or appropriate controls followed by admistration of CpG one day later. Draining lymph nodes were harvested one week after vaccination, single cell suspensions were prepared, cells were labelled with CFSE (1 µM) and plated at $1x10^6$ cells/ 200 µl in the presence of soluble *Leishmania* antigen (SLA). After 4 days of restimulation, cells were harvested and T cell proliferation was analysed by flow cytometry.

Vaccination with SLA induced both $CD4^+$ and $CD8^+$ T cell proliferation (Fig. 16), as determined by a significantly higher proliferation rate of both T cell subsets compared to vaccination with TAT-LACK or controls. The proliferation rate of $CD4^+$ and $CD8^+$ T cells after antigen-specific restimulation was ~8% and ~13%, respectively. LACK-vaccinated mice elicited higher proliferation of $CD4^+$ T cells, whereas vaccination with TAT-LACK induced higher proliferation of $CD8^+$ T cells, albeit the differences were not significant.

Summarizing the data presented in section 3.2.3 – 3.2.6, SLA seems to contain highly potent components, which are beneficial for mediating effective immune responses against *L. major* infections.

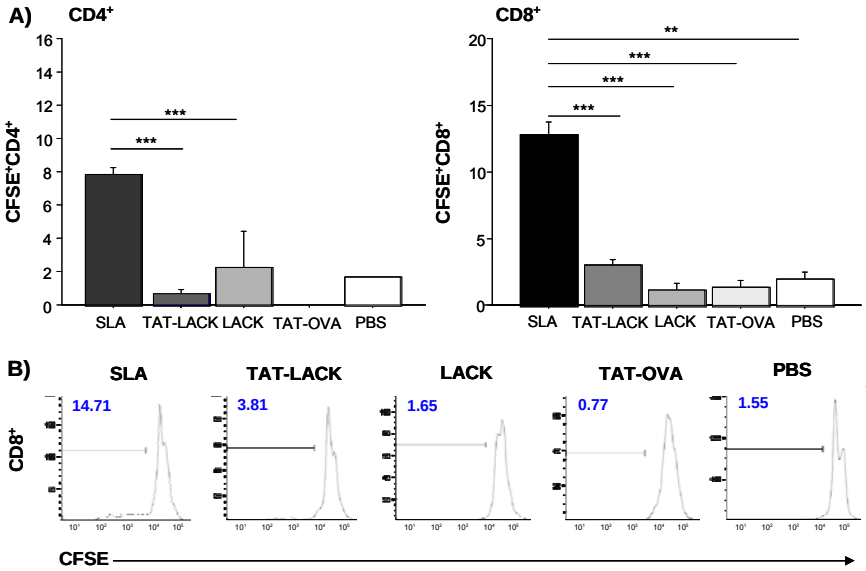

Fig. 16**: T cell proliferation after protein-based vaccination with TAT-LACK fusion proteins**
C57BL/6 mice were vaccinated with TAT-LACK or appropriate controls into the ear dermis on day -7 and day -6 (10 µg each). On day -5, 10 µg CpG 1826 was injected i.d. On day 0, draining LN were harvested, single cell suspensions were prepared and cells were stained with CFSE (1 µM). Labeled cells were plated into a 96 well round bottom plate ($1x10^6$ cells/ 200 µl) and stimulated antigen-specifically with soluble *Leishmania* lysate (SLA). **A)** After 4 days of stimulation, cells were harvested and the percentage of proliferating T cells was analyzed by FACS using monoclonal antibodies against CD4 and CD8. Percentages of SLA-specific proliferation minus control proliferation are expressed as mean ± SEM (pooled data from 2 independent experiments were shown, n≥3 mice/group, statistical differences bewteen vaccination groups are shown, **p≤0.005, ***p≤0.002). **B)** Histograms show percentages of $CD8^+$ T cell proliferation after restimulation as indicated. Background proliferation was subtracted. One representative experiment out of two is shown.

3.3 Role of the LMP7 domain of the immunoproteasome in murine leishmaniasis

3.3.1 Course of infection in $LMP7^{-/-}$ mice

To ultimately clear physiologically relevant low dose infection with *L. major* in mice mimicking the bite of a sand fly, both $CD4^+$ and $CD8^+$ T cells are necessary for effective establishment of protective immunity [18]. Priming of $CD8^+$ T cells in general requires proteasome-dependent degradation of antigens. We now investigated the role of the IFN-γ–inducible immunoproteasome in mice lacking the LMP7 subunit in *L. major* infections. Natural transmission was mimicked experimentally by intradermal infection of C57BL/6 mice or LMP7-deficient mice with ~1,000

metacyclic *L. major* promastigotes [26]. Both mouse strains developed comparable disease progression with regard to lesion sizes of infected ears. Small lesions were visible starting 3 weeks post infection, peaking at week 6 followed by lesion resolution (Fig. 17 *A*). Lesion sizes in week 6 and 9 correlated with parasite burdens (Fig. 17 *B*). The numbers of parasites isolated from infected ears did not differ between the strains investigated in week 6 or week 9 post infection, respectively.

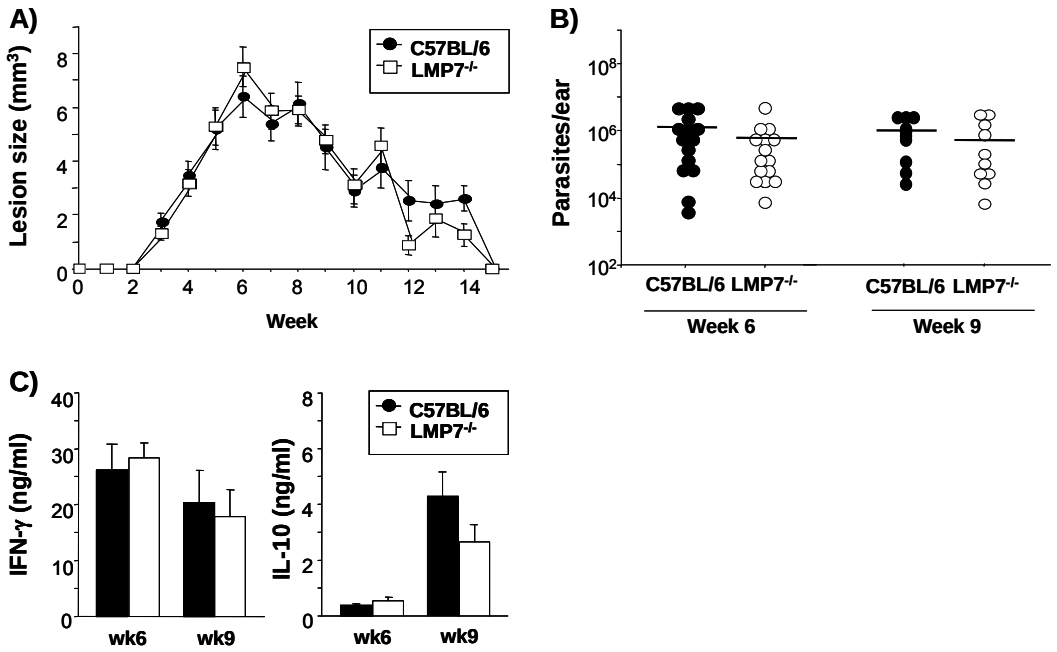

Fig. 17**: *Leishmania*-resistant LMP7$^{-/-}$ mice display no alterations in lesion sizes, parasite burdens and cytokine profiles in *L. major* infections.**
LMP7$^{-/-}$ and wild type C57BL/6 mice were infected intradermally with physiological low dose inocula of *L. major* (10^3 metacyclic promastigotes) in both ears. **A)** Lesion development was assessed weekly in 3 dimensions and expressed as mean±SEM (n≥5, pooled data from two independent experiments). **B)** Ears were harvested 6 and 9 weeks post infection and the number of parasites was assessed using a limiting dilution assay. Dots represent numbers of parasites in individual ears, bars indicate means. **C)** Lymph node cells were plated at 1x10^6 cells/200 µl and restimulated with soluble *Leishmania* lysate (SLA). Cytokine release was determined after 48 hrs by ELISA and expressed as mean±SEM (n≥9, pooled data from two independent experiments).

Next, draining lymph node cells of infected mice were harvested in week 6 and week 9 and antigen-specifically restimulated with soluble *Leishmania* antigen (SLA) for 48 hrs (Fig. 17 *C*). Cytokine levels were determined using cytokine-specific ELISA. Similar levels of IFN-γ were secreted by LN cells from C57BL/6 and LMP7-deficient mice in week 6 and 9 (Fig. 17 C). Compared to week 6, IFN-γ production from cells of both strains slightly decreased in week 9. In contrast, the Th2 cytokine IL-4 was

not detected at any time point in these mice on a *Leishmania*-resistant background. IL-10, a cytokine produced by Th2 cells as well as regulatory T cells (Treg), was detected only at low levels (Fig. 17 C). Strain-dependent differences between C57BL/6 mice or LMP7-deficient mice were not observed.

3.3.2 Phenotype and IL-12 release of *L. major*-infected LMP7$^{-/-}$ DC

Effective T cell mediated immune responses are induced upon recognition of antigen:MHC-complexes and co-stimulatory molecules present on the surface of DC. To investigate the surface expression pattern of DC generated from either C57BL/6 or LMP7-deficient mice, we generated bone marrow-derived DC and analysed their expression of MHC class I molecules (MHC I), MHC II, CD40, CD54 and CD86 by flow cytometry (Fig. 18 *A*). BMDC generated from LMP7$^{-/-}$ mice (open) showed a weaker expression of MHC I and MHC II than BMDC from C57BL/6 mice (shaded) as compared to isotype controls (dashed line). In addition, the expression of the co-stimulatory molecules CD54 and CD86 was slightly decreased on cell surface of BMDC generated from LMP7$^{-/-}$ mice. Only weak expression of CD40 was observed on BMDC from both mouse strains.

Prior pathogen/antigen uptake by DC is a prerequisite for presentation of antigen bound on MHC molecules to T cells. Therefore, we investigated the degree of parasite internalization of BMDC (Fig. 18 *B*) generated from C57BL/6 (black bars) and LMP7-deficient mice (open bars). BMDC were infected with *L. major* amastigotes at a MOI of 1:10 at 37°C and their infection rate was determined after 18 hrs. We did not observe significant differences in the infection rate of BMDC from both mouse strains; in each case an infection rate of ~20% was achieved.

Healing in cutaneous leishmaniasis is critically dependent on the development of Th1/Tc1 immunity [6]. Secretion of IL-12 from DC is required for the induction of Th1/Tc1-dependent immune responses in murine leishmaniasis [4;21;52;54;142;143] and, thus, we analyzed the secretion of IL-12p40 in DC supernatants by ELISA. Both LMP7$^{-/-}$ and wild type DC produced higher levels of IL-12p40 when co-cultured overnight with *L. major* amastigotes (Fig. 18 *C*; black bars) compared to non-infected controls (open bars). Obvious strain-dependent differences in IL-12 secretion were not observed.

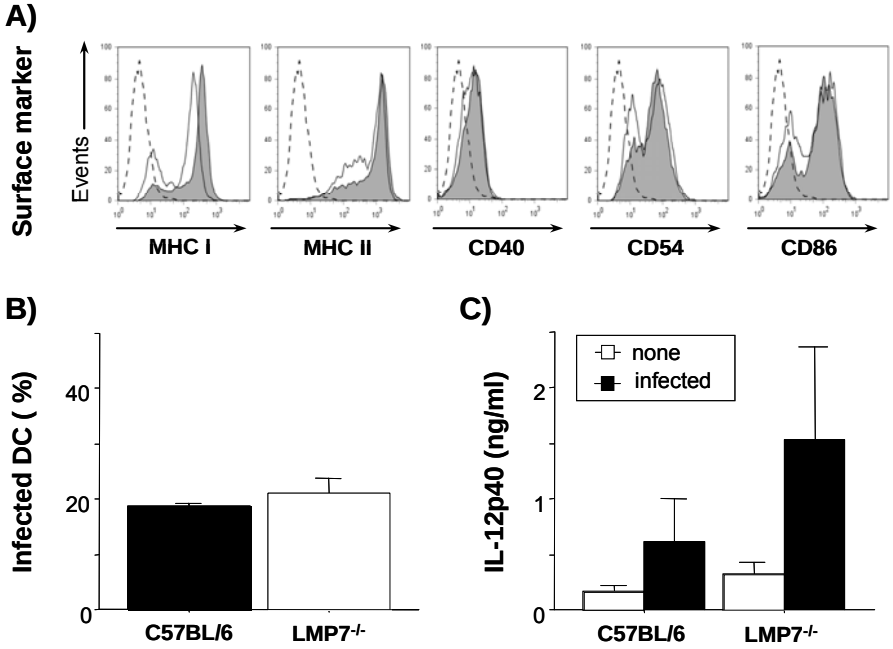

Fig. 18: ***L. major*-infected LMP7$^{-/-}$ DC are not impaired in parasite uptake and IL-12 secretion as compared to wild type DC.**
Bone marrow-derived DC were generated using rGM-CSF and rIL-4. **A)** Cells were harvested as immature DC on day 6. Surface expression of MHC I, CD40, CD54 and CD86 by C57BL/6 (shaded) or LMP7$^{-/-}$ (open) DC as compared to isotype control staining (dashed line) is presented. One representative staining of n=2 independent experiments is shown. **B)** DC (2x10^5/ml) were co-cultured with *L. major* amastigotes (10 parasites/cell) for 18 hrs. The percentage of infected DC was assessed on DiffQuick-stained cytospins by light microscopy. **C)** IL-12p40 secretion into 18 hr supernatants was assessed by ELISA. **B) + C)** All data are expressed as mean±SEM (n≥4).

3.3.3 Restimulation of antigen-specific CD8$^+$ T cells by *L. major*-infected LMP7$^{-/-}$ DC

To investigate the ability of BMDC from C57BL/6 or LMP7-deficient mice to efficiently restimulate Tc1 cells, we co-cultured infected BMDC from both mouse strains with isolated C57BL/6 CD8$^+$ T cells for 48 hrs (Fig. 19). Secretion of IFN-γ from CD8$^+$ T cells was determined after co-culture with *L. major* amastigote-infected DC or non-infected DC generated from C57BL/6 or LMP7-deficient mice (Fig. 19 A). No IFN-γ release of T cells co-cultured with non-infected DC was detected (open bars). Interestingly, similar IFN-γ levels from T cells were found upon co-culture with infected DC from either genetically immunoproteasome-deficient or wild type mice (black bars).

In addition, proliferation of the isolated *L. major*-specific CD8$^+$ T cell subset was analyzed after co-culture with infected or non-infected DC (Fig. 19 B). Proliferation of CD8 cells co-cultured with infected DC was

assessed by incorporation of ^{3}H-Thymidin. Despite the fact that the background T cell proliferation was higher when LMP7$^{-/-}$ DC were used, a significant increase of antigen-specific T cell proliferation was observed upon co-culture with infected BMDC in both mouse strains.

Thus, restimulation of *L. major*-specific CD8^{+} T cells by DC is independent of the immunoproteasome.

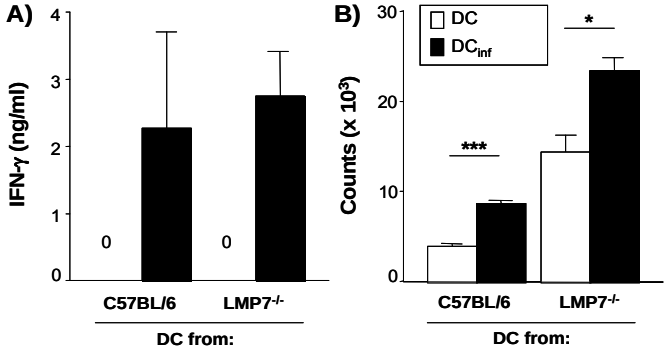

Fig. 19: ***L. major*-infected LMP7$^{-/-}$ DC show a restimulatory capacity of antigen-specific CD8^{+} T cells comparable to wild type DC.**
DC from LMP7$^{-/-}$ or C57BL/6 mice were infected overnight with amastigotes of *L. major* (MOI 10:1). Cells were harvested after 18 hrs, infection rates determined and DC plated at 1x10^{5}/ml. CD8^{+} T cells were isolated from *L. major*-infected C57BL/6 mice using micro beads. T cells were added to DC at 5x10^{5}/well (T cell/DC ratio 10:1). **A)** After 48 hrs, IFN-γ secretion by T cells was determined. **B)** CD8 T cell proliferation was determined by ^{3}H-Thymidin incorporation. ^{3}H-Thymidin was added for the last 18 hrs of the 48 hr co-culture. All data are expressed as mean±SEM (n=3, *=p≤0.05, ***=p≤0.002).

3.3.4 CD8^{+} T cell priming against *L. major* is not impaired in LMP7$^{-/-}$ mice

To investigate the priming of CD8^{+} T cells in the absence of a functional immunoproteasome *in vivo*, LMP7-deficient and C57BL/6 mice were infected with *L. major*. Draining lymph node cells were harvested after 4 weeks, labelled with CFSE and cultured with medium, or restimulated antigen-specifically with SLA. After 6 days, cells were stained with anti-CD4-APC or anti-CD8-APC antibody and analysed using flow cytometry (Fig. 20). In lymph node cells of C57BL/6 mice, we observed an increase of the antigen-specific proliferation in response to SLA of both CD4^{+} and CD8^{+} T cells, respectively, compared to unstimulated controls. We found similar proliferation of CD4^{+} and especially CD8^{+} T cells from LMP7-deficient mice after antigen-specific restimulation with SLA. These data indicate that priming and restimulation of CD8^{+} T cells in LMP7$^{-/-}$ mice is comparable to that in immunoproteasome-competent mice.

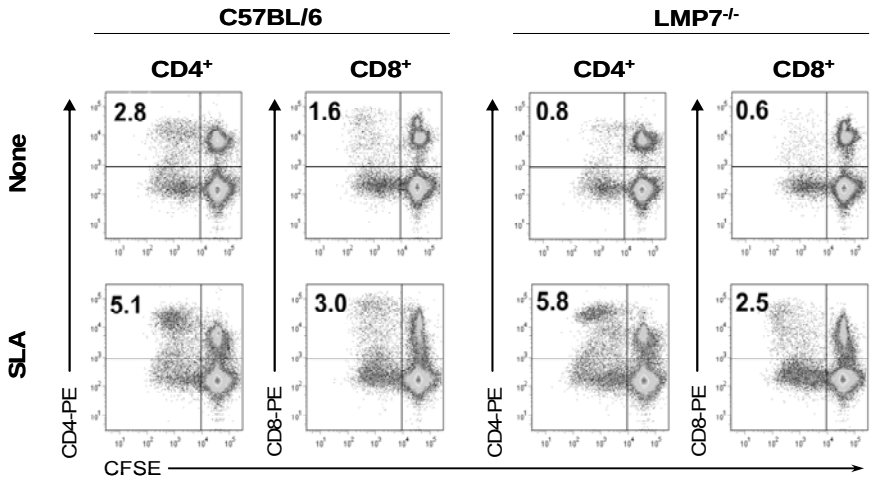

Fig. 20: **Priming of *L. major*-specific CD8$^+$ T cells is normal in infected immunoproteasome-deficient mice.**
Draining LN cells of C57BL/6 or LMP7$^{-/-}$ mice infected with 10^3 metacyclic promastigotes were harvested in wk 4 post infection, labeled with 0.5 µM CFSE, plated at 1x10^6/200 µl and restimulated with medium (none) or antigen-specifically with soluble *Leishmania* antigen (SLA, 25 µg/ml). Proliferation of CD4$^+$ and CD8$^+$ T cells was analyzed by flow cytometry on day 6 by CFSE dilution. One representative experiment of two with ≥3 mice/group is shown.

Thus, the processing of *L. major*-specific CD8$^+$ T cell epitopes is mediated in an immunoproteasome-independent pathway (Brosch et al. 2009, submitted).

3.4 *In vitro* epitope characterisation

SLA generated from promastigotes and in vitro generated amastigotes

It has already been shown that fractionation of SLA from *L. major* yields fractions that were able to stimulate T cells obtained from immunized susceptible BALB/c mice and to induce protective immunity [139]. In this work, fractions from promastigotes were generated by anion exchange liquid chromatography, a chemical separation of SLA based upon charge characteristics. By immunizing BALB/c mice with these fractions, only a single fraction stimulated significant immunity. Proteins (accounting for 1.3% of the total in SLA) appear to be responsible for the protection elicited with this fraction. Thus, a partially purified protective protein antigen fraction has been obtained and protection with this fraction correlated with cell-mediated immune responses.

In line with these results, we fractionated SLA generated from metacyclic promastigotes and *in vitro* cultured amastigotes by size exclusion chromatography. Parasites were lysed by freeze and thaw cycles and resulting lysate was analysed by HPLC. Lysates were fractionated by size exclusion chromatography, since this method was most promising prior to epitope characterization in a viral model (personal communication with AG Herr, Mainz), and performed in collaboration with this group. Size exclusion chromatography separates proteins according to differences in size as they pass through a gel filtration medium (cross-linked agarose) packed in a column [144]. Unlike ion exchange, proteins do not bind to the chromatography medium, so buffer composition (PBS) does not directly affect resolution. Thus, protein degradation by detergent-containing buffers was prevented. This might be an important step for subsequent cell culture experiments, such as restimulation of T cells with eluted proteins. Buffer (mobile phase) and sample move through the column. Proteins diffuse in and out of the pores of the matrix. Smaller proteins move further into the matrix and thus stay longer on the column. As buffer passes continuously through the column, proteins that are larger than the pores of the matrix are unable to diffuse into the pores and pass through the column. Smaller proteins diffuse into the pores and are delayed in their passage down the column. Finally larger proteins leave the column first followed by smaller proteins in order of their size. For size determination (in kDa), proteins from *Leishmania* lysate were compared to reference proteins.

Fractions of promastigote lysate

Gel-filtration chromatogram (Fig. 21) revealed separation of 50 µl pure promastigote lysate by size exclusion chromatography. Peptide bonds of analysed proteins were depicted at 215 nm (blue) and aromatic amino acid residues were depicted at 280 nm (red). Protein content is shown in mili absorption units (mAU) on the y-axis. Fractions were eluted by single fractions of 40 µl per minute, shown on the x-axis (green). For promastigotes (Fig. 21 A), the main amount of protein was found to be in a range from ~13 to less than 6.5 kDa in size compared to reference proteins (aldolase 185 kDa, ovalbumin 43 kDa, ribonuclease A 13.7 kDa and aprotinin 6.5 kDa). A certain amount of larger proteins was also found, correlating with a protein size of about 185 kDa.

