Effector-mediated subversion of proteasome activator (PA)28αβ enhances lysosomal targeting of *Legionella pneumophila* within cytokine-activated macrophages

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Keywords: PA28, proteasome, phagolysosomal fusion, cytokines, cell-autonomous immunity, *Legionella pneumophila*, LegC4, proteostasis, oxidative stress
Abstract

*Legionella pneumophila* is a natural pathogen of protozoa that causes Legionnaires’ Disease pneumonia via replication within macrophages using hundreds of translocated effector proteins. In metazoans, effectors can also enhance pathogen clearance via effector-triggered immunity. The effector LegC4 confers a fitness disadvantage on *L. pneumophila* uniquely within cytokine-activated macrophages, but the mechanism is unknown. Here, we demonstrate that LegC4 restriction occurs via subversion of proteasome activator (PA)28αβ, which is induced by cytokines and functions to resolve oxidative stress. LegC4 impaired resolution of oxidative stress and LegC4-mediated restriction was abolished within PA28αβ-deficient macrophages. Impaired proteasome activity upregulates lysosomal degradation pathways and, indeed, subversion PA28αβ by LegC4 enhanced lysosomal fusion with the *Legionella*-containing vacuole. PA28αβ has been traditionally associated with antigen presentation; however, our data support a new model whereby subversion of PA28αβ enhances macrophage cell-autonomous immunity against *L. pneumophila*. This work provides a solid foundation to evaluate induced proteasome regulators as mediators of innate immunity.
**Introduction**

Many intracellular bacterial pathogens cause disease by replicating within host macrophages (Mitchell et al., 2016). However, macrophages activated by Th1 proinflammatory cytokines, such as tumor necrosis factor (TNF) and interferon (IFN)-γ, are potently and broadly microbicidal. Activated macrophages are in a state of altered protein homeostasis (proteostasis), owing in part to robust production of reactive oxygen species (ROS), which cause collateral damage to cellular proteins. To cope with oxidative stress, cytokine signaling induces expression of proteasome activator (PA)28αβ, which engages proteolytic 20S proteasome core particles (CPs) and facilitates degradation of oxidant-damaged proteins (Pickering et al., 2010). Impaired proteasome activity causes compensatory upregulation of lysosomal degradation pathways, which are central to cell-autonomous immunity within activated macrophages (Dikic, 2016). PA28αβ has been traditionally associated with antigen presentation and adaptive immunity (Graaf et al., 2011; Preckel et al., 1999); however, the contribution of PA28αβ to antimicrobial innate host defense has not been investigated.

*Legionella pneumophila* is a natural pathogen of amoebae and the etiological agent of Legionnaires' Disease, a severe inflammatory pneumonia of the elderly and immunocompromised that results from uncontrolled bacterial replication within alveolar macrophages. *L. pneumophila* replication within macrophages has resulted from extensive co-evolution with their protozoan hosts (Gomez-Valero et al., 2019; Hugoson et al., 2022; Park et al., 2020). To establish its intracellular niche, the *Legionella*-containing vacuole (LCV), *L. pneumophila* employs hundreds of virulence factors, called effector proteins, that are translocated into infected host cells by a Dot/Icm type IV secretion system (T4SS) (Chauhan and Shames, 2021). Effectors are essential for virulence, but their activity can also trigger upregulation of pro-inflammatory cytokines by infected macrophages via effector-triggered immunity (ETI) (Asrat et al., 2014; Fischer et al., 2019; Fontana et al., 2011; Ngwaga et al., 2021). Pro-inflammatory cytokines are central to clearance of *L. pneumophila*.
pneumophila by healthy hosts, since they activate and potentiate the antimicrobial capacity of infected macrophages. Inability to thwart innate host defenses is likely due to the rarity of *L. pneumophila* transmission from person-to-person and consequent lack of selective pressure from the mammalian immune system. Thus, *L. pneumophila* serves as a well-established model pathogen to study mechanisms of antimicrobial innate immunity.

We discovered that the Dot/Icm-translocated effector LegC4 confers a fitness disadvantage on *L. pneumophila* in a mouse model of Legionnaires’ Disease (Shames et al., 2017). The in vivo LegC4 restriction phenotype is likely due to elevated levels of pro-inflammatory cytokines in the *L. pneumophila*-infected lung since LegC4 impairs *L. pneumophila* replication within cultured macrophages when activated with exogenous tumor necrosis factor (TNF) or interferon (IFN)-γ (Ngwaga et al., 2019; Shames et al., 2017). We also found that overexpression of *legC4* from a complementing plasmid is sufficient to attenuate *L. pneumophila* replication within BMDMs that produce TNF and signal through TNFR1 (Ngwaga et al., 2019). The evolutionary basis for retention of LegC4 by *L. pneumophila* stems from its importance for replication within its natural host, *Acanthamoeba castellanii* (Shames et al., 2017). Thus, LegC4 confers quantifiable opposing phenotypes in natural (protozoa) and ‘accidental’ (mammalian) hosts, which is noteworthy since the majority of *L. pneumophila* effectors are functionally redundant in laboratory models and have no obvious growth phenotypes (Ghosh and O’Connor, 2017; Shames et al., 2017). The specific augmentation of cytokine-mediated restriction by LegC4 represents a potentially novel mode of ETI; however, the mechanism by which LegC4 functions and enhances cytokine-mediated restriction of *L. pneumophila* is unknown.

Here, we investigated the mechanism of LegC4 function within macrophages. We found that LegC4 interacts with host PA28α and that the LegC4 restriction phenotype is abolished in PA28αβ complex deficient (*Psme1/2−/−*) bone marrow-derived macrophages. LegC4 also suppressed
resolution of oxidative stress and enhanced lysosomal fusion with *Legionella*-containing vacuoles (LCVs) in a cytokine- and PA28αβ-dependent manner. Overall, our data support a model whereby PA28α targeting by a translocated effector protein perturbs proteostasis and enhances cell-autonomous restriction of *L. pneumophila* within cytokine-activated macrophages. These data are the first to define a role for PA28αβ in cell-autonomous host defense against an intracellular bacterial pathogen and reveal a mechanism by which to enhance the antimicrobial activity of activated macrophages.

