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Immunogenicity and protective efficacy of *Burkholderia pseudomallei* BLF1-N and BLF1-C terminal domains against BLF1 toxin

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ABSTRACT

Burkholderia lethal factor 1 (BLF1), a glutamine deamidase, is a key virulence factor that plays significant role in *B. pseudomallei* pathogenesis. To elucidate the BLF1 immunological responses, two truncated BLF1 structural units, BLF1-C (90–211 amino acids) with structural similarity to *T. maritima* Chemoreceptor glutamine deamidase (CheD) protein, and BLF1-N (1–89 amino acids) disparate to CheD were identified from the 23 kDa BLF1 protein. Both the components were devoid of toxicity in mice and elicited an antibody titer of 1:16,000 that reacted with the respective truncated proteins and BLF1. A549 cell lines supplemented with anti BLF1-N and BLF1-C antibodies exhibited 73.47% and 83.24% survival when treated with BLF1 toxin. Passive i.p. transfer with antibodies elicited by BLF1-C that contained LSGC active site resulted in 80% protection while anti BLF1-N (devoid of LSGC) antibodies provided 51.4% protection, establishing the role of BLF1-N terminal also in deamidase action. The truncated proteins also elicited cell mediated immune responses through proliferation of CD4+ T cells, IFN-γ and IL-4 cytokines but with bias towards Th2 subsets. BLF1-C and BLF1-N immunization resulted in 80% and 60% active protection when challenged with BLF1 toxin while the sham immunized mice exhibited severe histopathological changes like necrosis in liver, lung, spleen and kidney similar to that observed in melioidosis and were killed within 7 days post challenge. The higher level of active and passive protection by BLF1-C protein could be attributed to the comparatively higher level of immune responses and inclusion of LSGC residues.

1. Introduction

Burkholderia pseudomallei is a Gram-negative saprophytic bacillus that causes melioidosis in human and animals. The clinical manifestation of human melioidosis vary from acute or chronic localized infection of skin and soft tissue abscesses, necrotizing fasciitis to fulminant septicemia in multiple organs and is a serious public health concern in endemic regions [1]. Aerosol dissemination, high mortality rate of over 80–95%, rapid development of resistance against broad spectrum antibiotics, unavailability of effective vaccines render the pathogen as a potential biothreat agent [2,3].

B. pseudomallei virulence factors that assist its intracellular survival have been targeted as possible subunit candidate vaccines to restrain the systemic spread of the bacterium. This includes surface antigens like ABC transporters [4–6], Outer Membrane Proteins (OMPs) [7,8], autotransporter-related proteins [9,10], secretion system proteins, adhesins [10], etc. or secretory molecules like proteases, lipases, lecithinases, catalases and hemolysins [11], lipopolysaccharide (LPS), and capsular polysaccharide [12–14]. However, none of them provide comprehensive protection owing to the involvement of multiple virulence factors and require further improvement. Alternatively, the heat-inactivated bacteria [15] or live-attenuated strains of *B. pseudomallei* demonstrated partial protection in murine models [16]. However, a range of serious side effects and impending threat to revert to their active pathogenic forms restrict the utility of such vaccines for human applications [17–19]. In such scenarios, subunit vaccines are generally considered safe, since they do not exhibit the risks associated with persistence, latency, or reactivation.

Recently, *Burkholderia* lethal factor 1 (BLF1), the first *B. pseudomallei* toxin with role in virulence has been identified and characterized. BLF1, a 23 kDa glutamine deamidase specifically deamidates Glu339 in the eukaryotic translation initiation factor eIF4A and its expression is upregulated by microbial virulence promoting conditions like 30% human serum, animal specific amino acid taurine and physiological insulin concentration. About 100 fold reduction in mortality was observed in mice infected with BLF1 deletion mutant and 100% lethality was observed in recombinant toxin administered mice (i.p.) with significant damage in liver, otherwise commonly observed during

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B. pseudomallei infections [20]. Identification of BLF1 toxin and its association with *B. pseudomallei* pathogenesis has highlighted need to identify protective immune response elicited by BLF1 domains. In the present study, efforts were made to determine the protective immune responses by rationally determined recombinant N-terminal (BLF1-N) and C-terminal regions (BLF1-C) of BLF1 toxin against lethal challenge of BLF1 toxin. The recombinant proteins were devoid of observable toxicity and induced humoral & cell-mediated immunity that provided significant levels of protection. The BLF1-C with the LSGC active region elicited comparatively higher levels of cytokines, CD4 + /CD8 + T cells and provided increased protection in mice against lethal BLF1 toxin challenge.

2. Materials and methods

2.1. Ethics statement

Female BALB/c mice (5 to 6 week-old) were obtained from Central Animal Facility, Defence Food Research Laboratory (D.F.R.L), Mysore and maintained under specific-pathogen-free conditions on a 12-h light/dark cycle with free access to food and water. All animal experiments were performed according to the guidelines approved by the Institutional Animal Ethical Committee, Defence Food Research Laboratory (DFRL/28/IAEC/CPCSEA), completely accredited by Committee for the Purpose of control and Supervision of Experiments on Animals (CPCSEA), India.

2.2. Bacterial strain and cultural conditions

The *B. pseudomallei* NCTC 10274 was routinely grown in Luria-Bertani (LB) medium at 37 °C with shaking for 18 h. The *E. coli* strain BL21 (DE3) was propagated in Luria-Bertani (LB) broth/agar and incubated overnight at 37 °C. All the bacteriological media used in this study were procured from Himedia, India; all the cell culture media, reagents and chemicals were procured from Sigma-Aldrich (Bangalore, India). All cultures were maintained as 15% glycerol stocks at −80 °C as described by Sambrook et al., 1989 [21].

2.3. DNA and plasmid extraction

Genomic DNA from *B. pseudomallei* NCTC 10274 was extracted following Marmur's (1961) [22] protocol. pRSET A Vector was used for cloning experiments. *E. coli* BL21 (DE3) cells were harvested for the isolation of plasmid DNA using Gen-Elute plasmid extraction kit (Sigma, Bangalore, India).

2.4. Cell culture and media

The lung epithelial cell line A549 was procured from National Centre for Cell Sciences (NCCS), Pune. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (Thermo Scientific Ltd, India), 5 mM L-glutamine, 1 mM sodium pyruvate, penicillin 100 U/ml, streptomycin 100 μg/ml and 0.2% $NaHCO_3$ (Sigma, India). Cultured cells were incubated with 5% CO_2 at 37 °C.

2.5. PCR amplification of N-terminal and C-terminal regions of BLF1 from *B. pseudomallei* NCTC 10274

Nucleotide regions corresponding to the N (1 to 89 amino acid) (BLF1-N) and C (90 to 211 amino acid) (BLF1-C) terminal regions of BPSL1549 were amplified from *B. pseudomallei* NCTC 10274 genomic DNA by using oligonucleotide primers listed in Table 1. PCR was performed in a final volume of 20 μl that contained 2 μl 10× reaction buffer with 1.5 mM $MgCl_2$ (Sigma Life Science), 30 ng *B. pseudomallei* genomic template, 1 μl deoxynucleotide mix (2 mM each of dNTP), 1 μl forward and reverse primers (25 pmoles each), 1.0 U of Taq polymerase (Sigma, India) and 12.8 μl of Milli-Q water. The PCR thermal cycling conditions consisted of initial denaturation at 94 °C for 4 min, followed by 30 cycles at 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 10 min.

