Visceral leishmaniasis – malaria co-infections
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Concomitant infections by multiple pathogen species represent a serious threat to human health. Affecting over a billion people worldwide, co-infections are an important cause of human morbidity and mortality, and a powerful driver of pathogen evolution. Their clinical and pathological spectrum reflects the ability of co-infecting pathogens to interact with each other, resulting in synergistic or antagonistic effects that may alter the course and clinical presentation of disease, posing significant diagnostic and therapeutic challenges.

Visceral leishmaniasis and malaria are two vector-borne diseases that co-exist in many areas around the world. Caused by parasites of the genus *Leishmania* and *Plasmodium*, respectively, visceral leishmaniasis and malaria share part of their environmental, clinical and pathogenetic features. This suggests that the two diseases could co-occur and cross-interact in the same host, although limited data are available on this subject. This thesis presents an overview of clinical and experimental studies focusing on the epidemiology, immunology and parasitology of visceral leishmaniasis – malaria co-infections. By combining clinical and field evidence gathered from across East Africa with in vitro data on the mutual effects of the two parasites on and through the immune system, this thesis provides evidence that visceral leishmaniasis and malaria frequently co-exist in patients living in co-endemic areas, and can interact at immunological and non-immunological level, with potential implications on the course and resolution of the two diseases.
VISCERAL LEISHMANIASIS – MALARIA CO-INFECTIONS

Epidemiological, immunological and parasitological aspects

Erika van den Bogaart
Colofon

VISCERAL LEISHMANIASIS – MALARIA CO-INFECTIONS
Epidemiological, immunological and parasitological aspects

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Epidemiological, immunological and parasitological aspects

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Faculteit der Geneeskunde
What you get by achieving your goals

is not as important as what you become by achieving your goals.

Zig Ziglar

to my family
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Chapter 1

Introduction
The challenge of co-infections and their biological implications

The term ‘co-infection’, or alternatively ‘concomitant infection’, describes the simultaneous infection of a host by multiple pathogens. In its broadest sense, the term applies to all cases of co-existence among genetically different microorganisms, including members of the same species (e.g., those belonging to a different strain or population), for which the term of mixed infections is often preferred.

In nature, co-infections are the rule and this has been recognized since the earliest recorded times, as confirmed by the discovery of eggs from multiple helminth species in human coprolites and other human remains from prehistoric sites. Little has changed since then. Helminth co-infections continue to affect an estimated 800 million people worldwide, mainly in developing countries, while the true prevalence of co-infection is likely to exceed one sixth of the global population, with co-infections outnumbering single infections in many communities. Many of these co-infections involve globally important diseases, such as acquired immunodeficiency syndrome (AIDS) caused by the human immunodeficiency virus (HIV), tuberculosis (TB) or malaria, which combine to devastating effect. In 2014 alone, for example, over 1 million lives were lost to HIV, one fifth of whom due to concomitant TB, the leading cause of death among people living with HIV.

As with any single infection, the epidemiological pattern of co-infections relies on a combination of ecological and biological factors, which shape transmission dynamics both at the community and the individual level. Competition for space and resources structures pathogen communities, selecting species that are better suited for survival in certain environments and promoting biodiversity, whereas host-mediated interactions drive variation in susceptibility and infectiousness within and between individual hosts. It is a truism that a host harboring any infectious agent is not the same as one that is uninfected, just as a host harboring bacteria is not the same as one harboring parasites or viruses. From the moment a host comes in contact with any infectious agent, its immune system begins to mount a characteristic array of responses that are qualitatively different for each pathogen. This diversity of immune responses is achieved through the concerted action of specialized cells and their cytokines that drive polarization of adaptive immunity into either cell- or antibody-mediated responses. Because these immune responses tend to be mutually exclusive (they antagonize each other’s actions by blocking polarized maturation of the opposite cell type or its receptor functions), any pre-existing condition able to trigger a defined cytokine milieu can potentially influence the response to a second stimulus. In other words, cytokines secreted in response to one pathogen may act synergistically, antagonistically or independently with those elicited by another pathogen, enhancing (cross-immunity), suppressing (immune-suppression) or not at all affecting the immune response to each of the two pathogens. Most of inter-microbial interactions result in immune-suppression (representative is the case of individuals developing Burkitt’s lymphoma as a result of their reduced resistance to the Epstein Barr virus following exposure to chronic malaria), but examples of negative interactions between concomitant pathogens abound in wild life as well as in experimental infections. To name just a few, infections with Plasmodium falciparum appear to be suppressed in patients...
co-infected with measles or influenza viruses,\textsuperscript{25} while the presence of an ongoing disease, such as malaria or schistosomiasis protects the host from being re-infected with other plasmodial or schistosome species, respectively.\textsuperscript{26-28}

Not all factors affecting the course and outcome of co-infections are immunological or host-derived. Changes in the microenvironment as a result of the first infection can indirectly affect the fitness of the second infectious agent,\textsuperscript{1} while a number of pathogen-associated molecules are known to directly modulate proliferation of certain co-existent micro-organisms (e.g., the \textit{Leishmania} surface molecule lipophosphoglycan induces transcription of HIV in CD4\textsuperscript{+} T cells).\textsuperscript{29} Timing represents another critical variable in determining the outcome of co-infection, as the nature of host-pathogen interactions varies according to the stage of infection.\textsuperscript{30} As an example, the outcome of dual infections with \textit{Babesia microti} and \textit{Trypanosoma brucei brucei} in mice varies according to when the piroplasm is administered in relation to the trypanosome, with an inhibitory effect that intensifies as the time between co-infection increases.\textsuperscript{31}

Despite the widespread acceptance that different organisms co-existing in the same hosts can, and do, influence one another directly or indirectly, health workers and microbiologists seldom consider more than a single organism at a time.\textsuperscript{1} Literature describing the extent and impact of co-infections remains scanty\textsuperscript{1,8} and much of the relevant scientific evidence continues to be generated under carefully controlled laboratory conditions, in which pathogenic infections typically occur in isolation. Several reasons may account for this contrived approach to reality. The first is that the mechanisms underlying co-infection dynamics are complex to understand, requiring an integrated approach that combines ecological, biological and immunological variables.\textsuperscript{1} The second is that diagnosing a co-infection can be challenging in the field, as most infections lack pathognomonic signs\textsuperscript{32-35} and can give rise to serodiagnostic cross-reactivity.\textsuperscript{36} Clinicians usually follow a differential approach in establishing their diagnoses, making use of diagnostic procedures that hardly detect more than a single infection at a time. All this results in a systematic underestimation of co-infection burden, particularly for those conditions that do not result in an increased mortality risk.\textsuperscript{35}

Recent studies on the epidemiology of poly-parasitism, however, have provided compelling evidence that co-infections are ubiquitous and no longer represented by the one host-one pathogen paradigm, advocating for a new way of conceiving host-pathogen interactions and designing disease control programs.\textsuperscript{37}

\textbf{Visceral Leishmaniasis and Malaria, the diseases}

Visceral leishmaniasis (VL) and malaria are two severe infectious diseases caused by parasites of the genus \textit{Leishmania} and \textit{Plasmodium}, respectively. Endemic in large areas of the tropics and subtropics, the two diseases share a similar vector-borne transmission, with female insects spreading infection by feeding on the blood of vertebrate hosts. Sand flies of the genus \textit{Phlebotomus} and \textit{Lutzomya} are responsible for transmitting VL in the Old and in the New World, respectively,\textsuperscript{38} whereas \textit{Anopheles} mosquitoes act as malaria vectors throughout all endemic regions.\textsuperscript{39}

Visceral leishmaniasis, also known as kala-azar, black fever or Dumdum fever, presents as one
and the most severe of several clinical syndromes associated with human leishmaniasis; the other ones being (diffuse) cutaneous leishmaniasis (CL), muco-cutaneous leishmaniasis (MCL) and post kala-azar dermal leishmaniasis (PKDL). Cutaneous leishmaniasis is the most common form of the disease and presents with skin lesions on exposed parts of the body, which may heal spontaneously leaving disfiguring scars. In MCL, patients suffer from progressively destructive ulcerations of the mucosa, extending from the nose and mouth to the pharynx and larynx. These lesions are not self-healing and usually appear months or years after a first episode of CL. Visceral forms of leishmaniasis arise as parasites disseminate throughout the reticulo-endothelial system and invade internal organs, particularly liver, spleen and bone marrow. These syndromes are typically fatal if left untreated. After treatment, VL may progress to PKDL, a dermatological sequel that is frequently observed in Sudan and more rarely in other East African countries and on the Indian subcontinent.

As many as 21 Leishmania species are pathogenic for humans, with clinical manifestations depending on complex interactions between the virulence characteristics of the infecting Leishmania species and the immune responses of its host. As a result, different species generally cause different clinical forms of the disease (e.g., members of the Leishmania tropica and Leishmania mexicana complexes usually cause CL, whereas Leishmania braziliensis is the etiological agent of most MCL cases), but a single Leishmania species can produce more than one clinical syndrome, and each syndrome can be caused by multiple species. Accordingly, several parasites causing CL can visceralize (e.g., L. tropica), but only two species routinely do so (Leishmania donovani and Leishmania infantum), and these are the causative agents of most human VL worldwide.

In contrast to the complex etiology of leishmaniasis, human malaria is caused by six species of Plasmodium only (P. falciparum, P. vivax, P. malariae, P. ovale curtisi, P. ovale wallikeri and P. knowlesi), of which P. falciparum and P. vivax are by far the most common. P. vivax is the most frequent and widely distributed cause of recurrent malaria, causing almost half the cases of malaria outside Africa. By contrast, P. falciparum, the deadliest of the six Plasmodium species, predominates across sub-Saharan Africa, where it accounts for the large majority of malaria infections and deaths. The other human malaria parasite species are less common, with P. ovale causing less than 5% of malaria cases, primarily in sub-Saharan Africa, and P. malariae being widespread but rare. Both species, along with most P. vivax infections, cause a milder and self-limiting form of malaria, known as ‘benign malaria’, that opposes to the potentially severe syndromes caused P. falciparum and P. knowlesi. The latter is a primate malaria parasite mostly found in South-East Asia that leads to severe malaria in about 2% of cases. It causes malaria in long-tailed macaques (Macaca fascicularis), but it may also infect humans, accounting for up to 70% of malaria cases in certain areas of South-East Asia.

Epidemiology of Visceral Leishmaniasis and Malaria

To date, a total of 80 countries and 1 territory on 5 continents reported endemic VL transmission (Figure 1). Of these, Bangladesh, India, Brazil, Sudan, South Sudan and Ethiopia harbor more than 90% of global VL cases. Overall, official case tolls total more than 58,000 cases of VL
per year, but this figure is believed to be a substantial underestimate of the actual disease incidence, due to poor reporting and detection of VL cases. Global estimates of VL occurrence and its underreporting levels across different endemic foci indicate that approximately 0.2 to 0.4 million people are affected by the disease each year, of whom 20,000 to 40,000 die, ranking VL as the second largest parasitic killer in the world after malaria.

Worldwide, malaria continues to take a huge toll on human health, both in terms of morbidity and mortality. Despite increasingly successful efforts to eliminate the disease, 97 countries still reported ongoing malaria transmission in 2014 (Figure 2), the majority of which (80%) occurred in just 18 countries across sub-Saharan Africa. Overall, an estimated 198 million malaria cases were reported in 2013 alone, resulting in 584,000 deaths, of which 78% were estimated to occur in children under the age of five. Whereas for P. vivax, three Asian countries (India, Indonesia and Pakistan) accounted for more than 80% of estimated cases, the global burden of malaria mortality and morbidity was dominated by P. falciparum malaria in sub-Saharan Africa: the Democratic Republic of the Congo and Nigeria together accounted for 39% of the global total of estimated malaria deaths and 34% of cases in 2013.

The epidemiological pattern of the two diseases varies upon the endemic region, reflecting differences in the parasite species, the ecology of its transmission sites and the individual susceptibility of its hosts. In the Mediterranean basin and in Latin America for example, where VL is caused by L. infantum (also known as Leishmania chagasi in South America), the
prevalence of the disease is low (≤1%) and transmission is anthropozoonotic, with domestic and wild canids acting as main reservoirs of infection.\textsuperscript{42,51} Peridomestic sandflies living in the yards of rural villages or suburbs are largely responsible for spreading the disease, although other routes of transmission, including parenteral, congenital and sexual exposures, have been occasionally reported.\textsuperscript{52,53} Children and an increasing rate of immunosuppressed individuals, such as HIV-infected patients\textsuperscript{54} and patients under immunosuppressive therapy,\textsuperscript{55,56} are mostly affected, whereas infected adults typically carry the parasite asymptomatically – between 2% and 40% of the population living in the Mediterranean region are carriers of \textit{Leishmania} parasites.\textsuperscript{57,58}

In contrast, in East Africa and on the Indian subcontinent, VL affects all age groups. CAUSED by \textit{L. donovani} \textit{sensu stricto}, the disease is usually considered to be an anthroponosis, with PKDL cases being the putative reservoir between epidemic cycles.\textsuperscript{51} The landscape epidemiology of \textit{L. donovani} transmission differs across the Asian and African continents:\textsuperscript{42} on the Indian subcontinent, most foci are in long-established villages with sedentary populations, whereas in East Africa, migratory populations are also at high risk, including cattle herdsmen and villagers displaced by drought and warfare.\textsuperscript{42} Migration, along with lack of control measures and HIV co-infection, have been recognized as being the main factors driving the increased incidence of VL worldwide\textsuperscript{41,59,60} and the severe outbreaks that have occurred in the past, like in South Sudan, where in a context of civil war and famine, VL killed an estimated 100,000 people out of a population of 280,000 between 1984 and 1994.\textsuperscript{61}

Malaria transmission is also widely heterogeneous around the world, both across and within...
countries, reflecting differences in the density, longevity, biting habits, and efficiency of the mosquito vector. Those that are long-lived and robust to environmental change, occur in high densities in tropical climates, breed readily, and preferentially bite humans are the most effective in transmitting the disease – e.g., the *Anopheles gambiae* complex in Africa. In most of Asia and South and Central America, where transmission is mainly low and seasonal, *P. falciparum* and *P. vivax* malaria have roughly an equal prevalence. In these areas, most people typically receive one or fewer infectious bites per year – the so-called entomological inoculation rate (EIR). By contrast, transmission intensities are much higher in much of sub-Saharan Africa and in parts of Oceania. Here, EIRs can be as high as 1,000 per year and transmission occurs all year round, resulting in a pronounced malaria morbidity and mortality among children and pregnant women, while the rest of the population has become immune. In the sub-Sahel region from Senegal to Sudan, transmission is intense but largely confined to the 3-4 month rainy season. In these areas where malaria transmission is unstable (low and erratic), full protective immunity is not acquired, and symptomatic disease can occur at all ages. In such settings, changes in environmental, economic, or social conditions – e.g., heavy rains after drought or large population movements – together with a breakdown in malaria control and prevention services (often because of armed conflicts) can result in epidemics, with substantial mortality in all age groups. Endemic *P. vivax* infections occur well beyond the tropics and subtropics with the exception of most of West and Central Africa, where the absence of the Duffy gene amongst the local populations prohibits the parasite from infecting them. Strong and complex linkages associates VL and malaria to poverty, as confirmed by the high disease prevalence among marginalized communities, in countries that are among the least developed in the world or in the poorest regions of so-called ‘middle-income’ countries (such as Bihar State in India). Poor housing conditions and environmental sanitation, along with lack of protective bed nets, increase the risk of acquiring the infection (sand flies breed in cracks of mud-plastered houses and moist soils and are attracted to crowded households), whereas malnutrition and immunosuppression increase the risk that an infection will progress to clinically manifested disease and result in severe complications.

**Biology of Visceral Leishmaniasis and Malaria**

**Parasite life cycle**

Both VL and malaria are caused by dixenic protozoan parasites that cycle between an insect and a vertebrate host (Figures 3 and 4). Inhabiting the arthropod vector as free-living organisms, *Leishmania* and *Plasmodium* parasites have developed unique adaptive mechanisms that enable them to survive as obligate intra-cellular organisms in the mammalian host, where they infect professional phagocytes (such as macrophages) and hepatocytes/erythrocytes, respectively.

The cycle begins with inoculation of motile forms – *Leishmania* flagellated promastigotes and *Plasmodium* sporozoites – into the dermis of the vertebrate host. Here, in the case of *Leishmania*, the metacyclic promastigotes from the initial inoculum (or those that have been released from infected neutrophils) are quickly internalized by tissue-resident phagocytes (macrophages and Langerhans cells) and by inflammatory monocyte-derived dendritic cells that infiltrate from
Figure 3. Life cycle of Leishmania parasites\textsuperscript{21} (adapted by permission from Macmillan Publishers Ltd © 2002).

the blood to the inoculation site, where they may facilitate parasite trafficking to the draining lymph nodes.\textsuperscript{68} Upon phagocytosis, metacyclic promastigotes transform into round-shaped, aflagellated amastigotes (mammalian stage) that undergo replication by binary fission, eventually overburdening the infected cell and causing its rupture. The so-released amastigotes proceed then to infect other surrounding phagocytes, perpetuating the infection to the reticuloendothelial system via the lymphatic and blood circulations and (in case of visceral disease only) causing infiltration of the bone marrow, spleen and liver.\textsuperscript{68}

Conversely, the Plasmodium sporozoites travel to the liver within few minutes from inoculation, invading the hepatocytes where they begin to multiply. After about a week, the liver schizonts burst, releasing into the bloodstream thousands of merozoites that infect the erythrocytes and begin the asexual cycle.\textsuperscript{62} Inside the red blood cells, parasites undergo several developmental stages (ring form, trophozoite and schizont), marked by a progressively increasing size, alterations in cell membrane composition to facilitate importation of nutrients (inserting new parasite-derived proteins and exposing cryptic surface antigens), and accumulation of malaria pigment to dispose of the toxic heme waste product. By the end of the intra-erythrocytic lifecycle, when most of the red blood cell contents have been consumed and several nuclear divisions have taken place, the erythrocytic schizont is ready to burst and release between 6 and 30 daughter merozoites, each of which can invade erythrocytes and repeat the cycle.\textsuperscript{62} Illness starts 6-8 days after parasites have emerged from the liver, when total asexual
parasite numbers in the circulation reach about 50/μL of blood (roughly 100 million parasites in the blood of an adult). In P. vivax and P. ovale infections, some intra-hepatic forms remain dormant as hypnozoites for between 2 weeks and more than a year (depending on geographic origin), before awakening to cause the relapses that characterize these infections.

Few days after the peak of asexual parasitaemia, some blood-stage parasites develop into longer-lived sexual forms (gametocytes) that, upon uptake by feeding anopheline mosquitoes, initiate sporogony in the insect midgut and produce infecting sporozoites.

Similarly, sandflies become infected by ingesting Leishmania-harboring cells during blood meals. After entering the sandfly midgut, amastigotes undergo several morphological transformations that culminate with the development of metacyclic promastigotes, ready to infect new vertebrate hosts.

**Hemozoin**

During its intra-erythrocytic cycle, the malaria parasite feeds on host cell hemoglobin, liberating toxic free heme that is detoxified via the malaria pigment, hemozoin (HZ). Hemozoin, a yellowish, optically birefringent crystal, structurally identical to synthetic β-hematin, is composed by heme dimers that are assembled in the digestive food vacuole of the parasite, where they accumulate and persist until schizont rupture. It is estimated that at the end of the intra-erythrocytic cycle, up to 80-90% of all heme iron is localized within the parasite food vacuole, resulting in as much as 0.2-2.0 grams of HZ being produced by P. falciparum after each cycle, assuming 1-10% parasitaemias.

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**Figure 4. Life cycle of Plasmodium parasites.**

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At the end of the asexual cycle, when the infected erythrocytes rupture, daughter merozoites are released into the blood stream along with HZ crystals, that are rapidly engulfed by the phagocytic cells in the peripheral blood and in the reticulo endothelial system. Here, HZ persists undigested, withstanding phagosome degradation and possibly outliving the cells in which it resides, as demonstrated by the persistence in the peripheral circulation of pigment-containing monocytes and pigment-containing neutrophils, and by the accumulation of HZ in various organs (including liver, spleen, and bone marrow) after extended periods of up to 270 and 196 days post-infection. Importantly, these studies noted that HZ appeared to be actively redistributed, both between organs (e.g., from liver to spleen) and inside organs (e.g., between red and white pulp in the spleen).

For a long time, HZ has been considered a metabolically inert side product of hemoglobin digestion. Recent research, however, has led to the recognition that HZ acts as a key factor in malaria-associated immunopathology, although an exact role is yet to be defined. The reported immunomodulating effects of HZ include: (1) changes in the production of pro- and anti-inflammatory cytokines, chemokines, and other effector molecules; (2) reduced phagocytic activity and oxidative burst in HZ-laden monocytes/macrophages; (3) changes in nitric oxide (NO) release, whose up- and down-regulation may contribute to the pathogenesis of severe malaria and malaria-mediated immunosuppression, respectively; (4) activation and migration of neutrophils; (5) induction of matrix metalloprotease 9, which causes increased inflammation and extravasation of blood cells; (6) down-regulation of cyclooxygenase-2 and prostaglandin E2, thought to contribute to cerebral malaria and anemia; (7) changes in the activation status of innate immune responses, that range from Toll-like receptor-9-mediated activation of dendritic cells to impaired maturation of dendritic cells and reduced expression of cell-surface markers on monocytes; (8) impaired T-cell and B-cell responses. Some of these studies examining the effect of HZ on host immune cells have resulted in conflicting evidence, as it is the case for activation-suppression of dendritic cells, which may be partly explained in terms of dose-dependency. Specifically, smaller quantities of HZ, at the beginning of the infection, may act by activating dendritic cells, whereas larger amounts may be associated with a suppressive effect. Finally, some doubts remain as to whether these immunomodulating effects are caused by the HZ alone or by HZ-associated lipids, proteins or Plasmodium DNA.

**Immunopathogenesis of Visceral Leishmaniasis and Malaria**

As for most successful pathogens, the remarkable ability of *Leishmania* and *Plasmodium* parasites to evade host immunity and exploit its machinery is the key to their success and pervasiveness. Immunological elusion, achieved through the controlled suppression of innate and cell-mediated immunities in the case of *Leishmania* spp. or via epigenetic switch and sequestration of infected erythrocytes in the case of *Plasmodium* spp., ensures the parasite may escape host clearance and combines with pathogenic manipulation of host pathways to preserve parasite fitness. While improving the chance that the parasite may complete its life cycle, these virulence determinants come at a cost to the host, who suffers the pathological
Introduction

consequences of the infection and of the immune response that ensues.\textsuperscript{117,118}

It is generally accepted that in the presence of an infection, naïve CD4\textsuperscript{+} T helper cells (Th)0 differentiate into either Th1 or Th2 lymphocytes, that – upon release of specific cytokine patterns – drive the immune system towards cell- or antibody-mediated responses, respectively. CD4\textsuperscript{+} Th1 cell-mediated responses involving type-1 cytokines (e.g., interferon-gamma (IFN-γ)) are ideally suited for clearing intracellular protozoa through activation of specific effector cells, whereas extracellular pathogens are predominantly controlled by CD4\textsuperscript{+} Th2 cells, through a network of type-2 cytokines (e.g., interleukin (IL)-4 and IL-5) that promote expansion of antibody-producing B cells.\textsuperscript{119} In addition, CD4\textsuperscript{+} Th0 cells may also differentiate into either regulatory T cells (Treg) or Th17 lymphocytes. Treg cells act by suppressing type-1 and pro-inflammatory responses through IL-10 and transforming growth factor-beta (TGF-β) signaling, whereas IL-17 and IL-6 released by Th17 lymphocytes promote inflammation (Figure 5).\textsuperscript{120}

Figure 5. Summary of the major CD4\textsuperscript{+} T cell differentiation pathways and related cytokines (boxes),\textsuperscript{222} with their impact on VL and malaria diseases.\textsuperscript{222} Cross-regulating cell- and cytokine-mediated mechanisms shape the immune reaction across the four response arms and prevent from deleterious polarizations. Skewing of the initial T cell differentiation towards a T helper cell (Th)1 response is pivotal for clearing Leishmania or early Plasmodium infections, but it requires a T regulatory (Treg) counterbalance to mitigate their clinical course. Failure to activate Th1 cells, e.g., due to the inhibiting effect of IL-10 and its producing cells, may result in the uncontrollable proliferation of L. donovani and P. falciparum parasites, the latter causing severe complications under a pro-inflammatory milieu (adapted with permission).
In human VL, manifestations range from asymptomatic infection to progressive and potentially fatal visceral disease, pending on the host’s immune status. Clinically silent infections require robust cell-mediated immune responses, mounted upon a network of Th1 cytokines (Figure 5) and enforced by classically activated macrophages through a battery of microbicidal mechanisms, that include release of pro-inflammatory cytokines, activation of natural killer (NK) cells, up-regulation of inducible nitric oxide synthase (iNOS), and production of nitrogen and oxygen radical species. The observation that peripheral blood mononuclear cells (PBMCs) isolated from individuals with asymptomatic or subclinical VL infections proliferate and release IL-2, IL-12 and IFN-γ in response to L. donovani stimulation confirms the central role played by the Th1 immunity in parasite killing. Conversely, failure to control L. donovani infections has been associated with a profound unresponsiveness of PBMCs and T cells to L. donovani and an increased release of IL-10 by Treg cells (Figure 5). Just this cytokine, possibly in combination with IL-4, IL-13 and IgE, is believed to act as one of the main immunosuppressive factors that drives replication of Leishmania, as confirmed by its progressive decrease in VL patients recovering from disease and the ability of anti-IL-10 antibodies to restore antigen-mediated proliferation of PBMCs and IFN-γ release in cells isolated from VL patients. Other potentially immunosuppressive agents reported during human VL include soluble IL-2 and IL-4 receptors, IL-G and TGF-β, as confirmed by their increased serum levels during symptomatic disease.

In addition to confer sterile protection against VL, cell-mediated immunity also plays a key role in controlling malaria infections, both at pre- and intra-erythrocytic stages. Early type-1 responses dominated by IFN-γ signaling have long been established as driving the pre-erythrocytic immunity against both murine and human malaria (Figure 5), as demonstrated by a wealth of in vivo and ex vivo evidence. By contrast, its protective role against blood-stage parasites has only recently been recognized, and is believed to be accomplished via macrophage activation. The observations that infected erythrocyte-specific IFN-γ responses correlate with protection against parasitaemia in animals as well as in patients, and that children with prior mild malaria display an enhanced ability to express iNOS over children with prior severe malaria converge towards an IFN-γ-led anti-parasitic effect. Consistent with this view is also the finding that tumor necrosis factor-alpha (TNF-α) and reactive oxygen intermediates induced by Plasmodium spp. contribute to the control and elimination of blood-stage parasites. However, whilst early activation of Th2 cells is deleterious for infection clearance and correlates with malaria severity, its involvement during the erythrocytic stage is essential for counterbalancing the Th1 cytokines and triggering antibody-mediated responses (Figure 5), important for protection against malaria. A prominent role in switching from Th1 to Th2 responses is attributed to IL-10, whose anti-inflammatory properties act by limiting the damage inflicted on normal tissues by excessive Th1 and pro-inflammatory responses, as seen in the immunopathology of severe malaria. Whilst uncontrolled release of IFN-γ, TNF-α and IL-12 caused by parasite-mediated inhibition of IL-10 and TGF-β signaling have been firmly associated with an increased risk of developing cerebral malaria, low IL-10/TNF-α ratios are considered predictive of malarial severe anemia.
Clinical presentation of Visceral Leishmaniasis and Malaria

Following an incubation period that generally lasts between 2 and 6 months, but that can be as short as a few weeks or as long as several years, VL patients present with symptoms and signs typical of a persistent systemic infection.\(^\text{41}\) Fever, at times intermittent and usually associated with rigor and chills, malaise, weakness, night sweats, loss of appetite and weight are common manifestations of VL, whose onset is usually insidious or subacute, with slow progression of symptoms over a period of months.\(^\text{147}\) In rare cases, acute febrile illness can occur with rapidly progressive symptoms. Hepato-splenomegaly resulting from parasite invasion of the reticulo-endothelial system, along with anemia caused by the persistent inflammation state, hypersplenism and/or bleeding are frequently observed, usually in combination with pancytopenia and hypergammaglobulinemia.\(^\text{41}\) Lymphadenopathy is frequent in East Africa, but rare outside this region,\(^\text{148,149}\) whilst melanocyte stimulation and xerosis typically occurred (today this symptom is uncommon) amongst VL patients from the Indian subcontinent, causing the characteristic skin hyper-pigmentation that has earned the disease the name of ‘kala-azar’ (black fever in Hindi).\(^\text{42}\)

As the disease advances, the risk of co-infections (such as pneumonia, diarrhea or tuberculosis) flaring up as a result of host immunosuppression increases, leading to potential misdiagnosis and worsened prognosis. Young malnourished children are most susceptible to developing progressive infection; those who present later in the course of the disease may present with edema caused by hypoalbuminemia, hemorrhage caused by thrombocytopenia, or growth failure caused by features of chronic infection.\(^\text{150}\) If the disease is left untreated, death usually occurs within 2 years as a result of massive bleeding (secondary to infiltration of the hematopoietic system), severe anemia, immunosuppression, and/or secondary infections.\(^\text{42}\) Importantly, infection with \textit{Leishmania} spp. does not always equate with clinical illness, as infected individuals, documented with a positive skin test to \textit{Leishmania} antigen (Montenegro test), can remain asymptomatic for life.\(^\text{41}\)

The non-specific nature of VL symptoms entails its differential diagnosis encompassing a variety of other infectious and febrile systemic illnesses, including malaria. In endemic areas, indeed, malaria is often the most common cause of fever,\(^\text{62}\) with characteristic spikes being described every day for \textit{P. knowlesi} (quotidian malaria), every 2 days for \textit{P. vivax}, \textit{P. ovale} and less commonly \textit{P. falciparum} (tertian malaria), and every 3 days for \textit{P. malariae} (quartan malaria). In practice though, this periodicity is rarely seen, particularly for \textit{P. falciparum}, and is most likely to occur if the infection is left untreated and becomes synchronous. Fever presents after an incubation period of about 2 weeks (range 6 to 40 days, depending on the parasite species), and is usually associated with a set of non-specific symptoms, such as chills, sweating, malaise, headache, body aches, nausea, vomiting and orthostatic hypotension.\(^\text{151}\) Physical findings may include mild anemia and a palpable spleen. Chronic anemia and splenomegaly are common among young children living in endemic areas, and are often due to multiple causes in addition to repeated malaria infections (including iron and other nutritional deficiencies as well as intestinal geoehelminth infection).\(^\text{37}\) Mild jaundice may also develop, but it is rather uncommon in children, who instead suffer from a more frequent enlargement of the liver. In individuals at risk, like non-immune persons, children...
to 36 months of age, immunocompromised patients (including splenectomized individuals) and pregnant women, *P. falciparum* malaria and more rarely *P. vivax* malaria may progress to severe disease, sometimes very rapidly, with manifestations depending on age.\textsuperscript{62,152} Severe anemia and hypoglycaemia are more likely to occur in children, whereas acute pulmonary edema, acute kidney injury, and jaundice are more common in adults. Coma (cerebral malaria) and acidosis, on the other hand, occur in all age groups.\textsuperscript{62} Manifestations of complicated malaria arise as the parasitaemia exceeds 2%, and mainly result from the ability of parasitized (and non-parasitized) erythrocytes to adhere to small blood vessels; a phenomenon known as cytoadherence that cause small infarcts, capillary leakage, and organ dysfunction.\textsuperscript{62} These syndromes can portend a grave prognosis, with fatal outcomes in nearly all cases that fail to receive treatment, and a mortality rate of 15% to 20% amongst patients properly managed in intensive care units.\textsuperscript{62,151}

**Treatment of Visceral Leishmaniasis and Malaria**

As an effective vaccine for VL or malaria has yet to be licensed,\textsuperscript{153} chemotherapy remains the mainstay for treatment and prevention of these two diseases (chemoprophylaxis for malaria only). Antimicrobial drugs selected upon local drug resistance patterns, treatment guidelines, tolerability, availability, and affordability are administered as part of the standard therapeutic approach in combination with supportive care to address concomitant anemia, hemorrhagic complications, malnutrition and secondary infections.\textsuperscript{41}

Pentavalent antimonials, the first-line treatment in most endemic areas since the 1940s, are toxic drugs that frequently cause adverse, sometimes life-threatening effects, such as cardiac arrhythmia and acute pancreatitis.\textsuperscript{41} Due to their high failure rates in some areas of the Indian subcontinent, their use as first-line treatment in these regions has been dismissed in favor of conventional amphotericin B.\textsuperscript{154} Amphotericin B has an excellent cure rate (up to 100%), but requires slow intravenous administration, that commonly cause infusion-related reactions – e.g., fever, chills, thrombophlebitis – and occasionally serious toxicity – e.g., hypokalemia, nephrotoxicity, myocarditis, and even death.\textsuperscript{155} Liposomal amphotericin B (AmBisome) – a relatively new drug that combines high efficacy with low toxicity – is currently the preferential treatment in high-income countries.\textsuperscript{156} Until recently, its use in developing countries was precluded by its high market price, but since it became available at a preferential pricing, the situation has partially improved. Currently, liposomal amphotericin B has been included as first-line VL treatment in East Africa in the 2010 revised World Health Organization (WHO) recommendations.\textsuperscript{51} Miltefosine, which was initially developed as an anti-cancer drug, is the first effective oral drug for VL.\textsuperscript{157} Registered for use in India since March 2002, the drug showed a final cure rate of 94-97% in India\textsuperscript{157-159} and of 85% in Bangladesh\textsuperscript{160} at the dose of 2.5 mg/kg/day for 28 days, while the results of clinical trials conducted in East Africa\textsuperscript{161} and Brazil\textsuperscript{162} are yet to be published (with the exception of one study conducted in north Ethiopia).\textsuperscript{163} Its main limitations include high cost, gastrointestinal adverse effects, occasional hepatic and nephrotoxicity, and teratogenicity (hence its preclusion to pregnant women).\textsuperscript{157} In addition, its long half-life also makes it vulnerable to rapid development of drug resistance, as confirmed by the high failure rates that are already
being reported across India and Nepal.\textsuperscript{164,165} Paromomycin (formerly known as aminosidine) is a broad-spectrum aminoglycosidic antibiotic with good anti-leishmanial activity.\textsuperscript{166} Registered in India in 2006, the drug showed excellent efficacy and safety in a Phase III clinical trial conducted in India,\textsuperscript{167} and an efficacy of 84.3\% in East Africa.\textsuperscript{168} Major advantages of this drug include its low cost and its good tolerability, while the need for a prolonged parenteral administration (three weeks) and for serum transaminase monitoring limits its desirability.\textsuperscript{166} Sitamaquine (WR-6026) is an oral 8-aminoquinoline drug at developmental stage that has completed phase II trials in India, Kenya and Brazil, where it showed cure rates ranging from 27\% to 87\%, and several cases of serious renal adverse events.\textsuperscript{169-171} Combination therapy is the suggested way forward to increase treatment efficacy, prevent the development of drug resistance, reduce treatment duration and perhaps decrease treatment costs.\textsuperscript{41} The association of sodium stibogluconate and paromomycin was found to be safe and effective in early trials conducted in India and East Africa,\textsuperscript{168,172,173} while drug combinations including liposomal amphotericin B and miltefosine have been studied in India\textsuperscript{174} and are currently under evaluation in Bangladesh\textsuperscript{175} and East Africa.\textsuperscript{176}

Artemisinin-based combination therapies (ACTs) are the cornerstone for the treatment of malaria nowadays. Recommended in 2001 by the WHO as the first-line treatment of uncomplicated \textit{P. falciparum} malaria (severe \textit{P. falciparum} malaria is treated initially with intravenously administered artesunate, followed by a suitable oral drug (combination)), these treatments have substantially contributed to the reduction of global malaria morbidity and mortality since their use became widespread approximately 10 years ago.\textsuperscript{177,178} In the 5 currently recommended ACTs, two active anti-malarial drugs with different mechanisms of action (one being an artemisinin derivative, such as artemether or artesunate) combine to improve treatment efficacy and minimize the spread of drug resistance, despite the first cases of reduced susceptibility have already been reported in South-East Asia.\textsuperscript{179,177} Artemisinin is a natural product that is isolated from \textit{Artemisia annua} (Asteraceae), a medicinal plant that has been used for over a thousand years in China. Its semi-synthetic derivatives artesunate and artemether are the two most widely used oral artemisinins, available either alone or in combination.\textsuperscript{181} Amodiaquine, mefloquine, chloroquine and their historic parent compound quinine are an important group of 4-aminoquinoline drugs that act as blood schizonticidal against all \textit{Plasmodium} species pathogenic to man, and as gametocytocidal against \textit{P. vivax} and \textit{P. malariae} in the case of mefloquine and quinine.\textsuperscript{177} Quinine is a quinidine alkaloid contained in the bark of the cinchona tree (\textit{Cinchona calisaya} and \textit{Cinchona succirubra}). Its use as an anti-malarial agent dates back as early as the 1600s, when malaria patients were cured with infusions of bark obtained from plants growing in the Peruvian Amazon. Nearly 400 years later, quinine remains an important drug for treating severe \textit{P. falciparum} malaria (in the absence of i.v. artesunate), despite its toxicity particularly when used for extended periods of time.\textsuperscript{182} Sulfadoxine and pyrimethamine are two schizonticidal that act by inhibiting the tetrahydrofolate synthesis pathway of the malaria parasite. Their use in fixed-dose associations (Fansidar) produces synergistic effects sufficient to cure sensitive malaria strains.\textsuperscript{181} The radical cure of \textit{P. vivax} and \textit{P. ovale} requires primaquine, an 8-aminoquinoline that targets all stages of the \textit{Plasmodium} life cycle (including hypnozoites), preventing late relapses.\textsuperscript{183} Prima-quin
metabolites, however, cause severe hemolytic anaemia and methemoglobinemia in patients who are genetically deficient in glucose-6-phosphate dehydrogenase (G6PD), imposing a pre-screening requirement for G6PD deficiency that limits its use. Tafenoquine, a novel 8-aminoquinoline with tissue-schizonticidal activity, currently still remains in clinical trials. \(^{183}\)

**The search for new drugs**

Current therapeutic options for VL rely on a handful of drugs that suffer from major limitations, such as severe toxicity, high costs, difficult route of administration and increasing inefficacy due to emergence and spread of resistance. New, safer and more effective oral treatments are urgently needed to improve clinical resolution of the disease and to reduce its transmission rates across endemic areas. Unfortunately, the lack of a profitable market and effective mechanisms related to public health policy, financing, and drug discovery and development expertise and capacity has largely inhibited the pharmaceutical industry from investing in the development of new drugs for VL and other neglected tropical diseases. \(^{185}\) – during 1975-2004, only 21 (1.3%) out of 1556 approved drugs were specifically developed to address neglected tropical diseases. \(^{186}\) Recent changes in the drug discovery model for these diseases (product development and private-public partnerships) have dramatically improved their drug Research & Development landscape. \(^{185,187}\) Nevertheless, the current pipeline for anti-leishmanial drugs remains substantially weak, with few new chemical entities expected to enter the market in the coming years. \(^{188}\)

One of the main difficulties that hinders the systematic search for new anti-leishmanial compounds is the complexity of performing adequate phenotypic screening. \(^{188}\) The *Leishmania* life cycle is composed of two stages, the free living promastigote stage found in the vector, and the obligate intracellular amastigote stage that lives in the vertebrate host. Whilst promastigotes can be easily adapted for *in vitro* culturing and testing due to their axenic requirements, *in vitro* maintenance of the clinically relevant stage of disease necessitates a suitable host cell model that inevitably interferes with the standard high-throughput parasite viability assays. As a result, screening of potential anti-leishmanial compounds has relied for decades upon the use of promastigotes, and only more recently of axenic amastigotes, for which simple and efficient *in vitro* assays have been made available. \(^{189-195}\) However, lack of consistency between insect and mammalian stage drug susceptibilities has been frequently reported, emphasizing the need of testing against the intracellular amastigote stage already at primary hit-discovery screens. \(^{196,197}\) Assessment of anti-leishmanial activity against the vertebrate host stage traditionally relies on phenotypic assays, with major problems related to data quality and poor performance. \(^{198,199}\) With the advent of new technologies, however, it has now become possible to increase the throughput of these very labor-intensive assays. Two main methods are in use for the detection of intracellular parasites: plate-reader-based methods that rely on reporter constructs \(^{197-199}\) and automated high-content microscopy-based counting. Both platforms have radically improved the screening capacity for leishmaniasis, \(^{202-205}\) but bear the disadvantages of using transgenic *Leishmania* spp. in the case of reporter-gene assays or requiring a complex technology in the case of automated microscopy. This precludes their broad implementation and routine application to clinical isolates, compelling
the development of a simpler and more widely applicable assay.

Phenotypic screens are also key to the discovery of new anti-malarial drugs, as confirmed by the observation that only a minority of compounds currently in clinical testing had molecularly defined targets from the outset. The majority, on the contrary, are derived from phenotypic screening, with their pharmacological targets being identified only at a later stage. This renaissance of phenotype-based screening in the drug discovery against malaria has followed the recent advances in assay automation, image capture and analysis technology, which altogether have noticeably reduced the costs of assaying a compound against primary human erythrocytes infected with \textit{P. falciparum}. As a result, a total of ~6 million compounds have been screened to date, of which, excitingly, more than 25,000 have shown half-maximal (IC\textsubscript{50}) activity at approximately 1 μM or lower against \textit{P. falciparum}. This, along with the new therapeutic agents currently in development, highlights how dramatically improved is the malaria drug pipeline over the past 5 years. It should be noted, however, that most of the medicines under development act as schizonticidal. In an era where the malaria research agenda is set towards eradication rather than control, different approaches are required, including the use of drugs that block transmission and kill the dormant liver stages of \textit{P. vivax} and \textit{P. ovale}. In neither case, unfortunately, the biology can be perfectly reproduced in high-throughput screening formats, as confirmed by the absence of models for the early-stage gametocytes and the primate dormant forms. For these reasons, it is important that the investment in the primary biology continues so that screening against all phases of gametogenesis and primate hypnozoites, or even human cells infected with \textit{P. vivax}, can eventually be performed. New classes of drugs for such stages of malaria parasites would truly be transformative and accelerate the decline of malaria – a change that is urgently needed over the next 20 years.

**Co-infections with Visceral Leishmaniasis and Malaria**

Although concomitant infections by multiple pathogens are ordinary events in nature, many of their fundamental patterns remain undescribed, including the mechanisms that govern the type and magnitude of co-infections and their burden on human health. Interest in unraveling these critical issues has raised in recent years, but the focus has remained disproportionately confined to a small group of infections, at the expense of the global killers that thrive amongst the world’s poorest. In this respect, co-infections with VL and malaria are no exception. Despite the anything-but-negligible co-infection rates that have been sporadically reported across various African and Asian countries, the literature is silent on this subject, and there is virtually no information on how often these co-infections occur and with which consequences for the patient. Given the high burden that VL and malaria impose worldwide, this knowledge gap appears somehow paradoxical, particularly in light of the fact that all conditions apply for the two infections to co-occur and cross-interact in the same host (Table 1).

The first of these conditions requires VL and malaria to share part of their geographical and ecological distribution. Malaria is widespread across much of the tropics and subtropics and so is VL to a lesser extent, resulting in an extensive overlap throughout most \textit{L. donovani} foci and
a part of *L. infantum* ones (Figure 6). Namely, people living in East Africa (Sudan, South Sudan, Ethiopia, Uganda, Kenya, Somalia and Eritrea) are at high risk of co-acquiring VL and malaria, as confirmed by the co-infection prevalences found amongst VL patients (20.8% and 6.4% in two studies conducted in east Uganda and 10.7% in Sudan), while a moderate to low risk exists for people inhabiting certain districts of Brazil and the Indian subcontinent (co-infection rates of 1.2% and 5.9% were found amongst Bangladeshi VL patients and Indian patients with fever and splenomegaly, respectively). The risk of co-acquiring VL and malaria is particularly high for children, who have not yet developed immunity to the diseases, and live in endemic communities where infected adults act as reservoirs of infection. Nomadic populations, and men who work in agricultural or pastoral settings are also at increased risk (particularly those who sleep outside or under acacia-balanite trees), due to the increased time spent outdoors and in proximity of vector breeding sites.

The current knowledge on the immuno-patho-genetic mechanisms of VL and malaria supports the view that the two parasites may interact with each other in the co-infected host, either directly

### Table 1. Selected features of VL and malaria forming the rationale for the co-occurrence and cross-interaction of these two infections.

| Visceral Leishmaniasis | Malaria |
|------------------------|---------|
| **Endemic in East Africa, in the Indian subcontinent and in Latin America.** | Endemic in most of tropics and subtropics. |
| Transmitted by sand flies that breed and shelter in moist soils (cracks of plastered houses, termite hills, etc.). | Transmitted by mosquitoes that breed on water and shelter either indoors (eg., *A. gambiense*) or outdoors (eg., *A. arabiensis*). |
| Transmission throughout the year, but higher during or shortly after the rainy season. | Transmission throughout the year where malaria is stable, during or shortly after the rainy season where unstable. |
| Incubation period averages 2 to 6 months. | Incubation period of 10 days to several months and longer. |
| Children and young adults at higher risk. | Children and pregnant women at higher risk in areas of stable malaria, all age groups where malaria is unstable. |
| Poor housing, crowded households, HIV and malnutrition are main risk factors. | Poor housing and crowded households are important risk factors. |
| Parasites infect phagocytes of the spleen, liver, and bone marrow. | Parasites infect hepatocytes and erythrocytes. Hemozoin accumulates throughout the reticulo-endothelial system. |
| Cell-mediated immunity and IFN-γ responses required for control of infection (asymptomatic infection). | Cell-mediated and IFN-γ responses required for pre-erythrocytic immunity and control of early blood stages. Humoral immunity required for clearing blood stages and contrasting inflammation. |
| Suppression of cell-mediated immune responses by IL-10 predisposes to development of clinical disease. | Suppression of cell-mediated immune responses by IL-10 predisposes to development of clinical disease, but is required to reduce the risk of severe malaria induced by excessive inflammation. |

The current knowledge on the immuno-patho-genetic mechanisms of VL and malaria supports the view that the two parasites may interact with each other in the co-infected host, either directly...
Figure 6. Overlap in the geographical distribution of VL- and malaria-endemic areas, as achieved by graphically overlapping Figure 1 and Figure 2.
or through the immune system. In humans, *Leishmania* and *Plasmodium* parasites have different host cell tropisms (phagocytes in the case of *Leishmania*, hepatocytes and erythrocytes in the case of *Plasmodium*), but the two infections share a number of microenvironments that either support the parasite life cycle or participate in the pathophysiology of disease. For example, the liver which hosts the pre-erythrocytic stage of the malaria cycle is scattered with HZ-laden macrophages (Kupffer cells) during acute malaria\(^7\) and with inflammatory granulomas surrounding infected Kupffer cells during VL.\(^213\) Again, deposits of HZ are found in the spleen,\(^8\) which in turn undergoes extensive micro-architectural remodeling following infections with visceralizing *Leishmania* spp.\(^214\) These alterations at cellular and tissue level, arisen in response to one infection, bear the potential to impact the course and pathophysiology of the other infection, and this holds true to a greater extent for any systemic effect or response (immunological and non) mounted by the host. For example, an ongoing pro-inflammatory and type-1 response initiated in response to a leishmanial infection could potentially improve clearance of *Plasmodium* liver forms, whereas the severe anemia induced by VL could act as a deterrent to blood-stage malaria, in line with previous evidence suggesting that anemia may offer protection against malaria infection.

