The fate and persistence of *Leishmania major* in mice of different genetic backgrounds: an example of exploitation of the immune system by intracellular parasites

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SUMMARY

Leishmania spp. are intracellular protozoan parasites that are delivered within the dermis of their vertebrate hosts. Within this peripheral tissue and the draining lymph node, they find and/or rapidly create dynamic microenvironments that determine their ultimate fate, namely their more or less successful expansion, and favour their transmission to another vertebrate host though a blood-feeding vector. Depending on their genetic characteristics as well as the genetic make-up of their hosts, once within the dermis *Leishmania* spp. very rapidly drive and maintain sustained T cell-dependent immune responses that arbitrate their ultimate fate within their hosts. The analysis of the parasitism exerted by *Leishmania major* in mice of different genetic backgrounds has allowed us to recognize some of the early and late mechanisms driven by this parasite that lead to either uncontrolled or restricted parasitism. Uncontrolled parasitism by *Leishmania major* characterizing mice from a few inbred strains (e.g. BALB/c) is associated with the expansion of parasite reactive Th2 CD4 lymphocytes and results from their rapid and sustained activity. In contrast, restricted parasitism characteristic of mice from the majority of inbred strains results from the development of a polarized parasite-specific Th1 CD4 response. This murine model of infection has already been and will continue to be particularly instrumental in dissecting the rules controlling the pathway of differentiation of T cells *in vivo*. In the long run, the understanding of these rules should contribute to the rational development of novel immunotherapeutic interventions against severe infectious diseases.

Key words: *Leishmania major*, T cells, persistance, mice.

INTRODUCTION

Leishmania spp. are protozoan parasites that require the mononuclear phagocytes of their vertebrate hosts to achieve their life-cycle. Briefly, once inoculated within the dermis by a blood-feeding vector they enter rapidly in resident macrophages. In this first, peripheral site, *Leishmania* parasites initiate complex processes resulting in a micro-environment that favours their setting in. These complex processes involve both innate and adaptive immune mechanisms (Lanzavecchia, 1993; Marrack & Kapler, 1994; Abbas, Murphy & Sher, 1996; Fearon & Locksley, 1996).

It has been known for a long time that *Leishmania* spp.-driven parasitism in their vertebrate hosts (human beings, rodents or dogs) can result in either asymptomatic or pathological processes (leishmaniasis) (Sacks, Louis & Wirth, 1993). Such different outcomes are highly indicative of genetic polymorphism of either the parasites or their invertebrate/vertebrate hosts (Lanzaro & Warburg, 1995; Ivens & Blackwell, 1996; Wincker *et al*. 1996). The analysis of the contribution of the genetic component of the vertebrate hosts has been greatly facilitated by devising experimental models based on the use of mice from genetically different inbred strains (Behin, Mauel & Sordat, 1979; Mitchell *et al*. 1983). In this context, the study of the murine model of parasitism (Russell, 1994) with *Leishmania major* has provided important insights in the understanding of the complex interactions between this intracellular parasite and the host genome, particularly the genes used by the host immune system (Blackwell, 1996).

In the present review, we will focus on recent approaches and results using this model pertaining to the characterization of both early and late immunological processes set in motion by *L. major*. Special emphasis will be given to mechanisms used by these parasites to exploit the host immune system, one of the end results being a persistence state probably favouring optimal transmission.

THE FATE OF *LEISHMANIA MAJOR* IN MICE OF DISTINCT GENETIC BACKGROUNDS: FROM THE CONCEPT OF RESISTANCE/SUSCEPTIBILITY TO THE NOTION OF THE EXPLOITATION OF THE IMMUNE SYSTEM

Following inoculation of *Leishmania major* promastigotes into the footpads, mice of the majority of inbred strains (CBA, C3H/He, C57BL/6, 129Sv/

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Ev, B10.D2) develop locally small lesions that spontaneously resolve after a few weeks (between 4 and 8 weeks). Since, once cured, these mice do not develop lesions after a second inoculation of *L. major*, they were designated as 'resistant' mice. In contrast, BALB/c mice develop locally severe lesions that do not resolve spontaneously; since BALB/c mice are not 'resistant' to secondary challenge (Behin *et al*. 1979; Handman, 1992) they were thus designated 'susceptible' mice.

