The *Leishmania mexicana* Cysteine Protease, CPB2.8, Induces Potent Th2 Responses


*J Immunol* 2003; 170:1746-1753; doi: 10.4049/jimmunol.170.4.1746

http://www.jimmunol.org/content/170/4/1746

**References**  
This article cites 59 articles, 29 of which you can access for free at:  
http://www.jimmunol.org/content/170/4/1746.full#ref-list-1

**Subscription**  
Information about subscribing to *The Journal of Immunology* is online at:  
http://jimmunol.org/subscription

**Permissions**  
Submit copyright permission requests at:  
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  
Receive free email-alerts when new articles cite this article. Sign up at:  
http://jimmunol.org/alerts
We have previously identified that *Leishmania mexicana* cysteine proteases (CPs) are virulence factors. We have now produced a recombinant *L. mexicana* CP, CPB2.8, which has similar enzymatic activity to native enzyme. Inoculation of CPB2.8 (≤5 μg) into the footpads of BALB/c mice not only up-regulated mRNA transcripts for IL-4 and IL-4 production in the draining popliteal lymph nodes, but also polarized splenocyte anti-CD3 stimulated responses toward a Th2 bias as measured by increased IL-5 production compared with controls. In agreement with promoting a Th2 response, CPB2.8 also induced strong specific IgE responses in treated mice as well as increasing whole IgE levels. Inhibition of the enzyme activity of CPB2.8 by treatment with E-64 ablated the enzyme’s ability to induce IgE. Significantly, infection of mice with CPB-deficient parasites failed to stimulate production of IgE, unlike infection with wild-type parasites. Furthermore, enzymatically active (<0.1 U/ml) but not E-64-inactivated CPB2.8 was able to proteolytically cleave CD23 and CD25, although not B220 or CD4 from murine lymphocytes. These properties are similar to those demonstrated by the house dust mite allergen Der p 1 and provide an explanation for the immunomodulatory activity of the CPB2.8 virulence factor. Vaccination with CPB2.8 enhanced *L. mexicana* lesion growth compared with control animals. Nevertheless, vaccination with IL-12 and CPB2.8 resulted in a degree of protection associated with inhibition of lesion growth and a Th1 response. Thus, CPB2.8 is a potent Th2-inducing molecule capable of significant vaccine potential if administered with a suitable adjuvant. *The Journal of Immunology*, 2003, 170: 1746–1753.

Protective immunity against *Leishmania* is generally acknowledged to be dependent on an IL-12-driven, type 1 response (1–4), although dendritic cells rather than macrophages are the likely primary source of this cytokine (4–7). NK cells activated by IL-12, which may be augmented by IL-18 (8), are the primary source of early IFN-γ which not only plays an important role in controlling early resistance to infection but is also influential in initiating type 1 responses (3). Later in the response, IFN-γ, primarily from Th1 CD4+ T cells, mediates protection by inducing NO synthase 2 expression and NO production by macrophages (reviewed by Liew and O’Donnell (9)).

Early studies, particularly with *L. major* (10–12), would suggest that an IL-4-driven type 2 response and associated cytokines counterregulate type 1 responses and consequently it would be expected that a type 2 response would be detrimental to the control of *Leishmania*. In susceptible BALB/c mice, a single T cell epitope derived from the parasite LACK Ag (*Leishmania* homolog of receptors for activated C kinase) has been shown to induce rapid IL-4 production by Vβ4Vα8CD4+ T cells which rendered T cells unresponsive to IL-12 and correlated with lesion development (13). Conversely, mice made tolerant to LACK by transgenic expression in the thymus exhibited both a diminished Th2 response and a healing phenotype (14). Although these results indicated that parasite Ags could promote the development of counterprotective Th2 responses, other studies suggested that early IL-4 production did not necessarily predict susceptibility to *L. major* (15). Furthermore, studies using IL-4−/− and IL-4Rα−/− mice have provided contrasting results as to the importance of IL-4 and the associated cytokine IL-13 in determining susceptibility to *L. major* (16–19).

