OmpL1 Is an Extracellular Matrix- and Plasminogen-Interacting Protein of *Leptospira* spp.


Leptospirosis is a zoonosis with multisystem involvement caused by pathogenic strains of the genus *Leptospira*. OmpL1 is an outer membrane protein of *Leptospira* spp. that is expressed during infection. In this work, we investigated novel features of this protein. We describe that OmpL1 is a novel leptospiral extracellular matrix (ECM)-binding protein and a plasminogen (PLG) receptor. The recombinant protein was expressed in *Escherichia coli* BL21(DE3) Star/pLysS as inclusion bodies, refolded, and purified by metal-chelating chromatography. The protein presented a typical β-strand secondary structure, as evaluated by circular dichroism spectroscopy. The recombinant protein reacted with antibodies in serum samples from convalescent leptospirosis patients with a high specificity compared to serum samples from individuals with unrelated diseases. These data strengthen the usefulness of OmpL1 as a diagnostic marker of leptospirosis. The characterization of the immunogenicity of recombinant OmpL1 in inoculated BALB/c mice showed that the protein has the capacity to elicit humoral and cellular immune responses, as denoted by high antibody titers and the proliferation of lymphocytes. We demonstrate that OmpL1 has the ability to mediate attachment to laminin and plasma fibronectin, with $K_D$ (equilibrium dissociation constant) values of 2,099.93 ± 871.03 nM and 1,239.23 ± 506.85 nM, respectively. OmpL1 is also a PLG receptor, with a $K_D$ of 368.63 ± 121.23 nM, capable of generating enzymatically active plasm in. This is the first report that shows and characterizes OmpL1 as an ECM-interacting and a PLG-binding protein of *Leptospira* spp. that may play a role in bacterial pathogenesis when expressed during infection.

Leptospirosis is considered an important reemerging infectious disease. It is a zoonosis caused by pathogenic *Leptospira* spp. that is transmitted from reservoir hosts to humans through water and soil contaminated with their urine (18). Symptoms of the disease include fever, vomiting, headache, diarrhea, and abdominal and generalized muscle pain. Progression to multiorgan system complications, known as Weil's syndrome, occurs in 5 to 15% of cases, with mortality rates of 5 to 40% (18, 30, 34, 55).

At present, available vaccines are based on inactivated whole-cell or membrane preparations of pathogenic leptospires. They confer protective responses mostly through the induction of antibodies against leptospiral lipopolysaccharide (1, 14). However, these vaccines do not induce long-term protection against infection and do not provide cross-protective immunity against leptospiral serovars not included in the vaccine preparation (1). Due to the large number of leptospiral serovars (8), conserved and protective antigens are being pursued.

Surface-exposed proteins are potential targets for inducing immune responses during infection and may also mediate the initial process of adhesion to host cells. Indeed, several of these proteins have been reported to be leptospiral adhesins (2, 4, 12, 28, 44, 49). Furthermore, well-conserved outer membrane proteins (OMPs) are promising vaccine targets, because they would have an advantage in inducing cross-protective immunity (31). After adherence, pathogens have to overcome host tissue barriers to reach blood circulation and organs. We reported previously that leptospires bind plasminogen (PLG) at their surface and that proteolytic activity is achieved due to the generation of plasm in (77). OmpL1 is a 31-kDa leptospiral transmembrane OMP containing 320 amino acid residues and was characterized previously by Haake and colleagues (23). Several studies pointed out the importance of OmpL1 as a serological marker for the diagnosis of human (17, 20, 47, 56, 67, 78) and canine (29, 48) cases of leptospirosis.

This work describes the expression and characterization of recombinant OmpL1, the immune response induced in BALB/c mice, and the reactivity of this protein with leptospirosis-positive serum samples. We also describe the ability of OmpL1 to mediate attachment to various extracellular matrix (ECM) and serum components. We report that OmpL1 is a novel surface adhesin that binds to laminin and plasma fibronectin, interacts with PLG, is expressed during infection (6), and may participate in leptospiral pathogenesis.

**MATERIALS AND METHODS**

**ECM and biological components.** Macromolecules, including the control protein fetuin, were purchased from Sigma-Aldrich (St. Louis, MO). Laminin-1 and collagen type IV were derived from the basement membrane of Engelbreth-Holm-Swarm mouse sarcoma; cellular fibronectin was derived from human foreskin fibroblasts; plasma fibronectin, human complement serum, and fibrinogen were isolated from human plasma;...
and collagen type I was isolated from rat tail. Native plasmologen, which was purified from human plasma, and human factor H were purchased from EMD Chemicals, Inc. (San Diego, CA). C4bP was obtained from Complement Technology, Inc. (Tyler, TX).

**Bacterial strains and serum samples.** The pathogenic, high-passage-number, nonviral Leptospira strains used were *L. interrogans* serovar Canicola strain Hound Utrech IV, *L. interrogans* serovar Copenagheni strain M 20, *L. interrogans* serovar Icterohaemorrhagiae strain RGA, *L. interrogans* serovor Pomona strain Pomona, *L. borgtrensenii* serovor Castellonis strain Castellon 3, *L. borgtrensenii* serovar Whitcombia strain Whitcomb, *L. kirschneri* serovar Grippotyphosa strain Moskva V, *L. kirschneri* serovar Cynoptery strain 3522C, *L. santarosai* serovar Shermanni strain 1342 K, and *L. noguchii* serovor Panama strain CZ 214, and the nonpathogenic *Lepapprox* strain used was *L. biflexa* serovar Patoc strain Patoc. The leptospires were cultured at 28°C under aerobic conditions in liquid EMJH medium (Difco, BD, Franklin Lakes, NJ) with 10% rabbit serum, which was enriched with 1-aspargine (0.015%, wt/vol), sodium pyruvate (0.001%, wt/vol), calcium chloride (0.001%, wt/vol), magnesium chloride (0.001%, wt/vol), peptone (0.03%, wt/vol), and yeast extract (0.022%, wt/vol) (69). Low-passage-number, virulent *L. interrogans* serovar Copenagheni strain FIOCRUZ L1-130 and *L. interrogans* serovar Kennewicki strain Pomona Fromm were also used. Recently weaned male Golden Syrian hamsters were infected intraperitoneally with 500 μl of approximately 1 × 10^9 virulent *L. interrogans* serovar Kennewicki strain Pomona Fromm leptospires. The animals were bled from the retro-orbital plexus after the appearance of symptoms such as loss of weight and mobility (approximately 5 days postinfection). *Leptospira* cultures were maintained in the Faculdade de Medicina Veterinaria e Zootecnia, Universidade de São Paulo (USP), São Paulo, Brazil. Serum samples from hamsters with confirmed leptospirosis were obtained by experimental infection with virulent leptospires. Human serum samples from patients with confirmed leptospirosis were obtained from the collection of the Instituto Adolfo Lutz, São Paulo, Brazil. Serum samples from patients with unrelated infectious diseases were obtained from the serum collection of the Laboratorio Imunoepidemiologia, Superintendência de Controle de Endemias (SUCEN), São Paulo, Brazil. *Escherichia coli* DH5α was used as the cloning host strain, and *E. coli* BL21 (DE3) Star/pLysS (66) was used as the host strain for the expression of the recombinant protein.

