



Article

# Functional Validation of the Proteome-Identified LIC\_13056 Putative Lipoprotein of *Leptospira interrogans* and the Potential Role in Pathogenesis

Giovanna M. Costa , João P. Gaspar, Aline F. Teixeira <sup>\*,†</sup> and Ana L. T. O. Nascimento <sup>\*,†</sup>

Laboratory of Vaccine Development, Butantan Institute, Vital Brasil Av., Sao Paulo 05503-900, SP, Brazil; giovannamatsukumi@alumni.usp.br (G.M.C.); j.inacio.proppg@proppg.butantan.gov.br (J.P.G.)

\* Correspondence: aline.rteixeira@butantan.gov.br (A.F.T.); ana.nascimento@butantan.gov.br (A.L.T.O.N.)

<sup>†</sup> These authors contributed equally to this work and share senior authorship.

## Abstract

Leptospirosis is a widespread zoonosis of human and veterinary concern. The etiological agent of the disease is the pathogenic bacteria of the genus *Leptospira*. Transmission typically occurs through mucosal contact and/or injured skin with the urine of infected animals or contaminated environmental sources. Understanding the biology and pathogenesis of leptospires is the main focus of our study. In this work, we characterized a novel protein encoded by the LIC\_13056 gene from *L. interrogans* serovar Copenhageni, having an OmpA-like domain. We show that this coding sequence (CDS), previously assigned as a hypothetical protein with an unknown function, is capable of binding to the cellular receptor  $\alpha 8$  integrin subunit, potentially contributing to kidney colonization. Additionally, the protein bound to both purified and normal human serum (NHS) plasminogen (PLG). In both conditions, PLG bound to protein was able to generate plasmin (PLA). Furthermore, rLIC\_13056 interacted with the complement system components C4b, C4BP, C8 and C9. The interaction of recombinant protein to the C9 had a negative impact on C9 polymerization. Taken together, the protein LIC\_13056, having an OmpA-like domain, appears to be involved in leptospiral pathogenesis via different stages of the infection process; PLA generation together with the inhibition of the membrane attack complex (MAC) may contribute to the immune evasion mechanism of *Leptospira*, thus facilitating the infection.

**Keywords:** *Leptospira*; leptospirosis; OmpA-like protein; host-pathogen interaction



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## 1. Introduction

Pathogenic species of the genus *Leptospira* are the etiological agents of leptospirosis, a globally distributed zoonosis that affects both humans and animals. The bacteria are usually transmitted via the contact between the mucosa and/or injured skin with urine of infected animals, or with contaminated water or soil [1]. Climate-related events, particularly increased rainfall and flooding, have heightened concern about leptospirosis as an emerging public health threat [2]. While leptospirosis is widespread in tropical and subtropical urban regions, in developed countries it is primarily an occupational disease, linked to professions like water and sewage workers or animal handlers [3]. Clinical manifestations vary widely, from asymptomatic to severe forms like Weil's syndrome or pulmonary hemorrhagic syndrome, with mortality rates ranging between 10% [4] and 50% [5–7] in severe cases. Since leptospirosis symptoms are non-specific, clinical diagnosis is difficult,

leading to wrong treatment [8]. Serology is the most used diagnostic method, which detects agglutinating antibodies that normally occur after 10 days from the onset of the disease [1].

Prophylactic measures are the best way to prevent the disease. Veterinary vaccines are available but they promote adverse reactions, present a short duration of efficacy, and lack protection against serovars that are not included in the vaccine preparations [3,5]. Licensed human vaccines exist in certain countries, with similar drawbacks as the veterinary ones. As outer membrane proteins (OMPs) are the first to interact with host components, they are often the main candidates to be studied. Proteins containing OmpA-like domains have emerged as promising targets due to their roles in leptospiral adhesion, infection and virulence. Since a study with the protein Loa22 [9], several findings with proteins having OmpA-like domains have been reported [10,11].

The present work aims to characterize a novel protein encoded by the LIC\_13056 gene from *L. interrogans* serovar Copenhageni, which contains an OmpA-like domain. This coding sequence was identified in comparative proteomics analyses [12] and has been assigned as hypothetical with an unknown function. In this work, we expressed the recombinant protein and show that it might affect leptospiral immune evasion in two different ways, by binding PLG and generating PLA, which can decrease opsonophagocytosis, or by inhibiting poly-C9 formation, and thus MAC generation.

## 2. Results

### 2.1. In Silico Analysis of LIC\_13056 CDS

According to the SignalP 6.0 software, the LIC\_13056 gene encodes a protein with a signal peptide cleavage site located between the amino acids 15 and 16 of the coding sequence, and it is recognized by the signal peptidase II enzyme. After cleavage, this enzyme may perform lipidation of cysteine residues, suggesting that LIC\_13056 encodes a putative lipoprotein. The alignment of LIC\_13056, with two lipoproteins, Loa22 and LipL46, shows the presence of a cysteine residue after the signal peptide (Cys<sub>+1</sub>), as expected for spirochetal lipoproteins [13] (Figure S1). The PFAM program identified the OmpA-like domain as the only conserved domain of this protein, located between positions 101 and 109 of the amino acid sequence, but NCBI genbank predicted a flagellar motor protein MotB. According to the CELLO program, there is higher probability that the LIC\_13056 is cytoplasmic followed by an outer membrane, but according to PSORTb program, it is likely to be in the cytoplasmic membrane. Prediction of the molecular masses for both the native and recombinant proteins, as well as their respective isoelectric points (pIs), was performed by the ProtParam tool. It predicted a molecular mass of approximately 25 kDa for the LIC\_13056 native, with theoretical pIs of 8.9 and 27 kDa for the recombinant protein, with a pI estimate of 8.95.

A multiple sequence alignment of the LIC\_13056 amino acid sequence with orthologs from different pathogenic, saprophytic, and intermediate *Leptospira* species was performed using BLASTp to observe the conservation of the proteins among them. A phylogenetic tree was then generated based on CDS alignments (Figure 1A) with the help of the Clustal Omega tool. Additionally, the conservation of the amino acid sequence corresponding to the OmpA-like domain was analyzed (Figure 1B). Although there was a high data bank coverage of the sequences amongst the aligned proteins, in both trees, a higher identity between LIC\_13056 and its orthologs from pathogenic and intermediate species was observed. Based on this information, it is suggested that the protein encoded by LIC\_13056 is likely to be present in pathogenic species, possibly playing a role in bacterial virulence, which is supported by previous research on the OmpA-like domain in leptospiral proteins [10,11].

