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

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Abstract

Legionella pneumophila is a natural pathogen of protozoa that causes Legionnaires' Disease pneumonia via replication within host macrophages using an arsenal of hundreds of translocated virulence factors termed effector proteins. Effectors are critical for intracellular replication but can also enhance pathogen clearance by mammalian hosts via effector-triggered immunity. The effector LegC4 confers a fitness disadvantage on *L. pneumophila* in mouse models of Legionnaires' Disease and uniquely potentiates the antimicrobial activity of macrophages activated with either tumor necrosis factor (TNF) or interferon (IFN)- γ . Here, we investigated the mechanism by which LegC4 enhances macrophage restriction of *L. pneumophila* and found a central role for the induced proteasome activator (PA)28 $\alpha\beta$. PA28 $\alpha\beta$ facilitates ubiquitin-independent proteasomal degradation of oxidant-damaged proteins to relieve oxidative stress and impaired proteasome activity results in compensatory upregulation of lysosomal degradation pathways. We found that LegC4 binds the conserved PA28 α subunit and that the LegC4 restriction phenotype is abolished within PA28 $\alpha\beta$ -deficient macrophages. Moreover, LegC4 impaired the resolution of oxidative proteotoxic stress and enhanced cytokine-dependent phagolysosomal fusion with the *Legionella*-containing vacuole. PA28 $\alpha\beta$ has been traditionally associated with antigen presentation and adaptive immunity; however, our data support a model whereby suppression of PA28 $\alpha\beta$ by LegC4 culminates in the cell-autonomous lysosomal killing of *L. pneumophila* within activated macrophages. This work provides a solid foundation on which to evaluate induced proteasome regulators as mediators of innate immunity.

Significance Statement

Pro-inflammatory cytokines induce antimicrobial host defense pathways within macrophages to control intracellular pathogens. We discovered that the *Legionella pneumophila* effector protein LegC4 potentiates pathogen clearance within cytokine-activated macrophages. Here, we investigated LegC4 function and found that host proteasome activator (PA)28αβ was required for LegC4 restriction within macrophages. PA28αβ is cytokine-induced and facilitates proteasomal degradation of oxidant-damaged proteins. We found that LegC4 binds PA28α and impairs the resolution of oxidative stress. Loss of proteasome activity causes compensatory upregulation of lysosomal degradation pathways and, concomitantly, LegC4 increases phagolysosomal fusion with *Legionella*-containing vacuoles. These findings support a model whereby pathogen subversion of host proteostasis machinery triggers lysosomal pathogen targeting within activated macrophages.

Introduction

Many intracellular bacterial pathogens cause disease by replicating within host macrophages (1). Macrophages activated by T_H1 proinflammatory cytokines, such as tumor necrosis factor (TNF) and interferon (IFN)- γ , are potently and broadly microbicidal. However, 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)28 $\alpha\beta$, which engages proteolytic 20S proteasome core particles (CPs) and facilitates degradation of oxidant-damaged proteins (2). Impaired proteasome activity causes compensatory upregulation of lysosomal degradation pathways, which are central to cell-autonomous immunity within activated macrophages (3). PA28 $\alpha\beta$ has been traditionally associated with antigen presentation and adaptive immunity (4, 5); however, the contribution of PA28 $\alpha\beta$ and oxidative proteotoxic stress to antimicrobial host defense has not been investigated.

Legionella pneumophila is a natural pathogen of freshwater amoebae and the causative agent of Legionnaires' Disease pneumonia, which results from bacterial replication within alveolar macrophages. 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) (6). Effectors are essential for virulence, but their activity can also trigger upregulation of pro-inflammatory cytokine production by infected macrophages via effector-triggered immunity (ETI) (7–9). We discovered that the *L. pneumophila* effector LegC4, which is a *bona fide* virulence factor in amoebae, confers a fitness disadvantage on *L. pneumophila* in a mouse model of Legionnaires' Disease (10). 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 activated with either tumor necrosis factor (TNF) or interferon (IFN)- γ (10, 11).