The hypothesis that resistance to secondary challenge could be the consequence of the development of protective T lymphocytes was formulated in the late 1970s (Mitchell *et al*. 1981; Handman, 1992 for review). The effector functions of protective T lymphocytes were expected to be targeted to the host-cells of *L. major*, namely the mononuclear phagocytes, and to operate at both the level of invasion and intracellular growth (Nacy *et al*. 1983). The first hypothesis was validated by results showing that adoptive transfer of T lymphocytes from 'resistant' mice to otherwise very susceptible syngenic nu/nu mice allowed them to control the disease process (Mitchell *et al*. 1981). The demonstration that T cell-derived cytokines lead to activation of macrophages to a parasiticidal state validated the second hypothesis (Nacy *et al*. 1983; Titus *et al*. 1984*a*). It took a longer time to pinpoint the pro-parasite functions of T lymphocytes in 'susceptible' BALB/c mice (Titus *et al*. 1984*b*). The generation of monoclonal antibodies discriminating different T cell subsets (Fitch *et al*. 1993) permitted identification of CD4 T lymphocytes as the major subset paradoxically contributing to both resistance and susceptibility to *L. major* (Titus *et al*. 1987; Liew, 1989).

In 1986, the recognition of the existence in mice of functionally distinct CD4 subsets named Th1, Th2 (Mossman & Coffman, 1989 for review) led to the seminal observation that Th1 cytokine (IFNγ) and Th2 cytokine (IL-4) were associated with 'resistance' and 'susceptibility', respectively (Locksley *et al*. 1987). This observation favoured the hypothesis along which *L. major* triggers distinct CD4 T cell subsets in genetically different hosts. The monitoring, through Northern blot analysis, of those CD4 cytokine transcripts would be expected to reveal the expansion of Th1 (IFNγ and IL2) and Th2 (IL-4, IL5 and IL10) lymphocytes; it clearly showed the development of Th1 cells in lymph nodes of C57BL/6 mice and Th2 cells in lymph nodes of BALB/c mice (Heinzel *et al*. 1991). Other studies have confirmed that spontaneous healing of the lesion in 'resistant' mice correlated with reduced amounts of IL-4 mRNA and reduced numbers of IL-4 producing cells (Morris *et al*. 1993). However, several studies have shown that drastic differences between 'susceptible' and 'resistant' mice in IFNγ-mRNA and numbers of IFNγ producing cells are only observed at late stages of infection (Kopf *et al*. 1996; Stenger *et al*. 1995). Together with the demonstration that IL-4 can inhibit the interferonγ-mediated activation of macrophages (Liew *et al*. 1989; Bogdan *et al*. 1996), these observations illustrate the ability of *L. major* to exploit the immune system of certain hosts (such as BALB/c mice) to their benefit.

THE MATURATION OF FUNCTIONALLY DISTINCT T LYMPHOCYTES IN MICE OF DIFFERENT GENETIC BACKGROUNDS: A CRUCIAL ROLE FOR CYTOKINES DURING THE EARLY STAGE OF *L. MAJOR* INFESTATION?