Although *L. mexicana* also induces cutaneous lesions, infections with this parasite appear to be under different host genetic controls to *L. major* and the majority of mouse strains are susceptible to infection and develop nonhealing disease (reviewed by Blackwell (20)). Unlike its inconclusive role in *L. major* infections, the paramount role of IL-4 in promoting nonhealing murine *L. mexicana* infections has been clearly demonstrated. Thus, inhibition of IL-4 production or IL-4-mediated signaling in susceptible mice results in induction of a Th1 response and failure to develop lesions following *L. mexicana* infection (21–23). Although *L. mexicana* expresses LACK Ag, this Ag is not involved in determining susceptibility to *L. mexicana* and thus the induction of IL-4 and a Th2 response must be under other influences (24). We have previously demonstrated that the multiple highly active *L. mexicana* cysteine proteases (CPs),4 many of which are stage regulated, are potent virulence factors (25, 26). Thus, *L. mexicana* mutants lacking CPB, a multicopy CP gene expressed in metacyclic promastigotes and amastigotes, not only have greatly reduced infectivity for mice but also generate a Th1 rather than a Th2 response. The effect was further enhanced in mutants additionally deficient in CPA, a single

---

1 A work was funded by The Wellcome Trust. K.G.J.P. was the recipient of a Medical Research Council postgraduate studentship. J.M.B. is in receipt of a Wellcome Trust Career Development Fellowship.

2 K.G.J.P. and K.S.M. contributed equally to this work.

3 Address correspondence and reprint requests to Dr. James Alexander, Department of Immunology, University of Strathclyde, The Todd Wing, John Arbuthnott Building, 27 Taylor Street, Glasgow G4 0NR, U.K. E-mail address: J.Alexander@Strath.ac.uk

4 Abbreviations used in this paper: CP, cysteine protease; HPRT, hypoxanthine-guanosine phosphoribosyltransferase.
copy CP gene. Significantly, it is well documented that CPs such as papain (27), the house dust mite allergen Der p I (28), and various schistosomal Ags (29, 30) are potent allergens inducing IL-4 and a Th2 response. Therefore, the following study was undertaken using recombinant, enzymatically active CPB (designated CPB2.8) to determine whether L. mexicana CPs could play a significant IL-4/Th2-promoting role during infection. Not only was CPB2.8 found to be a potent inducer of IL-4 and a Th2 response, but also significant IgE production was found to be associated with its enzymatic activity in a manner similar to that previously demonstrated for Der p I (31). Consequently, mice inoculated preinfection with CPB2.8 developed exacerbated lesion growth, while adjuvanting CPB2.8 with IL-12 was found to alter the Th1/Th2 balance and induce resistance.

Materials and Methods
Parasites and infection protocols
L. mexicana (MYNC/BZ/62/M379) was maintained by serial passage of amastigotes inoculated s.c. into the shaven rumps or footpads of BALB/c mice. Amastigotes for use in experimental studies were isolated and purified from lesions and enumerated as previously described (32). CPB-deficient mutants (Δcpb) used in this study are described in Motttram et al. (25).

Experimental groups consisted of at least five 8- to 10-wk-old female mice, which following vaccination protocols were infected with 10⁷ amastigotes by s.c. inoculation into the shaven rump. Disease progression was monitored by the measurement of lesion diameters at two weekly intervals up to the termination of experiments. Most experiments were terminated between 8 and 16 wk, but others continued for up to 6 mo. All vaccine experiments were repeated in C57BL/6 and C58/Ca mice and on occasions used promastigotes or axenic amastigotes for infection. Results were essentially similar. Individual experimental details are provided in the text.

Recombinant CPB expression, inactivation with E-64, and inoculation into mice
The recombinant CPB was expressed in Escherichia coli without the C-terminal domain and activated as described previously (33). The enzyme, designated CPB2.8 for this study, was stored frozen at −20°C until used.

A total of 500 µg of CPB2.8 enzyme was inactivated by incubation with 10 µM of E-64 (trans-epoxysuccinyl-l-leucylamido-(4-guanidino)-butane) at 37°C for 30 min with gentle agitation. Excess E-64 was removed by overnight dialysis against PBS, pH 6.0, at 4°C. Inhibition of the enzyme was assayed using gelatin SDS-PAGE (33). LPS levels were shown to be <1 ng LPS/mg protein by using an E-toxate assay. Mice were generally inoculated into the footpad (1-5 µg) either nonadjuvanted or adjuvanted with 1 µg rIL-12. Individual experimental details are given in the text.

