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Characterization of LIC11207, a novel leptospiral protein that is recognized by human convalescent sera and prevents apoptosis of polymorphonuclear leukocytes

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ABSTRACT

We report the study of a predicted outer-membrane leptospiral protein encoded by the gene *lic11207*. This protein is conserved in several pathogenic leptospiral strains but is absent in the saprophyte *Leptospira biflexa*. This putative outer-membrane protein has a domain of unknown function (DUF) 1565 found in several phylogenetically diverse bacteria and in the archaeon *Methanosarcina acetivorans*. The gene was cloned and expressed in *Escherichia coli* BL21 (SI) strain using the expression vector pDEST17. The 34 kDa recombinant protein was tagged with N-terminal hexahistidine and purified by metal-charged chromatography. The purified protein was used to assess: reactivity with human convalescent sera; *in vivo* expression; ability to activate endothelial cells (EC); and ability to modulate the apoptosis of polymorphonuclear cells (PMNs). The LIC11207 coding sequence was identified *in vivo* in the hamster renal tubules during experimental infection with *Leptospira interrogans*. The rLIC11207 showed significant antigenicity against human convalescent sera when compared with sera from healthy donors. The recombinant protein did not alter the surface expression of E-selectin or intercellular adhesion molecule 1 (ICAM-1) in EC and failed to induce the release of von Willebrand factor (vWF). Interestingly, rLIC11207 delayed apoptosis of PMNs suggesting a possible role of this protein during the infection.

1. Introduction

Leptospirosis is a re-emerging infectious disease with a global distribution, caused by pathogenic spirochetes of the genus *Leptospira*. The transmission of leptospirosis is associated with exposure of individuals to wild or farm animals [1,2]. Humans usually become infected through contact with urine-contaminated soil or water, with infected animal tissue, or from rat bites [3,4]. Consequently, in recent years the disease has become prevalent in cities with sanitation problems and a large population of urban rodent reservoirs [5].

Traditionally, *Leptospira* spp. have been subdivided and classified into numerous serovars grouped into serogroups as determined by shared antigens [1]. The extensive serological diversity of leptospires has inspired a search for conserved outer-membrane proteins (OMPs) that may have distinctive antigenic or even immunogenic properties [6–10]. Some leptospiral OMPs such as LipI41 and Loa22 are expressed during acute and chronic infection [11] and are recognized by serum from infected patients [12,13].

Leptospira spp. invasiveness is attributed to its ability to disseminate widely within the host during the early stage of infection but the mechanisms associated with this invasion are poorly understood [1]. Cell adhesion molecules (CAMs) are surface receptors present in eukaryotic cells that mediate cell–cell or cell–extracellular matrix interactions [14,15]. Some leptospiral proteins such as rLIC10365, rLIC10507, rLIC10508, and rLIC10509 of *Leptospira interrogans* promote upregulation of intercellular adhesion molecule 1 (ICAM-1) and E-selectin on human umbilical vein endothelial cells (HUVECs) [16,17]. This upregulation appears to be selective since another leptospiral protein, Lp95, activated E-selectin in a concentration-dependent fashion but not ICAM-1 [18]. The potential involvement of these proteins in pathogenesis has been suggested [16–19].

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The von Willebrand factor (vWF) is a glycoprotein synthesized and stored in endothelial cells, megakaryocytes, and platelets. It has an important role in adhesion of platelets to the subendothelial matrix and platelet aggregation [20]. An increased release of vWF has been proposed as an indicator of endothelial activation or damage [21].

Neutrophils, representing more than 95% of polymorphonuclear leukocytes (PMNs), are short-lived phagocytic leukocytes that are rapidly recruited from the bloodstream to the site of tissue infection [22]. Once they have reached their destination, they can efficiently kill many microbes via phagocytosis, extracellular release of granule contents, and the formation of neutrophil extracellular traps. They also actively produce cytokines and other mediators to promote or suppress inflammation, repair tissues, and to modulate both the innate and the adaptive immune-responses [23]. Neutrophils have the shortest lifespan among leukocytes in the circulation and die via apoptosis. Apoptosis of inflammatory neutrophils and their clearance are critical control points for an effective host defense and termination of the inflammatory response [24].

In the present work, we describe the cloning, expression, purification, and characterization of a predicted leptospiral outer-membrane protein encoded by the gene *lic11207*, identified by bioinformatics tools in the genome sequence of the *L. interrogans* serovar Copenhageni (LIC). LIC11207 was expressed *in vivo* in the renal tubules during experimental infection in hamsters and rLIC11207 was recognized by antibodies present in human convalescent sera. Although rLIC11207 did not trigger the expression of E-selectin or ICAM-1, or the release of vWF by HUVECs, it delayed PMN apoptosis suggesting that this protein might has a role during infection.

2. Materials and methods

2.1. Leptospira strains and culture conditions

The non-pathogenic *Leptospira biflexa* (serovar Patoc strain Patoc 1) and the pathogenics *L. interrogans* (serovars Canicola strain Hond Utrechet IV, Copenhageni strains M-20 and Fiocruz L1-130, Grippotyphosa strain Moskva V, Icterohaemorrhagiae strain RGA, Pomona strain Pomona) and *L. borgpetersenii* (serovars Hardjo strain Hardjoprajtino, Tarassovi strain Perepelicin and Ballum strain Castellonis Castellon 3) were cultured at 30 °C under aerobic conditions in liquid EMJH medium (Difco®) with 10% rabbit serum, enriched with L-asparagine (wt/vol: 0.015%), sodium pyruvate (wt/vol: 0.001%), calcium chloride (wt/vol: 0.001%), magnesium chloride (wt/vol: 0.001%), peptone (wt/vol: 0.03%), and meat extract (wt/vol: 0.02%) [25].

