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Evaluation of different strategies to promote a protective immune response against leptospirosis using a recombinant LigA and LigB chimera

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ABSTRACT

Leptospirosis is a zoonosis of worldwide distribution, caused by infection with pathogenic *Leptospira* species. The vaccines that are currently available are bacterins, with limited human use, that confer short-term, serovar-specific immunity. Lig proteins are considered to be the best vaccine candidates to date. Here, we aimed to construct a recombinant Lig chimera (LC) comprised of LigAni and LigBrep fragments, and to evaluate it as subunit or DNA vaccine using different administration strategies. Vaccines were formulated with 50 μg of recombinant LC associated with different adjuvants or with 100 μg of pTARGET/LC. Four-week-old hamsters received two doses of vaccine with different strategies and were challenged with 5 × DL_{50} *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130. The immune response generated by Lig chimera conferred 100% protection to hamsters treated with at least one dose of recombinant LC. Despite the high levels of antibodies that vaccinated animals produced, a sterilizing immunity was not achieved. The lack of a sterilizing immunity could indicate the importance of a mixed humoral and cellular immune response. The present study generated insights that will be useful in the future development of improved subunit vaccines against leptospirosis.

1. Introduction

Leptospirosis is a zoonosis caused by pathogenic *Leptospira* spp. that has spread throughout the world. Approximately 1 million human cases are diagnosed per annum [1], although this number may be higher as leptospirosis is a neglected disease [2]. An 74.6% increase in cases was observed between the years 1999 and 2014 [1,3]. Further to being a public health concern, leptospirosis may cause huge economic losses in livestock due to miscarriages and stillbirths [4]. Vaccination is the most indicated preventive measure against leptospirosis. The vaccines that are currently available are heat-killed leptospiras (bacterins) that have several side effects (e.g., local pain, fever, nausea, etc.) and afford serovar-specific protection only [5,6]. In light of these issues, recombinant subunit vaccines have become an attractive alternative as they can, theoretically, confer long-lasting cross-protection [7,8] and are relatively easy to produce.

Surface-exposed outer-membrane proteins (OMPs) are interesting vaccine targets because antibodies can readily access them. Furthermore, there is evidence these proteins are responsible for attachment and invasion in the early stages of infection, and some evidence also supports the hypothesis that OMPs may play a role in cross-protective immunity [9,10]. Several recombinant proteins have already been tested as vaccine candidates against leptospirosis, although a protein capable of conferring cross-protection is yet to be identified [7,8].

LigA non-identical fragment (LigAni) and LigB repetitive fragment (LigBrep) have been shown to induce a protective immune response against homologous challenge in the hamster model of acute leptospirosis [11–16]. There is also evidence of their protective potential as DNA vaccines [17]. Moreover, LigA and LigB are considered the best recombinant vaccine candidates so far [7]. However, even if the vaccinated animals do not develop the disease, they could still be infected and become asymptomatic carriers. It has been hypothesized that mixing different leptospiral antigens in one single vaccine could represent a means of obtaining sterilizing immunity [18–26]. Sterilizing immunity has been

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Table 1
Experimental design and results of rLC potency test in hamsters against lethal leptospirosis challenge.

Vaccine	Dose	Protection	Efficacy	Fisher test (two-tailed)	Log rank (Mantel-Cox)	Sterility (survivors)
Adj. neg. ctrl[A]	PBS	0%	–	–	–	NS[a]
pTARGET	100 μg	0%	–	–	–	NS
LC/Alum	50 μg	100%	100%	0,0003	<0,0001	12.5% (1/8)
LC prime boost	100 μg DNA/50 μg protein	100%	100%	0.0002	<0,0001	12.5% (1/8)
LC/Montanide	50 μg	100%	100%	0.0002	<0.0001	25% (2/8)
pTarget/LC	100 μg	25%	25%	0.4667	0.3063	ND[b]
Bacterin	10^8 cells	100%	100%	0.0286	0.028	50% (4/8)

[A] Adjuvant negative control; either Alum or Montanide were used.
[a] No survivors.
[b] Not determined as efficacy was below 80%.

described after hamster vaccination with recombinant LigBrep [15]. However, there are discrepancies in terms of this result as it is also been reported that LigBrep confers only up to 71% protection against lethal challenge [13] or conferred no protection at all [27].

Some studies have attempted to achieve a stronger protection against leptospirosis by fusing or co-administering multiple recombinant antigens in the same vaccine preparation [28,29]. More recently, this approach has been used with co-administered LigAni and LigBrep and aluminum hydroxide as adjuvant, although sterilizing immunity was not achieved [16]. In the present study, we created a recombinant chimera composed of LigAni (domains 11–13) and LigBrep (amino acids 131–645) and evaluated its protective potential as a DNA vaccine. We also evaluated the use of different adjuvants in the hamster model of acute leptospirosis. The humoral immune response was characterized, and histopathological damage and leptospiral load in kidneys, liver, and lungs were assessed.

2. Materials and methods

2.1. Ethics statement

Animal experimentation was approved by the Ethics Committee on Animal Experimentation of the Federal University of Pelotas under protocol number 7563.

