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Proteolytic activity of secreted proteases from pathogenic leptospires and effects on phagocytosis by murine macrophages

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ABSTRACT

Leptospirosis is a zoonosis caused by spirochete *Leptospira.* Pathogenic leptospires evade the Complement System, enabling their survival upon contact with normal human serum *in vitro*. In a previous study, we demonstrated that proteases secreted by pathogenic leptospires cleave several Complement proteins, including C3 and the opsonins C3b and iC3b. We hypothesize that these *Leptospira* proteases, such as thermolysin and leptolysin, may decrease the phagocytic activity of murine peritoneal macrophages. We observed decreased amounts of CR3 and CR4 using flow cytometry when these cells were treated with supernatant from the culture of pathogenic leptospires (SPL) for 24 h. Through confocal microscopy, we observed a reduction in TLR2, CD11b, and CD206 (mannose receptor) levels when these cells were treated with SPL or recombinant thermolysin for 24 h. Furthermore, opsonins such as C3b/iC3b deposited on the surface of pathogenic leptospires were clearly degraded in the presence of recombinant thermolysin or recombinant leptolysin. Consequently, when opsonized bacteria and macrophages were previously incubated with these proteases, phagocytic activity was diminished. These observations lead us to suggest that proteases secreted by pathogenic leptospires could degrade opsonins present in normal serum or deposited on the bacterial membrane, as well as cleave or inhibit macrophage surface molecules. Therefore, these proteases could interfere with the recognition and internalization by murine macrophages, favoring the spread of leptospires in the host.

1. Introduction

Leptospirosis is one of the primary zoonoses disseminated worldwide and carries a high mortality rate, particularly in patients who develop its most severe form. This disease is caused by bacteria of the genus *Leptospira*, which currently comprises 69 species divided into two main clades: pathogenic and saprophytic (non-pathogenic) [1–5]. The number of new cases of leptospirosis is estimated at approximately one million each year, resulting in 60,000 deaths. It is recognized as a serious public health problem [6].

This disease spreads through direct contact with the urine of infected animals or indirectly through water and soil contaminated with leptospires. Wild and domestic animals can serve as reservoirs, with rodents being the main transmitters. Leptospires colonize the proximal renal tubules of these animals and are subsequently shed in the urine [7,8]. Humans are considered accidental hosts, becoming infected through the penetration of leptospires through the mucous membranes or damaged skin. Once in the host, leptospires spread through the bloodstream, mainly lodging in organs such as the liver, kidneys, and lungs, thus establishing a systemic infection [8,9].

Macrophage membrane receptors play a significant role in phagocytosis. Pathogen recognition by Toll-like Receptors (TLR) triggers intracellular signaling through the activation of NFκB or AP-1 transcription factors, culminating in the production of pro-inflammatory

Abbreviations: (TLR), Toll-like Receptors; (DC), Dendritic Cells; (SPL), supernatants of pathogenic leptospires; (SNPL), supernatants of non-pathogenic leptospires; (DMEM), Dulbecco's Modified Eagle's Medium; (NMS), normal mice serum; (LPF), *L. interrogans* serovar Kennewicki strain Fromm; (LPS), lipopolysaccharide.

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cytokines [10–12]. In addition to TLRs, other receptors also participate in pathogen recognition, such as the mannose receptor (CD206), which is expressed mainly in tissue macrophages, dendritic cells (DC), and liver cells. This receptor recognizes certain terminal sugars present on the surfaces of various microorganisms, including bacteria, fungi, viruses, and parasites [13,14].

Another type of macrophage receptors (SR-AI/II and CD36) recognize a wide range of microbial products, including LPS, lipoteichoic acid, β-glucans, and proteins. Their role in innate immunity was demonstrated in studies by Thomas and colleagues (2000) [15], where mice deficient in these receptors showed increased susceptibility to *Staphylococcus aureus* infection [16]. In addition, SR-AI was identified as a significant non-opsonic phagocytic receptor involved in the phagocytosis of *Leptospira*. SR-AI also contributes to regulating pro-inflammatory cytokine responses to *Leptospira* infection by modulating the inflammatory response [17].

CR1 (CD35) is a glycoprotein present in several blood cells, such as erythrocytes, monocytes, neutrophils, and B lymphocytes. This receptor exhibits a higher affinity for binding to C3b than to C4b, iC3b, C1q, and MBL [18,19]. CR3 (CD11b/CD18, also known as Mac-1; αMβ2) and CR4 (CD11c/CD18, also known as p150,95; αXβ2) are transmembrane heterodimers belonging to the family of β2-integrins, sharing a common β chain. These receptors bind to fibrinogen and ICAM-1, facilitating cell adhesion, as well as the iC3b fragment. The well-characterized functions of these receptors include cell adhesion, pathogens clearance, and phagocytosis of apoptotic and tumor cells. The binding of CR3 and CR4 to fibrinogen enhances certain antimicrobial properties, such as the production of cytokines, including chemokines [20–22].

IgG Fc receptors (FcγRs) are present on the surfaces of lymphoid and myeloid cells. The functional and structural heterogeneity of FcγRs can generate opposing signals regarding the immune response of these cells. Activating receptors (FcγRI, III, IV in mice and FcγRIA, IIA, IIIA in humans) can trigger inflammatory, phagocytic, cytotoxic, and hypersensitivity responses. The FcγRIIb receptor (CD32b) can inhibit cellular activation dependent on the B lymphocyte antigen receptor (BCR), thereby limiting its expansion and regulating humoral immunity. FcγR-mediated phagocytosis by macrophages and dendritic cells can lead to antigen presentation, thereby amplifying the immune response [23–26].

Phagocytosis plays a crucial role in controlling microbial infections, particularly when particles are coated with opsonins. The most important opsonins include IgG and fragments generated through the activation of the Complement System (iC3b and C3b). However, little is known about the phagocytosis of leptospires by both macrophages and neutrophils. Some studies have suggested that pathogenic leptospires can survive within host cells such as peritoneal macrophages, microglia, Kupffer cells, and human peripheral blood monocytes. Nevertheless, the mechanism underlying this survival remains poorly understood [27–30].

