Visceral leishmaniasis – malaria co-infections
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While biomedical research has traditionally focused on isolated interactions between a single host and a single pathogen, there is now growing evidence indicating that co-infections are common in nature, and that their complex interplay in the co-infected host is likely to affect host pathology, pathogen transmission and virulence evolution.\textsuperscript{1-3} The increased susceptibility to deadly bacterial pneumonia in patients suffering from influenza,\textsuperscript{4} or to opportunistic infections such as cryptococcosis and pneumocystosis, causing much of HIV-associated morbidity and mortality\textsuperscript{5} are prime examples of how otherwise relatively harmless infections can turn into life-threatening conditions in the presence of other pathogens. Notably, co-infections do not only impact on disease burden at individual level (e.g., by improving or exacerbating disease morbidity); they also act at population level, shaping prevalence of single diseases as a result of their effects on host infectiousness and pathogen virulence.\textsuperscript{1,2,6,7} Consequently, a thorough understanding of the mechanisms governing nature and consequences of co-infection may not only benefit patients directly suffering from these conditions, but most importantly, it could help designing truly integrated disease control programs that effectively tackle multiple infections at a time.\textsuperscript{8-10} This holds true, particularly, for diseases sharing a similar clinical presentation and/or route of transmission, as in the case of VL and malaria. Those two parasitic infections, transmitted by phlebotomic insects that co-exist in many natural habitats,\textsuperscript{11,12} are co-endemic in many foci around the world, where they afflict mostly children (or young adults, depending on the exposure risk) and individuals suffering from malnutrition and immunosuppression.\textsuperscript{13-15} Given that both VL and malaria are known to increase patient susceptibility to secondary infections by causing immunosuppression,\textsuperscript{16-18} tackling VL in co-endemic areas may impact indirectly on the burden of malaria and vice versa.

**Clinical epidemiology of VL-malaria co-infections**

Despite the frequent anecdotal reporting of concomitant VL and malaria cases, the current literature on the extent of this co-infection is no more than just a fairly blank state. The only dedicated study on this subject dates back to 1995 and consists of a small cross-sectional study examining a few dozen patients with fever and splenomegaly in Bihar State, India.\textsuperscript{19} Ever since, few other figures on the rate of VL-malaria co-infection have incidentally appeared in reports profiling VL cases across various endemic settings,\textsuperscript{20-23} suggesting either that VL and malaria rarely co-exist in the same patients or that their co-infections are clinically unapparent. Nothing of this appears to be true, however, when taking a closer look to the medical records of VL patients hospitalized in East Africa [chapters 2 and 3].

A systematic examination of 4,225 laboratory-confirmed cases of VL spread across one hospital in Uganda (Amudat Hospital, 2000-2006) [chapter 2] and five different health centers in Sudan (Um-el-Kher and Kassab Hospitals, 1998; Al’Azaza kala-azar Clinic, Tabarakallah and Gedarif Teaching Hospitals, 2005-2010) [chapter 3] identified as many as 943 laboratory-confirmed cases of malaria co-infection, resulting in an overall co-infection rate of 22%. Thus, more than one in five VL patients living in the Pokot territories across the Ugandan-Kenyan border and in the Sudanese States of Gedarif and Sennar had malaria next to VL. Of these, the majority were children in areas of stable malaria transmission (Uganda/Kenya), whereas in Sudan where malaria is largely seasonal, the risk was
equally shared among all age groups. Reflecting the classical age distribution of malaria cases as defined by the transmission intensity pattern, this age-related susceptibility to the co-infection suggests that a pre-existing or concomitant VL infection neither disrupted nor boosted the naturally acquired immunity against malaria, in contrast with other co-infected cohorts (e.g., HIV-malaria co-infections), in which semi-immune adults suffered from an increased *P. falciparum* prevalence and intensity as a result of their HIV seropositivity. Similarly, a pre-existing or concomitant malaria infection neither prolonged nor shortened the incubation period of VL, as most co-infection cases presented at hospital during the dry season just as most VL mono-infected patients. Whilst this may be suggestive of malaria failing to cross-regulate immune mechanisms involved in controlling *Leishmania* replication, it should be noted that reports of primary VL with shortened incubation times have yet to be provided even in the case of the deadly gridlock marking HIV-VL co-infections.