2.2. Bacteria strains and cultivation

Leptospira spp. were grown in liquid or semi-solid EMJH (Difco) supplemented with 10% Leptospira Enrichment EMJH (Difco) and 200 μg/ml 5-fluorouracil at 30 °C without agitation. All *Escherichia coli* strains were grown in liquid (200 RPM) or solid LB medium at 37 °C. Ampicillin (100 μg/ml) was used for selection when necessary.

2.3. Molecular cloning, protein expression and purification

Coding sequences for LigAni and LigBrep were amplified from the genomic DNA of *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130 using the primers described in Table 2. The strain was maintained at the Biotechnology Center of the Federal University of Pelotas. All primers were designed in this study to contain cleavage sites for *Xho*I and *Nhe*I (*forward*) or *Avr*II and *Kpn*I (*reverse*). The compatibility between the cohesive ends of DNA fragments digested with *Nhe*I or *Avr*II enzymes allows the tandem cloning of both amplified fragments.

Reactions contained 300 nM of each primers, 1 μl of template DNA and 50% (v/v) of GoTaq master mix (Promega) in a final volume of 25 μl, as per manufacturer's instructions. The PCR programme consisted of initial denaturation (95 °C, 10 min), 35 cycles of denaturation (95 °C, 30 s), annealing (55 °C, 30 s), and extension (72 °C, 1 min), and a final extension step (72 °C, 10 min). PCR products were purified digested, and cloned in tandem into pAE *E. coli* expression vector, which adds a His-tag to the N terminus of the protein, creating pAE/LC (Ligs chimera) with LigAni in the N-terminus and LigBrep in the C-terminus. Ligs were also cloned individually into the same vector to obtain individual proteins to be used in ELISA. The entire LC sequence was amplified using LigAni forward primers and LigBrep reverse primer and cloned in the pTARGET plasmid following the manufacturer's instructions. All DNA manipulation steps were performed as previously described [30]. Recombinant plasmids were characterized by restriction analysis.

One clone of each construction (except for pTARGET) was used to transform *E. coli* BL21 (DE3) Star. When culture OD_{600} reached 0.6–0.8, expression was induced with IPTG (0.5 mM) for 3 h. Cells were harvested (6.000*g*, 10 min, 4 °C), resuspended in lysis buffer (NaH_2PO_4 0.2 M, NaCl 0.5 M, Imidazole 0.005 M, pH 8.0), and sonicated. Lysed cells were centrifuged (10,000*g*, 30 min, 4 °C) and the pellet was treated with lysis buffer containing urea (8 M, pH 8.0) for solubilization, which was confirmed by Western blot (please see Characterization of recombinant chimeras section below) using anti His-tag primary antibodies (Thermo Fischer). Solubilized, recombinant proteins were purified by nickel-affinity chromatography using HisTrap FF 1 ml column (GE Healthcare) and the ÄKTAprime following the manufacturer's instructions (GE Healthcare): 5 column volumes (CV) of solubilized protein were loaded on the column and washed with 15 CV of lysis buffer. Purified

Table 2
Description of the Lig fragments amplified and used to construct rLC. Primers used in the PCR are also shown.

Antigen	Molecular mass (kDa)	Primers[a]
LigA11-13[b]	31,1	F-CCGCTCGAGGTGGCTAGCAGAATAGCTTCAATC R-GGGGTACCTTACCTAGGCGATCCTCCACCTCCTGGCTCCGTTTTAATAGAGGCTAA
LigBrep[c]	53,7	F-CCGCTCGAGATCGCTAGCATTACCGTTACACCAGCC R-CGGGGTACCTTACCTAGGACTGCCGCCTCCGCCTGGAGTGAGTGTATTTGT

[a] All primers contain cleavage sites for *Xho*I and *Nhe*I (*forward*) or *Avr*II and *Kpn*I (*reverse*) and were designed in this study. Reverse primers contain a Gly_4Ser linker and a *stop codon* between restriction sites.
[b] Spans domains 11–13 of LigA protein (11).
[c] Spans residues I131-P649 of LigB proteins (15).

recombinant proteins were eluted using 10 CV lysis buffer containing 500 mM imidazole (pH 8,0) and dialyzed against PBS overnight (1:1000) at 4 °C, concentrated with PEG 20,000 using dialysis tubing (12 kDa, 25 mm width), and quantified using BCA kit. Purity of purified recombinant proteins was assessed by SDS-PAGE stained with Coomassie Blue. Reagents and materials used in these steps have been purchased from Sigma-Aldrich unless otherwise specified.

2.4. Characterization of recombinant chimera

Antigenicity of purified LC construct was evaluated by western blot using rabbit anti-LigAni or anti-LigBrep sera or monoclonal antibodies and anti-rabbit or anti-mouse peroxidase-conjugated secondary antibodies (Thermo Fischer Scientific). Primary antibodies were kindly provided by Dr. Leonardo Monte and Dr. Claudia Hartleben [31]. Briefly, samples were loaded onto SDS-PAGE (12%) and transferred to nitrocellulose membrane (0.45 μM – Bio-Rad), blocked with 5% non-fat dry milk overnight at 4 °C; antibody incubations lasted for 1 h at room temperature. Membrane was washed with PBS with Tween 20 (0.05% – v/v – PBS-T) 5 times between each step. Reagents were suspended or diluted in PBS with Tween 20 (0.05% – v/v). Reactions were reavealed using 3,3′-Diaminobenzidine (Sigma-Aldrich) and hydrogen peroxide.