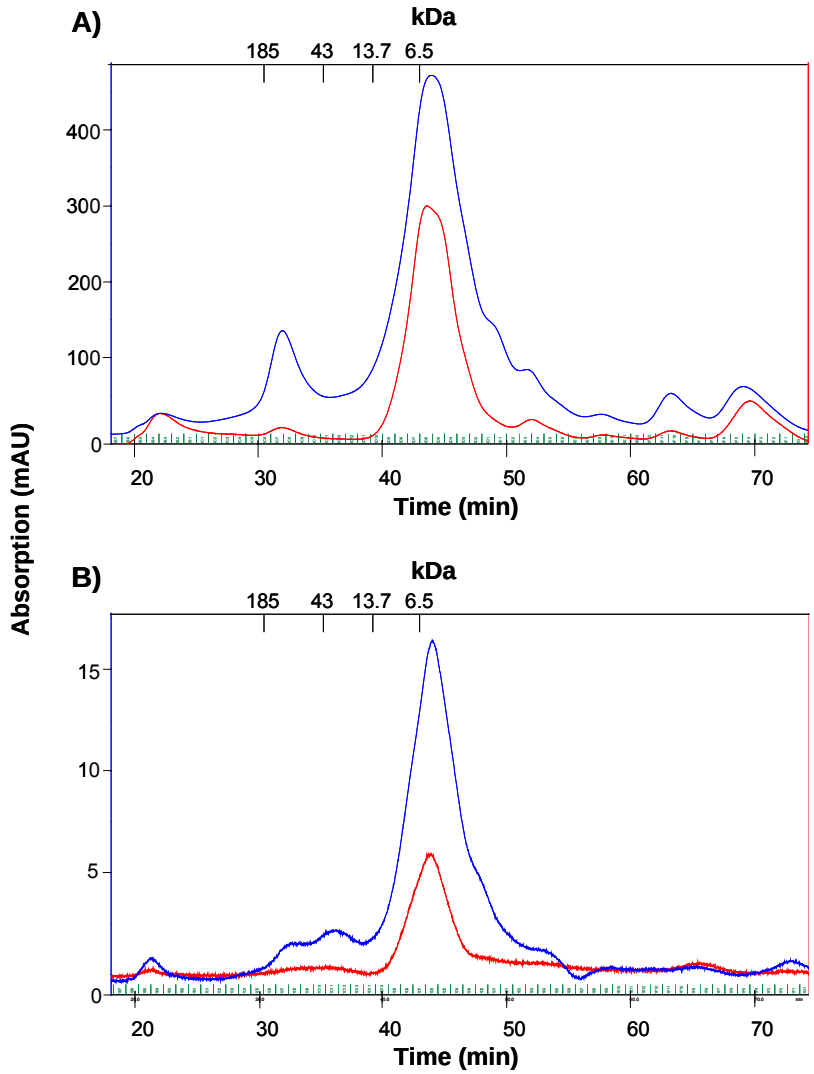

Fig. 21: **Chromatogram of SLA from promastigotes and *in vitro* generated amastigotes**
50 µl of SLA generated from either promastigotes **(A)** or *in vitro* generated amastigotes **(B)** were separated by size exclusion chromatography. The gel filtration chromatogram elicits protein amounts of abundant proteins of SLA depicted by UV-absorption spectrum. Protein bond is shown by 215 nm (blue) or by aromatic ammino acid residues (red) in mili absorption units (mAU) on the y-axis. The retention time of protein eluat is shown in minutes (min) on the x-axis. All samples were eluted with PBS over a period of 40 min with each fraction of 40 µl/min.

Fractions of amastigote lysate

Several studies using SLA in vaccination approaches against *L. major* were performed with lysate generated from the promastigote life form of the parasite [139;145;146]. It has to be considered that amastigotes rather than promastigotes are effciently phagocytosed by DC

[4;18]. The majority of DC-derived antigens presented to T cells, and especially $CD8^+$ T cells, might therefore be generated from the amastigote life form.

Thus, we fractionated SLA derived from amastigotes generated *in vitro* (van Zandbergen, manuscript in progress) as compared to mouse-derived amastigotes in order to obtain protein solution without contaminating non-parasite specific proteins. Gel-filtration chromatogram revealed separation of 50 µl *in vitro* generated amastigote lysate by size exclusion chromatography (Fig. 21 B). The absolute protein amount detected from amastigote SLA is lower compared to promastigote SLA.

In line with the promastigote results, we detected a smaller initiating peak correlating with low amounts of protein. A main peak consisting of mostly all amastigote proteins was detected after 40 to 50 minutes. This "bell-shaped" peak revealed a homogenous composition of proteins relating to protein size. We mainly detected proteins in between a range from ~13 to less than 6.5 kDa in size for SLA from amastigotes compared to reference proteins.

Fractions of SLA generated from either promastigotes or *in vitro* generated amastigotes will be analysed in further experiments for their capacities to induce T cell-mediated immune responses. IFN-γ secretion of T cells restimulted with these fractions will be analysed

In ongoing studies proteins/peptides identified from distinct fractions of *Leishmania* lysate can be analysed by tetramer analysis or *in vivo* vaccination studies. The analysis of immunodominant fractions of amastigote-derived SLA contributes to understanding efficient $CD8^+$ T cell priming and additionally will aim to identify of possible new candidates of protein-based vaccines against *L. major*.

3.5 Epitope prediction

3.5.1 In silico epitope prediction based on the entire *L. major* proteom

With the genome of *L. major* sequenced in 2005 [147], the prediction of potential antigenic peptides for the induction of T cell-mediated immune responses by computer-based algorithms has arisen as a powerful alternative for detecting immunodominant peptides. Different algorithms have been used so far for predicting CD4 and CD8 T cell epitopes. One of the most frequently used ones is the SYFPEITHI database (www.syfpeithi.de) [136]. The current assembly of the *Leishmania major*, clone VI (MHOM/IL/80/Friedlin) genome and corresponding proteom (version 5.2) can be found at GeneDB (www.genedb.org/leish), with the sequence accounting for 32,816,678 base pairs (genome size: 33.6 Mb) and about 8300 proteins arranged in 36 chromosomes [147].

To identify possible candidates of immunodominant peptides a mouse model with high potential relevance for human infections was chosen. Thus, we predicted CD8 T cell epitopes that bind to the corresponding haplotypes of MHC class I from C57BL/6 mice, namely H2-K^b and H2-D^b MHC class I molecules.

For the prediction of CD8 T cell epitopes of the entire *L. major* proteom taken from GeneDB, we used the matrix-based MHC-binding prediction obtained by the SYFPEITHI database. However, the SYFPEITHI algorithm was unable to process the entire protein sequence of every chromosome all at once. For effective prediction of peptides small fractions of protein sequences were analysed step by step.

SYFPEITHI predicts peptides based on binding to MHC class I molecules by affinity to distinct anchor residues. Results were shown as scores. The better peptide affinity to these anchor residues of the MHC class I molecule, the higher the resulting score.

Not to be restricted to only a single algorithm, we also used NetMHC (www.cbs.dtu.dk/services/NetMHC) to either verify SYFPEITHI-predicted peptides or to find other high ranking peptides based on artificial neural network-based predictions.

In contrast to SYFPEITHI, neural network-based prediction by NetMHC is much more complex. Prediction of peptide binding affinities to MHC class I molecules by NetMHC is given in a concentration (in nM), which is needed for saturation of 50% of correlating MHC class I molecules. Thus, peptides with highest affinities to MHC molecules correlated with lowest concentrations of peptide. Best binding peptides were depicted with a peptide concentration of ≤ 100 nM.

Not surprisingly, the identification of CD8 T cell epitopes from the entire proteom of *L. major* resulted in very high numbers of potential epitopes (Fig. 22). We limited the numbers of peptides by arbitrarily setting a cut-off for SYFPEITHI-predicted peptides at a score of 25 and for NetMHC-predicted peptides by 100 nM. The resulting number of peptides within these limitations is still very high. Using the SYFPEITHI algorithm, we predicted 6599 peptides for the H2-D^b haplotype and 869 epitopes for the H2-K^b haplotype. However, we detected only very few peptides with scores >30.

NetMHC predicted 2130 peptides for H2-D^b and 3619 peptides for H2-K^b haplotype. In contrast to SYFPEITHI, here, the best fitting peptides correlate with lowest concentrations. Thus, the most potent peptides would be those, which have a capacity to bind 50% of MHC class I molecules at a concentration of ≤ 10 nM.

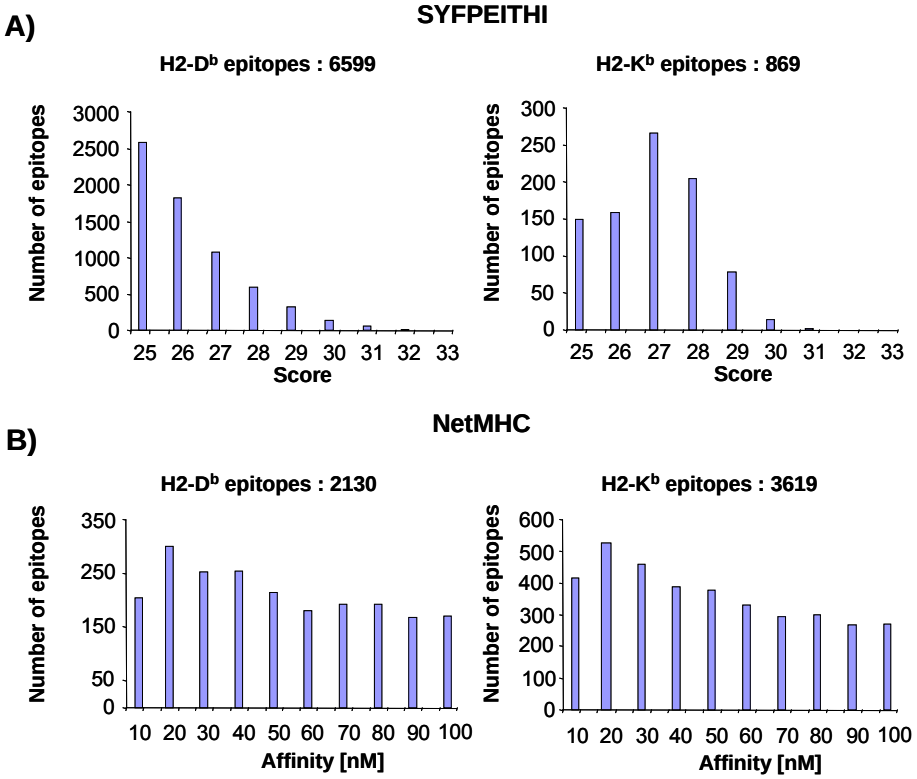

Fig. 22: **Epitope prediction from the entire *L. major* proteom**
Prediction of CD8 T cell epitopes from the entire proteom of *Leishmania major*, clone VI (MHOM/IL/80/Friedlin) was performed with computer-based algorithms (SYFPEITHI and NetMHC). **A)** The high-scoring peptides (score ≥ 25) determined by matrix-based MHC-binding prediction using SYFPEITHI are depicted for the H2-D^b haplotype (left panel) and for the H2-K^b haplotype (right panel). **B)** High-binding peptides (affinity ≤ 100 nM) predicted by neural network-based predictions utilizing NetMHC are shown for H2-D^b (left panel) and for the H2-K^b haplotype (right panel).

To determine if certain predicted peptides were found by both algorithms, we searched the high scored SYFPEITHI-predicted peptides (score > 30) for their occurance within the list of topranking (> 100 nM) peptides from NetMHC and the high scoring NetMHC-predicted peptides (> 10 nM) for their occurance within the topscoring peptides (score > 25) from SYFPEITHI (Fig. 23). For the H2-D^b peptides that were predicted by SYFPEITHI with a score > 30, we found 78 out of 216 peptides (36%) that were also predicted by NetMHC (> 100 nM), and for H2-K^b, we found 8 out of 15 peptides (53%), respectively. On the other hand, we detected 144 out of 205 peptides (70%) that were predicted by NetMHC (< 10 nM) within the topscoring peptides of SYFPEITHI-predicted peptides (score > 25) for the H2-D^b haplotype and 134 out of 412 peptides (33%) for H2-K^b, respectively. This data shows that different algorithms do not predict identical peptides and to further address possible immunodominant

peptides we initially focused on predictions made by the more frequently used SYFPEITHI algorithm.

A)

SYFPEITHI

NetMHC Affinity [nM]	H2-D^b # epitopes		H2-K^b # epitopes	
1-5	10		5	
6-10	21		2	
11-15	10	**78**	1	**8**
16-20	8		0	
21-100	29		0	
No entry (>100)	138		7	
	216		**15**	

B)

NetMHC

SYFPEITHI score	H2-D^b # epitopes		H2-K^b # epitopes	
32	3		0	
31	9		0	
30	20		7	
29	16		21	
28	35	**144**	46	**134**
27	25		28	
26	18		16	
25	18		16	
< 25	61		278	
	205		**412**	

Fig. 23: **Comparison of high scored predicted *L. major* epitopes from SYFPEITHI and NetMHC and *vice versa***
A) The number of 216 high scored SYFPEITHI-predicted epitopes (30-33) were analysed whether they were predicted with high affinity (≤100 nM) by NetMHC as well, and **B)** NetMHC epitopes with highest affinity (1-10 nM) were analysed whether also predicted with high scores (≥25) by SYFPEITHI.

3.5.2 Mass spectrometry-based analysis of protein expression in *L. major* promastigotes and amastigotes

L. major exists in two major developmental stages: free-living flagellated promastigotes and intracellular amastigotes residing in the phagolysosomes of macrophages in the mammalian host. *L. major* encounters distinct environmental changes during the developmental switch from the promastigote to the amastigote form and *vice versa*. These developmental stages display distinct morphologic and metabolic

characteristics consistent with a highly regulated level of differential protein expression [148].

Epitope prediction of the entire proteom of *L. major* resulted in a large number of possible peptides from all proteins of the parasite. In contrast, prediction of only life form-dependent expression of proteins might limit this large number dramatically. In addition, the prediction of *de facto* expressed proteins would be much more relevant for the development of possible vaccine candidates.

Thus, we first analysed the protein expression of both *L. major* life forms, promastigotes and amastigotes, and predicted epitopes by SYFPEITHI solely from *de facto* expressed proteins.

Metacyclic promastigotes and *in vitro* generated amastigotes were lysed mechanically and additionally treated with PBS containing 7 M urea for complete disruption of parasites. Analysis of parasite lysates were performed in collaboration with S. Tenzer, AG Schild, Inst. of Immunology, Mainz: 20 µg of total protein were precipitated and resolubilized in 20 µl digestion Buffer (0.1 % RapiGest). After reduction/alkylation to block cysteines, proteins were digested with trypsin. For mass spectrometry analysis, samples were diluted 1:3 with 1% formic acid and 2.5 µl were injected per UPLC-Run. For MS analysis, a Waters Q-TOF Premier was used in LC-MSE-Acquisition mode with a scan time of 0.7 sec and an interscan delay of 0.05 sec. Glu-Fib (m/z=785.8426, 500 pmol/µl, 500 nl/min via ASM) was used as lockmass and sampled every 30 sec.

For separation of tryptic peptides, a nano Acquity UPLC equipped with a 75µm x 150 mm BEH C18 1.7µm was used. Gradient: 7 - 35 % AcN in 120 min, flow 300 nl/min. All samples were analyzed in quintuplicates. Database searching and protein identification was performed using the Identity Search Engine of PLGS2.3 software using a combined database consisting of the murine and *Leishmania* RefSeq-Protein databases. The false positive rate of protein identification was below 0.3% based on a 5x randomized database.

Out of 8370 proteins in total, mass spectrometry analysis revealed 489 proteins (5.8%) being expressed in metacyclic promastigotes and 265 proteins (3.2%) in amastigotes, respectively (Fig. 24). 203 proteins were expressed in both life forms, which indicates 286 promastigote-specific expressed proteins and 62 amastigote-specific expressed proteins.

We next prdicted the $CD8^+$ T cell epitopes only from *de facto* expressed life form-specific proteins based on these mass spectrometric data. Analysis of a) promastigote-expressed proteins, b) amastigote-expressed proteins and c) overlapping proteins expressed by both life forms was performed by using the SYFPEITHI algorithm.

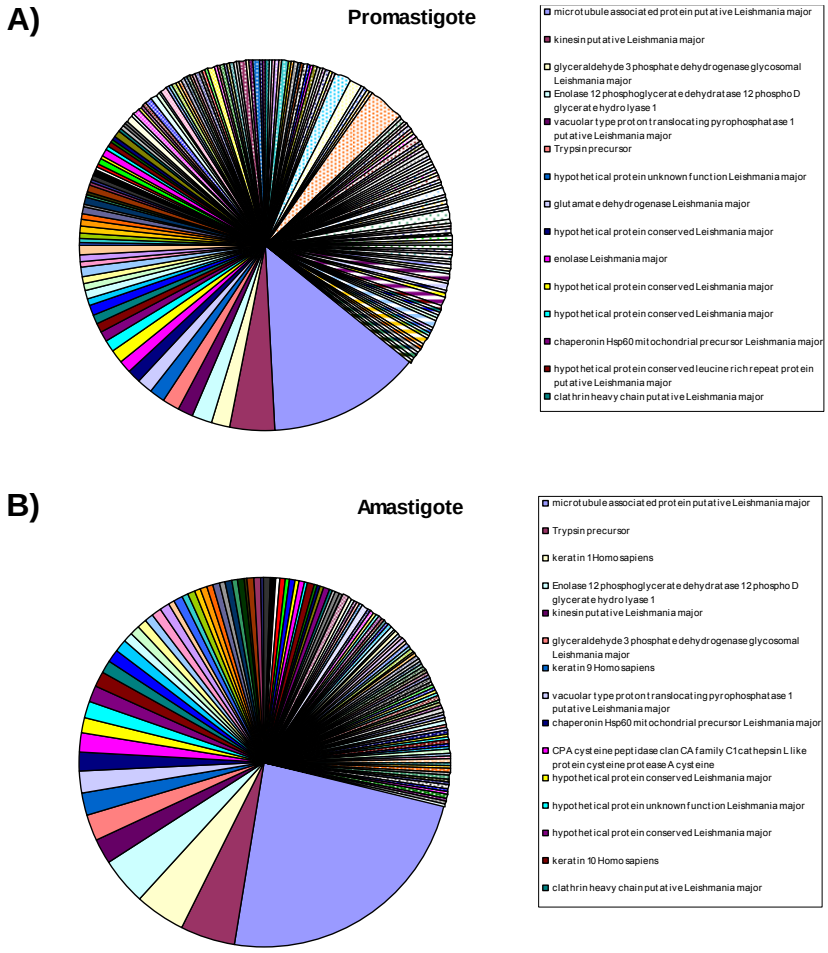

Fig. 24: **Mass spectrometry analysis of *L. major* promastigotes and amastigotes**
Promastigote **(A)** and *in vitro* generated amastigote lysate **(B)** was analysed by mass spectrometry for stage-specific expression of proteins of *L. major*. 489 proteins expressed by promastigotes and 265 proteins expressed by amastigotes were detected, respectively. The quantity of protein expression is depicted and the 15 most frequently expressed proteins for both life forms are shown; for complete list, see supplementary data (pooled data from two independent experiments are shown).

A SYFPEITHI-based prediction of promastigote proteins revealed 157 peptides for the H2-D^b haplotype and 7 peptides for the H2-K^b haplotype (Fig. 25). 59 peptides from amastigotes were predicted for the H2-D^b haplotype and only 7 epitopes for the H2-K^b haplotype. Additionally, we predicted 175 peptides for the H2-D^b haplotype from overlapping proteins expressed by promastigotes and amastigotes and 11 for the H2-K^b haplotype. However, these findings are restricted to again setting the analysis cutoff in SYFPEITHI arbitrarily to a score ≥ 25.

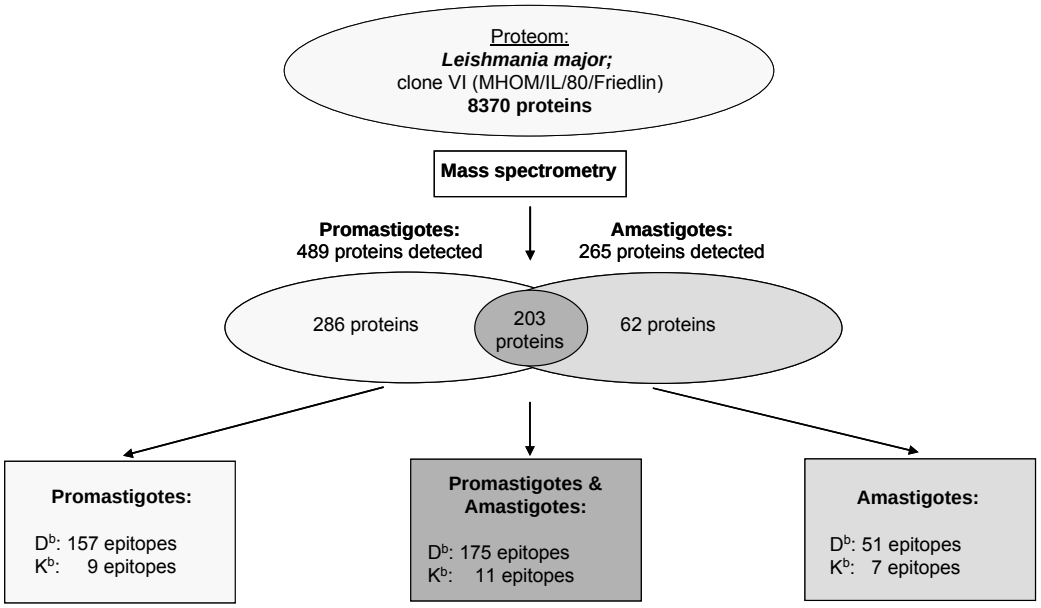

Fig. 25: **Overview of *L. major* epitope prediction: From proteom to peptides**
Based on mass spectrometry analysis of the entire proteom of *L. major*, 489 promastigote and 265 amastigote *de facto* expressed proteins were detected. Epitope prediction for both MHC class I haplotypes of C57BL/6 mice, H2-D^b and H2-K^b, was performed by using the SYFPEITHI algorithm.

Activation of DC for initiating protective T cell-mediated immunity is achieved by phagocytosis of the amastigote life form of the parasite and in addition, the activation of $CD8^+$ T cells is mediated by activated DC [4;18]. Thus, to further assess mainly the immunogenicity of predicted peptides we synthesized 300 peptides in total (JPT Peptide Technologies, Berlin). All predicted peptides from amastigote expressed proteins and the high scoring peptides from promastigotes expressed proteins were chosen (Fig. 26). Restimulation experiments of primed T cells with these peptides and analysis of secretion of Th1/Tc1 cytokines such as IFN-γ will be performed in ongoing studies to identify immunodominant peptides.