Overall, co-infection with VL and malaria resulted in a deterioration of patients’ clinical picture. At Amudat Hospital, malaise and to a lesser extent, anorexia, were found to be positively associated with the VL-malaria co-infection, while a similar association for weight loss and jaundice was identified amongst patients hospitalized in Sudan (2005-2010). Less obvious, conversely, appeared to be the impact of the co-infection on the severity of anemia and splenomegaly. Whilst at Amudat Hospital, the co-infection did not significantly change the level of hemoglobin among VL patients suffering from no or mild anemia (only if grouped together though; else it reduced the extent of anemia), the risk of developing moderate and severe anemia in Sudan was substantially higher in the co-infected vs. the VL mono-infected group. A similar association, however, could not be observed with regard to the intensity of splenomegaly, since enlargement of the spleen appeared to be reduced by the co-infection in Sudan, but not in Uganda (here, the effect on splenomegaly was minimal). Given that a very weak, but statistically significant correlation could only be identified between duration of illness and severity of splenomegaly in Sudanese VL mono-infected patients (data not shown), all hypotheses attempting to harmonize these apparently discordant findings remain purely speculative. Of those, perhaps the most conceivable is that co-infected patients in Sudan may have suffered from an acute malaria-related symptomatology (in addition to the VL one), that manifested with jaundice and aggravated anemia levels, and that prompted patients to seek medical help earlier than the VL controls (in Sudan, co-infected patients reported to hospital on average 10 days earlier than VL mono-infected patients). As a result, co-infected patients may have been hospitalized at a less advanced stage of VL compared to the VL controls, compatible with their reduced splenomegaly frequency and intensity (malaria only induces moderate splenomegaly, while VL causes massive spleen enlargement). After all, the possibility of a *Leishmania* attenuated parasitaemia causing milder splenomegaly in co-infected patients seems unlikely given the increased (albeit not significantly) parasite loads found in their lymph node and bone marrow aspirates. In the Pokot territories across Kenya and Uganda, on the other hand, malaria transmission is fairly stable, and individuals above the age of five are relatively immune to clinical malaria attacks, in line with the lack of malaria-specific symptoms highlighted amongst co-infected patients admitted at Amudat Hospital (over 80% of the examined patients consisted of individuals
aged five or above). Interestingly, both co- and mono-infected patients from Uganda and Kenya presented at hospital as early as 9 days on average after onset of symptoms (as opposed to the 30 day-illness preceding hospitalization at the MSF-managed hospitals of Um-el-Kher and Kassab, Sudan), suggesting a rapid progression of the disease possibly attributable to their poor nutritional conditions (the majority of patients admitted to Amudat Hospital suffered from malnutrition and anemia disorders).

When promptly identified and effectively treated, malaria proved to minimally affect VL patients’ prognosis, as confirmed by the similar case-fatality rate found amongst co- and mono-infected VL patients at Amudat Hospital and at the three Sudanese health centers (2005-2010). In striking contrast though, co-infected patients at Um-el-Kher and Kassab Hospitals, Sudan, exhibited a nearly four-and-a-half time higher risk of a fatal outcome as compared to the VL mono-infected patients. Given that no major difference, other than the proportion of patients with severe anemia and the type of anti-malarial drug received, distinguished these co-infected patients from the other cohorts, the prospect of a treatment failure attributable to either sulpadoxine-pyrimethamine or quinine as the cause of this increased mortality rate is not unlikely.

Clearly, albeit inevitable under these study settings (VL-dedicated treatment centers), the significance of the findings highlighted by the present studies is limited by the sampling bias that arises from the selection of VL mono-infected patients as control samples. Should these studies have been performed with healthy individuals or malaria mono-infected patients as controls, considerably different findings would have emerged, including a lower co-infection rate and, most likely, a more severe clinical picture associated with this condition. In addition, the lack of control for social-economic status and household infection clustering, along with the retrospective nature of the study, further reduces the statistical power of these observations. It is evident, therefore, that additional detailed and integrated studies are required to substantiate and investigate the VL-malaria interactions at population level.