Proteolytic inhibition assays have revealed that certain metalloproteases constitute one of classes of enzymes exclusively secreted by pathogenic leptospires, responsible for cleaving Complement proteins [31,32]. The pathogenic species *L. interrogans* serovar Copenhageni possesses four genes that code for metalloproteases of the thermolysin family (LIC10715, LIC13320, LIC13321 and LIC13332) [33].

Among the four *Leptospira* thermolysins, the protease encoded by the *LIC13322 gene* has a catalytic domain with the highest degree of identity and similarity with proteases produced by other pathogens such as aerolysin from *Staphylococcus aureus*, lambda toxin from *Clostridium perfringens*, elastase from *Pseudomonas aeruginosa* and gelatinase from *Enterococcus faecalis*, all of which exhibit proteolytic activity on Complement proteins [31,34–38]. Our group previously reported the expression and purification of two recombinant fragments of thermolysin encoded by the LIC13322 gene and demonstrated that one of them (PepSY-M4, 74 kDa) was able to cleave the C3 Complement molecule [31].

Human pappalysin is a multidomain metalloprotease initially described as a protein present in the plasma of pregnant women, with important roles such as follicular development, myogenesis, implantation of human embryos, and wound healing [39]. A homolog of human pappalysin in *Leptospira*, recently characterized and named leptolysin [40], was identified through exoproteomic analysis in the supernatant of pathogenic leptospires (SPL) [41]. While the saprophyte *L. biflexa* serovar Andamana strain CH11 *and L. biflexa* serovar Patoc strain Patoc I also secrete leptolysin, its concentration is proportionally lower compared to pathogenic species. This protein (~52 kDa) is highly conserved in pathogenic *Leptospira* species and belongs to the M23 family of zinc-dependent proteases [40,41].

Few studies have explored why pathogenic leptospires are more resistant to phagocytosis compared to non-pathogenic ones. Our objective is to assess whether proteases secreted by pathogenic leptospires could disrupt their internalization and neutralization by phagocytes. In this study, we aimed to determine whether these enzymes could cleave surface molecules of murine macrophages and remove opsonins present on the surface of pathogenic leptospires, thereby influencing the pathogen's internalization by host cells.

2. Material and methods

2.1. Leptospira strains and culture supernatants

Pathogenic leptospires, including *L. interrogans* serovar Kennewicki strain Fromm (LPF, pathogenic), *L. interrogans* L1 130 FIOCRUZ and non-pathogenic *L. biflexa* serovar Patoc strain Patoc I, was cultivated for seven days at 29 °C in modified Ellinghausen-McCullough-Johnson-Harris medium (EMJH) (BD DifcoTM, Franklin Lakes, New Jersey, United States). Following this incubation period, the bacteria were harvested by centrifugation (5.400×*g* at 15 min a 4 °C), washed twice with phosphate-buffered saline (PBS), pH 7.4, and then resuspended in PBS. The bacterial concentration was determined by counting using a Petroff-Hausser chamber under an Olympus BX51 optical microscope (Olympus, Shinjuku, Tokyo, Japan) with dark-field condenser Olympus U-DCD (Olympus). These strains were provided by the Laboratory of Bacterial Zoonoses, Faculty of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo. The supernatant of pathogenic leptospires used in this study was obtained exclusively from cultures of *L. interrogans* serovar Kennewicki strain Fromm (LPF). To investigate the degradation of opsonins deposited on the leptospiral membrane, we used whole pathogenic *L. interrogans* strain L1-130 FIOCRUZ.

2.2. Preparation of Leptospira culture supernatants and recombinant proteases

L. interrogans serovar Kennewicki strain Fromm and L. *biflexa* serovar Patoc strain Patoc I, was resuspended in PBS containing 1 x 10^9 bacteria and then incubated at 37 °C for 4 h. After centrifugation, the supernatants of pathogenic (SPL) and non-pathogenic (SNPL) leptospires were collected, filtered through a 0.22 μm membrane, aliquoted into Eppendorf tubes and stored at ˉ80°C for future functional tests. Recombinant thermolysin, encoded by the *LIC13322* gene of *L. interrogans* serovar Copenhageni was expressed in *E. coli* and purified as described by Fraga et al. (2014) [31]. Recombinant leptolysin, encoded by the *LIC13434* gene of *L. interrogans* serovar Copenhageni was also expressed in *E. coli* and purified as described by Courrol et al. (2022) [40].

2.3. Animal model

Male mice of the C57Black/6 strain 129S4–C3tm/Crr/J (C3 deficient; B6.C3$^{-/-}$) were obtained from The Jackson Laboratory. C57BL/6 mice (wild B6.C3$^{+/+}$) were provided by the Mouse Laboratory of the Immunology Department of the Institute of Biomedical Sciences of the University of São Paulo. All animals were aged 6–8 weeks. This Project was conducted following the Ethics Committee on the Use of Animals of

the Institute of Biomedical Sciences, registered under protocol number 48/2017.

2.4. Isolation of murine macrophages

C57BL/6 wild-type mice were injected with 1 ml of 4 % thioglycolate (Sigma-Aldrich) into the peritoneal cavity. After 3 days of stimulation, the animals were anesthetized and euthanized and injected intraperitoneally (i/p) with 5 ml of cold PBS to withdraw peritoneal cells. The suspended cells were collected and centrifuged at 600×*g* for 10 min at 4 °C. Subsequently, the cells were resuspended in 1 ml of complete Dulbecco's Modified Eagle's Medium (DMEM), containing 10 % heat-inactivated fetal bovine serum, 10 mM HEPES, 2 mM glutamine, 100 mg/ml penicillin, and 100 mg/ml streptomycin. Aliquots of 1ml/well of the suspension (2 x 10^5 cells) were added to cell culture plates (Costar) and kept under 5 % CO2 conditions for 2 h at 37 °C. After this period, the wells are washed three times with PBS to remove non-adherent cells, and DMEM was added to the plates containing mostly macrophages. The cell viability of murine macrophage cultures was determined using Trypan blue staining and was consistently equal to or greater than 90 % after all concentrations of SPL, SNPL, thermolysin, or leptolysin used in this study.

