Leptospira interrogans Lsa23 protein recruits plasminogen, factor H and C4BP from normal human serum and mediates C3b and C4b degradation

Gabriela H. Siqueira,1,2 Marina V. Atzingen,3 Gisele O. de Souza,4 Silvio A. Vasconcellos4 and Ana L. T. O. Nascimento1,2

Correspondence
Ana L. T. O. Nascimento
ana.nascimento@butantan.gov.br

1Centro de Biotecnologia, Instituto Butantan, Avenida Vital Brazil, 1500, 05503-900, São Paulo, SP, Brazil
2Instituto de Ciências Biomédicas, Universidade de São Paulo, Avenida Professor Lineu Prestes, 1730, 05508-900, São Paulo, SP, Brazil
3Instituto Adolfo Lutz, Avenida Doutor Arnaldo, 355, 01246-000, São Paulo, SP, Brazil
4Laboratório de Zoonoses Bacterianas do VPS, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Avenida Professor Dr Orlando Marques de Paiva, 87, 05508-270, São Paulo, SP, Brazil

It has been reported that pathogenic Leptospira are resistant to normal human serum (NHS) due to their ability to evade the complement immune system by interacting with factor H (FH) and C4b-binding protein (C4BP) regulators. Moreover, plasmin generation on the leptospiral surface diminishes C3b and IgG deposition, decreasing opsonophagocytosis by immune competent cells. We have previously reported that Lsa23 (LIC11360) is a multipurpose protein capable of binding purified extracellular matrix molecules, FH, C4BP and plasminogen (PLG)/plasmin in the presence of PLG activators. In this work, we provide further evidence that Lsa23 is located at the bacterial surface by using immunofluorescence microscopy. We show that Lsa23 has the ability to acquire FH, C4BP and PLG from NHS, and use these interactions to evade innate immunity. The binding with the complement regulators FH and C4BP preserves factor I (FI) activity, leading to C3b and C4b degradation products, respectively. C3b and C4b alpha-chain cleavage was also observed when Lsa23 bound to PLG generating plasmin, an effect blocked by the protease inhibitor aprotinin. Lsa23 also inhibited lytic activity by NHS mediated by both classical and alternative complement pathways. Thus, Lsa23 has the ability to block both pathways of the complement system, and may help pathogenic Leptospira to escape complement-mediated clearance in human hosts. Indeed, NHS treated with Lsa23 confers a partial serum resistance phenotype to Leptospira biflexa, whereas blocking this protein with anti-Lsa23 renders pathogenic L. interrogans more susceptible to complement-mediated killing. Thus, Lsa23 is a multifunctional protein involved in many pathways, featuring C4b cleavage by plasmin, knowledge that may help in the development of preventive approaches to intervene with human complement escape by this versatile pathogen.

INTRODUCTION

Leptospira are bacteria of the order Spirochaetales, and pathogenic strains are the aetiological agents of leptospirosis, a zoonosis of worldwide distribution and one of the most important public-health issues in Latin America (Picardeau, 2013). Reservoir animals harbour leptospires in proximal renal tubules and shed live bacteria in their urine. Humans are considered accidental and terminal hosts, and can present a broad spectrum of clinical symptoms ranging from a flu-like illness to severe haemorrhagic syndromes, such as pulmonary haemorrhagic leptospirosis and Weil’s syndrome.

Pathogens have developed many strategies to survive the host immune system attack, particularly innate immunity. It is known that pathogenic leptospires are resistant to the
bactericidal activity of normal human sera (NHS) (Johnson & Muschel, 1965) and can spread through the circulation of susceptible hosts until reaching target tissues. The resistance of leptospires to the innate immunity response is attributed to their ability of binding the soluble complement regulators factor H (FH), C4b binding protein (C4BP) and, more recently, vitronectin (Barbosa et al., 2009; da Silva et al., 2015; Meri et al., 2005; Verma et al., 2006). Another suggested strategy for leptospiral immune evasion is the recruitment of plasminogen (PLG) to the bacterial surface and plasmin generation. This broad-spectrum protease is able to reduce IgG and C3b deposition, possibly by degradation, and thereby decrease opsonophagocytosis by immune-competent host cells (Vieira et al., 2011).

Factor H is a globular glycoprotein that negatively regulates the alternative pathway of the complement system by acting as cofactor for factor I (FI) to mediate the cleavage of C3b, and binding C3b competing with factor B or displacing the Bb subunit from C3 convertase. Similarly to FH, C4BP acts as cofactor for FI-mediated cleavage of C4b by binding C4b, negatively regulating the classical pathway complement activation. PLG is the zymogen of plasmin, a serine protease responsible for the degradation of many plasma proteins. The main role of plasmin is acting on the fibrinolytic system in dissolution of fibrin clots maintaining the balance between coagulation and bleeding.

To date, a number of leptospiral proteins have been reported to interact with the complement regulators of the classical and alternative pathways, including LcpA, LenA, Lsa25, Lsa30, Lsa33, LigA and LigB, (Barbosa et al., 2010; Castiblanco-Valencia et al., 2012; Choy, 2012; Domingos et al., 2012; Souza et al., 2012; Verma et al., 2006). However, the mechanisms of complement inactivation have not yet been clearly elucidated. Recently, we reported a novel leptospiral surface adhesion protein encoded by the gene LIC11360, Lsa23, as a multifunctional protein with a possible role in the pathogenesis of leptospirosis. The coding sequence is predicted to be an outer/inner-membrane protein, peripheral, according to the Psort program (Nakai & Kanehisa, 1991). Lsa23 has been shown to interact with several host components. This includes purified extracellular matrix laminin and fibronectin molecules and serum components, such as the complement regulators FH and C4BP, and the zymogen PLG. Moreover, Lsa23 is recognized by antibodies present in positive leptospirosis patients and is probably expressed during infection (Siqueira et al., 2013). We thus decided to further characterize these interactions with the aim of gathering information on the role of Lsa23 in immune evasion strategies of Leptospira.