2.5. In vitro expression of the Lig chimera

Functionality of construct pTARGET/LC was assessed by expression in CHO.K1 cells *in vitro* as previously described [32]. Briefly, cells were grown to 80% confluence in DMEM supplemented with 10% fetal calf serum and 5% CO_2 at 37 °C in six-well plates. Plasmid DNA concentration was determined using Qubit Fluorimetric Quantification (Thermo Fischer Scientific) according to manufacturer's instructions. Cells were transfected using 2 μg of plasmid DNA and Nanofect (Qiagen) according to manufacturer's instructions. Recombinant LC expression was detected by immunofluorescence with rabbit anti-LigAni or anti-LigBrep antibodies and anti-rabbit FITC-conjugated antibody. Chromosomal DNA was stained with Hoechst 33258. Photos were taken at 100× magnification using a Leica TCS SP8 confocal microscope.

2.6. Vaccine formulation

Vaccines were formulated with 50 μg or recombinant LC and aluminum hydroxide (Aluminum hydroxide gel – Sigma-Aldrich) or Montanide ISA 50 V2 oil adjuvant (50% v/v – Seppic, São Paulo, Brazil) in a final volume of 100 μl per dose. Antigen and adjuvant were mixed overnight (16–18 h) at 4 °C.

The DNA vaccines contained 100 μg of pTARGET/LC in a final volume of 100 μl. One-hundred microliters of sucrose (50% w/v) was administered at the same vaccine site 30 min before injecting the recombinant plasmid.

2.7. Potency test and sample collection

Hamsters (*Mesocricetus auratus*) of four weeks of age were randomly segregated into 8 groups of 8 animals each and immunized intramuscularly at days 0 and 14 using disposable insulin syringe and needle (6 mm × 31G) at the medial portion of the thigh. Animals were challenged at day 28 with 5 × DL50 (approximately 200 cells) *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130 suspended in EMJH medium in a final volume of 1 ml, which was injected intraperitoneally. DL_{50} was ascertained as previously described [15]. Hamsters were monitored daily for signs of end-point criteria [11] and euthanized when these criteria were met or 28 days post-challenge. Serum samples were collected from the retro-orbital venous plexus one day before each vaccine dose. Kidney, lung, and liver samples were taken post-mortem for histopathological analysis and quantification of the leptospiral load.

2.8. Humoral immune response assessment

The humoral immune response was assessed by ELISA using individual proteins. Briefly, 96-well plates were coated with optimum protein concentration (200 and 50 ng per well of LigAni and LigBrep, respectively), as determined by a check-board, in coating buffer (bicarbonate/carbonate 100 mM, pH 9.6) for 16–18 h at 4 °C. Wells were blocked with 200 μl of 5% non-fat dry milk in PBS-T (1 h, 37 °C). Hamster sera were diluted 1:100 in PBS-T and incubated (1 h, 37 °C). Peroxidase-conjugated anti-hamster antibody was diluted 1:5000 in PBS-T and incubated (1 h, 37 °C). The reaction was revealed with o-Phenylenediamine dihydrochloride (OPD – Sigma) and hydrogen peroxide, stopped with 1 N sulfuric, acid and absorbance was measured at 492 nm. Plates were washed with 300 μl PBS-T three times between each step; before the last step, plates were washed five times.

The immune response was classified as Th1, Th2, or mixed Th1/Th2 according to IgG isotype, which was determined by ELISA with anti-hamster IgG 1, IgG2/3, or IgG3 (Southern Biotec) [15]. ELISA was performed as described above with the addition of one step to incubate the anti-isotype antibodies after adding hamster sera and peroxidase-conjugated anti-mouse was used instead.

2.9. Quantification of leptospiral load in organs

DNA was extracted from organs using the Wizard SV Genomic DNA Purification System (Promega) following the manufacturer's instructions. Quantitative real-time PCR was performed using Light Cycler 96 (Roche) and the results were analyzed using the accompanying software. Primers and probes are described in Table 3. The PCR program consisted of two initial denaturation steps (50 °C, 2 min; 95 °C, 10 min), and 40 cycles of denaturation (95 °C, 15 s) and annealing and extension (60 °C, 60 s) using GoTaq Probe qPCR Master Mix (Promega). Leptospiral load (relative quantification)

Table 3
Primers and probes used to quantify leptospiral load in kidneys, lungs, and livers of animals that survived challenge.

Primer/probe	Sequence (5′–3′)	Label	Final concentration (nM)
lipl32 forward	TTGGATCCGTGTAGAAAGAATGTC	–	300
lipl32 reverse	TCGTCCAATTTTTGAACTGGTTT	–	300
lipl32 probe	CCAAATCGCCAAAGCTGCGAAAGC	FAM/ZEN/ IowaBlack	250
ACTB forward	TTCAACACCCCWGCCATGTA	–	300
ACTB reverse	TCWCCGGAGTCCATCACRAT	–	600
ACTB probe	CCATCCAGGCYGTGCTGTCCCTG	VIC/ZEN/ IowaBlack	250

was calculated as the number of leptospiras compared to the number of hamster cells as determined by the copy number of *lipl32* and ACTB genes, respectively.

To access the sterilizing immunity promoted and to differentiate the presence of live and dead leptospires detected by qPCR, we also performed a culture of macerated kidneys after euthanasia as previously described [15].

