Original Article

Protective immunity of recombinant LipL21 and I-LipL21 against *Leptospira interrogans* serovar Autumnalis N2 infection

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Abstract

Introduction: Leptospirosis is a zoonotic disease caused by the spirochete of genus *Leptospira* with widespread distribution in tropical, subtropical and temperate zones. Leptospirosis is often confused with other febrile illnesses including jaundice, dengue, and malaria. Generally, the disease is often underdiagnosed or misdiagnosed. Though leptospirosis is curable with antibiotic treatment, the laboratory diagnosis of the disease is specialized and open to interpretation with multiple kits available to detect the different serological markers of *Leptospira*. Moreover, when leptospirosis is misdiagnosed, the disease can lead to multi-organ failure and may have fatal effects. There is a need for strategies to develop vaccines and prevent leptospirosis. In the present study, the immunogenic potential of leptospiral recombinant protein LipL21 (rLipL21) and its truncated form I-LipL21 (rI-LipL21) was evaluated.
Methodology: The recombinant proteins were established in cyclophosphamide treated BALB/c mice model infected with *L. interrogans* serovar Autumnalis strain N2.
Results: The vaccination study showed 66% and 83% survivability among mice immunized with rLipL21 and rI-LipL21 respectively and post-challenge with leptospiral strain N2 compared to control groups that showed 100% lethality. Additionally, a significant increase in antibody levels and cytokine levels (TNF-α, IFN-γ and IL-10) was observed evidencing a marked stimulation of both humoral and cell-mediated immune response in mice immunized with rLipL21/rI-LipL21 compared to whole cell leptospiral lysate (WCL).
Conclusions: This study evidenced protective immunization against leptospirosis with rLipL21 and rI-LipL21 recombinant proteins and are potential candidates for the development of leptospiral vaccine.

Key words: leptospirosis; LipL21; I-LipL21; vaccine.

Introduction

Leptospirosis is a neglected tropical disease of global importance, caused by pathogenic spirochetes of genus *Leptospira*. The infection generally occurs through direct or indirect contact with contaminated urine, soil or tissues. In the case of human leptospirosis, patients have influenza like symptoms, fever and the severity of disease is characterized by the presence of jaundice, acute renal or hepatic failure, and pulmonary hemorrhages even leading to death [1-3]. Likewise, in animal herds, leptospirosis causes severe economic loss due to abortion. Therefore, the best way to control leptospirosis is through vaccination and chemically inactivated leptospires are considered an authentic source of commercial vaccines in many countries [4].

Hamsters or guinea pigs that are sensitive to virulent leptospires are the generally used animal models for experimental infection of *Leptospira* [5-7]. However, the upkeep of these animals is laborious in comparison with mice and experimental data can vary as a result of genetic heterogeneity [8]. Maintenance of hamsters/guinea pigs in a laboratory animal house is very expensive compared to mice. Laboratory strains of mice are in general not susceptible to virulent leptospires. However, Adler and Faine [9], reported that BALB/c mice can be lethally infected with virulent *Leptospira* by pre-administration of cyclophosphamide (Cy), an alkylating agent that suppresses the primary humoral response of the host, at 2 days before challenge and that immunosuppressed mice treated with Cy could

possibly serve as an animal model for immunization studies. Masuzawa *et al.*, [8] further confirmed the usefulness of pre-administration Cy in mice as an experimental model of leptospiral lethal infection and compared the results of experimental infection in Cy treated mice with those in hamsters. These results indicated that Cy treated mice can be used in the experimental infection of *Leptospira* in place of hamsters or guinea pigs. Hence the animal experiments for immunization with mice model can be routinely used and recently LipL45 DNA vaccine has been studied and proven as being efficient using the mouse model [10]. Recently there has been several efforts for developing recombinant leptospiral vaccines using mice as the model system [10-13]. In a previous study, leptospiral outer membrane protein LipL21 and its truncated N-terminal immunogenic region (I-LipL21) were evaluated for diagnosis of leptospirosis and the outcome was promising [14]. The aim of this study was to determine the efficacy of rLipL21 and its truncated rI-LipL21 as a vaccine candidate to confer protective immunity in Cy treated BALB/c mice against *L. interrogans* serovar Autumnalis N2 infection.

Methodology

Bacterial strains and culture conditions

L. interrogans serovar Autumnalis strain N2 was maintained by regular sub-culturing in Ellinghausen-McCullough-Johnson-Harris (EMJH) bovine serum albumin-Tween 80 medium (Difco Laboratories, USA) at the Medical Microbiology Laboratory, Bharathidasan University, Tiruchirappalli, India. All procedures with *L. interrogans* were conducted using MACS (Mouse Adapted Challenge Strain) as previously described [15]. The *Escherichia coli* strains NovaBlue and BL21 (DE3) (Novagen, Madison, USA) were used for cloning and expression of recombinant proteins. Isolates were grown in Luria-Bertani (LB) medium (Sigma Aldrich, St Louis, USA) with the addition of ampicillin (100 µg/mL).