The first approach to address this question relies on the neutralization of IL-4 either at the onset of infection or in a sustained manner throughout infection with the monoclonal antibody raised by Paul *et al*. (Ohara & Paul, 1985). Briefly, in BALB/c mice receiving injections of anti-IL-4 within the first week of *L. major* infection, the local lesions resolve, leading to a dominant Th1 response (Sadick *et al*. 1990). These results indicated a crucial role of IL-4 in the rapid and sustained differentiation of naïve CD4 T lymphocytes towards the Th2 phenotype after *L. major* inoculation. Even though recent results have questioned the importance of IL-4 in both susceptibility of BALB/c mice to infection and Th2 cell maturation (Noben-Trauth, Kropf & Muller, 1996), other results also using IL-4 deficient mice have clearly shown that these mice exhibit an impaired Th2 cell development and a state of resistance to infection with *L. major* (Kopf *et al*. 1996). In parallel, the early and/or sustained neutralization of IFNγ in C3H/HeN mice was shown to promote the development of Th2 T lymphocytes as assessed by the synthesis of IL-4 by CD4 T lymphocytes restimulated *in vitro* with soluble *L. major* extracts (Scott, 1991). These results suggested a role of IFNγ in the promotion of Th1 cell development and the parallel prevention of Th2 cell expansion.

In agreement with this contention, it has been shown that C57BL/6 mice with disruption of the IFNγ gene develop a Th2 cell response following inoculation of *L. major* (Wang *et al*. 1994). In contrast, administration of high doses of IFNγ to BALB/c mice did not impede Th2 cell development as assessed by the presence of IL-4 transcripts in lymphoid organs (Sadick *et al*. 1990). Furthermore, 129/Sv/Ev mice with disruption of the gene encoding the ligand-binding chain of the heterodimeric IFNγ receptor mounted a polarized Th1 CD4 response similar to that of control wild type mice (Swihart *et al*. 1995). These apparently conflicting results concerning the role of IFNγ in Th1 cell development could reflect genetic differences in the regulation of CD4 T cell subset differentiation. Indeed, recent results from our laboratory have

shown a differential effect of neutralization of IFNγ at the initiation of the infection on the development of IL-4 transcription and secretion in 129/Sv/Ev and C57Bl/6 mice (P. Launois & J. A. Louis, unpublished).

Meanwhile, another cytokine, the heterodimeric IL-12, was identified as a triggering stimulus for IFNγ secretion by NK cells as well as CD4 and CD8 T cells (Chan *et al*. 1991). Using antibodies neutralizing the p35/p40 bioactive IL-12, and 129 Sv/Ev as well as C57Bl/6 mice with either p35 or p40 gene disruption, it was possible to establish the predominant role of this cytokine in the development of Th1 CD4 T lymphocytes in response to *L. major* (Mattner *et al*. 1996).

It is important to emphasize that the predominant role of both IL-4 and IL-12 at the initiation of antigenic stimulation in directing the differentiation of CD4 Naïve T lymphocytes towards the type 2 or type 1 functional phenotype has been most carefully established *in vitro* using naïve CD4 T cells from TCR $\alpha\beta$ transgenic mice (Paul & Seder, 1994; O'Garra & Murphy, 1996; Swain & Cambier, 1996). Interestingly, when both exogenous IL-4 and IL-12 are added to cultures of TCR $\alpha\beta$ transgenic in presence of antigen, but in absence of any other modulating reagents, the effect of IL-4 appears to predominate (Bradley & Watson, 1996; Swain & Cambier, 1996).

LEISHMANIA MAJOR RAPIDLY AND TRANSIENTLY INDUCES THE PRODUCTION OF CYTOKINES THAT SHAPE THE FUNCTIONAL CHARACTERISTICS OF THE T CELL RESPONSES, CONDITIONING THEIR ULTIMATE FATE/PERSISTENCE IN MICE

Using either neutralizing antibodies, exogenous recombinant cytokines and/or knock-out mice, four cytokines have been shown to play an early critical role in the differentiation of naïve T lymphocytes. Indeed, in addition to IL-4, IFNγ and IL-12 mentioned above, TGFβ also deserves attention (Locksley & Reiner, 1995). Therefore the subsequent steps were to compare directly, in mice of genetically different backgrounds, the production of critical cytokines in lymphoid organs during the initial period following *L. major* inoculation. This was possible to achieve due to the design of more quantitative methods by which to monitor directly mRNA transcripts or proteins, in cell suspensions, recovered from the lymphoid organs (Pannetier *et al*. 1993; Reiner *et al*. 1993; Openshaw *et al*. 1995).