**Cytokine profiling of VL-malaria co-infected patients**

Despite suffering from poor diagnostic specificity, characterization of circulating cytokine expression has proved to be valuable for assessing the stage and prognosis of certain diseases, including malaria and VL. Excessive systemic production of inflammatory cytokines, for example, mediates pathology of cerebral *P. falciparum* malaria, whereas a cytokine balance tipped towards regulatory patterns promotes survival of *L. donovani* parasites and predisposes to the development of disease. Typically, release of cytokines is triggered by specific pathogen-associated molecules, but the functional pleiotropy that characterizes their signaling pathways ensures that these molecules are capable of acting across a range of tissues and biologic systems, cross-regulating the immune responses generated either locally or systemically and shaping the outcome of host-pathogen(s) interactions. Accordingly, profiling of serum cytokines may assist in predicting susceptibility vs. resistance to infections, providing a potential key for understanding the interactions taking place in the co-infected host [Chapter 4]. Examination of circulating cytokines in Sudanese patients co- and mono-infected with VL and/
Discussion

or malaria [chapter 4] highlighted substantial variations in the immune response mounted upon co-infection, confirming the ability of *L. donovani* and *P. falciparum* to mutually interact at immunological level. Patients harboring both leishmanial and malaria parasites responded with an overall increase in type-1 and pro-inflammatory cytokine release, which partly reflected the effect elicited by VL (TNF-α, IFN-γ) and malaria (IL-2), and partly resulted from a synergistic interaction of the two diseases upon each other (IL-17A). Importantly, a significantly reduced *P. falciparum* parasitaemia was observed among co-infected patients as compared with malaria mono-infected patients, suggesting either a protective or non-detrimental effect of the co-infection on malaria.

Longer incubation times (usually two to six months) precede the onset of VL, as opposed to malaria (days to few weeks), whose transmission season in the sampling area (Gedarif State) peaks just few months after the *Leishmania* one.\(^ {38-40}\) As a result, in the study area, VL patients typically report to hospital during the dry season (November-May),\(^ {41}\) just as most VL-malaria co-infected patients [chapter 3]. This warrants the assumption that most co-infection cases enrolled in this study (conducted in February) may have resulted from *Leishmania*-infected patients acquiring malaria, rather than from *Plasmodium*-harboring individuals who got infected with *Leishmania* spp. Pre-existence of a pro-inflammatory and type-1 cytokine milieu has been shown to boost clearance of malaria parasites,\(^ {42-44}\) particularly during the pre-erythrocytic stage, when cell-mediated immunity is essential for control of infection. Hence, if the assumption on the sequence of infections holds true, the IFN-γ dominant response elicited by VL could have acted as a pre-priming stimulus upon *Plasmodium* infection, for the development of malaria adaptive immunity (e.g., via NKT cells) and the nitric oxide-dependent suppression of intra-hepatocytic forms. The leading presence of IFN-γ, followed by TNF-α and IL-4 in the sera of VL patients vs. the malaria ones, and the progressive polarization towards a type-1/pro-inflammatory cytokine pattern observed in co-infected patients along with their reduced malaria parasitaemias seem to support this view. A similar scenario could also apply to patients in whom malaria preceded infection with *L. donovani* (IL-12, IFN-γ and TNF-α are involved in resistance to blood stage malaria, too), provided that the VL-boosted raise in type-1/pro-inflammatory cytokines takes place sufficiently early in the immune response against malaria to not interfere with the Th1/Th2 switch required for clearing parasites at the latter stages of infection.

Evidence of an immune-mediated alteration in malaria frequency and severity abounds for HIV or helminthiasis co-infections,\(^ {25,45-48}\) although the consequences of these immunological interactions are not always straightforward. Equally unclear is also the effect of *Leishmania* infection on murine malaria, as the evidence provided by the very few animal studies conducted until now is far from being conclusive, not least as a result of the intrinsic differences that distinguish the different models used. Overall, *Leishmania*-infected hamsters exhibited an improved or unaltered resistance to *Plasmodium berghei*\(^ {49,50}\) and *Plasmodium yoelii*,\(^ {50}\) irrespective of the sequence of infection (with the exception of *P. yoelii* pre-infected hamsters receiving *Leishmania enrietti* and *P. berghei* 5 and 6 weeks later, respectively). In co-infected mice, conversely, the malaria infection was both enhanced and prolonged when *P. yoelii* was preceded by *L. mexicana*,\(^ {51}\) but not when *P. yoelii* and
Plasmodium chabaudi chabaudi were followed by L. mexicana and L. infantum, respectively,\textsuperscript{51-53} suggesting animal- and/or strain-specific mechanisms of parasite clearance. Interestingly, the increased Leishmania parasite loads detected in P. c. chabaudi and L. infantum-co-infected mice as compared to the mono-infected ones, appeared to be IL-4-mediated and IFN-γ independent, as splenic and hepatic expression of both cytokines was found to be enhanced among co-infected animals.\textsuperscript{51}

