Protective Immunity and Reduced Renal Colonization Induced by Vaccines Containing Recombinant *Leptospira interrogans* Outer Membrane Proteins and Flagellin Adjuvant

D. Monaris,[a,b] M. E. Sbrogio-Almeida,[c] C. C. Dib,[d] T. A. Canhamero,[e] G. O. Souza,[b] S. A. Vasconcellos,[b] L. C. S. Ferreira,[f] P. A. E. Abreu[a]

Laboratório de Bacteriologia, Instituto Butantan, São Paulo, SP, Brazil[a]; Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, SP, Brazil[b]; Centro de Biotecnologia, Instituto Butantan, São Paulo, SP, Brazil[c]; Laboratório de Tuberculose, Instituto Biológico, São Paulo, SP Brazil[d]; Laboratório de Imunogenética, Instituto Butantan, São Paulo, SP, Brazil[e]; Laboratório de Desenvolvimento de Vacinas, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, SP, Brazil[f]

Leptospirosis is a global zoonotic disease caused by different *Leptospira* species, such as *Leptospira interrogans*, that colonize the renal tubules of wild and domestic animals. Thus far, attempts to develop effective leptospirosis vaccines, both for humans and animals, have failed to induce immune responses capable of conferring protection and simultaneously preventing renal colonization. In this study, we evaluated the protective immunity induced by subunit vaccines containing seven different recombinant *Leptospira interrogans* outer membrane proteins, including the carboxy-terminal portion of the immunoglobulinlike protein A (LigA$_C$) and six novel antigens, combined with aluminum hydroxide (alum) or *Salmonella* flagellin (FliC) as adjuvants. Hamsters vaccinated with the different formulations elicited high antigen-specific antibody titers. Immunization with LigA$_C$, either with alum or flagellin, conferred protective immunity but did not prevent renal colonization. Similarly, animals immunized with LigA$_C$ or LigA$_C$ coadministered with six leptospiral proteins with alum adjuvant conferred protection but did not reduce renal colonization. In contrast, immunizing animals with the pool of seven antigens in combination with flagellin conferred protection and significantly reduced renal colonization by the pathogen. The present study emphasizes the relevance of antigen composition and added adjuvant in the efficacy of antileptospirosis subunit vaccines and shows the complex relationship between immune responses and renal colonization by the pathogen.

Leptospirosis is an important global public health problem, particularly in tropical and subtropical countries (1). It is a zoonotic disease caused by pathogenic leptospires that are maintained by persistent renal colonization of domestic and wild animal species. The infection may result from either direct contact with infected animals or indirect exposure to water or soil contaminated with the urine of infected animals (2). Annually, 500,000 cases of severe leptospirosis in humans have been reported worldwide, with a mortality rate of more than 10% (3, 4). Despite the gravity of the disease, the incidence of the illness is likely underestimated due to the nonspecific clinical manifestations and lack of an efficient method of diagnosis (4, 5). In the veterinary field, leptospirosis causes significant economic losses due to the effects on the reproductive potential of animals, including infertility, stillbirths, abortions, weak newborns, and reduced milk production in cattle and other ruminants (6, 7).

Commercially available leptospirosis vaccines have been widely used in livestock and are licensed for human use in a few countries (8). These vaccines consist of killed whole cells (bacterins) and suffer from several limitations, such as reduced protective efficacy that fails to prevent renal colonization and urinary shedding of the pathogen by vaccinated animals. The vaccines also fail to induce long-term immunity and confer protection only against the serovars present in the preparation. In addition, the presently available vaccines carry a number of contaminants that originate from the production process and are associated with rather serious side effects (8–10).

Subunit vaccines may be an alternative for leptospirosis prevention. Several leptospiral outer membrane proteins have been evaluated as potential vaccine antigens, including lipoproteins (LipL41 and LipL32), porin OmpL1, immunoglobulinlike proteins (LigA and LigB), and OmpA-like proteins (9–20). Previous evidence indicated that hamsters vaccinated with a combination of OmpL1 and LipL41 embedded in bacterial membranes developed protective immunity and resistance to renal colonization (11). Immunization with a DNA vaccine encoding the conserved amino-terminal regions of LigA and LigB provided partial protection, and most of the surviving animals showed sterilizing immunity (19). LigA belongs to the family of bacterial proteins characterized by the presence of immunoglobulinlike repeat domains. Three genes (*ligA*, *ligB*, and *ligC*) have been described in *Leptospira* spp. and are only present in pathogenic species. The *ligB* gene is found in several *Leptospira* species, whereas *ligA* is restricted to *L. interrogans* and *Leptospira kirschneri*, and *ligC* is a pseudogene (15,

21–23). LigA and LigB are expressed during infection and participate in the processes of adhesion of leptospires to host cells (15, 21, 22).

In a previous study, 238 putative surface-exposed or secreted leptospiral proteins were expressed in *Escherichia coli* and tested as potential antigens in vaccine formulations with aluminum hydroxide adjuvant (24). The purified recombinant proteins were immunogenic, but none could prevent renal colonization after challenge with *Leptospira borgpetersenii* (24). These results indicated that the induction of protective immunity and the simultaneous prevention of renal colonization remain unmet challenges for those dealing with the development of leptospirosis vaccines. For that purpose, the testing of new adjuvants may represent a key step toward the discovery of an effective leptospirosis vaccine.

Flagellin, the subunit protein of the flagellar filament, expressed by *Salmonella* as well as other bacterial species, represents an agonist of innate immunity and has been successfully used as a vaccine adjuvant (25–30). The inflammatory responses induced by flagellin, as well as other pathogen-associated molecular patterns (PAMPs), activate antigen-presenting cells and result in the release of cytokines and chemical mediators with direct effects on the adaptive immune response (31–33). This knowledge has been used in the development of new vaccine formulations by promoting the link between innate and adaptive immune responses through the incorporation of PAMPs with the target antigens (25–30).

In this study, we evaluated the induction of protective immunity in hamsters after immunization with leptospiral subunit vaccines containing seven different *L. interrogans* outer membrane proteins, including the carboxy portion of LigA, in combination with two different adjuvants, the *Salmonella enterica* serovar Typhimurium flagellin (FliC) and aluminum hydroxide (alum). Our results demonstrated that only animals immunized with the pool of antigens combined with flagellin mounted a protective immune response and controlled renal colonization by the pathogen.

MATERIALS AND METHODS

Leptospiral strain and growth conditions. The *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (ATCC BAA-1198) was cultivated at 29°C under aerobic conditions in liquid EMJH medium (Difco) with 10% rabbit serum, enriched with L-asparagine (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 meat extract (0.02% [wt/vol]). Virulence was maintained by iterative passages in hamsters (34).

***In silico* analysis.** The proteins encoded by the LIC10009, LIC10301, LIC10507, LIC10704, LIC11030, and LIC11087 genes were identified in the *L. interrogans serovar* Copenhageni lipoprotein database (http://mic.sgmjournals.org/content/journal/micro/10.1099/mic.0.28317-0#tab5) (35) on the basis of outer membrane localization as previously described (36). The standard protein-protein BLAST analysis (BLASTp) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify similarities between selected proteins in sequence databases. We considered only sequences that aligned to the query with an E value of zero or less.