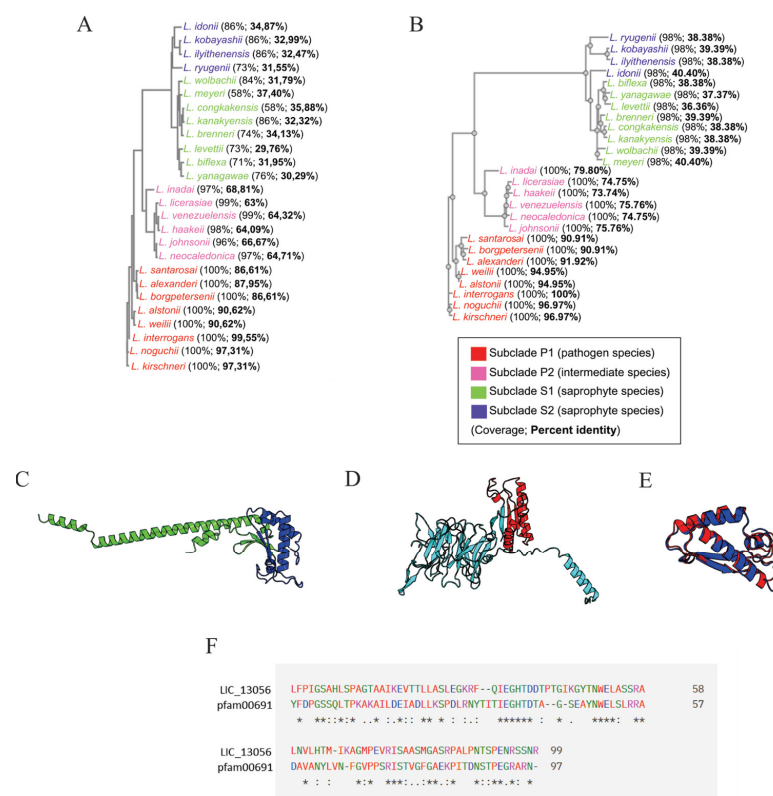


**Figure 1.** Conservation of LIC\_13056 coding sequences and OmpA-like domains among different *Leptospira* spp. Phylograms were obtained by the Clustal Omega software, showing LIC\_13056 (A) and OmpA-like domain (B) coding sequence conservation among pathogenic (red), intermediate (purple) species, and saprophytic strains S1 (green) and S2 (blue). In (C,D) are the three-dimensional structures of LIC\_13056 and OmpA-like domain and Lsa46, respectively, as predicted by the software AlphaFold 2. In (E) are the overlays of the OmpA-like domain three-dimensional structures LIC\_13056 and Lsa46, respectively. In (F) is the sequence alignment of the OmpA-like region of LIC\_13056 with the OmpA-like consensus sequence pfam00691.

The hypothetical structure of LIC\_13056 was generated by the AlphaFold 2 program with a confidence level of 90% or higher. As observed in Figure 1C, its structure is predominantly composed of  $\alpha$ -helices. Likewise, the OmpA-like domain also presents high  $\alpha$ -helix contents (Figure 1C). Using the PsiPred software, the secondary structures of the protein were analyzed and predicted to be composed mainly of  $\alpha$ -helices (55.6%) and regions with no defined conformation (30.5%). Consistent with the AlphaFold 2 prediction,  $\beta$ -sheets (13.9%) represent the smallest portion of the protein. While LIC\_13056 exhibits high  $\alpha$ -helix contents throughout its sequence, Lsa46, another OmpA-like domain containing protein, is predominantly composed of  $\beta$ -sheets (Figure 1D). Aiming to compare the structure conservation among domains LIC\_13056 and Lsa46, an overlay of sequences was performed. As can be seen in Figure 1E, both proteins have a conserved OmpA-like domain, characterized by an arrangement of  $\alpha$ -helices.

Analysis of the LIC\_13056 C-terminal OmpA-like domain with the OmpA-like consensus domain conserved in the C-terminal—pfam00691 [14]—showed a 39.39% identity and 98% coverage (Figure 1F). This result is very similar to the one obtained with Loa22 and the OmpA-like consensus sequence [9].

## 2.2. Protein Expression, Purification and Immunogenicity

The target gene was amplified by PCR from the genome of *L. interrogans* serovar Copenhageni and cloned into the pET-28a vector. The recombinant plasmid selected

was used to transform the *E. coli* C43 (DE3) strain for protein expression. The protein was expressed within an expected molecular weight of 27 kDa in its soluble form, and purification was performed successfully (Figure 2A, lane 1).

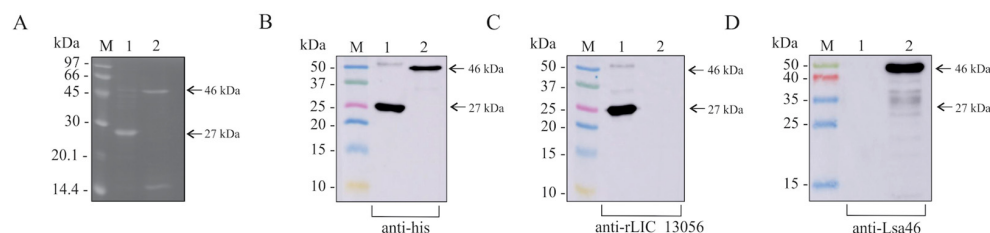


**Figure 2.** Specificity analysis of mouse serum anti-rLIC\_13056. The specificity of anti-rLIC\_13056 to recognize rLIC\_13056 was evaluated by Western blotting. Purified samples of rLIC\_13056 and rLIC\_13479, another OmpA-like domain-containing protein, were separated on a 15% SDS-PAGE gel and transferred onto 3 different nitrocellulose membranes. Each membrane was blocked and incubated separately with anti-His (1:10,000), anti-rLIC\_13056 (1:5000) or anti-rLIC\_13479 (1:5000). (A) SDS-PAGE gel stained with Coomassie blue, confirming the presence of both rLIC\_13056 and rLIC\_13479 in the samples used; (B) nitrocellulose membrane incubated with anti-His; (C) nitrocellulose membrane incubated with anti-rLIC\_13056; (D) nitrocellulose membrane incubated with anti-rLIC\_13479. Both membranes in (C,D) were developed after incubation with HRP-conjugated anti-mouse IgG (1:5000). The arrows indicate the expected molecular masses of approximately 27 kDa (rLIC\_13056) and 46 kDa (rLIC\_13479). (M): Molecular mass protein marker; (1): 2 µg of purified rLIC\_13056; (2): 2 µg of purified rLIC\_13479. The experiment was performed once.