2.5. Obtaining sera from B6.C3$^{+/+}$ and B6.C3$^{-/-}$ mice

Sera from B6.C3 $^{+/+}$ and B6.C3 $^{-/-}$ mice were used to perform the phagocytosis assay. The mice were anesthetized, via i/p, with 200 μl of a solution containing 125 mg/kg of ketamine and 12.5 mg/kg of xylazine. Blood was collected from the orbital plexus of 10 animals/group and, after 15–30 min at room temperature, was centrifuged at 1700×*g* for 15 min at 4 °C to separate the sera. The sera were pooled, and aliquots were stored at 80 °C for future use.

2.6. Degradation of opsonins by leptospiral proteases

To analyze the degradation of opsonins deposited on the *Leptospira* membrane, the pathogenic strain *L. interrogans* serovar Copenhageni Fiocruz L1-130, used exclusively in this experiment, was pre-opsonized with murine serum C3b and iC3b for 1 h at 37 °C, then washed with 1 x PBS. These opsonized bacteria were then incubated with purified recombinant thermolysin (0.5 μg) and leptolysin (0.1 μg) for 1 h and 24 h at 37 °C. The samples were then washed with PBS, and the degradation of opsonins was analyzed by Western blot using goat anti-mouse C3 antibody, followed by peroxidase-conjugated anti-goat IgG.

2.7. Analysis of macrophage surface molecules by flow cytometry

To analyze the effect of proteases on phagocyte surface receptors, murine macrophages (2 x 10^6 cells) were incubated with SPL (3 μg of total proteins) or PBS (negative control) for 24 h. At the end of the incubations, a cold buffer composed of PBS + EDTA (5 mM) was added to detach the cells from the culture plates. The cells were then incubated for 30 min at 4 °C in labeling buffer (PBS + 2 % heat-inactivated fetal bovine serum) with specific monoclonal antibodies: anti-CD21/CD35 Alexa Fluor 647 (CR2/CR1), anti-CD11b FITC (CR3), anti-CD11c Pe/Cy7 (CR4) and anti-CD64 Brilliant Violet 421(FcγRI) and 7AAD for cell viability with the same functional characteristics similar to Trypan Blue (Biolegend, San Diego, CA) in the dark. Cells were also labeled with anti-F4/80 antibody. Negative controls were labeled with isotype-matched PE- or FITC-conjugated antibodies and compensation were adjusted using the single-stained cell samples (After the incubation period, the murine macrophages were washed with 1 ml of labeling buffer. Cells were suspended in 200 μl of labeling buffer and analyzed by a flow cytometer FACS Canto II or FACSAria II (Becton Dickinson, Mountain View, CA). Data analysis was performed using FlowJo software (Tree Star) based on the population of macrophages considering size (FSC) and cell granularity (SSC).

2.8. Analysis of the presence of cell receptors by confocal microscopy

To examine the effect of thermolysin or SPL on cell receptors, a pool of peritoneal macrophages (n = 4) was grown in 20 mm × 20 mm glass coverslips placed in 6-well plates (Costar) at a concentration of 2 x 10^5 cells per well. The cell cultures were then treated with SPL (3 μg of total proteins) or recombinant thermolysin (0.5 μg) for 1 h and 24 h at 37 °C with 5 % CO2. Macrophage cultures were labeled with anti-CD282-PE (TLR2), anti-CD206-APC (mannose receptor), and anti-CD11b-FITC (CR3) (Biolegend, San Diego, CA), diluted in labeling buffer (PBS + 2 % heat-inactivated fetal bovine serum) in the dark. After 1 h of incubation, the cells were washed again with PBS and analyzed using confocal microscopy (LSM-780-NLO, Carl Zeiss, Jena, Germany) at the Core Facility to Support Research (CEFAP-USP). The mean fluorescence intensity of each cell in five different images per coverslip was measured after manual annotation of the cell area using Image J software and plotted as the percentage relative to the group PBS 1 h (100 %).

2.9. Analysis of phagocytosis of zymosan particles by murine macrophages in the presence of Leptospira supernatant and recombinant thermolysin

Zymosan particles (Sigma Chemical Co., St. Louis, MO, USA) were boiled for 15 min and washed 3x with PBS. A total of 2 x 10^6 particles were resuspended in 1 ml of GVB-EGTA-Mg^{++} buffer. Then, for opsonization, 10 % of mouse sera B6.C3$^{+/+}$ and B6.C3$^{-/-}$ were added to zymosan for 1 h at 37 °C. As a negative control, zymosan particles were treated only with PBS. After incubation, the zymosan particles were again washed 3x with PBS and resuspended with DMEM medium for use in the experiments. Macrophages from mice previously injected with thioglycolate were cultured on 20 × 20 mm glass coverslip. Then, the cell cultures were treated with SPL or SNPL (3 μg of total proteins), and recombinant thermolysin (0.5 μg) individually for 1 h at 37 °C with 5 % CO2. The zymosan preparations were added to the macrophage cultures and incubated for 1 h under the same conditions. After this period, the coverslips were washed 3x with PBS to remove excess non-phagocyted particles. Next, the coverslips were fixed and stained with Rapid Panotic kit (Laborclin) according to the manufacturer's instructions. Phagocytosis analysis was performed under an optical microscope (Nikon Eclipse E200). Initially, 200 total cells were counted, distinguishing those that were not able to phagocytize zymosan particles from those that were not. Then, among the population of cells that internalized up to 3 or more zymosan particles, quantification was performed.

2.10. Phagocytosis of pathogenic leptospires by murine macrophages in the presence of leptospiral proteases

Peritoneal macrophages (2 x 10^5) were incubated with 3 μg of total proteins of SPL, 0.5 μg of recombinant thermolysin, or 0.1 μg of recombinant leptolysin for 24 h at 37 °C 5 % CO2. Concomitantly, LPF suspensions were labeled with 10 μM of CFSE (Sigma-Aldrich) and washed with PBS. Then, *L. interrogans*-CFSE was incubated with 10 % NMS for 1h at 37 °C. After three washes, opsonized *L. interrogans*-CFSE were treated with proteases for 24 h at 37 °C. The bacteria suspensions were then added to macrophage cultures for 1 h at 37 °C, under 5 % CO2. As a control, macrophages and *Leptospira* were incubated for 24 h in the absence of proteases. To eliminate non-phagocytosed bacteria, cell cultures were treated with 25 μg/ml gentamicin (Sigma). At the end of the incubation, the macrophage cultures were fixed with 4 % paraformaldehyde (PFA) for 10 min and the samples were analyzed by flow cytometry.

