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Overexpression of the Natural Inhibitor of Cysteine Peptidases in Leishmania mexicana Leads to Reduced Virulence and a Th1 Response

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Leishmania mexicana cysteine peptidases (CPs) have been identified as important parasite virulence factors. More recently, a natural inhibitor of CPs (ICP) from L. mexicana has been characterized, and ICP mutants have been created. Infection of BALB/c mice with ICP null mutants or ICP reexpressing mutants resulted in nonhealing, progressively growing lesions albeit slightly attenuated compared with the growth of lesions produced by wild-type parasites. In contrast, BALB/c mice infected with mutants overexpressing ICP were able to significantly control lesion growth or heal. While BALB/c mice infected with wild-type parasites, ICP null mutants, or ICP reexpressing mutants produced significant antibody responses, including immunoglobulin E (IgE), no Th1 response, as indicated by antigen-induced splenocyte gamma interferon (IFN-γ) production, could be demonstrated. In contrast, BALB/c mice infected with mutants overexpressing ICP produced significantly less antibody, particularly IgE, as well as significantly reduced splenocyte interleukin-4 and enhanced IFN-γ production. BALB/c mice were able to resolve infection following infection with one ICP overexpressing clone, which was subsequently used for vaccination studies with BALB/c mice. However, no protection was afforded these mice when they were challenged with wild-type parasites. Nevertheless, two other mouse strains susceptible to L. mexicana, C3H and C57BL/6, vaccinated with overexpressing ICP mutants were able to control challenge infection associated with an enhanced Th1 response. This study confirms that L. mexicana CPs are virulence factors and that ICPs have therapeutic potential.

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Leishmaniasis encompasses a large spectrum of clinical diseases which, depending upon the parasite species and the host’s immune response, can have various outcomes (16). Our understanding of the factors that lead to this diversity of clinical symptoms has, in large part, come from studies using murine models and particularly Leishmania major. While the majority of mouse strains can control infection with L. major, virtually all develop nonhealing lesions full of parasites when infected cutaneously with L. mexicana (4). Studies with the BALB/c mouse have indicated that the nonhealing response of this mouse strain to infection with L. major is LACK antigen dependent (13), while the nonhealing response to L. mexicana is independent of this antigen (27). Conversely, our studies have implicated cysteine peptidases (CPs) as major virulence factors for L. mexicana (reviewed in reference 17). Information about the apparent functions and importance of CPs in the host-parasite interaction was obtained via the generation of mutants deficient in the CPA and CPB genes (Δcpa and Δcpb mutants). L. mexicana mutants deficient in the multicopy CPB gene array (Δcpb mutants) have reduced virulence, with poor lesion growth, for BALB/c mice (2, 18). Only the reexpression of multiple CPB genes from a cosmids significantly restored virulence (8), suggesting that the multiple genes have complementary functions. CPA CPB double null mutant parasites (Δcpa Δcpb mutants) were less infective for BALB/c mice than Δcpb mutants (2, 18), which not only implicates CPA as a virulence factor but also indicates that there is some redundancy in function between CPA and CPB. CPA, together with CPB1 and CPB2 (the CPB isoforms encoded by the first 2 genes of the 19-gene CPB tandem array), is expressed in metacyclic promastigotes, suggesting roles for these CPs in the virulence of this life cycle stage. Indeed, the L. mexicana Δcpa Δcpb mutants are defective in metacyclogenesis, due to an impairment of autophagy-dependent protein turnover required for differentiation (28).

