Pathogenic, but not non-pathogenic, *Rickettsia* evade inflammasome-dependent IL-1 responses to establish an intracytosolic replication niche.

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Author Contributions

O.H.V., M.S.R., and A.F.A. planned the research, analyzed, and interpreted the data; O.H.V., J.C., H.G., N.R.D., and M.S.R. performed the experiments; A.F.A., O.H.V., L.D., and R.S. contributed to the overall project administration and supervision; O.H.V., M.S.R., and A.F.A. wrote the manuscript; and all authors participated in editing the manuscript.

ABSTRACT

Rickettsia species (spp.) are strict obligate intracellular bacteria, with some being pathogenic in their mammalian host, including humans. One critical feature of these stealthy group of pathogens is their ability to manipulate hostile cytosolic environments to their benefits. Although our understanding of *Rickettsia* cell biology and pathogenesis are evolving, the mechanisms by which pathogenic *Rickettsia* spp. evade host innate immune detection remains elusive. Here, we showed that disease severity in wild-type (*WT*) C57BL/6J mice infected with *R. typhi* (etiologic agent of murine *typhus*) and *R. rickettsii* (etiologic agent of Rocky Mountain Spotted Fever), but not with non-pathogenic *R. montanensis*, correlated with levels of bacterial burden as detected in the spleens, as well as the serum concentrations of pro-inflammatory cytokine IL-1 α and to a lesser extent IL-1 β . Antibody-mediated neutralization of IL-1 α confirmed a key role in controlling mortality rates and bacterial burdens of rickettsiae-infected *WT* mice. As macrophages are a primary source of both IL-1 α and IL-1 β cytokines, we determined the mechanism of the anti-rickettsial activities using bone-marrow-derived macrophages. We found that pathogenic *R. typhi* and *R. rickettsii*, but not non-pathogenic *R. montanensis*, eluded pro-IL-1 α induction and benefited pre-dominantly from the reduced IL-1 α secretion, via a Caspase-11-Gsdmd-dependent pathway, to facilitate intracytosolic replication. Adoptative transfer experiments identified that IL-1 α secretion by macrophages was critical for controlling rickettsiosis in *WT* mice. In sum, we identified a previously unappreciated pathway by which pathogenic, unlike non-pathogenic, rickettsiae

preferentially target the Caspase-11-Gsdmd-IL-1 α signaling axis in macrophages thus supporting their replication within the host.

IMPORTANCE

Currently, no vaccines are available to prevent rickettsioses, while vector-borne rickettsial infections in humans are on the rise globally. In fact, the insufficient understanding of how pathogenic *Rickettsia* species circumvent host immune defense mechanisms has significantly hindered the development of more effective therapeutics. Here, we identified a previously unappreciated role for the Caspase-11-Gsdmd-IL-1 α signaling axis, to limiting the replication of pathogenic *R. rickettsia* and *R. typhi* species in murine macrophages and wild-type (WT) C57BL/6J mice. Adoptative transfer studies further identified IL-1 α -secreting macrophages as critical mediators in controlling rickettsial infection in WT mice. Collectively, these findings provide insight into the potential mechanism of how pathogenic, but not non-pathogenic *Rickettsia* spp., benefit from a reduction in the Caspase-11-Gsdmd-mediated release of IL-1 α to support host colonization.

INTRODUCTION

Invasive cytosolic bacteria, including *Listeria*, *Shigella*, *Burkholderia*, *Francisella*, *Orientia*, and *Rickettsia* species (spp.) have developed strategies to induce their own uptake by phagocytosis and to circumvent host innate immune defenses for their intracellular survival (1, 2). Human infection with *Rickettsia* spp. occurs via infected hematophagous arthropods such as fleas, ticks, and human body lice (3), either through the bite or deposited infected feces into skin and mucosal surfaces. Upon entry, *Rickettsia*

spp. encounter tissue-resident immune cells, like macrophages (MΦ). Activated MΦ play a crucial role in either terminating an infection at an early stage, which commonly is the fate of non-pathogenic *Rickettsia* spp., or succumbing to bacterial replication and pathogen colonization as well as host dissemination to distant organs (3). After internalization into host cells, *Rickettsia* spp. escape from phagosomes and subvert host cytosolic defense mechanisms (*i.e.*, autophagy and inflammasomes) to establish an intracytosolic replication niche. Recently, we reported that pathogenic *Rickettsia* spp. secrete effectors to promote host colonization by modulating endoplasmic reticulum structures or by hijacking the autophagic defense pathway (4–9). Subversion of autolysosomal destruction to colonize the host cytosol exposes *Rickettsia* spp. to another cytosolic host sensor-regulated defense mechanism, the inflammasomes (1, 10). Inflammasomes are immune signaling complexes categorized into canonical [Caspase (Casp)-1] and non-canonical [murine Casp-11 or human Casp-4/5] inflammasomes. The inflammasome complex assembly involves the adaptor protein ASC and upstream sensors including NLRP1, NLRP3, NLRC4, AIM2, and pyrin, which are primed by exogenous pathogen-associated molecular pattern molecules (PAMPs) and activated through endogenous damage-associated molecular pattern molecules (DAMPs). Initiation of the canonical inflammasome results in the activation of Casp-1. Active Casp-1 leads to the maturation of proinflammatory cytokines IL-1β and IL-18 and the activation of gasdermin D (Gsdmd), the executor of pyroptosis (11, 12). Of note, recent findings have also suggested that active Casp-11 is capable of activating Gsdmd (11, 12). Although IL-1β is released by both canonical and non-canonical inflammasome pathways, IL-1α is preferentially released by the non-canonical inflammasome pathway

(13–15). IL-1 α is expressed by a wide range of hematopoietic and non-hematopoietic cell types, whereas IL-1 β is primarily produced by myeloid cells (15). Importantly, although IL-1 α and IL-1 β signal through the same receptor, IL-1R, these two cytokines are not completely functionally redundant (15). Given the importance for both canonical and non-canonical inflammasome-mediated IL-1 signaling in limiting pathogen colonization, many bacteria have evolved strategies to block their activation (16–21). In fact, various pathogenic intracellular bacteria utilize their own effector repertoire to evade these pathways to successfully colonize and disseminate in their host cells (10, 22).