Purification of recombinant proteins and *Salmonella* flagellin. Open reading frames LIC10009 (encoding a protein designated Lp25, for leptospiral protein 25), LIC10301 (Lp11), LIC10507 (Lp21), LIC10704 (Lp22), LIC11030 (Lp35), and LIC11087 (leptospiral adhesion protein 30 [Lsa30]) were cloned without signal peptide tags into the pAE vector as previously described (36). The coding sequence of the carboxy-terminal portion of LigA (LigA$_C$), corresponding to nucleotides 1891 to 3675

(LIC10465), was amplified by PCR from genomic DNA of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 using the following primers: F, GGATCCCTTACCGTTTCCAACACAAACG, and R, CCATGGTTAC TCGAGTGGCTCCGTTTTAATAG. After digestion with BamHI and NcoI restriction enzymes, the fragment was cloned into the pAE vector. The expression and purification of recombinant proteins with an amino-terminal 6×His tag were performed as previously described (36, 37). Competent cells of the *E. coli* C43 strain were transformed with pAE constructs and cultivated until the optical density at 600 nm reached 0.6. The expression of recombinant proteins was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 37°C for 3 h. The cells were harvested by centrifugation, resuspended in 20 mM Tris-HCl and 500 mM NaCl at pH 8.0, and lysed by using a French pressure cell (Spectronic Instruments, Inc., Rochester, NY). The soluble and insoluble fractions were separated by centrifugation at 26,000 × g for 15 min. The His-tagged recombinant proteins Lp21 and Lp22, from the supernatant (soluble fraction), and Lp25, Lp11, Lp35, Lsa30, and LigA$_C$, from the inclusion bodies, (insoluble fraction) were purified by metal affinity chromatography. The soluble fractions were diluted (10-fold) in binding buffer (20 mM Tris-HCl and 500 mM NaCl at pH 8.0) containing 5 mM imidazole. Inclusion bodies were washed with binding buffer containing 2 mM β-mercaptoethanol, 1 M urea, and 1% Triton X-100 and were then solubilized with binding buffer containing 8 M urea and 5 mM β-mercaptoethanol for 16 h at 25°C. For refolding of proteins solubilized in the presence of urea, the suspensions were diluted (100-fold) with binding buffer containing 5 mM imidazole. Protein solutions obtained from the diluted soluble fraction and refolding fraction were loaded onto nickel-charged chelating Sepharose columns (GE Healthcare, United Kingdom). After adsorption of proteins, columns were washed sequentially with binding buffer mixtures containing 5, 20, 40, 60, and 100 mM imidazole. His-tagged proteins were eluted from the column with 1 M imidazole. Purified proteins were dialyzed extensively against phosphate-buffered saline (PBS), and samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The flagellin (FliC) was purified from *Salmonella enterica* serovar Typhimurium SL3261 as previously described (30). Briefly, the cells were harvested by centrifugation, suspended in PBS, and sheared in a bench mixer at maximal speed (a 1-min treatment repeated three times), followed by another centrifugation step to remove the bacterial cells. Broken flagella fragments were precipitated with acetone, suspended in PBS, and submitted to heat treatment (65°C for 30 min) to dissociate the flagellin monomers. The protein concentrations were determined using the Bradford assay (Pierce, Rockford, IL), and the purity of the protein preparations was monitored by SDS-PAGE. Removal of contaminating lipopolysaccharide was accomplished with Detoxi-Gel columns (Pierce, Rockford, IL) according to the manufacturer's instructions. Endotoxin levels, determined with the chromogenic Limulus amebocyte lysate assay (Lonza), were always below 2.0 endotoxin units/μg protein.

CD spectroscopy. Purified recombinant proteins were dialyzed against sodium phosphate buffer (pH 7.4). Circular dichroism (CD) spectroscopy measurements were performed at 20°C using a Jasco J-810 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan) equipped with a Peltier unit for temperature control. Far-UV CD spectra were measured at 0.5-nm intervals using a 1-mm-path-length cell. The spectra are presented as the averages of five scans recorded from 190 to 260 nm.

Hamster immunization and challenge. Groups of 10 male Syrian golden hamsters, 4 weeks of age, were immunized subcutaneously with six purified recombinant proteins or a pool of these proteins (20 μg of each protein) with or without 50 μg of LigA$_C$, as well as in combination with 5 μg of FliC or 500 μg of aluminum ion in the form of aluminum hydroxide (Alhydrogel). Control groups were immunized with PBS in addition to 5 μg of FliC or 500 μg of aluminum ion. The animals were boosted 15 days later with the same antigen preparation. One day before the second immunization and 1 day before the challenge, the hamsters were bled from the retro-orbital plexus, and the sera were analyzed by enzyme-linked immunosorbent assay (ELISA). On the 31st day, the hamsters were chal-

lenged by intraperitoneal inoculation of 2×10^3 leptospires, corresponding to 100-fold the 50% lethal dose (LD_{50}) calculated by the method of Reed and Muench (38). Animals surviving after 21 days were considered protected. Protection was calculated as the percentage of animals that were protected out of the total number of animals challenged. Blood was collected from survivors, and the collected sera were analyzed by the microagglutination test (MAT) using *L. interrogans* serovar Copenhageni antigen, as previously described (11). The surviving hamsters were euthanized, and the animals' kidneys were collected at necropsy. One kidney sample from each animal was prepared for bacteriological culture by gently homogenizing the tissue, and two serial 10-fold dilutions of the tissue homogenate were used to inoculate a semisolid medium. Cultures were periodically examined for the presence of bacteria using dark-field microscopy for up to 1 month before being designated negative. Another kidney sample was processed for histopathology with formalin fixation, paraffin embedding, sectioning, and staining using the Warthin-Starry method. The establishment of renal infection was measured using bacteriological culture and direct examination of leptospires in silver-stained tissue sections by microscopic analysis and MAT test. Figure S1 in the supplemental material presents representative examples of positive and negative cultures and microscopic examinations. An animal was considered positive for renal colonization when we were able to detect leptospires using at least one of the methods. The percentage of renal colonization was calculated as the number of positive animals divided by the total number of survivors. During all experiments, animals were supplied with food and water *ad libitum*, and experimental protocols were previously approved by the Ethical Committee for Animal Research of the Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, Brazil, under the license number 156108.

ELISA. Serum antibody responses after immunization with recombinant proteins and controls were quantified by ELISA. Microtiter plates (Nunc MaxiSorp; Thermo Fisher Scientific, Inc., Rochester, USA) were coated with recombinant proteins (100 µl at 10 µg/ml) overnight at 4°C. The wells were washed three times with PBS–0.05% Tween 20 (PBST) (pH 7.4), blocked with 10% nonfat dry milk in PBS for 2 h at 37°C, and incubated with serial dilutions of hamster sera in PBS for 1 h at 37°C. The plates were washed three times with PBST and incubated with rabbit anti-hamster IgG (1:5,000) and with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:5,000) (Sigma) in PBS–1% nonfat dry milk at 37°C for 1 h. The HRP substrate, *o*-phenylenediamine (0.04%) in citrate phosphate buffer (pH 5), plus 0.01% H_2O_2, was added, and the plates were incubated for an additional 15 min at room temperature in the dark. The reaction was interrupted by the addition of 50 µl of 8 M H_2SO_4. All samples were assayed in triplicate, and endpoint titers were defined as the inverse of the highest dilution that resulted in a reading 2 standard deviations above the background.