Preparation of soluble leishmanial Ag
Soluble leishmanial Ag for use in ELISA and T cell proliferation assays was prepared from stationary-phase promastigotes of L. mexicana cultured in Schneider’s Insect Medium (Sigma-Aldrich, Poole, U.K.) plus 20% (v/v) heat-inactivated FCS. Promastigotes were washed twice in ice-cold PBS and resuspended in hypotonic buffer consisting of 10 mM Tris-HCl (pH 7.8), 2 mM EDTA with 50 mM N-p-tosyl-l-lysine chloromethyl ketone, and 15 mM leupeptin (Sigma-Aldrich). Following 15 min of incubation on ice, the promastigotes were disrupted in a Brinkmann homogenizer (Braun, Kronberg, Germany) and centrifuged at 10,000 × g for 60 min at 4°C. The supernatant containing soluble leishmanial Ag was dialyzed against PBS (pH 7.4) overnight at 4°C and the protein concentration determined by the method of Bradford (34). Alternatively, L. mexicana lysate Ag was prepared by six cycles of freezing (−70°C) and thawing (37°C) at 10⁶/ml in PBS. After each thaw, the suspension was passed through a 25-gauge needle and the suspension centrifuged at 1,000 × g for 5 min and then sterilized by passing through a 2-µm filter. Ag preparations were stored at −20°C until required.

Detection of Leishmania- and CPB2.8-specific IgG1, IgG2, and IgE and whole plasma IgE by ELISA
Peripheral blood was obtained from experimental animals by tail bleeding into heparinized capillary tubes. All plasma samples were stored at −20°C before analysis for specific Ab content. Leishmania- or CPB2.8-specific IgG1 and IgG2a end-point titers were measured by ELISA as previously described (26). Briefly, each well of an Immulon-1 microtiter plate (Dynatech Laboratories, Billinghamurst, U.K.) was coated with leishmanial lysate Ag (freeze/thawed wild-type promastigotes in PBS, pH 7.4) or 50 µl of 2 µg/ml inactive recombinant CPB2.8 by overnight incubation at 4°C.

Following serial dilutions of plasma samples for 2 h at 37°C, bound Abs were detected by incubation with goat anti-mouse IgG1 and goat anti-mouse IgG2a HRP conjugate (Southern Biotechnology Associates, Birmingham, AL). Binding of conjugate was visualized with tetramethylbenzidine (0.06 mg/ml) in 0.1 M sodium acetate buffer (pH 5.5) containing 0.03% H₂O₂. The color reaction was stopped by adding 10% (v/v) sulfuric acid and OD₅₇₀ was measured as end-point dilutions where the end point is defined as the final plasma concentration which yielded an absorbance higher than a negative control plasma sample included in the assay. Statistical comparisons between groups of mice were made by using the Mann-Whitney U test.

Total and specific plasma IgE was determined by capture ELISA. Flat-bottom Immulon 1B plates (Dynatech Laboratories) were coated overnight at 4°C with 50 µl rat anti-mouse IgE mAb (R35-72; BD PharMingen, San Diego, CA) or 2 µg/ml inactivated recombinant CPB2.8 in PBS (pH 9.0), wells were blocked with 200 µl 10% (v/v) FCS in PBS for 60 min at 37°C. After washing as above, 100 µl samples of serially diluted plasma were added in duplicate to wells and incubated at 37°C for 2 h. After washing three times, 100 µl biotynolated anti-mouse IgE (Southern Biotechnology Associates) diluted in 0.1% (v/v) FCS in PBS (pH 7.2) was added and incubated for 60 min at 37°C. After washing as above, alkaline phosphatase-conjugated streptavidin (BD PharMingen) diluted 1/2000 was added to each well and incubated for 30 min at 37°C. Plates were washed three times as above and 100 µl p-nitrophenylphosphate (1.0 mg/ml; Sigma-Aldrich) in 0.1 M glycine buffer, pH 10.4, added per well. The color was then incubated for 60 min or overnight at 37°C in the dark before the resulting absorbances were read at 405 nm on a Spectramax Plus Reader (Molecular Devices, Sunnyvale, CA).

