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***Leishmania major* intracellular survival is not altered in SHP-1 deficient me^v or CD45^{-/-} mice**

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Abstract

Protozoan parasites of the genus *Leishmania* escape from the immune response by interfering with signal transduction pathways of its host cell, the macrophage, thereby establishing permissive conditions for intracellular survival. Inhibition of macrophage activation after *Leishmania* infection has been suggested to require activation of the host cell phosphatase SHP-1. However, by utilizing infections of SHP-1 deficient (me^v) and CD45 null mutant mice or macrophages, we provide evidence that intracellular survival of *Leishmania major* is not generally dependent on these cellular phosphatases.

Keywords

Leishmania major; immune subversion; host cell signaling pathways; macrophage; trypanosomatid protozoa; intracellular parasite; cellular phosphatases

1. Introduction

Protozoan parasites of the genus *Leishmania* are the causative agents of a range of important diseases in humans and animals termed leishmaniasis, which range from self-healing cutaneous to fatal visceral manifestations, afflicting more than 12 million people worldwide.

Leishmania parasites are transmitted by blood feeding sand flies that inoculate highly virulent metacyclic promastigotes into the mammalian host. Parasites are quickly ingested by host macrophages and differentiate inside fully acidified phagolysosomes into amastigotes, where they replicate and ultimately induce disease pathology. Immunocompetent humans and resistant mouse strains (e.g. C57BL/6) are able to control *Leishmania major* through the development of a protective Th1 response, leading to secretion of IFN γ and activation of

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macrophages to destroy intracellular parasites, which has been shown in murine experimental infection to occur through the production of leishmanicidal NO (Mauel, et al., 1991).

The success of *Leishmania* as an intracellular pathogen relies in part on its ability to interfere with host cell activation, and a potential role for cellular phosphatases in macrophage de-activation has been suggested. For example, *Leishmania*-infected animals treated with the phosphotyrosine phosphatase inhibitor peroxovanadium exhibit a partial rescue of the macrophage leishmanicidal activity, and *Leishmania* infection has been associated with increased activity of SHP-1, a cellular phosphatase implicated in inhibition of IFN γ -mediated host cell activation (Blanchette, et al., 1999, Forget, et al., 2006, Matte, et al., 2000, Nandan, et al., 2002, Olivier, et al., 1998). Together these data suggest a model where *Leishmania* activates cellular phosphatases, thereby promoting survival within the host cell macrophage.

The hypothesis that SHP-1 activation is required for intracellular survival predicts that a reduction in SHP-1 activity should significantly decrease parasite replication and disease pathology. Previously a role for this phosphatase in the intracellular survival of *Leishmania* was suggested by the use of SHP-1 deficient mice (Forget, et al., 2006, Forget, et al., 2001). *In vitro* studies using immortalized macrophage lines generated from SHP-1 deficient motheaten mice indicated that SHP-1 deficient macrophages quickly clear *Leishmania* infection in a nitric oxide-dependent manner, as compared to macrophage lines derived from littermate controls. The lifespan of motheaten mice is only 3 weeks, therefore *in vivo* studies assessing the role of SHP-1 during *Leishmania* infection were performed in viable motheaten (me^v) mice (Blanchette, et al., 2007, Forget, et al., 2006, Forget, et al., 2001, Matte, et al., 2000), as these mice live relatively longer (~9 weeks) and express a SHP-1 protein with 5-10% activity (Shultz, et al., 1993, Tsui and Tsui, 1994). In the *in vivo* studies above, me^v mice infected with *L. major* parasites only exhibited negligible, transient footpad swelling as compared to substantial, sustained swelling in littermate controls, consistent with the hypothesis that SHP-1 activation was important for parasite survival.

