THE STEPWISE INTERACTIONS ESTABLISHED BY LEISHMANIA *IN ITS HOSTS*

LES INTERACTIONS PARASITES EUCARYOTES-HÔTES : L'EXEMPLE DE *LEISHMANIA*

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SUMMARY

Parasitism is based on lasting and renewed interactions between the parasite's genome and that of the living organism on which it depends for its perpetuation. All parasites subvert and/or remodel the host's tissue(s) where they live and reproduce. The genus Leishmania *is used to illustrate the sequential nature and diversity of the processes occurring in the host. The life cycle of* Leishmania *requires two successive hosts : hematophagous insects and mammals, the latter also being essential to the survival of the insects. Novel* in vivo *models were designed to identify and characterise processes established by* Leishmania *(*L. major*) in the tissues (skin and draining lymph nodes) of laboratory mice. Homogenous populations of phagocytic leukocytes (macrophages, dendritic leukocytes, and sometimes neutrophils), obtained from laboratory mice, have helped to define how* L. amazonensis *and* L. donovani *subverts these leukocytes, respectively as host cells stricto sensu or shuttle cells. The objective of these studies is to improve our understanding of the pathogenesis of the transient skin lesions observed in mammalian hosts, and more importantly, of the conditions required for the perpetuation of* Leishmania *and their transmission from the mammalian host to the female sandfly, acting here as both host and vector.*

Key words : parasitism, parasite perpetuation, phagocytic leukocytes, tissue and cell remodelling.

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RÉSUMÉ

On sait actuellement que le parasitisme est basé sur une interaction durable et renouvelée entre le génome du parasite et celui de l'organisme vivant dont il dépend pour sa pérennité. Tout organisme parasite détourne et/ou remodèle le(s) tissu(s) de son hôte, pour pouvoir y vivre et s'y reproduire. Le genre *Leishmania* permet d'illustrer le caractère séquentiel et la diversité des processus se déroulant chez l'hôte. Le cycle de vie de *Leishmania* nécessite deux hôtes successifs : des insectes hématophages et des mammifères, ces derniers étant aussi nécessaires à la survie des insectes. Des modèles *in vivo* originaux ont été mis au point pour identifier et caractériser les processus établis par *Leishmania* (*L. major*) dans les tissus (peau et ganglions lymphatiques drainants) de souris de laboratoire. En parallèle, l'obtention, à partir des souris de laboratoire, de populations homogènes de leucocytes phagocytaires (macrophages, leucocytes dendritiques, et neutrophiles dans certains cas) a permis de préciser comment *L. amazonensis* et *L. donovani* détournent l'activité de ces leucocytes, pour qu'ils deviennent, respectivement, des cellules hôtes *stricto sensu* ou des cellules navettes. L'objectif de ces études est de mieux comprendre la pathogénie des lésions cutanées transitoires observées chez l'hôte mammifère, et surtout les conditions nécessaires à la pérennité des leishmanies et à leur transmission de l'hôte mammifère à la femelle phlébotome, qui agit à la fois comme hôte et comme vecteur.

Mots-clés : parasitisme, pérennité des parasites, leucocytes phagocytaires, remodelage tissulaire et cellulaire.

INTRODUCTION

Leishmania are protozoan parasites that are transmitted to mammals when a blood-feeding sand fly – that harbours mammal invasive *Leishmania* developmental stage in the anterior part of their gut lumen – is searching the blood meal on which relies its perpetuation (Alexander *et al.* 1999 ; Antoine *et al.* 2004 ; Lukes *et al.* 2007 ; Peacock *et al.* 2007 ; Smith *et al.*2007). There are many *Leishmania* species that have first been detected and characterized through the local or systemic pathogenic processes they can initiate in humans and in other mammals domesticated by humans – *e.g* dogs – (Ashford 2000 ; Desjeux 2004). Overall, these pathogenic processes – designated as leishmaniases – afflict ~12 million human individuals in 88 countries worldwide. Visceral leishmaniases – the most severe systemic diseases triggered by two species within the *L. donovani* complex – are diagnosed each year in ~ 500 000 humans primarily in India, Sudan, Nepal. The emergence of *L. donovani* strains that are resistant to pentavalent antimonials hampers the use of the first-line drugs otherwise known to display host-damaging side effects (Croft *et al.* 2006).