2.2. In silico protein analysis

The predicted coding sequence (CDS) of LIC11207 was selected from the *L. interrogans* serovar Copenhageni genome sequences [26] based on its cellular localization prediction by the PSORT program, http://psort.nibb.ac.jp [27,28] and the P-Classifier program, http://www.bii.a-star.edu.sg/index.php [29]. The SMART, http://smart.embl-heidelberg.de [30,31], PFAM, http://www.sanger.ac.uk/Software/Pfam/ [32], and LipoP, http://www.cbs.dtu.dk/services/LipoP/ [33] web servers were used to search for predicted functional and structural domains within the amino acid sequences of the selected sequences. The predicted sequence of the lipobox was evaluated using the SpLip program, as described by Setubal et al. [34]. Sequence analysis was performed with ClustalX [35] and a tree-display program by the Neighbor-Joining method [36].

2.3. DNA isolation and PCR analysis

Leptospira cultures were harvested by centrifugation at 11,500 *g* for 30 min and gently washed twice in sterile PBS. Genomic DNA was isolated from the pellets by the guanidine-detergent lysing method using DNAzol® Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. An 855-bp LIC11207 DNA fragment was amplified using oligonucleotides (5′–3′) LIC11207-F GGCATAATCGTCGCCATCTC and LIC11207-R CTTCCCGTT GAACCTTGACC designed according to *L. interrogans* serovar Copenhageni genome sequences (GenBank accession AE016823). PCR was performed in a reaction volume of 25 μl containing 100 ng of genomic DNA, 1× PCR buffer (20 mM Tris–HCl (pH 8.4), 50 mM KCl), 2 mM $MgCl_2$, 20 pmol of each specific primer, 200 μM of each dNTP, and 2.5 U Taq DNA Polymerase (Invitrogen). Cycling conditions were: 94 °C, 5 min, followed by 35 cycles at 94 °C, 20 s, 62 °C, 20 s, 72 °C, 50 s, and a final extension cycle of 2 min at 72 °C. PCR amplified products were loaded onto a 1% agarose gel for electrophoresis and visualization with ethidium bromide.

2.4. RNA extraction and RT-PCR analysis

For reverse transcription (RT)-PCR, total RNA was isolated by the acid guanidinium thiocyanate phenol–chloroform method using TRIzol® Reagent (Invitrogen) according to the manufacturer's recommendations. One microgram of RNA from each sample was treated with 1 U of DNAse I Amplification Grade (Invitrogen) for 15 min at room temperature. DNAse I was inactivated by the addition of 1 μl of 25 mM EDTA solution followed by an incubation at 65 °C for 10 min. DNAse-treated RNAs were reversely transcribed using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). One tenth of RT products were amplified in a 25 μl reaction mix using oligonucleotides LIC11207-F/LIC11207-R as described above. Sample quantity and integrity were verified by amplification of a 331-bp 16S ribosomal cDNA fragment using oligomers (5′–3′) 16S-F CATTCATGTTTCGAATCATTTCAAA and 16S-R GGCCCAAGTTCCTTCTAAAAG.

2.5. Cloning

Predicted *lic11207* was amplified by PCR from total *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 genomic DNA using the primer pairs described above and the PCR product was cloned into pENTR-TOPO vector (Invitrogen) followed by transfer/recombination of DNA insert into the *Escherichia coli* expression vector pDEST17 (Invitrogen) using the LR Clonase (Invitrogen). Vector pDEST17 allows the expression of recombinant protein with a minimal 6× His-tag at the N-terminus. All cloned sequences were confirmed by DNA sequencing with an ABI 3100 automatic sequencer (PE Applied Biosystems, Foster city, CA).

2.6. Expression and purification of rLIC11207

Protein expression was achieved in *E. coli* BL21 (SI) strain by the action of T7 DNA polymerase under control of the osmotic induced promoter *proU* [37]. *E. coli* BL21 (SI) containing recombinant plasmids were grown at 37 °C in Luria–Bertani without NaCl and with 100 mg/ml ampicillin with continuous shaking until an optical density at 600 nm of 0.6–0.8 was reached. Recombinant protein synthesis was induced by the addition of 300 mM NaCl. After 3 h (h), the cells were harvested by centrifugation, the bacterial pellets resuspended in lysis buffer (10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 100 mg/ml of lysozyme, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride). The bacterial cell pellets were lysed on ice with the aid of a sonicator (Ultrasonic Processor; GE Healthcare).

The insoluble fraction was washed five times with 20 ml of buffer (20 mM Tris–HCl (pH 8.0), 500 mM NaCl, 1 M urea, and 0.1% Triton X-100) and resuspended in a buffer containing 20 mM Tris–HCl (pH 8.0), 500 mM NaCl, and 8 M urea. The protein was then purified through metal-chelating chromatography in a Sepharose fast-flow column (GE Healthcare) and fractions were analyzed by 12% SDS-PAGE. The recombinant LIC11207 was refolded by dilution with 50 mM Tris–HCl (pH 8.0), and 500 mM NaCl and purified through metal-chelating chromatography in a Sepharose fast-flow column (GE Healthcare), and extensively dialyzed against phosphate-buffered saline (PBS; pH 7.4), 0.1% (wt/vol) glycine solution for 24–48 h.