Conflicting data have emerged from the very few studies that have examined the course of malaria and leishmaniasis co-infections so far. In 1954, the first experimental study ever conducted on this subject demonstrated that multiplication of *Plasmodium berghei* parasites in golden hamsters was inhibited by the concomitant infection with *L. infantum*, but not vice versa.\(^215\) Thirty years later, Coleman *et al.* observed that mice co-infected with *L. mexicana amazonensis* and *Plasmodium yoelii* suffered from an exacerbated course of leishmaniasis,\(^216,217\) which was subsequently attributed to the *Plasmodium chabaudi chabaudi*-triggered release of splenic IL-4 in mice co-infected with *L. infantum*.\(^218\)

Whatever the nature of these interactions taking place during *Leishmania*-*Plasmodium* co-infections, it is clear that their outcome depends upon multiple factors, amongst which the immunocompetency of the host, the EIR, and the stage and order with which the two infections superimpose. Consequently, an arrange of outcomes, rather than a single one, appears to be a more likely scenario, which may have partially contributed to making these co-infections poorly apparent and, as such, prone to neglect.

**Thesis outline**

Despite cases of co-infection with VL and malaria are frequently encountered across co-endemic areas and the two infections have been shown to cross-interact in co-infected animal models, little is known on the extent and course of these co-infections in humans, and even less is known on the nature of the interactions that may take place in the co-infected host.

It has been the aim of the research described in this thesis to provide more insights into the clinical epidemiology and bio-immunology of these co-infections. Designed on the initial, circumstantially-underpinned hypothesis that in co-endemic areas, malaria frequently superimposes in VL patients and exacerbates host pathology as a result of immunological interactions, this dissertation combines clinical and field evidence gathered from patients hospitalized in East Africa with *in vitro* data on the mutual effects of *L. donovani*
and *P. falciparum* or its waste product HZ on and through the innate immune system, respectively.

In relation to clinical epidemiology, the research aimed at exploring the prevalence, features and risk factors for VL-malaria co-infections amongst VL patients hospitalized in two different bioclimatic regions of East Africa, marked by high VL endemicity and different malaria transmission patterns. Accordingly, chapter 2 describes a case-control study performed on a dataset gathered by Médecins sans Frontières (MSF) at Amudat Hospital, north-east Uganda, whereas the results of a multi-center retrospective survey conducted in east Sudan are presented in chapter 3.

With regard to the immunological interactions taking place during these co-infections, an initial exploratory survey was conducted on naturally infected patients from Gedarif State, Sudan. This study, reported in chapter 4, compared the cytokine profiles of co-infected patients over the ones of VL and malaria mono-infected patients and healthy endemic controls. In chapter 5, the effect of *in vitro* concomitant exposure of *L. donovani* and *P. falciparum*-infected erythrocytes on the phenotype and function of human dendritic cells is presented.

One of the common obstacles when dealing with complex models such as those involving two or more pathogens, is the unsuitability or under-performance of existing methodologies, as it was our case when trying to measure by microscopy the growth of *Leishmania* intracellular amastigotes in the presence of other phagocytic meals. The problem was subsequently circumvented by developing a set of two new *Leishmania* viability assays. The first consists of a quantitative reverse-transcriptase PCR that simultaneously monitors viability of the *Leishmania* parasite and its host cells [chapter 6], while the second is a simple enzymatic assay that enables assessment of parasite viability at high throughput [chapter 7]. This latter assay was next applied to the study of HZ-mediated effects on the ability of human and murine macrophages to sustain *Leishmania* invasion and replication [chapter 8].
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Prevalence, features and risk factors for malaria co-infections amongst visceral leishmaniasis patients from Amudat Hospital, Uganda

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Abstract

Background and methodology
Due to geographic overlap of malaria and visceral leishmaniasis (VL), co-infections may exist but have been poorly investigated. To describe prevalence, features and risk factors for VL-malaria co-infections, a case-control analysis was conducted on data collected at Amudat Hospital, Uganda (2000-2006) by Médecins sans Frontières. Cases were identified as patients with laboratory-confirmed VL and malaria at hospital admission or during hospitalization; controls were VL patients with negative malaria smears. A logistic regression analysis was performed to study the association between patients’ characteristics and the occurrence of the co-infection.

Results
Of 2414 patients with confirmed VL, 450 (19%) were positively diagnosed with concomitant malaria. Most co-infected patients were males, residing in Kenya (69%). While young age was identified by multivariate analysis as a risk factor for concurrent VL and malaria, particularly the age groups 0-4 (odds ratio (OR): 2.44; 95% confidence interval (CI): 1.52-3.92) and 5-9 years (OR: 2.23; 95% CI: 1.45-3.45), mild (OR: 0.53; 95% CI: 0.32-0.88) and moderate (OR: 0.45; 95% CI: 0.27-0.77) anemia negatively correlated with the co-morbidity. VL patients harboring skin infections were nearly three times less likely to have the co-infection (OR: 0.35; 95% CI: 0.17-0.72), as highlighted by the multivariate model. Anorexia was slightly more frequent among co-infected patients (OR: 1.71; 95% CI: 0.96-3.03). The in-hospital case-fatality rate did not significantly differ between cases and controls, being 2.7% and 3.1%, respectively (OR: 0.87; 95% CI: 0.46-1.63).

Conclusions
Concurrent malaria represents a common condition among young VL patients living in the Pokot region of Kenya and Uganda. Although these co-morbidities did not result in a poorer prognosis, possibly due to early detection of malaria, a positive trend towards more severe symptoms was identified, indicating that routine screening of VL patients living in malaria-endemic areas and close monitoring of co-infected patients should be implemented.
Author summary

Visceral leishmaniasis (VL) and malaria are two major parasitic diseases sharing a similar demographic and geographical distribution. In areas where both diseases are endemic, such as Sudan, Uganda, India and Bangladesh, co-infection cases have been reported, but features and risk factors associated with these co-morbidities remain poorly characterized. In the present study, routinely collected data of VL patients admitted to Amudat Hospital, Uganda, were used to investigate the magnitude of VL-malaria co-infections and identify possible risk factors. Nearly 20% of the patients included in this study were found to be co-infected with VL and malaria, indicating that this is a common condition among VL patients living in malaria-endemic areas. Young age (≤9 years) was identified as an important risk factor for contracting the VL-malaria co-infection, while being anemic or carrying a skin infection appeared to negatively correlate with the co-morbidity. Co-infected patients presented with slightly more severe symptoms compared to mono-infected patients, but had a similar prognosis, possibly due to early diagnosis of malaria as a result of systematic testing. In conclusion, these results emphasize the importance of performing malaria screening amongst VL patients living in malaria-endemic areas and suggest that close monitoring of co-infected patients should be implemented.

Introduction

Due to extensive overlap in the geographical distribution of many infectious diseases, multiple infections appear to be the rule rather than the exception in many tropical and subtropical regions. Poly parasitism, in particular, may predominate in rural areas of developing countries, where poor sanitation and economic conditions allow the uninterrupted transmission of many parasites. Prevalences of multiparasite infections above 30% have been shown to occur regularly throughout South-East Asia and much of Central and West Africa, with communities harboring multiple parasites in up to 80% of their population. Importantly, the different combinations in which pathogens might co-exist in a certain population and their distribution therein, do not result from a random process, but they are rather part of a selection governed by a variety of ecological and host factors, which include the biological interactions of the parasites within the host. These interactions may affect the pathogenicity of the infective agents, resulting in a spectrum of effects on the polyparasitized host, ranging from exacerbation to amelioration of disease severity. Despite the recent upsurge in investigations targeting multiple helminth species and Plasmodium-helminth co-infections, the actual extent of polyparasitism and its pathological consequences remain largely unassessed. The scarcity of information is particularly striking for infections that are clinically not apparent or lack pathognomonic signs. As the tropics and sub-tropics are burdened with infectious diseases sharing similar clinical pictures, recognition of these diseases occurring in the same patient might be difficult in poor resource settings.

Visceral leishmaniasis (VL) is a life-threatening syndrome caused by protozoan parasites of the Leishmania donovani complex. Most cases occur in East Africa, South-East Asia and Brazil, where nearly 0.5 million people get infected each year, half of whom are children. Differential diagnosis...
of VL often includes malaria amongst other febrile splenomegalies, due to its geographical and clinical overlap. Malaria, in fact, is widespread in tropical and subtropical regions of the world, where it accounts for more than 250 million cases annually, the vast majority of which occurs among children under 5 years old. Transmission can occur throughout the year or be seasonal, depending on the region. In the latter case, transmission seasons for VL and malaria may not coincide, but the two diseases still overlap, due to the longer incubation period of VL. The overlap in disease distribution suggests the two diseases could co-occur in the same host. Nonetheless, figures describing the extent of VL and malaria co-infections are not readily available in literature.

To gather evidence on the occurrence of such co-morbidities, a systematic review of the present literature was first conducted (Figure S1). This review showed that cases of VL and malaria co-infections have been reported across various African and Asian countries, with the prevalence among VL patients ranging from 20.8% and 6.4% in Uganda to 10.7% in Sudan and 1.2% in Bangladesh and a rate of 5.9% among Indian patients with fever and splenomegaly. With the exception of the case-reports whose evidence remains anecdotal, no further details on these co-infections are described, preventing identification of possible risk factors and specific features associated with these co-morbidities. To address this issue, a retrospective case-control analysis was performed on clinical data of VL patients living in the Pokot territory of Kenya and Uganda and admitted to Amudat Hospital, northeast Uganda. The area, a semi-arid lowland region mainly inhabited by pastoralists of the Pokot tribe, is part of a large VL endemic focus, which includes Pokot County in Uganda and extends eastwards to Pokot North, West Pokot, East Pokot and Baringo Districts in Kenya. Here, VL is caused by *Leishmania donovani* and transmitted by the sandfly *Phlebotomus martini*. Malaria represents another major health problem in the region, with incidence rates ranging from 20% in Kenya to 30% in Uganda. Hookworms and other neglected tropical diseases such as lymphatic filariasis have also been identified as endemic in the area. Given such high disease burdens, it is hardly surprising that polyparasitism may represent a common condition.

The main goal of the present study was to describe the prevalence of VL and malaria co-infections amongst VL patients attending Amudat Hospital and identify risk factors associated with this condition. Recognition of the burden posed by these co-morbidities among different patient groups may contribute to improve the clinical management of VL in malaria-endemic areas.

### Methods

#### Ethics statement

The analysis was conducted on anonymized data, collected as part of routine patient care; no additional investigations were performed. Therefore, no prior informed consent from the patients was required. Ethical approval for the study was obtained from the MSF Ethics Review Board (8th April 2011).

#### Patients

Data of suspected VL patients admitted to Amudat Hospital, a 120-bed rural hospital located in Pokot County, Amudat District, Uganda, were collected between January 2000 and December 2006 by Médecins sans Frontières (MSF-Swiss Section). MSF support to Amudat Hospital medical activities included
the establishment of a VL control program, in which patients were provided with free diagnosis and treatment. Clinically suspected VL patients, defined as individuals with a history of prolonged fever (≥14 days) associated with either splenomegaly or wasting, were included in MSF’s program and further examined for VL. According to the diagnostic algorithm implemented, VL was confirmed either serologically and/or parasitologically. Serological tests included the direct agglutination test (DAT) and after 2004, the rk39 antigen-based DiaMed IT-Leish dipstick, while parasitological confirmation was obtained by microscopy examination of spleen or, more rarely, lymph node aspirates. Microscopic examination of thick and thin blood smears for malaria detection was systematically performed at hospital admission or during hospitalization. The diagnosis of other concomitant diseases was based on clinical suspicion, possibly supported by laboratory confirmation. Testing for HIV was performed only occasionally, due to the lack of voluntary counseling and testing facilities and the shortage of antiretroviral treatment in the district. First-line treatment for primary VL consisted of intramuscular meglumine antimoniate (Glucantime™), temporarily replaced, due to drug shortages, by intravenous amphotericin B deoxycholate or intramuscular sodium stibogluconate (Pentostam™). Second-line treatment based on amphotericin B deoxicholate was administered in case of relapse or intolerance to antimonials. Chloroquine in combination with sulfadoxine-pyrimethamine or alternatively quinine was administered as first-line treatment for malaria. Demographic and medical data of all VL suspected patients were collected by MSF and the local medical staff and entered in a Microsoft Excel data sheet. Besides outcomes of the performed laboratory tests, the database gathered information on patients’ medical history, clinical presentation on admission, symptoms, clinically suspected co-morbidities, treatment administered and relative outcome.

**Data analysis**

A retrospective case-control analysis was performed. Cases were identified as patients with a laboratory-confirmed diagnosis of both VL and malaria at hospital admission or during hospitalization; controls were patients similarly diagnosed with VL, whose blood smears tested negative for malaria. Descriptive analyses were conducted to feature the overall study population and the co-infected patients and to assess prevalence and mortality rates of the co-infection. Statistical analysis was performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). Univariate analyses exploring the association between the explanatory variables and the outcome were performed, using the Pearson Chi-square test. Specifically, risk factors, symptoms and the in-hospital death were examined for their association with the co-infection. Associations are shown as odds ratios (OR) with 95% confidence intervals (CI). Denominators can vary for each variable because of incomplete data in the database. Continuous variables were categorized into predefined groups: age (0-4, 5-9, 10-19, 20-29, ≥30 years) based on malaria risk, anemia on hemoglobin level (severe <5.3 g/dL, moderate 5.3-7.2 g/dL, mild 7.3-10.9 g/dL, none ≥11 g/dL) and spleen size beneath the costal margin by tertiles (<11 cm, 11-14 cm, >14 cm). Seasonality of the co-infection was examined by categorizing hospital admissions during wet season (from March to May, from October to November) and dry season (from December to February, from June to September), which reflects the climate in Uganda. Anthropometric indices WHZ, weight-for-height Z-score for children ≤5 years, BMIZ, body mass index Z-score
for children 6-19 years and BMI for patients >19 years, were used to categorize nutritional status, according to the WHO standards (severe malnutrition: WHZ and BMIZ < -3SD, BMI < 16 kg/m^2; moderate-mild malnutrition: WHZ and BMIZ < -2SD, BMI 16.0-18.4 kg/m^2; no malnutrition: WHZ and BMIZ > -2SD, BMI ≥18.5 kg/m^2). Self-reported symptoms experienced prior to hospitalization were included in the analysis, with the exception of the variable diarrhea, which combined episodes that occurred prior to and during hospitalization. In order to identify independent patients' characteristics associated with the co-infection, logistic regression models were constructed, using a manual stepwise backward procedure to control for confounding and describe statistical interactions. All variables with a P-value < 0.10 in the univariate analyses were entered stepwise in the multivariate models. If an additional variable significantly increased the model fit (as assessed by the -2 Log Likelihood test), the variable was retained in the multivariate analysis (P < 0.05).

Results

Prevalence, features and mortality rate of VL-malaria co-infections

Between 2000 and 2006, a total of 4428 VL suspected patients were admitted to Amudat Hospital, 57% of whom (n = 2511) were confirmed with VL. The diagnosis was confirmed by DAT in 1160 patients (46.2%), by DiaMed IT-Leish in 1115 patients (44.4%) and by parasitological evidence in a spleen or lymph node aspirate in 236 patients (9.4%). Of the 2511 VL-confirmed cases, 2461 (98%) were primary VL infections, while the remaining 50 were relapsing cases. 547 (22%) of the VL-confirmed patients were co-diagnosed with malaria at hospital admission or during hospitalization. Clinically-based diagnosis with negative or no microscopy confirmation occurred for 97 patients, who were excluded from the analysis. Only microscopy-confirmed cases of concomitant malaria were retained for analysis in the co-infected group, resulting in 19% of VL-confirmed patients (n = 450 out of 2414) co-infected with positively diagnosed malaria. Among the non-VL cases, 1107 patients (25%) were clinically diagnosed with probable hyper-reactive malaria splenomegaly, a severe syndrome frequently observed among patients exposed to persistent malarial antigenic stimulation. Most co-infected patients were males residing in Kenya (69%), with a median age of 10 years (inter quartile range 6-16) (Table 2). With the highest percentage of cases found among children aged between 0 and 4 years (26%, n = 82/316) and a progressive decrease with age (13%, n = 32/251 in the age group ≥30 years) (Table 1), the odds of detecting malaria in patients already diagnosed with VL appeared to be inversely related to age.

Table 1. Prevalence of VL-malaria co-infections, stratified by age, amongst VL patients from Amudat Hospital, Uganda, 2000-2006.

| Age group | %  | n cases/denominator |
|-----------|----|---------------------|
| 0-4 years | 25.9 | 82/316              |
| 5-9 years | 22.8 | 133/584             |
| 10-19 years | 17.4 | 145/832            |
| 20-29 years | 13.4 | 55/409              |
| ≥30 years | 12.7 | 32/251              |

* 22 missing values for the variable age

Nearly all co- and mono-infected patients were diagnosed with a degree of anemia (hemoglobin level <11 g/dL) on hospital admission, with a median hemoglobin level of 7.3 g/dL (inter
Table 2. Characteristics associated with visceral leishmaniasis-malaria co-infections at Amudat Hospital, Uganda, 2000-2006 (part I).

| Variable                      | Malaria-VL co-infection cases | Non-malaria VL cases | Crude Odds Ratio | 95% Confidence Interval |
|-------------------------------|-------------------------------|----------------------|-----------------|-------------------------|
|                               | n (%)                        | n (%)                |                 |                         |
| **Gender**                    |                               |                      |                 |                         |
| Male                          | 311 (69.1)                   | 1350 (68.7)          | 1               |                         |
| Female                        | 139 (30.9)                   | 614 (31.3)           | 0.98            | 0.79-1.23               |
| **Age**                       |                               |                      |                 |                         |
| Median (interquartile range)  | 10 (6-16)                    | 12 (7-21)            |                 |                         |
| 0-4 years                     | 82 (18.3)                    | 234 (12.0)           | 2.34            | 1.53-3.75*              |
| 5-9 years                     | 133 (29.8)                   | 451 (23.2)           | 2.02            | 1.33-3.07*              |
| 10-19 years                   | 145 (32.4)                   | 687 (35.3)           | 1.44            | 0.96-2.18*              |
| 20-29 years                   | 55 (12.3)                    | 354 (18.2)           | 1.06            | 0.67-1.70               |
| ≥30 years                     | 32 (7.2)                     | 219 (11.3)           | 1               |                         |
| **Country of origin**         |                               |                      |                 |                         |
| Uganda                        | 140 (31.1)                   | 533 (27.2)           | 1               |                         |
| Kenya                         | 310 (68.9)                   | 1429 (72.8)          | 1.21            | 0.97-1.51               |
| **Season on admission**       |                               |                      |                 |                         |
| Wet season                    | 177 (39.3)                   | 828 (42.2)           | 1               |                         |
| Dry season                    | 273 (60.7)                   | 1136 (57.8)          | 1.12            | 0.91-1.39               |
| **VL infection**              |                               |                      |                 |                         |
| Primary infection             | 442 (98.2)                   | 1922 (97.9)          | 1               |                         |
| Relapse                       | 8 (1.8)                      | 42 (2.1)             | 0.83            | 0.39-1.78               |
| **Previous VL treatment**     |                               |                      |                 |                         |
| No                            | 440 (97.8)                   | 1928 (97.7)          | 1               |                         |
| Yes                           | 10 (2.2)                     | 46 (2.3)             | 0.95            | 0.45-1.89               |
| **Malnutrition**              |                               |                      |                 |                         |
| No                            | 168 (39.6)                   | 638 (33.9)           | 1               |                         |
| Moderate-mild                 | 142 (33.5)                   | 744 (39.5)           | 0.73            | 0.57-0.93*              |
| Severe                        | 114 (26.9)                   | 500 (26.6)           | 0.87            | 0.66-1.13               |
| **Anemia degree on admission**|                               |                      |                 |                         |
| Median Hb g/dL (interquartile range) | 7.3 (6.7-9.7)     | 7.3 (6.7-8.7)       |                 |                         |
| None (Hb ≥11 g/dL)            | 26 (5.8)                     | 68 (3.5)             | 1               |                         |
| Mild (Hb 7.3-10.9 g/dL)       | 276 (61.7)                   | 1218 (62.8)          | 0.59            | 0.37-0.95*              |
| Moderate (Hb 5.3-7.2 g/dL)    | 130 (29.1)                   | 592 (30.5)           | 0.64            | 0.35-0.94*              |
| Severe (Hb <5.3 g/dL)         | 15 (3.4)                     | 60 (3.1)             | 0.91            | 0.32-1.35*              |
quartile range 6.7-9.7 for cases and 6.7-8.7 for controls) (Table 2). Splenomegaly, as detected here by palpation of the spleen below the left costal margin, was also a common feature among co-infected patients, but neither its frequency (75% for cases, 74% for controls) nor its severity (median spleen size below the costal margin 13.0 cm for cases and controls) were increased by the malaria co-infection (Table 2). More than half of the co-infected patients were co-diagnosed with a third infectious disease, reflecting the high prevalence of infections found in the study area. Acute respiratory infections followed by otorhinolaryngological and skin infections were the most common co-morbidities among both cases and controls (Table 3).

Of the 450 co-infected patients, 2.7% (n = 12) died while hospitalized. A similar in-hospital mortality rate was found in the control group (3.1%, n = 60) (OR: 0.87; 95% CI: 0.46-1.63) (data not shown).

**Risk factors for VL-malaria co-infections**

Associations between demographic and clinical variables and the co-infection, as described by the univariate analyses, are summarized in Tables 2 and 3. Young age (≤9 years) was identified as a risk factor for the VL-malaria co-infection: in particular, children in the age groups 0-4 (OR: 2.34; 95% CI: 1.53-3.75) and 5-9 years (OR: 2.02; 95% CI: 1.33-3.07) were more than two times more likely to be co-infected compared to adults ≥30 years. Transmission seasons for malaria and VL did not coincide in the study area. However, no significant difference was found in the seasonal distribution of cases’ and controls’ hospitalizations. VL-relapsing patients did not show different susceptibilities to the malaria co-morbidity, compared to patients with primary VL infections, and were therefore maintained in the relative groups (cases and controls). Malnourishment was relatively more common in the control group, resulting in patients with mild-moderate malnutrition being negatively associated with the co-infection, compared to the well-nourished patients (OR: 0.73; 95% CI: 0.57-0.93). A significant negative association was found between anemia and the co-infection: patients whose hemoglobin level was <11 g/dL, in fact, were less likely to be diagnosed with the co-infection compared to the normochromic group. Among concomitant diseases, patients diagnosed with a skin infection (OR: 0.44, 95% CI: 0.22-0.88) were less likely to have the VL-malaria co-infection. A trend towards a negative association was also observed for patients

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### Table 2. Continued

| Variable                        | Malaria-VL co-infection cases | Non-malaria VL cases | Crude Odds Ratio | 95% Confidence Interval |
|---------------------------------|------------------------------|----------------------|-----------------|-------------------------|
| spleen size on admission        | 443                          | 1943                 |                 |                         |
| Median spleen size below the costal margin (interquartile range) | 13.0 (10.0-15.0) | 13.0 (10.0-16.0) |                 |                         |
| <11 cm                          | 111                          | 513                  | 1.17            | 0.91-1.52               |
| 11-14 cm                        | 191                          | 752                  | 1.17            | 0.91-1.52               |
| >14 cm                          | 141                          | 678                  | 0.96            | 0.73-1.26               |

*P-value <0.10 based on Person Chi-square tests.

Hb = hemoglobin
Table 3. Characteristics associated with visceral leishmaniasis-malaria co-infections at Amudat Hospital, Uganda, 2000-2006 (part II).

| Variable                      | Malaria-VL  | Non-malaria VL | Crude Odds Ratio | 95% Confidence Interval |
|-------------------------------|-------------|----------------|------------------|-------------------------|
|                               | co-infection cases | cases          |                  |                         |
|                               | n (%)       | n (%)          |                  |                         |
| Concomitant diagnoses         |             |                |                  |                         |
| Acute respiratory infections  | 450 (36.7)  | 1964           | 1.09             | 0.94-1.44               |
| Ear, nose and throat infections | 46 (10.2) | 234 (11.9)     | 1.01             | 0.73-1.26               |
| Skin infections†              | 9 (2.0)     | 87 (4.4)       | 0.44             | 0.22-0.88*              |
| Bacterial infections‡         | 7 (1.6)     | 15 (0.8)       | 1.91             | 0.83-5.07               |
| Chicken pox                   | 3 (0.7)     | 19 (1.0)       | 2.04             | 0.20-2.33               |
| Tuberculosis                  | 2 (0.4)     | 7 (0.4)        | 0.75             | 0.26-6.03               |
| Brucellosis                   | 3 (0.5)     | 15 (0.8)       | 0.71             | 0.21-2.46               |
| Typhoid fever                 | 0           | 9 (0.5)        | 1.00             | 0.99-1.00               |
| Intestinal parasites§         | 2 (0.4)     | 34 (1.7)       | 0.25             | 0.06-1.06*              |
| Hepatopathy                   | 1 (0.2)     | 8 (0.4)        | 0.55             | 0.07-4.37               |
| Other infections              | 4 (0.9)     | 37 (1.9)       | 0.47             | 0.17-1.32               |
| Symptoms on admission         | 81          | 908            |                  |                         |
| Cough                         | 34 (42.0)   | 380 (41.9)     | 1.01             | 0.63-1.59               |
| Weight loss                   | 29 (35.8)   | 267 (29.4)     | 1.34             | 0.83-2.16               |
| Anorexia                      | 19 (23.5)   | 129 (14.2)     | 1.85             | 1.07-3.20*              |
| Bleeding                      | 15 (18.5)   | 192 (21.1)     | 0.85             | 0.47-1.52               |
| Headache                      | 13 (16.0)   | 164 (18.1)     | 0.87             | 0.47-1.61               |
| Malaise                       | 14 (17.3)   | 100 (11.0)     | 1.69             | 0.92-3.11*              |
| Diarrhea                      | 23 (5.1)    | 165 (8.4)      | 0.59             | 0.38-0.92*              |
| Vomiting                      | 3 (3.7)     | 12 (1.3)       | 2.87             | 0.79-10.39*             |
| Fatigue                       | 2 (2.5)     | 25 (2.8)       | 0.89             | 0.21-3.85               |
| Abdominal distension          | 1 (1.2)     | 11 (1.2)       | 1.02             | 0.13-8.00               |
| Edema                         | 1 (1.2)     | 26 (2.9)       | 0.42             | 0.06-3.17               |
| Abdominal pain                | 1 (1.2)     | 14 (1.5)       | 0.80             | 0.10-6.15               |
| Jaundice                      | 1 (1.2)     | 10 (1.1)       | 1.12             | 0.14-8.88               |
| Joint pain                    | 0           | 12 (1.3)       | 1.00             | 0.99-1.00               |
| Sweating                      | 1 (1.2)     | 8 (0.9)        | 1.41             | 0.17-11.39              |
| Palpitations                  | 0           | 3 (0.3)        | 1.00             | 0.99-1.00               |
| Skin rash                     | 0           | 3 (0.3)        | 1.00             | 0.99-1.00               |

*P-value <0.10 based on Person Chi-square tests.

*Ear, nose and throat infections include: otitis, pharyngitis, sinusitis, tonsillitis, parotitis, gingivitis and noma.
†Skin infections include bacterial and fungal infections.
‡Bacterial infections include: infections of the urinary tract, sepsis and other infections.
§Intestinal parasite infections include protozoan and helminthic infections.
suspected to harbor intestinal protozoa and/or helminthes, possibly indicating a mutual protection among the three diseases (OR: 0.25, 95% CI: 0.06-1.06). Finally, anorexia (OR: 1.85, 95% CI: 1.07-3.20), malaise (OR: 1.69, 95% CI: 0.92-3.11), and vomiting (OR: 2.87, 95% CI: 0.79-10.39) were identified as positively associated symptoms of the VL-malaria co-infection, while a negative association was found for diarrhea (OR: 0.59, 95% CI: 0.38-0.92).

All 9 variables associated with the co-infection in the univariate analyses were included in the multivariate logistic regression models. Two separate models were created, due to the high number of missing data in reporting the symptoms: model A, based on 2306 patients, which includes all variables except the symptoms and model B, based on 929 patients, which combines symptoms and all other variables. Model A (Table 4) shows that, after adjusting for the other variables in the model, mild-moderate malnutrition lost its statistical significance (P-value 0.18). However, the variable was kept in the final model as it was found to be a confounder for age. After adjusting for the other variables included in the model, age ≤9 years remained significantly associated with the co-infection. When compared to adults ≥30 years, the risk of being diagnosed with the co-morbidity was highest among children aged 0-4 years (OR: 2.44; 95% CI: 1.52-3.92) and progressively decreased with age (OR: 2.23; 95% CI: 1.45-3.45 for age group 5-9 years; OR: 1.48; 95% CI: 0.97-2.26 for age group 10-19 years). Moderate (OR: 0.45; 95% CI: 0.27-0.77) and mild anemia (OR: 0.53; 95% CI: 0.32-0.88) negatively correlated with the co-infection. Finally, the model highlighted a negative association between being co-infected with VL and malaria and carrying a skin infection (OR: 0.35; 95% CI: 0.17-0.72) or, to a lesser extent, intestinal parasites (OR: 0.28; 95% CI 0.07-1.19). Patients suspected to harbor such pathogens, in fact, were three times less likely to be diagnosed with the co-infection.

In model B (Table 5), logistic regression was performed on the sub-cohort of patients for whom symptoms, besides the other variables, were reported. As anemia was found to be a confounder in the association between age and the co-infection, the variable was kept in the model. Besides concomitant diagnoses, diarrhea, vomiting and malaise lost their significance and

| Variable          | Odds Ratio | 95% Confidence Interval | P-value |
|-------------------|------------|-------------------------|---------|
| Age               |            |                         |         |
| 0-4 years         | 2.44       | 1.52-3.92               | <0.010  |
| 5-9 years         | 2.23       | 1.45-3.45               | <0.010  |
| 10-19 years       | 1.48       | 0.97-2.26               | 0.067   |
| 20-29 years       | 1.09       | 0.67-1.76               | 0.730   |
| ≥30 years         | 1          |                         | <0.001  |
| Malnutrition      |            |                         |         |
| No                | 1          |                         | 0.295   |
| Moderate-mild     | 0.84       | 0.64-1.08               | 0.176   |
| Severe            | 1.01       | 0.77-1.33               | 0.955   |
| Anemia on admission |          |                         |         |
| None (Hb ≥11 g/dL)| 1          |                         | 0.035   |
| Mild (Hb 7.3-10.9 g/dL) | 0.53 | 0.32-0.88 | 0.014  |
| Moderate (Hb 5.3-7.2 g/dL) | 0.45 | 0.27-0.77 | 0.004  |
| Severe (Hb <5.3 g/dL) | 0.49 | 0.22-1.08 | 0.075  |
| Concomitant diagnoses |            |                         |         |
| Skin infections   | 0.35       | 0.17-0.72               | 0.005   |
| Intestinal parasites | 0.28 | 0.07-1.19 | 0.085  |
were consequently excluded from the model. The association between anorexia and the co-infection also lost its statistical significance in the multivariate analysis, but the variable was kept in the model as it increased the model fit. A positive association with this symptom can be seen (OR: 1.71; 95% CI: 0.96-3.03), indicating that co-infected patients more frequently reported anorexia among their symptoms.

Table 5. Model B: multivariate analysis of symptoms associated to VL-malaria co-infections among 929 Amudat Hospital patients.

| Variable            | Odds Ratio | 95% Confidence Interval | P-value |
|---------------------|------------|--------------------------|---------|
| Age                 |            |                          |         |
| 0-4 years           | 3.19       | 1.09-9.32                | 0.034   |
| 5-9 years           | 2.99       | 1.10-8.13                | 0.032   |
| 10-19 years         | 0.86       | 0.31-2.45                | 0.783   |
| 20-29 years         | 0.85       | 0.26-2.77                | 0.785   |
| ≥30 years           | 1          | <0.001                   |         |
| Malnutrition        |            |                          |         |
| No                  | 1          |                          | 0.522   |
| Moderate-mild       | 1.10       | 0.61-1.98                | 0.748   |
| Severe              | 1.41       | 0.77-2.60                | 0.265   |
| Anemia on admission |            |                          |         |
| None (Hb ≥11 g/dL)  | 1          |                          | 0.199   |
| Mild (Hb 7.3-10.9 g/dL) | 0.31 | 0.06-1.54 | 0.154 |
| Moderate (Hb 5.3-7.2 g/dL) | 0.24 | 0.05-1.21 | 0.083 |
| Severe (Hb <5.3 g/dL) | 0.19 | 0.03-1.38 | 0.101 |
| Symptoms            |            |                          |         |
| Malaise             | 2.14       | 1.12-4.07                | 0.021   |
| Anorexia            | 1.71       | 0.96-3.03                | 0.070   |

**Discussion**

Nineteen percent of the VL-confirmed patients admitted to Amudat Hospital between 2000 and 2006 were co-diagnosed with malaria. Young age (≤9 years) was identified as a risk factor for concomitant VL and malaria, while being anemic or having a skin infection appeared to negatively correlate with the co-infection. Anorexia was slightly more frequent among co-infected patients compared to the mono-infected ones. The in-hospital case-fatality rate did not significantly differ between cases and controls, indicating that VL-malaria co-infections did not result in a poorer prognosis.

Affecting nearly one out of five VL patients, VL and malaria co-infections appeared to be a common condition among VL patients living in the Pokot Region of Kenya and Uganda. Though in agreement with the findings of Mueller et al. who investigated the in-hospital mortality of VL within the same study population, the co-infection rate described in the present study (19%) is one of the highest so far reported, as identified by our systematic literature review. The main reason behind this variation may be found in the malaria prevalence characterizing the study areas (31% and 18% in Uganda and Kenya, respectively, 28% in Sudan, 0.31% in India and 2.0% in Bangladesh in the corresponding study years). This case, however, does not apply to the study of Kolaczinski et al., in which only 6.4% of the Amudat Hospital VL patients recruited between June and September 2006 were reported to be co-infected with malaria. Although different malaria diagnostics were used to define the two study populations (microscopy in the present study and the *Plasmodium falciparum*-specific Paracheck test in Kolaczinski’s study), the role played by the use of such diagnostic tools is
likely to be marginal, considering the high performance of the Paracheck test in Uganda,\textsuperscript{31} where \textit{P. falciparum} causes most of malaria infections.\textsuperscript{35} Most likely, the higher co-infection prevalence observed here resulted from recruiting a larger number of study subjects. When examining only patients hospitalized in the same four-month period of 2006, indeed, the co-infection rate remains significantly higher (19%), even in comparison with the malaria prevalence estimated by Kolaczinski \textit{et al.} (16%), emphasizing the importance of recruiting large numbers of participants.

The co-infected population described by the present study mainly consisted of male Kenyans (69%), with a median age of 10 years. Despite the similar demographic pattern found among the controls, young age appeared to be positively associated with the co-infection; children ≤9 years, in fact, were found to be more than two times more likely to be co-infected with malaria, compared to adults ≥30 years, reflecting the well-known age patterns of malaria.\textsuperscript{32}

Anemia, a hallmark of both VL and malaria, negatively correlated with the co-occurrence of malaria in VL patients. Given the bi-directional nature of such association, it is not possible to determine \textit{a priori} whether such correlation might reflect the reduced susceptibility of VL anemic patients to the malaria infection or simply results from the interaction of the two diseases upon each other. Splenic-associated hemolysis and reduced hematopoiesis as a result of abnormal iron retention by macrophages, have been implicated in the multifactorial origin of VL-induced anemia, where both hemoglobin and plasma iron levels are affected.\textsuperscript{33} Iron-deficiency, in particular, was associated with delay in the \textit{in vitro} intra-erythrocytic growth of \textit{Plasmodium} parasites\textsuperscript{34} and protection from mild clinical malaria \textit{in vivo}.\textsuperscript{35} Although in our study population, anemia was diagnosed on the basis of the hemoglobin levels only, iron deficiency might be equally common, due to poor nutritional status and ongoing diseases, providing temporary resistance to the malaria disease. A less severe course of VL as a result of concomitant malaria might represent a possible scenario, corroborated by the findings that severe splenomegaly and fatal outcome were slightly less common among co-infected patients. The milder anemia observed among co-infected patients compared to mono-infected patients could then be the result of a malaria-mitigating effect on VL. Such hypothesis, however, appears to be in contrast with previous observations obtained from animal models, in which the course of \textit{Leishmania} infections appeared to be unaffected or exacerbated by the concomitant presence of \textit{Plasmodium} parasites.\textsuperscript{36-39} In addition, the more frequent reporting of anorexia by co-infected patients seems to point towards an exacerbation of disease severity rather than alleviation. The hypothesis whereby co-infected patients may have been diagnosed at an earlier stage than VL mono-infected patients, due to more severe symptoms, is unlikely, as no difference was found in the ongoing sickness duration of cases and controls prior to hospitalization. The severe dehydration observed in some patients may have led to misleading (elevated) hemoglobin levels on hospital admission. However, the significant increase in hemoglobin level observed after treatment (+2.14 g/dL) suggests that severe dehydration may have played only a marginal role. Finally, it should be noted that the groups comprising the non-anemic patients consisted of relatively few patients for both cases and controls, which may limit the validity of the associations described. In fact, when patients
with no anemia and mild anemia (the most numerous group) are grouped together, the association between moderate anemia and the VL-malaria co-infection loses its significance (data not shown).

Unlike what may be observed in other co-morbidities, 40,41 co-infections with VL and malaria did not result in an exacerbated spleen enlargement compared to VL mono-infections. This may be related to splenic disorders promoted by VL, which might be hampering for the malaria-induced hyperplasia. Remarkably, while massive splenomegaly is a common feature of VL, it is more rarely associated to malaria infections, which instead induce only moderate spleen enlargement. Therefore, exacerbation of splenomegaly as promoted by concomitant malaria might have occurred but be undetectable due to the larger effect produced by VL on the spleen size. Finally, a possible malaria-induced attenuation of VL severity should not be excluded.

More than half of the patients among both cases and controls were co-diagnosed with one or more infections beyond VL and malaria. If this figure reflects the high infectious disease burdens found in the study area, we cannot exclude the development of co-morbidities to be related to the VL infection. Importantly, if VL might have favored the superimposing of other infections, malaria neither exacerbated the susceptibility of co-infected patients nor influenced the concomitant disease-pattern, with the exception of skin infections and, to a lesser extent, intestinal parasites. VL patients diagnosed with bacterial and fungal skin infections, in fact, appeared to be more than two times less likely to be co-diagnosed with malaria, although no evident explanation could be found. Similarly, a negative, but not significant association between co-infected patients and VL patients suspected to harbor intestinal parasites was identified, suggesting that the parasites involved (intestinal protozoa and/or helminthes, Leishmania and Plasmodium spp.) may be able to cross-regulate their host susceptibility. It should be noted, however, that diagnosis of intestinal parasitic infections for some patients might have been based on clinical suspicion only, with no laboratory confirmation, and that testing for these parasites might have not been performed systematically, as suggested by the very low prevalence of positive patients (1.5%). A dedicated study recruiting larger patient cohorts diagnosed with intestinal parasitic infections and performing systematic testing of all study subjects would be required to confirm this finding.

Anorexia was reported slightly more frequently in the co-infected group than in the mono-infected group, indicating that a more severe clinical picture may be associated with the overlapping of the two diseases. This symptom was frequently associated with the malaria infection; however, the significance of such finding remains questionable, given the relatively small number of patients which it is based on. Importantly, if VL-malaria co-infections were associated with a worse symptomatology, they did not result in a poorer prognosis among patients properly diagnosed and treated. The overall fatality-rate, in fact, was similar in the co-infected group as in the mono-infected one, suggesting that here, malaria and its treatment neither altered the severity of VL and its responsiveness to chemotherapy nor resulted in an increased toxicity of the antileishmanial drugs. A similar conclusion might be extended to the co-infected patients diagnosed with relapses rather than primary infections, as no death occurred among these patients. Whether the prognosis of co-infected patients...
in whom malaria remained undetected and/or untreated may be different, is hard to predict, but a higher mortality risk may be expected, as observed for late diagnosed malaria.

The findings highlighted by the present study may be generalized with caution to the VL-symptomatic population residing in the Uganda-Kenya VL foci. Evidence in support of this statement includes the large catchment area of Amudat Hospital, its specialization in VL and the local awareness of the disease. Until November 2006, when MSF transferred its medical activities to Kacheliba Health Centre in Kenya, Amudat Hospital was the only reliable facility available in the region for the diagnosis and treatment of VL. Furthermore, a recent study conducted in the same area indicated that VL is a well-known disease among members of the Pokot communities, who not only recognize its main clinical signs and poor prognosis, but are also aware that drug treatment is available and effective. This resulted in many individuals with symptoms of VL presenting voluntarily to the MSF’s VL-ward, where VL diagnosis and treatment were provided free of charge by MSF.

The main limitations of this study relate to data quality and lack of mono-infected malaria patients and healthy controls. It was not possible to assess the malaria prevalence in the study area, which prevented us from calculating the VL associated risk of being co-diagnosed with malaria. Comparison of study variables was only possible for VL co-and mono-infected patients, possibly reducing the significance of some of the associations found. The lack of quality control on malaria microscopy might have led to poor performance, possibly affecting the accuracy of the study results. Hemoglobin estimates were only semi-quantitative and possibly subject to interpretation, been based on the Lovibond method. Spleen size measurement might have suffered from poor standardization, with different physicians using slightly different techniques.

In conclusion, this study highlights for the first time that concurrent VL and malaria infection represents a common co-morbidity among young patients living in highly malaria-endemic areas, such as the Pokot territory of Kenya and Uganda. Based on these findings, we recommend that malaria screening be implemented for all VL patients residing in malaria-endemic areas in order to promptly initiate anti-malarial drug treatment. Future studies are needed to address the complicated question on whether the concurrency of such infections might influence the course of one or both diseases in humans. This may be of importance for the design of successful disease control programs.
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Authors’ contributions
Conceived and designed the experiments: EvdB ERA PFM HDFHS FC. Performed the experiments: EvdB MMZB ERA PFM MS ES DBM HDFHS FC. Analyzed the data: MMZB MS. Contributed reagents/materials/analysis tools: EvdB MMZB ES DBM MS FC. Wrote the paper: EvdB. Reviewed the manuscript: MMZB ERA PFM ES DBM MS HDFHS FC.

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Competing Interests
The authors have declared that no competing interests exist.
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Supporting Information

474 studies identified by searching Medline/Pubmed (1950-week 19, 2011), Embase (1947-week 19, 2011), Google Scholar, African Journals Online and African Index Medicus -Access to African Health Information- electronic databases. A combination of search terms tailored to individual database was used, with no restriction on study design or publication language. Search terms included: *Leishmania*, leishmaniasis, VL, malaria, *Plasmodium*, prevalence, incidence, risk factors, morbidity and mortality.

407 studies screened for their eligibility (titles and abstracts or full-texts) after removal of duplicates.
Criteria for eligibility included:
– concomitant exposure to VL and malaria
– exposure of human subjects

399 studies excluded

10 full-text articles assessed for eligibility

3 studies excluded.
Reasons for exclusion:
– Full-text cannot be retrieved: 2
– Lack of relevance: 1

7 studies included in the systematic review

5 additional studies identified through manual screening of conference proceedings and references of retrieved articles

3 studies excluded.
Reasons for exclusion:
– Full-text cannot be retrieved: 2
– Lack of relevance: 1

5 full-text articles assessed for eligibility

2 studies included in the systematic review

9 studies included in the systematic review: 5 cross-sectional studies and 4 case-report [16-24]

Figure S1. Search strategy and study selection used for the systematic literature review.
Concomitant malaria among visceral leishmaniasis in-patients from Gederif and Sennar States, Sudan: a retrospective case-control study

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Abstract

Background
In areas where visceral leishmaniasis (VL) and malaria are co-endemic, co-infections are common. Clinical implications range from potential diagnostic delay to increased disease-related morbidity, as compared to VL patients. Nevertheless, public awareness of the disease remains limited. In VL-endemic areas with unstable and seasonal malaria, vulnerability to the disease persists through all age groups, suggesting that in these populations, malaria may easily co-occur with VL, with potentially severe clinical effects.

Methods
A retrospective case-control study was performed using medical records of VL patients admitted to Tabarakallah and Gedarif Teaching Hospitals (Gedarif State) and Al'Azaza kala-azar Clinic (Sennar State), Sudan (2005-2010). Patients positively diagnosed with VL and malaria were identified as cases, and VL patients without microscopy-detectable malaria as controls. Associations between patients’ characteristics and the occurrence of the co-infection were investigated using logistic regression analysis. Confirmation of epidemiological outcomes was obtained with an independently collected dataset, composed by Médecins Sans Frontières (MSF) at Um-el-Kher and Kassab Hospitals, Gedarif State (1998).

Results
The prevalence of malaria co-infection among VL surveyed patients ranged from 3.8% to 60.8%, with a median of 26.2%. Co-infected patients presented at hospital with deteriorated clinical pictures. Emaciation (Odds Ratio (OR): 2.46; 95% Confidence Interval (95% CI): 1.72-3.50), jaundice (OR: 2.52; 95% CI: 1.04-6.09) and moderate anemia (OR: 1.58; 95% CI: 1.10-2.28) were found to be positively associated with the co-infection, while severity of splenomegaly (OR: 0.53; 95% CI: 0.35-0.81) and, to a lesser extent, hepatomegaly (OR: 0.52; 95% CI: 0.27-1.01) appeared to be reduced by concomitant VL and malaria. The in-hospital case-fatality rates did not significantly differ between co- and mono-infected patients (OR: 1.13; 95% CI: 0.59-2.17). Conversely, a significantly increased mortality rate (OR: 4.38; 95% CI: 1.83-10.48) was observed by MSF amongst co-infected patients enrolled at Um-el-Kher and Kassab Hospitals, who also suffered an enhanced risk of severe anemia (OR: 3.44; 95% CI: 1.68-7.02) compared to VL mono-infections.

Conclusions
In endemic VL areas with unstable seasonal malaria, like east Sudan, VL patients are highly exposed to the risk of developing concomitant malaria. Prompt diagnosis and effective treatment of malaria are essential to ensure that its co-infection does not result into poor prognoses.

Keywords
Visceral leishmaniasis, Malaria, Co-infection, Prevalence, Mortality, Risk factors
Background

In areas where malaria is co-endemic with visceral leishmaniasis (VL), co-infections with both diseases are common. Previous observations performed through cohorts of VL patients report the occurrence of this co-infection across major VL hot spots, with prevalences ranging from 31% in Sudan to 1.2% in Bangladesh. At Amudat Hospital, Uganda, where nearly one out of five VL-confirmed patients hospitalized between 2000 and 2006 was co-diagnosed with malaria, concomitant malaria was shown to exacerbate symptoms of VL patients, though with no implications for their prognosis. Children under 10 years of age, notoriously more vulnerable to both malaria and VL, exhibited a twofold higher risk of being co-infected compared to adults. The clinical relevance of the VL-malaria co-morbidity, as attested by its frequency and severity at Amudat Hospital, highlights the risks associated with disease co-endemicity and suggests that other malaria- and VL-endemic areas may experience similar co-infection burdens.

Of the 30,000 to 50,000 annual VL cases estimated in East Africa, the second largest VL focus after South Asia, half as many (15,700 to 30,300) are thought to occur in Sudan. Hyper-endemic foci are located in the east part of the country, stretching from the White Nile in the west to the Ethiopian border in the east, and from Kassala State in the north across the border of South Sudan up to Upper Nile State in the south. Here, the disease has been reported since the early 1900s, but it is in the past 30 years that it has reached dramatic proportions, resulting in recurrent epidemics which have claimed hundreds of thousands of lives. Currently, Gedarif State in Eastern State, together with the region of Sennar and Singa in Central State, accounts for the vast majority of VL cases in Sudan. The distribution of the disease within these areas is wide, erratic and variable, with highly endemic clusters situated near the banks of the Blue Nile River and its tributaries (Dinder, Rahad and Atbara Rivers) and the Dinder National Park. Here, during the outbreaks that cyclically recur (every 7-10 years), the disease can reach incidences of >50 cases per 1000 per year. Diagnostics and treatment services are available at several governmental hospitals and rural dispensaries. However, due to the high cost of treatment, many cases have been referred to Médecins sans Frontières-Holland (MSF-H), whose activities at Um-el-Kher and Kassab VL treatment centers have resulted in more than 24,000 VL patients treated over the last hyper-endemic period (1996-2003). In the early 2000s, after an initial decline in the disease incidence, a new upsurge in the number of VL cases was recorded, leading to the establishment in January 2010 of a new VL ward at Tabarakallah Hospital.