2.11. Statistical analysis

Data were analyzed using 2-way ANOVA and in all analyses, whenever necessary, multiple comparisons were carried out using the Tukey method. Data transformations were applied considering the homogeneity of variances and normality. The significance level adopted was $p < 0.05$. Statistical analysis of the data was performed using the Statgraphics Centurion XVI software. Data were expressed as the means or means±standard error of five or more independent measurements.

3. Results

3.1. Presence of molecules on macrophage surface after treatment with SPL

Phagocytosis can be enhanced by the binding of opsonins to specific receptors, such as FcγRI (for IgG) and CR1, CR3, and CR4 (for certain C3 fragments). Therefore, we decided to investigate whether leptospiral proteases could degrade or inhibit some macrophage surface molecules and impair phagocytosis. Peritoneal macrophage cultures were incubated for 1 h, 3 h, 6 h, and 24 h with 3 μg of total secreted proteins from SPL. These cells were then labeled with specific monoclonal antibodies against the following surface molecules: FcγRI, CR1, CR3, and CR4. Data analysis was performed using FlowJo software (Tree Star) based on the population of macrophages considering size (FSC) and cell granularity (SSC). As negative controls, macrophages were incubated with non-related antibody isotypes (Supplementary Fig. 1). We observed that the presence of FcγRI (Fig. 1A) and CR1 (Fig. 1B) were not affected by treatment with SPL. However, when macrophages were incubated with SPL for 24 h, a significant decrease in CR3 on the surface of these cells was observed (Fig. 1C). The amount of detectable CR4 was lower when cells were incubated with SPL compared to the control (Fig. 1D).

3.2. Analysis of macrophage receptors by confocal microscopy

The results obtained so far prompted us to investigate by confocal microscopy the impact of proteases present in SPL or recombinant thermolysin on the levels of TLR2, CD206, and CR3 in treated macrophages. Following the respective treatments, the cells were washed and labeled with specific antibodies against these receptors. As illustrated in Fig. 2, a significant decrease in the fluorescence of TLR2 (Fig. 2A and B) and CD206 (Fig. 2C and D) was observed after 1 h and 24 h of treatment, respectively, compared to the PBS control. Conversely, the presence of CR3 significantly decreased only when cells were treated with recombinant thermolysin (Fig. 2E and F). In this context, we hypothesize that proteases secreted by pathogenic leptospires may impact the presence of certain molecules on the surface of macrophages, thereby impairing their immune response.

3.3. Evaluation of the effect of proteases from pathogenic Leptospira on phagocytosis by macrophages

To evaluate whether the proteases present in the leptospiral supernatants could inhibit phagocytic activity, the macrophages were individually incubated with PBS, SPL, SNPL, or recombinant thermolysin for 1 h at 37 °C, followed by several washes of cell cultures with fresh PBS. Zymosan suspensions were treated with 10 % serum from B6.C3$^{+/+}$ or B6.C3$^{-/-}$ mice for opsonization, or without serum (negative control) for 1 h at 37 °C and added to the macrophage cultures. The cultures were further incubated for an additional 1 h at 37 °C. As anticipated, zymosan particles opsonized with serum from B6.C3$^{+/+}$ mice were more effectively internalized by macrophages when compared to serum from B6.C3$^{-/-}$ mice or without serum. Phagocytosis of zymosan was not significantly affected when macrophages were previously incubated with SPL (Fig. 3A) or SNPL (Fig. 3B). However, incubation of macrophages with recombinant thermolysin resulted in a significant decrease in zymosan phagocytosis irrespective of the treatment (serum from B6.C3$^{+/+}$ or B6.C3$^{-/-}$; PBS) (Fig. 3C). This suggests that this protease interferes with zymosan uptake by macrophages even in the absence of Complement activation.

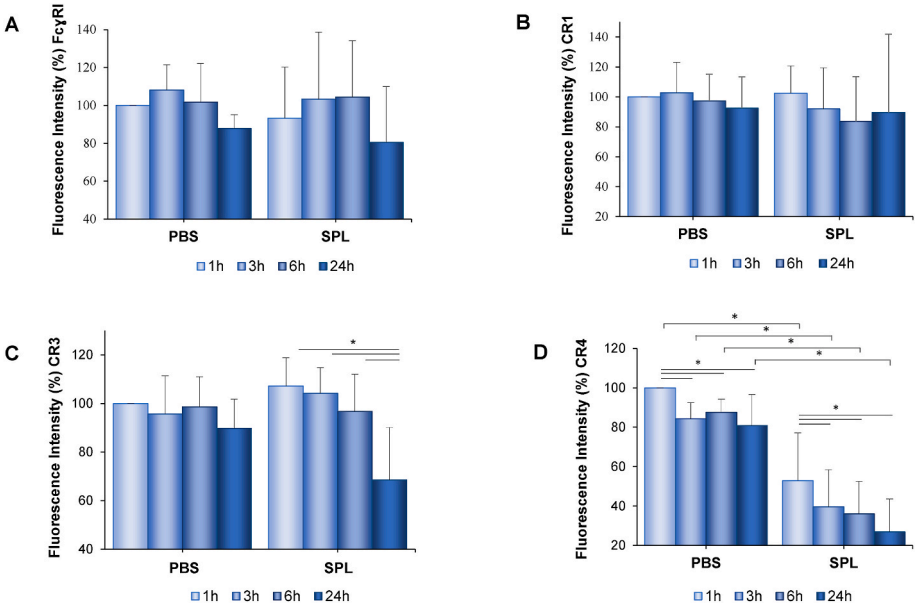

Fig. 1. Presence of receptors on the surface of murine macrophages treated with proteases from pathogenic leptospires. Murine peritoneal macrophages were incubated with culture supernatant from pathogenic leptospires (SPL, 3 μg) for 1 h, 3 h, 6 h and 24 h. SPL was obtained from the culture of *L. interrogans* serovar Kennewicki strain Fromm. The presence of **(A)** anti-CD64 (FcγRI) **(B)** anti-CD35 (CR1) **(C)** anti-CD11b (CR3) **(D)** anti-CD11c (CR4) were analyzed by flow cytometry using FACS Canto II. Macrophages were resuspended in a volume of 200 μl in the presence of specific monoclonal antibodies and 10,000 events were acquired per sample. The abundance of each receptor was expressed about the 1h control (100 %). Data analysis from three experiments were carried out by FlowJo software (Tree Star), and statistical analyses were performed using ANOVA *$p < 0.05$.