Western blot analysis. Cultures of leptospires were harvested by centrifugation, and the pellets were washed twice with PBS. After centrifugation, the cells were suspended in PBS, homogenized, and along with purified recombinant proteins, subjected to SDS-PAGE under reducing conditions; all proteins were then transferred to a nitrocellulose membrane. Nonspecific binding sites were blocked by using 10% (wt/vol) nonfat dried milk in PBST (pH 7.4) overnight at 4°C. Subsequently, the membrane was rinsed one time in PBST and incubated for 60 min at 25°C with hamster anti-recombinant protein antibody (diluted 1:500), mouse anti-His tag antibody (diluted 1:3,000), or sera from hamsters immunized with heat-killed leptospires (diluted 1:50 for Lp11, Lp21, Lp22, and LigA$_C$ and 1:100 for Lp25, Lsa30, and Lp35) in 5% (wt/vol) nonfat dried milk–PBST. After five washes with PBST, the membrane was incubated with secondary peroxidase-conjugated anti-mouse or anti-hamster IgG at a 1:5,000 dilution for 60 min at 25°C. After incubation, the membrane was washed with PBST four times. The positive signal was detected by enhanced chemiluminescence (West Pico; Pierce, Thermo Fisher Scientific, Inc.).

Statistics. Differences in rates of survival and the presence of renal colonization were analyzed by Fisher's exact test.

RESULTS

Selection and characteristics of the antigens. As surface proteins are potential targets of host immunity, our rationale for antigen selection was based on the localization of proteins at the outer membrane. Bioinformatics analyses using the *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 genome sequence were performed previously as described in a work published by our group (36). We selected six probable outer membrane proteins that could be expressed in *E. coli* and produced them for the immunization experiments. These proteins were assigned designations using Lp, for leptospiral protein, with the exception of the previously characterized protein named Lsa30 (leptospiral adhesion protein 30), which is able to bind to laminin and plasma fibronectin (39), and the protein encoded by the LIC10507 gene, herein named Lp21, which is involved in the upregulation of intercellular adhesion molecule 1 (ICAM-1) and E-selectin on endothelial cells (40). We also evaluated the amino acid sequence identities of the proteins in comparison to the amino acid sequences of proteins from other leptospire species. The names, GenBank accession numbers, and sequence identities of the proteins are presented in Table S1 in the supplemental material. Sequences identical or similar to the those of the six proteins were identified in NCBI databases by BLASTp analyses. All proteins were conserved in pathogenic strains of *Leptospira* spp., with identity values ranging from 30% to 100%. No sequences similar to those of these proteins were identified in saprophytic *Leptospira* species. The proteins did not exhibit an assigned function or specific structural domains. Outer membrane localization was experimentally confirmed for the proteins Lp21 (40), Lsa30 (39), and Lp35 (41) in previous studies. In addition to these six proteins, the LigA carboxy-terminal fragment, corresponding to repeat domains 7 to 13 (LigA$_C$), was included in the study based on previously reported protective effects, although LigA$_C$ has not been shown to confer sterilizing immunity (17, 18).

Purification, characterization, and antigenicity of recombinant proteins. The recombinant proteins were purified by immobilized metal ion affinity chromatography, and the homogenous protein bands were visualized by SDS-PAGE (see Fig. S2 in the supplemental material). The structural integrity of the purified proteins was assessed by circular dichroism (CD) spectroscopy. As illustrated in Fig. S3, the CD spectra reveal that the secondary structures of the recombinant proteins were maintained after the purification process. The presence of significant near-UV signals is a good indication that the proteins were folded into well-defined structures.

The antigenicity of the recombinant proteins was evaluated using the sera of hamsters immunized with heat-killed leptospires (*L. interrogans* serovar Copenhageni strain Fiocruz L1-130). The reactivity of the antileptospiral antisera to each recombinant protein was determined by ELISA and Western blot analysis (Fig. 1). Hamsters immunized with heat-killed leptospires developed serum IgG responses to all recombinant proteins tested. In contrast, the sham (PBS)-treated animal group developed low-level, nonspecific reactions to the tested proteins (Fig. 1A). Immunoblots were performed using the immobilized purified recombinant proteins and anti-His tag antiserum or sera from hamsters immunized with heat-killed leptospires. Figure 1B shows the results of reaction with the anti-His tag antibodies (Fig. 1B, lanes 1). The same figure also shows the results obtained with sera from ham-
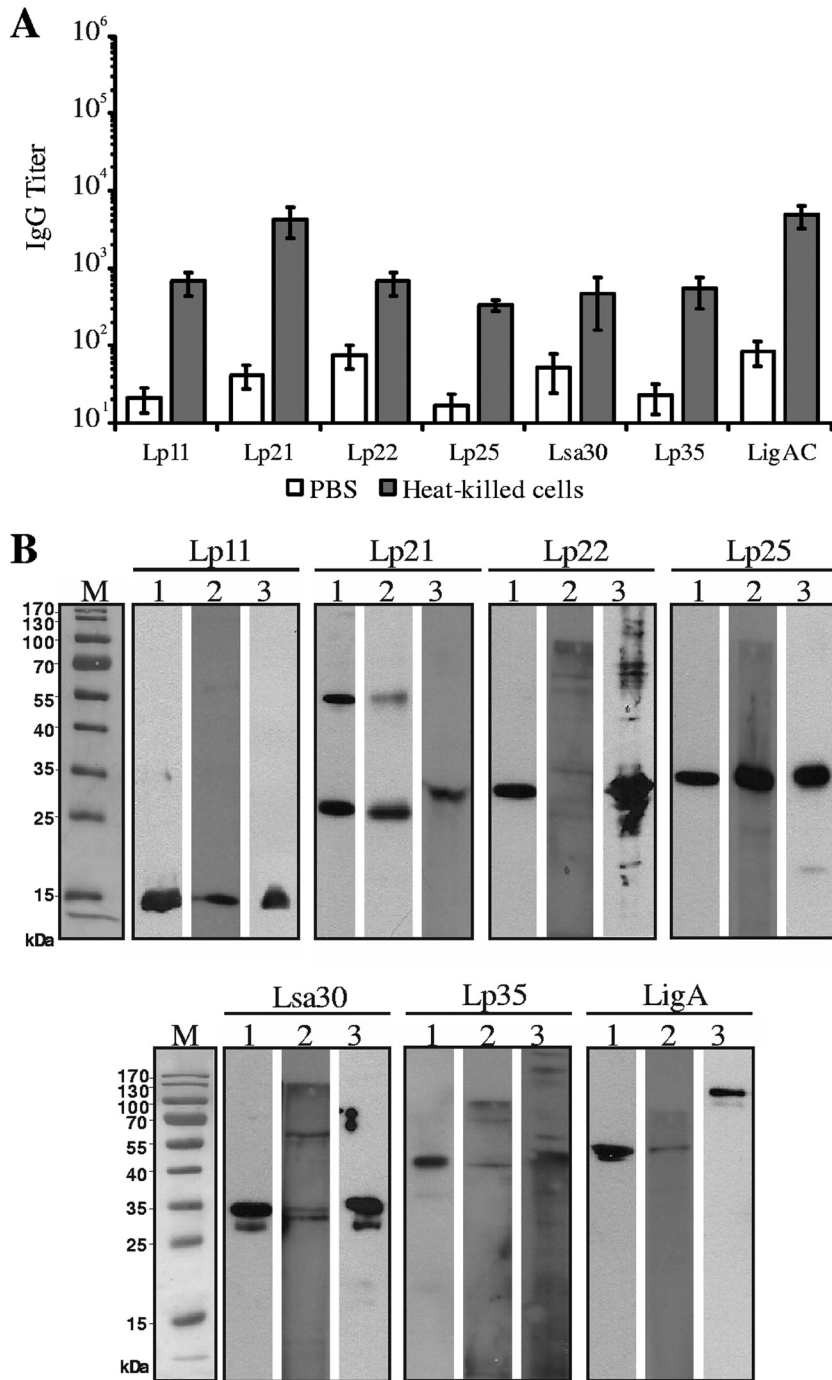
FIG 1 Antigenic properties of the recombinant *L. interrogans* antigens. (A) Antigen-specific antibody responses in sera from hamsters immunized with heat-killed leptospires and subsequently reacted with purified recombinant antigens in ELISAs. Hamsters were inoculated subcutaneously with two doses of 10^5 heat-killed *L. interrogans* serovar Copenhageni strain Fiocruz L1-130. A negative-control group was injected with PBS. On the 30th day after the first immunization, hamsters were bled, and the sera were analyzed by ELISA. Microtitration plates were coated with each recombinant protein and incubated with serial dilutions of serum from the immunized hamsters to measure specific reactions. Results are expressed as the means ± standard errors (SE). (B) Reactivities of sera with recombinant antigens detected in immunoblots. The samples used in the Western blot analyses were as follows: lanes M, prestained molecular mass markers; lanes 1, purified recombinant proteins probed with anti-His tag monoclonal antibodies; lanes 2, sera from hamsters immunized with heat-killed leptospires; lanes 3, whole-cell lysate of *L. interrogans* serovar Copenhageni probed with polyclonal antiserum raised with each of the recombinant proteins tested. The native LigA protein is ~130 kDa, and the recombinant form, LigA$_C$, is ~60 kDa.