Determination of T-Cell Epitope on LipL21

The immunogenic B-cell epitope of LipL21 flanking the N-terminal region was selected for the prediction of T-cell epitopes using MHCPred analysis (http://www.jenner.ac.uk/MHCPred). MHCPred uses the statistical models for both Class I alleles (HLA-A*0101, HLA-A*0201, HLA-A*0202, HLA-A*0203, HLA-A*0206, HLA-A*0301, HLA-A*1101, HLA-A*3301, HLA-A*6801, HLA-A*6802 and HLA-A*3501), Class II alleles (HLA-DRB*0101, HLA-DRB*0401, HLA-DRB*0701, HLA-I*Ab, HLA-I*Ad,

HLA-I*Ak, HLA-I*Eg, HLA-I*Ek, HLA-I*As, HLA-I*Ed) and other Transporter associated with antigen processing (TAP) [16]. The results of computational analysis included peptides and their corresponding IC_{50} value, which implies the binding affinity. Usually, peptides with predicted binding affinities < 500 nM are significant binders, whereas those with binding affinities > 500 nM are considered as non-binders and peptides with IC_{50} < 500 were selected.

Recombinant LipL21 and I-LipL21

As described previously [14], recombinant LipL21 and truncated N-terminal I-LipL21 were expressed and purified using Ni^{2+} metal affinity chromatography (Bio-Rad, Hercules, USA). The purified recombinant proteins were quantified by bicinchoninic acid (BCA) kit (Sigma, St. Louis, USA).

Challenge experiments of hamsters, mice and Cy mice

In order to demonstrate the Cy mice model as a susceptible host as per Adler and Faine [9], three groups were challenged intraperitoneally with MACS (LD_{50} dose). These groups were consisting of 6 hamsters, aged between 4 and 6 weeks (Group 1), 6 BALB/c mice of 4-6 week old without Cy (Group 2) and 6 BALB/c mice treated with cyclophosphamide (300 mg/Kg) (Group 3). Two independent experiments were conducted.

Immunization of Cy mice with rLipL21/rI-LipL21/WCL and challenge experiments

Four to six week old BALB/c mice were divided into five groups with 6 mice each. The mice from different groups were immunized subcutaneously thrice at 7 days' interval with 50µg each of rLipL21 / r-I-LipL21 / WCL. Control groups received phosphate buffered saline (PBS) or Alhydrogel (Sigma, St. Louis, USA) as the vehicle control. Prior to immunization, the recombinant proteins were mixed with Alhydrogel (Sigma, St. Louis, MO). Two independent experiments were conducted. Blood samples were collected on day 0 (pre immunization), 14th, 21st, 42nd and 72nd days after immunization and serum was separated and stored -80°C to measure the antibody response.

To perform challenge experiments, the immune response was suppressed 48h before challenge with cyclophosphamide (300mg/Kg). The immunized mice were challenged with 1.37 x 10^7 leptospires (LD_{50} dose) intraperitoneally at 42nd day post-immunization. The LD_{50} was determined for Autumnalis N2 as per the earlier procedures [17]. The mice were monitored daily and their survival /death recorded. Mice were sacrificed on 72nd day post immunization. Two independent

experiments were conducted. Blood was collected by cardiac puncture and serum was separated and stored at -80°C until use. The tissues from infected mice were taken aseptically for isolation of leptospires in EMJH semisolid medium as per earlier reports [18]. The Institutional Animal Ethics Committee (IAEC) of Bharathidasan University (BDU/IAEC/25/2013/09.04.2013) approved the study protocols. The methods were carried out in accordance with the relevant guidelines and regulations.

Isolation of leptospires from mice tissue

Mice kidney tissues were collected aseptically from the animals; a piece of tissue was inoculated in to EMJH medium containing 5-fluorouracil (100 μg/mL) and maintained at 30°C for 16 weeks in the dark [18]. Growth was monitored using a dark-field microscopy at regular intervals.

IgG-ELISA for anti r-LipL21, rI-LipL21 and WCL

Mice IgG antibodies against rLipL21 and rI-LipL21, WCL and PBS were evaluated for the presence of specific immunoglobulin G (IgG) by ELISA. The plates were coated with r-LipL21 / rI-LipL21 / WCL (1 μg/well) with carbonate coating buffer (pH9.6) and incubated overnight at 4°C. The plates were washed three times with PBS containing 0.05% Tween 20 (PBST). Mice sera, at a dilution of 1:100, was added to wells in triplicates and incubated at 37°C for 1hr and washed thrice with PBS-T. Anti-mouse IgG-HRP (Sigma, St. Louis, USA) at 1:5000 was used as secondary antibody conjugate and incubated at 37°C for 1hr and washed with PBST. To this the substrate Ortho-Phenylenediamine (Sigma, St. Louis, USA) was added and incubated in dark for 10-15 min and 2N H_2SO_4 was added to stop reactions and the plates were read at 490nm (Bio-rad, Hercules, USA).