Immunized BALB/c mice were bled via retro-orbital plexus after each 15-day interval, and the produced sera were analyzed by ELISA. Antibody titer after the third immunization was achieved, at 409,600, showing that the recombinant protein was able to stimulate the immune system of the animals, resulting in the production of polyclonal antiserum against rLIC\_13056. We selected another recombinant protein containing an OmpA-like domain, Lsa46 (rLIC\_13479, [11]), in order to determine if both sequences could share common immune epitopes. Both purified proteins were separated by SDS-PAGE (Figure 2A) and transferred to nitrocellulose membranes. Membrane-bound proteins were probed with anti-His (Figure 2B), anti-rLIC\_13056 (Figure 2C), or anti-Lsa46 antibodies (Figure 2D) for detection. Notably, anti-rLIC\_13056 and anti-Lsa46 antibodies exhibited exclusive specificity for their respective recombinant protein (Figure 2C,D), strongly suggesting a lack of epitope conservation between the two sequences. A complete sequence alignment performed between both sequences confirms the low similarity between them (Figure S2).

### 2.3. Reactivity Analysis of rLIC\_13056 with Leptospirosis Serum Samples

The most commonly used diagnostic method for leptospirosis is the MAT test, which detects the presence of agglutinating antibodies in leptospirosis serum samples [15]. Using MAT as the reference, we evaluated the reactivity of recombinant protein using 100 convalescent-phase serum samples (MAT+). The results showed that only 19% of the samples exhibited reactivity against the protein compared to NHS controls. Although, the proteins containing OmpA-like domains Lsa46 and Lsa77 [11] display high reactivity with positive serum samples, others, like the leptospiral protein Lsa66 [10], showed low immunoreactivity, similar to LIC\_13056.

### 2.4. Evaluation of rLIC\_13056 Expression in Virulent and Attenuated *Leptospira* strains

The expression of LIC\_13056 was evaluated by analyzing bacterial extracts of the *L. interrogans* serovar Copenhageni strain FIOCRUZ L1-130 (virulent), *L. interrogans* serovar Copenhageni strain M20 (culture-attenuated) and *L. biflexa* serovar Patoc 1, as well as

purified rLIC\_13056 as a positive control. Following SDS-PAGE separation (Figure 3A), the gel contents were transferred to a nitrocellulose membrane and the protein was probed with anti-rLIC\_13056 serum (Figure 3B). The results revealed that LIC\_13056 is indeed present in extracts of both virulent and culture-attenuated *L. interrogans*, but not in saprophytic *L. biflexa* (Figure 3). These findings corroborate with the in silico analysis data, which indicated a broader sequence conservation of this protein among pathogenic and intermediate species compared to saprophytic subclades.

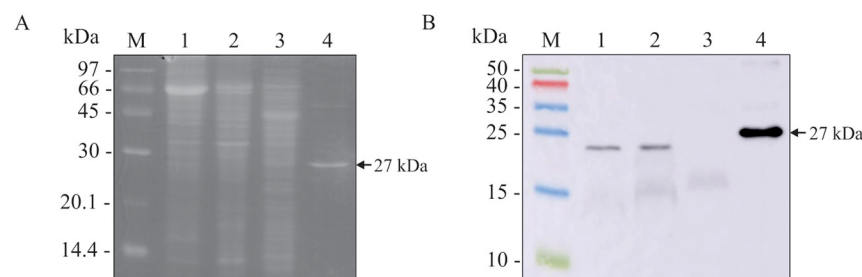


**Figure 3.** Presence of LIC\_13056 in leptospiral cellular extracts. Extracts of *L. interrogans* serovar Copenhageni strain FIOCRUZ L1-130 (virulent), *L. interrogans* serovar Copenhageni strain M20 (attenuated), and *L. biflexa* serovar Patoc 1 were normalized to an OD<sub>420nm</sub> of approximately 50 and separated on a 15% SDS-PAGE gel alongside a sample of rLIC\_13056 as control. The contents of the gel were then transferred to a nitrocellulose membrane, which was developed by chemiluminescence, as described in the Materials and Methods section. (A) SDS-PAGE gel stained with Coomassie blue to confirm that the samples used were viable; (B) nitrocellulose membrane incubated with anti-rLIC\_13056 and developed after incubation with HRP-conjugated anti-mouse IgG (1:5000). (M): Molecular mass protein marker; (1): virulent *L. interrogans* extract; (2): attenuated *L. interrogans* extract; (3): *L. biflexa* serovar Patoc 1 extract; (4): sample of purified rLIC\_13056.

### 2.5. Cellular Location of LIC\_13056 Coding Sequence on *L. interrogans*

The cellular location was assessed by flow cytometry, in which intact leptospires were fixed and then incubated with antiserum against rLIC\_13056 or the controls VapB (negative) [16] and LipL46 (positive) [17], followed by FITC-conjugated secondary antibodies. Cells treated with antiserum against LipL46 or rLIC\_13056 resulted in the higher mean fluorescence intensity (MFI) of the leptospiral population when compared to the negative cytoplasmic control VapB (Figure 4), suggesting surface exposure. The calculated MFI was of 64, 70 and 40% for LipL46, LIC\_13056, and VapB, respectively.

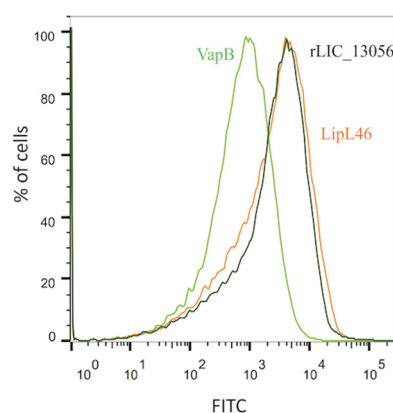


**Figure 4.** Cellular localization of LIC\_13056 performed by FACS. *L. interrogans* were incubated with primary antiserum against VapB, LipL46 or rLIC\_13056 for 1 h at 30 °C. After incubation, FITC-conjugated goat anti-mouse IgG (1:50) was added for 16 h at 4 °C. Fluorescence measurements were performed in a BD FACSCanto II. Data are representative of an experiment performed in triplicate.

## 2.6. Characterization of the Interaction Between rLIC\_13056 and Host Components

Outer membrane proteins (OMPs) of pathogenic *Leptospira* species have attracted significant research interest due to their surface localization and possible direct interaction with host components, contributing to the establishment of the infection. Thus, the ability of rLIC\_13065 to interact with cell adhesion receptors, plasma and complement system proteins was determined by ELISA. Among the cellular receptors tested, rLIC\_13056 demonstrated saturable dose-dependent binding to the  $\alpha 8$  integrin subunit (Figure 5 and Table 1), consistent with previous reports of integrin-binding leptospiral proteins [18,19].