2.10. Histopathology

Kidney, lung, and liver tissue were fixed in 10% buffered formalin (pH 7.2 – Synth) and embedded in paraffin (Synth), 4–5 μm thick sections were cut (LEICA RM2245 microtome) and stained with hematoxylin-eosin (Merck) and observed using a light microscope (Olympus BX 51 coupled to Olympus DT 72 camera). The kidneys were assessed for signs of congestion, hemorrhage, nephritis, and the presence of leukocytes; the liver tissues was evaluated for signs of congestion, hemorrhage, unusual liver tissue architecture, and hemosiderin; and the lungs were examined for signs of edema, congestion, hemorrhage, and leukocytes. According to the presence of each sign or how severe the damage was, 0–3 points were assigned to each criterion, 0 being the absence of tissue damage and 3 being severe damage.

2.11. Statistical analysis

Fisher and Log-rank were used to establish any significant difference in mortality and survival, respectively. Shapiro-Wilk test was used to assess normality of sample sets. ANOVA and Tukey's posttest were used to establish the difference among three or more groups with normal distribution. Kruskal-Wallis one-way AOV followed by all pairwise comparison was used to establish significant differences in groups with a non-normal distribution. Two-way ANOVA and Bonferroni's posttest were used to establish significant difference among two or more groups with two or more variables. Values of $P < 0.05$ were considered significant. Prism GraphPad and Statistix v8 were used to perform analyses and create graphs.

3. Results

3.1. Expression and characterization of recombinant Lig chimera (LC)

The recombinant Lig chimera (rLC) and individual Lig proteins were cloned and expressed in *E. coli*. Fig. S1 contains a schematic representation of the LC construct. The antigenicity of each component of LC was assessed by western blot, confirming that both domains are recognized by immune sera against these proteins (Fig. 1). The functionality of pTARGET/LC construct was assessed by *in vitro* expression in CHO.K1 cells. Once more, both domains were recognized in this assay (Fig. 2). Although there was a low expression level, it was significantly different to the negative control. The green spot in the negative control is considered to be background, as it does not seem to be a cell according to DNA Hoechst 33258 staining (it is stained differently to other cells in the same field).

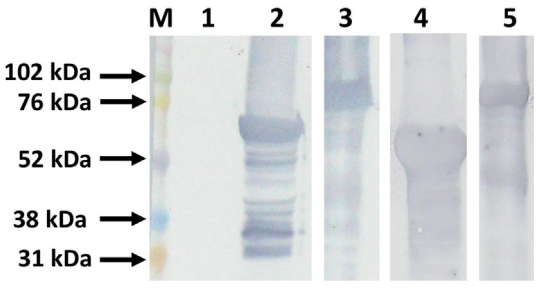

Fig. 1. Western blot analysis to characterize recombinant LC. rLC and individual LigAni or LigBep fragments (approximately 63 and 54 kDa, respectively) fragments were loaded onto a SDS-PAGE (12%), transferred to a nitrocellulose membrane, and probed with either anti-LigA (lanes 2, 3) or anti-LigB (lanes 4, 5) monoclonal antibodies. M, Amersham High-Range Rainbow ladder; 1, *E. coli* BL21(DE3) – negative control; 2, purified rLigA; 3, 5: purified rLC; 4, rLigB.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2019.02.010.

3.2. Protective potential of LC with different adjuvants or as DNA vaccine

Four-week-old hamsters were segregated into eight groups of eight animals each, received two doses of vaccine with different strategies (see Table 1), and were challenged two weeks after the second dose. Table 1 summarizes the results of the experiment together with the vaccination details and key statistics. All animals in the groups that were treated with at least one dose of recombinant LC achieved 100% protection against lethal challenge, showing a significant difference to the respective control groups. The group that received DNA vaccine only without a protein boost dose was not protected (only 25% of animals survived). The survival curve for this experiment is shown in Fig. 3.

3.3. Characterization of the humoral immune response

The humoral immune response of all animals was characterized in terms of their antibody levels against LigAni and LigBrep by ELISA (Fig. 4). Levels of IgG1, IgG2/3, and IgG3 were also quantified by ELISA (Fig. 5). All groups that received at least one protein dose developed significant levels of antibodies against both Ligs; even animals that received only one dose of protein (followed by a DNA vaccine dose) produced high antibody levels and developed protection. Animals that received the DNA-only vaccine did not develop antibodies against either Lig protein and were not protected against lethal challenge (pTARGET group).

The animals that were vaccinated with pTARGET exhibited no differences to those in the negative control group. All other groups, except LC/Alum against LigB, developed a similar humoral immune response against, characterized by a higher level of IgG2/3 compared to IgG1 or IgG3; The isotype levels of animals in the prime-boost strategy group, except IgG2/3 against LigA, did not differ from the negative control; also, the IgG3 levels of animals that received rLC with aluminum hydroxide were similar to the negative control. All other groups exhibited higher antibody levels than the negative control against either protein.