LegC4 is the only *L. pneumophila* effector that confers quantifiable opposing host-dependent fitness phenotypes and the specific augmentation of cytokine-mediated restriction represents a potentially novel mechanism of ETI. However, the mechanism by which LegC4 functions and enhances cytokine-mediated restriction of *L. pneumophila* is unknown.

Here, we investigated how LegC4 activity suppresses *L. pneumophila* replication within activated macrophages. We found that LegC4 interacts with host PA28 α and that LegC4-mediated restriction was abolished within PA28 $\alpha\beta$ -deficient (*Psme1/2^{-/-}*) bone marrow-derived macrophages. We further revealed that LegC4 impairs resolution of oxidative proteotoxic stress, which is sufficient to induce the LegC4 restriction phenotype, and that LegC4 induces phagolysosomal fusion with intracellular *Legionella*-containing vacuoles (LCVs). In sum, these results support a model whereby PA28 $\alpha\beta$ targeting by a translocated effector protein perturbs proteostasis and enhances lysosomal targeting and restriction of *L. pneumophila* within cytokine-activated macrophages.

Results

LegC4 potentiates redundant cytokine-mediated restriction of *L. pneumophila* We previously reported that LegC4 confers a fitness disadvantage on *L. pneumophila* in intranasal mouse models of Legionnaires' Disease (LD) and within cytokine-activated macrophages (10, 11). C57Bl/6 mice are restrictive to *L. pneumophila* due to flagellin-mediated activation of the NAIP5/NLRC4 inflammasome (12, 13). Thus, we leveraged a flagellin (FlaA)-deficient *L. pneumophila* (Δ *flaA*), an established model used to study FlaA-independent host responses within C57Bl/6 mouse models of infection (12, 13). Using competitive index experiments, we found that *L. pneumophila* Δ *flaA* Δ *legC4* mutant strains outcompeted parental Δ *flaA* strains in the lungs of C57Bl/6 wild-type (WT) mice (11). However, no LegC4 fitness phenotype was observed within isogenic WT bone marrow-derived macrophages (BMDMs) (11). *L. pneumophila* replicates within

alveolar macrophages in the lung, but we did not observe any LegC4-dependent differences in *L. pneumophila* replication within MH-S mouse alveolar macrophages (**Fig S1**). However, LegC4-mediated restriction is observed within BMDMs activated with either purified recombinant (r)TNF or rIFN- γ (11). Thus, the phenotype observed for LegC4 *in vivo* is likely due to enhanced levels of cytokines seen in the *L. pneumophila*-infected lung compared to cultured macrophages (14). Alternatively, a phenotype for LegC4 can be observed in non-cytokine activated (naïve) BMDMs by overexpressing *legC4* from an inducible promoter on a complementing plasmid (**Fig 1A**) (11). 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 1B**). 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*^{-/-} and *Tnfr1*^{-/-}, respectively) (**Fig 1C, 1D**) (11). IFN- γ signaling is sufficient for LegC4 restriction (11); however, the LegC4 restriction phenotype was retained within *Ifngr1*^{-/-} BMDMs (**Fig 1E**), indicating that the relatively low levels of IFN- γ secreted by macrophages are not necessary for restriction. Furthermore, the loss of any single cytokine was not sufficient to restore *L. pneumophila* fitness since LegC4 still conferred a fitness disadvantage on *L. pneumophila* within the lungs of TNFR1-, MyD88- and IFNGR1-deficient mice (**Fig 1F**). These data further support our previous work showing that LegC4 augments redundant cytokine-mediated pathogen restriction mechanisms (11). While overexpression of *legC4* is not necessary to observe a phenotype, this infection model is robust and useful for our goal to define LegC4 function and mechanisms of cytokine-mediated restriction within macrophages.