Lymphoproliferation assay

Splenocytes derived from mice on 42nd day of post-immunization were seeded in 96-well flat bottom plates at a concentration of 5×10^5 cells in RPMI medium (Sigma, St. Louis, USA). The splenocytes were stimulated with varying concentration of purified rLipL21, rI-LipL21 and WCL (0.01, 0.1, 0.5, 1.0, 10, 100 μg/mL) for 48hrs at 37°C with 5% CO_2. Splenocytes stimulated with PBS / Alhydrogel were treated as controls. The entire procedures were followed as per earlier reports [19,20]. Incorporation of bromodeoxyuridine (BrdU) was used as a measure of lymphoproliferation (Cell proliferation BrdU colorimetric kit, Roche Diagnostics). Optical density (OD) was measured at 490nm (ELISA-plate reader, Bio-rad, Hercules, USA). The results were expressed as stimulation indices (SI), calculated as the ratios between the mean OD of cells cultured with proteins versus the mean OD of cells cultured in medium alone.

RNA isolation, cDNA synthesis, and qRT-PCR

Total RNA was isolated from the spleen cells using the TRIzol reagent (Invitrogen, Carlsbad, USA) and cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Real-time PCR (qRT-PCR) reactions were performed on a CFX96 Touch Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA). The qPCR using SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA, USA) and primers [12,21] was carried out in a 25 μL reaction volume (50 ng cDNA, 12.5 μL Master Mix, 0.5 μM of each primer). Primer details are provided in Table 1. The cycling conditions consisted of 95°C for 10 min (denaturation), followed by target DNA amplification for 45 cycles (95°C for 5 s, 60°C or 61°C for 30 s, and a variable extension time at 72°C). The melting curves were analyzed immediately after amplification at a linear temperature transition rate of 0.1°C/s from 55 to

Table 1. List of primers used for qRT- PCR assays**.**

Gene name	Direction	Sequence (5'-3')	Product Size (bp)
TNF-α	Forward	GGACTAGCCAGGAGGGAGAA	162
	Reverse	CGCGGATCATGCTTTCTGTG	
GAPDH	Forward	AACGACCCCTTCATTGAC	191
	Reverse	TCCACGACATACTCAGCAC	
IL-10	Forward	GCCCTTTGCTATGGTGTCCT	179
	Reverse	TTTTCAGGGATGAAGCGGCT	
IFN-γ	Forward	ATTCAGAGCTGCAGTGACCC	162
	Reverse	GGAAGCACCAGGTGTCAAGT	
IL-4	Forward	CAAACGTCCTCACAGCAACG	156
	Reverse	AAGCCCGAAAGAGTCTCTGC	
IL-12p40	Forward	GGAAGCACGGCAGCAGAATA	180
	Reverse	AACTTGAGGGAGAAGTAGGAATGG	

95°C, with continuous fluorescence acquisition. The relative cycle threshold Ct ($\Delta\Delta$Ct) method was used to quantify cytokine gene expression [22]. Briefly, the fold change of each target mRNA was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene Ct (ΔCt), and compared to a calibrator sample, the same normalized gene in the pre-immune sera sample ($\Delta\Delta$Ct). The final value represents the mean of triplicate relative fold between immunized and non-immunized mice.

Statistics

The Fisher exact test and log-rank test were used to determine significant differences in mortality and survival rates, respectively, among the experimental groups. The Student's t-test was used to determine significant differences among the serological and mRNA relative expression analyses. Differences were considered significant at a p value of $\leq$ 0.05. The

analyses were carried out with GraphPad Prism 7 and SigmaPlot software.

Results

Determination of T-cell specific epitope on LipL21

Class I and II alleles were selected for binding affinity prediction. The MHCPred analysis predicted 33-peptide sequence with confidence prediction score of 1 and IC50 value <500nM. Peptides with the best predicted binding affinities for each allele are presented in Table 2.

Purification of recombinant LipL21/I-LipL21

The rLipL21 and rI-LipL21 proteins were expressed in inclusion bodies in *E. coli* with the expected size of 21 kDa and 18 kDa respectively as per a previous study [14]. Dialysis against PBS, solubilization in urea and purification of the recombinant proteins resulted in a yield of 9.6 mg/mL

Table 2. T-Cell specific epitopes of leptospiral LipL21.

