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1 # Deciphering the role of *Leptospira* surface protein LigA in
2 # modulating the host innate immune response

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25 **Short running title**: Role of surface protein LigA in *Leptospira* host interaction

26 **Keywords:** Surface proteins, LigA, *Leptospira*, immune evasion, Host-Pathogen Interaction

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29 **Abstract**

30 *Leptospira*, a zoonotic pathogen, is known to infect various hosts and can establish persistent
31 infection. This remarkable ability of bacteria is attributed to its potential to modulate the host
32 immune response by exploiting its surface proteins. We have identified and characterized the
33 domain of the variable region of *Leptospira* immunoglobulin-like protein A (LAV) involved
34 in immune modulation. The 11th domain (A_{11}) of the variable region of LigA (LAV) induces
35 a strong TLR4 dependent innate response leading to subsequent induction of humoral and
36 cellular immune responses in mice. A_{11} is also involved in acquiring complement regulator
37 FH and binds to host protease Plasminogen (PLG), there by mediating functional activity to
38 escape from complement-mediated killing. The deletion of A_{11} domain significantly
39 impaired TLR4 signaling and subsequent reduction in the innate and adaptive immune
40 response. It also inhibited the binding of FH and PLG thereby mediating killing of bacteria.
41 Our study discovered an unprecedented role of LAV as a nuclease capable of degrading
42 Neutrophil Extracellular Traps (NETs). This nuclease activity was primarily mediated by A_{11}.
43 These results highlighted the moonlighting function of LigA and demonstrated that a single
44 domain of a surface protein is involved in evading a myriad of host innate immune defenses,
45 which might allow the persistence of *Leptospira* in different hosts for a long term without
46 clearance.

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58

59 **Introduction**

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

71 Innate immune responses comprising of soluble factors like antimicrobial peptides and
72 complement proteins, pattern recognition receptors like Toll-like receptors (TLRs) and NOD-
73 like receptors (NLRs), and phagocytic cells such as Dendritic cells (DCs), neutrophils, and
74 macrophages contribute to the killing and removal of invading pathogens by a variety of
75 mechanisms[4]. Signaling through TLRs induces activation of innate immune cells leading to
76 secretion of pro-inflammatory cytokines (IL-6, TNF-α) and expression of surface molecules
77 (CD80, CD86, MHC-II), thereby enabling these cells to become efficient in subsequent
78 activation of adaptive response[5,6]. TLRs play a key role in promoting adaptive immune
79 responses and are also essential for T-cell expansion, differentiation, and memory formation[7].
80 The Complement system is a vital part of innate immune defense that promptly kills the
81 invading pathogen by opsonization and target lysis[8]. To prevent damage to the host cells, the
82 complement system is tightly regulated by soluble plasma proteins like Factor H (FH) and
83 C4b-binding protein (C4BP)[9]. FH and C4BP regulate the Alternative pathway (AP), Classical
84 pathway (CP), and Lectin pathway of complement activation. Plasmin, the enzymatically
85 active form of plasminogen (PLG) acts as a protease that potentially cleaves complement
86 factors C3b, C4b and C5[10]. Neutrophils are major phagocytic cells that utilize a combination
87 of reactive oxygen species (ROS), cytotoxic granules, antimicrobial peptides, and Neutrophil
88 Extracellular Traps (NETs) to kill and degrade the invading pathogen[11]. However, pathogens
89 have devised several strategies to escape from host innate immune defenses through a

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90 mechanism mediated by their surface proteins[12]. These proteins may be pro-inflammatory
91 where they can activate APCs like macrophages and DCs but might also enable the pathogen
92 to avoid recognition through innate receptors (TLRs) through downregulation of their
93 expression or causing antigenic variations to evade from host defences[13,14]. Pathogens escape
94 from complement-mediated killing by expressing surface proteins that acquire complement
95 regulators like FH and C4BP, act as proteases or acquire host proteases that can cleave
96 complement components[8,15]. They may avoid killing by phagocytes like neutrophils by
97 expressing surface proteins, which may help inevading extravasation and chemotaxis,
98 preventing opsonization and phagocytosis, promoting survival inside the neutrophil, and
99 inducing apoptosis or cell death and degrading NETs by virtue of their nuclease activity[16,17].

100 Like other pathogens, *Leptospira* has also evolved strategies to modulate the host's innate
101 immune response by exploiting the capacities of its surface proteins to favor their
102 pathogenesis[18-20]. Toll-like receptors like TLR2 and TLR4 play a major role in host defense
103 as mice lacking these receptors were highly susceptible to *Leptospira* infection[21]. These
104 bacteria likely modulate the expression of surface molecules (proteins, LPS) to avoid
105 recognition through protective TLR2 and TLR4 and establish infection in the host. Several
106 surface proteins of *Leptospira* have been identified as a potent activator of pro-inflammatory
107 response via signaling through both TLR2 and TLR4[22-24]. Besides that, several proteins have
108 been shown to acquire FH, C4BP and PLG on their surface or act as proteases to cleave
109 complement components to evade killing[25-30]. *Leptospira* is known to induce NET; hence it is
110 likely that it might express surface proteins/nucleases like other bacteria to evade NETosis[31].
111 Thus, identification and characterization of a surface protein involved in the modulation of
112 the host innate immune response will aid in designing a better strategy to combat this
113 bacterial zoonosis.

114 *Leptospira* immunoglobulin-like (Lig) proteins (LigA and LigB) are surface proteins having
115 12-13 immunoglobulin-like repeat domains similar to an invasin of *Yersinia* and intimin of
116 *E.coli*[32]. The N terminal region of LigA and LigB from domains 1 to 6.5 are conserved,
117 whereas C terminal regions from domains 6.5 to 13 are variable[32,33]. Lig proteins are
118 expressed during infection and have been shown to bind to multiple components of the host
119 extracellular matrix (ECM), thereby mediating attachment to host cells [34,35]. They are the
120 most promising vaccine candidate identified to date. Moreover, the variable region of LigA
121 (LAV) comprising domain 6.5-13 (LAV) was shown to be sufficient to induce protection
122 against challenge in the hamster model [36-41]. Despite various reports confirming the protective

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123 role of LAV, its involvement in the modulation of the host innate immune response has not
124 been studied extensively. Several groups demonstrated that Lig proteins bind to FH and
125 C4BP to inhibit lectin, classical and alternative pathways; however, specific domains
126 involved in binding to these regulators have not been characterized[27,29,30,42,43]. Further, the
127 role of the protein in activation of the innate response or evasion from killing by phagocytes
128 has not been reported so far. In the present study, we have demonstrated the role of LAV in
129 modulating the host innate immune response. Using various assays, we identified the
130 domain/s involved in activating of innate and subsequent adaptive immune response and
131 evasion from complement-mediated killing via binding to FH and host protease PLG.
132 Further, we demonstrated LAV's nuclease activity which might play a major role in evasion
133 from Neutrophil extracellular traps (NETosis).

134 **Results:**

135 **LAV induced TLR4 dependent activation of mouse macrophages**

136 To test whether the immunogenicity of Variable region of A (LAV) correlates to innate
137 immune response activation, we tested its ability to activate mouse macrophages. We cloned,
138 expressed, and purified LAV in a soluble form (Sup Fig. 1). We stimulated mouse
139 macrophages with varying doses (1, 2, and 5µg/ml) of the protein and our result shows that
140 LAV induced production of pro-inflammatory cytokines (IL-6, TNF-α) in dose-dependent
141 manner (Fig. 1A). Taking into account that the purified protein might have LPS
142 contamination, they were pre-treated twice with Polymyxin-B Agarose to remove the
143 endotoxin activity. 500ng/ml LPS pre-incubated with the same concentrations of PMB-
144 agarose was used as control. The concentration of LPS in final protein preparation varied
145 from (0.10–0.15ng/ml). The effect was protein-specific because Proteinase-K plus heating
146 abolished cytokine production (Sup Fig. 2). Besides, PMB inhibited the LPS induced
147 cytokine production but did not attenuate the levels induced by LAV, indicating that the
148 stimulatory effects observed were specific to protein and not due to contamination with LPS
149 (Sup Fig. 2). Next, we tested whether this LAV-induced activation was via signaling through
150 TLR2 or TLR4. As confirmed by confocal microscopy, LAV showed binding, specifically
151 with TLR4 and failed to bind to the TLR2 receptor (Fig. 1B). To verify that this binding leads
152 to activation and subsequent cytokine production, we stimulated WT, TLR2KO, TLR4KO
153 and DKO macrophages and HEK-293T cells expressing these receptors with LAV. Our result
154 shows that while WT and TLR2KO macrophages cells induced significant levels of IL-6 and
155 TNF-α, TLR4KO and DKO macrophages failed to induce these cytokines (Fig. 1C).

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156 Similarly, HEK-TLR4 stimulated with LAV produced significant levels of IL-8, whereas
157 HEK-TLR2 cells didn't produce a significant level of this cytokine (Fig. 1D). These results
158 indicate that LAV is a TLR4 ligand that induces signalling through this receptor for the
159 production of pro-inflammatory cytokines.

160 **11th domain of the variable region of LigA (A_{11}) is involved in signalling through TLR4**
161 **for the activation and maturation of macrophages**

162 Since LAV induced TLR4-dependent activation of mouse macrophages, we aimed to identify
163 and characterize the domain/s involved in activation. We cloned, expressed and purified the
164 individual domain (A_8-A_{13}) and tested their ability to activate mouse macrophages
165 (RAW264.7 cells). Our result shows that only 11th domain (A_{11}) induced a significant level of
166 IL-6 and TNF-α (Fig.2A). To confirm that A_{11} is involved in the production of cytokines, we
167 created domain deletion mutants of LAV($A\Delta_8$-$A\Delta_{13}$) and purified the proteins in the soluble
168 form (Sup Fig.1B). We tested the ability of these mutants to activate mouse macrophages,
169 and our result shows that all the deletion mutants of LAV induced production of IL-6 and
170 TNF-α except $A\Delta_{11,}$ further confirming that this domain is involved in the activation of
171 macrophages and subsequent production of cytokines (Fig. 2B). We tested its binding with
172 the receptor to confirm that A_{11} is involved in interaction and subsequent signaling via TLR4.
173 Confocal microscopy confirmed the binding of A_{11} with the mouse TLR4 as strong anti-A_{11}
174 fluorescence was observed on the surface of WT and TLR2KO cells but little fluorescence on
175 TLR4KO or DKO cells. Further, there was very little anti-$A\Delta_{11}$ fluorescence on the surface of
176 all cell types indicating that this protein failed to bind to the TLR receptor (Fig. 2C). To
177 confirm that this TLR4 binding leads to activation of these cells, we stimulated mouse WT,
178 TLR2KO, TLR4KO, and DKO macrophages with LAV, $A_{11,}$ and $A\Delta_{11,}$ and our results
179 indicate that A_{11} induced IL-6 and TNF-α production via signaling through TLR4 as
180 TLR4KO and DKO macrophages failed to induce any significant level of these cytokines.
181 Further, the inability of $A\Delta_{11}$ to induce substantial levels of cytokines in WT or TLR2 KO
182 macrophages indicates that the 11th domain is critical for signaling via TLR4 (Fig. 2D). To
183 confirm whether stimulation with A_{11} causes macrophage activation and maturation, we
184 analyzed the expression of costimulatory molecules (CD80, CD86, and CD40) and the
185 maturation marker (MHC-II) in RAW264.7 cells. Our Flow cytometry results show that LAV
186 and A_{11} significantly enhanced the expression of CD80, CD86, CD40, and MHCII, whereas
187 $A\Delta_{11}$ failed to upregulate them, indicating that this domain is involved in enhancing the
188 expression of these surface molecules (Fig. 2E). To understand whether deletion of the 11th
189 domain leads to structural changes in the protein, which might be contributing to impairing

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190 its innate immune activity, we did CD spectroscopy and our result shows that deletion of A_{11}
191 has reduced the helix and beta sheets and, in turn, distorted the structure but didn't have any
192 effect on proper folding of LAV (Sup. Fig. 3). In conclusion, our results demonstrate that A_{11}
193 is involved in TLR4 dependent activation and maturation of mouse macrophages.

194 **A_{11} induces immune activation via MAPK signaling involving the MyD88 adapter**

195 Since TLR4 involves both MyD88 and TRIF adapter for downstream signaling and A_{11}
196 induced immune activation through TLR4, we examined the adapter molecule involved in the
197 signaling. We stimulated MyD88KO, TRIFKO, and TMDKO macrophages with $A_{11,}$ and our
198 results show that the signaling pathway involves MyD88 adapter as MyD88KO macrophages
199 failed to induce significant levels of IL-6 and TNF-α. In contrast, there was no difference in
200 the production of these cytokines in TRIFKO macrophages (Fig. 3A). Because MAPKs are
201 critical factors involved in cellular responses to inflammatory stimuli, we examined the
202 activation of this pathway in response to $A_{11.}$ We stimulated mouse WT, TLR2KO,
203 TLR4KO, and DKO macrophages with A_{11} and analyzed the phosphorylation of P38, JNK,
204 ERK and degradation of IkBα (Fig. 3B). Next, to elucidate the functional role of these
205 kinases in A_{11} induced macrophage activation and maturation, we used pharmacological
206 inhibitors of these pathways and analyzed cytokines in RAW264.7 cells pre-treated with or
207 without inhibitors of NF-kB, JNK, p38MAPK or ERK. IL-6 and TNF-α production was
208 significantly blocked by p38 inhibitor ($P < 0.05$, 50% inhibition with 2μg/ml A_{11}) and by JNK
209 and NF-kB (P, <0.05, 30% inhibition with 2μg/ml A_{11}). ERK inhibitor didn't effect the
210 production of cytokine, indicating that this pathway is not involved in signalling. The
211 production of TNF-α was also significantly blocked by JNK, p38, and NF-κB inhibitor (P
212 <0.05, 60% inhibition). A combination of all inhibitors completely inhibited A_{11} induced
213 cytokine production (Fig. 3C). All these results suggest that A_{11} stimulates the production of
214 pro-inflammatory cytokines through p38, JNK and NF-kB pathways. The ability of A_{11} to
215 regulate innate responses was further investigated based on the expression of key
216 inflammatory cytokine and chemokine genes at various time points (4, 24, and 48h). WT,
217 TLR2KO, TLR4KO and DKO mouse macrophage were stimulated with $A_{11,}$ and expression
218 of mRNA transcript was analyzed by RT-PCR. A_{11} induced CXCL10, IL-1β, TNF-α, COX2,
219 iNOS, MCP-1, and IL-6 in WT and TLR2KO mouse macrophage at 4h and 24h time point
220 which was significantly reduced or down-regulated in TLR4KO and DKO macrophages (Fig.
221 3D). PAM3CSK4 (TLR2 ligand) and LPS (TLR4 ligand) showed significant upregulation of
222 genes involved in TLR2 and TLR4 signalling as expected. These results demonstrate that A_{11}
223 induced TLR4 dependent expression of innate response genes.