Small-molecule inhibitors of CPB have some efficacy against Leishmania, both in vitro and in vivo (24). These inhibitors are thought to be active against not only CPB but also CPA and CPC, and their toxicity to Leishmania may be a consequence of the inhibition of multiple enzymes. Nevertheless, these results confirm the importance of CPs in the host-parasite interaction. Treatment with a natural CP inhibitor, cystatin, promoted a protective response against Leishmania infection and a switch from a predominately Th2 to a Th1 response (7). Leishmania lacks cystatins, but the parasite possesses an unusual inhibitor of CPs (designated ICP), which does not occur in mammals (22). Leishmania ICP has been shown to be a potent inhibitor
of clan CA, family C1 CPS, such as CPB and mammalian cathepsin L (22), and the structure has recently been solved by nuclear magnetic resonance (25). Analysis of L. mexicana mutants lacking ICP implicated the inhibitor in the host-parasite interaction (3), but whether ICP plays an influential immune modulatory role awaited examination. The present study used ICP null mutants (Δicp mutants), ICP reexpressing parasites in which ICP had been expressed in the null mutant (Δicp::P_rRNA::ICP), and null mutant parasites overexpressing ICP (Δicp[pXG ICPI]) to determine the influence of ICP on the immune response and developing disease phenotype. Significantly, while ICP-deficient parasites remained comparatively virulent, ICP mutants overexpressing ICP did not promote significant lesion growth in BALB/c mice and developed a Th1 response. The vaccine potential of the mutant parasites was subsequently examined.

MATERIALS AND METHODS

Generation of L. mexicana Δicp null mutant promastigotes reexpressing and overexpressing ICP. All ICP transgenic cell lines were generated as described by Besteiro, Coombs, and Mottram (3). They include two independent ICP null mutants (Δicpl and Δicp3 mutants), where the two alleles coding for the gene were replaced by selection markers using homologous recombination. Clones of the mutants were subsequently used to produce reexpressing cell lines. One had an integrated single-gene copy at the rRNA locus conferring expression in both amastigote and promastigote stages of the parasite (i.e., Δicp::P_rRNA::ICP) and providing a level of expression of ICP similar to that of wild-type parasites. The second had extrachromosomal expression of ICP, via the P_gx vector expression plasmid, to yield ICP overexpressing cell lines (Δicp[pXG ICPI]).

Culture of mutant parasites. L. mexicana (MNYY/CZ/M579) promastigotes were cultured at 25°C in modified Eagle’s medium into the hind footpad. The lesion size was monitored weekly using a sliding-gauge micrometer. Lesions were excised from the right hind footpad. The lesion size was monitored weekly using a sliding-gauge micrometer. Depletion and parasite enumeration. In vivo depletion of IFN-γ was achieved by the intraperitoneal inoculation of 150 μg rat hybridoma R4-6A2 (PharmMingen) on day 0 and on days 4 and 8 postinfection of mice with 5 × 10⁵ L. mexicana amastigotes into the footpad. Three animals comprised each experimental group, and four samplings were taken from each footpad. Parasite numbers were quantified by limiting dilution as previously described (6).