Unstable seasonal malaria, due to Plasmodium falciparum, prevails in most areas of Gedarif and Sennar States, where prevalence rates of 1.6% and 1.1%, respectively, were confirmed among the communities surveyed in 2009. This epidemiological pattern of malaria transmission considerably affects the age related ability of local individuals to acquire immunity against clinical malaria, resulting into seasonal outbreaks that strike all age groups, albeit with different incidences. Subclinical infections are often harbored throughout the dry season, resulting into semi-immunity which ultimately develops within 2 to 3 decades. Concomitant exposure of these individuals to Leishmania parasites may, therefore, have dramatic consequences on
the ability to control both infections, potentially resulting in an aggravated clinical picture.

To describe the clinical impact of VL-malaria co-infections in patients with low malaria immunity, we investigated the epidemiology of VL-malaria co-infections in a VL endemic area characterized by unstable seasonal malaria. By surveying three strategically located hospitals, we obtained a semi-representative data collection of Gedarif and Sennar States (2005-2010), whereby the risk for local VL patients of acquiring a malaria co-infection was assessed. The high prevalence of this co-morbidity and its negative impact on patients’ clinical condition, as observed in this study, highlighted the existence of a clinically significant condition, whose life-threatening implication was indirectly confirmed by an independently collected dataset, gathered by MSF-H at Um-el-Kher and Kassab Hospitals, Gedarif State, in 1998.

Methods

Study area
The study area lies in Sudan, between the Blue Nile River in the central region of Sennar State and the lower Atbara River, which borders Gedarif State in the north-east. The Rahad River and the Dinder River, both tributaries of the Blue Nile River, flow across the region with seasonal regime, marked by major floods during the rainy season (from June to October) and long periods of dry-off throughout the rest of the year. Al’Azaza kala-azar Clinic (Sennar State), Gedarif Teaching Hospital and Tabarakallah Hospital (Gedarif State) (Figure 1) were selected as treatment centers for conducting the survey. The hospitals encompass a VL-dedicated ward, where diagnosis and treatment for VL are performed. Gedarif Teaching Hospital, situated in the city of Gedarif, receives patients from the town and surrounding areas. In addition, difficult clinical cases encountered at regional level are referred to the hospital, which therefore serves as Regional Reference Hospital. Al’Azaza kala-azar clinic, located along the Dinder River, in proximity to the Dinder National Park, is a rural hospital. Together with Gedarif Teaching Hospital, Kassab Hospital and 4 other health centers in Gedarif and Sennar States, the clinic received training and support from MSF-H, as part of a “restructuring program” (2001-2004) for improving local management of VL cases.

Tabarakallah Hospital, located in the East Atbara region, 20 km south from the Atbara River, is a rural hospital which houses an MSF VL treatment center from January 2010. After departure of MSF-H from the region in 2004, many of the medical staff who worked with MSF in an independent Zakat (Islamic charity)-funded clinic in Tabarakallah, were employed by the hospital.

Study design and population
A retrospective case-control study was performed on patients positively diagnosed for VL. Patients with a laboratory-confirmed diagnosis of both VL and malaria at hospital admission or during hospitalization were identified as cases, while controls were patients similarly diagnosed with VL, whose blood smears tested negative for malaria. Laboratory confirmation of VL was obtained either through microscopic examination of lymph node, bone marrow or spleen aspirates, or using serological tests, such as the direct agglutination test (DAT) (titer ≥1:6400) and the rk39 antigen-based dipstick (Kalazar Detect Rapid Test®), in combination with the clinical case definition of the World Health Organization (WHO). Intensity of Leishmania infection in lymph node or bone marrow aspirates was categorized according to the WHO-recommended grading system. Briefly, parasitaemias ranging from +1 to +4 were
defined as smears containing 1-10 parasites in 1000, 100, 10 or 1 field, respectively, while 10-100 and >100 parasites per field indicated a parasitaemia of +5 and +6, respectively. Malaria was confirmed by microscopic examination of blood smears or by Rapid Diagnostic Tests (RDTs) (SD Bioline Malaria Ag P.f/P.v). Diagnosis of other concomitant diseases was based on clinical suspicion only. Clinical examination of patients included assessment of hemoglobin (Hb) levels (Lovibond method), spleen size (measured in the anterior axillary line to the furthest point during quiet breathing) and liver size (measured in midclavicular line during quiet breathing). Nutritional status, as determined by Weight-for-High percentiles or Body Mass Index (BMI), was directly recorded. Standard treatment for all VL patients, including relapsing cases, consisted of generic sodium stibogluconate administered parenterally. For malaria, artemisinin derivatives (artemether or artesunate), as mono-therapy or in combination with sulfadoxine-pyrimethamine (SP) or lumefantrine, or alternatively quinine were administered as first-line treatment. Test of cure was regularly performed for VL, but not for malaria, by microscopy on lymph node or bone marrow aspirates.

**Data collection**

Medical records of patients hospitalized between January 2005 and December 2010 in the three hospitals, were retrospectively reviewed. Data from patients positively diagnosed with VL were collected manually, by using paper Case Record Forms (CRF) specially designed for the study. Data included patients’ demographic details, clinical signs and symptoms, test results, medical treatments and relative outcomes. Upon completion of the collection process, data
were single-entered using SPSS 15.0 (SPSS Inc., Chicago, USA) and Microsoft Excel softwares.

**Data analysis**

Descriptive analyses were conducted to examine the features of the overall study population and to assess prevalence and case-fatality rates of the co-infected patients. Statistical analysis was performed using SPSS 15.0 software. Characteristics of cases and controls were individually compared, using the Pearson Chi-square test or the Fisher Exact Probability test. Continuous variables were categorized in predefined groups: age (<5, 5-9, 10-19, 20-29 and ≥30 years) based on malaria risk, spleen size (0-3 cm, 4-5 cm and ≥6 cm) by percentiles and anemia based on hemoglobin level (no-mild ≥7.3 g/dL, moderate 5.3-7.2 g/dL and severe <5.3 g/dL). As only few patients (n = 22, 2.0%) were found to be non-anemic (Hb ≥11 g/dL), while the majority (n = 606, 55.1%) had mild anemia (Hb 7.3-10.9 g/dL), the groups with no and mild anemic patients were combined in one category, more truly representative of the patient set for anemia risk assessment. Seasonality of the co-infection was examined by categorizing hospital admissions during wet season (from June to October) or dry season (from November to May). Odds ratios (OR) with 95% confidence intervals (CI) were calculated to test whether a variable significantly differed between cases and controls. Multivariable logistic regression models were used to identify independent patients’ characteristics associated with the co-infection. All variables with a P-value <0.10 in the univariate analyses were entered stepwise in the multivariate analysis, whose final model only comprised significant variables (P-value <0.05) and variables which significantly increased the model fit, as assessed by the −2 Log Likelihood test.

**MSF’s dataset**

Demographic and clinical data of VL patients enrolled in a clinical trial conducted by MSF-H at Um-el-Kher and Kassab Hospitals (1998) were analyzed for comparison with the survey outcomes. Both hospitals lie in Gedarif State (Figure 1), in proximity to the Rahad River and the Gedarif town, respectively, where prevalence of malaria and VL averages the rates characterizing the survey sites. The trial conducted by MSF aimed to compare efficacy and safety of branded vs. generic sodium stibogluconate for the treatment of primary VL. Hence, the study population comprised patients with a laboratory-confirmed diagnosis of VL (DAT titer ≥1:6400 or demonstration of *Leishmania* on aspirates of spleen or lymph node) and no history of previous anti-leishmanial treatment. Microscopic search for malaria was also performed on all study subjects at enrollment. Treatment for VL consisted of either Pentostam or generic sodium stibogluconate given intramuscularly (20 mg/kg/day for 30 days), while SP and quinine were administered as first-line treatment for uncomplicated and severe malaria, respectively. Using the same case-control definition as in the survey, descriptive and statistical analyses (Person Chi-square test) were performed to assess prevalence and mortality rates of the VL-malaria co-infection and to measure its association with explanatory variables.

**Ethical approval**

Data were collected as part of routine patient care, with no need for additional investigations. Data extraction from medical records was performed by anonymizing the information recorded in the CRFs. Ethical approval for the study was obtained from the Health Directorates of Gedarif and Sennar States (3rd January 2011) and from the Ethics Review Board of MSF (19th July 2012).
Results

Prevalence of VL-malaria co-infections in Gedarif and Sennar States (2005-2010)

A total of 1324 medical records reporting on VL-diagnosed patients hospitalized in Gedarif Teaching Hospital, Al’Azaza kala-azar Clinic and Tabarakallah Hospital during the study period were reviewed for the survey. Of these, 1295 reported on patients with a laboratory-confirmed VL infection, who were included in the study, while the remaining 29 were excluded for lack of laboratory diagnostic evidence. Microscopy, mainly performed on lymph node (n = 848, 65.5%), bone marrow (n = 428, 33.1%) and/or spleen (n = 3, 0.2%) aspirates, was used for the diagnosis of VL. Serology was performed in 17 patients, 13 of whom were confirmed by DAT and 4 by rk39 antigen-based dipstick. Overall, 404 (31.2%) of the VL-confirmed patients were co-diagnosed with malaria at hospital admission or during hospitalization. In all cases but one, in which diagnosis was established with an RDT, malaria was confirmed by microscopy. Stratification of the study population by hospital resulted into an unevenly distributed co-infection rate, with a prevalence of 3.8% in Gedarif Teaching Hospital (n = 18), followed by Tabarakallah Hospital (n = 84) and Al’Azaza kala-azar Clinic (n = 302) with 26.2% and 60.8%, respectively. With the exception of one P. vivax-infection, all malaria cases for which the Plasmodium species was determined, were attributed to P. falciparum (n = 396, 98.0%).

Demographic and clinical features of VL-malaria co-infections in Gedarif and Sennar States (2005-2010)

With a median age of 12 years (interquartile range 5-23 years), nearly three quarters of VL-malaria co-infected patients were under 20 years old. Stratified by age, the percentage of cases was highest among children aged between 0 and 4 years (40.7%, n = 120/296) and progressively reduced until 29 years (27.0%, n = 50/185) (data not shown), indicating that the chance of detecting malaria in patients already diagnosed for VL declined as age increased. Residents of Sennar State appeared to be mostly stricken by the co-infection, particularly in the villages of Al’Azaza (28.0%), Om Bagraa (8.7%) and Jaldook (5.2%). Anemia (hemoglobin level <11 g/dL) was a hallmark of nearly all co- and mono-infected patients (91% and 79%, respectively), with most patients being diagnosed with mild or moderate anemia on hospital admission. Co-occurrence of malaria in VL patients resulted in an increased severity of the anemia status, as shown by the decrease in median hemoglobin level (7.0 g/dL, interquartile range 6.5-8.0 g/dL for cases and 8.0 g/dL, 7.0-9.0 g/dL for controls). Despite most co- and mono-infected patients presented with enlarged spleen, frequency (92% for cases, 95% for controls) and severity of splenomegaly (median spleen size below the costal margin 4.0 cm, interquartile range 2.0-6.0 cm for cases and 5.0 cm, 2.0-8.0 cm for controls) appeared to be slightly reduced by concomitant malaria.

In Al’Azaza kala-azar Clinic and Tabarakallah Hospital, most co-infections were detected among young boys (median age 9 and 10 years, interquartile range 3-23 years and 4-18 years, respectively) (Table 1). In Gedarif Teaching Hospital, conversely, cases unlike controls mainly consisted of girls (55.6%, n = 10/176) (Table 1), although the difference appeared to be statistically not significant. Seasonal distribution of hospital admissions was similar in all the three hospitals, with most patients presenting at hospital during the long dry season (November-May). Co-occurrence of malaria in VL patients resulted in a sharper division between dry and
Table 1. Characteristics of the overall VL population and VL-malaria co-infected patients, stratified by hospital, Sudan (2005-2010).

| Treatment center | Gedarif Teaching Hospital | Tabarakallah Hospital | Al’Azaza kala-azar Clinic |
|------------------|---------------------------|-----------------------|---------------------------|
|                  | Total population | Co-infected patients | Total population | Co-infected patients | Total population | Co-infected patients |
|                  | 468 (18) | 18 (3.8) | 321 (10) | 84 (26.2) | 497 (10) | 302 (60.8) |
| Gender           | 456 | 18 | 320 | 84 | 496 | 302 |
| Male             | 280 (61.4) | 8 (44.4) | 179 (55.8) | 49 (58.3) | 254 (51.1) | 155 (51.3) |
| Female           | 176 (38.6) | 10 (55.6) | 142 (44.2) | 35 (41.7) | 243 (48.9) | 147 (48.7) |
| Age              | 452 | 17 | 312 | 84 | 492 | 301 |
| 0-4 years        | 68 (15.5) | 2 (13.3) | 77 (24.0) | 21 (29.8) | 68 (13.8) | 42 (14.0) |
| 5-9 years        | 87 (19.8) | 3 (20.0) | 71 (22.1) | 19 (22.6) | 99 (20.1) | 55 (18.3) |
| 10-19 years      | 127 (28.9) | 5 (33.3) | 99 (30.8) | 28 (33.3) | 91 (18.5) | 59 (19.6) |
| 20-29 years      | 89 (20.2) | 2 (13.3) | 28 (8.7) | 6 (7.1) | 68 (13.8) | 42 (14.0) |
| ≥30 years        | 69 (15.7) | 3 (20.0) | 46 (14.3) | 10 (11.9) | 83 (16.9) | 48 (15.9) |
| Season           | 452 | 17 | 312 | 84 | 492 | 301 |
| Wet season       | 151 (33.4) | 3 (17.6) | 124 (38.6) | 32 (38.1) | 226 (46.3) | 131 (44.1) |
| Dry season       | 301 (66.6) | 14 (82.4) | 197 (61.4) | 52 (61.9) | 262 (53.7) | 166 (55.9) |
| Duration on-going sickness | 433 | 17 | 302 | 82 | 441 | 259 |
| Median (days)    | 30.0 | 30.0 | 14.0 | 12.0 | 30.0 | 21.0 |
| (interquartile range) | (15.0-40.0) | (13.5-76.0) | (9.0-21.0) | (7.0-14.0) | (14.0-61.0) | (14.0-61.0) |
wet season, particularly in Gedarif Teaching Hospital, where 82.4% of all co-infected patients were hospitalized between November and May, emphasizing the peculiar time-trend of these co-infections (in these regions malaria usually occurs during or shortly after the rains, while VL clinical cases peak during the dry season). Finally, with a median disease duration of 12 days from the initial onset of symptoms, co-infected patients presented at Tabarakallah Hospital earlier than at Al`Azaza kala-azar Clinic (21 days) or Gedarif Teaching Hospital (30 days).

Infection intensity and case-fatality rate of VL-malaria co-infections in Gedarif and Sennar States (2005–2010)

Intensity of VL infection, as determined by parasitaemia in lymph node or bone marrow aspirates, was directly related with the frequency of VL-malaria co-infections. Stratified by infection intensity, in fact, the ratio of VL-malaria co-infections was lowest in the groups with less severe VL \( (n = 339/757, 44.8\% \text{ as determined in } \text{lymph node aspirates and } n = 15/259, 5.8\% \text{ as determined in bone marrow aspirates}) \) and gradually increased as VL infection intensified \( (n = 3/4, 75.0\% \text{ and } n = 1/5, 20.0\% \text{ in lymph node and bone marrow aspirates, respectively, with the highest intensity}) \) (Table 2).

During hospitalization, 14 co-infected patients died, resulting in an overall case-mortality rate of 3.5%. A similar case-fatality rate was found among the controls \( (3.1\%, n = 27) \) (OR: 1.13; 95% CI: 0.59-2.17), indicating that concomitant malaria did not represent a risk factor for poor prognosis. When stratified by malaria treatment, in-hospital fatalities clustered amongst co-infected patients treated with artemether and quinine, whose mortality risk was 13-fold (OR: 13.34; 95% CI: 0.77-229.85) and 15-fold (OR: 15.35; 95% CI: 0.81-289.33) higher, respectively, than in patients receiving artesunate + SP (Table 3). Patients with a high density of *Leishmania* parasites were overrepresented among those who died, confirming previous findings, whereby high intensities of VL infection increased the mortality risk.\(^30\) Here, patients harboring high numbers of *Leishmania* parasites in their lymph node or bone marrow were found to be 13 (OR: 13.46; 95% CI: 1.34-135.77) and 15 (OR: 14.79; 95% CI: 2.24-97.74*).

Table 2. VL-malaria co-infections, stratified by VL infection intensity, and its association with the overall in-hospital death, Sudan (2005-2010).

| Infection intensity of VL in aspirates | n cases/denominators | % | In-hospital deaths n (%) | Discharged alive n (%) | Odds Ratio | 95% Confidence Interval |
|---------------------------------------|----------------------|---|--------------------------|------------------------|------------|------------------------|
| lymph node                            |                      |   |                         |                        |            |                        |
| +1                                    | 339/757              | 44.8 | 18 (85.7)               | 727 (92.0)             | 1          |                        |
| +2                                    | 24/44                | 54.5 | 2 (9.5)                 | 41 (5.2)               | 1.97       | 0.44-8.78              |
| +3                                    | 11/19                | 57.9 | 0                       | 19 (2.4)               | 1.00       | 0.99-1.00              |
| +4                                    | 3/4                  | 75.0 | 1 (4.8)                 | 3 (0.4)                | 13.46      | 1.34-135.77*           |
| bone marrow                           |                      |   |                         |                        |            |                        |
| +1                                    | 15/259               | 5.8 | 11 (84.6)               | 244 (98.8)             | 1          |                        |
| +2                                    | 1/5                  | 20.0 | 2 (15.4)               | 3 (1.2)                | 14.79      | 2.24-97.74*            |

*P-value <0.10 based on Fisher Exact Probability tests.
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CI: 0.26-0.55) compared with patients with no or minor spleen enlargement. In some patients, malaria displayed an exacerbating, although not significant, effect on VL infection, as revealed by the increased number of *Leishmania* parasites observed in their lymph node or bone marrow aspirates. Finally, while hepatomegaly was less frequently observed in co-infected patients compared to mono-infected patients (OR: 0.29; 95% CI: 0.18-0.48), jaundice (OR: 2.85; 95% CI: 1.43-5.66) and, particularly, weight loss (OR: 3.04; 95% CI: 2.37-3.90) were more commonly reported among co-infected cases.

All 11 variables associated with the VL-malaria co-infection in the univariate analysis were included in the multivariable models. Due to the high number of missing data in reporting clinical signs and symptoms, three different models were designed to explore the association between the selected variables and the VL-malaria co-infection: model A, in which the independent risk factors were identified, model B describing the association between clinical signs and symptoms and the co-infection and model C, which combines all variables (risk factors, clinical signs and symptoms) in one analysis. Model A (Table 5) shows that, after adjusting for the other variables

95% CI: 2.24-97.74) times more likely to die, respectively (Table 2).

**Risk factors for VL-malaria co-infections in Gedarif and Sennar States (2005-2010)**

Associations between demographic and clinical variables and the co-infection, as described by univariate analysis, are summarized in Table 4. Gender and age were identified as risk factors for the VL-malaria co-infection. Significant associations were also found to link the co-infected patients with the treatment center in which they have been hospitalized. Neither the rainy season nor the intake of anti-malarial drugs prior to hospitalization significantly altered the risk for VL patients of acquiring malaria. Malnourishment was relatively more common among cases, resulting in a positive association between severe malnutrition and the co-infection (OR: 2.21; 95% CI: 1.01-4.85). A similar positive association was found with moderately (OR: 1.73; 95% CI: 1.32-2.28) or severely anemic patients (OR: 1.50; 95% CI: 0.99-2.28). The likelihood of being diagnosed with concomitant VL and malaria decreased as spleen size increased: patients with massive splenomegaly (spleen size ≥6 cm below the costal margin) were found to be more than twice less likely to be co-infected (OR: 0.38; 95% CI: 0.26-0.55) compared with patients with no or minor spleen enlargement. In some patients, malaria displayed an exacerbating, although not significant, effect on VL infection, as revealed by the increased number of *Leishmania* parasites observed in their lymph node or bone marrow aspirates. Finally, while hepatomegaly was less frequently observed in co-infected patients compared to mono-infected patients (OR: 0.29; 95% CI: 0.18-0.48), jaundice (OR: 2.85; 95% CI: 1.43-5.66) and, particularly, weight loss (OR: 3.04; 95% CI: 2.37-3.90) were more commonly reported among co-infected cases.

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| Malaria treatment       | n in-hospital fatalities / denominator | %   | Odds Ratio | 95% Confidence Interval |
|-------------------------|---------------------------------------|-----|------------|-------------------------|
| Artesunate + SP         | 0/118                                 | 0   | 1          |                         |
| Artemether              | 10/186                                | 5.4 | 13.34      | 0.77-229.85*            |
| Quinine                 | 4/69                                  | 5.8 | 15.35      | 0.81-289.33*            |
| Artesunate              | 0/15                                  | 0   | -          | -                       |
| Artemether + lumefantrine| 0/9                                   | 0   | -          | -                       |

*P*-value <0.10 based on Fisher Exact Probability tests. For the statistical analysis, a value of 0.5 was added to each variable to ensure calculation of OR and 95% CI.

Table 3. VL-malaria co-infections, stratified by malaria treatment, and their association with in-hospital death, Sudan (2005-2010).
### Table 4. Univariate analysis of characteristics associated with VL-malaria co-infections, Sudan (2005-2010).

| Variable                                    | Cases $n$ (%) | Controls $n$ (%) | Crude Odds Ratio | 95% Confidence Interval |
|---------------------------------------------|---------------|------------------|------------------|--------------------------|
| Gender                                      |               |                  |                  |                          |
| Male                                        | 212 (52.5)    | 501 (57.6)       | 1                |                          |
| Female                                      | 192 (47.5)    | 369 (42.4)       | 1.23             | 0.97-1.56*               |
| Age                                         | 400           | 853              |                  |                          |
| <5 years                                    | 120 (30.0)    | 176 (20.6)       | 1.53             | 1.05-2.24*               |
| 5-9 years                                   | 77 (19.3)     | 180 (21.1)       | 0.96             | 0.64-1.44                |
| 10-19 years                                 | 92 (23.0)     | 225 (26.4)       | 0.92             | 0.62-1.35                |
| 20-29 years                                 | 50 (12.5)     | 135 (15.8)       | 0.83             | 0.53-1.30                |
| ≥30 years                                   | 61 (15.3)     | 137 (16.1)       | 1                |                          |
| Treatment center                            |               |                  |                  |                          |
| Gedarif Teaching Hospital                   | 18 (4.4)      | 450 (51.0)       | 1                |                          |
| Tabarakallah Hospital                       | 84 (20.8)     | 237 (26.9)       | 8.86             | 5.20-15.10*              |
| Al'Azaza kala-azar Clinic                   | 302 (74.8)    | 195 (22.1)       | 38.72            | 23.38-64.11*             |
| Season                                      | 398           | 863              |                  |                          |
| Dry season                                  | 232 (58.3)    | 528 (61.2)       | 1                |                          |
| Wet season                                  | 166 (41.7)    | 335 (38.8)       | 1.13             | 0.89-1.44                |
| Previous anti-leishmanial treatment         | 389           | 860              |                  |                          |
| No                                          | 373 (95.9)    | 806 (93.7)       | 1                |                          |
| Yes                                         | 16 (4.1)      | 54 (6.3)         | 0.64             | 0.36-1.13                |
| Previous anti-malarial treatment            | 396           | 864              |                  |                          |
| No                                          | 277 (70.0)    | 604 (69.9)       | 1                |                          |
| Yes                                         | 119 (30.0)    | 260 (30.1)       | 1.00             | 0.77-1.29                |
| Malnutrition                                | 322           | 575              |                  |                          |
| None                                        | 262 (81.4)    | 497 (86.4)       | 1                |                          |
| Mild                                        | 17 (5.3)      | 25 (4.3)         | 1.29             | 0.68-2.43                |
| Moderate                                    | 29 (9.0)      | 41 (7.1)         | 1.34             | 0.82-2.21                |
| Severe                                      | 14 (4.4)      | 12 (2.1)         | 2.21             | 1.01-4.85*               |
| Anemia degree on admission                  | 374           | 725              |                  |                          |
| Median Hb (interquartile range)             | 7.0 (6.5-8.0) | 8.0 (7.0-9.0)    |                  |                          |
| None-mild (Hb ≥7.3 g/dL)                    | 182 (48.7)    | 445 (61.4)       | 1                |                          |
| Moderate (Hb 5.3-7.2 g/dL)                  | 149 (39.8)    | 210 (29.0)       | 1.73             | 1.32-2.28*               |
| Severe (Hb <5.3 g/dL)                       | 43 (11.5)     | 70 (9.7)         | 1.50             | 0.99-2.28*               |
| Spleen size below the costal margin         | 238           | 563              |                  |                          |
| Median (interquartile range)                | 4.0 (2.0-6.0) | 5.0 (2.0-8.0)    |                  |                          |
| 0-3 cm                                      | 94 (39.5)     | 150 (26.6)       | 1                |                          |
| 4-5 cm                                      | 79 (33.2)     | 141 (25.0)       | 0.89             | 0.61-1.30                |
| ≥6 cm                                       | 65 (27.3)     | 272 (48.3)       | 0.38             | 0.26-0.55*               |
Table 4. (Continued)

| Variable                                | Cases n (%) | Controls n (%) | Crude Odds Ratio | 95% Confidence Interval |
|------------------------------------------|-------------|----------------|------------------|-------------------------|
| Infection intensity in lymph node aspirate |             |                |                  |                         |
| +1                                       | 339 (89.9)  | 418 (93.5)     | 1                |                         |
| +2                                       | 24 (6.4)    | 20 (4.5)       | 1.48             | 0.80-2.72               |
| +3                                       | 11 (2.9)    | 8 (1.8)        | 1.70             | 0.67-4.26               |
| +4                                       | 3 (0.8)     | 1 (0.2)        | 3.70             | 0.38-35.72              |
| Infection intensity in bone marrow aspirate | 16          | 248            |                  |                         |
| +1                                       | 15 (93.8)   | 244 (98.4)     | 1                |                         |
| +2                                       | 1 (6.2)     | 4 (1.6)        | 4.07             | 0.43-38.68              |
| Symptoms                                 |             |                |                  |                         |
| Hepatomegaly                             | 19 (4.7)    | 128 (14.5)     | 0.29             | 0.18-0.48*              |
| Jaundice                                 | 19 (4.7)    | 15 (1.7)       | 2.85             | 1.43-5.66*              |
| Weight loss                              | 278 (68.8)  | 370 (42.0)     | 3.04             | 2.37-3.90*              |

*P-value <0.10 based on Person Chi-square tests.

Hb = hemoglobin

Table 5. Model A: Multivariate analysis of risk factors for VL-malaria co-infections among VL patients, Sudan (2005-2010).

| n = 1253 | Odds Ratio | 95% Confidence Interval | P-value |
|----------|------------|-------------------------|---------|
| Treatment center                          |            |                         |         |
| Gedarif Teaching Hospital                  | 1          | –                       | <0.001  |
| Tabarakallah Hospital                      | 9.88       | 5.57-17.55              | <0.001  |
| Al’Azaza kala-azar Clinic                 | 44.85      | 25.89-77.71             | <0.001  |
| Age                                         |            |                         |         |
| <5 years                                    | 1.26       | 0.81-1.97               | 0.305   |
| 5-9 years                                   | 1.01       | 0.63-1.62               | 0.972   |
| 10-19 years                                 | 1.31       | 0.83-2.09               | 0.246   |
| 20-29 years                                 | 1.06       | 0.62-1.80               | 0.842   |
| ≥30 years                                   | 1          | –                       | 0.608   |
included in the model, the treatment center in
which co-infected patients were hospitalized,
and therefore its catchment area, remained the
most important factor associated with the risk of
being co-infected. After adjusting for the other
variables included in the model, gender and age
lost their statistical significance. The variable age,
however, was kept in the model as it significantly
increased its fit.

In model B (Table 6), logistic regression was
performed on the sub-cohort of patients for
whom symptoms, besides other features, were
reported. After adjusting for the other variables
included in the model, malnutrition lost its
statistical significance and was therefore excluded
from the model. The variable hepatomegaly
also lost most of its significance, but a slightly
negative association could still be recognized
(OR: 0.52; 95% CI: 0.26-1.01). The risk for VL
patients of developing jaundice (OR: 2.52; 95%
CI: 1.04-6.09) or undergoing weight loss (OR:
2.46; 95% CI: 1.72-3.50), on the other hand, was
doubled by the concomitancy of malaria.
Massive splenomegaly was less common among
co-infected patients (OR: 0.53; 95% CI: 0.35-0.81),
who instead suffered frequent exacerbations of
their anemic status, as observed in the group
with moderate anemia (OR: 1.58; 95% CI: 1.10-
2.28).

Combining all variables in one model (model
C, Table 7) resulted in the loss of significance
for all variables, except the one involving the
treatment center, which possibly displayed a
confounding effect on all other associations.
The model confirmed a key role for this variable,
which led to an 8- and 42-fold different likelihood
of being co-diagnosed with VL and malaria in
Tabarakallah Hospital (OR: 8.02; 95% CI: 3.68-
17.46) and Al’Azaza kala-azar Clinic (OR: 41.66;
95% CI: 20.01-86.72), respectively, compared to
Gedarif Teaching Hospital.

### Table 6. Model B: Multivariate analysis of clinical signs and symptoms associated to VL-malaria co-infections,
Sudan (2005-2010).

|                              | Odds Ratio | 95% Confidence Interval | P-value |
|------------------------------|------------|-------------------------|---------|
| Symptoms                     |            |                         |         |
| Hepatomegaly                 | 0.52       | 0.26-1.01               | 0.052   |
| Jaundice                     | 2.52       | 1.04-6.09               | 0.041   |
| Weight loss                  | 2.46       | 1.72-3.50               | <0.001  |
| Spleen size below the costal margin |            |                         |         |
| 0-3 cm                       | 1          | –                       | 0.007   |
| 4-5 cm                       | 0.90       | 0.60-1.36               | 0.629   |
| ≥6 cm                        | 0.53       | 0.35-0.81               | 0.003   |
| Anemia                       |            |                         |         |
| None – mild (Hb ≥7.3 g/dL)   | 1          | –                       | 0.440   |
| Moderate (Hb 5.3-7.2 g/dL)   | 1.58       | 1.10-2.28               | 0.013   |
| Severe (Hb <5.3 g/dL)        | 1.10       | 0.63-1.93               | 0.737   |

*Hb = hemoglobin*
Chapter 3

**MSF’s dataset**

Between November and December 1998, 516 primary VL cases were diagnosed in the MSF kala-azar treatment centers of Um-el-Kher and Kassab. Positive DAT results were obtained for most patients (n = 440, 86%), whose diagnosis was occasionally confirmed, as for all patients with borderline DAT titers (from 1:400 to 1:6400), by parasitological evidence in lymph node or spleen aspirates (n = 140, 27%). Microscopy-confirmed malaria was co-diagnosed in 89 VL patients (17.2%), 10 of whom died (11.2%). Given the lower case-fatality rate among controls (2.8%), here co-infections significantly increased the mortality risk of VL patients by four-and-a-half fold (OR: 4.38; 95% CI: 1.83-10.48) (Table 8). Of the 10 co-infection-related fatalities, 4 were considered to be caused by anemia, and one by cerebral malaria. Although no clear differences in the median hemoglobin level of VL-malaria co-infected patients (7.5 g/dL) vs. the mono-infected VL patients (8.2 g/dL) could be detected, the proportion of patients with severe anemia (Hb <5.3 g/dL) was significantly higher among cases (15.7%) than among controls (5.2%) (OR: 3.44; 95% CI: 1.68-7.02). No other major differences in nutritional status, spleen size below the costal margin and duration of on-going disease prior to hospitalization were observed between co- and mono-infected patients, except their distribution between the two treatment centers. With more than 80% of the co-infection cases recorded in Um-el-Kher Hospital, the risk here, of being co-diagnosed with VL and malaria was nearly double (OR: 1.94; 95% CI: 1.10-3.41) than at Kassab Hospital.

**Discussion**

This is the first multicenter retrospective survey ever undertaken in the field of VL-malaria co-infections. The study describes the epidemiology of concomitant malaria among VL in-patients from Gedarif Teaching Hospital, Tabarakallah Hospital and Al’Azaza kala-azar Clinic, east Sudan (2005-2010) and confirms its clinical relevance by

Table 7. Model C: Multivariate analysis of risk factors, clinical signs and symptoms associated to VL-malaria co-infections, Sudan (2005-2010).

| n = 690 | Odds Ratio | 95% Confidence Interval | P-value |
|--------|------------|-------------------------|---------|
| Treatment center |          |                         |         |
| Gedarif Teaching Hospital | 1 | – | <0.001 |
| Tabarakallah Hospital | 8.02 | 3.68-17.46 | <0.001 |
| Al’Azaza kala-azar Clinic | 41.66 | 20.01-86.72 | <0.001 |
| Spleen size below the costal margin |          |                         |         |
| 0-3 cm | 1 | – | 0.706 |
| 4-5 cm | 1.20 | 0.76-1.90 | 0.428 |
| ≥6 cm | 1.15 | 0.71-1.86 | 0.563 |
| Anemia |          |                         |         |
| None – mild (Hb ≥7.3 g/dL) | 1 | – | 0.219 |
| Moderate (Hb 5.3-7.2 g/dL) | 1.28 | 0.85-1.94 | 0.239 |
| Severe (Hb <5.3 g/dL) | 0.74 | 0.39-1.40 | 0.356 |

Hb = hemoglobin
comparing prevalence and mortality rates with an antecedent (1998), independently collected dataset from the same region (Um-el-Kher and Kassab Hospitals). Not only the risk of co-acquiring VL and malaria appears to be substantial in these areas, with a significant geographical variation, but the clinical implications deriving from being co-infected provide the evidence for a public health concern. Exacerbated clinical pictures and increased mortality risk, possibly due to inadequate anti-malarial treatment, were highlighted by this survey, suggesting that prompt diagnosis and effective therapy of concomitant malaria is needed to ensure positive resolution of the VL-malaria co-infection.

Ranging from 3.8% to 60.8% and with a median of 26.2%, the prevalence of malaria co-infection...
among VL surveyed patients (2005-2010) confirms the frequent superimposing of the two diseases in rural areas of Gedarif and Sennar States. Although these estimates may be higher than expected, based on the local malaria transmission rates, similar figures have been found in the MSF’s dataset from Um-el-Kher and Kassab Hospitals, where 19.7% and 11.3%, respectively, of the VL patients enrolled in the clinical trial (1998) were positive for malaria. Again at Um-el-Kher Hospital, clinical studies conducted between January 2004 and early 2005 revealed that 15% of pregnant VL women enrolled in the trial\(^{31}\) and 31% of Ambisome-treated VL patients\(^{6}\) were co-diagnosed with malaria, while a 4.8% rate was found in Kassab (2005-2006),\(^{32}\) where the malaria prevalence is notoriously lower. In agreement with previous observations performed at Amudat Hospital, Uganda (2000-2006),\(^{1}\) where a co-infection rate of 19% was reported using the same criteria as here, the frequent co-occurrence of malaria in VL patients suggests that these patients may have an increased susceptibility towards the malaria infection, possibly due to a VL-promoted impairment of the immune system. The clustering of most co-infection diagnoses in Al’Azaza kala-azar Clinic (74.8%), however, followed by Tabarakallah Hospital (20.8%) and Gedarif Teaching Hospital (4.4%) is rather unexpected. In Gedarif Teaching Hospital, only 3.8% of the VL-confirmed patients were co-diagnosed with malaria, a figure which may be explained by its function as Regional Reference Hospital, besides the low malaria burden found in this urban area. Difficult cases encountered in rural hospitals and referred to Gedarif Teaching Hospital, usually received, prior to admission to the Regional Hospital, a full course of anti-malarial treatment to exclude malaria as a possible complication. This may therefore have resulted in a lower percentage of VL patients having malaria on hospital admission. The longer disease duration described among VL patients hospitalized in Gedarif Teaching Hospital, compared to the other two study sites and their villages of origin, seem to confirm that a large number of these patients may not have presented to this hospital as a first-line action. If, therefore, a higher malaria-VL co-infection rate is to be expected in rural hospitals of Gedarif and Sennar States, the figure obtained in Al’Azaza kala-azar Clinic (60.8%) appears to somehow overestimate the burden posed by this co-morbidity. Given that higher malaria rates, favored by the proximity with the river and natural reserve, might have locally occurred, poor quality of malaria diagnosis in Al’Azaza kala-azar Clinic cannot be excluded.

Concomitant malaria partly exacerbated the clinical picture of VL patients, who presented with more frequent emaciation, icterus and moderate anemia. Two scenarios may be postulated: co-infected patients may either have suffered from malaria-associated symptoms which, in addition to the VL ones, have caused deterioration of their clinical condition and/or have run an exacerbated course of VL due to concomitant malaria, in which case symptoms may be related to VL rather than to malaria. If this latter hypothesis may find its rationale in the increased number of *Leishmania* parasites observed in aspirates of co-infected patients, the first speculation may be supported by the peculiar symptom pattern. Jaundice, in fact, is rarely described among VL patients, while it is not uncommon in *P. falciparum* malaria.\(^{33}\) Weight loss and anemia, on the other hand, are hallmark of both VL and malaria and an increased severity of the anemic status, as observed in co-infected patients, may therefore be the result of an added effect displayed by both diseases on the polyparasitized host. In apparent contradiction
is the finding, whereby co-infected patients suffered from less severe hepato-splenomegaly. Suggesting a less advanced state of the diseases in the co-infected patients, the result may be explained by their earlier hospitalization compared to mono-infected VL patients. Patients with concomitant VL and malaria, in fact, presented at hospital nearly 10 days earlier, on average, than those with only VL, possibly due to their more severe symptomatology. Importantly, this may have also had positive implications for their prognosis, which was found to be similar to the controls’ one.

In antithesis to the positive resolution of VL-malaria co-infections during the 2005-2010 survey, is the significantly higher fatality rate (P-value 0.001) associated with co-infected patients enrolled by MSF at Um-el-Kher and Kassab Hospitals in 1998. During this trial, co-infected patients were nearly four-and-a-half times more likely to die compared with the VL mono-infected patients, whose mortality (2.8%) on the other hand, compares well to what was found in the most recent survey (3.1%). Different anti-malarial regimens were used within the two study groups: SP and quinine in 1998 for uncomplicated and severe malaria, respectively; artemisinin derivatives (alone or in combination) and more rarely quinine between 2005 and 2010. Sudan’s choice to introduce artemisinin-based combination therapies (ACTs) for treatment of uncomplicated and severe malaria was implemented nation-wide in 2004, following increasing evidence of resistance against chloroquine, SP and quinine, for which failure rates up to 76.9%, 16.1% and 16.7%, respectively, were recorded in east Sudan prior to ACT era. The 11.2% mortality rate of VL-malaria co-infections observed in 1998 at Um-el-Kher Hospital may therefore have partially resulted from treatment failures attributable to either SP or quinine, besides the more severe malaria course in patients receiving quinine. In fact, no major differences for age, median Hb level, nutritional status, spleen size and duration of on-going disease distinguished the co-infected patients’ group at Um-el-Kher and Kassab Hospitals from the one surveyed in 2005-2010 and from its relative controls. The only exception is to be found in the significantly higher number of VL patients who developed severe anemia when co-infected with malaria, similarly to what was observed during the 2005-2010 survey, though to a lesser extent. Hence, concomitant malaria may not only cause aggravation of VL patients’ clinical condition, but it may also result in a poorer prognosis, if failed to be treated. Among co-infected patients surveyed in 2005-2010, an increased mortality risk, not ascribable to differences in Leishmania intensities, was observed when quinine (P-value 0.07) and artemether (P-value 0.04) were administered as anti-malarials, suggesting either increased malaria severity or inadequate drug treatment.

The population surveyed within this study consists of VL patients residing in over 300 different villages, mainly located in Gedarif and Sennar States. Within these districts, Tabarakallah Hospital and Al’Azaza kala-azar Clinic are two rural hospitals receiving patients from some of the worst-affected villages. It should be noted, however, that the cohort of VL patients surveyed here might be sub-representative of the local VL community, as the number of VL-related hospitalizations carried out by the two MSF’s treatment centers in Gedarif Sate (>4000 per year between 1997 and 1999) exceeds by far the one recorded by the three study hospitals (1324 in total between 2005 and 2010). Other limitations apply to this study, the most important
ones being the lack of non-VL malaria infected patients and the quality of diagnosis. Unlike VL, uncomplicated malaria infections are commonly treated on an out-patient basis in hospitals, clinics or simple practices, resulting in few data being systematically recorded by the different facilities. Moreover, malaria patients are rarely found in VL-dedicated hospitals, such as those surveyed in this study. This resulted into the lack of valid malaria controls, essential to investigate whether VL might predispose or rather protect towards a malaria-attack and whether it might influence its course and clinical presentation. In absence of quality control, quality of diagnosis remains questionable. Variable outcomes, as documented in medical records, may have suffered from poor standardization, due to the different techniques implemented by clinicians in the different treatment centers and the possible involvement of different health workers in filling these files.

Conclusion

Based on the results of this study, we conclude that VL patients living in areas with unstable seasonal malaria, such as east Sudan, are highly exposed to the risk of developing concomitant malaria. Large variation in the geographical distribution of co-infection cases highlights the presence of environmental and/or social factors, whose identity and relevance in the risk of co-acquiring VL and malaria still remain to be elucidated. Clinical concerns should arise when the two diseases co-occur in the same patients, as significant exacerbation of their clinical condition was observed, along with an increased mortality risk, possibly associated with inappropriate anti-malarial treatment. Local health care policies should take into account the high co-infection burden borne by VL foci with unstable malaria, by recommending systematic malaria screening for all VL patients and ACTs for treatment of malaria.

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Authors’ contributions

EvdB contributed in conceiving of the study, drafted the study protocol and the manuscript and participated in the data analysis. MB performed the data entry and the statistical analysis and helped to draft the manuscript. AN performed the collection and entry of data. PM and EA participated in conceiving of the study and reviewed the study protocol and the manuscript. AT participated in the collection and entry of data and helped to draft the study protocol. HA participated in the collection and entry of data. SA reviewed the study protocol and the manuscript. KR participated in the data analysis and reviewed the manuscript. BN coordinated the collection and entry of data, organized local logistics, and helped to review the study protocol and the manuscript. HS conceived the study and participated in its design, and reviewed the study protocol and the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.
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Cytokine profiles amongst Sudanese patients with visceral leishmaniasis and malaria co-infections

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Abstract

Background
The immune system plays a critical role in the development of co-infections, promoting or preventing establishment of multiple infections and shaping the outcome of pathogen-host interactions. Its ability to mediate the interplay between visceral leishmaniasis (VL) and malaria has been suggested, but poorly documented. The present study investigated whether concomitant infection with *Leishmania donovani* complex and *Plasmodium falciparum* in naturally co-infected patients altered the immunological response elicited by the two pathogens individually.

Results
Circulating levels of interferon (IFN)-γ, interleukin (IL)-2, IL-4, IL-6, IL-10, IL-12p70, IL-13, IL-17A and tumor necrosis factor (TNF) were assessed in sera of patients infected with active VL and/or malaria and healthy individuals from Gedarif State, Sudan. Comparative analysis of cytokine profiles from co- and mono-infected patients highlighted significant differences in the immune response mounted upon co-infection, confirming the ability of *L. donovani* and *P. falciparum* to mutually interact at the immunological level. Progressive polarization towards type-1 and pro-inflammatory cytokine patterns characterized the co-infected patients, whose response partly reflected the effect elicited by VL (IFN-γ, TNF) and malaria (IL-2, IL-13), and partly resulted from a synergistic interaction of the two diseases upon each other (IL-17A). Significantly reduced levels of *P. falciparum* parasitaemia (*P*<0.01) were detected in the co-infected group as opposed to the malaria-only patients, suggesting either a protective or a non-detrimental effect of the co-infection against *P. falciparum* infection.

Conclusions
These findings suggest that a new immunological scenario may occur when *L. donovani* and *P. falciparum* co-infect the same patient, with potential implications on the course and resolution of these diseases.

Keywords
Visceral leishmaniasis, Malaria, Co-infection, Cytokines, Sudan
Background

Co-infection of a host by multiple parasite species is a commonly observed condition, where individual susceptibility and infectiousness are affected at various levels.\textsuperscript{1-3} Inter-microbial competition along with micro-environmental and immunological conditioning govern the magnitude and type of interactions across polyparasitism, promoting or hampering establishment of multiple parasite infections and their ability to persist and spread to new patients.\textsuperscript{4} At the immunological level, cross-regulation of pathogen-associated pathways is achieved through cytokine signaling; an integrated network responsible for the controlled polarization and amplification of immune responses.\textsuperscript{5,6} As a result, cytokines secreted in response to one parasite species may act synergistically or antagonistically with those elicited by another species, enhancing (cross-immunity) or impairing (immune-suppression) simultaneous control of infections. Cytokine ability to shape the immune system into efficacious responses arises from their downstream actions on the effector mechanisms, with great variation across different host-parasite systems.\textsuperscript{6,7} Conversely, upstream cytokine functions are more stereotypical, while remaining predictive of effector efficacy. Hence, their characterization in polyparasitized models provide a valuable and convenient framework for understanding co-infection dynamics.\textsuperscript{5,8}

Visceral leishmaniasis (VL) and malaria are two major parasitic diseases which overlap geographically and may co-exist in the same patients.\textsuperscript{9,10} Partially sharing the same host tissue niches, the two infections have the ability of evading and subverting immune surveillance, with clinical outcomes largely dependent on the immunological status of the host. Obligate intracellular parasites of the visceralizing \emph{Leishmania donovani} complex successfully colonize macrophages and other reticuloendothelial cells of the lymphoid system, by altering signaling pathways associated with parasite killing and adaptive immunity engagement.\textsuperscript{11,12} As a result, phagocytes harboring \emph{Leishmania} parasites are incapacitated to function as cytolytic and T cell priming effectors, causing immune dysfunction and tissue injury. Resistance to infection is conferred by development of effective T helper cell 1-type (Th1) responses, mounted upon release of a pleiotropic interleukin (IL)-12 and interferon (IFN)-γ cytokine network, and boosted by pro-inflammatory (tumor necrosis factor (TNF), IL-23, IL-17A) and Th2-promoting (IL-4) mediators.\textsuperscript{13-17} Thus, in contrast to the classical Th1-Th2 paradigm suiting predictions of resistance/susceptibility to cutaneous leishmaniasis,\textsuperscript{12} clearance of \emph{L. donovani} appears to be blunted by induction of the regulatory T cell subset Tr1, rather than Th2 or Th3 clusters, through an IL-10 mediated mechanism.\textsuperscript{18-20} Anergic IL-10-producing T cells have also been detected in response to \emph{Plasmodium falciparum} infections,\textsuperscript{21-25} which account for the largest proportion of malaria disease. Complex, stage-specific networks of antibody-dependent and cell-mediated interactions provide immunity against \emph{Plasmodium} spp., with clinical implications depending on the type and timing of cytokine release. Early type-1 responses, dominated by IFN-γ, IL-2 and TNF, have been reportedly associated with inhibition of liver stage development,\textsuperscript{26-31} resolution of acute malaria parasitaemias\textsuperscript{32-34} and delay of re-infection,\textsuperscript{35} as confirmed by the absolute requirement of IFN-γ in the effector mechanism of sporozoite-induced protective immunity.\textsuperscript{35-38} Release of these cytokines, initiated by the innate immune system (natural killer (NK) cells, γδT-
\( \alpha\beta T \) cells)\(^{38-41} \) and sustained by \( \text{Plasmodium} \)-specific CD8\(^{+} \) and CD4\(^{+} \) cells,\(^{32,37,39} \) requires to be timely counterbalanced by a switch to type-2 responses which propagate humoral immunity against the erythrocytic stage, and limit the pathogenicity of pro-inflammatory cytokines.\(^{42,43} \) Similar symptom-suppressing activities appear to be mediated by IL-10 and transforming growth factor (TGF)-\( \beta \), which in the attempt to reduce immunopathology, can interfere with the protective effects of IFN-\( \gamma \) and TNF and allow the parasite to grow uncontrollably.\(^{21,44,45} \)

Despite VL and malaria co-infection cases being encountered across co-endemic areas, little work has been done so far to examine the dynamics of this co-infection and its effect on host immunity. Studies performed in co-infection murine models of \( \text{P. chabaudi} \) \( \text{chabaudi} \) and \( \text{L. infantum} \),\(^{46} \) and of \( \text{P. yoelii} \) and \( \text{L. mexicana amazonensis} \)\(^{47,48} \) have highlighted an exacerbating effect of the two diseases upon each other, particularly for leishmaniasis, whose enhanced parasite load was attributed to the \( \text{Plasmodium} \)-triggered release of splenic IL-4, as assessed by gene expression.\(^{46} \) Conversely, in golden hamsters pre-inoculation with different \( \text{L. infantum} \) strains resulted in a reduced proliferation of \( \text{P. berghei} \), with no aggravation of the \( \text{Leishmania} \) infection.\(^{49} \)

Whilst these discrepancies reflect the difficulty in extrapolating animal model data, particularly when dealing with multiple infections, they agree on recognizing the immune system as a major determinant of \( \text{Leishmania} \) and \( \text{Plasmodium} \) spp. interactions upon co-infection.