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224

225 **A_{11} is an immuno-dominant domain that induces a strong adaptive immune response**

226 To test whether the innate response induced by A_{11} correlates to subsequent induction of
227 adaptive response, we evaluated antigen-specific humoral, and cell-mediated immune
228 response in mice immunized with LAV and A_{11} in alum adjuvant. Our results show that mice
229 immunized with A_{11} with or without alum adjuvant-induced strong antibody response at day
230 28 post-immunization (Fig. 4A). The generation of high levels of IgG1 and significant levels
231 of IgG2c against A_{11}, indicates a mixed Th1 and Th2 response (Fig.4A). A_{11} failed to induce
232 a significant level of IgA. A_{11} induced proliferation generation of T cells secreting significant
233 levels of IL-4 and IL-10 (Th2 cytokines) and high levels of IFN-γ (Th1 cytokine) (Fig. 4B,
234 4C). There was no significant enhancement in antibody levels against A_{11} with the addition of
235 alum adjuvant; however, cells obtained from Alum-A_{11} immunized animals produced higher
236 levels of IL-10 and IFN-γ (Fig 4A, 4B, 4C). To test whether A_{11} is an immuno-dominant
237 domain, we analysed IgG response at day 28 in animals immunized with LAV or A_{11} or $A\Delta_{11}$
238 in Freund's adjuvant. Our results show that $A\Delta_{11}$ immunized animals induced significantly
239 lower levels of IgG than those immunized with LAVor A_{11} (Fig.4D). This result correlated to
240 a significant decrease in levels of IgG, cell proliferation, and induction of cytokines when
241 serum and lymphocytes isolated from LAV-Alum immunized animals were used to analyze
242 the response against $A\Delta_{11}$(Fig. 4E, 4F, 4G). Altogether, these results indicate that A_{11} is an
243 immuno-dominant domain, and its deletion significantly impairs the ability of LAV to induce
244 a robust adaptive immune response.

245

246 ***Leptospira* evades complement-mediated killing by acquiring complement regulators**
247 **through A_{11}**

248 *Leptospira* evades complement-mediated killing by acquiring complement regulators (FH
249 and C4BP) or host proteases (PLG) which involves binding with surface proteins. Lig
250 proteins, including LigA have been shown to bind to FH, C4BP and PLG. However, except
251 for C4BP the domain/s of LigA involved in binding to FH or PLG have not been
252 characterized. To identify and characterize the domain of LAV involved in binding to FH and
253 PLG, we screened the individual domains (A_8-A_{13}) and corresponding deletion mutants ($A\Delta_8$-
254 $A\Delta_{13}$) for their ability to bind with FH and PLG. Our dot blot result shows that only A_{11} and
255 all the deletion mutants except $A\Delta_{11}$ were able to bind to FH and PLG (Fig. 5A). This binding
256 was further confirmed by pulldown assay and ELISA, and our result shows that while A_{11} led
257 to strong binding, $A\Delta_{11}$ failed to bind to both FH and PLG (Fig. 5B, 5C). We further

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258 determined if the binding of A_{11} with FH is sufficient for its functional activity. Our result
259 shows that both LAV and A_{11} were able to bind to FH to cleave C3b in the presence of Factor
260 I (FI) as evidenced by cleavage fragments. In contrast, $A\Delta_{11}$ failed to do so, indicating that
261 the 11th domain is involved in binding with and mediating the cofactor activity (Fig. 5D). Our
262 ELISA result shows that A_{11} binds with PLG and converts it into active plasmin, whereas
263 $A\Delta_{11}$ failed to generate a significant level of plasmin (Fig.5E). Western blot analysis further
264 confirms that the released plasmin was able to cleavae C3b in presence of A_{11} whereas failed
265 to do so in presence of $A\Delta_{11}$ indicating that 11th domain is involved in PLG binding and
266 mediating subsequent plasmin activity (Fig.5E). To establish the role of A_{11} in the
267 complement-mediated killing, we incubated *E. coli* with 10% Normal Human Serum (NHS)
268 pre-incubated with A_{11} or LAV or $A\Delta_{11}$. Our results show that both LAV and A_{11} domains
269 could rescue bacteria from complement-mediated killing, but $A\Delta_{11}$ failed to do so, indicating
270 that the 11th domain is involved in evasion from complement-mediated killing (Fig. 5F, Sup.
271 Fig.3).
272
273 **LAV is a nuclease involved in the evasion of *Leptospira* from neutrophil extracellular**
274 **traps.**
275 Recently it has been shown that *Leptospira* induces NET; however, a protein with nuclease
276 activity to degrade NET has not been reported. To test whether LAV has nuclease activity,
277 which might have a role in evasion from NETs, we incubated plasmid or linear DNA with
278 varying concentrations of the protein (1 to 10µg), and our result shows that LAV was able to
279 degrade both plasmid and linear DNA in a dose-dependent manner indicating that its having
280 both endo and exonuclease activity (Fig 6A, 6B). To analyze if this activity is restricted to
281 any domain, we incubated the linear DNA with individual domains (A_8-A_{13}) or deletion
282 mutants ($A\Delta_8$-$A\Delta_{13}$) and our result shows that A_{11} and A_{13} were able to degrade DNA
283 whereas all the deletion mutants except $A\Delta_{11}$ degraded DNA with equal propensity (Fig. 6C,
284 6D). Further, $A\Delta_{11}$ was also not able to cause significant degradation of plasmid DNA (Fig.
285 6E). These results indicate that LAV's nuclease activity primarily resides in 11th domain, and
286 LAV is mediating this activity by utilizing this domain. To test whether LAV can cleave the
287 Neutrophil Extracellular Trap (NET) we stimulated the mouse neutrophils with PMA to
288 induce NET and then treated them with LAV (5µg/ml). Our confocal microscopy result
289 shows that LAV could degrade the PMA induced NET, further confirming its nuclease
290 activity and possible role in degrading NETs *in vivo* (Fig.6F). These results indicate that LAV
291 has nuclease activity that *Leptospira* might exploit to evade from NETosis.

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292

293 **Discussion**

294 The ability of *Leptospira* to cause persistent infection and efficient colonization in a variety
295 of hosts reflects its potential to subvert or thwart the innate immune response[15]. This ability
296 has been attributed to the procession of a wide variety of surface molecules like proteins,
297 lipopolysaccharide, etc., which are redundant in their function and may also undergo
298 structural variation to avoid recognition by the host immune system been observed in other
299 spirochetes [12]. Surface proteins, including lipoproteins from spirochetes like *Borrelia* and
300 *Treponema* play a critical role in immune evasion by limiting their expression or inducing
301 antigenic variation after infection, which greatly enhances host infectivity and
302 persistence[13,14,45]. *Leptospira,* like other pathogens, may voluntarily interact with TLRs (or
303 other innate receptors) through surface molecules (proteins, LPS) but might evade this
304 recognition through multiple mechanisms to establish infection or fitness in the host[46-48].
305 Both TLR2 and TLR4 receptors play a major role in host defense against *Leptospira*
306 infection[21]. It has been shown that TLR4 plays a critical role in controlling bacterial load and
307 developing severe leptospirosis in mice [49]. Thus, it is likely that those surface proteins and
308 LPS which are natural ligand of these receptors and can activate macrophages and DCs,
309 might modulate their expression or undergo variations thereby enabling the bacteria to evade
310 this innate recognition as has been reported for other pathogens. Several surface proteins of
311 *Leptospira* have been identified as ligands of TLR2 or TLR4 capable of activating the innate
312 immune response and are potential vaccine candidates [22,23,50-52]. Lig proteins are important
313 virulence factors, and their expression during infection or loss of expression during *in vitro*
314 culture has been correlated to virulence of the infecting serovar [34,53]. These proteins interact
315 with various host molecules, including extracellular matrix (ECM), coagulation cascade, and
316 complement regulators. LigA is the most promising vaccine candidate. It has been
317 demonstrated that the variable region of the protein comprising domains 10-13 ($LigA_{10-13}$) is
318 sufficient in inducing protection against challenge in the hamster model[36-38,40]. Thus, the
319 diverse functions of LigA prompted us to investigate whether their role is limited to binding
320 to host ECM and complement regulators, or they are also involved in the modulation of the
321 host innate immune response, thereby contributing to infection and persistence in the host.
322 Since several investigators have established the protective role of a variable region of LigA
323 (LAV), we chose to decipher its role in the modulation of the host innate immune response.
324 We cloned, expressed and purified the recombinant LAV and tested its ability to activate
325 mouse macrophages. Our result shows that LAV could activate macrophages, as evident from

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326 the production of pro-inflammatory cytokines (Fig.1). This effect was not due to
327 contaminating LPS as pretreatment with Polymixin B didn't attenuate, whereas digestion
328 with Proteinase K abrogated the cytokine production (sup Fig.1B). The LAV-induced
329 activation was TLR4 dependent as evident from its binding to the receptor, induction of IL-8
330 by TLR4 transfected HEK293 cells, and abrogation of cytokine production in TL4KO mouse
331 macrophages. We expected that LAV might not signal through TLR2 as it is devoid of the
332 signal sequence and hence lipidation. However, several non-acylated proteins like LcrV from
333 *Yersinia*, MPB83, and PPE18 from *Mycobacterium*, PorB from *Neisseria* and FimA from *P.*
334 *gingivalis* have been shown to signal through TLR2 [54-58]. Although rare, several proteins
335 from other bacterial pathogens have been reported to induce TLR4 dependent production of
336 pro-inflammatory cytokines and expression of surface markers [59,60]. Further, lipidated
337 recombinant proteins, which usually signal through TLR2 due to the lipid moiety, may signal
338 through TLR4 if unlipidated as has been observed in the case of Omp16 and Omp19 of
339 *Brucella* [61,62]. Moreover, recombinant unlipidated rBCSP31 from *Brucella abortus* and
340 rLsa21 from *Leptospira* have been shown to signal through both TLR2 and TLR4 and induce
341 activation of macrophages[22,63]. Since LAV is composed of several immunoglobulin-like
342 repeat domains, we attempted to identify the domain involved in innate immune activation.
343 Our results shows that A_{11} is involved in TLR4 dependent activation of mouse macrophages
344 leading to production of pro-inflammatory cytokines and expression of costimulatory
345 molecules and maturation marker (Fig. 2 and 3). Further, A_{11} modulated the expression of
346 several innate responses related to genes (cytokines, chemokines, and surface receptors)
347 involved in the activation and maturation of macrophages (Fig.3). Our results are in
348 accordance with previous reports, where several TLR4 ligands, including bacterial proteins,
349 have been shown to activate macrophages and DCs via signalling through the MAP kinase
350 pathway, leading to the induction of cytokines, expression of surface markers, and immune
351 response-related genes[59,64-67]. Since TLR, dependent activation of innate response, is essential
352 for T cell expansion, differentiation, and memory formation, we tested the adaptive response
353 induced by A_{11} in mice. Our result shows that the strong innate response induced by A_{11} also
354 correlated to the generaion of higher level of adaptive response (Fig. 4). Interestingly the
355 response generated against A_{11} was equivalent to LAV and was significantly decreased in
356 terms of antibody titer, cell proliferation, and cytokines in the absence of this domain,
357 indicating that A_{11} is the most immuno-dominant domain capable of inducing robust antibody
358 and T cell response. Although A_{11} induced mixed Th1 and Th2 response, high levels IFN-γ
359 may be correlated to strong activation of innate response, particularly innate B cells, which

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360 require investigation[68]. The strong adaptive response induced by A_{11} without any adjuvant
361 highlights its immunomodulatory potential. It suggests that TLR4 dependent signaling by A_{11}
362 might activate strong innate and subsequent adaptive response leading to clearance of
363 *Leptospira* from the host. We speculate that to evade this protective response, *Leptospira*
364 might limit expression or undergo antigenic variation in LigA to avoid recognition with
365 TLR4 and subsequent activation of the innate and adaptive response. However, this needs to
366 be tested, and experiments are ongoing. Further, the inability of $A\Delta_{11}$ to activate a strong
367 innate and subsequent adaptive response is not due to misfolding, as mutant protein retains
368 significant numbers of alpha-helix and beta sheets as revealed by structural analysis (sup Fig.
369 2).
370 It is known that pathogenic *Leptospira* is resistant to the bactericidal activity of normal
371 human serum (NHS)[69,70]. They can evade complement attack by using various strategies like
372 recruitment of the host complement regulators, acquisition of host proteases or secretion of
373 proteases that can cleave complement components on the bacterial surface and in its
374 surroundings[8]. Several surface proteins of *Leptospira* like LenA, LenB, LcpA, Lsa30,
375 including Lig proteins (LigA and LigB) have been shown to bind to various complement
376 regulators [20,25-30,42,43,71-74]. Moreover, both conserved and the variable (N and C terminal)
377 regions of LigA and LigB are involved in binding to FH and C4BP. Our results confirmed the
378 previous report of binding of LAV with FH and PLG and identified and characterized the
379 domain/s involved in binding and mediating subsequent co-factor or plasmin activity (Fig. 5).
380 Additionally, the rescue of *E. coli* from complement-mediated killing in NHS pre-incubated
381 with A_{11} further substantiates the critical role of this domain in complement evasion[75]
382 (Fig.5F). Thus, binding of FH and PLG to A_{11} reflects the ability of *Leptospira* to utilize this
383 domain for simultaneously inhibiting lectin and alternate pathway of complement-mediated
384 killing (Fig. 5 and sup Fig.4). To our knowledge, this is the first report that demonstrates that
385 a single domain of a surface protein is alone capable of recruiting FH and PLG directly from
386 NHS and prevents complement activation.
387 Apart from killing bacterial pathogens by intracellular ROS and phagocytosis, neutrophils
388 might release neutrophils extracellular traps (NETs) that capture and kill microbes in the
389 extracellular space in tissues (at sites of infection) or within blood vessels[17,76]. This
390 mechanism on killing extracellular bacteria by trapping outside the cell is independent of
391 phagocytosis and degranulation [17]. Several bacterial pathogens, including *Staphylococcus*
392 *aureus*, *Clostridium perfringens* and *Streptococcus pyogenes* have evolved sophisticated
393 mechanisms to suppress, escape, and/or resist NETs through surface proteins having nuclease