The work herein reports that Lsa23 can block activation of both the alternative and classical pathways of the complement system. We show that the recombinant protein is capable of recruiting FH, C4BP and PLG directly from NHS, and demonstrate that FH and C4BP individually preserve their cofactor activity for FI when bound to Lsa23. PLG bound to Lsa23 could be converted into plasmin, which in turn degrades C3b and C4b. These results suggest that Lsa23 might be involved in complement evasion processes by acting on three different mechanisms and could assist Leptospira to overcome lysis promoted by the membrane attack complex (MAC). To our knowledge, this is the first report of C4b cleavage by plasmin bound to a leptospiral surface protein, Lsa23, a mechanism that might inhibit the complement cascade through the classical pathway.

**METHODS**

**Biological components.** Native PLG, purified from human plasma, was purchased from EMD Chemicals. Human urokinase plasminogen activator (uPA) and aprotinin were from Sigma Aldrich. C4BP, FH, C4b, FI, C3b, isolated from NHS, anti-C3b9 antibody, gelatin veronal buffer (GVB+), GVBE (containing 5 mM EDTA), MgeGTA, NHS and antibody-sensitized sheep erythrocytes were purchased from Complement Technology.

**Leptospira strains.** The non-pathogenic Leptospira biflexa serovar Patoc strain Patoc 1, the pathogenic culture-attenuated Leptospira interrogans serovar Copenhageni strain M-20 and the low-passage virulent L. interrogans serovar Copenhageni strain FIOCRUZ L1-130 were cultured at 28 °C under aerobic conditions in liquid EMJH medium (Difco, BD) with 10% rabbit serum, enriched with L-asparagine (w/v, 0.001%), calcium chloride (w/v, 0.001%), magnesium chloride (w/v, 0.001%), peptone (w/v, 0.03%) and meat extract (w/v, 0.02%) (Turner, 1970). Leptospire cultures are maintained in the Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Brazil.

**RNA extraction.** Total RNA was isolated from leptospires cultures after 5 days of growth by the acid guanidine thiocyanate phenol/chloroform method using TRizol reagent (Invitrogen, Life Technologies), according to the manufacturer’s recommendations. The RNA purity was measured using the absorbance relation at 260 and 280 nm (A260/280). Two micrograms of RNA from each sample was treated in three steps with DNase I enzyme using the DNase I amplification grade kit (Invitrogen). DNase I was inactivated by addition of 25 mM EDTA solution, followed by incubation at 65 °C for 10 min. DNase-treated RNAs were reversely transcribed using the SuperScript III First-Strand Synthesis system (Invitrogen). Reactions without the SuperScript III enzyme were carried out in parallel as a control for DNA contamination (RT–).

**Real-time reverse transcriptase quantitative PCR (RT-qPCR).** RT-qPCRs were performed using the Applied Biosystems 7300 Real-Time PCR system equipment and SYBR Green I dye to detect the synthesized ds DNAs. Primers were designed according to the L. interrogans serovar Copenhageni genome sequence using Primer Express software (Applied Biosystems, Life Technologies), as follows: 16S ‘F’, 5'-CACGAAGCGTGGTGGATGTA-3'; 16S ‘R’, 5'-CAACG TTAGGGCGTGGATTA-3'; LIC11360 ‘F’, 5'-CGGTACATCATTT TGATTTCCATTYYT3'; LIC11360 ‘R’, 5'-CTGGGTCTATGGTCGTT TCTGA-3'. Standard curves were performed in order to obtain the primers’ optimum concentrations, as well as their amplification efficiency. RT-qPCR was performed in a reaction volume of 25 μl containing 25 ng cDNA, 300 nM each oligonucleotide (‘F’ and ‘R’) and 12.5 μl SYBR Green PCR Master Mix (Applied Biosystems), as recommended by the manufacturer. All reactions were performed in triplicate in 96-well optical plates. Negative controls using all the reagents except the cDNA were run in parallel (NTC, no template control). Cycling conditions were: 50 °C for 2 min, 95 °C for 10 min; followed by 40 cycles of (a) 95 °C for 15 s, (b) 60 °C for 30 s. Dissociation curves were obtained after each reaction by heating the
PCR products from 60 to 95 °C, in order to verify the amplification specificity. The relative gene expression among leptospira strains was analysed using the competitive 2-ΔΔCt method (Livak & Schmittgen, 2001). The housekeeping gene 16S was used as the internal control gene to normalize the gene expression.

**Gene cloning, recombinant protein expression and purification.** Detailed cloning, expression and purification of the recombinant proteins Lsa23 and Lsa36 have been previously described (Siqueira et al., 2013). Briefly, the gene sequences were amplified, without the signal peptide tag, by PCR from *L. interrogans* serovar Copenhageni strain M-20 genomic DNA using complementary primer pairs. DNA sequencing of constructs was verified on an ABI Prism 3730_L sequencer (Seq- Wright) with appropriate vector-specific T7 primers (F, 5′-TAATACGACTCACTATAAGGG-3′) and pAE (R, 5′-CAGCAGGCGCACTACGGTTCT-3′). Recombinant proteins were expressed in *E. coli* BL21 (SI) strain with a 6 × His-tag at the N-terminus and purified by metal-chelating chromatography after urea denaturation. The coding sequence LIC108289 was cloned and the protein expressed essentially as described for Lsa23 and Lsa36.

**Antiserum production against Lsa23, DnaK, LipL32 and Lsa36.** BALB/c mice (4–6 weeks old) were immunized subcutaneously with 10 µg recombinant protein mixed with 10 % (v/v) Alhydrogel [2 % Al(OH)₃; Brenntag Biosector] as an adjuvant. Negative control mice were injected with PBS mixed with adjuvant. Two weeks after each immunization, the mice were bled from the retro-orbital plexus, and the resulting pooled sera analysed by ELISA for the determination of antibody titres and concentration.

