Leptospira surface protein LigA plays a multifaceted role in modulating the host innate immune response

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Abstract

Leptospira, a zoonotic pathogen is known to infect a variety of hosts and capable of establishing persistent infection. This remarkable ability of bacteria is attributed to its potential to evade or modulate the host immune response by exploiting its surface proteins. We have identified and characterized the domain of *Leptospira* immunoglobulin-like protein A (LigA) that is involved in modulating the host innate immune response. We identified that the 11th domain (A_{11}) of the variable region of LigA (LAV) induces strong TLR4 dependent innate response in mouse macrophages via signalling through MAP kinase pathway leading to the production of pro-inflammatory cytokines (IL-6 and TNF-α) and expression of costimulatory molecules (CD80, CD86, CD40) and maturation marker (MHC-II). A_{11} is also involved in acquiring complement regulators like FH, C4b binding protein and Plasminogen and mediating functional activity to escape from both classical and alternate pathways of complement-mediated killing. The deletion of A_{11} significantly impaired TLR4 signalling and subsequent activation of innate immune cells and also inhibited the binding of complement regulators leading to the killing of bacteria. Our study discovered an unprecedented role of LAV as nuclease capable of degrading Neutrophil Extracellular Traps (NETs). This nuclease activity was mediated by A_{11} and was inhibited with anti-LAV antibodies. These results highlight the moonlighting function of LigA and demonstrates that a single domain of a surface protein is involved in evading a myriad of host innate immune defences, which might allow the persistence of *Leptospira* in different hosts for a long term without clearance.

Introduction

Leptospirosis is one of the most widespread bacterial zoonosis especially in developing countries like India and one of the major neglected infectious disease globally[1]. It is caused by pathogenic spirochete of genus *Leptospira* that can cause fatal infections involving multiple organs in human and animal hosts. According to WHO, there is a huge economic burden of human Leptospirosis that estimates 1.03 million cases with 58,900 deaths worldwide annually[2]. The actual burden may be much higher as lot of cases are not reported due to difficulties associated with diagnosis[2,3]. The major challenge in combating this zoonosis has been the unavailability of early diagnostics and potent vaccines that can induce cross-protection against various serovars [4,5]. Understanding how *Leptospira* escapes from host innate immune defenses to disseminate and colonize in multiple organs for establishing infection will aid in devising prophylactic strategies.

Innate immune responses are composed of multiple dynamic defense systems, comprising of soluble factors viz. antimicrobial peptides, complement proteins, pattern recognition receptors like Toll-like receptors (TLRs) and NOD-like receptors (NLRs) and phagocytic cells such as Dendritic cells (DCs), neutrophils and macrophages. All of these components contribute in the killing and removal of invading pathogens by a variety of mechanisms[6,7]. Signalling through TLRs induce activation of innate immune cells leading to secretion of pro-inflammatory cytokines (IL-6, TNF-α) and expression of surface molecules (CD80, CD86, MHC-II) thereby enabling these cells to become efficient in subsequent activation of adaptive response[8-10]. TLRs play a key role in promoting adaptive immune responses and are also essential for T-cell expansion, differentiation, and memory formation[11,12]. The Complement system is a vital part of innate immune defense which promptly kills the invading pathogen by opsonization and target lysis[13]. It induces inflammatory reactions that initiate additional cellular effector functions of both innate and adaptive immune response[14]. To prevent damage to the host cells, the complement system is tightly regulated by soluble plasma proteins like Factor H (FH), C4b-binding protein (C4BP) and plasminogen (PLG)[15]. FH and C4BP inactivate the Alternative pathway (AP) and Classical pathway (CP) of complement activation by binding with the complement components C3b and C4b respectively and facilitate their Factor I mediated cleavage. Plasmin, the enzymatically active form of PLG acts as a protease that cleaves complement factors C3b, C4b and C5[16]. Neutrophils are major phagocytic cells that utilize a combination of reactive oxygen species (ROS), cytotoxic granules, antimicrobial peptides and Neutrophil Extracellular Traps (NETs) to kill and

degrade the invading pathogen[17,18]. However, pathogens have devised several strategies to escape from host innate immune defences mainly through mechanism mediated by their surface proteins[19,20]. These proteins may be pro-inflammatory where they can activate APCs like macrophages and DCs but might also enable the pathogen to avoid recognition through innate receptors (TLRs) by downregulating their expression or causing antigenic variations to evade from host defences[21-24]. Pathogens escape from complement-mediated killing by expressing surface proteins that acquire complement regulators like FH and C4BP, act as proteases or acquire host proteases that can cleave complement components[13,25]. They may avoid killing by phagocytes like Neutrophils by expressing surface proteins which can evade extravasation and chemotaxis, prevent opsonization and phagocytosis, promote survival inside the neutrophil, induce apoptosis or cell death and degrades NETs by virtue of their nuclease activity[26-29].

Like other pathogens, *Leptospira* have also evolved strategies to modulate the host innate immune response by exploiting capacities of its surface proteins to favour their pathogenesis[30-32]. Toll-like receptors like TLR2 and TLR4 play a major role in host defence as mice lacking these receptors were highly susceptible to *Leptospira* infection[33]. It is likely that these bacteria modulate the expression of surface molecules (proteins, LPS) to avoid recognition through protective TLR2 and TLR4 and establish infection in the host. Several surface proteins of *Leptospira* have been identified as a strong activator of pro-inflammatory response via signalling through both TLR2 and TLR4[34-38]. Besides that, several Leptospiral proteins have been shown to acquire complement regulators like FH, C4BP and PLG on their surface or act as proteases to cleave complement components to evade from killing[39-49]. *Leptospira* is known to induce NET, hence it is likely that it might express surface proteins/nucleases like other bacteria to evade from NETosis[50,51]. Thus, identification and characterization of a surface protein involved in the modulation of host innate immune response will aid in designing a better strategy to combat this bacterial zoonosis.

Leptospira immunoglobulin-like (Lig) proteins (LigA and LigB) are surface proteins having 12-13 immunoglobulin like repeat domains similar to invasion of *Yersinia* and intimin of *E.coli*[52-54]. The N terminal region of LigA and LigB from domains 1 to 6.5 are conserved whereas C terminal region from domain 6.5 to 13 are variable[52,55]. Lig proteins are expressed during infection and have been shown to bind to multiple components of the host extracellular matrix (ECM) thereby mediating attachment to host cells [56-58]. They are the most promising vaccine candidate identified to date. Moreover, 10-13 domains in the variable

region of LigA is sufficient to induce protection against challenge in hamster model [59-64]. Despite various reports confirming the protective role of variable region of LigA (LAV) its involvement in the modulation of host innate immune response has not been studied extensively. Several groups have demonstrated that Lig proteins bind to various complement regulators like FH and C4BP to inhibit both classical and alternative pathway of complement activation, however, the specific domain involved in binding to these regulators have not been characterized [40,42,45,49,65]. Further, the proteins role in activation of the innate response or evasion from killing by phagocytes has not been reported so far. In the present study, we have demonstrated the role of LAV in modulating the host innate immune response. Using various assays, we identified the domain/s involved in the activation of innate response via signalling mediated through TLR4 receptor and evasion from complement-mediated killing via binding to various regulators like FH, C4BP and PLG. Further, we demonstrated LAV's nuclease activity which might play a major role in evasion from Neutrophil extracellular traps (NETosis).

Material & Methods

Animals, cell lines and reagents

C57BL/6 mice were obtained from the Animal Resource and Experimental Facility of NIAB, Hyderabad. The original breeding colonies were obtained from Jackson Labs, USA. The animals were maintained in a pathogen-free condition. All the procedures for animal experiments were approved by the Institutional Animal Ethics Committee (IAEC) and performed in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines. RAW264.7, HEK293 cell lines were originally purchased from the ATCC (Manassas, VA). Mouse macrophage WT (NR-9456), TLR2KO (TLR2-/-, NR-9457), TLR4KO (TLR4-/-, NR-9458), DKO (TLR2-/-/4-/-, NR-19975), TRIFKO (TRIF-/-, NR-9566), MyD88KO (MyD88-/-, NR-15633) and TMDKO (TRIF-/-MyD88-/-, NR-15632), cell lines were obtained from BEI Resources, USA. Cells were cultured in DMEM (Sigma, USA) supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA), penicillin (100 U/ml), and streptomycin (100 mg/ml) and maintained at 37˚C in a humidified incubator (5% CO2). Inhibitors of NF-kB (SN50), p38 (SB203580), ERK (U0126) and JNK (SP600125) were purchased from Invivogen. Mouse IL-6 and TNF-α Sandwich ELISA kit were from R&D Biosystems. PE-CY5–conjugated anti-MHC-II, PE-

conjugated CD80, PE vio conjugated CD86, BV 421 conjugated CD40, and Per Cp Cy5.5 conjugated MHC-II antibodies were procured from BD biosciences, US.

Cloning, expression and purification of recombinant proteins

The gene sequences for Lig Conserved (LC), Lig A variable (LAV), Lig B variable (LBV) were amplified, without the signal sequence, by PCR from *L. interrogans* serovar Pamona strain genomic DNA using specific primers and then cloned in His-Sumo tagged pET28a expression vector. Domains of LAV(A_8-A_{13}) and corresponding deletion mutants ($A\Delta_8$-$A\Delta_{13}$) were similarly cloned in pET28a vector. Various domain deletion mutants of LAV ($A\Delta_8$-$A\Delta_{13}$) were generated by PCR-based site-directed mutagenesis. All the clones were verified by sequencing. The plasmid was transformed into BL21DE3 Rosetta and resulting transformants were grown at 37°C overnight on LB broth containing 50µg/ml kanamycin and the expression of the protein was induced with 1 mM isopropyl β-D-1-thiogalactoside (IPTG). The cells were harvested by centrifugation at 10,000 rpm and the cell pellet was resuspended in 100mM Tris HCl, 150mM NaCl pH8.0 followed by sonication at constant pulses. The lysate was centrifuged to remove cell debris and the supernatant was subjected to affinity chromatography using Ni-NTA beads column (Takara). Sumo protease (Sigma, USA) was added in the column and kept on a rocker at 4°C overnight. The His-tag fusion protein was eluted with 50mM imidazole, dialyzed and checked for size and purity by SDS-PAGE. Eluted protein was dialysed against 1×PBS with four changes for 2days at 4°C. The protein was then passed through Detox- Gel (Pierce, USA) to remove any contaminating LPS from *E. coli* and a residual trace amount of LPS was monitored by Limulus amoebocyte lysate (LAL, Endotoxin Detection Kit, Pierce, Thermo, USA) assay following the manufacturer's instructions. The concentration of purified protein was estimated using Bradford reagent (Sigma, USA).