In the case of *Rickettsia*, our understanding of the role of inflammasomes in controlling host colonization is only now emerging (23–25). Specifically, how pathogenic *Rickettsia* spp. manipulate immune defenses to replicate within the host cytosol not only relies almost exclusively on data from tick-transmitted rickettsiae [e.g., members of spotted fever group (SFG) or transition group (TRG)], but also shows that these pathogens likely employ species-specific strategies to evade host cytosolic defense mechanisms. For instance, *R. australis*, a pathogenic TRG member, benefited from ATG5-mediated autophagy induction and suppression of inflammasome-dependent IL-1 β production to colonize both BMDM Φ (23) and mice (26). In contrast, *R. parkeri*, a mildly pathogenic member of SFG (see Fig. 1A), utilizes its surface cell antigen Sca5 (OmpB) for protection against autophagic recognition, and consequently benefits from inflammasome-mediated host cell death that antagonizes the action of Type I IFN in BMDM Φ and mice (24, 27). In contrast, our recent report on the flea-transmitted *R. typhi* [pathogenic member of typhus group (TG)], showed that *R. typhi* is ubiquitinated upon host entry and escapes autolysosomal fusion to establish an intracytosolic niche in non-phagocytic cells (9).

These unexpected findings on how members of SFG, TRG, and TG *Rickettsia* differentially promote intracytosolic host survival prompted us to explore the underlying mechanism(s) by which pathogenic, but not non-pathogenic *Rickettsia* spp., block immune defense responses to establish a replication niche in phagocytic cells, like MΦ. Specifically, we sought to test the hypothesis that pathogenic, but not non-pathogenic, *Rickettsia* spp. reduce inflammasome-mediated IL-1 responses thereby promoting their intracytosolic replication within host cells.

RESULTS

In vivo models of rickettsiosis.

At present, more than 30 *Rickettsia* spp. have been described globally, but less than a dozen is known to cause disease in humans, with some being notoriously pathogenic and associated with high morbidity and mortality, while others exert limited or no pathogenicity (Fig. 1A). We sought to test our hypothesis that pathogenic, but not non-pathogenic, *Rickettsia* spp. evade immune responses in host defense cells, like MΦ, to replicate and disseminate. We simultaneously evaluated the cytosolic host defense responses between pathogenic [*R. rickettsii* (Sheila Smith, SS) and *R. typhi* (Wilmington)] and non-pathogenic (*R. montanensis*) strains *in vivo*. We first established a mouse model of mild (~LD₂₅) or more severe (~LD₅₀) rickettsiosis in C57BL/6J *WT* mice. For both *R. rickettsii* and *R. typhi* LD₂₅ or LD₅₀ were achieved with doses of 10⁵ or 10⁶ PFU, respectively (Figs. 1B-C), however, *R. montanensis*-infected mice showed no signs of lethality at either 10⁵ or 10⁶ PFU (Fig. 1D). Bacterial burden in spleens from infected C57BL/6J *WT* mice (only dose of 10⁶ PFU is shown) confirmed successful infection with

all three *Rickettsia* spp. at day 3 post-infection, while *R. typhi*- and *R. rickettsii*-infected *WT* mice displayed significantly higher bacterial burden in the spleens as compared to splenic tissues from *R. montanensis*-infected mice at day 7 (Fig. 1E). This correlated with the observed differences in the spleen sizes and weights of the infected animals (Fig. S1). Given the earlier findings from other laboratories and ours (9, 23–28), we hypothesized that the observed dissimilarities in pathogenicity among *Rickettsia* spp. is likely linked to differences in host defense responses. Recent findings further suggest that the intracytosolic survival of different *Rickettsia* spp. is either supported or suppressed by immune defense responses (e.g., IFN-I, TNF- α , or IL-1 β) (23–28), and thus leaving the precise mechanism to be determined. Therefore, we first sought to evaluate immune defense responses at the level of IL-1 β and IL-1 α cytokine secretion in the sera of *R. typhi*-, *R. rickettsii*-, and *R. montanensis*-infected animals. The increase in mortality and elevated bacterial burden correlated with reduced serum levels of both IL-1 β and IL-1 α cytokines (Figs. 1F-G), suggesting that reduced activation of both IL-1 signaling responses is a potential mechanism for lethality and survival of pathogenic *Rickettsia* spp.

Anti-rickettsial activity of IL-1 α is involved in restricting *Rickettsia* infection.

To characterize further the role of IL-1 α and IL-1 β cytokines in restricting non-pathogenic and pathogenic *Rickettsia* spp. *in vivo*, IL-1 α or IL-1 β function was neutralized via tail vein (*i.v.*) injection, with anti(α)-IL-1 α , α IL-1 β , or α lgG-isotype control antibodies (Abs) into C57BL/6J *WT* mice in our established model of mild rickettsiosis (LD₂₅; $\sim 10^5$ PFU, Figs. 1B-D). Neutralization of IL-1 α , and to a much lesser extent IL-1 β , was

associated with a significant increase in the mortality of *R. typhi*-, *R. rickettsii*-, and *R. montanensis*-infected mice (Figs. 2A-C) and resulted in the development of splenomegaly (Fig. S2), which correlated with an increase in bacterial loads in the spleen (Fig. 2D). The efficiency of Ab-mediated blocking was confirmed by measuring the levels of IL-1 β and IL-1 α cytokine in the sera of the rickettsiae-infected mice (Figs. 2E-F). Next, we sought to determine the effect of administering recombinant (r)IL-1 α or rIL-1 β proteins on *Rickettsia* colonization *in vivo*. Accordingly, we administered (*i.v.*) endotoxin-free rIL-1 α and rIL-1 β proteins following infection with *R. typhi*, *R. rickettsii*, and *R. montanensis*. Pretreatment of mice with rIL-1 α , and to a lesser extent rIL-1 β , protected C57BL/6J *WT* mice from pathogenic *Rickettsia*-induced lethality (Figs. 3A-C), with both reduced splenomegaly (Fig. S3), and decreased splenic bacterial burdens (Fig. 3D). Moreover, the observed phenotypes correlated with the measured IL-1 α and IL-1 β serum concentrations (Figs. 3E-F). Collectively, these findings suggest that IL-1 α , and to a lesser extent, IL-1 β , are involved in restricting the replication and colonization of non-pathogenic and pathogenic *Rickettsia* spp. in C57BL/6J *WT* mice.