In the present study, the cytokine profiles of naturally co-infected patients were examined. Blood samples from patients actively infected with VL and/or malaria and from healthy individuals were collected during an exploratory survey conducted in Gedarif State, Sudan, and the level of nine different cytokines selected from across the four major response arms of the immune system were assessed simultaneously. The comparative analysis between co- and mono-infected groups highlighted substantial differences in the cytokine profile of these patients and their levels of \( \text{P. falciparum} \) parasitaemia, emphasizing the importance of immune-mediated interactions in polyparasitism.

**Methods**

**Study site, study cases and ethical considerations**

The sample collection was performed in February 2011 in the village of Tabarak Allah, an endemic area of \( \text{L. donovani} \), located in Gedarif State, Sudan. Patients were recruited at Tabarak Allah Hospital, which hosts a VL treatment center managed by Médecins sans Frontières since January 2010. Seasonal and unstable malaria prevails in the area, where co-infection rates of 18% to 45% were recorded amongst Tabarak Allah VL in-patients (2005-2010).\(^{10} \)

All individuals included in the study originated from Gedarif State and aged six years or above. Eligibility for the study was precluded to children up to age six, due to immature status of their immune system.\(^{50,51} \) Individuals with previous history of VL were also excluded to ensure relapse case were not enrolled in the study. Included patients reported no history of immune-related disorders, or of ongoing infectious diseases (other than VL and malaria). Clinical and laboratory examinations were performed, including assessment of hemoglobin levels (by HemoCue) and white blood cell (WBC) counts (by microscopy), and their outcomes recorded on anonymized case record forms. Plasma and
serum were obtained from peripheral blood and stored at -70°C until tested. None of the subjects received anti-leishmanial chemotherapy before collection of blood samples, while a minimum of two-week lapse from previous treatment was observed for anti-malarial drugs. Written informed consent was obtained from each study participant above 18 years of age or guardian who consented on their behalf, after providing information on the study aim and procedures in the local language. The survey was conducted with the approval of the Sudanese Minister of Health (National Research Ethics Review Committee), who granted National Ethical Clearance (Nr. 140-10-11).

**Diagnostic algorithm**
For categorization of study subjects, the following diagnostic algorithm was implemented. All patients presenting at the study hospital with symptoms of VL and/or malaria, including fever, weight loss, hepato-splenomegaly and anemia, were given physical examination. Finger-prick blood was assessed by microscopy for diagnosis of malaria and by direct agglutination test (DAT) for diagnosis of VL. Assessment of *P. falciparum* parasitaemia was performed by microscopy, counting the total number of parasites per 200 WBCs, as previously described.52 Artemisinin-based combination therapies were administered to patients positively diagnosed for malaria. The DAT was performed on filter paper-spotted blood, using freeze-dried antigen and control sera from the Royal Tropical Institute (Amsterdam, the Netherlands). A cut-off titer of 3,200 was used as previously established for the area.53 Accordingly, patients meeting the WHO clinical definition for VL (fever for >2 weeks with either anemia or splenomegaly),54 and having a DAT titer >3,200, but no history of VL were diagnosed with primary active VL and received a 30-day course of parenteral sodium stibogluconate, conforming to the national policy, along with the anti-malarial regimen, if required. Patients who tested negative for both VL and malaria were excluded from the study and referred to the hospital medical staff for alternative diagnoses. In total, 102 participants (77 VL and/or malaria confirmed patients and 25 healthy controls) were included at study entry. Prior to initiation of specific chemotherapies, peripheral blood was collected from enrolled participants and processed to obtain serum and plasma samples. A second subsequent evaluation of all specimens to confirm (or exclude in case of healthy controls) diagnosis of VL and/or malaria was independently performed at the Royal Tropical Institute, the Netherlands. Specific antibodies to *Leishmania* were measured in sera or, when unavailable, filter paper-spotted blood using the DAT and two commercially available rk39 tests, the DiaMed IT-Leish® (Diamed AG, Cressier sur Morat, Switzerland) and the Kalazar Detect™ (InBios International, Inc., Washington, USA). The following conditions were considered indicative of VL infection: a) DAT titers ≥3,200, with or without positive rk39 test outcomes; b) DAT titers =1,600 with at least one confirmatory rk39 test; c) DAT titers <1,600 with positive result in the field (>3,200) and a positive rk39 test. Samples that did not fulfill these criteria were excluded from the study (n = 8) or re-categorized (n = 3), if tested positive for malaria only. Thin and thick blood smears of all study participants were microscopically re-assessed to confirm or exclude presence of *P. falciparum* parasites. When slide re-examination resulted in discordant outcomes, a rapid test (SD Bioline, Standard Diagnostics, Inc., Korea) for detection of *P. falciparum* and *Plasmodium* spp. was performed on the corresponding serum sample. Positive results obtained with the serological test were
considered confirmatory of malaria cases \(n = 4\), while specimens which tested negative were excluded from the analysis \(n = 2\). In addition, five other cases (3 healthy controls, 1 co-infected patient and 1 malaria patient) were excluded from the study, due to poorly reliable test outcomes, missing samples or diagnosis of non-\(P. falciparum\) malaria. From the 102 participants included at study entry, 15 were excluded because they did not match the diagnostic criteria, narrowing the sample size to 87 cases.

**Clinical groups**

**Group 1**

Primary VL cases \(n = 29\), defined as VL-sero-positive individuals who fulfilled the clinical case definition of VL and tested negative for malaria.

**Group 2**

Clinical malaria patients \(n = 21\). This group included parasitologically-confirmed cases of \(P. falciparum\) malaria who presented at hospital with clinical symptoms, such as fever, hepatosplenomegaly and anemia, and lacked Leishmania-specific antibodies.

**Group 3**

VL and malaria co-infected patients \(n = 15\), defined as VL-seropositive individuals diagnosed with a \(P. falciparum\) malaria infection.

**Group 4**

Healthy endemic controls \(n = 22\) with a VL-sero-negative profile and no microscopically detectable malaria in peripheral blood.

**Cytokine measurement**

Cytokine levels in patients’ samples were determined using a 9-milliplex magnetic bead-based immunoassay (HCYTOMAG-60K, Millipore BV, Amsterdam, the Netherlands), performed according to manufacturer’s instructions. Briefly, 25 µL of magnetic beads internally labeled with multiple fluorophores and coated with specific capture antibodies against one of the nine cytokines (TNF, IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-13, IL-17A and IFN-γ) was added to 25 µL of patient sample and an equal amount of assay buffer. Standards and quality controls for each cytokine were mixed likewise. After an overnight incubation followed by extensive wash to remove unbound proteins, 25 µL of biotinylated detection antibodies was added and the fluorescence of the streptavidin-phycoerythrin complex was measured by a MAGPIX® (Luminex, Austin, USA). A minimum of 50 beads per cytokine was measured. Interpolation of sample concentrations using a five-parameter logistic standard curve was performed with the MILLIPLEX® Analyst 5.1 software (Merck Millipore, Billerica, USA). The lower detection limits of the assay were: 0.53 pg/mL for TNF, 0.54 pg/mL for IL-2, 0.34 pg/mL for IL-4, 0.97 pg/mL for IL-6, 0.59 pg/mL for IL-10, 0.74 pg/mL for IL-12-p70, 0.57 pg/mL for IL-13, 0.22 pg/mL for IL-17A and 0.17 pg/mL for IFN-γ. Comparative analysis of cytokine profiles included both serum \(n = 83\) and, when unavailable, plasma samples \(n = 5\), since exclusion of plasma assessments had no effect on the outcome of the analysis.

**Statistical analysis**

Group-wise comparison of cytokine values was performed using non-parametric statistics. Mann-Whitney U test and Kruskal Wallis test were used to examine whether continuous variables from two or multiple groups, respectively, originated from the same distribution, whilst comparison of categorical variables was performed using the Chi-square test. \(P\)-values <0.05 were considered indicative of statistical significance. Spearman’s \(r_s\) rank correlations were computed to assess
statistical dependence between cytokine levels and the corresponding patient’s demographic/clinical characteristics and between each cytokine pair. Data analysis was conducted with STATA software (College Station, TX, USA).

Results

Study population

The sex, age, hemoglobin level, WBC count and *P. falciparum* infection intensity of patients diagnosed with VL and/or malaria are summarized in Table 1. With the exception of malaria parasitaemia, which appeared significantly reduced amongst co-infected patients (*P* < 0.01), no major differences in the baseline distribution of these variables were observed, with patients sharing most of their demographic and clinical characteristics. Mild anemia with normal leukocyte counts characterized most of the actively infected population, who largely consisted of young boys. Co-infected patients displayed some intermediate features between the VL and the malaria groups, including lower male to female ratio, younger age and milder anemia as compared with the VL patients.

Circulating cytokine profiles in VL and malaria mono-infected patients

Significantly increased levels of pro-inflammatory cytokines characterized the VL group compared with the healthy individuals. Tumor necrosis factor, a marker of local and systemic inflammation, and the type-1-inducing cytokine IFN-γ were both strongly up-regulated amongst VL patients, as compared with healthy controls (*P* < 0.0001) (Figure 1A and B). Circulating IL-12p70 was also enhanced by the *Leishmania* infection (*P* < 0.001): undetectable in all, but one control, this interleukin reached or exceeded the detection limit (0.74 pg/mL) in nearly half of the VL patients (Figure 1C). Similarly, the serum level

| Table 1. Baseline characteristics of patients with VL and/or malaria recruited at Tabarak Allah Hospital, Sudan. |
| --- |
| Characteristics | Patient group | P-value |
| --- |
| Subjects (*n*) | 29 | 21 | 15 | Total |
| Male/Female (*n*) | 20/8 | 11/9 | 9/6 | 40/23 | 0.5 |
| Age (years) | 19 (9-29) | 8 (7-26) | 16 (9-21) | 15 (8-24) | 0.2 |
| Hb level (g/dL) | 9.6 (7.1-12.4) | 10.3 (9.1-11.2) | 9.9 (7.5-11.5) | 10.0 (8.4-11.4) | 0.8 |
| WBC count/mm³ | 5650 (4925-6900) | 6800 (5900-8000) | 5400 (4250-7150) | 6050 (4950-7400) | 0.2 |
| DAT titer | 25600 (6400-102400) | NA | 12800 (6400-102400) | 19200 (6400-102400) | 0.9 |
| *P*. *falciparum* parasitaemia (parasites/µL) | NA | 825 (355-2325) | 53 (38-585) | 370 (51-1113) | 0.005 |

When not otherwise indicated, data shown represent median and (interquartile range).

Hb = hemoglobin; WBC = white blood cell; DAT = direct agglutination test; *P*. *f.* = *Plasmodium falciparum*; NA = not applicable.

Groups 1 to 3 are: 1 = visceral leishmaniasis patients, 2 = malaria patients, 3 = visceral leishmaniasis-malaria co-infected patients. The Kruskall-Wallis test was used to calculate the P-values, except for the variable sex for which the Chi-Square test was used, and for the variables DAT titer and *P*. *f*. parasitaemia, for which the Mann-Whitney test was used.

1*P*-value refers to differences between groups 1 and 3 only.

2*P*-value refers to differences between groups 2 and 3 only.

Sex data based on 28 patients b 20 patients c 63 patients. Age data based on d 27 patients and e 63 patients. WBC count data based on f 18 patients, g 11 patients, h 13 patients and i 42 patients. *P*. *falciparum* parasitaemia data based on j 12 patients, k 10 patients and l 22 patients. Two co-infected patients received artemether i.m. three weeks prior to diagnosis and their data on *P*. *falciparum* parasitaemia were excluded to reduce possible bias.
of IL-6 was negligible in all healthy individuals (<0.97 pg/mL), but raised to a 10-fold higher value in the VL group ($P < 0.0001$) (Figure 1D). Systemic inflammation amongst VL patients was confirmed by IL-17A, whose circulating levels appeared to be significantly induced ($P < 0.001$) (Figure 1E). Visceral leishmaniasis cases also exhibited an increase in their anti-inflammatory and regulatory cytokine patterns, as shown by the higher amounts of IL-4, and particularly IL-10 ($P < 0.0001$) (Figure 1F and G). No difference in the circulating level of IL-2 and IL-13, on the other hand, distinguished VL-affected and healthy individuals, for whom the level of these two cytokine resulted mostly under the assay detection limit (0.54 pg/mL and 0.57 pg/mL, respectively) (Figure 1H and I).

Clinical malaria cases exhibited an immunological profile qualitatively similar to that of the VL patients, with increased concentrations of TNF, IL-6 and IL-10 ($P < 0.0001$) and, to a lesser extent, IFN-$\gamma$ and IL-17A ($P < 0.01$), IL-4 and IL-12p70 ($P < 0.05$) (Figure 1A-G). Interleukin-2 and IL-13 persisted at negligible levels (Figure 1H and I).

### Circulating cytokine profiles in VL and malaria co-infected patients

Co-occurrence of malaria and VL in the same patients deeply altered their immunological
response as compared with the single infections. Overall, co-infection of *Leishmania* and *Plasmodium* resulted in a marked elevation of type-1 and pro-inflammatory cytokine patterns, predominantly triggered by the *Leishmania* disease. Whereas comparable amounts of IFN-γ and TNF were detected in the sera of VL mono- and co-infected patients, the level of these two cytokines significantly raised when malaria patients were co-diagnosed with VL (*P* <0.001 and *P* <0.01, respectively) (Figure 1A and B). Up-regulation of pro-inflammatory IL-17A distinguished the co-infected patients from both VL and malaria mono-infection cases (*P* <0.05) (Figure 1E), indicating that synergistic interactions of the two diseases upon each other concurred to its release. Strikingly different IL-17A concentrations were measured amongst the co-infected patients, whose demographic and clinical characteristics, however, did not display any correlation with the cytokine level. In the co-infected cohort, IL-2 and IL-13 concentrations exceeded the detection limit with a higher frequency (4 out of 15 samples) than amongst VL patients (1 out of 29 samples), resulting in significant differences (*P* <0.05) between the two groups (Figure 1H and I).

To exclude that the cytokine profiles observed for VL and/or malaria infected patients may have been biased by differences in their demographic and clinical features, the study population was stratified by sex, age or *P. falciparum* parasitaemia (Table 2). For age, patients were matched by means of two groups (6-15 years, >15 years) selected to yield similar group-wise compositions, while for *P. falciparum* parasitaemia, an infection intensity >100 parasites/µL of blood was chosen (corresponding to >0.002%, the level above which patients may become symptomatic). None of the three variables displayed a systematic confounding effect on the cytokines examined here, whose variations amongst the three study groups remained overall stable (Figure 2). Increased concentrations of IFN-γ distinguished the co-infected from the malaria mono-infected patients in all of the matched groups, whereas significant differences in TNF levels were observed for men only and for patients above 15 years of age. Importantly, for TNF and IFN-γ,

**Table 2. Characteristics of patients with VL and/or malaria matched by sex, age or *P. falciparum* parasitaemia.**

| Characteristics                  | Patient group | 1   | 2   | 3   | Total |
|----------------------------------|---------------|-----|-----|-----|-------|
| Subjects (n)                     |               | 29  | 21  | 15  | 65    |
| Male/Female (n)                  |               | 20/8| 11/9| 9/6 | 40/23 |
| Age 6-15 years (n)               |               | 13  | 14  | 7   | 34    |
| Median (Interquartile range)     |               | 9 (7-13) | 8 (7-9) | 9 (7-10) |
| Age >15 years (n)                |               | 14  | 7   | 8   | 29    |
| Median (Interquartile range)     |               | 26 (20-35) | 27 (25-30) | 19 (17-27) |
| *P. f.* parasitaemia >100 p/µL (n) |       | NA  | 12  | 4   | 16    |
| Median (Interquartile range)     |               | NA  | 825 (355-2325) | 640 (290-1088) |

*P. f.* = *Plasmodium falciparum*; NA = not applicable.

Groups 1 to 3 are: 1 = visceral leishmaniasis patients, 2 = malaria patients, 3 = visceral leishmaniasis-malaria co-infected patients.
the lower *P. falciparum* infection intensity recorded amongst the co-infected cohort did not flaw the comparison with the malaria patients, as statistically significant differences still distinguished the two groups after parasitaemia stratification. Increase in co-infected patient IL-17A level appeared to be mainly triggered by malaria, as the difference with this group reduced after age and parasitaemia matching, but persisted towards VL patients in women only and in subjects older than 15 years. No significant differences were observed for the remaining six cytokines, including IL-2 and IL-13, for which most patients displayed negligible levels (data not shown).

**Correlation between circulating cytokines in VL and malaria co-infected patients**

Specific mechanisms cross-regulate the production of cytokines, with positive and negative feed-back loops to control secretion of signaling molecules. To understand the link interconnecting each of the cytokines examined...
here, their correlation was investigated using the Spearman’s correlation rank test. As shown in Table 3, multiple positive correlations were identified between pro-inflammatory (TNF vs. IL-6) and type-1 cytokines (IFN-γ vs. IL-12p70), as well as for type-1 vs. pro-inflammatory (IFN-γ & IL-12p70 vs. TNF, IL-6 & IL-17A), and type-2 vs. pro-inflammatory (IL-13 vs. TNF, IL-6 & IL-17A) and type-1 cytokines (IL-13 vs. IL-12p70 & IFN-γ).

Conversely, no correlation could be identified between the examined cytokines and the DAT titers as well as the P. falciparum parasitaemia of co-infected patients (data not shown).

Discussion

The ability of Leishmania and Plasmodium parasites to manipulate host immunity and co-inhabit part of the same lymphoid tissues suggests the possibility that the two diseases may interact with each other, when co-occurring in the same host. This is demonstrated for the first time in naturally co-infected patients by the pilot study presented here. Comparative analysis of cytokine profiles from co- and mono-infected patients highlighted substantial variations in the immune response mounted upon co-infection, confirming the ability of L. donovani and P. falciparum to mutually interact at the 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, IFN-γ) and malaria (IL-2), and partly resulted from a synergistic interaction of the two diseases upon each other (IL-17A). Secretion of IL-13 in co-infected patients significantly exceeded the amounts found in VL patients and displayed positive correlations with most of the examined cytokines. Although this trend can be seen as an attempt of the immune system to contain the effects elicited by type-1 and pro-inflammatory cytokines (these patients exhibited some of the highest concentrations of IFN-γ, TNF and IL-17A), the finding remains poorly representative, reflecting the response of 4 patients only, with the remaining co-infected patients (n = 11) displaying negligible levels of IL-13 just as most of VL and malaria patients.

Measurement of IL-17A levels allowed to distinguish the co-infected patients from both VL and malaria mono-infected counterparts, indicating that both diseases synergistically concurred to its up-regulation. Better known for its pro-inflammatory effects in allergic and autoimmune conditions, IL-17 has been recently implicated in the protective immunity towards bacterial, fungal and protozoan infections, where it is thought to mediate recruitment of neutrophils to the epithelial and mucosal surfaces and induce production of antimicrobial peptides. Its release by CD4+ Th17 cells has been associated with resistance to human VL and positive resolution of murine L. donovani infections, suggesting that Th17 and Th1 cytokines may play complementary roles in parasite clearance. Hence, the increased concentrations of IL-17A found in the co-infected vs. the VL mono-infected cohort, besides the already elevated IFN-γ and IL-12p70, may be indicative of a favorable, possibly improved prognosis for VL, though the present data do not allow to draw conclusions in this respect. In support of this speculation is the finding of a recent study conducted in Barbar el Fugarra, a Sudanese village situated only a few tens of kilometers away from Tabarak Allah Hospital (where patients in this study were recruited), in which peripheral blood mononuclear cells (PBMCs) isolated from VL-seropositive individuals who did not develop disease at any time during
In the 6-year survey, secreted significantly higher IL-17 levels when challenged with *L. donovani* in comparison with VL-seropositive individuals who became symptomatic within 6 months from the evaluation. Interestingly, malaria appeared as the major trigger of this IL-17A up-regulation in co-infected patients, given that no relationship between patients’ demographic and clinical variables and the corresponding IL-17A serum level could be identified in this group (nor in any other group). Expansion of IL-17-producing cells (either CD4+ T cells, CD8+ T cells or macrophages) and related cytokines (IL-17, IL-22 and IL-23) has been observed in *P. vivax* natural infections as well as *P. berghei*, *P. chabaudi* and *P. fragile* animal models, where these interleukins have been shown to reduce parasite intensity and protect against fatal outcomes. Conversely, a clear role of IL-17 immunity in *P. falciparum* infections is yet to be demonstrated. Transcriptional profiling of PBMCs isolated from *P. falciparum*-infected patients has recently highlighted a Th17/Thαβ-driven bias in the immune response mounted against malaria, with up-regulation of several Th17- and neutrophil-related genes and induction of a NK cell-mediated humoral response via interferon α and β. Triggering of this Thαβ immunity, in particular, was shown to inhibit the IL-12-driven Th1 response, necessary for boosting clearance of malaria parasites, particularly during the pre-erythocytic stage, when cell-mediated immunity is essential for control of infection. If induction of a Th17 response may, therefore, indirectly impair host ability to contain malaria through suppression of macrophage activities, the IFN-γ dominant response elicited by VL may partially compensate for this deficiency and act 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 above-shown data confirm the leading presence of IFN-γ (*P* <0.0001), followed by TNF (*P* <0.05) and IL-4 (*P* <0.05), in the sera of VL patients as compared with the malaria ones, and clearly identify a shift towards type-1/pro-inflammatory polarization when malaria co-occurred with VL. In addition, a significantly reduced *P. falciparum* infection intensity was observed among co-infected patients, suggesting improved tolerance of these individuals to the malaria disease. Whether this reduced susceptibility resulted from the VL-driven pre-immune response remains to be demonstrated. The pioneer work of Adler et al. on co-infected hamsters highlighted a reduced proliferation of *P. berghei* for effect of the *Leishmania* infection, supporting the idea of a VL-triggered cross-immunity against malaria, whereas the more recent mouse model data seem to suggest the opposite conclusion. It is worthy to note that animals were challenged with blood-stage parasites rather than with sporozoites, bypassing the naturally occurring liver phase against which cellular immunity is most effective and most likely to be developed in response to VL (*Leishmania* parasites visceralize in the liver, too). Moreover, mice and hamsters are not equally representative models of the VL disease, whose clinico-pathological features in humans are better reproduced by the golden hamster model.

The exploratory nature of this survey implies its design and findings are limited by the small sample sizes and the lack of subject matching between groups, although no significant difference in the distribution of patients’ demographic and clinical features was observed. Diagnosis of VL in clinical suspects was confirmed by serology, according to the national policy, precluding any analysis on parasite loads and their
link with cytokine profiles. Assessment of malaria parasitaemia, on the contrary, was performed on peripheral blood films, but the low sensitivity of microscopy observation inevitably limits its reliability as a quantitative assay. Moreover, in the absence of a molecular screening of the recruited individuals, the risk of sub-microscopy malaria infections being carried by the VL patients and/or apparently healthy controls cannot be excluded. Malaria mono- and co-infected patients exhibited different *P. falciparum* blood parasitaemias. Whether these differences are linked to their particular diagnosis, however, is unknown, as patients were recruited sequentially and discernment between clinical and sub-clinical co-infection cases is not possible if one of the two diseases manifests with symptoms. Therefore, recruitment of asymptomatic, but parasitaemic individuals for each of the two infections may be useful to control for non-homogeneous group-wise comparisons. Absence of pre-existent disorders was based on patient reporting only, with no diagnostic procedure performed, other than those ones aimed to confirm VL or malaria. Finally, longitudinal rather than cross-sectional assessments should be endorsed, as they could help identifying those fundamental associations between parasite load, cytokine response and clinical picture which are keys to the interpretation of present data. Similar studies may not only clarify the exact role of the VL-malaria co-infection on *P. falciparum* proliferation, but they would be pivotal for understanding the clinical implications that arise from the different cytokine profiles.

**Conclusions**

Immune-mediated interactions between *L. donovani* complex and *P. falciparum* appear to crucially shape the immunological response taking place in the co-infected host and possibly the intensity of infections that follow. Similar scenarios have been depicted with other malaria co-infections, indicating that the potential implications arising from multiple pathogen-host relations should be addressed when designing malaria vaccine trials. Careful consideration of parasite interplays should be taken when defining the best strategy for clinical management of VL-malaria co-infections, to ensure that immune homeostasis may be restored without harming patient’s clinical course.

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Authors’ contributions

EvdB designed the study, carried out the cytokine measurement and drafted the manuscript. AT recruited the study participants, performed collection of data and samples and participated to the sample analysis. MS performed the statistical analysis of the data. PM and EM conceived of the study, participated in its design, contributed to the sample analysis and helped to draft the manuscript. MG revised the critical content of the manuscript. BN participated to the study design and coordinated the work in the field. HS conceived of the study, participated in its design and coordination, contributed to the sample analysis and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.
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### Additional file 1 – Cytokine levels (pg/mL) as measured in all patient samples

| Sample     | Analyte | IFN-γ | IL-10 | IL-12p70 | IL-13 | IL-17A | IL-2 | IL-4 | IL-6 | TNF |
|------------|---------|-------|-------|----------|-------|--------|------|------|------|-----|
|            |         | pg/mL | pg/mL | pg/mL    | pg/mL | pg/mL  | pg/mL| pg/mL| pg/mL| pg/mL|
| Pt 1       |         | 74.02 | 233   | 4.37     | <0.57 | 1.42   | 29.56| 22.45| 43.13|      |
| Pt 4       |         | 10.37 | 52.51 | 0.74     | <0.57 | 6.21   | 21.18| 2.58 | 14.69|      |
| Pt 7       |         | 4.59  | 140   | <0.74    | <0.57 | 0.69   | <0.54| 55.41| 27.58| 38.06|
| Pt 8 plasma|         | 12.75 | 206   | 5.56     | <0.57 | 2.67   | <0.54| 9.7  | 5.32 | 0.61 |
| Pt 9       |         | 1.27  | 1.28  | 0.74     | <0.57 | 0.91   | <0.54| 298  | <0.97| 0.79 |
| Pt 10      |         | 15.98 | 45.59 | <0.74    | <0.57 | 0.54   | <0.54| 104  | <0.97| 30.15|
| Pt 11      |         | 536   | 973   | 1270     | 926   | 310    | 15.65| 21.18| 248  | 86.34|
| Pt 13      |         | 39.28 | 70.33 | <0.74    | <0.57 | 1.13   | <0.54| 3.35 | <0.97| 44.62|
| Pt 14      |         | 23.17 | 197   | 0.96     | <0.57 | 10.8   | <0.54| 135  | 11.18| 76.14|
| Pt 15 plasma|        | 19.41 | 131   | 1.46     | <0.57 | 3.35   | <0.54| 45.74| 12.31| 56.98|
| Pt 16 plasma|        | 440   | 658   | 7.69     | <0.57 | 1.79   | <0.54| 69.62| 60.8 | 77.51|
| Pt 22      |         | 6.02  | 2.01  | <0.74    | <0.57 | 0.64   | <0.54| 12.5 | <0.97| 6.59 |
| Pt 24      |         | 35.18 | 179   | <0.74    | <0.57 | 1.65   | <0.54| 182  | 24.97| 15.97|
| Pt 29      |         | 0.37  | <0.59 | <0.74    | <0.57 | 4.42   | <0.54| 33.4 | <0.97| 2.73 |
| Pt 30      |         | 38.88 | 202   | 0.81     | <0.57 | 3.9    | <0.54| 52.76| 28.85| 73.68|
| Pt 35      |         | 173   | 23.88 | 18.53    | <0.57 | 28.42  | <0.54| 8.19 | 2.55 | 58.35|
| Pt 37      |         | 0.75  | <0.59 | <0.74    | <0.57 | 0.67   | 74.22| <0.34| 1.64 | 0.97 |
| Pt 38      |         | 11.71 | <0.59 | <0.74    | <0.57 | 3.01   | <0.54| 21.18| <0.97| 5.1  |
| Pt 39      |         | 3.01  | 1.28  | 1.96     | <0.57 | 1.36   | <0.54| 59.51| <0.97| 6.09 |
| Pt 42      |         | 0.88  | <0.59 | <0.74    | <0.57 | 1.59   | <0.54| 12.34| <0.97| 10.38|
| Pt 43      |         | 51.69 | 320   | 2.01     | 21.96 | 6.79   | <0.54| 54.04| 5.6  | 125  |
| Pt 44      |         | 62.22 | 78.38 | <0.74    | <0.57 | 3.82   | <0.54| 44.54| 1.37 | 25.69|
| Pt 49      |         | 14.47 | 201   | <0.74    | <0.57 | 0.88   | <0.54| 19.34| 17.41| 41.35|
| Sample   | Analyte | IFN-γ | IL-10   | IL-12p70 | IL-13   | IL-17A | IL-2   | IL-4   | IL-6   | TNF   |
|----------|---------|-------|---------|----------|---------|---------|--------|--------|--------|-------|
|          |         | pg/mL | pg/mL   | pg/mL    | pg/mL   | pg/mL   | pg/mL  | pg/mL  | pg/mL  | pg/mL |
| Pt 51    |         | 43.83 | 192     | 18.67    | <0.57   | 11.36   | <0.54  | 104    | 15.76  | 87.42 |
| Pt 54    |         | 0.49  | <0.59   | <0.74    | <0.57   | 23.35   | <0.54  | 11.4   | <0.97  | 3.02  |
| Pt 55    |         | 31.06 | 170     | <0.74    | <0.57   | 0.79    | <0.54  | 107    | 97.58  | 66.29 |
| Pt 58 plasma |     | 15.31 | 165     | <0.74    | <0.57   | 19.44   | <0.54  | 77.15  | 48.29  | 76.95 |
| Pt 67    |         | 9.7   | 68.74   | <0.74    | <0.57   | 1.02    | <0.54  | 88.33  | 42.84  | 13.79 |
| Pt 69    |         | 35.75 | 302     | 4.02     | <0.57   | 1.3     | <0.54  | 120    | 16.05  | 27.41 |
| Pt 73    |         | 73.59 | 21.45   | <0.74    | <0.57   | 1.02    | <0.54  | 78.41  | 37.76  | 58.08 |
| Pt 74    |         | 41.82 | 188     | 2.61     | <0.57   | 1.02    | <0.54  | 78.41  | 37.76  | 58.08 |
| Pt 77    |         | 44.48 | 72.71   | <0.74    | <0.57   | 1.23    | 18.07  | <0.34  | 9.66   | 165   |
| Pt 20    |         | <0.17 | 9.97    | <0.74    | <0.57   | 5.38    | <0.54  | 43.51  | <0.97  | 7.62  |
| Pt 21    |         | 5.22  | 18.76   | 0.74     | <0.57   | 1.62    | <0.54  | 26.15  | 423    | 9.74  |
| Pt 26    |         | 1.18  | 103     | <0.74    | <0.57   | 0.94    | <0.54  | 16.21  | <0.97  | 11.92 |
| Pt 27    |         | 0.72  | 160     | <0.74    | <0.57   | 0.36    | <0.54  | 31.52  | 6.54   | 13.52 |
| Pt 28    |         | 2.17  | 29.15   | 0.81     | <0.57   | 0.76    | <0.54  | 23.7   | <0.97  | 11.59 |
| Pt 32    |         | 3.75  | 1426    | 1.73     | <0.57   | 0.94    | <0.54  | 19.84  | 17.01  | 22.46 |
| Pt 33    |         | 1.27  | 303     | <0.74    | <0.57   | 3.53    | <0.54  | 31.43  | 1.55   | 21.91 |
| Pt 34    |         | 11.37 | <0.59   | <0.74    | <0.57   | 49.09   | 9.9    | 69.71  | 21.83  | 3.86  |
| Pt 36    |         | 4.36  | 400     | <0.74    | <0.57   | 0.94    | <0.54  | 3.11   | 20.93  | 47.35 |
| Pt 45    |         | 3.16  | 72.1    | <0.74    | <0.57   | 3.61    | <0.54  | 1.74   | <0.97  | 23.82 |
| Pt 47    |         | 2.23  | <0.59   | <0.74    | <0.57   | 0.8     | <0.54  | 26.92  | <0.97  | 7.28  |
| Pt 60    |         | 1.9   | 331     | <0.74    | <0.57   | 4.06    | <0.54  | 5.61   | 2.58   | 12.79 |
| Pt 61    |         | 0.49  | 7.94    | <0.74    | <0.57   | 0.36    | <0.54  | 20.51  | <0.97  | 11.67 |
| Pt 63    |         | 5.98  | 1232    | <0.74    | <0.57   | 3.25    | <0.54  | 27.43  | 40.54  | 46.61 |
| Pt 64    |         | 1.1   | 1011    | <0.74    | <0.57   | 0.38    | <0.54  | 0.85   | 377    | 81.29 |
| Pt 65    |         | 0.33  | 23.76   | 18.74    | <0.57   | 0.53    | 54.42  | <0.34  | 5.6    | 2.27  |
### Cytokine profiles of VL-malaria co-infected patients

| Sample   | Analyte | IFN-γ | IL-10 | IL-12p70 | IL-13 | IL-17A | IL-2 | IL-4 | IL-6 | TNF |
|----------|---------|-------|-------|----------|-------|--------|------|------|------|-----|
|          |         | pg/mL | pg/mL | pg/mL    | pg/mL | pg/mL  | pg/mL| pg/mL| pg/mL| pg/mL|
| Pt 66    | 1.77    | 26.26 | 9.98  | 48.98    | 3.82  | <0.54  | 82.46| 27.05| 3.09 | 10.4 |
| Pt 68    | 4.06    | 461   | <0.74 | <0.57    | 1.17  | <0.54  | 47.45| 1.08 | 18.7 | 73.95|
| Pt 72    | 17.11   | 8262  | <0.74 | <0.57    | 0.88  | <0.54  | 16.87| 155  | 73.95|
| Pt 3     | 48.83   | 17.66 | <0.74 | <0.57    | 16.99 | <0.54  | 150  | 1.92 | 23.72|
| Pt 5     | 0.23    | 10.34 | <0.74 | <0.57    | 2.11  | <0.54  | 16.87| <0.97| 6.47 |
| Pt 6 plasma | 156 | 137  | 485   | 358      | 97.98 | <0.54  | 20.59| 10.06| 76.72|
| Pt 12    | 112     | 117   | 64.3  | 38.97    | 21.76 | <0.54  | 110  | 74.52| 48.28|
| Pt 17    | 14.72   | 82.46 | 0.85  | 19.12    | <0.54 | 1.01   | 4.77 | 20.44|
| Pt 18    | 32.41   | 67.76 | 0.74  | <0.57    | 29.55 | <0.54  | 1.94 | 18.98| 35.37|
| Pt 23    | 90.72   | 222   | <0.74 | <0.57    | 1.49  | 5.69   | 65.55| 2.29 | 28.25|
| Pt 31    | 10.37   | 18.93 | <0.74 | <0.57    | 0.34  | <0.54  | 3.53 | <0.97| 12.67|
| Pt 40    | 9.37    | 121   | <0.74 | <0.57    | 2.82  | <0.54  | 31.86| 2.45 | 55.33|
| Pt 46    | 0.58    | 5.63  | 0.89  | <0.57    | 1.08  | <0.54  | 51.48| <0.97| 3.64 |
| Pt 50    | 66.52   | 119   | <0.74 | <0.57    | 94.43 | <0.54  | 83.46| 4.38 | 32.54|
| Pt 52    | 382     | 56.68 | 1170  | 521      | 249   | 3.39   | 91.26| 207  | 230  |
| Pt 53    | 64.33   | 235   | 4.72  | 0.57     | 1.54  | <0.54  | 70.3 | 22.51| 29.24|
| Pt 70    | 2.58    | 341   | <0.74 | <0.57    | 13.41 | <0.54  | 40.25| 5.29 | 10.4 |
| C1       | 4.44    | <0.59 | <0.74 | <0.57    | <0.22 | <0.54  | 9.09 | <0.97| 5.54 |
| C2       | 0.45    | 1.16  | <0.74 | <0.57    | 0.55  | <0.54  | 1.01 | <0.97| 3.13 |
| C3       | <0.17   | <0.59 | <0.74 | <0.57    | <0.22 | <0.54  | 5.61 | <0.97| 5.08 |
| C4       | <0.17   | <0.59 | <0.74 | <0.57    | 0.35  | <0.54  | 5.34 | <0.97| 5.76 |
| C6       | 2.72    | <0.59 | <0.74 | <0.57    | 1.26  | <0.54  | <0.34| <0.97| 2.31 |
| C7       | 0.2     | <0.59 | <0.74 | <0.57    | <0.22 | <0.54  | 12.5 | <0.97| 3.9  |
| C8       | 0.49    | <0.59 | <0.74 | <0.57    | 9.97  | <0.54  | 15.23| <0.97| 2.8  |
| C9       | 0.77    | <0.59 | <0.74 | <0.57    | 0.55  | <0.54  | 17.52| <0.97| 3.64 |
### Chapter 4

#### Analyte Concentrations

| Sample | IFN-γ (pg/mL) | IL-10 (pg/mL) | IL-12p70 (pg/mL) | IL-13 (pg/mL) | IL-17A (pg/mL) | IL-2 (pg/mL) | IL-4 (pg/mL) | IL-6 (pg/mL) | TNF (pg/mL) |
|--------|---------------|---------------|-----------------|---------------|----------------|--------------|--------------|--------------|-------------|
| C10    | 0.77          | <0.59         | <0.74           | <0.57         | 0.53           | <0.54        | 23.03        | <0.97        | 3.2         |
| C11    | 0.62          | <0.59         | <0.74           | <0.57         | 0.28           | <0.54        | 34.43        | <0.97        | 5.55        |
| C12    | 4.91          | <0.59         | <0.74           | <0.57         | 36.65          | <0.54        | 36.99        | <0.97        | 3.49        |
| C13    | 0.67          | <0.59         | <0.74           | <0.57         | 1.17           | <0.54        | 0.56         | <0.97        | 7.7         |
| C14    | 0.2           | <0.59         | <0.74           | <0.57         | <0.22          | <0.54        | 3.65         | <0.97        | 2.66        |
| C16    | 0.23          | <0.59         | <0.74           | <0.57         | 0.57           | <0.54        | 38.88        | <0.97        | 2           |
| C17    | 1.24          | <0.59         | <0.74           | <0.57         | 29.32          | <0.54        | 16.87        | <0.97        | 0.79        |
| C19    | <0.17         | <0.59         | <0.74           | <0.57         | <0.22          | <0.54        | 3.78         | <0.97        | 2.62        |
| C20    | 1.21          | <0.59         | <0.74           | <0.57         | <0.22          | <0.54        | 18.18        | <0.97        | 6.78        |
| C21    | 0.53          | <0.59         | 0.89            | <0.57         | 0.69           | <0.54        | 3.78         | <0.97        | 3.46        |
| C22    | <0.17         | <0.59         | <0.74           | <0.57         | <0.22          | <0.54        | <0.34        | <0.97        | 4.42        |
| C23    | 0.49          | <0.59         | <0.74           | <0.57         | 23.74          | <0.54        | 27.43        | <0.97        | 3.2         |
| C24    | 0.82          | <0.59         | <0.74           | <0.57         | 0.4            | <0.54        | 11.63        | <0.97        | 3.05        |
| C25    | 0.26          | <0.59         | <0.74           | <0.57         | 0.35           | <0.54        | 3.53         | <0.97        | 2.69        |
| Median (interquartile range) group 1 | 23.2 (10.0-44.2) | 131.0 (22.7-199.0) | 0.0 (0.0-2.3) | 0.0 (0.0-0.0) | 1.6 (1.0-4.2) | 0.0 (0.0-0.0) | 52.8 (20.3-96.2) | 9.7 (0.0-26.3) | 41.4 (12.1-70.0) |
| Median (interquartile range) group 2 | 1.9 (0.7-3.9) | 72.1 (10.2-401.0) | 0.0 (0.0-0.4) | 0.0 (0.0-0.0) | 1.2 (0.8-3.7) | 0.0 (0.0-0.0) | 26.2 (16.5-41.9) | 2.6 (0.0-24.4) | 11.7 (6.9-22.2) |
| Median (interquartile range) group 3 | 48.8 (9.4-112.0) | 117.0 (18.9-222.0) | 0.0 (0.0-64.3) | 0.0 (0.0-38.9) | 19.1 (1.5-94.4) | 0.0 (0.0-5.7) | 21.2 (3.1-83.5) | 4.8 (1.9-22.5) | 32.5 (20.4-55.3) |
| Median (interquartile range) group 4 | 0.5 (0.2-0.9) | 0.0 (0.0-0.0) | 0.0 (0.0-0.0) | 0.0 (0.0-0.0) | 0.5 (0.0-1.2) | 0.0 (0.0-0.0) | 10.4 (3.6-19.4) | 0.0 (0.0-0.0) | 3.3 (2.7-5.2) |
Chapter 5

*Leishmania donovani* infection drives the priming of human monocyte-derived dendritic cells during *Plasmodium falciparum* co-infections

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Summary

Functional impairment of dendritic cells (DCs) is part of a survival strategy evolved by *Leishmania* and *Plasmodium* parasites to evade host immune responses. Here, the effects of co-exposing human monocyte-derived DCs to *Leishmania donovani* promastigotes and *Plasmodium falciparum*-infected erythrocytes were investigated. Co-stimulation resulted in a dual, dose-dependent effect on DC differentiation which ranged from semi-mature cells, secreting low interleukin-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, interleukin-10-producing DCs, that poorly responded to lipopolysaccharide stimulation. Conversely, *P. falciparum* blood-stage forms failed to activate DCs and only slightly interfered with lipopolysaccharide effects. Stimulation with high *L. donovani* concentrations triggered phosphatidylserine translocation, whose onset presented after initiating the maturation impairment process. When added in combination, the two parasites could co-localize in the same DCs, confirming that the leading effects of *Leishmania* over *Plasmodium* may not be due to mutual exclusion. Altogether, these results suggest that in the presence of visceral leishmaniasis-malaria co-infections, *Leishmania*-driven effects may overrule the more silent response elicited by *P. falciparum*, shaping host immunity towards a regulatory pattern and possibly delaying disease resolution.

**Keywords**

Co-infection, Dendritic cell, *L. donovani, P. falciparum*
Introduction

Malaria and visceral leishmaniasis (VL) are two major parasitic diseases sharing part of their geographical distribution across the globe (Figure 1).\textsuperscript{1,2} Transmitted through the bite of infected vectors with similar feeding habits and exacerbated by the lack of protective immunity found in children, the two diseases can co-occur in individuals living in co-endemic areas, with potential implications for their clinical course.\textsuperscript{3-7} Recent studies conducted in East Africa, where the co-infection prevalence in patients with VL ranged from 4% to 61%, highlighted a substantial increase in disease-related morbidity, with co-infected patients suffering from more frequent emaciation, jaundice and malaise, despite exhibiting a similar prognosis.\textsuperscript{6,7} Interestingly, VL and malaria have been shown to cross-interact in experimentally co-infected animals, shaping host susceptibility towards one or the other infection and the immune response elicited locally, albeit with substantial differences across the various studies.\textsuperscript{8-11} At the patient level, increased concentrations of Th1 and pro-inflammatory cytokines were observed amongst Sudanese co-infected individuals, demonstrating the ability of the two pathogens to modulate host immunity and possibly the severity of infections that follow.\textsuperscript{12}

Crucial to the immune evasion strategies evolved by several pathogens is their ability to interfere with the function of dendritic cells (DCs), a group of professional antigen-presenting cells that coordinate innate and adaptive immunities.\textsuperscript{13,14} The result is an impairment of DC activities which manifests with host inability to mount a full adaptive immune response and clear infection. Resistance to intracellular pathogens, such as Leishmania, requires induction of immune responses capable of activating cellular microbicidal mechanisms. To this end, priming of DCs to release interleukin(II)-12 is pivotal for promoting Th1 differentiation and the production of interferon-gamma (IFN-γ), the most potent cytokine for the induction of leishmanicidal activity in macrophages. Interestingly, despite the early finding that some IL-12 may be pre-stored and rapidly demobilized following Leishmania contact,\textsuperscript{15,16} in vitro studies have collectively indicated that DCs exposed to L. donovani promastigotes fail to mature in response to parasite engulfment and produce a range of IL-12p40 and IL-10 concentrations.\textsuperscript{17,18} Likewise, reduced responsiveness of L. donovani-infected DCs was observed following addition of exogenous stimuli, such as lipopolysaccharide (LPS)/tumor necrosis factor-alpha (TNF-α), IFN-γ, CD40 ligand or Mycobacterium tuberculosis,\textsuperscript{17-20} suggesting that Leishmania promastigotes may not only act by evading engagement of pathogen-recognition receptors (PRRs) associated with DC activation and T cell priming, but also by actively suppressing DC signaling pathways. Importantly, these findings appear to be model- and stage-specific, as evidence obtained in vivo or using the amastigote stage indicates up-regulation of DC maturation-associated cell-surface markers, release of cytokines and induction of Th1 responses in naïve CD4\textsuperscript{+} T cells.\textsuperscript{18,21} In further contrast, Leishmania dermatotropic species have been shown to produce a variety of effects on the biology and functions of DCs, ranging from a simple delay in DC maturation, as the one promoted by L. major,\textsuperscript{22} to a severe inhibition of DC signaling pathways by L. amazonensis and L. mexicana.\textsuperscript{23-25} Impairment of DC phenotype and activities is part of a pathogen survival strategy exploited by the Plasmodium malaria parasite for improving
its fitness.\textsuperscript{26,27} Consistent with its two phase-cycle (hepatic and intra-erythrocytic) in the mammalian host, control of \textit{Plasmodium} infection by the host requires a multifaceted immune response, consisting of cytotoxic CD8$^+$ T cells to suppress the liver forms and CD4$^+$ T lymphocytes to clear blood parasites, first \textit{via} cell-mediated immunity stimulated by Th1 differentiation and ultimately by antibody opsonization.\textsuperscript{27} DC activation of CD8$^+$ T cells seems to be unaffected by the pre-erythrocytic stage of infection,\textsuperscript{28-32} whereas blood-stage forms are known to deeply modulate the functional capacity of various DC subsets. Several \textit{in vitro} and \textit{in vivo} studies conducted with human myeloid DCs have shown reduced DC activity following exposure to erythrocytes infected with \textit{P. falciparum} or hemozoin, its by-product of hemoglobin degradation.\textsuperscript{33-37} The effect appears to be dose-dependent, with low parasite doses resulting in DC activation and high doses causing DC suppression \textit{via} apoptosis induction.\textsuperscript{34} Analysis of DC function in rodent \textit{Plasmodium} infections, however, has only partially confirmed these results, providing evidence that the functional capacity of splenic CD11c$^+$ DC populations changes over the course of infection and the lethality of the infecting strain.\textsuperscript{34,38-40} Whilst early on in infection when parasite density is still low, IL-12-mediated mechanisms induce IFN-γ-producing CD4$^+$ T cells, the phenotype of DCs may change as the infection progresses.\textsuperscript{39,41} With lethal strains, in particular, DCs may become refractory to Toll-Like Receptors (TLR) and other signaling events, inhibiting IL-12 and TNF-α secretion and promoting induction of regulatory IL-10-secreting T cells, deleterious for parasite clearance.\textsuperscript{40,42}

In the present study, the effect of \textit{in vitro} concomitant exposure to \textit{L. donovani} promastigotes and \textit{P. falciparum}-infected erythrocytes on DC function was examined. Expression of co-stimulatory molecules along with cytokine release was measured after co-stimulating monocyte-derived DCs (mo-DCs) with increasing amounts of parasite, in the presence or absence
of an exogenous stimulus (LPS). Cellular and transcriptional expression of various PRRs (DC-SIGN, TLR2 and TLR4) was assessed for each of the examined stimuli, whose effect on apoptosis was also examined through assessment of phosphatidylinerine (PS) externalization.

