Therapeutic epitopes of *Leptospira* LipL32 protein and their characteristics

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Two LipL32-specific mouse monoclonal antibodies (mAbLPF1 and mAbLPF2) which neutralized *Leptospira*-mediated hemolysis *in vitro* and rescued hamsters from lethal *Leptospira* infection were produced. In this communication, locations and characteristics of the protective epitopes of the mAbs were studied by using a truncated LipL32 recombinant protein based-immunoassay and phage consensus mimotope identification and multiple alignments. The mAbLPF1 epitope consisted of P243, L244, I245, H246, L252 and Q253 on the LipL32 protein; it is mapped on the surface-exposed region of non-continuous β13-turn and C-terminal amphipathic α6 helix with hydrophobic patch, contributing to phospholipid/host cell adhesion and membrane insertion on one side, and hydrophilic, acidic and basic amino acid residues on another side. The epitope peptide of the mAbLPF2 is linear 122PEEKSMPHW130 and located on surface-exposed α1 and α2 between β7 and β8 that bound to several host constituents. Both epitopes are highly conserved among the pathogenic and intermediately pathogenic *Leptospira* spp. and are absent from the LipL32 superfamily proteins of other microorganisms. This study not only enlightens the molecular mechanisms of the therapeutic mAbLPF1 and mAbLPF2, but also elaborates the potential of the two LipL32 regions as diagnostic and vaccine targets for leptospirosis.

Keywords: epitope mapping/ *Leptospira* adhesive matrices/ leptospirosis/mimotopes/neutralizing mAb

Introduction

Presently *Leptospira* bacteria are divided according to genetic relatedness based on 16S rRNA: pathogenic (*Leptospira australiensis*, *L. borgpetersenii*, *L. alstoni* [genospecies 1], *L. interrogans*, *L. kirschneri*, *L. kentui*, *L. noguchii*, *L. santarosai* and *L. weilii*), intermittently pathogenic (*L. brounii*, *L. jaimei*, *L. inadai*, *L. licerasiae* and *L. wolffii*) and saprophytic (*L. biflexa*, *L. meyeri*, *L. terprestrae* [genospecies 4], *L. vanthieli* [genospecies 3], *L. wolbachii* and *L. yanagawae* [genospecies 5]) (International Committee on Systematics of Prokaryotes, 2008; Adler and de la Pena-Moctezuma, 2010; Ganoza et al., 2010). LipL32 is regarded as a major outer membrane lipoprotein, which is highly conserved at both genetic and protein levels (Haake et al., 2000; Guerreiro et al., 2001; Murray, 2013). This protein was found to be *Leptospira* hemolysin and enhances the hemolytic activity of sphingomyelinase-H (SphH), and hence given its synonym, hemolysis-associated protein-1 (Hap-1) (Lee et al., 2000; Hauk et al., 2005). Pathogenicity-related *Leptospira* expresses LipL32 constitutively in both the *in vitro* culture and the infecting mammalian hosts (Haake et al., 2000; Sakolvaree et al., 2007). The protein is highly immunogenic; LipL32-specific IgG can be detected in acute and convalescing leptospirosis sera (Guerreiro et al., 2000; Sakolvaree et al., 2007) and has been identified as a member of *Leptospira* adhesive matrices (MSCRAMMs), responsible for binding to the extracellular matrix (ECM) molecules, including matrigel, laminin, collagens (I and IV) and intact as well as 30 and 45-kDa proteolytic fragments of fibronectin (FN) (Hauk et al., 2008; Hoke et al., 2008). LipL32 also binds to zymogen plasminogen to generate plasmin (Vieira et al., 2010) and adheres to the proteoglycan of human cell surface receptors (Breiner et al., 2009), cultured mammalian cells (Liu et al., 2007) and neutrophils (Wang et al., 1984). Thus, LipL32 is an attractive target for not only leptospirosis diagnosis (Saengjaruk et al., 2002; Bomfim et al., 2005; Toyokawa et al., 2011) but also broad-spectrum vaccines (Branger et al., 2005; Seixas et al., 2007; Aviat et al., 2010) and passive immunotherapy (Maneewatch et al., 2008, 2009).

Two murine hybridoma clones secreting monoclonal antibodies (mAbs) specific to the *Leptospira* LipL32 (mAbLPF1 and mAbLPF2) were produced previously. Both mAbs neutralized *Leptospira*-mediated hemolysis *in vitro* and rescued experimentally infected hamsters with heterologous *Leptospira* (Maneewatch et al., 2008, 2009). Moreover, single-chain variable fragments (mouse ScFv) and humanized-ScFv of mAbLPF1 retained the therapeutic effects of the intact mAb in hamsters infected with heterologous *Leptospira* spp. (Maneewatch et al., 2009). This study was conducted to determine peptide epitopes of mAbLPF1 and mAbLPF2 and their characteristics, to provide insight into the molecular mechanisms of *Leptospira* neutralizing antibodies.