3.4. Histopathological analysis and leptospiral load of kidney

The levels of histopathological damage were assessed in the kidneys, liver, and lungs of the surviving animals to identify possible damage reduction in comparison to the bacterin vaccine; this was only performed for groups that exhibited a significant level of protection (>80%); therefore, surviving animals vaccinated with pTARGET/LC were not evaluated. There was no significant difference in the levels of histopathological damage either in the kidneys or liver of any group in comparison to the bacterin control. On the other hand, animals vaccinated with the prime-boost strategy or rLC with Montanide as adjuvant exhibited significantly less histopathological damage to their lung tissue in comparison to the bacterin control group; animals that received rLC with aluminum hydroxide did not differ from the bacterin control group and bore more damage to the lungs in comparison to other experimental groups. The histopathological score data is shown in Fig. 6.

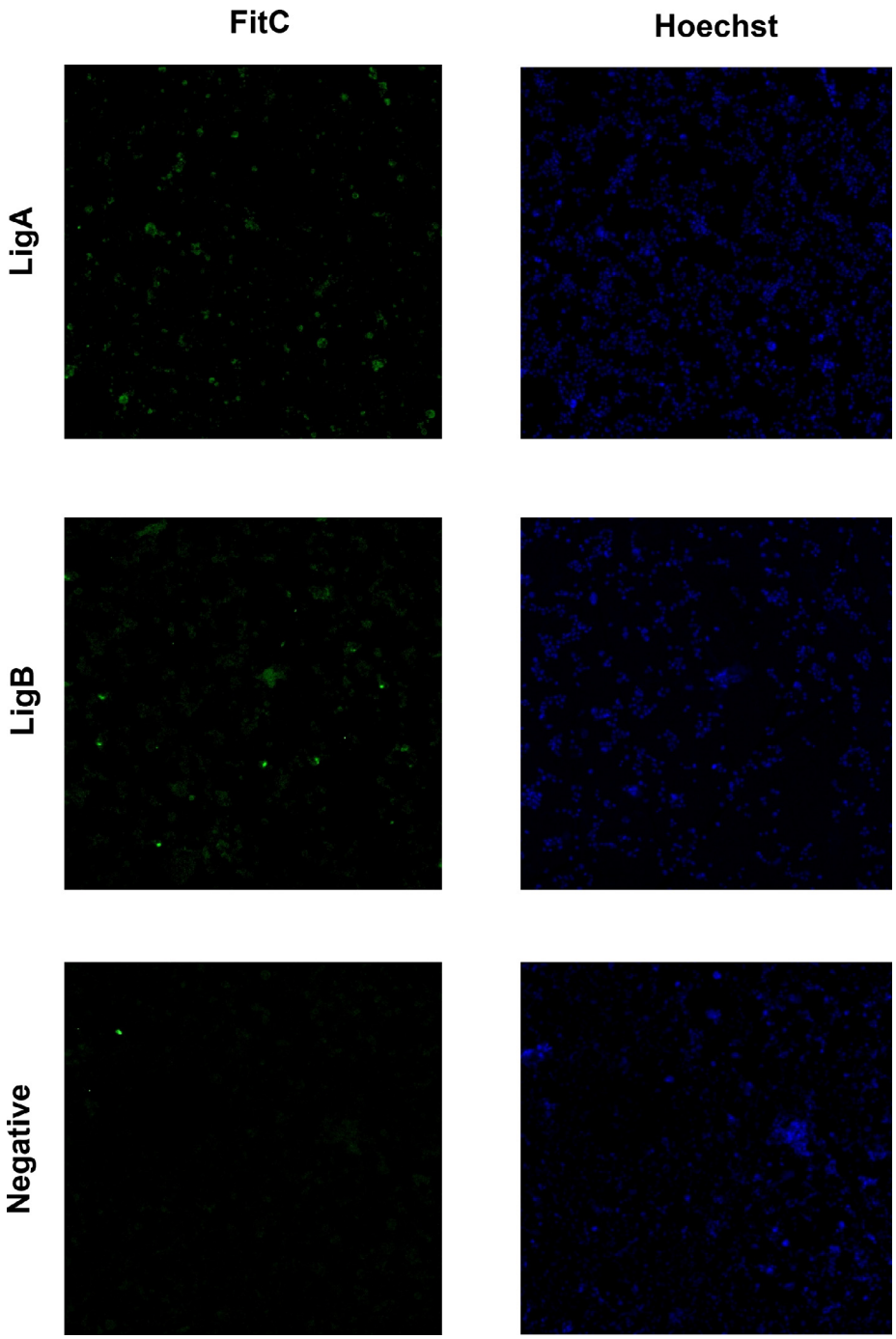

Fig. 2. Evaluation of the pTARGET/LC construct in CHO.K1 cells. Cells were grown to 80% confluence, transfected, and probed with either anti-LigA or anti-LigB monoclonal antibodies; PBS was used as negative control.

When checking the presence or absence of leptospira in the kidneys of surviving animals, the qPCR detection and renal culture of bacteria indicated that the vaccination approaches employed in the current study could not induce a sterilizing immunity (Fig. 7). Leptospiras were found in the kidneys of survivors in all groups, although with no significant difference. Animals that received a prime-boost strategy had significantly fewer leptospiras in their lungs than animals that belonged to other experimental groups. Reisolation of the pathogen was also possible through culture from kidneys maceration, confirming the presence of live leptospires in kidney in all groups.

4. Discussion

Immunity against leptospirosis is mainly due to thymus-independent, humoral immune response against the LPS, which is also responsible for antigenic differences among serovars [7]. Therefore, vaccination with *Leptospira* bacterins induce short-term, serovar-specific immunity. Despite their wide use in the veterinary field, bacterins are licensed for human use only in a few countries, mainly due to the serious side-effects they promote [6]. These issues show the importance of developing novel leptospirosis subunit vaccines with a low side-effect profile.