Pathogenic, but not non-pathogenic, *Rickettsia* species block IL-1 α secretion and avoid pro-IL-1 α induction to establish a replication niche in macrophages.

As M Φ are one of the cell types first encountered during infection by *Rickettsia* spp. and are considered to play a crucial role in either terminating the infection early at the skin site or allow initial pathogen colonization and subsequent dissemination within the infected host (3), we tested the hypothesis that pathogenic, but not non-pathogenic, rickettsiae suppress M Φ immune responses. In this effort, we determine the importance

of IL-1 α and IL-1 β in restricting the replication of *Rickettsia* spp. by infecting BMDM Φ derived from *WT*, *Il-1 β ^{-/-}* or *Il-1 α ^{-/-}* mice with pathogenic *R. typhi* and *R. rickettsii* or non-pathogenic *R. montanensis* and assessed the cytokine levels of IL-1 β and IL-1 α in cultured supernatants as well as bacterial burdens. Infection of *Il-1 β ^{-/-}* or *Il-1 α ^{-/-}* BMDM Φ with *Rickettsia* spp. did not result in the secretion of either IL-1 β or IL-1 α , respectively, when compared to infected *WT* BMDM Φ (Figs. 4A-F). Moreover, infections with *R. montanensis* resulted in overall higher IL-1 responses and lower bacterial burden when compared to infections performed with *R. typhi* or *R. rickettsii* in *WT*, *Il-1 β ^{-/-}* or *Il-1 α ^{-/-}* BMDM Φ (Figs. 4A-G). Of note, the bacterial burden of all three *Rickettsia* spp. in infected *Il-1 α ^{-/-}* BMDM Φ was higher than the levels detected in *Il-1 β ^{-/-}* or *WT* BMDM Φ (Fig. 4G), suggesting that IL-1 α and to significantly lesser extent IL-1 β , plays a role in restricting rickettsiae survival. In agreement with our *in vivo* data, infection assays using BMDM Φ also displayed an overall higher bacterial burden of both pathogenic *Rickettsia* spp., as compared to the non-pathogenic *Rickettsia* strain (Figs. 4G and 1E). We further tested the protein expression levels of pro-IL-1 β and -IL-1 α upon bacterial-infection of *WT* BMDM Φ and showed that pro-IL-1 β levels were induced by all three *Rickettsia* spp. to similar levels (~5-fold) when compared to uninfected *WT* BMDM Φ (Fig. 4H). Intriguingly, only *R. montanensis*-infected *WT* BMDM Φ produced significantly higher levels of pro-IL-1 α , as compared to *R. typhi*- or *R. rickettsii*-infected M Φ (Fig. 4H).

Intracytosolic replication of pathogenic *Rickettsia* species in macrophages depends on the inhibition of IL-1 cytokine secretion via a Caspase-11-Gsdmd-dependent pathway.

As our findings suggest that pathogenic, but not non-pathogenic, *Rickettsia* spp. prevent the activation of signaling pathways required for IL-1 α and IL-1 β production and release, we explored the mechanism of IL-1 signaling to a greater detail. As IL-1 signaling responses commonly involves the canonical and non-canonical inflammasome pathways, which in turn involves the proteolytic processing of both cytokines by activated Caspase (Casp)-1 (canonical) and Casp-11 (non-canonical) respectively, we assessed their potential role in regulating replication of non-pathogenic vs. pathogenic *Rickettsia* in BMDM Φ . In this effort, BMDM Φ derived from *WT*, *Casp-1*^{-/-}, *Casp-11*^{-/-}, and *Casp-1/11*^{-/-} mice were infected with *R. typhi*, *R. rickettsii*, and *R. montanensis* and the levels of IL-1 β and IL-1 α , cell death, as well as bacterial burdens, were evaluated over the course of infection. Our assays revealed that Casp-11 was involved in the secretion of cleaved IL-1 α upon infection of BMDM Φ with non-pathogenic and pathogenic *Rickettsia* spp. (Figs. 5A-B, D-E, and G-H). In addition, Casp-11-deficiency resulted in a significant decrease in host cell death (Figs. 5C, F, and I). Casp-1-deficiency (*Casp-1*^{-/-}) caused a significant decrease in IL-1 β production [Figs. 5A, D, and G (green lines)], while IL-1 α secretion as well as the level of cell death remained unaffected during infection with all three *Rickettsia* spp. [Figs. 5B-C, E-F, and H-I (green lines, symbols)]. Analysis of bacterial burdens further revealed a prominent role for Casp-11, but not for Casp-1, in restricting the replication of both pathogenic and non-pathogenic *Rickettsia* spp. (Fig. 5J; *WT* (square symbols); *Casp-1*^{-/-} (sphere symbols); *Casp-11*^{-/-} (triangles symbols); *Casp-1/11*^{-/-} (diamond symbols)). As our findings indicate that infections with *R. typhi*- and *R. rickettsii* resulted in a significant reduction of IL-1 α secretion, likely via Casp-11-dependent mechanism, we assessed the expression and activation status of Casp-1 and Casp-11

via Western blot analyses. *R. montanensis* infection resulted in a robust activation of Casp-1, as indicative of the detection of the Casp-1-p20 fragment (Fig. 5K). In contrast, infection with *R. typhi* and *R. rickettsii* spp. resulted in a lower activation of Casp-1 (~5-fold) (Fig. 5K). Intriguingly, only infection with *R. montanensis* resulted in a robust induction of Casp-11 (~8 fold) as compared to infection data using both pathogenic *Rickettsia* spp. (Fig. 5K). To test if IL-1 cytokine secretion dependent on the bacterial load, we heat-inactivated both pathogenic and non-pathogenic *Rickettsia* spp. and showed that IL-1 β and IL-1 α release was significantly impaired as compared to infections using viable *Rickettsia* spp., a phenotype more strongly observed in infections using *R. montanensis* (Fig. S4). As IL-1 cytokine secretion depends on Gsdmd, the pore-forming executor of pyroptosis (29–32), we assessed the proteolytic processing of Gsdmd and showed that only *R. montanensis* infection resulted in a robust cleavage of Gsdmd (~8 fold), as indicative by the detection of the Gsdmd-p30 fragment (Fig. 5K). In support of our findings, we showed that infection of *Gsdmd*^{-/-} BMDM Φ with non-pathogenic and pathogenic *Rickettsia* spp. released significantly lower levels of IL-1 β and IL-1 α , as compared to *WT* BMDM Φ (Figs. S5A-B). Furthermore, analysis of bacterial burdens provided additional evidence that Gsdmd plays a role in restricting the replication of *R. typhi*, *R. rickettsii*, and *R. montanensis* (Fig. S5C). These findings suggest that pathogenic, unlike non-pathogenic, *Rickettsia* spp. suppress IL-1 cytokine secretion via a Casp-11-Gsdmd-dependent pathway to support an intracytosolic replication in M Φ .