Cell stimulation assays by Cytokine ELISAs

Cytokine ELISA kits (R&D systems) were used to measure cytokine levels, following the manufacturer's instructions. HEK293T cells were cultured in complete DMEM medium for 24h at 37°C in presence of 5%CO_2 and transfected with TLR2, TLR4 and NF-kB reporter plasmids using X-fect Transfection reagent (Takara, Japan) following manufacturer's protocol. Cells were stimulated with LAV (2µg/ml) for 24h and then IL-8 levels were measured in the cell culture supernatant. RAW264.7 cells were stimulated with LC or LAV or LBV or (A_8-A_{13}) or corresponding deletion mutants (ΔA_8-$A\Delta_{13}$) (2µg/ml), PAM3CSK4

(20ng/ml), and *E. coli* LPS 0111-B4 (500ng/ml) for 24h at 37°C in presence of 5%CO_2 and cytokines (IL-6, TNF-α) were measured in the culture supernatant according to the manufacturer instructions. The proteins were pre-treated with Polymyxin B (20ng/mg protein) at 37°C for 1hr and Proteinase K (5µg/mg protein) at 65°C for 1hr followed by inactivation at 95°C for 5min before each assay to rule out endotoxin activity. In a separate experiment Wild type, TLR2KO, TLR4KO, DKO, MYD88KO, TRIFKO, TMDKO macrophage cell lines were stimulated with PAM3CSK4, LPS, LAV or A_{11} or $A\Delta_{11}$ for 24h at 37°C/5%CO_2 and cytokines (IL-6, TNF-α) in the culture supernatant were measured by ELISA kit as per manufacturer's instructions. To assess the signalling pathway involved additional experiments were done in which RAW264.7 cells were pre-treated for 30min at 37°C/5%CO_2 with inhibitors of NF-kB (SN50; 20µM) or JNK (SP600125; 40µM) or p38MAPK (SB203580; 30µM) or ERK (U0126; 20µM) followed by treatment with A_{11} (2µg/ml) for 24h at 37°C in presence of 5%CO_2 and cytokines were measured by ELISA kit.

Flow cytometric analysis

Cells were incubated in 6-well plates (0.3×10^6 cells/well) with PAM3CSK4 (20ng/ml), LPS (500ng/ml) or A_{11} (2µg/ml) for 24h at 37°C in the presence of 5%CO_2. Cells were harvested and washed with pre-chilled PBS and then incubated on ice for 1h in the dark with respective flourochrome conjugated antibodies against CD80, CD86, MHC-II and CD40. Cells were washed and then fixed with 1% paraformaldehyde and 50,000 total events/sample were acquired using a FACScan calibrator. The data were analysed using FlowJo software.

Preparation of Antisera

Female C57 BL6 mice (6-8 weeks) were immunized subcutaneously on days 0 with 20µg of LAV, A_{11}, $A\Delta_{11}$ in complete Freund's adjuvant (CFA) and then boosted on day 21 and 28 with 10µg of proteins in Incomplete Freund's adjuvant (IFA). Sera were collected one week after booster and titre were determined using ELISA. The mouse serum having anti-LAV, anti-A_{11} or anti-$A\Delta_{11}$ antibody was used in confocal microscopy.

Protein-Protein docking studies

The amino acid sequence of LAV (1-639), $A\Delta_{11}$(1-366 and 458-639) and A_{11} (from residues 367-457) was submitted to blastp to find a structural homolog as a template used for homology modelling. However, no significant structural homology was observed. Hence, the amino acid sequence of LAV, $A\Delta_{11}$ and A_{11} was submitted to the Swiss Model server (https://swissmodel.expasy.org/) with automated template search homology. We found 49.9%

,14.19% and 14.55 % of sequence or structural homology with templats 2mh4.1.A , 5ftx.1.A and 5ftx.1.A for A_{11}, $A\Delta_{11}$ and LAV respectively. We retrieved macromolecular 3D structures of tlr4 receptors from Mus musculus(3t6q), Homo sapiens(4g8a) and Bos taurus(3rg1) with sequence length 606, 635 and 612 respectively from protein data bank (PDB) https://www.rcsb.org/). We removed the additional compounds that are associated with tlr4 using auto dock tools to extract the purest form of tlr4 with chains A and B. To find protein-protein interaction with the LAV, $A\Delta_{11}$ and A_{11} domain we utilise HDOCK sever (http://hdock.phys.hust.edu.). It provides a suitable top 10 prediction for each protein-protein docking from which we selected the top predicted output with min energy involved and the best docking score.

TLR binding assay

WT, TLR2KO, TLR4KO and DKO cell lines were grown overnight on glass-bottom cell imaging dishes (Eppendorf) and then incubated for 30min at 37°C in presence of 5%CO_2 with LAV or A_{11} or $A\Delta_{11}$ (2µg/ml) in DMEM without FBS. The cells were washed with PBS and fixed for 15min using 4% paraformaldehyde followed by blocking with 5%FBS in PBS for 30min at RT. The cells were then incubated with anti- LAV or A_{11} or $A\Delta_{11}$ (mouse serum, 1:100 dilution) for 1h, washed three times with PBS and then stained with Alexa Flour 647 conjugated rabbit anti-mouse IgG (Biolegend, USA. Cells were extensively washed and mounted with VECTA SHIELD (containing DAPI) mounting medium (Biolegend) and observed under a confocal microscope (Leica SP8, Wetzlar, Germany).

RT-PCR

WT, TLR2KO, TLR4KO and DKO mouse macrophage cell lines were treated with A_{11} (2µg/ml), LPS (500ng/ml) or PAM3CSK4 (20ng/ml). After 4, 24 and 48h of treatment cells were recovered in 500µl of TRIzol (Invitrogen, Carlsbad, CA) and equal volumes of chloroform were added; samples were centrifuged at 12000 rpm for 15 min at 4°C. The aqueous phase was then passed through RNA easy mini columns (MN) and RNA was purified following the manufacturer's protocol. RNA quality was checked by running on a Formaldehyde gel for 18s and 28s RNA bands and analysed on Bioanalyser. The RNA quantity was assessed by UV spectroscopy and purity by 260/280 ratio. First-strand cDNA was synthesized using the superscript III-RT system (Invitrogen) following the manufacturer’s instructions. RT-PCR was performed in 96 well microtiter plates in a 10µl reaction volume containing 50ng cDNA, 10µM each primer (Table 1) and SYBR green (Bio-

Rad). Samples were run in triplicate and data was analysed with Sequence Detection System (Bio-Rad CFX-96). The experimental data were presented as fold changes of gene expression of stimulated cells at various time points relative to control. mRNA levels of the analyzed genes were normalized to the amount of GAPDH present in each sample.

Dot Blot binding Assay:

Dot blot binding assays were performed to confirm the binding of various domains of LAV (A_8-A_{13}) and also their single domain deletion mutants ($A\Delta_8$-$A\Delta_{13}$) with complement regulators FH, C4BP and PLG. 1µg of each protein (wild type, single domain and domain deletion mutant) were transferred onto NC membranes (0.2µ pore size; Bio-Rad). The membranes were kept for drying for 5-10 min at RT. The membranes were then blocked with 5% BSA in Tris-buffered saline-Tween 20 (TBS-T) for 2h at RT, washed three times with TBS-T, and incubated with 1% normal human serum (NHS) diluted in PBS with gentle shaking for 3h at RT. After extensive washing with TBS-T, the membranes were incubated with the corresponding primary antibody (Goat anti-FH, Mouse anti-PLG, Rabbit anti-C4BP; 1: 10,000 dilution) in TBS-T for 2h at RT. The membranes were then washed with TBS-T and incubated with a respective peroxidase-conjugated secondary antibody (1: 6,000) for 2h at room temperature. Reactive spots were developed using a chemiluminescence system with an exposure time of 10 sec.

ELISA binding assay:

Protein attachment to soluble complement regulators FH, C4BP and PLG were analysed by ELISA as described previously [42,45]. Briefly, micro titre plates were coated overnight at 4°C with 1 µg of domains (A_8-A_{13}) and their single domain deletion mutants ($A\Delta_8$-$A\Delta_{13}$). BSA and LAV were used as positive and negative control. The wells were washed three times with PBS containing 0.05 % Tween 20 (PBS-T), blocked with 300µl PBS/3 % BSA for 2h at 37°C, and incubated with 100µl 10 %NHS for 90 min at 37°C. After washing with PBST, goat anti-FH (1:1000), rabbit anti-C4BP (1:1000) or mouse anti-PLG (1:5000) was added and the plate was incubated for 1h at 37°C. After the usual steps of washing HRP-conjugated anti-goat/anti-mouse/anti-rabbit IgG was added and incubated for 1h at 37°C. The wells were washed again and TMB substrate was added (100µl/well). The reaction was stopped by the addition of 50 µl 2N H_2SO_4 and absorbance was read at 450 nm in a microplate reader.

Cofactor activity assay:

Cofactor activity was determined as described previously[45]. Briefly, 2µg of A_{11}, $A\Delta_{11}$ and LAV (positive control) or BSA (negative control) were coated on microplates overnight at

4°C. The wells were washed and blocked with PBS/2 % BSA for 2h at 37°C, followed by the addition of 2µg pure FH and further incubation for 90min at 37°C. Unbounded FH was removed by washing and then 250ng FI and 500ng C3b were added to the microtiter plate wells and incubated for 3-5h at 37°C. The supernatants were loaded onto a 10% SDS-PAGE gel and transferred onto a 0.22µ PVDF membrane. For the immunoblotting, membranes were blocked with 5 % BSA and then incubated with goat anti-human C3 (1:5000) for 2h at RT. After the usual steps of washing, the membranes were incubated with peroxidase-conjugated secondary antibody. The images were visualized under the Clarity Max Western ECL substrate (BIO-RAD, 1705062) using Syngene G: BOX Chemi XX6/XX9.

