Brucella Induces an Unfolded Protein Response via TcpB That Supports Intracellular Replication in Macrophages

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Abstract

Brucella melitensis is a facultative intracellular bacterium that causes brucellosis, the most prevalent zoonosis worldwide. The Brucella intracellular replicative niche in macrophages and dendritic cells thwart immune surveillance and complicates both therapy and vaccine development. Currently, host-pathogen interactions supporting Brucella replication are poorly understood. Brucella fuses with the endoplasmic reticulum (ER) to replicate, resulting in dramatic restructuring of the ER. This ER disruption raises the possibility that Brucella provokes an ER stress response called the Unfolded Protein Response (UPR). In this study, B. melitensis infection upregulated regulated expression of the UPR target genes BiP, CHOP, and ERdj4, and induced XBP1 mRNA splicing in murine macrophages. These data implicate activation of all 3 major signaling pathways of the UPR. Consistent with previous reports, XBP1 mRNA splicing was largely MyD88-dependent. However, upregulation of CHOP, and ERdj4 was completely MyD88 independent. Heat killed Brucella stimulated significantly less BiP, CHOP, and ERdj4 expression, but induced XBP1 splicing. Although a Brucella VirB mutant showed relatively intact UPR induction, a TcpB mutant had significantly compromised BiP, CHOP and ERdj4 expression. Purified TcpB, a protein recently identified to modulate microtubules in a manner similar to paclitaxel, also induced UPR target gene expression and resulted in dramatic restructuring of the ER. In contrast, infection with the TcpB mutant resulted in much less ER structural disruption. Finally, tauroursodeoxycholic acid, a pharmacologic chaperone that ameliorates the UPR, significantly impaired Brucella replication in macrophages. Together, these results suggest Brucella induces a UPR, via TcpB and potentially other factors, that enables its intracellular replication. Thus, the UPR may provide a novel therapeutic target for the treatment of brucellosis. These results also have implications for other intracellular bacteria that rely on host physiologic stress responses for replication.

Introduction

Brucellosis is a chronic debilitating disease with protein manifestations and insidious onset most frequently caused by the facultative intracellular bacteria Brucella melitensis [1]. With over 500,000 new infections per year, brucellosis is the most prevalent zoonosis worldwide [2]. Brucellosis is most often acquired by consumption of contaminated dairy products. Following ingestion, Brucella infects macrophages and dendritic cells that constitute the replicative reservoir [3]. The intracellular replicative niche thwart immune surveillance, complicates vaccine development, and renders the organism refractory to antibiotics [1]. A greater understanding of host-pathogen interactions is critical for elucidating disease pathogenesis and thus improving therapeutic strategies.

Brucella establishes its stealthy intracellular lifestyle through virulence factors. Brucella expresses a weakly endotoxic smooth LPS that directs bacterial uptake via class A scavenger receptor in lipid rafts [4–6]. Inside macrophages, ~90% of bacteria are killed within the first 4 h. However, some Brucella-containing vesicles (BCV) avoid end-stage lysosomes and ultimately fuse with the endoplasmic reticulum (ER) [7]. Fusion appears to involve an early ER to Golgi vesicular compartment, as GAPDH and the small GTPases Rab2 and Sar1 are essential for replication [8,9]. Replicative BCV contain ER markers including calnexin, calreticulin and sec61β [7]. Correct trafficking and ultimately replication depend upon de novo bacterial protein expression following cellular infection. In particular, BCV acidification activates the type IV secretion system encoded by the VirB operon [10]. VirB mutant BCV fail to fuse with the ER and VirB mutants are greatly attenuated in vivo [7]. Within 48 h of infection, Brucella induces a marked reorganization of the ER with ER membrane accretion around replicating bacteria [7]. The mechanism by which Brucella disrupts ER structure is currently unknown. Somehow, the host cell adapts to this perturbation, as Brucella infection inhibits apoptosis. Although the bacterial factors leading to successful infection are beginning to be clarified, the host pathways supporting replication remain poorly understood.