**In silico sequence analysis.** All sequences available in the GenBank database were employed to study the conservation and identity of OmpL1 using CLUSTAL 2.1 multiple-sequence alignment (http://www.ebi.ac.uk/Tools/msa/clustalw2/) (33).

**Cloning, expression, and purification of recombinant OmpL1.** The amplification of the OmpL1 gene was performed by PCR with *L. interrogans* serovar Copenagheni strain M20 genomic DNA using forward (F) primer 5′-GGATCCAAAACATGCAATGTTTG-3′ and reverse (R) primer 5′-GGATCCTTAGTTCGTTTATAACC-3′. PCR was performed with an initial denaturation step at 94°C for 5 min; 35 cycles at 94°C for 50 s, 60°C for 50 s, and 72°C for 2 min; and a final step at 72°C for 7 min. The reaction mix consisted 1× PCR buffer, 0.2 mM deoxynucleoside triphosphate (dNTP), 2 mM MgCl₂, 0.2 mM each primer (F and R), 2.5 U Taq polymerase, and 100 ng of genomic DNA for a final volume of 25 μl. The gene sequence was amplified without the signal peptide tag, which was determined by the use of SignalP 3.0 software (http://www.cbs.dtu.dk/services/SignalP/). The PCR fragment was cloned into the *E. coli* expression vector pAE (57) at the BamHI and KpnI restriction sites. Plasmid pAE-OmpL1, containing the correct DNA sequence, was used to transform *E. coli* BL21 (DE3) Star/pLysS. Protein expression was obtained by the inoculation of 10 ml of a culture grown overnight into 800 ml of Luria-Bertani medium containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol. The culture was grown with continuous shaking at 37°C to an optical density at 600 nm (OD<sub>600</sub>) of 0.6 and then induced for 3 h under constant agitation at 37°C in the presence of 0.1 mM IPTG (isopropyl-β-D-1-thiogalactopyranoside). The cells were harvested by centrifugation, and the bacterial pellet was resuspended in sonication buffer (20 mM Tris–HCl [pH 8.0], 200 mM NaCl, 200 mg/ml lysozyme, 2 mM phenylmethylsulfonyl fluoride [PMSF], and 1% Triton X-100) and lysed on ice with the aid of a sonicator tip (Ultrasonic processor; GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The insoluble fraction was resuspended in a buffer containing 20 mM Tris–HCl (pH 8.0), 500 mM NaCl, 8 M urea, and 1 mM β-mercaptoethanol. The protein was purified through Ni<sup>2+</sup>-charged beads of a chelating fast-flow chromatographic column (GE Healthcare) and extensively washed with buffer containing 20 mM Tris–HCl (pH 8.0), 500 mM NaCl, and increasing concentrations of imidazole (5 to 60 mM). Bound protein was eluted with buffer containing 1 M imidazole. The efficiency of the purification was evaluated by 12% SDS-PAGE. Fractions containing the recombinant protein were extensively dialyzed against PBS (phosphate-buffered saline) (pH 7.4) containing 0.1% glycine for 24 h.

**Circular dichroism spectroscopy.** Purified recombinant protein was dialyzed against sodium phosphate buffer (pH 7.4). Circular dichroism (CD) spectroscopy measurements were performed at 20°C by using a Jasco J-810 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan) equipped with a Peltier unit for temperature control. Far-UV CD spectra were measured by using a 1-mm-path-length cell at 0.5-nm intervals. The spectra were presented as averages of five scans recorded from 185 to 260 nm. The residual molar ellipticity is expressed in degrees × centimeter per decimole. Spectrum data were evaluated with K2D2 software (http://logic.ca/projects/k2d2/), using a method that calculated the secondary-structure content from the ellipticity experimental data (52).

**Microscopic agglutination test.** The microscopic agglutination test (MAT) was performed according to methods described previously by Faine et al. (18). In brief, 22 serovars of *Leptospira* spp. were employed as antigens: serovars Australsis, Autumnalis, Bataviae, Canicola, Castellonis, Celledoni, Copenhageni, Cytophthera, Djasiman, Grippotyphosa, Hardjo, Hebdomadis, Icterohaemorrhagiae, Javanica, Panama, Patoc, Pomona, Pyrogens, Sejroe, Shermanni, Tarassovi, and Woflli. All the strains were maintained in EMJH liquid medium at 29°C. A laboratory-confirmed case of leptospirosis was defined by the demonstration of a 4-fold increase of the microagglutination titer between paired serum samples. The probable predominant serovar was considered to be the one with the highest dilution that could cause 50% agglutination. The MAT was considered negative when the titer was below 100.

**Antiserum production against recombinant protein and isotype determination.** The recombinant protein was mixed with 10% (vol/vol) Alhydrogel [2% Al(OH)<sub>3</sub>; Brenntag Biosector, Denmark], which was used as an adjuvant. Ten micrograms of OmpL1 was subcutaneously administered to five female BALB/c mice (4 to 6 weeks old). Two subsequent booster injections of the same protein preparation were given at 2-week intervals. Negative-control mice were injected with PBS. Two weeks after each immunization, the mice were bled from the retro-orbital plexus, and the resulting sera were analyzed by an enzyme-linked immunosorbent assay (ELISA) for the determination of antibody titers. Prior to experiments, anti-recombinant protein sera were adsorbed into a suspension of *E. coli* cells to avoid reactivity with anti-*E. coli* antibodies (22). For isotype determinations, total IgG (immunoglobulin G), IgG1, and IgG2α (Southern Biotech, Birmingham, AL) levels were measured by the incubation of the pooled mouse sera with the recombinant protein, followed by incubation with horseradish peroxidase (HRP)-conjugated anti-mouse total IgG or goat anti-mouse IgG1 or IgG2α (1:2,000 dilution) and by incubation with HRP-conjugated anti-goat IgG (1:10,000). The OD<sub>405</sub> values exhibited by different dilutions of mouse serum were compared to a curve generated by coating the plates with different concentrations of mouse total IgG, IgG1, or IgG2α. IgM and IgA titers were also determined. In brief, plates were coated with recombinant protein (250 ng/well), and anti-OmpL1 sera and anti-PBS sera (negative control) were previously diluted (1:200 to 1:409,600), transferred into the OmpL1-coated wells, and incubated for 1 h at 37°C. Plates were washed, and HRP-conjugated goat anti-mouse IgA (1:5,000) (Sigma) was added and incubated as de-
scribed above. For IgM determinations, plates were incubated with goat anti-mouse IgM (1:5,000) (Sigma), followed by incubation with HRP-conjugated rabbit anti-goat IgG (1:50,000) (Sigma). The titers were considered the maximum dilution that gave OD492 values above 0.1.

