Influence of parasite encoded inhibitors of serine peptidases in early infection of macrophages with *Leishmania major*

Sylvain C. P. Eschenlauer,1† Marilia S. Faria,2† Lesley S. Morrison,1 Nicolas Bland,1 Flavia L. Ribeiro-Gomes,2 George A. DosReis,2 Graham H. Coombs,3 Ana Paula C. A. Lima2‡ and Jeremy C. Mottram1*‡

1Glasgow Biomedical Research Centre, Wellcome Centre for Molecular Parasitology and Division of Infection and Immunity, Faculty of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8TA, UK.
2Instituto de Biofisica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ 21949-900, Brazil.
3Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow G4 0NR, UK.

**Summary**

Ecotin is a potent inhibitor of family S1A serine peptidases, enzymes lacking in the protozoan parasite *Leishmania major*. Nevertheless, *L. major* has three ecotin-like genes, termed inhibitor of serine peptidase (ISP). ISP1 is expressed in vector-borne procyclic and metacyclic promastigotes, whereas ISP2 is also expressed in the mammalian amastigote stage. Recombinant ISP2 inhibited neutrophil elastase, trypsin and chymotrypsin with *K*<sub>i</sub> between 7.7 and 83 nM. *L. major* ISP2–ISP3 double null mutants (∆isp2/3) were created. These grew normally as promastigotes, but were internalized by macrophages more efficiently than wild-type parasites to BALB/c mice at the early stages of infection, but became equivalent as the infection progressed. These data support the hypothesis that ISPs of *L. major* target host serine peptidases and influence the early stages of infection of the mammalian host.

**Introduction**

*Leishmania* are trypanosomatid parasitic protozoa that cause a spectrum of human diseases, the leishmaniases, ranging from a lethal visceral infection to milder cutaneous ulcers. Their life cycle involves a flagellated procyclic promastigote stage that multiplies in the sandfly gut, a non-dividing flagellated metacyclic promastigote stage within the sandfly mouth parts, and a non-motile amastigote form that proliferates in mammalian macrophages. Peptidases play important roles in the pathogenicity of many parasitic protozoa, including *Leishmania* (Sajid and McKerrow, 2002; Mottram *et al*., 2004). The genome of *Leishmania major*, one of the causative agents of cutaneous leishmaniasis in the Old World, has been sequenced (Ivens *et al*., 2005), revealing the presence of at least 153 identifiable peptidases that can be classified into the major catalytic types (cysteine-, serine-, metallo-, threonine and aspartic-) and represent ~2% of the genome. One of the major mechanisms used to control the activity of peptidases in mammals is a tight interaction with natural peptidase inhibitors such as serpins, cystatins and tissue inhibitor of metalloproteinases (Rawlings *et al*., 2004). The *L. major* genome does not appear to contain any orthologues of these molecules, yet it is not devoid of natural peptidase inhibitors (Ivens *et al*., 2005). The first to be identified in *L. major* was an inhibitor of cysteine peptidases (ICP), which is a member of the chagasin family of inhibitors first identified in *Trypanosoma cruzi* (Monteiro *et al*., 2001) and subsequently found in a variety of bacterial and protozoan pathogens (Rigden *et al*., 2002; Sanderson *et al*., 2003). In *T. cruzi* and *Trypanosoma brucei* chagasin/ICP is a modulator of parasite differentiation (Santos *et al*., 2005; 2007),...
while *L. major* ICP is thought to play a role in the host–parasite interaction (Besteiro *et al*., 2004). *L. major* ICP and *T. cruzi* chagasin have an unusual immunoglobulin-like fold with a cystatin-like mechanism of inhibition, which distinguishes them from all other known peptidase inhibitors (Salmon *et al*., 2006; Smith *et al*., 2006).

A second group of putative peptidase inhibitors identified in the *L. major* genome are orthologues of the *Escherichia coli* serine peptidase (SP) inhibitor ecotin and have been termed inhibitor of serine peptidases (ISPs). Ecotin is an 18 kDa protein first isolated from the periplasm of *E. coli* (Chung *et al*., 1983). It forms dimers and inhibits a wide range of SPs from the S1A peptidase family (trypsin fold) of clan PA(S), which includes trypsin, chymotrypsin, neutrophil elastase (NE) and cathepsin G (Chung *et al*., 1983; McGrath *et al*., 1995). There are no reports that ecotin inhibits SPs of other families or catalytic classes. In addition, Chung *et al*. (1983) were unable to identify an *E. coli* peptidase sensitive to ecotin, suggesting that ecotin may protect the cell against exogenous S1A peptidases (Eggers *et al*., 2004), which are involved in processes such as coagulation, fibrinolysis and host defence (Stoop and Craik, 2003).

The structure of ecotin and its mechanism of SP inhibition have been investigated extensively (McGrath *et al*., 1991a; Perona and Craik, 1997; Yang *et al*., 1998). Ecotin has also been studied as a macromolecular scaffold to develop specific peptidase inhibitors with potential therapeutic applications (Stoop and Craik, 2003). Methionine is present in its reactive site (McGrath *et al*., 1991b), although it is not essential for ecotin’s inhibitory activity against trypsin (Seong *et al*., 1994). This dichotomy was resolved when the crystal structure of ecotin revealed that the protein forms a head to tail dimer that interacts with its substrate via multiple binding sites, including the active site of the peptidase (Shin *et al*., 1996; Yang *et al*., 1998). This also explains why a wide range of SPs from the S1A family are inhibited by ecotin.

Although *L. major* has 13 SPs belonging to six families, the parasite apparently lacks genes encoding SPs from the S1A family of clan PA(S) (Ivens *et al*., 2005). Three ecotin orthologous ISP genes are present in the *L. major* genome and while it is possible that the encoded ISPs could regulate the activity of *L. major* SPs other than family S1A, or those of other catalytic classes, it is likely that the ISPs, like ecotin, inhibit host SPs. This could be the trypsin and chymotrypsin-like peptidases found in the gut of the sandfly vector (Ramalho-Ortigao *et al*., 2003) or the abundance of S1A SPs that occur in the mammalian host. Postulated (*in vivo*) targets of bacterial ecotin include SPs expressed by cells of the innate immune system (i.e. neutrophils, mast cells, macrophages), such as NE, trypase and cathepsin G, as well as enzymes participating in the coagulation cascades (Eggers *et al*., 2004).

Activated neutrophils release SPs together with chromatin fibres forming extracellular traps that disarm pathogens and play a role in killing bacteria (Brinkmann *et al*., 2004). Ecotin protects *E. coli* from killing by neutrophils, primarily due to the inhibition of NE (Eggers *et al*., 2004).