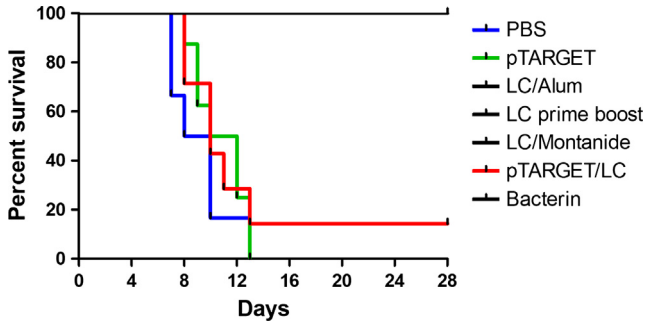

Fig. 3. Survival curve of groups of hamsters vaccinated and challenge with a lethal dose of *L. interrogans* strain Fiocruz L1-130. Groups with 100% survival are in black and overlapped. Control groups (blue and green) had 0% survival as expected. Animals vaccinated with DNA vaccine (red line) had only 25% protection. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

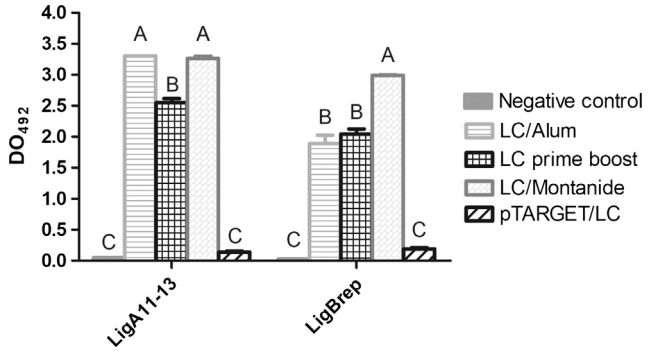

Fig. 4. Antibody levels produced after vaccination with recombinant LC at day 28. The levels of antibodies against either Lig component of the recombinant chimera were measured by ELISA and compared across each Lig component using ANOVA followed by Tukey's posttest.

Recombinant vaccines have gained ground over the last few decades as an alternative to develop a novel leptospirosis vaccine that is capable of inducing long-term, cross-protective immunity [9]. The main reason for this increasing popularity is that recombinant proteins are easy to produce and certain leptospiral antigens are conserved in pathogenic *Leptospira* spp.

Several recombinant proteins have been tested as vaccine candidates against leptospirosis in the last years, either as recombinant subunit vaccines or as DNA vaccines, as previously reviewed [7,8,24,33]. LigA and LigB proteins seem to be the most prominent antigens [24]. Lig proteins are higly conserved in pathogenic *Leptospira* spp. However, while the LigB protein is present in all pathogenic species, LigA is found only in *L. interrogans*, *L. kirschneri*, and *L. santarosai* [6,34]. There are reports of the proteins fragments LigAni and LigBrep achieving varying degrees of success as vaccines [11,17,19,27,35–38]. Chimeric antigens have also been evaluated as candidate antigens against leptospirosis, achieving 50–80% protection of subjects in experimental groups [28,29]. They have also been reported to be unable to confer protection against leptospirosis [35]. More recently, the co-administration of fragments of LigA and LigB in an attempt to achieve sterilizing immunity was unsuccessful [16]; however, a 100% survival rate was observed. In one study, sterilizing immunity was reported when using LigBrep alone [15], although other studies failed to achieve similar results [13,27,39]. In this study, we designed and expressed a recombinant chimeric protein comprised of domains 11–13 of LigA and the conserved region of LigB (residues 131–645). Fig. S1 shows a schematic representation of this construct. We reasoned that using a chimera instead of mixed recombinant proteins could be beneficial as only one biological process is required for production, and previous research has found that, despite fusing two proteins together, they are still capable of inducing a protective immune response [40,41]. The recombinant Lig chimera (rLC) was cloned and expressed in *E. coli* and inserted in the pTARGET DNA vaccine vector. After nickel-affinity chromatography purification of rLC, it was subject to characterization by western blot with anti-LigA and anti-LigB monoclonal antibodies (mAbs), which can recognize the native forms of both Lig proteins [31]. rLC was recognized by both mAbs, suggesting it can also be recognized by the immune system of rodents and would, therefore, be fit as a vaccine candidate (Fig. 1). Also, CHO.K1 mammalian cells were transfected with pTARGET/LC and subjected to indirect immunofluorescence with the same mAbs (Fig. 2). Although there was a poor transfection rate, some cells were detected by the mAbs, confirming expression of rLC in mammalian cells and suggesting pTARGET/LC would also be fit as a vaccine candidate. Even though there was some degree of background noise in the negative control, it is safe to state that it differed from probed cells. Nonetheless, sequencing confirmed the insertion of LC in the pTARGET vector in the correct frame.