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394 activity[77-79]. Recently, it has been shown that *Leptospira* can induce the NET, and its surface
395 protein LipL21 can modulate neutrophil function; however, nuclease capable of degrading
396 NET has not been reported[16,31]. Our study discovered the nuclease (DNase) activity of LAV
397 and demonstrated that A_{11} primarily or predominantly mediates this activity as it was able to
398 degrade DNA with the same propensity as LAV (Fig. 6). Our results further demonstrate that
399 LAV or A_{11} exhibits both endo and exonuclease activity. Although nuclease activity of LAV
400 is not restricted to $A_{11,}$ as significant activity was also mediated by A_{13} but our result clearly
401 shows that it is primarily mediated by A_{11}. The ability of LAV to degrade PMA induced NET
402 in mouse neutrophils and highlights the possible role of LigA in escaping the bacteria from
403 NETosis. To our knowledge, this is the first report of identification of nuclease activity of a
404 surface protein in *Leptospira* and also demonstrating its diverse role in modulating the host
405 innate response.

406 In conclusion, our results demonstrate that LigA is a multifunctional protein involved in
407 attachment to host cells to initiate infection, a TLR4 agonist which can activate a strong
408 innate response (possibly evading this TLR4 activation by antigen variation or
409 downregulating its expression upon infection in the host), binds to complement regulators to
410 evade complement-mediated killing and exhibit nuclease activity when *Leptospira* gets
411 entrapped in NET (Fig. 7). These features might contribute to its successful colonization in a
412 particular host. Interestingly, these functions are mediated primarily by a single domain (A_{11})
413 which lies in LAV. This promising vaccine candidate conferred protective immunity against
414 lethal infection in the hamster model of the disease. Thus, the protective efficacy of LAV
415 based vaccine may be correlated with its ability to induce the robust antigen-specific humoral
416 and T cell response that might lead to the generation of antibodies conceivably blocking
417 binding to host extracellular matrix, acquiring complement regulators and inhibiting DNase
418 activity all of these may aid in the clearance of bacteria from the host (Fig. 7). Our results
419 provide important insight into the role of LAV in host-pathogen interaction and also
420 established it as an immuno-modulator or adjuvant, which makes it an ideal candidate for
421 developing vaccines for this dreadful zoonosis[80].

422

423 **Material & Methods**

424 *Animals, cell lines and reagents*

425 Male C57BL/6J mice (6-8 weeks) were obtained from the Animal Resource and
426 Experimental Facility of NIAB, Hyderabad. The original breeding colonies were obtained

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427 from Jackson Labs, USA. The animals were maintained in a pathogen-free condition. All the
428 procedures for animal experiments were approved by the Institutional Animal Ethics
429 Committee (IAEC) and performed in accordance with the Committee for the Purpose of
430 Control and Supervision of Experiments on Animals (CPCSEA) guidelines. RAW264.7 and
431 HEK293 cell lines were originally purchased from the ATCC (Manassas, VA). Mouse
432 macrophage WT (NR-9456), TLR2KO (TLR2-/-, NR-9457), TLR4KO (TLR4-/-, NR-9458),
433 DKO (TLR2-/-/4-/-, NR-19975), TRIFKO (TRIF-/-, NR-9566), MyD88KO (MyD88-/-, NR-
434 15633) and TMDKO (TRIF-/-MyD88-/-, NR-15632), cell lines were obtained from BEI
435 Resources, USA. Cells were cultured in DMEM (Sigma, USA) supplemented with 10% FBS
436 (Invitrogen, Carlsbad, CA, USA), penicillin (100 U/ml), and streptomycin (100 µg/ml) and
437 maintained at 37˚C in a humidified incubator (5% CO2). Pharmalogical inhibitors of NF-kB
438 (SN50), p38 (SB203580), ERK (U0126) and JNK (SP600125) were purchased from
439 Invivogen. Mouse IL-6 and TNF-α Sandwich ELISA kits were from R&D Biosystems. APC
440 onjugated hamster antimouse-CD80, PE-conjugated rat antimouse-CD86, BV421 conjugated
441 rat antimouse-CD40 and Per Cp Cy5.5 conjugated rat antimouse-MHC-II antibodies were
442 procured from BD biosciences, US. Normal Human Serum (S1-100ml), Goat anti FH
443 (SAB2500260), Mouse anti PLG (SAB1406263-50UG), Plasmin substrate, UPA (SRP6273)
444 complement C3b (204860-250G) Complement factor I (C5938-1MG), Complement factor H
445 (C5813-1MG), plasminogen (SRP6518-1MG) were procured from Sigma Chemical Co,
446 USA. Polyclonal anti C3 was purchased from Complement Technology, USA.
447

448 *Cloning, expression, and purification of recombinant proteins*

449 The Lig A variable (LAV) gene sequence was amplified by PCR from *L. interrogans* serovar
450 Pomona strain genomic DNA using specific primers and then cloned in His-Sumo tagged
451 pET28a expression vector. Domains of LAV 8 to 13 and corresponding deletion mutants
452 ($A\Delta_8$-$A\Delta_{13}$) were similarly cloned in the pET28a vector. Various domain deletion mutants of
453 LAV ($A\Delta_8$-$A\Delta_{13}$) were generated by PCR-based site-directed mutagenesis. All the clones
454 were verified by sequencing. The plasmid was transformed into *BL21(DE3)* Rosetta. The
455 resulting transformants were grown at 37°C overnight on LB broth containing 50µg/ml
456 kanamycin, and the expression of the protein was induced with 1 mM isopropyl β-D-1-
457 thiogalactoside (IPTG). The cells were harvested by centrifugation at 10,000 rpm, and the
458 cell pellet was resuspended in 100mM Tris HCl, 150mM NaCl pH8.0, followed by sonication
459 at constant pulses. The lysate was centrifuged to remove cell debris, and the supernatant was

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460 subjected to affinity chromatography using Ni-NTA beads column (Takara). Eluted protein
461 was dialyzed against 1×PBS with four changes for two days at 4°C. The protein was then
462 passed through Detox- Gel (Pierce, USA) to remove any contaminating LPS from *E. coli,* and
463 a residual trace amount of LPS was monitored by Limulus amoebocyte lysate (LAL,
464 Endotoxin Detection Kit, Pierce, Thermo, USA) assay following the manufacturer's
465 instructions. The purified protein was checked for size and purity by SDS-PAGE, and
466 concentration was estimated using the Bradford reagent (Sigma, USA).

467 *Cell stimulation assays by Cytokine ELISAs*

468 Cytokine ELISA kits (R&D systems) were used to measure cytokine levels, following the
469 manufacturer's instructions. RAW264.7 cells were stimulated with LAV or (A_8-A_{13}) or
470 corresponding deletion mutants (ΔA_8-$A\Delta_{13}$) (2µg/ml), PAM3CSK4 (20ng/ml), and *E. coli*
471 LPS 0111-B4 (500ng/ml) for 24h at 37°C in the presence of 5%CO_2 and cytokines (IL-6,
472 TNF-α) were measured in the culture supernatant according to the manufacturer instructions.
473 The proteins were pre-treated with Polymyxin B (20ng/mg protein) at 37°C for 1hr and
474 proteinase K (5µg/mg protein) at 65°C for 1hr followed by inactivation at 95°C for 5min
475 before each assay to rule out endotoxin activity. In a separate experiment wild type,
476 TLR2KO, TLR4KO, DKO, MyD88KO, TRIFKO, TMDKO macrophage cell lines were
477 stimulated with PAM3CSK4, LPS, LAV or A_{11} or $A\Delta_{11}$ for 24h at 37°C/5%CO_2 and
478 cytokines (IL-6, TNF-α) in the culture supernatant were measured by ELISA kit as per
479 manufacturer's instructions. HEK-293T cells were cultured in a complete DMEM medium
480 for 24h at 37°C in the presence of 5%CO_2 and transfected with TLR2, TLR4, and NF-kB
481 reporter plasmids using X-fect Transfection reagent (Takara, Japan) following manufacturer's
482 protocol. Cells were stimulated with LAV (2µg/ml) for 24h, and then IL-8 levels were
483 measured in the cell culture supernatant. To assess the signaling pathway involved, additional
484 experiments were done in which RAW264.7 cells were pre-treated for 30min at 37°C/5%CO_2
485 with pharmacological inhibitors of NF-kB (SN50; 20µM) or JNK (SP600125; 40µM) or
486 p38MAPK (SB203580; 30µM) or ERK (U0126; 20µM) followed by treatment with A_{11}
487 (2µg/ml) for 24h at 37°C in the presence of 5%CO_2. Cytokine levels were measured by
488 ELISA kit.

489 *Flow cytometric analysis*

490 RAW264.7 cells were incubated in 6-well plates (0.3×10^6 cells/well) with PAM3CSK4
491 (20ng/ml), LPS (500ng/ml), LAV or A_{11} or $A\Delta_{11}$ (2µg/ml) for 24h at 37°C in the presence of
492 5%CO_2. Cells were harvested and washed with pre-chilled PBS and then incubated on ice for

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493 1h in the dark with respective fluorochrome conjugated antibodies against CD80, CD86,
494 MHC-II, and CD40. Cells were washed and then fixed with 1% paraformaldehyde, and
495 50,000 total events/sample were acquired using a BD Fortessa. The data were analyzed using
496 FlowJo software.

497 *Preparation of antisera*

498 Male C57BL/6J mice (6-8 weeks) were immunized subcutaneously on days 0 with 20µg of
499 LAV, $A_{11,}$ $A\Delta_{11}$ in complete Freund's adjuvant (CFA) and then boosted on day 21 with 10µg
500 of proteins in Incomplete Freund's adjuvant (IFA). Sera were collected one week after
501 booster (day 28) and titer were determined using ELISA. The mouse serum having anti-LAV,
502 anti-A_{11} or anti-$A\Delta_{11}$ antibodies were used in confocal microscopy.

503 *TLR binding assay*

504 WT, TLR2KO, TLR4KO and DKO cell lines were grown overnight on glass-bottom cell
505 imaging dishes (Eppendorf) and then incubated for 30 min at 37°C in the presence of 5%CO_2
506 with LAV or A_{11} or $A\Delta_{11}$ (2µg/ml) in DMEM without FBS. The cells were washed with PBS
507 and fixed for 15 min using 4% paraformaldehyde followed by blocking with 5%FBS in PBS
508 for 30min at RT. The cells were then incubated with anti-LAV or A_{11} or $A\Delta_{11}$ (mouse serum,
509 1:100 dilution) for 1h, washed three times with PBS, and then stained with Alexa Flour 647
510 conjugated rabbit anti-mouse IgG (Biolegend, USA). Cells were extensively washed and
511 mounted with VECTA SHIELD (containing DAPI) mounting medium and observed using a
512 63x oil objective on a confocal microscope (Leica SP8, Wetzlar, Germany).

513 *RT-PCR*

514 WT, TLR2KO, TLR4KO and DKO mouse macrophage cell lines were treated with A_{11}
515 (2µg/ml), LPS (500ng/ml) or PAM3CSK4 (20ng/ml). After 4, 24 and 48h of treatment cells
516 were recovered in 500µl of TRIzol (Invitrogen, Carlsbad, CA), and equal volumes of
517 chloroform were added; samples were centrifuged at 12000 rpm for 15 min at 4°C. The
518 aqueous phase was then passed through RNA easy mini columns (MN) and RNA was
519 purified following the manufacturer's protocol. RNA quality was checked by running on a
520 Formaldehyde gel for 18s and 28s RNA bands and analyzed on Bioanalyser. The RNA
521 quantity was assessed by UV spectroscopy and purity by 260/280 ratio. First-strand cDNA
522 was synthesized using the superscript III-RT system (Invitrogen) following the
523 manufacturer's instructions. RT-PCR was performed in 96 well microtiter plates in a 10µl

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524 reaction volume containing 50ng cDNA, 10μM each primer (Table 1) and SYBR green (Bio-
525 Rad). Samples were run in triplicate, and data was analyzed with Sequence Detection System
526 (Bio-Rad CFX-96). The experimental data were presented as fold changes of gene expression
527 of stimulated cells at various time points relative to control. mRNA levels of the analyzed
528 genes were normalized to the amount of GAPDH present in each sample.

529 *Circular Dichroism*

530 The proteins (LAV and $A\Delta_{11}$) were dialysed against sodium phosphate buffer and the CD
531 spectroscopy of the far-UV spectrum was obtained in a Jasco J-810 spectropolarimeter (Japan
532 Spectroscopic). The resulting spectra are presented as the averages of three scans recorded
533 from 190 to 260 nm. The residual molar ellipticity is expressed in degree cm2 dmol–1.
534 Spectral data were analyzed with the software BESTSEL (https://bestsel.elte.hu/) for
535 estimation of the secondary structure content.