**Immunofluorescence assay (IFA).** The localization of the corresponding Lsa23 protein in *Leptospira* was performed as described by Teixeira et al. (2015). A suspension of *L. interrogans* serovar Copenhageni M20 strain, containing approximately 10⁸ live leptospires per ml, was harvested at 3800 g for 15 min, washed twice with low salt PBS (lPBS) (with 50 mM NaCl), resuspended in 200 µl PBS with 2 % paraformaldehyde for 40 min at 30 °C. After incubation, the leptospires were washed gently with lPBS, blocked with 5 % BSA and incubated for 1 h at 30 °C with 1:50 dilution of mouse antiserum against Lsa23, PBS, LipL32 and DnaK at a 1:50 dilution. The leptospires were washed and incubated with goat anti-mouse IgG antibodies conjugated to FITC (Sigma Aldrich) at a dilution 1:50 for 45 min at 30 °C. After incubation with secondary antibody, the leptospires were washed and resuspended in lPBS-antifading solution (ProLong Gold; Molecular Probes) containing 0.03 µg propidium iodide (PI) µl⁻¹ (Sigma Aldrich). The immunofluorescence-labelled leptospires were examined using a confocal LSM 510 META immunofluorescence microscope (Carl Zeiss).

**Protein interaction assays.** Protein attachment to soluble complement regulators FH and C4BP, and PLG was analysed by ELISA. Microtitre plates were coated overnight at 4 °C with 1 µg Lsa23, rLIC10829 or BSA (negative control). The wells were washed three times with PBS containing 0.05 % Tween 20 (PBS-T), blocked with 200 µl PBS/5 % BSA for 2 h at 37 °C, and incubated with NHS diluted in 100 µl PBS (0–10 %) for 90 min at 25 °C. After washing, binding between Lsa23 and each component was detected using rabbit anti-FH and anti-C4BP (Sigma Aldrich), or goat anti-factor H, with 1:1000 dilution. As a control, 500 ng Fc was added together with 1 µg C4b or C3b. Incubation proceeded for 16 h at 37 °C. Reactions omitting FH and C4BP were run as negative controls, while reactions lacking the recombinant proteins were used as positive controls. The supernatants were loaded onto SDS-PAGE gels and transferred onto nitrocellulose membranes (Hybond ECL; GE Healthcare Bio-Sciences), or stained with silver using silver staining kit–protein (GE Healthcare). For the immunoblotting, membranes were blocked overnight at 4 °C with 10 % non-fat dried milk diluted in PBS-T and then incubated with mouse anti-human C4d mAb (Quidel) diluted 1:1000, or goat anti-human C3 polyclonal antibody diluted 1:5000, for 2 h at room temperature. After washing, the membranes were incubated with secondary peroxidase-conjugated antibodies. The reactivity was revealed by SuperSignal West Dura substrate (Thermo Scientific Pierce) using Carestream Molecular Imaging software (Equilab) with a Gel Logic 2200 PRO imaging system.

**Inhibition assays.** Microtitre plates were coated with 10 µg Lsa23 ml⁻¹ for 16 h at 4 °C. Then, plates were washed three times with PBS-T following a blockade with PBS/3 % BSA for 2 h at 37 °C. To evaluate the effect of ionic strength on the interaction of Lsa23 with host plasma components, increasing amounts of NaCl (50–550 mM) were incubated together with 1 µg purified FH, C4BP or PLG diluted in lPBS. In the case of heparin and C3b/C4b inhibition assays, the components were diluted in PBS. The incubation proceeded for 90 min at 37 °C. Unbounded FH, PLG and C4BP were washed away and the remaining bound proteins were detected using goat anti-factor H, mouse anti-PLG or rabbit anti-C4BPA, respectively, as described above. For statistical analysis, the interaction of each purified plasma component with Lsa23 was compared with lPBS (50 mM) and the absence of heparin and C3b or C4b by Student’s paired t-test.

**Competition assay.** Ninety-six-well plates were coated with 1 µg Lsa23 overnight at 4 °C, washed three times with PBS-T and blocked with PBS/3 % BSA for 2 h at 37 °C. Then, increasing concentrations of one component were incubated together with a fixed amount (1 µg) of the other one for 2 h at 37 °C. The interaction between Lsa23 and plasma components was detected with rabbit anti-C4BPA or goat anti-factor H or mouse anti-PLG, as described above. For statistical analysis, the interaction of one component with Lsa23 was compared in the presence and absence (0 µg) of the other component by Student’s paired t-test.

**Cofactor activity assay.** Two micrograms Lsa23 or Lsa36 were coated on microplates overnight at 4 °C. The wells were washed and blocked with PBS/3 % BSA for 2 h at 37 °C, following by an incubation of 90 min at 37 °C with 2 µg C4BP or FH (Complement Technology). The unbounded cofactor regulators were washed and 500 ng FI was added together with 1 µg C4b or C3b. Incubation proceeded for 16 h at 37 °C. Reactions omitting FH and C4BP were run as negative controls, while reactions lacking the recombinant proteins were used as positive controls. The supernatants were loaded onto SDS-PAGE gels and transferred onto nitrocellulose membranes (Hybond ECL; GE Healthcare Bio-Sciences), or stained with silver using silver staining kit–protein (GE Healthcare). For the immuno blotting, membranes were blocked overnight at 4 °C with 10 % non-fat dried milk diluted in PBS-T, and then incubated with mouse anti-human C4d mAb (Quidel) diluted 1:1000, or goat anti-human C3 polyclonal antibody diluted 1:5000, for 2 h at room temperature. After washing, the membranes were incubated with secondary peroxidase-conjugated antibodies. The reactivity was revealed by SuperSignal West Dura substrate (Thermo Scientific Pierce) using Carestream Molecular Imaging software (Equilab) with a Gel Logic 2200 PRO imaging system.

**Plasmin activity assay.** Microtitre plate wells were coated overnight with 2 µg Lsa23, or BSA, at 4 °C. The wells were washed with PBS-T and blocked for 2 h at 37 °C with PBS/3 % BSA. The blocking solution was discarded, and 2 µg human PLG was added, followed by an incubation for 90 min at 37 °C. Wells were washed three times with PBS-T, and then 3 U per well uPA was added together with human C3b or human C4b (1 µg). Plates were incubated for 20 h at 37 °C. As a control, 5 µg aprotinin was added together with C3b or C4b. Reaction mixtures were subjected to Western blotting, and the
degradation products were detected using a mouse anti-C4d mAb or a goat polyclonal anti-human C3 antibody, as described above.