Of note, though neglected by too many investigators, the *Leishmania* development in the human hosts does not always result in tissue-damaging processes. Thus we were curious to decipher the parasite – as well as host – dependent processes that result in stable and long term asymptomatic parasitism. *Leishmania* are indeed *bona fide* parasites *i.e* the perpetuation of these parasitic eukaryotic organisms strictly relies on other living organisms, (a) blood-feeding sand fly insects and (b) mammals. The latter population is not only subverted as hosts by *Leishmania* but is also subverted as blood sources by the insects-classified within the nematoceran subfamily, the Phlebotominae, of the family Psychodidae- : indeed, in absence of blood meal the insect gonotrophic cycle cannot proceed preventing the insect perpetuation.

Parasitism in the sand fly is initiated when sand flies take blood from a mammal, the dermis of which is hosting *Leishmania* amastigotes-loaded phagocytic leukocytes. The blood meal – loaded with intracellular *Leishmania* – is delivered to the posterior midgut, a compartment from where are extracted the blood derived nutrients and from where are generated the non trophic signalling molecules that contribute to the onset and progression of the insect progeny generation (Killick-Kendrick 1999 ; Lehane 2005). Thereafter, *Leishmania* amastigotes rapidly exit the mammal leukocytes and differentiate as procyclic promastigotes with very short flagella. Many distinct developmental stages are now known to proceed as the promastigotes migrate from the posterior midgut to (a) the stomodeal valve and downstream from this valve in the anterior part of the digestive tract (Rogers *et al.* 2004 ; Bates & Rogers, 2004 ; Kamhawi 2006 ; Bates 2007). Briefly, the first promastigote multiplication phase takes place in the early blood meal confined by the sand fly-derived peritrophic matrix. Promastigotes develop into large slender forms which (a) traverse the peritrophic membrane (b) anchor themselves to the epithelial cells lining the insect posterior midgut and (c) undergo the second multiplication phase before (d) migrating to the anterior part of the gut. Although two stages are observed at the stomodeal valve, haptomonads and metacyclic promastigotes, only the latter – otherwise known to have exited the cell cycle – are found upstream and behind the stomodeal valve and are highly adapted for successful transmission to the mammalian host (Volf *et al.* 2004 ; Kamhawi 2006 ; Bates 2007).

Although the metacyclogenesis process is still poorly understood within its natural insect host, it occurs in axenic promastigote culture, allowing the metacyclic promastigotes to be prepared for further *in vivo*, *ex vivo* or *in vitro* biologically relevant investigations. Model mammals such as laboratory mice are now known to act as (a) relevant sources of phagocytic leukocytes or phagocytic leukocyte progenitors (b) as *bona fide* hosts of at least the *Leishmania* species that are qualified as skin tropic, on the basis on the skin lesion that can develop at the site of inoculation. We refine an experimental system relying on C57BL/6 mice (Belkaid *et al*. 2000 ; Lang *et al*. 2005 ; Lecoeur *et al*. 2007) to decipher the parasite-driven processes and the host tissue remodelling. The objectives are to identify and characterize, at the tissue level, how *Leishmania* subvert and/or remodel both the skin and the draining lymph node from the initial time point post its delivery as metacyclic promastigotes to the later prolonged phase of the persistence of the parasite progeny that is transmissible to the insect. In this short review, only a few of the myriad of processes assessing the *Leishmania* developmental features in mice or mouse-derived phagocytic leucocytes will be highlighted. Other features of *Leishmania*-host interactions – not developed in the present contribution – are covered in the following reviews or original publications (Killick-Kendrick 1999 ; Alexander *et al*. 1999 ; Burchmore, Barrett 2001 ; Sacks & Kamhawi, 2001 ; Handman & Bullen, 2002 ; Bates & Rogers, 2004 ; McConville *et al*. 2007 ; Peacock *et al*. 2007 ; Smith *et al*. 2007 ; Naderer & McConville, 2008).