2.7. Antiserum against rLIC11207

Ten female BALB/c mice (4–6 weeks old) were immunized subcutaneously with 10 μg of rLIC11207 protein. The recombinant protein was adsorbed in 10% (vol/vol) Alhydrogel (2% $Al(OH)_3$, Brenntag Biosector, Denmark), used as an adjuvant. Two subsequent booster injections were given at two-week intervals with the same protein preparation. Negative-control mice were injected with PBS. One week after each immunization, the mice were bled from the retro-orbital plexus and the pooled sera were analyzed by enzyme-linked immunosorbent assay (ELISA) for determination of antibody titers. All animal studies were approved by the Ethics Committee of the Instituto Butantan, São Paulo, Brazil.

2.8. In vivo detection of LIC11207

In order to detect LIC11207 in kidney tissues, three weanling hamsters were inoculated intraperitoneally with 0.5 ml PBS (pH 7.2) containing 10^2 *L. interrogans* serovar Copenhageni Fiocruz L1-130. Animals were sacrificed when they presented 20% weight loss, and their kidneys were harvested and processed for routine histology as described in Ref. [17]. Mock-infected animals were used as controls. Immunostained sections were counterstained with hematoxylin. The slides were observed using a Nikon E200 photomicroscope. All animal studies were approved by the Ethics Committee of the Faculty of Exact Sciences, University of La Plata, Argentina.

2.9. Detection of human IgG antibodies against rLIC11207

Human IgG antibodies against rLIC11207 were detected by ELISA. The plates were covered with 250 ng rLIC11207 per well. All samples were diluted (starting at 1:20) and evaluated for total IgG using rabbit anti-human IgG antibodies (1:5000; Sigma) and a horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:5000; Sigma). The cut-off points were set at 3 SD above the mean A492 for sera from six humans that had not been exposed to leptospires.

2.10. Immunoblotting assay

Aliquots (1 μg) of recombinant protein were subjected to SDS-PAGE and then transferred to nitrocellulose membranes (Hybond ECL; GE Healthcare). Membranes were blocked with 10% non-fat dried milk in PBS containing 0.05% Tween 20 (PBS-T) and then incubated with a pool of sera from healthy and convalescent patients at 1:2000 dilution in 5% non-fat dried milk/PBS-T for 2 h at room temperature. After washing, the membrane was incubated with HRP-conjugated anti-human IgG (1:5000; Sigma) in 5% non-fat dried milk/PBS-T for 1 h. The bands were revealed with ECL reagent kit chemiluminescence substrate (GE Healthcare).

2.11. Limulus amebocyte lysate assay (LAL-test)

The chromogenic LAL assay for endotoxin activity of the protein samples was performed using the QCL-1000 kit (Bio-Whittaker Inc., Walkersville, MD), according to the manufacturer's instructions.

2.12. Endothelial cell cultures

HUVECs were isolated from umbilical cords obtained from normal full-term deliveries of healthy woman. This study was performed according to institutional guidelines (National Academy of Medicine, Buenos Aires, Argentina) and received approval of the institutional ethics committee and written consent from all the subjects. HUVECs were obtained by collagenase (GIBCO) digestion according to the method of Jaffe et al. [38] as described in Ref. [16]. Confluent HUVEC was used between the first and third passages. Cultured cells were identified as endothelial by their morphology and by vWF antibody binding. The experiments with recombinant proteins were performed in the presence of 7 μg/ml polymyxin B (PMB; Sigma) to rule out lipopolysaccharide (LPS) interference [16].

2.13. Expression of E-selectin and ICAM-1

HUVECs were incubated with 50 μg/ml purified recombinant proteins for 1 h in RPMI-1640 medium. The protein was

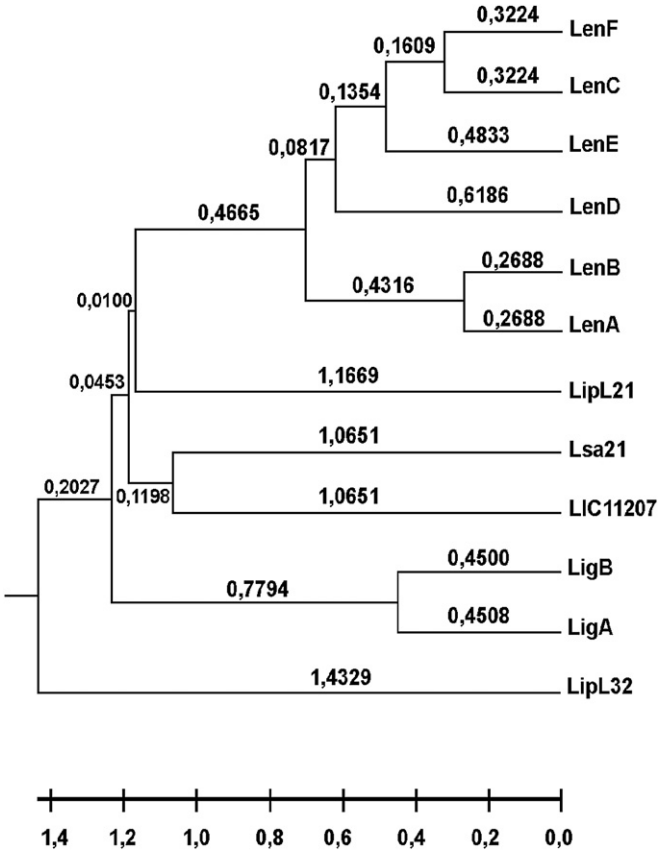

Fig. 1. Sequence comparison between *lic11207* encoded protein and other leptospira proteins. Unrooted phylogenetic tree of predicted amino acid sequences of the LIC11207, LipL32, Lsa21, LipL21 LigA/LigB, and Len family proteins. The tree was generated by the ClustalX program and displayed by NJ plot. Branch lengths are depicted where shorter distances indicate a stronger relationship between proteins. It can be observed that LIC11207 is only slightly related to Lsa21, with little relationship to the other proteins.