RESULTS

Western blot analysis of ICP mutant L. mexicana stationary-phase promastigotes. ICP null mutants were produced as described previously (3), and two independent clones (Δicp1 and Δicp3 mutants) were isolated. The two clones were used for producing ICP overexpressing cell lines (Δicp1[pXG ICPI] and Δicp3[pXG ICPI]), whereas clone 1 only was used to produce a cell line reexpressing the protein from a single ICP copy integrated in its genome (Δicp::P_rRNA::ICP). The expression of ICP in stationary-phase promastigotes from wild-type and transgenic L. mexicana cell lines was assessed by Western blot analysis (Fig. 1). This confirmed that no ICP was present in Δicp1 and Δicp3 promastigotes. Expression of ICP in the Δicp::P_rRNA::ICP mutant was similar to that in the wild type, whereas Δicp[pXG ICPI] and Δicp3[pXG ICPI] had a significantly higher level of ICP expression (~5-fold increase) (Fig. 1).
mastigotes into the hind footpad (Fig. 2a). However, lesion growth was significantly ($P < 0.05$) reduced, from week 8 postinfection, following inoculation with Δicp1 and Δicp3 parasites compared with that following inoculation with wild-type parasites. Reexpression of the ICP gene with the integrative construct restored some virulence, and 10 weeks elapsed before a significant difference in lesion growth kinetics was observed between BALB/c mice infected with Δicp::PrRNAICP and those infected with wild-type parasites. Overexpression of ICP resulted in a significant change in disease phenotype: BALB/c mice inoculated with Δicp3[pXG ICP] stationary-phase promastigotes developed small lesions which ceased to grow after week 5, whereas those infected with Δicp1[pXG ICP] stationary-phase promastigotes apparently resolved infections by week 13. Significantly reduced parasite burdens were also found in the lesions at 13 weeks postinfection of the BALB/c mice infected with Δicp1, Δicp3, Δicp3[pXG ICP], and Δicp::PrRNAICP parasites relative to the parasite burden for those infected with wild-type parasites (Fig. 2b). No parasites could be detected nor promastigotes derived from the site of infection with Δicp1[pXG ICP] stationary-phase promastigotes apparently resolved infections within the animal (which could lead to a loss of the plasmid expressing ICP). ~70% of the clones still expressed ICP, although at a level greatly reduced from the initial level (data not shown). When these parasites were used to infect mice, still in the absence of selection, they were able to produce lesions (data not shown), possibly due to their lowered ICP expression levels. Indeed, as discussed in our previous work (3), differences in virulence could be due to variations in ICP expression level between the cell lines. Indeed, wild-type ICP protein is downregulated in amastigotes (22), whereas the integrated re-expressing mutant Δicp::PrRNAICP has a constitutive expression throughout the stages. Hence, both cell lines have similar ICP expression levels in promastigotes, but in differentiated amastigotes the protein is likely to be slightly overexpressed in Δicp::PrRNAICP compared to the level in the wild type.

BALB/c mice infected with *L. mexicana* mutants overexpressing ICP develop a modified, Th1/Th2-biased response. At the termination of the experiment at week 13, no significant differences in *L. mexicana*-specific IgG1 or IgG2a levels were displayed in BALB/c mice inoculated with the Δicp1 mutant, the Δicp3 mutant, or Δicp::PrRNAICP compared with the levels displayed in BALB/c mice inoculated with $5 \times 10^6$ wild-type stationary-phase promastigotes (Fig. 3a). However, infection with Δicp1[pXG ICP] and Δicp3[pXG ICP] resulted in significantly ($P < 0.05$) lower levels of *L. mexicana*-specific IgG1 than did infection with wild-type parasites. Infection with Δicp1[pXG ICP] also resulted in significantly ($P < 0.05$) lower levels of IgG2a than did infection with wild-type parasites (Fig. 3a). Similarly, no significant difference was noted in the total IgE levels for BALB/c mice infected with the Δicp1 mutant, the Δicp3 mutant, or Δicp::PrRNAICP compared with the levels for BALB/c mice inoculated with $5 \times 10^6$ wild-type stationary-phase promastigotes, whereas ICP overexpressing cell lines produced significantly ($P < 0.05$) lower levels of IgE than did wild-type parasites (Fig. 3b). In addition, IgE levels were normalized against levels for noninfected control sera, and absorbances of greater than three times the level for the control sera were deemed indicative of significant IgE production. This revealed that while five of five BALB/c mice infected with wild-type parasites or Δicp::PrRNAICP and four of five mice infected with the Δicp1 or Δicp3 mutant had raised IgE levels. In vitro stimulation with *L. mexicana* antigenic lysate induced significantly ($P < 0.05$) increased levels of IFN-γ from splenocytes isolated from BALB/c mice inoculated with Δicp1[pXG ICP] or Δicp3[pXG ICP] that had raised IgE levels. In vitro stimulation with *L. mexicana* antigenic lysate induced significantly ($P < 0.05$) increased levels of IFN-γ from splenocytes isolated from BALB/c mice inoculated with Δicp1[pXG ICP] or Δicp3[pXG ICP] than...
vaccination with \( \Delta icp1[pXG ICP] \), BALB/c mice were challenged, along with naïve, sex- and age-matched counterparts, with wild-type \( L. \) mexicana metacyclic promastigotes. However, subcutaneous vaccination of BALB/c mice with \( 2 \times 10^5 \) \( \Delta icp1[pXG ICP] \) parasites 14 weeks prior to challenge inoculation did not induce resistance to infection with wild-type \( L. \) mexicana metacyclic promastigotes. No significant difference was noted in lesion development for vaccinated BALB/c mice infected with wild-type parasites in the contralateral footpad compared with that for similarly infected nonvaccinated BALB/c mice (Fig. 4a). Similarly, prior vaccination with \( \Delta icp1[pXG ICP] \) \( L. \) mexicana promastigotes did not alter the parasite burdens for the BALB/c mice at the termination of the experiment compared with those for infected, nonvaccinated BALB/c mice (Fig. 4b). There were also no differences in the immune responses between vaccinated and nonvaccinated BALB/c mice at the termination of the challenge infection (data not shown). Vaccination of BALB/c mice with \( \Delta icp3[pXG ICP] \) stationary-phase promastigotes also failed to induce protection against wild-type challenge (data not shown).