In summary, epitope prediction is a powerful tool to reduce the vast numbers of potential epitopes. In addition to a general epitope prediction of the entire proteom of *L. major*, we further diminished the number by prediction of peptides from only expressed proteins identified by mass spectrometry. The reduction from hypothetical 8370 proteins from *L. major* to 265 proteins in fact expressed by amastigotes and 489 proteins expressed by promastigotes and epitope prediction of only these proteins, may enhance the chance to identify possible candidates as protein/peptide-based vaccines against *L. major*

A) **Amastigote & promastigote; H2-D^b**

AAARNMPTM	AAGYNRHNM	AATTNSQLL	ACLENEELV	ACLSNEEFI	ADAENARRI
AEKENVKTL	AEVINLRSL	AEVSNLSVM	AGFMNFCTL	AGISNFAAI	AGLENLPNL
AGLLNKTSL	AGMENVLTF	AKNANGEYL	AKQGNIDLI	ALAKNLAQL	ALDTNGKEM
ALIANSEQL	ALPRNAHPM	AMAGNPVVI	AMGSNQCLL	AMVENTKEI	AQGTNPIDM
AQQENLLHL	ARGVNLCLL	ARNINLSLL	ASFRNVEEI	ATPYNPEDI	AVRFNAETL
AYARNLDTL	AAATNTLIM	CAQANVSLI	CNSVNRVEL	CSGENAARI	CTVTNAHTI
DAAKNQVAM	DALSNRDRV	DAMENPLSL	DLVNNLGTI	DMSTNAEYF	DQIRNMSVI
DSFPNSVTI	DSLYNEIRI	DSNENLLAL	EAAKNVECI	EAGVNRDDF	EALANDGSL
ECGVNPSEL	FAEDNFDEF	FAWVNLVHI	FFLINGPEI	FGDVNSPLL	FMPENYKLL
FNNVNMPFM	FQDDNYESL	FSLENVRLM	FTYDNLPEF	FVNENREKL	GAIYNAVNI
GGHMNLTML	GKGANLAEM	GRPKNMQAM	GSFVNPNTI	GMNVNVQRL	HGLANQEVL
HRPFNAKTL	IAGLNVLRI	IAGLNVVRI	IDPENKEQL	IGYDNFVEF	IPPTNVSTL
ISLDNYDSI	ISPGNKSDM	ITPFNFTAI	IAETNGREM	KAQRNPADL	KDPENFDHF
KDPNNTLYI	KEGENAASL	KENENLRVL	KGHTNDTFF	KGVQNMAEM	KGYANQTRI
KIIGNLTSM	KLGLNESEM	KLPTNSSAI	KLVMNKDGL	KNGRNLEDF	KSIENAACL
KSISNIVNI	KSLANTTTL	KTPENAEVL	KASFNDQQL	KMFSNKTSL	LAGYNAAVM
LAHFNDDNI	LALGNTVVL	LLVQNQGEM	LNVENLMDI	LSKKNIESI	MSKDNFDQL
NAKENSATL	NDVTNEEHI	NESSNADRL	NSLMNNHSI	PANPNFTDI	PAPTNAPEL
PSEENSDRM	PTLPNLHTL	QAVKNVNEI	QDAVNLGLM	QGIANAVGL	QGVQNIDSI
QMVYNQDEI	QQPTNTISI	RAMPNLQVI	RAVENADVV	RRLKNVTRM	RRVENAQFL
SADPNRMLI	SAPLNVENL	SAVLNLTLV	SAVRNCDEF	SAYENMETL	SDLNNLASI
SECHNPDTM	SEGGNDYEI	SELRNKVSL	SGASNSVTI	SLPQNQTQL	SMWGNLDQF
SPIVNCSDM	SPNSNFVTL	SPVWNSNLI	SQGNNMKFI	STIVNEGAL	SVEVNHQTM
SAVWNSNNI	TGDSNVKSI	TGKRNQITI	TIGFNVETL	TIPANATLL	TIVRNAIRL
TMLTNGVKL	TNPSNVVIL	TNVSNSRSI	TPISNIIRI	TRDPNLAYI	TRKDNLTLM
TSANNARSL	TSVDNIYAI	TTGLNAEAI	TVVRNLRKL	VALNNRGSI	VATVNAVTV
VELENGDVM	VEWRNPDTL	VHQVNLEYL	VKLSNRALI	VKQTNLSSL	VKVENQVRL
VLVVNGHRI	VNPANEEVI	VQAENGKEL	VQLTNRALI	VQQANLREL	VSAGNAKTL
VSFENPRAL	VSIRNSVHI	VSSTNLDRL	WGQTNAEKL	WMLRNIVTF	YASENVNKI
YMYTNLACI	YADENGDAI				

Amastigote & promastigote; H2-K^b

AMYPFLCL	ERYPYLSL	IDYPYVSL	IMYMYFAL		

B) **Amastigote specific; H2-D^b**

AAPQNAVSI	AASTNEVNL	AAVDNAVVV	AELRNGKKL	AGKTNAALI	AIPQNHALL
AQIYNHDFF	ASGTNPMDL	ASLNNIKTI	CYIENPRTL	DASFNASAI	DFIANTETI
DGGVNYNYI	EMLFNPSHL	ESITNVVRI	FALINYMRF	FLMVNADRM	FRPHNTSRI
FRRGNASTL	GAPVNEFAI	GGDGNLSAL	GMLYNFSEL	IGGVNVAGL	IMGDNAAFM
KAGVNLLPL	KESKNLTKM	KLRENAELM	KWLVNNVDI	LSYGYSIV	MLLENTSQM
MLVSNRREI	MPGDNRELI	QEPENRKVL	QLAANAEEL	RAHRNLSPI	SAIGNVCTV
SAVVNACSV	SGELNVSEI	SGKENKGAI	SGPGNLRLV	SWLSNVQKI	TGIGNRTSL
VAAHNAEAM	VAQHNASCI	VKLRNRVVM	VKVGNRSTL	VSAVNLLPL	VVDKNWEYL
YAGENYENL	YLVENTSHL	YQFMNFESL			

Amastigote specific; H2-K^b

AQYAYIEL	FFYFFFFL	IMYTYFAL	IYYSFGFL	LIYIFILI	MLYNFSEL
RKYFFPDL					

C) **Promastigote specific; H2-D^b**

ASGVNGDLL	EAPENAREL	SAIDNLKGM	ALIENADDL	KSLKNFITI	KALENPVNL
MKPSNADEL	NSVVNAVLL	SSMPNLQTL	VAAVNVERI	YKPVNVDTI	ASLVNIPVI
ASLTNAVIL	ATIMNLLEI	FGVVNCAGI	FKLVNTIYL	LAVDNVAEL	NGPTNTVLL
RAPVNSMSL	RGVVNMQYL	SLPVNGLHI	TMGVNACLL	VAPANYVGI	VGLVNCLLL
YGNGNLTTL	AAKRNKDVI	DALLNEENL	DAVRNAAAI	HAATNFREI	KEAENGDEL
KEPRNIQRI	LSPGNEEVM	RSPVNYNLL	SSSVNARNI	SHNENAYTM	VEIWNLVFM

Promastigote specific; H2-K^b

DAYDFLDL	ITYSYSRL	EPYGYDKL	KQYPYTGL

Fig. 26: **Top scored peptides predicted by SYFPEITHI from *in vitro* generated *L. major* amastigotes or promastigotes, respectively.**

4. Discussion

$CD4^+$ Th1 and $CD8^+$ Tc1 cells do play pivotal roles in immunity against *Leishmania major* infections in C57BL/6 mice by IFN-γ secretion [18].

No immunodominant, Tc1-inducing $CD8^+$ T cell epitope of *L. major* has been described so far and solely a single epitope for effective $CD4^+$ T cell-mediated immune responses has been identified from the LACK protein [1]. Thus, the goal of this work was to identify immunodominant $CD8^+$ T cell epitopes for utilization in *in vitro* assays, e.g. tetramer analysis or protein-based vaccination studies *in vivo.*

The work was divided in the following sections:

- Vaccination of C57BL/6 mice with TAT-LACK fusion protein confirming the role of $CD8^+$ T cell responses in *L. major* infections
- Role of the immunoproteasome for generation of $CD8^+$ T cell responses
- Fractionation of amastigote soluble *Leishmania* lysate for analysis of proteins with CD8-activating potential
- Epitope prediction by a combination of in silico and cell biological methods

4.1 TAT-LACK vaccination strategies

Protein-pulsed DC vaccination

Parasites and protein are engulfed by DC in different ways. Parasite internalization results from FcγRI/III-mediated phagocytosis [47], whereas protein uptake is the result of endocytosis. Notably, FcγR-mediated parasite uptake facilitates MHC class I and II antigen processing, while protein endocytosis more frequently favors MHC class II-predominant antigen presentation [18;47;149]. Thus, inefficient induction of CD8 T cells, rather than suboptimal CD4 priming, may be responsible for the inability of protein-pulsed DC to promote effective and long-term immunity as compared to infected DC.

The relevance of HIV-TAT in vaccination studies to enhance $CD8^+$ T cell activation has already been reported in principal for tumors [133;134;150]. A recent publication [151] reported additionally the ability of HIV-TAT containing whole protein in antigen-transduced DC for stimulation of Ag-specific $CD8^+$ and $CD4^+$ T cells in the tumor model.

The concept of effective induction of $CD8^+$ T cells is supported by findings in the *L. major* infection model by using protein-pulsed DC vaccination strategies (PhD-Thesis K. Kronenberg; 2006). In this approach, the vaccination efficacy of the leishmanial protein LACK fused to HIV-TAT was assessed.

Vaccination with DC pulsed with only LACK induced comparable CD4 priming as did DC pulsed with TAT-LACK, whereas CD8 priming was

weaker in mice vaccinated with DC pulsed with LACK protein alone. These findings show that fusion of HIV-TAT and LACK is more promising for vaccination strategies against *L. major* infections compared to vaccination with LACK alone.

However, beside the advantage of DC-based immunizations, such as high potency and efficacy, this form of vaccination is hardly applicable in *Leishmania*-endemic areas. Isolation of DC from humans or their generation from buffy coats is a very labour- and cost-intensive procedure. As a consequence, DC-based vaccination strategies can only serve as proof-of-principle experiments prior to the development of protein-based vaccines.

Protein-based vaccination

An alternative vaccination strategy to protein-pulsed DC vaccination that preferably targets DC *in vivo*, is the inoculation of purified proteins. In line with protein-pulsed DC vaccination results, the intradermal inoculation of purified TAT-LACK protein into the ear of susceptible BALB/c mice revealed similar results (Kronenberg et al, manuscript submitted). In that work we showed that the induction of $CD8^+$ T cell-dependent immune responses in direct vaccination with fusion proteins containing TAT was consistently superior in promoting protection against cutaneous leishmaniasis as compared to proteins without TAT.

However, in contrast to protein-pulsed DC vaccinations, direct injection of proteins showed only weak immunogenicity when inoculated without any co-stimulatory adjuvants [152]. In line with these results, it was shown in *L. major* low dose infections that utilization of TAT-LACK protein-based vaccinations together with co-administration of appropriate adjuvants such as CpG motifs the results were more promising (Kronenberg et al, manuscript submitted).

Adjuvants

Adjuvants in *Leishmania* vaccines should optimally modify or enhance the immune response towards protective Th1/Tc1 immunity. Synthetic oligonucleotides with unmethylated CpG dinucleotide motifs are strong Th1-promoting adjuvants. CpG ODN have been shown to activate macrophages and DC via TLR9 to synthesize Th1-associated cytokines including IL-12 and IFN-γ and to up-regulate costimulatory molecules such as CD40 and MHC class II molecules [153-158]. CpG is capable of inducing Th1/Tc1-mediated, long-lasting $CD4^+$ and $CD8^+$ T cell-dependent immune responses and is therefore one of the most frequently used adjuvant for murine vaccination studies against infection with *Leishmania spp*. so far.

Vaccination of C57BL/6 mice with TAT-LACK

The present work presented data obtained in C57BL/6 mice, which were used for the first time to confirm results from BALB/c mice.

C57BL/6 mice represent a mouse model with higher potential relevance for human infections. Therefore, vaccination was performed herein by direct intradermal administration of purified protein instead of protein-pulsed DC into the ears of C57BL/6 mice. The induction of both LACK-reactive $CD4^+$ and $CD8^+$ T cells contributed to protection against *L. major* infection after vaccination of resistent C57BL/6 mice with TAT-LACK fusion protein, as shown *in vivo* and *in vitro*. The superiority of TAT-LACK vaccination compared to LACK alone likely relates to the ability to induce CD8 priming in addition to CD4 priming, which was strikingly revealed *in vivo* by depletion of either $CD4^+$ or $CD8^+$ T cells prior to vaccination. In addition, these data are in line with recent findings of cross-talk of $CD4^+$ and $CD8^+$ T cells required for primary immunity in *L. major* infections [72].

In summary, these data demonstrate that induction of *Leishmania*-specific $CD8^+$ T cells, in addition to $CD4^+$ cells, is beneficial for the development of protective immunity in TAT-LACK protein-based vaccinated C57BL/6 mice. In line with findings in BALB/c mice, this work revealed the superiority of direct protein-based vaccination strategies in C57BL/6 mice as well. Immunization with TAT-LACK was uperior to injection of LACK alone. Thus, TAT-LACK represent a promising tool for the induction of protective CD8-dependent immunity against infectious diseases with intracellular pathogens.

4.2 Role of LMP7 in murine leishmaniasis

Priming of $CD8^+$ T cells for effective immune responses against many self/nonself antigens normally requires antigen processing in a proteasome/immunoproteasome-dependent manner [99;159]. Secretion of IFN-γ, which is required for the formation of the immunoproteasome, and of functional importance to the immunoproteasome itself has been demonstrated for the presentation of viral epitopes [160-162]. In the presence of immunoproteasomes, viral epitope generation was strongly enhanced and their function was tightly connected with the early phases of antiviral immune responses [95;96]. In murine cutaneous leishmaniasis both, IFN-γ-secreting $CD4^+$ and $CD8^+$ T cells are required for efficient immunity in natural infections [18;163-166]. The Th1/Tc1-dominated immune response in resistant mouse strains (e.g. C57BL/6) as well as humans is characterized by high levels of IFN-γ and therefore we postulated that these high amounts of IFN-γ will effectively induce formation of immunoproteasomes during *L. major* infections.

The present study revealed that *L. major*-specific $CD8^+$ T cell responses from mice with a functional knock-out in the chymotrypsin-like catalytic domain LMP7 of the immunoproteasome ($LMP7^{-/-}$) [167] do not differ from C57BL/6 mice. Both mouse strains elicited similar lesion development after low dose infection of *L. major* corresponding with similar parasite burdens. In addition, the cytokine profiles of draining lymph node

cells revealed comparably high levels of IFN-γ being produced in LMP7$^{-/-}$ compared to wild type lymph node cells after antigen-specific restimulation. Finally, CD8^{+} T cell antigen-specific priming was normal in LMP7$^{-/-}$ mice as assessed several weeks post infection. These findings are in line with results from a lymphocytic choriomeningitis virus (LCMV) infection model, in which mice deficient for LMP7 mounted largely normal CD8^{+} T cell responses compared to C57BL/6 wild type mice [168].

In our study, we found that DC generated from LMP7$^{-/-}$ mice expressed slightly decreased levels of MHC class I molecules, which is in line with a prior report that showed a reduced surface expression of both MHC class I haplotypes, H2-K^{b} and H2-D^{b}, of about 25 – 45% of wild type cells from littermate controls on all major lymphoid subpopulations of LMP7$^{-/-}$ mice [167]. The decreased surface expression of co-stimulatory molecules on DC generated on LMP7$^{-/-}$ DC as found in our study indicate a more immature state compared to wild type DC. Interestingly, DC from both strains internalized *L. major* amastigotes (via FcγRI- and FcγRIII-mediated uptake) [47] to a similar degree. We detected slightly higher amounts of secreted IL-12p40, which is crucial for the development of a Th1/Tc1 immune response [4;7] from infected LMP7$^{-/-}$ DC compared to wild type DC. This excess may have compensated for the decreased MHC/co-stimulatory molecule expression on the LMP7$^{-/-}$ DC. Overall, the restimulatory capacity of the infected DC generated from both mouse strains was not influenced by the absence of the immunoproteasome as indicated by similar IFN-γ secretion and proliferation of antigen-specific wild type CD8^{+} T cells.

Finally, proliferation of CD8^{+} T cells isolated from draining lymph nodes of LMP7$^{-/-}$ mice did not differ significantly compared to lymph nodes from C57BL/6 mice and we found more proliferating CD8^{+} T cells in both mouse strains if the lymph nodes were restimulated with antigen compared to medium controls.

Taken together we found that the immunoproteasome is dispensable for MHC class I-restricted CD8^{+} T cell priming in our experimental model of leishmaniasis *in vivo*. Our findings are in line with a recent paper by Bertholet *et al*. reporting that antigen presentation of *L. major* peptides to CD8^{+} T cells is proteasome/TAP-independent [169]. Considering the distinct proteolytic activities of both, the immunoproteasome and the constitutively expressed proteasome [90-93], presentation of *L. major* antigens to CD8^{+} T cells appears to be independent of these two major nonlysosomal protein degradation machineries.

Therefore, additional protein degradation pathways, such as tripeptidyl peptidase II or autophagy, for generating of *L. major* antigens have to be considered to participation in efficient priming of CD8^{+} T cells.

Tripeptidyl peptidase II

An alternative protease candidate is tripeptidyl peptidase II (TPPII) [170], which possesses aminopeptidase as well as endoproteolytic activities [171]. By analyzing the processing and presentation of viral epitopes [172], it was reported that TPPII was able to generate both the correct carboxyl and amino termini of a MHC I epitope by endoproteolytic cleavage independently of the proteasome system. Thus, this cytosolic protease might contribute to processing of *Leishmania*-specific antigens as well.

Autophagy

In addition to the proteasome, autophagy is the main catabolic pathway for the degradation of cytoplasmic constituents [173]. In both systems, hydrolytic activity is compartmentalized from the surrounding cytoplasm and recent evidence is emerging that the same post-translational modification, namely ubiquitinylation, might be involved in biodegradative targeting [174]. Proteasomal degradation is mainly used for the turnover of malfolded and short-lived proteins [175]. In contrast, lysosomal proteolysis targets long-lived proteins [176], damaged cell organelles [177] and endocytosed material in the steady state, but also breaks down cytoplasmic constituents more indiscriminatingly in times of metabolic stress such as starvation [178]. The structure in which primarily long-lived proteins are compartmentalized and in which they become substrates for autophagy are, in addition to cell organels, mainly protein aggregates [178]. This suggests that this degradation pathway is accessed by substrates that are too large for proteasomal degradation and/or cannot be kept soluble by chaperones for delivery to the proteasomal catalytic chamber. Along these lines, a variety of studies have demonstrated the macroautophagic degradation of depolarized mitochondria [177;179-181], expanded endoplasmatic reticulum [182] and protein aggregates [178;183]. For most of these cytoplasmic structures it remains unclear how they are recruited to isolation membranes for autophagy.

Autophagy degradation requires the formation of autophagosomes, which in turn fuse with endosomes and lysosomes for efficient cargo degradation [184]. Most microbes are probably efficiently cleared by autophagic and other immune mechanisms. However, a small subset of pathogens have developed strategies to outsmart host defense. Therefore, it is not too surprising that many also target autophagy. These pathogens target two main steps of autophagy, autophagosome formation and its fusion with lysosomes. *Leishmania* seem to have established its replicative niche in autophagosomes by preventing their maturation to degradative compartments [185]. Although most studies suggest that autophagy contributes primarily to MHC class II presentation of antigens [184], autophagy might also assist in antigen processing for classical and non classical MHC class I molecules [186;187].

In summary, our data indicate that despite the fact that CD8 responses are highly effective in protection against natural *L. major* infections [18;163-166] it remains unclear how antigens are processed in infected DC. It has been reported that some autoantigens required processing by the cytosolic proteasomes prior to transport by chaperone-mediated autophagy [188;189]. Thus, a complex interplay of different degradation pathways might contribute to *Leishmania*-antigen processing.

4.3 Epitope characterization from promastigote and amastigote SLA

Many attempts for vaccination of leishmaniasis have been performed so far, including whole or attenuated parasites, proteins, synthetic peptides, plasmids or glycolipids (all reviewed in [2;104]), but to date there are no effective vaccines available.

Parasite lysate (soluble *Leishmania* antigen (SLA)), generated by mechanical disruption of the whole parasite, has been shown to be one of the most promising antigenic mixtures [139]. This might be explained by the composition of various potential immunomodulatoric components of this whole parasitic lysate, such as proteins, peptides and many others. Highly immunodominant effects of SLA upon vaccination in the mouse model have already been reported [139-141].

It has already been shown that i.p. injection of parasite lysate protects BALB/c mice against infection with *L. major* as effectively as whole irradiated organisms [145]. Vaccination with lysate was found to induce both cell-mediated and humoral immune responses. Additionally, it has been reported that distinct fractions of SLA, generated from promastigotes of *L. major* by anion exchange liquid chromatography, revealed distinct antigen-specific properties [139]. Within fractions of parasite lysate, proteins and peptides as well as glycolipids can be found, all of which may play a role in enhancing parasite-specific immune responses. However, the role of glycolipids is discussed controversial. A protective capacity of glycolipid molecules from promastigotes has been reported [190], but in contrast, it has been shown that leishmanial glycolipids are rather immunosuppressive than protective depending on certain conditions [191].

In contrast to glycolipids, the existence of protective leishmanial protein antigens as a component of SLA fractions generated from promastigotes of *L. major* has been shown [139]. This is of importance for the identification of protective protein immunogens that opens the way for gene cloning and large scale immunogen production.

Further, the fractionation of pathogen lysate and characterization of immunodominant proteinogenic compounds from these fractions have been successfully performed in a viral model (Kleemann, personal communication).

Thus, our initial approach was to fractionate SLA of *L. major* generated from *in vitro* cultured promastigotes and, as a novel technical approach, from *in vitro* cultured amastigotes (van Zandbergen, manuscript in preparation) by size exclusion chromatography. This kind of chromatography was utilized for obtaining fractions containing proteins of a distinct size. In contrast to ion exchange, protein samples do not bind to the chromatography medium and the buffer composition (PBS) does not directly affect resolution. The fractionation of SLA from *in vitro* generated amastigotes reveals the advantage of high purity of parasitic compounds without contaminations of non-parasitic components.

To date, we obtained several fractions of SLA generated from either promastigotes or amastigotes. All fractions can be tested in ongoing studies for initiating Th1/Tc1 immune responses in *in vitro* assays, such as IFN-γ ELISpot or ELISA. Immunodominant fractions in turn can be directly used for *in vivo* vaccination studies in C57BL/6 mice. In parallel, these immunodominant fractions will be further analysed by mass spectrometry for their specific contents of proteins and peptides.

Vaccination with SLA might function solely as an initial attempt for *in vivo* vaccination trials, scince the reproducibility of SLA-based vaccines is limited. However, the analysis of SLA reveals an excellent method to identify immunogenic proteins/peptides and to further characterize $CD8^+$ T cell epiotpes from *de facto* expressed proteins of the parasite. This will aim to the identification of possible new candidates of protein-based vaccines against *L. major*.

4.4 $CD8^+$ T cell epitope prediction of *de facto* expressed, life form-specific proteins

Reverse vaccinology, and in particular T cell epitope prediction, is emerging as a very promising strategy to facilitate and rationalize vaccine development based on genome sequences [192;193]. However, while the concept is gaining acceptance, there are still many uncertainties regarding the actual reliability of these methods [194].

With a genome size of 33.6 Mb, *L. major* is considered to express about 8300 proteins [147], all of which are potential antigens. In fact, antigen identification represents an important issue in vaccine development against any pathogen, as this is usually achieved through rather empirical, time-consuming and labor-intensive *in vivo* and *in vitro* experiments. Efforts have thus been devoted to the development of novel strategies for a more rational and faster identification of antigens among a large number of proteins. For example, restricting the evaluation of immunogenicity to surface proteins [195] or to proteins overexpressed in a given stage of the pathogen have been used to identify novel antigens [196;197]. However, if only proteins of e.g. the cell surface were analysed, key antigens from cytosolically expressed proteins are likely to be missed

with these strategies. Other strategies like expression-library immunization have been proposed as very effective strategies to screen entire pathogen genomes for protective antigens [198-200]. Nonetheless, this strategy is still very labor intensive, time consuming and only very few studies have been able to identify protective antigens. The usefulness of expression-library immunization may thus be limited to pathogens with small genomes such as viruses and possibly bacteria.

T cell epitope prediction by bioanalytical analysis of protein sequences has been proposed as an alternative for rational vaccine development [192;193;201]. The prediction of $CD8^+$ T cell epitopes from a given protein sequence should be feasible in general, if antigen processing and presentation events follow rules that can be extracted and simulated by computer programs. This feasibility is based on the assumption that all of the antigen processing and presentation events are known; however, this is certainly not the case. Nevertheless, peptide binding to MHC molecules, peptide transport by TAP and peptide generation by proteasomes are key processing events that are crucial in determining the selection of protein fragments for the recognition by $CD8^+$ T cells [202]. Rules for these events have been described, making it possible to formulate prediction algorithms [171;203-206].