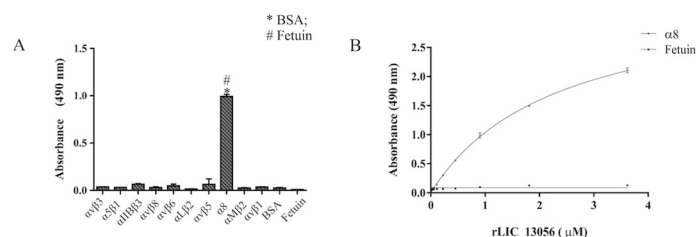


**Figure 5.** Analysis of integrin binding to rLIC\_13056. **(A)** Integrin binding assay. ELISA plates were coated with each integrin and incubated overnight. After blocking, the wells were incubated with the recombinant protein. Plates were developed following incubation with HRP-conjugated anti-mouse IgG (1:5000). Fetuin and BSA served as negative controls. Statistically significant binding was observed only for the  $\alpha 8$  subunit. **(B)** Dose–response curve analysis. The interaction between rLIC\_13056 and  $\alpha 8$  was further evaluated by dose–response analysis, indicating the binding saturation point. The graph was plotted using GraphPad Prism v.7 software. Statistical analysis was performed using a two-tailed paired Student’s *t*-test with a significance level of  $p < 0.05$  (\*; #). Data are representative of an experiment performed in duplicate.

**Table 1.** Dissociation constants ( $K_D$ ) of rLIC\_13056 interaction with host components.

| Host Components       | $K_D$ ( $\mu M$ ) |
|-----------------------|-------------------|
| Integrin              |                   |
| $\alpha 8$            | $2.332 \pm 0.140$ |
| Complement components |                   |
| C4b                   | $0.829 \pm 0.136$ |
| C4BP                  | $0.738 \pm 0.287$ |
| C8                    | $1.261 \pm 0.126$ |
| C9                    | $1.874 \pm 0.151$ |
| Plasma component      |                   |
| PLG                   | $0.034 \pm 0.001$ |

The interaction of rLIC\_13056 protein with human complement system components, was dose-dependent on the protein concentration, tending to reach saturation with C4b, C4BP, C8 and C9 (Figure 6A–E), with the corresponding  $K_D$  values listed in Table 1. Given that the recombinant protein was able to bind to C9, it was investigated whether it could interfere with the polymerization of this component. For this, rLIC\_13056 was pre-incubated with C9, and its polymerization was induced by  $ZnCl_2$ . The results (Figure 6F) revealed that, with only 1.25  $\mu g$  of the protein, there was no significant effect on poly-C9 formation, but with 5  $\mu g$ , polymerization was almost completely inhibited (Figure 6F—lane 7), therefore suggesting that rLIC\_13056 may function as an inhibitor of MAC formation and potentially contributing to immune evasion in vivo.

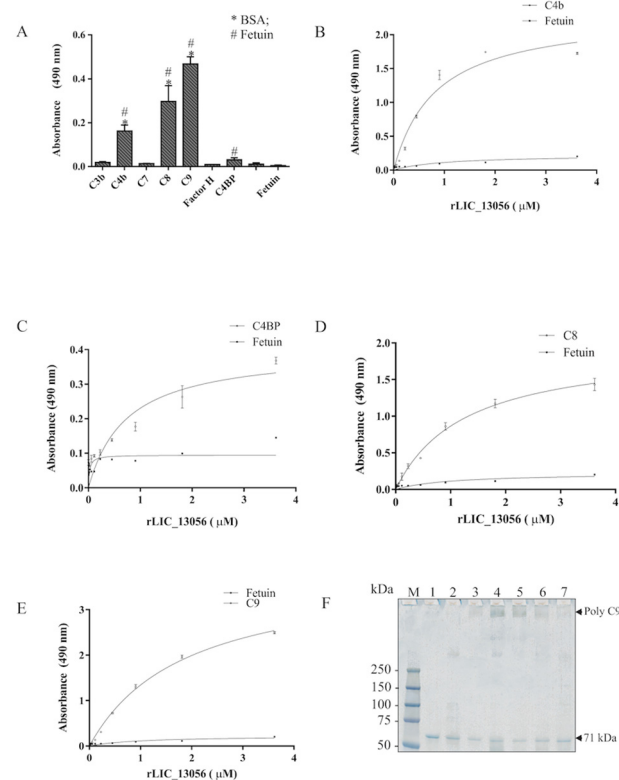


**Figure 6.** Characterization of the interaction between rLIC\_13056 and complement system components. **(A)** Binding assay of complement system components. ELISA plates were coated overnight with 1 μg of the components C3b, C4b, C7, C8, C9, Factor H, and C4BP, and incubated with the recombinant protein. The reaction developed after incubation with HRP-conjugated anti-mouse IgG (1:5000). Fetuin and BSA served as negative controls. Statistically significant binding was observed only for C4b, C8, C9, and C4BP. Statistical analysis was performed using a two-tailed paired Student's *t*-test with a significance level of  $p < 0.05$  (\*; #). **(B–E)** Dose–response curve analysis. Dose–response assays were performed as described for item **(A)** using increasing concentrations of rLIC\_13056. Graphs were plotted using GraphPad Prism software. **(F)** Poly-C9 inhibition assay. Samples of C9 were incubated for 40 min with increasing quantities of the recombinant protein (1.25, 2.5, and 5 μg), followed by ZnCl<sub>2</sub> incubation for 2 h at 37 °C. The samples were then separated on SDS-PAGE gradient gel (4–20%) to evaluate inhibition of C9's zinc-induced polymerization. Positive controls for the polymerization included a sample containing only C9 and ZnCl<sub>2</sub> and another containing fetuin (5 μg), C9 and ZnCl<sub>2</sub>. Negative controls consisted of a sample containing only C9 without ZnCl<sub>2</sub> and another containing C9, ZnCl<sub>2</sub>, and recombinant protein rLIC\_13259, known to inhibit C9's zinc-induced polymerization. Data are representative of an experiment performed in duplicate.

The reactivity of the recombinant protein with plasma components, PLG, plasma fibronectin, vitronectin, and fibrinogen showed that rLIC\_13056 bound only to PLG (Figure 7A). The interaction exhibited a dose-dependent and saturable profile (Figure 7B and Table 1). The participation of kringle domains in this interaction was further characterized using ACA, a lysine analog that competitively inhibits kringle domain-binding sites. Notably, ACA significantly reduced PLG binding to rLIC\_13056, strongly suggesting that PLG's kringle domains serve as the primary binding sites for this interaction. Furthermore, it was investigated whether rLIC\_13056 could recruit PLG directly from NHS. As shown in Figure 7D, the higher the NHS concentration, the greater the PLG capture, demonstrating that rLIC\_13056 can bind this component directly from NHS. Since previous studies have reported the conversion of PLG bound to the surface of *L. interrogans* into PLA in the presence of the host PLG activator uPA [20], we investigated whether rLIC\_13056-bound PLG similarly undergoes activation. When rLIC\_13056 was incubated either with purified

PLG (Figure 7E) or NHS 30% (Figure 7F), in the presence of uPA, it was demonstrated that both purified PLG and PLG acquired from NHS could be converted into PLA, indirectly measured using PLA substrate and detecting its product. These results demonstrate that the recombinant protein preserves native-like PLG activation kinetics, as observed in pathogenic *Leptospira* spp.