Ethics statement. All animal studies were approved by the Ethics Committee of the Instituto Butantan, São Paulo, Brazil, under protocol no. 767/10. The Committee in Animal Research of the Instituto Butantan adopts the guidelines of the Brazilian College of Animal Experimentation. Human serum samples from patients with confirmed leptospirosis were obtained from the collection of the Instituto Adolfo Lutz, São Paulo, Brazil. Serum samples from patients with unrelated infectious diseases were obtained from the serum collection of the Laboratório Imunopneumologia, SUCEN, São Paulo, Brazil; the Laboratorio Protozoologia, Instituto de Medicina Tropical de São Paulo (IMT), USP, São Paulo, Brazil; the Laboratorio Virologia, IMT, USP, São Paulo, Brazil; and Nucleo Estudos em Malária, SUCEN/IMT, USP, São Paulo, Brazil. Human sera from collections of the above-mentioned institutions were donated to be used for research purposes.

Immunoblotting assay. The purified recombinant protein was loaded onto 12% SDS-PAGE gels, and fractionated proteins were transferred onto nitrocellulose membranes (Hybond ECL; GE Healthcare) on semidy equipment. Membranes were blocked with 10% nonfat dry milk and 1% bovine serum albumin (BSA) in PBS containing 0.05% Tween 20 (PBS-T) and then incubated with anti-OmpL1 (1:1,600) mouse polyclonal serum or monoclonal anti-His tag antibodies (1:1,000) (GE Healthcare) for 2 h at room temperature. After washing, the membranes were incubated with HRP-conjugated anti-mouse IgG (1:5,000) (Sigma) in PBS for 1 h. The protein reactivity was revealed by use of the ECL reagent kit chemiluminescence substrate (GE Healthcare); the luminescence generated by the reaction was detected with the aid of a Carestream molecular imaging instrument (Equilib, Whitestone, NY) connected to Gel Logic 2200PRO.

OmpL1 genomic DNA and protein conservation among leptospiral strains. Leptospiral genomic DNAs were extracted as previously described (49), and sample integrity was verified by the amplification of the 16S rRNA gene. Cloning primers for OmpL1 were used for the DNA amplification of Leptospira spp., as described above. Fragments were visualized on a 1% agarose gel. For protein conservation, cultures of Leptospira spp. were harvested by centrifugation, washed three times with low-salt PBS containing 5 mM MgCl2, and resuspended in PBS. A sample of each strain was applied onto a 12% SDS-PAGE gel, and after separation, proteins were transferred onto a nitrocellulose membrane on semidy equipment (GE Healthcare). The membrane was blocked overnight at 4°C with 10% nonfat dry milk and 1% BSA in PBS-T. After three washes with PBS-T, the membrane was incubated for 3 h at room temperature with antisem against OmpL1 (1:500), produced in mice, followed by washes and incubation with HRP-conjugated goat anti-mouse IgG (1:5,000) for 1 h at room temperature. The membrane was then washed, and the reactivity was revealed as described above.

ELISA for detection of human and hamster antibodies. Human and hamster IgG antibodies against OmpL1 were detected by an ELISA, as previously described (50). In brief, MAT-negative and -positive serum samples from 32 patients with confirmed leptospirosis and 44 experimentally infected hamsters were diluted (1:100) and evaluated for total IgG by using HRP-conjugated anti-human IgG antibodies (1:5,000) (Sigma) and HRP-conjugated anti-hamster IgG antibodies (1:5,000). Cutoff values were set at 3 standard deviations above the mean OD492 of sera from 10 healthy individuals from the city of São Paulo, Brazil, or 5 noninfected hamsters. The reaction specificity was evaluated with serum samples from patients diagnosed with the following unrelated infectious diseases: dengue virus infection (n = 13), malaria (n = 12), HIV infection (n = 15), and Chagas' disease (n = 20). The sera were used at 1:100 dilutions, and the values for specificity were determined according to a method described previously by Galen and Gambino (21).

Lymphoproliferation assay. At the end of the immunization protocols, BALB/c mice were sacrificed, and their spleens were aseptically removed and suspended in RPMI 1640 medium containing 2 mM L-glutamine, 100 IU/ml of penicillin, 100 µg/ml of streptomycin, and 50 µg/ml of gentamicin. Spleens were macerated, and after erythrocyte lysis, splenocytes were resuspended in 1 ml of RPMI medium containing 10% fetal bovine serum and counted after staining with 0.4% trypan blue for viability. Splenocytes (5 × 106 cells/well) were plated in triplicate onto 96-well flat-bottom cell culture plates (Costar; Corning). Spleen cells were stimulated with 5 µg/ml of concanavalin A (ConA) (Sigma), which was employed as a positive control; 5 µg/ml of OmpL1; or medium alone, which was used as a negative control. Cells were cultured for 48 h at 37°C in 5% CO2 in a humidified atmosphere, and proliferative rates were determined as a function of DNA synthesis, measured by the incorporation of bromodeoxyuridine (BrdU) by a BrdU ELISA colorimetric kit (Roche Diagnostic, Indianapolis, IN). Cells were labeled for 2 h with 20 µl of BrdU labeling solution. Subsequently, anti-BrdU antibody conjugated with HRP (1:100) was added, and the mixture was incubated for 90 min. The enzyme substrate solution was added, and incubation was carried out at room temperature for 15 min. The enzymatic reaction was stopped by the addition of 1 M H2SO4 to the mixture, and the optical density at 450 nm was measured in a microplate reader. The stimulation index (SI) was calculated as the ratio between the mean OD of cells cultured with the antigen and the mean OD of cells cultured in medium only in the immunized group.

Evaluation of cytokine production. For analyses of secreted cytokines, spleen cells were isolated and cultured as described above, except that the culture was made in 24-well tissue culture plates, with each well containing 5 × 106 cells. After 48 h, cell-free culture supernatants were collected and stored short term at −20°C. Levels of interleukin-4 (IL-4), IL-10, gamma interferon (IFN-γ), and tumor necrosis factor alpha (TNF-α) were measured by an ELISA (PreproTech, Colonia Narvarte, Mexico) according to the manufacturer's instructions. Briefly, capture antibody was added to each well, and the plates were incubated overnight at 4°C, followed by incubation with blocking buffer for 1 h. Standard cytokines were diluted to create a concentration curve. One hundred microliters of each supernatant sample was then added, and plates were incubated for at least 2 h. After that, antibody detection solution was added (1:2,000), followed by 2 h of incubation; a substrate was added to each well; incubation proceeded at room temperature for reaction development; and readings were taken at 405 nm with the wavelength correction set at 650 nm in an ELISA plate reader. Results are expressed in pg/ml.

Binding of OmpL1 to ECM and to serum components. Protein attachment to individual macromolecules of the extracellular matrix and serum components was analyzed according to a previously reported protocol (4). Briefly, ELISA plates (Costar High Binding; Corning) were coated with 1 µg of the ECM component or BSA (negative control), gelatin, and fetuin (highly glycosylated attachment-negative control protein) in 100 µl of PBS for 3 h at 37°C and then blocked overnight at 4°C. One microgram of OmpL1 was added per well in 100 µl of PBS, and protein was allowed to attach to the different substrates for 2 h at 37°C. After washing six times with PBS-T, bound proteins were detected by the addition of an appropriate dilution of mouse antisem that gives an OD492 value of 1, determined by titration in 100 µl of PBS (1:1,600). Incubation proceeded for 1 h at 37°C, and after three washes with PBS-T, 100 µl of a 1:5,000 dilution of HRP-conjugated goat anti-mouse IgG in PBS was added per well and incubated for 1 h at 37°C. The wells were washed three times, and o-phenylenediamine (1 mg/ml) in citrate phosphate buffer (pH 5.0) plus 1 µl/ml H2O2 was added (100 µl per well). The reaction was allowed to proceed for 10 min and was interrupted by the addition of 50 µl of 8 M H2SO4 to the mixture. Readings were taken at 492 nm with a microplate reader (Multiskan EX; Thermo Fisher Scientific, Helsinki, Finland). For statistical analyses, the binding of the recombinant protein to ECM macromolecules was compared to its binding to all three negative controls by using Student’s two-tailed t-test, and the P value was determined by comparison to gelatin, which was used as the negative control for the following experiments. Binding was also confirmed by using HRP-
conjugated anti-His tag monoclonal antibodies previously titrated against the recombinant protein and used at a dilution that generates an OD$_{492}$ value of approximately 1.