Haemolytic assay. One per cent NHS was pre-incubated with Lsa23, BSA or Lsa36 (50 μg ml\(^{-1}\)) for 15 min at 37°C. Antibody-sensitized sheep erythrocytes (2 × 10\(^8\) cells ml\(^{-1}\)) were washed once with GVB\(^{2+}\) and incubated with 1 % NHS pre-incubated or not with recombinant proteins for 30 min at 37°C. The reactions were stopped with 1 ml GVB (containing 5 mM EDTA) and cells were centrifuged. Erythrocyte lysis was determined by measuring the release of haemoglobin at 412 nm. Erythrocyte lysis induced by NHS was compared in the absence (100 %) and in the presence of proteins.

Alternative pathway deposition. Zymosan (20 μg ml\(^{-1}\)) was immobilized in microplate wells for 16 h at room temperature. After three washes with PBS-T, residual binding sites were blocked with 1 % BSA diluted in PBS for 2 h at 37°C. NHS and heat-inactivated NHS (iNHS) were diluted (20 %) in GVB/MgEGTA (GVB\(^{2+}\) containing 5 mM EGTA and 5 mM MgCl\(_2\), pH 7.3). Serum samples were pre-incubated for 15 min at 37°C with Lsa23, Lsa36 or BSA (50 μg ml\(^{-1}\)), before addition to plates. Incubation proceeded for 1 h at 37°C, followed by washing. Activation of the alternative pathway was detected using rabbit anti-human C5b-9 antibody (1 : 2000) and a secondary HRP-conjugated anti-rabbit antibody (1 : 5000). Enzyme activity of HRP was detected following incubation with OPD, as previously described.

Evaluation of L. biflexa viability after exposure to NHS treated with Lsa23. NHS was pre-incubated with Lsa23 (50 μg ml\(^{-1}\)), or Lsa36 as a recombinant protein control, for 15 min at 37°C. Then, ~2 × 10\(^8\) L. biflexa serovar Patoc 1 cells were washed once with lPBS, following incubation with 20 % NHS pre-incubated or not with recombinant proteins in a final reaction volume of 100 μl. iNHS was also employed as a control. Viable leptospires were taken 1 and 2 h after challenge and counted under dark field microscopy using a Petroff–Hauser chamber. The results are expressed as percentages of the live motile cells observed in the cultures after treatment and compared to cell numbers at 0 h (100 %). Statistical analysis were performed comparing treated leptospires with untreated and iNHS with serum-sensitive cells by Student’s two-tailed t-test.

Evaluation of complement-mediated killing by NHS on L. interrogans (M-20) pre-treated with anti-Lsa23. L. interrogans serovar Copenhageni strain M-20 (~4 × 10\(^8\) cells ml\(^{-1}\)) was treated with heat-inactivated mouse anti-Lsa23 serum, or mouse anti-DnaK serum or mouse anti-Lsa36 serum (1 : 50), as an antibody control, for 1 h at 37°C. Then, treated and untreated bacteria were incubated, for 90 min at 37°C, with 20 % NHS as a complement source. Samples were incubated on ice for 1 min in order to stop the complement reaction. Leptospires were recovered by centrifugation, resuspended in lPBS and coated on microplate wells for 16 h at 4°C. The MAC deposition was evaluated by using rabbit anti-human C5b-9 antibody (1 : 2000), as described above. Absorbance values of leptospires treated with anti-Lsa23 were compared with untreated, anti-DnaK- and anti-Lsa36-treated leptospires by Student’s two-tailed t-test. iNHS was used as a negative and blank control.

Ethics statements. All animal studies were approved by the Ethics Committee of the Instituto Butantan, Sao Paulo, SP, Brazil, under protocol number 1193/14. The Committee in Animal Research in Instituto Butantan adopts the guidelines of the Brazilian College of Animal Experimentation.

RESULTS

Expression of the LIC11360 gene in L. interrogans serovar Copenhageni

The expression of the LIC11360 gene was assessed by RT-qPCR in low passage, virulent L. interrogans serovar Copenhageni strain FIOCRUZ L1-130, culture-attenuated L. interrogans strain M-20 and the saprophytic L. biflexa serovar Patoc strain Patoc 1. The results revealed the presence of LIC11360 mRNA in both L. interrogans strains, showing a tendency of an increase in expression in the virulent L1-130 strain, with no statistically significant difference. No mRNA level was found in the saprophytic strain Patoc 1 (data not shown).

Presence of the corresponding Lsa23 protein in Leptospira by IFA

To evaluate whether the corresponding Lsa23 protein was located at the leptospira membrane, we set out to analyse the protein position by IFA. Leptospires were visualized by PI staining (Fig. 1a) followed by protein detection with polyclonal mouse antiserum raised against the protein in the presence of anti-mouse IgG antibodies conjugated to FITC. Green fluorescence could be observed, as shown in Fig. 1(b), for Lsa23, and LipL32, an outer-membrane protein used as a positive control (Haake et al., 2000), but not with DnaK, a cytoplasmic protein (Guerrero et al., 2001), nor with non-immune serum, used as a negative control. The localization of the protein/green light lying on the leptospires was achieved by merging both fields and the results obtained are shown in Fig. 1(c). Although Pinne & Haake (2013) have shown that the majority of the LipL32 protein is subsurface, our data suggest that at least a portion of this protein may be presented on the Leptospira cell surface.

Recombinant protein Lsa23 can acquire FH, C4BP and PLG from NHS

We have previously shown the ability of Lsa23 to interact with purified FH, C4BP and PLG (Siqueira et al., 2013). Because we have demonstrated that virulent, culture-attenuated and saprophyte leptospiral strains interact with purified PLG, but only the virulent strain can acquire PLG from human plasma (Vieira et al., 2009), we then checked whether this protein could recruit these components from NHS. The binding of the recombinant protein was evaluated by ELISA employing serum as a unique source of these molecules. Lsa23 was immobilized on microtitre plates and incubated with different NHS concentrations. Binding of the protein was detected using specific antibodies against each component. As depicted in Fig. 2, Lsa23 featured a dose-dependent interaction with C4BP (Fig. 2a), FH (Fig. 2b) and PLG (Fig. 2c). No significant binding was detected between BSA and PLG, while in the case of FH and C4BP half of
the absorbance value was detected with BSA. We have employed BSA as a control because in our previous work BSA, fetuin and gelatin produced similar results (Siqueira et al., 2013). In addition, we have included the recombinant protein LIC10829 (rLIC10829), which does not bind PLG, as a control. At 5% serum concentration, binding saturation was achieved for Lsa23 and PLG, while with C4BP and FH, no saturation was reached even at 10% NHS, in the case of FH.