sters immunized with heat-killed leptospires (Fig. 1B, lanes 2). The Lsa30, Lp35, and LigA$_C$ proteins were detected less efficiently than the Lp11, Lp21, and Lp25 proteins, while Lp22 was not detected. The ~55-kDa fragment observed in the membrane probed

with anti-Lp21 antiserum probably corresponds to dimers of the protein. These results suggest that the reaction of Lp22 in ELISA may represent cross-reactivity with *E. coli* antigens.

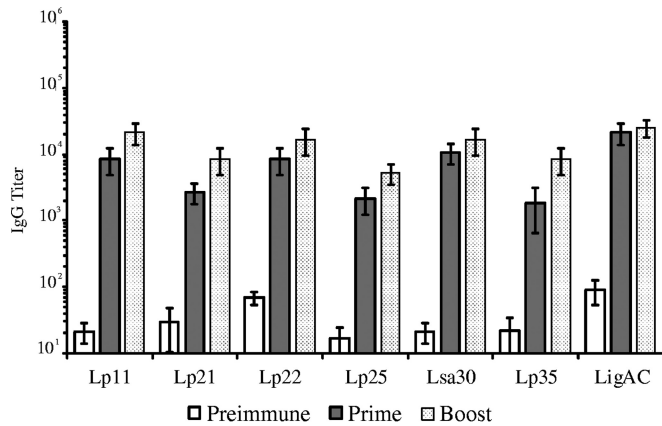To assess whether the tested proteins are expressed in culti-

FIG 2 Antibody responses induced in hamsters after immunization with the recombinant proteins. Hamsters were immunized subcutaneously with two doses of each purified recombinant protein adsorbed to alum at an interval of 15 days. One day before the second immunization (prime) and 1 day before the challenge (boost), the animals were bled, and the sera were analyzed by ELISA. Microtiter plates were coated with recombinant proteins and incubated with serial dilutions of the serum samples. Results are expressed as the means ± SE.

vated leptospires, we performed immunoblots using whole-cell lysates of *L. interrogans* serovar Copenhageni strain L1-130 incubated with antisera from hamsters immunized with each of the different recombinant antigens. The results in Figure 1B, lanes 3, show that the tested proteins are expressed in leptospires. These results are in agreement with previously published studies that demonstrate the expression of Lp22 (40), Lsa30 (39), and Lp35 (41) proteins in bacterial culture. Overall, these data demonstrate that the recombinant proteins preserve the antigenicity of the native leptospiral proteins.

Antibody responses, protective immunity, and renal colonization in hamsters immunized with subunit vaccines containing different recombinant leptospiral antigens. To evaluate the ability of recombinant proteins to promote protective immunity, hamsters were immunized with isolated antigens adsorbed to alum in two independent experiments. All experimental groups exhibited high serum IgG titers to the tested recombinant protein after the first and second immunizations (Fig. 2). However, no significant differences among the survival rates of immunized animals and sham-treated animals were recorded (see Table S2 in the supplemental material). Animals immunized with LigA$_C$ and alum survived the challenge and did not exhibit any symptoms of the disease (see Table S2). Nonetheless, as expected, hamsters immunized with LigA$_C$ could not prevent renal colonization by the pathogen.

Next, we investigated the immune responses and protective immunity elicited in hamsters immunized with the mixture of the recombinant proteins tested. Animals were immunized with the six purified recombinant proteins, with or without LigA$_C$, adsorbed to alum in three independent experiments. The vaccine formulations containing the recombinant Lps induced high antibody titers to all tested proteins, and no differences were observed with coadministration of LigA$_C$ (Fig. 3A and B). Hamsters immunized with LigA$_C$ alone or LigA$_C$ coadministered with the six Lps and alum adjuvant exhibited statistically significant immunoprotection (100% and 87%, respectively) compared to the level of protection in the animals inoculated with PBS and alum. Animals

immunized with the pool of Lps without LigA$_C$ were partially protected (50%) against the lethal challenge, whereas most of the animals inoculated with PBS and alum exhibited symptoms of leptospirosis and died (Fig. 3C; see also Table S3 in the supplemental material). In these experiments, animals immunized with LigA$_C$ or LigA$_C$ coadministered with the pool of Lps were positive (100%) for the presence of leptospira in the kidneys (Fig. 3D; see also Table S4). These data indicate that the combination of Lps did not enhance the protective immunity induced by LigA$_C$, nor did the combination confer sterilizing immunity when combined with alum as an adjuvant.

In the next step, we evaluated the role of adjuvant in the protective immunity conferred by the leptospirosis vaccine formulation containing the pool of Lps and LigA$_C$. For this purpose, we tested purified *Salmonella* FliC flagellin, previously shown to be a promising vaccine adjuvant for acellular vaccines (25–30). Hamsters immunized with LigA$_C$ alone or LigA$_C$ combined with the six Lps and coadministered with flagellin developed high serum IgG titers to the tested antigens. The incorporation of LigA$_C$ did not affect the antibody responses to the Lps (Fig. 4A and B). Nonetheless, animals immunized with LigA$_C$ plus FliC or LigA$_C$ plus the Lp mixture and FliC were protected against the lethal leptospiral challenge (93% for LigA$_C$ plus FliC and 86% for LigA$_C$, Lp pool, and FliC). In contrast, animals immunized with the Lp pool and FliC did not survive the challenge, despite the high serum antibody titers. Most of the animals inoculated with PBS and FliC showed symptoms of leptospirosis and died (Fig. 4C; see also Table S5 in the supplemental material). In three experiments, animals immunized with LigA$_C$ and FliC were positive for the presence of *Leptospira* in the kidneys. Nonetheless, animals immunized with LigA$_C$ coadministered with the Lp pool and FliC exhibited significant reductions in renal colonization (72% protection against renal colonization) (Fig. 4D; see also Table S6). These data indicate that incorporation of the *Salmonella* FliC flagellin as an adjuvant improves the protection against lethality and renal colonization in challenged hamsters.