Splenocyte and popliteal lymph node responses
Spleens and lymph nodes were aseptically removed at appropriate times post CPB2.8 injection or postinfection, as detailed for individual experiments, and cell suspensions prepared by gently teasing apart the tissue in RPMI 1640 supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.05 mM 2-ME, and 10% (v/v) heat-inactivated FCS (Life Technologies, Paisley, U.K.). Following centrifugation at 200 × g for 10 min at 4°C, spleen cells were resuspended in 3 ml of Boyle’s solution (0.17 M Tris-HCl, pH 7.2, 0.16 M ammonium chloride) at 37°C for 3 min to deplete RBCs. Lymph node and spleen cell suspensions were then centrifuged at 200 × g for 10 min at 4°C, resuspended, washed, and resuspended in 2 ml of complete RPMI 1640 medium (as described above). Viable cells were enumerated by trypan blue exclusion and the suspensions adjusted to 5 × 10⁶ cells/ml. A total of 100 µl aliquots of the cell suspension were added to 96-well, flat-bottom, tissue culture plates (Costar, Cambridge, MA) and 100 µl aliquots of Con A (5 µg/ml) or L. mexicana lysate Ag (50, 10, or 25 µg/ml protein/ml) or 1 µg/ml soluble anti-CPB2.8 Ab as appropriate. Cultures were then incubated in 5% CO₂/95% air for 48 h at 37°C and supernatants collected for quantification of cytokine production.

IFN-γ IL-4 and IL-5 assays
IFN-γ, IL-4, and IL-5 production by stimulated (Leishmania Ag or anti-CD3) and nonsensitized splenocytes or lymph node cells was measured by capture ELISA. Briefly, the wells of Immulon-1 microtiter plates (Dynatech Laboratories) were coated with capture Ab at 0.5 µg/ml (IFN-γ S51216, IL-5 S54393; BD PharMingen) (IL-4, 11B11; Genzyme, Cambridge, U.K.) in PBS (pH 9.0) by overnight incubation at 4°C. Wells were then washed three times with PBS, pH 7.4, 0.05% Tween 20 and blocked by incubation with 10% (v/v) FCS for 1 h at 37°C. The culture supernatants and appropriate recombinant standards (IFN-γ, IL-5; BD PharMingen) (IL-4, Genzyme) were then added to individual wells. For standard curves, IFN-γ (0–20 ng/ml), IL-5 (0–50 ng/ml), and IL-4 (0–1000 pg/ml) were used. Following incubations at 37°C for 2 h, the wells were washed three times with PBS, pH 7.4/0.05% Tween 20 and then biotynolated rat anti-mouse IFN-γ (used at 1 µg/ml; BD PharMingen), biotynolated rat anti-mouse IL-5 (used at 1 µg/ml; BD PharMingen), and biotynolated goat polyclonal IL-4 (used at 1 µg/ml; Genzyme) were added and incubated for 1 h at 37°C. For the detection of bound biotynolated rat Ab, 100 µl of streptavidin-alkaline phosphatase conjugate (diluted 1/1000; BD PharMingen) was added to each well for 45 min at 37°C, and following further washing, binding was visualized with substrate consisting of p-nitrophenylphosphate (1 mg/ml; Sigma-Aldrich) in glycine buffer (0.1 M, pH 10.4). The absorbance was subsequently measured at 405 nm on a Spectramax Plus Reader.

The Journal of Immunology

1747
For detection of bound biotinylated goat Ab, 100 μl of streptavidin-APR conjugate (diluted 1/500; Genzyme) was added to each well for 30 min at 37°C and following further washing was incubated with tetramethylbenzidine as described above. Cytokine concentrations in the cell cultures were determined from the standard curve. All assays were conducted in triplicate. Comparisons between groups were made using the Student’s t test. Values of p < 0.05 were considered significant.

**RNA extraction and RT-PCR**

At 24 h or 7 days after injection with 1 μg CPB2.8 or PBS into BALB/c footpads, popliteal nodes from these BALB/c mice were carefully removed. RNA was isolated following a protocol based upon the single-step acid guanidinium thiocyanate-phenol-chloroform RNA isolation method (35). cDNA was then produced from total RNA using Moloney murine leukemia virus reverse transcriptase (Life Technologies) according to manufacturer’s specifications. In a 90-μl reaction volume, 7 μg of RNA was combined with 18 μl of 5X first strand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl₂, 18 μl of dNTP mix (10 mM), 9 μl of 0.1 M DTT, 80 U of RNAsin RNase inhibitor (Promega, Madison, WI), 500 ng of random hexamer primers (Promega), and 1200 U of Moloney murine leukemia virus reverse transcriptase. Following a 10-min preincubation at 27°C, the mixture was incubated at 42°C for 60 min, followed by reaction termination by heating at 95°C for 5 min. All cDNA was stored at −20°C until used in PCR.