2.6. Cloning, expression and purification of proteins

The PCR amplicons were digested with BglII and HindIII restriction enzymes and ligated with pRSET-A vector predigested with the same enzymes. *E. coli* BL21 (DE3) transformed with ligation mixtures were selected on LB agar supplemented with 100 μg/ml ampicillin and screened by PCR using universal T7 forward and reverse primers listed in Table 1. The overnight culture of PCR positive clone were re-inoculated in LB medium supplemented with 100 μg/ml ampicillin, followed by incubation at 37 °C in shaking till the O.D. reached 0.6. Cells were induced with 1 mM Isopropyl-β-D-thiogalactopyranoside (IPTG) for 5 h at 37 °C and later pelleted. Expression of recombinant proteins was examined by SDS PAGE. Purification of recombinant BLF1-N and BLF1-C terminal proteins was performed with urea based denaturing buffers by immobilized metal affinity chromatography using Ni-NTA slurry (Qiagen, Germany) as per manufacturer's protocol. For challenge studies, the BLF1 was also expressed and purified using the above mentioned protocol (data not shown). The concentration of purified proteins was determined by Bradford method against known BSA standards and the proteins were stored at −20 °C until further use. The *Limulus* amoebocyte Lysate (LAL) test/assay (Lonza, India, Product # F245-065A) was used to determine the endotoxin level of recombinant BLF1-N and BLF1-C proteins according to manufacturer's instructions.

2.7. Immunization schedule

Two groups of 8 female (5- to 6-week-old) BALB/c mice were immunized subcutaneously with 50 μg of the recombinant proteins BLF1-N or BLF1-C at an interval of 14 days (0, 14, 28 and 42 days) for active protection studies. The first immunization was administered along with complete Freund's adjuvant (CFA) and 3 booster doses were administered with Freund's incomplete adjuvant. A group of control mice (8 No's) were given similar volume of adjuvant in 1X phosphate buffered saline (PBS). Sera collected at different time points of immunization were from the retro orbital sinuses of mice i.p. anesthetized with 200 μl of 0.5% xylazine and 0.2% ketamine mixture.

2.8. Humoral immune responses

Serum antibody titers specific to BLF1-C and BLF1-N proteins were estimated by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well flat bottomed polystyrene plates were coated with 10 μg/ml purified proteins in carbonate-bicarbonate buffer (100 μl) and incubated overnight at 4 °C. The plates were blocked with skimmed milk powder (5% w/v) in 1 × PBS for 2 h and washed twice with PBST. Two fold serially diluted test sera in PBS were added to the wells and maintained at 37 °C for 1 h, followed by similar incubation with Horse Radish Peroxidase (HRP)-conjugated goat anti-mouse IgG (Sigma Aldrich India). The chromogenic reaction was developed using the substrate O-phenylenediamine dihydrochloride (OPD) in presence of 0.4% H_2O_2 and the absorbance at 450 nm was measured using plate reader (Infinite M200 pro, Tecan, Grodig, Austria). The highest dilution of the serum having a mean OD_{450} two times greater than that of the sham immunized group serum at the lowest dilution were determined as the antibody endpoint titre. Antigen specific immunoglobulin subclasses IgG1, IgG2a, IgG2b, IgG3 and IgM were determined by using mouse isotyping kit (ISO-2KT, Sigma Aldrich, India) as per manufacturer's protocol.

Table 1
List of oligos used in this study.

Protein domain	Primer orientation	Oligo sequence (5′-3′)	Annealing temperature (°C)
BLF1-N Terminal	Forward	5′ CGTCAGATCTATGCCCAACTCACTCGAAGC 3′	50 °C
	Reverse	5′ GACGAAGCTTCTAGTAGGTGAAGAACAGCATGTC 3′	
BLF1-C Terminal	Forward	5′ CGTCAGATCTTACCTGAGCGGCTGCAAGG 3′	50 °C
	Reverse	5′ GCGAAAGCTTCTATTGCTTGCGCTGCTGGAG 3′	
T7	Forward	5′ TAATACGACTCACTATAGGG 3′	56 °C
	Reverse	5′ GGGGTTATGCTAGTTATTGC 3′	

2.9. SDS and Western blot analysis

SDS-PAGE was performed according to the Laemmli method (1970) [23] in Mini PROTEAN (Bio-Rad, Tetra System) followed by staining in Coomassie Blue R250 for visualization. For Western blot analysis, proteins separated in the SDS-PAGE gel were transferred on to nitrocellulose membrane at 70 V for 1 h. After electrophoretic transfer, membranes were blocked overnight with 5% skimmed milk in PBS and washed twice with PBST. The immunoblots were incubated with 1:1000 dilution of anti-BLF1-C and anti-BLF1-N serum for 1 h at 37 °C. The antigen-antibody interaction was detected by incubating the blots with goat anti-mouse IgG-HRP conjugate (Sigma chemicals, Bangalore) for 1 h at 37 °C, followed by subsequent development with Diaminobenzidine (DAB)-H_2O_2 substrate.

2.10. Toxin challenge

Groups of 8 mice immunized with BLF1-N or BLF1-C proteins and sham immunized mice were challenged with 250 μg of BLF1 toxin through i.p. route. The challenge mice were closely monitored over a 42 day's period to ascertain the mean survival time. At the end of the observation period, protective efficacy of BLF1-C and BLF1-N were estimated by Kaplan Meier's method.

2.11. Histopathology

For histopathological studies, the liver, lung, spleen, kidney samples were excised asceptically from dead sham immunized mice and humanely sacrificed BLF1-N and BLF1-C immunized mice that survived the observation period and fixed immediately in buffered neutral 10% formaldehyde solution. The specimens were paraffin embedded, processed and cut on a microtome in slices of 4–5 μm thin sections. The dehydrated tissue section were fixed on a glass slide and stained with Haematoxyline-Eosine, followed by visualization of the slide with a Nikon Eclipse Ni-E light microscope under 40× magnifications. Nis-Elements imaging software (Nikon, India) was used to capture images.

2.12. Cytokine profiling

The sera collected at 42nd day of immunization were assayed in triplicate for expression of IFN-γ, IL-2 and IL-4 by sandwich ELISA based cytokine estimation kit (Mabtech, Nacka, Sweden) as per manufacturer's protocol. Cytokine expression was expressed in pg/ml.

2.13. Flow cytometric analysis of CD4+ and CD8+ T cells from mice blood

Proportion of CD4+ and CD8+ T cells among the CD3+ cells gated from the total lymphocytes was analyzed from peripheral blood obtained from BLF1-N, BLF1-C and sham immunized groups of mice. The erythrocytes were lysed for 30 min in dark at 37 °C with 1:10 diluted lysis solution (FACS lysing solution, BD). Post incubation, cells were harvested and washed twice with Dulbecco's PBS. The expression of cell surface markers was determined by incubation of 10^6 cells with fluorescein isothiocyanate (FITC) (BioLegend, India) and phycoerythrin (PE) (BioLegend, India) conjugated MAb specific for CD4+ and CD8+ respectively, for 10 min in dark before FACS analysis. A minimum of twenty thousand events were obtained for each analysis using BD FACS Verse Flow Cytometer (Becton-Dickson, Singapore) and data were analyzed using Kaluza software version 3.1 v (Beckman Coulter, USA) in accordance with the manufacturer instructions. The electronic gating of percent activated CD3+ cells were performed among the total lymphocytes obtained and the CD4+ and CD8+ T cell populations in the gated CD3+ T cells were determined.

2.14. Cytotoxicity assay

The lung epithelial A549 cells were grown in 96-well plates (10^5 cells per well) with Dulbecco modified Eagle medium containing 10% fetal calf serum. The plates were maintained at 37 °C with 5% CO_2 allowing the cells to attach. The cells were exposed with BLF1 toxin along with 250 μg anti BLF1-C or anti BLF1-N polysera or sham sera. After 72 h incubation at 37 °C, the percentage cell viability in each treatment were measured by MTT (3-[4, 5-dimethyl thiazol-2-yl]-2, 5 diphenyl tetrazolium bromide) assay.