However, despite the fact that prediction of $CD8^+$ T cell epitopes by algorithms is influenced by proteasomal degradation and TAP transport prior to antigen presentation, a recent publication revealed promising $CD8^+$ T cell epitopes predicted from the *Leishmania major* genome [207]. Within this study, the authors analysed ~8200 sequences from the entire *L. major* genome to generate consensus predictions of potential T cell epitopes. Their analysis resulted in a total of 78 *L. major* proteins that were predicted to contain epitopes predicted with a very high consensus among the different algorithms used in this study. Interestingly, none of the known *L. major* antigens and current leading vaccine candidates such as gp63, LACK, histone 2B, LmSTI1, TSA, Pb, NH36 or ß-tubulin were included among the top predicted epitope-containing proteins. This lack of important proteins may explained by elimination of these proteins during a initial limiting step in the analysis procedure [207]. Most of the detected proteins from the entire genome/proteome of *L. major* within that work were identified as conserved, hypothetical proteins based on the prediction of mRNA expression levels by genome microarrays, which not necessarily correspond with identical protein expression levels.

It has already been shown in the murine cytomegalovirus (mCMV) model that screening of peptides based on SYFPEITHI predictions revealed promising results and allowed for identification of virus-specific $CD8^+$ T cell epitopes [208]. Epitope prediction was performed by the authors because of failure of protein identification by mass spectrometry analysis due to heterogeneity and low amount of peptides in the samples.

Predictions by computer-based algorithms are not limited in quantities of peptides and thus represent alternative methods for

identifying T cell epitopes. However, utilization of computer-based epitope prediction is limited by the size of the proteom. Viral and small bacterial proteoms can easily be analysed by this method. Analysis of a large proteome such as from *L. major* might be more critical and appropriate limitations have to be considered.

Analysis of the L. major proteom for de facto expressed proteins

Thus, the results presented within the present work were based on only in fact expressed proteins of the proteom of both life forms of *L. major* in contrast to predictions of the entire proteom [207]. Both life forms, promastigotes and amastigotes revealed different protein expression patterns of distinct amounts of proteins [148].

Herein the analysis of protein expression levels was determined by mass spectrometry. In contrast to the analysis of mRNA levels by microarrays, this method directly analyses *de facto* expressed proteins. In addition, frequencies of very low expressed protein levels can also be detected with very high sensitivity and specificity.

Mass spectrometry analysis of protein expression of *L. major* resulted in 489 proteins in fact being expressed by promastigotes and 265 proteins by amastigotes, respectively. Herein, we analysed protein expression from *in vitro* generated pure amastigote fractions. Protein expression studies using lesion derived amastigotes are compounded by the fact that it is difficult to obtain sufficient numbers of pure amastigotes that are entirely free from host derived proteins. As there was no current culture system for *L. major* amastigotes published so far, *L. infantum* cells were used in studies targeting the proteomic analysis [209]. The complete life cycle of *L. infantum* can be replicated in culture, and by several criteria the axenic amastigotes are very similar to lesion-derived amastigotes [210]. A recent publication identified only 91 proteins of *L. infantum* amastigotes by mass spectrometry analysis [209] compared to 265 proteins detected by mass spectrometry analysis of *in vitro* generated *L. major* amastigotes presented within the present work. Thus, our analysis of *de facto* expressed protein levels of *in vitro* generated amastigotes of *L. major* by mass spectrometry revealed higher number of proteins. Overall, with our approach, a higher number of potential relevant immunogenic peptides as possible vaccine candidates can be analysed.

Prediction of $CD8^+$ T cell epitopes from de facto expressed proteins

Therefore, we next predicted $CD8^+$ T cell epitopes of life-stage expressed proteins with the SYFPEITHI algorithm, based on peptide binding motifs [74;211]. A high number of $CD8^+$ T cell epitopes for both haplotypes of C57BL/6 mice were predicted. As a limitation of this large number, we arbitrarily set a cut off at ≥ 25 for the resulting SYFPEITHI scores. A number of 300 high scored peptides, including all amastigote-

derived peptides, has already been synthesized and will be tested for possible immunodominant candidates by IFN-γ ELISpot or ELISA. The ongoing studies will focus on amastigote-derived peptides, as this life form of the parasite is likely be engulfed by DC, which in turn are capable of efficiently inducing $CD8^+$ T cells-dependent immune responses.

It has to be considered that scores obtained from computer-based prediction algorithms do not necessarily correspond with functional results with these peptides. While peptides with a low score and thus with low MHC-I binding affinity are clearly inferior; a high score gives no guarantee of antigenicity. It has been reported that the immunogenicity of a viral peptide predicted with a high score of 31 was not confirmed by functional analyses [212]. On the other hand, an immunodominant peptide in that study was predicted with a relative low score of 26. In addition to binding to MHC-I molecules, further parameters are involved in determining antigenicity. Specifically, it is trivial that the respective protein must be expressed adequately for efficient priming of T cells. In addition, residues that flank the antigenic sequence in the protein can have a critical influence on the efficacy of proteasomal processing.

As a conclusion, a sensitive analysis method such as mass spectrometry was chosen for analysis of *de facto* expressed proteins of *in vitro* generated amastigotes from *L. major.* The epitope prediction and further the functional testing of high scored predicted epitopes from in fact expressed proteins will aim to further characterize $CD8^+$ T cell epitopes as potential vaccine candidates against this parasite.

4.5 Outlook

Within the present work, we showed that the priming of $CD8^+$ T cells is independent of the immunoproteasome. In a further approach, other protein degradation pathways such as autophagy or different cytosolic proteases such as TPP II has to be analysed for their contribution to effective activation of $CD8^+$ T cells.

Further, fractions of SLA generated from *in vitro* generated amastigotes have to be analysed for their immunogenic properties *in vivo* and *in vitro*. Each fraction can be used in direct *in vivo* vaccination studies of C57BL/6 mice in accordance with prior performed protein-based vaccination studies with TAT-LACK. Additionally, fractions will be tested *in vitro* for either the specific components by mass spectrometry analysis, or for their capacity to induce IFN-γ secretion of T cells.

Finally, top scoring epitopes predicted by computer based algorithms such as SYFPEITHI have already been synthesized and will be analysed for their restimulation properties of IFN-γ expression of $CD8^+$ T cells. As a further goal, promising candidates revealing appropriate secretion of IFN-γ secretion *in vitro* might be used in peptide-based vaccines in C57BL/6 mice *in vivo*.

5. Experimental procedures

5.1 Cell biological methods

5.1.1 Cultivation of promastigote *Leishmania major*

The infectious metacyclic promastigote life form of *Leishmania major* is associated with gut tissues in the sand fly. To mimic this habitat, promastigotes were cultured in specific *Leishmania*-medium under anaerobic conditions at 27°C [26]. For the generation of a new culture 10-20x10^6 freshly isolated amastigote parasites were inoculated in 5 ml *Leishmania*-medium in a 25 cm^2 culture bottle, which was sealed with parafilm afterwards. Log-phase replicating parasites were cultured in 10 ml medium and were diluted every 2 days by 1:3 or 1:5.

5.1.2 Isolation of metacyclic, promastigote *Leishmania major*

Cultured promastigotes are heterogenic with regard to their stages of development, but mice were exclusively infected with highly infectious metacyclic promastigotes. For the isolation, metacyclic promastigote parasites out of a 5-6 day old culture were purified with a biphasic ficoll gradient centrifugation step. Parasites were washed twice with sterile DMEM for 8 min and 200 g. Within this isolation step, parasites remained in the supernatant. Next, for sedimentation of the promastigotes, parasite containing supernatant was centrifuged for 8 min at 3,000 g and the resulting pellet was resuspended in 2 ml of DMEM. In a 15 ml centrifugation tube containing 2 ml of 20% ficoll and 2 ml of 10% ficoll, the resuspended parasites were loaded onto the ficoll gradient and were centrifuged at room temperature for 15 min at 2,000 g without brake. Metacyclic promastigotes were enriched in the upper two phases within this step and needed to be washed again with DMEM or PBS before counting.

5.1.3.1 Isolation of amastigote *Leishmania major*

Generation of a new culture of *Leishmania major* requires isolation of intracellular amastigote parasites as well as distinct experimental setups, e.g. infection of DC with *Leishmania major*. Amastigotes were directly isolated from foot pads of BALB/c mice [24], which were infected with 2x10^5 metacyclic promastigotes 3-4 weeks in advance. Infected foot pads were harvested in PBS/glucose/EDTA-buffer and the tissue was chopped with scissors in a first step. To isolate the intracellular parasites from the cell, cells were mechanically sheared within a sterile tissue grinder. The supernatant was passed through a 70 µm cell

strainer and washed twice with DMEM at 3,000 g for 8 min. The resulting pellet contained the amastigote life form of the parasite.

5.1.3.2 *In vitro* generation of amastigotes

In vitro generated amastigotes were a kindly gift from Ger van Zandbergen, Institute of Medical Microbiology and Hygene, Universitätsklinikum Ulm, Germany.

5.1.4 Generation of soluble *Leishmania* antigen (SLA)

SLA was used for antigen-specific restimulation of *Leishmania*-primed T cells. It is a highly complex mixture containing all *Leishmania* antigens in a degraded form. To generate SLA, *Leishmania major* promastigotes from a 6-day old culture or *in vitro* generated amastigotes were isolated, washed twice with sterile PBS for 8 min at 200 g and were pelleted at 3,000 g afterwards. Parasites were adjusted to $300x10^6$ parasites per ml in RPMI-complete or PBS to generate pure SLA without any contaminating proteins and degraded with repetitive freeze-thaw cycles as follows:

(1) Freeze parasites in liquid nitrogen
(2) Thaw in 50°C water-bath
(3) Resupend

Cycles (1) - (3) were repeated 10 times. Immunogenicity of SLA was determined in restimulation experiments by measuring secreted cytokines. If SLA was used for vaccination experiments, protein concentration was determined using a BCA-assay.

5.1.5 Intradermal infection in the ear

To infect mice with *Leishmania major*, isolated metacyclic promastigote parasites were injected intradermally in the ear of anaesthetised mice. In the low dose model, only 1,000 parasites were inoculated, whereas in the high dose model, $2x10^5$ parasites were inoculated into the ear.

5.1.6 Subcutaneous infection in the foot pad

To generate the amastigote life form of *Leishmania major*, anaesthetised BALB/c mice were infected s.c. with $2x10^5$ metacyclic parasites into the hind foot pad.

5.1.7 Determination of lesion development

Lesion development was assessed weekly for up to 4 months in three dimensions using a caliper, calculated as ellipsoids using the equitation (a/2 x b/2 x c/2) x 3/4π and expressed as mean ±SEM [213].

5.1.8 Determination of parasite burdens

Infected ears were harvested, split and incubated dermis-down for 2 hrs at 37°C and 5% CO_2 in collagenase A medium. Next, enzymatically fermented tissues were chopped with scissors and milled in a further step for 1 min mechanically with a pellet pestle. Cell suspensions were filtered through a 70 µm cell strainer and washed twice with sterile PBS. Liberated parasites were pelleted by centrifugation with 3,000 g for 8 min. Finally, parasites were cultured in a 96 well plate, using a limited dilution assay. Each well was pre-filled with 100 µl of Schneider´s drosophila medium and 100 µl of parasite-containing suspension were added to the first well. In a 1:2 dilution every following well was titrated down with parasites for three rows. The plate was sealed with parafilm and was incubated for 6-9 days at 27°C. Most of the amastigote life form transform to the promastigote form under these conditions. The well with the highest dilution containing a viable parasite indicates the number of absolute parasites in the infected ear.

5.1.9 Preparation of lymph nodes

Lymph nodes (LN) of naïve or infected mice were harvested and suspended by squeezing with the hilt of a sterile syringe. The suspension was filtered through a 70 µm cell strainer, washed with RPMI complete and cells were counted.

5.1.10 Cytokine profiles of restimulated lymph node cells

To determine the cytokine profiles of infected or vaccinated mice, draining lymph nodes cells were cultured with $1x10^6$ cells in 200 µl RPMI complete per well in a 96 well plate (flat bottom). Antigen specific restimulation was performed by addition of 10 µl soluble *Leishmania* antigen (SLA). As a positive control 20 µl of *staphylococcus* enterotoxine B (SEB; 1 mg/ml) was added or, as a negative control, cells were left untreated. All cells were incubated for 48 hrs at 37°C and 5% CO_2. Supernatants were harvested afterwards and cytokine secretion was assessed by cytokine-specific ELISA.

5.1.11 Generation of murine bone marrow derived dendritic cells (BMDC)

Generation of bone marrow derived DC from hematopoietic stem cells requires administration of recombinant IL-4 and recombinant GM-CSF [214]. Bone marrow was obtained from femur and tibia of C57BL/6 mice. Cell suspensions were washed with PBS and centrifuged at 200 g for 8 min. Cells were resuspended in 1 ml ACK-lysis buffer to lyse erythrocytes and after 1 min washed with PBS again. Up to $40x10^6$ cells were cultured in 20 ml RPMI complete containing 10 ng/ml IL-4 and GM-CSF in a cell culture flask. After two days of culture, DC precursor cells were adherent and medium needed to be changed. 15 ml of fresh RPMI complete with cytokines were added. On day four, all cells were in suspension and 5 ml of RPMI complete including cytokines were added. On day six, immature DC were harvested and characterized by flow cytometry.

5.1.12 Infection of dendritic cells with amastigotes

6 day old immature dendritic cells were adjusted to $1x10^6$ cells per ml DC-medium and were incubated for 18 hrs at 37°C and 5% CO_2 together with $5x10^6$ freshly isolated amastigotes. Prior to co-culture parasites were opsonized with 5% vol. normal mouse serum (NMS) of BALB/c mice for 10 min at 37°C in a water bath.

5.1.13 Restimulation of T cells with infected DC

To determine the cytokine profiles of distinct subpopulations of T cells, draining lymph node cells were incubated with microbead-labelled antibodies against CD4 or CD8 on the cell surface of T cells. DC were irradiated with 4,000 rad and co-cultured in a U-bottom plate with subfractionated T cells in a ratio of 1:10 (DC to T cells) for 48 hrs at 37°C and 5% CO_2. The maximum cell number was $1x10^6$ cells per well per 200 µl. After incubation, supernatants were harvested and cytokine release was determined by cytokine-specific ELISA.

5.1.14 Proliferation of T cells

CFSE

The dye carboxyfluorescin diacetate, succinimidyl ester (CFSE) is connected covalently with intracellular amines which leads to the formation of the fluorescent form of the dye. These protein-dye-conjugates remain within the cell, which consequently can be detected by flow cytometry. T cells divide upon activation and the intracellularly bound CFSE-conjugates are split up in both resulting daughter cells. Cell proliferation of CFSE-labelled T cells therefore was detected by

decreasing fluorescence intensity in the FL-1 channel. $5x10^6$ cells per ml PBS were mixed with 1 µM CFSE at 37°C in a water bath for 15 min. Cells were washed with RPMI complete medium and subsequently were used for additional restimulation experiments. After 4-6 days of incubation at 37°C and 5% CO_2, cell proliferation was measured by flow cytometry in the FL-1 channel.

^{3}H-Thymidin

Metabolic incorporation of tritiated thymidine (^{3}H-TdR) into cellular DNA is a widely used protocol to monitor rates of DNA synthesis and cell proliferation. Cell suspension containing DC and T cell subsets were cultured in a flat-bottom 96-well plate and ^{3}H-Thymidin was added in excess after 48 hrs and cells were cultured for 18 hrs at 37°C. Afterwards, cells were harvested onto a filter membrane that enriched solely intact cells. The filter membrane was dried and the amount of radioactivity (which corresponds to the number of cells in the well or the number of cell divisions during incubation) was counted in a liquid scintillation counter, (Wallac).

5.1.15 Isolation of T cells from the spleen

To isolate T cells from the spleen, the organ was passed through a 70 µm cell strainer in a 55 mm petri dish containing 5 ml sterile PBS. The tissue was grinded with sterile tweezers and the resulting cell suspension was washed with PBS and centrifuged at 200 g for 8 min. The pellet was resuspended in 1 ml ACK buffer to lyse erythrocytes and incubated for 1 min. Afterwards, 10 ml RPMI complete were added and the suspension was centrifuged again at 200 g for 8 min. Cells were counted and the T cell sub-fractions were purified by MACS in further isolation steps.

5.1.16 Cytospin

For quantification of isolated cells by microscopy, cells were spun down on a glass slide by cyto centrifuge. $4x10^5$ cells were centrifuged for 5 min at 500 rpm. To distinguish different cell types, slides were stained with Diff-Quick in accordance with the manufactors guidelines.

5.1.17 Vaccination with proteins

Vaccination with fusion proteins was accomplished together with immunostimulatoric CpG-oligonucleotides, which function as adjuvants. These oligonucletides contain non-methylated cytosin-guanosin-dinucleotides, which activate the immune system via toll-like receptor 9 (TLR9) [126]. For vaccination, 10 µg of each protein was

delivered intradermally into the ear of anesthetized mice repetitivly by day -14 and day -13. On day -12, 10 µg of CpG 1826 was injected in the same ear. Low dose inocula of *L. major* were injected on day 0 into the contralateral ear.

5.1.18 Depletion of T cells

In order to characterize the role of single T cell populations on protein-based vaccinations, T cells were depleted before immunisation with proteins via depleting antibodies. 125 µg anti-CD4-depletion antibody (GK 1.5) or 50 µg anti-CD8-depletion antibody (2.43) were injected intraperitoneally on day -15 in anesthesized mice. As a depletion control, rat IgG isotype antibody (GL113) was injected. The success of depletion was determined 3 days post antibody administration by flow cytometry analyzing blood cells by staining for $CD4^+$ or $CD8^+$ T cells. Infection with *L. major* was performed two weeks after T cell depletion, when T cells had recovered.

5.2 Immunological methods

5.2.1 Enzyme-linked Immuno Sorbent Assay (ELISA)

The ELISA is a biochemical method to detect the presence of an antigen in a sample. Here we used the “sandwich-ELISA”, which implies the coating of the antigen-specific antibody (capture antibody) onto the surface of a 96 well plate. Cytokines binds to the immobilized capture antibody and can be detected in a further step by binding the enzyme-linked detection antibody. This enzyme (HRP, horse-raddish peroxidase) catalyzes the conversion of a chromogenic substrate, which makes the immunocomplexes visible. The quantification of the antigen concentration in the sample can be obtained by photometric analysis in an absorbtion microplate reader.

ELISA-kits

The quantifications of secreted cytokines like IL-4, IL-10 IFN-γ, IL-12p40, were performed with commercial ELISA-kits. All assays were performed in accordance with the manufactures guidelines. Exceptions:

(1) Washing buffer (PBS) contained additionally 0.1% Tween 20
(2) In between washing steps, ELISA plates were washed only three times
(3) Volume of blocking buffer was 150 µl per well

5.2.2 Magnetic Cell Sorting (MACS)

Specific target cells can be isolated out of a mixture of different cell populations by using the MACS technique. Target cells are covalently bound with microbead-labeled antibodies specific for molecules on the surface of the cells. The heterogenous cell suspension is loaded onto a magnetic column and all unlabeled cells were eluted. After removing the column from the magnetic field, the purified fraction of target cells could be eluted. The MACS method was used herein in accordance with the manufactures guidelines for isolation of $CD4^+$ and $CD8^+$ T cells. Exception: A pellet of max. $1x10^7$ cells were resuspended in 45 µl MACS-buffer. For isolation of cells, 5 µl bead solution was added to suspension.

5.2.3 Fluorescence Activated Cell Sorting (FACS)

Flow cytometry is a technique for counting and characterizing single cells by suspending them in a stream of fluid and passing them by an electronic detection unit. A beam of laser light of a single wavelength is directed onto the hydrodynamically-focused stream of the cell suspension. Each cell from 0.2 to 150 micrometers passing through the beam scatters the light in a special way, and fluorescent dyes attached to the cell may be excited into emitting light at a longer wavelength than the original light source. This combination of scattered and fluorescent light is analysed by detectors. FSC correlates with the cell volume and SSC depends on the inner complexity of the particle. Cells were washed twice with FACS buffer and free Fc-receptors were blocked by incubation in 10 µl Fc-blocking antibody for 20 min at 4°C. Further, cells were incubated in a 10 µl solution with fluorescent-labelled antibodies for an additional 30 min at 4°C. If more than one fluorescent dye is used, cells were washed after the first incubation step twice with FACS buffer before staining with the second antibody.

5.2.4 BCA-assay for protein concentration

To determine the concentration of proteins, we used the BCA (bicinchoninic acid)-assay which combines the Biuret method with selective bicinchoninicacid-complex of Cu^+. Cu^{2+} is reduced to Cu^+ due to proteins in alkaline solutions. Two BCA molecules binds one Cu^+-ion and a chelat complex, which can be detected at 562 nm, is formed. Herein, we used the BCA Protein Assay Reagent Kit for determination of protein concentrations of TAT-LACK, LACK, TAT-OVA and SLA for vaccination. The assay was operated in accordance to the manufactures guidelines.

5.3 Statistical analysis and software

Statistical significance was determined using the unpaired Students-*t*-test using StatView® for Windows. Results are expressed as mean ±SEM, statistically significant differences are designated as *=p≤0.05, **=p≤0.005 and ***=p≤0.002
For prediction of $CD8^+$ T cell epitopes SYFPEITHI (www.syfpeithi.de) and NetMHC (www.cbs.dtu.dk/services/NetMHC) algorithms used. All flow cytometry results were obtained by using a LSR II (BD Bioscience) and FlowJo® software.

6. Materials and equipment

6.1 Parasites

Leishmania major, clone VI (MHOM/IL/80/Friedlin)

6.2 Animals

C57BL/6 mice	Zentrale Versuchstiereinrichtung, Johannes Gutenberg University, Mainz
BALB/c mice	Zentrale Versuchstiereinrichtung, Johannes Gutenberg University, Mainz
LMP7$^{-/-}$ mice	Institute of Immunology, Johannes Gutenberg University, Mainz

6.3 Antibodies

Unlabeled antibodies

anti-mouse CD16/CD32 (Fcγ receptor III/II), clone 2.4G2	BD Pharmingen, Heidelberg
anti-mouse CD4 mAb (depletion)	GK1.5 hybridoma cell line, ATCC
anti-mouse CD8 mAb (depletion)	2.43 hybridoma cell line, ATCC

FITC-conjugated antibodies

Isotype rat anti-mouse, IgG2b	BD, Pharmingen Heidelberg
Isotype hamster anti-mouse, IgG1	BD, Pharmingen Heidelberg
anti-MHC I, clone 2G5;	Serotec, Kidlington, UK
anti-MHC II, clone 2G9;	BD Pharmingen, Heidelberg
anti-CD40, clone 3/23;	BD Pharmingen, Heidelberg
anti-CD54, clone 3E2;	BD Pharmingen, Heidelberg
anti-CD86, clone GL1;	BD Pharmingen, Heidelberg
Neutrophil, clone 7/4	eBioscience, USA
anti-streptavidin	BD Pharmingen, Heidelberg

PE-conjugated antibodies

Isotype hamster anti-mouse IgG2a	Natutec, Frankfurt/Main
Isotype rat anti-mouse IgG2b	BD, Pharmingen Heidelberg
anti-CD11c clone N418;	eBioscience, USA
anti-F4/80, clone BM8	eBioscience, USA
anti-NK1.1, clone NK1.1	eBioscience, USA

APC/PerCP or otherwise conjugated antibodies

Isotype hamster anti-mouse IgG	eBioscience, USA

anti-mouse CD4 APC clone L3T4	BD Pharmingen, Heidelberg
anti-CD8 APC clone Ly-3	BD Pharmingen, Heidelberg
anti-CD11c clone HL3	BD Pharmingen, Heidelberg
anti-B220 PerCP, clone Ra3-6B2	BD Pharmingen, Heidelberg

6.4 Cytokine Kits

BCA Protein Assay Kit	Pierce, Perbio Science, Bonn
Diff-Quik staining	Dade Behring, Düdingen, CH
DuoSet ELISA mouse IFN-γ	R&D Systems, Wiesbaden
DuoSet ELISA mouse IL-12p40	R&D Systems, Wiesbaden
BD OptEIA ELISA mouse IL-4	BD Pharmingen, Heidelberg
BD OptEIA ELISA mouse IL-10	BD Pharmingen, Heidelberg

6.5 Buffers and solutions

PBS

PBS (10x): 1,38 M NaCl
0,1 M NaH_2PO_4 x 1 H_2O
in 1 l aqua dest., pH 6,6

PBS (1x): PBS (10x) was diluted with aqua dest, pH was adjusted to 7.2 – 7.3 and buffer was autoclaved

RPMI (complete)

5% FCS
1% Penicilin/streptomycin
1% Glutamine
1% HEPES
1% non essential amino acids
0.1% ß-mercaptoethanol
Ad 500 ml RPMI 1640

***Leishmania*-medium**

M199 (1x)	350 ml
M199 (10x)	14.25 ml
FCS	100 ml
HEPES (1 M)	20ml
Adenin (10 mM)	5 ml
Hemin (0.25%)	1 ml
Penicilin/Streptomycin	5 ml
Glutamin	5 ml

Adenin 10 mM Adenin

5% HEPES (1 M)
in aqua dest.