**Competitive RT-PCR for cytokine transcripts**

The level of IL-4 transcript was assessed by competitive PCR as described previously (36–38) using multispecific competitor plasmids pMEUS (39) and pQRs (40). To ensure that equal quantities of cDNA were used in PCR for each sample, levels were first normalized against hypoxanthine-guanosine phosphoribosyl transferase (HPRT). To achieve this, PCR was first performed for HPRT in the absence of competitor and samples adjusted until similar band intensities were observed. To confirm normalization, PCR for HPRT was then performed in the presence of a constant amount of competitor. Where necessary, samples were adjusted until the resulting amplification resulted in equal intensities of specific and competitor products for all the samples (38). All PCR were made to 25 μl and contained a final concentration of 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100), 1.5 mM MgCl₂, 200 μM of each dNTP, 0.5 μM of each primer, and 0.25 U Taq polymerase (Promega). Cycling conditions were an initial denaturation at 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min. Reactions finished with a final extension period for 10 min at 72°C. Primers used in these experiments are listed below. HPRT forward and reverse primers (5’-GGATTGGAATTCAGAC-3’ and 5’-GAGGTAGCTGGCCTA TGGCCT-3’), respectively, for use with qRAS; IL-4 forward and reverse primers (5’-TCGGCATTGTGAACGAGGTC-3’ and 5’-GAAAAAAC GAAAAGTGCCT-3’), are for mPUS. The amounts of specific and competitor product of each reaction were assessed from scanned images of ethidium bromide-stained gels using NIH Image software (http://rsb.info.nih.gov/nih_image/). The ratios of the total volume (density × area) of competitor and specific products were determined and statistical analysis performed using the Mann-Whitney U test.

**FACS analysis**

Peripheral lymph node cells from BALB/c mice were isolated RPMI and either directly treated with CPB2.8 for CD23 and B220 analysis, or incubated for 48 h with immobilized anti-CD3 (5 μg/ml) and then treated with CPB2.8 for CD4 and CD25 analysis. Cells were treated for 60 min with varying doses of active CPB2.8 (specific activity 3.8 U/mg) or CPB2.8 which had been enzymatically inactivated by addition of E-64. Fluorochrome-labeled Abs (all from BD Biosciences, Mountain View, CA) were used to determine the expression of CD4, CD23, CD25, and B220 by flow cytometry. Fc binding of Abs to cells was blocked with anti-CD16/CD32 Ab. Ten thousand events were collected for each sample using a FACScan (BD Biosciences) and analysis performed using CellQuest software (BD Biosciences). Results from duplicate cultures are presented as mean fluorescence intensity and are representative of two independent experiments.

**Results**

**CPB 2.8 induces a change in the Th1/Th2 balance**

Following a single inoculation of 1 μg CPB2.8 into the footpads of BALB/c mice, there was a significant (p < 0.05) up-regulation (>4-fold) of transcripts for IL-4 mRNA as measured by competitive RT-PCR 7 days posttreatment in the draining popliteal lymph nodes compared with PBS-treated controls (Fig. 1a). Increased IL-4 mRNA expression was evident but not significant 24 h after a single inoculation of CPB2.8 and was maintained above control levels for at least 7 days. Furthermore, inoculation of 5 μg CPB2.8 resulted in consistent significant (p < 0.02) spontaneous production of IL-4 protein by draining popliteal lymph node cells from mice inoculated s.c. 7 days previously with 5 μg CPB2.8. There was significant spontaneous IL-4 production (p < 0.02) compared with PBS inoculation. One of three independent experiments.