It is important to note, however, that in our study on VL-malaria co-infected patients, the link between malaria parasitaemia and cytokine pattern remains theoretical. Participants to this study were sequentially recruited, but this does not exclude the possibility that co-infected patients may have carried low-intensity or subclinical malaria infections also in the absence of VL, in which case the suggestion that VL exerts a protective role on malaria would lose its significance. To this end, enrollment of asymptomatic, but parasitaemic individuals for each of the two infections (VL and malaria) and repeated assessments of cytokine and parasitaemia levels over time would be important to control for non-homogeneous group-wise comparisons and to verify the legitimacy of the speculations made.

Importantly, if the mounting of a type-1/pro-inflammatory cytokine response in VL patients may potentially afford them some degree of anti-Plasmodium activity, the implications for disease pathogenesis may not be as much positive. Although this was not evident in our patients' cohort, down-regulation of type-1 and pro-inflammatory cytokines has been repeatedly associated with reduced pathology in malaria immune individuals,\textsuperscript{54} whose ability to timely balance between symptom-suppressing (IL-10 and TGF-β) and parasite-inhibiting (IFN-γ and TNF-α) cytokines determines the outcome of the infection. Similarly, enhancement in systemic inflammation may cause the VL patients to suffer from more severe symptoms, as confirmed in East Africa, where a worsened clinical picture was observed amongst co-infected patients as compared with VL mono-infected patients [\textit{chapters 2 and 3}].

\textbf{In vitro studies on the interaction between L. donovani and P. falciparum or its pigment hemozoin}

To assess the level at which host-pathogen interactions may take place during co-infections with VL and malaria, two different in \textit{vitro} studies were performed. The first one [\textit{chapter 5}], conducted with \textit{ex vivo} human monocytes differentiated into dendritic cells (mo-DCs), aimed at characterizing the effect of \textit{L. donovani} and \textit{P. falciparum} co-exposure on cell maturation and function, while in the second study [\textit{chapter 8}] the ability of hemozoin (HZ) to influence proliferation of \textit{L. donovani} in macrophage-like cells of human and murine origin was investigated.

As most pathogens, \textit{Leishmania} and \textit{Plasmodium} have evolved a variety of mechanisms to overcome host immune defenses, including a complex of strategies to modulate functioning of dendritic cells,\textsuperscript{55-59} a group of highly adept antigen-presenting cells responsible for the induction of adaptive immune responses.\textsuperscript{60} Importantly, the nature of these DC-modulating effects differs significantly between the two parasites and so does the mechanism that determines them. For example, whilst \textit{Leishmania} inhibits IL-12 production, leaving other pro-inflammatory
cytokine pathways (such as NF-κB) intact and inducing IL-10 to avoid clearance,\textsuperscript{61-63} *Plasmodium* spp. act on a dose- and species-dependent basis, with low parasite amounts causing DC activation and high amounts blocking release of IL-12 and TNF-α through induction of apoptosis.\textsuperscript{64-67} As the two parasites share part of their host tissue niches, the question as whether DCs co-exposed to *Leishmania* and *Plasmodium* may respond by acquiring a regulatory rather than a Th1/ pro-inflammatory phenotype appears crucial to predict the course of the co-infection and its pathological consequences.