DECODING FEATURES OF *LEISHMANIA*-DRIVEN PARASITISM IN THEIR NATURAL ECOCYSTEMS

In their natural ecosystems, *Leishmania amazonensis* (Antoine *et al*. 2004) and *Leishmania major* (Fichet Calvet *et al*. 2003) alternatively subvert blood-feeding sand flies and wild rodents as hosts. The two host populations are closely co-inhabiting within the same biotopes. The wild rodents are hosting intracellular amastigotes in the upper dermis of their ears, the latter skin sites being the main locations from where the female sand flies recover both their blood meal and the amastigotes-loaded host leukocytes. Of note, most frequently, in such stable ecosystems, the rodents do not display any ear lesion or scar, indicating that the successful amastigote transmission from the rodent mammals to sand flies is uncoupled from any clinically detectable tissue damage in wild rodents. Once in the sand fly digestive tract lumen, the major environmental changes – temperature drop, higher oxygen tension to mention some of the most easily detectable – result in a novel *Leishmania* developmental program, namely the differentiation as extracellular flagellated promastigotes, the population size of which fluctuates, the endpoint being a small size population of mammal invasive non cell-cycling metacyclic promastigotes. It is important to note that the metacyclic promastigotes-carrying sand fly is expected to deliver, in the rodent ear dermis, a number of parasites that range from 10 to 10,000.These features – illustrated in ***figure 1*** – led us (a) to design novel reliable laboratory mouse-based models (b) and to further identify and characterize the stepwise discrete processes that develop in mammals once *Leishmania* metacyclic promastigotes have been delivered in the ear dermis (Belkaid *et al*. 2000 ; Lang *et al*. 2005 ; Lecoeur *et al*. 2007).

DESIGNING RELIABLE MOUSE MODELS FOR DECIPHERING *IN VIVO*, AT THE TISSUE LEVEL, THE STEPWISE REMODELING OF THE TISSUE (S) HOSTING *LEISHMANIA*

The C57BL/6 that have received intra-dermally in the ear *Leishmania* metacyclic promastigotes, engineered to express firefly luciferase, offer the relevant experimental conditions for exploring in real time and simultaneously i) the parasite load fluctuations ii) the macroscopic ear features iii) transcriptional signatures captured – with quantitative real time PCR – from both the *Leishmania*-loaded ear and the ear-draining lymph node. In this latter context, while longitudinally probing transcriptional signatures in both tissues we expect to determine (a) the duration of the phase during which the *Leishmania* amastigote-hosting phagocytic leukocytes provide both nutrients and non trophic signals to the amastigotes allowing them to expand the size of their population (b) when dermis-protective regulatory T lymphocytes are subverted to help amastigotes hosted by phagocytic leucocytes to further expand the size of their population (c) when in the lymph node-draining the parasite-loaded ear, immunogenic signals are delivered to CD3 T lymphocytes (d) when both parasite-clearing potentially tissue-damaging CD3 lymphocytes and dermis and parasite-protective regulatory T lymphocytes are recruited, in a balanced manner, in the parasite-loaded ear, the latter process leading to the shaping of the optimal dermal niche where a low number of amastigotes does persist until the natural death of the mouse hosts. Many quantitative readout assays have been set up, standardized and carried out in real time. Although "tissue transcriptional signatures" of asymptomatic as well as of tissue-damaging transient processes have been identified, many processes still need to be clarified especially before and post the transient tissue damage that is deploying in mice according to their genotypes. Indeed post the intradermal inoculation of a low (number 100) dose of metacyclic promastigotes, the dermal macrophages hosting cell-cycling amastigotes are the main phagocytic leukocytes rapidly and directly contributing to the ear remodelling as a niche *per se*. While in C57BL/6 mice, the tissue-damaging immune functions are concomitant to the immune clearance of more than 95 per cent of the amastigote population, the tissue repair immune functions are concomitant to the dynamic remodelling of a unique niche where does persist a stable and low number of intracellular amastigotes. In BALB/c mice, the outcome is different: indeed, at the site of the promastigote inoculation, once the onset of the tissue-damaging processes is