**Vaccination with \( L. \) mexicana mutants overexpressing ICP protects C3H and C57BL/6 mice against challenge with wild-type parasites.** Previously, we have shown that other \( L. \) mexicana-susceptible mouse strains are more responsive to vaccination with CP mutants than BALB/c mice (2, 5). In addition, other mouse strains, such as C3H and C57BL/6, were able to control lesion growth with mutants that continued to cause nonhealing disease, albeit limited, in BALB/c mice (2, 6). Consequently, infection of these mouse strains with \( \Delta icp3[pXG ICP] \) and, to a lesser extent, \( \Delta icp1[pXG ICP] \) resulted in control of lesion growth. In a typical experiment, in contrast to the nonhealing response of C3H mice infected with wild-type parasites, mice infected with \( \Delta icp3[pXG ICP] \) developed small lesions that peaked at 4 to 6 weeks and consequently proceeded to heal (Fig. 5a). Parasite burdens were similar for wild-type- and \( \Delta icp3[pXG ICP] \)-infected mice up to 1 week postinfection (Fig. 5b). However, by week 2 postinfection parasite burdens were approaching 3 orders of magnitude greater than those for mice infected with \( \Delta icp3[pXG ICP] \) \( (P < 0.01) \). However, treatment of \( \Delta icp3[pXG ICP] \)-infected mice with neutralizing IFN-\( \gamma \) resulted in a significant increase in mutant parasite growth, also approaching 3 orders of magnitude greater (Fig. 5b). This not only confirmed the ability of \( \Delta icp3[pXG ICP] \) to multiply within the host but also confirmed that growth was limited due to the generation of IFN-\( \gamma \). Neutralizing IFN-\( \gamma \) also increased \( \Delta icp3[pXG ICP] \) growth in BALB/c mice, indicating that the role of IFN-\( \gamma \) in protection was independent of the mouse strain used (data not shown).

Consequently, we studied the ability of ICP overexpressing mutants to protect C3H and C57BL/6 mice against challenge with wild-type parasites, lesions were smaller in both \( \Delta icp1[pXG ICP] \)- and \( \Delta icp3[pXG ICP] \)-vaccinated C3H and C57BL/6 mice at the termination of the experiment, at week 10, than in nonvaccinated mice following challenge with \( 5 \times 10^5 \) wild-type stationary-phase promastigotes (Fig. 6a and b). However, only in C57BL/6 mice vaccinated with \( \Delta icp3[pXG ICP] \) was this difference significant \( (P < 0.05) \). No increase in lesion growth was detected from 5 weeks after challenge infection in vaccinated C3H mice, whereas continuous significant lesion growth was noted for the nonvaccinated challenged mice.
Control of the challenge infection due to prior vaccination of C3H mice with \( \Delta icp1 \) \([pXG ICP]\) or \( \Delta icp3 \) \([pXG ICP]\), and \( \Delta icp::PrRNA ICP \) stationary-phase promastigotes into the hind footpad \( (*, P < 0.05; **, P < 0.01) \). In vitro production of IFN-\( \gamma \) (c) and IL-4 (d) by splenocytes, in the presence of 10 \( \mu \)g/ml \( L. mexicana \) antigen and 0.5 \( \mu \)g/ml anti-CD3, respectively, from BALB/c mice infected with \( 5 \times 10^5 \) wild-type, \( \Delta icp1 \), \( \Delta icp3 \), \( \Delta icp::PrRNA ICP \), and \( \Delta icp::PrRNA ICP \) \( L. mexicana \) stationary-phase promastigotes at week 13 postinfection \( (*, P < 0.05) \). Bars represent standard errors of the means. Results are representative of three independent experiments.