S. No.	Query Sequence	Peptide Sequence	Allele	IC$_{50}$ value
1.	Epitope 1- ACSST DTGQKDATTVGDG	DT$_7$GQKDATT$_{14}$V	H2Kk	234.96
		TD$_6$TGQKDATT$_{14}$V	A0101	345.94
		SS$_4$TDTGQKDA$_{12}$T	A0101	350.75
		TD$_6$TGQKDATT$_{14}$V	A0203	487.53
		TD$_6$TGQKDATT$_{14}$V	A6802	142.89
		CS$_3$STDTGQKD$_{11}$A	DRB0101	15.78
		GQ$_9$KDATTVGD$_{17}$G	DRB0101	63.83
		GQ$_9$KDATTVGD$_{17}$G	DRB0701	61.24
		QK$_{10}$DATTVGDG$_{18}$	DRB0701	82.79
		SS$_4$TDTGQKDA$_{12}$T	IAk	361.41
		DT$_7$GQKDATTV$_{15}$G	IAd	35.24
		A$_1$CSSTDTGQ$_9$K	IAs	2.67
		GQ$_9$KDATTVGD$_{17}$G	IAs	36.39
		TG$_8$QKDATTVG$_{16}$D	IAs	57.68
		ST$_5$DTGQKDAT$_{13}$T	IAs	141.58
2.	Epitope 2- WGGPPEQRMDGKTPRDTN	WG$_2$GPPEQRM$_9$D	H2Kk	82.79
		MD$_{10}$GKTPRDT$_{17}$N	H2Kk	338.06
		W$_1$GGPPEQRM $_9$D	DRB0101	78.70
		GG$_3$PPEQRMDG$_{11}$K	DRB0701	286.42
		RM$_9$DGKTPRDT$_{17}$N	IAb	421.70
		W$_1$GGPPEQRM$_9$D	IAd	155.96
		WG$_2$GPPEQRMD$_{10}$G	IAd	234.42
		QR$_8$MDGKTPRD$_{16}$T	IAs	127.06
		RM$_9$DGKTPRDT$_{17}$N	IAs	352.37
		MD$_{10}$GKTPRDTN$_{18}$	IEd	497.94
		EQ$_7$RMDGKTPR$_{15}$D	TAP	44.67
3.	Epitope 3- VKGVGVYECKATGSGSDP	VK$_2$GVGVYECK$_{10}$A	A1101	32.04
		KG$_3$VGVYECKA$_{11}$T	DRB0101	153.11
		KG$_3$VGVYECKA$_{11}$T	DRB0701	328.10
		VK$_2$GVGVYECK$_{10}$A	DRB0701	368.13
		CK$_{10}$ATGSGSDP$_{18}$	IAk	32.28
		VG$_5$VYECKATG$_{13}$S	IAd	92.47
		YE$_8$CKATGSGS$_{16}$D	IAs	24.61