Secretion of IL-1 α by macrophages is crucial in restricting the replication of pathogenic and non-pathogenic *Rickettsia* species *in vivo*.

To examine whether secretion of IL-1 by MΦ limits the replication of *Rickettsia* spp. *in vivo*, we first injected (*i.v.*) *WT* mice with PBS- or dichloromethylene biphosphate (Cl₂MBP)-liposomes to deplete endogenous macrophages as described previously [37]. Next, PBS- or Cl₂MBP-liposomes treated *WT* mice were injected (*i.v.*) with BMDMΦ isolated from *WT*, *Il-1β*^{-/-} or *Il-1α*^{-/-} mice prior to infection with *R. typhi*, *R. rickettsii*, or *R. montanensis*. Strikingly, adoptive transfer of *Il-1α*^{-/-} BMDMΦ, but not *Il-1β*^{-/-}, or *WT* MΦ, significantly increased the mortality of Cl₂MBP- but not PBS-treated *WT* mice injected with all three *Rickettsia* spp., reaching levels similar to the survival percentages observed in IL-1α Ab-neutralization studies (Figs. 6A-D, Fig. S6, and Fig. 2). Moreover, transfer of *Il-1α*^{-/-} BMDMΦ resulted in the development of splenomegaly (Fig. S7) and an increase in bacterial burden in the spleens of Cl₂MBP-treated *WT* mice (Fig. 6E), which correlated with a decrease in IL-1α serum concentrations without affecting IL-1β serum levels (Figs. 6F-G). In contrast, transfer of *Il-1β*^{-/-} BMDMΦ, had an overall lesser effect on the severity of rickettsiosis, as evidenced by a lower mortality rate, smaller spleen size, and lower bacterial burden in Cl₂MBP-treated *WT* mice (Figs. 6A-E, and Fig. S7), which is in agreement with our IL-1β Ab-neutralization data (Fig. 2). In addition, transfer of *Il-1β*^{-/-} BMDMΦ, resulted in a decrease in IL-1β serum concentrations without affecting IL-1α serum levels (Figs. 6F-G). Collectively, these data suggest that modulation of expression and secretion of IL-1α secretion by macrophages is important to limit the replication of *Rickettsia* spp. *in vivo*.

Discussion

Obligate intracellular bacterial pathogens, which successfully reside and replicate within the host cell, overcome responses of innate immune defense surveillance (e.g., inflammasomes and autophagy). However, in the case of strict obligate intracytosolic *Rickettsia* spp., the roles for both inflammasome and autophagy to restrict their replication in endothelial cells and immune cells, like MΦ, is only now emerging, although without consistent mechanistical insights (23–28, 33). Given these knowledge gaps and our current lack of understanding on how TG *Rickettsia* spp. evade immune defense responses to facilitate host colonization, we established an animal model of rickettsiosis using C57BL/6J *WT* mice by comparing both mild (~LD₂₅) and more severe disease (~LD₅₀) development for two pathogenic spp., *R. rickettsii*, and *R. typhi*. In fact, data of disease severity correlated with the levels of bacterial burden detected in the mice spleens. We also observed that infections with pathogenic, but not non-pathogenic, *Rickettsia* spp. resulted in a reduced serum response of both pro-inflammatory cytokines, IL-1β and IL-1α, which is likely the result of pathogenic *Rickettsia* spp. to evade canonical and non-canonical inflammasome-dependent defense sensing, of which the former is in agreement with recent studies using *R. australis* (26). Thus, our data suggests that inhibition of both canonical and non-canonical inflammasome-dependent signaling contributes to the enhanced survival and colonization of pathogenic *Rickettsia* spp. in our *in vivo* experiments.

Our data further suggest that IL-1α, and to significantly lower extent IL-1β, plays a role in limiting rickettsial infection *in vivo*. By testing the putative anti-rickettsial capabilities of these cytokines through employing Ab-neutralization and recombinant protein assays, we showed that IL-1α, and to a much lesser level IL-1β, was able to restrict the replication

and colonization of both non-pathogenic and pathogenic *Rickettsia* spp. *in vivo*. Our current understanding by which *Rickettsia* spp. evade host-induced anti-bacterial activities, and in particular, how cytosolic rickettsiae overcome host immune surveillance in defense cells, like MΦ, is primarily based on reports that are not aligned with one another (23–25, 27). Thus, we sought to address the hypothesis that pathogenic *R. typhi*, and *R. rickettsii* spp. but not non-pathogenic *R. montanensis* evade innate immune defense responses, in order, to establish an intracytosolic replication niche in MΦ. In agreement with our *in vivo* infection models, *R. montanensis*-infected WT BMDMΦ produced higher levels of IL-1α and IL-1β cytokines and displayed reduced bacterial loads during the course of infection, compared to *R. typhi*- or *R. rickettsii*-infected BMDMΦ. These data support the notion that non-pathogenic, but not pathogenic, *Rickettsia* spp. are more efficiently cleared by MΦ, a mechanism that further supports the previously published findings with SFG *Rickettsia* using THP-1 cells, a human macrophage-like cell line (34). Collectively, our presented data strengthen our hypothesis that pathogenic, but not non-pathogenic, *Rickettsia* spp. suppress anti-rickettsial inflammasome-dependent IL-1 cytokine responses to establish an intracytosolic replication niche in MΦ.