**Active but not inactive CPB2.8 induces IgE production**

Two weeks postinjection of 5 μg CPB2.8 on days 0 and 7 into BALB/c footpads, no specific IgG1 or IgG2a was detected. However, whole plasma IgE was significantly increased (p < 0.01) in animals treated with active CPB2.8 compared with PBS-treated controls (Fig. 3a). Inactivation of CPB2.8 by treatment with E-64 ablated the ability of CPB2.8 to promote IgE production. In addition, a CPB2.8-specific IgE response was measured in active

![FIGURE 1. a. The effect of a single inoculation into BALB/c feetpads of 1 μg CPB2.8 on the expression in the poplilteal lymph nodes at day 7 of IL-4 mRNA, assessed by competitive RT-PCR. Samples were first normalized for HPRT expression. Expression of IL-4-specific product (lower band) was low or absent in control samples (inoculated with PBS), while expression in CPB2.8 was up-regulated (note increase in intensity of lower band). B. Densometric analysis of the PCR products indicated a >4-fold increase in IL-4 mRNA in CPB2.8-treated mice at 7 days postinfection (p < 0.05). In two replicates, similar results were obtained at 24 h and 7 days postinfection. b, IL-4 production by draining popliteal lymph node cells from mice inoculated s.c. 7 days previously with 5 μg CPB2.8. There was significant spontaneous IL-4 production (p < 0.02) compared with PBS inoculation. One of three independent experiments.](http://www.jimmunol.org/)
CPB2.8-inoculated mice although not mice inoculated with the inactive (E64-treated) CPB2.8 (Fig. 3b). IL-5 production from the draining lymph node cells of these mice indicated a Th2 response in animals receiving active but not inactive enzyme (Fig. 3c).

**Active CPB2.8 cleaves CD23 and CD25**

Incubation of the lymphocytes with active but not E-64-inactivated CPB2.8 resulted in reduced levels of CD23, the low-affinity IgE receptor found on mature resting B cells (Fig. 4a). This reduction required concentrations of enzyme in excess of 10 μg/ml (0.038 U/ml) and was specific for CD23, as expression of another B cell marker, B220/CD45R, was not affected by enzyme treatment (Fig. 4b). Similarly, levels of CD25 found on activated T cells were reduced by active but not inactive enzyme (Fig. 4c), while levels of CD4 expression were unaffected (Fig. 4d).

**CPB-deficient L. mexicana mutants fail to induce levels of IgE similar to wild-type parasites**

CPB-deficient *L. mexicana* mutants (Δcpb) have reduced infectivity for BALB/c mice and IL-4 production and lesion growth are inhibited compared with animals infected with wild-type parasites (26). IgE levels were significantly reduced in animals infected with Δcpb parasites compared with wild-type *L. mexicana* infections (Fig. 5). IgE levels increased progressively in wild-type parasite-infected mice as lesions increased in size. However, no corresponding increase in IgE levels was observed in Δcpb-infected mice as lesions developed slowly compared with mice infected with wild-type parasites (Fig. 5). Indeed, total IgE level in Δcpb-infected mice were similar to noninfected animals (see Fig. 3a).

The levels of CD25 expression in the draining lymph nodes of mice infected with wild-type or Δcpb parasites were measured by FACS analysis 3 and 6 wk postinfection. The CD25 levels were significantly reduced (*p < 0.01*) in the presence of CPB, although the number of CD25+ cells was similar (data not shown).

**Vaccination with CPB2.8 results in increased lesion growth**

Lesion growth following infection with 10⁶ *L. mexicana* amastigotes was significantly enhanced compared with nonvaccinated controls (*p < 0.01* at wk 12 postinfection) in mice vaccinated with CPB2.8 on 2 occasions, 2 wk apart, the second vaccination being 2 wk before challenge (Fig. 6a). IgG1 levels (Fig. 6b) were significantly increased in CPB2.8-vaccinated animals compared with nonvaccinated infected mice (*p < 0.01*), as were IgE levels (*p <
comparing with nonvaccinated animals (data not shown).

The speciﬁc activity of CPB2.8 in this study was 3.8

Reduced levels of CD25 were found on activated T cells following incubation with active but not inactive enzyme (Fig. 4). Incubation of lymph node cells with CPB2.8 reduced levels of CD23, while expression of another B-cell marker, B220/CD45R, was not affected (d), while levels of CD4 expression were unaffected (e). Levels of lymphocyte markers were determined by FACS analysis and are presented as mean ﬂuorescence intensity from duplicate incubations. Consistent results were found in two independent experiments. The speciﬁc activity of CPB2.8 in this study was 3.8 U/mg.