2.15. In vivo passive transfer studies

Two groups of 8 female BALB/c mice were i.p. injected with heat inactivated 250 μg anti-BLF1-C or anti-BLF1-N polysera, 2 h prior to challenge with BLF1. Control mice received equal amount of sham immunized mice sera. Each mouse was challenged intraperitoneally with 250 μg BLF1 toxin, diluted in PBS and closely observed for 42 days post challenge. To compare percentage survival, the toxin neutralization efficacy of anti-BLF1-C and anti-BLF1-N were estimated by Kaplan Meier's method.

2.16. Statistical analysis

The data were expressed as mean ± standard deviation (SD). Survival curves were constructed using Kaplan-Meier tests using GraphPad Prism (v 5.02) software. Statistical differences among groups were analyzed by Uni-variate/Oneway ANOVA applying with Dunnett' sand Tukey's post hoc test.

3. Results

3.1. Molecular cloning, expression and purification of recombinant BLF1-C-terminal and BLF1-N-terminal domains

The PCR amplified nucleotide sequences of 267 bp and 369 bp corresponding to BLF1-N (1–89 amino acids) and BLF1-C terminal domain (90–211 amino acids), respectively were cloned into *E. coli* BL21 (DE3) host cells using pRSET A vector. Sequencing analysis of recombinant vectors (pRSET A-BLF1-N and pRSET A-BLF1-C) revealed in-frame ligation of amplicons with intact nucleotide sequences (data not shown). The *E. coli* host harbouring the respective recombinant plasmids expressed BLF1-N and BLF1-C proteins of apparent molecular

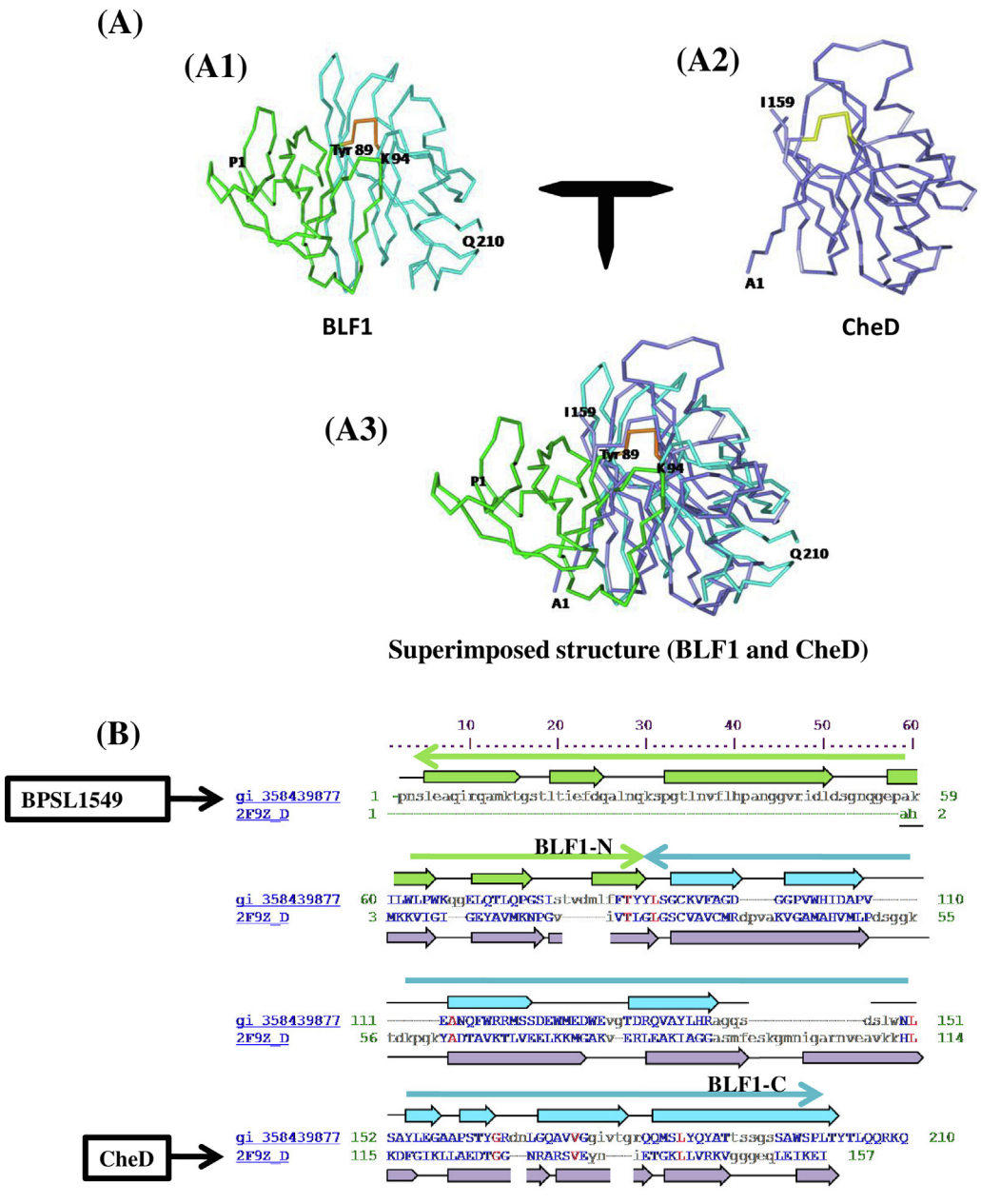

Fig. 1. Schematic representation of *Burkholderia* lethal factor 1 and CheD proteins structure. (A) An illustration of the structural folds and active site of BLF1 (A1) and CheD (A2) protein generated by Cn3D software to show that both have common active site but different peripheral loops and inserted regions (A3). The structural superimposition of BLF1 and CheD proteins to depict similarity in active site and highlighted with orange and violet colour respectively. (B) Structure based amino acid sequences alignment showing likely conserved region LSGC of BPSL1549 (gi 358439877) and CheD (2F9ZD). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

weight 15.1 kDa and 18.3 kDa, respectively upon induction with 1 mM IPTG (Fig. 2C). The initial localization studies showed accumulation of expressed recombinant proteins in inclusion bodies, which was solubilised and purified under denaturing conditions using immobilized metal affinity chromatography (Ni-NTA column). The purified proteins were confirmed by SDS-PAGE analysis (Fig. 2C). The BLF1-N and BLF1-C proteins did not exhibit any toxicity or reduction in all cell viability of A549 cell monolayer. The endotoxin level in BLF1-N and BLF1-C preparation were estimated and found to be 0.06 EU/ml that is far below permissible level (< 20 EU/ml).

3.2. Mouse immunization with recombinant BLF1-C and BLF1-N proteins induces humoral immune responses

The humoral immune response induced by BLF1-N and BLF1-C proteins was assessed by examining the antigen specific antibody titre elicited by the respective proteins. After first booster immunization, significant level of specific antibody titers were observed in both BLF1-N and BLF1-C mice groups (1:2000 each) and substantially increased until final immunization at 42nd day (1:16000 each) (Fig. 3B). The antibody subsets were determined by Isotyping analysis. The ratio of IgG1: IgG2 (IgG2a + IgG2b) was 1.2:1 and 1.5:1 in BLF1-N and BLF1-C immunized mice respectively (Fig. 3C). The IgG3 and IgM isotypes titres were observed to be insignificant in all groups. Mice when administered subcutaneously with these proteins did not exhibit any visible toxicity.

The Western blot analysis was performed to determine the immunereactivity of polyclonal sera with BLF1 toxin and its components. The anti-BLF1-N and BLF1-C polysera specifically reacted with 27.8 kDa BLF1 toxin and their corresponding recombinant BLF1-N (15.1 kDa) and BLF1-C (18.3 kDa) proteins respectively (Fig. 4).