Plasmin activity assay:

Plasmin activity was determined as described previously[45]. Briefly, Microtiter plate wells were coated overnight at 4°C with 2µg LAV (positive control), BSA (negative control), $A_{11,}$ $A\Delta_{11}$. The plate was washed with PBS-T and blocked for 2h at 37°C with 10% skim milk. After discarding the blocking solution, human PLG (2µg/well) was added, followed by incubation for 90 min at 37°C. After washing plates three times with PBS-T, 250µg/ml uPA was added together with the plasmin-specific substrate, *D*-valyl-leucyl-lysine 4-*p*-nitroanilide dihydrochloride (100µl/well) at a final concentration of 0.4mM in PBS. Plates were incubated for 24h at 37°C and absorbance were measured at 405nm using a microplate reader.

Bactericidal assay

Bactericidal activity was determined as described elsewhere[45,66].1.3 × 10^8 *E Coli (BL21 DE3)* cells were washed once with PBS and incubated with 10 % NHS with or without pre-incubation with recombinant proteins ($A_{11,}$ $A\Delta_{11}$, LAV at 20µg/ ml) in a final reaction volume of 100µl for 30 min at 37 °C. The samples were placed on ice to stop further bacteriolysis and then plated on LB agar plates. The plates were incubated at 37°C overnight. Survival was determined by counting bacterial colonies the following day.

Nuclease activity

To examine the DNase activity of LAV, 1µg of PCR product of 1100bp was incubated with different concentration (1 or 2 or 5 or 10µg) of LAV or 2µg domains (A_8-A_{13}) and 2µg domain deletion mutants ($A\Delta_8$-$A\Delta_{13}$) or DNase I (20IU, positive control) in DPBS with 5mM $MgCl_2$ in a PCR tube at 37°C for 2h. The reaction mixture was subjected to EtBr Agarose gel electrophoresis (1%) and then observed under the Gel doc.

Isolation of Neutrophils from murine bone marrow

Neutrophils were isolated from the bone marrow of C57BL/6J mice using standard procedure[67]. Briefly, bone marrow was flushed from femurs using a 26G needle, passed through a 30µm cell strainer and then cells were washed in complete RPMI-1640 twice at 1,400 rpm for 10min at 4°C. After lysis of RBCs using ACK lysis buffer, cells were washed with RPMI 1640 supplemented with 10% FBS, counted and resuspended in 1ml of ice-cold sterile PBS. Cells were overlaid on 3ml of Histopaque 1077/1119 mix in a 15ml conical tube and then centrifuged for 30min at 2,000 rpm at 25°C without brake. Neutrophils at the interface were collected and washed twice with complete RPMI-1640 medium, counted and suspended in the medium for specific assay. The viability was determined by Trypan blue exclusion assay.

NET assay

2 x 10^5 freshly isolated neutrophils in 300µl medium were added on to the imaging dish and kept at 37°C in the presence of 5%CO_2 overnight. Cells were treated with 3µl of DMSO or PMA (50ng/ml) or LPS (100ng/ml) and further incubated for 3h at 37°C/5%CO_2. Cells were washed thrice with DPBS and then incubated with LAV (2µg/ml) or DNase-I (20IU) or BSA (5µg/ml) in 5mM $MgCl_2$ containing PBS for 2h at 37°C/5%CO_2. Cells were washed with DPBS, fixed with 4% PFA (15min at RT) and then stained with Rat anti-mouse Ly6G (Alexa flour 647) for 30min. Cells were washed thoroughly with DPBS, mounted with VECTA SHIELD (with DAPI) mounting medium (Biolegend) and observed using a 63x oil objective on a confocal microscope (Leica SP8, Wetzlar, Germany).

Statistical Analysis

For all the experiments, wherever required, Student's t-test and one-way ANOVA were executed for the analysis of the results. The data were represented as the mean of triplicates ± SEM. $p < 0.05$ was considered significant.

Results:

Lig proteins induced TLR4 dependent activation and maturation of mouse macrophages

To test whether the immunogenicity of Lig proteins is correlated to activation of innate immune response we tested its ability to activate mouse macrophages. We cloned, expressed and purified LC, LAV, and LBV in a soluble form (Sup Fig. 1A). We stimulated mouse macrophages with varying doses (1, 2 and 5µg/ml) of the proteins and our results show that while LAV was stimulatory at a low dose (2µg/ml), LC and LBV induced production of pro-inflammatory cytokines (IL-6, TNF-α) at a higher dose of 5µg/ml (Fig. 1A). Taking into

account that these proteins might have LPS contamination, they were pre-incubated twice with (Polymyxin-B) PMB-agarose to remove the endotoxin activity. 500ng/ml LPS pre-incubated with the same concentrations of PMB-agarose was used as control. The estimated concentration of LPS in final protein preparation varied from (0.10–0.15ng/ml). The effect was protein specific because proteinase-K plus heating abolished the production of cytokines (Sup Fig. 1C). Besides, PMB inhibited the LPS induced cytokine production but did not attenuate the levels induced by proteins, indicating that the stimulatory effects observed were specific to protein and not due to contamination with LPS (Sup Fig. 1C). Since LAV induced a stimulatory effect at a low dose we chose this protein to test whether this activation was via signalling through TLR2 or TLR4. LAV showed binding specifically with TLR4 and failed to bind to the TLR2 receptor as confirmed by confocal microscopy (Fig. 1B). To confirm that this binding leads to activation and subsequent cytokine production, we stimulated WT, TLR2KO, TLR4KO and DKO macrophages and HEK cells expressing these receptors with LAV. Our result shows that while WT and TLR2KO macrophages cells induced significant levels of IL-6 and TNF-α, TLR4KO and DKO macrophages failed to induce these cytokines (Fig. 1C). Similarly, HEK-TLR4 stimulated with LAV produced significant levels of IL-8 whereas HEK-TLR2 cells didn't produce a significant level of this cytokine upon stimulation with LAV (Fig. 1D). These results indicate that LAV is a TLR4 ligand that induces signalling through this receptor for the production of pro-inflammatory cytokines.

11th domain of the variable region of LigA (A_{11}) is involved in signalling through TLR4 for the activation and maturation of macrophages

Since LAV induced TLR4 dependent activation of mouse macrophages, we aimed to identify and characterize the domain/s involved in activation. We cloned, expressed and purified the individual domain (A_8-A_{13}) and tested their ability to activate mouse macrophages (RAW264.7 cells). Our result shows that only 11th domain (A_{11}) was able to induce significant level of IL-6 and TNF-α (Fig.2A, Sup Fig.1A). To confirm that A_{11} is involved in production of cytokines we created domain deletion mutants of LAV($A\Delta_8$-$A\Delta_{13}$), purified the proteins in soluble form (Sup Fig.1B). We tested the ability of these mutants to activate mouse macrophages and our result shows that all the deletion mutants of LAV induced production of IL-6 and TNF-α except AΔ11 further confirming that this domain is involved in activation of macrophages and subsequent production of cytokines (Fig. 2B). To confirm that A_{11} is involved in interaction and subsequent signalling via TLR4, we tested its binding with the receptor first by docking the protein with TLR4 of mouse, human and bovine origin.

Our result of top-ranking docking scores structures shows that A_{11} was able to bind to the concave side of the TLR4 receptor of all the species with low binding energy whereas $A\Delta_{11}$ has shown binding on the convex side with high binding energy indicating that the deletion of this domain impaired the binding to the concave side which is essential for signalling (Fig. 2C Sup Fig.2). Confocal microscopy also confirmed the binding of A_{11} with mouse TLR4 as strong anti-LAV or anti-rA_{11} fluorescence was observed on the surface of WT and TLR2KO cells incubated with respective antibodies but little fluorescence on TLR4KO or DKO cells. Further, there was very little anti- $A\Delta_{11}$ fluorescence on the surface of all cell types indicating that this protein failed to bind to the TLR receptor (Fig. 2D). To confirm that this TLR4 binding leads to activation of these cells we stimulated mouse WT, TLR2KO, TLR4KO and DKO macrophages with LAV, A_{11} and $A\Delta_{11}$ and our results indicate that A_{11} induced IL-6 and TNF-α production via signalling through TLR4 as TLR4KO and DKO macrophages failed to induce any significant level of these cytokines. Further inability of AΔ11 to induce significant levels of cytokines in WT or TLR2 KO macrophages indicates that the 11th domain is critical for signalling via TLR4 (Fig. 2E). To confirm whether stimulation with A_{11} causes activation and maturation of macrophages we analysed the expression of costimulatory molecules (CD80, CD86 and CD40) and maturation marker (MHC-II) in RAW2645.7 cells. Our FACS results show that LAV and A_{11} significantly enhanced the expression of CD80, CD86 and CD40 which was significantly reduced in cells stimulated with $A\Delta_{11}$ indicating that this domain is involved in enhancing the expression of these surface molecules (Fig. 2F). These results demonstrate that A_{11} is involved in TLR4 dependent activation and maturation of mouse macrophages.

A_{11} induces immune activation via MAPK signalling involving the MyD88 adapter

Since TLR4 involves both MyD88 and TRIF adapter for downstream signalling and A_{11} induced immune activation through TLR4, we examined the adapter molecule involved in the signalling. We stimulated MyD88KO, TRIFKO and TMDKO macrophages with A_{11} and our result shows that signalling pathway involves MyD88 adapter as MyD88KO macrophages failed to induce significant levels of IL-6 and TNF-α whereas there was no difference in the production of these cytokines in TRIFKO macrophages (Fig. 3A). Because MAPKs are critical factors that are involved in cellular responses to inflammatory stimuli, we examined the activation of this pathway in response to $A_{11.}$ We stimulated mouse WT, TLR2KO, TLR4KO and DKO macrophages with A_{11} and analysed phosphorylation of P38, JNK, ERK and degradation of IkBα. The A_{11} mediated phosphorylation of p38 and JNK was

significantly reduced in TLR4KO and DKO macrophages indicating that p38 and JNK pathway are involved in signalling (Fig. 3B). Degradation of IkBα in WT and TLR2KO macrophages indicate that A11 is signalling via NF-kB pathway. Next, to elucidate the functional role of these kinases in A_{11} induced macrophage activation and maturation we used pharmacological inhibitors of these pathways and analysed cytokines in RAW264.7 cells pre-treated with or without inhibitors of NF-kB, JNK, p38MAPK or ERK. IL-6 and TNF-α production was significantly blocked by p38 inhibitor ($P < 0.05$, 50% inhibition with 2µg/ml A_{11}) and by JNK and NF-kB($P, < 0.05$, 30% inhibition with 2µg/ml A_{11}). ERK inhibitor didn't effect the production of cytokine indicating that this pathway is not involved in signalling. The production of TNF-α was also significantly blocked by JNK, p38 and NF-κB inhibitor ($P < 0.05$, 60% inhibition). A combination of all three inhibitors completely inhibited A_{11} induced cytokine production (Fig. 3C). All these results suggest that A_{11} stimulates the production of pro-inflammatory cytokines through p38, JNK and NF-kB pathways. The ability of A_{11} to regulate innate responses was further investigated based on the expression of key inflammatory cytokine and chemokine genes at various time points (4, 24 and 48h). WT, TLR2KO, TLR4KO and DKO mouse macrophage were stimulated with A_{11} and expression of mRNA transcript was analysed by RT-PCR. A_{11} induced significant upregulation (>5fold) of CXCL10, IL-1β, TNF-α, COX2, iNOS, MCP-1 and IL-6 in WT and TLR2KO mouse macrophage at 4hr and 24h time point which was significantly reduced or down-regulated in TLR4KO and DKO macrophages (Fig. 3D). PAM3CSK4 (TLR2 ligand) and LPS (TLR4 ligand) showed significant upregulation of genes involved in TLR2 and TLR4 signalling as expected. These results demonstrate that A_{11} induced TLR4 dependent expression of innate response genes.