Macrophages are the principle host cell for *Leishmania*, but neutrophils can act as their first residence in a mammalian host – with the infected neutrophils serving as vehicles for parasite dissemination (Laufs *et al*., 2002; Van Zandbergen *et al*., 2004; Van Zandbergen *et al*., 2007). Equally monocytes, like neutrophils express NE and cathepsin G (Kargi *et al*., 1990) and have plasma membrane receptors that enable the uptake of these enzymes from the surrounding environment (Campbell, 1982). *Leishmania* also primes mast cell degranulation following exposure to chymase and trypase (de Oliveira *et al*., 2005). Thus we hypothesize that the numerous SPs produced and/or taken up by cells of the immune system in the course of *Leishmania* infection are potential targets for the ISPs. We begin to address the physiological targets of the *Leishmania* ISPs by creating *L. major* mutants deficient in ISP2 and ISP3 and characterizing their phenotype during the early phases of macrophage infection.

### Results

**ISP genes of *L. major***

We identified three *ISP* genes in the *L. major* genome (http://www.genedb.org), *ISP1* (LmjF15.0300), *ISP2* (LmjF15.0510) and *ISP3* (LmjF15.0520). *L. major* ISP1 is located on the same transcription unit 5’ to ISP2 and ISP3, which are found in tandem (Fig. 1A). An ISP2 homologue could be identified in the syntenic locus for both *T. brucei* (Tb927.5.1880) and *T. cruzi* (Tc00.1047053508533.40), but no ISP3 gene was present in either of these species. ISP1 is also present in the syntenic locus in *T. brucei* (Tb927.5.1730), but the locus could not be found in the *T. cruzi* genome – possibly because the data set is incomplete.

*Leishmania major* ISP1 and ISP2 encode predicted proteins of 16.5 kDa and 17.5 kDa respectively, which is similar to the 16.1 kDa for the mature form of *E. coli* ecotin. ISP3 is predicted to encode a 41.8 kDa protein, with an ecotin-like domain at the N-terminal end of the protein. The C-terminal domain of the protein does not have sequence identity with known proteins or motifs. An alignment of ecotin with the three *L. major* ISPs showed that they have a shorter N-terminus compared with ecotin (Fig. 1B). *E. coli* ecotin is exported to the bacterial periplasm and the first 20 amino acids of the protein sequence act as an export signal peptide. The P1 reactive site methionine of ecotin occurs in *L. major* ISP2, but not ISP1 or ISP3 (Fig. 1B). The percentage identities between

---

© 2008 The Authors
Journal compilation © 2008 Blackwell Publishing Ltd, *Cellular Microbiology*, 11, 106–120
ecotin and ISP1, ISP2 and ISP3 are 32%, 32% and 30% respectively. Structural analysis of the trypsin–ecotin complex has revealed two secondary substrate-binding sites, both of which are surface loops (Yang et al., 1998).

The primary sequence alignment of ecotin and the L. major ISPs shows that the amino acids of these secondary binding sites are highly conserved between the aligned sequences (Fig. 1B). However, ecotin has a disulfide bond next to its P1 methionine (Shin et al., 1996) and the three L. major ISPs lack the cysteine residues that form this bond, although they are conserved in many bacterial ecotin sequences (Eggers et al., 2004).

Activity and stage-regulated expression of L. major ISPs

Recombinant ISP1, ISP2 and truncated ISP3 (rISP1, rISP2, rISP3), each with an N-terminal histidine tag, were expressed and purified from E. coli. rISP2 was found to inhibit the human SPs, NE, trypsin and chymotrypsin, with Ks of 7.7 (±1.4) nM, 83 (±2.3) nM and 19 (±4.2) nM respectively. Antibodies raised against rISP1 and rISP2, and an antipeptide antibody raised for rISP3, allowed the expression levels of the three L. major ISPs to be examined by Western blot analyses of three life cycle stages (Fig. 2). The purified ISP2 antibodies did not cross-react with ISP1 but the ISP1 antibodies still recognized ISP2. However, discrete sized ISP1 and ISP2 proteins could be resolved on 4–12% gradient SDS-PAGE. Expression of ISP1 could only be detected in the procyclic and metacyclic promastigotes. ISP2 was expressed in all three life stages of the parasite with the levels of expression being greater in metacyclic promastigote and amastigote stages compared with procyclic promastigotes. ISP3 was not detected in any of the life cycle stages of the parasite. The antipeptide antibody, however, was able to detect ISP3 when it was overexpressed in procyclic promastigotes using an extra-chromosomal construct (Fig. 2). Elongation factor 1 alpha was used as a loading control.
null mutants also differentiated to metacyclic promastigotes normally, as assessed by the peanut agglutinin agglutination assay (Sacks and Perkins, 1984) and the detection of the metacyclic marker HASPB (Fig. 3C). Analyses of the infectivity of stationary phase promastigotes to macrophages in vitro revealed that ∆isp2/3 mutants were internalized by elicited macrophages more efficiently than WT parasites whether infections were performed in the presence (Fig. 4A) or absence (Fig. 4B) of heat-inactivated fetal bovine serum. Importantly, the increased internalization of ∆isp2/3 was reduced to the WT levels in the presence of the SP inhibitor aprotinin or of rISP2 (Fig. 4B), indicating that the entry route used by ∆isp2/3 is dependent on the activity of trypsin-like SPs. Neither aprotinin nor rISP2 affected the uptake of WT parasites (Fig. 4B), suggesting that the SP-dependent entry route is selectively triggered by ISP-deficient L. major. The enhanced internalization of ISP-deficient parasites was reverted when aprotinin was incubated with macrophages before the addition of parasites, but not if this SP inhibitor was added at later times during interaction (Fig. 4C). These observations suggest that SPs are being triggered at the initial stages of the interaction between L. major and the host cell. The uptake of ∆isp2/3 by resident peritoneal macrophages was likewise higher in comparison with WT (data not shown), indicating that ISP2 and/or ISP3 modulate parasite internalization in both resting and activated macrophages.

The entry of L. major into macrophages is mediated in part by the interaction of the parasite with the complement 3-receptor (CR3, CD11b/CD18, Mac-1) (Moss and Edelson, 1985; Brittingham et al., 1999; Kedzierski et al., 2004), which acts in conjunction with the mannose-fucos receptor (MFR) for parasite engulfment, at least for L. donovani (Blackwell et al., 1985). Thus we sought to address if CR3 is involved in the uptake of ∆isp2/3 in our assay conditions, by blocking the CD11b subunit with MAB M1/70 (Fig. 4D). We observed a small reduction in the internalization of WT parasites upon CD11b blocking (Fig. 4D, white bars). However, blocking of CD11b dramatically decreased the uptake of ∆isp2/3, while pre-incubation of macrophages with control IgG did not affect parasite internalization (Fig. 4D, black bars). Uptake of ∆isp2/3 : ISP2–ISP3 parasites was not affected by CD11b blocking, strongly suggesting that enhanced uptake of ∆isp2/3 promastigotes is mediated by increased CR3-mediated phagocytosis. Mannan purified from Saccharomyces cerevisiae was used in order to verify if MFR was involved in parasite ingestion (Fig. 4E). As previously observed, blocking of CD11b reduced the uptake of ∆isp2/3 but mannan did not affect the uptake of the three parasite lines, suggesting that MFR does not contribute to the ingestion of L. major promastigotes in this system.
Fig. 3. Generation of ISP2 and ISP3 double null mutants.
A. Schematic representation of the ISP2–ISP3 locus in WT L. major (upper) and the constructs for gene deletion. ORFs are shown as grey arrows and 5′ FR and 3′ FR boxes represent the FR DNA sequences used for gene targeting. The predicted DNA fragment sizes after restriction digest are shown. HYG, hygromycin-resistance gene; BLE, bleomycin-resistance gene.
B. Southern blot of the WT L. major (lanes 1, 3 and 5) and Δisp2/3 (lanes 2, 4 and 6) digested with AgeI or EcoRI/BamHI and probed with radiolabelled 5′ FR, ISP3 ORF and 3′ FR probes.
C. Western blot of cell extracts from 0.5 × 10⁷ purified metacyclic promastigotes of WT L. major (lane 1), Δisp2/3 (lane 2) and Δisp2/3:ISP2–ISP3 (lane 3) using antibodies against ISP2, HASPB and EF1α.
ISP2 and ISP3 modulate macrophage phagocytic activity through the inhibition of SPs