The immune dysfunction arising from SHP-1 deficiency in me^v mice leads to increased accumulation of macrophages and neutrophils in some peripheral tissues (Shultz, et al., 1984, Tsui and Tsui, 1994) and severe footpad inflammation, even in the absence of infection (Fig. 1A upper panel; Tsui and Tsui, 1994, Wei, et al., 2004). Thus, the background inflammatory response evident in me^v footpad infections complicates the interpretation of the studies above. Here, we reasoned that infection at other sites not exhibiting inflammatory pathology would provide a better setting for testing the role of SHP-1 in microbial infections. Thus we employed an infection model where parasites were inoculated into the rump (Nabors, et al., 1995), which unlike footpads does not show signs of inflammation or cutaneous pathology in me^v mice (Fig. 1A, lower panel). In contrast to the previous studies utilizing the footpad model, we were unable to detect a substantial effect of SHP-1 deficiency on intracellular survival of *L. major* *in vivo* or *in vitro*.

2. Material and methods

2.1 Mice and parasites

me^v (Tsui and Tsui, 1994) and CD45 $^{-/-}$ mice (Trowbridge and Thomas, 1994) were kindly provided by Dr. Matthew Thomas and Dr. Eric Brown, respectively (Washington University School of Medicine). RAG1 $^{-/-}$ mice and isogenic control mice were purchased from Jackson Laboratories. *Leishmania major* strain Friedlin V1 (MHOM/JL/80/Friedlin) cells were grown in M199 medium at 26°C as previously described (Kapler, et al., 1990).

2.2 Mouse infection assays

Virulence was assessed following inoculation of 10^6 promastigote parasites maintained for 5 consecutive days, and thus at the early stage of stationary phase prior to much parasite death, into the shaved rump (Nabors, et al., 1995) or footpads of 3-6 weeks old female me^v , $RAG1^{-/-}$, $CD45^{-/-}$ mice, and isogenic controls. Infections were monitored by determining either the lesion area (me^v) for rump infections or footpad swelling ($CD45^{-/-}$, $RAG1^{-/-}$) using a Vernier caliper. Lesion parasite burdens were estimated by a limiting dilution assay as described (Späth, et al., 2000). Statistics for lesion size comparison was performed by the Student's t-test using SigmaPlot 8.0 software.

2.3 Macrophage isolation and infection

Peritoneal exudate macrophages (PEM) were isolated by peritoneal lavage using cold Dulbecco's modified Eagles medium (DMEM), washed and resuspended in DMEM/10% FBS. For infection, PEM were seeded in 12 well plates onto 18 mm glass cover slips (3×10^5 cells/ml), non adherent cells were removed by washing after 30 min incubation at 37°C in 5% CO₂ and adherent cells were infected with complement-opsonized promastigotes from day 5 of stationary growth (Racoosin and Beverley, 1997) at a multiplicity of infection of 10 parasites per macrophage. Following a 2 hour incubation at 33°C in DMEM 0.7% BSA under serum free conditions to prevent formation of cell aggregates, non-phagocytosed parasites were removed by multiple washing steps with DMEM without FBS and incubation was proceeded for another 5 days at 33°C. Growth of extracellular parasites was prevented during this period by washing the cells once a day. The number of intracellular parasites was monitored at 2h, 48h and 120h post-infection by nuclear staining and fluorescence microscopy as described (Späth, et al., 2000). Two independent triplicate experiments with similar outcomes were performed. All culture media were tested to be endotoxin-free using the Pyrotell LAL test (Associates of Cape Cod Inc., MA).

2.4 Determination of nitric oxide

NO-derived nitrite in culture supernatants was determined by the Griess reaction (Green, et al., 1982). Briefly, 100 μ l were removed from conditioned medium, incubated with an equal volume of Griess reagent (1% sulfanilamide/0.1% naphthyl ethylene diamine dihydrochloride/2.5% H₃PO₄) at room temperature for 10 min, and the NO₂⁻ concentration was determined in spectrophotometrically at 550 nm using NaNO₂ as a standard. Two independent triplicate experiments with similar outcome were performed.

3. Results

One standard murine infection model for *L. major* involves introduction of infective stage parasites into the footpad; in genetically susceptible mice (BALB/c), parasite replication is uncontrolled leading to progressive disease pathology, while in genetically resistant mice (C57BL/6) a productive immune response leads to parasite containment and healing. Previously, SHP-1 deficient me^v mice were used to probe the role of SHP-1 in *L. major* infections *in vivo* (Forget, et al., 2001), using the footpad model of infection. However, we observed that the use of the mouse footpad model is problematic with me^v mice, because of the severe footpad inflammation exhibited by these animals (Fig. 1A upper panel) (Tsui and Tsui, 1994). Furthermore macrophage activation also is associated with this defect and the increased levels of nitric oxide detected in me^v footpads (Forget, et al., 2001) suggested to us that this site was not well suited for parasite infections, as multiple studies demonstrate that *Leishmania* is not able to survive in macrophages activated prior to infection (Bogdan, et al., 2000, Mael, et al., 1991).