Figure 1. A) Immunoblots showing the results of inducing rLipL21 and rI-LipL21 expression with 0.1 (Lane 1,2), 1.0 (Lane 3,4), and 10 mM (Lane 5,6) isopropylthio-b-D-galactoside and probed with specific sera raised against rLipL21 and rI-LipL21, B) and the similar recognition of the native LipL21 protein of serovars Australis, Autumnalis, Icterohaemorrhagiae, Grippotyphosa, Pomona, Hebdomadis, Hardjoprajitno, and Javanica (Lane 1-8) whole cell lysate (WCL) against the rLipL21 specific sera and C) rI-LipL21 specific sera.
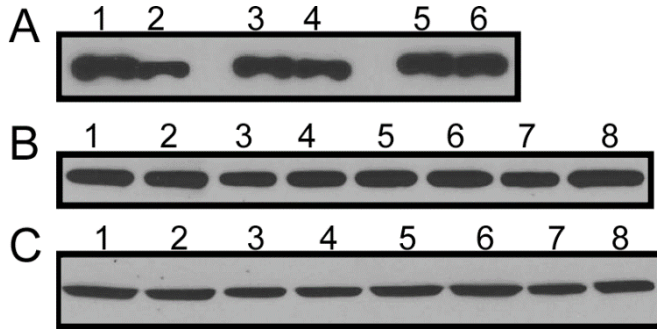
Figure 2. MACS specific challenge experiments of hamsters, mice and Cy mice. Y-axis percent survival of Hamster, Cy mice and mice after lethal challenge and X-axis days post challenge. The results are a summary of two independent experiments.
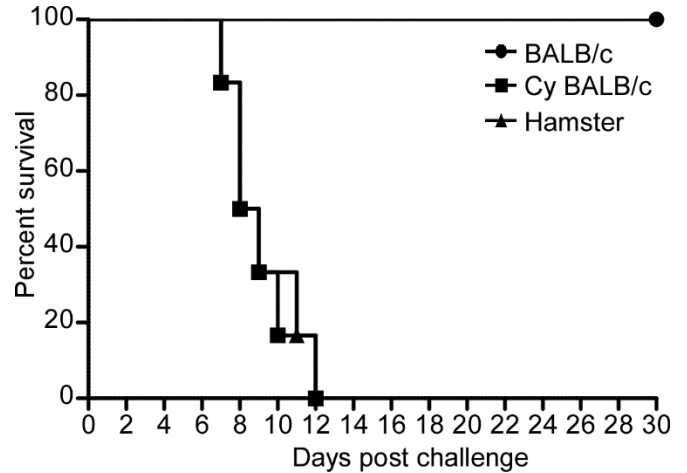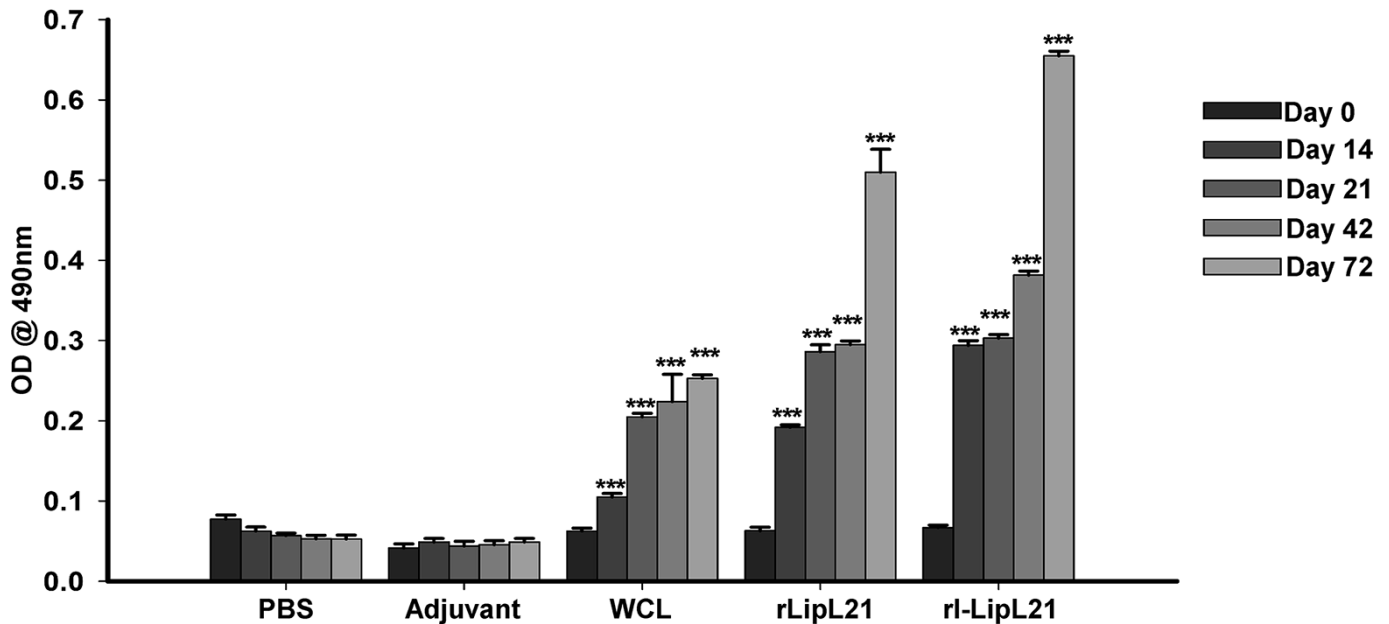

Figure 3. IgG antibody quantification by ELISA of immunized BALB/c mice with WCL, rLipL21 and rI-LipL21 on day 0 (pre-immunization), and 14, 21, 42 72 day of post immunization. Y-axis $OD_{490\ nm}$ and X-axis different groups. *** (p < 0.001).

and 5.3 mg/mL respectively for rLipL21 and rI-LipL21. The specificity of the purified proteins was evaluated by Western blot analysis using specific antibodies (Figure 1A). Additionally, the native LipL21 protein was observed to be conserved among different *Leptospira* serovars as observed by the Western blot analysis with the whole cell lysate (WCL) probed with specific antibodies (Figure 1B and C).

MACS specific challenge experiments of hamsters, mice and Cy mice

Following the MACS specific challenge of hamsters and Cy mice, the survivability of the animals was not successful after 4-7 days post challenge. However, the mice group which received the MACS without Cy treatments survived (Figure 2). These results supported the susceptibility of the Cy mice and their utility for the challenge experiments for vaccine trials for leptospirosis.