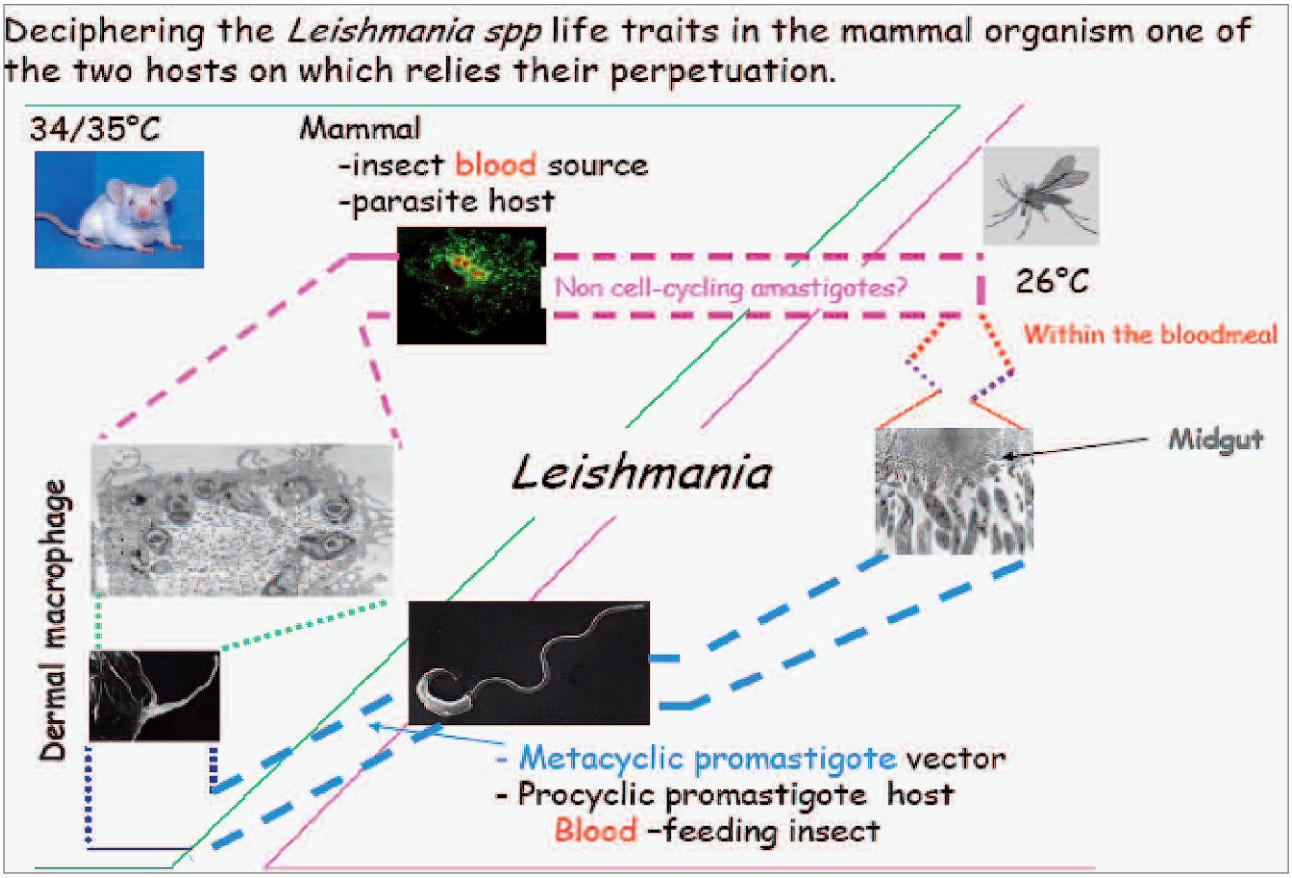

Figure 1 : *Schematic representation of the* Leishmania amazonensis *development in both the laboratory mammal-BALB/c mouse-and the laboratory insect sand fly.*

occurring, the latter progress and are never attenuated (Sacks & Noben-Traub, 2002). For *L. major* and *L. amazonensis* we still do not know whether, in BALB/C mice, there is/are dermal niche(s) – distant from the primary inoculation site – that is/are loaded with persisting amastigotes. Though many more leukocyte lineages seem to participate to the dermal niche where a stable number of amastigotes does persist, the respective contribution of each lineage, their turnover are still far from being established: in addition to the parasite-loaded mononuclear phagocytic leukocytes – very likely displaying dendritic cell features – are indeed contributing in a balanced and dynamic manner both parasite-reactive regulatory CD4 T lymphocytes and parasite-reactive T lymphocytes (Belkaid *et al.* 2002; Belkaid *et al.* 2006). Of note while a disruption of the balance in favour of regulatory T lymphocytes will result in re-expansion of the amastigote population, a disruption in favour of the effector population will result in the complete clearance of the persisting amastigotes, preventing the dynamic "premunition" status to be sustained (Sergent & Sergent, 1956).