3.3. BLF1-C and BLF1-N immunization induces expression of Th1 and Th2 cytokines

After 42nd day of scheduled immunization, significantly higher ($P < 0.001$) expression of pro-inflammatory cytokine (IFN-γ) and anti-inflammatory cytokine (IL-4) were observed in BLF1-N and BLF1-C

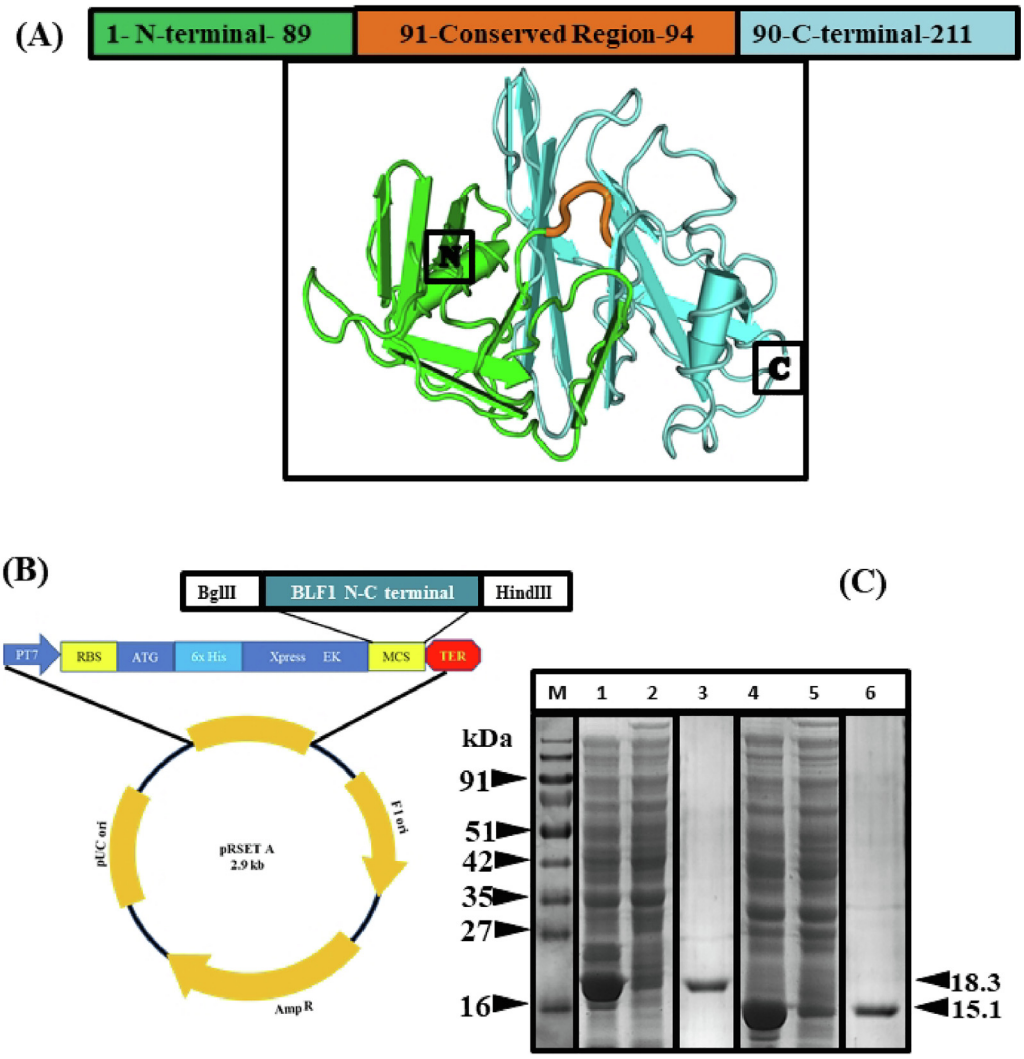

Fig. 2. Construction of the recombinant expression vector pRSET A-BLF1- N/C. (A) The crystal structure of BLF1 toxin drawn by Cn3D to depict BLF1-N (Green) and BLF1-C (Cyan) domain. The four amino acid motif LSGC active site is highlighted in orange. (B) The Schematic model of a plasmid construct for protein expression. Nucleotide sequences corresponding to BLF1-N or BLF1-C domains were cloned in pRSET A for the expression of recombinant proteins. (C) SDS-PAGE profile depicting expression and purification of proteins. 12% SDS-PAGE stained with coomassie blue shows expression and purification of BLF1-C and BLF1-N proteins. Lane M: Pre-stained protein molecular mass marker; Lane 1 and 4: Whole cell lysates of IPTG induced BLF1 C-terminal and BLF1 N-terminal clones; respectively. Lane 2 and 5: whole cell lysate of BL21 (DE3). Lane 3 and 6: Purified BLF1-C and BLF1-N recombinant proteins. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

immunized mice. The r-BLF1-N and r-BLF1-C immunized mice exhibited a maximum increase in IL-4 (10.23 and 13.05 fold respectively) and IFN-γ (3.97 and 5.08 fold respectively) compared to sham immunised mice. A moderate induction of IL-2 (1.6 fold) was also recorded in sera obtained from both the groups. These results indicated that immunization with the BLF1-N and BLF1-C proteins elicited both Th1 and Th2 cytokines (Fig. 5).

3.4. Immunization with BLF1-C and BLF1-N elicits CD4+ and CD8+ T cells expression

The blood samples collected from immunized and sham immunized groups of mice were analyzed for CD4+ and CD8+ T cells. The flow cytometric analysis showed predominant levels of CD4+ T cells in mice immunized with both BLF1-N and BLF1-C proteins. The BLF1-C showed significantly higher percentage of both CD4+ (45.3%) and CD8+ (20.2%) T cell population as compared to CD4+ (34.1%) and CD8+ (18.5%) of BLF1-N immunized mice. Sham immunized groups of mice showed no significant proliferation of CD4+ and CD8+ T cells (Fig. 8A; B).

3.5. Anti-BLF1-N and BLF1-C antibodies prevent BLF1 mediated cytotoxicity

Antibody mediated protection of A549 cells treated with BLF1 toxin in the presence of sham immunized mice sera (control) or BLF1-N and BLF1-C specific polysera was assessed *in vitro*. After 72 h of exposure, lung epithelial cells A549 treated with sham immunized mice sera resulted in cell rounding (Fig. 6B) with 18.95% survival (Fig. 6A). In contrast, the anti-BLF1-N and anti-BLF1-C antibodies provided significant protection ($P < 0.001$) against BLF1 toxin with 73.47% and 83.24% survival, respectively for 40 μg/ml (Fig. 6A).

3.6. In vivo active and passive protection studies

Mice immunized with r-BLF1-N and r-BLF1-C proteins and sham immunized mice (control) were i.p. challenged with 5 × LD_{50} (250 μg per mouse) dose of BLF1 toxin. Sham immunized mice showed a significant weight loss and succumbed to death of within 7 days post challenge. BLF1 mediated toxicity resulted in enlargement of liver lobules with discoloration and haemorrhagic necrosis, hepatic lobular congestion with central vein dilation, portal triad distortion and moderate baloonic hepatocytic degeneration (Fig. 7C. i). Lung tissue exhibited multiple areas of complete bronchoalveolar distortion with thickened alveolar septa, chronic inflammatory cells and ruptured alveolar walls with hemorrhagic foci. Numerous dark stained neutrophils, macrophages and lymphocytes infiltrating into the alveolar space scattered repetitively in tissues sections (Fig. 7C. iv). Sham mice also showed progressive splenomegaly wherein densely stained lymphocytes infiltrated marginally and arranged around macrophage cells. Moreover, distorted and dilated splenic sinusoids with few showing haemorrhage has also been observed (Fig. 7C. vii). The altered