Results

LegC4 enhances restriction of *L. pneumophila* within cytokine-activated macrophages and in vivo. We previously reported that LegC4 confers a fitness disadvantage on *L. pneumophila* in intranasal C57Bl/6 mouse models of Legionnaires’ Disease (LD) and within cytokine-activated macrophages (Ngwaga et al., 2019; Shames et al., 2017). C57Bl/6 mice are restrictive to *L. pneumophila* due to flagellin (FlaA)-mediated activation of the NAIP5/NLRC4 inflammasome (Molofsky et al., 2006; Ren et al., 2006). Thus, to study FlaA-independent host responses within C57Bl/6 mice, we leverage FlaA-deficient *L. pneumophila* (∆flaA) strains. Using competitive index experiments, we found that *L. pneumophila* ∆flaAΔlegC4 (herein called ΔΔ) mutant strains outcompeted parental ∆flaA strains in the lungs of C57Bl/6 wild-type (WT) mice (Ngwaga et al., 2019). The fitness advantage associated with loss of legC4 is likely due to enhanced levels of cytokines seen in the *L. pneumophila*-infected lung compared to cultured macrophages (Liu et al., 2020) since the LegC4-mediated fitness defect was phenocopied within macrophages activated with exogenous recombinant (r)TNF or rIFN-γ (Ngwaga et al., 2019). We also found that LegC4-mediated restriction can be observed in non-cytokine activated (naïve) BMDMs by over-expressing legC4 from an inducible promoter on a complementing plasmid (Ngwaga et al., 2019). Thus, LegC4 is deleterious to *L. pneumophila* in a dose-dependent manner within cytokine-activated macrophages. The LegC4 restriction phenotype is not exclusive to mouse macrophages...
since LegC4 confers a comparable fitness disadvantage on *L. pneumophila* within human THP-1 macrophages (**Fig 1A-B**). Further support and utility of the LegC4 overexpression model was evidenced by loss of LegC4-mediated restriction within BMDMs unable to produce or respond to endogenous TNF (*Myd88<sup>−/−</sup>* and *Tnfr1<sup>−/−</sup>*, respectively) (**Fig 1C, 1D**) (Ngwaga et al., 2019). IFN-γ signaling is sufficient for LegC4 restriction (Ngwaga et al., 2019); however, the LegC4 restriction phenotype was retained within *Ifngr1<sup>−/−</sup>* BMDMs (**Fig 1E**), indicating that the relatively low levels of IFN-γ secreted by macrophages are not sufficient for restriction.

We subsequently evaluated how cytokine signaling impacts the fitness of LegC4-deficient bacteria *in vivo*. We confirmed our previous results showing that LegC4 is deleterious to *L. pneumophila* within the lungs of WT and TNFR1-deficient mice (**Fig 1F**). We also found that LegC4 still conferred a fitness disadvantage on *L. pneumophila* within the lungs of MyD88- and IFNGR1-deficient mice (**Fig 1F**). However, the fitness advantage associated with loss of LegC4 was less pronounced in the lungs of *Myd88<sup>−/−</sup>* mice compared to WT, *Tnfr1<sup>−/−</sup>* and *Ifngr1<sup>−/−</sup>* (**Fig 1F**), which is mainly due to the relatively low levels of MyD88-independent IFN-γ produced in the *L. pneumophila*-infected lung (Archer and Roy, 2006). These data support our previous work showing that LegC4 augments redundant cytokine-mediated pathogen restriction mechanisms (Ngwaga et al., 2019) and demonstrate relevance to human cells. While overexpression of *legC4* is not necessary to observe a phenotype (Ngwaga et al., 2019), this infection model is robust and useful for our goal to define LegC4 function and mechanisms of cytokine-mediated restriction within macrophages.

**LegC4 interacts with host proteasome activator (PA)<sub>28a</sub>**. To gain insight into the mechanism of LegC4 function, we conducted a yeast two-hybrid (Y2H) interactomics screen using LegC4 as bait and genomic (g)DNA from mouse splenocytes as prey. This screen revealed interactions between LegC4 and several regulators of eukaryotic proteasomes, including proteasome activator (PA)<sub>28a</sub>, PA28γ, and Ecm29 (**Table S1**). The highest-confidence interactor was PA28α.
with 28 individual clones, all of which encoded a minimal overlapping region in the PA28α C-terminal domain (amino acid residues 105-249) (Table S1). These data suggest a direct interaction between LegC4 and host PA28α, which is conserved in amoebae and induced by cytokines in macrophages.

We initially validated the PA28α-LegC4 interaction by reciprocal co-immunoprecipitation and immunofluorescence microscopy on ectopically produced epitope tagged fusion proteins. Ectopic expression of PA28α was necessary in these experiments since it is expressed only in response to cytokine signaling (Fabunmi et al., 2001; Seifert et al., 2010). We found that PA28α-Myc co-immunoprecipitated with 3xFLAG-LegC4 (Fig 2A) and that 3xFLAG-LegC4 co-immunoprecipitated with GFP-PA28α (Fig 2B). We also found that ectopically produced 3xFLAG-LegC4 co-localized with GFP-PA28α within transfected HeLa cells (Fig 2C). Furthermore, LegC4 influenced GFP-PA28α localization since GFP-PA28α localized to the cell nucleus in the absence of LegC4 (Fig 2C-D) (Soza et al., 1997). Finally, we quantified co-localization by calculating Pearson Correlation Coefficients (PCC). PCC values indicated a significant positive correlation between LegC4 and PA28α (PCC = 0.768 ± 0.028) compared to the GFP control (PCC = 0.468 ± 0.172) (Fig 2E). Together, these data show that LegC4 interacts with host PA28α, which has not been previously identified as a target of bacterial effectors.

**LegC4-mediated restriction of *L. pneumophila* is abolished in PA28αβ-deficient BMDMs.** Based on the interaction between PA28α and LegC4, we tested the hypothesis that PA28α is important for LegC4-mediated restriction of *L. pneumophila* within BMDMs. PA28α-deficient (*Psm1*<sup>-/-</sup>) mice are not commercially available, so we evaluated the role of PA28α using BMDMs derived from PA28αβ-deficient (*Psm1/2*<sup>-/-</sup>) mice (Preckel et al., 1999). PA28α associates with PA28β to form the PA28αβ (11S) proteasome regulator (Mott et al., 1994). PA28β is homologous to PA28α but is dependent on PA28α for activity and is hypothesized to enhance the
interaction of PA28α with 20S proteolytic proteasome core particles (CPs) (Wilk et al., 2000). We
found that PA28αβ was necessary for LegC4-mediated restriction of L. pneumophila since the no
growth attenuation was associated with legC4 overexpression compared to control strains within
Psme1/2−/− BMDMs (Fig 2F). Thus, our data suggest that PA28αβ activity is important for LegC4-
mediated restriction and support a model whereby LegC4 binds and regulates the function of
PA28α.

LegC4 impairs resolution of oxidative proteotoxic stress. In cells of the innate immune
system, PA28αβ is produced in response to oxidative stress, TNF, or IFN-γ (Fabunmi et al., 2001;
Halleralm et al., 2001; Mott et al., 1994; Pickering et al., 2010), which provides an explanation
as to why LegC4 confers a growth phenotype within cytokine-activated cells. Induced PA28ab
associates with proteasome core particles (CPs) to generate antigenic peptides and resolve oxida-
tive stress by facilitating ubiquitin-independent proteasomal degradation of oxidant-damaged
(carbonylated) proteins (Graaf et al., 2011; Li et al., 2010). Since LegC4 binds PA28α and the
LegC4 restriction phenotype was dependent on PA28αβ (see Fig 2F), we hypothesized that LegC4
regulates PA28αβ activity.