For those *Leishmania* species (*L. infantum*, *L. donovani*) which are classified as viscero-tropic -on the basis of the severe systemic disease they can trigger in mammals such as dogs and humans - major limitations of the present experimental settings are still preventing us to capture the stepwise complex processes that deploy post the metacyclic promastigote inoculation in the skin. Considering the severity of *L. donovani*-driven pathogenic processes in humans – when the latter, known as visceral leishmaniasis, do develop – we wish to address the issues of the innovative models that need to be set up and further validated. It is indeed urgent to design such models for capturing the maximal number of (a) *L. donovani* or *L. infantum* life traits in the different tissues – distant from the cutaneous site where the metacyclic promastigotes were initially delivered – (b) the signatures unique to each of the tissues-the liver, the spleen the bone marrow – the parasite subvert as more or less optimal niches. For setting up this next generation of relevant models, we shall benefit from the following recent exciting observations: Massberg *et al.* (Massberg *et al.* 2007) did recently demonstrate *in vivo* that some hematopoietic stem cells (HSCs) otherwise known to be present in the bone marrow not only circulate in the blood in steady state conditions but also travel through the lymphatic system. Furthermore, migration of HSCs – which express microbial sensors such as the Toll-like receptors – allows microbial molecules to be sensed in peripheral tissues, thereby promoting the local and accelerated generation of leucocytes displaying phagocytic activity at the site of microbe entry and development. Some of these HSCs triggered to rapidly dif-

ferentiate in peripheral tissues – such as the skin – exit the latter site and home back to the bone marrow. Such HSC programs – dedicated to peripheral tissue surveillance – could have been subverted by *L. donovani*, *L. infantum* to reach the bone marrow, the latter tissue becoming the main site from where regularly emigrate, at low frequency, amastigote- loaded phagocytic leucocytes that could further enter peripheral tissues such as skin sites distant from the primary inoculation site. Of note the HSC population could also rapidly sense endogenous signalling molecules released at the primary skin site where the parasite inoculum was initially delivered.

DESIGNING RELIABLE *IN VITRO* CULTURE SYSTEMS FOR EXTRACTING UNIQUE TRANSCRIPTIONAL SIGNATURES DISPLAYED BY THE MAMMAL PHAGOCYTIC LEUCOCYTE LINEAGES THAT ARE SUBVERTED AS *LEISHMANIA* AMASTIGOTE HOST CELLS

Although we are aware that each discrete phase, in the *Leishmania*-loaded dermis or the non dermal sites distant from the primary site of inoculation, assesses the otherwise versatile activities of many different leukocyte lineages [macrophages, macrophage related dendritic cells/DCs], we decided to first focus on the phagocytes the *Leishmania* amastigotes subvert as *bona fide* host cells namely the mouse macrophages left unexposed to IFNγ, TNF or so called microbial molecular pattern molecules but only exposed to carefully prepared amastigotes recovered from nude mice *i.e* amastigotes without any mouse antibody on their surface (Osorio y Fortea *et al*. 2007). Twenty four hours post exposure to this amastigote population in such mouse macrophages, the *Leishmania amazonensis* amastigotes enter a cell-cycling phase within a communal parasitophorous vacuole. We were curious to extract the macrophage transcriptional signatures at this early time point comparing to the same number of macrophages left unexposed to any amastigote. Thus total RNAs were extracted from these two cultures and once controlled for their quality, they were further processed according to the Affymetrix GeneChip® array technology. Compared to the amastigotes-free macrophage cultures, many transcriptional signatures were indeed extracted from the amastigotes-loaded macrophage cultures, indicating that cell-cycling amastigotes do (a) exploit some of the macrophage metabolic pathways – such as the sterol synthesis pathway, the polyamine pathway – to multiply efficiently, and (b) induce a unique sub-cellular organelle known as the communal parasitophorous vacuole to expand the size of their population. In addition, these macrophages do display, at least transiently, the transcriptional signatures of parasite'host cells able to autonomously shape the early dermal environment toward a parasite-protective niche where the amastigotes not only survive but where they expand the size of their population: indeed we demonstrate the presence of transcripts whose products are expected to be tissue-protective and the absence of transcripts whose products are expected to recruit inflammatory leucocytes. We did not yet extend the present analysis of transcriptional profiling to downstream processes *i.e* when the first generation of the amastigote progeny will be handled by other local mononuclear phagocytic leukocytes, the origin of which is still not clarified. For addressing the latter important issue, it will be interesting to add to our robust culture system – at different time points post the amastigote addition – the circulating HSCs (briefly introduced above) and to check whether these HSCs can rapidly differentiate as unique phagocytic leukocyte populations displaying properties that assess their ability to sense the amastigote-loaded macrophages at early time point of the culture or that assess their ability to handle free amastigotes *i.e* when the progeny of the latter is expected to be released at later time points of the culture. In the first scenario, will these HSC-derived DLs allow natural regulatory T lymphocytes to be retained and to display the tissue-protective functions they are expected to express thus contributing to the up-scaling of the amastigote-permissive and dermis-protective micro-environment ? In the second scenario with the amastigote-handling, do DLs derived from HSCs maintain their unique migratory properties through lymphatic vessels, allowing them to stop in secondary lymphoid organ, a specialized environment where they are expected to deliver signals to T lymphocytes that will display tissue-protective functions, thus again up-scaling the local parasite-favourable environments in both the ear-draining lymph node and the ear where the first wave of amastigotes could have been generated in absence of any tissue-protective T lymphocyte-dependent signals ? These questions need to be extended to other *Leishmania* species especially the *L. donovani* and *L. infantum/chagasi* species : indeed the features of the latter species could result, at the initial dermal point of delivery, in the immediate subversion of the HSCs as parasite-handling shuttling leukocytes that deliver the *Leishmania* very rapidly firstly in the bone marrow ; the bone marrow will be the key compartment from where regularly renewed phagocytic mononuclear leucocytes or local HSC- derived phagocytic leucocytes could be subverted as shuttling cells delivering amastigotes in every peripheral tissue which is known to be under the sustained surveillance of patrolling or residing mononuclear phagocytic leucocytes.