Materials and methods

Isolation of human monocytes and DC generation

Human monocytes derived from healthy donors (Sanquin, Amsterdam, the Netherlands; Approval Nr. NVT0224.01-04) who had signed informed consent for research purposes, were isolated from fresh buffy coats, by centrifugation over a gradient of Ficoll-Paque Plus, followed by 46% iso-osmotic Percoll (both from GE Healthcare, Uppsala, Sweden). After isolation, monocytes were cultured at a final concentration of 1.5 X 10^6 cells/mL in RPMI-buffered medium (RPMI 1640 medium containing 25 mM HEPES and 2 mM L-glutamine) (Gibco, Bleiswijk, the Netherlands), supplemented with 10% fetal calf serum (FCS, Sigma-Aldrich, St Louis, USA), 100 IU/mL penicillin and 100 μg/mL streptomycin. Differentiation into immature mo-DCs was achieved in the presence of 20 ng/mL of IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (both from Life Technologies, Bleiswijk, the Netherlands), which were added to the above-mentioned culture medium (DC culture medium). After 4 or 5 day-differentiation, immature mo-DCs were transferred to 96-well round-bottom culture plates (1X10⁴ cells/well) and stimulated for 24-h (unless otherwise stated) with increasing doses of L. donovani 1S promastigotes, uninfected red blood cells (RBCs), P. falciparum-infected RBCs or a combination of L. donovani 1S promastigotes and P. falciparum-infected RBCs, followed by 48-h in the presence or absence of LPS (from E. coli 0111:B4, Sigma-Aldrich Co.). The amounts of parasite used for mo-DC (co)-stimulation were determined based on previous studies exploring the effect of malaria and VL mono-infections on DC function.¹⁷,¹⁹,³⁴,⁴³,⁴⁴

Cultivation of P. falciparum-infected RBCs and enrichment of late-stage parasite cultures

Parasites of the P. falciparum strain 3D7 (MR4/ATCC: MRA-102) were grown at 5% hematocrit (0° human erythrocytes) in RPMI-buffered medium supplemented with 10% heat-inactivated human AB+ serum (Sanquin) and 50 μg/mL gentamycin. Cultures were maintained at 37°C in candle jars, as described by Trager and Jensen,⁴⁵ under continuous agitation at 40 rpm/min and with daily refreshment of medium. When parasitaemia exceeded 7-8%, sub-cultures were made.

For stimulation of immature mo-DCs, highly enriched, late-stage P. falciparum cultures were prepared according to Wahlgren et al.,⁴⁶ with minor modifications. Briefly, mature-stage parasite cultures were washed three times with RPMI-buffered medium and fractionated at 10% hematocrit onto a 60% Percoll gradient. After centrifugation at 1500 g, 4°C for 15 min, late-stage P. falciparum-infected RBCs (trophozoite and schizont forms) were harvested from the top of the Percoll solution, while uninfected and ring-containing RBCs pelleted at its bottom. Infected RBCs were washed three times with phosphate-buffer saline pH 7.2 (PBS) to remove residual Percoll and re-suspended in DC culture medium at a final concentration of 2.5 X 10⁸ RBCs/mL. The enriched fractions had a mean parasitaemia purity of 75%, as assessed by microscopic examination of Field'-stained smears. As controls, uninfected RBCs were cultured for 3 to 4 days in the same conditions and similarly processed onto
the 60% Percoll solution, from which they were retrieved at the lowest layer. The uninfected RBCs were then washed and re-suspended as for the enriched *P. falciparum*-infected cells.

**Cultivation of *L. donovani* promastigotes**

Promastigotes of the Sudanese *L. donovani* strain 1S (MHOM/SD/1968/1S) were cultured as previously reported, with minor modifications. Briefly, promastigotes were maintained at 27°C with a weekly passage in RPMI-buffered medium supplemented with 15% heat-inactivated FCS, 100 IU/mL penicillin and 100 μg/mL streptomycin. Parasites used for cell stimulation were harvested from 5-day-old cultures (stationary phase) and re-suspended in DC culture medium at a final concentration of 2.5 X 10⁸ parasites/mL.

**Flow cytometric analysis**

Fluorescence-activated cell sorting (FACS) analysis was used to analyze cell-surface expression of DC marker CD11c, maturation markers CD80, CD86, CD40 and HLA-DR, the adhesion molecule CD209 (DC-SIGN), and the PRRs CD282 (TLR-2) and CD284 (TLR-4). Prior to immunostaining, cells were treated with an RBC lysing buffer to remove non-internalized RBCs, else interfering with the FACS analysis, and incubated for 30 min at 4°C with a mixture of the following fluorescent-labeled monoclonal anti-human antibodies (all from BioLegend, San Diego, USA), according to the manufacturer’s instructions: fluorescein isothiocyanate (FITC)-anti-CD11c, phycoerythrin (PE)-anti-CD80, Pacific Blue-anti-CD86, allophtycocyan (APC)-anti-CD40, allophtycocyanin-cyanine 7 (APC-Cy7)-anti-HLA-DR, peridin chlorophyll protein-cyanine 5.5 (PerCP-Cy5.5)-anti-CD209, Alexa Fluor 647-anti-CD282 and PE-anti-CD284. Thereafter, cells were fixed with 2% paraformaldehyde in PBS, re-suspended in FACS buffer (PBS pH 7.2 supplemented with 0.5% bovine serum albumin and 0.02% azide) and acquired on an LSR Fortessa (BD Biosciences, San Jose, USA). A minimum of 10,000 events was measured per sample. A gate based on forward and side scatters was set to exclude cell debris and non-internalized parasites. The mean fluorescence of gated cells was determined, and the results were analyzed using the FlowJo software (Tree Star Inc., Ashland, USA). Annexin V staining was used to assess cell-surface expression of PS, a phospholipid normally confined to the inner leaflet of the plasma membrane, which serves as a ligand for receptor-mediated phagocytosis. Although not limited to, cells undergoing apoptotic cell death typically externalize PS, whose detection serves as apoptotic marker. Briefly, cells were treated as described above, incubated for 30 min at 4°C with FITC-labeled annexin V (Bio-Legend) and after fixation, re-suspended in binding buffer (10 mM HEPES, 140 mM NaCl, 25 mM CaCl₂). Monitoring of necrotic cells was not performed due to incompatibility between the propidium iodide assay and cell fixation, required for parasite inactivation prior to FACS analysis.

**Cytokine measurements**

For detection of cytokines, DC culture supernatants were harvested at different time points after stimulation and frozen at -20°C until analysis. Levels of IL-10 and IL-12p70 were measured by enzyme-linked immunosorbant assay (ELISA) using Legend Max™ ELISA kits (BioLegend), performed according to manufacturer’s instructions. Detection of human TNF-α was performed by ELISA using an antibody sandwich pair (Invitrogen, Bleiswijk, the Netherlands). Plates were first coated overnight at 4°C with the capture antibody diluted in 0.05 M Na₂CO₃. Thereafter, plates were washed in PBS/0.02%
Tween® 20 (Merck KGaA, Darmstadt, Germany) and blocked with PBS/1% bovine serum albumin fraction V (Roche Diagnostics, Basel, Switzerland), prior to the addition of the sample together with the detection antibody. The reaction was monitored at 450 nm with an Infinite M200Pro multimode plate reader (Tecan, Männendorf, Switzerland), after halting the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) catalyzed by streptavidin-horseradish peroxidase with 0.8 M sulfuric acid (H₂SO₄).

mRNA isolation and complementary DNA (cDNA) synthesis
Real-time quantitative Reverse Transcriptase-PCR (qRT-PCR) was performed to assess mRNA expression of TLR2, TLR4 and DC-SIGN. Total DNA and RNA was isolated from (un)stimulated DCs, as described by Boom et al., and DNase-digested using the Turbo DNA-free kit (Ambion, Austin, USA), according to the manufacturer’s procedures. To confirm the complete removal of genomic DNA, qPCR targeting two of the five target genes was performed after each DNase treatment on a randomly selected set of samples. Prior to real-time quantification, 20 µL of cDNA synthesis mix consisting of 0.03 µg/µL random hexamers primers (Roche Diagnostics), 5 mM MgCl₂, 0.48 mM dNTP (Thermo Scientific, Waltham, USA), 0.08 U/µL RNasinPlus RNase inhibitor (Promega, Madison, USA), 0.4 U/µL SuperScript II reverse transcriptase (Life Technologies) in 1X CBM buffer was mixed with 80 µL of each DNA-free RNA sample, and incubated at 42°C for 30 min or longer to enable synthesis of cDNA from the RNA templates. Samples were subsequently stored at -70°C until further analysis.

qRT-PCR
Amplification of TLR2, TLR4 and DC-SIGN sequences was performed in separate reactions, each of which included the household single copy gene β-2-microglobulin (β-2M) to correct for exact cell number and to control for test performance. Primers (Eurogentec, Maastricht, the Netherlands) targeting TLR2, TLR4 and DC-SIGN mRNA were designed to amplify intra-exon sequences, while intron-spanning primers were used to selectively amplify β-2M cDNA over its genomic counterpart, providing an internal control for cDNA synthesis (Table 1). Detection of DNA amplification was performed by sequence-specific probes (Eurogentec) labeled with the fluorophore FAM (Table 1). The qPCR reaction mix for TLR2, TLR4 and β-2M sequences (final volume 25 µL) consisted of 2.5 µL of 10X PCR buffer (Qiagen, Venlo, the Netherlands), 200 nM forward primer, 200 nM reverse primer, 40 nM probe, 0.5 U Hotstart Taq polymerase (Qiagen), 400 nM dNTP, 18.3 µL water and 2.5 µL cDNA. For DC-SIGN, the qPCR reaction mix was similarly composed, except that it contained 2 mM MgCl₂ (Qiagen) and 17.8 µL water. The qPCR was performed in a Biorad CFX96 real-time detection system (Bio-Rad, Hercules, USA) using the following cycling conditions: 10 min at 95°C, followed by 40 cycles, each consisting of 20 s at 95°C and 1 min at 60°C, during which the emitted fluorescence is measured. In each qPCR experiment, a negative control (water), an extraction control and a 5-step dilution series of parasite or cell cDNA were simultaneously assessed to ensure for high-quality DNA extraction and good performance/efficiency of the PCR. Percentage expression of target genes TLR2, TLR4 and DC-SIGN was calculated against unstimulated and LPS-treated cells, respectively, after computing a relative expression ratio against the reference gene β-2M on the basis of
the PCR efficiency and crossing point deviation of the investigated transcripts.\textsuperscript{51}

**Statistical analysis**

Expression of co-stimulatory molecules and DC-SIGN in response to *L. donovani* and/or *P. falciparum* stimulation was measured on mo-DCs obtained from five different donors, three of whom were examined for cytokine release, too. Statistical analysis and data shown in the present manuscript refer to these three donors for whom phenotypic data were matched by cytokine release activity (a substantially similar trend in phenotype was evident with mo-DCs from these 3 as well as from all 5 donors).

Statistical analyses were performed with the software package GraphPad Prism (Software Inc., San Diego, USA). For paired comparisons of two groups, a paired sample t-test was performed. For other parametric data, a one-way analysis of variance (ANOVA) was performed, followed by post hoc Dunnett’s multiple comparison test. A *P* value <0.05 was considered statistically significant. *P* <0.05, **P** <0.01, ***P** <0.001 indicate values that are significantly different from the unstimulated DCs. *P* < 0.05, **P** < 0.01, and ***P** < 0.001 indicate values that are significantly different from the LPS-stimulated DCs.

**Results**

*L. donovani* promastigotes promote semi-mature, anergic IL-10-producing mo-DCs that interfere with LPS-triggered activation.

To investigate the effect of VL on DC function, the activation status of mo-DCs obtained from three different donors and exposed to various amounts of *L. donovani* 1S promastigotes was examined through their phenotype and cytokine patterns. Immature mo-DCs responded to stimulation with *Leishmania* parasites in a dual fashion (Figure 2a, 51). At lower parasite amounts (mainly 1 to 10 promastigotes per mo-DC), mo-DCs up-regulated co-stimulatory molecules CD80 (*P* <0.05 with Ld\textsuperscript{5}) and CD86 (*P* <0.05 with Ld\textsuperscript{10} and Ld\textsuperscript{25}), with a little decrease in CD40 and HLA-DR cell levels. This phenotype interposed between the one of immature and LPS-activated cells, indicating that DCs stimulated with low amounts of *L. donovani* promastigotes only reached a semi-mature state. At higher parasite-to-cell ratios, conversely, a decrease for most of the examined maturation markers was observed (*P* <0.05 for CD80 and CD40 with Ld\textsuperscript{25}, *P* <0.01 for LPS with Ld\textsuperscript{10} and Ld\textsuperscript{25}), with a little decrease in CD40 and HLA-DR cell levels. This phenotype interposed between the one of immature and LPS-activated cells, indicating that DCs stimulated with low amounts of *L. donovani* promastigotes only reached a semi-mature state. At higher parasite-to-cell ratios, conversely, a decrease for most of the examined maturation markers was observed (*P* <0.05 for CD80 and CD40 with Ld\textsuperscript{25}, *P* <0.01 for LPS with Ld\textsuperscript{10} and Ld\textsuperscript{25}), with a little decrease in CD40 and HLA-DR cell levels. This phenotype interposed between the one of immature and LPS-activated cells, indicating that DCs stimulated with low amounts of *L. donovani* promastigotes only reached a semi-mature state. At higher parasite-to-cell ratios, conversely, a decrease for most of the examined maturation markers was observed (*P* <0.05 for CD80 and CD40 with Ld\textsuperscript{25}, *P* <0.01 for LPS with Ld\textsuperscript{10} and Ld\textsuperscript{25}), with a little decrease in CD40 and HLA-DR cell levels. This phenotype interposed between the one of immature and LPS-activated cells, indicating that DCs stimulated with low amounts of *L. donovani* promastigotes only reached a semi-mature state. At higher parasite-to-cell ratios, conversely, a decrease for most of the examined maturation markers was observed (*P* <0.05 for CD80 and CD40 with Ld\textsuperscript{25}, *P* <0.01 for LPS with Ld\textsuperscript{10} and Ld\textsuperscript{25}), with a little decrease in CD40 and HLA-DR cell levels.

### Table 1. Nucleotide sequences of the PCR primers and probes used to assay gene expression by qRT-PCR.

| Gene            | Forward primer | Reverse Primer   | Probe                          |
|-----------------|----------------|-----------------|--------------------------------|
| TLR2            | 5’ GGCCAGCAAAT | 5’ GAGCAGGAATCA | 5’ FAM-CTCTCTGGT               |
|                 | TACCTGTGTTG 3’ | GCGGAAACAG 3’   | GTCGGAATGT-BHQ1 3’             |
| TLR4            | 5’ CAGAGCCGCTG | 5’ TCCACCTCCAG 3’ | 5’ FAM-TGAATATGAGA             |
|                 | GTGTATC 3’     | GTAAGTGTGTT 3’   | TGTCTCAGACCT-BHQ1 3’           |
| DC-SIGN         | 5’ GTCCCTCAGTG | 5’ GCCCTGAAGAA   | 5’ FAM-CTCTGCTGGCG             |
|                 | GAGCAAGTT 3’   | GCGGAAACGAA 3’   | TTTCT-BHQ1 3’                  |
| β-2-Microglobulin | 5’ GGCTATCCAGC | 5’ GATGAAACCCA | 5’ FAM-CTCACGTCGA             |
|                 | GTACTCCAA 3’   | GACATAGCA 3’     | TCCAGCAGAG-BHQ1 3’            |

…
release (Figure 3a) was characterized by up-regulation of IL-10 production and a substantial, although statistically not significant, decrease in IL-12p70 levels. These changes were greatest in immature mo-DCs stimulated with equal amounts of *Leishmania* parasites (parasite-to-cell ratio of 1:1). Secretion of TNF-α followed a dose-dependent increase up to 10 parasites per mo-DC, but the raise lacked statistical power.

When mo-DCs pre-incubated with *L. donovani* were stimulated with LPS, only the lowest parasite doses (parasite-to-cell ratios of 1:1 and 5:1 partially) displayed a slight additional effect on LPS-induced expression of co-stimulatory molecules, whereas higher parasite amounts caused the expression of all maturation markers to progressively decrease to nullify the effect of LPS (*P* <0.05 for CD80 with *Ld* 25, *P* <0.01 for CD80 and CD40 with *Ld* 50 and *Ld* 25, respectively and *P* <0.001 for CD40 with *Ld* 50) (Figure 2a, S1). *L. donovani*-mediated interference of LPS effects was also evident on IL-10 and IL-12p70, whose levels in cell supernatants decreased following a dose-dependent fashion (up to 10 parasites per mo-DC for IL-12p70) (Figure 3a).

**P. falciparum fails to activate mo-DCs and partially interferes with LPS-triggered activation**

Immature mo-DCs exposed to allogeneic erythrocytes responded with an overall, minimal increase in the expression of DC maturation markers (Figure 2b, S2a). Similarly, a modest increase in IL-10, TNF-α and particularly IL-12p70 release was observed for almost all concentrations of RBCs (Figure 3b), but no statistically significant difference could be observed. Importantly, uninfected RBCs displayed a stable effect on both phenotype and cytokine profiles, with most changes exhibiting a dose-independent pattern. When the same allogeneic erythrocytes were infected with *P. falciparum*, exposure to immature mo-DCs (isolated from the same donors as for stimulation with uninfected RBCs and *L. donovani*) barely altered their phenotypic maturation, with the strongest effects being displayed on CD80 (*P* <0.05 with *Pf*RBC 10 and *P* <0.01 with *Pf*RBC 25) and HLA-DR (*P* <0.05 with *Pf*RBC 50) (Figure 2c, S2b). Little effects were observed on cytokine release, too, which registered a modest, dose-dependent increase in IL-10 and TNF-α (starting from *Pf*RBC5) and a more pronounced, but highly variable, enhancement in IL-12p70 (Figure 3c). Comparison of mo-DCs stimulated with the same *P. falciparum*-infected and uninfected erythrocyte populations (isolated from the same donor) demonstrated a partial inhibition of RBC-triggered effects (Figure 2b and 2c, S2a and S2b). Whilst a dose-dependent increase in CD80 and CD86 levels was observed, sufficient to outnumber only RBC-induced CD80 (*P* <0.01 with *Pf*RBC 5), expression of CD40 and HLA-DR progressively diminished as the parasite-to-cell ratio augmented (*P* <0.05 with *Pf*RBC 50) (statistical data not shown).

A similar *P. falciparum*-triggered interference was observed with regard to the LPS-induced activation. Mo-DCs stimulated with uninfected RBCs 24-h prior to LPS displayed similar features to the LPS-exposed cells, both in terms of phenotype (Figure 2b, S2a) and cytokine production (Figure 3b). By contrast, mo-DCs pre-incubated with *P. falciparum*-infected RBCs failed to fully mature in response to LPS stimulation (*P* <0.05 for CD40 with *Pf*RBC 5 and *P* <0.001 with *Pf*RBC 25 and *Pf*RBC 50), whose effects were partially inhibited in a dose-dependent fashion (Figure 2c, 3c, S2b).
Figure 2. Expression of surface markers on monocyte-derived dendritic cells (mo-DCs) in response to parasite stimulation. Immature mo-DCs were cultured alone or in the presence of either *L. donovani* promastigotes (a), uninfected RBCs (b), *P. falciparum*-infected RBCs (c) or *L. donovani* promastigotes and *P. falciparum*-infected RBCs (d) at different parasite-to-cell ratios (ranging from 1:1 to 50:1) for 24-h, followed by 48-h of incubation in the presence or absence of LPS (20 ng/mL). Thereafter, cells were harvested, and expression of maturation markers was measured by flow cytometry. Data represent means + SEMs (error bars) of three independent experiments performed with DCs from different donors and are indicated as the percent fold-change of activity vs. the unstimulated DCs (100%), calculated by pooling the percent fold-change of each individual experiment. *Ld* = *L. donovani*; *PfRBC* = *P. falciparum*-infected RBC; *Ld-PfRBC* = *L. donovani* and *P. falciparum*-infected RBC. *P < 0.05, **P < 0.01, ***P < 0.001 for values that are significantly different from the unstimulated mo-DCs. *P < 0.05, **P < 0.01, and ***P < 0.001 for values that are significantly different from the LPS-stimulated mo-DCs.
Figure 3. Cytokine secretion profiles of mo-DCs in response to parasite stimulation. Immature mo-DCs were cultured alone or in the presence of either *L. donovani* promastigotes (a), uninfected RBCs (b), *P. falciparum*-infected RBCs (c) or *L. donovani* promastigotes and *P. falciparum*-infected RBCs (d) at different parasite-to-cell ratios (ranging from 1:1 to 50:1) for 24-h, followed by 48-h of incubation in the presence or absence of LPS (20 ng/mL). Thereafter, cell supernatants were collected and levels of IL-10, IL-12p70 and TNF-α were measured by ELISAs. Data represent means ± SEMs (error bars) of three independent experiments performed with DCs from different donors and are indicated as the percent fold-change of activity vs. the unstimulated DCs (100%), calculated by pooling the percent fold-change of each individual experiment. Cytokine control levels of the three donors varied between 14.6 and 195.8 pg/mL for IL-10 (mean 100% value = 78.3 pg/mL), 2.6 and 51.5 pg/mL for IL-12p70 (mean 100% value = 18.5 pg/mL), 262.7 and 10062.0 pg/mL for TNF-α (mean 100% value = 4672.6 pg/mL). *Ld* = *L. donovani*; *Pf* RBC = *P. falciparum*-infected RBC; *Ld*-*Pf* RBC = *L. donovani* and *P. falciparum*-infected RBC. No statistically significant difference was found.
**Leishmania drives the priming of mo-DCs co-exposed to L. donovani promastigotes and P. falciparum-infected RBCs**

Co-exposure of mo-DCs (isolated from the same donors as for the above stimulation) to *L. donovani* promastigotes and *P. falciparum*-infected RBCs resulted in a phenotype largely mirroring the *Leishmania*-driven scenario (Figure 2d, S3). Whilst low doses of parasites triggered a semi-maturation state, marked by a significant up-regulation of CD80 (*P* <0.05 with *Ld*-PfRBC 5) and CD86 levels (*P*<0.05 with *Ld*-PfRBC 10), higher amounts of parasite significantly decreased the expression of CD80 (*P* <0.05 with *Ld*-PfRBC 25 and *P* <0.01 with *Ld*-PfRBC 50) and CD40 (*P*<0.05 with *Ld*-PfRBC 10, *P* <0.01 with *Ld*-PfRBC 25 and 50). This dual, dose-dependent effect was evident in the cytokine release, too, which peaked at parasite-to-cell ratios of 10:1 for IL-10 and TNF-α, and of 5:1 for IL-12p70, while reaching its minimum with the highest parasite dose (Figure 3d). None of the examined stimuli, however, was sufficient to significantly induce or inhibit secretion of the three cytokines.

Cell response to LPS stimulation 24 hour after parasite exposure consisted in an unaltered or slightly increased expression of maturation markers at the lowest doses (parasite-to-cell ratios of 1:1 and 5:1 for CD80 and CD86 only), followed by a progressive, dose-related inhibition of phenotypic maturation (*P* <0.05 for CD86 with *Ld*-PfRBC 5 and for CD40 with *Ld*-PfRBC 25 and 50, *P* <0.01 for CD80 with *Ld*-PfRBC 25 and 50) (Figure 2d, S3). Similarly, pre-stimulation of mo-DCs with the two parasites prior to LPS negatively affected their IL-10 and TNF-α production in a dose-dependent fashion (Figure 3d).

**L. donovani and P. falciparum co-localize in mo-DCs and modulate expression of cellular receptor DC-SIGN**

Immature mo-DCs have been shown to successfully phagocytose antigens from the surrounding environment, including parasites of the *Leishmania* and *Plasmodium* spp.\textsuperscript{16,52,53} This was evident in our experiments, too (data not shown). In addition, mo-DCs co-exposed to *L. donovani* promastigotes and *P. falciparum*-infected RBCs were shown to be potentially permissive towards both parasites, being able to internalize and co-harbor *Leishmania* as well as *Plasmodium* late-stage forms (trophozoites and schizonts) (Figure 4).

To investigate whether the driving effect displayed by *L. donovani* on DC differentiation may have resulted from the inability of *Plasmodium* parasites to engage its effector receptors, expression levels of DC-SIGN, TLR2 and TLR4 were examined. Mo-DCs (isolated from the three donors as for the previous experiments) exposed to increasing doses of *L. donovani* promastigotes progressively reduced the amount of C-type lectin DC-SIGN expressed on their surface (Figure 5a, S4). The decrease was evident at low parasite amounts already (*P* <0.05 with *Ld* 5 and 10), but it reached high statistical significance power (*P* <0.001) only at the highest parasite-to-cell ratios (25:1 and 50:1). A similar effect (*P* <0.05 with *Ld* 5, *P* <0.001 with *Ld* 10, 25 and 50) was observed in the presence of *L. donovani* and *P. falciparum*-infected erythrocytes, as confirmed by the comparable levels of DC-SIGN on mo-DCs co-exposed to the two parasites, which reflected the poor ability of uninfected and *P. falciparum*-infected RBCs to modulate DC-SIGN surface levels (Figure 5a, S4). LPS-induced maturation of mo-DCs significantly reduced the overall expression of DC-SIGN (*P* <0.05), as to be expected, but
Figure 4. Co-localization of *L. donovani* and *P. falciparum* in mo-DCs. Mo-DCs were co-cultured with *L. donovani* promastigotes and *P. falciparum*-infected RBCs. After 12-h of incubation, cells were harvested and optical microscope images (Field'-stained, viewed with a 100X objective) were taken. Several hemozoin crystals (some of which are indicated by the arrows) are visible in the cell, where they persist as remnants of late-stage malaria parasites, along with several *Leishmania* parasites (some of which are indicated by the asterisks), recognizable through their nucleus (pink colored-mass) and kinetoplast (deeply stained, purple organelle).

Figure 5. Expression of DC-SIGN and TLR2 on mo-DCs in response to parasite stimulation. Immature mo-DCs were cultured alone or in the presence of either *L. donovani* promastigotes, uninfected RBCs, *P. falciparum*-infected RBCs or *L. donovani* promastigotes and *P. falciparum*-infected RBCs at different parasite-to-cell ratios (ranging from 1:1 to 50:1) for 24-h, followed by 48-h of incubation in the presence or absence of LPS (20 ng/mL). Thereafter, cells were harvested, and expression of DC-SIGN (a) or TLR2 (b) was measured by flow cytometry. Data represent means + SEMs (error bars) of three independent experiments performed with mo-DCs from different donors and are indicated as the percent fold-change of activity vs. the unstimulated mo-DCs (100%), calculated by pooling the percent fold-change of each individual experiment. *Ld = L. donovani; PfRBC = P. falciparum*-infected RBC; *Ld-PfRBC = L. donovani and P. falciparum*-infected RBC. *P <0.05 and ***P <0.001 for values that are significantly different from the unstimulated mo-DCs. *P <0.05 and **P <0.01 for values that are significantly different from the LPS-stimulated mo-DCs.
the progressive decrease caused by *L. donovani* was still clearly visible (*P* <0.01 with *Ld* 25 and 50 and with *Ld-PfRBC* 25 and 50) (Figure 5a, S4). Transcriptional profiling of DC-SIGN (Table S1) showed its diminished detection on cellular surface could not be attributed to a reduction in gene expression, corroborating the prospect of a rapid DC-SIGN complex internalization upon *Leishmania* binding.

Cellular expression of TLR2 and TLR4 was also investigated, as these receptors have been shown to play an important role in recognition of parasite glycolipids, such as *P. falciparum* glycosylphosphatidylinositol (GPI) and *Leishmania* lipophosphoglycan (LPG), abundantly expressed on promastigote surface. When surface expression of TLR2 was analyzed (Figure 5b), a significant decrease was observed with *P. falciparum* (*P* <0.05 with *PfRBC* 5 and 25), but the effect appeared to be partially nullified by the subsequent addition of LPS. When TLR2 mRNA levels were examined, no clear trend for any of the analyzed stimuli could be identified (Table S2), suggesting that the changes in cellular expression of TLR2 observed here may not have resulted from an enhanced or decreased gene transcription. Similarly, analysis of cellular and transcriptional expression of TLR4 after exposure to *L. donovani* and/or *P. falciparum* parasites did not highlight any clear effect from these stimuli on the receptor (data not shown).

**L. donovani promastigotes induce PS externalization at high doses**

Pathogen-induced apoptosis of DCs has been described as a mechanism of immune dysfunction used by several micro-organisms, including *Plasmodium* spp., to evade adaptive immunity.\(^5\)\(^-\)\(^9\)

To investigate whether a similar event may account for the failure of our mo-DCs to mature and fully respond to LPS following exposure to *L. donovani* and *P. falciparum*, translocation of PS on the outer cell membrane was assessed by annexin V-FITC binding (Figure 6). Exposure of mo-DCs to the highest doses of *L. donovani* promastigotes resulted in a progressive rise in the proportion of PS-expressing cells (annexin V-positive), comparable to the effects induced by staurosporine, a commonly used initiator of apoptosis. Whilst this increase was evident (but not statistically significant) at the doses of 25 and 50 parasites/cell, no effect was displayed at lower parasite-to-cell ratios. On the contrary, *P. falciparum* and uninfected RBCs did not increase the number of annexin V-positive cells. When mo-DCs were co-exposed to the two parasites simultaneously, a more pronounced cytotoxicity appeared to affect the cells, as confirmed by the increased number of PS-expressing cells in samples stimulated with lower parasite-to-cell ratios. On the contrary, *P. falciparum* and uninfected RBCs did not increase the number of annexin V-positive cells. When mo-DCs were co-exposed to the two parasites simultaneously, a more pronounced cytotoxicity appeared to affect the cells, as confirmed by the increased number of PS-expressing cells in samples stimulated with lower parasite-to-cell ratios. Similar results with *Leishmania* and/or *Plasmodium* parasites were obtained after a 24 and 48-h incubation (data not shown), providing evidence that the marked reduction in the number of gated cells observed at the highest parasite doses is consistent with an increased proportion of non-internalized parasites remaining in the sample (a fixed number of 10,000 events was measured per sample with non-internalized parasites being set out of the gate), rather than with a rise in the number of dead, down-sized (and as such not-gated) DCs, as it was in the case of staurosporine stimulation.
Figure 6. Dose-dependent apoptosis of mo-DCs cultured with *L. donovani* promastigotes, *P. falciparum*-infected RBCs or a combination of the two stimuli. Immature mo-DCs were cultured alone or in the presence of either *L. donovani* promastigotes, uninfected RBCs, *P. falciparum*-infected RBCs or *L. donovani* promastigotes and *P. falciparum*-infected RBCs at different parasite-to-cell ratios (ranging from 1:1 to 50:1) for 72 h. Thereafter, cells were harvested and expression of annexin V was measured by flow cytometry. Expression levels of annexin V are indicated in each scatter plot, whose data are representative of three independent experiments performed with mo-DCs from different donors. In the bar chart, the mean percentage of apoptotic cells + SEMs (error bars) are shown for two independent experiments. *Ld* = *L. donovani*; *PfRBC* = *P. falciparum*-infected RBC; *Ld-PfRBC* = *L. donovani* and *P. falciparum*-infected RBC.
**Kinetics of mo-DC maturation inhibition and mo-DC apoptosis by L. donovani promastigotes**

To verify whether the lack of mo-DC maturation induced by high doses of *L. donovani* in the presence of LPS may be caused by a cytotoxic effect solely, expression of DC-SIGN, DC maturation markers (CD80 and CD86) and PS translocation were monitored at earlier time points (Figure 7). Pre-incubation of mo-DCs with increasing amounts of *L. donovani* promastigotes prior to LPS stimulation reduced the surface expression of DC-SIGN (*P* < 0.05 for *Ld* 25 and 50) and displayed an inhibitory effect on DC maturation, regardless of the duration of the pre-incubation step (Figure 7a). Thus, 1-h incubation with 25 or 50 *L. donovani* promastigotes per mo-DC inhibited the LPS-triggered expression of CD80 and CD86 to a comparable, dose-dependent extent as a 12-h or a 24-h incubation (*P* < 0.01 with *Ld* 25 and *P* < 0.001 with *Ld* 50 for CD80, *P* < 0.05 with *Ld* 50 for CD86) (Figure 7a). When *L. donovani* promastigotes (parasite-to-cell ratio of 25:1) and LPS were added simultaneously to the mo-DCs, a reduced expression of DC-SIGN, CD80 and CD86 was already evident after 1-h incubation and became more visible after 12-h (Figure 7b) (*P* < 0.05 for CD80), confirming that the *Leishmania*-mediated interference of LPS maturation already starts at very early stages. When PS externalization was measured by annexin V binding, a dose-related increase above the baseline level (unstimulated cells) could be observed only after 24-h for *Ld* 25 and *Ld* 50, and after 48-h for all three doses tested (Figure 7c) (*P* < 0.05 for *Ld* 25 and *P* < 0.01 for *Ld* 50 after 48 h).

**Discussion**

In the present study, the effect of VL-malaria co-infections on DC function was explored by examining the activation of mo-DCs co-exposed to increasing concentrations of *L. donovani* promastigotes and *P. falciparum*-infected RBCs. The obtained data indicate that co-stimulation with low amounts of parasites results in the induction of partially anergic DCs, characterized by semi-mature phenotypes and progressive failure to release IL-12p70. Similar outcomes were observed in the presence of *L. donovani* only, in contrast to the more silencing *P. falciparum*, suggesting a dominating effect of *Leishmania* during co-stimulation.

In absence of serum opsonization, *Leishmania* parasites are generally believed to be devoid of DC-activating signals, as DCs incubated with either promastigote or amastigote stages have been shown to retain an immature phenotype and produce little IL-12p70, although with variable effects upon the different DC subsets and *Leishmania* species. Nevertheless, earlier studies conducted with bone marrow-derived DCs have demonstrated that whereas the majority of cells remained immature following infection with *L. major*, a small, but consistent fraction of DCs containing parasites or parasite debris slowly up-regulated the expression of maturation markers, like CD86, CD40 and HLA-DR. A similar scenario seems to apply to our model, as moderate up-regulation of CD80 and CD86 levels following *L. donovani* exposure was observed in a restricted proportion of mo-DCs only. This indicates that the *Leishmania* entry, or the presence of its intracellular forms, may not have remained completely silent in these cell populations. Importantly, we found that this partial activation of DCs was accompanied by a significant down-regulation in IL-12p70 release.
and by an enhanced IL-10 secretion, suggesting these DCs may act by preferentially priming IL-10-producing CD4+ T cells, deleterious for disease resolution. Our results, however, contrast with those of earlier studies showing release of IL-12 immediately following DC contact with *L. donovani*, despite being in agreement with others in which *L. donovani* promastigotes failed to induce IL-12p70 secretion from DCs. Failure to detect IL-12p70 in our culture supernatants could also have resulted from its autocrine effects upon mo-DCs via constitutively expressed IL-12 receptors, which might explain the semi-mature phenotype detected in a fraction of these cells. Future work will have to prove the actual capacity of these DCs to stimulate proliferation of T cells and drive their differentiation towards regulatory T cells rather than Th1 cells. In contrast to this low-dose *Leishmania*-driven induction of semi-mature, IL-10-producing DCs, *P. falciparum* appeared to partially inhibit the slightly activating effects triggered by the uninfected RBCs, most likely caused by their allogeneic source, and only induced a marginal increase in co-stimulatory molecule expression. Lack of *in vitro* DC maturation upon *Plasmodium* exposure is a well-described phenomenon, although low parasite doses have been recently shown to induce slight up-regulation of DC maturation markers, in full compliance with our results. Interestingly, this *P. falciparum*-mediated inhibition appeared not to affect the phenotypic pattern triggered by the *Leishmania* parasites in co-stimulated DCs. Mutually exclusive internalization of the two parasites as a conceivable explanation was ruled out as remnants of *P. falciparum*-infected RBCs were found to co-localize with *L. donovani* parasites at cellular level. In addition, DC contact with *L. donovani*, but not *P. falciparum*, resulted in a sharp, dose-dependent reduction in the surface expression of DC-SIGN, not attributable to transcriptional down-regulation, confirming the strong role played by this C-type lectin in DC-*Leishmania* interactions. TLR2 expressed on monocytes/macrophages and natural killer cells has been shown to mediate recognition of *P. falciparum* GPI as well as *Leishmania* surface LPG. Therefore, we investigated whether internal competition for TLR2 occupancy or parasite-driven effects on its expression may have accounted for the *Leishmania*-driven priming of DCs during co-stimulation. In agreement with some previous findings, we found that *L. donovani* parasites were able to slightly increase the surface presence of TLR2 in a dose-dependent fashion, although transcriptional profiling did not yield clear results. Whilst these data are limited to draw conclusions on whether this parasite-mediated effect may or may not have influenced DC response to *L. donovani* and *P. falciparum* co-exposure, they provide evidence that malaria failure to inhibit *Leishmania*-driven priming of mo-DCs did not result from the leishmanial parasite avidity towards TLR2 nor from a paucity in its expression.

Inhibition of LPS-induced DC maturation by either *L. donovani* promastigotes, *P. falciparum*-infected RBCs or a combination of the two was shown to be dose-dependent. Whilst low parasite amounts (ranging from 1 to 5) did not affect LPS-induced maturation of mo-DCs, higher parasite-to-cell ratios resulted in a progressively increasing inhibition of LPS effects which intensified when both *L. donovani* and *P. falciparum* were present. Intriguingly, a similar pattern was observed for the proportion of cells displaying PS translocation, suggesting a close association between the two phenomena. Incubation with *L. donovani* at ratios of 25 and 50 promastigotes per DC triggered a pronounced increase in the expression of annexin V and enhanced, although not significantly, the percentage of PS-expressing cells, conceivably with the suppressing activity...
displayed on their maturation. On the contrary, *P. falciparum*-infected RBCs were able to induce a similar effect only at higher amounts (50 parasites per DC). Incubation with both parasites, on the other hand, exacerbated the cytotoxic effect elicited by the two stimuli, resulting in an increased, but statistically non-significant proportion of PS-expressing cells at parasite-to-cell-ratios of 5:1 and above. Apoptosis as a driver of unresponsiveness to LPS stimulation in mo-DCs exposed to high (50-100 parasites per DC), but not low (10 parasites per DC), doses of *P. falciparum* has been previously reported\textsuperscript{34,62,63} and our results fully comply with the respective literature. However, as PS translocation can also be apoptosis-independent,\textsuperscript{48} an ultimate role for this cell death mechanism as a result of DC-*P. falciparum* interactions remains to be confirmed. Conversely, an intrinsic ability to delay apoptosis induction in various cell subsets, including macrophages, mo-DCs and polymorphonuclear neutrophil granulocytes, has been described for promastigotes and amastigotes of several *Leishmania* spp.,\textsuperscript{64-69} supporting the speculation that the *Leishmania* parasite may deliberately manipulate host cell apoptotic machinery to improve its survival and pathogenicity. This activity, however, arose when cells were challenged with a pathogen multiplicity equal to or lower than 10 parasites per cell, disregarding the potential cytotoxic effect that higher parasite numbers may display. Natural transmission models currently suggest the *Leishmania* inoculation size to vary between 10 and 100,000 promastigotes per sand fly bite.\textsuperscript{70} This makes it difficult to establish the precise physiological ratio of promastigotes in the spleen or liver, where DCs are likely to be co-exposed to *Leishmania* and *Plasmodium* parasites. The same challenge applies to *P. falciparum*-infected RBCs, although the recent finding that circulating DCs from individuals harboring low-level *P. falciparum* infection and CD8\textsuperscript{+} splenic DCs harvested from *P. chabaudi*-infected mice displayed apoptotic features clearly supports a role for the programmed cell death in malaria *in vivo* infections.\textsuperscript{38,62} While further studies are required to establish the physiological

\textbf{Figure 7. Kinetics of mo-DC maturation inhibition and mo-DC apoptosis by *L. donovani* promastigotes.} (a) Immature mo-DCs were cultured alone or in the presence of *L. donovani* promastigotes at different parasite-to-cell ratios (ranging from 5:1 to 50:1) for different time intervals (ranging from 1-h to 12-h), followed by 48-h of incubation in the presence or absence of LPS (20 ng/mL). Thereafter, cells were harvested, and expression of DC-SIGN, CD80 or CD86 was measured by flow cytometry. (b) Immature mo-DCs were cultured alone or in the presence of *L. donovani* promastigotes (parasite-to-cell ratio of 25:1) and LPS (20 ng/mL) for different time intervals (ranging from 1-h to 12-h). Thereafter, cells were harvested, and expression of DC-SIGN, CD80 or CD86 was measured by flow cytometry. (c) Immature mo-DCs were cultured alone or in the presence of *L. donovani* promastigotes at different parasite-to-cell-ratios (ranging from 5:1 to 50:1) for different time intervals (ranging from 5-h to 48-h). Thereafter, cells were harvested, and expression of annexin V was measured by flow cytometry. For charts a and b, data represent means + SEMs (error bars) of two independent experiments performed with mo-DCs from different donors. For chart c, data represent means + SEMs (error bars) of three independent experiments performed with mo-DCs from different donors. For all chart, data are indicated as the percent fold-change of expression vs. the unstimulated mo-DCs (100%), calculated by pooling the percent fold-change of each individual experiment. *Ld* = *L. donovani*. *P* < 0.05 and **P < 0.01 for values that are significantly different from the unstimulated mo-DCs. *P < 0.05, ##P < 0.01 and ###P < 0.001 for values that are significantly different from the LPS-stimulated mo-DCs.
relevance of the observations highlighted by the present study, particularly with respect to the use of amastigotes as a better proxy for the study of VL-malaria interactions and of in vivo-resembling parasite levels, the possibility that the DC apoptosis observed in the presence of large numbers of parasites may have resulted from culture medium exhaustion, as previously suggested, should not be excluded. Indeed in the present model, both parasites were used at concentrations (2.5-5 X 10^7 parasites/mL for ratios of 25-50 parasite per DC) that largely exceed the densities found in standard, long-term cultures. Interestingly, a dose-dependent inhibition of LPS-mediated DC phenotypic maturation by L. donovani promastigotes began to appear at very early stages, prior to the translocation of PS on the outer membrane layer, suggesting that specific mechanisms rather than a generalized toxicity may be responsible for this differentiation impairment.

In conclusion, we found that L. donovani promastigotes exerted a dose-dependent effect on mo-DCs differentiation, which ranged from semi-mature cells producing low IL-12p70, but high IL-10 levels to a complete lack of phenotypic maturation in the presence of high parasite doses. The effect preceded the onset of apoptosis and was displayed against unstimulated as well as LPS-stimulated cells. Conversely, P. falciparum-infected RBCs failed to activate mo-DCs and only slightly interfered with their LPS-triggered activation. When mo-DCs were stimulated with both parasites simultaneously, cells displayed an overall phenotype reflecting the Leishmania trigger. L. donovani was shown to co-localize with P. falciparum in the same DCs, interact with them via the C-type lectin DC-SIGN and slightly increase cellular expression of TLR2, important for DC recognition of surface glycolipids. This suggests that in the presence of a VL-malaria co-infection, Leishmania-driven effects may overrule the more silent response elicited by P. falciparum, shaping host immunity towards regulatory patterns and possibly delay resolution of the two diseases. Additional testing exploring the effect of asynchronous L. donovani and P. falciparum co-stimulation on various DC subsets, including ex vivo DCs isolated from tissues that are likely to co-encounter the two parasites or their products, such as blood, spleen or liver, will be required to support this conclusion and provide new insights into the immunobiology of VL-malaria co-infections.

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Author's contributions

EvdB, HMdB, PPSB, IvD and HDFHS conceived and designed the experiments. EvdB, HMdB and PPSB performed the experiments. EvdB, HMdB, PPSB, IvD and HDFHS analyzed the data. EvdB, HMdB, PPSB, PFM, ERA, MPG, IvD and HDFHS interpreted the data. EvdB, HMdB, PPSB, PFM, ERA, MPG, IvD and HDFHS wrote and approved the final version of the manuscript.

Competing interests

The authors have no competing interests.
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Figure S1. *L. donovani* promastigotes promote semi-mature, anergic IL-10-producing moDCs that interfere with LPS-triggered activation.
Figure S2. *P. falciparum* fails to activate moDCS and partially interferes with LPS-triggered activation.
Figure S3. *L. donovani* drives activation of moDCs co-stimulated with *L. donovani* promastigotes and *P. falciparum*-infected RBCs, by promoting semi-mature, anergic IL-10-producing moDCs that interfere with LPS-triggered activation.

Figure S4. *L. donovani* alone or in combination with *P. falciparum* interacts with DC-SIGN.
Table S1. *L. donovani* alone or in combination with *P. falciparum* does not affect DC-SIGN transcriptional levels.

| CD209 mRNA | - LPS | + LPS |
|------------|-------|-------|
| **Medium** | 100%  | 100%  |
| *Ld* 5     | 95 ± 22% | 123 ± 41% |
| *Ld* 25    | 79 ± 12% | 177 ± 34% |
| *Ld* 50    | 80 ± 12% | 200 ± 27% |
| *RBC* 5    | 123 ± 35% | 179 ± 76% |
| *RBC* 25   | 51%    | 199 ± 103% |
| *RBC* 50   | 109 ± 23% | 114 ± 23% |
| *PRBC* 5   | 90 ± 15% | 144 ± 69% |
| *PRBC* 25  | 155 ± 8% | 164 ± 82% |
| *PRBC* 50  | 112 ± 23% | 189 ± 94% |
| *Ld*-*PRBC* 5 | 114 ± 60% | 154 ± 80% |
| *Ld-*PRBC* 25 | 128 ± 11% | 244 ± 8% |
| *Ld-*PRBC* 50 | 154 ± 33% | 359 ± 71% |

Table S2. *L. donovani* alone or in combination with *P. falciparum* does not affect TLR2 transcriptional levels.

| TLR2 mRNA | - LPS | + LPS |
|-----------|-------|-------|
| **Medium** | 68% | 100% |
| *Ld* 5     | 37 ± 5% | 87 ± 29% |
| *Ld* 25    | 61 ± 16% | 64 ± 16% |
| *Ld* 50    | 27 ± 8% | 28 ± 5% |
| *RBC* 5    | 74 ± 7% | 468 ± 263% |
| *RBC* 25   | 55% | 232 ± 113% |
| *RBC* 50   | 58 ± 7% | 153 ± 39% |
| *PRBC* 5   | 108 ± 41% | 184 ± 49% |
| *PRBC* 25  | 37 ± 22% | 106 ± 9% |
| *PRBC* 50  | 59 ± 22% | 70 ± 14% |
| *Ld-*PRBC* 5 | 101 ± 14% | 111 ± 28% |
| *Ld-*PRBC* 25 | 45 ± 13% | 104 ± 54% |
| *Ld-*PRBC* 50 | 38 ± 23% | 85 ± 57% |
Duplex quantitative Reverse-Transcriptase PCR for simultaneous assessment of drug activity against *Leishmania* intracellular amastigotes and their host cells

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Abstract

Currently available drugs for treatment of Leishmania infections are highly toxic and drug resistance to first-line therapies has been observed. New, safer and more effective drugs are urgently needed to improve clinical resolution of the disease and reduce the risks associated with it. High-throughput screening of new compounds against cultured promastigotes is easy to perform, but the results are poorly predictive of in vivo efficacy. Intra-macrophage amastigote models provide a better proxy of the clinically relevant stage of disease and should be routinely implemented in the search for new anti-leishmanial agents, despite being labor-intensive.