LegC4 binds host proteasome activator (PA)28 α . 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)

28 α , PA28 γ , and Ecm29 (**Table S1**). The highest-confidence interactor was PA28 α with 28 individual clones, all of which encoded a minimal overlapping region in the PA28 α C-terminal domain (amino acid residues 105-249) (**Table S1, Fig S2**). We initially evaluated the PA28 α -LegC4 interaction by reciprocal co-immunoprecipitation from lysates of HEK 293T cells ectopically producing epitope-tagged PA28 α and LegC4. We found that PA28 α -Myc co-immunoprecipitated with 3xFLAG-LegC4 and that 3xFLAG-LegC4 co-immunoprecipitated with GFP-PA28 α (**Fig 2A**). We also found that ectopically produced GFP-PA28 α and 3xFLAG-LegC4 localize within transfected HeLa cells (**Fig 2B**). Ectopic expression of PA28 α was necessary since it is normally expressed only in response to cytokine signaling (15, 16). Together, these data suggest that LegC4 binds host PA28 α , which has not been previously identified as a target of bacterial effectors and provides an explanation as to why LegC4 only has a phenotype within cytokine-activated cells.

PA28 $\alpha\beta$ contributes to LegC4-dependent and -independent macrophage restriction of *L. pneumophila*. Based on the interaction between PA28 α and LegC4, we tested the hypothesis that PA28 α contributes to LegC4-mediated restriction of *L. pneumophila* within BMDMs. PA28 α -deficient (*Psme1*^{-/-}) mice are not commercially available, so we evaluated the role of PA28 α using BMDMs derived from PA28 $\alpha\beta$ -deficient (*Psme1/2*^{-/-}) mice. PA28 α associates with PA28 β to form the PA28 $\alpha\beta$ (11S) proteasome regulator. PA28 β is homologous to PA28 α , but is strictly dependent on PA28 α for activity and is hypothesized to enhance the interaction of PA28 α with 20S proteolytic proteasome core particles (CPs) (17). We found that PA28 $\alpha\beta$ was necessary for LegC4-mediated restriction of *L. pneumophila* since it was abolished within *Psme1/2*^{-/-} BMDMs (**Fig 2C**). Moreover, replication of *L. pneumophila* Δ *flaA* was attenuated within naïve and cytokine-activated *Psme1/2*^{-/-} BMDMs (**Fig 2D**), which phenocopies LegC4 and supports the hypothesis that LegC4 impairs PA28 α activity. Since autocrine and paracrine TNF signaling is sufficient for the LegC4 restriction phenotype, we tested whether PA28 $\alpha\beta$ or LegC4 enhance TNF

secretion from *L. pneumophila*-infected BMDMs. However, we found no LegC4- or PA28 $\alpha\beta$ -mediated differences in TNF secretion from infected BMDMs (**Fig S3**). We also found that viability of *L. pneumophila*-infected BMDMs was unaffected by either LegC4, PA28 $\alpha\beta$, or exogenous rTNF compared to untreated cells (**Fig S4**). These data suggest that the LegC4 restriction phenotype is dependent on PA28 $\alpha\beta$ and occurs independently of increased cytokine secretion or cell death, which support a novel role for PA28 $\alpha\beta$ in cell-autonomous immunity against an intracellular bacterial pathogen.

LegC4 impairs resolution of oxidative proteotoxic stress. In cells of the innate immune system, PA28 α is produced in response to oxidative stress, TNF, or IFN- γ and forms a heteroheptameric complex with PA28 β to assemble the 11S (PA28 $\alpha\beta$) proteasome regulator (2, 16, 18, 19). PA28 $\alpha\beta$ associates with proteasome CPs to generate antigenic peptides and resolve oxidative proteotoxic stress by facilitating ubiquitin- and ATP-independent proteasomal degradation of oxidant-damaged (carbonylated) proteins (4, 20). Since PA28 $\alpha\beta$ was required for LegC4-mediated *L. pneumophila* restriction within BMDMs (see Fig 2C), we tested the hypothesis that LegC4 impairs resolution of oxidative proteotoxic stress by quantifying protein carbonyls in cell lysates.