Overall, co-stimulation of mo-DCs with *L. donovani* promastigotes and *P. falciparum*-infected erythrocytes resulted in a dual, dose-dependent effect on DC differentiation, which ranged from semi-mature cells, secreting low IL-12p70 levels to a complete lack of phenotypic maturation in the presence of high parasite amounts. The effect was mainly triggered by the *Leishmania* parasites, as illustrated by their ability to induce semi-mature, IL-10-producing DCs that poorly responded to lipopolysaccharide (LPS) stimulation. Conversely, *P. falciparum* blood-stage forms failed to activate mo-DCs and only slightly interfered with LPS effects. This suggests that at low parasite amounts, DC-mediated control of *L. donovani* may not be affected by the presence of concomitant malaria, whereas the regulatory milieu shaped by *L. donovani*-primed mo-DCs could either trigger *P. falciparum* proliferation or dampen its immunopathological effects, pending on the host susceptibility and the infection timing. Future work will have to assess the actual capacity of these mo-DCs to polarize T cell-responses and elicit a protective or detrimental effect against VL and/or malaria. It is worth noticing that notwithstanding the distinct level of complexity that characterizes this and the other study on co-infected patients’ cytokines [chapter 4], the present findings on DC maturation as described here may suggest that the increased levels of Th1/pro-inflammatory cytokines observed amongst VL mono- and co-infected patients are likely to share a similar cellular source, which has to be sought outside the CD4\(^+\) T lymphocyte population, provided that the *L. donovani*-stimulated mo-DCs are confirmed capable of activating Treg cells as suggested by their regulatory phenotype. Various cell subsets could be singled out in this respect as potential sources of Th1/pro-inflammatory cytokines, including gamma delta T cells (γδ T cells) and CD8\(^+\) cytotoxic T cells (NK and NKT cells, the major sources of IFN-γ rely on IL-12p70 for their activation). Gamma delta T cells are a group of unconventional innate-like lymphocytes that function as early sources of pro-inflammatory cytokines (TNF-α, IFN-γ and/or IL-17) upon recognition of specific (un)processed antigens, co-stimulatory signals or cytokines.\textsuperscript{68} A putative role for these cells in the immune clearance of *Leishmania* has been suggested by their increased number in the peripheral blood of VL patients and by their ability to specifically proliferate *in vitro*, following stimulation with *Leishmania* antigens.\textsuperscript{69-71} Similarly, CD8\(^+\) cytotoxic T cells which recognize antigens presented by MHC class I molecules (constitutively expressed in all nucleated cells), are believed to participate to the resolution of *Leishmania* infections by activating bystander macrophages of *Leishmania*-infected cells through the release of cytokines, like IFN-γ.\textsuperscript{72,73} Although the involvement of γδ\(^+\) and CD8\(^+\) T cells in the immune responses mounted against VL has been repeatedly documented\textsuperscript{69-73} and could account for the IL-12p70-independent increase of Th1/pro-inflammatory cytokine levels observed
amongst VL mono- and co-infected patients, it should be noted that most IFN-γ-producing T cells isolated from VL patients are, in fact, CD4+ T cells. This does not exclude a role for γδ+ and CD8+ T cells, obviously, but strongly advocates for the search of actual evidence in support of this IL-12p70-independent release of IFN-γ and TNF-α, as speculated here. After all, the low levels of IL-12p70 detected in the mo-DC supernatants and in patients’ sera may also have resulted from the cytokine being engaged in specific signaling pathways (and, as such, not available in solution) rather than from failure of DCs to release it. The characterization of DC priming activity on T cells and their functional profiling will be essential to clarify the significance of these findings.

While *L. donovani* and *P. falciparum* interplay at different levels of the host immunity, other non-immunological factors at both cellular and tissue levels are likely to play a role in shaping the co-infection dynamics and its outcome. Hemozoin, for example, which is produced during the parasite blood-stage, is avidly internalized by the host phagocytes (neutrophils, monocytes/macrophages and DCs), where it impairs a number of cellular functions that may be important for controlling proliferation of *Leishmania* amastigotes. Hence, exploring the effect of HZ ingestion on the infection rate of *L. donovani* in macrophages [chapter 8] may help providing valuable insights into the course of the *L. donovani-P. falciparum* co-infection and its mechanisms of interaction.