CONCLUDING REMARKS AND PERSPECTIVES

The long term objectives of the ongoing exploration are to characterize, in the amastigotes-loaded mouse ears, the successive host signals that operate a) when *Leishmania* amastigotes first develop intra-cellularly without any restriction b) when the size of the amastigote population is drastically reduced c) when the size of the persisting amastigote population no more fluctuates and is even be known to be present in many different dermal sites (a) distant from the primary sites (b) where any tissue damage will be prevented to occur.

In mammals, like in other organisms, phagocytosis *per se* and/or post phagocytosis transduction signalling processes – otherwise known to be mainly displayed by different professional phagocytic leukocyte subsets, including the HSC-derived ones – are key processes that contribute to the maintenance of tissue steady state as well as to any physiological tissue remodelling. Professional phagocytes, mainly macrophages and dendritic leucocytes, internalize apoptotic cells or bodies contributing to the physiological clearance of senescent cells. Recent evidence still to consolidate indicates that these latter processes are subverted by *L. donovani* species (Gueirard *et al.* 2008). Depending on the *Leishmania* species a fascinating diversity of phagosomes inhabited by intracellular cell-cycling *Leishmania* amastigotes is indeed observed and is under active exploration (McConville *et al.* 2007 ; Osorio *et al.* 2007).

The host cell population(s) hosting persisting amastigotes in the upper dermal sites – the one that was initially permissive to extensive amastigote population size expansion as well as the distant ones where such an amastigote population size expansion does not occur or does rarely occur – deserve further in depth and extended investigations. Indeed we need to unveil the host signals they rely for their persistence. We hypothesize that in unique environments, such as those where parasite-reactive natural regulatory T lymphocytes operate (Belkaid *et al.* 2006), either the macrophages, the steady state non HSC-derived DL or the HS-derived DL that differentiate in the tissue-protected dermis loaded with persisting amastigotes, could be the cells where non-cycling amastigotes persist. Why such a hypothesis ? Many observations make this hypothesis biologically sound. Briefly, when the insect sand fly is no more subverted as a *Leishmania* host only but is acting as *Leishmania* vectors, unique *Leishmania* developmental stages are known to be present in the anterior part of the gut lumen : they have exited or are exiting the cell cycle and display developmental features that pre-adapt them to the mammals the second hosts on which relies the perpetuation of this parasite genus. The developmental stage – known as extra-cellular stumpy *Trypanosoma brucei* that ensures the transmission from the mammal to the blood-feeding *Glossina* spp insect – is known to have exited the cell cycle (Szoor *et al.* 2006). The *Plasmodium* gametocytes hosted by circulating red blood cells are known to have exited the cell cycle… These three examples – others could have been deployed – have led me to propose that the persisting amastigote populations that will further develop in the sand fly gut lumen have also exited the cell cycle. We would expect to detect these non cell-cycling persisting amastigote population not only in the healed primary inoculation site but also in many dermal sites – distant from the primary site –, the latter having being loaded by a limited number of *Leishmania*-housing shuttling leucocytes that are under the active control of tissue-protective natural T regulatory lymphocyte populations. Attempting to characterize these *L. amazonensis in situ* at different stages of the developmental programs of *Leishmania* is a future challenge that will benefit from the additional approaches we also design such as the macrophage microculture system set up for high throughput microscopy (Osorio *et al.* 2007)

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