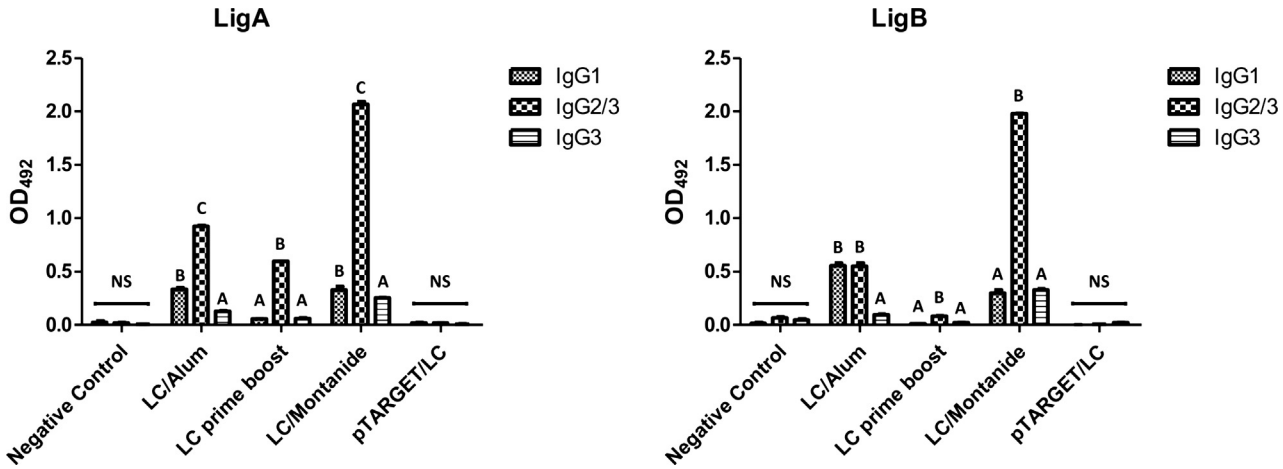

Fig. 5. IgG isotype levels against each Lig component or rLC in each group. Isotype levels were measured by ELISA to characterize the type of immune response induced by each vaccination strategy in hamsters. Levels of each isotype were compared within each group (letters on top of groups of bars) and against the negative control to establish significant difference (not shown).

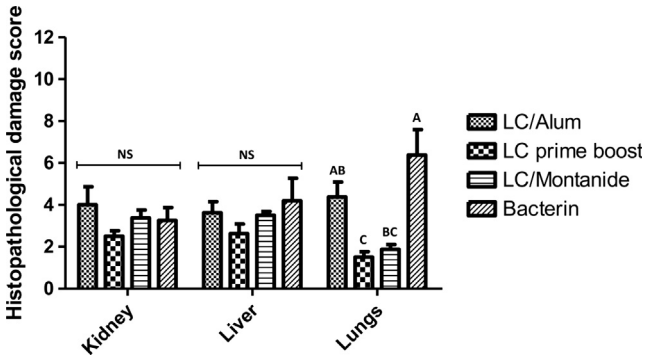

Fig. 6. Histopathological damage score for the kidneys, livers, and lungs of surviving animals; only animals in groups that had >80% efficacy were considered. Statistical significance was established by Kruskal-Wallis. NS, not significant.

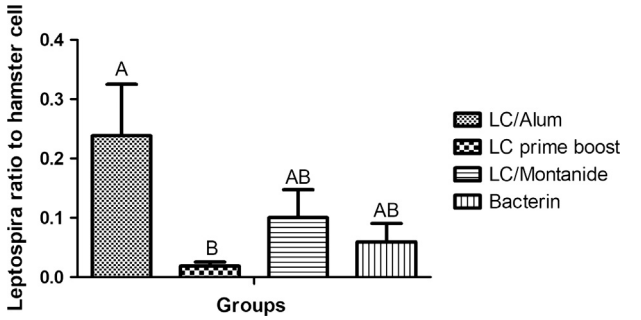

Fig. 7. Quantification of the leptospiral load in the kidneys of animals that survived lethal leptospirosis challenge; only animals of groups with >80% efficacy were considered. The leptospiral load was determined by qPCR as the ratio of leptospira cells (represented by the number of *lipl32* gene copy) over the number of hamster cells (represented by the number of ACTB gene copy).

Because the use of a combined Lig vaccine with aluminum hydroxide as adjuvant has already been assessed and failed to achieve sterilizing immunity [16], we evaluated whether different vaccine approaches (using Montanide oil as adjuvant [42] prime-boost strategy [32,39,43] or DNA vaccine [33] with Lig vaccines would be capable of inducing a more robust immune response.

DNA vaccines present advantages when compared with traditional vaccines, such as the potential to induce a broad immune response with stimulation of a balanced cellular and humoral response. Despite considerations about the safety of genetic immunizations, DNA vaccines have been licensed for veterinary use for more than a decade, and several studies have evaluated this approach to induce protection against leptospirosis [33]. We believe comparing different approaches is useful, considering it could provide insights that might help understanding what the ideal immune response to achieve a protective and sterilizing immunity would be.

Our results show that all animals that received at least one protein dose produced an immune response strong enough to survive lethal challenge, while animals that were administered a DNA-only vaccine did not (see Table 1, Fig. 3). An assessment of the humoral immune response revealed that one protein dose alone was sufficient to induce high antibody levels against LigA, although these antibody levels were not as high as when two protein doses were used. Equal levels of anti-LigB antibodies as those induced by two doses or rLC and aluminum hydroxide, but lower than rLC and oil adjuvant (Montanide) were also achieved, as shown in Fig. 4. Likewise, animals that developed a humoral immune response were protected against lethal challenge, while animals that didn't were not (group that received the DNA vaccine). It is worth noting that there were two survivors in the DNA vaccine group despite the absence of a humoral immune response, suggesting cellular immune response may play a role in protection [6] and should be mixed with a humoral immune response.