Phagocytosis of native (HZ) and synthetic (β-hematin, BH) malaria pigment by mouse RAW 264.7 macrophage-like cells prior to *L. donovani* infection increased the parasite burden therein, whereas human THP-1 cells differentiated with phorbol-12-myristate-13-acetate (PMA) displayed similar infection rates with or without pre-ingestion of malarial pigment. The effect in RAW 264.7 cells proved to be dose-dependent, malarial pigment-specific (phagocytosis of latex beads, as a control, did not influence *Leishmania* burden) and NO-independent, as equal quantities of NO were measured in all cell supernatants (with or without pre-treatment with phagocytic meals). Given this discrepancy of results between the human and the murine model, the significance of the present findings remains unclear, leaving room for speculation.

THP-1 is a human leukemia monocyctic cell line extensively used to assess modulation of monocyte and macrophage functions. Upon treatment with PMA, THP-1 monocytes differentiate into inflammatory macrophages that display most of the PBMC-derived macrophage features, such as cell adherence, high phagocytic capacity, expression of differentiation-dependent cell surface markers and ability to respond to LPS stimulation through release of cytokines and prostaglandin E2. This high level of similarity corroborates the use of THP-1 cells as a simplified, suitable and reliable model to study monocyte and macrophage functions in response to the surrounding environment, and suggests that the lack of HZ-induced effects on the infection burden of *L. donovani*, as observed here, may be representative of what happens in vivo to patients co-infected with VL and malaria. Albeit plausible, a similar conclusion drawn in the absence of further evidence, however, may be oversimplifying, as substantial differences exist between the in vitro and the in vivo environments which macrophages are exposed to. For example, it was demonstrated that human cells, including human phagocytes, can express the gene encoding for the inducible nitric oxide synthase (iNOS) and release
conspicuous amounts of NO.\textsuperscript{80,81} In sharp contrast though, \textit{in vitro} stimulation of PBMC as well as of THP-1 monocyte-derived macrophages with conventional stimuli (e.g., LPS/IFN-γ) neither induces the expression of iNOS nor the release of NO,\textsuperscript{81} as confirmed by the present study, too. This conflicts with the high responsiveness of mouse macrophages, including the RAW 264.7 cell line, which display features of activated macrophages and promote expression of iNOS protein and activity upon LPS/IFN-γ \textit{in vitro} stimulation.\textsuperscript{82} Hence, in the absence of appropriate \textit{in vitro} stimuli capable of inducing the human iNOS-encoding gene, murine macrophages may serve as a complementary tool for studying the effect/response of human macrophages to specific conditions.\textsuperscript{72}

It should be noted, however, that here, no evidence was found to link the increased \textit{Leishmania} infection burden as seen in RAW 264.7 cells with a change in NO levels. Interestingly, recent evidence on the fate of HZ in phagocytic cells suggests that upon trafficking into the phagolysosome, the malaria pigment destabilizes the integrity of this organelle, causing the leakage of lysosomal contents into the cytosol, where cell function is believed to be modulated both biochemically – through the hematin core – and immunologically – through the malarial DNA that HZ carries along.\textsuperscript{83} Given the inherent differences that mark phagocytosis and maturation of \textit{Leishmania}-containing phagosomes in macrophages of different sources (primary vs. immortalized cells, human vs. mouse cells),\textsuperscript{84,85} it is somehow not surprising that the complex array of HZ-mediated effects may result in different outcomes on \textit{Leishmania} growth, pending on the specific host cell genotype and phenotype. Future investigations should attempt to clarify the relevance of these findings (e.g., by repeating similar experiments in other cell types) and provide mechanistic insights in the way HZ improves cell hospitability and/or permissiveness to \textit{L. donovani}.