Here, infective *L. major* promastigotes were inoculated into the rumps of groups of me^v mice and congenic C57BL/6 controls, and lesion development was monitored. Parasites elicited a progressive response in WT mice, with onset of lesion formation 7 days after inoculation and a maximum lesion size obtained 3 weeks post-infection (Fig. 1B, upper panel). In contrast, lesion pathology was slightly delayed in me^v mice, not appearing until 12 days post-infection, and reaching maximum size by 5 weeks (Fig. 1B, upper panel). As lesion pathology is not always proportional to parasite numbers, we quantified parasite load by a limiting dilution assay. After 3 weeks, a time where WT and me^v infections showed a three-fold difference in lesion size, the parasite burden was similar in both mice (Fig. 1B, lower panel). Thus in the rump infection model, parasite replication is not dependent on SHP-1, although the lesion pathology was moderately reduced (discussed further below).

We obtained similar results in CD45 null mutant mice, inactivated for a second cellular phosphatase implicated in macrophage activation (Trowbridge and Thomas, 1994). Unlike me^v , CD45-deficient animals do not exhibit any spontaneous skin pathology, allowing for parasite inoculation into the footpads. As expected, infected WT C57BL/6 mice developed small lesions that resolved by 50 days post-infection (Fig. 1C). Similar to the situation in me^v mice, the appearance of lesion pathology in infected CD45^{-/-} mice was delayed with significant lesion development observed 25 days after the infected wild-type control, again there was no significant difference in parasite burden (data not shown). Ultimately, CD45^{-/-} mice failed to control parasite replication lesions pathology.

A complicating factor in these models is that me^v and CD45^{-/-} mice exhibit multiple defects in both innate and acquired immune responses arising from defects in lymphocyte development (Kishihara, et al., 1993, Tsui and Tsui, 1994). To discriminate amongst these, we compared the results from the two phosphatase deficient mice with those obtained in infections of RAG1^{-/-} deficient mice, which are defective only in the generation of mature T and B cells and the acquired immune response (Spanopoulou, 1996). In the rump-infection model, the results were very similar to those obtained with the SHP-1 deficient mice, in that the emergence and progression of lesion pathology was delayed, while parasite numbers were not significantly different (Figs. 1B, 2A). In the footpad infection model, the results were similar to those obtained with the CD45^{-/-} mice, with a delay in the emergence and progression of lesion pathology, but with an ultimate failure to control the disease (Fig. 2B).

These data suggest that the altered disease progression observed in me^v or CD45^{-/-} mouse infections likely arises from the T and B cell developmental deficiencies associated with SHP-1 and CD45 deficiency, rather than defects in innate macrophage defenses. To confirm this supposition, we tested the ability of *L. major* to survive in macrophages in culture. Resident PEMs from WT C57BL/6, CD45^{-/-} and me^v mice were infected with complement-opsonized *L. major* promastigotes. In WT macrophages, parasites survived and replicated over the 5 day infection period (Fig. 3A). Similar results were obtained with infections of macrophages derived from either me^v or CD45^{-/-} mice, confirming that *L. major* survival and replication in macrophages was not compromised by these two phosphatase deficiencies (Fig. 3A). Furthermore, no difference was observed in infections with the LPG-deficient *L. major* mutant *lpgI*⁻ (data not shown). To rule out the possibility that the macrophages were intrinsically deficient in microbicidal functions, we performed infections of macrophages which had been previously activated by treatment with IFN γ /LPS 2 h prior to infection. This resulted in the complete destruction of intracellular parasites, independent of the host cell phenotype (Fig. 3A, right panel), and was associated with the induction of similar levels of nitric oxide in both control and phosphatase-deficient PEMs (Fig. 3B). Thus neither wild-type SHP-1 activity nor CD45 are necessary for intracellular parasite survival or their elimination following macrophage activation.