536 *Adaptive immune response*

537 Male C57BL/6J mice (6-8 weeks) were immunized subcutaneously on days 0 with 20μg and
538 on day 21 with 10μg of LAV or A_{11} with or without Alum adjuvant. Animals vaccinated with
539 PBS were used as control. Mice bled at various time points a (day 0, 21 and 28), and the
540 serum was analyzed for antigen-specific antibodies. Animals were euthanized on day 28 and
541 blood, and spleens were collected for evaluation of antigen-specific immune responses. To
542 determine antibody response, serum samples from individual mice were collected on the day
543 before immunization and then on day 21 and 28. Total IgG, IgG1, IgG2a, and IgA
544 concentrations were evaluated using ELISA using the standard procedure. For cell
545 proliferation assay, splenocytes prepared from different mice groups were stimulated with
546 varying concentrations (1, 2 and 10 μg/ml) of LAV or A_{11} or $A\Delta_{11.}$ Cells were counted
547 under an inverted microscope at 24h, 48h and 72h post-stimulation. To determine Th1 and
548 Th2 cytokines, culture supernatant was collected at 48-72h post antigen stimulation and were
549 used to estimate the IL-4, IL-10 and IFN-γ using cytokine ELISA kits (R&D systems)
550 following the manufacturer's instructions.

551 *Dot Blot binding Assay:*

552 Dot blot binding assays were performed to confirm the binding of various LAV domains (A_8-
553 A_{13}) and their single domain deletion mutants ($A\Delta_8$-$A\Delta_{13}$) with FH and serine protease PLG.
554 1μg of each protein (wild type, single domain, and domain deletion mutant) was immobilized
555 onto NC membranes (0.2μ pore size; Bio-Rad). The membranes were kept for drying for 5-
556 10 min at RT. The membranes were then blocked with 5% BSA in Tris-buffered Saline-

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557 Tween 20 (TBS-T) for 2h at RT, washed three times with TBS-T, and incubated with 1%
558 normal human serum (NHS) diluted in PBS with gentle shaking for 3h at RT. After extensive
559 washing with TBS-T, the membranes were incubated with the corresponding primary
560 antibody (Goat anti-FH, Mouse anti-PLG 1: 10,000 dilution) in TBS-T for 2h at RT. The
561 membranes were then washed with TBS-T and incubated with a respective peroxidase-
562 conjugated secondary antibody (1: 6,000) for 2h at room temperature. Reactive spots were
563 developed using a chemiluminescence system with an exposure time of 10 sec.

564 *Pull down assay*

565 Each domain deletion mutants of LAV ($A\Delta_8$-$A\Delta_{13}$) along with 10% heat-inactivated NHS,
566 were incubated with 15 µL of Ni-NTA agarose beads (Takara) overnight at 4°C. Agarose
567 beads were washed five times with PBS containing 40mM imidazole and then interacting
568 proteins were eluted with PBS containing 250 mM imidazole. Each elutes boiled in reducing
569 Laemmli buffer and subjected to SDS-PAGE. The proteins were transferred to nitrocellulose
570 membrane followed by western blot against Anti- FH, and Anti-PLG, respectively.

571 *ELISA binding assay:*

572 Protein binding to soluble complement regulators FH, C4BP and PLG were analysed by
573 ELISA as described previously [27,29]. Briefly, micro titre plates were coated overnight at 4°C
574 with 1µg of domains (A_8-A_{13}) and their single domain deletion mutants ($A\Delta_8$-$A\Delta_{13}$). BSA
575 and LAV were used as positive and negative control. The wells were washed three times with
576 PBS containing 0.05 % Tween 20 (PBS-T), blocked with 300µl PBS/3 % BSA for 2h at
577 37°C, and incubated with 100µl 10 %NHS for 90 min at 37°C. After washing with PBST,
578 goat anti-FH (1:1000), rabbit anti-C4BP (1:1000) or mouse anti-PLG (1:5000) was added and
579 the plate was incubated for 1h at 37°C. After washing, HRP-conjugated anti-goat or anti-
580 mouse or anti-rabbit IgG was added and incubated for 1h at 37°C. The wells were washed
581 and TMB substrate was added (100µl/well). The reaction was stopped by the addition of 50µl
582 2N H_2SO_4 and absorbance was measured at 450nm in a microplate reader.

583 *Cofactor activity assay:*

584 Cofactor activity was determined as described previously[29]. Briefly, 2µg of A_{11}, $A\Delta_{11}$ and
585 LAV (positive control) or BSA (negative control) were coated on microplates overnight at
586 4°C. The wells were washed and blocked with PBS/2 % BSA for 2h at 37°C, followed by the
587 addition of 2µg pure FH and further incubation for 90 min at 37°C. Unbounded FH was
588 removed by washing and then 250ng FI and 500ng C3b were added to the microtiter plate
589 wells and incubated for 3-5h at 37°C. The supernatants were loaded onto a 10% SDS-PAGE

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590 gel and transferred onto a 0.22µ PVDF membrane. For the immunoblotting, membranes were
591 blocked with 5 % BSA and then incubated with goat anti-human C3 (1:5000) for 2h at RT.
592 After the usual steps of washing, the membranes were incubated with a peroxidase-
593 conjugated secondary antibody. The images were visualized under the Clarity Max Western
594 ECL substrate (BIO-RAD) using Syngene G: BOX Chemi XX6/XX9.

595 *Plasmin activity assay:*

596 Plasmin activity was determined as described previously[29]. Briefly, Microtiter plate wells
597 were coated overnight at 4°C with 2µg LAV (positive control), BSA (negative control), $A_{11,}$
598 $A\Delta_{11}$. The plate was washed with PBS-T and blocked for 2h at 37°C with 10% skim milk.
599 After discarding the blocking solution, human PLG (2µg/well) was added, followed by
600 incubation for 90 min at 37°C. After washing plates three times with PBS-T, 250µg/ml uPA
601 was added with the plasmin-specific substrate, *D*-Val-Leu-Lys 4-*p*-nitroanilide
602 dihydrochloride (100µl/well) at a final concentration of 0.4mM in PBS. Plates were
603 incubated for 24h at 37°C, and absorbances were measured at 405nm using a microplate
604 reader.

605 *Bactericidal assay*

606 Bactericidal activity was determined as described elsewhere[29].1.3□××10^8 *E Coli (BL21DE3)*
607 cells were washed once with PBS and incubated with 10□% NHS with or without pre-
608 incubation with recombinant proteins ($A_{11,}$ $A\Delta_{11}$, LAV at 20µg/ ml) in a final reaction
609 volume of 100µl for 30□min at 37°C. The samples were placed on ice to stop further
610 bacteriolysis and then plated on LB agar plates. The plates were incubated at 37°C overnight.
611 Survival was determined by counting bacterial colonies the following day.

612 *Nuclease activity*

613 To examine the DNase activity of LAV, 200ng of 700bp DNA was incubated with different
614 concentration (1 or 2 or 5 or 10µg) of LAV or 2µg domains (A_8-A_{13}) and 2µg domain
615 deletion mutants ($A\Delta_8$-$A\Delta_{13}$) or DNase I (20IU, positive control) in DPBS with 5mM $MgCl_2$
616 in a PCR tube at 37^oC for 2h. The reaction mixture was subjected to EtBr Agarose gel
617 electrophoresis (1%) and then observed under the Gel doc. In a separate experiment, a
618 plasmid was used as a substrate to check endonuclease activity of various concentrations (
619 2ug, 5ug, and 10ug) of rLAV or $rA\Delta_{11.}$ EDTA is a nuclease activity inhibitor, was used as
620 control.

621

622 *Isolation of Neutrophils from murine bone marrow*

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623 Neutrophils were isolated from the bone marrow of C57BL/6J mice using the standard
624 procedure[44]. Briefly, bone marrow was flushed from femurs using a 26G needle, passed
625 through a 30µm cell strainer, and then cells were washed in complete RPMI-1640 twice at
626 1,400 rpm for 10 min at 4°C. After lysis of RBCs using ACK lysis buffer, cells were washed
627 with RPMI-1640 supplemented with 10% FBS, counted, and resuspended in 1ml of ice-cold
628 sterile PBS. Next, cells were overlaid on 3ml of Histopaque 1077/1119 mix in a 15ml conical
629 tube and then centrifuged for 30min at 2,000 rpm at 25°C without braking. Neutrophils at the
630 interface were collected and washed twice with a complete RPMI-1640 medium, counted and
631 suspended in the medium for the specific assay. The viability was determined by Trypan
632 blue exclusion assay.

633 *NET assay*

634 2×10^5 freshly isolated neutrophils in 300µl medium were added to the imaging dish and kept
635 at 37°C in the presence of 5%CO_2 overnight. Cells were treated with 3µl of DMSO or PMA
636 (50ng/ml) or LPS (100ng/ml) and further incubated for 3h at 37°C/5%CO_2. Cells were
637 washed thrice with DPBS and then incubated with LAV (2µg/ml) or DNase-I (20IU) or BSA
638 (5µg/ml) in 5mM $MgCl_2$ containing PBS for 2h at 37°C/5%CO_2. Cells were washed with
639 DPBS, fixed with 4% PFA (15min at RT), and then stained with Rat anti-mouse Ly6G
640 (Alexa flour 647) for 30min. Cells were washed thoroughly with DPBS, mounted with
641 VECTA SHIELD (with DAPI) mounting medium and observed using a 63x oil objective on a
642 confocal microscope (Leica SP8, Wetzlar, Germany).

643 *Statistical Analysis*

644 For all the experiments, wherever required one way or two-way ANOVA were executed to
645 analyze the results. The data were represented as the mean of triplicates ± SEM.
646 $p < 0.05$ was considered significant.

647

648 **Data Availability**

649 The data that support the findings of this study are available from the corresponding author
650 upon reasonable request.

651

652

653

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664 ## Legends to Figures

665 **Fig.1: Variable region of LigA (LAV) induced TLR4 dependent activation of mouse**
666 **macrophages.**
667 (**A**) *Screening of pro-inflammatory response of LAV in RAW264.7 cells.* RAW264.7 cell
668 lines were stimulated with LAV in varying concentrations 1 or 2 or 5µg/ml along with PMB
669 for 24h and supernatant was collected to measure levels of TNF-α and IL-6 cytokines by
670 using ELISA. **(B)** *Binding of LAV with TLR4.* WT, TLR2KO, TLR4KO, or DKO
671 macrophages were incubated with LAV (2µg/ml) for 30 min. After washing, cells were fixed
672 and stained with then stained with Alexa Flour 647 conjugated rabbit anti-mouse IgG and
673 analysed by the confocal microscope as described in materials and methods. **(C)** *Pro-*
674 *inflammatory response of mouse macrophages stimulated with rLig proteins*. WT, TLR2KO,
675 TLR4KO and DKO macrophages cell lines were treated LAV at 2µg/ml for 24h at 37°C in
676 presence of 5%CO_2 and levels of IL-6 and TNF-α in the supernatants were measured by
677 ELISA. **(D)** *IL-8 response in HEK293-TLR4 cells stimulated with LAV*. HEK293T cells
678 transfected with TLR2, TLR4 and NF-kB reporter plasmids were stimulated with rLAV
679 (2µg/ml) for 24h and IL-8 was measured in the culture supernatant by ELISA. *E. coli* LPS
680 (500ng/ml) or PAM3CSK4(20ng/ml) as TLR4 and TLR2 ligands respectively were used as
681 positive controls in all experiments wherever indicated. Data are representative of three
682 different experiments. Significant differences were calculated using the one or two way
683 ANOVA(****, ***, **, * indicates $P < 0.0001$, < 0.001, $P < 0.01$ and $P < 0.05$
684 respectively).
685
686 **Fig.2: 11th domain of LigA (A_{11}) is involved in binding to TLR4 and subsequent**
687 **signalling leading to activation and maturation of mouse macrophages**
688 **(A)** *Screening of pro-inflammatory response of individual domains of LAV in RAW264.7 cells*
689 RAW264.7 cells were incubated with individual domains (A_8-A_{13}) at concentration of 2µg/ml
690 pre-treated with Polymixin B for 24h at 37°C in presence of 5%CO_2 and supernatant was
691 collected to measure levels of IL-6 and TNF-α by ELISA. **(B)** *Screening of pro-inflammatory*
692 *response of domain deletion mutants of LAV in RAW264.7 cells.* RAW264.7 cells were
693 stimulated with LAV or corresponding deletion mutants ($A\Delta_8$-$A\Delta_{13}$) at a concentration of
694 2µg/ml pre-treated with Polymixin B for 24h at 37°C in the presence of 5%CO_2 and
695 supernatant was collected to measure levels of IL-6 and TNF-α by ELISA. **(C)***Binding of A_{11}*

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696 *with the mouse TLR4*. WT, TLR2KO, TLR4KO or DKO macrophages cell lines were
697 incubated with A_{11} and $A_{\Delta 11}$ (2µg/ml) for 30□min. After washing, cells were fixed and
698 stained with respective antibodies and analysed by confocal microscope as described in
699 materials and methods. **(D)** *The pro-inflammatory response of mouse macrophages*
700 *stimulated with A_{11}*. WT, TLR2KO, TLR4KO and DKO bone marrow-derived macrophages
701 cell lines were treated with LAV, $A_{11,}$ $A\Delta_{11}$ (2µg/ml), LPS (500ng/ml) or
702 PAM3CSK4(20ng/ml) for 24h at 37°C in presence of 5%CO_2 and levels of IL-6 and TNF-α
703 in the supernatants were measured with ELISA. **(E)** *A_{11} enhanced the expression of surface*
704 *markers in mouse macrophages. RAW*264.7 cells were incubated with LPS (500 ng/ml) or
705 PAM3CSK (20ng/ml) or LAV or A_{11} or $A\Delta_{11}$ (2µg/ml) for 24h at 37°C in presence of
706 5%CO_2. Cells were stained with fluorochrome-conjugated antibodies and then analyzed by
707 Flow cytometry as described in materials and methods. Control indicates uninduced or
708 unstimulated cells, *E. coli* LPS (500ng/ml) or PAM3CSK4(20ng/ml) as TLR4 and TLR2
709 ligands respectively were used as positive controls in all experiments wherever indicated.
710 Data are representative of three different experiments. Significant differences were calculated
711 using the one or two way ANOVA(****, ***, **, * indicates P < 0.0001, < 0.001, P < 0.01
712 and P□<□0.05 respectively).
713

