

1 **The Dot/Icm-translocated effector LegC4 potentiates cytokine-mediated restriction of**  
2 ***Legionella* within accidental hosts**

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4 Running title: LegC4 augments cytokine-mediated host defense

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14

15 **Abstract**

16 Abstract

17 *Legionella pneumophila* is ubiquitous in freshwater environments where it replicates  
18 within unicellular protozoa. However, *L. pneumophila* is also an accidental human pathogen that  
19 can cause Legionnaires' Disease in immunocompromised individuals by uncontrolled replication  
20 within alveolar macrophages. To replicate within eukaryotic phagocytes, *L. pneumophila* utilizes  
21 a Dot/Icm type IV secretion system to translocate a large arsenal of over 300 effector proteins  
22 directly into host cells. In mammals, translocated effectors contribute to innate immune  
23 restriction of *L. pneumophila*. We found previously that the effector LegC4 is important for *L.*  
24 *pneumophila* replication within a natural host protist but is deleterious to replication in a mouse  
25 model of Legionnaires' Disease. In the present study, we used cultured mouse primary  
26 macrophages to investigate how LegC4 attenuates *L. pneumophila* replication. We found that  
27 LegC4 enhanced restriction of *L. pneumophila* replication within macrophages activated with  
28 tumor necrosis factor (TNF) or interferon (IFN)- $\gamma$ . Specifically, TNF-mediated signaling was  
29 required for LegC4-mediated attenuation of *L. pneumophila* replication within macrophages. In  
30 addition, expression of *legC4* was sufficient to restrict *L. longbeachae* replication within TNF- or  
31 IFN- $\gamma$ -activated macrophages. Thus, this study demonstrates that LegC4 contributes to *L.*  
32 *pneumophila* clearance from healthy hosts by potentiating cytokine-mediated host defense  
33 mechanisms.

34

35 Importance

36 *Legionella* are natural pathogens of protozoa and accidental pathogens of humans. Innate  
37 immunity in healthy individuals effectively controls *Legionella* infection due in part to rapid and

38 robust production of pro-inflammatory cytokines resulting from detection of Dot/Icm-  
39 translocated substrates, including effectors. Here, we demonstrate that the effector LegC4  
40 enhances pro-inflammatory host restriction of *Legionella* from macrophages. These data suggest  
41 that LegC4 may augment pro-inflammatory signaling or antimicrobial activity of macrophages, a  
42 function that has not previously been observed for another bacterial effector. Further insight into  
43 LegC4 function will likely reveal novel mechanisms to enhance immunity against pathogens.  
44

## 45 **Introduction**

46 *Legionella* are natural pathogens of unicellular protozoa and accidental pathogens of  
47 humans that can cause in a severe inflammatory pneumonia called Legionnaires' Disease, which  
48 results from uncontrolled bacterial replication within alveolar macrophages. To replicate within  
49 eukaryotic phagocytes, *Legionella* subvert normal endocytic signaling by establishing a  
50 specialized compartment called the *Legionella* containing vacuole (LCV). To form the LCV and  
51 replicate intracellularly, *Legionella* employ a Dot/Icm type IV secretion system (T4SS) to  
52 translocate virulence factors - called effector proteins - into host cells (1). Although >15  
53 *Legionella* species are capable of causing human disease, the overwhelming majority is caused  
54 by *L. pneumophila* (2, 3). In healthy individuals, *L. pneumophila* infection is efficiently  
55 controlled and human-to-human transmission is incredibly rare (4). This is due to efficient  
56 detection and subsequent clearance of *L. pneumophila* by the mammalian innate immune system.  
57 Consequently, *L. pneumophila* is a well-established model pathogen used to characterize  
58 mechanisms of host defense against bacterial pathogens.

59 Innate immune detection of bacterial pathogens is facilitated by host pattern recognition  
60 receptors (PRRs) that recognize pathogen associated molecular patterns (PAMPs). Surface toll-  
61 like receptors (TLRs) are PRRs critical for host defense against *L. pneumophila*. The majority of  
62 TLRs signal through the adaptor MyD88 to activate pro-inflammatory gene expression. Mice  
63 lacking MyD88 are highly susceptible to *L. pneumophila* infection, which is mostly due to lack  
64 of interleukin (IL)-1 and TLR2-mediated signaling (5-7). Intracellular PRRs such as Nod1,  
65 Nod2, and inflammasomes also contribute to innate immune restriction of *L. pneumophila* in  
66 macrophages [reviewed in (8, 9)]. Leakage of PAMPs through the Dot/Icm pore amplifies cell-  
67 autonomous restriction of *L. pneumophila* within immune phagocytes. Specifically, recognition

68 of *L. pneumophila* flagellin monomers (*flaA*) by the NAIP5/NLRC4 inflammasome is sufficient  
69 to restrict *L. pneumophila* replication within macrophages (10, 11). In addition, translocation of  
70 peptidoglycan, nucleic acids, and lipopolysaccharide into the host cell cytosol through the  
71 Dot/Icm pore also enhances restriction of *L. pneumophila*. Engagement of both extracellular and  
72 intracellular PRRs results in a robust pro-inflammatory response mediated by secretion of  
73 cytokines by infected and bystander immune phagocytes (5, 12-16). In particular, tumor necrosis  
74 factor (TNF) and interferon (IFN)- $\gamma$  are critical for restriction of pulmonary *L. pneumophila*  
75 infection (17-21). TNF and IFN- $\gamma$  both promote cell autonomous defense against *L. pneumophila*  
76 within macrophages and mediate bacterial killing by increasing phagolysosomal fusion (22, 23).

77 In addition to canonical PAMPs, translocated effectors can augment pro-inflammatory  
78 responses in *L. pneumophila*-infected macrophages. For example, effector-mediated inhibition of  
79 host protein translation results in increased expression of pro-inflammatory genes in  
80 macrophages (24-27). In addition, macrophage pro-inflammatory gene expression was decreased  
81 during infection with *Legionella* that possess a functional Dot/Icm T4SS but are unable to  
82 translocate a subset of effectors due to a mutation in the *icmS* effector chaperone gene (28).  
83 These studies elaborate the concept of effector-triggered immunity in animal cells (29) and  
84 provide further evidence for the contribution of effectors to innate immune restriction of *L.*  
85 *pneumophila*.

86 We recently demonstrated that the effector LegC4 attenuates *L. pneumophila* fitness in a  
87 mouse model of Legionnaires' disease (30). Loss-of-function mutation of the *legC4* gene  
88 conferred a fitness advantage on *L. pneumophila* in the mouse lung as evidenced by increased  
89 pulmonary bacterial burden and the ability to outcompete the wild-type strain (30). However, the  
90 *legC4* mutation had no effect on *L. pneumophila* replication in primary bone-marrow derived

91 macrophages (BMDMs) and impaired replication within a natural amoeba host, *Acanthamoeba*  
92 *castellanii* (30). Furthermore, expression of *legC4* from a plasmid further attenuated *L.*  
93 *pneumophila* fitness in the mouse lung compared to the wild-type strain. Thus, we hypothesized  
94 that LegC4 is deleterious to *L. pneumophila* in the presence of cell-mediated innate immunity.

95         The present study was designed to determine how LegC4 augments restriction of *L.*  
96 *pneumophila* in mammalian hosts. Expression of *legC4* from a plasmid was sufficient to  
97 attenuate *L. pneumophila* replication in BMDMs, which relied on TNF secretion and subsequent  
98 signaling. Moreover, a  $\Delta$ *legC4* mutant exhibited increased replication in cytokine-activated  
99 BMDMs. Interestingly, expression of *legC4* was sufficient to attenuate *L. longbeachae*  
100 replication within TNF- and INF- $\gamma$ -activated BMDMs. These results suggest that LegC4  
101 enhances macrophage cell autonomous defense against *Legionella* by potentiating cytokine-  
102 mediated restriction.

103

## 104 **Materials and Methods**

### 105 Bacterial strains, plasmids, primers, growth conditions

106 *Legionella pneumophila* Philadelphia-1 SRS43 (30), SRS43 *flaA*::Tn (30), Lp02  $\Delta$ *flaA* (10),  
107 Lp03 (31), and *Escherichia coli* strains were gifts from Dr. Craig Roy (Yale University). *L.*  
108 *longbeachae* NSW 150 was a gift from Dr. Hayley Newton (University of Melbourne).  
109 *Escherichia coli* strains used for cloning (Top10, Invitrogen) and *L. pneumophila* mutagenesis  
110 [DH5 $\alpha$ - $\lambda$ *pir*, (32)] were maintained in Luria-Bertani (LB) medium supplemented with 25  $\mu$ g  
111 mL<sup>-1</sup> chloramphenicol (pJB1806 and pSN85) or 50  $\mu$ g mL<sup>-1</sup> kanamycin (pSR47s). *Legionella*  
112 strains were cultured on supplemented charcoal N-(2-Acetamido)-2-aminoethanesulfonic acid  
113 (ACES)-buffered yeast extract (CYE) and grown at 37°C as described (33). *L. pneumophila*

114 Lp02 strains were maintained on CYE supplemented with 100  $\mu\text{g mL}^{-1}$  thymidine. Liquid  
115 cultures were grown at 37°C with aeration in supplemented ACES-buffered yeast extract (AYE)  
116 as described (33, 34). When necessary, media were supplemented with 10  $\mu\text{g mL}^{-1}$   
117 chloramphenicol (plasmid maintenance), 10  $\mu\text{g mL}^{-1}$  kanamycin (allelic exchange) or 1 mM  
118 isopropyl- $\beta$ -D-1-thiogalactopyranoside (IPTG).