This study describes the use of a duplex quantitative Reverse-Transcriptase PCR (qRT-PCR) for assessment of drug activity against Leishmania intracellular amastigotes and their host cells. The assay simultaneously quantifies Leishmania 18S ribosomal RNA and the human β2-microglobulin (β-2M) mRNA, used for monitoring drug cytotoxicity and test performance. Accurate determination of parasite viability by the newly developed qRT-PCR was confirmed by parallel assessment of compound performance against standard microscopy. Highly reproducible anti-leishmanial activities were obtained with a set of structurally- and pharmacologically-diverse compounds, whose toxicity against host cells correlated with a low β-2M amplification. Sensitive and versatile, this duplex qRT-PCR offers a valuable tool for assessment of drug activities against Leishmania amastigotes and their host cells.

Keywords
Leishmaniasis, Polymerase chain reaction, Drug screening, Cell viability
Introduction

Leishmaniasis is caused by protozoan parasites of the genus *Leishmania*. In humans, the parasite causes a wide spectrum of clinical syndromes, from self-healing skin ulcers to severe, life-threatening disease, following visceralization in the lymphoid organs. Approximately 12 million people are currently infected with the disease and an estimated 2 million new cases occur each year, of which 1.5 million of cutaneous leishmaniasis and 0.2-0.4 million of visceral leishmaniasis.\(^1\) Chemotherapy remains the only effective way to treat all forms of disease, but current drugs are toxic, expensive and increasingly inefficacious due to parasite resistance, compelling the search for new anti-leishmanial agents.\(^2-4\)

Development of new drugs relies on the screening of large numbers of compounds, for which robust and highly performing assays are required. Axenic, insect stage promastigotes provide a simple model for evaluation of anti-leishmanial drug activity at high-throughput, but the resulting data have little value in animals, due to the metabolic and ecological differences that distinguish promastigotes from the mammalian amastigote stage.\(^5,6\) Macrophages carrying *Leishmania* amastigotes resemble the clinically relevant phase of disease, and have therefore been recognized as most predictive of clinical efficacy.\(^6,7\) Traditionally, drug activity against this stage is measured by microscopy counting of the percentage of infected macrophages and the number of amastigotes therein, a method which is labor-intensive and time-consuming.\(^8\) In addition, establishment of parasite viability through staining procedures is difficult and often subjective, leading to potential inaccuracies in potency estimation.\(^6\) Recent introduction of reporter gene assays for engineered *Leishmania* spp.\(^9-12\) has overcome this problem, but the use of parasite-recombinant markers that is associated with these techniques require stringent culturing conditions and prevent from assessing macrophage viability. Automated image-based assays provide a valuable alternative in this respect, enabling high-content examinations of compound activity against both *Leishmania* amastigotes and their host cells.\(^13\) Their implementation across non-dedicated facilities, however, remains limited, due to the sophisticated equipment required. PCR technology is nowadays widely available and has proven useful in monitoring anti-leishmanial drug efficacy at both *in vitro* and *in vivo* level.\(^14,15\) Several real-time quantitative PCR (qPCR) assays have been developed for detection of viable *Leishmania* parasites and assessment of drug performance,\(^16-18\) either through SYBR Green or fluorogenic probe technology. Importantly, these tests lacked an internal control that surveyed for test performance and monitored drug toxicity against the mammalian host cells.

The present study validates the use for drug screening purposes of a duplex Reverse-Transcriptase qPCR (qRT-PCR) simultaneously assessing for toxicity against *Leishmania* intracellular amastigotes and their host cells. Amplification of *Leishmania*-specific 18S ribosomal RNA was combined with the human β2-microglobulin (β2-M) mRNA, producing a sensitive and highly accessible tool to accurately measure anti-proliferative effects against the relevant stage of leishmaniasis.
Materials and Methods

Compounds
Amphotericin B (Calbiochem, Nottingham, UK), miltefosine, paromomycin, H-89 dihydrochloride hydrate and imipramine hydrochloride (all from Sigma-Aldrich Co., St. Louis, USA) were used for pharmacological validation of the qRT-PCR, alongside with the newly synthesized compounds (VUF11852, VUF11854, VUF11856, VUF11857 and VUF13577) kindly provided by Dr. K. Orrling (VU University, Amsterdam, the Netherlands) within the framework of a collaborative project (T4-302, Ti Pharma) for developing new phosphodiesterase inhibitors as potential anti-leishmanial drugs. Drug stock solutions (10 mM) were constituted in water, ethanol or DMSO, according to the compound solubility. Further dilutions of the drugs were performed in culture medium, possibly supplemented with the corresponding solvent to yield equal medium composition over the dilution series. Untreated (containing no drugs) and uninfected (containing no parasites and no drugs) controls were included in all drug experiments using the same culture medium. Two independent experiments testing a total of 10 different compounds were performed in triplicate for PCR and in duplicate for microscopy.

Leishmania promastigotes
Leishmania donovani 1S promastigotes (MHOM/SD/68/1S) were maintained axenically at 27°C, by serial passage in RPMI 1640 medium (Gibco, Bleiswijk, the Netherlands) supplemented with 25 mM HEPES, 2 mM L-glutamine and 10% fetal calf serum (FCS, Sigma-Aldrich Co.).

Leishmania intracellular amastigote model
THP-1 cells (human acute monocyte leukemia cell line) were cultured in RPMI 1640 medium supplemented with 25 mM HEPES, 2 mM L-glutamine, 10% FCS, penicillin (50 IU/mL) and streptomycin (50 µg/mL), to a density of 500,000 cells per mL. Cells (100 µL) were transferred to a 96-well culture plate for later testing by PCR and to a Lab Tek 16-well chamber-slide (Nunc, Waltham, USA) for microscopy counting. Next, 100 µL of cell culture medium containing 20 ng/mL of phorbol myristate acetate (PMA, Sigma-Aldrich Co.) were added to each well in order to differentiate the THP-1 cells into adherent macrophage-like cells. After 2 days of incubation at 37°C, 5% CO₂, cells were washed 3 times with culture medium to remove non-adherent cells. Infection of macrophages was performed with 200 µL of L. donovani 1S promastigotes at a density of 2.5 million parasites/mL. After 18-h of incubation at 37°C, 5% CO₂, cells were washed to remove excess of promastigotes and further incubated for 3 days with culture medium containing the appropriate concentration of test compound. Untreated (containing no drugs) and uninfected (containing no parasites and no drugs) controls were included in all drug experiments using the same culture medium. Two independent experiments testing a total of 10 different compounds were performed in triplicate for PCR and in duplicate for microscopy.

Analysis of intracellular amastigote content by microscopy
After 72-h of incubation at 37°C, 5% CO₂, the slides were removed from the culture chambers, methanol-fixed and Field'-stained. Their examination was performed with a light microscope (Leica, Rijswijk, the Netherlands), using a 100X objective. Results were expressed by mean of the parasite index (percentage of infected macrophages × mean number of amastigotes per macrophage), after examining a minimum of 100 macrophages per sample. Infection was judged to be adequate if more than...
70% of the macrophages in the untreated control were infected.

**Analysis of intracellular amastigote content by qRT-PCR**

After removal of culture medium, 0.75 mL of Trizol® (Invitrogen, Bleiswijk, the Netherlands) was added to all wells for cell lysis and the lysates were transferred to 1.5 mL tubes. RNA was Trizol®-extracted and purified according to manufacturer’s instructions. Residual DNA was further removed from all extracts by DNase digestion with Turbo DNase (Ambion, Invitrogen), performed according to the manufacturer’s instructions. A duplex qRT-PCR was performed for quantification of both *Leishmania* and human RNAs. The assay targeted the 18S small sub unit (SSU) rRNA conserved in *Leishmania* spp. and the human β-2M mRNA. The *Leishmania* qRT-PCR consisted of the forward primer 5’-CCAAAGTGTGGAGATCGAAG-3’, the reverse primer 5’-GGCCGGTAAAGGCCGAATAG-3’ and the probe 5’-6FAM-ACCATTGTAGTCCACACTGC-3’-NFQ conjugated to a minor groove binder (MGB) to increase the binding affinity of the probe to the target. For β-2M, the sense primer 5’-GGCTATCCAGCGTACTCCAA-3’ and the anti-sense primer 5’-GATGAAACCCAGACACATAGCA-3’ located within exon 1 and 2 were used, together with the fluorescent probe 5’-GATGAAACCCAGACACATAGCA-3’, labeled at the 5’-terminus with the reporter dye Texas red and at the 3’-terminus with the quenching dye. Tenfold lower quantities of β-2M primers and probe with respect to *Leishmania* 18S ones were added to the reaction mixture, to avoid sensitivity of the 18S PCR being affected by competition for β-2M amplification. For the PCR reaction, 1.25 μL of isolated RNA sample was added to 11.25 μL of amplification mix (Bio-Rad iScriptTM One-Step RT-PCR Kit for Probes, Hercules, USA) containing 2X PCR reaction reagent (20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.8 mM dNTPs, 3 mM MgCl₂ and 0.6 U/μL iTaq DNA polymerase), reverse transcriptase, 0.8 μM of each *Leishmania* primer and 0.2 μM of FAM-MGB probe, 80 nM of each β-2M primer and 20 nM of probe. Amplification and real-time measurement were performed with the CFX-1 Cycler (Bio-Rad) using the following conditions: 10 min at 50°C, 5 min at 95°C followed by 45 cycles, each consisting of 30 s at 95°C and 45 s at 60°C.

Toxicity thresholds for macrophage viability were set according to 90% cytotoxic concentrations (CC₉₀) of tested drugs, corresponding to a 3 Ct value increase in β-2M amplification over the controls. Consistent reduction of β-2M signal over the triplicate samples and its dose-related dependency from drug concentration confirmed low RNA contents being caused by drug toxicity rather than poor test performance. Inter-replicate variation >3 Ct values for either *Leishmania* 18S or β-2M amplification was considered indicative of poor RNA quality or extraction performance, resulting in the exclusion of these samples.

**Data analysis**

The inhibition of parasite growth was determined by comparing the amount of viable parasites detected in the drug-treated samples with that of the untreated controls. Non-linear regression analysis (Graph-Pad Software Inc., San Diego, USA) was used for curve fitting and calculation of 50% inhibitory concentrations (IC₅₀). The relationship between the two assays was studied by use of the individual logIC₅₀ values. Correlation between data obtained with the two assays was assessed by the Pearson’s correlation test, while the Bland and Altman plot was used to study the level of agreement between the two methods and to show dispersion of data.
Results

Performance of the duplex qRT-PCR

The qRT-PCR targeting the *Leishmania* 18S rRNA has previously been validated for quantification of *Leishmania* parasites.\(^2^3\) The standard curve for amplification was linear over a 6-fold \(\log_{10}\) range of *L. donovani* 1S RNA.

In the present study, a dilution series of Trizol®-extracted RNA from *L. donovani* 1S promastigotes was used as a standard for the duplex qRT-PCR in a background of 100 ng of THP-1 cell RNA extract, which corresponds to the average amount of human RNA found in each microtiter well seeded with *Leishmania* amastigotes. The *Leishmania* 18S amplification reaction was confirmed linear with a correlation coefficient of 0.995 over the range of 1 ng to 1 fg RNA, corresponding to 10,000-0.01 parasites (Figure 1A, blue curves, and Figure 1B). To exclude that sensitivity of the *Leishmania* 18S PCR could be negatively affected by competition for β-2M amplification, the standard samples were tested with and without β-2M primers in the PCR mix. Comparison of the two reaction mixes showed equally good amplification of *Leishmania* 18S for all standard samples, with no loss of sensitivity due to competition for β-2M amplification (data not shown). The β-2M mRNA signals derived from amplification of background THP-1 cell RNA in the standard samples were all comparable (Ct values ranging from 23.3 to 23.7), regardless of the amount of *Leishmania* RNA present (Figure 1A, red curves). Only the standard samples with the highest parasite content (1 ng and 100 pg parasite RNA) showed slightly reduced amplifications of β-2M (Ct values of 25.6 and 24.1, respectively), due to internal competition (Figure 1A, red curves 1-2).

Pharmacological validation

To validate the use of the qRT-PCR for assessment of drug efficacy against *Leishmania* intracellular amastigotes, the PCR assay was compared with standard microscopy by a blind, side-by-side evaluation. A set of 10 compounds marked...
by different potencies and mechanisms of action was used to validate the performance of the duplex qRT-PCR. The compound panel included a series of protein kinase-, protein transporter- and phosphodiesterase-inhibitors.\textsuperscript{19} Next to these compounds, three reference anti-leishmanial drugs were also assessed by the developed assay. A high correlation was found between the parasite load estimated by qRT-PCR and microscopy counting, regardless of which compound and concentration the parasites were exposed to (Figure 2 and Table 1). The IC\textsubscript{50} values yielded by the two assays during two independent experiments showed good agreement for most of the tested compounds (Table 1). Few discrepancies were observed (imipramine, VUF11852 and VUF11856), in which either the qRT-PCR or the microscopy data were marked by poor reproducibility with the replicate experiment. The Pearson’s correlation test confirmed a significant correlation between the logIC\textsubscript{50} values obtained by the two assays ($r = 0.95$, $n = 21$, $P < 0.0001$) (Figure 3A). In the Bland and Altman plot (Figure 3B), the mean difference in the logIC\textsubscript{50} values of the 10 compounds yielded by the qRT-PCR and microscopy was -0.03 (limits of agreement -0.30 and 0.24). No tendency for
a greater or smaller difference between the two methods was shown as the IC₅₀ values increased.

Table 1. Comparison of IC₅₀ values (µM) of test drugs against *Leishmania donovani* 1S intracellular amastigotes, as measured by the qRT-PCR and microscopy in two independent experiments. Data are shown with three significant digits.

| Test compound | qRT-PCR (µM)   | Microscopy (µM) |
|---------------|----------------|-----------------|
|               | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 |
| Amphotericin B | 0.0590 | 0.233  | 0.0200 | 0.180  |
| Miltefosine    | 1.19   | 0.639  | 0.820  | 0.590  |
| Paromomycin    | 81.8   | 53.4   | 41.9   | 112    |
| H-89           | 10.8   | 6.90   | 14.6   | 9.92   |
| Imipramine     | 25.6   | 5.50   | 17.8   | 9.92   |
| VUF 11852      | 21.0   | 27.6   | 8.93   | 31.6   |
| VUF 11854      | 15.0   | 13.3   | 13.5   | 16.7   |
| VUF 11856      | 4.82   | 27.6   | 20.6   | 40.9   |
| VUF 11857      | 4.39   | 5.88   | 6.21   | 7.95   |
| VUF 13577      | 6.70   | 9.01   | 7.34   | 7.05   |

The mean Ct value for β-2M amplification ranged from 24.5 in the first experiment to 22.7 in the second experiment for untreated *Leishmania*-harboring macrophages and uninfected cells. When serial dilutions of the test compounds were incubated with the *Leishmania*-infected cells, a dose-related increase in β-2M Ct values could be observed (Figure 4), suggesting reduced viability of macrophages as a result of drug exposure. The cytotoxic effect displayed by these compounds and indicated by an at least 10-fold lower β-2M amplification (corresponding to ≥3 Ct value increase over the controls) corroborated microscopy observation, whereby poor or no cell morphology was confirmed (data not shown). Based on these microscopy observations, a 10-fold lower β-2M amplification was considered indicative of overall cell toxicity.

Figure 3. (A) Correlation between the logarithm of the individual IC₅₀ values (in µM) measured by the qRT-PCR and the microscopy assays for 10 different compounds (*n* = 21; *r* = 0.94; *P* <0.0001). (B) Bland and Altman graph of the mean logIC₅₀ values, as determined by qRT-PCR and microscopy for 10 different compounds, plotted against their logIC₅₀ difference. The estimated mean difference (-0.03) and its confidence interval (± 2 standard deviation) are shown as horizontal lines.
Discussion

In vitro amastigote-based assays for screening of novel anti-leishmanial compounds have been widely recognized as highly predictive of in vivo drug efficacy, although experimental constrains still limit their broad implementation. New technologies for improving the quality of these assays have been developed, including several molecular tools for evaluation of anti-leishmanial drug activities in both in vitro and in vivo models. The present work describes a further advancement of these genetic assays, in which viability of Leishmania intracellular forms, the amastigotes, and their host cells is simultaneously assessed by a duplex qRT-PCR, targeting the Leishmania-specific 18S rRNA and the human β-2M mRNA, which serves as internal control for test performance and drug cytotoxicity. Pharmacological validation of the assay, performed with a 10-compound set blindly assessed against standard microscopy, resulted in a good correlation between anti-leishmanial dose-response curves obtained by the two assays and the corresponding IC₅₀ values, even when considering the highly subjective and inaccurate nature of microscopy scoring. Duplicate experiments showed fair intra-assay agreement.

Figure 4. Drug dose-response curves of Leishmania donovani 1S intracellular amastigotes, as measured by the qRT-PCR assay (closed circles, left y axis) and the corresponding Ct values for β-2M amplification (open rhombus, right y axis). Values indicating Leishmania growth (closed circles) are normalized by use of the upper and the lower best-fit values as 100 and 0% responses, respectively. Data are plotted as the means ± standard errors of the means of three replicates for the qRT-PCR assay and two replicates for microscopy. Representative data of one out of two experiments are shown.
for both PCR and microscopy, although for few compounds, a 4- to 5-fold difference in IC₅₀ values was observed, possibly caused by differences in the infection load. Intra-assay variation of parasite burdens is not uncommon when using this amastigote-macrophage model, as parasites need to actively enter the host cells and proliferate therein, upon conversion into amastigotes. This may have important implications for the IC₅₀ outcome, as confirmed by the critical role played by the number of promastigotes used to infect the cells, the particular Leishmania strain they belong to and the cell line bearing the infection.²⁴

Compared to alternative drug screening assays, microscopy counting offers the advantage of enabling host cell morphology to be visualized, so that both anti-leishmanial activity and drug cytotoxicity can be simultaneously assessed. In this respect, this duplex qRT-PCR represents a valuable alternative, as it allows for simultaneous detection of parasite and cell viability. Substantial drug cytotoxicity, microscopically confirmed by the poor morphology of human macrophages, was detected by the qRT-PCR, which yielded an at least ten-fold reduced β-2M amplification rate as opposed to the untreated controls.

Extensive debate on the use of DNA over RNA for drug resistance studies¹⁶,²⁰,²⁵ has resulted in the general belief that DNA remains stable over a long period of time, pending on environmental conditions, and it is therefore unsuited for testing viability of micro-organisms, including Leishmania spp. Our preliminary experiments conducted with leishmanicidal doses of amphotericin B indicated that 18S DNA levels of L. donovani 1S-infected macrophages differed less than a factor 10 from the untreated controls (data not shown), whereas a 1000-fold difference in 18S rRNA content was observed when the same samples were assayed by qRT-PCR and microscopy. Though in agreement with the general opinion, these findings contradict what was previously observed by others, who used real-time PCR to amplify DNA extracts and assess anti-leishmanial drug activity. Gomes et al.¹⁸ targeted the Leishmania-18S DNA in a Leishmania infantum amastigote-mouse model, obtaining amphotericin B IC₅₀ values (0.02 µM) that corroborate our and previously published results. Whether these data resulted from a comparable reduction in parasite amount, however, is unknown since the report does not mention absolute parasite numbers. Ordóñez-Gutiérrez et al.¹⁷ were able to demonstrate by PCR only a 3-fold decrease in Leishmania amastigote DNA content, as opposed to the 10-fold difference observed by microscopy during exposure to cystatin. Finally, Prina et al.²⁶ also used a DNA-based protocol for testing parasite viability and thereby demonstrate that Leishmania DNA is rapidly degraded in vitro following parasite death. These observations, however, were based on the use of L-Leucine methyl ester only, an experimental drug inducing rapid death of Leishmania mexicana and Leishmania amazonensis spp. Commercially available anti-leishmanial drugs, such as those tested in this study, are instead known to act progressively over a longer period of time,⁴ resulting in potentially different effects on parasite/host cell viability and corresponding metabolisms. Furthermore, the use in the three above-mentioned studies of mouse cells as a macrophage source rather than the human THP-1 cell line employed here, may partially account for the discrepancies observed, given that profound differences between rodent and human macrophages have been described.²⁷
The present study was conducted on a selection of compounds, for which comparable anti-leishmanial activities were obtained by qRT-PCR and microscopy. Further evaluation of the assay is now required to establish its performance with different *Leishmania* models and testing compounds. Efforts to automatize the technique are also equally crucial to ensure this qRT-PCR may be suitable for high-throughput drug screening. Accurate RNA isolation is key to this assay, but several automated RNA extraction systems capable of yielding high-quality RNA are nowadays available on the market. This opens important avenues for the further development of this assay.

In conclusion, the present results demonstrate the value of this qRT-PCR as a sensitive and reliable tool for assessing drug activities against *Leishmania* intracellular amastigotes and their host cells, simplifying the use of the clinically relevant stage of leishmaniasis for drug discovery programs.

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**Competing interests**

The authors declare that no competing interests exist.
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Corrigendum to ‘Duplex quantitative Reverse-Transcriptase PCR for simultaneous assessment of drug activity against *Leishmania* intracellular amastigotes and their host cells’ [Int. J. Parasitol. Drugs Drug Resist. 4 (2014) 14–19]

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The authors regret that there was an error in a sentence in the section 2.5. The corrected sentence is as below:

For β-2M, the sense primer 5′-GGCTATCCAGTGACTCCAA-3′ and the antisense primer 5′-GATGAAACCCAGACATAGCA-3′ located within exon 1 and 2 were used, together with the fluorescent probe 5′-ACTCACGTCATCCAGAG-3′, labeled at the 5′-terminus with the reporter dye Texas red and at the 3′-terminus with the quenching dye.

The authors would like to apologise for any inconvenience caused.
Simple colorimetric trypanothione reductase-based assay for high-throughput drug screening against *Leishmania* intracellular amastigotes

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Abstract

Critical to the search for new anti-leishmanial drugs is the availability of high-throughput screening (HTS) methods to test chemical compounds against the relevant stage for pathogenesis, the intracellular amastigotes. Recent progress in automated microscopy and genetic recombination has produced powerful tools for drug discovery. Nevertheless, a simple and efficient test for measuring drug activity against *Leishmania* clinical isolates is lacking. Here we describe a quantitative colorimetric assay in which the activity of a *Leishmania* native enzyme is used to assess parasite viability. Enzymatic reduction of disulphide trypanothione, monitored by a microtiter plate reader, was used to quantify the growth of *Leishmania* parasites. An excellent correlation was found between the optical density at 412 nm and the number of parasites inoculated. Pharmacological validation of the assay was performed against the conventional alamarBlue method for promastigotes and standard microscopy for intracellular amastigotes. The activity of a selected-compound panel, including several anti-leishmanial reference drugs, demonstrated high consistency between the newly developed assay and the reference method and corroborated previously published data. Quality assessment with standard measures confirmed the robustness and reproducibility of the assay, which performed in compliance with HTS requirements. This simple and rapid assay provides a reliable, accurate method for screening anti-leishmanial agents, with high-throughput. The basic equipment and manipulation required to perform the assay make it easy to implement, simplifying the method for scoring inhibitor assays.
Introduction

The global scope of leishmaniasis and the inadequacy of present treatments support the need for improved chemotherapies. Undermined by severe toxicity and high failure rates, besides poor compliance and high costs, current anti-leishmanial treatment relies on a few drugs, of which toxic antimonials remain the mainstay 70 years after their introduction. Assessment of novel drugs or treatment regimens for leishmaniasis requires reliable methods for high-throughput screening (HTS) against the clinically relevant stage of the disease, the intracellular amastigotes. For decades, anti-leishmanial drug discovery has rested upon the use of insect stage, free living promastigotes, for which simple and efficient in vitro assays have been made available. Nevertheless, failure to identify all active compounds and selection of numerous false-positive hits have recently been associated with the use of promastigotes in primary screenings, suggesting that host cell-mediated effects and stage-specific chemosensitivities should be addressed already at early stages of drug discovery programs. Testing on intra-macrophage amastigotes, therefore, remains crucial, though challenging, given the lack of standardized methods that characterize the culturing of these parasite forms and the biological diversity exhibited by the different strains and species. Technically difficult and laborious, assessment of anti-leishmanial activity against the vertebrate host stage traditionally relies on microscopic quantification of parasite burdens, with major problems related to data quality and poor performance. Establishment of parasite viability through staining procedures is difficult and error-prone, due to the uneven distribution of parasite loads throughout the sample and the difficulty to recognize individual amastigotes within heavily infected host cells.

Recent progress in automated image-based screening assays and genetic engineering of Leishmania spp. has opened important avenues for the high-throughput search of compounds targeting the intracellular stage. Nonetheless, advanced technology and intense manipulation are required to perform these assays, precluding their broad implementation and routine application to clinical isolates.

We report the development and validation of a simple, one-step assay for measuring drug activity against Leishmania intracellular amastigotes grown in human macrophages. Trypanothione reductase (TryR), an essential component of the kinetoplast-unique thiol-redox metabolism, was used to assess the viability of the Leishmania parasites by monitoring its 5,5'-dithiobis 2-nitrobenzoic acid (DTNB)-coupled reducing activity. This reaction combines the TryR-catalyzed reduction of trypanothione disulfide (T\(\text{S}_2\)) with its in situ regeneration through DTNB, resulting in a colorimetric assay with improved sensitivity and efficiency (Figure 1). No cross-reactions with the host cells were observed, as a result of the poor affinity of substrate for the mammalian homologue. The assay was designed for microtiter plate format and colorimetric detection, enabling automation and high-throughput measurements with basic equipment. With its simplicity and robustness, this assay offers a valuable tool for the screening of new anti-leishmanial drugs, simplifying the method for assessing inhibitory activities at intracellular amastigote stage and improving accessibility to drug susceptibility testing with the ability to implement in most laboratories.
Materials and Methods

Chemicals and drugs
All chemicals and drugs were purchased from Sigma-Aldrich Chemical Company (St. Louis, USA), unless stated otherwise. Triton X-100 and T[S] were obtained from BDH Laboratory Supplies (Poole, United Kingdom) and Bachem AG (Bubendorf, Switzerland), respectively. alamarBlue was purchased from AbD Serotec (Düsseldorf, Germany). The test compounds (VUF series of catecholpyrazolinones and the dibenzylmethylene amine derivative IOTA 0058) are proprietary compounds of the VU University, Amsterdam, the Netherlands, and were provided within the framework of a collaborative project for development of new phosphodiesterase inhibitors (T4-302; TI Pharma). Amphotericin B and sodium stibogluconate were purchased from Merck KGaA (Darmstadt, Germany). Stock solutions of NADPH and T[S] were prepared at a concentration of 8 and 10 mM, respectively (NADPH in Tris (0.5 M) buffer, pH 7.5; T[S] in water), stored at -70°C, and freshly thawed before use. DTNB was dissolved in ethanol at a concentration of 25 mM prior to each experiment. Further dilutions of NADPH, T[S], and DTNB were performed in Tris (0.05 M) buffer, pH 7.5. Stock solutions of the test drugs were prepared at concentrations of 5 to 10 mM in dimethyl sulfoxide (DMSO), water, or ethanol, according to the corresponding solubility.

Cell cultures
Promastigotes of Leishmania donovani (strains MHOM/SD/1968/1S and antimony-resistant MHOM/IN/2010/BHU814) and Leishmania major (strain MHOM/IR/1972/NADIM5) were maintained at 27°C in RPMI 1640 medium supplemented with 15% heat-inactivated fetal bovine serum (FBS) (Sigma Aldrich Co.), 25 mM HEPES, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin. The human monocyte leukemia cell line THP-1 (ATCC no. TIB 202) was grown in RPMI 1640 medium supplemented with 10% FBS, 25 mM HEPES, 2 mM L-glutamine, and 1% antibiotics in a 5% CO₂ atmosphere at 37°C. Intra-macrophage amastigotes of L. donovani were cultured as previously described, with minor modifications. Briefly, prior to infection, THP-1 monocytes (2.5 X 10⁵ cells/mL) were differentiated into adherent, non-dividing macrophage-like cells by a 48-h incubation with 10 ng/mL phorbol 12-myristate 13-acetate (PMA). After elimination of PMA by 3 washes, differentiated THP-1 cells were incubated overnight with stationary-growth-phase L. donovani promastigotes at a parasite/cell ratio of 10/1. Non-internalized promastigotes were then removed by washing adherent cells three times in excess of medium, and the cultures reincubated at 37°C in 5% CO₂ for an additional 72 h. All cell culture reagents were purchased from Gibco (Bleiswijk, the Netherlands), unless otherwise stated.

TryR-based assay
Assessment of TryR activity was performed in THP-1 cell and Leishmania parasite lysates. After a prewash with phosphate-buffered saline to remove potentially interfering compounds, samples were chemically lysed by 15 min incubation with a lysis buffer (200 μL/well), consisting of EDTA (1 mM),
HEPES (40 mM), Tris (50 mM; pH 7.5), and Triton X-100 (2% vol/vol). Immediately prior to use, the buffer was supplemented with the protease inhibitor phenylmethanesulfonyl fluoride at a final concentration of 1 mM. TryR activity was measured in 75 µL of sample lysate, dispensed into the test well of a 96-well plate. NADPH (25 µL/well), T[S]₂ (75 µL/well), and DTNB (25 µL/well) were sequentially added to the sample lysate to yield final concentrations of 200, 75 and 100 µM, respectively. A blank was set for each sample, consisting of sample lysate supplemented with the reaction mixture described above, in which the substrate T[S]₂ had been replaced by Tris (0.05 M) buffer, pH 7.5. After a serial 27°C incubation ranging from few minutes to 3-h, absorbance was measured with an Infinite M200Pro multimode plate reader from Tecan (Männendorf, Switzerland) at a wavelength of 412 nm. The optical density, as measured in the blank, was subtracted from the corresponding sample signal, yielding the TryR activity responsible for 2-nitro-5-thiobenzoate (TNB²-) production.

Promastigote drug susceptibility assay

Exponentially growing promastigotes, seeded in 96-well tissue culture plates at an initial concentration of 1 X 10⁶/mL, were allowed to grow for 72-h at 27°C in the presence of a series of drug concentrations, ranging from 0 to a maximum of 10⁻³ M. Test drugs were serially diluted (in triplicate for each concentration) in promastigote culture medium supplemented with the corresponding drug solvent to yield equal concentrations of solvent over the dilution series. Untreated controls consisting of parasites incubated with medium only (with drug solvent) were included in triplicate in each test plate. Drug activity, as measured with the TryR-based assay, was compared with the previously described alamarBlue method. For each test drug, plates for both assay methods were prepared in parallel with the same cells and medium. For the alamarBlue assay, 20 µL (10% vol/vol) of alamarBlue reagent was added to each well. After 5-h of incubation at 27°C, fluorescence was measured with a Tecan Infinite M200Pro multimode plate reader, with excitation and emission wavelengths of 560 and 590 nm, respectively, and a plate-tailored optimal gain setting. For the TryR-based assay, samples were processed as described above.

Intracellular amastigote drug susceptibility assay

THP-1 monocytes were plated in 16-well chamber slides (Lab-Tek, Nunc) or in 96-well culture plates for microscopic and enzymatic assessment, respectively. Differentiation and subsequent infection of cells were carried out as described above. After removal of non-internalized promastigotes by extensive washing with RPMI 1640 medium, parasitized cells were incubated with medium (untreated control) or a range of drug concentrations, as described for the promastigote drug assay. A negative control consisting of uninfected cells with no drug exposure was also included in each plate. After 72-h of incubation at 37°C in 5% CO₂, total parasite burdens (calculated as the percentage of infected macrophages × mean number of amastigotes per macrophage) were microscopically assessed on Field'-stained slides and compared to the burdens of the untreated infected controls. At least 100 macrophages were counted per sample, and infection was judged to be adequate if 70 to 80% of the macrophages present in the untreated controls were infected. For the TryR-based assay, infection of cells and subsequent testing of drugs was carried out as described above. After 72-h of incubation
at 37°C, 5% CO₂ cells were washed and lysed prior to being tested with the TryR-based assay. Results were expressed as the percentage of activity reduction in the treated sample vs. the untreated control. No microscopic assessment of macrophage infection rate was performed on these samples.

**Statistical analysis**
The inhibition of parasite growth was determined by comparison of the signal produced by drug-treated parasites with that of untreated control parasites. Non-linear regression analysis (GraphPad Prism, version 5.03; Software Inc., San Diego, USA) was used for curve fitting and calculation of 50% inhibitory concentrations (IC₅₀) and 90% inhibitory concentrations (IC₉₀). Analysis of correlation and agreement between data obtained from the TryR-based assay and microscopy was performed after transformation of assay results to log scale. Transformed data from the two assays were used for obtaining a scatter plot in which one set of assay results was plotted against the other on a log-log scale. The level of correlation between these data was assessed by the Pearson’s correlation test (GraphPad Prism), in which the null hypothesis stated that no linear relationship linked the measurements obtained by the two methods. To determine the level of agreement between the two tests, the transformed data from both assays were graphed in a Bland-Altman plot (GraphPad Prism), where the difference of IC₅₀ values obtained for each compound by the two assays was plotted as a function of the mean IC₅₀ between microscopy and the TryR-based assay. Two standard deviations (SD) of the mean IC₅₀ difference between two assays were defined as the acceptable boundary range, as previously described, and were incorporated in the Bland-Altman plot to visualize the acceptable variation for the agreement. For validation of the TryR-based assay, the mean ratio (MR) and its confidence limits, the minimum significant ratio (MSR) and its limits of agreement (LsA), and the Z’ factor were calculated with Microsoft Excel software. The MR with its confidence limits and the MSR with its LsA were used to characterize the reproducibility in replicate experiments of average and individual compound potency estimates, respectively. The MR, whose ideal value should be 1.0, represents the average potency ratio across two concentration-response assay runs. When included within statistical limits of 1.0, the MR indicates no statistically significant differences in compound activity between runs. The MSR describes the largest potency ratio that can be considered a random change within a run of the assay and its LsA identify statistical limits for individual potency ratios. Typically, an assay is considered acceptable if the MSR is small enough and both the LsA are close to 1.0. For our purpose, acceptance criteria were set at ≤3 for MSR and at 0.33 and 3.0 for the LsA, similarly to what was previously described by Eastwood et al. For assessment of Z’ factor values, the signal data yielded by untreated control *L. donovani* BHU814-infected and uninfected host cells were used. The Z’ factor, a characteristic parameter for the quality of the assay itself, is commonly used to validate the reliability of single-concentration HTS, as its values predict the rates of false positives and negatives to be expected with an assay. The categorization of screening assay quality by the value of the Z’ factor has been described elsewhere. Typically, a Z’ factor value >0.5 is defined as acceptable for an assay to be used in compound testing.
Results

Development of a TryR-based assay to quantify Leishmania spp.

A TryR-based assay was developed, adapting the previously described TryR DTNB-coupled reaction\textsuperscript{24} for use with the non-purified native enzyme contained in parasite lysates. The kinetics of the TryR-catalyzed reaction was monitored spectrophotometrically at 412 nm, after correcting for the non-trypanothione (T[SH]\textsubscript{2}) mediated reduction of DTNB. Free thiols stoichiometrically convert colorless DTNB into yellow TNB\textsuperscript{2-}, whose absorbance is commonly used to quantify the number of sulfhydrylic groups in biological samples. To monitor the T[SH]\textsubscript{2}-mediated production of TNB\textsuperscript{2-} solely, a blank sample, consisting of lysate incubated with all reagents but substrate, was set for each sample, and its optical density was subtracted from the signal of the corresponding test sample. DTNB was reduced by the T[SH]\textsubscript{2} produced in lysates of *L. donovani* 1S and *L. major* NADIM5 promastigotes at a constant speed, until exhaustion of one or more reagents (Figure 2A). Conversely, no reduction of T[S]\textsubscript{2} was observed with host cells (Figure 2A), confirming the low affinity of the glutathione reductase for trypanothione-disulfide. Promastigotes of *L. donovani* and *L. major* exhibited striking different reaction rates in terms of DTNB reduction, highlighting species-specific features of the TryR metabolism. Under optimized conditions, a linear relationship ($R^2 = 0.9973-0.9999$) between plating density and absorbance was observed when different numbers of promastigotes (ranging from $4.1 \times 10^4$ to $2.5 \times 10^7$ parasites/mL, depending on the reaction time) were incubated with the reaction mixture for 0 to 6-h (Figure 2B). Longer incubation times (3 to 6-h) resulted in increased sensitivity of the assay due to higher optical densities, and, when linear, in enhanced signal differences between serial dilution points, due to increase of the curve slopes. However, they also caused the curves to plateau at higher parasite numbers, due to depletion of substrate and/or coenzyme. After 3 or more hours of

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**Figure 2.** Time- and dose-dependent TryR activity. (A) Kinetics of TryR activity in parasite and cell lysates. Equal amounts ($5 \times 10^6$/mL) of *Leishmania* promastigotes (*L. donovani* 1S and *L. major* NADIM5) and THP-1 cells were lysed, and their TryR-mediated reduction of DTNB was assessed at intervals of 15 min. (B) Dose-dependent TryR-mediated reduction of DTNB in *L. donovani* 1S promastigote lysate.
incubation, only a small increase in absorbance was observed with $3 \times 10^3$ promastigotes, which therefore represents the lower detection limit of this assay.

**Assessment of standard drug efficacies against Leishmania promastigotes**

The suitability of the TryR-based assay for pharmacological studies was initially confirmed on promastigotes, by assessing the efficacies of four anti-leishmanial reference drugs. Amphotericin B, miltefosine, pentamidine and paromomycin were tested against promastigotes of *L. donovani* and *L. major*, two major species causing visceral and cutaneous leishmaniasis, respectively. The inhibitory activities of the four drugs were measured with the novel TryR-based assay and compared to the activities obtained with the conventional alamarBlue method. All drugs exhibited a dose-dependent effect on parasite proliferation, with dose-response curves and IC$_{50}$ and IC$_{90}$ values showing high correlation between the alamarBlue determination and the novel TryR-based assay (Figure 3 and Table 1). Different susceptibilities to pentamidine isethionate were observed for *L. donovani* and *L. major* parasites, with the two methods in agreement with each other.

**Assessment of standard drug efficacies against Leishmania intracellular amastigotes**

To validate the use of the TryR-based assay for assessing viability of the *Leishmania* intra-macrophage forms, the activity of the four reference drugs was measured against *L. donovani* 1S and *L. major* NADIM5 intracellular amastigotes. Light microscopy was used as the conventional method to measure drug efficacies, due to incompatibility of the alamarBlue assay with the intracellular model. The TryR-catalyzed reaction was allowed to proceed for 3 hours prior to measurement, because of the lower parasite loads compared to the axenic tests. Similarly to the promastigote stage, the amastigote activity of the TryR reflected the dose-dependent effects of the drugs, resulting in growth curves and IC$_{50}$ and IC$_{90}$ values largely consistent with the microscopic assessments (Figure 4 and Table 2). Species-specific chemosensitivities were observed with pentamidine isethionate, whose potency against *L. donovani* visceralizing forms was 20 times as high as against *L. major* ones (responsible for cutaneous leishmaniasis), similarly to what was measured in promastigote stages.

**Pharmacological and HTS validation**

To assess the pharmacological relevance of this assay and analyze its robustness, two independent tests were performed with a 19-compound set and the corresponding activities blindly assessed by microscopy and the TryR-based assay (Table 3). The compound panel included a variety of chemicals with different potencies and mechanistic actions, ranging from TryR, protein kinase and phosphodiesterase inhibitors to G protein-coupled receptor ligands and various antiprotozoals, including a few anti-leishmanial reference drugs (Table 2). Overall, data assessed by microscopy resulted in noisier curves than data from the TryR-based assay, affecting the quality of fitting results and their reproducibility therein. As a consequence, estimation of IC$_{50}$s for compounds displaying weak anti-leishmanial activities and poorly fitted dose-response curves, such as efornithine, metergoline, and carmustine, was not always feasible. Poor solubility of compound H-89 resulted in precipitation at the highest concentrations and very steep dose-response curves. Nifurtimox and suramin exhibited insufficient inhibition during the first test but yielded complete dose-response curves.
in the second run when higher concentrations were tested. The level of correlation and agreement between the TryR-based assay and the microscopic assessments was examined with the Pearson’s correlation test and the Bland-Altman plot. The scatter plot (Figure 5A) showed a significantly high level of correlation between the results from the two assays (coefficient correlation $r = 0.88; P <0.0001$), with the line of best fit (slope = 0.97 ± 0.09) approximating the line of equivalence, which can be expected if the data from each assay were identical (i.e., the line $x = y$). In the Bland-Altman plot (Figure 5B), all points were scattered around the 0 and fell within the limits shown (upper limit of the agreement = 0.495; lower limit = -0.563), with a negative bias of -0.034 towards the TryR-based assay, indicating that data can be normalized from the TryR-based assay to microscopy by a factor of 0.95. No tendency toward a greater or smaller difference between the two methods was seen as the IC$_{50}$s increased.

For the TryR-based assay, the inter-assay activity variation for compounds ($n = 11$) devoid of solubility problems and exhibiting complete inhibitory curves over the tested dose range resulted in an MR of 1.10 with confidence limits of 0.88 and 1.39, indicating that there was no systematic difference in potency estimates between the two runs. Similarly, with an MSR of 2.70 and LsA of 0.55 and 2.98, and with no compound within the 11-compound sub-cohort used for statistical assessment lying outside the LsA, the assay met the acceptance criteria described above. To monitor for signal stability, the following measurements were performed. Three independent plates, each containing 30 replicates of uninfected and $L. donovani$ BHU814-infected cells (edging wells were excluded), were sequentially measured over a 6-week period, enabling calculation of assay windows. Z’ factor values of 0.502, 0.559 and 0.646, respectively, were obtained for the three measured plates, confirming the good performance of the assay and its compliance with single-dose screen requirements (acceptance limit for the Z’ factor value was >0.5).

**Discussion**

Critical to the success of drug discovery strategies against leishmaniasis is the use of the intracellular amastigote stage for primary screenings, which minimizes selection of falsely active compounds and prevents true hits from being undetected. New technologies for improving the quality and throughput of these assays have been developed but remain difficult to implement with clinical isolates or in non-dedicated laboratories. Here, we describe a simple alternative assay for assessing the viability of $Leishmania$ intracellular forms and screening anti-leishmanial drugs at high throughput. The activity of a native, kinetoplast-unique enzyme was measured by a one-step colorimetric assay and linearly related to the amount of parasites. TryR, the NADPH-dependent flavoprotein responsible for regeneration of the trypanothione pool, is essential to survival of trypanosomatids and, as such, is constitutively expressed throughout the parasite life cycle. Its absence from the mammalian host makes it a highly attractive target for drug development and a valuable biomarker for parasite viability, as shown in this report. The previously described DTNB-coupled reaction for quantification of TryR activity in enzyme binding assays was applied to the complex environment of axenic and intramacrophage parasite cultures. The reaction was initiated after chemical disruption of cell membranes by a non-ionic detergent-containing buffer, to ensure access of the substrate to the
Figure 3. Dose-response curves of *L. donovani* (A and B) and *L. major* (C and D) promastigotes to four anti-leishmanial reference drugs (amphotericin B, miltefosine, pentamidine and paromomycin), as measured by the TryR-based assay (A and C) and the conventional alamarBlue method (B and D). Values are normalized by use of the upper and the lower best-fit values as 100 and 0% responses, respectively, and are plotted as the means ± standard errors of the means for three replicates. Two independent determinations were performed with both methods run in parallel, and representative data of one of the two experiments are shown.

Figure 4. Dose-response curves of *L. donovani* (A and B) and *L. major* intracellular amastigotes (C and D) to four anti-leishmanial reference drugs (amphotericin B, miltefosine, pentamidine, and paromomycin), as measured by the TryR-based assay (A and C) and standard microscopy (B and D). Values are normalized by use of the upper and the lower best-fit values as 100 and 0% responses, respectively, and are plotted as the means ± standard errors of the means of three replicates for the TryR-based assay and two replicates for microscopy. Two independent determinations were performed with both methods run in parallel, and representative data from one of the two experiments are shown.
Table 1. Comparison of IC$_{50}$ and IC$_{90}$ values of four anti-leishmanial reference drugs against *L. donovani* and *L. major* promastigotes, as measured by the TryR-based assay and the alamarBlue method.

| Reference drugs     | IC$_{50}$ (µM)$^a$ | IC$_{90}$ (µM)$^a$ |
|---------------------|--------------------|--------------------|
|                     | TryR-based assay   | alamarBlue method  | TryR-based assay   | alamarBlue method  | TryR-based assay   | alamarBlue method  | TryR-based assay   | alamarBlue method  |
| Amphotericin B      | 0.133 ± 0.0410     | 0.135 ± 0.0475     | 0.143 ± 0.0647     | 0.140 ± 0.0671     | 0.227 ± 0.00735    | 0.195 ± 0.0443     | 1.03 ± 0.839       | 1.02 ± 0.844       |
| Miltefosine         | 10.6 ± 6.16        | 11.8 ± 8.58        | 6.70 ± 1.07        | 4.24 ± 1.02        | 16.4 ± 9.10        | 23.6 ± 15.15       | 13.7 ± 0.420       | 8.52 ± 0.0585      |
| Pentamidine         | 0.836 ± 0.159      | 1.64 ± 1.11        | 5.60 ± 1.63        | 6.51 ± 3.75        | 7.53 ± 1.44        | 5.12 ± 0.396       | 9.21 ± 0.135       | 18.2 ± 12.6        |
| Paromomycin         | 161 ± 32.4         | 123 ± 18.7         | 123 ± 53.6         | 95.3 ± 84.7        | 292 ± 115          | 343 ± 46.2         | 581 ± 278          | 218 ± 33.0         |

$^a$ Values are shown with three significant digits and are the means ± standard errors of the means from two independent parallel determinations run in triplicate.