Leptospira evades killing by both classical and alternate pathways by acquiring complement regulators through A_{11}

Leptospira evades complement-mediated killing by acquiring complement regulators which involves binding with surface proteins. Lig proteins including LigA have been shown to bind to various complement regulators. To identify and characterize the domain of LAV we screened the individual domains (A_8-A_{13}) and corresponding deletion mutants ($A\Delta_8$-$A\Delta_{13}$) for their ability to bind with complement regulators (FH, C4BP and PLG). Our dot blot result shows that A_{11} was involved in binding to FH and PLG whereas $A_{8,}$ A_9 A_{10} and A_{11} are involved in binding to C4BP (Fig. 4A, sup Fig. 3). This was further confirmed by ELISA (Fig. 4B). We further determined if binding of A_{11} with complement regulators is sufficient

for its functional activity. Our result shows that both LAV and A_{11} were able to bind to FH to cleave C3b in presence of Factor I (FI) as evidenced by cleavage fragments, whereas $A\Delta_{11}$ failed to do so indicating that the 11th domain is involved in binding with and mediating the cofactor activity (Fig. 4C). Our result also shows that A_{11} binds with PLG and induces subsequent plasmin activity as evidenced by cleavage of C3b (Fig.4D). To establish the role of A_{11} in the complement-mediated killing, we incubated *E. coli* with 10% Normal Human Serum (NHS) pre-incubated with A_{11} or LAV or $A\Delta_{11}$. Our results show that both LAV and A_{11} domain were able to rescue bacteria from complement-mediated killing but $A\Delta_{11}$ failed to do so indicating that the 11th domain is involved in evasion from complement-mediated killing (Fig. 4E).

LAV is a nuclease involved in the evasion of *Leptospira* from Neutrophil extracellular traps.

Recently it has been shown that *Leptospira* induces NET, however, whether it expresses protein having nuclease activity to degrade the NET has not been reported. To test whether LAV has nuclease activity which might have a role in evasion from NETs, we incubated the DNA (PCR product) with varying concentration of the protein (1 to 10µg) and our result shows that LAV was able to degrade DNA in a dose-dependent manner indicating its nuclease activity (Fig 5A). To analyse if this activity is restricted to any particular domain we incubated the DNA with individual domains (A_8-A_{13}) or deletion mutants ($A\Delta_8$-$A\Delta_{13}$) and our result shows that none of the domains except A_{11} were able to degrade DNA whereas all the deletion mutants except $A\Delta_{11}$ degraded DNA with equal propensity (Fig. 5B, 5C). These results indicate that LAV's nuclease activity resides in 11th domain and LAV is mediating this activity by utilizing this domain (Fig. 5B, 5C). To test whether LAV can cleave the NET we stimulated the mouse neutrophils with PMA and then treated with LAV (5µg/ml). Our result shows that LAV was able to degrade the PMA induced NET further confirming its nuclease activity and possible role in degrading NETs *in vivo* (Fig.5D). To investigate if the antibodies against LAV can inhibit its nuclease activity we incubated the protein with anti-LAV mouse serum and then tested the activity. Our results show that anti-LAV antibodies inhibited the nuclease activity (degradation of DNA) in a dose-dependent manner which was detectable up to 1:1000 dilution of serum. In contrast, normal serum (without anti-LAV antibodies) failed to inhibit the nuclease activity even at 1:10 dilution (Fig. 5E). These results indicate that LAV has nuclease activity that *Leptospira* might exploit to evade from NETosis.

Discussion

The ability of *Leptospira* to cause persistent infection and efficient colonization in a variety of host reflects its potential to subvert or thwart the innate immune response[25 68,69]. This ability has been attributed to the procession of a wide variety of surface molecules like proteins, Lipopolysaccharide, etc. which are not only redundant in their function but may also undergo structural variation to avoid recognition by the host immune system as has been observed in other spirochetes [20,70]. Surface proteins including lipoproteins from spirochetes like *Borrelia* and *Treponema* play a critical role in immune evasion by limiting their expression or inducing antigenic variation after infection which greatly enhances host infectivity and persistence[21-23]. *Leptospira* like other pathogens may voluntarily interact with TLRs (or other innate receptors) through surface molecules (proteins, LPS) but might evades this recognition through multiple mechanisms to establish infection or fitness in the host. Both TLR2 and TLR4 receptors play a major role in host defence against *Leptospira* infection[33]. It has been shown that TLR4 plays a critical role in controlling bacterial load and the development of severe leptospirosis in mice [71].Thus, it is likely that those surface proteins and LPS which are natural ligands of these receptors and can activate macrophages and DCs, might undergo variations thereby enabling the bacteria to evade this innate recognition and adhere to the host. Several surface proteins of *Leptospira* have been identified as ligands of TLR2 or TLR4 that are capable of activating innate immune response and are potential vaccine candidates [34-37,72]. Lig proteins are important virulence factors and its expression during infection or loss of its expression during *in vitro* culture has been correlated to virulence of the infecting serovar [52,56,73]. These proteins interact with various host molecules, including proteins of the extracellular matrix (ECM), coagulation cascade and also complement regulators. LigA is the most promising vaccine candidate and it has been demonstrated that the variable region of the protein comprising of domains 10-13 ($LigA_{10-13}$) is sufficient in inducing protection against challenge in hamster model[59-61,63]. Thus the diverse functions of LigA prompted us to investigate whether their role is limited to binding to host ECM and complement regulators or they are also involved in the modulation of host innate immune response thereby contributing to infection and persistence in the host.

We truncated the Lig proteins (LigA and LigB) into conserved (LC) and the variable region of A (LAV) and B (LBV), expressed the recombinant proteins without signal sequence and screened for their ability to activate mouse macrophages. Our result demonstrated that out of three fragments of Lig proteins tested, LAV induced production of pro-inflammatory cytokines via signalling through TLR4 (Fig.1). This effect was not due to contaminating LPS

but specific to protein as incubation with PMB didn't attenuate whereas digestion with Proteinase K abrogated the cytokine production (sup Fig.1C). We expected that LAV might not signal through TLR2 as it is devoid of the signal sequence and hence lipidation, although several non-acylated proteins like LcrV from *Yersinia*, MPB83 and PPE18 from *Mycobacterium*, PorB from *Neisseria* and FimA from *P. gingivalis* have shown to signal through TLR2 [74-78]. LAV signalling through TLR4 is also not unusual as several proteins from other bacterial pathogens have shown to induce TLR4 dependent production of pro-inflammatory cytokines and expression of surface markers [79,80]. Further, lipidated recombinant proteins which usually signal through TLR2 due to lipid moiety may signal through TLR4 if unlipidated as has been observed in the case of Omp16 and Omp19 of *Brucella* [81,82]. Moreover, recombinant unlipidated rBCSP31 from *Brucella abortus* and rLsa21 from *Leptospira* have shown to signal through both TLR2 and TLR4 and induce activation of macrophages[34,83]. Since LAV induced strong TLR4 dependent innate immune activation and demonstrated protective immunity we chose this region to identify and characterize the domain involved in modulation (activation or evasion) of the innate response. Our results clearly demonstrate that the 11th domain (A_{11}) which lies in LAV was able to bind to the TLR4 receptor of mouse, human and bovine origin. This binding stimulated the mouse macrophages and subsequent activation of MAP kinase involving p38 and JNK pathway for the production of pro-inflammatory cytokines and enhanced the expression of costimulatory molecules and maturation marker (Fig. 2and 3). Further, A_{11} modulated the expression of several innate responses related genes (cytokines, chemokines and surface receptors) involved in the activation and maturation of macrophages (Fig.3). The inability of $A\Delta_{11}$ to induce pro-inflammatory cytokines and express surface markers (Fig. 2 and sup Fig.2) may be correlated to its inability to bind to the concave side of the receptor which is required for signalling and subsequent activation of cells[84]. Our results are in accordance with previous reports where several TLR4 ligands including bacterial proteins have shown to activate macrophages and DCs via signalling through the MAP kinase pathway leading to the induction of cytokines, expression of surface markers and immune response-related genes[79,85-88]. Our results suggest that TLR4 dependent signalling by A_{11} might activate antigen-presenting cells (APCs) to produce bactericidal effectors and cytokines which would eventually enhance its phagocytic activity and subsequent activation of B cells to produce antibodies for clearance of *Leptospira* from the host. We speculate that to evade this protective response, *Leptospira* might limit expression or undergo antigenic

variation in LigA to avoid recognition with TLR4 and subsequent activation of the innate response. However, this need to be tested and experiments are ongoing.