Given that IL-1 signaling is modulated through inflammasome-dependent Casp-1, Casp-11, and Gsdmd activation, we tested the role of both caspases as well as Gsdmd, and found that non-pathogenic, but not pathogenic, *Rickettsia* spp. ensued Casp-1 activation and Casp-11 induction, which ultimately resulted in the proteolytic processing of Gsdmd and release of IL-1α and IL-1β cytokines. Intriguingly, the lack of Casp-11 induction by pathogenic, but not non-pathogenic, *Rickettsia* spp. suggest that the membrane-bound LPS of *R. typhi*, and *R. rickettsii* spp. is likely less immunogenic than

that of *R. montanensis*, which is further supported by our recent reports (35, 36). Our findings further suggest that pathogenic, as compared to non-pathogenic, *Rickettsia* spp., benefit from evasion of the Casp-11-Gsdmd-IL-1 α signaling axis to establish a replication niche in M Φ as evidenced by the increased replication in M Φ from *Casp-11*^{-/-}, *Casp-1/11*^{-/-}, or *Gsdmd*^{-/-} mice compared to *WT* and *Casp-1*^{-/-} BMDM Φ . Finally, we sought to determine the role of IL-1 cytokine responses by M Φ in restricting the replication of *Rickettsia* spp. and showed that transfer of *Il-1 α* ^{-/-} BMDM Φ , and to much lesser extent the administration of *Il-1 β* ^{-/-} BMDM Φ , exacerbated the disease progression in *WT* mice injected with either pathogenic or non-pathogenic *Rickettsia* spp. It is worth noting that the observed differences in IL-1 α release could be partially attributed to alternative mechanisms, including the retainment of IL-1 α in the cytosol or nucleus, the dependance of IL-1 β , and/or the association with the decoy receptor of IL-1 (IL-1R2), and future experiments are underway to address these possibilities (15, 29, 37–40). Also, IL-1 α is produced by other immune cells, such as neutrophils (15). Although our study did not evaluate a potential contributing role of neutrophils, preceding findings suggest that neutrophils did not alter the course of rickettsiosis or contribute to the restriction of bacterial growth (28).

Importantly, preceding findings suggest that intracellular pathogens, like rickettsiae, not only encounter inflammasome-dependent defense mechanisms but are also confronted by another cytosolic defense pathway, autophagy (23, 25). Both responses not only are key to mount the appropriate host defense responses (16, 18) but are also functionally interconnected. In fact, recent reports indicated that autophagy acts on intracellular microbes upstream of the inflammasome and thereby functions as a negative

regulator by degrading inflammasome components (10, 16, 18, 22). In the case of rickettsiae, however, the role of autophagy in regulating inflammasome responses to facilitate their host colonization remains inconclusive. For instance, *R. australis*, a pathogenic TRG member, benefited from ATG5-dependent autophagy induction and suppression of inflammasome-dependent IL-1 β production to colonize M Φ (23, 25). In contrast, *R. parkeri*, a mildly pathogenic member of SFG, demonstrated that its surface protein OmpB is critical for protecting against autophagic recognition, while evasion of autophagy was critical for invasion of BMDM Φ and *WT* mice by *R. parkeri* (24, 27). Intriguingly, our recent report on *R. typhi* showed that *R. typhi* is ubiquitinated upon host entry, induces autophagy, but escapes autophagolysosomal maturation for intracellular colonization in non-phagocytic cells (9). Given these reports by others and our recent findings, it is tempting to speculate that pathogenic, but not non-pathogenic, *Rickettsia* spp. induce autophagy to down-regulate inflammasome-dependent IL-1 β and IL-1 α cytokine responses to establish an intracytosolic replication niche *in vitro* and *in vivo*, and our future research will address this possibility.

Overall, our findings present a previously unappreciated model of host invasion by which pathogenic, but not non-pathogenic, *Rickettsia* spp., avoid the activation of signaling pathways required for IL-1 α production and release; likely via the suppression of the Casp-11-Gsdmd signaling pathway, to facilitate their intracytosolic replication in M Φ and ultimately cause host colonization (Fig. 6H).

Materials and Methods

Animals

All experiments were conducted in fully AAALAC accredited program using 8-10 weeks old female C57BL/6J *WT* mice in a specific pathogen-free environment according to the University of Maryland School of Medicine Institutional Animal Care and Use Committee (IACUC) in compliance with the National Institutes of Health Guide.

Antibodies and reagents

Anti(α)-IL-1 α (clone ALF-161), α IL-1 β (clone B122), or an isotype control IgG (Armenian hamster IgG) antibody (Ab) were purchased from BioXCell. α Caspase (Casp)-1 Ab was purchased from Adipogen, while α Casp-11 [EPR18628] and α Gsdmd [EPR19828] Abs were obtained from Abcam. Elongation factor Ts (EF-Ts) Ab was obtained from Primm Biotech as previously described (9), while the α Gapdh (FL-335) Ab was purchased from Santa Cruz Biotechnology. Halt protease and phosphatase inhibitor cocktail was obtained from Thermo Fisher Scientific. Endotoxin-free recombinant mouse IL-1 α and IL-1 β proteins were purchased from BioLegend.

Bacterial strains, cell culture, and infection

Vero76 cells (African green monkey kidney, ATCC, RL-1587) were maintained in minimal Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C with 5% CO₂. *R. montanensis* strain M5/6 and *R. rickettsia* strain Sheila Smith (SS) were obtained from Dr. Ted Hackstadt (Rocky Mountain Laboratories, NIH, MT, USA) and *R. typhi* strain Wilmington was obtained from CDC. All *Rickettsia* strains were propagated in Vero76 cells grown in DMEM supplemented with 5% FBS at 34°C with 5% CO₂. All *Rickettsia* were purified as previously described (7).

For infection of BMDMΦ, purified *Rickettsia* spp. were used at a multiplicity of infection (MOI) of 50, to ensure the presence of enough bacteria at early stage of infection, for host response (5, 8, 9). For infection using heat-inactivated bacteria, purified *Rickettsia* spp. were heated at 90°C for 20 min (41). Rickettsiosis in mice was induced by tail vein injection (*i.v.*) of purified *Rickettsia* (10^5 - 10^6 PFU), resuspended in PBS. At Days 1, 3, and 7 after administration, blood was collected, and serum cytokine levels were measured by flow cytometry. Splenic tissue specimens were collected at the indicated times and used for bacterial burden analysis by qPCR described below.