Table 2. Comparison of IC$_{50}$ and IC$_{90}$ values of four anti-leishmanial reference drugs against *L. donovani* and *L. major* intracellular amastigotes, as measured by the TryR-based assay and light microscopy.

| Reference drugs     | IC$_{50}$ (µM)$^a$ | IC$_{90}$ (µM)$^a$ |
|---------------------|--------------------|--------------------|
|                     | TryR-based assay   | Microscopy         | TryR-based assay   | Microscopy         | TryR-based assay   | Microscopy         | TryR-based assay   | Microscopy         |
| Amphotericin B      | 0.0606 ± 0.0280    | 0.100 ± 0.0780     | 0.414 ± 0.275      | 0.010 ± 0.00150    | 0.363 ± 0.223      | 0.183 ± 0.124      | 0.763 ± 0.469      | 0.231 ± 0.177      |
| Miltefosine         | 0.760 ± 0.355      | 0.708 ± 0.115      | 1.01 ± 0.173       | 0.930 ± 0.0310     | 3.37 ± 1.26        | 1.80 ± 0.609       | 3.25 ± 0.380       | 5.20 ± 0.576       |
| Pentamidine         | 0.607 ± 0.0755     | 0.284 ± 0.237      | 5.91 ± 2.52        | 1.38 ± 0.344       | 5.46 ± 0.679       | 0.527 ± 0.102      | 204 ± 29.5         | 27.6 ± 10.5        |
| Paromomycin         | 59.1 ± 9.97        | 77.0 ± 35.1        | 46.2 ± 3.09        | 32.6 ± 0.656       | 334 ± 93.7         | 507 ± 249          | 297 ± 91.2         | 189 ± 110          |

$^a$ Values are shown with three significant digits and are the means ± standard errors of the means of two independent parallel determinations run in triplicate for the TryR-based assay and in duplicate for microscopy.
Table 3. Comparison of IC$_{50}$ and IC$_{90}$ values of test drugs against *L. donovani* intracellular amastigotes, as measured by the TryR-based assay and light microscopy.

| Test drugs | IC$_{50}$ (µM)$^a$ | IC$_{90}$ (µM)$^a$ | TryR-based assay | Microscopy | TryR-based assay | Microscopy |
|------------|-------------------|-------------------|-----------------|------------|-----------------|------------|
|            | *L. donovani* promastigotes | *L. donovani* promastigotes |                 |            |                 |            |
| Imipramine | 15.6 ± 2.84       | 18.5 ± 0.640      | 29.7 ± 1.97     | 27.9 ± 3.30 |
| Carmustine | 15.2              | 8.80              | 66.0            | 41.2       |
| H-89       | 7.30 ± 4.42       | 12.2 ± 2.33       | 75.2 ± 30.3     | 110 ± 21.0 |
| Metergoline| 8.18              | 17.1              | 22.0            | 41.4       |
| Naloxonazine| 5.67 ± 0.384     | 19.0 ± 5.97       | 27.9 ± 14.7     | 96.4 ± 21.3 |
| VUF 11852  | 29.2 ± 1.02       | 20.2 ± 11.3       | 63.1 ± 0.240    | 54.0 ± 40.6 |
| VUF 11854  | 25.6 ± 0.570      | 15.1 ± 1.59       | 59.9 ± 0.395    | 37.0 ± 12.7 |
| VUF 11856  | 17.3 ± 3.17       | 30.8 ± 10.1       | 40.4 ± 4.64     | 66.7 ± 18.3 |
| VUF 11857  | 15.0 ± 7.87       | 7.08 ± 0.870      | 104 ± 59.2      | 30.8 ± 19.8 |
| VUF 13577  | 9.29 ± 0.732      | 7.20 ± 0.147      | 20.0 ± 0.380    | 12.1 ± 2.82 |
| IOTA 0058  | 3.25 ± 0.0730     | 5.56 ± 1.26       | 18.7 ± 7.45     | 23.6 ± 14.2 |
| Quinacrine | 1.80 ± 0.365      | 0.522 ± 0.315     | 4.06 ± 1.74     | 4.15 ± 3.52 |
| Eflornithine| 13.0 ± 11.5       | 34.1 ± 14.6       | 127             | 176        |
| Nifurtimox | 23.9              | 23.9              | 38.3            | 37.9       |
| Suramin    | 319               | 404               | 1344            |            |

$^a$ Values are shown with three significant digits and are the means ± standard errors of the means of two independent parallel determinations run in triplicate for the TryR-based assay and in duplicate for microscopy. For carmustine, metergoline, nifurtimox and suramin, data obtained from one parallel determination run in triplicate for the TryR-based assay and in duplicate for microscopy are shown. For eflornithine, IC$_{90}$ values could be derived from one parallel determination, due to insufficient inhibition in one of the two tests. Insufficient inhibition for determining the IC$_{90}$ value was observed for suramin, too, when it was tested with the TryR-based assay only.

Figure 5. Analysis of agreement between the TryR-based assay and microscopy by a scatter plot (A) and a Bland-Altman plot (B) ($n = 34$ determinations). The mean difference of the Bland-Altman plot is -0.034. The upper and lower limits of agreement (mean ± 2SD) are 0.495 and -0.563, respectively.
target enzyme (T[S]₂ is membrane impermeable). The low affinity of T[S]₂ for the glutathione reductase guarantees minimal cross-reactivity with the mammalian counterpart, which mainly contributes to the signal yield with its other thiols. Under optimized assay conditions, the T[SH]₂-mediated reduction of DTNB proved to be linear for up to 6-h, depending on the initial parasite inoculum (Figure 2). Whereas for promastigote drug assays, the reaction was fast enough to enable immediate detection, more extended reaction times were required with intracellular amastigotes. The main reason behind this kinetic variability is most likely the >50-fold difference in parasite density between the two models, although stage-specific expression levels of TryR cannot be excluded. Differential rates of TryR-mediated reduction were also observed between *L. donovani* and *L. major* promastigotes, though with little effect on drug activities, suggesting that assay output may vary from one species to another, without affecting compound potency estimates. This does not exclude, however, the possibility that assay optimization might be required when the test is implemented with other *Leishmania* species or in other laboratory facilities.

A collection of structurally and pharmacologically diverse compounds was used to validate the assay, which performed robustly with drugs that are effective in the micro- and nanomolar ranges (Table 1, 2 and 3). Reference anti-leishmanials exhibited highly correlated potencies when tested with the new TryR-based assay and a standard method (alamarBlue assay for promastigotes, microscopy for intracellular amastigotes) (Figures 3 and 4), which corroborated literature data. Statistical analysis confirmed the high correlation and good agreement that characterized the results obtained with the TryR-based assay and microscopy, suggesting their interchangeability as drug screening methods. In agreement with previously published data, though not extensively addressed in the present study, was the observation that pentamidine and miltefosine exhibited differential effects toward different parasite species (*L. donovani* vs. *L. major*) and developmental stages (promastigote vs. amastigote), respectively. Comparable activities were also observed with the other tested compounds, including imipramine and carmustine, for which inhibitory activity toward TryR has been abundantly reported. It should be noted, however, that neither of the two drugs specifically targets the trypanosomatid enzyme, supporting the possibility that other mechanisms, as already reported in the literature, may have equally contributed to the mild toxicity displayed against the parasite (IC₅₀ = 15.6 µM and 12.6 µM, respectively) and its host cells. TryR-specific inhibitors with reduced toxicity for the mammalian cells should be tested to exclude possible interference with the assay, but at present, only a few compounds with the required features have been identified and their access remains limited.

Compounds devoid of solubility problems and exhibiting complete dose-response curves at the tested concentrations yielded consistent IC₅₀ when assayed with the TryR-based test (but not with microscopy), as confirmed by meeting of the statistical parameters (MR, MSR and LoA). Conversely, less reproducible potency estimates were obtained for drugs showing poor or excessive inhibition, possibly resulting from inaccuracies related to either poor IC₅₀ curve fitting or initial parasite loads rather than from the assay itself. As for most cytotoxicity assays, determination of IC₅₀ by the TryR-based assay requires only an intra-experiment comparison of
enzyme activities between the test samples and the controls, enabling the signals to be corrected for inter-test variability. Nevertheless, differences in parasite growth can result in substantial effects on the IC$_{50}$s of some compounds, as previously described $^{38,39}$. The cellular model used for validation of the TryR-based assay requires the *Leishmania* promastigotes to actively invade the macrophages and, upon conversion to amastigotes, proliferate therein. Though standardized in every step, the model is rather sensitive to variables like inoculum size, developmental stage, and handling procedures, each of which may influence the parasite growth rate. This was particularly true of *L. donovani* strain 1S, whose tendency to grow at 37°C intracellularly but also extracellularly resulted in variable and at time inadequate assay windows. However, when the recently isolated strain BHU814, for which much-improved infectivity rates were observed was used, the Z' values ranged from 0.50 to 0.65, indicating that robust performance of the assay to fulfill single-dose HTS requirements partly relies on the choice of appropriate *Leishmania* strains. Gene expression profiling of antimony-susceptible and -resistant strains recently highlighted important differences in the thiol metabolism of these isolates, with an overall, though at times inconsistent, overexpression of thiol metabolizing enzymes among antimony-resistant parasites $^{40-43}$. While this might have contributed to the improved signal window yielded by the strain BHU814 (TryR overexpression, however, is yet to be demonstrated in this resistant strain), it is unlikely to interfere with drug activity assessment if the strains stably overexpress the TryR. Further evaluation with biochemically-defined antimony-resistant and -sensitive isolates should be performed to exclude falsely enhanced IC$_{50}$ values as a result of increased enzymatic activity.

Equally suitable for *Leishmania* promastigotes, albeit less convenient than existing methods, the TryR-based assay described here represents a significant advancement over the current techniques in assessing drug susceptibility at the intracellular amastigote stage, particularly in clinical isolates. Unlike standard microscopy, which remains the traditional screening method, the newly developed assay is fast, accurate and operator independent. Time-wise, the benefit is remarkable: while microscopic assessment of a duplicate 8-point drug dilution series requires a minimum of 1-h examination for a single compound, as many as 20 compound dilution series may be analyzed by the TryR-based assay in the same timeframe (excluding the incubation step with the TryR reagents). The suitability of the assay for HTS applications, as confirmed by compliance with widely accepted statistical parameters rather than by actual use as high-throughput assay (prevented by the lengthy performance of the reference method) makes it a valuable tool, along with other (semi)automated techniques (microscopy and reporter-gene assays), compared to which it offers greater simplicity and applicability. Implementable in virtually every culture laboratory where a microplate reader is available, the test can be applied to both laboratory and field isolates, with no need for genetic manipulation, enabling drug susceptibility testing to be promptly and extensively performed. Its lowest detection limit makes it as sensitive as other *in vitro* screening methods $^{7,8,11,19}$ but less powerful than some *Leishmania* recombinants for which very low sensitivities (up to 10 promastigotes) have been described $^{20}$. Further limitations of the TryR-based assay include the relatively high costs associated with the use of the substrate T[S]$_2$ and the lack of morphological visualization, which characterizes the microscopic assessment. 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. Interestingly, recognition of the trypanothione pathway in other trypanosomatids, like *Trypanosoma cruzi*, whose life cycle also encompasses an intracellular stage, provide potential new applications to the assay for improving the search of new anti-trypanosomatid agents.

In conclusion, this report demonstrates the feasibility of a simple, rapid TryR-based assay for use in high-throughput drug screening against *Leishmania* intracellular amastigotes. Further optimization and implementation are required to determine the ultimate role of this method in anti-leishmanial drug discovery and establish its suitability for drug susceptibility testing.

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Phagocytosis of hemozoin by RAW 264.7 cells, but not THP-1 cells, promotes infection by *Leishmania donovani* with a nitric oxide-independent mechanism

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Abstract

During its intra-erythrocytic development, the malaria parasite *Plasmodium falciparum* synthesizes insoluble hemozoin (HZ) crystals that are released into the circulation upon rupture of parasitized red blood cells, and rapidly phagocytized by host mononuclear cells. Here, HZ persists undigested, causing functional impairment and possibly leading to increased host susceptibility to secondary infections. In patients with malaria and visceral leishmaniasis (VL) co-infections, HZ-loaded macrophages are likely to co-harbor *Leishmania donovani* parasites, but whether this might influence the course of the *Leishmania* infection is unknown. In this study, *L. donovani* amastigote growth was monitored in mouse RAW 264.7 macrophages and PMA-differentiated THP-1 cells previously exposed to increasing amounts of HZ or its synthetic analogue β-hematin (BH). Latex beads were used as a phagocytic control. Data demonstrate that phagocytosis of HZ and BH by RAW 264.7 cells promoted infection therein by *L. donovani* parasites in a dose-dependent fashion. Similar results were not observed when using THP-1 cells, despite a clear persistence of undigested heme up to 48-h after phagocytosis.

Conditioning with lipopolysaccharide (LPS)/interferon (IFN)-γ prior to *Leishmania* infection triggered the release in RAW 264.7 cells of nitric oxide (NO), a highly leishmanicidal metabolite. However, neither HZ nor BH pre-ingestion were able to inhibit NO production following stimulation with LPS/IFN-γ, suggesting that the HZ- and BH-promoting effect on *L. donovani* infection occurred with an NO-independent mechanism. In conclusion, these preliminary findings highlight a possible detrimental effect of HZ on the course of VL, warranting further investigation into the clinical relevance of the current models.

**Keywords**
Hemozoin, *Leishmania donovani*, *Plasmodium falciparum*, Macrophage, Co-infection, Nitric oxide.
Introduction

Immunosuppression is a major hallmark of *Plasmodium falciparum* malaria. Its effects range from an impaired response to certain vaccines to an increased susceptibility for bacterial (most notably Gram negative bacteria), viral and protozoal infections. Data on all-cause child mortality across Sub-Saharan Africa suggest that the indirect burden of malaria, largely caused by its secondary infections, may equal the direct burden of disease with young children bearing the brunt due to their immunological immaturity. Interestingly, whilst immunopathology of severe malaria predominantly arises from excessive host inflammation, the risk of developing malaria co-morbidities mainly relies on its immunosuppressive effects.

To date, no single mechanism can explain the immunomodulating effects of malaria completely, but several parasite-driven abnormalities in the innate immune function have been shown to compromise host ability to clear infections. Phagocytic cell functions, in particular, were found to be deranged by the ingestion of malaria pigment (hemozoin, HZ), an insoluble polymer of β-hematin crystallites produced by the malaria parasite to dispose of toxic free heme. While growing inside the red blood cells (RBCs), *P. falciparum* digests considerable amounts of host cell hemoglobin, releasing free heme that accumulates in the food vacuole upon conversion to HZ. At schizont burst, the pigment is expelled into the blood stream where it is avidly phagocytized, along with HZ-containing late parasite stages (trophozoites and schizonts), by circulating and resident phagocytes (granulocytes, monocytes and macrophages). Evidences of HZ accumulation in the microvasculature of deep organs and their tissues (spleen, liver, bone marrow, kidney, lung and brain) confirm that the malaria pigment persists undigested within the host leukocytes, where it exerts a variety of biological effects. Hemozoin-loaded monocytes, in particular, appear to be viable, but functionally impaired, failing to repeat phagocytosis, to generate oxidative burst upon appropriate stimulation, or to kill ingested bacteria, fungi or tumor cells. In addition, ingestion of HZ causes the monocytes/macrophages to release large amounts of cytokines (interleukin (IL)-1β, IL-12, tumor necrosis factor (TNF)-α, and IL-10), chemokines (macrophage inflammatory protein (MIP)-1α and MIP-1β) and nitric oxide, while altering membrane translocation and activity of protein kinase C (PKC), major histocompatibility complex (MHC) class II-dependent antigen presentation and differentiation and maturation to monocyte-derived dendritic cells (DCs). Whether those effects are caused by the HZ itself, however, remains controversial, as recent studies suggest that other HZ-associated molecules, such as *Plasmodium* DNA, host fibrinogen or RBC lipids, may be responsible for the immunomodulating activity of the malaria pigment.

Pathogenic manipulation of the host immune system is not unique to *P. falciparum* infection; on the contrary, it is a common feature of several microbial survival strategies. In some cases, as for the macrophage-tropic parasites of the *Leishmania donovani* complex, the pathogen has evolved mechanisms that enable it to resist intracellular destruction and to proliferate within the reticuloendothelial system. Following transmission to the vertebrate host, *L. donovani* promastigotes...
are largely engulfed by macrophages of the inner organs (spleen, liver and bone marrow especially), where they differentiate into obligate intracellular amastigotes that reside within the phagolysosomal compartment. Here, *Leishmania* parasites disrupt several key functions involved in the microbicidal activities of macrophages, including their ability to produce NO and radical oxygen species (ROS), to perform proteolytic digestion and to activate adaptive immunity via antigen presentation and cytokine release. This suggests the possibility that subversion of macrophage functions by other pathogens or related products, such as the *Plasmodium* HZ, might benefit the survival of *Leishmania* parasites.

In areas where malaria and visceral leishmaniasis (VL) are co-endemic, co-infections with the two pathogens have been documented. However, little is known on how the two infections impact each other in the co-infected host. Early studies conducted in animal models have produced conflicting results on the effect of the two diseases upon each other, with mice suffering from an exacerbated leishmanial disease course and golden hamsters remaining unaffected. In particular, doubts remain as whether HZ accumulation in the macrophages may affect *L. donovani* growth therein. The present study represents the first attempt to address this question. In this experimental setting, human and murine macrophage-like cells were allowed to phagocytize increasing amounts of *P. falciparum* native HZ and its synthetic form (β-hematin, BH), prior to being infected with *L. donovani* promastigotes. After removal of non-internalized parasites, intracellular amastigote growth was monitored using the previously described trypanothione reductase (TryR)-based assay. The possible involvement of the NO pathway was assessed by stimulating the cells with lipopolysaccharide (LPS)/ interferon (IFN)-γ, and by measuring the levels of nitrites/nitrates in the corresponding supernatants. Finally, persistence of HZ and BH heme was monitored over time in phorbol 12-myristate 13-acetate (PMA)-differentiated THP-1 cells and compared to a control of anti-Rh(D) immunoglobuline G (anti-D IgG) treated RBCs.

**Materials and Methods**

**Cell and parasite cultures**

The murine RAW 264.7 macrophage cell line (ATCC: TIB-71) and the human THP-1 monocyte cell line (ATCC: TIB 202) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma Aldrich Co., St Louis, USA), HEPES 25 mM, L-glutamine 2 mM, penicillin 100 IU/mL and streptomycin 100 µg/mL, in a 5% CO₂ atmosphere at 37°C. Prior to each experiment, cells were subcultured once in IMDM medium supplemented with 10% heat-inactivated FBS, HEPES 25 mM, L-glutamine 2 mM, penicillin 100 IU/mL and streptomycin 100 µg/mL. *In vitro* cultures of *P. falciparum* were adapted from Trager and Jensen. Briefly, the chloroquine-sensitive *P. falciparum* strains NF54 and 3D7 (MR4/ATCC: MRA-1000 and MRA-102) were grown at 5% hematocrit (0+ human erythrocytes) in RPMI buffered medium supplemented with 10% heat-inactivated FBS, HEPES 25 mM, L-glutamine 2 mM, penicillin 100 IU/mL and streptomycin 100 µg/mL. Hematocrit (0+ human erythrocytes) in RPMI buffered medium supplemented with 10% heat-inactivated human AB+ serum (Sanquin, Amsterdam, the Netherlands) and 50 µg/mL of gentamycin. Cultures were maintained at 37°C in candle jars under continuous agitation at 40 rpm/min and with daily refreshment of medium. When parasitaemia exceeded 7-8%, sub-cultures were made.
The antimonial-resistant *L. donovani* strain MHOM/IN/2010/BHU814, isolated by Prof. S. Sundar at the Banaras Hindu University, Varanasi, India, was kindly provided by Prof. J. C. Dujardin, Institute of Tropical Medicine, Antwerp, Belgium. Promastigotes were maintained at 27°C with a weekly passage in RPMI 1640 medium supplemented with 15% FBS, HEPES 25 mM, L-glutamine 2 mM and 1% antibiotics. For infection of RAW 264.7 and THP-1 cells, promastigotes were diluted in IMDM medium supplemented with 10% heat-inactivated FBS, HEPES 25 mM, L-glutamine 2 mM, penicillin 100 IU/mL and streptomycin 100 µg/mL. All cell culture reagents were purchased from Gibco (Bleiswijk, the Netherlands), unless otherwise stated.

**Preparation of native HZ and synthetic BH**

For isolation of native HZ, mature-stage *P. falciparum* cultures were washed three times with serum-free RPMI-buffered medium and fractionated at 25% hematocrit on a discontinuous Percoll (GE Healthcare, Uppsala, Sweden)/4% sorbitol (wt/vol) gradient (0, 40, 80%). After centrifugation at 2500 rpm, HZ was collected at the top of the gradient, 0–40% interphase, washed three times with phosphate-buffered saline (PBS) and stored at -20°C. No further purification of HZ was undertaken to prevent removal of adherent molecules, such as membranes necessary for HZ opsonization and phagocytosis or *P. falciparum* DNA, involved in immunomodulation. Synthetic malaria pigment was donated by Prof. D. Taramelli, University of Milan, Italy. β-hematin was synthesized from a solution of hematin precipitated in acidic conditions as previously described, and its purity was confirmed by infrared spectroscopy. The heme content of a weighed amount of HZ and BH dissolved in 1 M NaOH was determined against a standard curve of hemin (Sigma-Aldrich Co.), by reading its absorbance at 405 nm (Soret band) with an Infinite M200Pro multimode plate reader (Tecan, Männedorf, Switzerland).

**Phagocytic meals**

Hemozoin and BH along with latex beads or RBCs as controls were used as phagocytic meals. Latex beads served as phagocytic control when assessing the effects of HZ and BH on *Leishmania* intracellular growth and NO production, whereas RBCs were used as a control for the chemiluminescent assay measuring phagocytosis of heme and its persistence over time. The concentrations of HZ and BH used in the experiments were determined according to their heme content, while RBCs and latex beads were added in equivalent amounts, based on the assumption that one RBC contains approximately 2 fmol of heme. Immediately before the phagocytosis experiment, washed HZ, BH and latex beads were sonicated and opsonized with human plasma at 37°C, 5% CO₂ for 30 min to enable binding to plasmatic fibrinogen. Thereafter, they were diluted to 10% hematocrit (or 10% vol/vol in the case of latex beads) in PBS supplemented with glucose 10 mM (PBS-G) and added to the cells. Washed uninfected RBCs were suspended at 20% hematocrit in PBS-G and incubated for 30 min at 37°C, under a 5% CO₂ atmosphere with human anti-D IgGs according to the manufacturer’s instructions (AbD Serotec, Oxford, UK). Afterwards, RBCs were washed three times with PBS-G, re-suspended at 10% hematocrit in PBS-G and kept at 4°C until used.

**Cell treatment**

THP-1 monocytes (2.5 X 10⁵ cells/mL) were differentiated into adherent, non-dividing macro-phage-like cells by a 72-h incubation...
with 10 ng/mL of PMA (Sigma-Aldrich Co.), subsequently removed by three washes in culture medium. On day three of THP-1 differentiation, RAW 264.7 cells were seeded at 5 X 10^5 cells/mL in 96-well flat bottom tissue culture microplates (Greiner Bio-One, Alphen aan de Rijn, the Netherlands) and allowed to adhere for 4–5 h at 37°C, 5% CO₂. Pre-treatment with phagocytic meals was performed by incubating adherent THP-1 and RAW 264.7 cell monolayers overnight (unless otherwise stated) at 37°C, 5% CO₂, with increasing amounts of opsonized HZ, BH or latex beads or with culture medium as a control. After removal of non-ingested material by washing once briefly with water and twice with medium, part of the cells were stimulated for 8 hours with 1 µg/mL of LPS (from *E. coli* 0111:B4, Sigma-Aldrich Co.) and 100 IU/mL of human (Sigma-Aldrich Co.) or mouse (BioLegend, San Diego, USA) recombinant IFN-γ, prior to being incubated overnight with stationary-growth phase *L. donovani* promastigotes at a parasite/cell ratio of 10/1. Non-internalized promastigotes were then removed by washing cells three times in excess of medium and the cultures were re-incubated at 37°C, 5% CO₂ for an additional 72 h. A number of non-infected control cells treated as described above was also included in each experiment for both cell lines. In all experiments, RPMI 1640 medium (used for long-term culturing) was replaced by IMDM medium, due to incompatibility of the former with NO measurements (RPMI-1640 medium contains large amounts of nitrites/nitrates).

**Assessment of Leishmania growth**

The growth of *L. donovani* parasites in THP-1 and RAW 264.7 cells was assessed by measuring the activity of a parasite-specific enzyme, the TryR, as previously described.²⁴ Briefly, after a pre-wash with PBS, cells were chemically lysed by a 15 min incubation with a lysing buffer (200 µL/well), consisting of EDTA (1 mM), HEPES (40 mM), Tris (50 mM; pH 7.5) and Triton X-100 (2% vol/vol) (BDH Laboratory Supplies, Pool, UK), and supplemented immediately prior to use with the protease inhibitor phenylmethanesulfonyl fluoride (1 mM). Enzymatic reduction of trypanothione-disulfide (T[S]₂) (Bachem AG, Bubendorf, Switzerland) was monitored in a 200 µL reaction mixture consisting of 75 µL of sample lysate, 25 µL of NADPH 1.6 mM (Sigma-Aldrich Co.), 75 µL of T[S]₂ 200 µM and 25 µL of DTNB 800 µM (Sigma-Aldrich Co.). A blank was set for each sample, consisting of sample lysate supplemented with the reaction mixture described above, in which the substrate T[S]₂ had been replaced by Tris 0.05 M buffer, pH 7.5. After 2-h of incubation for the RAW 264.7 cells and 3-h of incubation for the THP-1 cells at 27°C, absorbance was measured with an Infinite M200Pro multimode plate reader at a wavelength of 412 nm. The optical density, as measured in the blank, was subtracted from the corresponding sample signal, yielding the enzyme-specific activity responsible for reduction of the DTNB.

**Microscopy**

Microscopic examinations were performed with an optical microscope (Leitz, Wetzlar, Germany), using a 10X ocular and a 100X oil-immersion objective. Cells were plated onto 16-well or 4-well chamber slides (Lab-Tek, Nunc, Waltham, USA) at the same concentrations as for the culture plates, and treated as described above. After 72-h of incubation with Leishmania parasites or culture medium (for uninfected cells), cell supernatants were discarded and the slides were subjected to methanol fixation and Field’ staining. Pictures were acquired with a MikroCam microscope camera (Bresser
Effect of HZ ingestion on *L. donovani* infection

Microscopic counts of parasites were expressed by mean of the parasite index (percentage of infected macrophages × mean number of amastigotes per macrophage), after examining a minimum of 100 macrophages per sample in two replicate samples. The average number of cells containing HZ, BH or latex beads was assessed on the same slides, after examining a minimum of 100 macrophages per sample in the two replicate samples.

**Assessment of cell viability**

Viability of cells exposed to the various phagocytic meals was measured by monitoring the reduction of the alamarBlue reagent (AbD Serotec). In this assay, the non-fluorescent resazurin dye contained in the alamarBlue reagent is reduced by the activity of viable cells, resulting into a highly fluorescent product (resorufin) that serves as an indicator of cell growth. The assay can also be used to monitor cytotoxicity, as non-viable cells are unable to convert resazurin into resorufin following loss of their enzymatic activity, and they can be easily distinguished from living cells. Uninfected RAW 264.7 and PMA-differentiated THP-1 cells exposed to increasing amounts of opsonized HZ, BH or latex beads, or to culture medium as a control, in the presence or absence of LPS/IFN-γ, were incubated with the alamarBlue reagent (10% vol/vol) at 37°C, 5% CO₂. After 2-h of incubation, fluorescence was measured with an Infinite M200Pro multimode plate reader, with excitation and emission wavelengths of 560 and 590 nm, respectively, and a plate-tailored optimal gain setting. Measurements were taken on the same day that the TryR-based assay was performed to assess the growth of *Leishmania* in infected cells. Viability of infected cells was not determined, as the *Leishmania* parasites contribute to reduction of resazurin, thereby interfering with the assay.

**Nitric oxide measurement in cell supernatants**

Nitric oxide determination in cell supernatants was based on quantification of total nitrite and nitrate contents by the Griess reaction, performed according to manufacturer’s instructions (Assay Designs, Inc., Ann Arbor, USA). Briefly, aliquots of (pre-diluted) cell-free supernatants were diluted with NADH (25 µL) and nitrate reductase (25 µL) for conversion of nitrates into nitrites. Samples were then incubated with 50 µL of 2% sulfanilamide and 0.2% N-(1-Naphthyl)ethylenediamine in hydrochloric acid 2M, at room temperature, for 10 min. Total nitrite concentration was determined against a standard curve by measuring the optical density at 540 nm with an Infinite M200Pro multimode plate reader.

**Assessment of HZ and BH phagocytosis**

Phagocytosis of HZ and BH by the adherent cells was quantified by measuring the chemiluminescence generated by the peroxidase activity of the heme. At alkaline pH, heme catalyzes the production of chemiluminescence by luminol and tert-butylhydroperoxide in amounts that are proportional to the heme concentration. For this purpose, cells were incubated for 3 hours with anti-D IgG treated RBCs as a control (30 RBCs/cell) or with opsonized HZ or BH in equivalent amounts, as determined by their heme content. After removal of non-internalized material, cells were chemically lysed and assessed for their chemiluminescent signal (time point 0-h) or re-incubated for later assessments (15, 24 or 48-h). The chemiluminescent assay was performed as previously described, with some modifications. Briefly, adherent cells were lysed in 100 µL NaOH 0.1 N containing 0.05% (vol/vol) Triton X-100 and EDTA 3mM. The lysate
was then assayed in 96-well flat bottom white plates (Greiner Bio-One) containing 100 µL luminol (Fluka, Sigma-Aldrich Co.) 1 mg/mL, prepared by diluting a 10 mg/mL stock solution of luminol in DMSO with NaOH 0.1 N/EDTA 3 mM. Chemiluminescence was elicited by injecting 100 µL of a tert-butylhydroperoxide (Sigma-Aldrich Co.)/EDTA solution (80% tert-butylhydroperoxide in di-tert-butyl peroxide/water 3:2 diluted 2.2 times in 0.1 N NaOH/EDTA 3 mM), which triggered photon emission and counting. Photon counting was performed with an Infinite M200Pro multimode plate reader, using an integrated photon counting time set at 2000 milliseconds. Photon counts per cell were transformed into a number of RBCs ingested per cell (RBC equivalents) by comparison with the signal produced by a known amount of heme contained in the HZ and BH samples. Each phagocytosis value represents the average of data obtained from three different wells.

**Data analysis**

Data analysis was performed using Microsoft Excel (Microsoft Office Inc., Seattle, USA) and Prism V5.03 software (GraphPad Prism, Software Inc., San Diego, USA). Graphically displayed data were examined by mean of visual analysis only, as the sample size was too small for any meaningful statistical analysis.

**Results**

**Phagocytosis of HZ, BH and latex beads**

Exposure of RAW 264.7 and PMA-differentiated THP-1 cells to increasing amounts of opsonized HZ, BH or latex beads resulted in the internalization of these three phagocytic meals, as confirmed by the microscopic (Figure 1A) and chemiluminescent (Figure 1B) assessments performed 96-h later. Cells displaying HZ crystals were more numerous than those containing BH – a result that can be easily explained by the tendency of BH to aggregate and form bigger clusters – but the amount of heme persisting in the sample, albeit dose-dependent in the case of HZ, did not always exceed the one in BH-laden cells (Figure 1B). Latex beads were more avidly phagocytized than were HZ and BH, as judged by the number of macrophages containing this phagocytic meal, but the overall number of beads in the sample was not quantified.

**Phagocytosis of HZ and BH promotes growth of L. donovani amastigotes in RAW 264.7 cells, but not in THP-1 cells**

To assess the potential effect of malaria pigment ingestion on *L. donovani* growth, RAW 264.7 and PMA-differentiated THP-1 cells were incubated with increasing amounts of opsonized HZ, BH or latex beads prior to being infected with *L. donovani* promastigotes. Intra-macrophage amastigote growth was then assessed 72-h later, by measuring the activity of TryR. This enzyme acts as a good indicator of *Leishmania* growth, as demonstrated by the high level of correlation between enzymatic activities and microscopic counts in infected THP-1 cells (Figure 2). For infected RAW 264.7 cells, a similar dose-dependent curve was obtained when plotting the TryR activities against the number of seeded host cells (data not shown), but the enzymatic activities could not be related to the microscopic counts, as the different seeding densities resulted in different cell growth rates that disproportionally affected the parasite indexes – these indexes express the relative, rather than absolute, parasite loads and are directly influenced by the total number of host cells in the sample.
Effect of HZ ingestion on *L. donovani* infection

Figure 1. (A) *L. donovani*-infected RAW 264.7 cells (a-c) and PMA-differentiated THP-1 cells (d-f) pre-exposed to HZ (a, d), BH (b, e) or latex beads (c, f). Cells were allowed to phagocyte 50 μg/mL of HZ, BH or an equivalent amount of latex beads overnight, prior to be washed and infected with *L. donovani* BHU814. After 72-h of incubation, microscopy slides were methanol-fixed, Field’-stained and examined with an oil immersion microscope (1000X magnification). The percentage of cells containing HZ, BH or latex beads was assessed after examining a minimum of 100 macrophages per sample in two replicate samples and the corresponding average values are indicated in the box at the top of each image (a-f). (B) Phagocytosis of HZ and BH by RAW 264.7 and PMA-differentiated THP-1 cells. Cells were incubated overnight with increasing amounts of HZ or BH, prior to be washed and re-incubated with culture medium. Phagocytosis was assessed 96-h later (corresponding to the moment in which *Leishmania* growth was measured in infected cells) by measuring the chemiluminescence generated by the heme moieties. Data were calculated as the number of RBC equivalents (each RBC equivalent corresponds to 2 fmol of heme) ingested in each microplate well and plotted as the means of three replicates. Data refer to one of the two experiments shown in Figures 3-5.
Phagocytosis of HZ and BH by RAW 264.7 cells substantially increased the uptake and/

or intracellular replication of *L. donovani*, as confirmed by its enhanced enzymatic activity in cells pre-exposed to HZ and BH vs. the control infected cells (Figure 3). The effect followed a dose-dependent course and became more pronounced at the concentrations of 25 and 50 µg/mL. Conversely, ingestion of latex beads by the RAW 264.7 cells resulted in a slight or no alteration of *L. donovani* loads in comparison with the control infected cells. When PMA-differentiated THP-1 cells were pre-treated with HZ, BH or latex beads, no clear effect was observed on parasite growth, for which comparable signals to the control infected cells were displayed (Figure 3).

Activation of macrophages by LPS/IFN-γ following exposure to phagocytic meals partly inhibited the parasite-promoting effect displayed by HZ and BH, leading to a dose-dependent, but highly variable improvement of *Leishmania* growth in RAW 264.7 cells containing the malaria pigment (Figure 3). Addition of LPS/IFN-γ to THP-1 cells resulted in no clear pattern on the *L. donovani* loads in cells pre-treated with phagocytic meals, despite an overall, but considerably variable increase in parasite numbers per effect of HZ (Figure 3).

**Phagocytosis of HZ, BH or latex beads does not affect cell viability**

To exclude that HZ- and BH-laden cells may have suffered from potential cytotoxic effects upon internalization of the malaria pigment, viability of RAW 264.7 and PMA-differentiated THP-1 cells was investigated. Overall, phagocytosis of HZ, BH or latex beads appeared to neither reduce nor improve survival of macrophage-like cells as compared to the control cells, regardless of whether cells were subsequently treated with LPS/IFN-γ or not (Figure 4), indicating that in our experiments, none of the phagocytic meals was cytotoxic. Notwithstanding, a dose-dependent, but highly variable increase in resazurin reduction rate was evident for RAW 264.7 cells exposed to opsonized HZ, BH or latex beads, suggesting either an increased anti-oxidant capacity triggered by the phagocytic process or, more likely, a delay in cell apoptosis.
Effect of HZ ingestion on \textit{L. donovani} infection

![Figure 3](image)

**Figure 3.** Relative load of \textit{L. donovani} BHU814 amastigotes in cells pre-exposed to increasing doses of HZ, BH or latex beads, in the presence or absence of LPS/IFN-γ. RAW 264.7 and PMA-differentiated THP-1 cells were allowed to phagocytize increasing amounts of HZ, BH or latex beads overnight, prior to be washed and stimulated for 8-h with culture medium or LPS/IFN-γ. Thereafter, cells were infected with \textit{L. donovani} promastigotes at a ratio of 10 to 1. After an overnight incubation, non-internalized parasites were washed away and cells were re-incubated for an additional 72-h, prior to be measured by the TryR-based assay. Data were calculated as the percentage of TryR activity in the treated sample vs. the untreated control and plotted as the means ± standard error of the means (SEM) of two independent experiments, each one performed in triplicate.

**HZ and BH promote infection by \textit{L. donovani} with an NO-independent mechanism**

Nitric oxide is a major mammalian secretory product involved in the microbicidal activity of macrophages against a number of intracellular pathogens, including \textit{Leishmania} parasites. Its release from LPS- and/or IFN-γ-stimulated macrophages has been shown to be impaired or potentiated, respectively, following ingestion of malaria pigment by immortalized or primary murine macrophages,\textsuperscript{28,55,56} supporting a possible NO-mediated mechanism for the promoting effect displayed by HZ on \textit{L. donovani} growth. To verify this hypothesis, the total content of two stable NO breakdown products, nitrites and nitrates, was measured in cell supernatants, providing an indirect assessment of NO interaction in the biological system.

Infection of RAW 264.7 and PMA-differentiated THP-1 cells by \textit{L. donovani} did not trigger release of NO, as confirmed by the comparable amounts of nitrites and nitrates detected in the supernatant of uninfected and infected cells (Table 1). Similarly, pre-exposure of both RAW 264.7 and PMA-differentiated THP-1 cells to HZ, BH or latex beads did not alter cellular NO production either in \textit{L. donovani}-infected
cells (Figure 5) or in uninfected cells (data not shown). Activation of cells by LPS/IFN-γ resulted in considerable differences between RAW 264.7 and PMA-differentiated THP-1 cells. Whilst the latter showed no change in the supernatant level of nitrites and nitrates following addition of LPS/IFN-γ (Table 1), neither after ingestion of phagocytic meals nor in the presence of \textit{L. donovani} parasites (Figure 5, Table 1), in line with previous reports\textsuperscript{57-59} but in contrast with others.\textsuperscript{60,61} RAW 264.7 cells markedly up-regulated NO release in response to the immunomodulating agents (Table 1). This increased production of NO did not affect infection by \textit{L. donovani}, for which comparable, although highly variable, parasite loads were observed in unstimulated and LPS/IFN-γ-stimulated RAW 264.7 cells (Table 1). Conversely, consistently decreased parasite loads were observed in LPS/IFN-γ-stimulated THP-1 cells (Table 1). Cell exposure to HZ, BH or latex beads prior to LPS/IFN-γ stimulation did not reduce NO release from RAW 264.7 cells neither in uninfected nor in \textit{L. donovani}-infected cells. On the contrary, it displayed a slightly stimulating effect, which was evident with latex beads and, to a lesser extent, with BH (Figure 5).

Figure 4. Viability of uninfected cells exposed to increasing concentrations of HZ, BH or latex beads, in the presence or absence of LPS/IFN-γ. RAW 264.7 and PMA-differentiated THP-1 cells were incubated overnight with increasing amounts of HZ, BH or latex beads, prior to be washed and stimulated for 8-h with culture medium or LPS/IFN-γ. Thereafter, cells were re-incubated with culture medium for an additional 96-h, after which reduction of the alamarBlue reagent was measured (corresponding to the moment in which \textit{Leishmania} growth was assessed in infected cells). Data were calculated as the percentage of resazurin reduction in the treated sample vs. the untreated control and plotted as the means ± standard error of the means (SEM) of two independent experiments, each one performed in triplicate.
Effect of HZ ingestion on *L. donovani* infection

Persistence of HZ and BH over time

To investigate whether the lack of effects displayed by HZ and BH in THP-1 cells might have been due to a reduced internalization of the malaria pigment and/or its degradation, cellular phagocytosis of HZ and BH was assessed over time in PMA-differentiated THP-1 cells, by measuring the chemiluminescence generated by the peroxidase activity of the heme group (Figure 6). Measurement of heme content immediately after removal of non-internalized material revealed that THP-1 cells avidly phagocytized BH as well as HZ, despite a 3-fold difference in the number of RBC equivalents per cell. The difference became particularly striking with respect to the anti-D IgG treated RBCs, for which less than 2 RBC equivalents/cell were measured. When measured over time, the heme contained in the HZ and BH showed to persist undigested within the cells, whereas the heme contained in the anti-D IgG treated RBCs was progressively degraded.

**Discussion**

The present study demonstrates that phagocytosis of native (HZ) and synthetic (BH)
malaria pigment by RAW 264.7 cells promotes infection therein by *L. donovani* with an NO-independent mechanism. This conclusion is based on the following observations: (a) ingestion of HZ and BH, but not latex beads, prior to *L. donovani* infection resulted in a dose-dependent increase in the number of amastigotes; (b) infection with *L. donovani* did not trigger NO release neither in control nor in HZ- and BH-fed cells; (c) LPS/IFN-γ pre-conditioning did not hamper *L. donovani* infection despite triggering NO release and (d) activation by LPS/IFN-γ partly inhibited the Leishmania-promoting effect displayed by HZ and BH, with no effect on the NO levels, as measured in the supernatant of LPS/IFN-γ-stimulated cells fed with HZ and BH vs. the unstimulated control cells. These results were not observed when PMA-differentiated human THP-1 cells were used instead of mouse RAW 264.7 macrophages, providing evidence of a species- and/or cell-specific effect.

Accumulation of HZ in host leukocytes and organs is a characteristic, though non-pathognomonic, feature of blood-stage malaria. Its sequestration within the mononuclear system begins upon rupture of parasitized RBCs and continues throughout multiple generations of phagocytes, leading to the aggregation of undigested HZ crystals from different phagosomes and the functional derangement

### Table 1. Nitric oxide (NO) release and relative burden of *L. donovani* BHU814 in RAW 264.7 and PMA-differentiated THP-1 cells, after stimulation with culture medium or LPS/IFN-γ.

|                  | NO release (µM) | % relative load of *L. donovani* |
|------------------|-----------------|-------------------------------|
|                  | medium          | LPS/IFN-γ                     | medium          | LPS/IFN-γ                     |
| **RAW 264.7 cells** |                 |                               |                 |                               |
| uninfected       | 1.00 ± 0.93     | 29.35 ± 2.98                  | 29.35 ± 2.98    |                               |
| *L. donovani*-infected | 0.71 ± 0.71     | 31.04 ± 2.12                  | 100%            | 90.03 ± 49.07%                |
| **THP-1 cells**  |                 |                               |                 |                               |
| uninfected       | 0.47 ± 0.46     | 0.40 ± 0.03                   | 0.40 ± 0.03     |                               |
| *L. donovani*-infected | 0.47 ± 0.46     | 0.42 ± 0.06                   | 100%            | 68.45 ± 0.02%                 |

Data are shown as the means ± standard error of the means (SEM) of two independent experiments, each one performed in triplicate.

**Figure 6.** Time-dependent course of heme digestion in PMA-differentiated THP-1 cells exposed to anti-D IgG treated RBCs, HZ or BH. Data were calculated as the number of RBC equivalents ingested per cell (each RBC equivalent corresponds to 2 fmol of heme) and plotted as the means ± standard error of the means (SEM) of two independent experiments, each one performed in triplicate. For anti-D IgG treated RBCs, data refer to a single experiment, performed in triplicate.
of their host cells. Accordingly, human monocytes fed with HZ failed to respond to IFN-γ stimulation, repeat the phagocytic cycle and kill ingested bacteria, fungi and tumor cells, raising the question that prompted this study, as whether HZ accumulation during malaria may alter the course of secondary infections. In this regard, associations between increased levels of HZ in pulmonary tissue and malaria-associated acute respiratory distress syndrome or between HZ and increased susceptibility of mice to Mycobacterium tuberculosis have been reported in the literature. Here, we provide evidence of a HZ- and BH-mediated enhancement of L. donovani infection load in RAW 264.7 cells. The effect in our study was shown to be dose-dependent and malaria pigment-specific, as phagocytosis of latex beads failed to trigger a similar outcome. Both HZ and BH exhibited Leishmania-promoting activities, but at equal content of heme, HZ was more effective in stimulating L. donovani growth. Multiple explanations may account for this observation, the most likely of which recognizes a cell-modulating activity to the non-hematin content of HZ (fibrinogen excluded, as both HZ and BH were plasma-opsonized). Addition of LPS/IFN-γ to the culturing medium resulted in the release by RAW 264.7 cells of large quantities of NO, the most powerful leishmanicidal agent produced by phagocytic cells. Intriguingly, production of this metabolite appeared not to affect L. donovani infection rates, in agreement with literature data reporting cross-resistance of Leishmania isolates to antimonial drugs and NO toxicity, but in contrast to the widely accepted notion that NO is detrimental to Leishmania survival. Additional testing examining the effect of macrophage activation before and after infection with Leishmania, using both susceptible and resistant strains, will be required to confirm this finding, particularly given the fact that highly variable infection rates were obtained in LPS/IFN-γ-stimulated control RAW 264.7 cells. When cells were pre-treated with HZ and BH prior to LPS/IFN-γ stimulation, an increased production of NO was evident, but the effect was neither significant nor pigment-specific, as phagocytosis of latex beads appeared to trigger a similar outcome. Whilst this HZ- and BH-sustained release of IFN-γ-inducible NO has been clearly demonstrated in a number of murine phagocytic cell lines, it finds no evidence in studies performed with mouse peritoneal macrophages, where a reduction in cytokine- and/or LPS-induced NO production was observed. This differential susceptibility of macrophages with different tissue origin has been attributed to the HZ-mediated induction of ROS, whose signaling pathway has been found to lead to ROS-dependent transcription factor up- or down-regulation in different cell types, according to their susceptibility to ROS.

To investigate whether degradation of the malaria pigment could have accounted for the lack of effects observed on L. donovani growth in THP-1 cells, the HZ and BH cell content was followed over time. In human monocytes, persistence of HZ results from inability of proteolytic enzymes to de-polymerize the HZ moiety and release free heme, while retaining full activity, as confirmed by the degradation of HZ-associated proteins. A similar scenario seemed to apply to our PMA-differentiated THP-1 cell model. When monitored up to 48-h after phagocytosis, in fact, these cells retained 36% and 100% of their initial HZ and BH heme content, respectively, whilst no RBC-derived heme could be detected 15-h post-incubation already. This partial decrease in the HZ-associated heme content is likely to be caused by degradation of HZ-bound heme proteins, given that the HZ used here was subjected to minimal purification, thus increasing the likelihood of...
a native-resembling pigment carrying proteins and RBC remnants. However, whether the differential phagocytic efficiency manifested by the cells towards the three meals may have partly biased the results is unclear, as cells were incubated with equal amounts of HZ, BH and RBCs, as established on the basis of their heme content, but appeared to have internalized as much as 26 and 3 times more BH than RBCs and HZ, respectively. Importantly, these findings on the intracellular stability of the malaria pigment appear to comply with the relative literature, showing persistence of undigested HZ within human monocytes, while they contrast with a previous report in which BH appeared to be degraded by RAW 264.7 macrophages.

In the present study, Leishmania growth was measured indirectly, through the activity of a parasite-specific enzyme, rather than by conventional microscopic counting, as visualization of Leishmania amastigotes was hindered by the presence of phagocytic meals in the host cells. This may cast doubt on whether the HZ- and BH-mediated increases in TryR activity truly arose from higher parasite burdens rather than from enzymatic up-regulation. Trypanothione reductase is constitutively expressed in Leishmania parasites. Its activity levels are not rate-limiting for the enzymatic regeneration of the thiol pool, which explains why overexpression of TryR does not alter the in vitro sensitivity of L. donovani to pro-oxidant agents, such as nifurtimox. Failure to amplify TryR in response to a number of compounds, including nifurtimox, was also evident in a previous study conducted by our group, wherein microscopy and the presently used TryR-based assay yielded comparable growth curves for the drug-sensitive L. donovani 1S strain. A linear correlation between TryR activities and parasite numbers as assessed by microscopy was also demonstrated here using a dilution series of differentiated THP-1 cells harboring L. donovani intracellular amastigotes. This supports the assumption that the increased TryR activities, as they were measured here, truly resulted from an improved growth of the antimonial-resistant strain L. donovani BHU814, with a rise in enzymatic activity likely resembling the increase in parasite numbers. It should be noted that increased levels of TryR have been described in L. donovani isolates unresponsive to sodium stibogluconate as compared with sensitive strains, but this feature appears to be part of a stable antimony resistance-associated phenotype, with little or no evidence of a transient TryR up-regulation in response to thiol depletors or TryR inhibitors. In any case, confirmation of increased parasite rates in HZ- and BH-fed cells using alternative assays, such as reporter gene-based methods, the promastigote transformation growth assay or quantitative PCR, would be beneficial to banish all doubts on the effect of the malaria pigment on TryR activity.