We directly evaluated how LegC4 impacts resolution of oxidative proteotoxic stress by quantifying protein carbonyls within RAW 264.7 cells stably producing 3xFLAG-LegC4 (RAW-LegC4). We confirmed production of 3xFLAG-LegC4 by confocal microscopy (**Fig 3A**) and Western blot (**Fig 3B**). Immunoprecipitation of 3xFLAG-LegC4 was necessary due to its low abundance. However, we validated that ectopically produced LegC4 was active, since replication of *L. pneumophila* $\Delta flaA$ within RAW-LegC4 cells was attenuated compared to control (RAW-Cntrl) cells (**Fig 3C**). To test the hypothesis that LegC4 impairs resolution of oxidative proteotoxic stress, we quantified protein carbonyls using ELISA within lysates of RAW-LegC4 and -Cntrl cells treated with hydrogen peroxide (H₂O₂). H₂O₂ is commonly used to induce oxidative stress since

superoxide radical ($O_2^{\cdot-}$) (precursor to H_2O_2) is unstable in solution (21–23). We found that the concentration of protein carbonyls in lysates of H_2O_2 -treated RAW-LegC4 cells was significantly greater than control cells (**Fig 3D**) and that LegC4 alone did not affect the concentration of protein carbonyls in untreated cells (**Fig 3D**). These data suggest that LegC4 is sufficient to impair resolution of oxidative proteotoxic stress.

We subsequently evaluated how LegC4 impacts the abundance of protein carbonyls within *L. pneumophila*-infected cells. To control for LegC4-mediated differences in bacterial replication within WT BMDMs, we leveraged *Tnfr1*^{-/-} BMDMs (see Fig 1). *Tnfr1*^{-/-} BMDMs were infected for 4 h in the presence or absence of H_2O_2 and protein carbonyls were visualized by Western blot. We found that the abundance of protein carbonyls was increased within H_2O_2 -treated BMDMs infected with *L. pneumophila* $\Delta flaA \Delta legC4$ (*plegC4*) relative to $\Delta flaA$ and empty vector control strains (**Fig 3E**). Within untreated BMDMs, infection with the avirulent *dotA*::Tn strain increased the abundance of protein carbonyls (**Fig 3E**), which is likely a consequence of increased global ROS production by macrophages infected with avirulent compared to virulent *L. pneumophila* (24). 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.

Oxidative stress is sufficient for LegC4-mediated restriction. 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 which LegC4 is not restrictive (see Fig 1C), in the presence or absence of a sublethal concentration of H_2O_2 to induce oxidative stress. We found that replication of *L. pneumophila* $\Delta flaA \Delta legC4$ (*plegC4*) strain was significantly attenuated within H_2O_2 -treated *Tnfr1*^{-/-} BMDMs compared to *L. pneumophila* $\Delta flaA$ and

$\Delta flaA \Delta legC4$ 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 μ M) of H₂O₂. It is unlikely that growth attenuation was due to direct *L. pneumophila* killing by H₂O₂ since *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) (22, 24–26). Thus, these data suggest that oxidative stress is sufficient for the LegC4 restriction phenotype.

LegC4 increases phagolysosomal fusion with *Legionella*-containing vacuoles. Our current data show that LegC4 functions through PA28 $\alpha\beta$ and confers a fitness disadvantage under oxidative stress conditions. Impaired proteasome activity causes compensatory upregulation of lysosomal degradation pathways to cope with proteotoxic stress (3, 27). Based on our data showing that oxidative proteotoxic stress is sustained in LegC4-producing cells, we tested the hypothesis that LegC4 increases lysosomal targeting of *L. pneumophila* within activated macrophages. Virulent *L. pneumophila* establish a replicative *Legionella*-containing vacuole (LCV) by blocking endocytic maturation of their phagosome (28, 29). However, avirulent (*dotA::Tn*) *L. pneumophila* 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 (28) (**Fig S5**). 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 $\Delta flaA$ and empty vector control strains compared to the avirulent control (**Fig 4A**). There was a significant increase in the percentage of LAMP1⁺ LCVs harboring $\Delta flaA \Delta legC4$ (*plegC4*) compared to $\Delta flaA$ and empty vector control strains within both WT and *Tnfr1*^{-/-} BMDMs (**Fig 4A**). However, within WT BMDMs, LAMP1 localization to LCVs harboring $\Delta flaA \Delta legC4$ (*plegC4*) did not differ significantly from the avirulent *dotA::Tn* strain (**Fig 4A**), which was surprising since plasmid expression of