Our yeast two-hybrid data suggest that LegC4 binds the CTD of PA28α, which is involved
in PA28αβ docking on proteolytic proteasome core particles and consequent proteasomal degra-
dation of oxidant damaged (carbonylated) proteins. We rationalized that LegC4-mediated inhibi-
tion of PA28αβ activity would impair degradation of carbonylated proteins and tested this by
quantifying protein carbonyls within macrophages. We first tested this within uninfected RAW
264.7 cells stably producing 3xFLAG-LegC4 (RAW-LegC4). Stable production of 3xFLAG-LegC4
was confirmed by confocal microscopy (Fig 3A) and Western blot (Fig 3B). Immunoprecipita-
tion was necessary to visualize LegC4 since it is produced by macrophages at low abundance.
However, this amount of ectopically produced LegC4 was sufficient to attenuate L. pneumophila
ΔflaA replication when compared to vector-transfected control (RAW-Cntrl) cells (Fig 3C). Attenuated growth was observed at 48 h post-infection, similar to the kinetics of LegC4 restriction within BMDMs (Fig 1A), which suggests that RAW-LegC4 cells are suitable to evaluate whether LegC4 impacts the concentration of carbonylated proteins within macrophages under oxidative stress conditions.

We quantified the abundance of carbonylated proteins within lysates of uninfected RAW-LegC4 and -Cntrl cells in the presence or absence H$_2$O$_2$ to induce oxidative stress. Under native conditions, the concentration of carbonylated proteins within RAW-LegC4 and -Cntrl cells did not differ (Fig 3D). However, under oxidative stress conditions, the concentration of protein carbonyls in RAW-LegC4 cells was significantly greater than control cells (Fig 3D). These data suggest that cells producing LegC4 are impaired for resolution of oxidative proteotoxic stress and support a model whereby LegC4 impairs PA28α activity.

**Oxidative stress is sufficient for LegC4-mediated restriction.** We subsequently evaluated how LegC4 impacts the abundance of protein carbonyls within *L. pneumophila*-infected cells. To control for differences in bacterial replication within WT BMDMs, we leveraged Tnfr1$^{-/-}$ BMDMs, in which LegC4 is not restrictive (see Fig 1C). Tnfr1$^{-/-}$ BMDMs were infected for 4 h in the presence or absence of H$_2$O$_2$ and protein carbonyls were visualized by Western blot. We found that the abundance of carbonylated proteins was increased within H$_2$O$_2$-treated BMDMs infected with *L. pneumophila* ΔflaAΔlegC4 (plegC4) relative to ΔflaA and empty vector control strains (Fig 3E). Since no LegC4-mediated differences were observed within untreated Tnfr1$^{-/-}$, it is unlikely that the increase in protein carbonyls observed is due to a LegC4-mediated upregulation global ROS production by infected BMDMs. Together, these data support our hypothesis that LegC4 impairs resolution of oxidative proteotoxic stress within macrophages.

Based on our data showing that LegC4 impairs resolution of oxidative proteotoxic stress, we hypothesized that oxidative stress is sufficient for LegC4-mediated restriction within BMDMs.
To test this hypothesis, we quantified *L. pneumophila* replication within *Tnfr1*⁻/⁻ BMDMs in the presence or absence of H₂O₂ to induce oxidative stress. We found that replication of the *L. pneumophila* ΔflaAΔlegC4 (*plegC4*) strain was significantly attenuated within H₂O₂-treated *Tnfr1*⁻/⁻ BMDMs compared to *L. pneumophila* ΔflaA and ΔflaAΔlegC4 empty vector (pEV) control strains (Fig 3F). These differences represent a 78% decrease in colony forming units (CFU), which is striking since experimental limitations necessitated a low concentration (10 µM) of H₂O₂. It is unlikely that growth attenuation was due to direct *L. pneumophila* killing by H₂O₂ since (1) replication of control *L. pneumophila* strains did not differ between H₂O₂-treated and untreated BMDMs; and (2) *L. pneumophila* has evolved multiple complimentary strategies to protect against oxidative stress and can withstand up to 2 mM H₂O₂ *in vitro* (orders of magnitude greater than intracellular concentrations) (Bandyopadhyay and Steinman, 2000; Bandyopadhyay et al., 2003; Flynn and Swanson, 2014; Harada et al., 2007). These data suggest that oxidative stress is sufficient for the LegC4 restriction phenotype.

**PA28αβ-deficient BMDMs are restrictive to *L. pneumophila***. Our current data suggest that LegC4 binding impairs PA28αβ activity. Thus, we hypothesized that LegC4-mediated restriction of *L. pneumophila* would be phenocopied in PA28αβ-deficient BMDMs. To test this hypothesis, we infected WT or *Psme1/2*⁻/⁻ BMDMs with *L. pneumophila* ΔflaA in the presence or absence of rTNF or rIFN-γ. We found that replication of *L. pneumophila* ΔflaA was significantly attenuated within *Psme1/2*⁻/⁻ BMDMs compared to WT BMDMs (Fig 3G). However, when BMDMs were treated with either rTNF or rIFN-γ, impaired bacterial replication was observed within 24 h of infection whereas restriction was observed after 48 h in untreated BMDMs (Fig 3H-I). These data show that loss of PA28αβ enhances macrophage restriction of *L. pneumophila*, which is more pronounced under inflammatory conditions and suggest a novel role for PA28αβ in antimicrobial innate immunity.
Neither PA28αβ nor LegC4 increase TNF secretion from L. pneumophila-infected BMDMs or induce cell death. We then evaluated whether differences in cytokine secretion or cell death account for differences in PA28αβ- or LegC4-mediated restriction of L. pneumophila within Psme1/2−/− compared to WT BMDMs. Autocrine and paracrine TNF signaling within L. pneumophila-infected BMDMs is sufficient for the LegC4 restriction phenotype (Ngwaga et al., 2019) (see Fig 1D); thus, we tested whether growth differences were due to increased TNF secretion by infected BMDMs. At 8h post-infection, there were no differences in TNF secretion from Psme1/2−/− BMDMs compared WT BMDMs infected with either L. pneumophila ∆flaA or ∆Δ (pEV); however, there was less TNF secreted from Psme1/2−/− BMDMs infected with L. pneumophila ∆ (plegC4) compared to WT BMDMs (Fig S1A). However, at 24 h post-infection, there was significantly less TNF secreted from Psme1/2−/− BMDMs infected with L. pneumophila ∆flaA or ∆Δ (pEV) compared to WT BMDMs but no differences in secretion from BMDMs infected with L. pneumophila ∆ (plegC4) (Fig S1B). These data suggest that LegC4- and PA28αβ-mediated growth phenotypes are not due to enhanced TNF production by infected BMDMs.

We subsequently evaluated whether LegC4 or PA28αβ affect viability of L. pneumophila-infected BMDMs using lactate dehydrogenase release (LDH) assay. We found no LegC4- or PA28αβ-mediated differences in LDH release from L. pneumophila-infected BMDMs (Fig S1C). These data suggest neither LegC4 nor PA28αβ restriction phenotypes are a consequence of host cell death.

Phagolysosomal fusion contributes to LegC4-mediated restriction within BMDMs.