119 Where indicated, recombinant mouse interferon- $\gamma$  (rIFN- $\gamma$ ; Thermo Fisher Scientific),  
120 recombinant mouse tumor necrosis factor (rTNF; Gibco), rat  $\alpha$ - mouse TNF antibody ( $\alpha$ -TNF;  
121 R&D Systems), or normal rat IgG control (Rat IgG; R&D Systems) were used at a concentration  
122 of 50ng  $\text{mL}^{-1}$ .

123 A complete list of oligonucleotide primers used in this study is shown in Table 1.

124

#### 125 Molecular cloning, plasmid construction and generation of *Legionella* strains

126 In-frame deletions of *legC4* were generated by allelic exchange. Plasmids pSR47s:: $\Delta$ *legC4* and  
127 pSR47s:: $\Delta$ *flaA*, gifts from Dr. Craig Roy, were conjugated into SRS43 or Lp02 followed by  
128 selection for *L. pneumophila* deletions as described (35, 36). Sucrose-resistant, kanamycin-  
129 sensitive colonies were screened by PCR using *legC4*KO-up/*legC4*KO-down and *flaA*KO-  
130 up/*flaA*KO-down primer pairs for  $\Delta$ *legC4* and  $\Delta$ *flaA* deletions, respectively.

131 To express *legC4* on a plasmid under its endogenous promoter, *legC4* plus the 300 base-pair  
132 upstream region was amplified with the LegC4BglII-F/LegC4XbaI-R primer pair and cloned as a  
133 BglII/XbaI fragment into BamHI/XbaI-digested pJB1806 (pJB) (37).

134 For IPTG-mediated expression of *legC4*, the *legC4* gene was amplified using the primer pair  
135 LegC4BamHI-F/LegC4XbaI-R and cloned as a BamHI/XbaI fragment into BamHI/XbaI-  
136 digested pSN85, a gift from Dr. Craig Roy (pEV; N-terminal 3XFLAG epitope tag fusion (38)).

137 Sequence-confirmed pJB1806::*plegC4* (pJB::*plegC4*) and pSN85::*legC4* (*plegC4*) plasmids and  
138 empty vectors were transformed into *Legionella* strains as previous described (39). IPTG-  
139 induced expression of *3xFLAG-legC4* was confirmed by Western blot analysis (data not shown).

140

#### 141 Mice and BMDMs

142 C57BL/6 wild-type, *Myd88*<sup>-/-</sup>, and *Tnfr1*<sup>-/-</sup> breeding pairs were purchased from the Jackson  
143 Laboratories (Bar Harbor, Maine) and in-house colonies were maintained in specific pathogen-  
144 free conditions at Kansas State University. All experiments involving animals were approved by  
145 the Kansas State University Institutional Animal Care and Use Committee (protocol #4022) and  
146 performed in compliance with the Animal Welfare Act and NIH guidelines.

147 Bone marrow was harvested from mice as previous described (40). Bone marrow-derived  
148 macrophages were generated by differentiation in RPMI supplemented with 20% heat-  
149 inactivated fetal bovine serum (HI-FBS) (Gibco) and 15% L929 cell supernatant for 6 days prior  
150 to seeding for infection.

151

#### 152 Competitive index (CI) experiments in mice

153 Six- to ten-week old age and sex-matched C57BL/6 mice were infected for competitive index  
154 experiments as previously described (30). Mixed bacterial inoculums (1:1) were diluted and  
155 plated on selective medium (5  $\mu\text{g mL}^{-1}$  for *flaA*::Tn and 10  $\mu\text{g mL}^{-1}$  for plasmid selection). At 48  
156 h p.i., mice were euthanized and whole lung tissue was harvested. Lung tissue was homogenized  
157 in 300  $\mu\text{L}$  of sterile water using a Bullet Blender (Next Advance) as described (41) and dilutions  
158 were plated on selective medium as above. Colony forming units (CFUs) were enumerated and  
159 used to calculate CI values  $[(\text{CFUcm}^{\text{R}}_{48\text{h}}/\text{CFUwt}_{48\text{h}})/(\text{CFUcm}^{\text{R}}_{\text{IN}}/\text{CFUwt}_{\text{IN}})]$ .



160

161 Quantification of *Legionella* replication within macrophages

162 Differentiated BMDMs were maintained in RPMI supplemented with 10% HI-FBS  
163 (Gibco) and 7.5% L929 cell supernatant. BMDMs were seeded  $2.5 \times 10^5$ /well in 24-well plates  
164 one day prior to infection. BMDMs were infected with the indicated strains of *L. pneumophila* or  
165 *L. longbeachae* at a multiplicity of infection (MOI) of 1 in the presence or absence of 1 mM  
166 IPTG and/or recombinant cytokine as indicated. At 1 h p.i., cell monolayers were washed three  
167 times with PBS<sup>-/-</sup> and fresh supplemented medium was added. Infections were allowed to  
168 proceed for up to 72 h or for 48 h, as indicated. To enumerate CFUs, BMDMs were lysed in  
169 sterile water for 8 mins followed by repeat pipetting. Lysates were diluted as appropriate and  
170 plated on CYE agar plates, which were then incubated at 37° for 4 days. For growth curve  
171 experiments, bacteria were enumerated after 1 h of infection and every 24 h thereafter for up to  
172 72 h. To quantify fold replication, BMDMs were infected for 1 h and 48 h and fold replication  
173 was enumerated by normalization of the 48 h CFU counts to the 1 h CFU counts.

174

175 Enzyme-linked immunosorbent assay (ELISA)

176 BMDMs were seeded in a 24-well plate at  $2.5 \times 10^5$ /well 1 day prior to infection. The indicated  
177 Lp02 or SRS43 strains were used to infect the BMDMs ( $n = 3$ ) at an MOI of 30 or 10,  
178 respectively. Infections with Lp02 strains were performed in the absence of exogenous  
179 thymidine to prevent bacterial replication. One-hour p.i., media were aspirated and cells were  
180 washed 3 times with PBS. Media were replaced and supernatants were collected after 5 h.  
181 Supernatants were either used fresh or stored at -20°C for up to 1 week followed by

182 quantification of TNF using a Mouse TNF ELISA Kit (BioLegend) following manufacturer's  
183 instructions.

184

#### 185 Western blot

186 To confirm production of 3XFLAG-LegC4 protein from *Legionella*, suspensions of strains  
187 harboring either pSN85 alone (pEV) or pSN85::*legC4* (*plegC4*) induced with IPTG were lysed  
188 by boiling in 3X Laemmli buffer. Proteins were separated by SDS-PAGE followed by transfer to  
189 polyvinylidene difluoride (PVDF) membrane (ThermoFisher) using a wet transfer cell (BioRad).  
190 Membranes were incubated in blocking buffer [5% non-fat milk dissolved in Tris-buffered  
191 saline/0.1% Tween 20 (TBST)]. Anti-FLAG (clone M2, Sigma) was diluted at 1:1000 in  
192 blocking buffer and incubated with membranes either overnight at 4°C or at ambient temperature  
193 for 3 h with rocking. Wash steps were performed 3 times for 10 min each in TBST. HRP-  
194 conjugated goat- $\alpha$ -mouse HRP (Sigma) was diluted in blocking buffer at 1:5000 and incubated  
195 with membranes for 1-2 hours at room temperature with rocking. Membranes were washed,  
196 incubated with ECL substrate (Amersham) and imaged by chemiluminescence using a c300  
197 Azure Biosystems Darkroom Replacer.

198

#### 199 Statistical analysis

200 Statistics were performed with GraphPad Prism software using either Mann-Whitney U test or  
201 Students' *t*-test, as indicated, with a 95% confidence interval. In all experiments, error bars  
202 denote standard deviation ( $\pm$  S.D.) of samples in triplicates.