DISCUSSION

In this study, we evaluated the induction of protective immunity in hamsters after immunization with seven different *L. interrogans* outer membrane proteins, including LigA$_C$, in combination with flagellin of *Salmonella* Typhimurium and alum. Due to its ability to confer protective immunity, LigA$_C$ has been considered to be one of the most promising antigen candidates for a subunit vaccine (15–18). Nonetheless, sterilizing immunity remains an unmet challenge because immunization with LigA$_C$, as well as other antigens, has not yet succeeded in inhibiting renal colonization by leptospires. Our results demonstrated that hamsters immunized with the pool of Lp antigens and LigA$_C$ combined with flagellin not only survived the lethal challenge with *L. interrogans* but also exhibited a significant reduction in renal colonization. Altogether, the results of the present study indicate that the combination of LigA$_C$, additional Lp antigens, and FliC deserves consideration as an approach for the development of leptospirosis vaccines capable of inducing immunological mechanisms involved in both protection against mortality and prevention of kidney colonization.

We selected six outer membrane proteins that are conserved in pathogenic strains of *Leptospira* spp. as potential vaccine antigens. Most recombinant proteins were expressed in an insoluble form
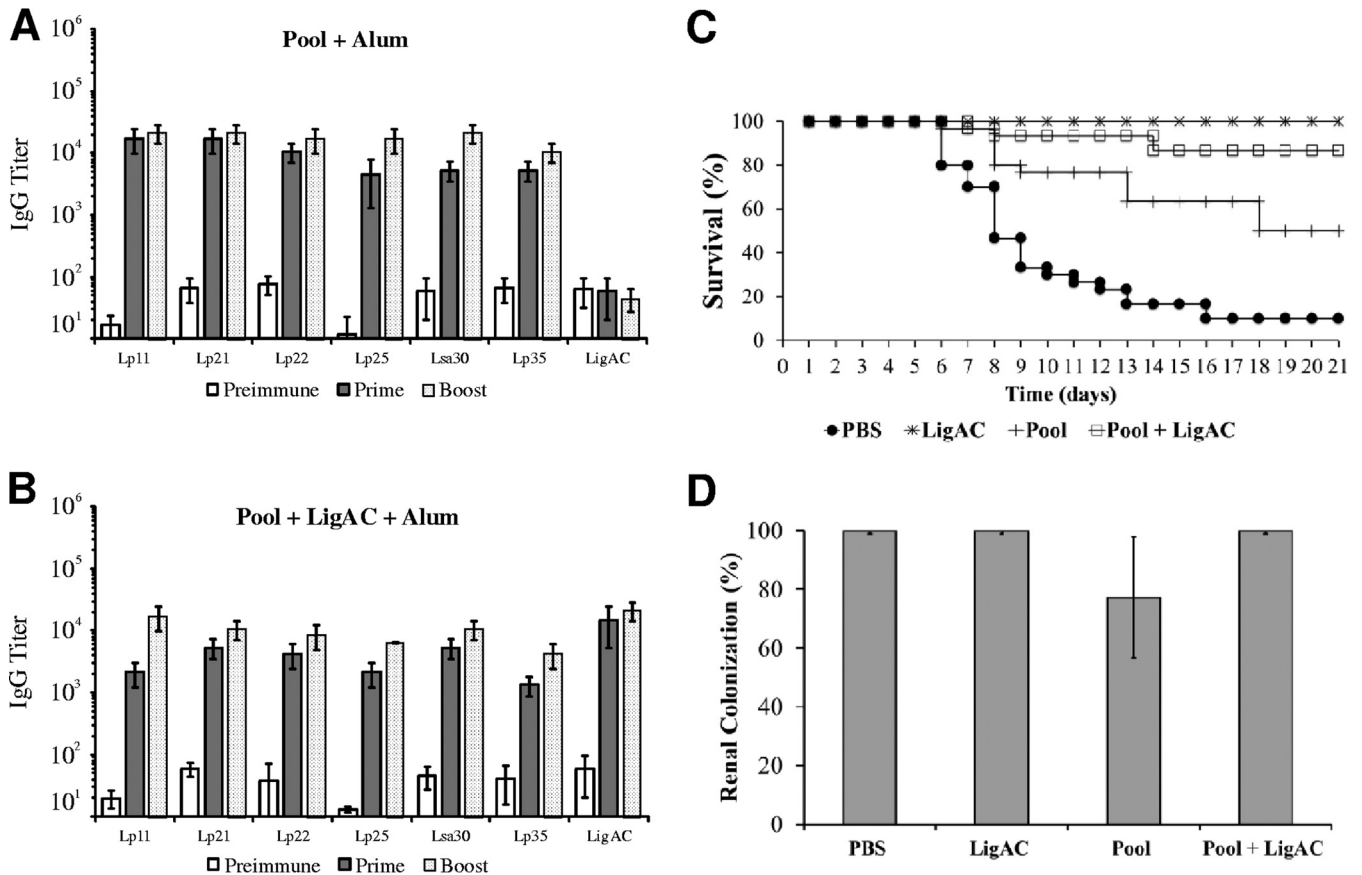
FIG 3 Antibody response and protective immunity induced in hamsters after immunization with recombinant proteins plus alum as an adjuvant. Hamsters were subcutaneously immunized twice with the pool of recombinant protein, at an interval of 15 days. One day before the second immunization (prime) and 1 day before the challenge (boost), the hamsters were bled, and the sera were analyzed by ELISA. Microtitration plates were coated with recombinant proteins and incubated with serial dilutions of serum samples collected from the immunized hamsters to measure the antigen-specific IgG responses. On the 31st day, animals were challenged by intraperitoneal inoculation of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130. (A) Antibody responses after immunization with the pool of recombinant proteins without LigA$_C$ but with alum. (B) Antibody responses after immunization with the pool of recombinant proteins and LigA$_C$ plus alum. (C) Survival of hamsters after the lethal challenge. The percentage of hamsters that survived was calculated as the number of survivors divided by the total number of animals challenged in three experiments (detailed results are in Table S3 in the supplemental material). (D) Renal colonization in animals surviving the challenge. The percentage of animals with renal colonization was calculated as the number of hamsters positive for kidney infection divided by the number of surviving hamsters in three experiments (detailed results are in Table S4). Results are expressed as the means ± SE.

and had to be denatured and resolubilized before being tested as vaccine antigens. The optimal conditions for solubilization and refolding were specific for each protein and had to be determined experimentally. The circular dichroism data analyses revealed that the established purification and refolding protocols were suitable for the generation of the recombinant antigens with good yields, purity, and preservation of structural conformation. The structural integrity of the antigens may be critical for inducing protection against leptospirosis, because previous studies have reported that immunization of hamsters with denatured LigA$_C$ did not protect against lethal challenge (18, 42).