legC4 did not impair biogenesis of a replicative LCV (≥ 5 bacteria/vacuole) compared to *L. pneumophila* $\Delta flaA$ and $\Delta flaA \Delta legC4$ (pEV) control strains (**Fig 4B**). As expected, the avirulent control strain was significantly attenuated for replicative LCV formation compared to the parental *L. pneumophila* $\Delta flaA$ strain (** $P < 0.01$, **Fig 4B**). These data correlate with our CFU data showing that that LegC4 restriction is observed after 24 h of infection within BMDMs (Fig 1A) and challenge the dogma that LAMP1 localization and *L. pneumophila* intracellular replication are mutually exclusive. Moreover, these data support our hypothesis that LegC4 enhances phagolysosomal fusion with LCVs.

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 (30). 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 (31); however, intracellular replication is minimally impacted within BMDMs treated with a low concentration (12.5 nM) of BAF (32). We found that the LegC4 restriction phenotype was abolished within BAF-treated BMDMs (**Fig 4C**), suggesting that phagolysosomal fusion and lysosomal acidification are important for LegC4-mediated restriction. Together, our data support a central role for phagolysosomal fusion and vacuolar acidification in LegC4-mediated restriction of *L. pneumophila* within BMDMs.

Discussion

This study supports a novel role for proteasome activator (PA)28 $\alpha\beta$ in cell-autonomous host defense against an intracellular pathogen. We found that the *L. pneumophila* effector LegC4 enhances restriction of *L. pneumophila* within macrophages activated with either TNF or IFN- γ in a PA28 $\alpha\beta$ -dependent manner. Our results suggest a model whereby LegC4 impairs resolution of

oxidative proteotoxic stress and promotes lysosomal restriction of *L. pneumophila* by subverting PA28 $\alpha\beta$ activity (**Fig S6**). This model is supported by our data showing that LegC4 binds PA28 α , PA28 $\alpha\beta$ is required for the effects of LegC4 on host cells, LegC4 impairs resolution of oxidative proteotoxic stress, and the contribution of both oxidative stress and phagolysosomal fusion to LegC4-mediated restriction of *L. pneumophila*. This work is the first describe a role for PA28 $\alpha\beta$ in innate and cell-autonomous host defense.

L. pneumophila is an accidental pathogen of humans that rarely transmits between infected individuals. Thus, an evolutionary basis for the LegC4-PA28 α interaction can be explained by LegC4 targeting the PA28 α homolog in amoebae. Indeed, the natural host *A. castellanii*, in which LegC4 is a *bona fide* virulence factor (10), encodes a PA28 α homolog (AcPA28) that shares 34% identity with PA28 α . Sequence identity between PA28 α and AcPA28 is primarily in the C-terminal domain, which is important for engaging 20S CPs and was contained in all PA28 α clones identified in our yeast two-hybrid screen (Fig 2A; Table S1). These data together with our observation that the LegC4 phenotype is recapitulated in PA28 $\alpha\beta$ -deficient macrophages have led us to speculate that LegC4 binding prevents PA28-CP complex formation. While the function of AcPA28 has not been described, structural studies have shown that mammalian PA28 α and evolutionary distant PA28s are similar and all associate with proteolytic proteasome core particles, suggesting that it likely also functions as a proteasome regulator (33, 34). 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 mammalian cells 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_2O_2) was sufficient for the LegC4 restriction phenotype. H_2O_2 is routinely used to induce oxidative stress in laboratory models since both superoxide ($O_2^{\cdot-}$) and hydroxyl ($OH^{\cdot}$) 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 (24, 35, 36). 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 (24, 35). 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_2O_2 *in vitro* (orders of magnitude greater than intracellular concentrations) (22, 24–26). Moreover, *Tnfr1*^{-/-} BMDMs treated with H_2O_2 did not suppress replication of *L. pneumophila* control strains compared to untreated BMDMs (see Fig 3F). This suggests that the concentration of H_2O_2 used in our study is insufficient for direct *L. pneumophila* killing. Future studies will reveal whether LegC4 or PA28 $\alpha\beta$ 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 pathogen restriction within IFN- γ -activated macrophages involves immunity-related GTPases (IRGs), which localize to and facilitate lysosomal trafficking to pathogen-containing phagosomes (37). Although IRGs may contribute to restriction of LegC4-producing *L. pneumophila* strains within IFN- γ -activated macrophages (38), we found that IFNGR1 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 (36). 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 (39). Autophagy is also upregulated to prevent aberrant pyroptotic cell death below a certain threshold of inflammasome agonist (40). The NLRC4 inflammasome is dispensable for LegC4-mediated restriction (10, 11), but a role for the NLRP3 inflammasome is possible since it is activated by proteotoxic stress (41). Inflammasome components and pro-inflammatory cytokines are selectively degraded below a threshold of agonist. We are currently evaluating the role of autophagy and the NLRP3 inflammasome in PA28 $\alpha\beta$ - and LegC4-mediated restriction of *L. pneumophila*.