Hemin 50% Triethanolamin
0.25% Hemin
in aqua dest.

Ficoll
20% Ficoll in aqua dest.

PBS/Glucose/EDTA-buffer
0.2% Glucose
2 mM EDTA (pH 6.5)
in sterile PBS

Collagenase-medium
1% Penicilin/Streptomycine
0.1% Collagenase A
in sterile DMEM

Schneider´s medium
2% urine (human)
10% FCS
2% Glutamin
1% Penicilin/Streptomycin
0.5% HEPES (1 M)
in Schneider´s drosophila medium

FACS-buffer
2% FCS
50 µl NaN_3 (10% sol.)
in sterile PBS

Dendritic cell medium
10 ng/ml recombinant IL-4
10 ng/ml recombinant GM-CSF
in sterile RPMI-complete medium (Biochrom)

Mass spectrometry-lyse buffer
(gift from Inst. of Immunology, Mainz)
PBS (1x) containing 7 M urea

6.6 Reagents and chemicals

ACK lysis buffer	BioWhittaker/Cambrex BioScience, Verviers, Belgium
Adenin	Carl Roth, Karlsruhe

Alkopharm 70 (Ethanol 70%)	Brüggemann Alcohol, Heilbronn
CD4 (L3T4) MicroBeads, mouse	Miltenyi Biotec, Berg. Gladbach
CD8 (Ly-2) MicroBeads, mouse	Miltenyi Biotec, Berg. Gladbach
CFDA SE Cell Tracer Kit (CFSE)	Invitrogen, Karlsruhe
Collagenase, Clostridiopeptidase A	Sigma-Aldrich, Taufkirchen
CpG 1826	Sigma-Aldrich
DMEM w/o pyruvate	Merck, Darmstadt
EDTA (Ethylendiamintetraacetat)	Sigma-Aldrich, Taufkirchen
FACS Clean Solution	BD Pharmingen, Heidelberg
FACS Flow Sheath fluid	BD Pharmingen, Heidelberg
FACS Rinse Solution	BD Pharmingen, Heidelberg
Ficoll 400	Sigma.-Aldrich, Taufkirchen
FCS, fetal calf serum	PAA Laboratories, Cölbe
Forene (Isoflurane)	Abbott, Wiesbaden
Glucose (D-(+)-Glucose, p.a.)	Carl Roth, Karlsruhe
L-Glutamin	Gibco, Invitrogen, Karlsruhe
Helipur H plus N	B. Braun, Melsungen
Hemin(chlorid), 98%	Carl Roth, Karlsruhe
HEPES Pufferan, 99.5%	Carl Roth, Karlsruhe
Hydrochloride acid (HCl)	Merck, Darmstadt
Immidazol Pufferan, 99.5%	Carl Roth, Karlsruhe
Immersionoil (microscopy)	Merck, Darmstadt
Isopropanol	Hedinger, Stuttgart
Ketamin Hydrochloride	Sigma-Aldrich, Taufkirchen
Liberase CI Purified Enzyme Blend	Roche Diagnostics, Mannheim
M199 Medium	Gibco, Invitrogen, Karlsruhe
MEM non-essential amino acids	Gibco, Invitrogen, Karlsruhe
ß-mercaptoethanol, 99% p.a.	Carl Roth, Karlsruhe
Paraformaldehyde	Merck, Darmstadt
Protein plasmids	Gift from Dr. M. Udey, NIH, Bethesda, MD, USA
Recombinant murine GM-CSF	PeproTech, Offenbach
Recombinant murine IL-4	PeproTech, Offenbach
Rotiszint, Scintillation solution,	Carl Roth, Karlsruhe
RPMI 1640 medium	Biochrome, Berlin
Schneiders Drosophila Medium	BioWhittaker/Cambrex Bioscience
Sodium azide (NaN_3)	Carl Roth, Karlsruhe
Sodiumcarbonate (Na_2CO_3)	Carl Roth, Karlsruhe
Sodiumchlorid (NaCl)	Carl Roth, Karlsruhe
Sodiumdihydrogenphosphate, (NaH_2PO_4 x 1 H_2O)	Carl Roth, Karlsruhe
Sodiumhydrogencarbonate ($NaHCO_3$)	Carl Roth, Karlsruhe
Staphylococcus enterotoxine B	Sigma-Aldrich, Taufkirchen
Triethanolamin	Sigma-Aldrich, Taufkirchen
Trypanblau solution	Sigma-Aldrich, Taufkirchen
Tween 20	Sigma-Aldrich, Taufkirchen

6.7 Expendable materials

Canula, 23Gx1¼, 0.6x30	B. Braun, Melsungen
Canula, 26Gx1½, 0.45x12	B. Braun, Melsungen
Canula, 23Gx1½, 0.3x13	BD, Heidelberg
Cell culture bottle, 25 cm^2	Greiner Bio-One, Frickenhausen
Cell culture bottle, 75 cm^2	Greiner Bio-One, Frickenhausen
Cell culture plate 6 well, flat bottom	Greiner Bio-One, Frickenhausen
Cell culture plate 12 well, flat bottom	Greiner Bio-One, Frickenhausen
Cell culture plate 24 well, flat bottom	Greiner Bio-One, Frickenhausen
Cell culture plate 48 well, flat bottom	Greiner Bio-One, Frickenhausen
Cell culture plate 96 well, flat bottom	Greiner Bio-One, Frickenhausen
Cell culture plate 96 well, u-bottom	Falcon, Fisher Scientific, Schwerte
Cell strainer, 70 µm	Falcon, Fisher Scientific, Schwerte
Cryotubes, 1.8 ml	Nunc, VWR, Darmstadt
Finntip 300, 5-300 µl	Thermo Lab, VWR Darmstadt
Greiner Tubes, 15 ml	Greiner-One, Frickenhausen
Greiner Tubes, 50 ml	Greiner-One, Frickenhausen
MACS Cell separation column MS	Miltenyi Biotec, Berg. Gladbach
MaxiSorp Nunc-Immuno plate 96 well	Nunc, Brandt, Wertheim
Medicon, 50 µm sterile	BD, Heidelberg
Parafilm M Laboratory Film	Pechiney Plastic, Chicago, USA
Petridishes	Greiner Bio-One, Frickenhausen
Pipett-Tips, 0.1 – 10 µl	Carl Roth, Karlsruhe
PD Tips Plastibrand, 0.5 ml	Brand, Wertheim
PD Tips Plastibrand, 2.5 ml	Brand, Wertheim
PD Tips Plastibrand, 5.0 ml	Brand, Wertheim
Pellet pestler, grinders PP	Sigma-Aldrich, Taufkirchen
Scalpel	B. Braun, Melsungen
Syringe, 1 ml	B. Braun, Melsungen
Syringe, 2 ml	B. Braun, Melsungen
Syringe, 5 ml	BD, Heidelberg
Syringe, 10 ml	BD, Heidelberg
Syringe, 20 ml	BD, Heidelberg
ZapCap-S Bottel-top filters, 0.2 µm	Schleicher & Schuell, Darmstadt

6.8 Electronic equipment

Absorptions Microplate Reader ELx808	BioTEK Instruments, Bad Friedrichshall
Calliper, digital, 150 mm	Rheinwerkzeuge, Mainz
Centrifuge Biofuge pico	Heraeus, Kendro, Langenselbold
Centrifuge Mutlifuge 3 L-R	Heraeus, Kendro, Langenselbold
CO_2-Incubator	Heraeus, Kendro Laboratories, Langenselbold

FACSCalibur, Flow Cytometer	BD, Heidelberg
Medimachine	BD, Heidelberg
Microscope Diaplan	Leitz, Wetzlar
Microscope Diavert	Leitz, Wetzlar
Milli-Q water systems	Millipore, Schwalbach
Pellet pestle, cordless motor	Sigma-Aldrich, Taufkirchen
Shandon Cytospin centrifuge	Thermo Electron, Langenselbold
Scintillation counter	
Sonorx, Super RK 103	Bandelin Electronics, Berlin
Varioclav	H+P Labortechnik, Oberschleißheim
Vortex	Merck Eurolab, Darmstadt
Water bath, type GFL-1003, 14 l	Gesellschaft für Labortechnik

7. Supplementary data

Name	Amastigotes (ppm)	Promastigotes (ppm)
microtubule associated protein putative Leishmania major	245014	157456
Trypsin precursor	48777	16707
keratin 1 Homo sapiens	46917	6270
Enolase 1 2 phosphoglycerate dehydratase 1 2 phospho D glycerate hydro lyase 1	43786	18047
kinesin putative Leishmania major	23956	45225
glyceraldehyde 3 phosphate dehydrogenase glycosomal Leishmania major	21821	21624
keratin 9 Homo sapiens	20735	2853
vacuolar type proton translocating pyrophosphatase 1 putative Leishmania major	19459	17588
chaperonin Hsp60 mitochondrial precursor Leishmania major	18771	10230
CPA cysteine peptidase clan CA family C1 cathepsin L like protein cysteine protease A cysteine	17902	6962
hypothetical protein conserved Leishmania major	14701	14026
hypothetical protein unknown function Leishmania major	14403	15863
hypothetical protein conserved Leishmania major	14339	12785
keratin 10 Homo sapiens	13193	
clathrin heavy chain putative Leishmania major	12260	9875
cysteine peptidase Clan CA family C2 putative cytoskeleton associated protein CAP5 5 putative	11374	8059
glucose regulated protein 78 putative Leishmania major	10408	7991
chaperonin Hsp60 mitochondrial precursor Leishmania major	9584	6760
succinyl CoA ligase GDP forming beta chain putative Leishmania major	8914	6574
heat shock protein putative Leishmania major	8803	8927
citrate synthase putative Leishmania major	8680	5812
aconitase putative Leishmania major	8233	7221
succinyl coA 3 ketoacid coenzyme A transferase mitochondrial precursor putative Leishmania major	8083	5503
hypothetical protein conserved Leishmania major	7414	7440
glycoprotein 96 92 putative Leishmania major	7321	4337
glutamate dehydrogenase Leishmania major	6852	14934
hypothetical protein conserved Leishmania major	5950	4834
malate dehydrogenase Leishmania major	5929	4439
glutamate dehydrogenase putative Leishmania major	5876	1332
hypothetical protein conserved Leishmania major	5710	
10 kDa heat shock protein putative Leishmania major	5668	3169
CPC cysteine peptidase Clan CA family C1 Cathepsin B like Leishmania major strain Friedlin	5490	4040
hypothetical protein conserved Leishmania major	5447	8331
hypothetical protein conserved leucine rich repeat protein putative Leishmania major	5330	10409
peroxidoxin tryparedoxin peroxidase Leishmania major	5116	3551
hypothetical protein conserved Leishmania major	5088	3435
dihydrolipoamide dehydrogenase putative Leishmania major	4945	4192
transketolase putative Leishmania major	4843	5833
hypothetical protein conserved Leishmania major	4738	4711
hypothetical protein conserved Leishmania major	4720	12098
peptidylprolyl isomerase like protein Leishmania major	4676	1783
ATP dependent RNA helicase putative Leishmania major strain Friedlin	4641	7915
ATP dependent RNA helicase putative Leishmania major	4625	7895
hypothetical protein unknown function Leishmania major	4604	
enolase Leishmania major	4313	13832

hypothetical protein conserved Leishmania major	4290	
ribosomal protein l3 putative Leishmania major	4169	5797
branched chain amino acid aminotransferase putative Leishmania major strain Friedlin	4101	2645
isocitrate dehydrogenase NADP mitochondrial precursor putative Leishmania major	3872	3133
fructose 1 6 bisphosphate aldolase Leishmania major	3796	6522
myo inositol 1 phosphate synthase Leishmania major	3717	
poly a binding protein putative Leishmania major strain Friedlin	3635	3703
hypothetical protein conserved Leishmania major	3554	5502
triosephosphate isomerase Leishmania major	3550	2590
hypothetical protein unknown function Leishmania major	3524	4021
universal minicircle sequence binding protein putative Leishmania major	3460	5768
hypothetical protein unknown function Leishmania major strain Friedlin	3392	
Transitional endoplasmic reticulum ATPase putative valosin containing protein homolog Leishmania	3390	5020
hypothetical protein LT 05 Leishmania major strain Friedlin	3362	3194
hypothetical protein conserved Leishmania major	3327	3051
aminopeptidase putative metallo peptidase Clan MF Family M17 Leishmania major	3304	2070
carnitine choline acetyltransferase putative Leishmania major strain Friedlin	3301	1840
hypothetical protein conserved Leishmania major	3276	5986
thiolase protein like protein Leishmania major	3238	2230
ATP dependent DEAD box RNA helicase putative Leishmania major strain Friedlin	3168	7381
heat shock protein 20 putative Leishmania major strain Friedlin	3143	767
ascorbate dependent peroxidase putative Leishmania major	3107	
hypothetical protein unknown function Leishmania major	3062	
GP63 leishmanolysin metallo peptidase Clan MA M Family M8 Leishmania major	3062	
glucose 6 phosphate isomerase Leishmania major	3047	4729
splicing factor ptsr1 like protein Leishmania major	3017	3324
hypothetical protein conserved Leishmania major	3012	
aldehyde dehydrogenase mitochondrial precursor Leishmania major	2992	3467
stress induced protein sti1 Leishmania major	2943	2846
vacuolar ATP synthase subunit B putative Leishmania major	2911	2277
elongation factor 1 gamma Leishmania major	2892	5282
hypothetical protein conserved Leishmania major	2858	1613
heat shock protein DnaJ putative Leishmania major strain Friedlin	2849	4550
4 coumarate coa ligase like protein Leishmania major	2780	2525
hypothetical protein conserved Leishmania major	2723	3178
dihydrolipoamide acetyltransferase precursor putative Leishmania major	2722	2464
delta 1 pyrroline 5 carboxylate dehydrogenase putative Leishmania major	2688	4629
ATP dependent zinc metallopeptidase putative metallo peptidase Clan MA E Family M41 Leishmania	2652	1744
acetyl CoA synthetase putative Leishmania major	2621	
acyl coa dehydrogenase putative Leishmania major	2571	2290
phosphoprotein phosphatase putative Leishmania major	2567	2701
40S ribosomal protein S6 putative Leishmania major	2561	4298
40S ribosomal protein S14 Leishmania major	2536	2920
heat shock protein putative Leishmania major	2533	1498
transaldolase putative Leishmania major	2493	2458
pyruvate dehydrogenase E1 component alpha subunit putative Leishmania major	2458	1500
calmodulin like protein containing EF hand domain hypothetical protein conserved Leishmania major	2447	2502
pyruvate kinase putative Leishmania major strain Friedlin	2371	3310
glucose 6 phosphate dehydrogenase Leishmania major	2365	

hypothetical protein conserved Leishmania major	2353	1338
trypanothione reductase Leishmania major	2351	2142
tryparedoxin Leishmania major strain Friedlin	2315	2242
inosine 5 monophosphate dehydrogenase Leishmania major	2234	2348
hypothetical protein conserved Leishmania major	2233	2590
carboxypeptidase putative metallo peptidase Clan MA E family 32 Leishmania major	2174	2407
cytochrome c oxidase subunit iv Leishmania major	2153	3592
calpain like cysteine peptidase Clan CA family C2 calpain like cysteine peptidase putative Leis	2150	1684
vacuolar ATP synthase catalytic subunit a putative Leishmania major	2146	2387
hypothetical protein conserved Leishmania major	2108	1807
S adenosylhomocysteine hydrolase Leishmania major	2107	4337
ribonucleoprotein p18 mitochondrial precursor putative Leishmania major	2089	3659
GTP binding protein putative Leishmania major strain Friedlin	2086	2711
protein disulfide isomerase Leishmania major	2024	2290
small GTP binding protein Rab1 putative Leishmania major strain Friedlin	2018	1848
translation elongation factor 1 beta putative Leishmania major	2006	3619
i 6 autoantigen like protein Leishmania major	1971	2623
hypothetical protein conserved Leishmania major	1953	2615
ATP synthase epsilon chain putative Leishmania major	1931	1456
actin a Leishmania major	1903	3113
long chain fatty Acyl CoA synthetase putative Leishmania major	1892	2928
hypothetical protein conserved Leishmania major strain Friedlin	1889	2087
nucleoside diphosphate kinase b Leishmania major	1878	5521
hypothetical protein conserved Leishmania major	1865	1906
hypothetical protein conserved Leishmania major	1864	1603
regulatory subunit of protein kinase a like protein Leishmania major	1840	2328
60S ribosomal protein l36 putative Leishmania major	1824	1749
metallo peptidase Clan ME Family M16 Leishmania major strain Friedlin	1820	4751
hypothetical protein conserved Leishmania major	1802	2335
pyruvate dehydrogenase E1 beta subunit putative Leishmania major	1785	1521
poly zinc finger protein 2 putative Leishmania major	1742	1851
iron superoxide dismutase putative Leishmania major	1742	
6 phosphogluconate dehydrogenase decarboxylating putative Leishmania major strain Friedlin	1722	
map kinase putative mitogen activated protein kinase putative Leishmania major	1701	2103
protein kinase putative Leishmania major	1699	
RNA binding protein putative Leishmania major strain Friedlin	1691	1817
calpain like cysteine peptidase putative cysteine peptidase Clan CA family C2 putative Leishma	1682	2514
elongation factor TU putative Leishmania major	1633	
electron transfer flavoprotein putative Leishmania major	1628	824
phosphomannomutase putative Leishmania major	1564	1175
succinate dehydrogenase flavoprotein putative Leishmania major	1552	
hypothetical protein conserved Leishmania major	1552	1321
epsin putative Leishmania major	1533	1319
hypothetical protein conserved Leishmania major	1532	4818
protein disulfide isomerase putative Leishmania major	1511	1321
t complex protein 1 gamma subunit putative Leishmania major	1479	2509
mevalonate kinase putative Leishmania major	1464	5158
hypothetical protein unknown function Leishmania major	1447	3361
hypothetical protein conserved Leishmania major strain Friedlin	1429	

metallo peptidase Clan ME Family M16 mitochondrial processing peptidase alpha subunit putative	1399	1449
20S proteasome beta 6 subunit putative proteasome beta 6 subunit putative Leishmania major	1377	1603
hypothetical protein conserved Leishmania major	1324	
hypothetical protein conserved Leishmania major	1276	1888
14 3 3 protein like protein Leishmania major	1265	3343
peptidyl prolyl cis trans isomerase cyclophilin 40 putative Leishmania major strain Friedlin	1246	1852
co chaperone GrpE putative Leishmania major	1236	
ATP synthase putative Leishmania major	1201	858
hypothetical protein conserved Leishmania major	1182	1293
adp ribosylation factor putative Leishmania major	1101	1349
kinetoplast associated protein p18 2 putative Leishmania major	1099	
hs1vu complex proteolytic subunit like hs1vu complex proteolytic subunit like threonine peptidase	1098	
14 3 3 protein putative Leishmania major	1095	2820
adenylate kinase putative Leishmania major	1093	998
hypothetical protein conserved Leishmania major	1022	
hypothetical protein conserved Leishmania major	1017	1333
proteasome alpha 1 subunit putative Leishmania major	1012	1404
40S ribosomal protein S12 putative Leishmania major	1010	2385
fibrillarin putative Leishmania major	967	
la RNA binding protein putative Leishmania major	934	
hypothetical protein unknown function Leishmania major	907	
60S acidic ribosomal protein P2 putative Leishmania major	850	1512
ubiquinone biosynthesis methyltransferase putative Leishmania major	845	1666
dihydrolipoamide acetyltransferase precursorlike protein Leishmania major	818	982
cytosolic malate dehydrogenase putative Leishmania major	732	
hypothetical protein unknown function Leishmania major	666	1621
proteasome alpha 7 subunit putative Leishmania major strain Friedlin	657	936
hypothetical protein conserved Leishmania major	652	1500
macrophage migration inhibitory factor like protein Leishmania major	650	
ATP dependent DEAD H RNA helicase putative Leishmania major		1499
translation initiation factor like protein Leishmania major		1011
nucleolar protein putative Leishmania major		1073
3 methylcrotonoyl CoA carboxylase beta subunit putative Leishmania major		989
60S ribosomal protein l21 putative Leishmania major		1497
tyrosyl or methionyl tRNA synthetase like protein Leishmania major		635
bifunctional aminoacyl trna synthetase putative prolyl trna synthetase putative Leishmania major		1818
mitogen activated protein kinase putative protein kinase putative Leishmania major		1063
aminopeptidase putative metallo peptidase Clan MG Family M24 Leishmania major		1193
nucleosome assembly protein putative Leishmania major		640
proteasome alpha 2 subunit putative Leishmania major		945
proteasome regulatory ATPase subunit 5 putative Leishmania major		1716
3 ketoacyl coa thiolase like protein Leishmania major		1205
cytochrome c oxidase VII putative Leishmania major		793
ATPase beta subunit putative Leishmania major		13759
hypothetical protein conserved Leishmania major		1252
hypothetical protein conserved Leishmania major		962
heat shock 70 related protein 1 mitochondrial precursor putative Leishmania major		13058
hypothetical protein conserved Leishmania major		1202
60S ribosomal protein L9 putative Leishmania major		1303

biotin lipoate protein ligase like protein Leishmania major	905
nucleosome assembly protein like protein Leishmania major	594
3 2 trans enoyl CoA isomerase mitochondrial precursor putative Leishmania major	1151
ubiquinol cytochrome c reductase like protein Leishmania major	847
ribosomal protein S25 Leishmania major	654
40S ribosomal protein S19 protein putative Leishmania major	1841
EIF3 interacting protein like protein Leishmania major	2746
polyubiquitin putative Leishmania major	37446
hypothetical protein conserved Leishmania major	1509
hypothetical protein conserved Leishmania major	1107
hypothetical protein conserved Leishmania major	2020
eukaryotic translation initiation factor 3 subunit 8 putative Leishmania major	1964
elongation initiation factor 2 alpha subunit putative Leishmania major	829
aspartate aminotransferase putative Leishmania major strain Friedlin	2207
ubiquitin conjugating enzyme E2 putative Leishmania major strain Friedlin	1079
threonyl tRNA synthetase putative Leishmania major strain Friedlin	3669
60S ribosomal protein L32 Leishmania major strain Friedlin	923
high mobility group protein homolog tdp 1 putative Leishmania major strain Friedlin	1081
tryparedoxin putative Leishmania major strain Friedlin	725
radial spoke protein 3 putative Leishmania major strain Friedlin	1048
possible RNA binding protein Leishmania major	1358
eukaryotic translation initiation factor 2 subunit putative Leishmania major	1760
bifunctional aminoacyl trna synthetase putative prolyl trna synthetase putative Leishmania major	2739
hexokinase putative Leishmania major	3167
Ca2 binding EF hand protein centrin putative Leishmania major	669
alanyl tRNA synthetase putative Leishmania major	3506
hypothetical protein conserved Leishmania major	1572
40S ribosomal protein S2 Leishmania major	1785
hypothetical protein conserved Leishmania major	3453
Serum albumin precursor Allergen Bos d 6 BSA	3828
cytochrome c1 heme protein mitochondrial precursor putative Leishmania major	1122
hypothetical protein conserved Leishmania major	2824
inosine 5 monophosphate dehydrogenase Leishmania major	2256
UDP galactopyranose mutase Leishmania major	2955
phosphoglycerate kinase C glycosomal Leishmania major	2141
calpain like cysteine peptidase Clan CA family C2 calpain like cysteine peptidase putative Leis	1063
hexokinase putative Leishmania major	4932
t complex protein 1 delta subunit putative Leishmania major	2026
adenylate kinase putative Leishmania major	1490
RNA helicase putative Leishmania major	1495
cytochrome C oxidase subunit VI putative Leishmania major	1055
mannose 1 phosphate guanyltransferase Leishmania major	1388
endoribonuclease L PSP pb5 putative Leishmania major	1682
acetyl CoA synthetase putative Leishmania major	2507
ribosomal protein S25 Leishmania major	623
trifunctional enzyme alpha subunit mitochondrial precursor like protein Leishmania major	2004
cytochrome c oxidase subunit V putative Leishmania major	1410
2 oxoglutarate dehydrogenase E2 component dihydrolipoamide succinyltransferase putative Leishman	1601
pyridoxal kinase putative Leishmania major	1428