Additional testing that could confirm the findings reported here and help unveil the mechanisms behind them will be required, as current data do not provide sufficient evidence to truly speculate on them. In particular, emphasis should be given on understanding whether the improved Leishmania growth, as observed here, resulted from a reduced leishmanicidal activity of RAW 264.7 cells or from an enhanced parasite replication rather. Monitoring Leishmania growth over time along with the release of NO and ROS may help addressing this important question, as it could clarify whether the increased Leishmania growth begins with an enhanced phagocytosis of promastigotes or arises only during the exponential growth phase of the parasites.
Supported by the geographical overlapping of malaria- and VL-endemic areas and the occurrence of co-infection cases, the true biological relevance of the present model roots in the visceral tropism of *L. donovani* spp. Within their mammalian host, in fact, parasites of the *L. donovani* complex infect the mononuclear phagocytes of liver, spleen and bone marrow, where most of the HZ accumulates and displays its cytotoxic effects. This supports the possibility that in a co-infected patient, HZ-fed macrophages may become host of *L. donovani* amastigotes. However, whether these cells will develop increased *Leishmania* parasitaemias, as observed with RAW 264.7 cells here, remains to be elucidated, particularly in the light of the discrepancies towards the THP-1-derived results. RAW 264.7 and PMA-differentiated THP-1 cells are commonly used surrogates of mouse and human macrophages, respectively, due to their high phagocytic activity, adherence capability and ease of culturing, but their interchangeability and equivalence to primary macrophages remains limited. This holds true, particularly, with respect to the hosting of *Leishmania* parasites, as some of the cellular pathways responsible for parasite clearance *in vivo* (e.g., NO release) remain silent in human monocytic cell lines when applying standard stimulation *in vitro*. Replication of the present experiments, including monitoring of HZ and BH degradation over time, in primary macrophages as well as in other cell lines (e.g., U937, Mono Mac 6, J774 A.1 and IC-21) is, therefore, necessary to corroborate the findings presented in our study and clarify their biological relevance with respect to human infections. Importantly, even in the absence of a HZ-mediated effect on human VL, a deep understanding of its effects on host cell biology may prove very useful for providing new insights into the cellular mechanisms of the host-parasite interaction, given that many of the cell functions impaired by HZ ingestion are likely to play a role in the intracellular parasitism of *Leishmania* spp.

**Conclusions**

In conclusion, if the RAW 264.7 cell line may be considered as a model of the human macrophage behavior and the amounts of HZ and BH used be close to the physiological situation, it could be justly concluded that a recent or concomitant infection with *P. falciparum* is likely to exacerbate the course of VL in co-infected patients. On the other hand, if the PMA-differentiated THP-1 macrophages may more closely resemble the cells of the reticuloendothelial system, this would imply that data originating from mouse macrophage cell lines should be handled with great caution, as they may lead to misleading conclusions. In either case, further research is required to substantiate these preliminary findings and demonstrate their applicability to the complex reality of the malaria-VL co-infection.
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Chapter 9

Discussion
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.\(^1,3\) The increased susceptibility to deadly bacterial pneumonia in patients suffering from influenza,\(^4\) or to opportunistic infections such as cryptococcosis and pneumocystosis, causing much of HIV-associated morbidity and mortality\(^5\) 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.\(^1,2,6,7\) 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.\(^8-10\) 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,\(^11,12\) 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.\(^13-15\) Given that both VL and malaria are known to increase patient susceptibility to secondary infections by causing immunosuppression,\(^16-18\) tackling VL in co-endemic areas may impact indirectly on the burden of malaria and viceversa.

**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.\(^19\) 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,\(^20-23\) 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,\textsuperscript{15} 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 \textit{P. falciparum} prevalence and intensity as a result of their HIV seropositivity.\textsuperscript{24,25} 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 \textit{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).\textsuperscript{26} After all, the possibility of a \textit{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,\textsuperscript{27} 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

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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 sulfadoxine-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 \textit{P. falciparum} malaria, whereas a cytokine balance tipped towards regulatory patterns promotes survival of \textit{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/
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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, 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.

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, 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 [chapters 2 and 3].

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 vitro studies were performed. The first one [chapter 5], conducted with ex vivo human monocytes differentiated into dendritic cells (mo-DCs), aimed at characterizing the effect of L. donovani and P. falciparum co-exposure on cell maturation and function, while in the second study [chapter 8] the ability of hemozoin (HZ) to influence proliferation of L. donovani in macrophage-like cells of human and murine origin was investigated.

As most pathogens, Leishmania and Plasmodium have evolved a variety of mechanisms to overcome host immune defenses, including a complex of strategies to modulate functioning of dendritic cells, a group of highly adept antigen-presenting cells responsible for the induction of adaptive immune responses. 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 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.\textsuperscript{74,75} 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 \textit{L. donovani} and \textit{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 \textit{Leishmania} amastigotes.\textsuperscript{76-78} Hence, exploring the effect of HZ ingestion on the infection rate of \textit{L. donovani} in macrophages [chapter 8] may help providing valuable insights into the course of the \textit{L. donovani}-\textit{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 \textit{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 \textit{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.\textsuperscript{79} 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.\textsuperscript{80} 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 \textit{L. donovani}, as observed here, may be representative of what happens \textit{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 \textit{in vitro} and the \textit{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. In sharp contrast though, 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, 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 in vitro stimulation. Hence, in the absence of appropriate 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.

It should be noted, however, that here, no evidence was found to link the increased 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. Given the inherent differences that mark phagocytosis and maturation of Leishmania-containing phagosomes in macrophages of different sources (primary vs. immortalized cells, human vs. mouse cells), it is somehow not surprising that the complex array of HZ-mediated effects may result in different outcomes on 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 L. donovani.

**Alternative assays for detection of 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. Experimental models can provide a simplifying tool to this end, but have to deal with the poor availability of assays optimally suited for use in such complex systems, as in the case of Leishmania parasites grown in HZ-laden macrophages. In this in vitro model mimicking the VL-malaria co-infection, microscopic assessment of parasite burden, the standard method to quantify 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 Leishmania parasites that cannot be readily purchased. To circumvent this problem, two novel assays were developed: one consisting of a quantitative Reverse Transcriptase PCR (qRT-PCR) for measuring viability of the Leishmania parasite and its host cells; the other one being a simple enzymatic reaction with colorimetric readout that assesses the activity of a Leishmania-specific enzyme as a proxy of parasite viability. Both assays are applicable to all strains of 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.\textsuperscript{88-92} Some of these include an internal control surveying for test performance\textsuperscript{88} or correcting for cDNA synthesis/RNA input amount variation (normalization to a housekeeping gene),\textsuperscript{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 \textit{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 \textit{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 \textit{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 \textit{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\(^9\) 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.
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Concomitant infections by multiple pathogens pose one of the greatest challenges to global health due to their staggering burden and severe consequences. Affecting over a billion people worldwide, mainly among the global poor, co-infections are an important cause of human morbidity and mortality and a powerful driver of pathogen evolution and disease dynamics. Their effects across multiple biological systems reflect their ability to interact with each other either directly or through the host, resulting in potentially synergistic, additive or antagonistic effects that can alter transmission, clinical progression and control of diseases. Surprisingly, despite compelling evidence that co-infections outnumber single infections in many communities and can have dire effects on human health, little is known about the extent and outcome of these conditions, and the mechanisms implicated in their pathogenesis. This knowledge gap is particularly striking for co-infections involving childhood and neglected tropical diseases. In this respect, co-infections with visceral leishmaniasis (VL) and malaria are no exception. Caused by parasites of the genus *Leishmania* and *Plasmodium*, respectively, VL and malaria are two major vector-borne diseases that co-exist in many foci around the world. Their concomitancy in people living across co-endemic areas and their ability to cross-interact within co-infected animal models has been reported; however, very little focused attention has been paid to this condition so far.

In this thesis, different aspects of the VL and malaria co-infection are addressed with respect to its clinico- and immuno-epidemiology across East Africa and its pathological mechanisms as observed in vitro, using newly developed tools. The research began with a systematic review of the available literature in search of evidence for the occurrence of VL-malaria co-infections [chapter 2]. As only one VL-malaria cohort study was identified in this literature review, along with a few anything-but-negligible figures on the rate of co-infection among VL patients, two retrospective case-control studies were undertaken in VL endemic areas with different malaria transmission patterns. The first of these studies, described in chapter 2, was performed on data routinely collected by Médecins sans Frontières at Amudat Hospital, in the north-east of Uganda, where both VL and malaria are stably endemic. The analysis revealed that nearly one out of five VL patients hospitalized between 2000 and 2006 suffered from a malaria co-infection, with children under the age of ten incurring a twofold higher risk of being co-infected than adults, in line with the local age patterns of malaria. Prognosis was similar for both co- and mono-infected VL patients, as confirmed by their analogous in-hospital mortality rates, but co-infected patients presented with slightly compounded symptoms, suggesting that routine screening for malaria of VL patients living in co-endemic-areas and close monitoring of co-infected patients should be implemented.

A similar case-control study performed in an area with unstable and seasonal malaria transmission is presented in chapter 3. Medical data of patients admitted to Tabarakallah and Gedarif Teaching Hospitals (Gedarif State) and Al’Azaza kala-azar Clinic (Sennar State), East Sudan (2005-2010), were examined for their association with the VL-malaria co-infection using logistic regression analysis and the resulting outcomes were compared with an antecedent (1998), independently collected dataset from the same
region (Um-el-Kher and Kassab Hospitals). Not only the risk of co-acquiring VL and malaria was shown to be substantial for all age groups and in all surveyed areas, albeit with a significant variation among the different health centers, but the clinical implications deriving from being co-infected appeared somehow worrisome. Exacerbated clinical pictures, marked by increased emaciation, jaundice and anemia rates, characterized the co-infected patients with respect to the VL mono-infected patients, whose prognoses, however, were similar, provided that malaria was adequately treated. This confirms that prompt diagnosis and effective therapy of concomitant malaria is needed to ensure positive resolution of its co-infection with VL.

Supported by this evidence of a co-infection-induced deterioration of patients’ clinical pictures, the hypothesis of an immune-mediated interaction between the two pathogens was explored. Blood samples from patients actively infected with VL and/or malaria and from healthy individuals were collected during a pilot immuno-epidemiological survey conducted in Gedarif State, Sudan, and the level of nine different cytokines selected from across the four major response arms of the immune system were assessed simultaneously [chapter 4]. Comparative analysis highlighted substantial variations in the immune response mounted upon co-infection, confirming the ability of *Leishmania donovani* and *Plasmodium falciparum* to mutually interact at immunological level. Progressive polarization towards type-1 and pro-inflammatory cytokine patterns characterized the co-infected patients, who harbored significantly reduced levels of *P. falciparum* parasitaemias as compared to the malaria mono-infected patients, indicating that co-existence of VL and malaria resulted in either a protective or non-detrimental effect against *P. falciparum* infection.

This immune-mediated interaction between VL and malaria was subsequently examined in further detail, by assessing the effect of *in vitro* concomitant exposure of human monocyte-derived dendritic cells (mo-DCs) to *L. donovani* and *P. falciparum* [chapter 5]. 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 interleukin-12p70 levels to a complete lack of phenotypic maturation in the presence of high parasite amounts. Similar outcomes were observed in the presence of *L. donovani* only, whose effects were shown to precede the onset of apoptosis and affect unstimulated as well as lipopolysaccharide-stimulated cells. In contrast, *P. falciparum*-infected erythrocytes failed to activate mo-DCs and only slightly interfered with their lipopolysaccharide-triggered activation, suggesting that in the presence of a VL-malaria co-infection, *Leishmania*-driven effects may overrule the more silent response elicited by *P. falciparum*, shaping host immunity towards regulatory patterns and possibly delay resolution of the two diseases.

To further explore the potential for interaction between *Leishmania* and *Plasmodium* spp., two novel *Leishmania* viability assays were developed with the aim of replacing standard microscopic counting, whose applicability to our co-infection model is limited by the visual hindrance that follows cell ingestion of the malaria parasite and its products. The first of these two assays consisted of a duplex Reverse-Transcriptase qPCR (qRT-PCR) for simultaneous assessment of drug toxicity against *Leishmania*
in intracellular amastigotes and their host cells [chapter 6]. The assay combines amplification of *Leishmania*-specific 18S ribosomal RNA with human β2-microglobulin (β2-M) mRNA as an internal control for test performance and drug cytotoxicity. Pharmacological validation, performed with a set of structurally- and pharmacologically-diverse compounds blindly assessed against standard microscopy, resulted in highly reproducible anti-leishmanial activities and a good level of correlation between host cell toxicity and β-2M amplification. The result is a sensitive and versatile tool that can be used to accurately measure anti-proliferative effects against *Leishmania* intracellular amastigotes and their host cells, simplifying the use of the clinically relevant stage of leishmaniasis for drug discovery programs.

Chapter 7 describes the development and validation of a simple, one-step assay for measuring drug activity against *Leishmania* intracellular amastigotes grown in human macrophages. The assay relies on the the trypanothione reductase, a native, kinetoplastid-unique enzyme, whose activity correlates linearly with the growth of *Leishmania* parasites. By coupling the chromogenic properties of a detection dye with its substrate-regenerating capacity, this reaction allows the intracellular parasite viability to be monitored colorimetrically, 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 requirements.

This colorimetric assay was subsequently applied to the study of hemozoin-mediated effects on *Leishmania* infection burden [chapter 8]. In patients suffering from concomitant VL and malaria, the malaria waste product, hemozoin (HZ), is likely to co-localize with *Leishmania* parasites in the same mononuclear cells. To investigate the effect of this co-localization, *L. donovani* amastigote growth was monitored in mouse RAW 264.7 macrophages and human differentiated THP-1 cells previously exposed to increasing amounts of HZ or its synthetic analogue β-hematin (BH). Data demonstrated that phagocytosis of HZ and BH by RAW 264.7 cells, but not THP-1 cells, promoted infection therein of *L. donovani* parasites. The result proved to be dose-related, malaria pigment-specific and nitric oxide-independent, highlighting a possible detrimental effect of HZ on the course of VL, for which further investigation is warranted.

Embracing different aspects of a very complex topic, which is the VL-malaria co-infection, the research described in the present thesis provides evidence that, albeit its neglect, malaria co-infection is common among VL patient living in co-endemic areas, where it tends to compound patient symptomatology. Skewing of host serum cytokines with a possibly DC-independent mechanism and modulation of the two parasites’ survival marks the co-existence of *L. donovani* and *P. falciparum* in vitro as well as in vivo, suggesting a mutual interaction of the two pathogens at both immunological and non-immunological levels. Future studies examining the course of the co-infection prospectively and under multiple exposure conditions will be required to validate these findings and help unraveling the complex mechanisms that take place in the VL-malaria co-infected host.
Samenvatting

Co-infecties, gelijktijdig veroorzaakt door verschillende pathogenen, vormen vanwege de ziekte last en de ernstige gevolgen, wereldwijd een grote bedreiging voor de volksgezondheid. Meer dan een miljard, met name arme, mensen worden getroffen door deze co-infecties die een belangrijke oorzaak zijn van morbiditeit en mortaliteit en een sterke drijfkracht vormen voor pathogene evolutie en ziekte dynamiek. De impact op deze verschillende biologische systemen weerspiegelt de wisselwerking tussen de co-infecterende ziekteverwekkers, hetzij rechtstreeks of via een gastheer, welke kan leiden tot synergetische, additieve of antagonistische effecten die de transmissie, de klinische progressie en de bestrijding van individuele ziekten kan beïnvloeden. Ondanks overtuigend bewijs dat er in veel gemeenschappen meer co-infecties dan mono-infecties voorkomen, en dat deze enorme gevolgen voor de gezondheid kunnen hebben, is er van deze aandoeningen weinig bekend over de mate, de impact en de mechanismen die voor de pathogenese van co-infecties verantwoordelijk zijn. Dit gebrek aan kennis is in het bijzonder opvallend voor co-infecties waarbij kinderen en verwaarloosde tropische ziekten betrokken zijn en in dit opzicht vormen parasitaire co-infecties met viscerale leishmaniasis (VL) en malaria geen uitzondering. VL, veroorzaakt door *Leishmania* parasieten en malaria, veroorzaakt door *Plasmodium* soorten, zijn belangrijke door vectoren overgedragen ziekten die in veel regio’s in de wereld naast elkaar kunnen voorkomen. VL-malaria co-infecties zijn in mensen gerapporteerd, evenals de wisselwerking tussen deze parasieten in diermodellen, maar tot nu toe is hiervoor weinig gerichte aandacht voor geweest.

In dit proefschrift zijn verschillende aspecten van VL-malaria co-infecties onderzocht; de epidemiologie in Oost-Afrika, klinische aspecten, immunologische interacties en *in vitro* mechanismen die betrokken zijn bij de pathologie, waarvoor speciaal voor dit doel, in ons laboratorium ontwikkelde testen zijn gebruikt. Als eerste is een systematische evaluatie van de beschikbare literatuur uitgevoerd om bewijs te zoeken voor het voorkomen van deze co-infecties [hoofdstuk 2]. Er werd slechts één VL-malaria cohortstudie gevonden in deze evaluatie, naast enkele, niet te verwaarlozen, gegevens over het aantal co-infecties onder VL patiënten. Dit heeft geleid tot twee retrospectieve case-control studies die zijn uitgevoerd in co-endemische gebieden met verschillende malaria transmissie patronen. De eerste studie, beschreven in *hoofdstuk 2*, betreft de analyse van routine-matig verzameld gegevens door Artsen zonder Grenzen in het Amudat Ziekenhuis, in het Noordoosten van Oeganda, waar zowel VL en malaria stabiel endemisch zijn. Hierin werd aangetoond dat bijna één op de vijf VL patiënten die in het ziekenhuis werden opgenomen in de periode 2000-2006, aan een malaria co-infectie leed. Kinderen jonger dan tien jaar hadden een tweemaal hoger risico van co-besmetting dan volwassenen, hetgeen in overeenstemming is met de lokale leeftijd verdeling van malaria. De prognose, weergegeven als sterftecijfer, is vergelijkbaar voor zowel co-geïnfecteerde als mono-geïnfecteerde VL patiënten, maar de symptomen van de co-geïnfecteerde patiënten waren heftiger. Dit leidt tot de aanbeveling om routinematige malaria screening van VL patiënten
in co-endemisch gebieden en intensieve monitoring van co-geïnfecteerde patiënten te implementeren.

Een vergelijkbare ‘case-control’ studie is uitgevoerd in een regio met instabiele en seizoensgebonden malaria transmissie en wordt in hoofdstuk 3 beschreven. Medische gegevens van patiënten van de Tabarakallah en Gedair Academisch Ziekenhuizen (Gedair Provincie) en de Al’Azaza kala-azar Kliniek (Sennar Provincie), in Oost-Soudan (2005-2010), werden middels logistische regressie analyse geanalyseerd om de associatie met VL-malaria co-infectie te bepalen. De daaruit voortvloeiende resultaten werden vergeleken met een in 1998 onafhankelijke verzamelde dataset uit dezelfde regio (Um-el-Kher en Kassab Ziekenhuizen). Het risico voor het oplopen van VL en malaria bleek significant te zijn voor alle leeftijdsgroepen en in alle onderzochte gebieden, ondanks dat er variaties tussen de verschillende gezondheidscentra waren. Echter de klinische implicaties die uit de co-besmetting voortvloeien waren zorgelijk. Verslechterde ziektebeelden, gekarakteriseerd door toenemend gewichtsverlies, geelzucht en bloedarmoede, waren kenmerkend voor de co-geïnfecteerde patiënten ten opzichte van de VL mono-geïnfecteerde patiënten. De prognoses waren echter vergelijkbaar, mits malaria adequaat werd behandeld. Dit bevestigt dat een snelle diagnose en effectieve therapie voor malaria in co-geïnfecteerde patiënten noodzakelijk is voor een goede uitkomst van de co-infectie met VL.

Ondersteund door het gevonden bewijs dat een co-infectie een verslechtering van het ziektebeeld van de patiënten veroorzaakt, werd de hypothese van een immuun-gemedieerde interactie tussen de twee pathogenen bestudeerd in een immuun-epidemiologisch onderzoek uitgevoerd in Gedair State, Soudan. Bloedmonsterstrans van patiënten met een actieve VL en/of malaria infectie of van gezonde individuen werden kwantitatief geanalyseerd voor de aanwezigheid van negen verschillende cytokines, elke geselecteerd uit de vier verschillende reactie typen van het immuunsysteem [hoofdstuk 4]. Deze vergelijkende analyse liet aanzienlijke variaties zien in de immuunrespons geïnitieerd door de co-infectie. Hiermee werd de wederzijdse interactie op immunologische niveau tussen *Leishmania donovani* en *Plasmodium falciparum* bevestigd. De co-geïnfecteerde patiënten werden gekenmerkt door een progressieve polarisatie van cytokines naar type-1 en pro-inflammatoire patronen, en een significante vermindering van de *P. falciparum* infectie (uitgedrukt in aantallen parasieten) ten opzichte van de malaria mono-besmette patiënten. Dit geeft aan dat co-existentie van VL en malaria resulteerde in een beschermende of tenminste niet-schadelijk werking tegen *P. falciparum* infectie.

Deze immuun-gemedieerde interactie tussen VL en malaria werd vervolgens nader onderzocht door *in vitro* het effect van gelijktijdige blootstelling van menselijke monocyt-afgeleide dendritische cellen (mo-DCs) aan *L. donovani* en *P. falciparum* te meten [hoofdstuk 5]. De gelijktijdige stimulatie van mo-DCs met *L. donovani* promastigoten en *P. falciparum*-geïnfecteerde erytrocyten resulteerde in het algemeen in een dosis-afhankelijk effect op DC differentiatie, variërend van half gerijpte cellen, die lage hoeveelheden interleukine-12p70 afscheiden, tot aan een compleet gebrek aan fenotypische rijping in aanwezigheid van hoge parasiet aantallen. Vergelijkbare resultaten werden waargenomen in de aanwezigheid van alleen *L. donovani*, terwijl de Mo-DCs werden...
Samenvatting

Hoofdstuk 10

niet geactiveerd door *P. falciparum*-geïnfecteerde erytrocyten en slechts in geringe mate werd hun lipopolysaccharide-gestimuleerde activering beïnvloed. Dit wekt de suggestie dat, bij een VL-malaria co-infectie, het effect van de *Leishmania* infectie domineert over de immunologische reactie veroorzaakt door *P. falciparum* en dat dit kan leiden tot een verandering in de immuniteit van de gastheer, waardoor mogelijk de genezing van de co-infectie wordt vertraagd.

Om de interactie tussen *Leishmania* en *Plasmodium* spp. verder te onderzoeken, werden twee nieuwe testen ontwikkeld die de vitaliteit (of levensvatbaarheid) van de *Leishmania* parasiet kunnen bepalen. Deze twee testen kunnen de standaard microscopische telling vervangen, die door de lastige zichtbaarheid van de malaria parasieten niet toepasbaar was voor ons co-infectie model. De eerste test bestond uit een duplex Reverse-Transcriptase qPCR (qRT-PCR) voor de gelijktijdige bepalingen van de toxiciteit van geneesmiddelen tegen intracellulaire *Leishmania* amastigotes en humane gastheer cellen [hoofdstuk 6]. Deze test combineert amplificatie van het *Leishmania*-specifieke 18S ribosomale RNA met humaan β2-microglobulin (β2-M) mRNA als interne controle voor de test en voor de toxiciteit van het te testen geneesmiddel op cellen. De farmacologische validatie van deze test werd blind uitgevoerd met een panel van structureel en farmacologisch-diverse stoffen die vergeleken werden met standaard microscopie. Dit resulteerde in reproduceerbare anti-*Leishmania* activiteiten en een goede correlatie tussen gastheer cel toxiciteit en β2-M amplificatie. Het resultaat is een gevoelige en veelzijdig test die gebruikt kan worden voor nauwkeurige metingen van anti-proliferatieve effecten tegen *Leishmania* intracellulaire amastigoten en de bijbehorende gastheer cellen. Dit vereenvoudigt het gebruik van de klinisch relevante fase van leishmaniasis voor “drug discovery” programma’s.

Hoofdstuk 7 beschrijft de ontwikkeling en validatie van de tweede vitaliteitstest voor het meten van de geneesmiddel activiteit tegen *Leishmania* intracellulaire amastigoten gekweekt in humane macrofagen. De bepaling is gebaseerd op trypanothione reductase, een kinetoplastid-uniek enzym, waarvan de activiteit lineair correleert met de groei van *Leishmania* parasieten. Door het koppelen van chromogene eigenschappen van een detectie kleurstof met zijn substraat-regenererende capaciteit, kan de levensvatbaarheid van intracellulaire parasieten door deze reactie via een kleurreactie (colorimetrisch) gevolgd worden. Deze vereenvoudiging van de inhibitie testen maakt het uitproberen van de geneesmiddel-gevoeligheid meer toegankelijk. De farmacologische validatie van de bepaling, uitgevoerd met een panel van geselecteerde stoffen, toonde aan dat deze nieuw ontwikkelde techniek en de referentiemethoden consistent waren aan elkaar en bevestigde de robuustheid en de reproduceerbaarheid van de test, die aan ‘high-throughput’ eisen voldeed.

Deze colorimetrische assay werd vervolgens toegepast in een studie waarin de hemozoon (HZ)-gemediëerde effecten op *Leishmania* infectie werd bestudeerd [hoofdstuk 8]. In patiënten met VL-malaria co-infecties, kan het metabolische afvalproduct van malaria HZ, zich in dezelfde mononucleaire cellen als de *Leishmania* parasieten bevinden. Om te onderzoeken wat het effect van deze co-lokalisatie is, werd de groei van *L. donovani* amastigoten bepaald in muis RAW 264.7 macrofagen en in humane gedifferentieerde THP-1 cellen die voorafgaand waren blootgesteld aan toenemende hoeveelheden HZ of het synthetisch analoog
β-hematin (BH). De verkregen data toonde aan dat fagocytose van HZ en BH door RAW 264.7 cellen, maar niet door THP-1 cellen, de infectie met *L. donovani* parasieten bevorderd. Het effect bleek dosis-afhankelijk, malaria pigment-specifiek en stikstof monoxide-onafhankelijk te zijn, en benadrukken een mogelijke nadelige effect van HZ op het verloop van VL waarvoor nog verder onderzoek gerechtvaardigd is.

Het in dit proefschrift beschreven complexe onderzoek naar VL-malaria co-infectie laat zien dat deze aandoening veel voorkomt onder de VL patiënten die in malaria co-endemische gebieden wonen. De co-infectie kan een verergering van de ziekten symptomen veroorzaken. Zowel *in vitro* als ook *in vivo* hebben beide parasieten gedurende een co-infectie een sterk effect op het profiel van de cytokinen die in het serum van de gastheer gevonden kunnen worden. Deze modulatie is waarschijnlijk een DC-onafhankelijk mechanisme dat de overleving en co-existentie van *L. donovani* en *P. falciparum* in *vitro* en *in vivo* beïnvloed, hetgeen suggereert dat een onderlinge interactie van de twee ziekteverwekkers zowel op immunologische en niet-immunologische niveaus kan plaatsvinden. Toekomstige prospectieve studies naar het verloop van de co-infectie zal nodig zijn om deze bevindingen verder te valideren en om de complexe mechanismen die in de VL-malaria co-geïnfecteerde gastheer plaatsvinden te helpen ontrafelen.
Le co-infezioni da più agenti patogeni rappresentano una sfida importante per la salute pubblica, causa l’elevata incidenza e le gravi conseguenze che ne derivano. Con oltre un miliardo di casi al mondo, la maggior parte dei quali concentrati tra le popolazioni più povere, le co-infezioni sono una causa importante di morbilità e mortalità, nonché un elemento chiave nel processo evolutivo dei micro-organismi patogeni e nella dinamica della trasmissione delle malattie. Il loro impatto a carico dei diversi sistemi biologici riflette la capacità degli agenti co-infettanti di interagire tra loro direttamente o attraverso l’organismo ospite, scatenando possibili effetti di tipo sinergistico, additivo o antagonistico, capaci di alterare la trasmissione, la progressione clinica e il controllo delle singole patologie coinvolte. Paradossalmente, nonostante l’esistenza di prove convincenti circa la tendenza delle co-infezioni, in molte comunità, a prevalere sulle mono-infezioni e ad arrecare danni pesantissimi alla salute umana, poco è noto circa l’entità e la prognosi di queste condizioni, nonché i meccanismi implicati nella loro patogenesi. Tale carenza di informazioni diviene ancor più evidente nel caso di co-infezioni con patologie infantili o malattie tropicali neglette e, in tal senso, la co-infezione da leishmaniosi viscerale (VL) e malaria non fanno eccezione. Causate da parassiti dei generi *Leishmania* e *Plasmodium*, rispettivamente, la VL e la malaria sono due patologie a trasmissione vettoriale, che coesistono in molti dei loro focolai. La loro concomitanza in individui residenti in aree co-endemiche è stata descritta, così come la capacità dei due parassiti di interagire tra loro in modelli animali co-infetti; malgrado ciò il problema ha ricevuto finora ben poca attenzione.

Questa tesi tratta diversi aspetti della co-infezione da VL e malaria, tra cui la sua epidemiologia clinica e immunologica in Africa orientale e la sua patogenesi, studiata in vitro con dei saggi di nuova concezione appositamente sviluppati. La ricerca ha avuto inizio con una revisione sistematica della letteratura scientifica in cerca di evidenze sulla presenza di tali co-infezioni [capitolo 2]. Poiché da tale revisione è emerso un solo studio di coorte VL-malaria, ma delle percentuali di co-infezione in pazienti VL tutt’altro che trascurabili, due studi retrospettivi caso-controllo sono stati condotti in aree co-endemiche a diverso regime di trasmissione malarica. Il primo di questi, descritto nel capitolo 2, consiste in un’analisi di dati clinici raccolti da Medici senza Frontiere all’Ospedale di Amudat, nel nord-est dell’Uganda, dove sia la VL che la malaria sono stabilmente endemiche. Lo studio ha evidenziato che tra i pazienti VL ospedalizzati nel periodo 2000-2006, quasi uno su cinque era co-infetto da malaria. Di questi, la maggior parte erano bambini di età inferiore ai 10 anni, il cui rischio di co-infezione si è rivelato due volte superiore a quello degli adulti, in accordo con l’andamento demografico locale dell’infezione malarica. La prognosi è apparsa simile sia per i pazienti VL co-infetti che per quelli mono-infetti, come testimoniano i tassi di mortalità registrati nel nosocomio; tuttavia, il quadro sintomatologico è risultato essere aggravato nei pazienti co-infetti, suggerendo l’importanza di sottoporre a screening malarico tutti i pazienti VL che risiedono in aree co-endemiche e di effettuare uno stretto monitoraggio dei pazienti co-infetti.

Un secondo studio caso-controllo, condotto in una regione a trasmissione malarica instabile e stagionale, è descritto nel capitolo 3. In quest’analisi, i dati clinici di pazienti ricoverati tra
il 2005 e il 2010 presso l’Ospedale Universitario di Gedarif e l’Ospedale di Tabarakallah (regione Gedarif) e presso la Clinica kala-azar Al’Azaza (regione Sennar), nel Sudan orientale, sono stati esaminati mediante analisi di regressione logistica per la presenza di associazioni con la co-infezione da VL e malaria; i risultati ottenuti sono stati poi comparati a quelli di un’altra serie di dati della stessa regione (Ospedali Um-el-Kher e Kassab), raccolti antecedentemente (1998) da una fonte indipendente. Non solo il rischio di contrarre entrambe la VL e la malaria è risultato essere considerevole per tutte le fasce d'età e in tutte le zone esaminate, sebbene con una notevole variabilità tra i vari centri ospedalieri, ma le implicazioni cliniche derivanti dall’essere co-infetto sono apparse piuttosto preoccupanti. Quadri clinici caratterizzati da aumentati livelli di emaciazione, ittero e anemia, sono apparsi più frequenti tra i pazienti co-infetti, le cui prognosi tuttavia si sono dimostrate simili a quelle dei pazienti VL mono-infetti, a condizione che la malaria fosse stata trattata efficacemente. Questo conferma che la diagnosi precoce di co-infezione malarica in soggetti VL, unitamente ad un suo trattamento efficace, è indispensabile per assicurarne una risoluzione positiva.

Supportata da tali evidenze sul decorso della co-infezione da VL e malaria, l’ipotesi di un’interazione immuno-mediata tra i due parassiti è stata analizzata mediante uno studio pilota immuno-epidemiologico condotto in Sudan, nella regione Gedarif. Campioni di sangue prelevati da pazienti sintomatici infetti da VL e/o malaria e da individui sani sono stati esaminati per la presenza di nove diverse citochine selezionate dai quattro maggiori bracci del sistema immunitario [capitolo 4]. L’analisi comparativa ha evidenziato variazioni sostanziali nella risposta immunitaria indotta dalla co-infezione, confermando la capacità di Leishmania donovani e Plasmodium falciparum di interagire tra loro a livello immunologico. In particolare, una progressiva polarizzazione dei profili citochinici verso risposte di tipo-1 e pro-infiammatorio è apparsa evidente tra i pazienti co-infetti, i cui livelli di parasitemia da P. falciparum sono risultati essere significativamente inferiori a quelli dei pazienti malarici mono-infetti, indicando un possibile effetto protettivo o quanto meno non-dilatero da parte della VL sull’infezione da P. falciparum.

Questa interazione immuno-mediata tra la VL e la malaria è stata poi ulteriormente esaminata valutando l’effetto della co-esposizione in vitro a L. donovani and P. falciparum di cellule dendritiche ottenute da monociti umani (mo-DCs) [capitolo 5]. Complessivamente, la co-stimolazione delle mo-DCs con promastigoti di L. donovani ed eritrociti infetti da P. falciparum ha evidenziato un duplice effetto, dose-dipendente, sulla differenziazione delle DCs, che va da cellule semi-mature secernenti bassi livelli di interleuchina 12p70 ad una completa assenza di maturazione fenotipica in presenza di elevate quantità di parassiti. Risultati simili sono stati ottenuti in presenza dei soli parassiti di L. donovani, mentre nessun effetto differenziativo e solo una leggera interferenza nel processo di attivazione delle mo-DCs indotto dal lipopolisaccaride sono stati osservati in presenza di eritrociti infetti da P. falciparum, suggerendo come durante una co-infezione VL-malaria, gli effetti indotti dalla Leishmania possano prevalere sulla risposta più silente evocata da P. falciparum, orientando la risposta immunitaria dell’organismo ospite verso profili regolatori e ritardando in tal modo la risoluzione delle due patologie.
Al fine di analizzare ulteriormente le possibili interazioni tra *Leishmania* e *Plasmodium*, due nuovi saggi per misurare la vitalità dei parassiti di *Leishmania* sono stati sviluppati con l’obiettivo di sostituire la tradizionale conta microscopica, la cui applicabilità al nostro modello di co-infezione è limitata dall’interferenza visuale che segue l’ingestione del parassita malarico e dei suoi prodotti da parte delle cellule. Il primo di questi due saggi consiste in una qPCR mediante transcrittasi inversa (RT-qPCR) per la simultanea valutazione della tossicità da farmaci sugli amastigoti intracellulari di *Leishmania* e le loro cellule ospite [capitolo 6]. Il saggio combina l’amplificazione di un target specifico per la *Leishmania*, l’RNA ribosomiale 18S, con uno umano, l’mRNA della β2-microglobulina (β2-M), che funge da controllo interno per valutare la prestazione del test e la citotossicità dei farmaci. La validazione farmacologica, compiuta con un set di composti strutturalmente e farmacologicamente eterogenei esaminati in cieco mediante microscopia standard, ha evidenziato un’elevata riproducibilità delle attività anti-leishmaniosi, nonché un buon livello di correlazione tra citotossicità e amplificazione del gene β2-M. Ciò dimostra come questa RT-qPCR sia un tool sensibile e versatile che si presta alla misurazione accurata degli effetti anti-proliferativi contro gli amastigoti intracellulari di *Leishmania* e le loro cellule ospiti, semplificando l’uso dello stadio clinicamente rilevante della leishmaniosi (l’amastigote) nella ricerca di nuovi farmaci.

Il capitolo 7 descrive lo sviluppo e la validazione di un secondo saggio per misurare l’attività dei farmaci contro gli amastigoti intracellulari di *Leishmania* coltivati in macrofagi umani. Il saggio si basa sulla tripanotione reduttasi, un enzima nativo, unico della famiglia dei kinetoplastidi, la cui attività è linearmente correlata alla crescita dei parassiti di *Leishmania*. Sfruttando le proprietà cromogeniche del reagente con le sue capacità rigeneranti nei confronti del substrato, questa reazione consente di monitorare colorimetricamente la vitalità dei parassiti intracellulari, semplificando la metodologia per effettuare saggi di inibizione e migliorando l’accessibilità ai tests di sensibilità farmacologica. La validazione farmacologica del saggio, condotta con un pannello di composti opportunamente selezionati, ha dimostrato un’elevato grado di concordanza tra la nuova tecnica e i metodi di riferimento e ha confermato la robustezza e la riproducibilità del saggio, in conformità ai requisiti di high-throughput.

Questo saggio colorimetrico è stato successivamente applicato allo studio degli effetti dell’emozoina sulla carica parassitaria della *Leishmania* [Capitolo 8]. Nei pazienti co-affetti da VL e malaria, infatti, è verosimile che l’emozoina (HZ), un prodotto di scarto del metabolismo malarico, co-localizzi con i parassiti di *Leishmania* all’interno delle medesime cellule mononucleari. Per esaminare l’effetto di tale co-localizzazione, la crescita degli amastigoti di *L. donovani* è stata monitorata in macrofagi murini (linea cellulare RAW 264.7) e umani (cellule differenziate della linea THP-1), previamente esposti a quantità crescenti di HZ o del suo analogo sintetico β-ematina (BH). I dati hanno dimostrato che la fagocitosi di HZ e BH da parte delle cellule RAW 264.7, ma non delle cellule THP-1, promuove l’infezione ai loro interni dei parassiti di *L. donovani*. Il risultato è apparso essere dose-dipendente, pigmento malarico-specifico e ossido nitrico-non mediato, evidenziando un possibile effetto peggiorativo dell’emozoina sul decorso della VL, meritevole di ulteriori approfondimenti.
Trattando vari aspetti di un argomento assai complesso, quale è la co-infezione VL-malaria, la ricerca descritta nella presente tesi conferma che per quanto negletta, questa condizione è frequente nei pazienti VL residenti in aree co-endemiche, in cui tende ad aggravarne il profilo clinico e sintomatologico. L’alterazione dei quadri citochinici mediante un possibile meccanismo DC-indipendente e la modulazione della carica parassitaria caratterizzano la coesistenza di *L. donovani* e *P. falciparum* sia *in vitro* che *in vivo*, suggerendo la capacità dei due patogeni di interagire tra loro sia a livello immunologico che non. Ulteriori studi volti ad esaminare il corso della co-infezione in maniera prospettica e sotto molteplici condizioni di esposizione saranno necessari per validare i risultati ottenuti finora e per comprendere i complessi meccanismi che hanno luogo nell’organismo co-infetto da VL e malaria.
Addendum

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This thesis reports part of the research I have been conducting at KIT Biomedical Research in Amsterdam. Based on what was initially conceived as the subject of a short research fellowship, this research is the result of intensive efforts aimed at exploring the complexity of the VL-malaria co-infection from multiple perspectives and translating it into a coherent set of studies. Many people have taken part to these efforts, some formally, many others in a less official way. To all of them I owe my most heartfelt gratitude. James Allen once said: ‘No duty is more urgent than that of returning thanks’. I believe nothing could be more true, and I will try to fulfill my duty to the best I can.

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Thank you | Bedankt | Grazie!

Amsterdam, September 2016

Erika


PhD Portfolio

Poster and Presentations

Oral presentation: ‘Epidemiology of visceral leishmaniasis-malaria co-infections in East Africa’.
van den Bogaart E, Berkhout M, Mens P, Adams E, Ritmeier K, Chappuis F, Nour B, Schallig H.
Invited speaker at investigative workshop “Malaria-Leishmania Co-infection”, The National Institute for Mathematical and Biological Synthesis, Knoxville, USA, 26-28 May 2015.

Oral presentation: ‘Malaria-visceral leishmaniasis co-infections in East Africa’.
van den Bogaart E, Berkhout M, Mens P, Adams E, Chappuis F, Ritmeier K, Nour B, Schallig H.
International Health Dutch Society for Parasitology (NVP) Spring Meeting, Utrecht, the Netherlands, 22 May 2014.
8th European Congress on Tropical Medicine, Copenhagen, Denmark, 10-13 Sep 2013.

Oral presentation: ‘Simple colorimetric assay for high-throughput screening of drugs against Leishmania intracellular amastigotes’.
van den Bogaart E., Schoone GJ, England P, Faber D, Orrling KM, Adams E, Schallig HDFH.
15th Edition of the Figon Dutch Medicine Days, Ede, the Netherlands, 30 Sep-2 Oct 2013.

Poster presentation: ‘Design, synthesis and pharmacological evaluation of potent inhibitors of trypanosomal phosphodiesterase B1’.
Orrling KM, Blaazer AR, Jansen C, Shanmugham A, England P, Bailey D, Balmer V, Bregy P, Lan Vu X, Cos P, Maes L, Adams E, van den Bogaart E, Chatelain E, Ioset J-R, Veerman J, Seebeck T, Sterk GJ, Leurs R, de Esch IJP.
15th Edition of the Figon Dutch Medicine Days, Ede, the Netherlands, 30 Sep-2 Oct 2013.

Poster presentation: ‘A novel colorimetric assay for screening anti-leishmanial drugs’.
van den Bogaart E, Faber D, Schoone G, Schallig H, Adams E.
TiPharma Spring Meeting, Utrecht, the Netherlands, 17 Apr 2012.
British Society Spring Meeting 2012, Glasgow, UK, 2-5 Apr 2012.

Oral presentation: ‘Co-infections with visceral leishmaniasis and malaria: clinico-epidemiological setting and biological implications’.
van den Bogaart E.
TropenCentrum, Academic Medical Centrum (AMC), Amsterdam, the Netherlands, 25 Mar 2012.
Courses

Laboratory Animal Science (article 9), University of Utrecht, the Netherlands, 27 Jun – 8 Jul 2016.

Short Course on Teaching of Teachers, KIT, Amsterdam, the Netherlands, 8 & 10 Apr 2014.

Basic Safety & Security Course and Security for Female Travelers Course, Centre for Safety and Development, Amersfoort, the Netherlands, 28 – 30 Jan & 3 Mar 2010.

Developing a Cochrane Diagnostic Test Accuracy Review, Cochrane Collaboration, Amsterdam, the Netherlands, 2010.

Teaching

Rapid Diagnostics, practical, Summer Course to Students from Jazan University (Saudi Arabia), KIT, Amsterdam, the Netherlands, 2015.

Supervision of MSc. students’ scientific internship (PB, MB, DF, AS), KIT, Amsterdam, the Netherlands, 2011–2012.

(International) National Conferences, Symposia and Workshops

Panel debate on the theme ‘From Innovation to Impact’, KIT, Amsterdam, the Netherlands, 26 Jan 2016.

Investigative Workshop ‘Malaria-Leishmania Co-infection’, NIMBioS, Knoxville, USA, 26 – 28 May 2015.

4th International Conference on Bio-Sensing Technology, Lisbon, Portugal, 10 – 13 May 2015.

Mini-symposium ‘The nature of the bug: do certain Mycobacterium tuberculosis genotypes have an intrinsic ability to cause multidrug-resistant clusters?’, KIT, Amsterdam, the Netherlands, 2014.

15th Edition of the Figon Dutch Medicine Days, Ede, the Netherlands, 30 Sep – 2 Oct 2013.

ISNTD Bites 2013 ‘Integrated disease surveillance & vector control’, Royal Geographic Society, London, United Kingdom, 15 Oct 2013.

Workshop on ‘Diagnostics for Disease Surveillance: TwistDx’, Royal Geographical Society, London, United Kingdom, 15 Oct 2013.
50th Spring Meeting of the British Society for Parasitology, Glasgow, United Kingdom, 2 – 5 Apr 2012.

TiPharma Spring Meeting, Utrecht, the Netherlands, 17 Apr 2012.

Mini-symposium on Human African Trypanosomiasis, KIT, Amsterdam, the Netherlands, 10 Dec 2012.

‘Parasite to prevention: Advances in the understanding of malaria’, Edinburgh, United Kingdom, 20 – 22 Oct 2010.
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*Antimicrob Agents Chemother* 2014, 58(1):527-35.

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Concomitant malaria among visceral leishmaniasis in-patients from Gedarif and Sennar States, Sudan: a retrospective case-control study.  
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*BMC Public Health* 2013, 13:332.
Catechol pyrazolinones as trypanocidals: fragment-based design, synthesis, and pharmacological evaluation of nanomolar inhibitors of trypanosomal phosphodiesterase B1.
Orrling KM, Jansen C, Vu XL, Balmer V, Bregy P, Shanmugham A, England P, Bailey D, Cos P, Maes L, Adams E, van den Bogaart E, Chatelain E, Ioset JR, van de Stolpe A, Zorg S, Veerman J, Seebeck T, Sterk GJ, de Esch IJ, Leurs R.
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van den Bogaart E, Berkhout MM, Adams ER, Mens PF, Sentongo E, Mbulamberi DB, Straetemans M, Schallig HD, Chappuis F.
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About the author

Erika van den Bogaart was born on August 3rd 1979 in Bergamo, Italy, and grew up in a small village on the Italian Prealps. After graduating from pre-university education in 1998, she continued her studies in Medicinal Chemistry and Technology at the University of Milan, for which she received her MSc in 2007. During this study, driven by her interest for tropical diseases, she served a 2-year internship at the Parasitology group of the University of Milan, where she gained extensive experience in the field of antimalarial drug discovery and pharmacodynamics. She continued working on the characterization of the mechanism of action of 4-aminoquinolines as a postgraduate fellow.

In November 2008, after having been entitled with her license to practice Pharmacy, she was awarded with a one-year personal fellowship from the University of Milan, by which she joined the Parasitology group at KIT Biomedical Research, Amsterdam. Here, she was introduced to the fascinating field of leishmaniasis, and developed the initial concept of what later has become the subject of her PhD study. In 2009, she began working on a project for the development of new phosphodiesterase inhibitors as novel therapeutic agents against leishmaniasis and trypanosomiasis, supported by the Top Institute Pharma. Meanwhile, on a part-time basis, she pursued her studies on the visceral leishmaniasis–malaria co-infection, under supervision of Dr. Henk Schallig and Prof. dr. Martin Grobusch (Academic Medical Center). She collaborated with multiple international (Médecins sans Frontières, Blue Nile National Institute for Communicable Diseases in Sudan, IOTA Pharmaceuticals Ltd in the UK) and national (VU University in Amsterdam) partners and supervised several MSc students during their internship at KIT Biomedical Research. She presented her research at various national and international academic meetings, and prepared several publications for peer-review journals which have resulted in this thesis. In 2012, she obtained a position as research fellow at the Rapid Diagnostics group of KIT Biomedical Research, where she has been responsible for the development and evaluation of simple diagnostic tests for poverty-related infectious diseases, and the application of novel technology platforms for diagnostic assays. In July 2016, she joined the Mondial Diagnostics Foundation as senior scientist, continuing her research activities on the development of simplified diagnostic tools for poverty-related diseases.

She lives together with Issa and their little son Rayan.