Leishmania major *rapidly induces a burst of IL-4 transcripts in BALB/c mice*

The kinetics of IL-4 mRNA expression were carefully compared in BALB/c and C57BL/6 mice during the first days of infection. In contrast to C57BL/6 mice, BALB/c mice exhibited (Fig. 1) in their draining lymph nodes, a burst of IL-4 transcripts peaking at 16 h after parasite inoculation (Launois *et al*. 1995). After returning to baseline level by 48 h, a second increase in IL-4 transcripts was observed in BALB/c mice from days 4 to 5 which remained elevated during the course of the parasitic process (Launois *et al*. 1997*a*) consistent with the development of the type 2 response. In C57BL/6 mice a small and gradual, but consistently 5 times lower than in BALB/c mice, production of IL-4 transcripts was observed peaking at day 4 and subsequently declining at day 7 to the baseline level of non-infected control mice (Launois *et al*. 1995). Another report has also documented the presence of IL-4 transcripts in CD4 lymphocytes recovered from lymph nodes 4 days after inoculation of *L. major*. Although in this study the *L. major* triggered a level of IL-4 transcripts which was usually higher in BALB/c than in C57BL/6 mice, the authors reported that they were similar in some experiments (Reiner *et al*. 1994). These differences in IL-4 transcript production 4 days after infection could be related to the use of *L. major* of different origins.

Is the production of IFNγ, IL-12, rapidly triggered by L. major *in mice of different genetic backgrounds? A still open issue*

Whatever the genetic background of mice inoculated with *L. major*, and in contrast to IL-4, it is difficult to document significant transcription of IFNγ in their lymph nodes during the first 5 days (Reiner *et al*. 1994; Launois *et al*. 1997*a*). However, the data documenting the detection of IFNγ at the protein level deserves comment (Scharton-Kersten *et al*. 1995). Lymph node cells recovered from C3H and BALB/c mice 2 days after inoculation of *L. major* were cultured in the absence of *L. major*-derived antigens, but in presence or absence of anti-IL-4, anti-IL10 and anti-TGFβ antibodies: 72 h later, IFNγ production was determined in the culture supernatants. While in the absence of anti-cytokine(s) neutralizing antibodies, C3H lymph node cells release 6000 pg/ml of IFNγ, BALB/c lymph node cells produce only 700 pg/ml. Interestingly, neutralization of TGFβ resulted in the release of larger amounts of IFNγ by BALB/c cells ($\simeq$ 13·000 pg/ml). IFNγ release was further increased in presence of the three antibodies neutralizing IL-4, IL10 and TGFβ. As far as IL-12 is concerned, the only available published data point out the delayed and low level of IL-12 p40 transcripts in both C57BL/6 and BALB/c lymph nodes as assessed from day 0 to day 7 following *L. major* inoculation (Reiner *et al*. 1994). Monitoring the 24 h production of IL-12 p40 immunoreactive protein in supernatant of BALB/c and C3H lymph node cells