Materials and methods

**Leptospira cultures and antigens**

*Leptospira* spp. were cultured at 30°C under aerobic conditions in liquid Ellinghausen-McCullough–Johnson–Harris...
Recombinant full-length LipL32 protein (LipL32-FL), consisting of 272 amino acids, was produced from a transformed BL21(DE3) *Escherichia coli* clone carrying lipl32-FL-pET23a(+) plasmid (Maneewatch et al., 2008). The bacterial colonies were inoculated into the Luria-Bertani (LB) broth containing 100 μg/ml ampicillin and cultured at 37°C with shaking at 200 rpm until the OD_{600} nm was 0.2. Expression of the recombinant protein was induced by adding 0.2 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) to the culture and bacteria were grown for 3 h. The recombinant protein in the *E. coli* lysate was purified using Co²⁺ metal-resin and gradients of imidazole solutions. The purified recombinant LipL32-FL was able to bind to ECM, plasma FN and heparin sulfate (HS), which were the inherent property of the native LipL32 (Hauk et al., 2008). The recombinant protein was also tested for binding to cell membrane lipid components, i.e. cholesterol (C), phosphatidylcholine (PC), as well as mammalian (Madin-Darby canine kidney, MDCK) cell surface. Two N-terminally truncated LipL32 recombinant proteins (LipL32-1 and LipL32-2) were prepared; these contained amino acid residues 91–272 and 171–272, respectively. Two N-terminally truncated LipL32 recombinant proteins (LipL32-1 and LipL32-2) were prepared; these contained amino acid residues 91–272 and 171–272, respectively. Two truncated LipL32 segments were individually amplified by PCR from lipl32-FL-pET23a(+) plasmid DNA template, using the reverse primer sequence p23/R specific to the 3'-end of the forward and 3'-end of the reverse primers to facilitate subsequent gene cloning. PCR amplicons were purified, digested with NdeI endonuclease and subsequently ligated using T4 DNA ligase (NEB, MA, USA). The resulting plasmids, lipl32-1-pET23a(+) and lipl32-2-pET23a(+), were introduced separately into competent *E. coli* by a heat-shock method. The presence of individual recombinant plasmids in the transformed *E. coli* was verified by DNA sequencing. Recombinant LipL-32-1 and LipL-32-2 were purified from the appropriate transformed *E. coli* lysates as for the LipL32-FL. All of the three recombinant LipL32 proteins were subjected to 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred onto nitrocellulose membranes (NC), blocked with blocking buffer (3% bovine serum albumin, BSA, in PBST), incubated with mouse monoclonal anti-6×-His antibody (1 : 3,000) and subsequently incubated with goat anti-mouse IgG immunoglobulin-alkaline phosphatase (AP) conjugate (diluted 1 : 3,000) (Southern Biotechnology, AL, USA). Antigen-antibody reactive bands were revealed using a BCIP/NBT chromogenic substrate (KPL, MA, USA).

**Bioactivities of the recombinant LipL32-FL protein**

Interaction of the recombinant LipL32-FL protein with various host components was studied by using an indirect enzyme-linked immunosorbtent assay (ELISA) (Barbosa et al., 2006; Tung et al., 2010), including plasma FN (affinity purified from human plasma by melting the gelatin immobilized on membrane as described previously by Chifflet et al., 2004), matrigel ECM basement membrane (containing majority of laminin, collagen I and entacin isolated from Engelbreth–Holm–Swarm mouse sarcoma) and HS (Becton Dickinson, Canada), which was the reported inherent property of the native Lip32 protein. Major lipid components of mammalian (MDCK) cell membrane, i.e. C and PC (egg yolk L-α-PC from Sigma, MO, USA) were also included in the LipL32-FL binding assays. Recombinant HlpA sugar transferase produced from *L. interrogans* serovar Copenhageni and non-acetylated fraction V bovine serum albumin (Thermo Fisher Scientific, USA) served as irrelevant *Leptospira* protein and background binding controls, respectively. Triplicate wells of ELISA plate (Nunc-Immuno Plate MaxiSorp Surface) were coated separately with the test materials: 100 μl of phosphate buffered saline (PBS) containing 1 μg of matrigel ECM/FN/BSA, 100 μl of PBS containing 1.2 μg of HS and the plate was kept at 4°C overnight, and 100 μl of absolute ethanol containing 10 μg of C/PC and kept at 37°C until dried. After the unbound materials were washed away with 0.05% Tween-20 in PBS (PBST) and the unoccupied sites on the plastic surface blocked with 1% BSA in PBS, 150 μl of recombinant LipL32-FL protein or 1 μg of HlpA sugar transferase in PBS was added individually and to appropriate wells. The plate was then kept at 37°C for 1 h. The recombinant LipL32-FL protein was used as a positive control. The recombinant LipL32-FL protein was used as a positive control. The recombinant LipL32-FL protein was used as a positive control.