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 (31, 42). 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. Since we observed no attenuation in replicative LCV formation or intracellular replication by CFU assay by 24 h post-infection, these data suggest that *L. pneumophila* intracellular replication and LAMP1 localization to LCVs are not mutually exclusive. It is tempting to speculate that *L. pneumophila* replication within a LAMP1⁺ LCV may result from translocated effectors that block v-ATPase activity, such as SidK (43–46). Interestingly, loss of TNFR1-mediated signaling did not fully abrogate LegC4-mediated LAMP1 localization to LCVs. We think this may be a consequence of sustained oxidative stress downstream of pattern recognition receptors. However, phagolysosomal fusion with LCVs is not sufficient for LegC4 to impair replication within Tnfr1^{-/-} BMDMs, further supporting our observation that LegC4 restriction occurs in a dose-dependent manner. The properties of a replication

permissive LAMP1⁺ LCV, the contribution of additional translocated effectors, and the contribution of canonical autophagic degradation pathways are currently being investigated in our lab.

In summary, we have uncovered a new role for PA28 $\alpha\beta$ and oxidative proteotoxic stress in cell-autonomous pathogen restriction. This work is also the first to show bacterial pathogen targeting of PA28 α and suggests that suppression of PA28 $\alpha\beta$ activity may be a pathogen-triggered host defense mechanism. Moreover, our data suggest that inhibition of PA28 $\alpha\beta$ may enhance innate immunity against intracellular pathogens by potentiating existing inflammatory responses. Future studies will provide further insight into the ubiquity and mechanism(s) of pathogen restriction induced by proteotoxic stress and suppression of PA28 $\alpha\beta$ 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 (47). Liquid cultures were grown overnight with shaking at 37°C in supplemented ACES-buffered yeast extract (AYE) medium, as described (11, 48). For plasmid maintenance, CYE was supplemented with 10 $\mu\text{g mL}^{-1}$ chloramphenicol and *legC4* expression was induced with 1 mM isopropyl- β -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

All mice were on a C57BL/6 background and 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 (49). 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.

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 5×10^6 bacteria were diluted and plated on selective medium ($10 \mu\text{g mL}^{-1}$ chloramphenicol for plasmid selection). At 48 h post-infection, mice were euthanized, and lung homogenates were plated on selective media as described (11). CFU were enumerated and used to calculate CI values $[(\text{CFUcm}^{\text{R}}_{48\text{h}}/\text{CFUwt}_{48\text{h}})/(\text{CFUcm}^{\text{R}}_{\text{IN}}/\text{CFUwt}_{\text{IN}})]$.

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 (50). To produce GFP-PA28 α , *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/BamHI fragment into pEGFPC1 (Clontech). Plasmid DNA was transfected into mammalian cells as described below.

Cell Culture and Transfections

HEK 293T, HeLa cells, 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 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 (51). 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.