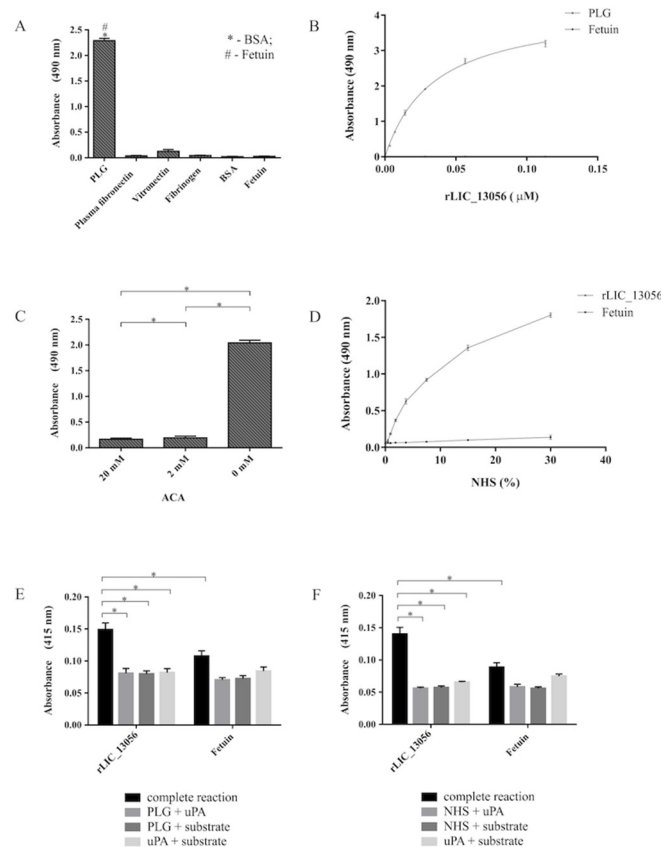


**Figure 7.** Characterization of the interaction between rLIC\_13056 and PLG. (A) Plasma components binding assay. ELISA plates were coated with 1  $\mu$ g of PLG, plasma fibronectin, fibrinogen, or 250 ng of vitronectin. The plates were incubated with the recombinant protein and developed after incubation with HRP-conjugated anti-mouse IgG (1:5000). Fetuin and BSA served as negative controls. Statistically significant binding was observed only for PLG. (B) Dose–response curve analysis. Dose–response assays were performed as described for item (A) using increasing concentrations of rLIC\_13056. (C) PLG binding inhibition assay. ELISA plates were coated with 1  $\mu$ g of PLG, followed by incubation with rLIC\_13056, diluted in solutions containing different concentrations of ACA (0, 2, and 20 mM). The inhibition of the interaction was assessed by measuring the absorbance after the development step. (D) Recruitment assay of PLG present in the NHS. ELISA plates were coated with 1  $\mu$ g of the recombinant protein, followed by incubation with increasing concentrations of NHS. The binding of PLG to the protein was evaluated by measuring the absorbance after the development step. A proportional increase in absorbance was observed with higher NHS concentrations. (E,F) Enzymatic assay demonstrating the conversion of purified PLG to PLA. ELISA plates were coated with 1  $\mu$ g of the recombinant protein and incubated with purified PLG in (E), while in (F), the plates were incubated with NHS. Fetuin was used as a negative control. Subsequently, a solution containing uPA and a chromogenic substrate was added and incubated overnight at 37 °C. Additional negative controls were prepared by omitting one component of the reaction (NHS, PLG, uPA, or substrate). The conversion of PLG to PLA was evaluated by measuring the absorbance after the developing step. For panels (A,C,E,F), the statistical analysis was performed by a two-tailed paired Student's *t*-test, with a significance level of  $p < 0.05$  (\*; #). The graph was plotted using GraphPad Prism software. Data are representative of an experiment performed in duplicate.

### 3. Discussion

In vitro studies have shown that several *L. interrogans* OMPs are possibly responsible for playing a role in one or more stages of the infection, including proteins containing OmpA-like domains [10,11,21–24]. Consequently, these proteins receive significant attention due to their potential to directly interact with host cellular components. Based on these findings, the hypothetical protein encoded by the gene LIC\_13056 from *L. interrogans* serovar Copenhageni, which contains an OmpA-like domain, was evaluated for its capacity to interact with host components, which could contribute to the virulence of *Leptospira*.

BLASTp v. 2.17.0 and Clustal Omega v.1.2.4 software indicated that LIC\_13056 CDS is conserved among pathogenic and intermediate species of *Leptospira* spp., which was confirmed by immunoblotting with cell extracts of the *L. interrogans* strain FIOCRUZ L1-L130 and the *L. interrogans* strain M20. The NCBI protein database showed that LIC\_13056 has an OmpA-like domain and a flagellar motor protein domain, MotB. Though the in silico data were not consistent with the cellular location of the coding sequence, our experimental results demonstrated an overlap in the cell population of LIC\_13056 with the outer membrane protein LipL46 [17] when compared with the cytoplasmic protein VapB [16], suggesting that it is at least partially located in the outer membrane. Although FACS analysis provides a relative measure of fluorescence intensity, which reflects the amount of labeled protein, this technique has been also used to suggest the possible location of bacteria proteins [17,25–27]. Even though the in silico prediction of LIC\_13056 cellular localization diverges from the experimental results, it does not exclude the possibility that this protein could be present in more than one cellular compartment, as is reported for LipL41 [28].

Despite being present in the bacterial outer membrane, rLIC\_13056 was only detected by 19% of the MAT+ serum samples tested, indicating its low expression during the infection, especially when compared to the highly expressed antigens such as LipL32 and LigA [12]. It also suggests that this protein has low diagnostic potential.

The rLIC\_13056 exhibited dose-dependent and saturable binding to a variety of host components, including integrin subunit  $\alpha 8$ , PLG, and complement system proteins C4b, C8, and C9. It has already been suggested that the interaction between leptospires and the host fibrinolytic system may play a significant role in the virulence by facilitating dissemination within the host [29]. The generation of PLA on the surface of *Leptospira* enables their penetration by degrading ECM components [20]. Additionally, PLA can reduce the deposition of complement C3b and IgG on the bacterial surface, decreasing opsonophagocytosis and conferring immune evasion capabilities [30]. The results of this study also demonstrated that rLIC\_13056 binds to PLG, both in its purified form and in NHS, in a specific and saturable manner. Thus, it is possible that generated PLA may probably enhance bacterial invasion and host immune system evasion.