The efficacy of the recombinant proteins as potential vaccine antigens was tested in hamsters, which develop acute lethal leptospirosis. All experiments were performed with 10 animals per group to allow an appropriate statistical analysis. In addition, the dose of 2×10^3 leptospires, corresponding to 100 times the LD$_{50}$, was determined to be an optimal challenge inoculum for *L. interrogans* serovar Copenhageni strain L1-130. Intraperitoneal inoculation with this dose caused 90 to 100%

mortality in the control groups of hamsters, with typical manifestations of leptospiral infection. This inoculation protocol was maintained during the study both to measure the protective immunity conferred by the tested vaccine formulations and to evaluate the renal colonization in hamsters surviving the lethal challenge.

Hamsters immunized with heat-killed leptospires or with each purified recombinant protein produced serum IgG responses to the recombinant proteins, suggesting that these proteins are antigenic and expressed by living leptospires. However, none of the six recombinant proteins was individually capable of inducing a protective immune response in hamsters. As previously reported, there seems to be no correlation between high levels of antibodies to leptospiral antigens and protection (18, 24). In contrast, animals immunized with LigA$_C$ survived the challenge, confirming previously reported results (15–18). Hamsters immunized with LigA$_C$ or LigA$_C$ administered with the pool of proteins, both in the presence of alum, were protected against the lethal challenge. Interestingly, in the presence of alum, animals immunized with the pool of Lps exhibited partial pro-
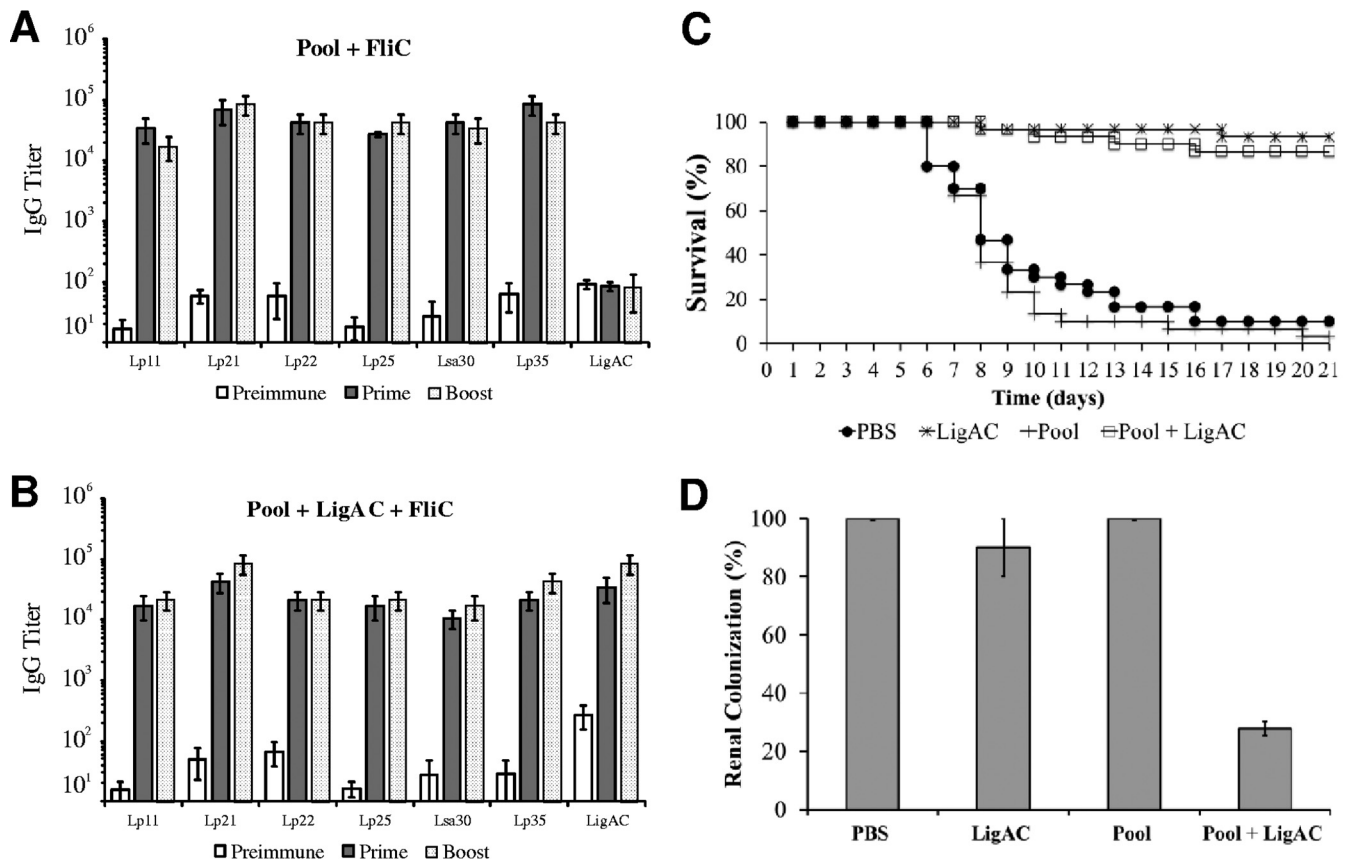
FIG 4 Antibody response and efficacy of protective immunity induced in hamsters after immunization with the pool of recombinant proteins plus flagellin as an adjuvant. Hamsters were subcutaneously immunized twice with the pool of recombinant proteins at an interval of 15 days. One day before the second immunization (prime) and 1 day before the challenge (boost), the hamsters were bled, and the sera were analyzed by ELISA. Microtitration plates were coated with recombinant proteins and incubated with serial dilutions of serum samples collected from the immunized hamsters to measure the antigen-specific IgG responses. On the 31st day, animals were challenged by intraperitoneal inoculation of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130. (A) Antibody responses after immunization with the pool of recombinant proteins without LigA$_C$ but with FliC. (B) Antibody responses after immunization with the pool of recombinant proteins and LigA$_C$ plus FliC. (C) Survival of the hamsters after the lethal challenge. The percentage of hamsters that survived was calculated as the number of survivors divided by the total number of animals challenged in three experiments (detailed results are in Table S5 in the supplemental material). (D) Renal colonization in animals surviving the challenge. The percentage of animals with renal colonization was calculated as the number of hamsters positive for kidney infection divided by the number of surviving hamsters in three experiments (detailed results in are Table S6). Results are expressed as the means ± SE.

tection (50%) against the challenge with *L. interrogans*. Synergistic effects generated from combinations of different leptospiral antigens have been reported previously for other antigens, such as the combination of OmpL1 and LipL41, which conferred enhanced protection in hamsters (11), and three probable outer membrane proteins of *L. interrogans* serovar Pomona (rLp1454, rLp1118, and recombinant MceII), which exhibited greater protective efficacy when administered together (43).