![Diagram](image-url)
LipL32-FL/HlpA protein bound to the immobilized test materials/controls, and was detected by adding 100 μl of mouse monoclonal anti-6×-His antibody (1:3000 in PBS) and incubated at 37°C for 1 h. Goat anti-mouse/rabbit IgG-horseradish peroxidase (HRP) conjugate (Southern Biotechnology, USA) and ABTS (KPL, MD, USA) substrate were used for the color reaction. After terminating the enzymatic reaction by adding 50 μl of 1% SDS to each well, the OD_{405 nm} of content in each well was measured against a reagent blank using a Multiscan EX microplate reader (Labsystems, Helsinki, Finland). Three independent experiments were performed.

LipL32-FL was also tested for their binding to the surface of mammalian cells using HlpA as the irrelevant Leptospira protein control. MDCK cells in Dulbecco’s modified Eagle medium (DMEM; Invitrogen, USA) containing supplements and antibiotics were seeded into individual wells of a 96-well tissue culture plate (Costar) (1 × 10^5 cells/well), which was then kept in a humidified 5% CO2 atmosphere until the cell monolayer was obtained. LipL32-FL and HlpA proteins were added separately into the MDCK cell monolayer in triplicate. Negative control wells with PBS instead of LipL32-FL/HlpA protein were included. After incubation, all wells were washed thoroughly and subjected to the indirect ELISA procedure described above. Significant binding of the LipL32-FL to the mammalian cell surface above the binding of the HlpA protein and the PBS controls was noted. The mean ± SD of OD_{405 nm} of the contents of the nine LipL32-FL test wells was compared with that of the HlpA and the BSA control wells. P < 0.05 was considered significantly different (Student’s two-tailed t test).

**Mouse mAbs**

Mouse mAbs used in this study included mAbs specific to LipL32 lipoprotein of pathogenic *Leptospira* spp.; mAbLPF1 and mAbLPF2 (Maneeewatch et al., 2008) and control mAb204D3 specific to Antigen 9 in LPS of Group D *Salmonella* Typhi (Chaicumpa et al., 1988). The hybridomas were cultured either in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (HyClone, UK), 2 mM L-glutamine, penicillin and streptomycin (100 U/ml and 100 μg/ml, respectively) (Invitrogen) or in protein-free CD hybridoma medium supplemented with 2 mM L-glutamine (Invitrogen) at 37°C in humidified 5% CO2. The monoclonal immunoglobulin in the hybridoma spent CD medium was concentrated and buffer exchanged to PBS, pH 7.4, using Amicon Ultra-tubes (Millipore, Billerica, MA, USA). The IgG content of each preparation was determined at OD_{280 nm}.

**LipL32 subdomains bound by the mAbLPF1 and mAbLPF2**

The LipL32-FL, LipL32-1 and LipL32-2 proteins were subjected to 15% SDS–PAGE and the separated components were blotted onto NC. Unoccupied sites on the NC were blocked with 3% BSA in PBS. After washing, all membranes were placed in individual mAb solutions (spent RPMI-1640 culture medium) and kept at 25°C for 1 h. After washing thoroughly with PBST, the membranes were placed in a solution of goat anti-mouse immunoglobulin-AP conjugate (diluted 1:3,000). Immunoreactive bands were revealed using a BCIP/NBT substrate.