Our current data suggest that LegC4 may impair PA28αβ activity, but how this leads to bacterial restriction is unknown. Loss of cellular proteasome activity causes compensatory upregulation of lysosomal degradation pathways to cope with proteotoxic stress (Dikic, 2016; Pandey et al., 2007).
Based on our data showing that oxidative proteotoxic stress is sustained in LegC4-producing cells, we tested the hypothesis that LegC4 restriction involves lysosomal targeting of *L. pneumophila*. Virulent *L. pneumophila* establish a replicative Legionella-containing vacuole (LCV) by blocking endocytic maturation of their phagosome (Roy et al., 1998; Swanson and Isberg, 1995). However, avirulent Dot/Icm-deficient *L. pneumophila* (∆dotA) are unable to establish a replicative LCV and phagosomes rapidly undergo endocytic maturation, as evidenced by localization of the lysosomal membrane marker LAMP1 to *L. pneumophila*-containing phagosomes (Roy et al., 1998) (Fig S2). We initially tested the hypothesis that LegC4 increases LAMP1 localization to LCVs by immunofluorescence microscopy and blinded quantification of LAMP1+ LCVs. As expected, there were significantly fewer LAMP1+ LCVs harboring ∆flaA and empty vector control strains compared to the avirulent control within WT BMDMs (Fig 4A). However, there were no differences in the percentage of LAMP1+ LCVs harboring ∆∆ (plegC4) strain compared to the ∆dotA control (Fig 4A), which was surprising since plasmid expression of *legC4* does not usually impair *L. pneumophila* intracellular replication until after 24 h of infection. We found that LCVs harboring *L. pneumophila* ∆∆ (plegC4) had significantly less LAMP1 staining compared to the ∆dotA strain within both TNFR1- and PA28αβ-deficient BMDMs (Fig 4B-C). These data suggest that LegC4 upregulates lysosomal fusion with LCVs in a cytokine- and PA28αβ-dependent manner.

We subsequently tested the hypothesis that phagolysosomal fusion is important for LegC4-mediated restriction by quantifying *L. pneumophila* replication within BMDMs treated with Bafilomycin A1 (BAF). BAF is a pharmacological inhibitor of the H+ type vacuolar (v)-ATPase that blocks vacuolar acidification and impairs phagolysosomal fusion (Matsumoto and Nakanishi-Matsui, 2019). Thus, we evaluated the contribution of phagolysosomal fusion to the LegC4 restriction phenotype by quantifying *L. pneumophila* replication within BAF-treated BMDMs. LCV acidification is important for late stages of the *L. pneumophila* lifecycle (Sturgill-Koszycki and Swanson, 2000); however, intracellular replication is minimally impacted within BMDMs treated with a low concentration (≤12.5 nM) of BAF (Khweek et al., 2013). We found that replication of
\textit{L. pneumophila} \( \Delta \Delta \) (\textit{plegC4}) was not attenuated compared to \textit{L. pneumophila} \( \Delta \text{flaA} \) and \( \Delta \Delta \) (pEV) strains within BAF-treated BMDMs (Fig 4D, left). As expected, \textit{L. pneumophila} \( \Delta \Delta \) (\textit{plegC4}) replication was significantly attenuated compared to \( \Delta \text{flaA} \) and \( \Delta \Delta \) (pEV) within vehicle (DMSO)-treated BMDMs (Fig 4D, right). These data suggest a role for phagolysosomal fusion and/or lysosomal acidification contribute to LegC4-mediated restriction. Together, our data support a model whereby LegC4-mediated subversion of PA28\( \alpha\beta \) enhances lysosomal pathogen targeting within activated macrophages.

**Inflammasome activation is dispensable for LegC4-mediated restriction.** Our current data suggest that LegC4-mediated restriction occurs via lysosomal fusion with LCVs under oxidative proteotoxic stress conditions. However, sustained oxidative stress and proteostasis perturbations can activate the NLRP3 inflammasome, which culminates in activation of caspase-1, an effector caspase responsible for pyroptotic cell death and inflammatory cytokine secretion. However, pharmacological inhibition of the NLRP3 inflammasome with MCC950 did not affect LegC4-mediated restriction since \textit{L. pneumophila} \( \Delta \Delta \) (\textit{plegC4}) was still attenuated for replication compared to \textit{L. pneumophila} \( \Delta \Delta \) (pEV) and \( \Delta \text{flaA} \) within WT BMDMs (Fig S3A). We also found that LegC4-mediated restriction was preserved within BMDMs derived from caspase-1-deficient (\textit{Casp1}\(^{-/-}\)) mice (Fig S3B). Within cultured macrophages, pyroptotic cell death is responsible for \textit{L. pneumophila} restriction downstream of inflammasome activation. Our data suggest that LegC4 does not enhance LDH release from infected BMDMs (Fig S3C); however, pyroptotic cell death is more pronounced within BMDMs primed with toll-like (TLR) receptor agonists (Bergsbaken et al., 2009; Feuvre et al., 2002). Since TLR2 is important for host defense against \textit{L. pneumophila} (Archer and Roy, 2006), we evaluated LDH release from \textit{L. pneumophila}-infected BMDMs pretreated with the TLR2 agonist PAM3CSK4. Consistent with our previous observation (Fig S1C), LDH release from BMDMs primed with PAM3CSK4 was unaffected by LegC4 (Fig S3C). These
data suggest that LegC4-mediated restriction occurs independently of inflammasome activation and pyroptotic cell death.

Discussion

This study supports a novel role for proteasome activator (PA)PA28αβ in effector-mediated host defense against an intracellular bacterial pathogen. Our data show that LegC4 binds PA28αβ, subverts resolution of oxidative proteotoxic stress and promotes lysosomal restriction of *L. pneumophila*. Thus, we propose a model whereby prolonged oxidative proteotoxic stress resulting from LegC4 subversion of PA28αβ enhances lysosomal targeting of *L. pneumophila* within cytokine-activated macrophages (Fig S4). This model is supported by our data showing that (1) LegC4 binds PA28α; (2) PA28αβ is required for the effects of LegC4 on host cells; (3) LegC4 impairs resolution of oxidative proteotoxic stress; and (4) that oxidative stress and phagolysosomal fusion contribute to LegC4-mediated restriction of *L. pneumophila*. This work is the first describe PA28α as a target for a bacterial effector protein and a role for PA28αβ in effector-mediated host defense against an intracellular bacterial pathogen.

*L. pneumophila* is an accidental pathogen of humans that rarely transmits between infected individuals. Thus, an evolutionary basis for the LegC4-PA28α interaction can be explained by LegC4 targeting the PA28α homolog in amoebae. Indeed, the natural host *A. castellanii*, in which LegC4 is a *bona fide* virulence factor (Shames et al., 2017), encodes a PA28α homolog (AcPA28) that shares 34% identity with mouse PA28α. Sequence identity between PA28αβ and AcPA28 is primarily in the C-terminal domain, which is important for engaging 20S CPs and was contained in all PA28α clones identified in our yeast two-hybrid screen (see Table S1). These data have led us to speculate that LegC4 binding prevents PA28-CP complex formation. The function of AcPA28 has not been described; however, structural studies have shown that mammalian PA28α and
evolutionary distant PA28s are similar and all associate with proteolytic proteasome core particles, suggesting that it likely also functions as a proteasome regulator (Lesne et al., 2020; Xie et al., 2019). The constitutive 26S proteasome system is central to *L. pneumophila*’s virulence strategy and its integrity may be preserved by sequestering PA28 from CPs. We are currently investigating the molecular mechanism by which LegC4 regulates PA28 activity within macrophages and amoebae and how this activity is advantageous to *L. pneumophila* in the natural host.