$K_d$, values for binding of OmpL1 to ECM and to serum components.

First, ELISA plates were coated with 100 µl of 10 µg/ml ECM or serum components, which were allowed to adhere for 3 h at 37°C. Plates were then blocked overnight, and increasing concentrations of purified OmpL1 were added (100 µl/well in PBS) and incubated for 2 h at 37°C. The assessment of bound protein was performed by incubation for 1 h at 37°C with the antiserum raised against the protein at the dilution of 1:1,600, followed by HRP-conjugated goat anti-mouse IgG (1:5,000 in PBS) (Sigma). The ELISA data were used to calculate the equilibrium dissociation constant ($K_d$), according to a method described previously (37), based on the equation $A = A_{max} [\text{protein}]/(K_d + [\text{protein}])$, where $A$ is the absorbance at a given protein concentration, $A_{max}$ is the maximum absorbance for the ELISA plate reader (equilibrium), [protein] is the protein concentration, and $K_d$ is the equilibrium dissociation constant for a given absorbance at a given protein concentration (ELISA data point).

**Antibody inhibition assay.** We assessed the effect of anti-OmpL1 antibodies on the binding of the recombinant protein to laminin, plasma fibronectin, and plasminogen in a dose-dependent manner. As a control, preimmune serum was employed. Briefly, plates were coated with 1 µg of each component and blocked with 10% nonfat dry milk. At the same time, 1 µg of OmpL1 was incubated with different concentrations of antiserum raised in mice against the recombinant protein or preimmune serum (from 1:50 to 1:400 dilutions) for 2 h at 37°C. After that, the recombinant protein was allowed to interact with the coated component for 2 h at 37°C. After washing with PBS-T, HRP-conjugated anti-His tag monoclonal antibodies, previously titrated against the recombinant protein and used at a dilution that generates an OD$_{492}$ value of approximately 1, were added. The reaction was allowed to proceed for 10 min and was interrupted by the addition of 50 µl of 8 M H$_2$SO$_4$ to the mixture. Reactions were taken at 492 nm in a microplate reader. For statistical analyses, the percent binding of the recombinant protein was compared to its binding in the treatment in which no anti-OmpL1 antiserum was added (100% binding) by Student’s two-tailed t test.

**Characterization of binding of OmpL1 to laminin and PLG.** In order to evaluate the contribution of the sugar moiety to the recombinant protein’s interaction, microplates were coated with 1 µg of laminin in 50 mM sodium acetate buffer (pH 5.0) and incubated for 16 h at 4°C. Wells were washed three times with the same buffer, and immobilized laminin was treated with different concentrations of sodium metaperiodate (5 to 100 mM) for 15 min at 4°C in the dark. After washing with 50 mM sodium acetate buffer, wells were blocked with 200 µl of PBS-T–10% (wt/vol) nonfat dry milk for 2 h at 37°C. The binding of OmpL1 (1 µg per well in PBS) to periodate-treated laminin was assessed as described above. The effect of the ionic strength on the binding of OmpL1 to PLG was assessed by increasing the NaCl concentration from 137 mM, which is the concentration in PBS solution, to 437 mM. To determine the role of lysines in the PLG-recombinant protein interactions, the lysine analog 6-aminocaproic acid (ACA) (Sigma-Aldrich), together with the recombinant protein at a final concentration of 2 mM or 20 mM, was added to the PLG-coated wells. The detection of bound protein was performed as described above.

**Plasmin enzymatic activity assay.** Ninety-six-well ELISA plates were coated overnight with 10 µg/ml recombinant protein or BSA and Lsa63 (73), as a negative control, in PBS at 4°C. Plates were washed once with PBS-T and blocked for 2 h at 37°C with PBS plus 10% (wt/vol) nonfat dry milk. The blocking solution was discarded, and 100 µl/well of 10 µg/ml human PLG was added, followed by incubation for 2 h at 37°C. Wells were washed three times with PBS-T, and 4 ng/well of human urokinase-type PLG activator (uPA; Sigma-Aldrich) was then added. Subsequently, 100 µl/well of the plasmin-specific substrate $\nu$-valyl-$\nu$-leucyl-$\nu$-lysine-$\nu$-nitroanilide dihydrochloride (Sigma-Aldrich) was added at a final concentration of 0.4 mM in PBS. Plates were incubated overnight at 37°C, and substrate degradation was measured by taking readings at 405 nm.

**Binding interference of OmpL1 with ECM and PLG.** Ninety-six-well plates were coated with 100 µl of 10 µg/ml laminin, plasma fibronectin, or PLG in PBS overnight at 4°C; washed three times; and blocked with 10% (wt/vol) nonfat dry milk for 2 h at 37°C. Concomitantly, OmpL1 (1 µg in 100 µl of PBS) was incubated with increasing concentrations (0 to 1 µg in 100 µl of PBS) of the other components separately and then transferred onto the coated plates, which were then incubated for 2 h at 37°C. After four washes, recombinant protein binding was quantified by specific antibodies, as described above.

Inhibition of binding of live leptospires to laminin, plasma fibronectin, and PLG by OmpL1.

ELISA plates were coated with laminin, plasma fibronectin, or PLG (1 µg/well). The plates were washed and blocked with 10% nonfat dry milk in PBS-T for 2 h at 37°C. The blocking solution was discarded, and the wells were incubated with increasing concentrations of recombinant protein (0 to 1.5 µM) for 2 h at 37°C. After three washes, 100 µl/well of 4 × 10^11 live *L. interrogans* serovar Copenhageni strain M20 leptospires in low-salt PBS (containing 50 mM NaCl) was added for 90 min at 37°C. The unbound leptospires were washed with low-salt PBS, and the quantification of bound leptospires was performed indirectly by the detection of anti-LipL32 antibodies produced in mice (14:000); due to the fact that LipL32 is a major outer membrane leptospiral protein (24), the procedure was followed by the detection of HRP-conjugated anti-mouse IgG antibodies essentially as described previously by Barbosa et al. (4). Detection was performed by o-phenylenediamine (OPD), as described above.

**Statistical analysis.** All results are expressed as means ± standard errors of the means (SEM). Student’s paired t test was used to determine the significance of differences between means, and P values lower than 0.05 were considered statistically significant.