Differentiation of bone marrow-derived macrophages

Bone marrow cells were isolated from femurs and tibias of *WT*, *Il-1β*^{-/-} or *Il-1α*^{-/-}, *Gsdmd*^{-/-}, *Casp-1*^{-/-}, *Casp-11*^{-/-}, *Casp-1/Casp-11*^{-/-} mice. Femurs from *Casp-1*^{-/-}, *Casp-11*^{-/-}, *Casp-1/Casp-11*^{-/-} were kindly provided by Dr. Amal Amer (The Ohio State University, OH, USA), while bones from *Il-1β*^{-/-} or *Il-1α*^{-/-} mice were obtained from Dr. Thirumala-Devi Kanneganti (St. Jude Children's Research Hospital, TN, USA). Femurs from *Gsdmd*^{-/-} were kindly provided by Dr. Matthew Welch (University of California, Berkeley, CA, USA). Differentiation was induced by culturing bone marrow cells in RPMI 1640 medium supplemented with 10% FBS and 30% L929-conditioned medium (a source of macrophage colony stimulating factor) and cultured for 7 days as described previously (42).

Measurement of cytokines and chemokines

IL-1 cytokine concentrations in the sera of mice or supernatants from cultured BMDMΦ were assessed using the Legendplex mouse inflammation kit (BioLegend) following the manufacturer's instructions as described previously (42).

RNA isolation and quantitative real-time PCR

BMDMΦ samples were collected at 2, 6, 24, and 48 h post-infection, while spleens were collected at day 3 or 7 post infection. RNA was extracted from 1×10^6 BMDMΦ or 100 μl of organ homogenate using the Quick-RNA miniprep kit (ZymoResearch). The iScript Reverse Transcription Supermix kit (Bio-Rad; 1708841) was used to synthesize cDNAs from 200 ng of RNA according to the manufacturer's instructions. Quantitative real-time PCR was performed using SYBR Green (Thermo Fisher Scientific), 2 μl cDNA and 1 μM each of the following oligonucleotides for rickettsial citrate synthase gene (*gltA*) forward (F): 5'-CATAATAGCCATAGGATGAG-3'; reverse (R): 5'-ATGATTTATGGGGAACCTACC-3' and analyzed as described previously (25). Oligonucleotides for *Gapdh* were obtained from Qiagen.

Extract preparation, and Western blot analysis.

Rickettsia-infected BMDMΦ cells were lysed for 2 h at 4°C in ice-cold lysis buffer (50 mM HEPES [pH 7.4], 137 mM NaCl, 10% glycerol, 1 mM EDTA, 0.5% NP-40, and supplemented with protease and phosphatase inhibitory cocktails) as described previously (42). Equal amounts of protein were loaded for SDS-PAGE and membranes were probed with αCasp-1, αCasp-11, αGsdmd, αIL-1α, αIL-1β, αEF-Ts, and αGapdh

Abs, followed by enhanced chemiluminescence with secondary Abs conjugated to horseradish peroxidase.

Neutralization of endogenous IL-1 α and IL-1 β

For *in vivo* neutralization of IL-1 α and IL-1 β , C57BL/6J *WT* mice were *i.v.* injected with 250 mg of α IL-1 α (clone ALF-161, BioXCell), α IL-1 β (clone B122, BioXCell), or an IgG-isotype control (Armenian hamster IgG, BioXCell) Ab 24 h before the induction of mild rickettsiosis using 10⁵ PFU of *R. typhi*, *R. rickettsii*, or *R. montanensis*.

Adoptive transfer of bone marrow-derived macrophages

C57BL/6J *WT* mice were injected (*i.v.*) twice with PBS- or dichloromethylene biphosphate (Cl₂MBP)-liposomes 72 and 48 hours prior to macrophage transfer as described previously (42). Next, C57BL/6J *WT* mice were injected (*i.v.*) with *WT*, *Il-1 β ^{-/-}* or *Il-1 α ^{-/-}* BMDM Φ [5 x 10⁶ cells/mouse] followed by infection (24 hours post-M Φ transfer) with *R. typhi*, *R. rickettsii*, *R. montanensis*, or PBS (dose 10⁵ PFU).

Statistical analysis

Endpoint studies of mice subjected to mild (10⁵ PFU) and severe (10⁶ PFU) rickettsiosis were analyzed by using Kaplan-Meier survival curves and the log-rank test (GraphPad Prism Software, version 8). The statistical significance was assessed using ANOVA with Tukey's multiple comparisons post-test (GraphPad). Data are presented as mean $\pm$ SEM, unless stated otherwise. Alpha level was set to 0.05.

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Conflicts of Interest

The authors declare no conflict of interest. The funding sources had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

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Figure Legend

Fig. 1 *In vivo* models of rickettsiosis.

(A) *Rickettsia* spp. and their level of pathogenicity to humans (3, 43).

(B-D) Establishment of a model of rickettsiosis (LD₂₅ and LD₅₀) for *R. typhi* [B], *R. rickettsii* [C] or *R. montanensis* [D] in C57BL/6J *WT* mice. Animals were injected via tail vein (*i.v.*) with different doses [10^5 - 10^6 PFU] of *R. typhi*, *R. rickettsii*, *R. montanensis*, or PBS (n=12 for each treatment). Survival was monitored for 7 days.

(E) Bacterial burden was tested in spleens of *R. typhi*-, *R. rickettsii*-, *R. montanensis*-, or PBS-injected *WT* mice (n=5 per each treatment) shown in (B-D) by *GltA* RT-qPCR at day 3 and 7 (n=5 for each treatment) using the host housekeeping gene, *Gapdh*, for normalization.

(F-G) Serum samples from rickettsiae-infected mice described in (B-D) were analyzed for IL-1 β (F) and IL-1 α (G) production using the Legendplex kits (BioLegend) followed by flow cytometry analysis.

Error bars in (E-G) represent means $\pm$ SEM from five independent experiments; NS, non-significant; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.005$; **** $p \leq 0.001$.

Fig. 2 Neutralization of IL-1 α activity augments mortality of pathogenic and non-pathogenic *Rickettsia*-induced rickettsiosis.

(A-C) C57BL/6J *WT* mice were injected via tail vein (*i.v.*) with 10^5 PFU of *R. typhi* [A], *R. rickettsii* [B], or *R. montanensis* [C] (A-C; n=12 for each treatment) followed by a subsequent injection (*i.v.*) with α IL-1 β , α IL-1 α or IgG-isotype control antibody (Ab) [250 μ g Ab/mouse]. Survival was monitored for 7 days.