**Leptospiral surface ELISA**

Double antibody sandwich ELISA (Matsunaga et al., 2006) was used for determining the reactivity of mAbs to native LipL32 on intact leptospires. Whole cells of *L. interrogans* serovar Icterohaemorrhagiae were harvested, washed twice with EMJH base medium and immobilized with 75 mM sodium azide. A *Leptospira* suspension containing 10^5 cells in 100 μl was added to each well of a Maxisorb ELISA plate (Nunc, Germany) which had been coated with a rabbit anti-*Leptospira* polyclonal antibody (diluted 1:5,000) and incubated at 37°C for 2 h. After washing and blocking the well surface with 3% BSA in PBST, 100 μl of mAbLPF1, mAbLPF2 and irrelevant mAb204D3 was added individually to the wells and incubated at 37°C for 1 h, followed by washing three times with the PBST. Each well was then filled with 100 μl of goat anti-mouse IgG-HRP conjugate (diluted 1:3,000) (Southern Biotechnology) at 37°C for 1 h. ABTS substrate (KPL) was added (50 μl/well). Enzyme–substrate reaction was allowed to occur at 25°C in the dark for 15 min; the reaction was stopped by adding 50 μl of 1% SDS. The OD_{405 nm} of the content of each well was determined against the blank.

**Phage mimotopes and LipL32 epitopes bound by the mAbs**

A Ph.D.-12™ phage display library (NEB, MA, USA) containing 2.7 × 10^12 random 12-mer peptide sequences (~1.5 × 10^8 pfu/ml) was used for searching phage display peptides that bound to mAbLPF1 and mAbLPF2 (phage mimotopes). Briefly, mAb IgG of individual hybridomas from serum-free CD medium (10 μg in 100 μl of 1× TBS) was coated onto ELISA wells (Costar) and kept at 4°C overnight. Unoccupied sites were blocked with 3% BSA in PBST at 25°C for 1 h. After washing with TBS containing 5% Tween-20 (TBST), the 12-mer peptide phage display library (100 μl containing ~4 × 10^8 pfu/ml) was added to each well and kept at 25°C for 1 h. After washing away the unbound phages, the mAb-bound phages were eluted out with 0.2 M glycine–HCl, pH 2.2, and immediately neutralized with few drops of Tris, pH 9.0. The eluted phages were amplified in log-phase grown ER2738 *E. coli*. Phage particles in individual *E. coli* culture supernatants were concentrated by PEG/NaCl precipitation and used in the next panning round. Three consecutive rounds of panning were performed. Phages recovered from the final panning round were used to transfect ER2738 *E. coli* in top agarose overlay on the LB/X-Gal/IPTG agar plate. After over-night incubation at 37°C, 15 isolated blue plaques were picked randomly from each plate and propagated in the ER2738 *E. coli*. The culture supernatants containing phage particles were added with the PEG/NaCl solution. Single-stranded DNA were extracted from the precipitated phages, and the respective DNA insert in each phage clone was sequenced with the −96 gIII sequencing primer as described in the instruction manual and deduced into peptide sequences using translate tool (http://web.expasy.org/translate/).

The phage displayed peptide sequences (phage mimotopes) were multiply aligned using the MIMOX program (Huang et al., 2006) with manual adjustment to identify the mimotope consensus sequences. The consensus mimotopes were then aligned with the LipL32 amino acid sequence to locate the presumptive peptide sequences of LipL32 bound by the mAbs (LipL32 epitopes), using multiple alignments ClustalW2.1 Server.

Phage clones displaying the mimotopes that bound to mAbLPF1 and mAbLPF2 were validated by testing the binding inhibition capacity of the respective mAbs to immobilized-LipL32. Phages were propagated in ER2738 *E. coli* and the
Prevalence of LipL32 epitopes among Leptospira spp. and LipL32 protein superfamily

Peptide epitopes of mAbLPF1 and mAbLPF2 were multiply aligned through ClustalW2.1 Server with LipL32 peptide sequences of several genomospecies of pathogenic and intermediate Leptospira spp. in the NCBI database, using BLASTP 2.2.26+ Server (http://www.ncbi.nlm.nih.gov/Entrez/) (Altschul et al., 1997). Prevalence of the epitopes was determined. Moreover, the epitope peptides were also aligned with the orthologous proteins of the LipL32 superfamily, retrieved from the annotated conserved domain database (http://www.ncbi.nlm.nih.gov/Entrez/) (Marchler-Bauer et al., 2009).

Structural characterization of LipL32 epitopes

Three-dimensional (3D) structures of LipL32 fragments containing epitopes bound by mAbLPF1 and mAbLPF2 were constructed by using a homology model, available from the PDB (ID 2WFK) database via SWISS-MODEL Server (Arnold et al., 2006). The reliability of the constructed models was verified by QMEAN Z-score calculation (Benkert et al., 2011) and visualizing using the POLYVIEW visualization server (Porollo and Meller, 2007). Alpha-helix conformation of the protein was analyzed by HELIQUEST server (Gautier et al., 2008). Lysine acetylation (K-Ac) sites were predicted from the LipL32 sequence by PAIL server.