**RESULTS**

**Cloning, expression, and purification.** Oligonucleotides for PCR amplification (see Materials and Methods) were designed based on genome sequences of *L. interrogans* serovar Copenhageni, excluding the signal peptide. The amplified coding sequence was cloned into an *E. coli* pAE vector (57), and the protein was expressed with a 6×His tag at the N terminus. The recombinant protein was expressed in the bacterial pellet, in its insoluble form, as inclusion bodies (Fig. 1A, lane 4). We failed to obtain the protein in its soluble form, possibly due to its structure, which is predicted to have 10 β-sheet transmembrane segments (63). Protein was recovered from inclusion bodies after solubilization with 8 M urea. Purification was performed by metal-chelating chromatography after refolding for the removal of urea and was evaluated by SDS-PAGE, as shown in Fig. 1A, lane 6. The recombinant protein bands were confirmed by probing Western blots with polyclonal antibodies raised in mice against OmpL1 (Fig. 1B, lane 1) and with anti-His tag monoclonal antibodies (Fig. 1B, lane 3). The calculated 32.4-kDa molecular mass of the recombinant OmpL1 protein comprises the vector fusion plus the encoded amino acid sequence. The structural integrity of the purified protein was assessed by circular dichroism (CD) spectroscopy. The method evaluates the secondary structure content of protein, and it is an important datum to obtain after protein refolding. As depicted in Fig. 1C, the CD spectrum shows the minima at 215 to 220 nm and the maximum at approximately 196 nm, which is typical of the β-strand secondary structure content of the recombinant protein. The predominance of the OmpL1 β-strand was also confirmed by an analysis of the spectrum data by K2D2 software, which showed 34% β-strand content and 14% alpha-helix content (52). The data are in agreement with the structure of the native protein, which
was predicted previously to have a predominance of β-strands (59).

Genomic DNA and protein conservation among leptospiral strains. The integrity of genomic DNA was confirmed by the amplification of the 16S rRNA gene. A single band corresponding to the OmpL1 gene was observed for all pathogenic strains tested, while no band was found for genomic DNA of the saprophytic strain (Fig. 2B, lanes 9 and 11). This might be due to some sequence problems with the gene or because the analysis was performed with different isolates.

Evaluation of immunological responses elicited by OmpL1 in mice. The antibody immune responses induced by OmpL1 in mice were analyzed by an ELISA on a quantitative basis. As depicted in Fig. 3A, IgG1 and IgG2a antibodies against OmpL1 were found, with a predominance of the IgG1 isotype. The major Ig response was that of IgG, with a titer of 100,400; the IgM titer was 6,400 (data not shown), and IgA was not detected. Statistically significant values for lymphocyte proliferation were achieved with animals immunized with OmpL1 and stimulated with the recombinant protein (P < 0.05) (Fig. 3B), with an average stimulation index of 2.8. A high proliferation level was obtained when cells were treated with ConA, which was employed as a positive control for the experiment (not shown). The addition of OmpL1 to lymphocytes from animals that had not been primed with the recombinant protein produced nonsignificant levels of proliferation (data not shown). Supernatants of cultured spleen cells from OmpL1-immunized mice were analyzed for the presence of the cytokines IL-10, IL-4, IFN-γ, and TNF-α, selected to discriminate cellular Th1 (IFN-γ and TNF-α) and humoral Th2 (IL-10 and IL-4) immune responses (58, 65). The cytokine profiles obtained showed a predominance of IL-10 and IFN-γ, with both occurring at high levels (Fig. 3C and D) similar to those for the ConA positive control (not shown). A statistically significant cytokine level was obtained for the OmpL1-primed group when cells were stimulated with OmpL1 (P < 0.001 for IL-10 and P < 0.0001 for IFN-γ); a lower value, although statistically significant, was observed for the TNF-α level (P < 0.05) when the cells from the OmpL1-primed group were induced or not with the recombinant protein (Fig. 3E). In contrast, no IL-4 stimulation was achieved when spleen cells from animal groups under the same experimental conditions were compared (Fig. 3F). Measurements of the same parameters with spleen cells from control animals immunized with PBS and either stimulated or not with the recombinant protein produced negligible results (not shown).

Reactivity of OmpL1 with human and hamster leptospirosis serum samples. We have performed ELISAs to analyze whether OmpL1 is recognized by antibodies present in serum samples from cases of confirmed leptospirosis in humans and experimentally infected hamsters. We employed 32 paired samples from patients in the early (MAT negative) and convalescent (MAT positive) phases of the disease and 44 samples of MAT-positive sera from hamsters. Our data show that the recombinant protein was able to recognize specific IgG antibodies in 90% of the tested sera from infected hamsters (Fig. 4A). A high level of responders (75%) was obtained for IgG in human MAT-positive sera, while a total of 9% of serum samples presented IgG antibodies against OmpL1 in the early phase of the disease (MAT negative) (Fig. 4B). The cutoff
value calculated with healthy human sera was 0.374. Due to the nonspecific clinical symptoms of leptospirosis, we analyzed the reactivity of the recombinant OmpL1 protein with serum samples from patients with unrelated infectious diseases who did not have a history of leptospirosis, including dengue virus infection ($n$ = 13), malaria ($n$ = 12), Chagas’ disease ($n$ = 20), and HIV infection ($n$ = 15). The reactivity obtained with OmpL1 and these serum samples was similar to that obtained with serum samples from healthy donors (Fig. 4B). The specificity of OmpL1 was calculated to be 100% for all unrelated diseases tested, except for dengue virus, for which the specificity was calculated to be 92.3%.

**Adhesion of OmpL1 to ECM components.** As native OmpL1 has surface-exposed domains (23, 54), we decided to evaluate whether this protein could mediate host colonization by adhering to extracellular matrix proteins. Thus, laminin, collagen type I, collagen type IV, cellular fibronectin, plasma fibronectin, ECM gel, elastin, and the control proteins gelatin, BSA, and fetuin were immobilized on 96-well microdilution plates, and recombinant protein attachment was assessed by an ELISA, as previously described (4). As shown in Fig. 5A, the OmpL1 protein exhibited statistically significant adhesiveness for laminin and plasma fibronectin ($P < 0.01$). The adherence of OmpL1 to ECM gel was expected, as laminin is one of the components (see Materials and Methods). No statistically significant adhesiveness was observed with the OmpL1 protein when wells were coated with collagen types I and IV, cellular fibronectin, elastin, or control proteins. Binding was confirmed by performing a similar assay but using anti-His tag monoclonal antibodies (Fig. 5B). The interaction of OmpL1 with laminin and with plasma fibronectin was also assessed on a quantitative basis by fixing the ECM components and changing the protein concentrations, as depicted in Fig. 5C and D, respectively. A binding saturation level was achieved with a protein concentration of $5 \mu$M for laminin and plasma fibronectin, with $K_D$ values of 2,099.93 ± 871.03 nM and 1,239.23 ± 506.85 nM, respectively. The role of the sugar moiety in laminin was also investigated by incubating this component with increasing concentrations of sodium metaperiodate. A reduction of 20% of the binding was found only with the highest concentration tested, suggesting that the carbohydrate moieties do not have a major role in the binding of OmpL1 to laminin (Fig. 5E). The effect of mouse anti-OmpL1 polyclonal serum on the interaction of the recombinant protein and ECM was also evaluated. A low antiserum dilution (1:50) almost abolished the binding of OmpL1 with laminin (90%), while a 73% binding reduction was obtained with fi-
bronectin (Fig. 5F). Preimmune serum at the same dilutions had only a moderate effect on the binding of the recombinant protein with ligands, with 21 and 17% reductions in binding for laminin and plasma fibronectin, respectively, reinforcing the interaction of OmpL1 and the two ECM components (Fig. 5F).