**Effect of NaCl, heparin, C3b and C4b concentration on the binding of Lsa23 with purified FH, PLG and C4BP**

In order to evaluate the effect of ionic strength, increasing NaCl concentrations ranging from 50 (lsPBS) to 550 mM were employed and the binding of Lsa23 with FH, PLG and C4BP was assessed. The results show that there was a statistically significant reduction in the binding of Lsa23 with FH, PLG and C4BP as the NaCl concentration increased.

**Fig. 1.** Localization of Lsa23 protein in *L. interrogans* by IFA. Confocal microscopy was performed with *L. interrogans* using antisera specific for Lsa23 and LipL32 (exposed protein), with DnaK (cytoplasmic protein) and non-immune serum (as negative controls). (a) Leptospires were identified by PI staining of the DNA. (b) FITC-conjugated secondary antibodies were used to detect the surface-bound antibodies. (c) Co-localization is shown in the merged images.
and with C4BP at a concentration of 100 and 200 mM NaCl, respectively (Fig. 3a). Increasing NaCl concentration had no effect on the binding of Lsa23 to PLG (Fig. 3a), which might suggest that the interaction between Lsa23 and this component is non-polar. Since FH and C4BP have heparin and C3b or C4b binding sites, respectively, we decided to assess whether these sites are involved in the binding of FH and C4BP with Lsa23. Heparin, from 0 to 1.0 mg concentration, had no effect on the binding of Lsa23 with FH or with C4BP, suggesting that these interactions do not involve heparin binding sites (Fig. 3b). The presence of C3b had no effect on the binding of Lsa23 to FH at the concentration range tested (0 to 3.8 μg) (Fig. 3c), suggesting that Lsa23 binding sites do not co-overlap to the C3b binding sites of FH. Contrasting with these results, increasing amounts of C4b reduced the binding of Lsa23 to C4BP, suggesting that the binding is at the C4b site of this compound (Fig. 3d).

In order to evaluate whether C4BP, FH or PLG competes for the same binding site of Lsa23, the recombinant protein was immobilized on a microtitre plate and incubated with a fixed concentration of one component (1 μg), while increasing amounts of the other component were added to the reaction mixtures. The interaction was detected with antiseraum against C4BP (a), FH (b) or PLG (c). Each point represents the mean absorbance at 492 nm ± SD of three replicates and is representative of two independent experiments.

**Cofactor activity of FH and C4BP with Lsa23**

It has been reported that FH and C4BP bound to *Leptospira* retain their ability to act as cofactors for the protease FI in the cleavage of C3b and C4b (Meri et al., 2005). We thus decided to assess whether these regulators bound to Lsa23 preserve their functionality as cofactors. For this, Lsa23 and the negative control Lsa36 – a protein that does not bind complement regulators (Siqueira et al., 2013) – were immobilized and incubated with purified FH or C4BP. Unbound components were washed away, and C3b or C4b was added together with FI. Cleavage fragments of C3b and C4b were visualized by silver staining following SDS-PAGE (left panels of Fig. 4a, b, respectively). The positive cleavage products control, in which FH, FI and C3b were present in the reaction mixture, shows C3b α-chain fragments at around 68 kDa and between 45 and 66 kDa (C⁺) (Fig. 4a, left panel). Comparable cleavage products are shown when Lsa23, FH and FI were contained in the reaction mixture (Fig. 4a, left panel). The positive cleavage products control, in which FH, FI and C3b were present in the reaction mixture, shows C3b α-chain fragments at around 68 kDa and between 45 and 66 kDa (C⁺) (Fig. 4a, left panel). Comparable cleavage products are shown when Lsa23, FH and FI were contained in the reaction mixture (Fig. 4a, left panel).
Comparable cleavage products were detected when Lsa23 was added in the reaction mixture, but not when one of the reagents was absent or the control Lsa36 protein was used (Fig. 4a, right panel). Reaction mixtures containing C4b/ C4BP/FI, as a positive control (C\textsuperscript{+}), or Lsa23 or the negative control, Lsa36, were fractionated by electrophoresis (Fig. 4b, left panel). A comparable protein band profile could be observed in the positive control (C\textsuperscript{+}) and when Lsa23 was added to the mixture, including a low molecular protein band of \(~30\) kDa, which was absent when one of the reagents was omitted or Lsa36 was employed (Fig. 4b, left panel). Western blotting of reaction mixtures probed with anti-C4d identified a 45 kDa C4d cleavage fragment in the positive control (C\textsuperscript{+}) and in the presence of Lsa23 (Fig. 4b, right panel), but this was absent when one of the reagents was omitted or Lsa36 was employed (Fig. 4b, left panel). Our results clearly show that the cofactor activity of FH or C4BP is retained when bound to Lsa23.

**Cleavage of C3b and C4b by plasmin bound to Lsa23**

Plasmin is a serine protease that is able to cleave extracellular matrix components and complement proteins, such as C3b and C5 (Barthel et al., 2012a; Law et al., 2013). Our group has shown that PLG bound to leptospires can be activated into plasmin, cleaving C3b and IgG, thus decreasing their deposition on the bacterial surface (Vieira et al., 2011). Because Lsa23 can bind PLG and