It is known that pathogenic *Leptospira* are resistant to the bactericidal activity of normal human serum (NHS). They are capable of evading complement attack by using various strategies like recruitment of host complement regulators, acquisition of host proteases or secretion of proteases that can cleave complement components on the bacterial surface and also in its surroundings[13]. Several surface proteins of *Leptospira* like LenA, LenB, LcpA, Lsa30 including Lig proteins (LigA and LigB) have shown to bind to various complement regulators [32,39-49,65,89]. Moreover, both conserved and the variable (N and C terminal) region of LigA and LigB are involved in binding to FH and C4BP. Our results not only confirm the previous report of binding of LAV with FH and C4BP but also demonstrated that this part of the protein is capable of binding to host plasminogen (PLG). Our study further identified and characterized the domain/s involved in binding FH, C4BP and PLG and mediating subsequent co-factor or plasmin activity (Fig. 4). Additionally, the rescue of *E.coli* from complement-mediated killing in NHS pre-incubated with A_{11} further substantiates the critical role of this domain in complement evasion (Fig.4E). Thus binding of FH, C4BP and PLG to A_{11} reflects the ability of *Leptospira* to utilize this domian for simultaneously inhibiting both classical and alternate pathway of complement-mediated killing (Fig. 4 and sup Fig.3). To our knowledge, this is the first report that demonstrates that a single domain of a surface protein is alone capable of recruiting FH, C4BP and PLG directly from NHS and prevent complement activation.

Neutrophils apart from killing bacterial pathogens by intracellular ROS and phagocytosis might release Neutrophils extracellular traps (NETs) that capture and kill microbes in the extracellular space in tissues (at sites of infection) or within blood vessels[29,90]. This mechanism of killing extracellular bacteria by trapping outside the cell is independent of phagocytosis and degranulation [29]. Several bacterial pathogens including *S. aureus*, *Clostridium perfringens* and *S. pyogenes* have evolved sophisticated mechanisms to suppress, escape, and/or resist NETs through surface proteins having nuclease activity[51,91,92]. Recently it has been shown that *Leptospira* can induce NET and its surface protein LipL21 can modulate neutrophil function, however, nuclease capable of degrading NET has not been reported[28,50]. Our study discovered the nuclease (DNase) activity of LAV. Our result shows that this nuclease activity is mediated by A_{11} as this domain was able to degrade DNA with the same propensity as LAV (Fig. 5). The ability of LAV to degrade PMA induced NET in mouse neutrophils and the ability of anti-LAV antibodies to inhibit DNA degradation *in vitro*

highlights the possible role of LigA in escaping the bacteria from NETosis. To our knowledge, this is the first report of identification of nuclease activity of a surface protein in *Leptospira* and also demonstrating its diverse role in modulating the host innate response.

In conclusion, our results clearly demonstrate that LigA is multifunctional protein involved in attachment to host cells to initiate infection, a TLR4 agonist which can activate a strong innate response (possibly evading this TLR4 activation by antigen variation or downregulating its expression upon infection in the host), binds to complement regulators to evade complement-mediated killing and exhibit nuclease activity when *Leptospira* gets entrapped in NET (Fig. 6). These features might contribute to its successful colonization in a particular host. Interestingly, these functions are mediated by single domain (A_{11}) which lies in LAV, a promising vaccine candidate that conferred protective immunity against lethal infection in the hamster model of the disease. Thus the protective efficacy of LAV based vaccine may be correlated to its ability to induce strong antigen-specific innate and adaptive response that might lead to the generation of antibodies conceivably blocking binding to ECM, acquiring complement regulators and also inhibiting DNase activity, all of these may aid in the clearance of bacteria from the host (Fig. 6). Our results provide important insight into the role of LAV in host-pathogen interaction and also establishes it as an immuno-modulator or adjuvant which makes it an ideal candidate for developing vaccines for this dreadful zoonosis[93].

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Author's contribution

SMF conceived the idea and designed the experiments. AK, VPV, SK, MA, PV, MAT performed the experiments. AK, VPV, SK and SMF analyzed the data. VPV made all the figures of the manuscript and did the statistical analysis. YFC contributed reagents and input in experiment design and data analysis. SMF, AK and VPV wrote the initial draft and SMF edited the manuscript. All authors approved the final version of the manuscript.

Competing interests

The authors declare no competing financial interests.

Legends to Figures

Fig.1: Variable region of LigA (LAV) induced TLR4 dependent activation of mouse macrophages.

(**A**) *Screening of pro-inflammatory response of rLig proteins in RAW264.7 cells.* RAW264.7 cell lines were stimulated with LPS (500ng/ml) or PAM3CSK4(20ng/ml) or rLig proteins (LC, LAV, LBV) in varying concentration 1 or 2 or 5μg/ml along with PMB for 24h and supernatant was collected to measure levels of TNF-α and IL-6 cytokines by using ELISA. **(B)** *Binding of LAV with TLR4.* WT, TLR2KO, TLR4KO or DKO macrophages were incubated with rLAV (2μg/ml) for 30min. After washing, cells were fixed and stained with respective antibodies and analysed by confocal microscope as described in materials and methods. **(C)** *IL-8 response in HEK293-TLR4 cells stimulated with LAV.* HEK293T cells transfected with TLR2, TLR4 and NF-kB reporter plasmids were stimulated with rLAV (2μg/ml) for 24h and IL-8 was measured in culture supernatant by ELISA **(D)** *Pro-inflammatory response of mouse macrophages stimulated with rLig proteins.* WT, TLR2KO, TLR4KO and DKO macrophages cell lines were treated with rLig proteins (LC or LAV or LBV at 2μg/ml), LPS (500ng/ml) or PAM3CSK4(20ng/ml) for 24h at 37°C in presence of 5%CO_2 and levels of IL-6 and TNF-α in the supernatants were measured by ELISA. Data are representative of three different experiments. Significant differences were calculated using the Student's t-test (***, **, * indicates $P < 0.001$, $P < 0.01$ and $P < 0.05$ respectively).

Fig.2: 11th domain of LigA (A_{11}) is involved in binding to TLR4 and subsequent signalling leading to activation and maturation of mouse macrophages

(A) *Screening of pro-inflammatory response of individual domains of LAV in RAW264.7 cells* RAW264.7 cells were incubated with LPS (500ng/ml) or PAM3CSK4(20ng/ml) or individual domains (2μg/ml) with PMB for 24h at 37°C in presence of 5%CO_2 and supernatant was collected to measure levels of IL-6 and TNF-α by ELISA. **(B)** *Screening of pro-inflammatory*

response of domain deletion mutants of LAV in RAW264.7 cells. RAW264.7 cells were stimulated with LPS(500ng/ml) or PAM3CSK4(20ng/ml) or LAV or corresponding deletion mutants ($A\Delta_8$-$A\Delta_{13}$) at a concentration of (2μg/ml) along with PMB for 24h at 37°C in presence of 5%CO_2 and supernatant was collected to measure levels of IL-6 and TNF-α by ELISA. **(C)** *Molecular modelling to show the interaction of A_{11} with TLR4.* The interaction of A11 (yellow) with mouse, human and bovine TLR4 LRR domain was modelled using various tools as described in the methodology. The top 10 ranking structures were selected based on the docking score and binding energy. **(D)** *Binding of A_{11} with mouse TLR4.* WT, TLR2KO, TLR4KO or DKO macrophages cell lines were incubated with LAV, $A_{11,}$ AΔ11 (2μg/ml) for 30 min. After washing, cells were fixed and stained with respective antibodies and analysed by confocal microscope as described in materials and methods. **(E)** *The pro-inflammatory response of mouse macrophages stimulated with A_{11}.* WT, TLR2 KO, TLR4 KO and DKO macrophages cell lines were treated with LAV, $A_{11,}$ $A\Delta_{11}$ (2μg/ml), LPS (500ng/ml) or PAM3CSK4(20ng/ml) for 24h at 37°C in presence of 5%CO_2 and levels of IL-6 and TNF-α in the supernatants were measured with ELISA. **(F)** *A_{11} enhanced the expression of surface markers in mouse macrophages. RAW*264.7 cells were incubated with LPS (500ng/ml) or PAM3CSK (20ng/ml) or LAV or A_{11} or $A\Delta_{11}$ (2μg/ml) for 24h at 37°C in presence of 5%CO_2. Cells were stained with fluorochrome-conjugated antibodies and then analysed by Flow cytometry. as described in materials and methods. UI indicates uninduced or unstimulated cells, PAM indicates PAM3CSK4 (20ng/ml) and LPS is *E. coli* Lipopolysaccharide (500ng/ml). Data are representative of three different experiments. Significant differences were calculated using the Student's t-test (***, **, * indicates $P < 0.001$, $P < 0.01$ and $P < 0.05$ respectively).

Fig.3: 11th domain of LigA (A_{11}) produces pro-inflammatory cytokines via signalling through MAP kinase involving p38 and JNK pathway

(A) *A_{11} signals through TLR4 involving the MyD88 adapter*. WT, MyD88 KO, TRIF KO and TMDKO macrophages cell lines were treated with LPS (500 ng/ml) or PAM3CSK (20 ng/ml) or LAV or A_{11} or $A\Delta_{11}$ (2μg/ml) for 24h at 37°C in presence of 5%CO_2 and levels of IL-6 and TNF-α in the supernatants were measured by ELISA. **(B)** *Pharmacological inhibitors of p38 and JNK significantly reduces A_{11} mediated cytokine response*. RAW 264.7 cells were pre-treated for 30min with NF-kB inhibitor (SN50; 20μM), JNK inhibitor (SP600125; 40μM) or p38MAPK inhibitor (SB203580; 30μM) or ERK (U0126; 50μM) or all four inhibitors together and then stimulated with A_{11} (2μg/ml) for 24h at 37°C in presence of 5%CO_2 and supernatant was collected to measure levels of IL-6 and TNF-α by ELISA. **(C)** *A_{11} signals through TLR4 via the MAP kinase pathway involving phosphorylation of p38 and JNK.* WT or TLR2KO or TLR4KO or DKO macrophages cell lines were stimulated with A11 (2μg/ml) for 24h at 37°C in presence of 5%CO_2. Levels of phosphorylated p38, JNK, and ERK1/2 induced by A11 were analyzed by western blot as described in materials and methods. (D) *Analysis of expression of immune response-related genes in mouse macrophages stimulated with A_{11}.* WT, TLR2KO, TLR4KO and DKO mouse macrophage cell lines were treated with A11 (2μg/ml), LPS (500 ng/ml) or PAM3CSK4 (20ng/ml). After treatment at various time points (4, 24 and 48h) cells were recovered, RNA was isolated, converted to cDNA and gene expression was analysed by RT-PCR as described in material and methods. The data were presented as fold changes between stimulated cells Vs control and normalized to GAPDH. Significant differences were calculated between control and treatment groups using the Student's t-test (*Indicates $P < 0.05$). UI indicates uninduced or

unstimulated cells, PAM indicates PAM3CSK4(20ng/ml) and LPS is *E. coli* Lipopolysaccharide (500ng/ml). All data are representative of three independent experiments. Significant differences were calculated using the Student's t-test (***, **, * indicates $P < 0.001$, $P < 0.01$ and $P < 0.05$ respectively).