Recombinant leptospiral OmpL1 binds to human PLG. In our previous work, we have shown that leptospires bind PLG on their surface (77), and we have also described several proteins that are probably PLG receptors (73). Based on this assumption, we decided to evaluate whether OmpL1 can also adhere to human PLG in vitro. In addition, we investigated whether the recombinant protein can also bind factor H, human complement, and C4bp, as previously reported for other recombinant proteins (4, 5, 11, 16, 71). Our data show that OmpL1 binds to human PLG ($P < 0.01$), while no or a very low level of reactivity was detected with the other components and the control proteins (Fig. 6A). This binding was also confirmed by using anti-His monoclonal antibodies (Fig. 6B). The interaction between OmpL1 and PLG was also assessed on a quantitative basis, by keeping the PLG concentration constant and changing the protein concentration, as shown in Fig. 6C. A binding saturation level was reached with 2 $\mu$M OmpL1, with a $K_D$ of 368.63 ± 121.23 nM. It is well known that PLG kringle domains frequently mediate interactions with lysine residues of the bacterial receptors (32). These domains were shown previously to participate in the binding of PLG and intact live L. interrogans serovar Copenhageni strain L1-130 cells, since the derivative and analogue of lysine, ACA, almost totally inhibited binding (77). Based on these findings, the participation of lysine residues in the binding of the recombinant protein was evaluated by the addition of ACA to the assay mixture. As depicted in Fig. 6D, an increase of the NaCl concentration from 137 mM, which is the concentration in PBS solution, to 437 mM had no effect on the OmpL1-PLG binding, suggesting that ionic interactions were not important. In contrast, when 2 mM ACA was added to the reaction mixture, the binding of the protein to PLG was almost completely abolished ($P < 0.01$), strongly suggesting the participation of these domains in the OmpL1 interaction with PLG. We further evaluated the interaction of PLG with the recombinant protein by adding several dilutions of mouse anti-OmpL1 polyclonal serum to the binding reaction mixture. An inhibitory effect on the interaction of OmpL1 with PLG was observed, which was dependent on the serum dilution, being almost totally eliminated at the lowest serum dilution (86%), in contrast to the 17% binding reduction obtained with preimmune serum (Fig. 6E).

Plasmin generation from bound PLG. It was demonstrated previously that enzymatically active plasmin is generated by PLG

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**FIG 3** Evaluation of immune responses of BALB/c mice immunized with OmpL1. (A) Total IgG antibodies and subclasses in sera from BALB/c mice immunized with OmpL1 were determined. OD values were obtained from different pooled serum dilutions and compared against a concentration curve of total IgG, IgG1, and IgG2a. (B) The lymphocyte proliferation response was analyzed by using spleens of mice immunized with OmpL1. Cells were cultured, followed by a pulse with medium alone, ConA (5 $\mu$g/ml), or OmpL1 (5 $\mu$g/ml). Lymphocytes stimulated with medium alone were used as negative controls, and those stimulated with ConA were used as positive controls. The proliferative response was measured by a colorimetric BrdU ELISA. The data represent the mean OD values of three determinations ± standard deviations from two independent experiments. For statistical analysis, OD values for the OmpL1-immunized group treated with the recombinant protein were compared with those for the OmpL1-immunized group treated with medium alone by the two-tailed t test (*, $P < 0.05$). Spleen cells were isolated and cultured in 24-well tissue culture plates, with each well containing 5 x 10^6 cells. (C to E) After 48 h, cell-free culture supernatants were collected, and levels of the cytokines IL-10 (C), IFN-γ (D), TNF-α (E), and IL-4 (F) were measured by a sandwich ELISA according to the manufacturer's instructions. For statistical analysis, concentration values for the OmpL1-immunized group pulsed with the recombinant protein were compared with those for the OmpL1-immunized group treated with medium alone by the two-tailed t test (*, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$).
bound to the surface of \textit{L. interrogans} when its activator is present (77). To assess whether the PLG bound to OmpL1 generates proteolytic activity, as previously reported for other recombinant proteins (44, 49, 70, 71, 73), a microplate was coated with OmpL1, blocked, and then incubated with PLG. Unbound PLG was washed away, and a uPA (urokinase)-type PLG activator together with a plasmin-specific chromogenic substrate were added. The reaction was carried out overnight, and the plasmin activity was evaluated by measuring the cleavage of the plasmin-specific substrate D-valyl-leucyl-lysine-\(p\)-nitroanilide dihydrochloride at 405 nm. The PLG bound to the OmpL1 protein could be converted into plasmin, as indirectly demonstrated by the specific proteolytic activity (Fig. 6F). Control reaction mixtures lacking PLG, uPA, or the chromogenic substrate showed no significant enzymatic activity. The adhesin Lsa63, which is not a PLG-binding protein and therefore does not generate plasmin (73), was also included as a negative control (Fig. 6F).

\textbf{Inhibition of \textit{L. interrogans} attachment to laminin, PLG, or plasma fibronectin by OmpL1.} It was reported previously that several recombinant proteins (4, 44, 49, 53) exhibit an inhibitory effect on the binding of leptospires to PLG and ECM macromolecules. We thus carried out similar experiments to evaluate whether OmpL1 has a similar effect on the adherence of leptospires to laminin, PLG, and plasma fibronectin. The results show that the addition of increasing concentrations of OmpL1 reduced the binding of leptospires to laminin, PLG, and plasma fibronectin. The reduction in the number of leptospires adhering to PLG was statistically significant with 0.1 \(\mu\)M OmpL1 (\(P < 0.05\)), while for laminin, significance was reached with 1 \(\mu\)M protein (\(P < 0.05\)), and for plasma fibronectin, significance was reached with 1.5 \(\mu\)M protein (\(P < 0.05\)) (Fig. 7A). These results suggest that OmpL1 competes with the native leptospiral protein for the component-binding sites. The experiment was performed in triplicate, and Fig. 7 shows one representative data set from two independent experiments.

\textbf{Binding interference among OmpL1 ligands.} As OmpL1 showed significant binding to PLG, laminin, and plasma fibronectin, we decided to evaluate whether these macromolecules compete for the same protein-binding sites. Thus, we evaluated the competition among the components in combinations of two each time. Increasing concentrations of laminin or PLG had no effect on the binding of OmpL1 to plasma fibronectin (Fig. 7B). Similarly, the addition of laminin or plasma fibronectin caused no changes in the binding of the recombinant protein to PLG (Fig. 7C). The binding of laminin to OmpL1 was not affected by the addition of plasma fibronectin (Fig. 7D), in contrast with the effect produced by the addition of PLG, which, unexpectedly, caused an increased interaction (Fig. 7D).