We found that inducing oxidative stress with hydrogen peroxide (H$_2$O$_2$) was sufficient for the LegC4 restriction phenotype. H$_2$O$_2$ is routinely used to induce oxidative stress in laboratory models since both superoxide (O$_2^-$) and hydroxyl (OH$^-$) radicals are highly reactive and unstable in solution. Interestingly, reactive oxygen species (ROS) are important for *L. pneumophila* restriction by neutrophils but play a negligible role in macrophages (Harada et al., 2007; Price et al., 2021; Ziltener et al., 2016). This difference has been attributed to suppression of global intracellular ROS production and NADPH oxidase (NOX2) trafficking to the LCV by virulent *L. pneumophila* within macrophages (Harada et al., 2007; Price et al., 2021). Direct oxidative killing of *L. pneumophila* within activated macrophages is unlikely since *L. pneumophila* has evolved multiple complimentary strategies to protect against oxidative stress and can withstand up to 2 mM H$_2$O$_2$ *in vitro* (orders of magnitude greater than intracellular concentrations) (Bandyopadhyay and Steinman, 2000; Bandyopadhyay et al., 2003; Flynn and Swanson, 2014; Harada et al., 2007). Moreover, *Tnfr1*<sup>−/−</sup> BMDMs treated with H$_2$O$_2$ did not suppress replication of *L. pneumophila* control strains compared to untreated BMDMs (see Fig 3F). This suggests that the concentration of H$_2$O$_2$ used in our study is insufficient for direct *L. pneumophila* killing. Future studies will reveal whether LegC4 or PA28αβ impact the abundance or source of ROS within *L. pneumophila*-infected macrophages.
Phagolysosomal fusion and autophagy are cytoprotective under inflammatory conditions and central to cytokine-mediated cell-autonomous pathogen restriction. Lysosomal restriction within IFN-γ-activated macrophages involves immunity-related GTPases (IRGs), which localize to and facilitate lysosomal trafficking to pathogen-containing phagosomes (MacMicking, 2012). Although IRGs may contribute to restriction of LegC4-producing *L. pneumophila* strains within IFN-γ-activated macrophages (Naujoks et al., 2016), we found that IFNγR1 signaling is dispensable for LegC4 restriction (Fig 1E), which suggests that IRGs are not central drivers of LegC4-mediated *L. pneumophila* restriction. A role for TNFR1-mediated signaling in lysosomal pathogen restriction has not been well established. Canonically, TNF signaling activates extrinsic cell death pathways; however, TNFR1-mediated signaling induces lysosomal fusion with LCVs within 3 h of *L. pneumophila* infection within primary mouse BMDMs, which is more rapid compared to BMDMs activated with IFN-γ (Ziltener et al., 2016).

The mechanism by which TNF signaling enhances phagolysosomal fusion with LCVs is poorly understood; however, upregulation of autophagy genes is cytoprotective against TNF-induced death in myeloid cells (Orvedahl et al., 2019). Autophagy is also upregulated to prevent aberrant inflammasome-mediated pyroptotic cell death below a certain threshold of inflammasome agonist (Byrne et al., 2013). The NLRC4 inflammasome is dispensable for LegC4-mediated restriction (Ngwaga et al., 2019; Shames et al., 2017) and we also found no role for the NLRP3 inflammasome, which is activated by both oxidative and proteotoxic stress (Shin et al., 2013). Whether autophagy contributes to LegC4-mediated restriction is unknown, but this possibility is intriguing since several *L. pneumophila* effectors impair global autophagy in host cells (Choy et al., 2012; Rolando et al., 2016b, 2016a).

Virulent *L. pneumophila* establish a replicative LCV by avoiding endosomal maturation of their phagosome. The current dogma dictates that lysosomal fusion with LCVs at early time points
during infection (≤ 16 h) prevents *L. pneumophila* intracellular replication (Marra et al., 1992; Sturgill-Koszycki and Swanson, 2000). We found that plasmid expression of *legC4* induced robust LAMP1 localization to LCVs within WT BMDMs, similar to what is seen for an avirulent a Dot/Icm-deficient strain. This is intriguing since plasmid expression of *legC4* does not completely abolish *L. pneumophila* intracellular replication (Ngwaga et al., 2019). Loss of TNFR1 did not fully abolish LegC4-mediated LAMP1 localization to LCVs; however, this relatively modest level of LAMP1 localization may result from ROS produced in response to pattern recognition receptors (West et al., 2011). The abrogation of LegC4-mediated LAMP1-LCV localization within *Psme1/2* supports our model that subversion of PA28αβ by LegC4 enhances lysosomal fusion with LCVs.

Here, we show a new role for PA28αβ and oxidative proteotoxic stress in cell-autonomous pathogen restriction. This work is the first to show bacterial pathogen targeting of PA28α and suggest that suppression of PA28αβ activity and aberrant proteostasis may be a pathogen-triggered host defense mechanism and mode of potentiating existing inflammatory responses. The ability enhance antimicrobial potential of activated macrophages has exciting implications for development of host-centric innate immunological therapeutics and future studies will provide further insight into the ubiquity and mechanism(s) of pathogen restriction induced by proteotoxic stress and suppression of PA28αβ activity.

**Materials & Methods**

**Bacterial Strains, Plasmids, and Culture conditions**

*Legionella pneumophila* strains used in this study (*Table S2*) were cultured on supplemented charcoal N-(2-Acetamido)-2-aminoethanesulfonic acid (ACES)-buffered yeast extract (CYE) and grown at 37°C as described (Feeley et al., 1979). Liquid cultures were grown overnight with shaking at 37°C in supplemented ACES-buffered yeast extract (AYE) medium, as described (Ngwaga et al., 2019; Saito et al., 1981). For plasmid maintenance, CYE was supplemented with 10 µg mL⁻¹
chloramphenicol and \textit{legC4} expression was induced with 1 mM isopropyl-\textnormal{\textbeta}-D-1-thiogalactopyranoside (IPTG) where indicated. Plasmids used in this study are listed in Table S3. Oligonucleotide primers are listed in Table S4.

**Mice and bone marrow-derived macrophages**

Wild-type, \textit{Tnfr1}\textsuperscript{−/−}, \textit{Ifngr1}\textsuperscript{−/−}, \textit{Myd88}\textsuperscript{−/−}, and \textit{Psme1/2}\textsuperscript{−/−} on a C57BL/6 background were purchased from the Jackson Laboratories (Bar Harbor, Maine) and in-house colonies were maintained in specific pathogen-free conditions at Kansas State University. All experiments involving animals were approved by the Kansas State University Institutional Animal Care and Use Committee (Protocols 4022, 4501, and 4386) and performed in compliance with the Animal Welfare Act and NIH guidelines.

Bone marrow was harvested from seven- to twelve-week-old mice as described (Case and Roy, 2013). BMDMs were generated by differentiation in RPMI supplemented with 20\% heat-inactivated fetal bovine serum (HI-FBS) (Biowest, Riverside, MO) and 15\% L929 cell supernatant for 6 days prior to seeding for infection. Femurs from C57Bl/6 \textit{Casp1}\textsuperscript{−/−} mice were a gift from Dr. Russell Vance (University of California, Berkeley).