Fig.4: Characterization of the domain of LAV involved in evasion from complement-mediated killing

(A) *Binding of LAV domains as analysed by Dot blot.* Purified proteins LAV (positive control), BSA (negative control), individual domains (A_8-A_{13}) and corresponding deletion mutants ($A\Delta_8$-$A\Delta_{13}$) were immobilized on nitrocellulose membranes and then incubated with 1% NHS (as a source of FH and PLG). FH and PLG were detected with specific antibodies by Western blot. **(B)** *Binding of LAV domains as analysed by ELISA.* Microtitre plates were coated with 1µg of proteins LAV, domains (A_8-A_{13}) and corresponding deletion mutants ($A\Delta_8$-$A\Delta_{13}$) and 10% HI-NHS was added to each well. The binding was detected with specific antibodies against FH and PLG as described in materials and methods. **(C)** *Plasmin activity.* LAV, A_{11}, $A\Delta_{11}$ (2µg/ml) and BSA (2µg/well) were immobilized on microtiter plates followed by the addition of PLG, uPA and specific plasmin substrate. The plate was incubated for 48h and absorbance was read at 405nm as described in materials and methods. In another experiment, C3b was incubated with activated plasmin in the presence or absence of A_{11} and cleavage products were visualized using Western blot. **(D)** *Co-factor activity.* LAV, A_{11}, $A\Delta_{11}$ (2µg/ml) and BSA (2µg/well) were immobilized on microtiter plates and incubated with purified FH. After washing, C3b and factor I (FI) were added and the plate was incubated for 4h at 37°C. The products were analysed by SDS PAGE, and the cleavage fragments of C3b was detected by Western blot using anti-human C3 polyclonal antibodies as described in materials and methods. **(E)** *Bactericidal assay.* 1.3×10^8 *E Coli (BL21 DE3)* cells were incubated with 10% NHS with or without pre-incubation with A_{11} or $A\Delta_{11}$ or LAV at 20µg/ml) for 30 min at 37 °C. The samples were plated on LB agar plates and incubated at 37°C overnight. Survival was determined by counting bacterial colonies the following day. All data are representative of three independent experiments. Significant differences were calculated using the Student's t-test (***, **, * indicates $P < 0.001$, $P < 0.01$ and $P < 0.05$ respectively.

Fig.5: LAV is nuclease capable of degrading Neutrophil Extracellular Trap (NET)

(A) *DNase activity of the LAV.* 700bp DNA (200ng) was incubated with different concentrations of LAV (2, 5 and 10µg) in DPBS with 5mM $MgCl_2$ at 37°C for 3h followed by visualised using the Agarose gel electrophoresis. Lane 1: DNA alone, Lane 2; DNA with 2µg of LAV, Lane 3; DNA with 5µg of LAV, Lane 4; DNA with10µg of LAV, Lane M: 100bp DNA ladder. **(B)** *DNase activity of domains of LAV.* DNA (200ng) with incubated with various LAV domains (A_8-A_{13}) at 5µg in DPBS with 5mM $MgCl_2$ incubated at 37°C for 3h followed by visualised using the EtBr- Agarose gel electrophoresis. Lane M- DNA ladder, Lane 2- DNA alone(C1), Lane 3- DNase (D), Lane 4- DNA with reaction mixture (C2), Lane 5-10- DNA with domains A_8-A_{13}. **(C)** *DNase activity of domain deletion mutants of LAV.* DNA (200ng) with incubated with various domain deletion mutants of LAV ($A\Delta_8$-$A\Delta_{13}$) at 5µg in DPBS with 5mM $MgCl_2$ incubated at 37°C for 3h followed by visualised using the EtBr- Agarose gel electrophoresis Lane M- DNA ladder, Lane 2- DNA alone(C1), Lane 3- DNase (D), Lane 4- DNA with reaction mixture (C2), Lane 5-10- DNA with deletion mutants $A\Delta_8$-$A\Delta_{13}$. **(D)** *Inhibition of DNase activity of LAV.* LAV(5µg) was pre-incubated with

different dilutions (1:10- 1:100000) of anti-LAV polyclonal antibodies for 30min at 37°C followed by the addition of DNA in DPBS with 5mM $MgCl_2$ and further incubated at 37°C for 2h followed by visualised using the EtBr- Agarose gel electrophoresis. Lanes C-DNA without LAV, D- DNA with DNase-I, WT-DNA with LAV, Lane 4-8- DNA with LAV pre-incubated with different dilutions of anti-LAV mouse serum Lane M; 100bp Ladder (C) *NETosis assay:* Mouse Neutrophils were cultured on glass coverslips stimulated with DMSO or LPS (500ng) or PMA (0.5µM) for 3.5h and then treated with DNase-I (positive control) or BSA(negative control) or LAV (5ug) for 2h at 37°C and visualized under 63X of Leice microscopy. DAPI; staining of the complete DNA content (Nuclear and released), Ly6G; neutrophil marker, BF; Bright field. All data are representative of three independent experiments.

Fig.6. **Schematic presentation of role of LigA in modulation of host immune response. (A)** *Immune evasion.* LigA expressed during infection might acquire complement regulators (FH, C4BP, PLG) to inhibit both the classical and alternate pathways of complement-mediated killing. *Leptospira* might utilize the nuclease activity of LigA to escape from NET. Upon interaction with host innate immune cells (DCs, macrophages), LigA might undergo antigenic variation or downregulate its expression to evade recognition through TLR4 and subsequent activation of the innate response. **(B)** *Immune activation.* LigA (LAV) can activate strong innate and subsequent adaptive immune response leading to the production of antibodies that may block binding to complement regulators, inhibit nuclease activity and enhance phagocytosis, all of which may contribute to the killing of bacteria and clearance from the host.

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Fig. 1

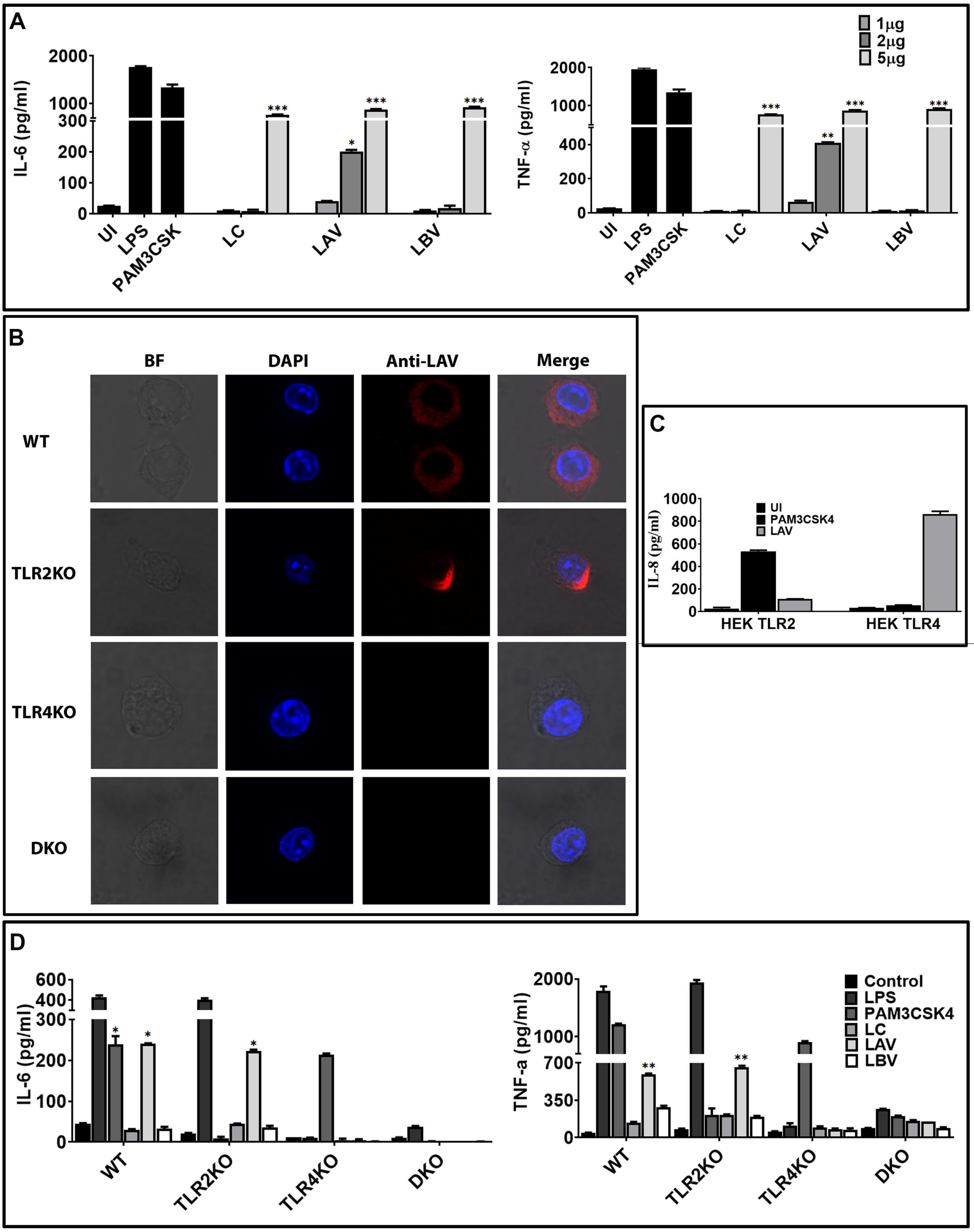
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Fig. 2