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 Na₄P₂O₇, 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 α-FLAG M2 antibody (Sigma Aldrich) or rabbit α-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 Laemmli sample buffer for Western blot analysis.

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 a-FLAG (Sigma-Aldrich, F1804), rabbit a-Myc (Cell Signaling, 2278S), rabbit a-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.

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 5×10^5 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 μ M H_2O_2 . After 24 h, cells were washed twice in PBS and lysed in ice-cold 500 μ L 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 μ g 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 2.5x10⁵ one day prior to infection with indicated *L. pneumophila* strains at an MOI of 50 in the presence or absence of 10 μM H₂O₂. 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 H₂O₂. 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 α-β-actin antibody (1:1000; Cell Signaling Technology) followed by goat-α-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* (11) 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 (52) and pP6 is based on the pGADGH plasmid (53).

60 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-a*) yeast strains as previously described (52). 303 His⁺ colonies were selected on a medium lacking tryptophan, leucine and histidine, and supplemented with 2mM 3-aminotriazole to handle bait auto-activation. 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 (54).

Immunofluorescence Microscopy

To quantify LAMP1-LCV co-localization and the number of *L. pneumophila* per LCV, 1x10⁵ BMDMs were seeded on poly-L-lysine-coated glass coverslips in 24-well plates and infected with *L. pneumophila* at a multiplicity of infection (MOI) of 30. Coverslips were fixed with 4% paraformaldehyde (PFA; ThermoFisher) and permeabilized in ice cold methanol. Coverslips were stained 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). Slides were blinded and LAMP1 localization to LCVs were scored on a Leica DMiL LED inverted epifluorescence microscope ($n=150$ cells).

To image ectopically expressed LegC4 and PA28 α , 5x10⁵ HeLa cells were seeded on poly-L-lysine-coated glass coverslips in 24-well tissue culture plates and transfected for 24 h. Coverslips were fixed in 4% PFA and stained with mouse α -FLAG M2 (1:1,000) and rabbit α -GFP (1:5,000) primary antibodies and 1:5,00 Alexa546-conjugated goat α -mouse and Alexa488-conjugated goat α -rabbit secondary antibodies, as described above. Images were acquired on a Zeiss AxioPlan LSM-5 laser-scanning confocal microscope (KSU Division of Biology Microscopy Facility). Images were assembled using Fiji-ImageJ and Adobe Photoshop software.

L. pneumophila intracellular growth curves

To quantify *L. pneumophila* intracellular replication, macrophages were seeded in 24-well tissue culture plates and infected at an MOI of 1 the next day. BMDMs were seeded at 2.5×10^5 in seeding media (RPMI, 10% HIFBS, 7.5% L929 cell supernatant), THP-1 cells were seeded at 5×10^5 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 2.5×10^5 in DMEM 2.5% HIFBS. MH-S cells were cultured in RPMI 10% HIFBS and seeded at 2.5×10^5 . 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 (11). Where indicated, 25 ng mL⁻¹ rTNF (Gibco), 5 ng mL⁻¹ rIFN- γ (Gibco), 10 μ M H₂O₂ (VWR), 12.5 nM bafilomycin A1 (BAF; ApexBio), or 100 nM ONX 0914 (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, 2.5×10^5 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 at 2.5×10^5 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 μ L of seeding media (RPMI/10% HIFBS/7.5% L929

cell supernatant) and incubated for 10 h. 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.

To quantify cellular metabolic activity, an MTT colorimetric assay was used. BMDMs were seeded in a 96-well tissue culture plate at 1×10^5 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 μ M 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 Mann-Whitney U test, Students *t*-test, or two-way ANOVA as indicated with a 95% confidence interval. Unless otherwise indicated, data are presented as mean $\pm$ standard deviation (s.d.) and statistical analysis was performed on samples in triplicates.

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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 $\pm$ standard deviation (s.d.). Asterisks denote statistical significance by Student's *t*-test (**P*<0.05, ***P*<0.01). (F) Competitive index (CI) of *L. pneumophila* $\Delta flaA\Delta legC4$ (pJB $legC4$) or $\Delta flaA\Delta legC4$ (pJB) versus *L. pneumophila* $\Delta flaA$ in the lungs of the indicated mice at 48 h post-infection. Each symbol represents an individual animal and asterisks denote statistical significance by Mann-Whitney U test (***P*<0.01). Data shown are representative of at least three independent experiments.