4. Discussion

Previous studies using phosphatase inhibitors suggested a model where intracellular *L. major* or *L. donovani* evade host cell activation through the induction of cellular phosphatases (Matte, et al., 2000, Olivier, et al., 1998). However, *Leishmania* parasites also express cellular phosphatases, many of which may be important for virulence (Nascimento, et al., 2006), raising the possibility that these inhibitors target essential pathways of both the host and pathogen. Indeed, treatment of *Leishmania amazonensis* amastigotes with the phosphatase inhibitor orthovanadate prior to host cell infection has been shown to significantly decrease parasite virulence (Martiny, et al., 1999). To assess specific host cellular phosphatases we employed genetic approaches, utilizing phosphatase deficient mice.

Previously, *L. major* infections were performed by footpad inoculation in the SHP-1 deficient me^v mouse model (Forget, et al., 2001), which as emphasized above introduces ambiguity into the interpretation due to the severe inflammation in the feet and digits of these mice (Tsui and Tsui, 1994, Wei, et al., 2004). Indeed, the study by Forget *et al* (Forget, et al., 2001) noted the occurrence of spontaneous inflammatory lesions at the footpads with high levels of leishmanicidal nitric oxide and were able to completely reverse the resistant phenotype by the depletion of neutrophils. This fact compromises the specific assignment of SHP-1 activity in the survival of *Leishmania* parasites *in vivo*, as opposed to the nonspecific inflammatory response occurring in the footpads of these animals. In contrast, nonspecific inflammation was not a factor in the two assays used in our study, neither the *in vivo* rump infection nor the *in vitro* cultured peritoneal macrophage model. Under these conditions, *L. major* replication, as assayed by quantitation of parasite burden, was not compromised in the cellular phosphatase deficient me^v or CD45^{-/-} animals or macrophages.

As expected, we did observe differences in the progression of lesion pathology *in vivo* between the WT and the two phosphatase-deficient mice. These differences likely arise from the fact that beyond their effects on macrophage signaling, both SHP-1 and CD45 deficiencies are accompanied by other defects in cellular immune pathways, including T and B cell development and in granulocytes and natural killer cells (Kishihara, et al., 1993, Tsui and Tsui, 1994). Indeed, comparisons of *L. major* infections of SHP-1- and CD45-deficient mice with those of RAG1-deficient mice, that are defective only in T and B cell development (Spanopoulou, 1996), revealed remarkably similar profiles of parasite replication and lesion progression and pathology (Fig. 2). These data suggest that the specific effects of SHP-1 and CD45 deficiency on parasite lesion progression arise primarily through effects downstream of the initial steps of macrophage survival and replication, presumably through T and B cell defects associated with these mutations. Thus our data, obtained in an *in vivo* infection model not compromised by nonspecific inflammatory models, show that there was no alteration in parasite replication in me^v mice.

For our studies we utilized the well established model, *L. major* strain Friedlin V1 for our *in vivo* infections, similar to the study of Forget *et al* (Forget, et al., 2001). Contrary to the previous studies exploring the role of SHP-1 during macrophage infections *in vitro* (Blanchette, et al., 1999, Forget, et al., 2006, Nandan, et al., 2002), we also used this strain for infections of peritoneal exudate macrophages from WT, SHP-1 deficient me^v or CD45^{-/-} mice. While we observed no effect on parasite replication in this model, it is known that me^v mice possess residual levels of SHP-1 activity (5-10 % WT; Shultz, et al., 1993). This residual level potentially is sufficient for *Leishmania* survival in this macrophage infection model. Thus, our data allow us only to conclude that normal levels of SHP-1 activity are not required for *L. major* survival in macrophages.