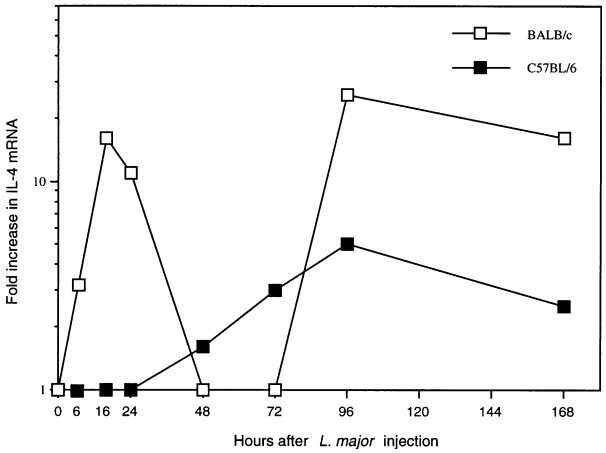

Fig. 1. Kinetics of IL-4 mRNA expression in popliteal lymph nodes following s.c. injection of *L. major* into the hind footpads. BALB/c and C57BL/6 mice were injected s.c. with 3×10^6 stationary phase *L. major* promastigotes. At various times following injection, the relative levels of IL-4 mRNA were determined by competitive RT–PCR (Reiner *et al.* 1993). Results in this figure are expressed as the increase in IL-4 mRNA in mice injected with *L. major* as compared to non-injected mice (reproduced with permission from *European Journal of Immunology* 1995, **25**, 3298).

obtained from day 1 to day 14 after parasite inoculation and cultured in absence of restimulation, a complex pattern was observed. While throughout the period under study the IL-12 p40 level increased by a factor 12 in C3H/mice, there was only a transient increase in BALB/c mice 1 day after infection ($\simeq$ 3 fold) (Scharton-Kersten *et al.* 1995).

IL-12 prevents the early IL-4 burst triggered by Leishmania major

The administration of antibodies neutralizing the bioactive p40/p35 IL-12 to C57BL/6 mice not only allowed the occurrence of the first IL-4 burst, but also and as expected, the second and sustained IL-4 wave, reflecting the type 2 differentiation (Fig. 2). Together, these data suggest that IL-12 produced either constitutively or rapidly in response to *L. major* down-regulates the early burst of IL-4, thus preventing the subsequent Th2 differentiation of T lymphocytes.

Conversely, treatment of BALB/c mice with recombinant IL-12 abolished the burst of IL-4 transcription (Launois *et al.* 1995) preventing the second and sustained wave of IL-4 production (Launois *et al.* 1997*a*).

Experiments were designed to define the time during which treatment of BALB/c mice with IL-12 is efficient in preventing the development of a type 2 cytokine profile as assessed by monitoring IFNγ and IL-4 production. Results have shown that IL-12 has to be given sooner than 48 h after *L. major* inoculation (Launois *et al.* 1997*a*).

The importance of the early burst of IL-4 in directing the subsequent CD4 lymphocyte differentiation towards Th2 in BALB/c mice

The early burst of IL-4 transcripts detected in BALB/c mice lymph nodes was found to be independent of endogenous (i.e. neutralizable) IL-4. However, the subsequent and sustained IL-4 mRNA transcription by CD4 T lymphocytes reflecting Th2 commitment was found to be strictly dependent upon the IL-4 produced as the result of the early burst (16 h) of IL-4 mRNA transcription (Launois *et al.* 1997*a*).

Results from experiments designed to define the time during which the IL-4 must be present in order to drive the subsequent Th2 differentiation of CD4 T lymphocytes have revealed a narrow temporal window of less than 48 h after *L. major* inoculation (Launois *et al.* 1997*a*).

The early L. major*-driven IL-4 burst rapidly induces unresponsiveness to IL-12 in BALB/c CD4 T lymphocytes*

The rapid ($<$ 48 h after *L. major* inoculation) loss of effectiveness of IL-12 in hampering Th2 commitment could be the result of the induction of IL-12 unresponsiveness in CD4 T lymphocytes. This hypothesis was validated by results from experiments showing that CD4 T lymphocytes recovered 72 hours after *L. major* inoculation do indeed loose their reactivity to IL-12 *in vitro* as assessed by IFNγ production. This acquired state of unresponsiveness to IL-12 by CD4 T lymphocytes was a direct consequence of the burst of IL-4 since it was prevented by treatment with anti-IL-4 neutralizing antibody at the initiation of infection (Launois *et al.* 1997*a*).