The entry of L. major into macrophages is a largely passive process for the parasite, which relies on the phagocytic capacity of host cells (reviewed in Rittig and Bogdan, 2000). Thus we examined if the overall phagocytic activity of macrophages was affected in the presence of the parasites, using fluorescein-coupled latex beads (FITC-beads) as a tracer to measure phagocytosis. The phagocytosis of FITC-beads was similar in the absence (Fig. 5A) or in the presence of WT stationary phase promastigotes. Stationary phase promastigotes were incubated with elicited peritoneal macrophages from BALB/c mice in RPMI supplemented with 10% (v/v) FCS (A) or 0.1% (v/v) BSA (B) at a 3:1 parasite : macrophage ratio for 3 h. Extracellular parasites were removed with three washes and the adhered cells were fixed and subsequently stained with Giemsa. In (B), the interaction was performed in the presence of 4 μg ml⁻¹ of purified rISP2 or aprotinin (APT). In (C), 10 μg ml⁻¹ of aprotinin was incubated with macrophages for 5 min prior to incubation with Δisp2/3, or 15, 30 or 60 min after addition of parasites and was present for the duration of the assay. In (D), macrophages were pre-incubated with 10 μg ml⁻¹ of anti-CD11b M1/70 or with control rat anti-mouse IgG2b for 30 min at 37°C, followed by three washes, before addition of promastigotes. In (E) macrophages were incubated as in (D) or with 1 mg ml⁻¹ of purified Saccharomyces cerevisiae mannan. The number of intracellular parasites was estimated by counting using light microscopy. The experiments were performed in triplicate, five independent times in A and B and two independent times in (C to E), the data are means ± SD. Key: white bars, WT parasites; black bars, Δisp2/3; grey bars, Δisp2/3 : ISP2–ISP3, M: anti-CD11b M1/70. In (A) the asterisk indicates statistical significance at P < 0.05 in relation to WT and P < 0.01 in relation to rISP2, in (B) the asterisk indicates significance in relation to all other samples at P < 0.01. In (D) the significant p values between samples are indicated in the graph, in (E) the asterisk indicates significance at P < 0.001 from all other samples without asterisk.
phase promastigotes (Fig. 5B), suggesting that the entry of WT *Leishmania* does not alter the basal phagocytic activity of macrophages. In contrast, the uptake of beads by macrophages increased significantly in the presence of the ∆isp2/3 mutants (Fig. 5C) but not in the presence of the ∆isp2/3 : ISP2–ISP3 line (Fig. 5D), indicating that exposure and/or attachment to ∆isp2/3 selectively enhances the phagocytic activity of macrophages. Quantification of the average number of intracellular beads revealed that macrophages internalized twofold more beads in the presence of the mutants (Fig. 5E), and that the increase was reversed by addition of aprotinin or rISP2. These observations suggest that the deletion of ISP2 and ISP3 rendered *Leishmania* capable of upregulating the phagocytic activity of macrophages, resulting in more efficient uptake of the mutant parasites. Furthermore, the mechanism underlying this enhanced phagocytosis requires the activity of family S1A SP.

ISP2 and ISP3 are required for the normal development of *L. major* in macrophages in vitro

The engulfment of *Leishmania* by macrophages is followed by the *Leishmania*-containing phagosome developing into a parasitophorous vacuole, a process that involves the acquisition of some late endosomal and lysosomal characteristics (Antoine *et al.*, 1998; Kima, 2007). The persistence of intracellular parasites is believed to rely on the differentiation of the engulfed promastigotes to amastigotes, which are resistant to the acidic environment and hydrolases (Kima, 2007). We examined the ability of ∆isp2/3 to establish productive infections in macrophages in vitro by comparing the number of intracellular parasites soon after entry (3 h) and after a 3 day period (Fig. 6). As before, higher numbers of intracellular ∆isp2/3 than WT were detected 3 h after infection (Fig. 6A). Also as expected, the number of intracellular WT amastigotes present at 73 h after infection...
(Fig. 6B) was significantly higher (44/100 macrophages) than the number of intracellular promastigotes present 3 h after infection (9/100 macrophages) (Fig. 6A). In contrast, the number of intracellular $\Delta$isp2/3 amastigotes (defined as amastigotes based on morphology) 73 h after infection (18/100 macrophages) was similar to that of intracellular promastigotes found 3 h after infection (22/100 macrophages) (Fig. 6B), indicating that the deletion of ISP2 and ISP3 affected the parasites’ ability to multiply inside macrophages. The number of intracellular $\Delta$isp2/3 : ISP2–ISP3 amastigotes was equivalent to that of WT at 73 h after infection (46/100), corroborating that the defect in parasite multiplication inside macrophages over a 3 day period was caused by the lack of ISP function. We observed release of $\Delta$isp2/3 parasites from infected macrophages after several days (not shown), showing that the intracellular parasites were viable.

Importantly, addition of rISP2 or aprotinin during the initial promastigote–macrophage interaction restored the ability of intracellular $\Delta$isp2/3 to develop normally in both elicited (Fig. 6B) and resident macrophages (data not shown). These observations suggest that the inability of intracellular $\Delta$isp2/3 to multiply is a result of the lack of control of host cell SP activity at the initial stages of parasite–macrophage interaction. We asked if blocking of CR3 by M1/70 at the entry stage of $\Delta$isp2/3 would improve the subsequent parasite intracellular survival and/or development in macrophages (Fig. 6C), as seen for the inhibition of SPs (Fig. 6B). We found reduced numbers of intracellular $\Delta$isp2/3 at 3 days post infection as compared with WT and to $\Delta$isp2/3 : ISP2–ISP3, even when binding to CR3 was blocked before parasite uptake, suggesting that signals triggered by CR3 are not responsible for the downmodulation of parasite growth. To analyse this more
fully, we monitored the growth of intracellular parasites every 24 h after infection. This revealed that the numbers of intracellular Δisp2/3 had significantly declined by 24 h after infection and then multiplied only slightly up to 73 h, while WT or the re-expressing parasites increased steadily in numbers over a 3 day period (Fig. 6D). These data show that the growth of Δisp2/3 parasites was delayed but not totally impaired.