Many of the previous studies were performed using *L. donovani* infections of immortalized macrophage-like cell lines, generated from SHP-1^{-/-} motheaten mice that lack the residual activity observed in me^v (Forget, et al., 2006, Shultz, et al., 1993). Moreover, these cell lines were derived from motheaten mice on a C3HeB/FeJ genetic background, a mouse strain known to express the *Nramp*^r allele, important for resistance to *L. donovani* infection (Crocker, et al., 1984). As many microbicidal and cellular functions have been attributed to Nramp it is possible that these functions are enhanced in the total absence of SHP-1. The me^v mice utilized in our study express the *Nramp*^s that allows for *L. donovani* replication. Host immune responses to cutaneous *L. major* strains and visceral *L. donovani* strains are known to differ, as do the antimicrobial responses of different macrophage subtypes and/or cell lines. Potentially, the differences observed between our data and the previous studies may reflect the use of different systems and/or models. Assuming any of these factors underlie the discrepant results, nonetheless our data minimally imply that normal SHP-1 activity is not a general requirement for intracellular survival of all *Leishmania* in all macrophage types.

We conclude that for *L. major*, deficiencies in the two cellular phosphatases studied here have little effect on the intracellular survival of *L. major* in murine macrophages. These data suggest that wild-type SHP-1 activity may not be a general requirement for intracellular survival of all *Leishmania* species or in all tissues. While our studies do not exclude the possibility that SHP-1 and CD45 phosphatases play some role in *L. major* infection, perhaps in conjunction with other cellular activities, the data clearly establish that normal levels of neither the SHP-1 nor CD45 phosphatases, nor their activation, are required either generally or absolutely for intracellular *Leishmania* survival. These findings have important relevance for current working models on the mechanisms underlying the evasion of INF γ -induced microbicidal NO by intracellular *Leishmania*.

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Fig.1.

Reduced lesion formation following infections of *L. major* in me^v and $CD45^{-/-}$ mice. (A) Micrographs of footpad and rump areas in C57BL/6 controls and congenic me^v mice. (B) Rump infection. Groups of me^v (◆) and congenic wild-type controls (○, C57BL/6) were inoculated into the shaved rump with 10^6 stationary phase *L. major* Friedlin V1 promastigotes. Lesion development was compared to infected C57BL/6 controls by measuring the lesion area with a Vernier caliper. Parasite burden was determined three weeks post-infection by limiting dilution assay (left panels). (C) Footpad infection. Groups of $CD45^{-/-}$ (■) and C57BL/6 WT control (○) were inoculated with 10^6 stationary phase *L. major* promastigotes and lesion development was

determined by measuring the footpad swelling with a Vernier caliper. (*), p value < 0.05, n = 5-7. After day 35 the me^v but not WT lesions continued to progress (not shown).

Fig. 2.

Delay of *Leishmania* pathogenesis in T cell deficient RAG1^{-/-} mice. Rump infection (A) and footpad infection (B) of T cell deficient RAG1^{-/-} mice (●) and C57BL/6 controls (○), and determination of parasite burden three weeks post-infection was performed as described in legend of Figure 1B. (*), p value < 0.05, n = 5-7.

Fig. 3.

L. major survives normally in phosphatase-deficient macrophages. (A) *Macrophage infection*. *L. major* Friedlin V1 stationary phase promastigotes were complement-opsonized and incubated at a ratio of 10 parasites per host cell with quiescent peritoneal exudate macrophages (left panel), or cells activated 2 h prior to infection with 100 ng/ml LPS and 100 U/ml IFN γ (right panel). The number of intracellular parasites 2h, day 2 and day 5 post-infection was estimated by nuclear staining and fluorescence microscopy. The standard deviation of two to four independent experiments is denoted by the bars. Relative parasite burden was calculated by normalizing values to the initial parasite burden at 2 h post-infection (Racoosin and Beverley, 1997, Späth, et al., 2000). (B) *Nitric oxide determination*. Peritoneal exudate macrophages from me^v and CD45^{-/-} mice, and congenic C57BL/6 (B6) controls were incubated for 24 h in the absence (non-activated, nact.) or the presence (activated, act.) of 100 ng/ml LPS (Sigma, *E. coli* serotype 026:B6) and 100 U/ml IFN γ (from Sigma Chemical Co., St. Louis, MO), and nitrite release into the supernatant was determined by the Griess reaction (Green, et al., 1982). Two independent triplicate experiments were performed. One representative experiment is shown, with standard deviation denoted by the error bars.