0.01) (Fig. 6c). At 12 wk postinfection, Ag- (both parasite lysate and CPB2.8) induced splenocyte IL-4 and IFN-γ production was similar in all groups and Th2 biased (data not shown).

Vaccination with CPR2.8 plus IL-12 inhibits disease progression

It is well documented that Ags such as LACK or adjuvants such as Alum, which normally induce a strong Th2 response, can promote T1 responses with the addition of IL-12 to the inoculum (41, 42). In mice vaccinated with CPB2.8 along with IL-12, the onset of lesion growth was signiﬁcantly inhibited and lesions were signiﬁcantly smaller, p < 0.05 up to 12 wk postinfection. Resistance in the vaccinated animals was associated with signiﬁcantly reduced IgG1 (p < 0.01) and IgE levels (p < 0.05) compared with nonvaccinated controls (Figs. 7, b, and c). In addition, vaccinated mice demonstrated signiﬁcantly increased Ag- (both parasite lysate and CPB2.8) speciﬁc splenocyte IFN-γ production and reduced IL-4 compared with nonvaccinated animals (data not shown).

Discussion

The cathespin L-like CP, CPB, has previously been demonstrated to be a virulence factor for L. mexicana and parasites deﬁcient for this enzyme have reduced infectivity for mice (26, 25). Although this reduced virulence in vivo was thought initially to be related to the reduced ability of mutant parasites to infect macrophages (25), recent studies suggest that this property is associated with promastigotes and not amastigotes; once transformed, Δcpb mutant amastigotes are equivalent to wild-type amastigotes in their ability to parasiteise macrophages (43). However, Δcpb amastigotes continue to have reduced infectivity for mice (43), demonstrating that macrophage innate effector functions have little impact on Δcpb initial infectivity and consequently that wild-type parasite CPs are operating as virulence factors downstream to initial parasite host cell invasion. The present study suggests that CPB2.8 may be facilitating nonhealing disease by promoting a Th2-biased immune response.

Previous studies have demonstrated CPs such as Der p I, the house dust mite allergen, to be potent inducers of IL-4 and IgE production (28). These properties are related to the ability of Der p I to cleave both the IL-2R, CD25, and consequently inhibit Th1 responses, and the low-afﬁnity IgE receptor, CD23, to enhance IgE levels and IL-4 (31, 44). Inactivation of Der p I CP by treatment with E-64 abolishes the ability of this Ag to enhance IL-4 and IgE levels (31, 44). We have now demonstrated that CPB2.8 has similar Th2-generating properties to Der p I and demonstrated that this activity, as well as the ability to speciﬁcally cleave CD25 and CD43, is also lost by treatment with E-64. Collectively, these results would suggest that L. mexicana CPs, via their ability to induce IL-4 and a Th2 response, may in large part be responsible for susceptibility to L. mexicana. Although studies with other Leishmania species suggest that nonhealing responses may develop independently of the involvement of IL-4 (15, 45), work on L. mexicana has demonstrated susceptibility is largely if not wholly related to this cytokine and signaling via IL-4Rα and STAT-6 (21–23, 46). Our previous studies (22) using SCID mice reconstituted with IL-4−/− or wild-type lymphocytes demonstrate that in the absence of IL-4-producing lymphocytes an innate source of IL-4 can initiate lesion growth. However, not only is lesion development dependent on lymphocyte responding to IL-4 (46), but...
lesions heal in the absence of lymphocytes producing IL-4. This indicates that elements of both innate and acquired immunity are involved in the induction and maintenance of a Th2 response following natural infection. Thus, although CPB clearly induces a switch in the Th1/Th2 bias, we cannot rule out an innate early nonlymphoid source of IL-4 induced by CPB2.8 driving this response. Significantly, while Der p I has been shown to induce mast cell IL-4 production (47), it has also been shown to induce CD4 and CD8 T cell IL-4 production (44). Although studies on L. mexicana demonstrate CPB to be contained primarily within large organelles called megasomes, an examination of parasite-induced lesions demonstrated large quantities of CPB in the extracellular milieu (48). Thus, CPs leaking from the site of infection could well influence the immunological Th1/Th2 balance of the host. The dramatically reduced ability of Δcpb mutants to induce IgE and IL-4 production and down-regulate CD25 expression (26) compared with wild-type parasites would certainly suggest that CPB directly influences the immune response in this way.