203

#### 204 **Results**

205

206 LegC4 confers a fitness disadvantage on non-flagellated *L. pneumophila* in wild-type mice

207 We found previously that the *L. pneumophila* effector LegC4 is detrimental to bacterial  
208 replication in the mouse lung (30). These experiments were performed using mice and  
209 macrophages deficient for production of the NLRC4 inflammasome (*Nlrc4*<sup>-/-</sup>) to prevent  
210 flagellin-mediated restriction of *L. pneumophila* replication (10, 30, 42). To confirm that LegC4-  
211 mediated phenotypes were not due to loss of NLRC4, we examined fitness of a *legC4*-deficient  
212 *L. pneumophila* ( $\Delta$ *legC4*) strain in the lungs of wild-type C57BL/6 mice using competitive index  
213 (CI) experiments. To prevent NLRC4-mediated restriction of bacterial replication we generated  
214 flagellin (*flaA*) loss-of-function mutations in our wild-type ( $\Delta$ *flaA*) and *legC4* mutant  
215 ( $\Delta$ *flaA* $\Delta$ *legC4*) strains (see *Materials and Methods*). We also used a previously generated  
216 *flaA*::Tn mutant to facilitate selective plating since the transposon confers resistance to  
217 chloramphenicol (30). Mice were infected intranasally with a 1:1 mixture of *L. pneumophila*  
218  $\Delta$ *flaA* $\Delta$ *legC4* and *flaA*::Tn for 48 h. Lung tissue was subsequently homogenized and plated on  
219 selective media for CFU enumeration and calculation of CI values (see *Materials and Methods*).  
220 The  $\Delta$ *flaA* $\Delta$ *legC4* strain significantly outcompeted the *flaA*::Tn mutant in the lungs of wild-type  
221 mice, as evidenced by average CI values significantly greater than 1.0 ( $P < 0.01$ ; **Fig 1A**). Our  
222 previous study also revealed that expression of *legC4* from a multi-copy plasmid conferred a  
223 fitness disadvantage on *L. pneumophila* compared to the wild-type strain (30). To confirm these  
224 results in wild-type mice, we generated a strain of *L. pneumophila*  $\Delta$ *flaA* $\Delta$ *legC4* harboring a  
225 plasmid encoding *legC4* under control of its endogenous promoter (pJB::p*legC4*). Plasmid  
226 expression of *legC4* (pJB::p*legC4*) resulted in significantly impaired fitness defect compared  
227 with the  $\Delta$ *flaA* parental strain, which was not observed for a  $\Delta$ *flaA* $\Delta$ *legC4* strain harboring vector

228 alone (pJB) ( $P < 0.05$ , **Fig 1B**). These data demonstrate that NLRC4 does not affect LegC4-  
229 mediated attenuation of *L. pneumophila* replication in the mouse lung. To fully evaluate LegC4-  
230 mediated phenotypes, the remainder of our study was performed using *flaA*-deficient strains and  
231 bone marrow-derived macrophages (BMDMs) derived from wild-type mice.

232

### 233 Plasmid expression of *legC4* attenuates *L. pneumophila* replication in BMDMs

234 Since plasmid expression of *legC4* attenuated *L. pneumophila* fitness in the mouse lung,  
235 we examined whether this also occurred in macrophages *ex vivo* using BMDMs derived from  
236 wild-type mice. We quantified bacterial replication within BMDMs over 72 h. Consistent with  
237 our previous study, loss of endogenous *legC4* ( $\Delta flaA \Delta legC4$ ) does not affect replication of *L.*  
238 *pneumophila* within primary mouse BMDMs compared to the parental strain ( $\Delta flaA$ ) (30) (**Fig**  
239 **2A**). However, *L. pneumophila*  $\Delta flaA \Delta legC4$  (pJB::*plegC4*) was significantly attenuated for  
240 replication in BMDMs at 48 and 72 h p.i. compared to the empty vector control strain (**Fig 2B**).  
241 Furthermore, IPTG-induced expression of *legC4* from a plasmid (*plegC4*) also resulted in  
242 impaired *L. pneumophila* replication compared to the control strain (pEV) ( $P < 0.01$ , **Fig 2C**).  
243 Fitness defects associated with plasmid expression of *legC4* were specific to intracellular  
244 replication since replication in rich media *in vitro* was unaffected (data not shown). These data  
245 demonstrate that increased levels of LegC4 are detrimental to *L. pneumophila* intracellular  
246 replication in macrophages.

247

### 248 LegC4-mediated restriction of *L. pneumophila* replication is dependent on cytokine production.

249 We subsequently investigated the mechanism by which plasmid expression of *legC4*  
250 attenuates *L. pneumophila* replication. In BMDMs, *L. pneumophila* infection results in

251 production of pro-inflammatory cytokines through engagement of TLRs by bacterial ligands.  
252 Indeed, we previously reported that BMDMs infected with *L. pneumophila* expressing *legC4*  
253 secreted increased levels of interleukin (IL)-12 (30). However, increased levels of IL-12 would  
254 likely not be sufficient to attenuate *L. pneumophila* intracellular replication within BMDMs. Like  
255 IL-12, tumor necrosis factor (TNF) is a pro-inflammatory cytokine expressed downstream of  
256 toll-like receptors (TLRs) in macrophages. TNF is important for host defense against *L.*  
257 *pneumophila* in mice and humans and TNF-mediated signaling is sufficient to restrict *L.*  
258 *pneumophila* intracellular replication within macrophages (17-19, 23, 43). Thus, increased TNF  
259 signaling could account for LegC4-mediated attenuation of *L. pneumophila* intracellular  
260 replication.

261 We hypothesized that plasmid expression of *legC4* would be sufficient to increase TNF  
262 secretion from *L. pneumophila*-infected BMDMs. Wild-type BMDMs were infected with  $\Delta$ *flaA*,  
263  $\Delta$ *flaA $\Delta$ *legC4*,  $\Delta$ *flaA $\Delta$ *legC4* (*p**legC4*), or  $\Delta$ *flaA $\Delta$ *legC4* (*p*EV) for 6 h and secreted TNF was  
264 quantified by ELISA (see *Materials and Methods*). Significantly greater concentrations of TNF  
265 were present in the supernatants of cells infected with *L. pneumophila* expressing *legC4* from a  
266 plasmid ( $P < 0.01$ , **Fig 3A**). Increased TNF secretion was not due to differences in bacterial  
267 replication, since all strains replicated to similar levels at 6 h p.i. (**Fig 3B**). We observed the  
268 same phenotype following infection with strains constructed in the Lp02 background, which is a  
269 thymidine auxotroph (*thyA*<sup>-</sup>) that is metabolically active but does not replicate in the absence of  
270 exogenous thymidine (**Fig S1**). Thus, overexpression of *legC4* results in enhanced TNF secretion  
271 from *L. pneumophila*-infected BMDMs.***

272 To determine if TNF secretion contributed LegC4-mediated attenuation of intracellular  
273 replication, we evaluated *L. pneumophila* replication within *Myd88*<sup>-/-</sup> BMDMs. Since attenuated

274 *L. pneumophila* replication within BMDMs associated with LegC4 were observed at 48 h p.i., we  
275 quantified fold replication of the indicated strains at this time point (see *Materials and Methods*).  
276 Plasmid expression of *legC4* impaired *L. pneumophila* intracellular replication within wild-type,  
277 but not *Myd88*<sup>-/-</sup>, BMDMs (**Fig 3B**). As expected, TNF was not secreted from *Myd88*<sup>-/-</sup> BMDMs  
278 under any of our experimental conditions [(44) & data not shown]. Together, these data suggest  
279 that pro-inflammatory cytokine production contributes to LegC4-mediated attenuation of *L.*  
280 *pneumophila* intracellular replication in BMDMs.

281 To further characterize LegC4-mediated restriction of *L. pneumophila* replication within  
282 BMDMs, *L. pneumophila* replication was evaluated in the absence of TNF signaling. To  
283 determine if TNF signaling contributed to *legC4*-mediated attenuation of *L. pneumophila*  
284 replication, we neutralized TNF in the supernatants of infected wild-type BMDMs using an  $\alpha$ -  
285 TNF antibody. Wild-type BMDMs were infected with *L. pneumophila*  $\Delta$ *flaA* $\Delta$ *legC4* harboring  
286 *plegC4* or pEV in the presence of either  $\alpha$ -TNF, Rat IgG isotype control antibody, or neither and  
287 fold replication at 48 h p.i. was quantified. Plasmid expression of *legC4* resulted in significantly  
288 attenuated *L. pneumophila* replication within untreated and Rat IgG-treated BMDMs ( $P < 0.05$ );  
289 however,  $\alpha$ -TNF antibody neutralization of TNF restored replication of the *legC4*  
290 overexpressing strain to wild-type levels (**Fig 4A**). We subsequently examined replication of  
291 these strains in BMDMs deficient for signaling from TNF receptor-1 (TNFR1). *Tnfr1*<sup>-/-</sup> BMDMs  
292 were infected with *L. pneumophila*  $\Delta$ *flaA* $\Delta$ *legC4* harboring either *plegC4* or pEV and fold  
293 replication was quantified at 48 h p.i. As previously observed, over-expression of *legC4* impaired  
294 intracellular replication within wild-type BMDMs ( $P < 0.05$ , **Fig 4B**). However, there was no  
295 difference in fold replication of the *legC4*-overexpressing strain compared to the empty vector  
296 control within *Tnfr1*<sup>-/-</sup> BMDMs (**Fig 4B**). Interestingly, overexpression of *legC4* resulted in

297 significantly increased *L. pneumophila* replication within *Tnfr1*<sup>-/-</sup> BMDMs ( $P < 0.01$ , **Fig 4B**).