**Competitive Index**

Six- to twelve-week-old sex- and age-matched mice were infected as previously described. Mixed bacterial inoculums (1:1) containing a total of 5 × 10\textsuperscript{6} bacteria were diluted and plated on selective medium (10 µg mL\textsuperscript{−1} chloramphenicol for plasmid selection). At 48 h post-infection, mice were euthanized, and lung homogenates were plated on selective media as described (Ngwaga et al., 2019). CFU were enumerated and used to calculate CI values \([\text{CFU}_{\text{cm}R_{48h}}/\text{CFU}_{\text{wt}48h}]/(\text{CFU}_{\text{cm}R_{IN}})/(\text{CFU}_{\text{wt}_{IN}})\].

**Molecular Cloning**
Plasmids were generated for stable and transient ectopic production in mammalian cells. For production of 3xFLAG-LegC4, legC4 was amplified from L. pneumophila genomic (g)DNA using LegC4BamHI-F/LegC4NotI-R primer pairs and cloned as a BamHI/NotI fragment into 3xFLAG 4/TO (Ingmundson et al., 2007). To produce GFP-PA28a, psme1 was amplified from pCMV-3Tag-4a::psme1 (purchased from Genscript, Piscataway, New Jersey) using Psme1Sal1-F/Psme1BamHI-R primer pairs and cloned as a Sal1/BamH1 fragment into pEGFPC1 (Clontech).

Plasmid DNA was transfected into mammalian cells as described below.

**Cell Culture and Transfections**

HEK 293T, HeLa, RAW 264.7 cells, and MH-S cells (gifts from Dr. Craig Roy, Yale University) were maintained at 37°C and 5% CO₂ in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% HI-FBS. THP-1 cells were purchased from the American Type Culture Collection (ATCC) and maintained in RPMI supplemented with 10% HIFBS and differentiated with 100 nM phorbol 12-myristate 13-acetate (PMA) for 4 days prior to infection with L. pneumophila (see below). All cell lines were used between passage 4-20.

HEK 293T cells were transfected with purified plasmid DNA (Table S2) using calcium phosphate as described (Shames et al., 2010). Lysates were generated from transfected cells after 48 h and used for immunoprecipitation and Western blot analysis (see below). HeLa cells were seeded one day prior to transfection with jetPRIME transfection reagent according to manufacturer’s guidelines. Two hours before transfection, HeLa cells were washed and incubated in low-serum media (DMEM 4% HIFBS. Media were replaced with DMEM 10% HIFBS four hours post-transfection and assayed 24 h post-transfection.

To generate RAW 264.7 cells with stable plasmid integrations, cells were transfected with pcDNA::3xflag-legC4 or pcDNA::3xflag vector using a Nucleofector 2b electroporator (Lonza, Basel, Switzerland) and cultured over 14 days with Zeocin selection for 14 days (200 -1000 µg mL⁻¹). Stable RAW 264.7 cells were maintained in culture in the presence of 200 µg mL⁻¹ Zeocin. For
*L. pneumophila* infections, cells were seeded one day prior to infection in the absence of Zeocin. Production of ectopic 3xFLAG-LegC4 was confirmed by Western blot and confocal microscopy using α-FLAG antibodies (see below).

**Western blot**

Boiled protein samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane using a BioRad TransBlot semidry transfer apparatus. Membranes were incubated in blocking buffer [5% non-fat milk powder dissolved in Tris-buffered saline-0.1% Tween-20 (TBST)]. Primary antibodies [rabbit α-FLAG (Sigma-Aldrich, F1804), rabbit α-Myc (Cell Signaling, 2278S), rabbit α-GFP (Abcam, ab6556)] were used at 1:1,000 in blocking buffer and detected with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5,000; ThermoFisher). Membranes were washed in TBST followed by addition of enhanced chemiluminescent (ECL) reagent (GE Amersham) and visualization using an Azure c300 Darkroom Replacer.

**Immunoprecipitation**

Transiently transfected HEK 293T cells or RAW 264.7 cells were washed with phosphate-buffered saline and lysed in ice-cold NP-40 buffer [1% non-iodet P40 (v/v), 20 mM Tris pH 7.5, 150 mM NaCl, 10 mM Na₃P₂O₇, 50 mM NaF, complete protease inhibitor (Roche)]. Lysates were clarified and added to magnetic Protein G-conjugated Dynabeads that had been pre-incubated with either mouse a-FLAG M2 antibody (Sigma Aldrich) or rabbit a-GFP antibody (Abcam, ab6556) according to manufacturer’s instructions. Input samples were collected from cell lysates prior to incubation with beads. Beads and input samples were resuspended in 3x Laemmlli sample buffer for Western blot analysis.

**Protein carbonyl assays**
Protein carbonyls were quantified using the OxiSelect™ Protein Carbonyl ELISA Kit (Cell Biolabs) following manufacturer’s instructions. Briefly, RAW 264.7 cells were seeded at $5 \times 10^5$ in a 6-well plate in DMEM 10% HIFBS. The next day, media were aspirated, and cells were incubated in DMEM/2.5% HIFBS in the presence or absence of 10 µM H$_2$O$_2$. After 24 h, cells were washed twice in PBS and lysed in ice-cold 500 µL of detergent-free lysis buffer [25 mM HEPES pH 7.4, 150 mM NaCl, 10 mM MgCl$_2$, 1 mM EDTA, 2% glycerol (v/v)] supplemented with complete protease inhibitor cocktail (Roche). Cell lysates were scraped into a pre-chilled microcentrifuge tube and incubated with agitation for 30 min at 4°C followed by sonication at 40% intensity three times in 10 second intervals. Lysates were clarified by centrifugation at 4°C for 20 min at 12,000 rpm. Supernatants were transferred to a fresh tube, snap frozen in liquid nitrogen for 2 min and stored at -80°C until use. Protein concentrations were quantified using a Coomassie Plus (Bradford) Protein Assay (Pierce) and diluted to 10 µg mL$^{-1}$. Samples and standards were adsorbed to a 96-well protein binding plate and ELISA was performed according to manufacturer’s instructions. Absorbance at 450 nm was quantified on a BioTek Epoch2 microplate reader.