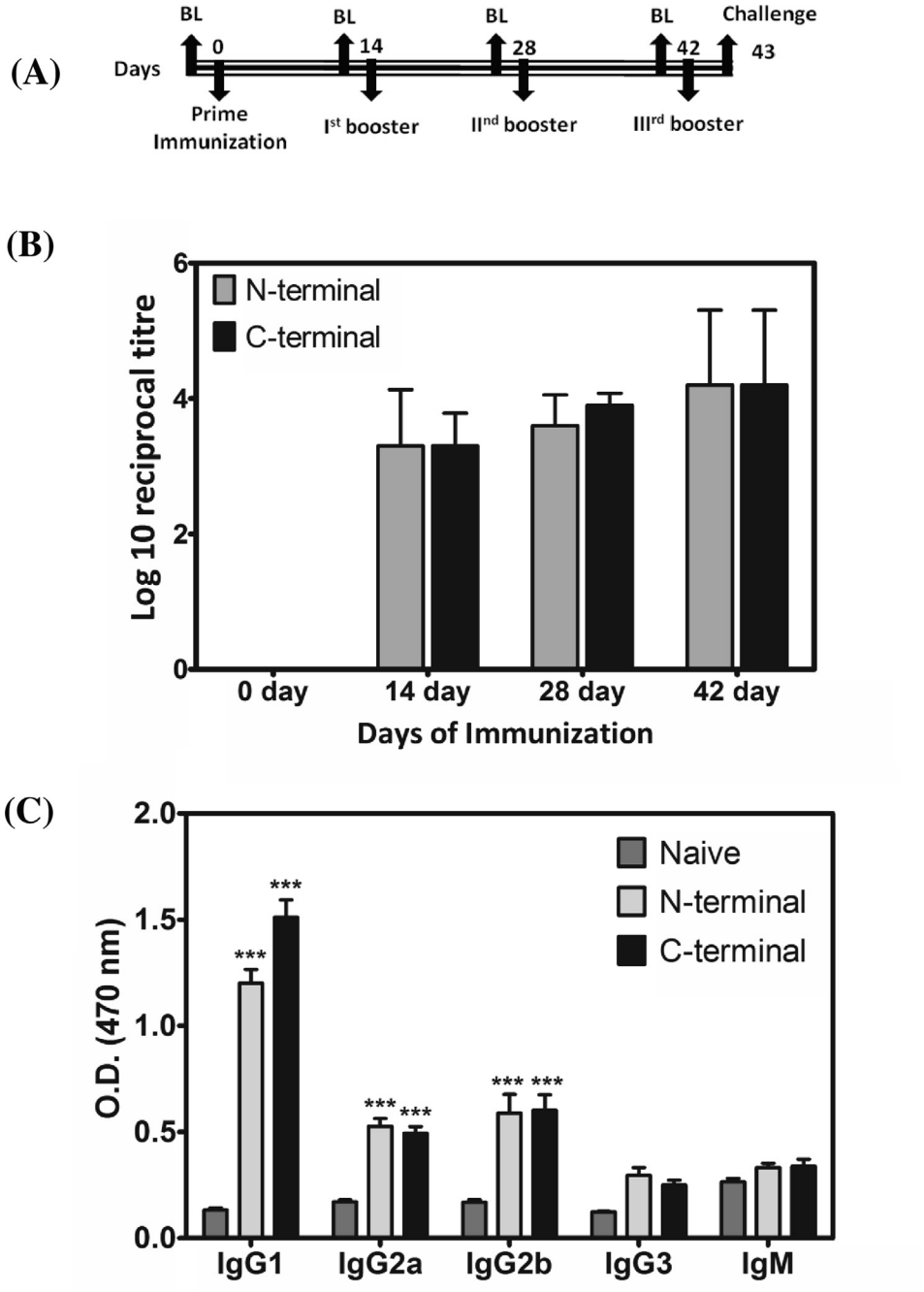

Fig. 3. Humoral immune response. (A) Schematic design of mice immunization schedule for antibody titre. (B) Comparative analysis of serum antibody titer response induced by purified recombinant proteins BLF1-C and BLF1-N in BALB/c mice. Sera collected at different time points of immunization were tested for antigen specific antibody titers by indirect-ELISA. Antibody titers are expressed as the log10 values of reciprocal endpoint titers. (C) Analysis of antibody isotypes in immunized BALB/c mice. Mice were immunized with 50 μg of BLF1-C and BLF1-N proteins. The serum samples were collected, pooled and the levels of IgG and IgM were analysed by using mouse isotyping kit.

glomerular and thickened tubular lining along with haemorrhagic lesions observed in kidney of control mice could be attributed to the necrotizing nature of BLF1 (Fig. 7C. x). In contrast the BLF1-C and BLF1-N immunized mice group displayed minimal or no conspicuous signs of infection indicating the ability of the later to inhibit BLF1 toxicity (Fig. 7C). The r-BLF1-N and r-BLF1-C immunized mice showed 60% and 80% survival as compared to control/sham mice challenged with BLF1 toxin (Fig. 7B).

For the passive protection experiments, antisera generated against BLF1-N and BLF1-C proteins were transferred through IP injection and mice were then challenged with BLF1 toxin. The control group of mice were injected with equal amount of sham immunized mice sera. Mice administered with anti-BLF1-N and anti-BLF1-C polysera were found protective with 51.4% and 80% survival, respectively over an observation period of 6 weeks post challenge. All the control group of animals challenged with BLF1 toxin died of within 8 days of toxin challenge. The results obtained confirm the protective role of anti-BLF1-N and anti-BLF1-C specific antibodies against lethal challenge of BLF1 toxin (Fig. 7A).

4. Discussion

Studies to decipher the pathogenesis mechanism of *B. pseudomallei* have earlier revealed that the infection initiates with the adherence of bacteria followed by invasion and replication inside the host epithelial and macrophage cells. The molecular mechanisms underlying this complex infection process and virulence factors determining the pathogenicity of *B. pseudomallei* are yet to be completely elucidated, despite the discovery of many factors with role in the pathogenesis [24,25]. The BLF1 discovered lately by Cruz et al., 2011, is the first toxigenic virulence factor to be reported from this opportunistic pathogen and kills mice upon intraperitoneal administration. It inhibits

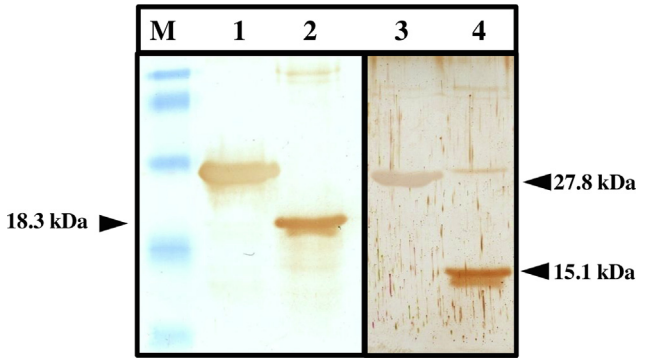

Fig. 4. Western immunoblotting analysis of BLF1 toxin (*Burkholderia* lethal factor 1) and BLF1 domains. Western blot showing reactivity of anti-BLF1-C (Lanes: 1, 2) and anti-BLF1-N (Lanes: 3, 4) antibodies with purified recombinant proteins. Lane M: Pre-stained protein marker, Lane 1 and 3: BLF1, Lane 2: BLF1-C and Lane 4: BLF1-N respectively.