**Alternative assays for detection of \textit{Leishmania} intra-macrophage viability**

It is well recognized that co-infection studies are inherently difficult to conduct, in part because of the many different skills required to perform and interpret this type of research and in part because of the high level of complexity that characterizes the co-infected system and the interactions taking place within.\textsuperscript{86,87} Experimental models can provide a simplifying tool to this end,\textsuperscript{83} but have to deal with the poor availability of assays optimally suited for use in such complex systems, as in the case of \textit{Leishmania} parasites grown in HZ-laden macrophages. In this \textit{in vitro} model mimicking the VL-malaria co-infection, microscopic assessment of parasite burden, the standard method to quantify \textit{Leishmania} survival, is hindered by the presence of intracellular phagocytic meals (hematin crystals) that hide from view the amastigotes growing therein. Alternative, non-microscopy based assays are available, but remain hard to access, requiring genetically modified \textit{Leishmania} parasites that cannot be readily purchased. To circumvent this problem, two novel assays were developed \textit{ad hoc}: one consisting of a quantitative Reverse Transcriptase PCR (qRT-PCR) for measuring viability of the \textit{Leishmania} parasite and its host cells [\textit{chapter 6}]; the other one being a simple enzymatic reaction with colorimetric readout that assesses the activity of a \textit{Leishmania}-specific enzyme as a proxy of parasite viability [\textit{chapter 7}]. Both assays are applicable to all strains of \textit{Leishmania}, including clinical isolates, and rely
on commonly available technologies, resulting in highly accessible tools for measuring parasite viability and assessing drug performance against the relevant stage of disease, the intracellular amastigote.

Several molecular tools for evaluation of anti-leishmanial drug activities at both in vivo and in vitro levels have been developed prior to this duplex qRT-PCR.\(^88-92\) Some of these include an internal control surveying for test performance\(^88\) or correcting for cDNA synthesis/RNA input amount variation (normalization to a housekeeping gene),\(^89,91\) but none of the tests developed so far makes use of an internal control to monitor for drug toxicity. In this respect, the qRT-PCR developed here [chapter 6] represents a significant advancement over the current genetic assays. By targeting the Leishmania-specific 18S ribosomal RNA (rRNA) and the human β-2-microglobulin (β-2M) mRNA, this duplex qRT-PCR simultaneously assesses the viability of the Leishmania intracellular amastigotes and their host cells, resulting in highly reproducible anti-leishmanial activities as measured against standard microscopy, and a good level of correlation between compound cytotoxicity and β-2M amplification. The result is a sensitive and versatile tool that can be used to monitor parasite and cell viability at high as well as at low infection rates (e.g., in the case of in vitro poorly adapted strains) and screen for compound toxicity, provided that efforts to upscale the throughput of this technique are made.

Another major step forward in obtaining a simple, non-microscopy based assay for assessment of intracellular Leishmania viability was achieved with the development of the trypanothione reductase (TryR)-based assay [chapter 7], a quantitative enzymatic assay monitoring the reduction of the kinetoplast-unique thiol trypanothione disulfide (T[S]₂). By coupling the chromogenic properties of the 5,5’-dithiobis-2-nitrobenzoic acid (DTNB) with its T[S]₂-regenerating capacity, this reaction allows intracellular parasite viability to be colorimetrically monitored, simplifying the methodology for scoring inhibitor assays and improving accessibility to drug-susceptibility testing. Pharmacological validation of the assay, performed with a panel of selected compounds, demonstrated high consistency between the newly developed technique and the reference methods and confirmed the robustness and reproducibility of the assay, which performed in compliance with high-throughput (HTS) requirements. It is undeniable, however, that benefits such as reduced performance time, universal applicability and wide accessibility of the test come at the cost of other important assay properties, such as sensitivity and output content which are fairly poor in comparison to genetically-based methods (e.g., recombinant parasites and PCR) and microscopic examination, respectively. To this end, establishment of suitable, user-tailored signal thresholds for assessing minimum satisfactory infection rates in the control samples is a prerequisite for a future implementation of the assay as a microscopy replacement. Further validation of the assay will require testing of a compound collection, independently assembled for control of test quality.

Both assays – the duplex qRT-PCR and the TryR-based assay – may be suitable to quantify the growth of Leishmania amastigotes in HZ-laden macrophages, each with its own set of advantages and disadvantages. However, as the TryR-based assay allows for a multiple condition screening in less time and with less resources than the qRT-PCR, this test was preferred over
the molecular one for assessing the *Leishmania* viability in our study on HZ-laden macrophages [chapter 7], where sensitivity of the assay did not represent an issue of concern.

**Future studies**

From the above discussion, it is clear that additional integrated studies are required to validate and fully understand the interactions taking place between VL and malaria.