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

716 **(A)** *A_{11} signals through TLR4 involving the MyD88 adapter*. WT, MyD88 KO, TRIF KO and
717 TMDKO bone marrow-derived macrophages cell lines were treated with LAV or A_{11} or $A\Delta_{11}$
718 (2µg/ml) for 24h at 37°C in the presence of 5%CO_2 and levels of IL-6 and TNF-α in the
719 supernatants were measured by ELISA. **(B)** *A_{11} signals through TLR4 via the MAP kinase*
720 *pathway involving p38 and NFkb.* WT or TLR2KO or TLR4KO or DKO macrophages cell
721 lines were stimulated with A_{11} (2µg/ml) for 24h at 37°C in the presence of 5%CO_2. Levels of
722 phosphorylated p38, JNK, and ERK1/2 induced by A_{11} were analyzed by western blot as
723 described in materials and methods. **(C)** *Pharmacological inhibitors of p38 and NFkb*
724 *significantly reduces A_{11} mediated cytokine response*. RAW 264.7 cells were pre-treated for
725 30min with NF-kB inhibitor (SN50; 20µM), JNK inhibitor (SP600125; 40µM) or p38MAPK
726 inhibitor (SB203580; 30µM) or ERK (U0126; 50µM) or all four inhibitors together and then
727 stimulated with A_{11} (2µg/ml) for 24h at 37°C in the presence of 5%CO_2 and supernatant was
728 collected to measure levels of IL-6 and TNF-α by ELISA. **(D)** *Analysis of expression of*
729 *immune response-related genes in mouse macrophages stimulated with A_{11}*. WT, TLR2KO,
730 TLR4KO and DKO mouse macrophage cell lines were treated with 2µg/ml A_{11} for 4h or 24h
731 or 48h. Cells were recovered, RNA was isolated, converted to cDNA and gene expression
732 was analyzed by RT-PCR as described in material and methods. The data were presented as
733 fold changes between stimulated cells vs control and normalized to GAPDH. *E. coli* LPS
734 (500ng/ml) or PAM3CSK4(20ng/ml) as TLR4 and TLR2 ligands respectively were used as
735 positive controls in all experiments wherever indicated. All data are representative of three
736 independent experiments. Significant differences were calculated using the one or two way
737 ANOVA(****, ***, **, * indicates P < 0.0001, < 0.001, P < 0.01 and P□<□0.05
738 respectively).
739
740

741 **Fig.4: A_{11} is an immuno-dominant domain that induces robust antibody and T cell**
742 **response in mice**

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743 (A) *Antibody response*. The antibody response (Total IgG, IgG1, IgG2c and IgA) on day 28
744 in various immunized groups (PBS, A_{11}, A_{11}-Alum) was evaluated by ELISA as described in
745 materials and methods. (B) *Lymphocyte proliferation*. The proliferation of splenocytes
746 isolated from various groups was analyzed by stimulating with recall antigen (A_{11}) and
747 counting cells after 72h. **(C)** *Cytokine analysis*. Culture supernatant from spleenocytes
748 stimulated with A_{11} for 72h were analyzed for IL-4, IL-10 and IFN-γ by using a sandwich
749 ELISA kit following manufacturer's instructions. **(D)** *Total IgG response at day 28 in*
750 *animals immunized with LAV, A_{11}, and $A\Delta_{11}$ in Fruend's adjuvant.* Total IgG at day 28 was
751 analysed in serum from animals immunized LAV, $A_{11}$and $A\Delta_{11}$ in Fruend's adjuvant by
752 ELISA as described above. **(E)** *Antibody response against A_{11} and $A\Delta_{11}$ in serum of animals*
753 *immunized with LAV-Alum.* Total IgG was analyzed in serum from LAV immunized animals
754 (diluted at 1:10000 and 1:100000) against A_{11} and $A\Delta_{11}$ by ELISA as described above **(F)**
755 *Cell proliferation* and **(G)** *Cytokine analysis of lymphocytes isolated from LAV-Alum*
756 *immunized animals after in-vitro stimulation with A_{11} and $A\Delta_{11}$*. Lymphocytes isolated at day
757 28 from animals immunized with LAV-Alum were stimulated with A_{11} and $A\Delta_{11}$ for 48-72h
758 and analysed for proliferation and cytokines in the culture supernatant were determined as
759 described above. All data are representative of three independent experiments. Significant
760 differences were calculated using the one or two way ANOVA(****, ***, **, * indicates $P <$
761 0.0001, < 0.001, $P < 0.01$ and $P < 0.05$ respectively).
762
763

764 **Fig.5: Characterization of the domain of LAV involved in evasion from complement-**
765 **mediated killing**

766 **(A)** *Binding of LAV domains as analyzed by Dot blot.* Purified proteins LAV (positive
767 control), BSA (negative control), individual domains (A_8-A_{13}), and corresponding deletion
768 mutants ($A\Delta_8$-$A\Delta_{13}$) were immobilized on nitrocellulose membranes and then incubated with
769 1% NHS (as a source of FH and PLG). FH and PLG were detected with specific antibodies
770 by Western blot. **(D)** *Binding of LAV deletion mutants as analysed by Pull down assay*. Bead
771 bound LAV deletion mutants($A\Delta_8$-$A\Delta_{13}$) were incubated with 10% HI NHS and protein –
772 protein interaction was detected by western blot using Anti FH (157 kDa) or PLG antibody
773 (45 kDa) as described in methodology **(C)** *Binding of LAV domains as analyzed by ELISA.*
774 Microtiter plates were coated with 1µg of proteins LAV, domains (A_8-A_{13}), and
775 corresponding deletion mutants ($A\Delta_8$-$A\Delta_{13}$) and 10% HI-NHS was added to each well. The
776 binding was detected with specific antibodies against FH and PLG as described in materials
777 and methods. **(D)** *Co-factor activity*. LAV, A_{11}, $A\Delta_{11}$ (2µg/ml), and BSA (2µg/well) were
778 immobilized on microtiter plates and incubated with purified FH. After washing, C3b and
779 factor I (FI) were added, and the plate was incubated for 4h at 37°C. The products were
780 analysed by SDS-PAGE, and the cleavage fragments of C3b was detected by Western blot
781 using anti-human C3 polyclonal antibodies as described in materials and methods. **(E)**
782 *Plasmin activity*. LAV, A_{11}, $A\Delta_{11}$ (2µg/ml), and BSA (2µg/well) were immobilized on
783 microtiter plates followed by the addition of PLG, uPA, and specific plasmin substrate. The
784 plate was incubated for 48h, and absorbance was read at 405nm as described in materials and
785 methods. In another experiment, C3b was incubated with activated plasmin in the presence or
786 absence of A_{11} and cleavage products were visualized using Western blot. **(F)** *Bactericidal*
787 *assay*. 1.3×10^8 *E Coli BL-21(DE3)* cells were incubated with 10 % NHS with or without
788 pre-incubation with A_{11} or $A\Delta_{11}$ or LAV at 20µg/ml for 30 min at 37 °C. The samples
789 were plated on LB agar plates and incubated at 37°C overnight. Survival was determined by
790 counting bacterial colonies the following day. All data are representative of three independent

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791 experiments. Significant differences were calculated using the one or two way
792 ANOVA(****, ***, **, * indicates P < 0.0001, < 0.001, P < 0.01 and P < 0.05
793 respectively).
794
795

796 **Fig.6: LAV is nuclease capable of degrading Neutrophil Extracellular Trap (NET)**
797 **(A)** *Exonuclease activity of the LAV*. 700bp DNA (200ng) was incubated with different
798 concentrations of LAV (2, 5 and 10μg) in DPBS with 5mM $MgCl_2$ at 37°C for 3h followed
799 by visualized using the Agarose gel electrophoresis.**(B)** *Endonuclease activity of the LAV.*
800 Plasmid DNA (200ng) was incubated with different concentrations of LAV (2, 5 and 10μg)
801 in DPBS with 5mM $MgCl_2$ at 37°C for 3h followed by visualized using the Agarose gel
802 electrophoresis. **(C)** *Exonuclease activity of the LAV domains.* 700bp DNA (200ng) with
803 incubated with various LAV domains (A_8-A_{13}) at 5μg in DPBS with 5mM $MgCl_2$ incubated
804 at 37°C for 3h followed by EtBr- Agarose gel electrophoresis. **(D)** *Exonuclease activity of*
805 *domain deletion mutants of LAV*. DNA (200ng) with incubated with various domain deletion
806 mutants of LAV ($A\Delta_8$-$A\Delta_{13}$) at 5μg in DPBS with 5mM $MgCl_2$ incubated at 37°C for 3h
807 followed by EtBr- Agarose gel electrophoresis **(E)** *Endonuclease activity of the* $A\Delta_{11}$.
808 Plasmid DNA (200ng) was incubated with different concentrations of $A\Delta_{11}$ (2, 5 and 10μg) in
809 DPBS with 5mM $MgCl_2$ with or without EDTA at 37°C for 3h followed by visualized using
810 the Agarose gel electrophoresis. C and C1 indicates DNA alone, D indicatres DNA treated
811 with DNAse, C2 indicates DNA with reaction mixture, M is 100bp or 1kb DNA ladder in all
812 experiments wherever indicated **(F).** *NETosis assay:* Mouse Neutrophils were cultured on
813 glass coverslips stimulated with DMSO or LPS (500ng) or PMA (0.5μM) for 3.5h and then
814 treated with DNase-I (positive control) or BSA (negative control) or LAV (5μg) for 2h at
815 37°C and visualized under 63X of Leica microscopy. DAPI; staining of the complete DNA
816 content (Nuclear and released), Ly6G; neutrophil marker, BF; Bright field. All data are
817 representative of three independent experiments.

818

819 **Fig.7. Schematic presentation of the role of LigA in the modulation of host immune**
820 **response. (A)** *Immune evasion.* LigA expressed during infection might acquire complement
821 regulators (FH, C4BP, PLG) to inhibit both the classical and alternate pathways of
822 complement-mediated killing. *Leptospira* might utilize the nuclease activity of LigA to
823 escape from NET. Upon interaction with host innate immune cells (DCs, macrophages),
824 LigA might undergo antigenic variation or downregulate its expression to evade recognition
825 through TLR4 and subsequent activation of the innate response. **(B)** *Immune activation.*
826 LigA (LAV) can activate strong innate and adaptive immune responses leading to the
827 production of antibodies that may block binding to complement regulators, inhibit nuclease
828 activity and enhance phagocytosis, all of which may contribute to the killing of bacteria and
829 clearance from the host.

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1120 **Author's contribution**

1121 SMF conceived the idea and designed the experiments. AK, VPV, SK, MA, PV, MAT
1122 performed the experiments. AK, VPV, SK and SMF analyzed the data. VPV made all the
1123 figures of the manuscript and did the statistical analysis. YFC contributed reagents and
1124 input in experiment design and data analysis. SMF, AK, and VPV wrote the initial draft,
1125 and SMF edited the manuscript. All authors approved the final version of the manuscript.
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1128 **Competing interests**