As previously reported, animals immunized with LigA$_C$ were positive for the isolation of leptospires (15–18), and none of these vaccine compositions in the presence of alum was able to confer sterilizing immunity despite the higher titers of antibodies generated in the hamsters. To date, no study has evaluated the adjuvant effects of flagellin in combination with protective leptospiral antigens. Inoculating hamsters with LigA$_C$ or LigA$_C$ plus the pool of purified Lps and flagellin conferred immunoprotection after challenge and induced robust antibody responses to the recombinant proteins. In contrast, animals inoculated with the Lp pool and FliC without LigA$_C$ did

not survive after the challenge, despite the high titers of antibodies generated. More remarkably, only animals inoculated with LigA$_C$ plus the pool of Lps and FliC exhibited a significant reduction of renal colonization by leptospires. These data suggest that the combination of antigens and the incorporation of *Salmonella* flagellin as adjuvant represent the only vaccine formulation that could confer protective immunity capable of impacting leptospiral renal colonization in hamsters, a feature not previously reported for leptospirosis vaccines.

To summarize, the results presented here suggest that a multivalent vaccine against leptospirosis, consisting of seven recombinant outer membrane proteins plus *Salmonella* flagellin as an adjuvant, represents a promising step toward the development of leptospirosis vaccines capable of triggering immune responses involved with more efficient control of leptospiral infection. Although the present evidence did not disclose the specific features of the immune responses leading to the more efficient control of the pathogen, the study provides valuable information for future studies in the field.

REFERENCES

1. **Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, Lovett MA, Levett PN, Gilman RH, Willig MR, Gotuzzo E, Vinetz JM.** 2003. Leptospirosis: a zoonotic disease of global importance. Lancet Infect Dis **3:**757–771. http://dx.doi.org/10.1016/S1473-3099(03)00830-2.

2. **Adler B, De la Peña-Moctezuma A.** 2010. Leptospira and leptospirosis. Vet Microbiol **140:**287–296. http://dx.doi.org/10.1016/j.vetmic.2009.03.012.

3. **Vijayachari P, Sugunan AP, Shriram AN.** 2008. Leptospirosis: an emerging global public health problem. J Biosci **33:**557–569. http://dx.doi.org/10.1007/s12038-008-0074-z.

4. **World Health Organization.** 2011. Leptospirosis: an emerging public health problem. Wkly Epidemiol Rec **86:**45–50.

5. **Guerra MA.** 2013. Leptospirosis: public health perspectives. Biologicals **41:**295–297. http://dx.doi.org/10.1016/j.biologicals.2013.06.010.

6. **Ellis WA.** 1994. Leptospirosis as a cause of reproductive failure. Vet Clin North Am Food Anim Pract **10:**463–478.

7. **Grooms DL.** 2006. Reproductive losses caused by bovine viral diarrhea virus and leptospirosis. Theriogenology **66:**624–628. http://dx.doi.org/10.1016/j.theriogenology.2006.04.016.

8. **Faine SB, Adler B, Bolin C, Perolat P.** 1999. Leptospira and leptospirosis. MediSci, Melbourne, Australia.

9. **Dellagostin AO, Grassmann AA, Hartwig DD, Félix SR, Dilva EF, McBride AJA.** 2011. Recombinant vaccines against leptospirosis. Hum Vaccin **7:**1215–1224. http://dx.doi.org/10.4161/hv.7.11.17944.

10. **Ko AI, Goarant C, Picardeau M.** 2009. Leptospira: the dawn of the molecular genetics era for an emerging zoonotic pathogen. Nat Rev Microbiol **7:**736–747. http://dx.doi.org/10.1038/nrmicro2208.

11. **Haake DA, Mazel MK, McCoy AM, Milward F, Chao G, Matsunaga J, Wagar EA.** 1999. Leptospiral outer membrane proteins OmpL1 and LipL41 exhibit synergistic immunoprotection. Infect Immun **67:**6572–6582.

12. **Branger C, Sonrier C, Chatrenet B, Klonjkowski B, Ruvoen-Clouet N, Aubert A, André-Fontaine G, Eliot M.** 2001. Identification of the hemolysis-associated protein 1 as a cross-protective immunogen of *Leptospira interrogans* by adenovirus-mediated vaccination. Infect Immun **69:**6831–6838. http://dx.doi.org/10.1128/IAI.69.11.6831-6838.2001.

13. **Branger C, Chatrenet B, Gauvrit A, Aviat F, Aubert A, Bach JM, André-Fontaine G.** 2005. Protection against *Leptospira interrogans* sensu lato challenge by DNA immunization with the gene encoding hemolysin-associated protein 1. Infect Immun **73:**4062–4069. http://dx.doi.org/10.1128/IAI.73.7.4062-4069.2005.

14. **Seixas FK, da Silva EF, Hartwig DD, Cerqueira GM, Amaral M, Fagundes MQ, Sossa RG, Dellagostin OA.** 2007. Recombinant *Mycobacterium bovis* BCG expressing the LipL32 antigen of *Leptospira interrogans* protects hamsters from challenge. Vaccine **26:**88–95. http://dx.doi.org/10.1016/j.vaccine.2007.10.052.

15. **Koizumi N, Watanabe H.** 2004. Leptospiral immunoglobulin-like proteins elicit protective immunity. Vaccine **22:**1545–1552. http://dx.doi.org/10.1016/j.vaccine.2003.10.007.

16. **Palaniappan RU, McDonough SP, Divers TJ, Chen CS, Pan MJ, Matsumoto M, Chang YF.** 2006. Immunoprotection of recombinant leptospiral immunoglobulin-like protein A against Leptospira interrogans serovar Pomona infection. Infect Immun **74:**1745–1750. http://dx.doi.org/10.1128/IAI.74.3.1745-1750.2006.

17. **Silva EF, Medeiros MA, McBride AJ, Matsunaga J, Esteves GS, Ramos JG, Santos CS, Croda J, Homma A, Dellagostin OA, Haake DA, Reis MG, Ko AI.** 2007. The terminal portion of leptospiral immunoglobulin-like protein LigA confers protective immunity against lethal infection in the hamster model of leptospirosis. Vaccine **25:**6277–6286. http://dx.doi.org/10.1016/j.vaccine.2007.05.053.

18. **Coutinho ML, Choy HA, Kelley MM, Matsunaga J, Babbitt JT, Lewis MS, Aleixo JA, Haake DA.** 2011. A LigA three-domain region protects hamsters from lethal infection by *Leptospira interrogans*. PLoS Negl Trop Dis **5:**e1422. http://dx.doi.org/10.1371/journal.pntd.0001422.

19. **Forster KM, Hartwig DD, Seixas FK, Bacelo KL, Amaral M, Hartleben CP, Dellagostin OA.** 2013. A conserved region of leptospiral immunoglobulin-like A and B proteins as a DNA vaccine elicits a prophylactic immune response against leptospirosis. Clin Vaccine Immunol **20:**725–731. http://dx.doi.org/10.1128/CVI.00601-12.

20. **Yan W, Faisal SM, McDonough SP, Chang CF, Pan MJ, Akey B, Chang YF.** 2010. Identification and characterization of OmpA-like proteins as novel vaccine candidates for leptospirosis. Vaccine **28:**2277–2283. http://dx.doi.org/10.1016/j.vaccine.2009.12.071.

21. **Matsunaga J, Barocchi MA, Croda J, Young TA, Sanchez Y, Siqueira I, Bolin CA, Reis MG, Riley LW, Haake DA, Ko AI.** 2003. Pathogenic Leptospira species express surface-exposed proteins belonging to the bacterial immunoglobulin superfamily. Mol Microbiol **49:**929–945. http://dx.doi.org/10.1046/j.1365-2958.2003.03619.x.