Δisp2/3 inhibited the production of nitric oxide by lipopolysaccharide (LPS)-stimulated macrophages to the same levels as WT (not shown), removing the possibility that inability of Δisp2/3 to develop in the macrophage was due to defects in its capacity to induce suppression of reactive nitrogen species. Likewise, Δisp2/3 retained the capability to block LPS-induced expression of IL-12 by macrophages (not shown). These observations suggest that the deficient development of intracellular Δisp2/3 is not linked to alterations in the ability to suppress macrophage activation. Moreover, Δisp2/3 amastigotes isolated from mice lesions were able to infect macrophages in vitro and multiply at the same level as WT (data not shown), suggesting that the defect in intracellular development of Δisp2/3 is entirely restricted to the initial stages of infection with promastigotes.

**Infection of mice with ISP mutants**

The consequences of the deficiency in ISP2 and ISP3 to Leishmania infection in the host were evaluated by the inoculation of purified metacyclic promastigotes into the footpads of susceptible mice, followed by the determination of parasite burden for 1–5 days post infection (Fig. 7). Following 24 h of infection, the parasite burden in animals infected with the mutant line was about fourfold higher than those of mice infected with WT or the re-expressing parasites (Fig. 7A). The higher burden of Δisp2/3 parasites in mice was sustained until day 3 after infection (Fig. 7B), but became approximately equivalent to that of WT by day 5 (Fig. 7C). We also monitored the progression of lesions in infected mice over a 21 day period and...
observed that the lesions of animals infected with metacyclic promastigotes of Δisp2/3, the re-expressing line or WT developed similarly (Fig. 7D).

Discussion

In the present study we have characterized L. major ISP genes that encode proteins with sequence similarity to the bacterial SP inhibitor ecoltin. The distribution of ecoltin is unusual in that it has been found in a very limited number of bacteria; just 15 genera including *Escherichia*, *Pseudomonas*, *Salmonella* and *Yersinia*, from the 335 fully sequenced bacterial genomes available in the Comprehensive Microbial Resource (http://cmr.jcvi.org/cgi-bin/CMR/shared/Genomes.cgi) and in trypanosomatids (*Leishmania* and *Trypanosoma*). The ISP genes may therefore have arisen in trypanosomatids due to a horizontal gene transfer event from a bacterium into an organism that preceded the divergence of *Trypanosoma* and *Leishmania* (Oppendoes and Michels, 2007). There is also divergence in the trypanosomatid *ISP* gene family as *ISP3* is only found in *Leishmania* and not *Trypanosoma*. Its proximity to *ISP2* and the unusual C terminal extension encoded indicates that *ISP3* may have arisen through an *ISP2* gene duplication event, after the divergence of *Leishmania* and *Trypanosoma*.

Ecoltin is a potent inhibitor of S1A family (trypsin-fold) SPs, yet *E. coli* lacks these enzymes. This also appears to be the case for *Leishmania*, as the parasite lacks S1A family SPs but contains ISP2, which we have demonstrated to be a potent inhibitor of different mammalian S1A family SPs including trypsin and NE. In the absence of S1A family SPs in *Leishmania*, the ISPs could potentially target host trypsin-type SPs. The possible role of *Leishmania* ISPs in protecting the parasite from the digestive SPs in the sandfly gut is currently under investigation, while in this study we investigated the role of ISPs in the early stages of infection in the mammalian host. Unlike ecoltin, *Leishmania* ISPs lack an N-terminal signal sequence, suggesting that they are not targeted into the secretory pathway, although they may be secreted via a non-classical secretory mechanism or released by apoptotic promastigotes (Van Zandbergen et al., 2006; 2007). Thus, our hypothesis incorporates the idea of inhibitor release by the parasite causing an effect on mammalian SPs.

ISP1 expression could be detected in both procyclic promastigotes and metacyclic promastigotes, but not amastigotes – consistent with the protein having a role in the sandfly vector or early in infection of the mammalian host. In contrast, ISP2 is expressed in all life cycle stages, but with a higher level of expression in metacyclic promastigotes and amastigotes, suggestive of a role in initiation and persistence of infection of the mammalian host. Here we have investigated the roles of *ISP2* and *ISP3* by generating *ISP2/ISP3*-deficient *L. major*. As expression of ISP3 was not detected in either metacyclic promastigotes or amastigotes, it is likely that the phenotypes observed in this study reflect *ISP2* and *ISP3* are primarily due to ISP2.

The phenotypic analyses of the Δisp2/isp3 mutants showed that the lack of these genes enhanced parasite infectivity in vitro, as a result of increased parasite uptake by peritoneal macrophages. We also showed that exposure of macrophages to Δisp2/3 increased the phagocytosis of inert particles, suggesting that upregulation of macrophage phagocytic activity is responsible for increased infectivity of Δisp2/3 in vitro. Increased uptake of Δisp2/3 was observed in the presence or absence of serum, indicating that a common mechanism susceptible to regulation by ISPs is involved. CR3 was found to be the predominant receptor used by Δisp2/3, but not by WT or the re-expressing parasites. Although the fixation of complement components at the surface of *Leishmania* mainly requires the presence of serum as a source of C3 (Mosser and Edelson, 1987), the C3 endogenously produced by macrophages might represent an alternative source of complement in its absence. Additionally, LPG and gp63 have binding sites for CD11b (Russell and Wright, 1986; 1987; Talamas-Rohana et al., 1990). We observed that Δisp2/3 adhere to macrophages more than WT at low temperature, in the absence of serum (M.S. Faria and A.P.C.A. Lima, unpubl. obs.), suggesting that more efficient adhesion contributes to increased infectivity. Although CR3-mediated phagocytosis, without FMR association, seems to be the main mechanism driving the internalization of ISP-deficient *Leishmania*, we cannot discount the possibility that additional receptors are involved in the uptake of the mutant lines.