removed by washing, and endothelial cells were further incubated in RPMI-1640 with 10% FBS. ICAM-1 expression was assessed after 12 h. HUVECs were detached by treatment with 0.25% trypsin and 0.02% EDTA solution and incubated in the dark at 4 °C for 15 min with PBS containing 1% FBS and PE-mouse anti-human CD54 (clone HA58, BD Pharmingen) in the case of ICAM-1. For E-selectin expression, cells were harvested after 4 h, combined with a primary anti-CD62E monoclonal antibody (MoAb, clone 1.2B6, Immunotech) and a secondary FITC-conjugated fragment F $(ab')_2$ anti-mouse IgG (Immunotech). For non-specific binding, anti-CD54 or anti-CD62E were replaced by a corresponding concentration of irrelevant isotype-matched IgG1. After labeling, cells were washed, fixed with 1% paraformaldehyde and analyzed by flow cytometry in a FACScan cytofluorimeter (Becton Dickinson, Mountain View, CA). Appropriate settings of forward- and side-scatter gates were used to examine 10,000 cells per experiment. The percentage of positive cells was determined by the thresholds set using isotypic controls.

2.14. Endothelial von Willebrand factor release assay

Cultures of HUVECs were treated with 50 μg rLIC11207. After 1 h, the vWF released into the culture medium was measured by ELISA (Research Diagnostics, NJ, USA). Results are expressed in ng/ml and were extrapolated from a standard curve plotted from serial dilutions of normal pooled plasma, assuming a 10 μg/ml vWF concentration [39]. Thrombin was used as a positive control.

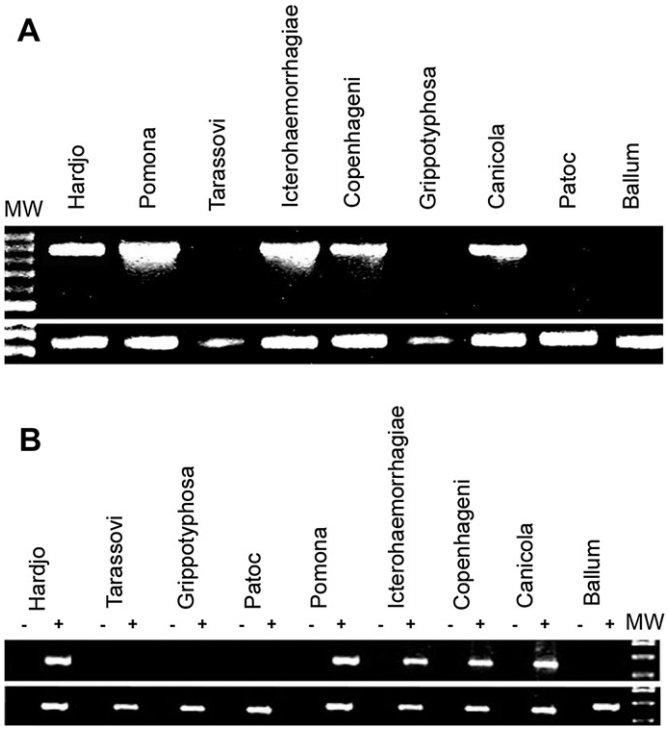

Fig. 2. Distribution and expression of *lic11207* gene in saprophytic and pathogenic leptospires. (A) Genomic DNA from *L. biflexa* Patoc and from eight serovars belonging to the pathogenic species *L. interrogans* and *L. borgpetersenii* was subjected to PCR analysis with *lic11207* specific primers designed according to *L. interrogans* serovar Copenhageni genome sequences. The expected size of the PCR product was 855-bp. No DNA was added to the negative-control reaction (−). (B) RT-PCR analysis of *lic11207* transcripts in high-passage *L. interrogans* strains. Reactions were performed with the same primer pairs mentioned above. Samples quantity and integrity were verified by amplification of a 331-bp 16S ribosomal cDNA fragment. RT+: reverse transcriptase present; RT−: reverse transcriptase omitted; M: molecular mass markers (bp).

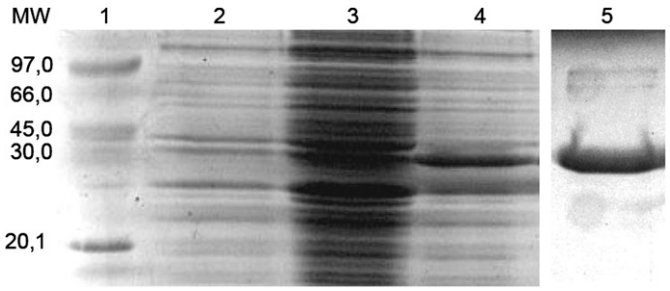

Fig. 3. Analysis of rLIC11207 from NaCl-induced *E. coli* Bl21-SI by SDS-12% PAGE. Lane 1, molecular mass protein marker; lane 2, non-induced culture at 0 h; lane 3, non-induced culture at 3 h; lane 4, induced culture at 3 h; lane 5, purified protein eluted from Ni^{+2}-charged chelating sepharose column with 1 M imidazole. Protein bands were visualized by *Coomassie blue* staining. Positions of molecular mass standards are indicated to the left (kDa).