298 These data demonstrate that TNF signaling contributes to LegC4-mediated attenuation of *L.*

299 *pneumophila* replication within BMDMs.

300

301 Endogenous LegC4 exacerbates TNF-mediated restriction of *L. pneumophila* from BMDMs.

302 To further characterize LegC4-mediated restriction of *L. pneumophila* from BMDMs, we

303 examined replication of *L. pneumophila* in BMDMs activated with recombinant mouse TNF

304 (rTNF). Wild-type BMDMs were infected with *L. pneumophila*  $\Delta$ *flaA* or  $\Delta$ *flaA* $\Delta$ *legC4* in the

305 presence or absence of 50 ng/mL rTNF and fold replication was quantified at 48 h p.i. *L.*

306 *pneumophila*  $\Delta$ *flaA* $\Delta$ *legC4* replicated to significantly greater levels than the parental  $\Delta$ *flaA* strain

307 in rTNF treated BMDMs ( $P < 0.01$ , **Fig 4C**). As reported above, loss of endogenous *legC4* does

308 not affect *L. pneumophila* replication within untreated BMDMs (**Fig 4C**). These data show that

309 endogenous levels of LegC4 can augment TNF-mediated restriction of *L. pneumophila*

310 replication.

311

312 LegC4 impairs *L. pneumophila* replication in interferon (IFN)- $\gamma$  activated BMDMs.

313 Interferon (IFN)- $\gamma$  plays a major role in host defense against *L. pneumophila* in the lung

314 (20, 45). To determine if LegC4-mediated impairment of *L. pneumophila* intracellular replication

315 was specific to TNF, we examined bacterial replication within IFN- $\gamma$ -activated BMDMs. Fold

316 replication of *L. pneumophila*  $\Delta$ *flaA*,  $\Delta$ *flaA* $\Delta$ *legC4*,  $\Delta$ *flaA* $\Delta$ *legC4* (pEV) or  $\Delta$ *flaA* $\Delta$ *legC4*

317 (*p**legC4*) within wild-type BMDMs activated with recombinant mouse IFN- $\gamma$  (rIFN- $\gamma$ ) was

318 quantified. We found that overexpression of *legC4* significantly attenuated *L. pneumophila*

319 replication within IFN- $\gamma$ -activated BMDMs ( $P < 0.05$ ; **Fig 5A**). The *L. pneumophila*

320  $\Delta flaA \Delta legC4$  mutant replicated to higher levels in IFN- $\gamma$ -activated BMDMs compared to the  
321 parental  $\Delta flaA$  strain (**Fig 5B**). Although the replication difference was not statistically  
322 significant ( $P=0.0918$ ), the trend was consistently observed. Together, these data suggest that  
323 IFN- $\gamma$ -mediated restriction of *L. pneumophila* replication is also augmented by LegC4.

324 Macrophage activation by IFN- $\gamma$  results in increased TNF production from macrophages  
325 (46, 47). To determine if LegC4-mediated restriction of *L. pneumophila* within IFN- $\gamma$  activated  
326 macrophages was due to TNF signaling, we quantified *L. pneumophila* replication within *Tnfr1*<sup>-/-</sup>  
327 BMDMs treated with IFN- $\gamma$ . Overproduction of LegC4 resulted in significantly decreased *L.*  
328 *pneumophila* replication in IFN- $\gamma$ -activated TNFR1<sup>-/-</sup> BMDMs compared to the control strain  
329 ( $P<0.001$ ; **Fig 5C**). Thus, LegC4 can also augment non-TNF-mediated restriction of *L.*  
330 *pneumophila* replication through IFN- $\gamma$ .

331

### 332 LegC4 impairs *L. longbeachae* replication within cytokine-activated BMDMs

333 *Legionella longbeachae* replicates within eukaryotic phagocytes using a Dot/Icm  
334 secretion system (39). Although the *L. longbeachae* Dot/Icm secretion system is highly similar to  
335 that of *L. pneumophila*, their effector repertoires are quite distinct and *L. longbeachae* does not  
336 encode a homolog of *legC4* (48, 49). Importantly, *L. longbeachae* is more virulent than *L.*  
337 *pneumophila* and lethal in a mouse model of infection. To determine if LegC4 could attenuate  
338 bacterial replication in non-*pneumophila* *Legionella*, we generated *L. longbeachae* strains either  
339 expressing *legC4* (*plegC4*) or harboring the empty vector (pEV). Wild-type BMDMs were  
340 infected with these *L. longbeachae* strains and fold-replication at 48 h was quantified.  
341 Expression of *legC4* did not impair *L. longbeachae* replication within BMDMs; however, *legC4*  
342 expression did result in significantly attenuated *L. longbeachae* replication within rTNF- and



343 rIFN- $\gamma$ -treated BMDMs compared to the control strain ( $P < 0.001$ ; **Fig 6**). These data suggest that  
344 LegC4 can augment cytokine-mediated restriction of non-*pneumophila* *Legionella* within  
345 BMDMs.

346

## 347 **Discussion**

348 The data presented in this study support the hypothesis that LegC4 potentiates cytokine-  
349 mediated host defense against *Legionella*. Our previous work (30) identifying LegC4 as  
350 contributing to *L. pneumophila* clearance from the lung was performed using flagellated *L.*  
351 *pneumophila* in a NLRC4-deficient (*Nlrc4*<sup>-/-</sup>) mouse model. To fully evaluate the mechanisms of  
352 LegC4-mediated clearance, utilized wild-type mice and BMDMs. Consistent with our previous  
353 study (30), we found that loss-of-function mutation in the *legC4* gene ( $\Delta$ *legC4*) conferred a  
354 fitness advantage on *L. pneumophila*  $\Delta$ *flaA* within the wild-type mouse lung. Moreover,  
355 complementation of the  $\Delta$ *legC4* mutation by a plasmid encoding *legC4* *in trans* conferred a  
356 fitness disadvantage on *L. pneumophila* compared to the parental strain. Also consistent with our  
357 previous report, *L. pneumophila*  $\Delta$ *flaA* $\Delta$ *legC4* replication within BMDMs did not differ from  
358 replication of the  $\Delta$ *flaA* strain. However, in the present study, we found that plasmid expression  
359 of *legC4* was sufficient to attenuate *L. pneumophila* replication within BMDMs. Although *legC4*  
360 was expressed downstream of its endogenous promoter, an exaggerated phenotype likely  
361 occurred due to expression from a multi-copy plasmid, suggesting a potential dose-response.  
362 Importantly, this strain provided us with a tool to increase the magnitude of LegC4-mediated  
363 fitness attenuation within cultured cells. These phenotypes were corroborated by the observation  
364 that endogenous LegC4 was deleterious in cytokine-activated BMDMs. We further found that  
365 the fitness disadvantage associated with plasmid expression of *legC4* was abolished in

366 macrophages deficient for TNF-mediated signaling, suggesting that LegC4 is able to exacerbate  
367 cytokine-mediated antimicrobial responses. Finally, we determined that LegC4 could impair  
368 replication of *L. longbeachae* in cytokine-activated macrophages. Together, these data suggest  
369 that LegC4 potentiates cytokine-mediated restriction of *L. pneumophila* within macrophages.

370 Inflammation is mediated primarily through cytokine secretion, which is critical for  
371 restriction of *L. pneumophila* replication *in vivo*. This has been evidenced by the inability of  
372 *Myd88*<sup>-/-</sup> mice to control *L. pneumophila* replication. Specifically, *Myd88*<sup>-/-</sup> BMDMs will not  
373 secrete TNF during infection. The inability of plasmid-expressed *legC4* to attenuate *L.*  
374 *pneumophila* replication in *Myd88*<sup>-/-</sup> BMDMs is likely due to lack of TNF signaling. In addition,  
375 LegC4-mediated increases in TNF secretion may amplify *Tnf* expression, which would further  
376 restrict *L. pneumophila* replication.

377 Pro-inflammatory cytokines contribute to host defense against *L. pneumophila in vivo*  
378 and in cultured macrophages (8, 50). Mice deficient for TNF-mediated signaling have increased  
379 pulmonary bacterial burdens and can succumb to infection (23, 43). TNF can signal through both  
380 TNFR1 and TNFR2; however, TNFR1-mediated signaling is primarily responsible for *L.*  
381 *pneumophila* restriction within alveolar macrophages *in vivo* (23) and is potentiated by LegC4.  
382 In the lung, multiple cell types contribute to TNF production, a consequence of which would be  
383 higher local TNF concentrations (16, 18, 23, 51). In addition, production of IFN- $\gamma$  during *L.*  
384 *pneumophila* infection *in vivo* is mediated primarily by circulating natural killer (NK) cells (45,  
385 52).