1129 The authors declare no competing financial interests.

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Fig. 1

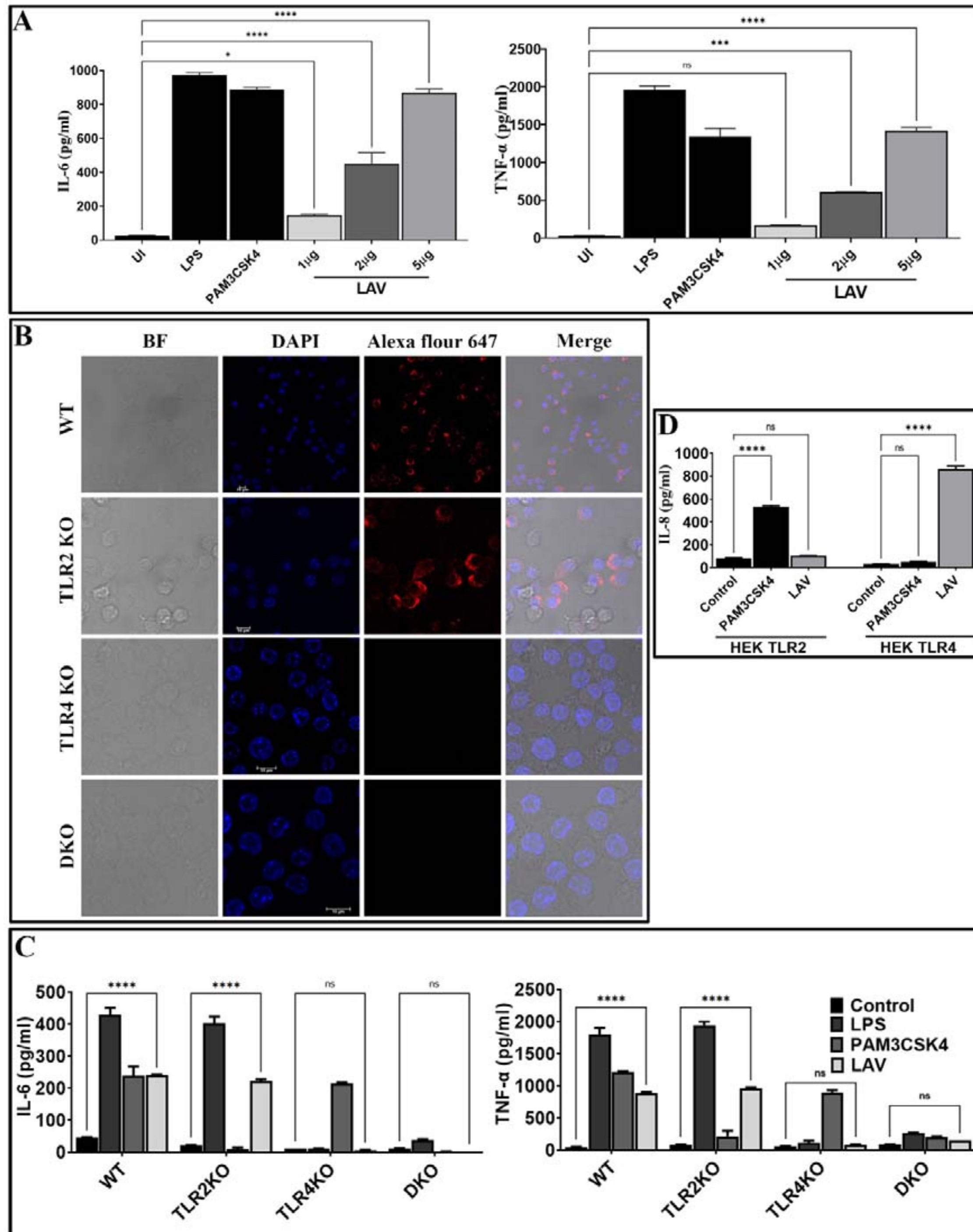

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Fig. 2

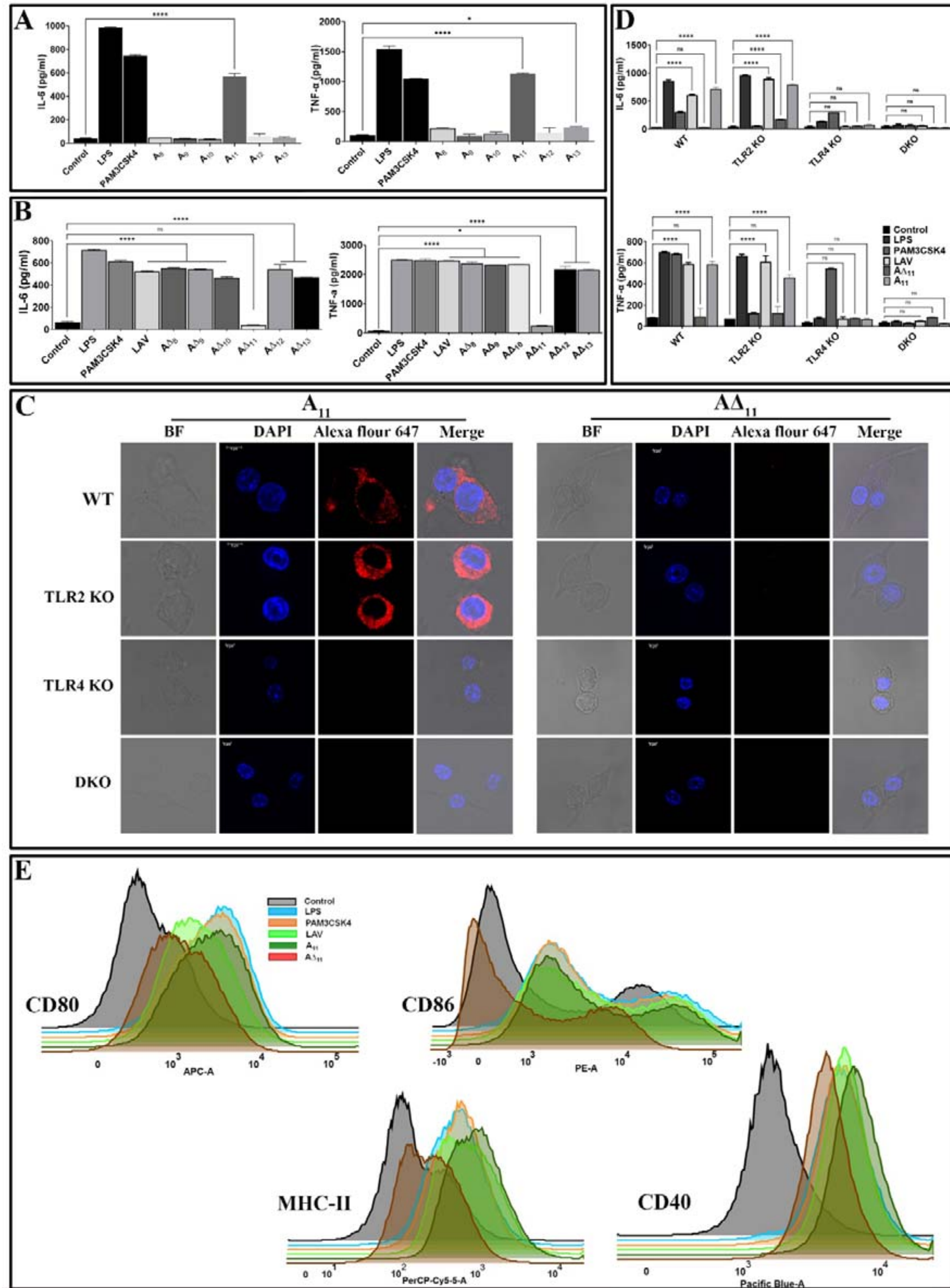

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Fig. 3

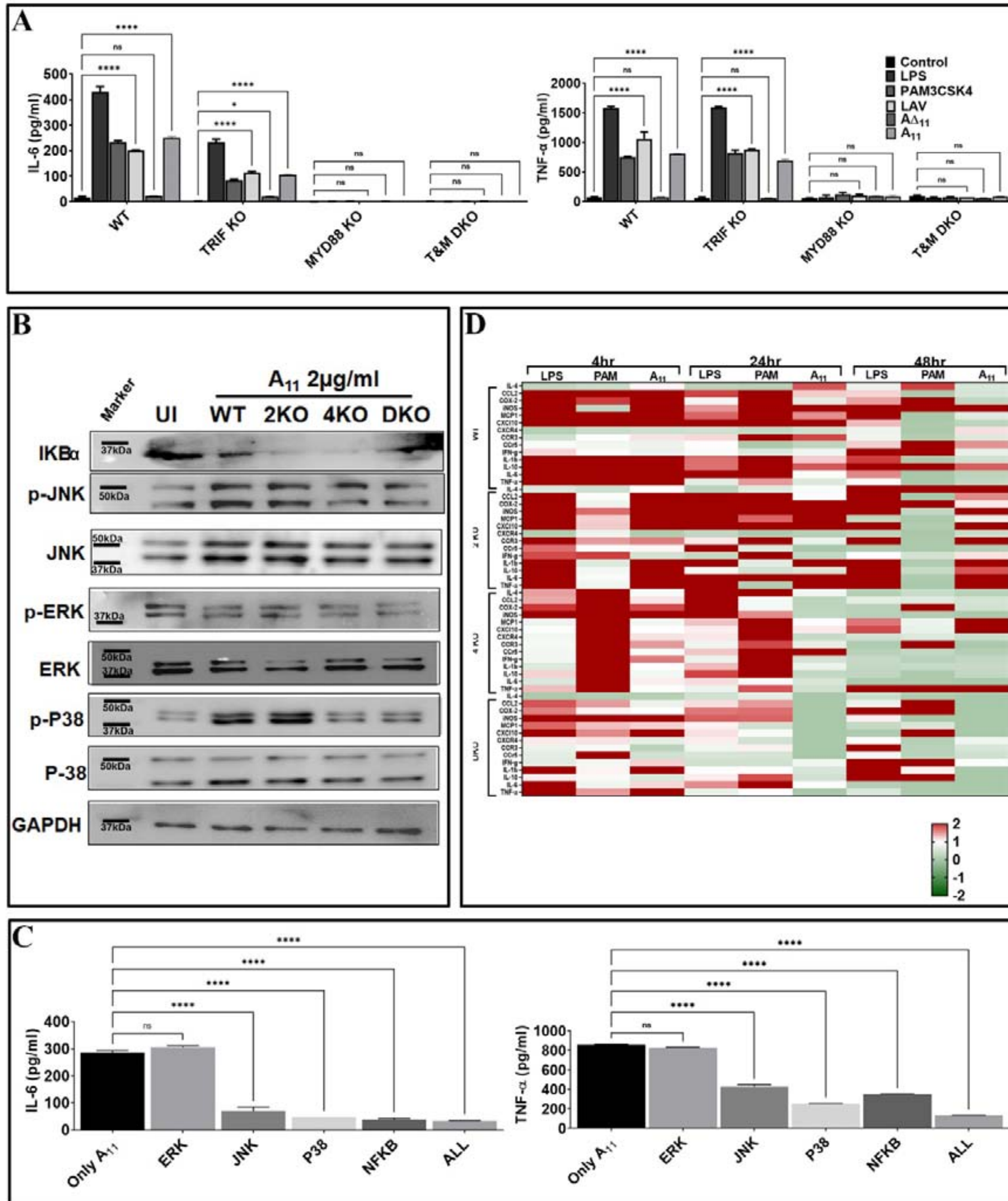

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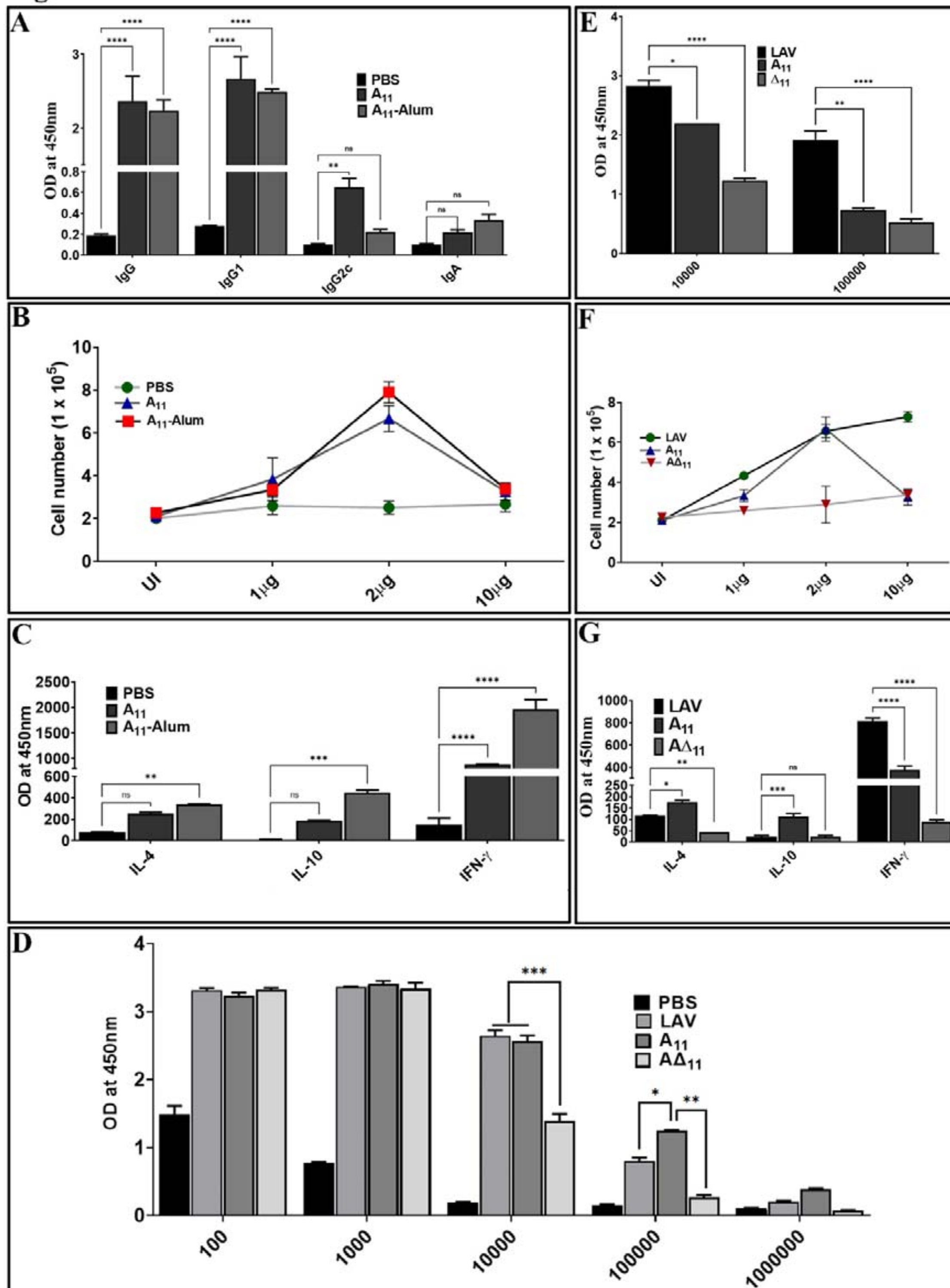
Fig. 4