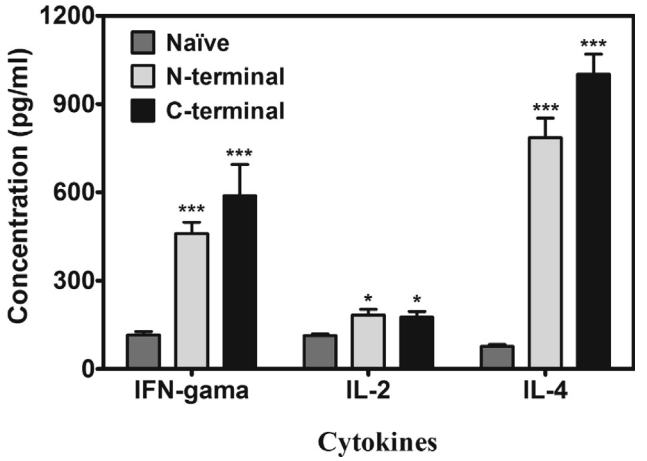

Fig. 5. Cytokines profile in immunized BALB/c mice. The concentrations of IL-4, IL-2 and IFN-gama were determined by sandwich ELISA in sera samples collected from r-BLF1-N and r-BLF1-C immunized mice after 42nd day of scheduled immunization.

cellular protein (synthesis) translation by irreversible enzymatic deamidation of Gln^{339} to Glu^{339} disrupting the RNA helicase activity of translation initiation factor eIF4A. The BLF1 share structural similarity in the B sheet core catalytic/deamidase pocket with that of cytotoxic necrotizing factor 1 (CNF1) catalytic domain and abolition of CNF1 deamidase activity with Cys 866 mutation to serine led to the development of BLF1 mutant (BPSL1549C94S) that retained similar structure [20]. The C94S point mutation in BLF1 inhibited glutamine deamidase activity in lesser concentration, however eIF4A binding and inhibitory properties were observed/restored in higher concentration and exhibited toxicity to J774 cells [20]. Alternatively, the Glu339 elF4 variant similar to BLF1 deamidated elF1 retained 53% helicase activity with wild type levels of ATPase and could bind to both RNA and ATP [20]. The predominant role played by this toxin in the *B. pseudomallei* pathogenesis thus establishes the importance to have molecules that prevent the deamidation/action of BLF1 on eIF4A. Traditionally, candidate subunit vaccines against toxins include non-toxic immunodominant domains or regions [26–28], although, in few cases whole protein is also used [29]. However, notwithstanding the major structural similarity with *E. coli* CNF1 catalytic domain, BLF1 lack receptor binding or translocation domains and no functional domains were identified in BLF1 that can be utilized to develop subunit vaccines.

The catalytic domain of Chemoreceptor glutamine deamidase (CheD) of *T. maritima* resembles with that CNF1 and possess glutamine deamidase activity similar to that of former [30]. With an objective to understand the immunological responses elicited by the domains in BLF1, we utilized manually-curated strategy of visually comparing the crystal structure models of BLF1 and *T. maritima* deamidase CheD proteins for structural unit identification (Fig. 1). The relatively smaller molecular size of CheD protein (16 kDa) and the presence of interaction sites with non-deamidation substrates like CheY via β6 - α2 loop [31] signified that if BLF1 and CheD were to share a structural similarity, then the homologous sequence in BLF1 might form a distinct structural unit. Thus, two segments of BLF1 (i.e.) BLF1-C with LSGC active region similar to that of CheD and BLF1-N lacking LSGC active region which is distinct from CheD were synthesized and characterized for their protective immune response against BLF1 toxin. Both were immunogenic devoid of any toxic effects when administered subcutaneously in mice. The antigen specific serum antibody titers (1:16000) progressively increased with booster immunizations and the lucid reactivity of anti BLF1-N and anti BLF1-C antibodies with the respective truncated proteins (15.1 and 18.3 kDa) and full length BLF1 (27.8 kDa) as observed by Western blot analysis demonstrated the possible structural similarity and preservation of epitopes in the truncated proteins with biologically active 27.8 kDa toxin. Absence of antibody cross reactivity with the alternate truncated BLF1 fragment indicated exclusiveness of the epitopes in BLF1-N and BLF1-C (Fig. 4).

The impact of anti BLF1-N and BLF1-C antibodies binding on BLF1 neutralization was examined by *in vitro* studies using A549 cell line. Substantial protection with 83.24% and 73.47% cell viability was observed with convalescent sera obtained from BLF1-C and BLF1-N immunized mice respectively, demonstrating cytotoxicity neutralization ability of the antibodies. However higher survival percentage (80%) was observed with anti-BLF1-C antibodies against 51.4% protection with anti-BLF1-N in *in-vivo* passive transfer studies. The binding of antibodies to toxin does not alter the antigen but bring about stearic interference in binding to host receptor or substrates in case of toxins with enzymatic property. The BLF1-N protein was devoid of the LSGC residues while these were maintained in BLF1-C protein which could be the possible reason for higher level of protection observed with anti BLF1-C antibodies that can bind to key residues of catalytic site involved in deamidation of translation initiation factor eIF4A (Fig. 7A). From the results it is evident that, though the BLF1-N lacked the LSGC residues, the antibodies elicited by it reduced BLF1 functionality to bring about 51.4% protection which indicates the influence of N terminal domain on the BLF1 toxicity. Recent study by Rust et al., 2015 showed that non-specific fluid phase uptake of BLF1 toxin occurs by means of macropinocytosis i.e. a process which mediates the non-selective uptake of solute molecules, nutrients and antigens. The enzymatic activity of BLF1 inside the cell was unhindered even as a m-Cherry fusion protein with a molecular size of 50 kDa instead of native protein with 24 kDa size [32]. This makes us hypothesise that binding of antibodies to BLF1 did not possibly hinder the macropinocytosis process while differentially altered the binding of eIF4A thereby conferring different levels of protection.

We further examined the cell mediated immune responses of r-BLF1-C and r-BLF1-N proteins. About 3 and 2.25 fold proliferation of CD4+ T cells was observed in our study when immunized with the former and later proteins respectively. The CD4 lymphocytes otherwise known as helper T cells aid the MHC class II antigen presenting cells through activation of intracellular kinases during cellular interactions, activation of B cells and the cytokines they produce [33,34]. The BLF1 activity is directed towards the intracellular target enzyme eIF4 albeit its extracellular phase before its entry into the host cell [20]. This underlines the requirement for both Th1 and Th2 subtypes of helper cells that otherwise act as cells host immunity effectors against intracellular and extracellular pathogens respectively [35]. The present study showed that the 5.08 and 3.97 fold upregulation of IFN-g and 13.05 and 10.23 fold upregulation of IL-4 in BLF1-C and BLF1-N groups respectively indicated the proliferation of both Th1 and Th2 subsets. However, the IFN-g: IL-4 and IgG1: IgG2 ratios of 0.389 (BLF1-C) or 0.388