Using CD4 T cells specific for I-A-^{d}-restricted ovalbumin peptide obtained from α/β TCR transgenic BALB/c and B10.D2 mice, elegant studies have been performed with the aim of comparing the intrinsic tendencies of T cells from different genetic backgrounds to develop towards either Th1 or Th2 cells (Hsieh *et al.* 1995). Upon specific priming *in vitro* under neutral conditions, i.e. in the absence of exogenous cytokines, CD4$^+$ cells from α/β transgenic BALB/c mice develop a Th2 phenotype in contrast to similarly treated cells from B10.D2 mice. Interestingly, during priming, CD4$^+$ T cells from BALB/c mice lost the capacity to respond to IL-12 in terms of IFNγ production during secondary stimulation. In contrast, B10.D2 CD4$^+$ T cells remained responsive to IL-12 (Güler *et al.* 1996). Based on these results, Güler *et al.* have proposed a model of '*L. major* resistance' based on maintenance of the IL-12 signalling pathway rather than on a differential regulation of IL-4 regulation. However, our results rather suggest that the ability of *L. major* to set in,

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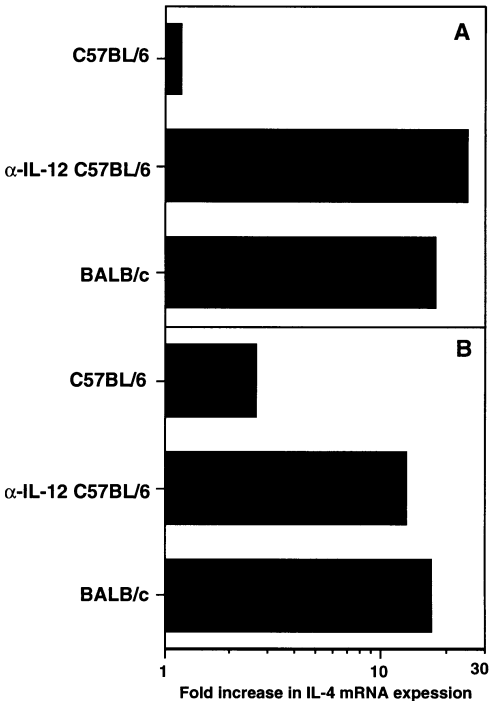

Fig. 2. Anti-IL-12 treatment results in an *L. major*-induced increase in IL-4 mRNA levels in resistant C57BL/6 mice. Mice were injected i.p. with 250 μg anti-IL-12 (polyclonal sheep anti-murine IL-12) 18 h and 2 h prior to s.c. injection of 3×10^6 *L. major*. Mice were killed 16 h (A) and 10 days (B) after injection of parasites, RNA was extracted from popliteal lymph nodes and the relative levels of IL-4 mRNA were determined by competitive RT–PCR (Reiner *et al.* 1993). For mice treated with anti-IL-12 the results are expressed as the fold increase in IL-4 mRNA in mice treated with anti-IL-12 and infected with *L. major* as compared to mice treated with the antibody but not infected with parasites.

in BALB/c mice, is primarily based on an up-regulation of IL-4 production which in turn induces IL-12 unresponsiveness in CD4 T lymphocytes. In a lymphoid environment devoid of IL-4, presumably as a result of the activity of IL-12, it is likely that CD4 T lymphocytes from C57BL/6 mice maintain their responsiveness to IL-12 resulting in their differentiation towards the Th1 phenotype.