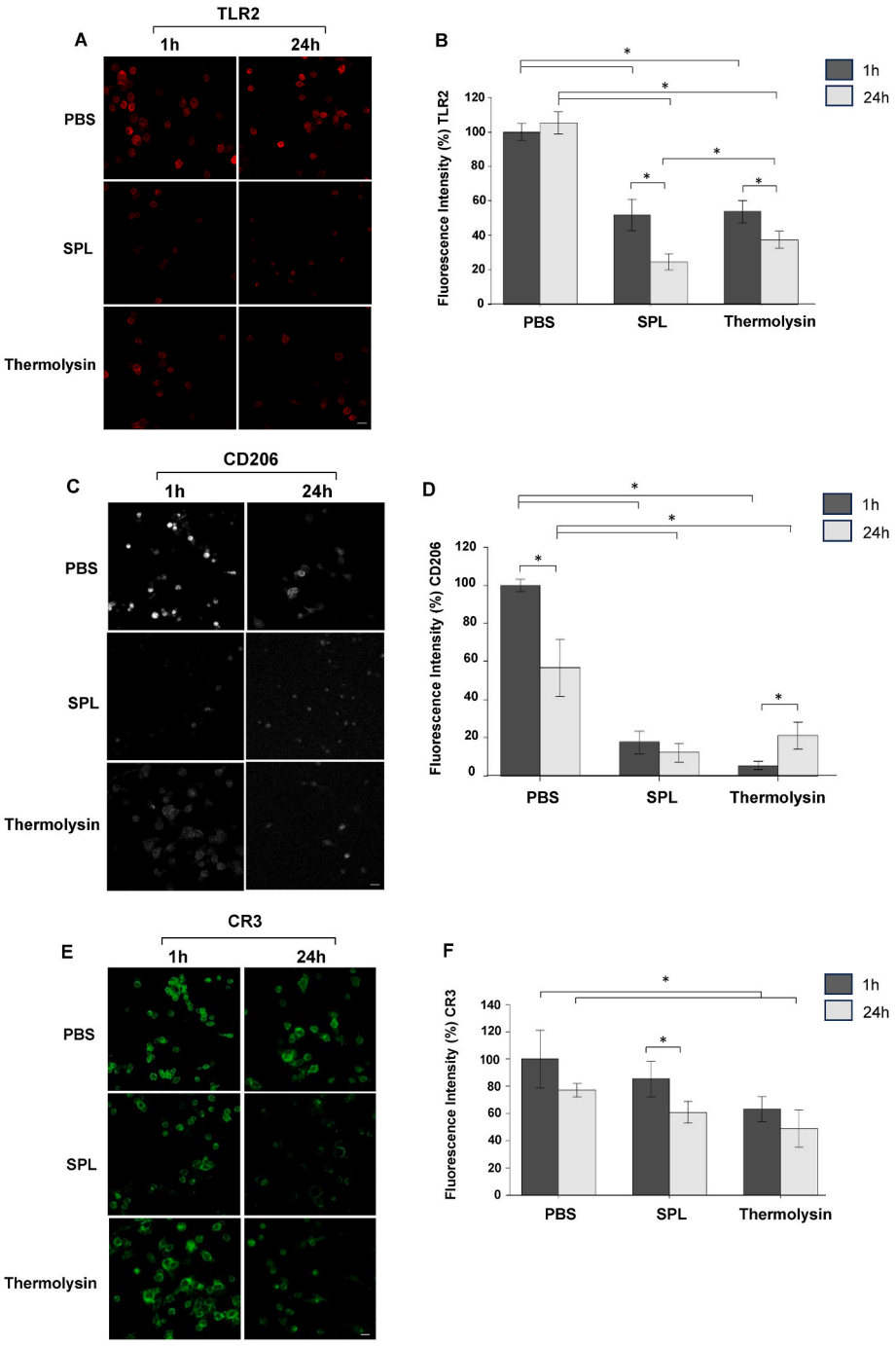

Fig. 2. Effect of enzymatic action of pathogenic leptospires proteases on macrophage receptors TLR2, CR3 and CD206. Murine peritoneal macrophages were treated with culture supernatant from pathogenic *L. interrogans* (SPL, 3 μg of total secreted proteins) or recombinant thermolysin (0.5 μg) for 1 h and 24 h at 37 °C with 5 % CO2. Then, these cells were labeled with anti-CD-282 Pe (TLR2) (**A, B**); and concomitantly with anti-CD206 APC (mannose receptor) **(C, D)** and anti-CD11b FITC (CR3) **(E, F)**. SPL was obtained from the culture of *L. interrogans* serovar Kennewicki strain Fromm. Images were captured using confocal microscopy and statistical analysis was performed using ANOVA *$p < 0.05$. These results were obtained from a single experiment and the fluorescence intensity was calculated after evaluating five different areas for each coverslip. Bar: 20 μm.