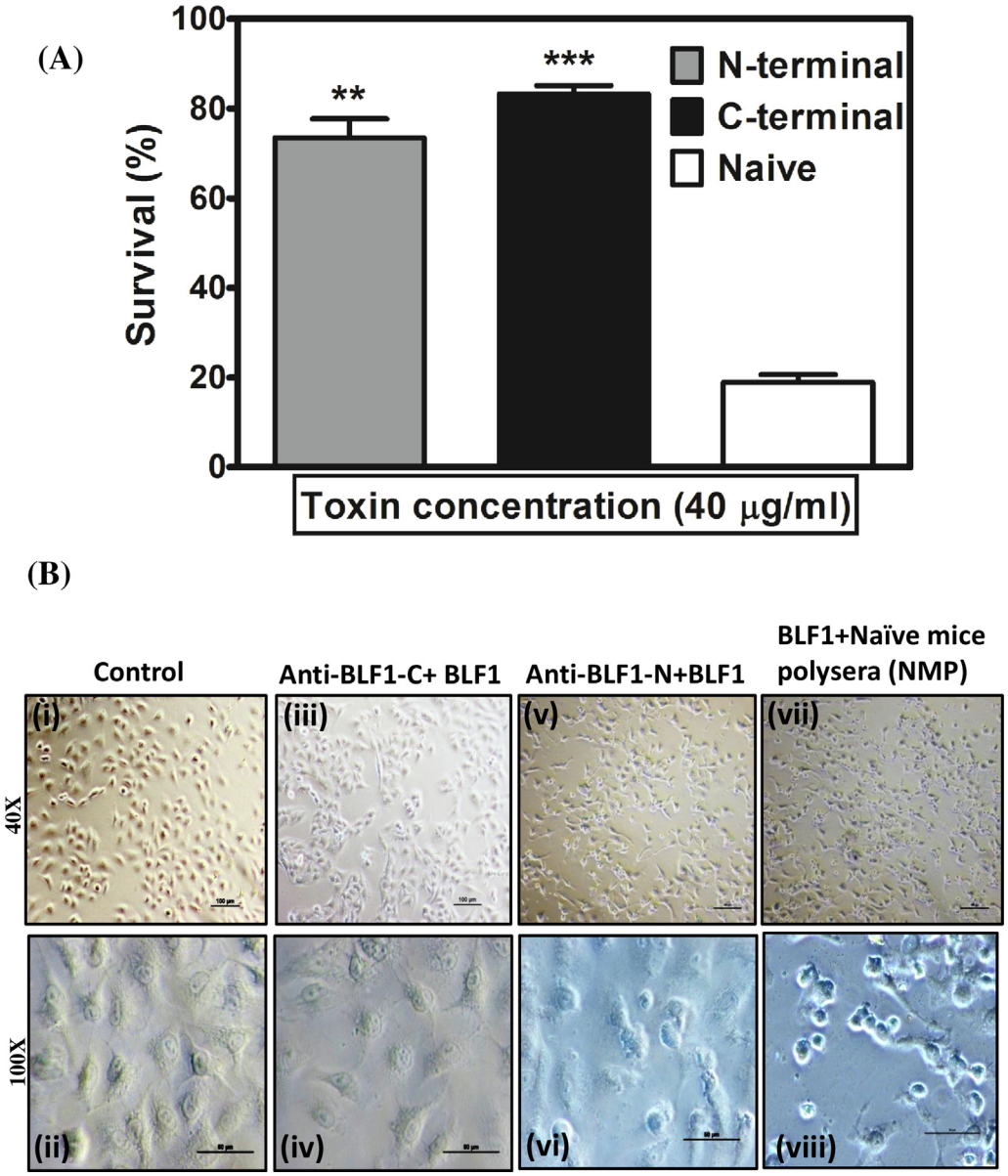

Fig. 6. Protection of cell line against toxin (*Burkholderia* lethal factor 1) of *B. pseudomallei*. (A) The lung epithelial A549 cell line were cultured to confluence in 96-well plates, washed, and medium was replaced with serum-free DMEM. Cells mixed with anti-BLF1-C or anti-BLF1-N sera raised in BALB/c mice were exposed to BLF1 toxin. Cell viability was determined after 72 hrs of incubation by MTT assay. (B) Morphological changes in lung epithelial A549 cells induced by BLF1 toxin in presence of anti-BLF1-C sera (iii & iv), anti-BLF1-N sera (v & vi) and naïve mice polysera (NMP) (vii & viii) are shown. The control lung epithelial A549 cells are shown in (i) and (ii). The representative photographs (i, iii, v, vii) and (ii, iv, vi, viii) were observed at 40× and 100× magnification, respectively.

(BLF1-N) and 1.5:1 (BLF1-C) or 1.2:1 (BLF1-N) respectively indicated a bias towards Th2 response [8,36,37]. The IL-4 cytokine is associated with stimulation of activated B cells and their proliferation into plasma cells that produce large amount of specific antibodies and T cell proliferation [38]. The increase in CD4+ and CD8+ T cells with generation of high titre antibodies (Fig. 8) observed in the study could be correlated with the higher levels of IL-4. With the development of antigen specific immunity, the CD4 Th1 helper cells and CD8 cytotoxic cells produce IFN-g that induces the expression of MHC II molecule [39,40], production of IgG2a [41], differentiation of more naïve CD4 cells to Th1 cells [42,43]. Active immunization with BLF1-C and BLF1-N resulted in 80% and 60% protection respectively owing to the Th1 and Th2 immune responses elicited by the truncated recombinant proteins. Sham immunized mice that received BLF1 intraperitoneally (I.P.) revealed histopathological changes in liver, lung, spleen and kidney (Fig. 7C). The hepatomegaly with severe hemorrhagic necrosis and discoloration of liver, splenomegaly with moderate necrosis, bronchoalveolar distortion with hemorrhagic foci in lungs and haemorrhagic lesions observed in kidney of sham immunized mice have been reported earlier in human cases of acute melioidosis and makes us speculate that BLF1 could be the possible factor for these pathology [44]. The cytoplasmic movement of BLF1 in these organs possibly through macropinocytosis offsets the absence of translocation/receptor binding domains in the BLF1 protein that otherwise facilitate its intracytoplasmic movement.

The present study describes the protective immune responses elicited by BLF1-C and BLF1-N proteins fragmented from the BLF1 toxin. The higher level of protection by BLF1-C immunization could be attributed to the comparatively elevated levels of immune responses that include CD4+, CD8+ T cells and cytokines elicited in this group. We speculate that the cytotoxic CD8+ T cells that otherwise aid in protection by killing cells infected with intracellular pathogens may also have a role in purging the cells internalised with BLF1. In this study we describe the humoral and T cell immune responses elicited by N and C terminal regions of BLF1 and level of protection provided against the toxin challenge. We also report that BLF1 cause pathological changes in lungs, spleen and kidney similar to that observed in cases of melioidosis.

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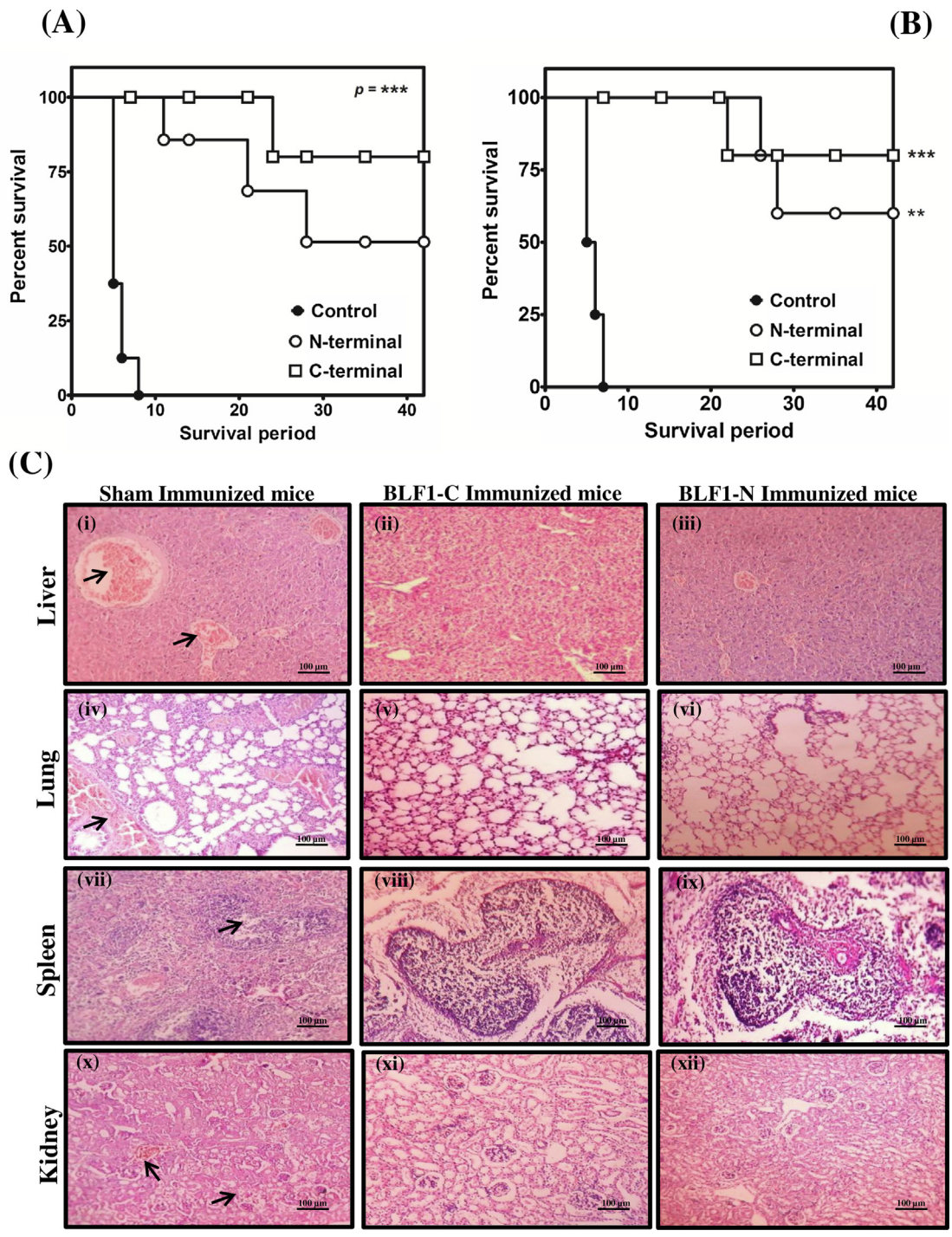