Well-conceived, randomized placebo control trials provide the most robust study design to investigate the association between multiple infections<sup>93</sup> and should be adopted to unveil the mutual effect of VL and malaria. In particular, future studies should address questions like: (1) does concomitant malaria increase the risk of developing active VL? (2) Does anti-leishmanial treatment increase the prevalence and/or intensity of malaria? And (3) what is the effect of age and malaria transmission intensity on the interactions between malaria and VL? In addition to investigating the effect of VL on susceptibility to clinical malaria and vice versa, further studies on the haematological, nutritional and organ-pathological impact of co-infection are also warranted.

As much of the interaction taking place between VL and malaria is likely to have an immunological basis, future efforts will have to focus on characterizing the immune response to one disease in relation to the other. Animal models provide a unique opportunity to this end, as they offer the required complexity to mimic natural co-infection hosts while allowing to control for a number of individual and environmental variables that are likely to crucially impact the outcome of co-infection. Part of these models has already been object of investigation by the very few studies investigating the VL-malaria co-infection, but with little or no attempt to characterize the cellular and immunological mechanisms involved, partly owing to their pioneering nature, too (most of these studies were conducted between the 1950s and the 1980s). Renewed research designed to embrace the latest advances in polyparasitism models and to employ state-of-the-art technology is, therefore, advocated. In particular, experiments testing the importance of co-infection timing, dose and route of administration in addition to the host genetic susceptibility are required to more fully understand the generality of these effects across a more natural range of co-infection scenarios. Importantly, these investigations should be combined with immuno-epidemiological surveys aimed at characterizing the humoral and cellular immune response to crude and defined antigens of *Leishmania* in relation to different levels of malaria infection and disease, and vice versa. These studies should: (1) take place in co-endemic areas with different transmission intensity patterns; (2) focus on the interaction between specific *Leishmania* and *Plasmodium* infections and diseases; (3) utilize different types of leishmanial and malaria antigens; (4) take place in age- and infection intensity-stratified groups; (5) examine the immune response before and after treatment; and (6) compare individuals from sympatric ethnic groups.
Concluding remarks

The evidence gathered in the present thesis confirms our initial hypothesis whereby malaria and VL frequently co-exist in patients living across co-endemic areas, exacerbating host pathology and cross-modulating immune responses. The high percentage of malaria co-infections detected among VL patients hospitalized throughout East Africa, along with their clinical deterioration, testifies how likely and insidious is the risk of contracting malaria while infected with VL or vice versa, although the question as whether this risk is truly increased by pre-exposure to either of these two diseases remains to be answered. Further studies on the extent and distribution of this co-infection will also have to clarify whether a similar risk exists in other co-endemic areas outside Africa, such as Brazil, India and Bangladesh. Importantly, effective treatment of malaria ensured that co-infected patients did not suffer increased mortality risks as compared with VL mono-infected patients, emphasizing the importance of early detection and adequate management of these conditions.

Initial investigations on the co-infection in natural hosts and in vitro models demonstrated the ability of *L. donovani* and *P. falciparum* to cross-interact at immunological and non-immunological level, skewing host serum cytokines with a possibly DC-independent mechanism and modulating each other’s survival within the corresponding host cells. In this latter regard, it is interesting to note that co-infected patients exhibited significantly lower *P. falciparum* parasitaemias as compared with malaria mono-infected patients, possibly to be associated with their increased immune polarization towards type-1/pro-inflammatory responses. If this association proves true, it could signify that treatment of VL in the absence of a proper anti-malarial regimen may have an impact on the intensity and/or clinical presentation of *P. falciparum* co-infections, as anti-leishmanial chemotherapy is known to alter the profile of circulating cytokines. Pending proper evidence that could confirm or rule against this notion, routine malaria screening should be adopted for all VL patients living in co-endemic areas, along with a set of specific treatment guidelines for effective management of these co-infections.

Beyond any direct implication that the present findings may have, it is undeniable that altogether, these data raise more questions than they answer. It is also equally undeniable, however, that these data do have the merit of shedding light on an important and yet deeply neglected condition, whose pathogenesis are worthy to all effects of further investigation.
References


42. Lumsden JM, Schwenk RJ, Rein LE. Protective immunity induced with the RTS,S/AS vaccine is associated with IL-2 and TNF-alpha producing effector and central memory CD4 T cells. *PLoS ONE* 2011, 6(7):e20775.