Intriguingly, our results show that the receptor(s) involved in the differential engulfment of Δisp2/isp3 are associated, either directly or indirectly, with the activity of SPs as internalization of the parasites were brought to WT levels in the presence of aprotinin or rISP2. We have not addressed which SPs are mediating increased activation of phagocytosis, but NE is one candidate. It has been reported that in polymorphonuclear leukocyte NE is an endogenous ligand for CR3, and that its binding, via an active site interaction, to CR3 modulates leukocyte adhesion via competition with other endogenous ligands (Cai and Wright, 1996). NE is also expressed by macrophages, although its association with CR3 has not been shown in this cell type. Furthermore, the activity of NE triggers toll-like receptor 4-mediated responses (Devaney et al., 2003), suggesting that the activity of SPs might be tightly associated with the modulation of leukocyte receptors. Another S1A family SP, factor X, has also been shown to associate with CR3, in an interaction involving peptidase activation (Altieri et al., 1988) and so this could also be a target of ISPs.
The binding to and activation of CR3 by \textit{L. major} promastigotes leads to the inactivation of infected cells, contributing to the subsequent intracellular survival of the parasite and allowing amplification as intracellular amastigotes at sites of infection (Mosser and Edelson, 1987). We observed that intracellular \(\Delta\text{isp2/3} \) were partially eliminated within 24 h of infection, and slowly recovered growth after 48 h. Importantly, \(\Delta\text{isp2/3} \) grew normally inside macrophages when SP inhibitors were added to the initial stages of the promastigote–macrophage interaction, suggesting that the activity of SPs triggered during parasite contact is responsible for the subsequent defect in the intracellular development of ingested parasites. The production of nitric oxide (NO) has been implicated in the control of the growth of \textit{L. major} in mice (Wei \textit{et al}., 1995), but we observed no difference in the capacity of \(\Delta\text{isp2/3} \) parasites to inhibit LPS-induced NO production by macrophages as compared with WT (data not shown), suggesting that this reactive nitrogen species is not mediating selective killing of \(\Delta\text{isp2/3} \). However, we cannot exclude that SPs engaged by \(\Delta\text{isp2/3} \) during interaction with macrophages trigger the production of reactive oxygen species, which could be responsible for the control of parasite growth. These observations strongly suggest that although ISP deletion enhances parasite entry to macrophages, this is not in fact beneficial as ISP function at the initial stages of parasite–macrophage interaction is required to prevent premature death and enable normal intracellular growth.

The defect in intracellular development of \(\Delta\text{isp2/3} \) \textit{in vitro} was different from the result observed \textit{in vivo}, where mice infected with \(\Delta\text{isp2/3} \) had a higher parasite burden at the early stages of infection (up to three days) in comparison with those infected with WT or with the re-expressing lines. It has been reported that the phagocytosis of apoptotic neutrophils by macrophages infected with \textit{L. major} enhances the intracellular multiplication of parasites in cells derived from BALB/c mice due to the induction of TGF-\(\beta\) (Ribeiro-Gomes \textit{et al}., 2004). The depletion of polymorphonuclear leukocyte prior to infection resulted in lower parasite burden 13 days later, indicating that neutrophils are required for the optimal development of \textit{L. major} \textit{in vivo}. More recently, studies \textit{in vivo} using intravital microscopy suggest that neutrophils recruited to the site of infection rapidly engulf \textit{L. major} and are the primary host cells for the parasites within the first 48 h (Peters \textit{et al}., 2008). \textit{In vivo}, it is possible that, similar to what was observed in macrophage infections \textit{in vitro}, \(\Delta\text{isp2/3} \) could be internalized by neutrophils at higher numbers in comparison with WT parasites and survive in these cells, leading to higher parasite burden in the first 24 h. Alternatively, we could hypothesize that the signals triggered by \(\Delta\text{isp2/3} \) during the parasite–macrophage contact, which resulted in the downregulation of intracellular para-site replication observed \textit{in vitro}, are overcome \textit{in vivo} by the deactivating stimulus given by neutrophils. In the absence of such downregulation, \(\Delta\text{isp2/3} \) that were internalized at higher numbers by macrophages can develop normally, leading to enhanced parasite burden, which is controlled later on. \(\Delta\text{isp2/3} \) was capable of generating lesions in the footpad of mice, although the lesions were slightly smaller in size in the first 11 days. The production of IFN-\(\gamma\), IL-4, IL-10 and TNF-\(\alpha\) in lymph nodes of mice 15 days after parasite inoculation was similar between infections with the different lines, suggesting that there was no major alteration in the development of the adaptive immune response. ISP1 is also expressed in metacyclic promastigotes and might compensate in part for the loss of ISP2 in the early infection process, a hypothesis that can be tested with \(ISP1/ISP2/ISP3\) triple mutants that we have recently generated. ISP1 expression could not be detected in amastigotes, so is unlikely to compensate for ISP2 after establishment of infection. Thus ISPs would not appear to be required for dissemination or persistence in the mammalian host.

At the initial stages of \textit{Leishmania} infection \textit{in vivo}, neutrophils are the first cells recruited to the site of infection (Lima \textit{et al}., 1998; Peters \textit{et al}., 2008) and they have been shown to exert an important modulatory role on the outcome of the immune response in susceptible mice (Chen \textit{et al}., 2005). In response to microbial components, neutrophils secrete the contents of azurophilic granules containing a high density of SPs, such as NE, cathepsin G and PR3, and extracellular NETs, which present trapped active NE to the surrounding milieu (Brinkmann \textit{et al}., 2004). Therefore, it is likely that \textit{Leishmania} are exposed to large amounts of SPs at the site of infection, which could be modulated by the ISPs.

Taken together, the results in this study suggest that the inhibition of host SPs by ISP2 at the initial stages of the parasite–macrophage interaction is required for the optimal initial intracellular adaptation of \textit{L. major} for living in macrophages, which is crucial for the establishment of productive infections in the host. Investigations into the role of ISP in the interaction of \textit{Leishmania} and neutrophils are ongoing and should reveal more on the role of ISPs in the infection process.

\textbf{Experimental procedures}

\textbf{Parasites}

\textit{Leishmania major} Friedlin (MHOM/JL/80/Friedlin) were grown as promastigotes in modified Eagle’s medium (designated HOMEM medium) supplemented with 10% heat-inactivated fetal calf serum (FCS) at 25°C as described previously (Besteiro \textit{et al}., 2006). The following antibiotics were used at the indicated concentration for the selection of transfectants: 50 \(\mu\)g ml\(^{-1}\) Hygromycin B (Roche), 25 \(\mu\)g ml\(^{-1}\) G418 (Invitrogen), 10 \(\mu\)g ml\(^{-1}\).
Genomic DNA (gDNA) was isolated from the WT and mutant cell lines with the DNeasy kit (Qiagen). Three micrograms of gDNA was digested with appropriate restriction enzymes, electrophoresed through a 0.8% agarose gel and blotted onto Hybond C Super (Amersham Pharmacia). The DNA probes were radiolabelled with a random priming kit (Stratagene) and the blots were hybridized at 65°C overnight. The membranes were washed twice with 2× SSC with 0.1% SDS for 15 min at 65°C and rinsed once with 0.2× SSC at 65°C. Phosphorstorage screens were exposed overnight and the membranes visualized using a Typhoon 8610 variable mode imager (Molecular Dynamics).

**Protein expression, Western blotting and antibodies**

*Leishmania major* ISP1 and ISP2 were amplified by PCR from gDNA using OL1315-OL1316 and OL1660-OL1318 respectively. The PCR products were subcloned into pPCRscript (Stratagene). The inserts were released from pPCRscript by Ndel/BamHI digest and ligated into similarly digested pET-15b (Novagen). The constructs pGL998 (ISP1/pET15b) and pGL1179 (ISP2/pET15b) were used to express N-terminal histagged ISP1 and ISP2 respectively. The proteins were isolated using nickel agarose affinity purification, ion-exchange chromatography. LPS was removed using Detoxi-Gel (Pierce) according to manufacturer’s instructions. Purified recombinant ISP1 and ISP2 were used to raise antisera in sheep. The antibodies were purified from the serum using immobilized recombinant protein or peptide onto AminoLink Plus coupling gel (Pierce). To remove possible cross-reaction between ISP1 and ISP2 antibodies, anti-ISP1 antisera was exposed to immobilized recombinant ISP2, and vice versa, prior to purification. ISP3 antibodies were produced in rabbit against a peptide corresponding to the last 20 amino acids of the encoded protein (ASASSTKSGNSKADPVDGK). The antibodies were purified from the antiserum using immobilized peptide onto AminoLink Plus coupling gel (Pierce).