To better characterize and understand the type of humoral immune response induced by our vaccines, we profiled the IgG isotypes against each protein by indirect ELISA. In every case but one (rLC/Alum against LigB, which had equal levels of IgG1 and IgG2, suggesting a mixed Th1/Th2 response), we observed higher IgG2 levels than IgG1 levels. There were no significant levels of IgG3 (Fig. 5). This isotype profile suggests the development a Th1 immune response, which is thought to be responsible for protection against leptospirosis and has been observed in other studies [15,28,29]. It has also been shown that vaccination with bacterin induces a strong IgG2 response and almost undetectable levels of IgG1 and IgG3 [15]. Of note, even though the prime-boost group exhibited considerably low levels of antibodies, it also showed a higher level of IgG2 in comparison to IgG1 and IgG3; IgG2 was also higher in the prime-boost group than in the negative control. These results suggest that even low levels of IgG2 antibodies or a weak Th1 immune response may be capable of conferring protection against lethal challenge, although we did not observe sterilizing immunity in our experiments. Moreover, further investigation is required to elucidate the roles played by each isotype in protecting against virulent *Leptospira* spp., as Th1 immune response is classically understood as being against intracellular pathogens, while *Leptospira* spp. are extracellular, although they might invade cells occasionally [44,45].

To further evaluate the protective potential of the Lig chimera vaccine in combination with different adjuvants and antigen presentation systems, we analyzed histopathological damage to kidneys, liver, and lungs as well as the presence of bacteria in the kidneys by qPCR. Scores were assigned to each organ based on the presence or absence of a set of criteria, as described in the methodology. There was no significant histopathological damage reduction in the kidneys and liver of the surviving animals either group in comparison to the bacterin control group. There was, however, a much higher degree of histopathological damage to the kidneys of animals that survived challenge and the vaccine was not efficient (pTARGET/LC group – not shown). The only organs that exhibited a significant difference in terms of histopathological damage were the lungs; the prime-boost strategy and rLC with Montanide as adjuvant resulted in the least damage in comparison to the bacterin control group, while rLC with aluminum hydroxide showed no difference from the control. Likewise, it was the prime-boost strategy that cleared leptospiras the most from the kidneys (Fig. 6), despite not reducing damage to the organ. Similarly, in previous studies, a reduction in lung histopathological damage has been reported following vaccination with LigBrep [15,39]. However, vaccination with LigAni didn't seem to protect against lung hemorrhage [36]. It is difficult to compare results between the current study and existing literature because most previous studies did not quantify the severity of histopathological damage; rather, the presence or absence of it was verified. Furthermore, in the current study, the group vaccinated with the prime-boost strategy was also the group that presented the lowest ratio of leptospiras per kidney cell (Fig. 7). However, no experimental group was different to the control group; experimental groups differed only among themselves, the rLC with aluminum hydroxide group having the highest bacterial load. It is also difficult to compare the leptospiral load identified in this study with previous studies because existing research has employed different methodologies that have different sensitivities (e.g., culture, microscopy, real-time PCR with SybrGreen or probes) and different units (e.g., cell ratio, genome equivalents per mg tissue or μg DNA). On the other hand, this might also explain, among

other reasons, why some authors found sterilizing immunity and others did not. Nonetheless, there is a requirement to standardize the methods employed to assess sterilizing immunity in novel leptospirosis vaccines.

Attention should be brought to the fact that the group immunized with the prime-boost strategy displayed both the lowest level of histopathological damage to the lungs and the lowest leptospiral load in the kidneys. However, when the immune response from this group was characterized, it was very similar to the remaining groups, except for the fact it had fewer antibodies and received a DNA dose, which might have prompted a cellular response. These observations highlight the hypothesis that a mixed humoral and cellular immune response might be necessary to confer a fully protective, sterilizing immune response. Studies suggest an important role of macrophages in *Lesptospira* killing [46]. Activation of macrophages by Th1 cells and the enhancement of phagocytosis by bacterial opsonization could be fundamental mechanisms for achieving sterilizing immunity, showing the importance of a mixed immune response.

Our results emphasize the different outcomes associated with various vaccination strategies and the use of different adjuvants. This information could aid future work on the development of enhanced subunit vaccine against leptospirosis, although some points should be considered more carefully. Additional replicates of the challenge experiments are needed to enhance the study confiability. However, the protection results previously achieved with Lig vaccines, reported by several research groups, help ensure the protection capability of our chimeric protein. By presenting these results, we also allow other research groups to replicate the experiments described here, contributing to increase the knowledge about Lig proteins and rLC chimera or other antigen combinations. Nonetheless, it would be beneficial to identify new targets and evaluate novel adjuvants.

In conclusion, the immune response generated by the Lig chimera (LC) was able to confer 100% protection to hamsters treated with at least one dose of the recombinant protein. Different adjuvants and strategies can be used with this Lig chimera to achieve a protective immune response. However, no treatment has promoted a sterilizing immune response.

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Conflict of interest statement

The authors declare no conflict of interest.

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