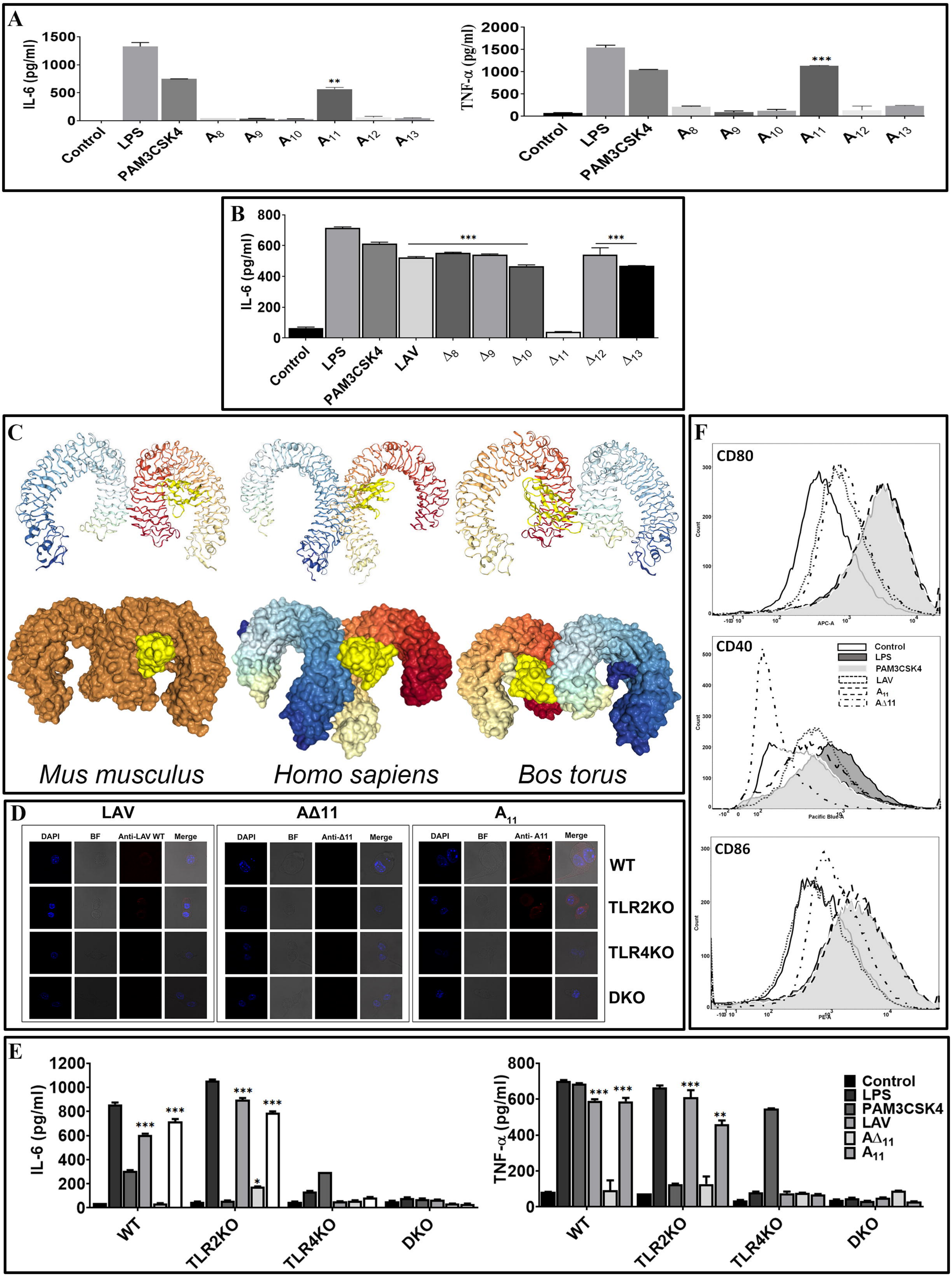
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Fig. 3

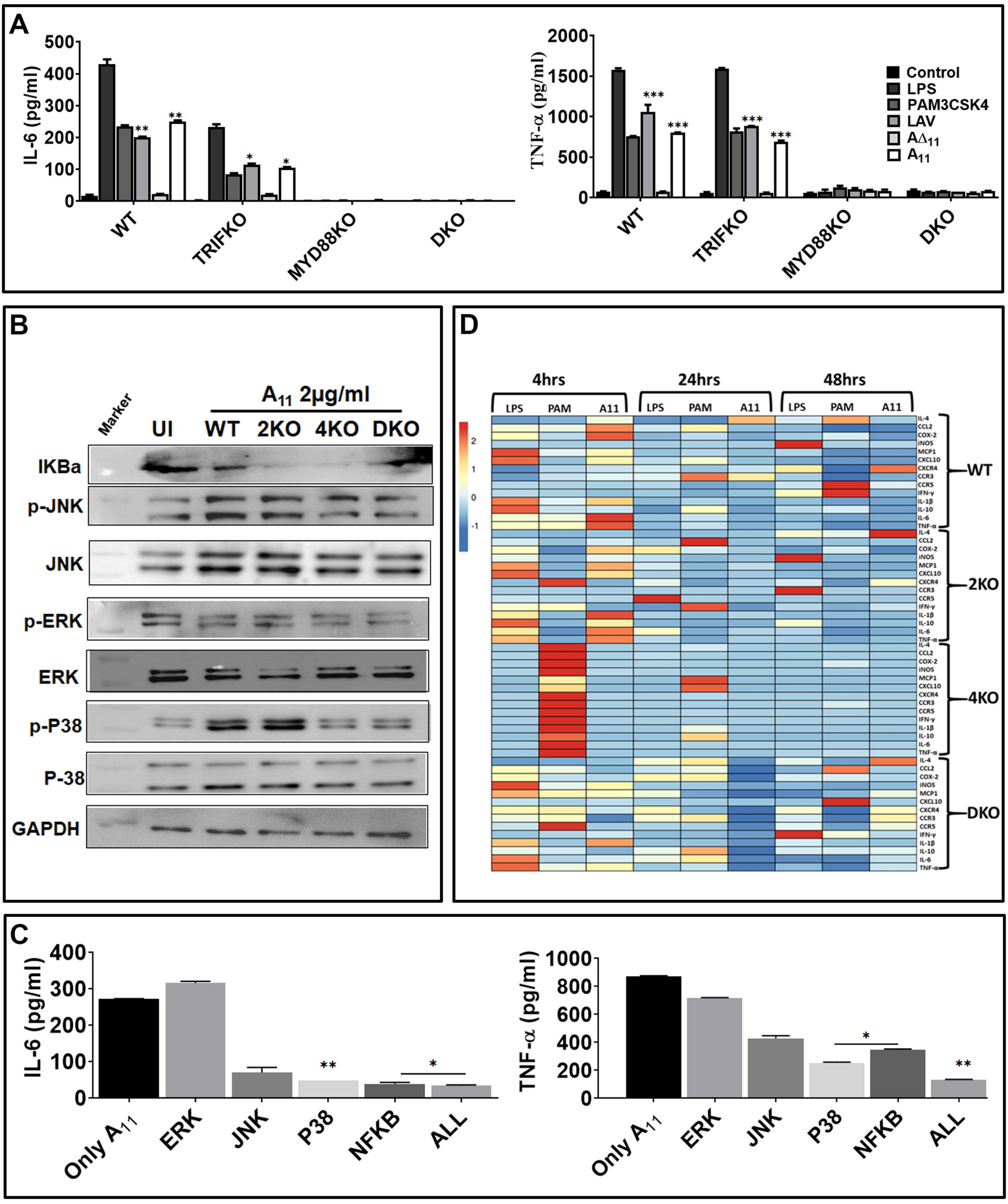
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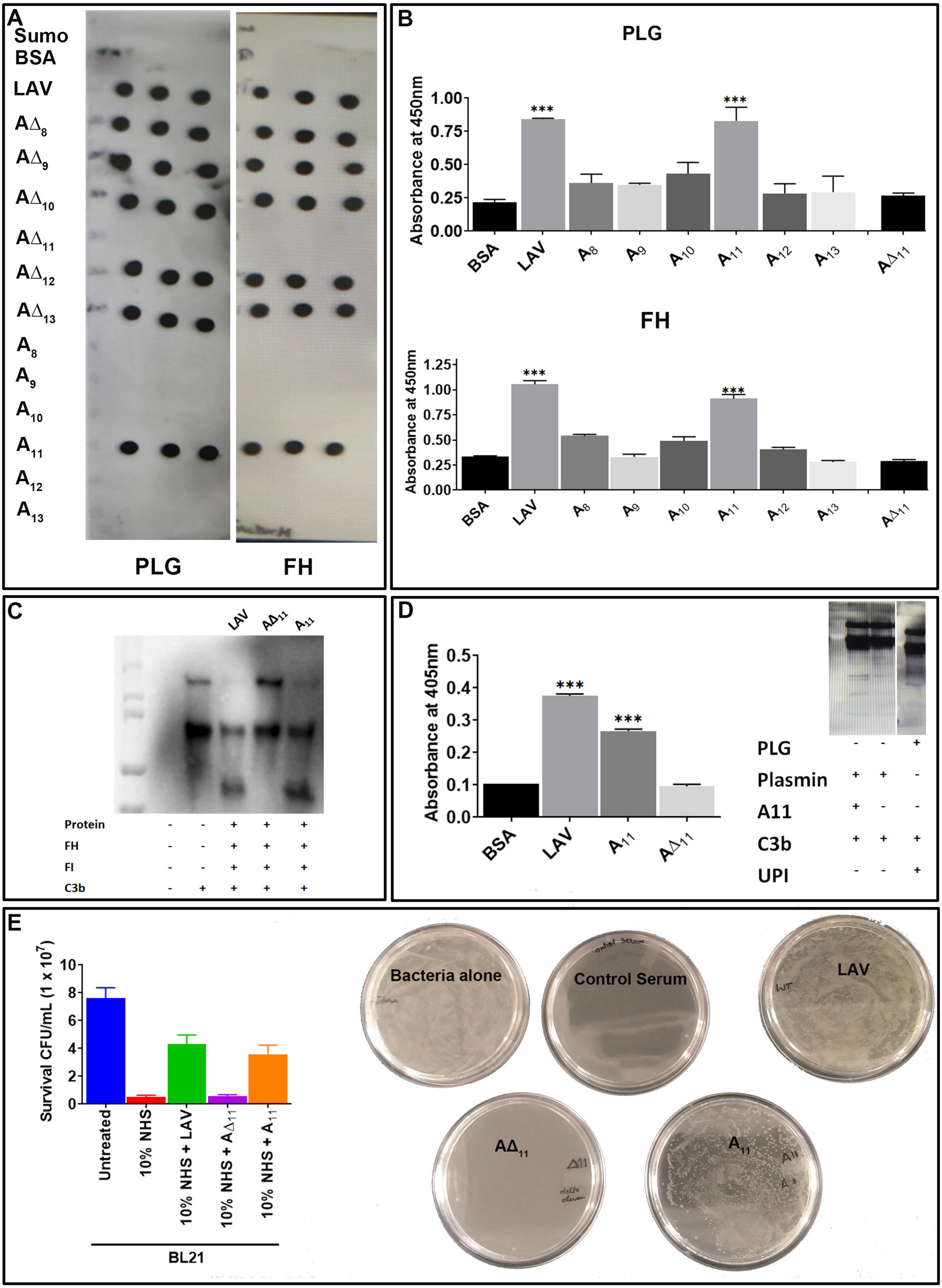
Fig. 4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Survival CFU/mL (1 x 10^7)
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Untreated
10% NHS
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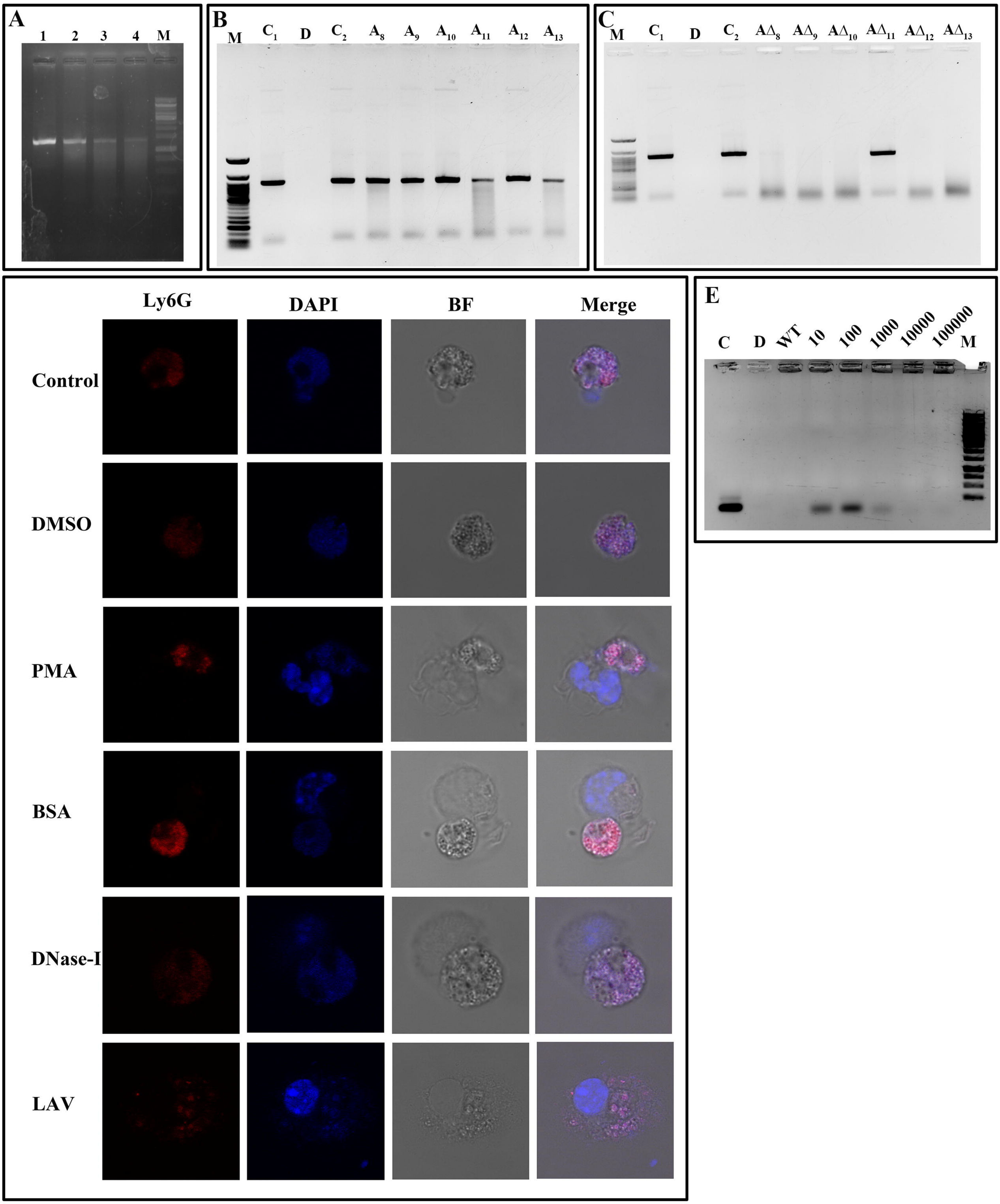
10% NHS + AΔ11
10% NHS + A11
BL21
Bacteria alone
Control Serum
LAV
AΔ11
A11