Integrins are cell adhesion receptors composed of  $\alpha$  and  $\beta$  subunits, each containing transmembrane and cytoplasmic domains [31–33]. These receptors promote bidirectional signaling between extracellular and intracellular environments. In the context of pathogen–host interactions, binding to integrins may serve as an adhesion and/or invasion mechanism for the microorganism. It is known that the  $\alpha 8$  subunit is an RGD receptor [32], which is the most common peptide sequence responsible for cell adhesion to the ECM, triggering intracellular signaling and cytoskeletal rearrangements that can promote microbial internalization [34,35]. Notably, a variety of pathogenic bacteria utilize RGD motifs as virulence factors, such as *Helicobacter pylori* [36], *Bordetella pertussis* [37], *Mycobacterium tuberculosis* [38], and the spirochete *Borrelia burgdorferi* via its P66 protein [39]. Moreover, it has already been shown that RGD-containing proteins identified in *Leptospira* spp., such as the protein rLIC\_12254, bind to integrin  $\alpha V \beta 1$  and the  $\alpha 8$  subunit through its RGD

domain [40]. Additionally, Hartner and colleagues (1999) reported that the  $\alpha 8$  subunit is strongly and specifically expressed in the glomerular mesangium of humans and rodents [41]. Given that the kidneys are among the primary organs affected by leptospirosis, this observation suggests a potential correlation between the binding to the  $\alpha 8$  subunit and disease progression, although further studies are required to confirm this hypothesis. Nonetheless, the ability of rLIC\_13056 to interact with the  $\alpha 8$  subunit highlights its possible contribution to the pathogenesis of leptospirosis.

The ability of a microbial pathogen to successfully establish an infection depends heavily on its capacity to evade the host's immune system. The first line of defense is mediated by innate immunity, which is non-specific and targets a wide range of microorganisms [42]. The complement system is one of the key mechanisms of innate immunity. It is a sophisticated pathway consisting of a coordinated and efficient proteolytic cascade that results in pathogen neutralization [42,43]. Complement activation can occur through three distinct routes: the classical, lectin and alternative pathways. All pathways converge to form the terminal C5 convertase complex, followed by a cascade of reactions generating the C5b-7 complex. This complex enables interaction with cell membranes, and upon incorporation of C8, it is inserted into the lipid bilayer. Finally, the C5b-8 complex mediates C9 polymerization, forming the MAC and leading to cell lysis [44].

Various pathogens, including *Borrelia* spp., *Treponema* spp. and *Leptospira* spp., develop evasion strategies to overcome the host immune system. One of their well-documented evasion strategies involves the expression of surface proteins capable of recruiting complement regulators, such as Factor H (FH) and C4BP. Examples include *Borrelia* CspA [45,46], *Treponema* FhbB [47], *Leptospira* LigA/B [48], Lsa33, and Lsa25 [49], which interfere with complement activation. The OmpA-like domain-containing protein Loa22 has been reported to bind Factor H, C4BP and PLG/PLA, but C9 polymerization inhibition was not observed [50]. Other evasion mechanisms include reducing C3b and IgG deposition through binding to PLG/PLA, as previously described, or inhibiting the terminal pathway by sequestering C7, C8, or C9 proteins, thereby preventing MAC assembly. Additionally, CspA has been shown to bind simultaneously to C7 and C9, suppressing  $Zn^{2+}$ -induced C9 polymerization [51]. Moreover, the recombinant protein rLIC\_13259 from *L. interrogans* has also demonstrated binding capacity to C9, inhibiting poly-C9 formation [21]. Similarly, rLIC\_13056 also has the ability to interact with the terminal component C9 and inhibit  $Zn^{2+}$ -induced polymerization, suggesting a potential role in preventing MAC establishment on the cell membrane.

#### 4. Materials and Methods

The macromolecules laminin (L6274), cellular fibronectin (2518), elastin (F5881), collagen I (C3867) and IV (C7521), PLG (P7999), plasma fibronectin (F2006), vitronectin (V8379), fibrinogen (F4883), fetuin (F3385), and BSA (A7906) were purchased from Sigma-Aldrich, St. Louis, MI, USA. C3b (A114), C4b (A108), C6 (A123), C7 (A124), C8 (A125), C9 (A126), Factor H (A137), and C4BP (A109) were purchased from Complement Technology (Tyler, TX, USA). Integrins  $\alpha 8$ ,  $\alpha V\beta 3$ ,  $\alpha V\beta 5$ ,  $\alpha 5\beta 1$ ,  $\alpha V\beta 6$ ,  $\alpha V\beta 8$ ,  $\alpha L\beta 2$ ,  $\alpha IIb\beta 3$ ,  $\alpha M\beta 2$ , and  $\alpha V\beta 1$  were purchased from R&D Systems (Minneapolis, MN, USA). The commercial antibodies used were peroxidase (HRP)-conjugated anti-mouse IgG (A9044) and mouse monoclonal anti-polyhistidine-peroxidase antibody (A7058), acquired from Sigma-Aldrich, St. Louis, MI, USA. Human serum samples were obtained from the Adolfo Lutz Institute, São Paulo, Brazil, and approved by the Ethics Committee (protocol no. 2.834.589). The National Research Ethics Commission (CONEP) approval is registered in "Plataforma Brasil site" (protocol CAAE 78770117.0.0000.8098).

#### 4.1. In Silico Analysis

The coding sequence (CDS) of the gene LIC\_13056 was obtained through the NCBI database [52]. Web servers PSORTb [53], LipoP [54], and CELLO [55,56] were used to predict the protein's cellular location. The program PFAM [57] was also used to show the conserved domains; SignalP [58], WebCutter [59], PsiPred [60], and AlphaFold 2 [61] were used to predict the signal peptide, restriction enzymes, and the secondary and tertiary structures, respectively. The phylogenetic tree was performed using the multiple-sequence alignment, made with NCBI blastp [62] and Clustal Omega [63].

Cloning of gene LIC\_13056: The CDS LIC\_13056 was amplified, without the peptide signal sequence, by a pair of oligonucleotides, designed with the software Snapgene v. 8.2.1, using the *L. interrogans* serovar Copenhageni strain M20 genomic DNA as template. Specific primers are presented in Table 2. The restriction sites were added to these oligonucleotides with the restriction enzymes BamHI and HindIII. The CDS was ligated into the vector pET-28a, which adds 6X histidine residues at the N-terminal region. The recombinant plasmid produced was used to transform *E. coli* DH5 $\alpha$ . The chain termination method was used in order to check the correct in-frame insertion and the absence of mutation in an automatic sequencer, the ABI (Applied Biosystems, Foster City, CA, USA).