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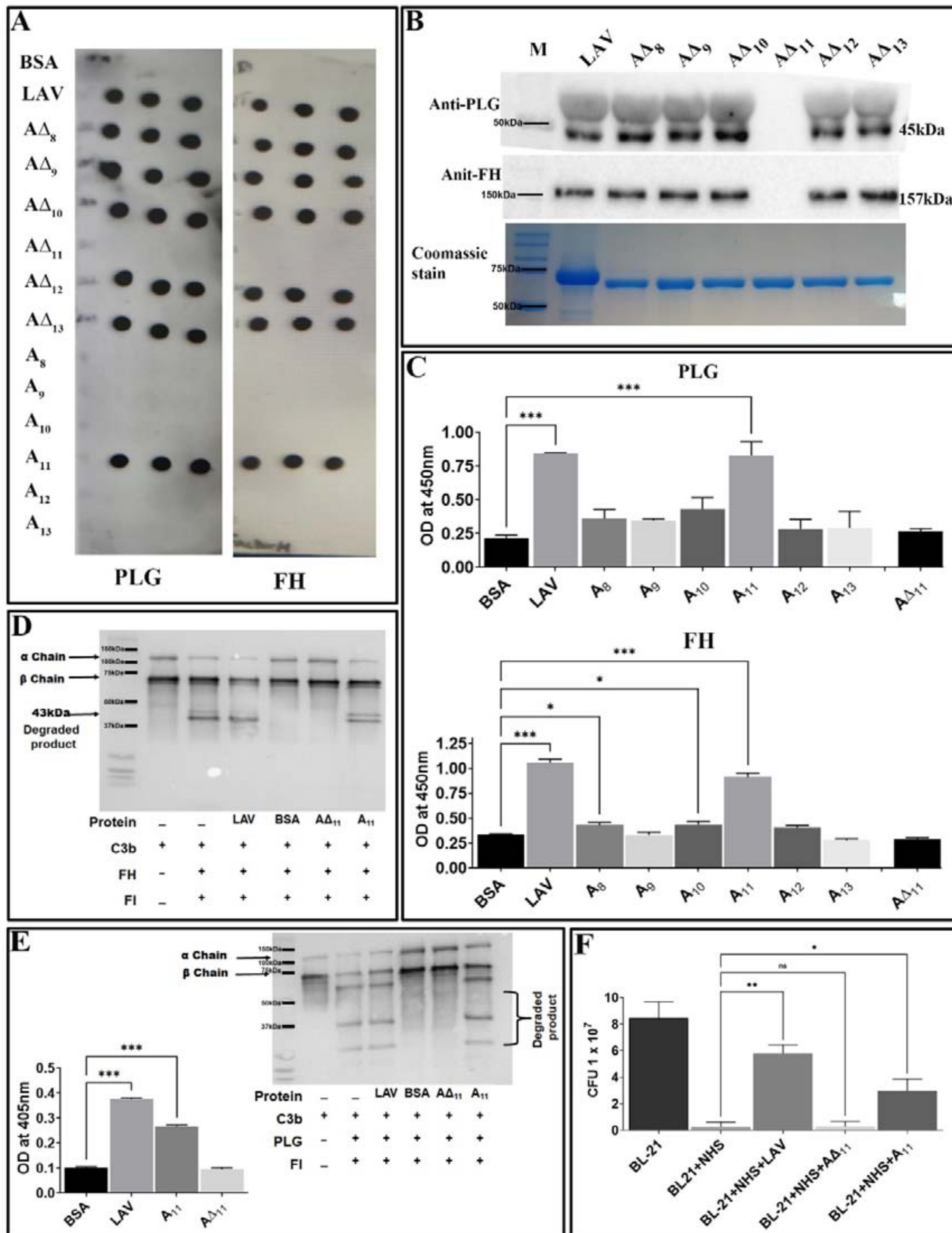

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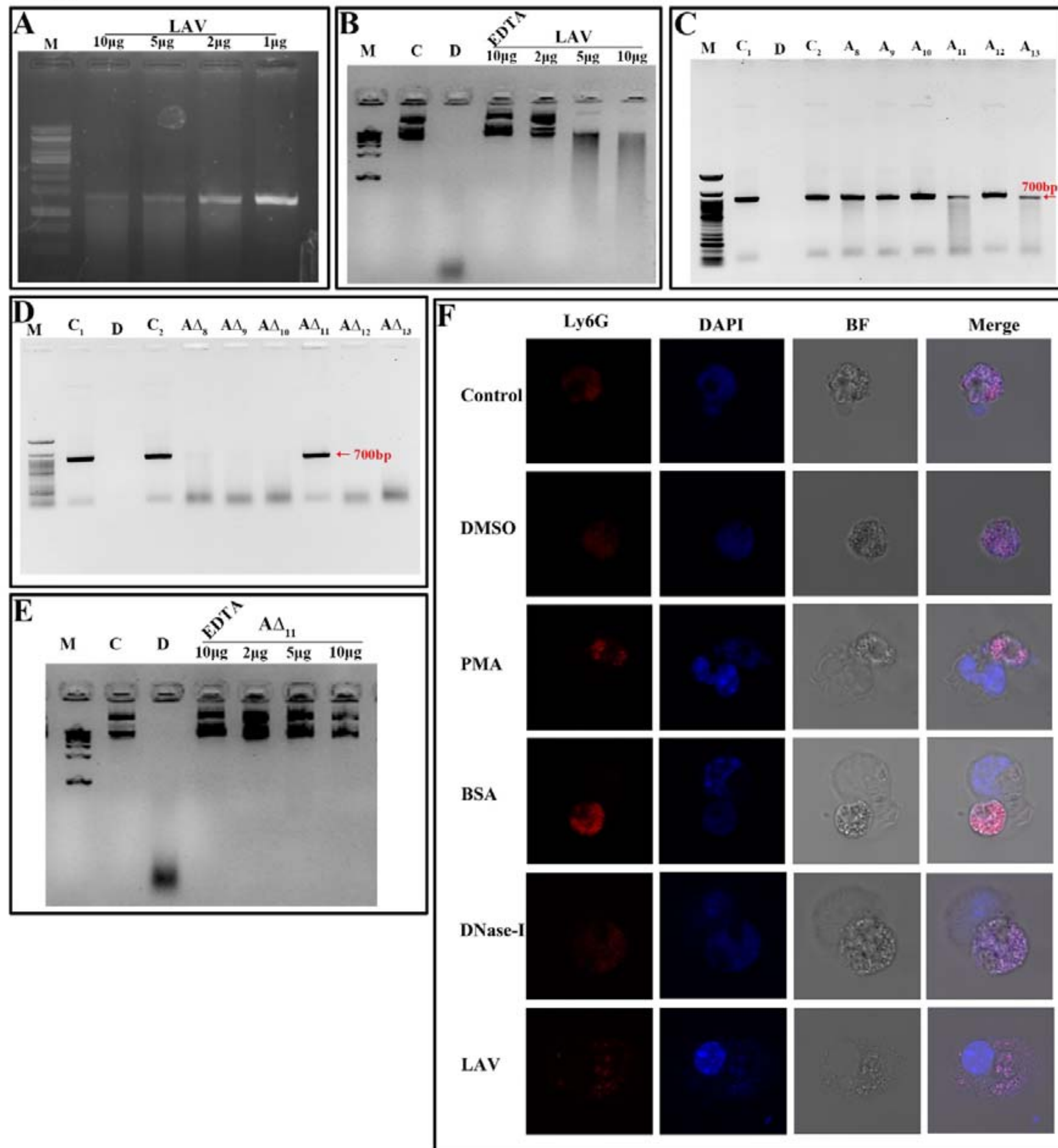

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1152 **Fig. 7**

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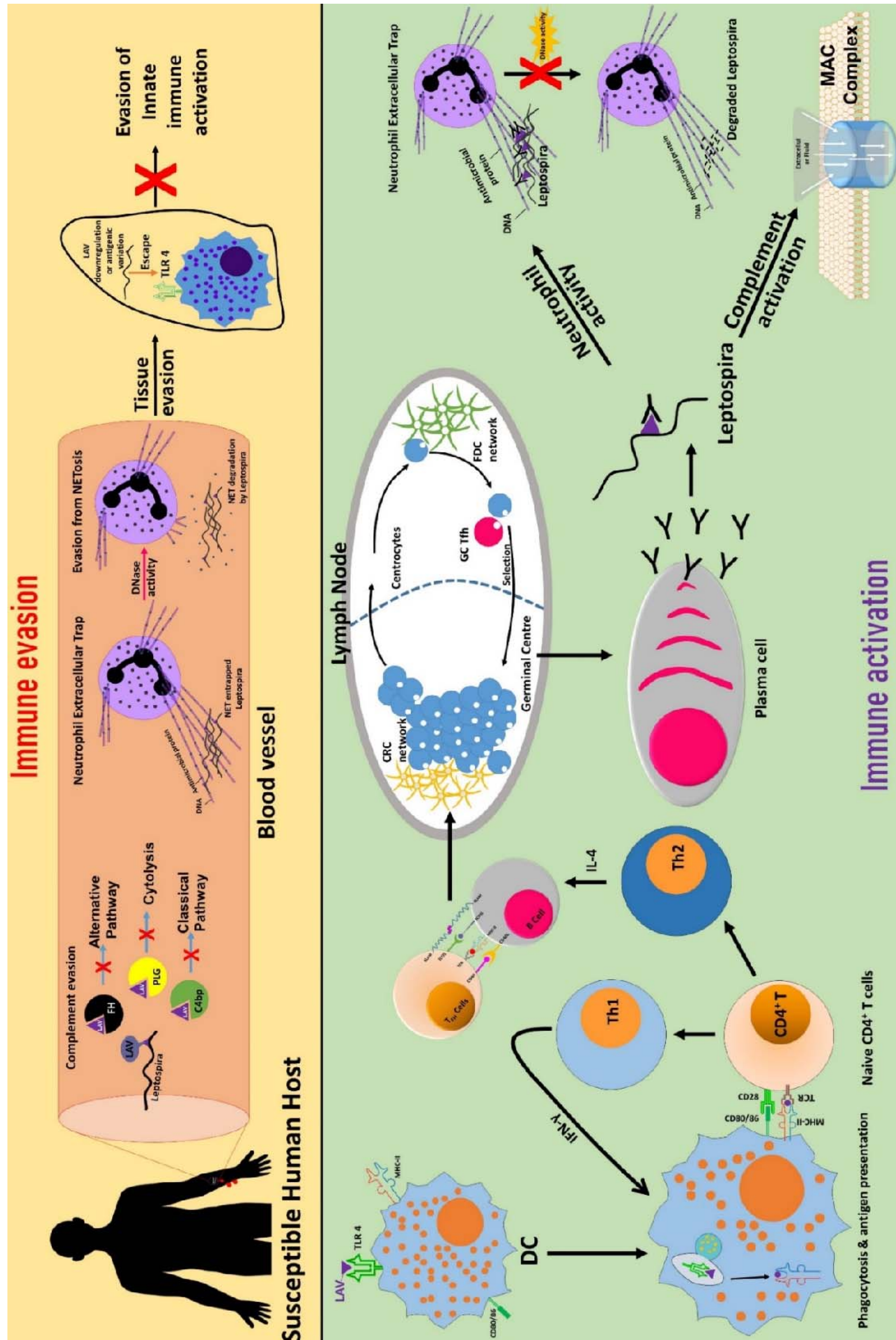

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1154 # Supplementary Materials

1155

1156 **Title:** Deciphering the role of *Leptospira* surface protein LigA in modulating the host innate
1157 immune response

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1164 **The supplementary file contains-**

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1166 **Figure S1-** Purification of recombinant proteins.

1167 **Figure S2-** Effect on TLR activity after pre-treatment of purified recombinant proteins with
1168 Polymixin B and Proteinase K

1169 **Figure S3-** Circular dichroism spectra of the recombinant proteins.

1170 **Figure S4-** Bacterial survival assay

1171 **Table 1-** Primers used for RT-PCR

1172 **Table 2-** Primers used for creating deletion domains of LAV

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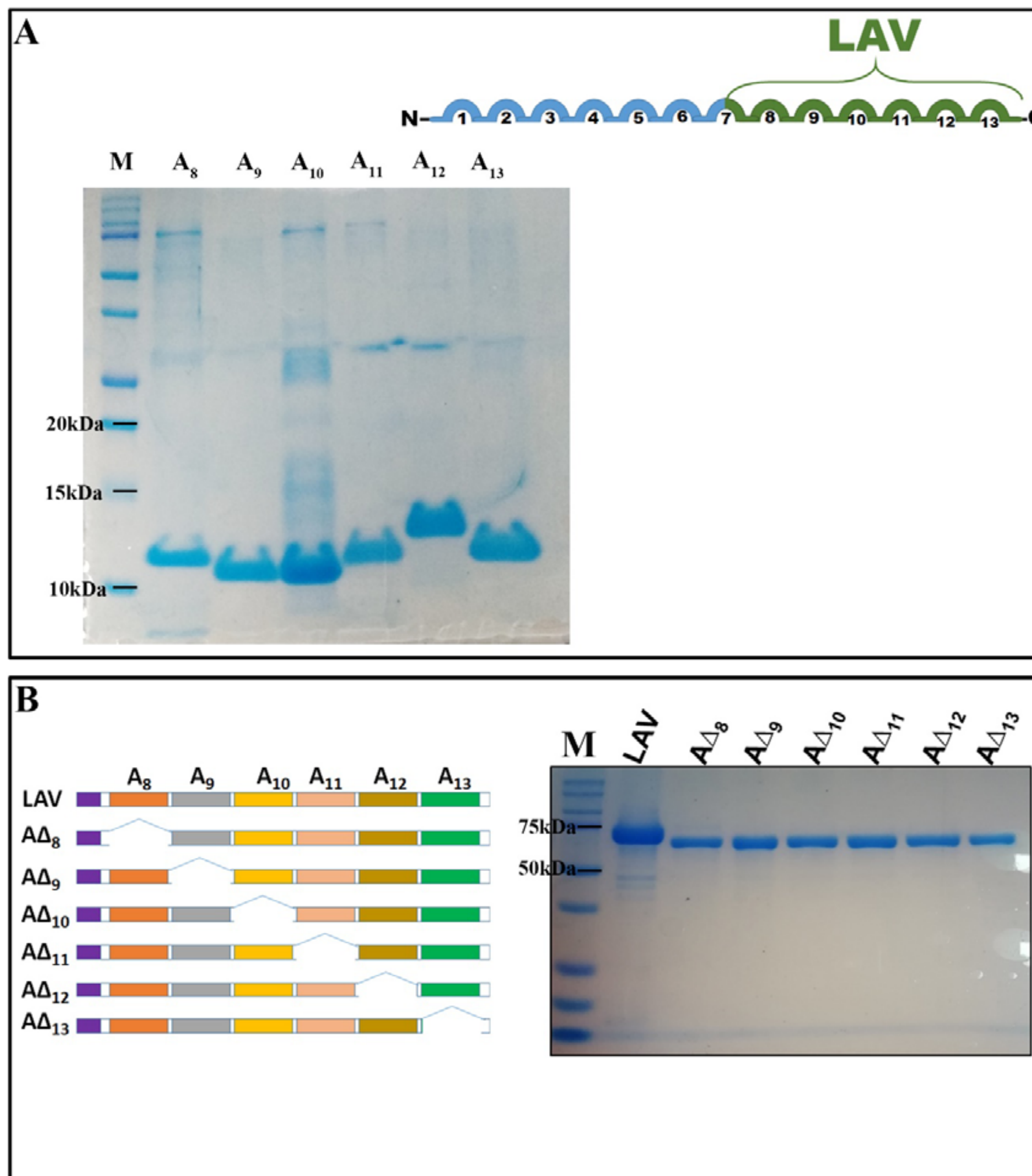
1184 # Figure S1



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1186 **Sup. Fig. 1: Purification of recombinant proteins.**

1187 **(A)** *SDS PAGE profile of domains of Variable A (LAV).* The recombinant proteins were
1188 purified as His-sumo fusion proteins as described in materials and methods. The expected
1189 molecular weight of each domain ranges from 11-12kd . **(B)** *Schematic presentation of*
1190 *strategy of creating LAV domain deletion mutants ($A\Delta_8$- $A\Delta_{13}$) by PCR based site-directed*
1191 *mutagenesis and SDS PAGE profile of purified proteins.* The recombinant proteins were
1192 purified as His-sumo fusion proteins as described in materials and methods The expected
1193 molecular weight of LAV was 73kd and each domain deletion mutant was ~63kd. Data are
1194 representative of three independent experiments. (*Indicates P□<□0.05).