2.15. Isolation of human PMNs

PMNs were isolated from peripheral blood drawn from healthy donors. PMNs were isolated by Ficoll Hypaque ($\delta = 1077$) gradient

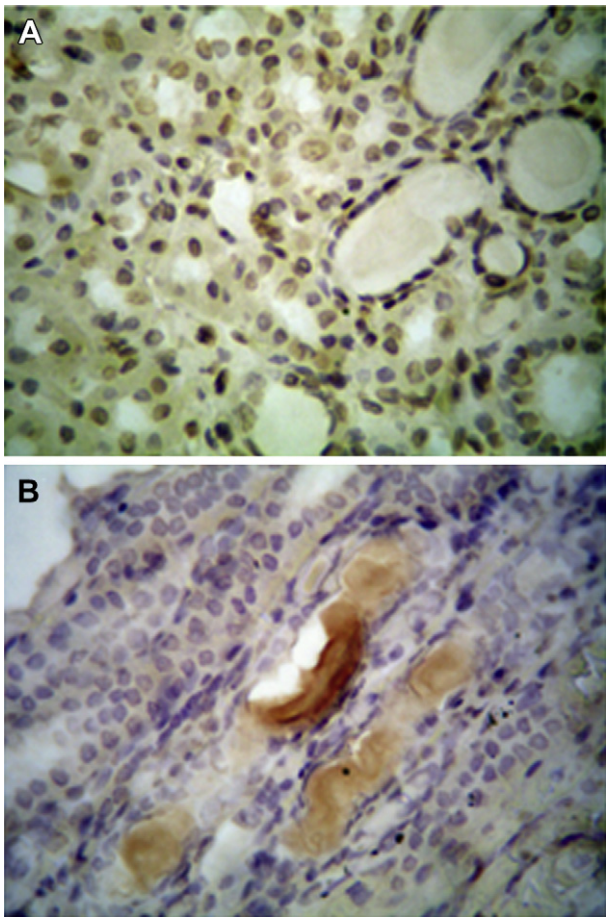

Fig. 4. Immunohistochemistry. Kidney tissues obtained from hamsters after infection with *L. interrogans* virulent strain Fiocruz L1-130, were probed with serum raised against rLIC11207. (A) No reactivity was observed when the antiserum was employed in kidney from uninfected animals. (B) LIC11207 antigens recognition within the renal tubular lumen of infected animals. Slides were counterstained with hematoxylin. Magnification ×450.

centrifugation (Ficoll; BIAcore, Uppsala, Sweden. Hypaque; Winthrop Products, Buenos Aires, Argentina), and dextran sedimentation, as described previously [40]. Cell suspensions contained >96% neutrophils, as determined by May-Grünwald-Giemsa-stained cytopreps, and the level of monocyte contamination was <0.2%, as evaluated by CD14 staining and FACS analysis. The cells were suspended in RPMI-1640 medium (Invitrogen) supplemented with 2% fetal calf serum (Invitrogen) at a concentration of 2.5×10^6/ml.

2.16. PMN apoptosis and viability assay

PMN suspension (100 μl aliquots) was placed in 96-well flat-bottom microplates in a humidified incubator at 37 °C and 5% CO_2 and were treated or not with 50 μg rLIC11207. After 18 h, cells were analyzed for changes in morphology and viability by labeling with a mixture of the fluorescent DNA binding dyes: 100 μg/ml acridine orange (AO) to determine the percentage of cells that had undergone apoptosis and 100 μg/ml ethidium bromide (EB) to differentiate between viable and non-viable cells as previously described [41]. Necrotic cells never exceeded 2%.

In addition, apoptosis was further studied by phosphatidylserine expression detected by Annexin-V binding and propidium iodide (PI) staining using a commercial kit (Sigma–Aldrich). Briefly, 18 h after different treatments, 1.5×10^5 PMNs were washed and incubated for 30 min with Annexin-V-FITC at 37 °C. Cells were then resuspended in 400 μl of binding buffer containing PI and immediately analyzed by flow cytometry.

2.17. Nucleotide sequence

The NCBI Reference Sequence number for CDS LIC11207 is YP 001177.1. The protein can also be accessed by the genome nomenclature for the gene locus, LIC number (*L. interrogans* Copenhageni).

3. Results

3.1. Bioinformatic analysis

The gene *lic11207* was identified in chromosome I by analysis of the probable ORFs present in the genome of *L. interrogans* serovar Copenhageni [26,42]. LIC11207 is predicted to be an OMP (>80%), based on the search for sequence motifs by the servers PSORT [28] and P-Classifier [29]. The LipoP server predicted LIC11207 CDS to be a lipoprotein with a cleavage site for signal peptidase II at amino acids 18–19 [33] in agreement with the result obtained with the SpLip program [34]. This predicted protein has a DUF1565, according to the PFAM web server [32]. Similar predicted CDS LIC11207 was identified in *L. interrogans* serovar Lai (99% similarity with LA2823) [43] and in *Leptospira borgpetersenii* (75% similarity) [44] but is absent in *L. biflexa* [45] genome sequences.

3.2. Sequence comparison between LIC11207 and other reported leptospiral outer-membrane proteins

To evaluate the similarity between LIC11207 with other previously identified leptospiral proteins [18,46–50], we proceeded with a sequence analysis using the ClustalX program and a tree-display NJ plot [35,36]. The calculated tree derived from sequence alignment is depicted in Fig. 1, where shorter distances indicate a stronger relationship between proteins. It can be observed that LIC11207 is only slightly related to Lsa21, with little relationship to others such as LipL21, LipL32, or the LEN family proteins, which have a role protecting against host immune-responses during mammalian infection [46].