---

Fig. 3. Effect of increasing concentrations of NaCl, heparin, C3b and C4b on the binding of Lsa23 with FH, PLG and C4BP. Recombinant Lsa23 (10 \(\mu\)g ml\(^{-1}\)) was coated onto microtitre plates for 16 h at 4 °C. (a) Increasing concentrations of NaCl were added together with FH, PLG or C4BP (10 \(\mu\)g ml\(^{-1}\)) in IsPBS. (b) Lsa23 was incubated with 1 \(\mu\)g FH or C4BP and increasing amounts of heparin. (c, d) 1 \(\mu\)g FH or C4BP were incubated with increasing amounts of C3b or C4b, respectively. Bound FH, PLG and C4BP were detected using specific antibodies. Each bar represents the mean absorbance at 492 nm \(\pm\) SD of three replicates and is representative of two independent experiments. Statistical analyses were performed by comparing the interaction of Lsa23 with the components in the presence and absence of NaCl, heparin or C3b/C4b, using Student’s paired \(t\)-test (**\(P<0.05\)).
generate plasmin in the presence of PLG-activator uPA (Siqueira et al., 2013), we decided to explore whether plasmin bound to Lsa23 is capable of degrading C3b and C4b, as previously described for other pathogens (Barthel et al., 2012b; Koch et al., 2012). Lsa23 immobilized on a microplate was incubated with PLG. After several washes, uPA was added together with C3b or C4b. The reaction mixtures and several controls in which one of the reagents was omitted, or aprotinin, a serine protease inhibitor, was included, were subjected to SDS-PAGE and the

---

**Fig. 4.** Cofactor activity of FH and C4BP bound to Lsa23. Two micrograms of recombinant protein Lsa23 or Lsa36 (control) were immobilized on microtitre plates and incubated with two micrograms of purified FH or C4BP. Free components were removed and 1 μg C3b or C4b and 500 ng FI were added and incubated for 16 h at 37 °C (a, b, left panels). The reaction mixtures were analysed by SDS-PAGE, and the cleavage fragments (*) of C3b or C4b were detected by immunoblotting (right panels) with a polyclonal anti-human C3 antibody (a, right panel) or C4d mAb (b, right panel). Reactions omitting FH or C4BP were performed as negative controls, while reactions lacking Lsa23 were performed as positive controls (C+), and non-degraded C3b (a) or C4b (b) controls were also included. Recombinant Lsa36 was used as a negative control for binding because this protein does not bind FH or C4BP. M, Protein standard molecular mass markers. WB, Western blot.
fractionated proteins were blotted onto membrane and probed with anti-C3 (Fig. 5a) or anti-C4d (Fig. 5b). Similar reactions were performed wherein Lsa23 was replaced by BSA. Proteolytic activity of plasmin generated when PLG was bound to Lsa23, or adhered to the well, was observed when anti-C3 was used, as demonstrated by degradation of the α-chain of this component and the generation of α’ 68 kDa (approximately) protein band (Fig. 5a, top), as previously reported (Barthel et al., 2012a). This band was not observed when aprotinin was added to the reaction or when BSA was employed (Fig. 5a, lane 7, top and bottom, respectively). In the case of C4b, the degradation of the α-chain, indicated by a decrease of its band, was seen in the reaction mixtures in which plasmin was generated, when the membrane was probed with anti-C4d (Fig. 5b, top). No C4b degradation took place in the presence of aprotinin, or when BSA was employed (Fig. 5b, lane 7, top and bottom, respectively). When plasmin was generated in the absence of protein, a partial degradation of the C4b α-chain could be seen (Fig. 5b, top). Cleavage fragments generated by plasmin acting on C3b were identified as α’ 68, α’ 40, α’ 30 and α’ 17 bands, and except for the α’ 68, fragments are distinct from those derived by FI (Barthel et al., 2012a). This might be the reason why anti-C3b recognizes the band generated by plasmin (Fig. 5a). Plasmin-cleaved C4b most probably produces fragments different from those generated by FI, and this might explain why anti-C4d does not

---

**Fig. 5.** Analysis of plasmin/Lsa23 degradation fragments of C3b and C4b by immunoblotting. Two micrograms of recombinant protein Lsa23 was immobilized on microtitre plates and incubated with 2 μg of purified PLG. After washing, 1 μg C3b (a) or C4b (b) and 3 U uPA were added and incubated for 20 h at 37 °C. Controls of the reactions were performed omitting one of the reagents or in the presence of the serine protease inhibitor aprotinin or by using BSA. The supernatants of the reaction mixtures containing C3b (a) or C4b (b) were separated by SDS-PAGE, transferred to a nitrocellulose membrane and probed with specific antibodies against C3 and C4d, respectively. WB, Western blot.
detect the C4b degradation products (compare Fig. 5b with Fig. 4b, right panel).

**In vitro inhibition of alternative and classical complement pathways by Lsa23**

Because it has been demonstrated that Lsa23 has the ability to interact with purified C4BP and FH (Siqueira et al., 2013) and, in this work, is capable of recruiting them directly from NHS, we set out to further evaluate whether this protein is able to inhibit the activation of the complement cascade. The effect of Lsa23 on the classical complement pathway was analysed indirectly by the haemolytic activity promoted by NHS using antibody-sensitized sheep erythrocytes. As shown in Fig. 6(a), Lsa23 was able to inhibit the classical pathway-mediated haemolysis. Lsa23 (50 µg ml⁻¹) blocked erythrocyte lysis by 83 %, while BSA and Lsa36, control proteins that do not bind complement regulators, did not significantly affect the haemolytic activity induced by NHS. In fact, there was a slight increase of erythrocyte lysis, suggesting that Lsa36 might possess haemolytic activity. In order to assess the interference of Lsa23 on the alternative pathway independently, we employed zymosan to activate the complement cascade, and MgEGTA to prevent its activation by the classical and lectin pathway. Using rabbit anti-human C5b-9-specific antibodies, we compared the deposition of C5b-9 between NHS pre-incubated with or without Lsa23, Lsa36 or BSA (Fig. 6b). Lsa23 also prevented complement activation by the alternative pathway, reducing C5b-9 deposition by 90 %. Thus, Lsa23 is a leptospiral protein that is able to inhibit in vitro complement activation by both the alternative and classical pathways.