386 Our observation that the *L. pneumophila*  $\Delta$ *legC4* mutant had fitness advantage compared  
387 to wild-type in the mouse lung but not in cultured macrophages suggested that LegC4 was  
388 detrimental to replication under specific environmental conditions. This was supported by the

389 observation that attenuated *L. pneumophila* replication was correlated with increased TNF  
390 secretion from BMDMs. Since the *L. pneumophila*-infected lung is an inflammatory  
391 environment, we examined whether cytokine-mediated restriction was exacerbated by LegC4.  
392 Abrogation of signaling from TNFR1 was sufficient to alleviate LegC4-mediated restriction of  
393 intracellular replication. Increased replication of the  $\Delta legC4$  mutant within rTNF-treated  
394 BMDMs strongly suggests that pro-inflammatory responses are exacerbated by LegC4. This  
395 conclusion was corroborated by the observation that the  $\Delta legC4$  mutant consistently replicated to  
396 higher levels within IFN- $\gamma$ -activated macrophages compared to untreated macrophages.

397         Similar to *L. pneumophila*, *L. longbeachae* replicates within an LCV by employing a  
398 Dot/Icm secretion system and a repertoire of translocated effector proteins (39). Despite high  
399 levels of homology between the Dot/Icm secretion systems of these two organisms, the effector  
400 repertoires are quite diverse and *L. longbeachae* does not encode a homolog of *legC4* (48, 49). In  
401 contrast to *L. pneumophila*, *L. longbeachae* is highly virulent in a mouse model of Legionnaires'  
402 disease (53, 54). Lethality in mice is likely due to *L. longbeachae* being poorly  
403 immunostimulatory and failing to induce substantial levels pro-inflammatory cytokines during  
404 infection. However, pro-inflammatory cytokines contribute to host defense against *L.*  
405 *longbeachae* in BMDMs and in vivo (53). Since inter-species translocation of Dot/Icm effectors  
406 by *Legionella* has been previously observed (39, 55), we introduced *legC4* into *L. longbeachae*.  
407 Production of LegC4 by *L. longbeachae* resulted in significantly attenuated replication within  
408 cytokine treated, but not untreated, BMDMs. These data reinforce our previous observations and  
409 demonstrate that LegC4-mediated restriction is not specific to *L. pneumophila*. Since *L.*  
410 *longbeachae* infection does not induce appreciable TNF secretion from BMDMs (53), it is likely  
411 that the concentration of TNF secreted by these cells is too low to permit LegC4-mediated

412 restriction. Together with relatively low levels of effector translocation by *L. longbeachae*  
413 compared to *L. pneumophila* (39), the amount of translocated LegC4 may be insufficient to  
414 restrict bacterial replication within untreated BMDMs. However, LegC4 is sufficient to attenuate  
415 *L. longbeachae* replication within BMDMs activated with either rTNF or rIFN- $\gamma$ . Whether  
416 LegC4 can protect mice from *L. longbeachae*-mediated lethality will be the subject of a future  
417 study.

418 Multiple effectors contribute to the innate immune response to *L. pneumophila* infection  
419 [reviewed in (56)]. Together with our data, these studies point to a complex interplay between  
420 effectors during *Legionella* infection of mammalian host. The effectors LnaB and LegK1  
421 enhance NF- $\kappa$ B activation, which augments immune signaling (57, 58). Since mammals are a  
422 dead-end host for *Legionella*, the evolutionary basis for effector modulation of NF- $\kappa$ B is  
423 intriguing. Interestingly, the effector EnhC enhances *L. pneumophila* replication in TNF-  
424 activated macrophages (59), the opposite of what we have observed for LegC4. Thus, it is  
425 tempting to speculate that there may be interplay between EnhC and LegC4 within *L.*  
426 *pneumophila* infected cells. Future investigations will reveal whether LegC4-mediated  
427 phenotypes are dependent on other Dot/Icm-translocated effectors.

428 In summary, we found that the Dot/Icm effector LegC4 can augment cytokine-mediated  
429 restriction of *Legionella* replication within macrophages. These data add to the growing body of  
430 literature on effector triggered immunity in animal cells. As an accidental pathogen that did not  
431 co-evolve under the selective pressure of an innate immune system, *L. pneumophila* continues to  
432 provide insight into novel mechanisms of innate immunity towards intracellular bacterial  
433 pathogens. Consequently, further understanding of LegC4 function will reveal strategies to  
434 augment pro-inflammatory signaling. Thus, this study has provided the foundation for future

435 investigations into the molecular mechanism by which LegC4 enhances host defense against  
436 intracellular bacterial pathogens.

437

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446

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625

626

## 627 **Tables**

628 **Table 1.** Oligonucleotide primers used in this study

<b>Name</b>	<b>Sequence<sup>a</sup></b>
legC4KO-up	ttgtggacaatagctcttgg
legC4KO-down	atacgtggctatagcacc
flaAKO-up	ccagttcagtactgtaaagc
flaAKO-down	tattctgccgtgactatcg
LegC4BglII-F	tgg <b>agat</b> ctaataagagtaagaaggatccg
LegC4BamHI-F	tgg <b>ggatc</b> cttttgattcattatgtatccttg
LegC4XbaI-R	att <b>tctag</b> attatagcttaatatcaaaag

629 <sup>a</sup>Restriction endonuclease cleavage sites are bolded

630

## 631 **Figure Legends**

632 **Figure 1. LegC4 attenuates *L. pneumophila* fitness in wild-type mice.** (A) Competitive index  
633 (CI) of  $\Delta$ *flaA* $\Delta$ *legC4* vs. *flaA*::Tn [chloramphenicol(Cm)<sup>R</sup>] from the lungs of wild-type mice.

634 (B) CI of  $\Delta$ *flaA* $\Delta$ *legC4* (pJB::p*legC4*) or  $\Delta$ *flaA* $\Delta$ *legC4* (pJB) vs. *flaA* from the lungs of wild-  
635 type mice. Each symbol represents an individual animal and the line represents the mean CI

636 values. Asterisks denote statistical significance by Mann-Whitney U test (\*\**P*<0.01). Data are

637 representative of at least two independent experiments.

638

639 **Figure 2. Plasmid expression of *legC4* impairs *L. pneumophila* replication within BMDMs.**

640 (A) Growth of *L. pneumophila* (A)  $\Delta flaA$  and  $\Delta flaA\Delta legC4$ , (B)  $\Delta flaA\Delta legC4$  (pJB) and  
641  $\Delta flaA\Delta legC4$  (pJB::*plegC4*) or (C)  $\Delta flaA\Delta legC4$  (pEV) and  $\Delta flaA\Delta legC4$  (*plegC4*) in BMDMs  
642 over 72 h. Expression of *legC4* from *plegC4* was induced with 1 mM IPTG as described (see  
643 *Materials & Methods*). Data are shown as mean  $\pm$  S.D. of samples in triplicates. Asterisks denote  
644 statistical significance by Students' *t*-test (\*\* $P < 0.01$ ) and data are representative of three  
645 independent experiments.

646

647 **Figure 3. Role of TNF secretion in LegC4-mediated attenuation of *L. pneumophila***

648 **replication.** (A) ELISA for TNF secretion from wild-type BMDMs infected with the indicated  
649 strains. (B) Enumeration of *L. pneumophila* strains from BMDMs assayed in (A). (C) Fold  
650 replication (48 h) of the indicated *L. pneumophila* strains within wild-type or *Myd88*<sup>-/-</sup> BMDMs.  
651 Expression of *legC4* was induced with IPTG. Data shown are mean  $\pm$  S.D. of samples in  
652 triplicates. Asterisks denote statistical significance (\*\* $P < 0.01$ ) by Students' *t*-test. Data are  
653 representative of at least two independent experiments.

654

655 **Figure 4. LegC4 augments TNF-mediated restriction of *L. pneumophila* replication. (A)**

656 Fold replication (48 h) of the indicated *L. pneumophila* strains within wild-type BMDMs treated  
657 with 50 ng mL<sup>-1</sup>  $\alpha$ -TNF, isotype control (Rat IgG) or left untreated (see *Materials & Methods*).

658 (B) Fold replication (48 h) of the indicated *L. pneumophila* strains within wild-type or *Tnfr1*<sup>-/-</sup>

659 BMDMs. Expression of *legC4* was induced with IPTG. (C) Fold replication (48 h) of *L.*

660 *pneumophila*  $\Delta flaA$  and  $\Delta flaA\Delta legC4$  within wild-type BMDMs the presence or absence of 50 ng

661 mL<sup>-1</sup> recombinant mouse TNF (rTNF). Data shown are mean  $\pm$  S.D. of samples in triplicates.

662 Asterisks denote statistical significance (\* $P < 0.05$ , \*\* $P < 0.01$ , n.s., not significant) by Students' *t*-  
663 test. Data are representative of at least two independent experiments.