3.3. Distribution of the lic11207 gene and comparative transcriptional analysis among Leptospira strains

The presence of the *lic11207* gene in eight pathogenic strains and in one saprophytic strain of *Leptospira* was examined by PCR

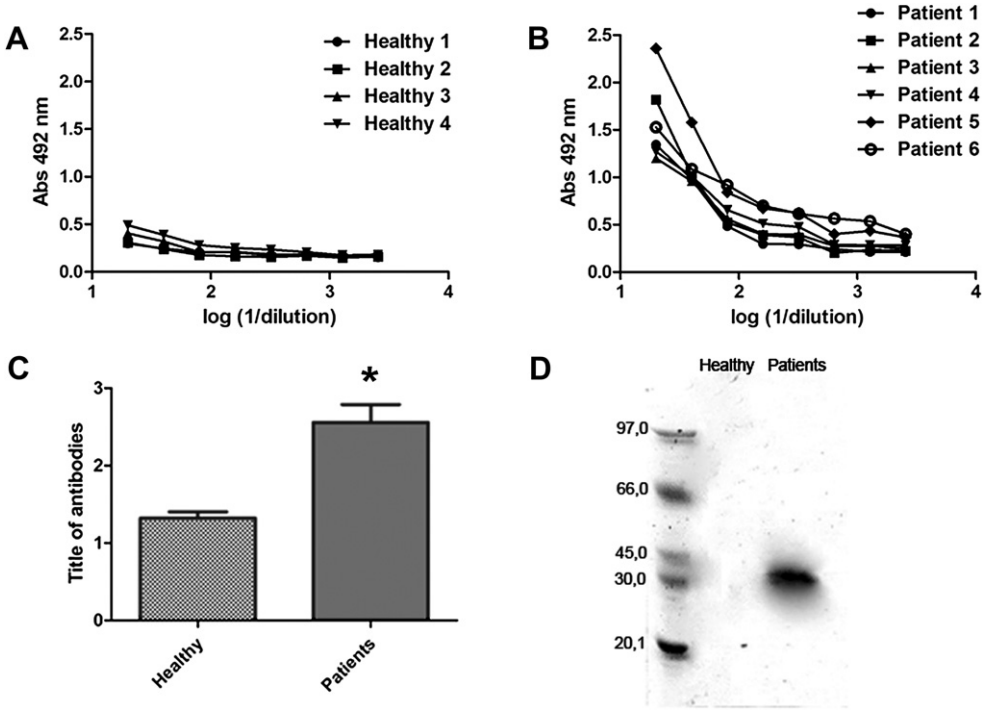

Fig. 5. Reactivity of infected patients sera against rLIC11207. An ELISA plate coated overnight with rLIC11207 showing reactivity against (A) sera dilutions of healthy donors, and (B) convalescent patients, respectively. (C) The mean of antibody titer defined as the reciprocal of the dilution corresponding to a half of the maximum absorbance ± SD. The * indicates a value of $p < 0.05$. (D) Immunoblotting of rLIC11207 reactivity using a pool of healthy and convalescent patients, respectively.

with a pair of primers designed according to *L. interrogans* serovar Copenhageni genome sequences. An 855-bp DNA fragment of *lic11207* was amplified by PCR in four strains belonging to the pathogenic specie of *L. interrogans* (serovars Canicola, Copenhageni, Pomona, and Icterohaemorraghie) and *L. borgpetersenii* serovar Hardjo (Fig. 2A). No amplification product was detected in the strains belonging to the serovars Grippotyphosa, Ballum, Tarassovi, or in the non-pathogenic strain Patoc 1 (*L. biflexa* serovar Patoc; Fig. 2A). The transcriptional distribution of *lic11207* among different strains of leptospires was evaluated by PCR amplification of reverse transcribed total RNA. The results obtained revealed the presence of *lic11207* transcripts in all five strains mentioned above (Fig. 2B). DNA contamination was ruled out as no amplification was observed in the absence of reverse transcriptase. The integrity of total RNA used in the RT-PCR experiment was assured by the presence of a 331-bp 16S ribosomal cDNA fragment in all samples (Fig. 2B).

3.4. Cloning, expression, and purification of rLIC11207

The *lic11207* gene was amplified, without the signal peptide sequence, and the DNA insert cloned and expressed as a full-length protein in *E. coli*. Recombinant protein was expressed with 6× His-tag at the N-terminal, purified by metal-chelating chromatography, and an aliquot of each step of the process was analyzed by SDS-PAGE. The expected protein band of 34 kDa is shown in NaCl-*E. coli* BL21 (SI)-induced culture. rLIC11207 was consistently recovered from the column in the absence of urea as a single major band indicating that most of the contaminants had been removed (Fig. 3).

3.5. In vivo expression

To evaluate whether the LIC11207 is expressed during infection, kidney tissues obtained from experimentally infected hamsters were probed with a serum raised against rLIC11207. The rLIC11207 antiserum did not show reactivity to normal hamster kidney sections (Fig. 4A) but revealed the presence of LIC11207 antigens located at the renal tubular lumen (Fig. 4B). In addition, some LIC11207 antigens were also observed at the sites of interstitial inflammatory cell infiltrates.

3.6. Antigenicity analysis

Sera from six patients convalescing from leptospirosis, previously confirmed by micro-agglutination test (MAT), were evaluated by ELISA for the presence of IgG antibodies against rLIC11207 together with sera from six healthy donors (Fig. 5A–B). Sera were considered positive when the mean values at A492 were above the mean + 3 SD of sera from healthy donors. Results showed that although rLIC11207 was recognized by both sera, the titer of antibodies present in the sera of convalescent patients was significantly higher ($p < 0.05$; Fig. 5C). The reactivity of confirmed leptospirosis human sera was confirmed by Western blot analysis using a pool of sera of each group exposed to a nitrocellulose membrane containing rLIC11207. Bands were revealed with an ECL reagent kit (Fig. 5D).