60S ribosomal protein L18a putative Leishmania major	1849
dynein putative Leishmania major	2693
hypothetical protein conserved Leishmania major	687
40S ribosomal protein S27 1 putative Leishmania major	721
glucose transporter lmgt2 putative Leishmania major	6672
2 3 bisphosphoglycerate independent phosphoglycerate mutase 2 3 bisphosphoglycerate independentphos	1780
glycerol kinase glycosomal putative Leishmania major strain Friedlin	1739
mitochondrial phosphate transporter putative Leishmania major strain Friedlin	1790
cofilin like protein Leishmania major strain Friedlin	911
heat shock protein 90 putative glucose regulated protein 94 putative Leishmania major strain Fri	1846
ADP ribosylation factor 3 putative Leishmania major strain Friedlin	1246
protein kinase putative Leishmania major strain Friedlin	970
hypothetical protein conserved Leishmania major strain Friedlin	738
40S ribosomal protein S15a putative Leishmania major	1042
OMPDCase OPRTase putative orotidine 5 phosphate decarboxylase orotate phosphoribosyltransferase p	1817
3 oxo 5 alpha steroid 4 dehydrogenase putative Leishmania major	1885
adenosine kinase putative Leishmania major	2141
universal minicircle sequence binding protein Leishmania major	10734
chaperone protein DNAJ putative Leishmania major	1303
hypothetical protein conserved Leishmania major	2433
chaperonin TCP20 putative Leishmania major	2556
hypothetical protein conserved Leishmania major	2929
sucrose phosphate synthase like protein Leishmania major	2525
hypothetical protein conserved Leishmania major	856
metallo peptidase Clan ME Family M16 mitochondrial processing peptidase alpha subunit putative	2041
ATP synthase F1 subunit gamma protein putative Leishmania major	1510
protein phosphatase putative Leishmania major	2073
electron transfer flavoprotein alpha polypeptide putative Leishmania major	1597
n acyl l amino acid amidohydrolase putative Leishmania major	2350
hypothetical protein conserved Leishmania major	1012
hypothetical protein conserved Leishmania major	1488
hypothetical protein conserved Leishmania major	2294
aldose 1 epimerase putative Leishmania major strain Friedlin	2260
reiske iron sulfur protein precursor putative Leishmania major strain Friedlin	2155
galactokinase like protein Leishmania major strain Friedlin	1777
hypothetical protein conserved Leishmania major strain Friedlin	2842
kinetoplast associated protein like protein Leishmania major strain Friedlin	4002
hypothetical protein conserved Leishmania major strain Friedlin	1799
T complex protein 1 beta subunit putative Leishmania major strain Friedlin	2637
arginyl tRNA synthetase putative Leishmania major strain Friedlin	2723
hypothetical protein conserved Leishmania major strain Friedlin	1057
dipeptidyl peptidase III putative metallo peptidase Clan M Family M49 Leishmania major	3216
paraflagellar rod component putative Leishmania major	2490
pyruvate phosphate dikinase putative Leishmania major	7226
surface antigen protein putative Leishmania major	2612
hypothetical protein conserved Leishmania major	2467
hypothetical protein conserved Leishmania major	4402
cyclophilin a Leishmania major	3483
hypothetical protein conserved Leishmania major	1941

GTP binding protein putative Leishmania major	1637
eukaryotic translation initiation factor putative Leishmania major	1929
5 methyltetrahydropteroyltriglutamate homocyst ei nemethyltransferase putative Leishmania major	8722
prostaglandin f2 alpha synthase Leishmania major	3615
RNA binding protein putative Leishmania major	2216
chaperonin containing t complex protein putative Leishmania major	2036
chaperonin alpha subunit putative Leishmania major	2504
60S ribosomal protein L2 putative Leishmania major	1446
hypothetical protein conserved Leishmania major	2824
carboxypeptidase putative metallo peptidase Clan MA E Family M32 Leishmania major	2926
hypothetical protein conserved Leishmania major	3733
hypothetical protein conserved Leishmania major	1174
stress inducible protein STI1 homolog Leishmania major	2085
hypothetical protein conserved Leishmania major	2839
hypothetical protein conserved Leishmania major	1844
T complex protein 1 theta subunit putative chaperonin putative Leishmania major	3034
NADH dependent fumarate reductase putative Leishmania major strain Friedlin	4642
cystathione gamma lyase putative Leishmania major strain Friedlin	4698
glycosomal membrane protein Leishmania major strain Friedlin	3573
t complex protein 1 eta subunit putative Leishmania major strain Friedlin	2444
hypothetical protein conserved Leishmania major strain Friedlin	1465
hypothetical protein conserved Leishmania major strain Friedlin	3317
ribosomal protein L1a putative Leishmania major strain Friedlin	2932
Serine peptidase putative Leishmania major strain Friedlin	4635
hypothetical protein conserved Leishmania major strain Friedlin	2417
trypanothione synthetase putative Leishmania major strain Friedlin	2887

Supplementary data: **Analysis of *de facto* expressed proteins from *L. major***
Mass spectrometry analysis of *L. major*, clone VI (MHOM/IL/80/Friedlin) lysate was performed of both life forms of the parasite. Detected proteins were analysed for species specificity with *Leishmania* RefSeq-Protein databases. Quantities of d*e facto* expressed proteins were depicted in ppm for amastigotes and promastigotes.

8. References

1. **Mougneau,E., Altare,F., Wakil,A.E., Zheng,S.C., Coppola,T., Wang,Z.E., Waldmann,R., Locksley,R.M., and Glaichenhaus,N.,** Expression Cloning of A Protective Leishmania Antigen. *Science* 1995. **268**: 563-566.

2. **Handman,E.,** Leishmaniasis: Current status of vaccine development. *Clinical Microbiology Reviews* 2001. **14**: 229-+.

3. **Roberts,L.J., Foote,S.J., and Handman,E.,** A new standard for the assessment of disease progression in murine cutaneous leishmaniasis. *Parasite Immunol.* 2000. **22**: 231-237.

4. **von Stebut,E. and Udey,M.C.,** Requirements for Th1-dependent immunity against infection with Leishmania major. *Microbes.Infect.* 2004. **6**: 1102-1109.

5. **Gradoni,L., Scalone,A., and Gramiccia,M.,** HIV-Leishmania co-infections in Italy: serological data as an indication of the sequence of acquisition of the two infections. *Trans.R.Soc.Trop.Med.Hyg.* 1993. **87**: 94-96.

6. **Sacks,D. and Noben-Trauth,N.,** The immunology of susceptibility and resistance to Leishmania major in mice. *Nat.Rev.Immunol.* 2002. **2**: 845-858.

7. **Cascio,A., di,M.L., Occorsio,P., Giacchino,R., Catania,S., Gigliotti,A.R., Aiassa,C., Iaria,C., Giordano,S., Colomba,C., Polara,V.F., Titone,L., Gradoni,L., Gramiccia,M., and Antinori,S.,** A 6 day course of liposomal amphotericin B in the treatment of infantile visceral leishmaniasis: the Italian experience. *J.Antimicrob.Chemother.* 2004. **54**: 217-220.

8. **Bommer,W., Eibl,H.J., Engel,K.R., Kuhlencord,A., Sindermann,H., Sundar,S., and Zappel,H.,** [Leishmaniasis--oral treatment with hexadecylphosphocholine]. *Wien.Klin.Wochenschr.* 2004. **116 Suppl 4**: 24-29.

9. **Gossage,S.M., Rogers,M.E., and Bates,P.A.,** Two separate growth phases during the development of Leishmania in sand flies: implications for understanding the life cycle. *Int.J.Parasitol.* 2003. **33**: 1027-1034.

10. **Bates,P.A. and Rogers,M.E.,** New insights into the developmental biology and transmission mechanisms of Leishmania. *Curr.Mol.Med.* 2004. **4**: 601-609.

11. **Warburg,A. and Schlein,Y.,** The effect of post-bloodmeal nutrition of Phlebotomus papatasi on the transmission of Leishmania major. *Am.J.Trop.Med.Hyg.* 1986. **35**: 926-930.

12. **Sacks,D. and Noben-Trauth,N.,** The immunology of susceptibility and resistance to Leishmania major in mice. *Nat.Rev.Immunol.* 2002. **2**: 845-858.

13. **Reiner,S.L. and Locksley,R.M.,** The regulation of immunity to Leishmania major. *Annu.Rev.Immunol.* 1995. **13**: 151-177.

14. **Mosser,D.M. and Edelson,P.J.,** The mouse macrophage receptor for C3bi (CR3) is a major mechanism in the phagocytosis of Leishmania promastigotes. *J.Immunol.* 1985. **135**: 2785-2789.

15. **Scott,P. and Farrell,J.P.,** Experimental cutaneous leishmaniasis: induction and regulation of T cells following infection of mice with Leishmania major. *Chem.Immunol.* 1998. **70**: 60-80.

16. **Lohoff,M., Gessner,A., Bogdan,C., and Rollinghoff,M.,** The Th1/Th2 paradigm and experimental murine leishmaniasis. *Int.Arch.Allergy Immunol.* 1998. **115**: 191-202.

17. **Heinzel,F.P., Sadick,M.D., Holaday,B.J., Coffman,R.L., and Locksley,R.M.,** Reciprocal expression of interferon gamma or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. *J.Exp.Med.* 1989. **169**: 59-72.

18. **Belkaid,Y., von Stebut,E., Mendez,S., Lira,R., Caler,E., Bertholet,S., Udey,M.C., and Sacks,D.,** CD8(+) T cells are required for primary immunity in C57BL/6 mice following low-dose, intradermal challenge with Leishmania major. *Journal of Immunology* 2002. **168**: 3992-4000.

19. **Locksley,R.M., Heinzel,F.P., Fankhauser,J.E., Nelson,C.S., and Sadick,M.D.,** Cutaneous host defense in leishmaniasis: interaction of isolated dermal macrophages and epidermal Langerhans cells with the insect-stage promastigote. *Infect.Immun.* 1988. **56**: 336-342.

20. **Blank,C., Fuchs,H., Rappersberger,K., Rollinghoff,M., and Moll,H.,** Parasitism of epidermal Langerhans cells in experimental cutaneous leishmaniasis with Leishmania major. *J.Infect.Dis.* 1993. **167**: 418-425.

21. **von Stebut,E., Belkaid,Y., Jakob,T., Sacks,D.L., and Udey,M.C.,** Uptake of Leishmania major amastigotes results in activation and interleukin 12 release from murine skin-derived dendritic cells:

implications for the initiation of anti-Leishmania immunity. *J.Exp.Med.* 1998. **188**: 1547-1552.

22. **Konecny,P., Stagg,A.J., Jebbari,H., English,N., Davidson,R.N., and Knight,S.C.,** Murine dendritic cells internalize Leishmania major promastigotes, produce IL-12 p40 and stimulate primary T cell proliferation in vitro. *Eur.J.Immunol.* 1999. **29**: 1803-1811.

23. **Henri,S., Curtis,J., Hochrein,H., Vremec,D., Shortman,K., and Handman,E.,** Hierarchy of susceptibility of dendritic cell subsets to infection by Leishmania major: inverse relationship to interleukin-12 production. *Infect.Immun.* 2002. **70**: 3874-3880.

24. **von Stebut,E., Belkaid,Y., Jakob,T., Sacks,D.L., and Udey,M.C.,** Uptake of Leishmania major amastigotes results in activation and interleukin 12 release from murine skin-derived dendritic cells: implications for the initiation of anti-Leishmania immunity. *J.Exp.Med.* 1998. **188**: 1547-1552.

25. **Sacks,D.L., Hieny,S., and Sher,A.,** Identification of cell surface carbohydrate and antigenic changes between noninfective and infective developmental stages of Leishmania major promastigotes. *J.Immunol.* 1985. **135**: 564-569.

26. **Belkaid,Y., Mendez,S., Lira,R., Kadambi,N., Milon,G., and Sacks,D.,** A natural model of Leishmania major infection reveals a prolonged "silent" phase of parasite amplification in the skin before the onset of lesion formation and immunity. *Journal of Immunology* 2000. **165**: 969-977.

27. **Nabors,G.S., Nolan,T., Croop,W., Li,J., and Farrell,J.P.,** The influence of the site of parasite inoculation on the development of Th1 and Th2 type immune responses in (BALB/c x C57BL/6) F1 mice infected with Leishmania major
1. *Parasite Immunol.* 1995. **17**: 569-579.

28. **Constant,S.L., Lee,K.S., and Bottomly,K.,** Site of antigen delivery can influence T cell priming: pulmonary environment promotes preferential Th2-type differentiation
2. *Eur.J.Immunol.* 2000. **30**: 840-847.

29. **Kamhawi,S., Belkaid,Y., Modi,G., Rowton,E., and Sacks,D.,** Protection against cutaneous leishmaniasis resulting from bites of uninfected sand flies
3. *Science* 2000. **290**: 1351-1354.

30. **Noben-Trauth,N., Paul,W.E., and Sacks,D.L.,** IL-4- and IL-4 receptor-deficient BALB/c mice reveal differences in susceptibility to Leishmania major parasite substrains. *J.Immunol.* 1999. **162**: 6132-6140.

31. **Belkaid,Y., Jouin,H., and Milon,G.,** A method to recover, enumerate and identify lymphomyeloid cells present in an inflammatory dermal site: a study in laboratory mice. *J.Immunol.Methods* 1996. **199**: 5-25.

32. **Belkaid,Y., Kamhawi,S., Modi,G., Valenzuela,J., Noben-Trauth,N., Rowton,E., Ribeiro,J., and Sacks,D.L.,** Development of a natural model of cutaneous leishmaniasis: powerful effects of vector saliva and saliva preexposure on the long-term outcome of Leishmania major infection in the mouse ear dermis. *J.Exp.Med.* 1998. **188**: 1941-1953.

33. **Bogdan,C., Donhauser,N., Doring,R., Rollinghoff,M., Diefenbach,A., and Rittig,M.G.,** Fibroblasts as host cells in latent leishmaniosis. *J.Exp.Med.* 2000. **191**: 2121-2130.

34. **Prina,E., Jouanne,C., de Souza,L.S., Szabo,A., Guillet,J.G., and Antoine,J.C.,** Antigen presentation capacity of murine macrophages infected with Leishmania amazonensis amastigotes. *J.Immunol.* 1993. **151**: 2050-2061.

35. **Kima,P.E., Soong,L., Chicharro,C., Ruddle,N.H., and McMahon-Pratt,D.,** Leishmania-infected macrophages sequester endogenously synthesized parasite antigens from presentation to CD4+ T cells. *Eur.J.Immunol.* 1996. **26**: 3163-3169.

36. **Courret,N., Prina,E., Mougneau,E., Saraiva,E.M., Sacks,D.L., Glaichenhaus,N., and Antoine,J.C.,** Presentation of the Leishmania antigen LACK by infected macrophages is dependent upon the virulence of the phagocytosed parasites. *Eur.J.Immunol.* 1999. **29**: 762-773.

37. **Reiner,N.E., Ng,W., and McMaster,W.R.,** Parasite-accessory cell interactions in murine leishmaniasis. II. Leishmania donovani suppresses macrophage expression of class I and class II major histocompatibility complex gene products. *J.Immunol.* 1987. **138**: 1926-1932.

38. **Kaye,P.M., Rogers,N.J., Curry,A.J., and Scott,J.C.,** Deficient expression of co-stimulatory molecules on Leishmania-infected macrophages. *Eur.J.Immunol.* 1994. **24**: 2850-2854.

39. **Saha,B., Das,G., Vohra,H., Ganguly,N.K., and Mishra,G.C.,** Macrophage-T cell interaction in experimental visceral leishmaniasis: failure to express costimulatory molecules on Leishmania-infected macrophages and its implication in the suppression of cell-mediated immunity. *Eur.J.Immunol.* 1995. **25**: 2492-2498.

40. **Reiner,S.L., Zheng,S., Wang,Z.E., Stowring,L., and Locksley,R.M.,** Leishmania promastigotes evade interleukin 12 (IL-12) induction by macrophages and stimulate a broad range of cytokines from CD4+ T cells during initiation of infection. *J.Exp.Med.* 1994. **179**: 447-456.

41. **Carrera,L., Gazzinelli,R.T., Badolato,R., Hieny,S., Muller,W., Kuhn,R., and Sacks,D.L.,** Leishmania promastigotes selectively inhibit interleukin 12 induction in bone marrow-derived macrophages from susceptible and resistant mice. *J.Exp.Med.* 1996. **183**: 515-526.

42. **Sartori,A., Oliveira,M.A., Scott,P., and Trinchieri,G.,** Metacyclogenesis modulates the ability of Leishmania promastigotes to induce IL-12 production in human mononuclear cells. *J.Immunol.* 1997. **159**: 2849-2857.

43. **Belkaid,Y., Butcher,B., and Sacks,D.L.,** Analysis of cytokine production by inflammatory mouse macrophages at the single-cell level: selective impairment of IL-12 induction in Leishmania-infected cells. *Eur.J.Immunol.* 1998. **28**: 1389-1400.

44. **Liew,F.Y., Wei,X.Q., and Proudfoot,L.,** Cytokines and nitric oxide as effector molecules against parasitic infections. *Philos.Trans.R.Soc.Lond B Biol.Sci.* 1997. **352**: 1311-1315.

45. **Jacobs,T., Andra,J., Gaworski,I., Graefe,S., Mellenthin,K., Kromer,M., Halter,R., Borlak,J., and Clos,J.,** Complement C3 is required for the progression of cutaneous lesions and neutrophil attraction in Leishmania major infection. *Med.Microbiol.Immunol.* 2005. **194**: 143-149.

46. **Maurer,M., Lopez,K.S., Siebenhaar,F., Moelle,K., Metz,M., Knop,J., and von Stebut,E.,** Skin mast cells control T cell-dependent host defense in Leishmania major infections. *FASEB J.* 2006. **20**: 2460-2467.

47. **Woelbing,F., Kostka,S.L., Moelle,K., Belkaid,Y., Sunderkoetter,C., Verbeek,S., Waisman,A., Nigg,A.P., Knop,J., Udey,M.C., and von Stebut,E.,** Uptake of Leishmania major by dendritic cells is mediated by Fcgamma receptors and facilitates acquisition of protective immunity. *J.Exp.Med.* 2006. **203**: 177-188.

48. **Sato,N., Ahuja,S.K., Quinones,M., Kostecki,V., Reddick,R.L., Melby,P.C., Kuziel,W.A., and Ahuja,S.S.,** CC chemokine receptor (CCR)2 is required for langerhans cell migration and localization of T helper cell type 1 (Th1)-inducing dendritic cells. Absence of CCR2 shifts the Leishmania major-resistant phenotype to a susceptible state dominated by Th2 cytokines, b cell outgrowth, and sustained neutrophilic inflammation. *J.Exp.Med.* 2000. **192**: 205-218.

49. **Moll,H.,** The role of dendritic cells at the early stages of Leishmania infection. *Adv.Exp.Med.Biol.* 2000. **479**: 163-173.

50. **Schonlau,F., Scharffetter-Kochanek,K., Grabbe,S., Pietz,B., Sorg,C., and Sunderkotter,C.,** In experimental leishmaniasis deficiency of CD18 results in parasite dissemination associated with altered macrophage functions and incomplete Th1 cell response
4. *Eur.J.Immunol.* 2000. **30**: 2729-2740.

51. **Moll,H.,** Epidermal Langerhans cells are critical for immunoregulation of cutaneous leishmaniasis
4. *Immunol.Today* 1993. **14**: 383-387.

52. **von Stebut,E., Belkaid,Y., Nguyen,B.V., Cushing,M., Sacks,D.L., and Udey,M.C.,** Leishmania major-infected murine langerhans cell-like dendritic cells from susceptible mice release IL-12 after infection and vaccinate against experimental cutaneous Leishmaniasis. *Eur.J.Immunol.* 2000. **30**: 3498-3506.

53. **Flohe,S., Lang,T., and Moll,H.,** Synthesis, stability, and subcellular distribution of major histocompatibility complex class II molecules in Langerhans cells infected with Leishmania major
1. *Infect.Immun.* 1997. **65**: 3444-3450.

54. **Flohe,S.B., Bauer,C., Flohe,S., and Moll,H.,** Antigen-pulsed epidermal Langerhans cells protect susceptible mice from infection with the intracellular parasite Leishmania major. *Eur.J.Immunol.* 1998. **28**: 3800-3811.

55. **Ahuja,S.S., Reddick,R.L., Sato,N., Montalbo,E., Kostecki,V., Zhao,W., Dolan,M.J., Melby,P.C., and Ahuja,S.K.,** Dendritic cell (DC)-based anti-infective strategies: DCs engineered to secrete IL-12 are a potent vaccine in a murine model of an intracellular infection. *J.Immunol.* 1999. **163**: 3890-3897.

56. **Berberich,C., Ramirez-Pineda,J.R., Hambrecht,C., Alber,G., Skeiky,Y.A., and Moll,H.,** Dendritic cell (DC)-based protection against an intracellular pathogen is dependent upon DC-derived IL-12 and can be induced by molecularly defined antigens. *J.Immunol.* 2003. **170**: 3171-3179.

57. **Hunter,C.A.,** New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions
5. *Nat.Rev.Immunol.* 2005. **5**: 521-531.