Protein carbonyls were visualized using the OxiSelect™ Protein Carbonyl Immunoblot Kit (Cell Biolabs). Tnrf1$^{-/-}$ BMDMs were seeded in 24-well plates at $2.5 \times 10^5$ one day prior to infection with indicated *L. pneumophila* strains at an MOI of 50 in the presence or absence of 10 µM H$_2$O$_2$. One hour after infection, cells were washed 3x with PBS$^{-/-}$, media were replaced and cells were incubated for an additional 3 h in the presence or absence of H$_2$O$_2$. Cells were washed in ice-cold PBS$^{-/-}$ and lysed in 120 µL ice-cold NP-40 lysis buffer (see above). Lysates were diluted in 3x Laemmli sample buffer and boiled for 10 min. Proteins were separated on a 4-20% gradient SDS-PAGE gel and transferred to PVDF membrane using a BioRad TransBlot semi-dry transfer apparatus. Membranes were processed according to manufacturer’s instructions using 10 min wash steps. Blots were stripped and re-probed with rabbit a-b-actin antibody (1:1000; Cell Signaling Technology) followed by goat-a-rabbit-HRP (1:5000; ThermoFisher). Blots were visualized as described above and densitometry was performed using ImageJ.
Yeast Two-Hybrid Analysis

Yeast two-hybrid screening was performed by Hybrigenics Services, S.A.S., Evry, France (http://www.hybrigenics-services.com). The legC4 coding sequence was PCR-amplified from pSN85::legC4 (Ngwaga et al., 2019) and cloned into PB66 as a C-terminal fusion to Gal4 DNA-binding domain (Gal4-C4). The construct was checked by sequencing and used as a bait to screen a random-primed Mouse Spleen library constructed into pP6. PB66 derives from the original pAS2ΔΔ vector (Fromont-Racine et al., 1997) and pP6 is based on the pGADGH plasmid (Bartel and Fields, 1995).

Sixty million clones (6-fold the complexity of the library) were screened using a mating approach with YHGX13 (Y187 ade2-101::loxP-kanMX-loxP, mat-α) and CG1945 (mat-α) yeast strains as previously described (Fromont-Racine et al., 1997). 303 His+ colonies were selected on a medium lacking tryptophan, leucine and histidine, and supplemented with 2mM 3-aminotriazole to handle bait autoactivation. The prey fragments of the positive clones were amplified by PCR and sequenced at their 5’ and 3’ junctions. The resulting sequences were used to identify the corresponding interacting proteins in the GenBank database (NCBI) using a fully automated procedure. A confidence score (PBS, for Predicted Biological Score) was attributed to each interaction as previously described (Formstecher et al., 2005).

Immunofluorescence Microscopy

To quantify LAMP1-LCV co-localization and the number of L. pneumophila per LCV, 1x10⁵ BMDMs were seeded on poly-L-lysine-(PLL)coated glass coverslips in 24-well plates and infected with L. pneumophila at a multiplicity of infection (MOI) of 30 in triplicates. Coverslips were fixed with 4% paraformaldehyde (PFA; ThermoFisher) and permeabilized in ice cold methanol. Coverslips were incubated with 1:1,000 rabbit α-L. pneumophila (Invitrogen, PA17227), rat α-LAMP1 (Developmental Studies Hybridoma Bank) primary antibodies and 1:500 Alexa 488-
conjugated goat α-rabbit and Alexa594-conjugated goat α-rat secondary antibodies (ThermoFisher) in blocking buffer [0.1% saponin (w/v), 1% HIFBS (v/v), 0.5% bovine serum albumin (BSA; w/v)] in PBS. Nuclei were stained with Hoechst (ThermoFisher) at 1:2,000. Coverslips were mounted on glass slides with ProLong Gold Antifade Mountant (ThermoFisher). LAMP1 localization to LCVs were scored blind on a Leica DMiL LED inverted epifluorescence microscope (n=300 cells/strain).

For confocal microscopy, HeLa cells were seeded on PLL-coated coverslips and transfected as described above. Coverslips were fixed in 4% PFA and processed as described above using mouse α-FLAG M2 (Sigma; 1:1,000) and rabbit α-GFP (Abcam; ab6556; 1:5,000) primary antibodies and Alexa546-conjugated goat α-mouse and Alexa488-conjugated goat α-rabbit antibodies (ThermoFisher; 1:500). Images were captured on a Zeiss LSM-5 PASCAL laser scanning confocal microscope. Pearson Correlation Coefficients were calculated (n=4) with Fiji software and images were processed using Adobe Photoshop and Fiji software.

*L. pneumophila* intracellular growth curves

To quantify *L. pneumophila* intracellular replication, macrophages were seeded in 24-well tissue culture plates and infected in triplicates at an MOI of 1 the next day. BMDMs were seeded at 2.5x10^5 in seeding media (RPMI, 10% HIFBS, 7.5% L929 cell supernatant), THP-1 cells were seeded at 5x10^5 and differentiated with 100 nM PMA in RPMI 10% HIFBS for three days. Cells were washed with PBS and incubated in the absence of PMA for one day prior to infection. Stable RAW 264.7 cells were seeded at 2.5x10^5 in DMEM 2.5%HIFBS. MH-S cells were cultured in RPMI 10% HIFBS and seeded at 2.5x10^5. At 1 h post-infection, cells were washed 3x with PBS followed by addition of fresh media. Macrophages were lysed in sterile water and colony forming units (CFU) were enumerated at the indicated time points as described (Ngwaga et al., 2019). Where indicated, 25 ng mL^-1 rTNF (Gibco), 5 ng mL^-1 rIFN-γ (Gibco), 10 µM H_2O_2 (VWR), 10 nM bafilomycin A1 (BAF; ApexBio), or 1 µM MCC950 (ApexBio) were added at the time of infection and...
maintained throughout. DMSO was used as a vehicle control where indicated. Fold replication was quantified by normalizing colony forming units to internalized bacteria at 1h post-infection.

Enzyme-linked immunosorbent assay (ELISA)

To quantify TNF secretion, 2.5 x 10^5 BMDMs were seeded in 24-well tissue culture plates and infected with the indicated *L. pneumophila* strains at an MOI of 10. After 1 h of infection, cells were washed with PBS, and media were replaced. Supernatants were at 8 h post-infection and used fresh or stored at -20°C for up to 1 week and TNF was quantified using mouse TNF-α ELISA MAX kit following manufacturer’s instructions. Absorbance at 450 nm was quantified on a BioTek Epoch2 microplate reader.

Quantification of cell death and metabolic activity

To quantify cell death, lactate dehydrogenase activity in cell supernatants was quantified. BMDMs were seeded in triplicates at 2.5x10^5 in a 24-well tissue culture plate for 24 h and infected with the indicated *L. pneumophila* strains at an MOI of 10 in 500 µL of seeding media (RPMP/10% HIFBS/7.5% L929 cell supernatant) and incubated for the indicated times. Supernatants were transferred to a 96-well plate and centrifuged at 200 r.c.f. for 10 min. LDH was quantified using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) according to manufacturer’s instructions. Absorbance at 490 nm was quantified on a BioTek Epoch2 microplate reader and percent cytotoxicity was calculated by normalizing absorbance values to cells treated with lysis buffer. Where indicated, cells were primed with 1 µM PAM3CSK4 (Tocris) for 24 h prior to infection.

To quantify cellular metabolic activity, an MTT colorimetric assay was used. BMDMs were seeded in a 96-well tissue culture plate at 1x10^5 and infected the next day with the indicated *L. pneumophila* strains at an MOI of 10. Cells were treated with 10 ng mL-1 rTNF and/or 10 µM staurosporine (Abcam), as indicated. Cells were assayed using the MTT Cell Proliferation Assay Kit (Colorimetric; BioVision) at 2 h and 24 h post-infection according to manufacturer’s
instructions. Absorbance at 590 nm was quantified on a BioTek Epoch2 microplate reader and percent viability was calculated by normalizing absorbance values to uninfected cells (100% viability).