3.7. Lack of endothelial activation by rLIC11207

To determine whether endothelial cells were activated by exposure to rLIC11207, cultures of HUVECs were treated with the indicated concentrations of rLIC11207 and the surface levels of E-selectin and ICAM-1 were evaluated by FACS analysis. Although the LPS content of the sample was estimated to be 1.1×10^{-4} μg/ml (LAL assay), we ruled out any effect of LPS by performing the assay in the presence of PMB (7 μg/ml) that effectively blocked E-selectin expression triggered by LPS (1 μg/ml; Fig. 6A–B). As an additional

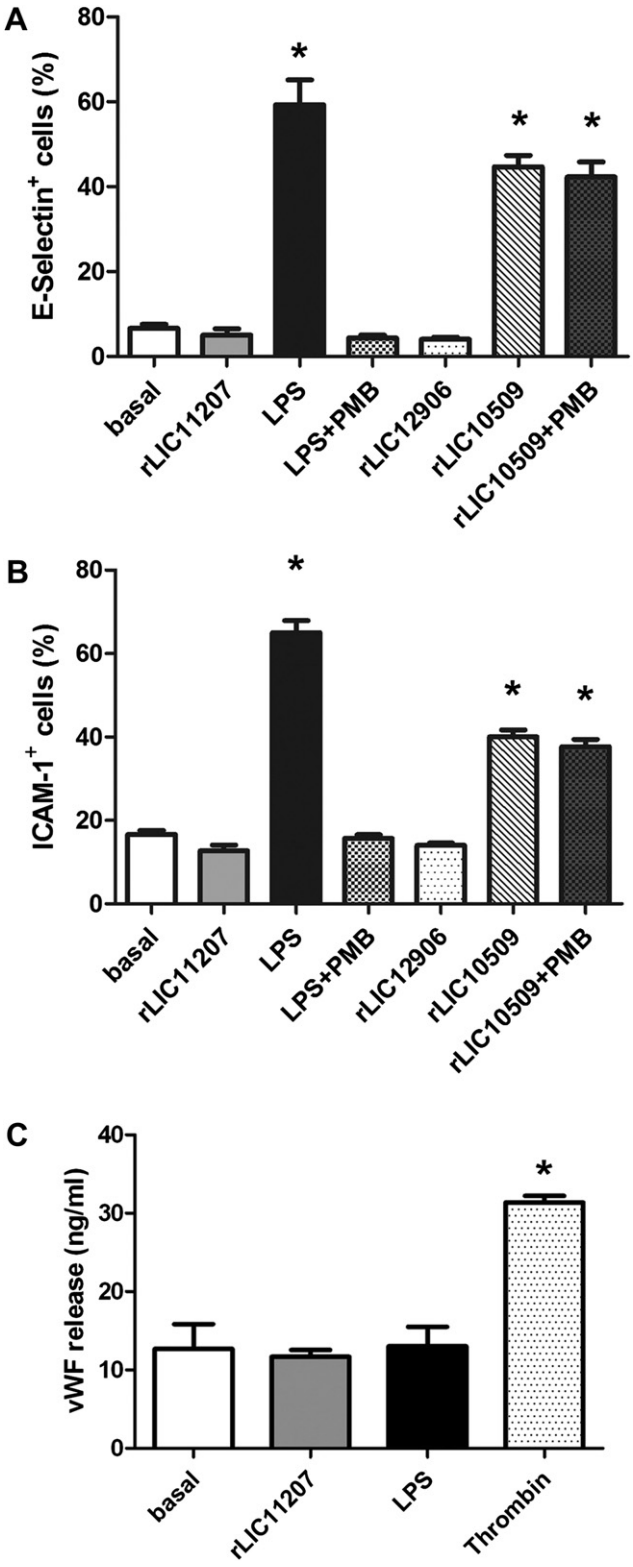

Fig. 6. Stimulation of E-selectin and ICAM-1 expression and vWF release on HUVECs by LIC11207. Confluent monolayers of HUVECs were treated for 1 h with rLIC11207 or rLIC12906 (50 μg/ml) in the presence of PMB (7 μg/ml). LPS (1 μg/ml) and rLIC10509 with or without PMB were used as a positive control. Thrombin was used as positive control for vWF release. Then medium was removed and cells were further cultured for 4 h with RPMI-1640 supplemented with 10% FBS. Data shown are mean ± SEM of three independent experiments, (A) Expression of E-selectin, and (B) ICAM-1. (C) vWF release measure by ELISA. No differences were observed between rLIC11207 and negative controls. The * indicates a value of $p < 0.05$ compared with basal levels.

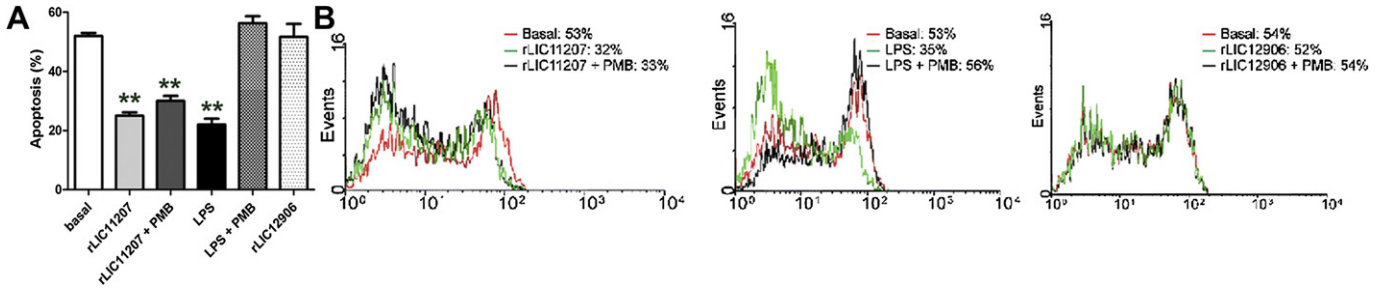

Fig. 7. Quantitation of PMN apoptosis. (A) PMNs were treated with 50 μg of rLIC11207 in presence or absence of PMB (7 μg/ml). After 18 h, an AO–BE staining was performed to determine the percentage of apoptotic cells. LPS was used as a positive control with and without PMB, and rLIC12906 was used as negative control. The results, expressed as percentage of apoptosis are the mean ± 3SD of three independent experiments. rLIC11207 inhibits apoptosis of PMNs significantly. The ** or * indicates a value of $p < 0.01$ or $p < 0.05$ compared with basal levels, respectively. (B) Under similar treatment, percentage of PMNs showing Annexin-V-positive cells was determined by flow cytometry analysis. Results showed that rLIC11207 inhibits significantly phosphatidylserine expression in PMN.