**NHS complemented with Lsa23 contributes to serum-sensitive L. biflexa survival**

Since Lsa23 promotes an inhibition of complement-mediated haemolysis by NHS, we asked whether NHS treated with Lsa23, when added to the saprophyte *L. biflexa* strain, was able to decrease the bacterial clearance...
promoted by the complement system. Lsa23 was first added to NHS, followed by incubation with serum-sensitive *L. biflexa*. Viable leptospires were counted 1 and 2 h after the challenge with NHS, iNHS (employed as a positive control), NHS treated with Lsa23 and NHS treated with Lsa36 (employed as negative control). The data from three independent experiments (Fig. 7) showed that 1 h after the challenge, ~69 % viable leptospires were rescued from iNHS and NHS/Lsa23, against almost total clearance (~5 %) from NHS and NHS/Lsa36. This type of experiment is subject to experimental errors due to viable cell counting, which may explain the slight difference in viable cells observed at 1 and 2 h after the exposure with iNHS (61 %) and NHS/Lsa23 (47 %). An almost

![Fig. 8. Blocking pathogenic *L. interrogans* serovar Copenhageni (M-20) with anti-Lsa23 increased cell susceptibility to complement attack. Leptospires (~4 x 10⁸ cells ml⁻¹) were incubated with inactivated anti-Lsa23, anti-DnaK or anti-Lsa36 serum, all at 1:50 dilution for 1 h, followed by the addition of NHS. Bacteria were harvested by centrifugation and used to coat microtitre plates. MAC deposition was evaluated using anti-human C5b-9 antibody. Absorbance values obtained for leptospires pre-incubated with anti-Lsa23 were compared with those of cells pre-incubated with anti-DnaK, with anti-Lsa36 and with untreated cells by Student’s two-tailed *t*-test (*P*<0.01; **P**<0.005). The bars represent the mean absorbance at 492 nm ± SD from three replicates and are representative of three independent experiments.](image)

**Fig. 8.** Blocking pathogenic *L. interrogans* serovar Copenhageni (M-20) with anti-Lsa23 increased cell susceptibility to complement attack. Leptospires (~4 x 10⁸ cells ml⁻¹) were incubated with inactivated anti-Lsa23, anti-DnaK or anti-Lsa36 serum, all at 1:50 dilution for 1 h, followed by the addition of NHS. Bacteria were harvested by centrifugation and used to coat microtitre plates. MAC deposition was evaluated using anti-human C5b-9 antibody. Absorbance values obtained for leptospires pre-incubated with anti-Lsa23 were compared with those of cells pre-incubated with anti-DnaK, with anti-Lsa36 and with untreated cells by Student’s two-tailed *t*-test (*P*<0.01; **P**<0.005). The bars represent the mean absorbance at 492 nm ± SD from three replicates and are representative of three independent experiments.

![Fig. 9. Enhanced representation illustrating the interplay of Lsa23 with FH, C4BP and PLG/plasmin taking place on the surface of pathogenic *Leptospira*. Lsa23 can recruit the complement regulators FH and C4BP from NHS, promoting C3b and C4b degradation products via Fii; Lsa23 is also able to acquire PLG from NHS, generating C3b and C4b degradation products by the action of plasmin. Through different mechanisms, these interactions inhibit the MAC formation, helping the bacteria to overcome the host immune system. F, Endoflagella; IM, inner membrane; OM, outer membrane; PG, peptidoglycan; PS, periplasmic space.](image)

**Fig. 9.** Enhanced representation illustrating the interplay of Lsa23 with FH, C4BP and PLG/plasmin taking place on the surface of pathogenic *Leptospira*. Lsa23 can recruit the complement regulators FH and C4BP from NHS, promoting C3b and C4b degradation products via Fii; Lsa23 is also able to acquire PLG from NHS, generating C3b and C4b degradation products by the action of plasmin. Through different mechanisms, these interactions inhibit the MAC formation, helping the bacteria to overcome the host immune system. F, Endoflagella; IM, inner membrane; OM, outer membrane; PG, peptidoglycan; PS, periplasmic space.
total cell clearance was detected with NHS and NHS/Lsa36 (1 and 1.5%, respectively). These results clearly indicate a tendency of Lsa23 to promote an increase in L. biflexa survival by inhibiting the complement-mediated killing by NHS.

**Pathogenic L. interrogans** serovar Copenhageni (M-20) blocked with anti-Lsa23 showed increased bacterial cell susceptibility to complement attack

To further illustrate the involvement of Lsa23 in immune evasion, we decided to evaluate whether the presence of anti-Lsa23 was able to block the protein on the bacterial surface, enhancing the sensitivity of leptospires to complement-mediated killing. Pathogenic *L. interrogans* serovar Copenhageni strain M-20 (∼4 × 10⁶ cells ml⁻¹) treated for 1 h with inactivated anti-Lsa23, or the controls anti-DnaK and anti-Lsa36 serum (all at 1:50 dilution), and untreated cells were subjected to NHS. Cells harvested by centrifugation were used to coat microtitre plates, and MAC deposition was assessed with anti-human C5b-9 antibody (1:2000). Anti-Lsa23-treated leptospires showed statistically significant higher levels of MAC deposition as compared to untreated or anti-DnaK- and anti-Lsa36-treated cells (Fig. 8). Bacteria treated with anti-DnaK or with anti-Lsa36 presented a similar C5b-9 deposition level to that of untreated cells. Our data clearly show that leptospiral Lsa23 is involved in complement immune evasion by inhibiting the formation of MAC. In summary, we show that Lsa23 can acquire PLG/plasmin and the complement regulators FH and C4BP from NHS, leading to C3b and C4b degradation, inhibiting MAC formation, and thereby helping pathogenic *Leptospira* to evade the immune system (Fig. 9).

**DISCUSSION**

To survive in the human host, pathogens have developed many different strategies to evade the innate immune response so as to reach organs and target tissues in order to establish an infection. About 50 years ago, researchers observed that surviving the bactericidal activity of non-immune human serum was an exclusive ability of pathogenic strains of *Leptospira* (Johnson & Muschel, 1965). Years later, it was demonstrated that fully and intermediately resistant strains were isolated from human patients, while the most sensitive strain was the saprophyte *L. biflexa*. The killing was revealed to be dependent on the complement system, and resistant strains were more competent to bind FH than the sensitive strain (Meri et al., 2005). More recently, it has been demonstrated that pathogenic *Leptospira* can acquire the soluble human complement regulators FH, FH related protein 1 and C4BP (Barbosa et al., 2009; Verma et al., 2006). Several pathogenic micro-organisms have evolved the capacity to bind FH and employ its properties to circumvent complement-mediated killing. Complement regulator-acquiring proteins have been described for the spirochaetes *Borrelia* and *Treponema*: Fhbb protein of *Treponema denticola* (McDowell et al., 2007), and OspE (Hellwage et al., 2001) and CRASPs of *Borrelia burgdorferi* (Kraiczky et al., 2001). Protein receptors for factor H and C4BP have been identified for pathogenic *Leptospira*, as LfhA/Lsa24/LenA (Verma et al., 2006) and Lig proteins A and B (Castiblanco-Valencia et al., 2012). Our research group has characterized four leptospiral surface adhesins, which are able to bind C4BP. They are Lsa33 and Lsa25 (Domingos et al., 2012), Lsa30 (Souza et al., 2012) and Lsa23 (Siqueira et al., 2013). In addition, Lsa23 is also able to interact with FH and PLG.