Binding inhibition of recombinant and native LipL32 to host components and mammalian cells by mAbs

Both mAbLPF1 and mAbLPF2 were tested for their ability to inhibit binding of the recombinant and native LipL32 in whole-cell lysate to C, PC and MDCK cell monolayer. MAb204D3 was used as irrelevant control. Two micrograms of the recombinant LipL32 and the L. interrogans Copenhageni lysate were separately incubated with individual mAbs (2 μM) and kept at 25°C for 2 h prior to adding the mixtures into the immobilized FN, ECM, HS, C, PC or the MDCK cell monolayer in ELISA wells and kept at 25°C for 1 h. After washing, mouse anti-6×-His and rabbit anti-Leptospira polyclonal antibodies were used for detecting LipL32 in the wells. ELISA color reactions were performed. The OD405 nm of the content of each well was determined against the reagent blank. Percent binding inhibition mediated by the mAb was calculated: % binding inhibition = [(OD405 nm of the LipL32 binding to the ligand-OD405 nm of the mAb-treated LipL32 binding to the ligand)/(OD405 nm of the LipL32 binding to the ligand)] × 100.

Results

Recombinant LipL32 proteins

Recombinant LipL32-FL (Residues 1–272), LipL32-1 (Residues 91–272) and LipL32-2 (Residues 171–272) were successfully expressed as fusion proteins with an N-terminal T7 peptide tag and C-terminal 6×-histidine tag in transformed BL21(DE3) E. coli under IPTG induction. All proteins were found in both soluble and insoluble E. coli fractions (data not shown). Western blot patterns of the three recombinant proteins with the relative molecular sizes of 29.6, 20.0 and 13.7 kDa, respectively, are shown in Lanes 1–3 of Supplementary Fig. S1. The recombinant LipL32-FL was found to have native LipL32 bioactivities in binding to tested host components, including FN, matrigel ECM and HS. The protein also bound to C, PC and MDCK cells and gave the OD405 nm above the HlpA protein and BSA controls (P < 0.05) (Supplementary Fig. S2).

Recombinant LipL32 subdomains bound by mAbLPF1 and mAbLPF2

SDS–PAGE and Coomassie Brilliant Blue G250 stained patterns of LipL32-FL, LipL32-1 and LipL32-2 are shown in Fig. 2A. Western blot analysis revealed that mAbLPF1 bound to full-length LipL32 as well as to the two truncated LipL32 proteins (Fig. 2B), while mAbLPF2 recognized LipL32-FL and LipL32-1 (Fig. 2C) only. The results of mAb reactivity assessed by Western blotting were conformed to the ELISA results (data not shown).

Binding of mAbs to Leptospira spp.

The leptospiral surface ELISA results showed that both mAbLPF1 and mAbLPF2 bound to intact Leptospira bacteria (ELISA signals at OD405 nm above the irrelevant control were 0.427 ± 0.59 and 0.447 ± 0.32, respectively; and ELISA signal of the binding by the irrelevant mAb204D3 was 0.06 ± 0.15). There was no significant difference in Leptospira binding between the two mAbs.

Phage peptides (mimotopes) that bound to mAbs

From the 15 randomly picked phage clones that appeared on the selective agar plate spread with mAbLPF1 bound phages, three mimotope types (KPHSIPLLDLTR, KPPQMPLYNLSA, and NVLPLLESSLQY) were found in two phage clones each (Table I). Moreover, the epitope peptides were also aligned with the orthologous proteins of the LipL32 superfamily, retrieved from the annotated conserved domain database (http://www.ncbi.nlm.nih.gov/Entrez/) (Marchler-Bauer et al., 2009). Lysine acetylation (K-Ac) sites were predicted from the LipL32 sequence by PAIL server.

Binding inhibition of recombinant and native LipL32 to host components and mammalian cells by mAbs

Both mAbLPF1 and mAbLPF2 were tested for their ability to inhibit binding of the recombinant and native LipL32 in whole-cell lysate to C, PC and MDCK cell monolayer. MAb204D3 was used as irrelevant control. Two micrograms of the recombinant LipL32 and the L. interrogans Copenhageni lysate were separately incubated with individual mAbs (2 μM) and kept at 25°C for 2 h prior to adding the mixtures into the immobilized FN, ECM, HS, C, PC or the MDCK cell monolayer in ELISA wells and kept at 25°C for 1 h. After washing, mouse anti-6×-His and rabbit anti-Leptospira polyclonal antibodies were used for detecting LipL32 in the wells. ELISA color reactions were performed. The OD405 nm of the content of each well was determined against the reagent blank. Percent binding inhibition mediated by the mAb was calculated: % binding inhibition = [(OD405 nm of the LipL32 binding to the ligand-OD405 nm of the mAb-treated LipL32 binding to the ligand)/(OD405 nm of the LipL32 binding to the ligand)] × 100.
that the phage mimotopes carried many amino acid residues analogous to LipL32 which validated the mimotope search results.