Figure 2. LegC4 binds and functions through PA28 $\alpha\beta$. **(A)** Western blot of co-immunoprecipitation of PA28 α -Myc with 3xFLAG-LegC4 on α -FLAG conjugated magnetic beads (top) or 3xFLAG-LegC4 with GFP-PA28 α on α -GFP-conjugated magnetic beads (bottom) from lysates of transfected HEK 293T cells. **(B)** Confocal microscopy to image HeLa cells co-transfected to ectopically produce 3xFLAG-LegC4 (red) and either GFP-PA28 α or GFP (green). Scale bar represents 10 μ m. **(C)** Fold replication of indicated *L. pneumophila* strains within *Psme1/2^{-/-}* (left) or WT (right) BMDMs. Plasmid expression of *legC4* was induced with 1 mM IPTG. **(D)** Fold replication of *L. pneumophila* (*L.p.*) $\Delta flaA$ within WT or *Psme1/2^{-/-}* BMDMs at 48 h post-infection. Where indicated BMDMs were treated with 25 ng mL⁻¹ recombinant mouse (r)TNF, 5 ng mL⁻¹ rIFN- γ , or left untreated (UT). Macrophages were infected in triplicates and data are presented as

691 mean $\pm$ s.d. Asterisks denote statistical significance (** $P < 0.01$) by Students' *t*-test. Data shown
692 are representative of at least two independent experiments.
693

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 μ m. **(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* Δ flaA within RAW-LegC4 and -Cntrl cells infected for up to 48 h. **(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 μ M H₂O₂. **(E)** Western blot for protein carbonyls and actin (loading control) in lysates of *Tnfr1*^{-/-} BMDMs infected for 4 h with the indicated strains. $\Delta\Delta$: Δ *flaA* Δ *legC4*; plasmid expression of *legC4* was induced with 1 mM IPTG. **(F)** Fold replication of *L. pneumophila* strains at 48 h post-infection within *Tnfr1*^{-/-} BMDMs in the presence or absence of 10 μ M H₂O₂ as indicated. Plasmid expression of *legC4* was induced with 1 mM IPTG. All quantitative data are presented as mean $\pm$ s.d. from samples in triplicates and are representative of at least two independent experiments. Asterisks denote statistical significance by Students' *t*-test (***P*<0.01; n.s., not significant).

Figure 4. Role of phagolysosomal fusion in LegC4-mediated restriction of *L. pneumophila*. **(A)**

Blinded immunofluorescence scoring of LAMP1 localization to LCVs within WT or *Tnfr1*^{-/-}

BMDMs after 9 h of infection. Plasmid expression of *legC4* was induced with 1 mM IPTG. Data

are representative of at least two independent experiments ($n \geq 150$ per experiment) and letters

denote statistical significance by Students' *t*-test ($P < 0.05$). letters not significant) of values com-

pared to Δ*flaA*. **(B)** WT BMDMs were infected with the indicated *L. pneumophila* strains and the

number of bacteria per LCV was quantified by blinded immunofluorescence scoring. Data are

representative of at least two independent experiments ($n \geq 150$ per experiment) and asterisks

denote statistical significance by Students' *t*-test (** $P < 0.01$; n.s., not significant) of values com-

pared to Δ*flaA*. Plasmid expression of *legC4* was induced with 1 mM IPTG. **(C)** Growth of

725 indicated *L. pneumophila* strains within WT BMDMs treated with 12.5 nM bafilomycin A1 (BAF)
 726 or DMSO (vehicle control). Plasmid expression of *legC4* was induced with 1 mM IPTG. Data are
 727 presented as mean $\pm$ s.d. of samples in triplicates and are representative of at least two independ-
 728 ent experiments. Asterisks denote statistical significance by Students' *t*-test (** $P < 0.01$).
 729