The following *L. major* cell lysates were used for the Western blots: log phase promastigote 0.5 × 10^7 cells per well, purified metacyclic promastigotes 0.5 × 10^7 cells per well and purified lesion amastigote lysates 10^7 cells per well. For the ISP3 Western blot, a log phase promastigote lysate overexpressing ISP3 was used.
(0.5 × 10⁷ cells per well). The cell lysates were separated by 4–12% gradient SDS-PAGE and the proteins were transferred onto a PVDF membrane. Affinity-purified anti-ISP1 and anti-ISP2 sheep antibodies and affinity-purified anti-ISP3 rabbit antibodies were used in Western blots to detect each individual ISP. The antibodies were used at 1/1000 dilution. A secondary anti-sheep or anti-rabbit IgG antibodies coupled to horseradish peroxidase (Santa Cruz Biotechnology) were used at a 1 in 4000 dilution. Rabbit polyclonal antiserum specific for HASPB (a gift from Prof. D. Smith, York University) was used at 1/1000 dilution. Following each Western blot, the membranes were stripped and re-probed with anti-elongation factor 1 alpha monoclonal antibody (Upstate Cell Signalling Solutions) as a loading control. This antibody was used at 1 in 5000 dilution and secondary anti-mouse IgG antibody coupled to horseradish peroxidase (Promega) was used at 1 in 4000 dilution. The Western blots were revealed by soaking the membrane in SuperSignal West Pico chemiluminescent substrate (Pierce) exposing the membranes to X-ray films.

**K. determination**

The human SPs trypsin (0.21 µM), chymotrypsin (5 nM) and NE (3 µM) were incubated in 100 mM Tris (pH 8) with varying concentrations of ISP2 (100 pM to 30 µM) for 20 min prior to the addition of the appropriate substrate, Bz-R-AMC, Suc-AAPF-l and MeOsSuc-AAPF-AMC and MeO-Suc-AAPF-AMC respectively (Calbiochem). Substrates were used at a saturating concentration, which was determined experimentally (data not shown). Activity was determined by the change in fluorescence (λex = 355 nm, λem = 460 nm) at 21°C using an EnVision 2102 plate reader (Perkin Elmer, Bucks). The effect on the initial velocity was calculated and curves were fit using FigP (FigPsoft Corporation, Canada). Relative activity was calculated from the ratio of initial velocity in the presence of inhibitor to that of uninhibited controls. Experiments were carried out in triplicate and results are expressed as the mean ± SEM. K values were calculated using the equation for tight binding inhibitors in equilibrium (K = (K × [S])/([S] + Ks)).

**Macrophage infection assays**

Peritoneal macrophages from BALB/c mice were elicited by injection of 1 ml of 1% thiglycolate in the peritoneal cavity and collected 3 days later upon injection of 5 ml of ice-cold RPMI 1640-medium (Sigma-Aldrich, Egham, UK) in the peritoneal cavity, followed by gentle massage. The cells were washed, plated onto glass coverslips in a 24-well tissue culture plate at a density of 3 × 10⁵ ml⁻¹ and cultivated overnight in RPMI supplemented with 10% of FCS (Cultilab, Campinas, SP, Brazil). The cells were washed three times with Hank’s balanced salt saline (HBSS) and incubated with stationary phase promastigotes in RPMI, supplemented with 0.1% bovine serum albumin (BSA) (Sigma-Aldrich, St Louis, MO, USA), at 37°C for 3 h. The coverslips were washed for the removal of extracellular parasites, fixed with Bouin overnight and stained with Giemsa (Laborclin, Pinhais, PR, Brazil). The number of intracellular parasites was determined by counting at least 100 cells per replicate under the light microscope. Where indicated in the figure legends, the infections were performed in the presence of 10% FCS. Aprotinin (Sigma-Aldrich, St., MO, USA) or rISP2 (4 µg ml⁻¹ each) were added to macrophages 5 min prior to addition of the parasites and remained during the 3 h of interaction. Anti-mouse CD11b monoclonal antibodies (M1/70) or rat IgG2 (BD Bioscience Pharmingen, San Jose, CA, USA) were incubated at 10 µg ml⁻¹ with macrophages in RPMI-BSA for 1 h, and removed by extensive washing before the addition of promastigotes in RPMI-BSA. For the survival assays, the macrophages were infected as described above for 3 h and after the removal of extracellular parasites the cells were cultured at 37°C, in RPMI supplemented with 10% FCS for 24, 48 or 72 h.

**Phagocytosis of latex beads by macrophages**

Elicited peritoneal macrophages were plated onto glass coverslips and cultivated overnight in RPMI-10% FCS. The cells were washed with HBSS and adherent cells were incubated with Fluorescein-coupled latex beads of 500 nm diameter (Molecular Probes, USA) diluted 1:2000, in RPMI-0.1% BSA, for 30 min at 37°C. Extracellular beads were removed by washing six times with HBSS and the cells were fixed in paraformaldehyde 4%, washed with phosphate-buffered saline pH 7.2, incubated with DAPI and mounted on N-propylgalacto glass slides. The slides were observed in an Axiosplan fluorescence microscope for estimation of the average number of beads per macrophage. Where indicated, L. major stationary phase promastigotes were co-incubated with the latex beads and the macrophages at a 3:1 parasite–host cell ratio.

**In vivo assay of L. major infectivity**

Stationary phase promastigotes were centrifuged at 2000 g, re-suspended in 0.5 ml of HOMEM and incubated with 50 µg ml⁻¹ peanut lectin for 20 min at room temperature for the agglutination of procyclic promastigotes. The cells were centrifuged at 100 g for 5 min, the supernatant containing metacyclic promastigotes was recovered and the cells were washed with HBSS. BALB/c mice (4–6 weeks) were infected in the footpads with 2 × 10⁵ metacyclic promastigotes and the parasite burden in paws and in the draining lymph nodes were evaluated between 1 and 10 days. The mice were sacrificed, the bottom tissue of the footpad was removed and ruptured to homogeneity with the back of a syringe through a nylon membrane in 2 ml of Schneider’s medium supplemented with 10% FCS, 2% urine. The popliteal lymph nodes were collected and submitted to rupture as described above. One hundred microlitres of the cell homogenates was submitted to serial dilutions in 24-well plates and cultivated at 27°C for 5–7 days. The number of parasites in the highest dilution was estimated by counting in a Neubauer chamber. The parasite concentrations in the footpad or lymph nodes were subsequently calculated as: number of parasites/ml × well × dilution factor in 24-well plate × dilution factor of the initial homogenate.