\textbf{DISCUSSION} Leptospiral outer membrane proteins are the main focus of our research. Because of their location, these proteins may be involved in mechanisms of leptospiral pathogenesis and protective immunity. We reported that leptospires have the ability to adhere to laminin, cellular fibronectin, plasma fibronectin, and collagens I and IV (4), and to date, several leptospiral adhesin ECM-binding proteins have been identified. These proteins include the 36-kDa fibronectin-binding protein (45), LfhA/Lsa24 (4, 71), the LigA and LigB proteins (12, 36), Len family proteins (64), Lsa21 (2), LipL32 (27, 28), Lsa27 (38), Lp95 (3), TlyC (10), LipL53 (51), Lsa63 (74), OmpL37 (53), Lsa66 (49), Lsa20 (44), Lsa25, and Lsa33 (16). After adherence, leptospires rapidly disseminate to several organs, which is thought to be mediated by rapid translocation across host cell monolayers (7). Besides motility and che-
motaxis, *Leptospira* invasion may be mediated by a proteolytic activity capable of cleaving host cell membranes. Indeed, we have found that leptospires acquire host-derived plasmin by receptor-bound PLG and can degrade fibronectin and laminin (77a), which could help bacterial dissemination (73, 75, 77).

OmpL1 is a transmembrane outer membrane protein of *Leptospira* first described by Haake and colleagues (23). This protein was identified in pathogenic *Leptospira* spp. by proteomics (40, 46, 61, 76), exhibited a synergistic immunoprotective capacity in combination with LipL41 in the Golden Syrian hamster model of leptospirosis (25), and exhibited partial protective immunity as a recombinant protein (17) or as a DNA vaccine (41), and it has been studied by several groups for its human and veterinary diagnostic capabilities (17, 20, 29, 47, 48, 56, 67, 78). In this work, we report that OmpL1, encoded by the gene LIC10973, is an adhesin of *Leptospira* that interacts with laminin and plasma fibronectin, is a PLG-binding receptor capable of generating plasmin, and is specifically recognized by antibodies in serum samples from cases of confirmed leptospirosis in humans.

The DNA coding sequence of OmpL1 is conserved among all serovars of *L. interrogans* and other pathogenic species tested but is absent in the saprophytic species *L. biflexa*. These results corroborate...
FIG 6 OmpL1 binds to PLG and generates active plasmin. (A) Wells were coated with 1 μg of PLG, factor H, complement mix, C4bp, and the control proteins gelatin, BSA, and fetuin. One microgram of the recombinant protein was added per well, and binding was measured by an ELISA. Data represent the means ± standard deviations from three independent experiments. For statistical analyses, the attachment of the recombinant protein to the serum components was compared to its binding to all negative controls by the two-tailed t test, although the P value given here is relative to a comparison against gelatin (**, P < 0.01). (B) Significant binding activity was confirmed by an ELISA employing anti-His monoclonal antibodies. Data represent the means ± standard deviations from three independent experiments. For statistical analyses, the attachment of the recombinant protein was compared to its binding to gelatin by the two-tailed t test (**, P < 0.01; ***, P < 0.001). (C) OmpL1 dose-dependent binding experiments. Each point represents data determined in triplicate, and results are expressed as the mean absorbance values at 492 nm ± standard errors for each point. Gelatin was included as a negative control. The equilibrium dissociation constant (K_D) is depicted and was calculated based on ELISA data for the recombinant OmpL1 protein that reached equilibrium at a given concentration. (D) Binding of OmpL1 to PLG was carried out in the presence of a NaCl concentration from 137 to 437 mM and in presence or absence (no inhibition) of the lysine analogue 6-aminocaproic acid (ACA). Bound PLG was detected by specific antibodies and quantified. Bars represent the mean absorbances at 492 nm ± standard deviations of triplicate determinations and are representative of two independent experiments. For statistical analyses, the attachment of the recombinant protein in the presence of ACA was compared to its binding to PLG without ACA (no inhibition) by the two-tailed t test (**, P < 0.01). (E) Effect of preimmune and mouse polyclonal anti-OmpL1 serum dilution upon binding of OmpL1 with PLG, compared to binding in the absence of antibodies (*, P < 0.05; **, P < 0.01). (F) Plasmin generation by PLG bound to recombinant protein, measured indirectly by the cleavage of the specific plasmin substrate, was assayed by a modified ELISA. The immobilized recombinant OmpL1 protein received the following treatment: PLG, uPA, and the specific plasmin substrate (PLG+uPA+S) or controls lacking one of the three components (PLG+uPA, PLG+S, and uPA+S). BSA and Lsa63 were employed as negative controls. Bars represent the mean absorbance values at 405 nm, as a measure of the relative substrate cleavage, ± standard deviations from three replicates for each experimental group and are representative of two independent experiments. Statistically significant differences in comparison to BSA are shown (*, P < 0.05).
orate previously reported findings showing that this protein is expressed in high-passage-number pathogenic and low-passage-number virulent strains of *Leptospira* (23, 25, 26, 79). The LIC10973 gene was cloned and expressed in *E. coli* as a 32.4-kDa full-length recombinant protein, which comprises the native sequence of OmpL1 plus a 6×His tag at the N terminus. The purified protein exhibited a single major band in SDS-PAGE gels and was recognized by monoclonal anti-His tag antibodies and its homologous antibodies from mice immunized with the recombinant protein. An assessment of the secondary structure of the recombinant protein after the purification process was performed by CD spectroscopy, which showed a typical β-strand secondary structure content in the recombinant protein, which is in agreement with the predicted native structure of OmpL1, characterized as an outer membrane-spanning protein whose topological model contains 10 amphipathic transmembrane β-strands (23, 63).

Several researchers have suggested the importance of OmpL1 as a serological antigen for the diagnosis of leptospirosis in humans (17, 20, 47, 56, 67, 78) and canines (29, 48). Our data showed a high percentage of anti-OmpL1 IgG responders when serum samples from experimentally infected hamsters or MAT-positive human serum samples were used, which is in agreement with data from previously reported studies. Moreover, the results corroborate previously reported findings that this protein is expressed during infection (6). Further evaluations of OmpL1 with human serum samples from patients with confirmed unrelated infectious diseases revealed a high specificity of this antigen, which reinforces the importance of OmpL1 as an antigen for the development of a leptospirosis diagnostic kit.