LEISHMANIA MAJOR-DRIVEN EARLY CYTOKINE PRODUCTION: POSSIBLE CELLULAR ORIGIN

Although cell types other than $CD4^+$ T cells, i.e. mast cells, basophils, eosinophils, $\gamma\delta$T cells, have been reported to produce IL-4, recent results from our laboratory and the other available data (Bogdan *et al.* 1996) have clearly identified $CD4^+$ T cells themselves as the source of the IL-4 burst directing the subsequence Th2 cell differentiation (Launois *et al.* 1995). These $CD4^+$ T cells do not belong to the minor NK1.1 positive $CD4^+$ subset which has been shown to produce IL-4 under other conditions (Yoshimoto & Paul, 1994; Yoshimoto *et al.* 1995). Indeed the IL-4 mRNA expression rapidly seen in BALB/c mice after infection with *L. major* did not occur in the $CD4^+$ T cell expressing either the Vβ8, Vβ7, or Vβ2 TCR β chains (Launois *et al.* 1995) which constitute the NK 1.1^+ $CD4^+$ subset (Lantz & Bendelac, 1994). Furthermore, cells from the $NK1.1^+$ subset were neither required for Th2 cell development nor progressive disease in BALB/c mice infected with *L. major* (Brown *et al.* 1996; von der Weid *et al.* 1996). More recent results have revealed this rapid IL-4 production, required for subsequent Th2 cell development, results from the activation, after recognition of a single antigen from *L. major*, of a restricted population of $CD4^+$ T cells expressing only the Vβ4-Vα8 TCR heterodimer (Launois *et al.* 1997*b*).

The other cytokines, namely IL-12 and IFNγ, could be rapidly produced and/or released by many different leucocytes once triggered by *L. major*-derived products. For IL-12, these include mononuclear phagocytes, dendritic leucocytes, neutrophils (Trinchieri, 1995). For IFNγ, these include non T leucocytes, such as NK cells, mast cells, as well as $\gamma\delta$ and $\alpha\beta$ T cells (Billiau, 1996).

CONCLUDING REMARKS

Since the first demonstration, in mice, of the crucial role of T lymphocytes in the healing or progression of lesions at the sites of *L. major* inoculation (Mitchell *et al.* 1983) critical reagents and quantitative readout assays have been developed allowing us to dissect the parameters controlling the T cell differentiation and functions. As a result, it is now possible to decipher when and how these *intracellular parasites* can exploit their host immune system to their benefit. We have described the available data indicating that *L. major* is able to trigger extremely rapidly the production of cytokines that determine the pathway of differentiation of naïve T cells of the host, ultimately conditioning the outcome of this parasite/host interplay.

The persistence of *Leishmania* spp. in their hosts, even at a time when cutaneous lesions are completely healed, represents another critical issue, particularly in relation to the transmission of these parasites. The persistence of *L. major* in resistant mice having recovered from a primary lesion has been well documented and, in this context, it has been recently shown that treatment of C57BL/6 mice with an inhibitor of the iNOS, at a time when the cutaneous lesion is resolved, results in an expansion, in the original lesion site, of those parasites which were either quiescent or replicating very slowly (Stenger *et al.* 1996). Although the subtle mechanisms allowing the persistence of *Leishmania* in immune

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mice are far from being elucidated, it is possible that the parasites persisting at the dermal sites in cured hosts reside in dermal macrophages continuously renewed from circulating bone-marrow-derived monocytes harbouring living parasites. Indeed, *Leishmania* spp., even those inducing pathological processes only at the level of the skin, have been detected within bone-marrow cells (Aebischer, Moody & Handman, 1993). Furthermore, among bone-marrow cells, stromal macrophages have been shown to become preferentially parasitized (Leclerq *et al*. 1996) in a murine model of infection with *L. infantum*. Since it has been shown that stromal bone marrow macrophages transiently bind monocyte precursors as well as neutrophils (Crocker *et al*. 1992), the monocyte precursors are therefore in the position of becoming randomly parasitized when contacting parasites harbouring stromal macrophages. These hypotheses will have to be carefully challenged with the proper tools and experimental approaches. If validated, such a pathway will indicate another subtle series of events pertaining to the ability of *Leishmania* spp. to exploit the immune system.

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