3.4. Degradation of opsonins by proteases secreted by pathogenic Leptospira

The proteolytic activity of recombinant leptolysin on C3 was confirmed, as shown in Supplementary Figs. 2A and 2B. This activity is relatively potent, as we observed the cleavage of C3 within 1 h of incubation with 300 ng, and degradation of the C3 protein with only 50 ng after 24 h. Opsonization is one of the most important biological functions, with the C3b and iC3b fragments responsible for covalently binding to the surface of different pathogens and facilitating their internalization by phagocytic cells. In this context, we decided to investigate whether proteases secreted by pathogenic leptospires could degrade opsonins present in mouse serum, as well as those deposited on the membrane of pathogenic leptospires (Supplementary Fig. 3), thus impairing their internalization by macrophages. To explore this, *L. interrogans* L1 130 FIOCRUZ was opsonized with C3b and iC3b present in NMS for 1 h at 37 °C and subsequently washed with 1x PBS. The opsonized bacteria were then incubated with recombinant thermolysin

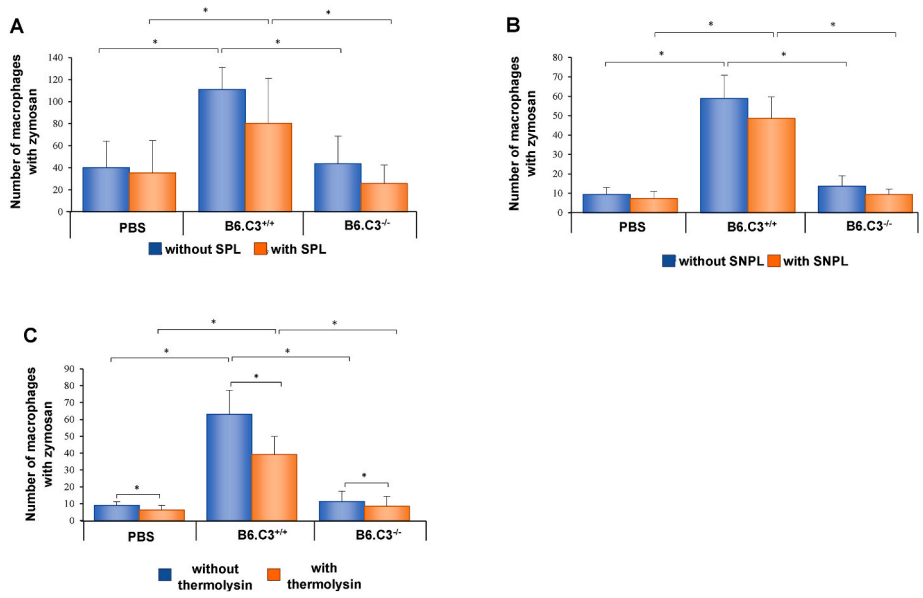

Fig. 3. Zymosan phagocytosis by peritoneal macrophages treated with SPL, SNPL, or recombinant thermolysin. Peritoneal cells obtained from mice previously stimulated with thioglycolate were incubated with **(A)** cell culture supernatant from pathogenic *L. interrogans* (SPL, 3 μg), **(B)** from non-pathogenic *L. biflexa* (SNPL, 3 μg) or **(C)** recombinant thermolysin (0.5 μg) for 1 h at 37 °C. SPL was obtained from the culture of *L. interrogans* serovar Kennewicki strain Fromm. Macrophage cultures incubated with only PBS were used as a negative control. Zymosan particles were treated with PBS or with 10 % serum from B6.C3$^{+/+}$ or B6.C3$^{-/-}$ mice before being added to the macrophage cultures and incubated for 1 h at 37 °C, 5 % CO2. A total of 200 adherent cells were counted. The number of macrophages that had phagocytosed more than 3 zymosan particles was quantified. Data was obtained from three experiments carried out in triplicate and statistical analysis was performed using ANOVA $*p < 0.05$.

or recombinant leptolysin for 1 and 24 h at 37 °C. The degradation of opsonins present on the surface of leptospires was analyzed by Western blot.

When opsonized leptospires with C3b/iC3b were exposed to 0.5 μg of recombinant thermolysin for 1 h and 24 h, we observed almost complete degradation of the α/α′ chains (115/105 kDa), β chain (75 kDa), and a 45 kDa fragment, possibly part of the opsonin iC3b, deposited on the bacterial membrane. Recombinant leptolysin (0.1 μg) also exhibited proteolytic activity on both chains, but with lower intensity (Fig. 4). These results suggest that the phagocytosis of leptospires may be impaired, at least in part, by the cleavage of opsonins deposited on the membrane of these bacteria through the enzymatic action of secreted proteases.

3.5. Phagocytosis of pathogenic leptospires by macrophages in the presence of proteases

Finally, we decided to investigate the phagocytosis of pathogenic leptospires by macrophages when both were exposed to the presence of these proteases. Macrophages were treated with SPL, recombinant thermolysin, or recombinant leptolysin for 24 h at 37 °C. Similarly, the pathogenic strain *L. interrogans* serovar Kennewicki strain Fromm was pre-opsonized by incubation with 10 % NMS for 1 h at 37 °C, followed by a wash with PBS. These opsonized bacteria were then incubated with the same proteases for 24 h at 37 °C. After incubation, the bacteria were added to the macrophage cultures for 1 h at 37 °C. As expected, we observed an increase in the phagocytosis of leptospires opsonized with NMS by macrophages. However, when these opsonized bacteria were also incubated with the proteases, a decline in leptospires phagocytosis was observed (Fig. 5A–C). This suggests that proteolytic activity may impair phagocytosis by peritoneal macrophages, primarily through the degradation of opsonins deposited on the membrane of leptospires.

4. Discussion

Over the last few years, our group has been investigating the interaction between pathogenic leptospires and the Complement System, contributing to several discoveries regarding bacterial evasion mechanisms. This research line originated from the observation that pathogenic leptospires are resistant to treatment with normal human serum,

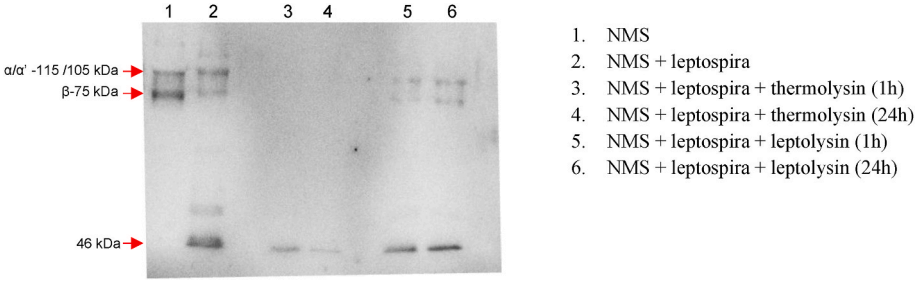

Fig. 4. Cleavage of opsonins by recombinant proteases in normal mouse serum or deposited on the membrane of pathogenic leptospires. *L. interrogans* L1 130 FIOCRUZ was opsonized by serum C3b and iC3b (lane 2). The bacteria were then washed and exposed to recombinant thermolysin for 1 h and 24 h (lanes 3 and 4), and recombinant leptolysin for 1 h and 24 h (lanes 5 and 6). Lane 1 (negative control) contained only NMS. Cleavage products were analyzed by Western blot using polyclonal anti-mouse C3 antibodies.