### Humoral immune response in immunized mice

The specificity of the antibody response in immunized mouse groups was assessed. An indirect

Figure 4. Lymphoproliferative response to WCL, LipL21 and I-LipL21. The data represents mean SI of two determinations ± SD. Y-axis denotes the stimulation index (SI) and X-axis denotes antigens used at different concentrations.
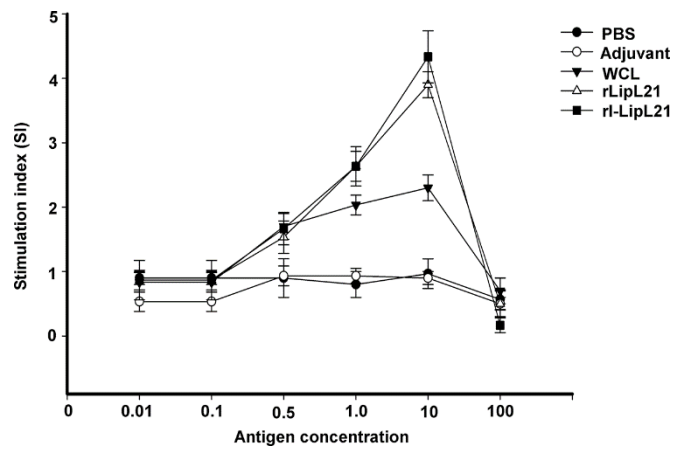
ELISA was performed with serum samples collected from each animal on days 0 (pre-immune), 14, 21, 42 and 72 post-immunization (pi), using rLipL21/rI-Lip21/WCL as the immobilized antigen. The antibody levels were found to be significantly elevated in serum

Figure 5. Relative mRNA expression levels of (A) TNF-α, (B) IL-12p40 (C) IL-10, (D) IL-4, and (E) IFN-ϒ in mice pooled blood samples. The relative Ct (ΔΔ Ct) method was used to quantify cytokine gene expression: Cts were normalized against the GAPDH gene Ct (Δ Ct) and compared. The values represent grouped results of two independent experiments. *(p < 0.05).
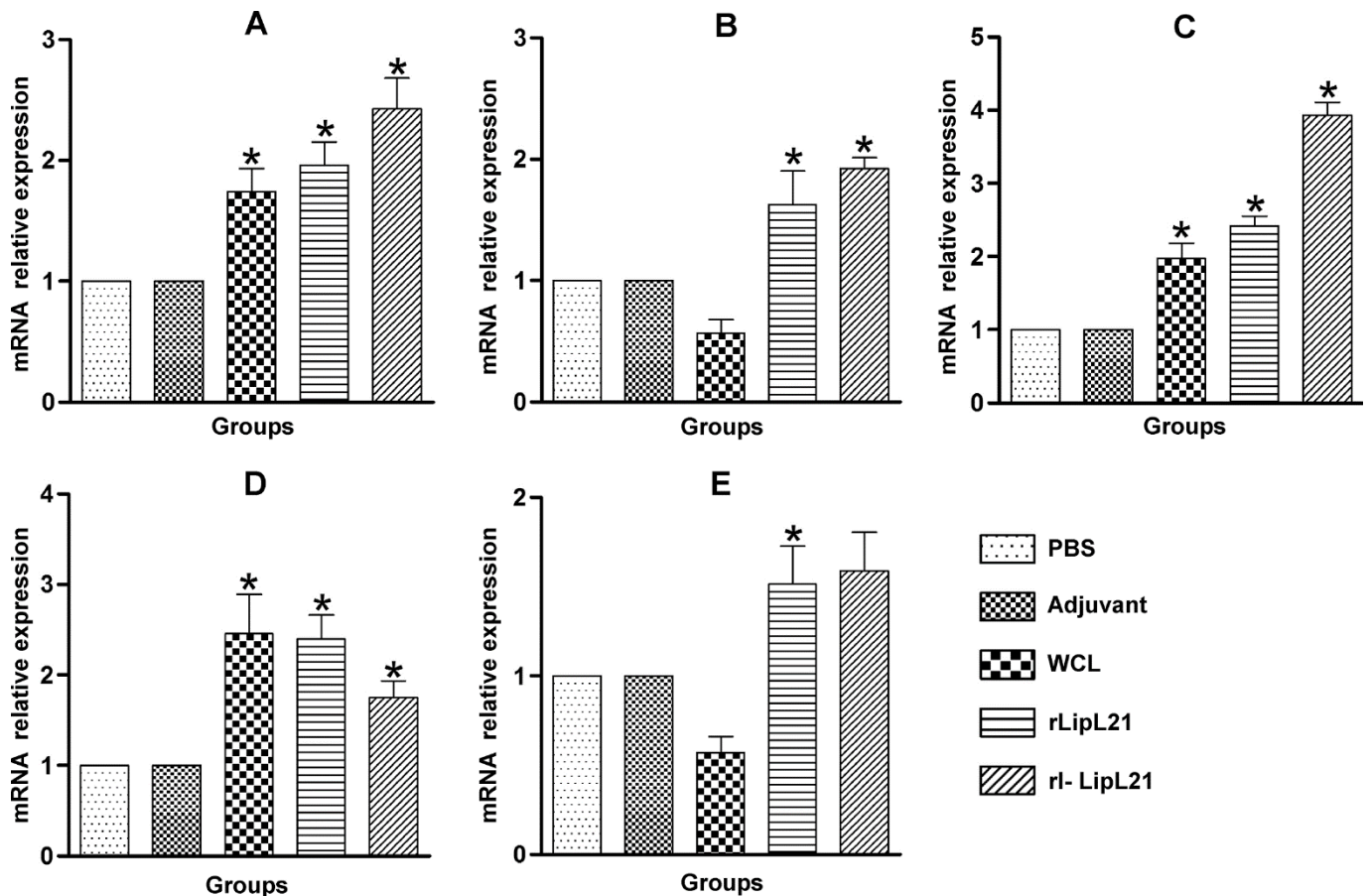
samples from mice immunized with rI-LipL21 followed by rLipL21, and WCL from 21-day post immunization (Figure 3). No significant levels of IgG were detected in animals immunized with PBS or adjuvant alone (p > 0.05).