58. **Shibuya,K., Robinson,D., Zonin,F., Hartley,S.B., Macatonia,S.E., Somoza,C., Hunter,C.A., Murphy,K.M., and O'Garra,A.,** IL-1 alpha

and TNF-alpha are required for IL-12-induced development of Th1 cells producing high levels of IFN-gamma in BALB/c but not C57BL/6 mice. *J.Immunol.* 1998. **160**: 1708-1716.

59. **Filippi,C., Hugues,S., Cazareth,J., Julia,V., Glaichenhaus,N., and Ugolini,S.,** CD4+ T cell polarization in mice is modulated by strain-specific major histocompatibility complex-independent differences within dendritic cells
3. *J.Exp.Med.* 2003. **198**: 201-209.

60. **Reis e Sousa, Hieny,S., Scharton-Kersten,T., Jankovic,D., Charest,H., Germain,R.N., and Sher,A.,** In vivo microbial stimulation induces rapid CD40 ligand-independent production of interleukin 12 by dendritic cells and their redistribution to T cell areas
2. *J.Exp.Med.* 1997. **186**: 1819-1829.

61. **Muller,I.,** Role of T cell subsets during the recall of immunologic memory to Leishmania major. *Eur.J.Immunol.* 1992. **22**: 3063-3069.

62. **Muller,I., Kropf,P., Louis,J.A., and Milon,G.,** Expansion of gamma interferon-producing CD8+ T cells following secondary infection of mice immune to Leishmania major. *Infect.Immun.* 1994. **62**: 2575-2581.

63. **Muller,I., Kropf,P., Etges,R.J., and Louis,J.A.,** Gamma interferon response in secondary Leishmania major infection: role of CD8+ T cells
2. *Infect.Immun.* 1993. **61**: 3730-3738.

64. **Milon,G., Titus,R.G., Cerottini,J.C., Marchal,G., and Louis,J.A.,** Higher frequency of Leishmania major-specific L3T4+ T cells in susceptible BALB/c as compared with resistant CBA mice
5. *J.Immunol.* 1986. **136**: 1467-1471.

65. **Titus,R.G., Milon,G., Marchal,G., Vassalli,P., Cerottini,J.C., and Louis,J.A.,** Involvement of specific Lyt-2+ T cells in the immunological control of experimentally induced murine cutaneous leishmaniasis. *Eur.J.Immunol.* 1987. **17**: 1429-1433.

66. **Wang,Z.E., Reiner,S.L., Hatam,F., Heinzel,F.P., Bouvier,J., Turck,C.W., and Locksley,R.M.,** Targeted activation of CD8 cells and infection of beta 2-microglobulin-deficient mice fail to confirm a primary protective role for CD8 cells in experimental leishmaniasis. *J.Immunol.* 1993. **151**: 2077-2086.

67. **Overath,P. and Harbecke,D.,** Course of Leishmania infection in beta 2-microglobulin-deficient mice
5. *Immunol.Lett.* 1993. **37**: 13-17.

68. **Huber,M., Timms,E., Mak,T.W., Rollinghoff,M., and Lohoff,M.,** Effective and long-lasting immunity against the parasite Leishmania major in CD8-deficient mice. *Infect.Immun.* 1998. **66**: 3968-3970.

69. **da Conceicao-Silva,F., Perlaza,B.L., Louis,J.A., and Romero,P.,** Leishmania major infection in mice primes for specific major histocompatibility complex class I-restricted CD8+ cytotoxic T cell responses
1. *Eur.J.Immunol.* 1994. **24**: 2813-2817.

70. **Smith,L.E., Rodrigues,M., and Russell,D.G.,** The interaction between CD8+ cytotoxic T cells and Leishmania-infected macrophages
1. *J.Exp.Med.* 1991. **174**: 499-505.

71. **Colmenares,M., Kima,P.E., Samoff,E., Soong,L., and McMahon-Pratt,D.,** Perforin and gamma interferon are critical CD8+ T-cell-mediated responses in vaccine-induced immunity against Leishmania amazonensis infection
1. *Infect.Immun.* 2003. **71**: 3172-3182.

72. **Kropf,P., Herath,S., Weber,V., Modolell,M., and Muller,I.,** Factors influencing Leishmania major infection in IL-4-deficient BALB/c mice. *Parasite Immunology* 2003. **25**: 439-447.

73. **Yewdell,J.T.W. and Bennink,J.R.,** Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. *Annual Review of Immunology* 1999. **17**: 51-88.

74. **Falk,K., Rotzschke,O., Stevanovic,S., Jung,G., and Rammensee,H.G.,** Allele-Specific Motifs Revealed by Sequencing of Self-Peptides Eluted from Mhc Molecules. *Nature* 1991. **351**: 290-296.

75. **Reits,E.A., Benham,A.M., Plougastel,B., Neefjes,J., and Trowsdale,J.,** Dynamics of proteasome distribution in living cells
1. *EMBO J.* 1997. **16**: 6087-6094.

76. **Neefjes,J.J., Momburg,F., and Hammerling,G.J.,** Selective and ATP-dependent translocation of peptides by the MHC-encoded transporter
2. *Science* 1993. **261**: 769-771.

77. **van Endert,P.M., Saveanu,L., Hewitt,E.W., and Lehner,P.,** Powering the peptide pump: TAP crosstalk with energetic nucleotides
2. *Trends Biochem.Sci.* 2002. **27**: 454-461.

78. **Yewdell,J.W., Reits,E., and Neefjes,J.,** Making sense of mass destruction: quantitating MHC class I antigen presentation
1. *Nat.Rev.Immunol.* 2003. **3**: 952-961.

79. **Ortmann,B., Copeman,J., Lehner,P.J., Sadasivan,B., Herberg,J.A., Grandea,A.G., Riddell,S.R., Tampe,R., Spies,T., Trowsdale,J., and Cresswell,P.,** A critical role for tapasin in the assembly and function of multimeric MHC class I-TAP complexes
1. *Science* 1997. **277**: 1306-1309.

80. **Garbi,N., Tan,P., Diehl,A.D., Chambers,B.J., Ljunggren,H.G., Momburg,F., and Hammerling,G.J.,** Impaired immune responses and altered peptide repertoire in tapasin-deficient mice
1. *Nat.Immunol.* 2000. **1**: 234-238.

81. **Dick,T.P., Bangia,N., Peaper,D.R., and Cresswell,P.,** Disulfide bond isomerization and the assembly of MHC class I-peptide complexes
8. *Immunity.* 2002. **16**: 87-98.

82. **Kloetzel,P.M.,** Antigen processing by the proteasome. *Nature Reviews Molecular Cell Biology* 2001. **2**: 179-187.

83. **Groll,M., Ditzel,L., Lowe,J., Stock,D., Bochtler,M., Bartunik,H.D., and Huber,R.,** Structure of 20S proteasome from yeast at 2.4 angstrom resolution. *Nature* 1997. **386**: 463-471.

84. **Kloetzel,P.M. and Ossendorp,F.,** Proteasome and peptidase function in MHC-class-I-mediated antigen presentation
6. *Curr.Opin.Immunol.* 2004. **16**: 76-81.

85. **Frentzel,S., Pesoldhurt,B., Seelig,A., and Kloetzel,P.M.,** 20-S Proteasomes Are Assembled Via Distinct Precursor Complexes - Processing of Lmp2 and Lmp7 Proproteins Takes Place in 13-16-S Preproteasome Complexes. *Journal of Molecular Biology* 1994. **236**: 975-981.

86. **Groettrup,M., Standera,S., Stohwasser,R., and Kloetzel,P.M.,** The subunits MECL-1 and LMP2 are mutually required for incorporation into the 20S proteasome. *Proceedings of the National Academy of Sciences of the United States of America* 1997. **94**: 8970-8975.

87. **Nandi,D., Woodward,E., Ginsburg,D.B., and Monaco,J.J.,** Intermediates in the formation of mouse 20S proteasomes: Implications for the assembly of precursor beta subunits. *Embo Journal* 1997. **16**: 5363-5375.

88. **Schmidt,M., Zantopf,D., Kraft,R., Kostka,S., Preissner,R., and Kloetzel,P.M.,** Sequence information within proteasomal prosequences mediates efficient integration of beta-subunits into the 20 S proteasome complex. *Journal of Molecular Biology* 1999. **288**: 117-128.

89. **Heink,S., Ludwig,D., Kloetzel,P.M., and Kruger,E.,** IFN-gamma-induced immune adaptation of the proteasome system is an accelerated and transient response. *Proceedings of the National Academy of Sciences of the United States of America* 2005. **102**: 9241-9246.

90. **Cardozo,C. and Kohanski,R.A.,** Altered properties of the branched chain amino acid-preferring activity contribute to increased cleavages after branched chain residues by the "immunoproteasome". *Journal of Biological Chemistry* 1998. **273**: 16764-16770.

91. **Eleuteri,A.M., Kohanski,R.A., Cardozo,C., and Orlowski,M.,** Bovine spleen multicatalytic proteinase complex (proteasome) - Replacement of X, Y, and Z subunits by LMP7, LMP2, and MECL1 and changes in properties and specificity. *Journal of Biological Chemistry* 1997. **272**: 11824-11831.

92. **Boes,B., Hengel,H., Ruppert,T., Multhaup,G., Koszinowski,U.H., and Kloetzel,P.M.,** Interferon-Gamma Stimulation Modulates the Proteolytic Activity and Cleavage Site Preference of 20S Mouse Proteasomes. *Journal of Experimental Medicine* 1994. **179**: 901-909.

93. **Gaczynska,M., Rock,K.L., and Goldberg,A.L.,** Gamma-Interferon and Expression of Mhc Genes Regulate Peptide Hydrolysis by Proteasomes. *Nature* 1993. **365**: 264-267.

94. **Schultz,E.S., Chapiro,J., Lurquin,C., Claverol,S., Burlet-Schiltz,O., Warnier,G., Russo,V., Morel,S., Levy,F., Boon,T., Van den Eynde,B.J., and van der Bruggen,P.,** The production of a new MAGE-3 peptide presented to cytolytic T lymphocytes by HLA-B40 requires the immunoproteasome. *Journal of Experimental Medicine* 2002. **195**: 391-399.

95. **Schwarz,K., van den Broek,M., Kostka,S., Kraft,R., Soza,A., Schmidtke,G., Kloetzel,P.M., and Groettrup,M.,** Overexpression of the proteasome subunits LMP2, LMP7, and MECL-1, but not PA28 alpha/beta, enhances the presentation of an immunodominant lymphocytic choriomeningitis virus T cell epitope. *Journal of Immunology* 2000. **165**: 768-778.

96. **Sijts,A.J.A.M., Standera,S., Toes,R.E.M., Ruppert,T., Beekman,N.J.C.M., van Veelen,P.A., Ossendorp,F.A., Melief,C.J.M., and Kloetzel,P.M.,** MHC class I antigen processing of an Adenovirus CTL epitope is linked to the levels of immunoproteasomes in infected cells. *Journal of Immunology* 2000. **164**: 4500-4506.

97. **Gileadi,U., Moins-Teisserenc,H.T., Correa,I., Booth,B.L., Dunbar,P.R., Sewell,A.K., Trowsdale,J., Phillips,R.E., and**

Cerundolo,V., Generation of an immunodominant CTL epitope is affected by proteasome subunit composition and stability of the antigenic protein. *Journal of Immunology* 1999. **163**: 6045-6052.

98. **Morel,S., Levy,F., Burlet-Schiltz,O., Brasseur,F., Probst-Kepper,M., Peitrequin,A.L., Monsarrat,B., Van Velthoven,R., Cerottini,J.C., Boon,T., Gairin,J.E., and Van den Eynde,B.J.,** Processing of some antigens by the standard proteasome but not by the immunoproteasome results in poor presentation by dendritic cells. *Immunity* 2000. **12**: 107-117.

99. **Yewdell,J.W. and Bennink,J.R.,** Cut and trim: generating MHC class I peptide ligands. *Current Opinion in Immunology* 2001. **13**: 13-18.

100. **von Stebut,E. and Sunderkotter,C.,** [Cutaneous leishmaniasis]. *Hautarzt* 2007. **58**: 445-458.

101. **Reithinger,R., Dujardin,J.C., Louzir,H., Pirmez,C., Alexander,B., and Brooker,S.,** Cutaneous leishmaniasis
4. *Lancet Infect.Dis.* 2007. **7**: 581-596.

102. **Greenblatt,C.L.,** The present and future of vaccination for cutaneous leishmaniasis
5. *Prog.Clin.Biol.Res.* 1980. **47**: 259-285.

103. **Kellina,O.I.,** Problem and current lines in investigations on the epidemiology of leishmaniasis and its control in the U.S.S.R
2. *Bull.Soc.Pathol.Exot.Filiales.* 1981. **74**: 306-318.

104. **Kronenberg,K., Brosch,S., and von,S.E.,** Vaccinations against cutaneous Leishmania infection
1. *G.Ital.Dermatol.Venereol.* 2008. **143**: 125-137.

105. **Stobie,L., Gurunathan,S., Prussin,C., Sacks,D.L., Glaichenhaus,N., Wu,C.Y., and Seder,R.A.,** The role of antigen and IL-12 in sustaining Th1 memory cells in vivo: IL-12 is required to maintain memory/effector Th1 cells sufficient to mediate protection to an infectious parasite challenge. *Proc.Natl.Acad.Sci.U.S.A* 2000. **97**: 8427-8432.

106. **Jaafari,M.R., Badiee,A., Khamesipour,A., Samiei,A., Soroush,D., Kheiri,M.T., Barkhordari,F., McMaster,W.R., and Mahboudi,F.,** The role of CpG ODN in enhancement of immune response and protection in BALB/c mice immunized with recombinant major surface glycoprotein of Leishmania (rgp63) encapsulated in cationic liposome
1. *Vaccine* 2007. **25**: 6107-6117.

107. **Connell,N.D., Medina-Acosta,E., McMaster,W.R., Bloom,B.R., and Russell,D.G.,** Effective immunization against cutaneous leishmaniasis with recombinant bacille Calmette-Guerin expressing the Leishmania surface proteinase gp63
40. *Proc.Natl.Acad.Sci.U.S.A* 1993. **90**: 11473-11477.

108. **Solioz,N., Blum-Tirouvanziam,U., Jacquet,R., Rafati,S., Corradin,G., Mauel,J., and Fasel,N.,** The protective capacities of histone H1 against experimental murine cutaneous leishmaniasis
1. *Vaccine* 1999. **18**: 850-859.

109. **Spitzer,N., Jardim,A., Lippert,D., and Olafson,R.W.,** Long-term protection of mice against Leishmania major with a synthetic peptide vaccine
4. *Vaccine* 1999. **17**: 1298-1300.

110. **Handman,E., Symons,F.M., Baldwin,T.M., Curtis,J.M., and Scheerlinck,J.P.,** Protective vaccination with promastigote surface antigen 2 from Leishmania major is mediated by a TH1 type of immune response
2. *Infect.Immun.* 1995. **63**: 4261-4267.

111. **Khamesipour,A., Rafati,S., Davoudi,N., Maboudi,F., and Modabber,F.,** Leishmaniasis vaccine candidates for development: a global overview
4. *Indian J.Med.Res.* 2006. **123**: 423-438.

112. **Zadeh-Vakili,A., Taheri,T., Taslimi,Y., Doustdari,F., Salmanian,A.H., and Rafati,S.,** Immunization with the hybrid protein vaccine, consisting of Leishmania major cysteine proteinases Type I (CPB) and Type II (CPA), partially protects against leishmaniasis
2. *Vaccine* 2004. **22**: 1930-1940.

113. **Melby,P.C., Yang,J., Zhao,W., Perez,L.E., and Cheng,J.,** Leishmania donovani p36(LACK) DNA vaccine is highly immunogenic but not protective against experimental visceral leishmaniasis
4. *Infect.Immun.* 2001. **69**: 4719-4725.

114. **Launois,P., Maillard,I., Pingel,S., Swihart,K.G., Xenarios,I., AchaOrbea,H., Diggelmann,H., Locksley,R.M., MacDonald,H.R., and Louis,J.A.,** IL-4 rapidly produced by V beta 4 V alpha 8 CD4(+) T cells instructs the development and susceptibility to Leishmania major in BALB/c mice. *Immunity* 1997. **6**: 541-549.

115. **Kelly,B.L., Stetson,D.B., and Locksley,R.M.,** Leishmania major LACK antigen is required for efficient vertebrate parasitization
1. *J.Exp.Med.* 2003. **198**: 1689-1698.

116. **Gurunathan,S., Prussin,C., Sacks,D.L., and Seder,R.A.,** Vaccine requirements for sustained cellular immunity to an intracellular parasitic infection. *Nat.Med.* 1998. **4**: 1409-1415.

117. **Frankel,A.D. and Pabo,C.O.,** Cellular uptake of the tat protein from human immunodeficiency virus
1. *Cell* 1988. **55**: 1189-1193.

118. **Green,M. and Loewenstein,P.M.,** Autonomous functional domains of chemically synthesized human immunodeficiency virus tat trans-activator protein
1. *Cell* 1988. **55**: 1179-1188.

119. **Sodroski,J., Rosen,C., Wong-Staal,F., Salahuddin,S.Z., Popovic,M., Arya,S., Gallo,R.C., and Haseltine,W.A.,** Trans-acting transcriptional regulation of human T-cell leukemia virus type III long terminal repeat
11. *Science* 1985. **227**: 171-173.

120. **Dayton,A.I., Sodroski,J.G., Rosen,C.A., Goh,W.C., and Haseltine,W.A.,** The trans-activator gene of the human T cell lymphotropic virus type III is required for replication
4. *Cell* 1986. **44**: 941-947.

121. **Schwarze,S.R., Hruska,K.A., and Dowdy,S.F.,** Protein transduction: unrestricted delivery into all cells?
1. *Trends Cell Biol.* 2000. **10**: 290-295.

122. **Wadia,J.S., Stan,R.V., and Dowdy,S.F.,** Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis
3. *Nat.Med.* 2004. **10**: 310-315.

123. **Schwarze,S.R., Ho,A., Vocero-Akbani,A., and Dowdy,S.F.,** In vivo protein transduction: delivery of a biologically active protein into the mouse
1. *Science* 1999. **285**: 1569-1572.

124. **Pantano,S., Tyagi,M., Giacca,M., and Carloni,P.,** Amino acid modification in the HIV-1 Tat basic domain: insights from molecular dynamics and in vivo functional studies
1. *J.Mol.Biol.* 2002. **318**: 1331-1339.

125. **Nagahara,H., Vocero-Akbani,A.M., Snyder,E.L., Ho,A., Latham,D.G., Lissy,N.A., Becker-Hapak,M., Ezhevsky,S.A., and Dowdy,S.F.,** Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27Kip1 induces cell migration
1. *Nat.Med.* 1998. **4**: 1449-1452.

126. **Vocero-Akbani,A., Lissy,N.A., and Dowdy,S.F.,** Transduction of full-length Tat fusion proteins directly into mammalian cells: analysis of T cell receptor activation-induced cell death
1. *Methods Enzymol.* 2000. **322**: 508-521.

127. **Kwon,H.Y., Eum,W.S., Jang,H.W., Kang,J.H., Ryu,J., Ryong,L.B., Jin,L.H., Park,J., and Choi,S.Y.,** Transduction of Cu,Zn-superoxide dismutase mediated by an HIV-1 Tat protein basic domain into mammalian cells
1. *FEBS Lett.* 2000. **485**: 163-167.

128. **Vocero-Akbani,A., Chellaiah,M.A., Hruska,K.A., and Dowdy,S.F.,** Protein transduction: delivery of Tat-GTPase fusion proteins into mammalian cells
1. *Methods Enzymol.* 2001. **332**: 36-49.

129. **Jin,L.H., Bahn,J.H., Eum,W.S., Kwon,H.Y., Jang,S.H., Han,K.H., Kang,T.C., Won,M.H., Kang,J.H., Cho,S.W., Park,J., and Choi,S.Y.,** Transduction of human catalase mediated by an HIV-1 TAT protein basic domain and arginine-rich peptides into mammalian cells
1. *Free Radic.Biol.Med.* 2001. **31**: 1509-1519.

130. **Yoon,H.Y., Lee,S.H., Cho,S.W., Lee,J.E., Yoon,C.S., Park,J., Kim,T.U., and Choi,S.Y.,** TAT-mediated delivery of human glutamate dehydrogenase into PC12 cells
7. *Neurochem.Int.* 2002. **41**: 37-42.

131. **Cao,G., Pei,W., Ge,H., Liang,Q., Luo,Y., Sharp,F.R., Lu,A., Ran,R., Graham,S.H., and Chen,J.,** In Vivo Delivery of a Bcl-xL Fusion Protein Containing the TAT Protein Transduction Domain Protects against Ischemic Brain Injury and Neuronal Apoptosis
25. *J.Neurosci.* 2002. **22**: 5423-5431.

132. **Harada,H., Hiraoka,M., and Kizaka-Kondoh,S.,** Antitumor effect of TAT-oxygen-dependent degradation-caspase-3 fusion protein specifically stabilized and activated in hypoxic tumor cells
54. *Cancer Res.* 2002. **62**: 2013-2018.

133. **Shibagaki,N. and Udey,M.C.,** Dendritic cells transduced with protein antigens induce cytotoxic lymphocytes and elicit antitumor immunity
1. *J.Immunol.* 2002. **168**: 2393-2401.

134. **Shibagaki,N. and Udey,M.C.,** Dendritic cells transduced with TAT protein transduction domain-containing tyrosinase-related protein 2 vaccinate against murine melanoma
1. *Eur.J.Immunol.* 2003. **33**: 850-860.

135. **Wang,H.Y., Fu,T., Wang,G., Zeng,G., Perry-Lalley,D.M., Yang,J.C., Restifo,N.P., Hwu,P., and Wang,R.F.,** Induction of CD4(+) T cell-dependent antitumor immunity by TAT-mediated tumor antigen delivery into dendritic cells
35. *J.Clin.Invest* 2002. **109**: 1463-1470.

136. **Rammensee,H., Bachmann,J., Emmerich,N.P., Bachor,O.A., and Stevanovic,S.,** SYFPEITHI: database for MHC ligands and peptide motifs
2. *Immunogenetics* 1999. **50**: 213-219.

137. **Krieg,A.M., Yi,A.K., Matson,S., Waldschmidt,T.J., Bishop,G.A., Teasdale,R., Koretzky,G.A., and Klinman,D.M.,** CpG motifs in bacterial DNA trigger direct B-cell activation
3. *Nature* 1995. **374**: 546-549.

138. **Wagner,H.,** Toll meets bacterial CpG-DNA
50. *Immunity.* 2001. **14**: 499-502.

139. **Scott,P., Pearce,E., Natovitz,P., and Sher,A.,** Vaccination against cutaneous leishmaniasis in a murine model. II. Immunologic properties of protective and nonprotective subfractions of soluble promastigote extract. *J.Immunol.* 1987. **139**: 3118-3125.

140. **Bhaumik,S.K., Naskar,K., and De,T.,** Complete protection against experimental visceral leishmaniasis with complete soluble antigen from attenuated Leishmania donovani promastigotes involves Th1-immunity and down-regulation of IL-10
9. *Eur.J.Immunol.* 2009. **39**: 2146-2160.

141. **Ali,S.A., Rezvan,H., McArdle,S.E., Khodadadi,A., Asteal,F.A., and Rees,R.C.,** CTL responses to Leishmania mexicana gp63-cDNA vaccine in a murine model
1. *Parasite Immunol.* 2009. **31**: 373-383.