Studies demonstrating that LACK-tolerant BALB/c mice remain susceptible to L. mexicana while developing resistance to L. major confirm that there are alternative/additional mechanisms in inducing susceptibility to L. mexicana (24). This observation has been confirmed by the fact that mouse strains such as CBA/Ca that do not have LACK-specific IL-4-producing CD4+ T cells and are resistant to L. major remain susceptible to L. mexicana. However, the LACK Ag is expressed by L. mexicana and is recognized by BALB/c CD4 T cells that produce IL-4 (24). Significantly, we have found that while Δcpb mutants do grow slowly in LACK-responsive BALB/c mice, they fail to grow in LACK-nonresponsive CBA/Ca mice (data not shown), indicating that in the absence of CPs the LACK Ag may operate as a virulence factor for L. mexicana in the BALB/c mouse. These results collectively would suggest that CPs are the virulence factors making the majority of mouse strains susceptible to this parasite.

FIGURE 6. Vaccination s.c. with 5 μg CPB2.8 on two occasions, 2 wk apart, exacerbated the growth of a challenge infection with 10⁶ L. mexicana amastigotes given 2 wk after the final vaccination (a). IgG1 levels (b) and IgE levels (c) were significantly higher in animals vaccinated with CPB2.8 compared with controls (p < 0.01) 6 wk postinfection. One of two independent experiments.

FIGURE 7. Vaccination s.c. with 5 μg CPB2.8 adjuvanted with 0.5 μg IL-12 significantly inhibited lesion growth (a). Animals were vaccinated on two occasions, 2 wk apart and 2 wk before challenge infection with 10⁶ L. mexicana amastigotes. Vaccinated mice had reduced IgG1 (b) and IgE production (c) compared with control animals at the termination of the experiment. One of three independent experiments.
CPs have also been implicated in the inhibition of Ag presentation by degrading MHC class II molecules in the parasitophorous vacuole (49, 50). Consequently, a number of studies have demonstrated that treatment with CP inhibitors promotes a protective response against Leishmania infection (51–53). Maekawa et al. (53) showed that a mammalian cathepsin B–specific inhibitor could switch CD4+ differentiation from Th2 to Th1, suggesting that mouse cathepsin B is involved in Ag processing. The inhibitor had little effect on parasite growth in vitro or in vivo (53), so the effect observed was unlikely to have been due to inhibition of the parasitic cathepsin B. This conclusion is supported by the finding that cathepsin B–deficient mutants of *L. mexicana* (Δcpc) are viable in mice and do not appear to alter significantly the Th1/Th2 balance (54). In contrast, it has recently been reported that the cathepsin B–like CPs of visceral leishmaniasis, *Leishmania donovani* and *Leishmania chagasi*, influence the immune response adversely by activating a latent form of TGF-β (55). Thus, both the *Leishmania* cathepsin L– and cathepsin B–like CPs may serve as parasite virulence factors to promote a Th2 response.

Previous studies have demonstrated that the Th2 response induced by Ags such as Der p 1 (56) or LACK (42) can be polarized toward Th1–type responses by the coadministration of IL-12, acting as an adjuvant. In addition, BALB/c mice vaccinated with LACK plus IL-12 demonstrated increased resistance against *L. major* (42). Similarly, we have demonstrated that vaccination with CPB2.8 and IL-12 also increases resistance against *L. mexicana* associated with a switch in the Th1/Th2 bias compared with nonvaccinated animals. Protective responses against *L. major* (57) and *L. mexicana* (58), using recombinant or native CPs, have previously been demonstrated in combination with suitable adjuvants. However, the reactivity of IL-12 in humans may prove an obstacle to its use (59). Perhaps this can be resolved by the use of DNA vaccines (42), which have been shown to be equally if not more effective at inducing a protective response against LACK.

Although the LACK Ag has been identified as the major virulence factor for *L. major* in susceptible mice, this and our previous studies (26) demonstrate the CP CPB to be influential in promoting IL-4 and a type 2 response and to be a major virulence factor for *L. mexicana*.

## References