664

665 **Figure 5. LegC4 enhances IFN- $\gamma$ -mediated restriction of *L. pneumophila* replication.** Fold  
666 replication (48 h) of the indicated *L. pneumophila* strains within **(A, B)** wild-type BMDMs or  
667 **(C)** *Tnfr1*<sup>-/-</sup> BMDMs in the presence or absence of 50 ng mL<sup>-1</sup> recombinant mouse IFN- $\gamma$  (rIFN-  
668  $\gamma$ ) as indicated. Expression of *legC4* was induced with IPTG. Data shown are mean  $\pm$  S.D. of  
669 samples in triplicates. Asterisks denote statistical significance (\*\* $P < 0.01$ ) by Students' *t*-test.  
670 Data are representative of at least two independent experiments.

671

672 **Figure 6. Replication of *L. longbeachae* producing LegC4 in cytokine-treated BMDMs.**

673 Fold replication (48 h) of the *L. longbeachae* (*Llo*) harboring the indicated plasmids within wild-  
674 type BMDMs the presence or absence of 50 ng mL<sup>-1</sup> rTNF or rIFN- $\gamma$ , as indicated. Expression of  
675 *legC4* was induced with IPTG. Expression of *legC4* was induced with IPTG. Data shown are  
676 mean  $\pm$  S.D. of samples in triplicates. Asterisks denote statistical significance (\*\* $P < 0.01$ , n.s.,  
677 not significant) by Students' *t*-test. Data are representative of at least two independent  
678 experiments.

679

680 **Supplemental information**

681

682 **Figure S1. TNF secretion from BMDMs infected with Lp02 strains.** (A) ELISA for TNF  
683 secreted from wild-type BMDMs infected the indicated Lp02 strains for 2 h or 6 h in the absence

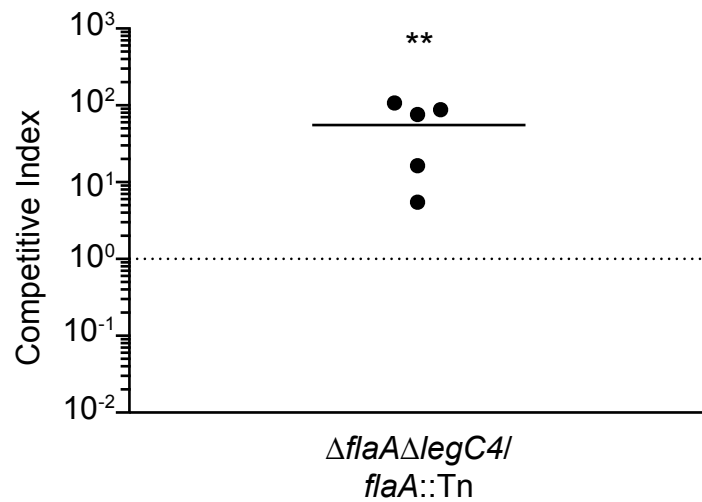


684 of exogenous thymidine. Asterisks denote statistical significance ( $*P < 0.05$ ) by Students' *t*-test.

685 Data are representative of two independent experiments.

Figure 1

A



B

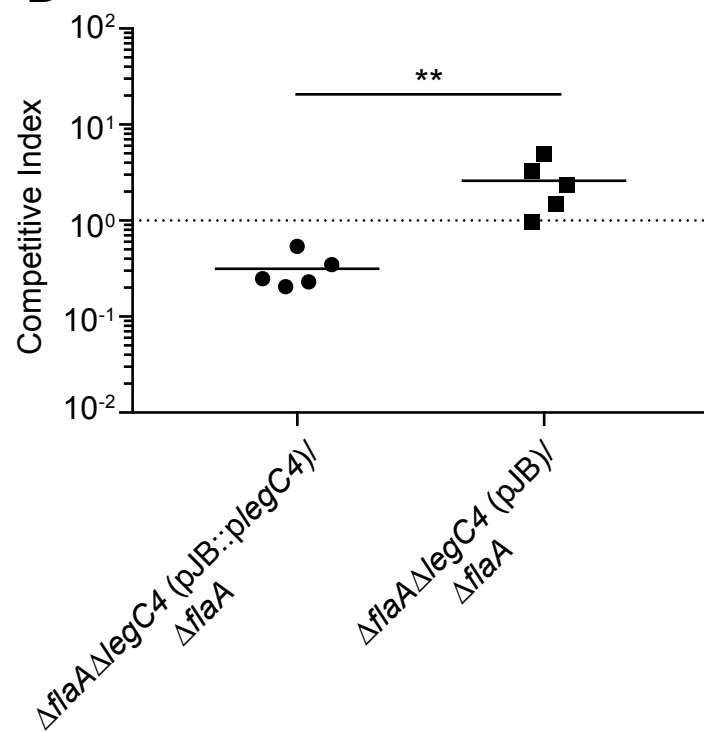
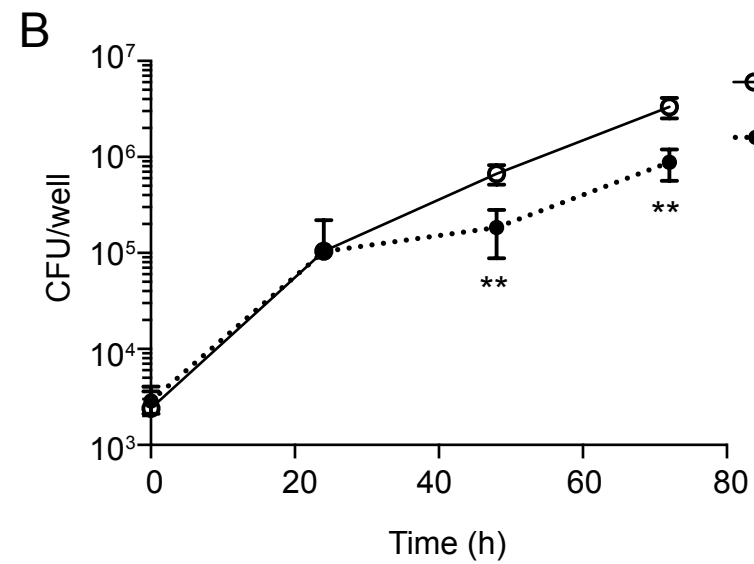
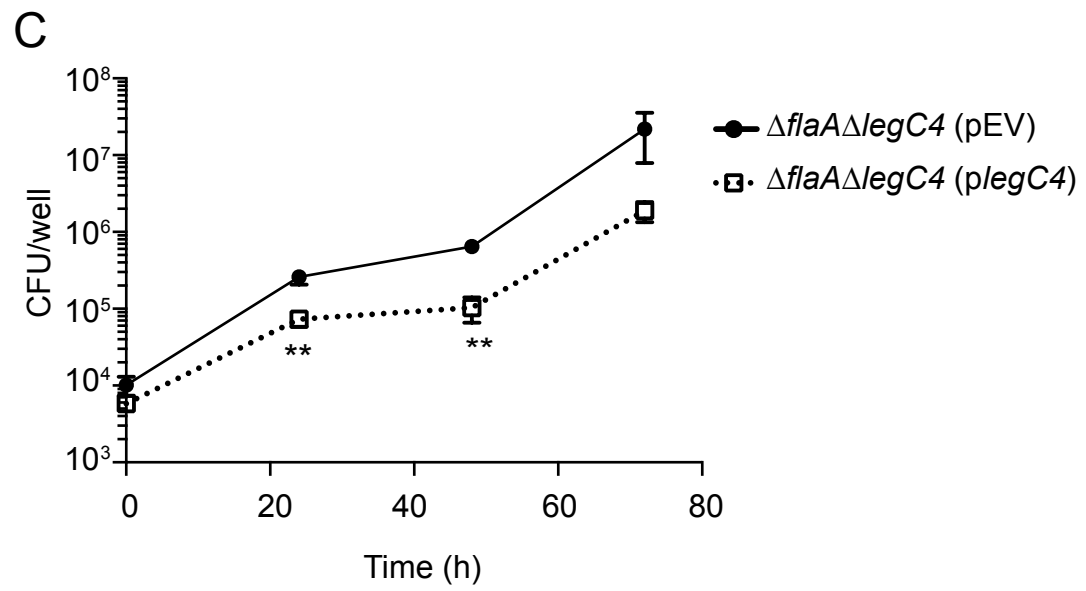
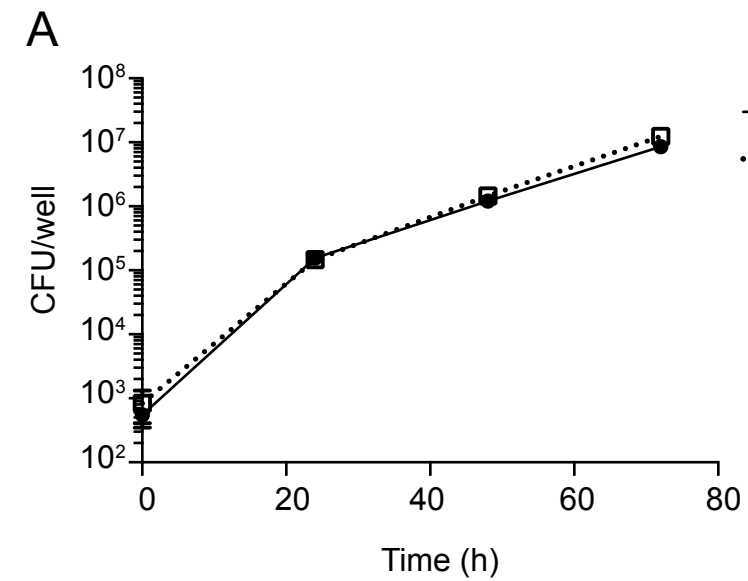
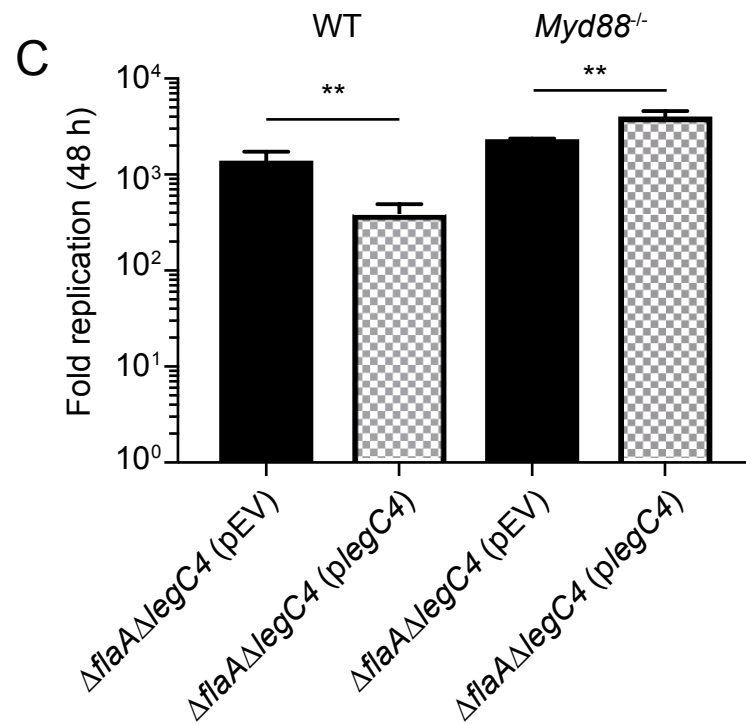
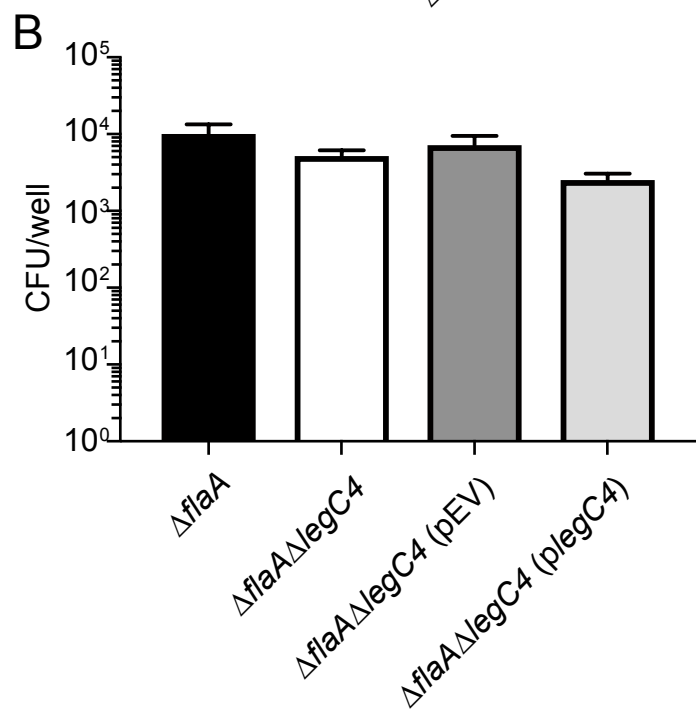
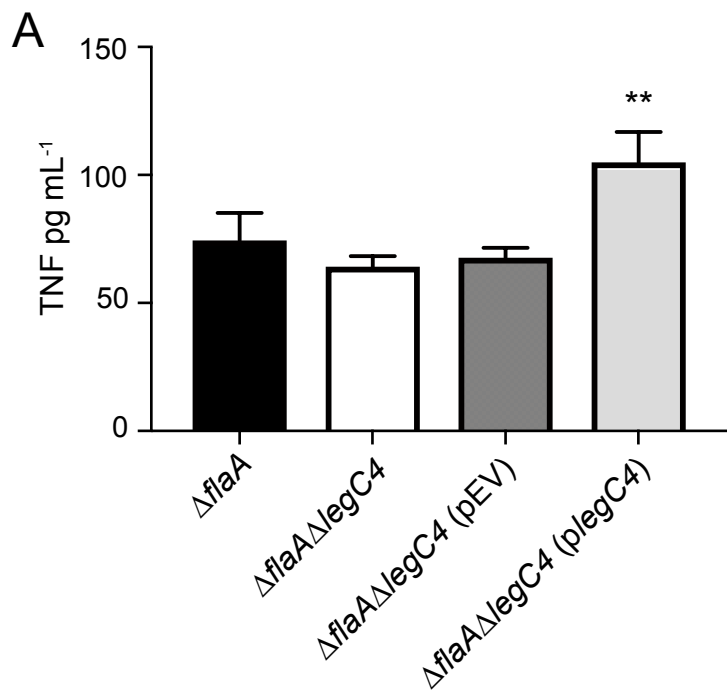