**Table 2.** Gene ID, given name, GenBank reference number, sequence of the primers used for DNA amplification, and expected molecular mass of the recombinant protein.

| Gene ID   | Given Name | Genbank    | Primer Sequence (Restriction Site Bolded)                                                                                | Molecular Mass (kDa) |
|-----------|------------|------------|--------------------------------------------------------------------------------------------------------------------------|----------------------|
| LIC_13056 | rLIC_13056 | AAS71605.1 | F: 5' gtacg <b>GGATCCTT</b> TAATTTTGTATTTTATTTTAATCAACTGCG 3'<br>R: 5' gtacg <b>AAGCTTTT</b> AGTTCTGAATACTTTTGCAACTCT 3' | 27                   |

#### 4.2. Expression and Purification of the Recombinant Protein

The plasmids purified with the Cytiva Illustra PlasmidPrep Mini Spin Kit (Cytiva, Marlborough, MA, USA) were used to transform *E. coli* C43 (DE3) to express the recombinant protein. The bacterial cell culture was induced with 1 mM IPTG for 16 h at 18 °C under constant agitation and in the presence of 50  $\mu$ g/mL of kanamycin. The purification process was done through chelating chromatography using a Ni<sup>2+</sup>-loaded Sepharose (GE) column. Contaminants were washed away with 5 washes of a buffer solution of 500 mM NaCl and 20 mM Tris/HCl (pH 7.4) with an imidazole gradient of 5 mM to 100 mM. Then, the protein was eluted with 500 mM and dialyzed against a buffer solution of 500 mM NaCl and 20 mM Tris/HCl (pH 7.4) to remove the imidazole. The purification's efficiency was analyzed with a 15% SDS-PAGE. The protein concentration was estimated through densitometry via the software ImageJ v. 1.54p by comparing the band intensity of determined concentrations of BSA.

#### 4.3. Production of Polyclonal Antibodies Against rLIC\_13056

Five female BALB/c mice, weighing between 18 and 22 g, were randomly grouped into boxes. They were subcutaneously immunized with 10  $\mu$ g of rLIC\_13056 mixed with 25  $\mu$ L of the adjuvant Al(OH)<sub>3</sub> and 125  $\mu$ L of 1 $\times$  phosphate-buffered saline (PBS) at a pH of 7.4, resulting in a total volume of 200  $\mu$ L per dose for each mouse. The negative control group (n = 5) received PBS combined with the adjuvant. Immunizations were administered three times at two-week intervals; prior to each dose, blood samples were collected from the retro-orbital plexus to obtain serum. The serum samples were titrated using ELISA, as detailed by Passalia et al. (2021) [27]. In this procedure, 250 ng of each recombinant protein was immobilized in the wells for 16 h at room temperature (RT) and subsequently blocked with a PBS solution containing 0.05% Tween 20 and 10% skimmed milk. The

diluted animal sera were then added to the wells and incubated for 1 h. Following this, the plates were washed and incubated with an anti-mouse IgG secondary antibody conjugated to peroxidase. Reactivity was measured by adding 1 mg/mL of OPD (o-phenylenediamine) along with 1  $\mu$ L/mL of H<sub>2</sub>O<sub>2</sub>. After a 10 min incubation, the reaction was halted by adding 50  $\mu$ L of 2 M H<sub>2</sub>SO<sub>4</sub>, and the absorbance was recorded at 492 nm using a Multiskan-FC microplate reader (Thermo Fisher Scientific, Helsinki, Finland). The antibody titer was calculated as the inverse of the dilution that produced an absorbance value of 0.1 at 492 nm. Blank controls without serum were included in all experiments, and the study was replicated three times.

#### 4.4. Immunoblotting Assay

A sample of the purified recombinant protein was loaded into a 15% SDS-PAGE and immunoblotted onto a nitrocellulose membrane. The membrane was blocked with 10% non-fat dried milk in PBS containing 0.05% of PBS-T overnight at 4 °C. Afterwards, the membrane was incubated with anti-rLIC\_13056 (1:5000) mouse polyclonal serum for 1 h and 30 min at RT and under constant and gentle agitation. The membrane was washed 3 times with PBS-T and incubated with HRP-conjugated anti-mouse IgG (1:5000) for 1 h at RT. The membrane was washed 5 times and the protein reactivity was developed by an ECL reagent kit (GE Healthcare, Piscataway, NJ, USA).

#### 4.5. Specificity Analysis of Anti-rLIC\_13056 Polyclonal Antibody

Samples of the proteins rLIC\_13056 and Lsa46 [11], which also contained OmpA-like domains, were separated on a 15% SDS-PAGE. The gel contents were subsequently blotted onto a nitrocellulose membrane, as previously described. The membrane was incubated for 2 h at RT with anti-rLIC\_13056 antiserum (1:5000). To confirm the presence of both proteins and to individually detect Lsa46, two additional membranes were incubated with monoclonal anti-His antiserum (1:10,000) and polyclonal anti-Lsa46 antiserum (1:5000), respectively. After 1 h, incubation was performed with HRP-conjugated anti-mouse IgG (1:5000). The membranes were washed 5 times and developed following the same protocol described above.

#### 4.6. Identification of LIC\_13056 in *Leptospira* spp. Extracts

Cell extract samples of three *Leptospira* spp., the virulent *L. interrogans* serovar Copenhageni strain FIOCRUZ L1-130, culture attenuated *L. interrogans* serovar Copenhageni strain M20, and *L. biflexa* serovar Patoc 1 strain Patoc1, along with a sample of LIC\_13056, were loaded into a 15% SDS-PAGE. Afterwards, the proteins were blotted onto a nitrocellulose membrane. The membrane was blocked with a solution of 10% non-fat dried milk in PBS-T overnight at 4 °C. Afterwards, the membrane was incubated with anti-rLIC\_13056 (1:5000) mouse polyclonal serum for 2 h at RT under constant and gentle agitation. Subsequently, the experiment was conducted as described above.

#### 4.7. Reactivity with *Leptospirosis* Serum Samples

Leptospirosis serum samples was diagnosed by the microscopic agglutination test (MAT) with 4-fold titer increases between samples at 10- to 15-day intervals. The serum samples corresponded to convalescent phases (MAT-positive; MAT+) of the disease. The cutoff value was determined by the mean absorbance plus 3 times the standard deviation of the values obtained with commercial NHS, as previously described [11].

#### 4.8. Flow Cytometry

*L. interrogans* serovar Copenhageni strain M20 cultures were centrifuged (2000 $\times$  g for 15 min) and resuspended in PBS. The leptospires were incubated with anti-rLIC\_13056

(1:100), anti-LipL46 (positive surface control) and anti-VapB (negative cytoplasmic control) for 1 h at 30 °C. After the incubation cells were washed, secondary FITC-conjugated goat anti-mouse IgG (1:50) was added for 2 h at 30 °C. Cells were then washed again and fixed with 2% paraformaldehyde. The measurements of fluorescence were performed in a BD FACSCanto II and data were expressed as median fluorescence intensity (MFI).