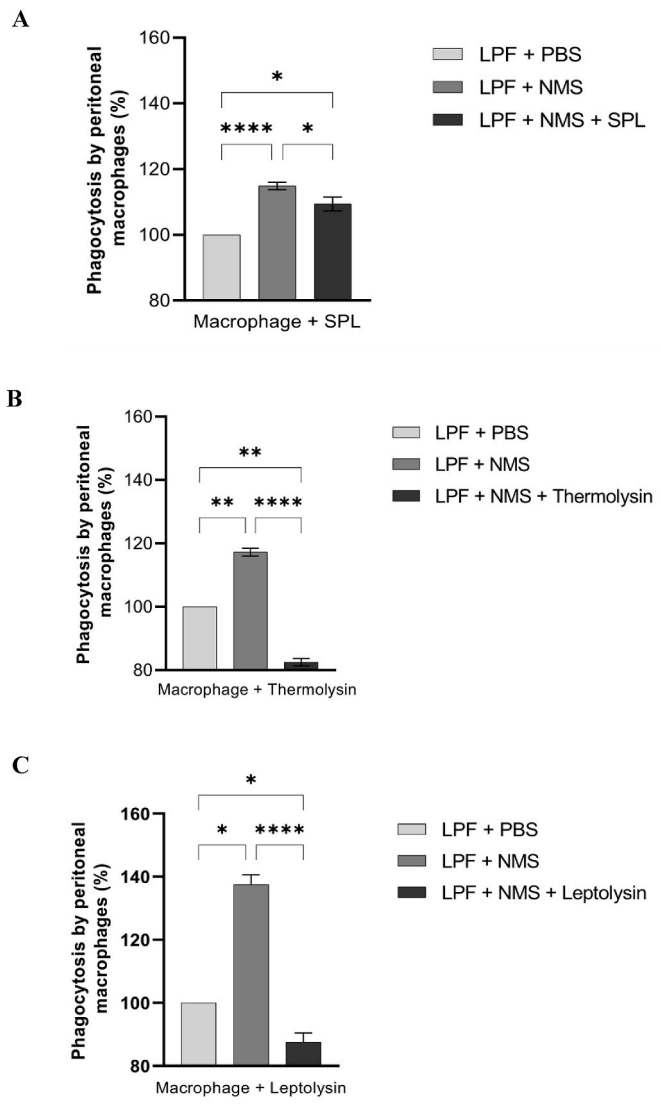

Fig. 5. Phagocytosis of pathogenic leptospires by peritoneal macrophages in the presence of SPL, recombinant thermolysin, or leptolysin. Peritoneal macrophages were incubated with **(A)** 3 μg of culture supernatant (SPL), which was obtained from the culture of *Leptospira interrogans* serovar Kennewicki strain Fromm (LPF) **(B)** 0.5 μg of recombinant thermolysin (thermo) or **(C)** 0.1 μg of leptolysin (lepto) for 24 h at 37 °C. Pathogenic bacteria LPF were labeled with 10 μM of CFSE and treated with 10 % normal serum for 20 min. As a control, bacteria were incubated with PBS alone. Subsequently, the opsonized bacteria were treated with proteases for 24 h at 37 °C and then incubated with macrophage cultures for 1 h at 37 °C, 5 % CO2. Three independent experiments were performed. Statistical analysis was performed using ANOVA (*$p < 0.05$).

whereas saprophytes are rapidly eliminated. Saprophyte killing is remarkably dependent on the Complement System since the inactivation of serum at 56 °C for 30 min promotes bacterial survival [40].

Previous studies by other groups have shown that pathogenic *L. interrogans* serovar Lai strain Lai can survive and replicate within human macrophages but not in murine macrophages [27,28]. However, the aspects of the interaction of leptospires with macrophages are still not fully elucidated. To address this, we investigated the action of proteases secreted by pathogenic leptospires on macrophage phagocytosis. We observed an approximately 20 % decrease in phagocytosis of zymosan-FITC particles when murine peritoneal macrophages were treated with recombinant thermolysin, whereas incubation with SPL caused a slight increase in phagocytosis. This difference in zymosan phagocytosis by murine macrophages treated with proteases may be due to a more potent proteolytic action of the purified recombinant thermolysin compared to other proteases present in the SPL.

Phagocytosis is enhanced when particles are coated with opsonins, the most important being IgG and fragments generated by Complement System activation (iC3b and C3b). The process is increased by the interaction of these opsonins with specific receptors such as FcγRI, II, and III (for IgG) and CR1, CR3, and CR4, present on the surfaces of phagocytic cells such as neutrophils and macrophages [41–44].

CR4 is more susceptible to cleavage by SPL than CR3 compared to untreated macrophages decreased over 24 h of incubation with SPL. However, the presence of FcγRI and CR1 was unaffected, indicating that these leptospiral metalloproteases exhibit a certain degree of specificity. As pointed out in Fig. 3C, thermolysin diminished the ingestion of zymosan in the absence of serum C3. Considering that fragments C3b, iC3b, and C4b bind to CR1, a lower percentage of zymosan phagocytosis would be expected if these particles were incubated with C3-deficient serum.

Furthermore, certain leptospiral proteases may contribute to the evasion of pathogenic *Leptospira* from macrophages, as receptor degradation, would reduce opsonophagocytosis. Non-pathogenic strains, which lack significant proteolytic activity on these substrates, are rapidly eliminated by macrophages [25,28].