**Prevalence of the LipL32 epitopes**

The epitope peptide sequences of the mAbLPF1 (PLIH-----LQ) and mAbLPF2 (PEEKSMPHW) were multiply aligned with 70 LipL32 peptide sequences of *Leptospira* spp. belonging to 11 genospecies: *L. interrogans* (serovars Australis, Autumnalis, Balico, Canicola, Copenhageni, Grippotyphosa, Hardjo, Hebdomadis, Icterohaemorrhagiae, Jalna, Javanica, Kremastos, Lai, Manilae, Mini, Paidjan, Pomona, Pyrogenes and Wolffi), *L. noguchii* (serovar Pomona), *L. kirschneri* (serovar Bim), *L. borgpetersenii* (serovars Ballum, Hardjo-bovis, Javanica, Mini and Tarassovi), *L. weilii* (serovar Manhao II), *L. santarosai* (serovar Shermani), *L. alexanderi*
(serovar Manhao 3), L. licerasiae (serovar Varillal), L. inadai (serovar Lyme), L. broomii and L. kemiyi (serovar Malaysia), deposited in the NCBI database (accessed on Aug 2012). It was found that both epitope peptides were highly conserved (Tables II and III). There are four variants of the mAbLPF1 epitope (Variants 1–4) among the 70 LipL32 NCBI database sequences (Table II). Variant 1 (PLIH-----LQ) was predominant (62 of 70) among the pathogenic Leptospira spp., Variant 2 (PAVH-----LQ) was found in one strain of pathogenic L. interrogans, Variant 3 (PIVH-----LQ) was found in two species of intermediately pathogenic Leptospira spp. (L. inadai and L. inadai) and Variant 4 (PILH-----LE) was found in two strains of the intermediately pathogenic L. licerasiae.

There were six variants of the mAbLPF2 epitope (Variants 1–6) (Table III). Variant 1 (122PEEKSMPHW 133) was predominant (59 of 70) among the pathogenic species. Variant 2 (P122R) and Variant 3 (P122L) were found in two strains and one strain, respectively, of the pathogenic Leptospira spp. Variant 4 (H129S) was found in L. kemiyi. Variant 5 (H129N) was found in both pathogenic and intermediately pathogenic species. However, Variants 2–5 had one conserved polar amino acid substitution. Variant 6 had S126C and two amino acid deletions at positions 127–128 and was found in one LipL32 sequence of L. interrogans.

Multiple alignments were also performed between the epitope sequences and orthologous proteins of the LipL32 superfAMILY including three outer membrane proteins (OMPs) of bacteria of the genus Pseudolalteromonas: P. tunicata-D2 (Holmstrom et al., 1998), P. rubra (ATCC29570) and P. spongiae (UST010723-006); two proteins of the genus Cellulophaga: C. lytica (DSM7489) and C. algicola (DSM14237) (Bowman, 2000); LipL32 proteins from Moritella sp. (PE36), Marinomonas mediterranea (MBB-1), unidentified Eubacterium (SCB49); and the OMP of Treponema brennaborense (DSM12168). The LipL32 epitope sequences were not found in any of the orthologous proteins of the LipL32 superfAMILY derived from other microorganisms (Fig. 3).

**Locations and structures of the peptide epitopes**

The mAbLPF1-specific epitope, i.e. P243, L244, I245, H246, L252 and Q253, was located at the previously reported β13 region and C-terminal α-helix (α6) of the LipL32 crystal structures (Vivian et al., 2009) (Fig. 4A). The α6 helix contained potential internal lysine acetylation (K-Ac) sites, i.e. K254, K264 and K265. Analysis of this helix using HELIQUEST server revealed that the helix had surface-exposed hydrophilic residues (Q253, Q255, I257, S262) and electrostatic (K254, K264 and K265) and negative patch (E250, E251, E260, E261) on one side and hydrophobic residues (P249, L252, A256, A259, A266) on another side (Fig. 4B). The amphipathic helix is important in membrane binding and insertion (Thiyagarajan et al., 2004).