Figure 2





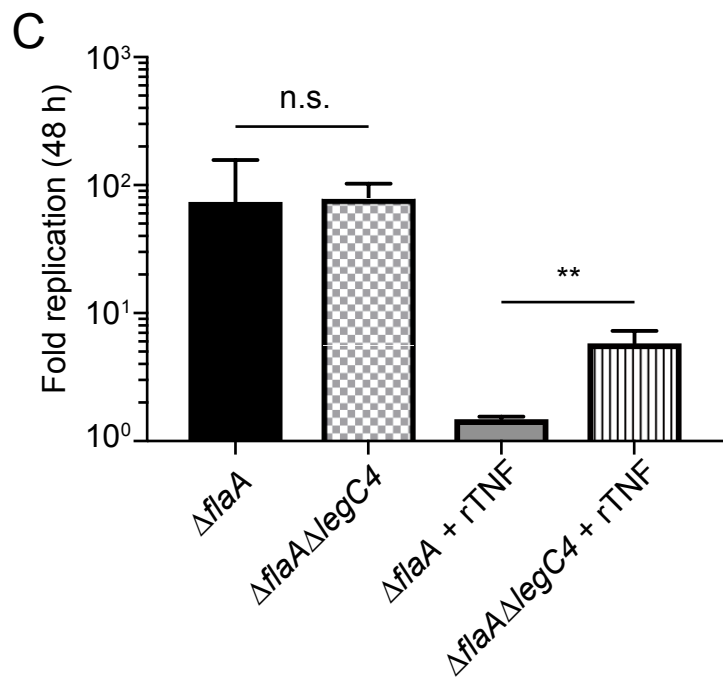
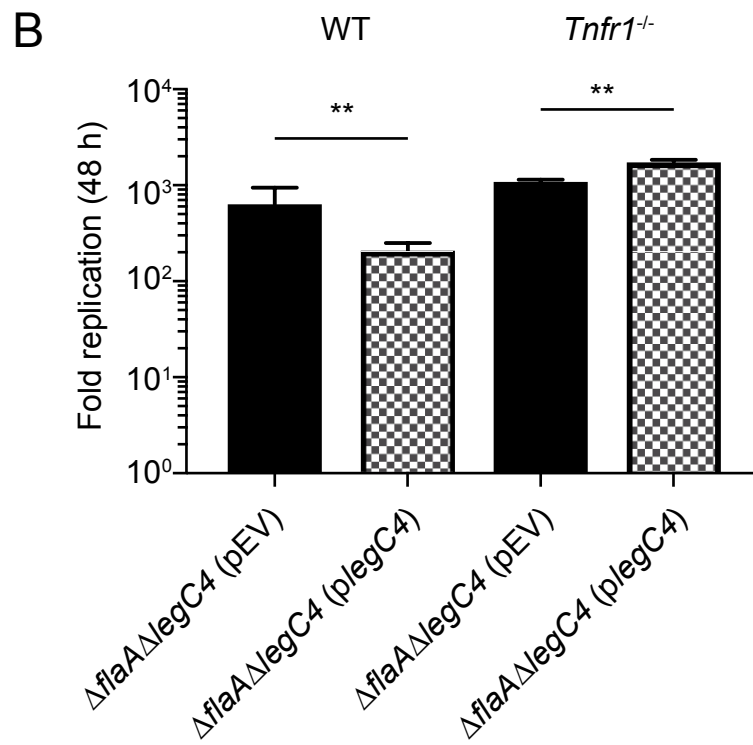
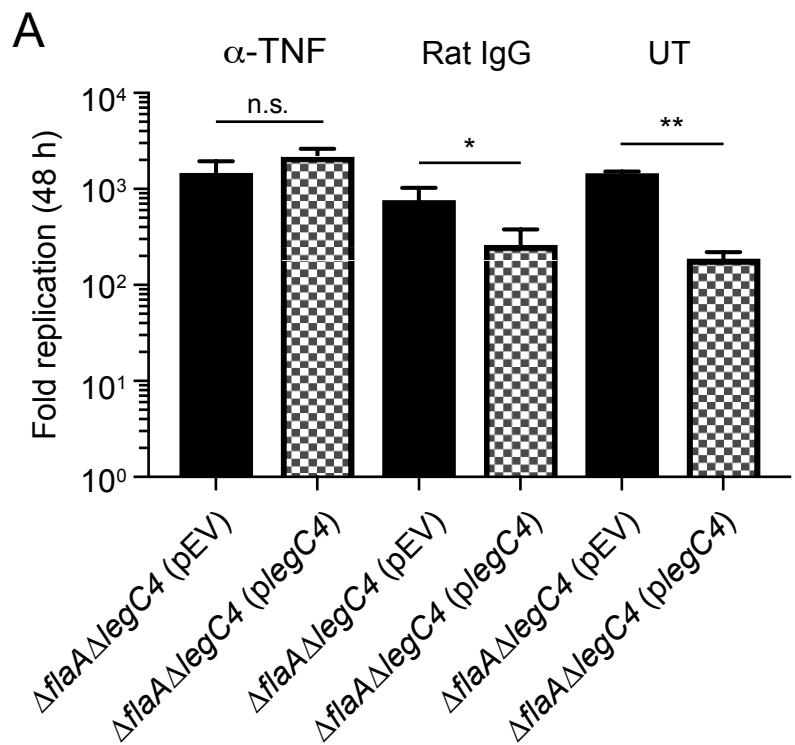