22. **Palaniappan RU, Chang YF, Jusuf SS, Artiushin S, Timoney JF, McDonough SP, Barr SC, Divers TJ, Simpson KW, McDonough PL, Mohammed HO.** 2002. Cloning and molecular characterization of an immunogenic LigA protein of *Leptospira interrogans*. Infect Immun **70:**5924–5930. http://dx.doi.org/10.1128/IAI.70.11.5924-5930.2002.

23. **Cerqueira GM, McBride AJ, Picardeau M, Ribeiro SG, Moreira AN, Morel V, Reis MG, Ko AI, Dellagostin OA.** 2009. Distribution of the leptospiral immunoglobulin-like (lig) genes in pathogenic Leptospira species and application of ligB to typing leptospiral isolates. J Med Microbiol **58**(Pt 9):1173–1181. http://dx.doi.org/10.1099/jmm.0.009175-0.

24. **Murray GL, Lo M, Bulach DM, Srikram A, Seemann T, Quinsey NS, Sermswan RW, Allen A, Adler B.** 2013. Evaluation of 238 antigens of *Leptospira borgpetersenii* serovar Hardjo for protection against kidney colonisation. Vaccine **31:**495–499. http://dx.doi.org/10.1016/j.vaccine.2012.11.028.

25. **Newton SM, Jacob CO, Stocker BA.** 1989. Immune response to cholera toxin epitope inserted in *Salmonella* flagellin. Science **244:**70–72. http://dx.doi.org/10.1126/science.2468182.

26. **Newton SM, Kotb M, Poirier TP, Stocker BA, Beachey EH.** 1991. Expression and immunogenicity of a streptococcal M protein epitope inserted in *Salmonella* flagellin. Infect Immun **59:**2158–2165.

27. **Sbrogio-Almeida ME, Mosca T, Massis LM, Abrahamsohn IA, Ferreira LC.** 2004. Host and bacterial factors affecting induction of immune responses to flagellin expressed by attenuated *Salmonella* vaccine strains. Infect Immun **72:**2546–2555. http://dx.doi.org/10.1128/IAI.72.5.2546-2555.2004.

28. **Lee SE, Kim SY, Jeong BC, Kim YR, Bae SJ, Ahn OS, Lee JJ, Song HC, Kim JM, Choy HE, Chung SS, Kweon MN, Rhee JH.** 2006. A bacterial flagellin, Vibrio vulnificus FlaB, has a strong mucosal adjuvant activity to induce protective immunity. Infect Immun **74:**694–702. http://dx.doi.org/10.1128/IAI.74.1.694-702.2006.

29. **Huleatt JW, Nakaar V, Desai P, Huang Y, Hewitt D, Jacobs A, Tang J, McDonald W, Song L, Evans RK, Umlauf S, Tussey L, Powell TJ.** 2008. Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin. Vaccine **26:**201–214. http://dx.doi.org/10.1016/j.vaccine.2007.10.062.

30. **Braga CJ, Massis LM, Alencar BC, Rodrigues MM, Sbrogio-Almeida ME, Ferreira LC.** 2008. Cytotoxic T cell adjuvant effects of three *Salmonella enterica* flagellins. Braz J Microbiol **39:**44–49. http://dx.doi.org/10.1590/S1517-83822008000100011.

31. **Takeda K, Kaisho T, Akira S.** 2003. Toll-like receptors. Annu Rev Immunol **21:**335–376. http://dx.doi.org/10.1146/annurev.immunol.21.120601.141126.

32. **Akira S, Takeda K, Kaisho T.** 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol **2:**675–680. http://dx.doi.org/10.1038/90609.

33. **Akira S, Takeda K.** 2004. Toll-like receptor signalling. Nat Rev Immunol **4:**499–511. http://dx.doi.org/10.1038/nri1391.

34. **Barbosa AS, Abreu PA, Neves FO, Atzingen MV, Watanabe MM, Vieira ML, Morais ZM, Vasconcellos SA, Nascimento AL.** 2006. A newly identified leptospiral adhesin mediates attachment to laminin. Infect Immun **74:**6356–6364. http://dx.doi.org/10.1128/IAI.00460-06.

35. **Setubal JC, Reis M, Matsunaga J, Haake DA.** 2006. Lipoprotein computational prediction in spirochaetal genomes. Microbiology **152**(Pt 1):113–121. http://dx.doi.org/10.1099/mic.0.28317-0.

36. **Barbosa AS, Monaris D, Silva LB, Morais ZM, Vasconcellos SA, Cianciarullo AM, Isaac L, Abreu PA.** 2010. Functional characterization of LcpA, a surface-exposed protein of *Leptospira* spp. that binds the human

complement regulator C4BP. Infect Immun **78**:3207–3216. http://dx.doi
.org/10.1128/IAI.00279-10.

37. **Castiblanco-Valencia MM, Fraga TR, Silva LB, Monaris D, Abreu PA, Strobel S, Józsi M, Isaac L, Barbosa AS.** 2012. Leptospiral immunoglobulin-like proteins interact with human complement regulators factor H, FHL-1, FHR-1, and C4BP. J Infect Dis **205**:995–1004. http://dx.doi.org/10.1093/infdis/jir875.

38. **Reed LJ, Muench H.** 1938. A simple method of estimating fifty percent endpoints. Am J Hyg **27**:493–497.

39. **Souza NM, Vieira ML, Alves IJ, de Morais ZM, Vasconcellos SA, Nascimento AL.** 2012. Lsa30, a novel adhesin of *Leptospira interrogans* binds human plasminogen and the complement regulator C4bp. Microb Pathog **53**:125–134. http://dx.doi.org/10.1016/j.micpath.2012.06.001.

40. **Gómez RM, Vieira ML, Schattner M, Malaver E, Watanabe MM, Barbosa AS, Abreu PA, de Morais ZM, Cifuente JO, Atzingen MV, Oliveira TR, Vasconcellos SA, Nascimento AL.** 2008. Putative outer membrane proteins of *Leptospira interrogans* stimulate human umbili-cal vein endothelial cells (HUVECS) and express during infection. Microb Pathog **45**:315–322. http://dx.doi.org/10.1016/j.micpath.2008.08.004.

41. **Mendes RS, Von Atzingen M, de Morais ZM, Gonçales AP, Serrano SM, Asega AF, Romero EC, Vasconcellos SA, Nascimento AL.** 2011. The novel leptospiral surface adhesin Lsa20 binds laminin and human plasminogen and is probably expressed during infection. Infect Immun **79**:4657–4667. http://dx.doi.org/10.1128/IAI.05583-11.

42. **Lucas DS, Cullen PA, Lo M, Srikram A, Sermswan R, Adler B.** 2011. Recombinant LipL32 and LigA from *Leptospira* are unable to stimulate protective immunity against leptospirosis in the hamster model. Vaccine **29**:3413–3418. http://dx.doi.org/10.1016/j.vaccine.2011.02.084.

43. **Chang YF, Chen CS, Palaniappan RU, He H, McDonough SP, Barr SC, Yan W, Faisal SM, Pan MJ, Chang CF.** 2007. Immunogenicity of the recombinant leptospiral putative outer membrane proteins as vaccine candidates. Vaccine **25**:8190–8197. http://dx.doi.org/10.1016/j.vaccine.2007.09.020.