Lymphoproliferative response

The lymphocytes obtained from the animals of various groups proliferated significantly (p ≤ 0.05) when stimulated *in vitro* with varying concentrations of antigens. The proliferation response occurred in a dose dependent manner. The proliferation response of cells exposed with rI-LipL21 antigen showed significant (p ≤ 0.05) response, followed by rLipL21 and WCL. The maximum proliferation in each group was obtained when the cells were stimulated with a concentration of 10 μg/mL (Figure 4). The lymphocytes obtained from control animals that were immunized with PBS or Alhydrogel adjuvant the proliferation was found to be insignificant (p > 0.05).

Cytokine expression profile in mice

The relative mRNA expression of TNF-α, IL-12p40, IL-10, IL-4, and IFN-γ, was evaluated by qRT-PCR [22]. TNF-α, IL-10 and IL-4 showed elevated mRNA levels in lymphocytes immunized with rLipL21, rI-LipL21 and WCL (p<0.05) compared to control groups. IL-12p40, and IFN-γ mRNA levels were specifically elevated during immunization with rLiPL21 and rI-LipL21 and not WCL. (Figure 5A, B, C, D, E).

Efficacy of rLipL21 and rI-LipL21 in challenge experiments

The protective efficacy of rLipL21 and rI-LipL21 in mice against N2 strain was determined in two independent experiments. The survival analysis showed 83% and 66% survival among mouse groups immunized with rI-LipL21 and rLipL21 respectively (Figure 6 and Table 3). Though immunization with WCL showed 100% protection against leptospiral challenge, the use of recombinant purified protein can reduce side-effects that may arise due to the presence of

Figure 6. Survival of mice immunized with WCL, rLipL21 and rI-LipL21 after lethal challenge. Survival curves were compared using log-rank analysis. Y-axis percent survival and X-axis days post challenge. The results are a summary of two independent experiments.
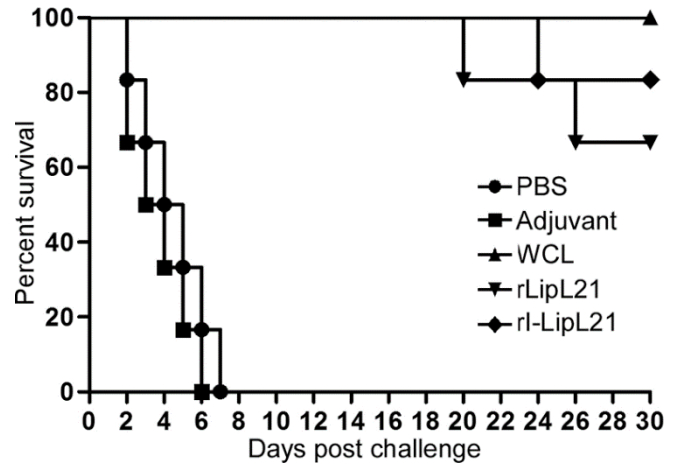
LPS in the WCL. Also protected animals showed 40% clearance of leptospires from kidney tissues compared to PBS/alhydrogel treated mice.