(D) Bacterial burden was tested in spleens of Ab-treated *R. typhi*-, *R. rickettsii*-, and *R. montanensis*-injected *WT* mice shown in (A-C) by *GltA* RT-qPCR at day 7 (n=5 for each treatment) using the host housekeeping gene, *Gapdh*, for normalization.

(E-F) Serum samples from mice described in (A-C) were analyzed for IL-1 β (E) and IL-1 α (F) production at day 7 (n=5 for each treatment) using the Legendplex kits (BioLegend) followed by flow cytometry analysis.

Error bars in (D-F) represent means $\pm$ SEM from five independent experiments; NS, non-significant; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.005$; **** $p \leq 0.001$.

Fig. 3 Administration of recombinant IL-1 α rescues fatal *Rickettsia*-induced rickettsiosis.

(A-C) C57BL/6J *WT* mice were injected *i.v.* with recombinant (r)IL-1 β or rIL-1 α protein [500 ng/mouse] followed by infection (24 h post protein injection) with 10⁶ PFU of *R. typhi* [A], *R. rickettsii* [B], *R. montanensis* [C], or PBS (A-C; n=15 for each treatment). Survival was monitored for 7 days.

(D) Bacterial burden was tested in spleens of protein-treated *R. typhi*-, *R. rickettsii*-, *R. montanensis*- or PBS-injected *WT* mice shown in (A-C) by *GltA* RT-qPCR at day 7 (n=5 for each treatment) using the housekeeping gene, *Gapdh*, for normalization.

(E-F) Serum samples from mice described in (A-C) were analyzed for IL-1 β (E) and IL-1 α (F) production at day 7 (n=5 for each treatment) using the Legendplex kits (BioLegend) followed by flow cytometry analysis.

Error bars in (D-F) represent means $\pm$ SEM from five independent experiments; NS, non-significant; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.005$; **** $p \leq 0.001$.

Fig. 4 IL-1 α , but not IL-1 β , contributes to the survival of *Rickettsia* species in macrophages.

(A-F) BMDM Φ from *WT*, *Il-1 β ^{-/-}* or *Il-1 α ^{-/-}* mice were infected with *R. typhi*, *R. rickettsii*, or *R. montanensis* [MOI=50] for 0, 6, 24, and 48 h. Culture supernatants were analyzed for production of IL-1 β (A, C, and E) and IL-1 α (B, D, and F) using Legendplex kits (BioLegend) followed by flow cytometry.

(G) Bacterial burden in rickettsiae-infected BMDM Φ from *WT*, *Il-1 β ^{-/-}* or *Il-1 α ^{-/-}* mice was evaluated at 2 and 24 h post-infection by *GltA* RT-qPCR. Expression of the housekeeping gene *Gapdh* was used for normalization.

(H) BMDM Φ from *WT* mice were either left uninfected (-) or infected with *R. typhi*, *R. rickettsii*, or *R. montanensis*, [MOI=50] for 24 h. Lysates were immunoblotted with α IL-1 α , α IL-1 β , α ET-Ts, and α Gapdh Abs. Densitometry was performed using Fiji software and data represent the fold-change ratios of pro-IL-1 β /Gapdh or pro-IL-1 α /Gapdh between uninfected and infected cells are shown. Immunoblot data are a representative of three independent experiments.

Error bars in (A-G) represent means $\pm$ SEM from five independent experiments; NS: non-significant; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.005$; **** $p \leq 0.001$.

Fig. 5 Pathogenic, but not non-pathogenic, *Rickettsia* species limit Caspase-1- and Caspase-11-dependent IL-1 signaling to facilitate their intracellular replication in macrophages.

(A-I) BMDMΦ from *WT*, *Casp-1^{-/-}*, *Casp-11^{-/-}*, or *Casp-1/11^{-/-}* mice were infected with *R. typhi* (A-C), *R. rickettsii* (D-F) or *R. montanensis* (G-I) [MOI=50] for 0, 6, 24, and 48 h. Culture supernatants were analyzed for production of IL-1β (A, D, G) and IL-1α (B, E, H) using Legendplex kits (BioLegend) followed by flow cytometry. BMDM cell death at 24 h post-infection, was measured by LDH release assay (C, F, I).

(J) Bacterial burdens in infected BMDMΦ were evaluated 2 and 24 h post-infection by *GltA* RT-qPCR. Expression of the host housekeeping gene *Gapdh* was used for normalization.

(K) Western analysis of Casp-1, Casp-11, and Gsdmd induction and processing at 24 h post-infection with *R. typhi*, *R. rickettsii*, or *R. montanensis* using αCasp-1, αCasp-11, and αGsdmd Abs. Reblotting with rickettsial specific αEF-Ts and host cell specific αGapdh Abs served as infection and equal loading controls respectively. Densitometry was performed using Fiji software and data represent the fold-change ratios of Casp-1-p20/FL-Casp-1; Gsdmd-p30/FL-Gsdmd; or Casp-11/Gapdh between uninfected and infected cells are shown.

Error bars in (A-J) represent means ± SEM from five independent experiments; NS: non-significant; **p* ≤ 0.05; ***p* ≤ 0.01; ****p* ≤ 0.005; *****p* ≤ 0.001. Immunoblot data are a representative of three independent experiments.

Fig. 6 Macrophages-dependent secretion of IL-1α contributes more than IL-1β for controlling the survival and colonization of pathogenic and non-pathogenic *Rickettsia* species.

(A-D) Dichloromethylene biphosphate (Cl₂MBP)-treated C57BL/6J *WT* mice were injected *i.v.* with *WT*, *Il-1β*^{-/-} or *Il-1α*^{-/-} BMDMΦ [5 x 10⁶ cells/mouse] followed by infection (24 h post-MΦ transfer) with *R. typhi* [B], *R. rickettsii* [C], or *R. montanensis* [D] (dose 10⁵ PFU) (B-D; n=12 for each treatment). Survival was monitored for 7 days.

(E) Bacterial burden was tested in spleens from mice described in (B-D) by *GltA* RT-qPCR at day 7 (n=5 for each treatment) using the housekeeping gene, *Gapdh*, for normalization.

(F-G) Serum samples from mice described in (B-D) were analyzed for IL-1β (F) and IL-1α (G) production at day 7 (n=5 for each treatment) using the Legendplex kits (BioLegend) followed by flow cytometry analysis.