#### 4.9. Binding of the Recombinant Protein to Host Components

Purified components of the extracellular matrix, plasma, complement system, and integrin receptors were used to evaluate the interaction of rLIC\_13056 with host components. Primarily, ELISA plates (Costar High Binding; Corning Inc., Kennebunk, ME, USA) were coated overnight at 4 °C with 1 µg of each component, using BSA and fetuin as negative controls, in 100 µL of PBS. The experiments were done in triplicate plus a blank control. On the next day, the plates were blocked for 2 h at 37 °C with a solution of 10% non-fat dried milk in PBS-T, and washed 3 times before adding the recombinant protein, followed by incubation under the same conditions as before. After that, the polyclonal antibodies (1:5000) against rLIC\_13056 were incubated for 1 h at 37 °C, followed by the addition of HRP-conjugated anti-mouse IgG (1:5000). The binding was detected with OPD substrate (1 mg/mL) in 100 µL of citrate phosphate buffer (pH of 5.0) with the addition of 1 µL/mL H<sub>2</sub>O<sub>2</sub> per well. The plates were incubated for 10 min at RT, and the reaction was stopped with 50 µL of 4 N H<sub>2</sub>SO<sub>4</sub>. The absorbance was measured at 490 nm, and then compared with the negative-control groups.

#### 4.10. Dose–Response Curves

Dose–response assays were performed with the components with which the recombinant protein interacted. Therefore, ELISA plates were coated overnight at 4 °C with 1 µg of PLG, complement system components C4b, C4BP, C8, and C9, and the integrin subunit α8. Fetuin was used as the negative control. The plates were then blocked, and increasing concentrations of the recombinant protein were added, followed by a 2 h incubation at 37 °C. The remainder of this assay was carried out as previously described in the section above. GraphPad Prism software v. 7 was utilized to plot the dose–response curves in a “non-linear regression” model, considering the binding type as “one site-specific binding”. The statistical analysis was performed using a two-tailed, paired Student’s *t*-test, with a significance level of *p* < 0.05.

#### 4.11. C9 Polymerization Inhibition Assay

The effect of the recombinant protein on poly-C9 formation was assessed based on the methodology described by Zhang et al. (2011) [64]. In brief, different quantities of rLIC\_13056 (1.25 µg, 2.5 µg, and 5 µg) were incubated with 3 µg of C9 in 20 mM Tris-HCl buffer (pH of 7.4) for 40 min at room temperature. ZnCl<sub>2</sub> was then added to induce C9 polymerization during a 2 h incubation at 37 °C. The samples were subsequently separated by SDS-PAGE gradient gel (4–20%) (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and stained with Coomassie blue overnight. Positive controls for the polymerization consisted of a sample containing only C9 and ZnCl<sub>2</sub>, and another containing fetuin (5 µg), C9 and ZnCl<sub>2</sub>. Other controls included a sample containing only C9 without ZnCl<sub>2</sub> and another containing C9, ZnCl<sub>2</sub>, and a recombinant protein, rLIC13259, which is known to inhibit C9 polymerization [21].

#### 4.12. Evaluation of PLG Binding Inhibition of Aminocaproic Acid (ACA)

Following the previously described protocol [20], an ELISA plate was coated with 1 µg of PLG in triplicate and incubated overnight at 4 °C. The plate was then washed and blocked, as previously mentioned. Subsequently, an incubation was performed for

an hour and 30 min at 37 °C with 1 µg of rLIC\_13056 in the presence of three different concentrations of ACA: 0, 2, and 20 mM. The reaction was developed as previously detailed, and the bindings were analyzed based on the absorbance values obtained.

#### 4.13. Recruitment Assay of PLG from NHS

The ability of the recombinant protein to recruit PLG from the NHS was evaluated, as previously reported in the literature [30]. In brief, an ELISA plate was incubated overnight at 4 °C with 1 µg of rLIC\_13056, using fetuin as the negative control. The plate was then washed and increasing concentrations of NHS diluted in blocking solution were added, followed by incubation for 1 h at 37 °C. Afterward, anti-human IgG antibody was added. The reaction was then developed as previously described, and the results were analyzed.

#### 4.14. Enzymatic Assay of PLG Conversion to PLA

To assess whether the PLG bound to rLIC\_13056 could be converted into PLA, the procedure proposed by Vieira et al. (2009) [20] was performed. An ELISA plate was coated with 1 µg of the recombinant protein in quadruplicate and incubated overnight at 4 °C. Fetuin was used as a negative control. The plate was then washed and incubated with NHS or purified PLG for 2 h at 37 °C. Subsequently, a blocking solution containing 4 ng of urokinase PLG activator (uPA) and 0.4 mM of the chromogenic substrate D-Val-Leu-Lys 4-nitroanilide dichloride was added, and incubation continued for 16 h at 37 °C. Negative controls were prepared by omitting one component at a time from the complete reaction (NHS, PLG, uPA, or the substrate). The generation of PLA was observed indirectly through its substrate degradation, measured at 415 nm. Statistical analysis was done as previously described.

## 5. Conclusions

In conclusion, the OmpA-like domain-containing protein LIC\_13056 appears to be involved in leptospiral pathogenesis via different stages of the infection process. Interaction with the  $\alpha 8$  integrin subunit, which is expressed in glomerular mesangial cells, suggests that the protein may play a role during kidney colonization, and thus deserves further study. Additionally, our results indicate that LIC\_13056 could improve the ability of *L. interrogans* to evade the host immune system, through different mechanisms, by generating PLA and decreasing bacteria phagocytosis or by inhibiting C9 polymerization, and consequently MAC formation.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms27052086/s1>.

**Author Contributions:** Conceived and designed the experiments: G.M.C., J.P.G., A.F.T. and A.L.T.O.N. Performed the experiments: G.M.C. and J.P.G. Analyzed the data: G.M.C., J.P.G., A.F.T. and A.L.T.O.N. Contributed with reagents/materials/analysis tools: A.F.T. and A.L.T.O.N. Wrote the paper: G.M.C., J.P.G. and A.L.T.O.N. Revised the paper: G.M.C., J.P.G., A.F.T. and A.L.T.O.N. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** This study was performed according to the guidelines outlined by the Brazilian National Council for Control of Animal Experimentation (CONCEA), which

follows international guidelines for animal welfare and the principles of the 3Rs. Experimental protocols complied with the ARRIVE guidelines and were approved by the Ethic Committee on Animal Use of the Butantan Institute, São Paulo, Brazil, on 12 December 2023, under the protocol no. 8793301023. Mice were housed in a BSL1 animal facility, in microisolators with individual ventilation and temperature and light cycle control. Animals received food and water *ad libitum* and manipulation was performed by trained personnel.

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