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1196 # Figure S2

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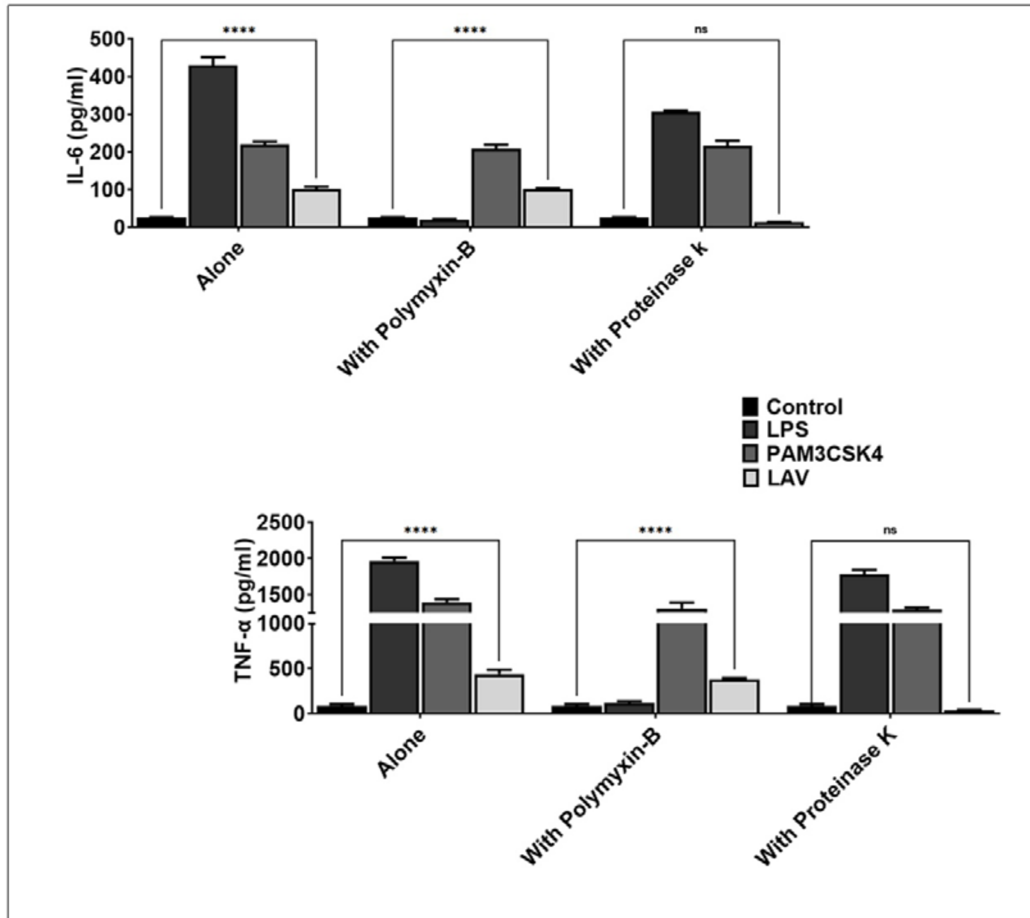

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1201 **Sup. Fig. 2: Effect on TLR activity after pre-treatment of purified recombinant proteins**
1202 **with Polymixin B and Proteinase K.** RAW264.7 cells were incubated with 2µg/ml of
1203 purified LAV pre-treated with Polymyxin B or Proteinase- K as mentioned in materials and
1204 methods and supernatant was collected to measure levels of IL-6 and TNF-α by ELISA.

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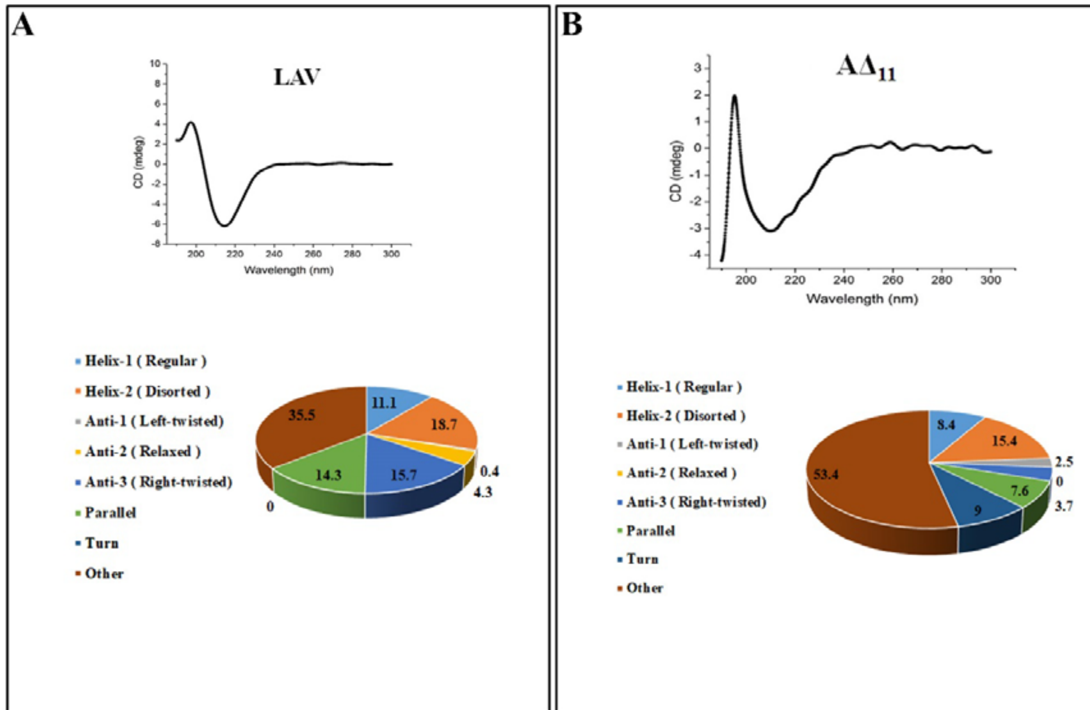
Figure S3



Sup Fig. 3 Circular dichroism spectra of the recombinant proteins. CD spectra of recombinant proteins LigA WT and LigAΔ11. Far-UV CD spectra are presented as an average of five scans recorded from 190 to 300 nm.

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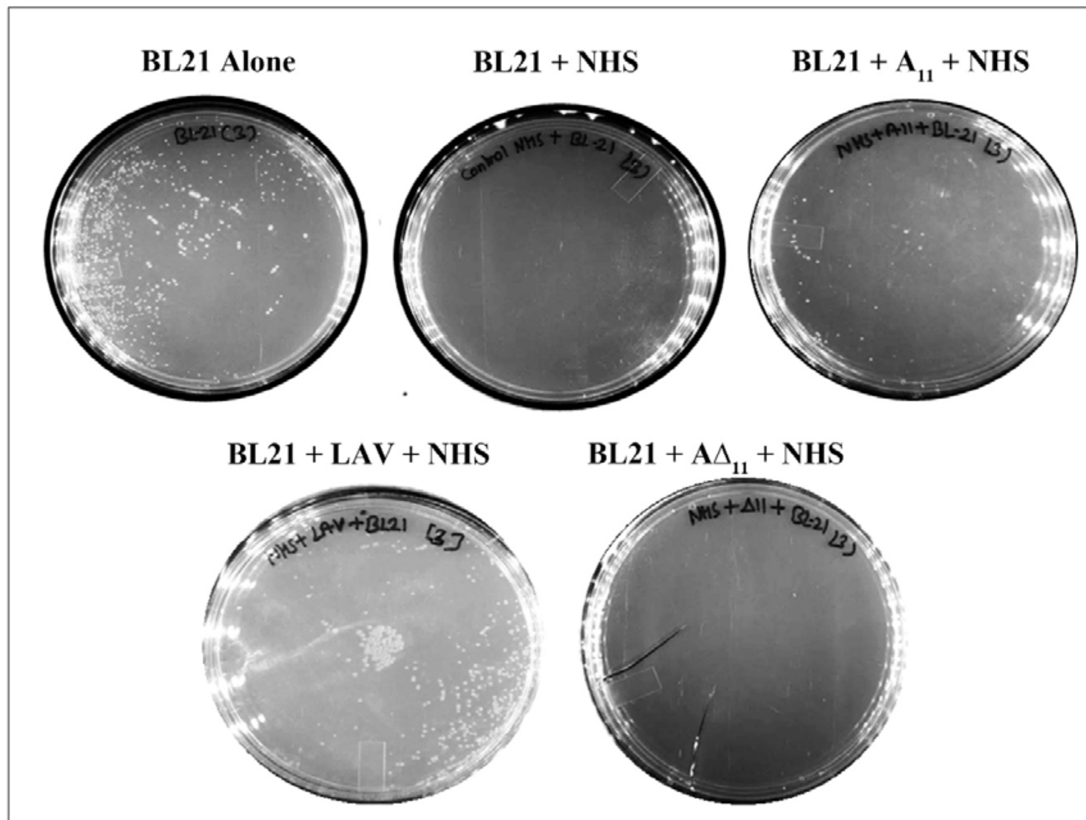
Figure S4



Sup Fig. 4. Bacterial survival assay. Photographic images of survival assay bacterial colonies formed by BL-21 *E. coli* after the treatment with NHS, which is pre-incubated with LigAvWT, Δ11 and A11.

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1252 **Table1. Primers used for RT-PCR**

S.NO	Gene	Primer Sequence
1	GAPDH	F- GCCTGGAGAAACCTGCC R- ATACCAGGAAATGAGCTTGACA
2	IL-4	F- AGTTGTCATCCTGCTCTTC R- GTGTTCTTCGTTGCTGTG
3	CCL2	F- ACGTGTTGGCTCAGCCAGA R- ACTACAGCTTCCTTTGGGACACC
4	COX-2	F- TCTGGAACATTGTGAACAACATC R- AAGCTCCTTATTTCCCTTCACAC
5	iNOS	F- CAGCCCAACAATACAAGATGACCC R- CAGTTCCGAGCGTCAAAGACCTGC
6	MCP1	F- GGAAAAATGGATCCACACCTTGC R- TCTCTTCCTCCACCACCATGCAG
7	CXCL10	F- CATGGTCCTGAGACAAAAGT R- TGATGACACAAGTTCTTCCA
8	CXCR4	F- GAAGTGGGGTCTGGAGACTATG R- AGGGGAGTGTGATGACAAAGAG
9	CCR3	F- CAACTTGGCAATTTCTGACCTG R- GCAAACACAGCATGGACGATAG
10	CCR5	F - ACACTCAGTATCATTTCTGG R- GGATCAGGCTCAAGATGACC
11	IFN-γ	F- ACTCAAGTGGCATAGATGTGGAAG R- GACGCTTATGTTGTTGCTGATGG
12	IL-1b	F- GCCTTGGGCCTCAAAGGAAAGAATC R- GGAAGACACAGATTCCATGGTGAAG
13	IL-10	F- GCCAGAGCCACATGCTCCTA R- GATAAGGCTTGGCAACCCAAGTAA
14	IL-6	F- TGGAGTCACAGAAGGAGTGGCTAAG R- TCTGACCACAGTGAGGAATGTCCAC
15	TNF-α	F- ATAGCTCCCAGAAAAGCAAGC R- CACCCCGAAGTTCAGTAGACA

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1260 **Table 2. Primers used for domain deletions**

S.NO	Name	Primer Sequence
1	AΔ8	F- ACCGTCACACAGGCGACTATTGCAGTTGGAAAACAT R- ATGTTTTCCAACTGCAATAGTCGCCTGTGTGACGGT
2	AΔ9	F- TCAAATCAGTCCTGTAAAAGATTATCCGTTACCGCA R- TGCGGTAACGGATAATCTTTTACAGGACTGATTTGA
3	AΔ10	F- GCAGCGGAACTTATTGAGCAAGTTACTCCGGCTAAA R- TTTAGCCGGAGTAACTTGCTCAATAAGTTCCGCTGC
4	AΔ11	F- CAAGTTACTCCGGCTAAATTGAATGTCACTCCAGCG R- CGCTGGAGTGACATTCAATTTAGCCGGAGTAACTTG
5	AΔ12	F- ACTCCAGCGCTTCTTCGTCCAGTTACGGTTACGGAA R- TTCCGTAACCGTAACTGGACGAAGAAGCGCTGGAGT
6	AΔ13	F- GTTACGGAAAGTGGTATAGTAACTCCAGAAATATTA R- TAATATTTCTGGAGTTACTATACCACTTTCCGTAAC

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