| Table II. Variants of LipL32 epitope at the location that interacted with the mAbLPF1 among different pathogenic and intermediately pathogenic Leptospira spp. |
|----------------------------------------|---------------------------------|-------------------|-------------------|
| Epitope variant | Amino acids at the mAbLPF1 epitope (underlined) | Frequency | Accession no. | Leptospira spp. | Clade |
| 1 | 236 PGIPUVSPLHNSPRELG 253 | 62 | 62 different accession no. | Pathogenic species | P |
| 2 | 236--CGNAV--253 | 1 | ADC80912.1 | L. interrogans | P |
| 3 | 236--T--LIV--E--253 | 2 | ZP_00536517.1 | L. broomii | I |
| 4 | 236--V--L--IL--A--A--253 | 2 | ZP_09259465.1 | L. licerasiae | I |
| 5 | * | * | * | * | * |

*From 70 LipL32 sequences of the database (accessed on August 2012). There were three LipL32 database sequences that had C terminal deletions (Δ243–272, Δ248–272 and Δ252–272).

**Table III. Variants of LipL32 epitopes at the location of interaction with the mAbLPF2 among different pathogenic and intermediately pathogenic Leptospira spp.**

<table>
<thead>
<tr>
<th>Epitope variant</th>
<th>Amino acids at the mAbLPF2 epitope (underlined)</th>
<th>Frequency</th>
<th>Accession no.</th>
<th>Leptospira spp.</th>
<th>Clade</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>120 ATPEEKSMPHFDFT 133</td>
<td>59</td>
<td>59 different accession nos.</td>
<td>Pathogenic species</td>
<td>P</td>
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<tr>
<td>2</td>
<td>120--R--133</td>
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<td>L. interrogaens</td>
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<td>1</td>
<td>ZP_10521264.1</td>
<td>L. kemiyi</td>
<td>P</td>
</tr>
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</tr>
<tr>
<td>6</td>
<td>*</td>
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</table>

*From 70 sequences of LipL32 of the NCBI database (accessed on August 2012).

Dash indicates amino acid identity. (•) identical amino acid; (•) conserved amino acid substitution; and (•) semi-conserved amino acid substitution.
The linear epitope of mAbLPF2 (122PEEKSMPHW130) was located at the surface-exposed α1 and α2 of β7–β8 (green shade in Fig. 4C).

**MAbs mediated the inhibition of binding of recombinant and native LipL32 proteins to host components**

The irrelevant mAb204D3 did not inhibit the binding of recombinant LipL32 or the *Leptospira* lysate to the test materials (data not shown). Fig. 5A shows the percentage mAb-mediated inhibition of the recombinant LipL32-FL binding to C, PC and MDCK cells. Binding of the LipL32-FL to C, PC and MDCK cells was inhibited by mAbLPF1 at rates of 72, 54 and 24%, respectively. The binding inhibition mediated by mAbLPF2 was at rates of 56, 39 and 30%, respectively. The two mAbs did not show significant binding inhibition of rLipL32-FL to FN, ECM and HS ligands when compared with inhibition by the irrelevant mAb204D3 control.
Leptospira lysate containing native LipL32 to the C, PC and toxic material released from the lysed bacteria (Watt and occur in antibiotic-treated cases due to the massive amounts of leptospirosis. Occasionally, Jarisch–Herxheimer reaction Penicillin is the primary drug of choice for the treatment of leptospirosis, especially in antibiotic-allergic subjects. In this study, locations, structural characteristics and biofunctions of peptide epitopes of both mAbs were studied in order to elucidate the molecular mechanisms of the mAb in mediating Leptospira’s neutralizing effects.

Recombinant full-length LipL32 (LipL32-FL, 272 amino acids) were produced and purified. LipL32-FL was found to have an inherent molecular matrix adhesive property of the Leptospira native counterpart, i.e. it bound readily to the previously reported host components, including plasma FN, matrigel ECM and HS (Hauk et al., 2008; Hoke et al., 2008). This study also noted the binding of the recombinant protein to C and PC, which are the predominant membrane lipid components of mammalian cells, and to intact MDCK cells. The most important prerequisite step in the pathogenesis of many microorganisms is their ability to bind to the host component/cell/tissue, and Leptospira spp. is not an exception (Patti et al., 1994; Cinco, 2010).