**Statistical analyses**

The analysis of significance of the data was performed by analysis of variance, using the GraphPad Prism 4.0 Program. The data were analysed by one-way ANOVA using the Bonferroni post-test, at a significance level of 5%. In Fig. 6D the data were analysed by two-way ANOVA using the Bonferroni post-test, at a signifi-
cance level of 5%. The scores showing statistical significance are indicated in the figures with asterisks and the P-values are indicated in the legends.

Acknowledgements

This work was funded by the Medical Research Council (Grant Numbers G9722968, G0000508, G0700127), The Wellcome Trust (Grant Numbers 072349, 081877), CNPq and FAPERJ. A.P.C.A.L. and G.A.D.R. are Research Fellows from CNPq. We thank Alan Scott for help with purification of recombinant proteins, Edna Lopes for technical assistance in parasite culture and the Laboratório Hertha Mayer de Ultra Estrutura e Biologia Celular for help with the fluorescence microscopy.

References

Altieri, D.C., Morrissey, J.H., and Edgington, T.S. (1988) Adhesive receptor Mac-1 coordinates the activation of factor X on stimulated cells of monocytic and myeloid differentiation: an alternative initiation of the coagulation protease cascade. Proc Natl Acad Sci USA 85: 7462–7466.

Antoine, J.C., Prina, E., Lang, T., and Courret, N. (1998) The biogenesis and properties of the parasitophorous vacuoles that harbour Leishmania in murine macrophages. Trends Microbiol 6: 392–401.

Besteiro, S., Coombs, G.H., and Mottram, J.C. (2004) A potential role for ICP, a leishmanial inhibitor of cysteine peptidases, in the interaction between host and parasite. Mol Microbiol 54: 1224–1236.

Besteiro, S., Williams, R.A.M., Morrison, L.S., Coombs, G.H., and Mottram, J.C. (2006) Endosome sorting and autophagy are essential for differentiation and virulence of Leishmania major. J Biol Chem 281: 11384–11396.

Blackwell, J.M., Ezekowitz, R.A., Roberts, M.B., Channon, J.Y., Sim, R.B., and Gordon, S. (1985) Macrophage complement and lectin-like receptors bind Leishmania in the absence of serum. J Exp Med 162: 324–331.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., et al. (2004) Neutrophil extracellular traps kill bacteria. Science 303: 1532–1535.

Brittingham, A., Chen, G., McGwire, B.S., Chang, K.P., and Mosser, D.M. (1999) Interaction of Leishmania gp63 with cellular receptors for fibrocin. Infect Immun 67: 4477–4484.

Cai, T.Q., and Wright, S.D. (1996) Human leukocyte elastase is an endogenous ligand for the integrin CR3 (CD11b/CD18, Mac-1, alpha M beta 2) and modulates polymorphonuclear leukocyte adhesion. J Exp Med 184: 1213–1223.

Campbell, E.J. (1982) Human leukocyte elastase, cathepsin G, and lactoferin: family of neutrophil granule glycoproteins that bind to an alveolar macrophage receptor. Proc Natl Acad Sci USA 79: 6941–6945.

Chen, L., Zhang, Z.H., Watanabe, T., Yamashita, T., Kobayakawa, T., Kaneko, A., et al. (2005) The involvement of neutrophils in the resistance to Leishmania major infection in susceptible but not in resistant mice. Parasitol Int 54: 109–118.

Chung, C.H., Ives, H.E., Almeda, S., and Goldberg, A.L. (1983) Purification from Escherichia coli of a periplasmic protein that is a potent inhibitor of pancreatic proteases. J Biol Chem 258: 11032–11038.

Devaney, J.M., Greene, C.M., Taggart, C.C., Carroll, T.P., O’Neill, S.J., and McElvaney, N.G. (2003) Neutrophil elastase up-regulates interleukin-8 via toll-like receptor 4. FEBS Lett 544: 129–132.

Eggers, C.T., Murray, I.A., Delmar, V.A., Day, A.G., and Craik, C.S. (2004) The periplasmic serine protease inhibitor ecotin protects bacteria against neutrophil elastase. Biochem J 379: 107–118.

Garami, A., and Illg, T. (2001) The role of phosphomannose isomerase in Leishmania mexicana glycoconjugate synthesis and virulence. J Biol Chem 276: 6566–6575.

Hart, D.T., Vickerman, K., and Coombs, G.H. (1981) A quick, simple method for purifying Leishmania mexicana amastigotes in large numbers. Parasitology 82: 345.

Ivens, A.C., Peacock, C.S., Worthey, E.A., Murphy, L., Aggarwal, G., Berriman, M., et al. (2005) The genome of the kinetoplastid parasite, Leishmania major. Science 309: 436–442.

Kargi, H.A., Campbell, E.J., and Kuhn, C., III (1990) Elastase and cathepsin G of human monocytes: heterogeneity and subcellular localization to peroxidase-positive granules. J Histochem Cytochem 38: 1179–1186.

Kedzierski, L., Montgomery, J., Bullen, D., Curtis, J., Gardiner, E., Jimenez-Ruiz, A., et al. (2004) A leucine-rich repeat motif of leishmanial parasite surface antigen 2 binds to macrophages through the complement receptor 3. J Immunol 172: 4902–4906.

Kima, P.E. (2007) The amastigote forms of Leishmania are experts at exploiting host cell processes to establish infection and persist. Int J Parasitol 37: 1087–1096.

Laufs, H., Müller, K., Fleischer, J., Reiling, N., Jahnke, N., Jensenius, J.C., et al. (2002) Intracellular survival of Leishmania major in neutrophil granulocytes after uptake in the absence of heat-labile serum factors. Infect Immun 70: 826–835.

Lima, G.M., Vallochi, A.L., Silva, U.R., Bevilacqua, E.M., Kiffer, M.M., and Abrahamsohn, I.A. (1998) The role of polymorphonuclear leukocytes in the resistance to cutaneous Leishmaniasis. Immunol Lett 64: 145–151.

McGrath, M.E., Hines, W.M., Sakanari, J.A., Fletterick, R.J., and Craik, C.S. (1991a) The sequence and reactive site of ecotin. A general inhibitor of pancreatic serine proteases from Escherichia coli. J Biol Chem 266: 6620–6625.

McGrath, M.E., Hines, W.M., Sakanari, J.A., Fletterick, R.J., and Craik, C.S. (1991b) The sequence and reactive site of ecotin. A general inhibitor of pancreatic serine proteases from Escherichia coli. J Biol Chem 266: 6620–6625.

McGrath, M.E., Gillmor, S.A., and Fletterick, R.J. (1995) Ecotin: lessons on survival in a protease-filled world. Protein Sci 4: 141–148.

Monteiro, A.C.S., Abrahamson, M., Lima, A.P.C.A., Vannier-Santos, M.A., and Scharfstein, J. (2001) Identification, characterization and localization of chagasin, a tight-binding cysteine protease inhibitor in Trypanosoma cruzi. J Cell Sci 114: 3933–3942.