Lin and colleagues (35) previously evaluated combined B and T cell epitopes of OmpL1 and of LipL41 conserved regions for their capacities to promote immune responses in BALB/c mice and reported that the selected epitopes can induce a Th1 response. It had already been shown that OmpL1 is highly immunogenic in animal models, but these antibodies are probably not protective, because OmpL1 alone did not protect hamsters against challenge with virulent leptospires (25). The high levels of OmpL1 IgG antibodies detected in sera from immunized BALB/c mice were mainly of the IgG1 subclass, indicating a Th2-type response. Our data show that recombinant OmpL1 was also capable of activating a cell-mediated immune response, indicated by lymphocyte proliferation in OmpL1-immunized mice. Moreover, OmpL1 was capable of inducing both cellular and humoral immune response, as demonstrated by the levels of the cytokines IFN-γ, TNF-α (Th1),

**FIG 7** Inhibition of binding of leptospires to ECM or PLG by OmpL1 and competition assays of OmpL1 binding to ECM and PLG. (A) Laminin, plasma fibronectin, or PLG (1 µg/well) was adsorbed onto microtiter plates, followed by incubation with increasing concentrations of OmpL1 (0 to 1.5 µM) for 90 min at 37°C. After washes, live leptospires (100 µl/well of 4 × 10⁷ leptospires) were added, and the quantification of bound leptospires was performed indirectly with anti-LipL32 antibodies produced in mice (1:4,000 dilution), followed by HRP-conjugated anti-mouse IgG antibodies. Each point represents the mean absorbance value at 492 nm ± the standard deviation of three replicates. Data are representative of two independent experiments (*, P < 0.05). The effect of the component on every other interaction with OmpL1 was determined by competition assays with the addition of increasing concentrations of different components (0 to 1.00 µg in 100 µl PBS). (B) The effect of PLG or laminin on the binding of OmpL1 (10 µg/ml) to immobilized plasma fibronectin (10 µg/ml) was determined with the addition of increasing PLG or laminin concentrations (0 to 1.00 µg in 100 µl PBS). (C and D) Effect of laminin and plasma fibronectin on the binding of OmpL1 to immobilized PLG (C) and effect of PLG and plasma fibronectin on the binding of OmpL1 to immobilized laminin (D). Statistically significant interference was determined by the two-tailed t test (**, P < 0.01).
and IL-10 (possibly Th2). These results might be explained in part by the effects exerted by the aluminum salt employed as an adjuvant, which is known to stimulate both Th1 and Th2 immune responses (42). In fact, Vernel-Pauillac and Merien (72) demonstrated previously that pathogenic leptospirases can elicit Th1 responses together with antilipopolysaccharide antibodies in an animal model. Moreover, the protective immune mechanism of the LigA DNA vaccine in immunized animals was evaluated and was shown to be conferred by both Th1 and Th2 immune responses (19). It is worth mentioning that the recombinant LipL32 protein, a major leptospiral antigen, failed to confer protection against virulent bacteria in several challenge studies (9, 39). However, protection with this antigen was achieved when hamsters were immunized with recombinant Mycobacterium boris BCG (rBCG) expressing LipL32 (62). Recombinant BCG has the ability to elicit strong cellular as well as humoral immune responses (15). Thus, it is possible that not only humoral but also cellular immune responses are important for immunoprotection against leptospirosis. An understanding of the immune responses elicited by recombinant leptospiral antigens may provide new insights into the mechanisms of anti-Leptospira immunity and may facilitate the design of an improved recombinant-based vaccine.

OmpL1 is a surface-exposed protein that is expressed during infection. It is therefore possible that it may play a role in attachment to the host. Indeed, the OmpL1 protein exhibits extracellular matrix-binding properties. This protein binds laminin and plasma fibronectin, in contrast to the previously reported Lsa24 (4), Lsa27 (38), and Lsa20 (44) adhesins, which are laminin-binding adhesins, but similar to other previously reported adhesins, namely, Len family proteins (64), the LigA and LigB proteins (12), Lsa21 (2), LipL32 (27, 28), TlyC (10), OmpL37 (53), and Lsa66 (49), which showed broader-spectrum binding with ECM. The calculated $K_d$ values for the binding of OmpL1 to laminin and to plasma fibronectin (2,099.93 ± 871.04 and 1,239.23 ± 506.85 nM, respectively) are, however, higher than the $K_d$ values obtained with Lsa66 and the same ECM components (55.4 ± 15.9 nM and 290.8 ± 11.8 nM) (49). Nevertheless, the kinetics of the dose dependence of each ligand with OmpL1 are specific, and saturable binding to immobilized components was observed, fulfilling the properties of a typical receptor-ligand interaction.

An interaction with host PLG, a key component of the host fibrinolytic system, has been shown for several invasive Gram-positive and Gram-negative bacteria (13, 68). We reported previously that *Leptospira* species were also capable of binding PLG and generating active plasmin on their surface (77). Moreover, we have demonstrated that the generation of this proteolytic activity makes virulent *L. interrogans* bacteria capable of degrading purified extracellular matrix fibronectin and laminin (77a), a step which may contribute to leptospiral invasiveness (76). In addition, we have identified proteins that act as leptospiral PLG receptors (73). OmpL1 also shows PLG-binding activity that seems to occur via PLG kringle domains, with a $K_d$ of 368.63 ± 121.23 nM. This $K_d$ value is of the same order of magnitude as the one reported previously for the adhesin Lsa20 (44) but higher than the ones reported previously for several recombinant proteins in our laboratory (73). As previously shown for other protein-PLG receptors (44, 49, 70, 71, 73), plasmin is actively generated in the presence of the urokinase activator by PLG-bound OmpL1. Thus, it is possible that this protein may contribute to leptospiral infectiveness. The low binding affinity of OmpL1 for ECM and PLG could be compensated for by the high copy number reported previously for this protein in *L. interrogans* (40), which was shown to increase after 7 days of serum treatment (61). The fact that mouse polyclonal anti-OmpL1 serum had a strong reduction effect on the binding of OmpL1 with laminin, plasma fibronectin, and PLG, along with the fact that preimmune serum had only a moderate effect, strengthens the data for the reactivity between the recombinant protein and these ligands. No interference of the binding of OmpL1 with these ligands was observed, except for the increased binding effect produced by the increased PLG concentration on the binding of recombinant protein to laminin, suggesting that PLG is probably binding directly to immobilized laminin. Indeed, the interaction of laminin with PLG was reported previously (60).

OmpL1 exhibited an inhibitory effect on the binding of intact *L. interrogans* to immobilized laminin, plasma fibronectin, or PLG, as was previously observed for the ECM- and PLG-interacting proteins Lsa24 (4), Lsa63 (74), LigA/LigB (12), OmpL37 (53), and Lsa20 (44).

In conclusion, in this work, we report for the first time that OmpL1 is an ECM- and PLG-binding protein of *Leptospira* spp. The interaction of OmpL1 with PLG in the presence of a host activator can generate plasmin, rendering the bacteria with proteolytic activity capable of degrading host cell membranes. Thus, as an ECM- and PLG-binding protein, OmpL1 may promote the attachment of the bacteria to mammalian hosts and may help the leptospires to disseminate during the infection process. The high percentage of human leptospirosis (MAT-positive) responders associated with the specificity of the IgG antibody response to OmpL1 among serum samples from patients with unrelated febrile diseases such as dengue virus, malaria, HIV, and Chagas’ disease strengthens the potential of OmpL1 as a diagnostic marker for leptospirosis. OmpL1 promotes the proliferation of lymphocytes and activates both Th1 and Th2 cytokines from immunized BALB/c mice. It is conceivable that OmpL1 administered with an adequate immune modulator might exhibit protective activity against lethal challenges.

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**REFERENCES**

Ompl1 Binds ECM and PLG

32. Pinne M, Haake DA. 2009. A comprehensive approach to identification of surface-exposed, outer membrane-spanning proteins of Leptospira interro-