To analyze the peptides of the LipL32 neutralizing epitopes bound by the mAbs, a search of conventional protein–protein interaction by Western blotting and phage mimotope from the random 12-mer peptide phage display library was conducted (Perera et al., 2008; Thongsaksrikul et al., 2010; Pissawong et al., 2013; Yodsheewan et al., 2013). Two recombinant N-terminally Δ90- and Δ170-LipL32 proteins were produced for determining the LipL32 portions bound by mAbLPF1 and mAbLPF2. The results of western blot analysis suggested that the mAbLPF1 bound to the epitope located between amino acids 171–272, which has been shown to be immunogenic and important for LipL32 binding to host components (Hauk et al., 2008; Hoke et al., 2008). Although mAbLPF2 did not bind to the recombinant Δ170 LipL32 protein, it bound to the recombinant Δ90 protein. Crystallographic analysis had identified that the β6–β8 region of LipL32 protein (residues 92–139), where epitope of the mAbLPF2 is located (α1 and α2), might have the protein–protein/protein–ligand interaction functions (Hauk et al., 2009; Vivian et al., 2009).

Mapping epitope peptides, by searching for phage mimotope peptide consensus sequence and multiple alignments of the sequence with the linear amino acid sequence of LipL32 of the database, not only confirmed the locations of the mAb epitopes guided by the Western blot results, but also revealed the amino acid residues and features of the epitopes. The epitope sequence of mAbLPF1 (PLIH-----LQ) was conformational, consisting of many hydrophobic residues (243PLIN/H246) in the β13-turn (Table I) or P249, L256A and I263L of the α6 hydrophobic patch (Fig. 4A) (Hauk et al., 2009; Vivian et al., 2009). Previous study has shown that the amphipathic α helix is involved in membrane binding and membrane insertion (Thiyagarajan et al., 2004). LipL32 protein has been found to have hemolytic activity (Lee et al., 2000; Hauk et al., 2005). Thus, this epitope may have a functional role in the hydrophobic interaction and/or hemolytic activity of LipL32. It has also been shown that mAbLPF1 inhibited Leptospira-mediated hemolysis and rescued Leptospira-infected hamsters from death (Maneewatch et al., 2008). It is therefore plausible to conclude that mAbLPF1 exerted the neutralizing/therapeutic activity by interfering with the pathogenic activities of the LipL32 epitope. The mimotope consensus sequence of the

**Discussion**

Penicillin is the primary drug of choice for the treatment of leptospirosis. Occasionally, Jarisch–Herxheimer reaction occurs in antibiotic-treated cases due to the massive amounts of Leptospira spp., the antibodies were able to rescue the animals from lethal leptospirosis. They also inhibited pathogenic Leptospira-mediated human erythrocyte lysis in vitro (Maneewatch et al., 2008, 2009). Thus, the antibodies, particularly the humanized-ScFv, have a high potential for further development as a safe adjunctive, if not sole, remedy for use in the treatment of leptospirosis.
mAbLPF2 matched with linear 122PEEKSMPHW130 residues of the LipL32 α1 and α2 between β7 and β8. This portion is surface exposed and easily accessible by the interacting partner(s) as well as specific antibodies. By inhibiting protein interaction with the host component(s), mAbLPF2 may neutralize a targeting effect of Leptospira infectivity and pathogenicity.

There were four mAbLPF1 epitope variants among the pathogenic and intermediately pathogenic clades of Leptospira spp. (Table II). Most of the LipL32 sequences (62 of 70) of the database carried the Variant-1 epitope. Few strains, however, had amino acids L244A and I245V of the Variant 2, and L244I and I245V of the Variant 3. Nevertheless, these amino acid substitutions are conserved non-polar residues, which retain the hydrophobic activity of the amino acids 244LIH246 of Variant-1. The overall findings indicate that the mAbLPF1 epitope is highly conserved among the pathogenic and intermediately pathogenic species of Leptospira spp. There were six mAbLPF2 epitope variants due to one amino acid substitution (P122R, P122L, S126C, H129N and H129S of Variants 2–6, respectively). However, these substituted amino acids of Variants 4–5 should retain the hydrophilic properties of mAbLPF2 epitope Variant 1 located at the hydrophilic pocket, which has been shown to be the polypeptide binding groove of the LipL32 protein (Hauk et al., 2009).

In conclusion, epitopes of mAbLPF1 and mAbLPF2 are highly conserved among the pathogenic and intermediately pathogenic Leptospira spp., and are absent from proteins of the LipL32 superfamily of other microorganisms. The locations, tertiary structures and amino acid compositions indicated that both epitopes are involved in the interaction of LipL32 protein with the host factors/cells, which is a prerequisite step in Leptospira infectivity and pathogenicity. This study not only enlightens the molecular mechanisms of the neutralizing mAbLPF1 and mAbLPF2, i.e. prevents LipL32 binding to host constituents, but also elaborates the potential of the two LipL32 regions as diagnostic and vaccine targets for leptospirosis.

Supplementary data
Supplementary data are available at PEDS online.

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References