Statistical analysis
Statistical analyses were performed with GraphPad Prism 9 software using either Students’ *t*-test, or two-way ANOVA, as indicated, with a 95% confidence interval, as indicated. Unless otherwise indicated, data are presented as mean ± standard deviation (s.d.) and statistical analyses was performed on samples in triplicates.

Acknowledgements
We thank Drs. Mary Weber, Kalyani Pyaram, and Philip Hardwidge for critical feedback on the manuscript and Dr. Russell Vance for femurs from Casp1−/− mice and helpful discussions. We also thank Andrew Haskell for assistance within blinded microscopic scoring of *L. pneumophila*-infected cells. This work was funded by NIH/NIGMS P20GM130448 (to S.R.S.); Kansas State University Johnson Cancer Research Center Summer Stipend Award (to T.N.) and Faculty Expansion Award (to S.R.S.); a Kansas-INBRE Postdoctoral Fellowship (P20GM103418 to D.C.); Kansas-INBRE Semester Scholar Award (P20GM103418 to A.G.S.); and startup funds from Kansas State University (to S.R.S.).
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Figure 1. LegC4 attenuates *L. pneumophila* replication within cytokine-activated macrophages and the mouse lung. Growth of indicated *L. pneumophila* strains within (A) WT BMDMs, (B) THP-1 cells, (C) Myd88−/− BMDMs, (D) Tnfr1−/− BMDMs, or (E) Ifngr1−/− BMDMs over 48 h. Plasmid expression of *legC4* was induced with 1 mM IPTG. Macrophages were infected in triplicates and data are presented as mean ± standard deviation (s.d.). Asterisks denote statistical significance by Student’s t-test (*P<0.05, **P<0.01) and data are representative of three independent experiments. ∆Δ: ∆*flaA*/∆*legC4*. (F) Competitive index (CI) of *L. pneumophila* ∆Δ (pJBlegC4) or ∆*flaA*/∆*legC4* (pJB) versus *L. pneumophila* ∆*flaA* in the lungs of the indicated at 48 h post-infection. Mice were infected with a total of 5x10^6 bacteria in a 1:1 mixture and CI was calculated as
described (see Materials and Methods). Each symbol represents an individual animal and asterisks denote statistical significance by Two-way ANOVA (\(*P<0.001\), \(*P<0.05\)). Data are representative of three independent experiments. ∆∆: ΔflaΔlegC4
**Figure 2.** LegC4 interacts with PA28α and PA28αβ is important for LegC4-mediated restriction.

(A) Western blot of co-immunoprecipitation of PA28α-Myc with 3xFLAG-LegC4 on α-FLAG conjugated magnetic beads (A) or 3xFLAG-LegC4 with GFP-PA28α on a-GFP conjugated magnetic beads (B) from lysates of transfected HEK 293T cells. Data are representative of two independent
experiments. (C) Confocal microscopy to image HeLa cells co-transfected to ectopically produce 3xFLAG-LegC4 (red) and either GFP-PA28α or GFP (green). Scale bar represents 10 µm. (D) Confocal micrograph of HeLa cells producing GFP-PA28α (arrowhead) or GFP-PA28α and 3xFLAG-LegC4 (arrows). Scale bar represents 50 µm. (E) Pearson Correlation Coefficients for cells producing 3xFLAG-LegC4 and either GFP-PA28α or GFP alone (n=4). PCC ≥ 0.7 (dashed line) indicates a high confidence association. Asterisks denote statistical significance by Students’ t-test (**P<0.01). Data shown are representative of two independent experiments. (F) Fold replication of indicated L. pneumophila strains within WT (left) or Psme1/2−/− (right) or BMDMs. Plasmid expression of legC4 was induced with 1 mM IPTG. Macrophages were infected in triplicates and data are presented as mean ± s.d. Asterisks denote statistical significance by Students’ t-test (**P<0.01). Data shown are representative of three independent experiments. ∆Δ: ΔflaAΔlegC4
Figure 3. LegC4 impairs resolution of oxidative stress, which is sufficient for *L. pneumophila* restriction. (A) Confocal microscopy to visualize 3xFLAG-LegC4 (red) production by stable RAW 264.7 cells (RAW-LegC4). Mock transfected cells (RAW-Cntrl) were used as a control and nuclei were imaged with Hoechst. Scale bar represents 10 µm. (B) Western blot analysis of 3xFLAG-LegC4 immunoprecipitated from lysates of RAW-LegC4, RAW-Cntrl or untransfected (UT) RAW cells using α-FLAG-conjugated beads. Arrow indicates 3xFLAG-LegC4 (~84 kDa). (C) Growth of *L. pneumophila* ΔflaA within RAW-LegC4 and -Cntrl cells. Data shown representative of three experiments. (D) ELISA quantification of protein carbonyls in lysates of RAW-LegC4 and -Cntrl cells incubated for 24 h in the presence or absence of 10 µM H2O2. Data are representative of two independent experiments. (E) Western blot for protein carbonyls and actin (loading control) in lysates of Tnfr1−/− BMDMs infected for 4 h with the indicated strains at an MOI of 50. ΔΔ: ΔflaAΔlegC4; plasmid expression of legC4 was induced with 1 mM IPTG. Data are representative of three independent experiments. (F) Fold replication of *L. pneumophila* strains at 48 h post-infection within Tnfr1−/− BMDMs in the presence or absence of 10 µM H2O2 as indicated. Plasmid expression of legC4 was induced with 1 mM IPTG. Data are representative of three independent experiments. (G-I) Fold replication of *L. pneumophila* (L.p.) ΔflaA within WT or Psme1/2−/− BMDMs untreated (G) or treated with (H) 5 ng mL−1 rIFN−γ or (I) 25 ng mL−1 rTNF. Macrophages were infected in triplicates and data are presented as mean ± s.d. Asterisks denote statistical significance by Student's *t*-test (***P<0.01). All quantitative data are presented as mean ± s.d. from samples in triplicates. Asterisks denote statistical significance by Student's *t*-test (***P<0.01; ns., not significant). Data are representative of two independent experiments. ΔΔ: ΔflaAΔlegC4
Figure 4. LegC4 induces lysosomal fusion with LCVs. Blinded immunofluorescence scoring of LAMP1 localization to LCVs within BMDMs derived from WT (A), Tnfr1<sup>−/−</sup> (B), or Psme1/2<sup>−/−</sup> (C) mice after 9 h of infection. Macrophages were infected in triplicates (n=300 per strain; moi of 30) and LAMP1 localization to LCVs was quantified over two independent experiments. Plasmid expression of legC4 was induced with 1 mM IPTG. Asterisks denote statistical significance by Students’ t-test (**P<0.01; ns, not significant). (D) Growth of <i>L. pneumophila</i> strains within WT BMDMs treated with 10 nM bafilomycin A1 (BAF) (left panel) or DMSO (vehicle control; right panel). Plasmid expression of legC4 was induced with 1 mM IPTG. Asterisks denote statistical significance by Students’ t-test (**P<0.01). All data are presented as mean ± s.d. of samples in triplicates and are representative of three independent experiments. ΔΔ: ΔflaAΔlegC4