Error bars in (E-G) represent means ± SEM from five independent experiments; NS, non-significant; **p* ≤ 0.05; ***p* ≤ 0.01; ****p* ≤ 0.005; *****p* ≤ 0.001.

(H) Proposed working model on how pathogenic *Rickettsia* spp. suppress Casp-1 and Casp-11-dependent IL-1 signaling responses to establish a replication niche *in vitro* and *in vivo*. Of note, the majority of non-pathogenic *Rickettsia* spp. is likely destroyed by phagolysosomal fusion, while a subpopulation may escape lysosomal fusion, ultimately allowing for the induction of inflammasome mediated IL-1 responses.

Fig. S1. Splenic data during infection of pathogenic and non-pathogenic *Rickettsia* species.

(A) Representative images of spleens (at day 7) from C57BL/6J *WT* mice injected *i.v.* with *R. typhi*, *R. rickettsii*, *R. montanensis*, or PBS (dose 10⁶ PFU).

(B) Spleen weight from injected animals was evaluated at day 7 day (n=5).

Error bars in (B) represent means $\pm$ SEM from five independent experiments; $*p \leq 0.05$; $***p \leq 0.005$.

Fig. S2. Splenic data after IL-1 signaling neutralization during infection of pathogenic and non-pathogenic *Rickettsia* species.

(A) Representative images of spleens (day 7) from C57BL/6J *WT* mice injected *i.v.* with *R. typhi*, *R. rickettsii*, or *R. montanensis* (dose 10^5 PFU) followed by administration of α IL-1 β , α IL-1 α , or IgG isotype control antibody (Ab) [250 μ g Ab/mouse] (24 h post-Ab injection).

(B) Spleen weight from injected animals was evaluated at day 7 (n=5).

Error bars in (B) represent means $\pm$ SEM from five independent experiments; $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.005$.

Fig. S3 Splenic data after administration of recombinant IL-1 α and IL-1 β proteins during infection of pathogenic and non-pathogenic *Rickettsia* species.

(A) Representative images of spleens (day 7) from C57BL/6J *WT* mice injected *i.v.* with rIL-1 β or rIL-1 α protein followed by administration of *R. typhi*, *R. rickettsii*, *R. montanensis* or PBS (dose 10^6 PFU) at day 7.

(B) Spleen weight from injected animals was evaluated at day 7 day (n=5).

Error bars in (B) represent means $\pm$ SEM from five independent experiments; $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.005$.

Fig. S4 Heat-inactivation of *Rickettsia* species resulted in a reduced IL-1 α and IL-1 β cytokine release by macrophages.

(A-B) BMDM Φ from *WT* mice were infected with heat-inactivated or non-heat treated *R. typhi*, *R. rickettsii*, or *R. montanensis* [MOI=50] for 24 h. Culture supernatants were analyzed for production of IL-1 β (A) and IL-1 α (B) using Legendplex kits (BioLegend) followed by flow cytometry.

(C) Bacterial burden in rickettsiae-infected BMDM Φ from *WT* mice was evaluated at 2 and 24 h post-infection by *GltA* RT-qPCR. Expression of the housekeeping gene *Gapdh* was used for normalization.

Error bars in (A-C) represent means $\pm$ SEM from five independent experiments; NS: non-significant; ** $p \leq 0.01$; *** $p \leq 0.005$; **** $p \leq 0.001$.

Fig. S5 Gsdmd is involved in the release of IL-1 α and IL-1 β by macrophages upon *Rickettsia* infection.

(A-B) BMDM Φ from *WT* and *Gsdmd*^{-/-} mice were infected with *R. typhi*, *R. rickettsii*, or *R. montanensis* [MOI=50] for 24 h. Culture supernatants were analyzed for production of IL-1 β (A) and IL-1 α (B) using Legendplex kits (BioLegend) followed by flow cytometry.

(C) Bacterial burden in rickettsiae-infected BMDM Φ from *WT* and *Gsdmd*^{-/-} mice was evaluated at 2 and 24 h post-infection by *GltA* RT-qPCR. Expression of the housekeeping gene *Gapdh* was used for normalization.

Error bars in (A-C) represent means $\pm$ SEM from five independent experiments; NS: non-significant; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.005$.

Fig. S6 Survival data after adoptive transfer of *WT*, *Il-1 β ^{-/-}* or *Il-1 α ^{-/-}* macrophages and infection of pathogenic and non-pathogenic *Rickettsia* species into PBS-liposome-treated C57BL/6J *WT* mice. (A-D) PBS-treated C57BL/6J *WT* mice were injected *i.v.* with *WT*, *Il-1 β ^{-/-}* or *Il-1 α ^{-/-}* BMDM Φ [5×10^6 cells/mouse] followed by infection (24 h post-M Φ transfer) with *R. typhi* [B], *R. rickettsii* [C], *R. montanensis* [D], or PBS (dose 10^5 PFU) (B-D; n=12 for each treatment). Survival was monitored for 7 days.

Fig. S7. Splenic data after adoptive transfer of *WT*, *Il-1 β ^{-/-}* or *Il-1 α ^{-/-}* macrophages and infection of pathogenic and non-pathogenic *Rickettsia* species into dichloromethylene biphosphate (Cl₂MBP)-treated C57BL/6J *WT* mice.

(A) Representative images of spleens (day 7) from Cl₂MBP-treated C57BL/6J *WT* mice injected *i.v.* with *WT*, *Il-1 β ^{-/-}* or *Il-1 α ^{-/-}* BMDM Φ [5×10^6 cells/mouse] followed by administration (24 h post-M Φ transfer) of *R. typhi*, *R. rickettsii*, *R. montanensis*, or PBS (dose 10^5 PFU).

(B) Spleen weight from injected animals was evaluated at day 7 (n=5).

Error bars in (B) represent means $\pm$ SEM; from five independent experiments; NS, non-significant; * $p \leq 0.05$; ** $p \leq 0.01$.

Fig. 1

A

B

C

D

E

F

G

Fig. 2

Fig. 3

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Fig. 5

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Fig. 6

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A

B

C

D

E

F

G

H

Fig. S1

A

B

Fig. S2

A

B

Fig. S3

Fig. S4

Fig. S5

Fig. S6

A

B

C

D

Fig. S7

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A

B