142. **Zahn,S., Wirtz,S., Birkenbach,M., Blumberg,R.S., Neurath,M.F., and von,S.E.,** Impaired Th1 responses in mice deficient in Epstein-Barr virus-induced gene 3 and challenged with physiological doses of Leishmania major
5. *Eur.J.Immunol.* 2005. **35**: 1106-1112.

143. **Ahuja,S.S., Reddick,R.L., Sato,N., Montalbo,E., Kostecki,V., Zhao,W., Dolan,M.J., Melby,P.C., and Ahuja,S.K.,** Dendritic cell (DC)-based anti-infective strategies: DCs engineered to secrete IL-12 are a potent vaccine in a murine model of an intracellular infection. *J.Immunol.* 1999. **163**: 3890-3897.

144. **Yoshida,H., Yusin,M., Ren,I., Kuhlenkamp,J., Hirano,T., Stolz,A., and Kaplowitz,N.,** Identification, purification, and immunochemical characterization of a tocopherol-binding protein in rat liver cytosol 1. *J.Lipid Res.* 1992. **33**: 343-350.

145. **Scott,P., Pearce,E., Natovitz,P., and Sher,A.,** Vaccination against cutaneous leishmaniasis in a murine model. I. Induction of protective immunity with a soluble extract of promastigotes. *J.Immunol.* 1987. **139**: 221-227.

146. **Stacey,K.J. and Blackwell,J.M.,** Immunostimulatory DNA as an adjuvant in vaccination against Leishmania major 5. *Infect.Immun.* 1999. **67**: 3719-3726.

147. **Ivens,A.C., Peacock,C.S., Worthey,E.A., Murphy,L., Aggarwal,G., Berriman,M., Sisk,E., Rajandream,M.A., Adlem,E., Aert,R., Anupama,A., Apostolou,Z., Attipoe,P., Bason,N., Bauser,C., Beck,A., Beverley,S.M., Bianchettin,G., Borzym,K., Bothe,G., Bruschi,C.V., Collins,M., Cadag,E., Ciarloni,L., Clayton,C., Coulson,R.M., Cronin,A., Cruz,A.K., Davies,R.M., De,G.J., Dobson,D.E., Duesterhoeft,A., Fazelina,G., Fosker,N., Frasch,A.C., Fraser,A., Fuchs,M., Gabel,C., Goble,A., Goffeau,A., Harris,D., Hertz-Fowler,C., Hilbert,H., Horn,D., Huang,Y., Klages,S., Knights,A., Kube,M., Larke,N., Litvin,L., Lord,A., Louie,T., Marra,M., Masuy,D., Matthews,K., Michaeli,S., Mottram,J.C., Muller-Auer,S., Munden,H., Nelson,S., Norbertczak,H., Oliver,K., O'neil,S., Pentony,M., Pohl,T.M., Price,C., Purnelle,B., Quail,M.A., Rabbinowitsch,E., Reinhardt,R., Rieger,M., Rinta,J., Robben,J., Robertson,L., Ruiz,J.C., Rutter,S., Saunders,D., Schafer,M., Schein,J., Schwartz,D.C., Seeger,K., Seyler,A., Sharp,S., Shin,H., Sivam,D., Squares,R., Squares,S., Tosato,V., Vogt,C., Volckaert,G., Wambutt,R., Warren,T., Wedler,H., Woodward,J., Zhou,S., Zimmermann,W., Smith,D.F., Blackwell,J.M., Stuart,K.D., Barrell,B., and Myler,P.J.,** The genome of the kinetoplastid parasite, Leishmania major 5. *Science* 2005. **309**: 436-442.

148. **Papadopoulou,B.,** *Leishmania After the Genome*. 2008.

149. **Amigorena,S.,** Fc gamma receptors and cross-presentation in dendritic cells 8. *J.Exp.Med.* 2002. **195**: F1-F3.

150. **Kim,D., Jeon,C., Kim,J.H., Kim,M.S., Yoon,C.H., Choi,I.S., Kim,S.H., and Bae,Y.S.,** Cytoplasmic transduction peptide (CTP): new approach for the delivery of biomolecules into cytoplasm in vitro and in vivo 11. *Exp.Cell Res.* 2006. **312**: 1277-1288.

151. **Mitsui,H., Inozume,T., Kitamura,R., Shibagaki,N., and Shimada,S.,** Polyarginine-mediated protein delivery to dendritic cells presents antigen more efficiently onto MHC class I and class II and elicits superior antitumor immunity
6. *J.Invest Dermatol.* 2006. **126**: 1804-1812.

152. **Singh,M. and O'Hagan,D.,** Advances in vaccine adjuvants
1. *Nat.Biotechnol.* 1999. **17**: 1075-1081.

153. **Klinman,D.M., Yi,A.K., Beaucage,S.L., Conover,J., and Krieg,A.M.,** CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma
7. *Proc.Natl.Acad.Sci.U.S.A* 1996. **93**: 2879-2883.

154. **Sparwasser,T., Koch,E.S., Vabulas,R.M., Heeg,K., Lipford,G.B., Ellwart,J.W., and Wagner,H.,** Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells
5. *Eur.J.Immunol.* 1998. **28**: 2045-2054.

155. **Hartmann,G., Weiner,G.J., and Krieg,A.M.,** CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells
5. *Proc.Natl.Acad.Sci.U.S.A* 1999. **96**: 9305-9310.

156. **Sparwasser,T., Vabulas,R.M., Villmow,B., Lipford,G.B., and Wagner,H.,** Bacterial CpG-DNA activates dendritic cells in vivo: T helper cell-independent cytotoxic T cell responses to soluble proteins
1. *Eur.J.Immunol.* 2000. **30**: 3591-3597.

157. **Walker,P.S., Scharton-Kersten,T., Rowton,E.D., Hengge,U., Bouloc,A., Udey,M.C., and Vogel,J.C.,** Genetic immunization with glycoprotein 63 cDNA results in a helper T cell type 1 immune response and protection in a murine model of leishmaniasis. *Human Gene Therapy* 1998. **9**: 1899-1907.

158. **Walker,P.S., Scharton-Kersten,T., Krieg,A.M., Love-Homan,L., Rowton,E.D., Udey,M.C., and Vogel,J.C.,** Immunostimulatory oligodeoxynucleotides promote protective immunity and provide systemic therapy for leishmaniasis via IL-12- and IFN-gamma-dependent mechanisms
7. *Proc.Natl.Acad.Sci.U.S.A* 1999. **96**: 6970-6975.

159. **Cresswell,P.,** Intracellular surveillance: Controlling the assembly of MHC class I-peptide complexes. *Traffic* 2000. **1**: 301-305.

160. **York,I.A., Goldberg,A.L., Mo,X.Y., and Rock,K.L.,** Proteolysis and class I major histocompatibility complex antigen presentation. *Immunological Reviews* 1999. **172**: 49-66.

161. **Witt,E., Zantopf,D., Schmidt,M., Kraft,R., Kloetzel,P.M., and Kruger,E.,** Characterisation of the newly identified human Ump1 homologue POMP and analysis of LMP7(beta 5i) incorporation into 20 S proteasomes. *Journal of Molecular Biology* 2000. **301**: 1-9.

162. **Ramos,P.C., Hockendorff,J., Johnson,E.S., Varshavsky,A., and Dohmen,R.J.,** Ump1p is required for proper maturation of the 20S proteasome and becomes its substrate upon completion of the assembly. *Cell* 1998. **92**: 489-499.

163. **Scott,P., Natovitz,P., Coffman,R.L., Pearce,E., and Sher,A.,** Immunoregulation of cutaneous leishmaniasis. T cell lines that transfer protective immunity or exacerbation belong to different T helper subsets and respond to distinct parasite antigens. *J.Exp.Med.* 1988. **168**: 1675-1684.

164. **Scott,P., Caspar,P., and Sher,A.,** Protection against Leishmania major in BALB/c mice by adoptive transfer of a T cell clone recognizing a low molecular weight antigen released by promastigotes. *J.Immunol.* 1990. **144**: 1075-1079.

165. **Mor,G., Klinman,D.M., Shapiro,S., Hagiwara,E., Sedegah,M., Norman,J.A., Hoffman,S.L., and Steinberg,A.D.,** Complexity of the Cytokine and Antibody-Response Elicited by Immunizing Mice with Plasmodium-Yoelii Circumsporozoite Protein Plasmid Dna. *Journal of Immunology* 1995. **155**: 2039-2046.

166. **Moll,H., Scollay,R., and Mitchell,G.F.,** Resistance to Cutaneous Leishmaniasis in Nude-Mice Injected with L3T4+ T-Cells But Not with Ly-2+T-Cells. *Immunology and Cell Biology* 1988. **66**: 57-63.

167. **Fehling,H.J., Swat,W., Laplace,C., Kuhn,R., Rajewsky,K., Muller,U., and Vonboehmer,H.,** Mhc Class-I Expression in Mice Lacking the Proteasome Subunit Lmp-7. *Science* 1994. **265**: 1234-1237.

168. **Nussbaum,A.K., Rodriguez-Carreno,M.P., Benning,N., Botten,J., and Whitton,J.L.,** Immunoproteasome-deficient mice mount largely normal CD8(+) T cell responses to lymphocytic choriomeningitis virus infection and DNA vaccination. *Journal of Immunology* 2005. **175**: 1153-1160.

169. **Bertholet,S., Goldszmid,R., Morrot,A., Debrabant,A., Afrin,F., Collazo-Custodio,C., Houde,M., Desjardins,M., Sher,A., and Sacks,D.,** Leishmania antigens are presented to CD8+ T cells by a transporter

associated with antigen processing-independent pathway in vitro and in vivo. *J.Immunol.* 2006. **177**: 3525-3533.

170. **Tomkinson,B.,** Tripeptidyl peptidases: enzymes that count. *Trends in Biochemical Sciences* 1999. **24**: 355-359.

171. **Geier,E., Pfeifer,G., Wilm,M., Lucchiari-Hartz,M., Baumeister,W., Eichmann,K., and Niedermann,G.,** A giant protease with potential to substitute for some functions of the proteasome. *Science* 1999. **283**: 978-981.

172. **Seifert,U., Maranon,C., Shmueli,A., Desoutter,J.F., Wesoloski,L., Janek,K., Henklein,P., Diescher,S., Andrieu,M., de la Salle,H., Weinschenk,T., Schild,H., Laderach,D., Galy,A., Haas,G., Kloetzel,P.M., Reiss,Y., and Hosmalin,A.,** An essential role for tripeptidyl peptidase in the generation of an MHC class I epitope. *Nature Immunology* 2003. **4**: 375-379.

173. **Ciechanover,A.,** Intracellular protein degradation: from a vague idea thru the lysosome and the ubiquitin-proteasome system and onto human diseases and drug targeting
2. *Cell Death.Differ.* 2005. **12**: 1178-1190.

174. **Mukhopadhyay,D. and Riezman,H.,** Proteasome-independent functions of ubiquitin in endocytosis and signaling
24. *Science* 2007. **315**: 201-205.

175. **Ciechanover,A., Finley,D., and Varshavsky,A.,** Ubiquitin dependence of selective protein degradation demonstrated in the mammalian cell cycle mutant ts85
1. *Cell* 1984. **37**: 57-66.

176. **Henell,F., Berkenstam,A., Ahlberg,J., and Glaumann,H.,** Degradation of short- and long-lived proteins in perfused liver and in isolated autophagic vacuoles--lysosomes
2. *Exp.Mol.Pathol.* 1987. **46**: 1-14.

177. **Sandoval,H., Thiagarajan,P., Dasgupta,S.K., Schumacher,A., Prchal,J.T., Chen,M., and Wang,J.,** Essential role for Nix in autophagic maturation of erythroid cells
2. *Nature* 2008. **454**: 232-235.

178. **Mizushima,N. and Klionsky,D.J.,** Protein turnover via autophagy: implications for metabolism
16. *Annu.Rev.Nutr.* 2007. **27**: 19-40.

179. **Kundu,M., Lindsten,T., Yang,C.Y., Wu,J., Zhao,F., Zhang,J., Selak,M.A., Ney,P.A., and Thompson,C.B.,** Ulk1 plays a critical role in the autophagic clearance of mitochondria and ribosomes during reticulocyte maturation
8. *Blood* 2008. **112**: 1493-1502.

180. **Schweers,R.L., Zhang,J., Randall,M.S., Loyd,M.R., Li,W., Dorsey,F.C., Kundu,M., Opferman,J.T., Cleveland,J.L., Miller,J.L., and Ney,P.A.,** NIX is required for programmed mitochondrial clearance during reticulocyte maturation
1. *Proc.Natl.Acad.Sci.U.S.A* 2007. **104**: 19500-19505.

181. **Twig,G., Elorza,A., Molina,A.J., Mohamed,H., Wikstrom,J.D., Walzer,G., Stiles,L., Haigh,S.E., Katz,S., Las,G., Alroy,J., Wu,M., Py,B.F., Yuan,J., Deeney,J.T., Corkey,B.E., and Shirihai,O.S.,** Fission and selective fusion govern mitochondrial segregation and elimination by autophagy
2. *EMBO J.* 2008. **27**: 433-446.

182. **Bernales,S., McDonald,K.L., and Walter,P.,** Autophagy counterbalances endoplasmic reticulum expansion during the unfolded protein response
1. *PLoS.Biol.* 2006. **4**: e423.

183. **Rubinsztein,D.C.,** The roles of intracellular protein-degradation pathways in neurodegeneration
4. *Nature* 2006. **443**: 780-786.

184. **Munz,C.,** Enhancing immunity through autophagy
11. *Annu.Rev.Immunol.* 2009. **27**: 423-449.

185. **Schaible,U.E., Schlesinger,P.H., Steinberg,T.H., Mangel,W.F., Kobayashi,T., and Russell,D.G.,** Parasitophorous vacuoles of Leishmania mexicana acquire macromolecules from the host cell cytosol via two independent routes
3. *J.Cell Sci.* 1999. **112 (Pt 5)**: 681-693.

186. **Qu,X., Zou,Z., Sun,Q., Luby-Phelps,K., Cheng,P., Hogan,R.N., Gilpin,C., and Levine,B.,** Autophagy gene-dependent clearance of apoptotic cells during embryonic development
65. *Cell* 2007. **128**: 931-946.

187. **Mellen,M.A., de la Rosa,E.J., and Boya,P.,** The autophagic machinery is necessary for removal of cell corpses from the developing retinal neuroepithelium
2. *Cell Death.Differ.* 2008. **15**: 1279-1290.

188. **Lich,J.D., Elliott,J.F., and Blum,J.S.,** Cytoplasmic processing is a prerequisite for presentation of an endogenous antigen by major histocompatibility complex class II proteins
17. *J.Exp.Med.* 2000. **191**: 1513-1524.

189. **Zhou,D., Li,P., Lin,Y., Lott,J.M., Hislop,A.D., Canaday,D.H., Brutkiewicz,R.R., and Blum,J.S.,** Lamp-2a facilitates MHC class II presentation of cytoplasmic antigens
99. *Immunity.* 2005. **22**: 571-581.

190. **Handman,E. and Mitchell,G.F.,** Immunization with Leishmania receptor for macrophages protects mice against cutaneous leishmaniasis
3. *Proc.Natl.Acad.Sci.U.S.A* 1985. **82**: 5910-5914.

191. **Mitchell,G.F. and Handman,E.,** The glycoconjugate derived from a Leishmania major receptor for macrophages is a suppressogenic, disease-promoting antigen in murine cutaneous leishmaniasis
11. *Parasite Immunol.* 1986. **8**: 255-263.

192. **Flower,D.R.,** Towards in silico prediction of immunogenic epitopes
4. *Trends Immunol.* 2003. **24**: 667-674.

193. **Lundegaard,C., Lund,O., Kesmir,C., Brunak,S., and Nielsen,M.,** Modeling the adaptive immune system: predictions and simulations
1. *Bioinformatics.* 2007. **23**: 3265-3275.

194. **Andersen,M.H., Tan,L., Sondergaard,I., Zeuthen,J., Elliott,T., and Haurum,J.S.,** Poor correspondence between predicted and experimental binding of peptides to class I MHC molecules
22. *Tissue Antigens* 2000. **55**: 519-531.

195. **Bhatia,V., Sinha,M., Luxon,B., and Garg,N.,** Utility of the Trypanosoma cruzi sequence database for identification of potential vaccine candidates by in silico and in vitro screening
8. *Infect.Immun.* 2004. **72**: 6245-6254.

196. **Almeida,R., Norrish,A., Levick,M., Vetrie,D., Freeman,T., Vilo,J., Ivens,A., Lange,U., Stober,C., McCann,S., and Blackwell,J.M.,** From genomes to vaccines: Leishmania as a model
30. *Philos.Trans.R.Soc.Lond B Biol.Sci.* 2002. **357**: 5-11.

197. **Stober,C.B., Lange,U.G., Roberts,M.T., Gilmartin,B., Francis,R., Almeida,R., Peacock,C.S., McCann,S., and Blackwell,J.M.,** From genome to vaccines for leishmaniasis: screening 100 novel vaccine candidates against murine Leishmania major infection
2. *Vaccine* 2006. **24**: 2602-2616.

198. **Barry,M.A., Lai,W.C., and Johnston,S.A.,** Protection Against Mycoplasma-Infection Using Expression-Library Immunization. *Nature* 1995. **377**: 632-635.

199. **Johnston,S.A. and Barry,M.A.,** Genetic to genomic vaccination 8. *Vaccine* 1997. **15**: 808-809.

200. **Barry,M.A., Howell,D.P., Andersson,H.A., Chen,J.L., and Singh,R.A.,** Expression library immunization to discover and improve vaccine antigens 6. *Immunol.Rev.* 2004. **199**: 68-83.

201. **Davies,M.N. and Flower,D.R.,** Harnessing bioinformatics to discover new vaccines 6. *Drug Discov.Today* 2007. **12**: 389-395.

202. **Nussbaum,A.K., Kuttler,C., Tenzer,S., and Schild,H.,** Using the World Wide Web for predicting CTL epitopes 3. *Curr.Opin.Immunol.* 2003. **15**: 69-74.

203. **Beninga,J., Rock,K.L., and Goldberg,A.L.,** Interferon-gamma can stimulate post-proteasomal trimming of the N terminus of an antigenic peptide by inducing leucine aminopeptidase 1. *J.Biol.Chem.* 1998. **273**: 18734-18742.

204. **Portaro,F.C., Gomes,M.D., Cabrera,A., Fernandes,B.L., Silva,C.L., Ferro,E.S., Juliano,L., and de Camargo,A.C.,** Thimet oligopeptidase and the stability of MHC class I epitopes in macrophage cytosol 2. *Biochem.Biophys.Res.Commun.* 1999. **255**: 596-601.

205. **Stoltze,L., Schirle,M., Schwarz,G., Schroter,C., Thompson,M.W., Hersh,L.B., Kalbacher,H., Stevanovic,S., Rammensee,H.G., and Schild,H.,** Two new proteases in the MHC class I processing pathway 1. *Nat.Immunol.* 2000. **1**: 413-418.

206. **Saric,T., Beninga,J., Graef,C.I., Akopian,T.N., Rock,K.L., and Goldberg,A.L.,** Major histocompatibility complex class I-presented antigenic peptides are degraded in cytosolic extracts primarily by thimet oligopeptidase 21. *J.Biol.Chem.* 2001. **276**: 36474-36481.

207. **Herrera-Najera,C., Pina-Aguilar,R., Xacur-Garcia,F., Ramirez-Sierra,M.J., and Dumonteil,E.,** Mining the Leishmania genome for novel antigens and vaccine candidates 1. *Proteomics.* 2009. **9**: 1293-1301.

208. **Holtappels,R., Thomas,D., Podlech,J., Geginat,G., Steffens,H.P., and Reddehase,M.J.,** The putative natural killer decoy early gene m04 (gp34)

of murine cytomegalovirus encodes an antigenic peptide recognized by protective antiviral CD8 T cells
5. *J.Virol.* 2000. **74**: 1871-1884.

209. **Leifso,K., Cohen-Freue,G., Dogra,N., Murray,A., and McMaster,W.R.,** Genomic and proteomic expression analysis of Leishmania promastigote and amastigote life stages: the Leishmania genome is constitutively expressed
2. *Mol.Biochem.Parasitol.* 2007. **152**: 35-46.

210. **Sereno,D., Cavaleyra,M., Zemzoumi,K., Maquaire,S., Ouaissi,A., and Lemesre,J.L.,** Axenically grown amastigotes of Leishmania infantum used as an in vitro model to investigate the pentavalent antimony mode of action
1. *Antimicrob.Agents Chemother.* 1998. **42**: 3097-3102.

211. **Rammensee,H.G., Friede,T., and Stevanoviic,S.,** MHC ligands and peptide motifs: first listing
7. *Immunogenetics* 1995. **41**: 178-228.

212. **Holtappels,R., Thomas,D., and Reddehase,M.J.,** Identification of a K(d)-restricted antigenic peptide encoded by murine cytomegalovirus early gene M84
2. *J.Gen.Virol.* 2000. **81**: 3037-3042.

213. **von Stebut,E., Ehrchen,J.M., Belkaid,Y., Kostka,S.L., Molle,K., Knop,J., Sunderkotter,C., and Udey,M.C.,** Interleukin 1 alpha promotes Th-1 differentiation and inhibits disease progression in Leishmania major - Susceptible BALB/c mice. *Journal of Experimental Medicine* 2003. **198**: 191-199.

214. **Inaba,K., Inaba,M., Romani,N., Aya,H., Deguchi,M., Ikehara,S., Muramatsu,S., and Steinman,R.M.,** Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J.Exp.Med.* 1992. **176**: 1693-1702.

9. Abbreviations

1x	onefold
10x	tenfold
100x	hundredfold
20x	twentyfold
4x	fourfold
α-	anti
APC	antigen presenting cell
BMDC	bone marrow-derived dendritic cell(s)
BSA	bovine serum albumin
CD	cluster of differentiation
CFSE	carboxylfluorescein diacetate succinimidyl ester (CFDA-SE)
CTL	cytotoxic lymphocyte
DC	dendritic cell
dLN	draining lymph node
DMEM	Dulbecco Mod´s Eagle Medium
DMSO	dimethylsulfoxyde
ELISA	enzym-linked immuno sorbent assay
et al.	et alii (and others)
FACS	fluorescent activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FSC	forward light scatter
GM-CSF	granulocyte macrophage colony stimulating factor
h	hours
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxyidase
i.d.	intradermal
i.p.	intraperitoneal
i.v.	intravenuously
IFN-γ	interferon gamma
IL	interleukine
L.	leishmania

l	liter
LACK	leishmania homologue of receptors for activated C kinase
LC	Langerhans cells
LN	lymph node
LPS	lipopolysaccharide
µg	microgramm
µl	microliter
µm	micrometer
µM	micromolar
M	molar
MACS	magnetic cell sorting
mAb	monoclonal antibody
MFI	mean fluorescence intensity
mg	milligramm
MHC	major histocompatibility complex
min	minute
ml	milliliter
mM	millimolar
n	numbers
nm	nanometer
nM	nanomolar
ng	nanogramm
o.n.	overnight
p	p-value of student´s t-test
p.a.	per analysis
PBS	phosphate buffered saline
PE	phycoerythrin
PFA	paraformaldehyde
pg	picogramm
rpm	rounds per minute
RT	room temperature
s.c.	subcutaneous
SEB	staphylococcus enterotoxine B
SEM	standard error of the mean
SLA	soluble *Leishmania* antigen
spp.	species
SSC	side scatter
tab.	Table

Tc	cytotoxic T cell
Th	helper T cell
Treg	regulatory T cell
vs.	*versus*
wk	week
wt	wild type