**DISCUSSION**

Previous studies of the characterization and function of \( L. mexicana \) ICP have suggested that it may play a role in protecting the parasite from the degradative activity of not only parasite CPB and CPA in particular but also host CPs which are found colocalized with ICP in the parasite endocytic vesi-

icles (3). Consequently, ICP-deficient \( L. mexicana \) mutants lose some virulence for BALB/c mice, as measured by slightly reduced lesion growth, which was somewhat restored by reexpressing normal levels of ICP in the mutants (3). In spite of the reduced virulence of the \( \Delta icp \) mutant, there are no discernible immunological consequences for the host or parasite, as demonstrated in the present study by antibody levels as well as IFN-\( \gamma \) and IL-4 production. In contrast, we demonstrate not only that parasites overexpressing ICP have greatly reduced virulence for mice but also that this is associated with a switch from a Th2- to a Th1-biased response. Consequently, when ICP is overexpressed by mutant parasites, it influences the infectivity of the parasite in ways other than those merely associated with efficient housekeeping and fitness activities.

Our previous studies utilizing mutants of \( L. mexicana \) deficient in the CPs have revealed the importance of both clan CA, family C1 peptidases CPB and CPA for virulence. \( \Delta cpa \) \( \Delta cpb \) mutant amastigotes, although equally as infective for macrophages in vitro as wild-type parasites (10), have a reduced ability to induce lesion growth in BALB/c mice (2, 8). \( \Delta cpa \) \( \Delta cpb \) mutants were less infective for BALB/c mice than \( \Delta cph \) mutants (2, 18), which also implicates CPA as a virulence factor. As ICP has been shown to preferentially inhibit clan CA, family C1,
cathepsin L-like peptidases, such as CPB (22), and to bind specifically to CPA and CPB (3), this could be the likely mechanism by which ICP overexpressing mutants lose virulence. Although expressed primarily in wild-type promastigotes rather than amastigotes (22), ICP is released in significant quantities by the amastigotes of overexpressing clones Δicp1[pXG ICP] and Δicp3[pXG ICP] (3). The virulence of L. mexicana for BALB/c mice has been associated with the ability of CPB/CPA to induce a Th2 response in BALB/c mice. In infected mice, Δcpb and Δcpa Δcpb mutants induce a Th1 response characterized by increased IFN-γ production, and consequently, lesion growth is inhibited (2, 5). Similar immunological and disease phenotypes were found in mice infected by ICP overexpressing mutants in the present study, suggesting that ICP acts to inhibit CPB/CPA immunomodulatory activity.