Discussion

Currently, the leptospiral vaccine preparations using bacterin for immunization of humans and animals suffer from several limitations including severe side effects, short-term immunity and restricted-serovar protection. This is due to the presence of serovar specific LPS in its preparations as major antigenic component. In recent years, several studies have evaluated different formulations of recombinant vaccine candidates with the intention of improving leptospiral vaccines. Among them the leptospiral immunoglobulin-like proteins (Lig) evidenced a promising candidate. Mice immunized with LigA/LigB survived lethal challenge, showing 90–100% of protection [23,24]. Using *in vivo* expressed protein Lig-domain proteins may have a significant role in inflammatory responses in addition to its protective immunity [25]. In a recent study leptospiral outer membrane protein LipL21 and its truncated N-terminal immunogenic region (I-LipL21) were evaluated for diagnosis of leptospirosis [14]. The use of rLipL21 or

Table 3. Effect of immunization with rLipL21, rI-LipL21 and leptospiral WCL antigens in mice.

Treatment group	No. Survivors / Total (% protection)			(%) Culture Positive
	# Exp 1	# Exp 2	# Total	
I-Lipl21	5/6(83.3)	5/6(83.3)	10/12(83.3)	2/5(60)
LipL21	4/6(66.6)	4/6(66.6)	8/12(66.6)	3/4(75)
WCL	6/6(100)	6/6(100)	12/12(100)	3/6(50)
Freund's adjuvant	0/6(0)	0/6(0)	0/12	6/6(100)
PBS	0/6(0)	0/6(0)	0/12	4/4 (100)

rI-LipL21 in the form of dot blot or ELISA diagnostics showed a significant sensitivity and specificity for diagnosis of leptospirosis [14]. Considering the diagnostic potential of rLipL21 and rI-LipL2,1we hypothesize that these proteins might be strong immunogens. In line with this studies hypothesis, the immunization of Cy mice with rLipL21/rI-LipL21 elevated the lymphoproliferation (Figure 4), and pro-inflammatory cytokine mRNA levels (Figure 5). The *in vitro* lymphoproliferative assay showed optimal spleenocyte proliferation at a concentration of 10μg/mL. Whereas the use of 100μg/mL antigens succumbed the spleenocyte proliferation evidencing a cytotoxic effect. This highlights the use of optimal concentration of antigens for effective immunization against leptospirosis. Additionally, this study showed immunization with rLipL21/rI-LipL21 activated innate immune responses, resulting in the production of a vast spectrum of pro-inflammatory cytokines. These pro-inflammatory cytokines are known to trigger activation of immune cells and pathogen clearance thereby reducing infection and inflammation of host tissues. From this studies survival analysis, the activation of both humoral and cell-mediated immune response by rLipL21/rI-LipL21 was predicted that enabled the protective immunity against leptospiral infection as shown in the results (Figure 6).

Due to the maintenance difficulties and supply of hamsters or guinea pigs for routine experimentation, mice, as an alternate, are being considered for leptospiral vaccines assessment and immunogenicity [10]. Since there was limited access to golden Syrian hamsters during this study, Cy mice was used as an alternate as used in previous studies [8,9,10]. In the case of non-availability of hamsters, the Cy mice could be an ideal experimental model which has been further evidenced in this study through MACS specific challenge experiments in comparison with hamsters and BALB/c mice. Moreover, Syrian hamsters have certain limitations for the measurement of cytokine profile at the transcriptional level [26,27]. The use of Cy mice as a susceptible model was adequately validated in studies from other groups [8,9]. These studies have substantiated the usage of mice as an animal model for immunization and challenge experiments for leptospires and its products. Immunization with rLipL21/rI-LipL21 significantly elevated the antibody levels, substantiating an effective humoral immune response for these proteins in the immunized mouse model.

The cytokine profile of the immunized mice showed increased circulating TNF-α at 42nd day post immunization. Delayed and sustained TNF-α production has been associated with a poor prognosis during infection [28]. IL-12p40 acts as a chemoattractant for macrophages and promotes the migration of bacterially stimulated dendritic cells. It is associated with several pathogenic inflammatory responses but it is also protective in a mycobacterial model. The independent function of IL-12p40 is important for improving understanding of both protective and pathogenic immune responses [29]. The anti-inflammatory cytokine IL-10 and IL-4 was also up regulated in rI-LipL21/rLipL21 immunized mice, demonstrating stimulated activation of B-cell and T-cell. IFN-γ production was related to protection in cattle vaccinated with monovalent serovar Hardjo vaccines [30]. In the present study, however, significantly increased IFN-γ mRNA levels (Figure 5E) were found in mice immunized with rLiPL21 and rI-LipL21, demonstrating the indication of protective immune response for these proteins. This has been further substantiated by the significant increase in lymphoproliferative response against rLipL21 and rI-LipL21.

Conclusions

In summary, the combined B and T cell-mediated immune response in this study offered protective immunity in mice immunized with rI-LipL21/rLipL21 against the lethal infection of Autumnalis N2 in Cy mice during challenge experiments. It is recommended that rI-LipL21 may be an ideal vaccine candidate for the protective immunity against leptospirosis.

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