control we used two other recombinant proteins, rLIC12906 and rLIC10509. The former was chosen as a negative control because, although it contained higher LPS levels than rLIC11207 (8.8×10^{-3} μg/ml), it is a protein that is not structurally related to rLIC11207. The rLIC10509 was used as a positive control because we have previously demonstrated its ability to trigger CAM upregulation [17]. The results show that while treatment with either rLIC11207 or rLIC12906 (Fig. 6A–B) did not result in an increase in the basal expression of E-selectin or ICAM-1, exposure of HUVECs to rLIC10509 was able to induce a significant augmentation in the expression levels of both CAMs.

Failure of rLIC11207 to induce endothelial activation was also confirmed by the inability of this protein to trigger vWF release (Fig. 6C).

3.8. Analysis of PMNs apoptosis

In order to evaluate the ability of rLIC11207 to inhibit apoptosis of PMNs purified from peripheral blood were incubated with rLIC11207 for 18 h. After incubation, apoptosis was tested by evaluating both, morphological nuclear changes using fluorescence microscopy and the fluorescent DNA binding dyes AO and EB (Fig. 7A) and the expression of phosphatidylserine using flow cytometry and Annexin-V-FITC (Fig. 7B). Since LPS is a well-known inhibitor of PMN apoptosis it was used as a positive control [51]. To determine the potential effect due to the contaminating LPS, assays were performed in the absence or presence of PMB. Incubation of PMNs with either rLIC11207 or LPS resulted in a marked reduction of leukocyte apoptosis. While PMB barely reduced the anti-apoptotic effect of rLIC11207, it completely suppressed the increased viability of PMN mediated by LPS. Moreover, the percentage of PMN apoptosis in the presence of rLIC12906, was similar to control samples and was not affect by PMB.

Taken together, our data indicate that rLIC11207 positively modulates PMN lifespan.

4. Discussion

Predicted OMPs have been identified during genome annotation of pathogenic *L. interrogans* serovars Lai [43] and Copenhageni [26], and *L. borgpetersenii* [44]. The majority of predicted OMPs are assigned as hypothetical proteins of unknown function but, due to their putative location, are believed to play a role in the host–pathogen interactions.

The protein encoded by the gene *lic11207* is predicted to be an OMP (>80%) with a DUF1565. This domain is found in several phylogenetically diverse bacteria, among them some families of cyanobacteria phylum like nostocaetales and gloeobacterales, and in the leptospiraceae family. This motif is also present in the archaeon *Methanosarcina acetivorans*.

The LIC11207 CDS was identified by mass spectrometry-based proteomics of *L. interrogans*, but the number of copies of this protein was below the detection limit of the method [52].

The LIC11207 CDS sequence is well-conserved among five pathogenic leptospires strains but could not be detected in the saprophytic strain *L. biflexa* serovar Patoc and in some pathogenic strains. Its transcript is found in the same pathogenic strains—i.e., *L. interrogans* serovars, Copenhageni, Icterohaemorraghie, Canicola y Pomona y *L. borgpetersenii* serovar Hardjo—and absent in the serovars Grippotyphosa, Ballum, Tarassovi, and *L. biflexa* serovar Patoc.

As observed for other leptospiral antigens [17,18,48,53], the LIC11207 antigen was identified in the tubular lumen and associated with inflammatory cell infiltrates in kidneys from experimentally infected hamsters, indicating that this protein is expressed during infection of hamsters.

Several OMPs have been recognized by serum from human patients [12,13]. We observed that sera from patients convalescing from leptospirosis had a moderate but significant antibody response to rLIC11207, suggesting that this protein is expressed during infection in humans.

It has previously been demonstrated that several leptospiral recombinant proteins induce endothelial cell activation [16–18]. In contrast, our results showing that neither CAM expression nor vWF release was observed after HUVECs stimulation indicate that rLIC11207 did not trigger endothelial activation.

As expected, LPS had a pronounced inhibitory effect on PMN apoptosis [54]. In contrast, our results suggest that rLIC11207 has a specific anti-apoptotic effect on PMN leukocytes. Since the role of neutrophils during leptospirosis is still unknown, the physiopathological meaning of a prolongation of its lifespan remains to be further investigated. It could be speculated that the delay in neutrophil apoptosis triggered by the pathogen in infected host cells might modulate microbial replication and the persistence of infection [55]. In this regard, an enhanced neutrophil survival could contribute to the breakdown of tissue barriers resulting in a more efficient pathogen spread in the host [56,57]. However, more experiments are necessary to clarify whether LIC11207 has a role during the *in vivo* infection.

5. Conclusion

We describe LIC11207 as a new leptospiral protein. The absence of its homolog in the saprophyte strain, the fact that is recognized by the sera of convalescent patients, its expression during infection, and the anti-apoptotic properties shown in PMNs, suggest that LIC11207 might have a role during the disease.

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