Fig. 5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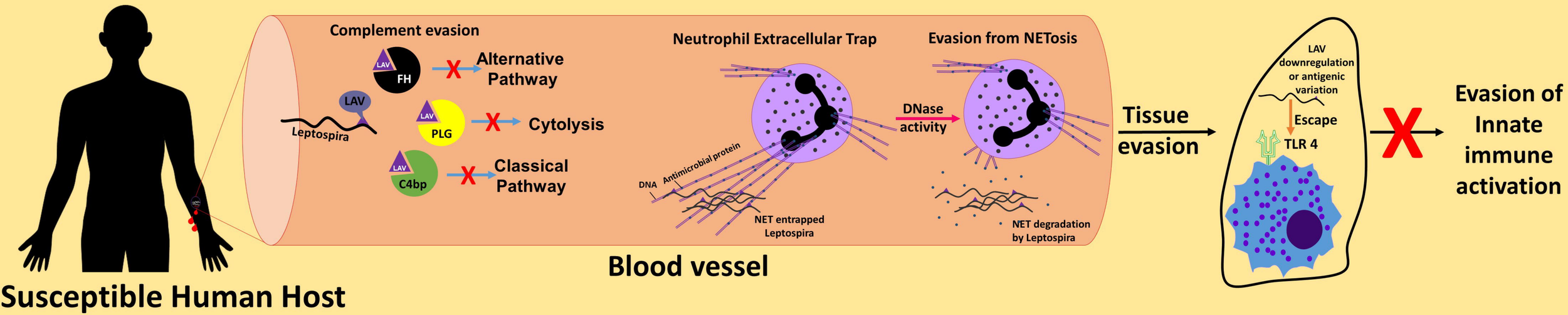
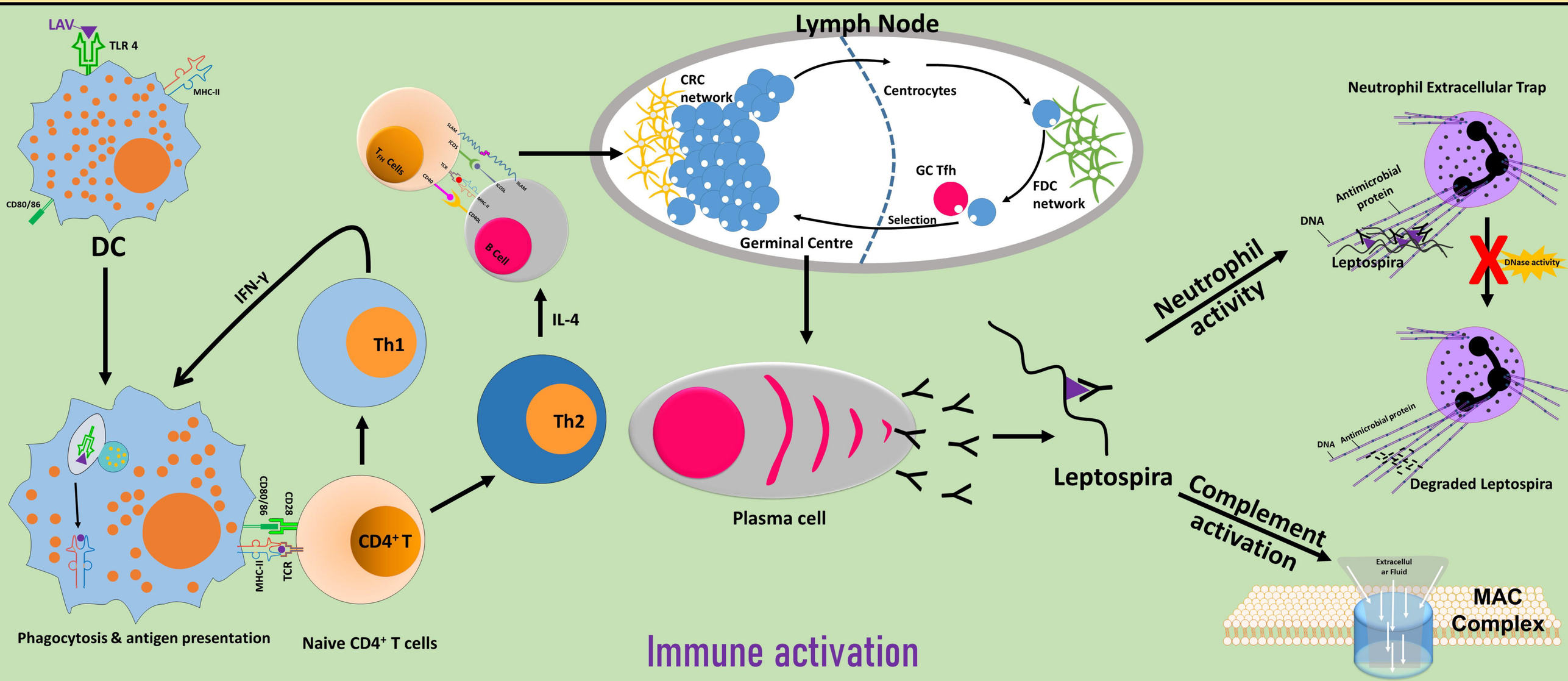
C1
D
C2
A8
A9
A10
A11
A12
A13
C
M
C1
D
C2
AΔ8
AΔ9
AΔ10
AΔ11
AΔ12
AΔ13
Ly6G
DAPI
BF
Merge
Control
DMSO
PMA
BSA
DNase-I
LAV
E
C
D
WT
10
100
1000
10000
100000
M

Immune evasion