Confocal microscopy revealed that the amount of TLR2, CR3, and CD206 was reduced in macrophages after 1 and 24 h of treatment with SPL. CD206 is a transmembrane glycoprotein belonging to the C-type lectin family, mainly expressed in macrophages and dendritic cells. This receptor recognizes a series of carbohydrates present on the surfaces of microorganisms such as *Candida albicans*, *Pneumocystis carin*ii and *Leishmania donovani*. After recognition, the pathogens are internalized and degraded by the phagocytic pathway [45,46]. The amount of CD206 decreased after treatment of macrophages with leptospiral proteases, Similar decrease was observed with TLR2 and CR3, suggesting that these enzymes could favor leptospires dissemination in the host.

This suggests that the secretion of proteases by pathogenic leptospires may be an important evasion mechanism, not only from the Complement System but also from macrophages. This process hinders the recognition of leptospires, preventing an efficient immune response.

Degradation of opsonins by the metalloprotease GelE, including C3b and iC3b, has been shown to inhibit phagocytosis of *Enterococcus faecalis* by human polymorphonuclear leukocytes [34,35]. Our group previously demonstrated that native C3 can be cleaved by leptospiral proteases, generating several degradation fragments. Furthermore, using purified Complement components, we observed that C3b and iC3b fragments are also cleaved by leptospiral proteases. These opsonins mediate phagocytosis by binding to CR1 and CR3 receptors, respectively [29].

The pathogenic LPF was incubated with NMS for opsonization by C3b/iC3b generated by Complement System activation. Subsequently, leptospires were incubated with SPL, recombinant thermolysin, or leptolysin. The proteases secreted by pathogenic leptospires degraded opsonins deposited on the bacterial membrane after 1 h of incubation, with greater intensity after 24 h. In addition, when NMS was incubated with these proteases and then added to the cultures of pathogenic leptospires, we observed complete degradation of the C3 α/α′ chain (115/105 kDa) and recombinant thermolysin was also able to degrade the 75 kDa β chain within just 1 h of incubation. These findings suggest that pathogenic strains of *Leptospira* could evade phagocytosis by macrophages by inactivating the Complement-mediated opsonins and certain Complement receptors. This evasion strategy likely contributes to their prolonged survival in the host and facilitates systemic dissemination. When *Leptospira* was treated with NMS, an increase in macrophage phagocytosis was observed, due to the action of opsonins deposited on the bacterial membrane. However, when macrophages and *Leptospira* were incubated with SPL, recombinant thermolysin, and recombinant leptolysin, phagocytosis significantly decreased. This supports previous findings regarding the potential degradation of CR3 and CR4 receptors (Fig. 1) and the cleavage of opsonins deposited on the bacterial

membrane, as shown in Fig. 4.

Since the recombinant proteins (thermolysin and leptolysin) used in this study were expressed in *E. coli*, one concern could be the influence of lipopolysaccharide (LPS) contamination on the expression of some macrophage receptors and phagocytosis. Several studies have shown that LPS enhances the expression of FcγRI [47], CR1 and CR3 [43,44,48], TLR-4 and TLR2 [49,50]. However, we believe that any potential residual LPS contamination in the recombinant protein preparations did not affect our conclusions, as the number of these receptors on the surface of peritoneal macrophages was either similar to or lower than those in untreated cells.

Thermolysin LIC13322 is present at low levels in the culture supernatants of several pathogenic *Leptospira* species but is absent in saprophytic species [29]. The contribution of the other three thermolysins LIC10715, LIC13330, and LIC13321 remains to be further investigated. Fraga et al. (2014) and Amamura et al. (2017) demonstrated that only the metalloprotease inhibitor 1,10-phenanthroline effectively inhibited the cleavage of complement molecules by proteases secreted by pathogenic leptospires. The observed effects on CR3 and CR4 are likely attributable to thermolysin, a known metalloprotease, which has been identified in leptospires supernatants. In addition, Courrol et al. (2022) observed similar results when leptolysin was inhibited with 1,10-phenanthroline. Additional secreted proteases may be involved and warrant further investigation in future studies. We believe that it is crucial to validate our *in vitro* findings using infected C3H/HeJ mice since this mouse strain is considered susceptible to *Leptospira* infection due to its TLR4-deficient [51,52]. The relative concentrations of leptolysin and thermolysin during different phases of *in vivo Leptospira* infection remains to be further investigated. Additionally, an important aspect to be explored in future studies is the extent to which these proteases are secreted by different serovars, as well as the influence of host-specific factors and bacterial load in the blood and target organs.

In conclusion, the secretion of proteases by pathogenic leptospires may serve as an evasion mechanism, both from the Complement System through the cleavage of its molecules and from phagocytosis by macrophages. These proteases can degrade opsonins on leptospiral membranes, inhibiting their internalization by phagocytes. In addition, the proteolytic action of SPL, recombinant thermolysin, or recombinant leptolysin reduced the presence of recognition and phagocytosis receptors on the surface of macrophages. This degradation or inhibition of these receptors may impair the immune response against leptospires, facilitating their dissemination in the body of the infected host.

CRediT authorship contribution statement

Thais A. Amamura: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Daniella dos S. Courrol:** Validation, Resources, Investigation. **Angela S. Barbosa:** Writing – review & editing, Resources, Data curation. **Ildefonso A. Silva-Junior:** Writing – review & editing, Methodology. **Tiago F. da Silva:** Writing – review & editing, Methodology. **Leonardo M. Midon:** Writing – review & editing, Formal analysis. **Mario C. Cruz:** Writing – review & editing, Methodology, Investigation. **Marcos B. Heinemann:** Writing – review & editing, Resources. **Rosa M. Chura-Chambi:** Writing – review & editing, Resources. **Ligia Morganti:** Writing – review & editing, Resources. **Lourdes Isaac:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Ethics statement

The animal study was evaluated and approved by the ETHICS COMMITTEE ON ANIMAL USE, Institute of Biomedical Science, University of São Paulo (CEUA- ICB/USP) # 48/2017.

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Conflict of interests

The authors have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micinf.2025.105469.

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