The present work strengthens previous data showing that Lsa23 is a surface leptospiral protein (Siqueira et al., 2013) and that this protein has the capacity to recruit the complement regulators FH, C4BP and PLG directly from NHS. Most important, both regulators, FH and C4BP, retain their cofactor activity for FI when bound to Lsa23, leading to the degradation of C3b and C4b, respectively. However, when Lsa36 was employed, C3b and C4b degradation products were not observed (see Fig. 4a, b). The spirochaete *B. burgdorferi* possesses numerous FH-binding proteins to avoid complement killing (Brissette et al., 2009; Hallström et al., 2010; Hammerschmidt et al., 2014; Kraiczky et al., 2003; Siegel et al., 2010). Leptospiral immunoglobulin-like proteins LigA and LigB are also capable of interacting with FH and C4BP (Castiblanco-Valencia et al., 2012), but surface expression of LigB on saprophyte *L. biflexa* exhibits a partial protection from NHS bactericidal activity (Choy, 2012). These are multifunctional leptospiral proteins that may act in concert contributing to resistance to complement attack by alternative and classical/lectin pathways.

Another mechanism of immune evasion used by many pathogens is to express or acquire proteases to cleave components of the immune system (Zipfel et al., 2013). We have demonstrated that *Leptospira* spp. can bind to PLG, generate plasmin on the surface and degrade laminin and fibronectin (Vieira et al., 2009, 2012; Vieira & Nascimento, 2015). A number of PLG-binding proteins of *Leptospira* have been reported (Vieira & Nascimento, 2015), but, to date, only a few of those proteins were reported to interact with complement regulators. PLG, when bound to Lsa23, can be converted into plasmin, in the presence of PLG activators, as indirectly demonstrated by degradation of a specific substrate (Siqueira et al., 2013). Lsa23 is capable of recruiting PLG directly from NHS, generating plasmin, and able to degrade C3b and C4b. This activity was completely abolished by the presence of serine protease inhibitor aprotinin. The use of plasmin by leptospires to cleave C3b was reported for the first time by our group. We have shown that plasmin bound to the leptospiral surface degrades IgG and C3b, probably decreasing opsonophagocytosis by immune competent cells (Vieira et al., 2011). The leptospiral moonlight protein factor Tu, identified as PLG-binding protein (Vieira et al.,
Lsa23 exhibited an inhibitory effect on the haemolysis of antibody-sensitized sheep erythrocytes by NHS, while no inhibitory effect was observed with the control protein Lsa36. On the contrary, it appears that Lsa36 has haemolytic activity towards sheep erythrocytes. Considering that Lsa36 also binds to PLG (Siqueira et al., 2013), the effect observed may be due to the interaction of Lsa23 with C4BP. The absence of plasmin activity probably is due to the fact that PLG bound to Lsa23 needs the host uPA to become active (Vieira et al., 2009). The inhibitory effect produced by Lsa23 was similar to that exhibited by LigB of L. interrogans (Choy, 2012), and to that exerted by CspA, a surface protein of B. burgdorferi that interferes with the complement cascade at different levels, such as by binding FH and C7 (Hallström et al., 2013). We hypothesize that Lsa23 can use other mechanisms to inhibit the classical complement pathway. The same profile can be observed in the deposition assay using MgEGTA buffer, which blocks complement activation by the classical and lectin pathways. Lsa23 inhibited almost all C5b-9 deposition on zymosan complement activation; thus, binding of Lsa23 to factor H probably is not the only mechanism used by Lsa23 to inhibit complement activation by the alternative pathway.

B. burgdorferi CspA protein is a complement inhibitor. When this protein was employed to treat NHS before challenging serum-sensitive Borrelia garinii, these bacteria remained viable for 7 days (Hallström et al., 2013). Viable serum-sensitive L. biflexa were rescued after challenge with NHS supplemented with Lsa23, strengthening the involvement of this protein in immune evasion. Unsurprisingly, NHS/Lsa23 confers only partial serum resistance taking into account that other proteins and/or pathways are involved in this process. As expected, pathogenic L. interrogans became more susceptible to complement-mediated killing by MAC when the bacteria were previously blocked with anti-Lsa23 inactivated serum. Knock-in experiments using L. biflexa as surrogate are planned to be performed in our laboratory and should elucidate the functional role of Lsa23 in Leptospira.

Taken together, our data show that Lsa23 is a multifunctional surface protein of L. interrogans that might contribute to the serum resistance phenotype of pathogenic Leptospira. Lsa23 has the ability to bind FH, C4BP and PLG from NHS to mediate innate immunity evasion. Lsa23 promoted C3b and C4b degradation either by binding to FH and C4BP, via FI, or by binding PLG, via plasmin generation. The reduction of the amount of C3b and C4b might obstruct MAC formation by classical and alternative pathways, as observed by haemolysis inhibition and decreased C5b-9 deposition, respectively. The Lsa23 complement-inhibitory activity mediated by plasmin leading to C3b and C4b degradation offers an interesting strategy to understand how this resourceful pathogen uses PLG/plasmin.

ACKNOWLEDGEMENTS

The following Brazilian agencies, FAPESP (grant 12/23913-9), CNPq (grants 302758/2013-5 and 441449/2014-0) and Fundacao Butantan, financially supported this work; G. H. S. has a post-doctoral fellowship from FAPESP (2014/03792-8). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES


lipoprotein expressed during mammalian infection. *Infect Immun* 68, 2276–2285.


Edited by: D. Demuth