Mosser, D.M., and Edelson, P.J. (1985) The mouse macrophage receptor for C3bi (CR3) is a major mechanism in the phagocytosis of Leishmania promastigotes. J Immunol 135: 2785–2789.
Mosser, D.M., and Edelson, P.J. (1987) The third component of complement (C3) is responsible for the intracellular survival of Leishmania major. Nature 327: 329–331.

Mottram, J.C., Coombs, G.H., and Alexander, J. (2004) Cysteine proteases as virulence factors of Leishmania. Curr Opin Microbiol 7: 375–381.

de Oliveira, M.P., Lima, M.C., Calheiros, A.S., Martins, M.A., Antas, P.R., De Luca, P.M., et al. (2005) Leishmania (Viannia) braziliensis: human mast cell line activation induced by logarithmic and stationary promastigote-derived-lysates. Exp Parasitol 109: 72–79.

Oppendoes, F.R., and Michels, P.A. (2007) Horizontal gene transfer in trypanosomatids. Trends Parasitol 23: 470–476.

Perona, J.J., and Craik, C.S. (1997) Evolutionary divergence of substrate specificity within the chymotrypsin-like serine protease fold. J Biol Chem 272: 29987–29990.

Peters, N.C., Egen, J.G., Secundino, N., Debrabant, A., Kimblin, N., Kamhawi, S., et al. (2008) In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. Science 321: 970–974.

Ramalho-Ortigao, J.M., Kamhawi, S., Rowton, E.D., Ribeiro, J.M.C., and Valenzuela, J.G. (2000) Cloning and characterization of trypsin- and chymotrypsin-like proteases from the midgut of the sand fly vector Phlebotomus papatasi. Insect Biochem Mol Biol 33: 163–171.

Rawlings, N.D., Tolle, D.P., and Barrett, A.J. (2004) Evolutionary families of peptidase inhibitors. Biochem J 378: 705–716.

Ribeiro-Gomes, F.L., Otero, A.C., Gomes, N.A., Moniz-de-Souza, M.C., Cysne-Finkelstein, L., Arnholdt, A.C., et al. (2004) Macrophage interactions with neutrophils regulate Leishmania major infection. J Immunol 172: 4454–4462.

Rigden, D.J., Mosolov, V.V., and Galperin, M.Y. (2002) Sequence conservation in the chagasin family suggests a common trend in cysteine proteinase binding by unrelated protein inhibitors. Protein Sci 11: 1971–1977.

Rittig, M.G., and Bogdan, C. (2000) Leishmania–host–cell interaction: complexities and alternative views. Parasitol Today 16: 292–297.

Russell, D.G., and Wright, S.D. (1988) Complement receptor type 3 (CR3) binds to an Arg-Gly-Asp-containing region of the major surface glycoprotein, gp63, of Leishmania promastigotes. J Exp Med 168: 279–292.

Sacks, D.L., and Perkins, P.V. (1984) Identification of an infective stage of Leishmania promastigotes. Science 223: 1417–1419.

Sacks, D.L., Hiény, S., and Sher, A. (1985) Identification of cell surface carbohydrate and antigen changes between non-infective and infective developmental stages of Leishmania major promastigotes. J Immunol 135: 564–569.

Sajid, M., and McKerrow, J.H. (2002) Cysteine proteases of parasitic organisms. Mol Biochem Parasitol 120: 1–21.

Salmon, D., do Aido-Machado, R., Diehl, A., Leidert, M., Schmetzer, O., de, A.L.A., et al. (2006) Solution structure and backbone dynamics of the Trypanosoma cruzi cysteine protease inhibitor chagasin. J Mol Biol 357: 1511–1521.

Sanderson, S.J., Westrop, G.D., Scharfstein, J., Mottram, J.C., and Coombs, G.H. (2003) Functional conservation of a natural cysteine protease inhibitor in protozoan and bacterial pathogens. FEBS Lett 542: 12–16.

Santos, C.C., Sant’Anna, C., Terres, A., Cunha-e-Silva, N., Scharfstein, J., and de, A.L. (2005) Chagasin, the endogenous cysteine protease inhibitor of Trypanosoma cruzi, modulates parasite differentiation and invasion of mammalian cells. J Cell Sci 118: 901–915.

Santos, C.C., Coombs, G.H., Lima, A.P.C.A., and Mottram, J.C. (2007) Role of the Trypanosoma brucei natural cysteine protease inhibitor ICP in differentiation and virulence. Mol Microbiol 66: 991–1002.

Seong, I.S., Lee, H.R., Seol, J.H., Park, S.K., Lee, C.S., Suh, S.W., et al. (1994) The P1 reactive site methionine residue of ecotin is not crucial for its specificity on target proteases. A potent inhibitor of pancreatic serine proteases from Escherichia coli. J Biol Chem 269: 21915–21918.

Shin, D.H., Song, H.K., Seong, I.S., Lee, C.S., Chung, C.H., and Suh, S.W. (1996) Crystal structure analyses of uncomplexed ecotin in two crystal forms: implications for its function and stability. Protein Sci 5: 2236–2247.

Smith, B.O., Picken, N.C., Westrop, G.D., Bromek, K., Mottram, J.C., and Coombs, G.H. (2006) The structure of Leishmania mexicana ICP provides evidence for convergent evolution of cysteine peptidase inhibitors. J Biol Chem 281: 5821–5828.

Stoop, A.A., and Craik, C.S. (2003) Engineering of a macro-molecular scaffold to develop specific protease inhibitors. Nat Biotechnol 21: 1063–1068.

Talamas-Rohana, P., Wright, S.D., Lennartz, M.R., and Russell, D.G. (1990) Lipophosphoglycan from Leishmania mexicana promastigotes binds to members of the CR3, p150,95 and LFA-1 family of leukocyte integrins. J Immunol 144: 4817–4824.

Van Zandbergen, G., Klinger, M., Mueller, A., Dannenberg, S., Gebert, A., Solbach, W., et al. (2004) Cutting edge: neutrophil granulocyte serves as a vector for Leishmania entry into macrophages. J Immunol 173: 6521–6525.

Van Zandbergen, G., Bollinger, A., Wenzel, A., Kamhawi, S., Voll, R., Klinger, M., et al. (2006) Leishmaniasis disease development depends on the presence of apoptotic promastigotes in the virulent inoculum. PNAS 103: 13837–13842.

Van Zandbergen, G., Solbach, W., and Laskay, T. (2007) Apoptosis driven infection. Autoimmunity 40: 349–352.

Wei, X.Q., Charles, I.G., Smith, A., Ure, J., Feng, G.J., Huang, F.P., et al. (1995) Altered immune responses in mice lacking inducible nitric oxide synthase. Nature 375: 408–411.

Yang, S.Q., Wang, C.I., Gillmor, S.A., Fletterick, R.J., and Craik, C.S. (1998) Ecotin: a serine protease inhibitor with two distinct and interacting binding sites. J Mol Biol 279: 945–957.