Fig. 7. Protection of BALB/c mice against toxin (*Burkholderia* lethal factor 1) of *B. pseudomallei*. (A) Groups of BALB/c mice were injected i.p. with sham immunized mice sera or anti-BLF1-C antisera or anti-BLF1-N terminal antisera 2 h prior to challenge with BLF1 toxin and observed for 42 days. The survival percentage was estimated by Kaplan Meier's method. (B) BLF1-C, BLF1-N and sham immunized groups of BALB/c mice were challenged with BLF1 and observed for 42 days. The percentage survival was estimated by Kaplan Meier's method. The BLF1-C and BLF1-N immunized mice group showed significant levels of survival as compared to sham immunized mice. (C) Histopathological analysis. Tissue sections of liver, lung, spleen and kidney harvested from mice groups that were sham immunized or with BLF1-C or BLF1-N were stained with haematoxylin-eosin and observed under 40X magnification. The sham immunized mice showed histopathological signs of necrosis in liver, lung, spleen and kidney as evidenced by (i) hepatic lobular congestion and central vein dilatation, (iv) bronchoalveolar distortion with thickened alveolar septa, (vii) Dilated splenic sinusoids with few showing haemorrhage and (x) altered glomerular and tubular architecture whereas BLF1-C and BLF1-N immunized mice showed minimal sign of infection. Photomicrograph represents a typical view of haematoxylin and eosin (H&E) section of Liver (i, ii, iii); Lung (iv, v, vi); Spleen (vii, viii, ix); and Kidney (x, xi, xii) from sham immunized, BLF1-C and BLF1-N immunized group respectively, at a microscopic scale bar of 100 µm. Arrows indicate to the various sign of damages in tissue section.

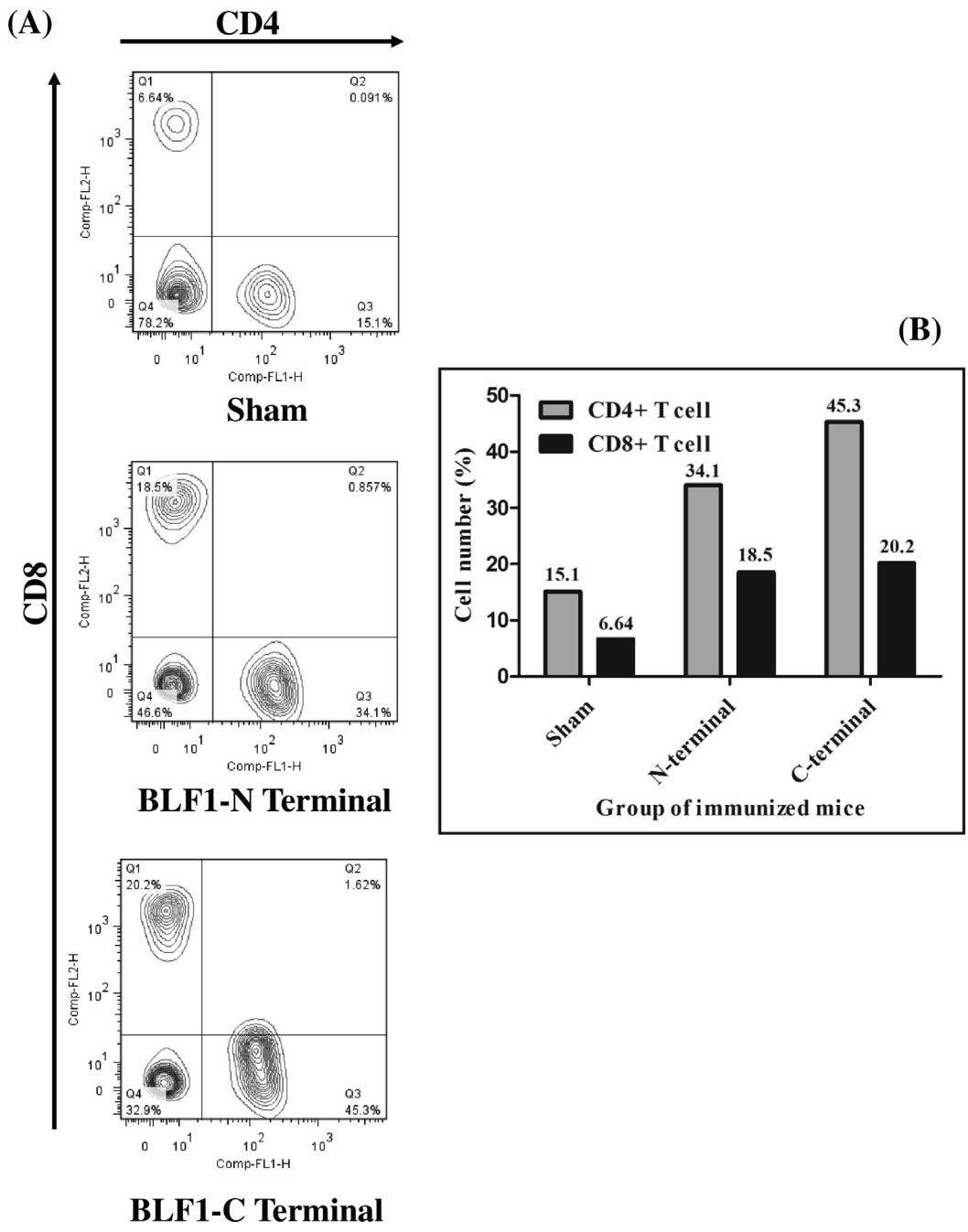

Fig. 8. T Cells analysis in BLF1-N and BLF1-C terminal immunized mice. The fluorescent tagged anti-CD4 (FITC) and anti-CD8 (PE) monoclonal antibodies were used to analyse the relative expression of CD4+ and CD8+ T cells from peripheral blood cells. After 42nd day of immunization with r-BLF1-C and r-BLF1-N proteins, blood samples drawn from respective mice groups were used for the assay. (A). The gating strategy along with percentage of CD4+ and CD8+ T cells in sham, BLF1-N and BLF1-C immunized mice have been shown. CD8+ T cell % (Q1); CD4+ and CD8+ T cell % (Q2); CD4+ T cell % (Q3); $CD4^{-ve}/CD8^{-ve}$ T cell % (Q4). (B) The graph shows relative expression percentage of CD4+ and CD8+ T cell population in sham, BLF1-N and BLF1-C-terminal immunized mice blood samples.

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Declaration of Competing Interest

The authors declare that there are no conflicts of interest to disclose.

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