Figure 5

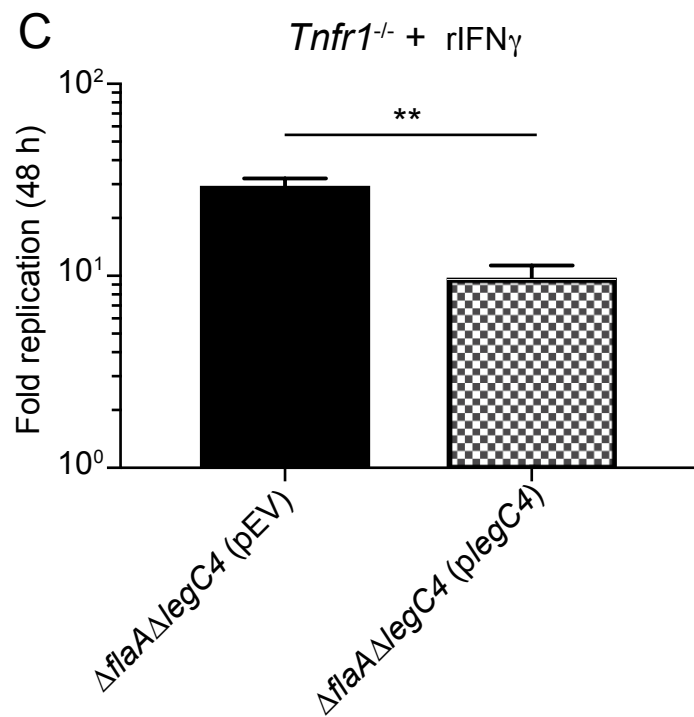
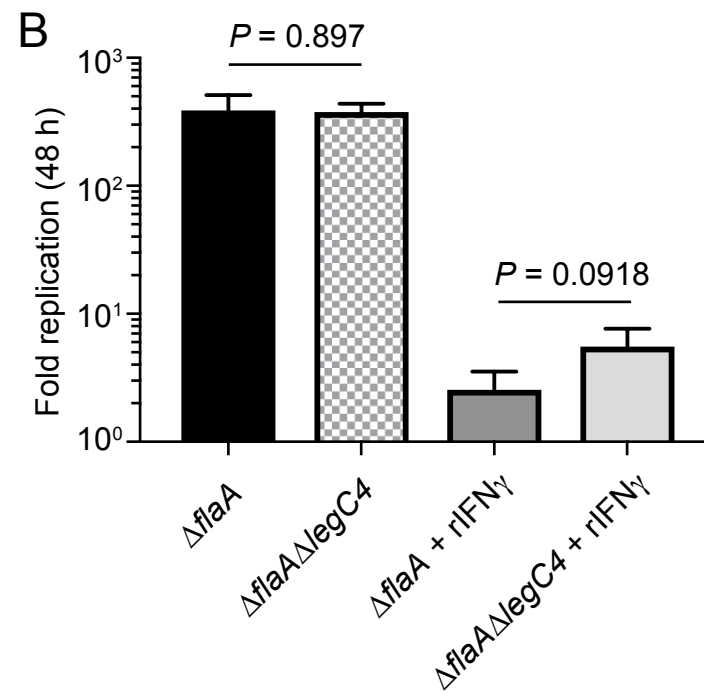
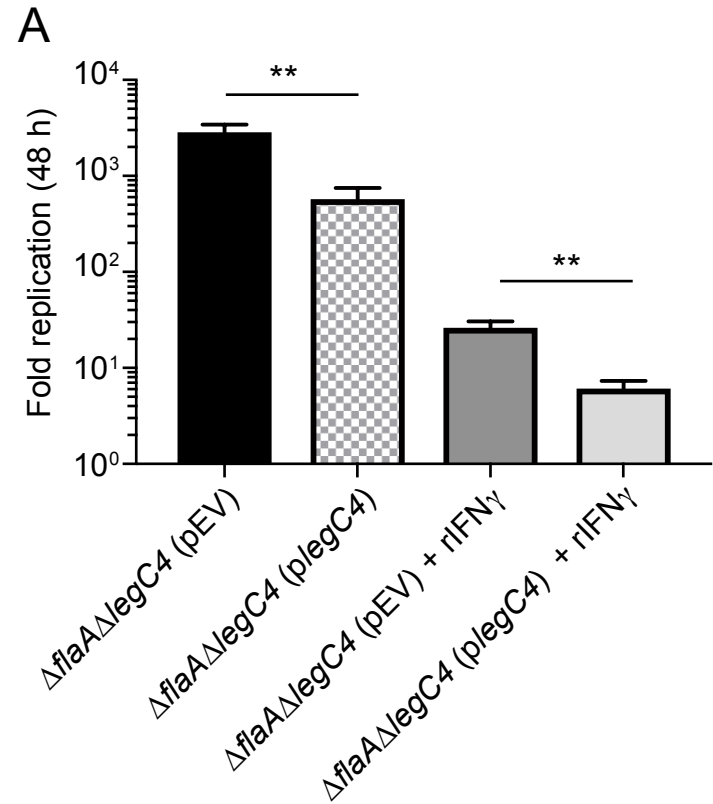


Figure 6

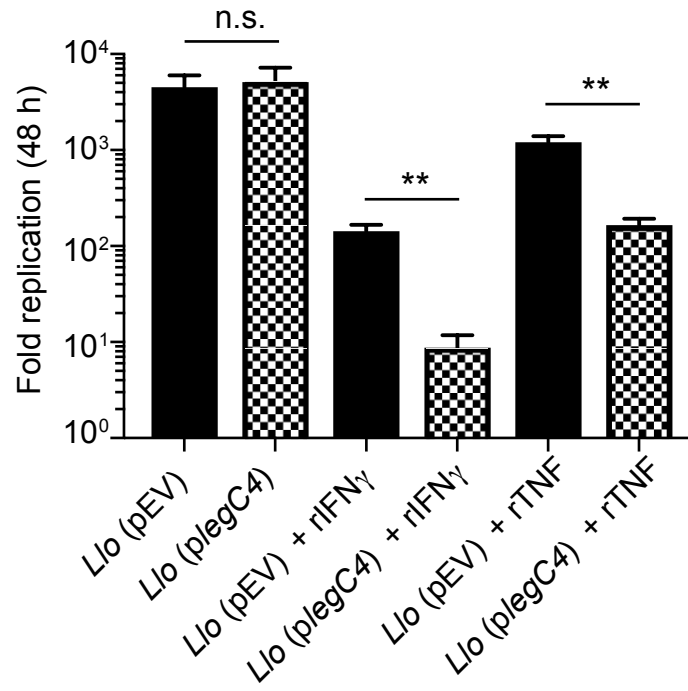


Figure S1