Small chemical inhibitors of clan CA CPs have previously been shown to inhibit the growth of several species of Leishmania, including L. donovani (7), L. chagasi (11, 15, 26), L. tropica (15), and L. major (14, 21), and a number of mechanisms have been implicated. However, ICPs may not necessarily operate by targeting parasite CPs directly but may act by inhibiting the crucial role of host CPs in the processing of antigen via major histocompatibility complex (MHC) class II. For example, studies have shown that chemically inhibiting cathepsins B, which are involved in antigen processing, switches L. major-infected BALB/c mice from producing a nonhealing Th2 to a healing Th1 response (14), while inhibiting cathepsins L promotes a Th2 response and increased susceptibility to the parasite (19, 29). However, ICP preferentially binds and inhibits mammalian cathepsin L and has 1/1,000 less affinity for cathepsin B (22). Consequently, this specificity probably negates a role for ICP from overexpressing mutants in directly modulating host cell antigen-presenting pathways. However, L. mexicana and L. amazonensis CPs have been implicated in the inhibition of antigen presentation by degrading MHC class II molecules in the parasitophorous vacuole (9). ICP in overexpressing mutants in this study could therefore function to promote MHC class II antigen presentation indirectly by inhibiting the degradative activity of parasite CPs. Inhibitors of cathepsin L-like CPs have also been associated with modulating host cell production of nitric oxide upon infection with L. donovani (7) or L. major (21), production of tumor necrosis factor alpha upon infection with L. major (21), and production of IL-12 upon infection with L. major (21) or

FIG. 4. Mean increases in hind-footpad diameter (a) and parasite burdens (b) following subcutaneous infection with 5 × 10⁵ L. mexicana stationary-phase promastigotes into naïve BALB/c mice and BALB/c mice vaccinated with Δicp1[pXG ICP]. Bars represent standard errors, and results are representative of three independent experiments.

FIG. 5. (a) Mean increases in hind-footpad thickness for C3H mice infected with wild-type (WT) and Δicp3[pXG ICP] promastigotes. (b) The parasites in the footpad were quantified by limiting dilution for up to 2 weeks postinfection, and the effects of intraperitoneal anti-IFN-γ treatment (150 µg rat hybridoma R4-6A2 on day 0, day 4, and day 8) on the growth of Δicp3[pXG ICP] were assessed. Bars represent standard errors of the means. Results are representative of two independent experiments.
All of these products are associated with, or would promote, a Th1 response and protection. There is strong evidence that \textit{L. mexicana} effects on host cell IL-12 production are, in part, a consequence of CPB degrading host cell NF-κB signaling pathways and that this degradation does not occur upon infection with \textit{Δcpb} mutant parasites or in the presence of cell-permeable CP inhibitors (6). Overexpressed ICP could inhibit this previously characterized CPB activity, and in addition, it could be released into the extracellular milieu (3), as previously demonstrated for CPB (12). Hence, ICP could inhibit the ability of the CP to generate IL-4 and a Th2 response (20).
As infection of BALB/c mice with *L. mexicana* metacyclic promastigotes of overexpressing clones Δicp1[pXG ICP] and Δicp3[pXG ICP] induced immune responses that favor resistance to infection, these genetically modified parasites were considered as vaccine candidates. Δicp1[pXG ICP] was considered the most suitable candidate for the development of a vaccine, as this mutant induced no detectable lesion progression or parasite replication. However, subcutaneous vaccination with Δicp1[pXG ICP] did not confer protection to subsequent challenge with wild-type *L. mexicana* promastigotes. No significant differences in lesion development or parasite burdens or subsequent immune responses were detected (data not shown) following infection of vaccinated mice with wild-type parasites or infection of naive mice. Significantly, we have demonstrated previously that only limited resistance was conferred on BALB/c mice vaccinated with Δcpe Δcpb mutant parasites and that this required extremely large experimental groups of 20 mice to demonstrate (2). However, while the nonhealing responses of BALB/c mice for *L. mexicana* have been related to CP-induced IL-4 (8, 20), by contrast, the virulence of *L. mexicana* for the *C. major*-resistant C3H and C3HBL/6 strains has been associated with the ability of CPB to inhibit Th1 responses rather than to induce Th2 responses (5).

The *C. major*-induced IL-4 (8, 20), by contrast, the virulence of *L. mexicana* for the *C. major*-resistant C3H and C3HBL/6 strains has been associated with the ability of CPB to inhibit Th1 responses rather than to induce Th2 responses (5).

ICP, a leishmanial inhibitor of cysteine peptidases, in the interaction between host and parasite. Mol. Microbiol. 54:1224–1236.