The requirement for ER fusion and dramatic restructuring of the ER suggest Brucella most likely disrupts ER homeostasis. To
cific drug tauroursodeoxycholic acid significantly diminished replication in physiologic host cells (e.g. macrophages) [7]. We observed ER fragmentation and condensation even within 24 h of infection [12]. Induction of the Brucella UPR was detectable as early as 24 h following infection (Figure 1). The replicative requirement for ER-BCV fusion and extracellular replication of the bacterium differs greatly from other intracellular pathogens such as M. tuberculosis [19]. However, the relationship between infection and host response was not clear. In one report, intracellular bacteria Francisella, Listeria, and Mycobacteria induced XBP1 mRNA splicing via toll like receptor (TLR) signaling [20]. Deficiency of the TLR-adaptor protein myeloid differentiation primary response gene 88 (MyD88) ablated TLR2 and decreased TLR4-dependent XBP1 splicing. TLR-dependent XBP1 splicing was not accompanied by downstream UPR target gene induction, although there was evidence supporting a role for XBP1 in synergistic cytokine induction. XBP1 was essential for optimal cytokine production and immune control of Francisella in vivo. The exact mechanism underlying this selective XBP1 pathway activation is unknown. Extracellular Listeria monocytogenes has also been shown to induce a more complete UPR, involving all three signaling axes, via production of listeriolysin [21]. However, the intracellular life cycle of this bacterium differs greatly from Brucella.

In this study, we evaluated induction of the host UPR by Brucella infection in macrophages. We detected activation of all three axes of the UPR, stemming from activation of IRE1, PERK and ATF6, as evident by increased UPR target gene expression and XBP1 mRNA splicing. Although XBP1 splicing appeared to be largely MyD88-dependent, UPR gene expression was independent of the TLR-signaling adaptor molecule. Optimal UPR target gene induction required both live bacteria and expression of the microtubule-modulating Brucella protein TcpB. Finally, tauroursodeoxycholic acid (TUDCA), a pharmacologic chaperone that inhibits the UPR, substantially decreased replication. Together these data suggest Brucella actively induces a UPR that enables its intracellular replication within the ER in macrophages.

Results

Brucella induces the UPR

A previous study documented dramatic reorganization of the ER within 48 h of Brucella infection [7]. We observed ER fragmentation and condensation even within 24 h of infection (Figure 1). The replicative requirement for ER-BCV fusion and the ER structural reorganization following infection raised the possibility that Brucella triggers the host cell UPR. The UPR directs an adaptive program through the induction of target gene transcription. Although the three primary biochemical signaling pathways have overlapping functions, several of the UPR gene targets appear to be relatively pathway specific; thus activation of PERK, IRE1, and ATF6 can be detected by downstream induction of mRNA for CHOP, ER localized DnaJ homologue 4 (ERdj4), and BiP, respectively [22–24]. XBP1 spliced and unspliced mRNA species can be resolved by high-percentage agarose gel electrophoresis, and this method is often used to detect IRE1 endonuclease activity [25]. To test for UPR activation, RAW264.7 macrophages were infected with B. melanitiss for 24 h (Figure 2). Of the genes examined, CHOP showed the most robust induction and mRNA expression correlated with a marked increase in CHOP protein. The induction of BiP, CHOP and ERdj4 mRNA and evidence for XBP1 mRNA splicing supports the hypothesis that Brucella induces a UPR involving all three primary signaling axes in macrophages in vitro.

To determine if Brucella infection induces a detectable UPR in vivo, splenic CD11b+ cells (containing macrophages) were isolated 24 h following infection and UPR gene expression assessed by qPCR (Figure 3). TNF-α expression served as a positive control
that is expected to increase early with infection. Induction of BiP, CHOP, and ERdj4 expression was evident following in vivo infection, consistent with activation of the UPR in vivo.

**Brucella-induced XBP1 mRNA splicing is MyD88 dependent**

A recent study described XBP1 splicing in response to TLR2 and TLR4 agonists (Pam3csK4 and LPS) as well as various intracellular bacteria [20]. Interestingly, although XBP1 was required for optimal TLR-stimulated cytokine production, TLR ligation decreased BiP and CHOP induction by pharmacologic UPR inducers. Thus the TLR-MyD88-XBP1 pathway appears antagonistic towards the rest of the ER stress response. Another report also documents selective suppression of ER stress signaling by LPS [26]. *Brucella* stimulates both TLR2 and TLR4 and the TLR adaptor MyD88 appears to be essential for controlling infection in vivo [27]. To further elucidate the role of TLR-MyD88 signaling in UPR induction by *Brucella*, XBP1 splicing and UPR target gene expression was examined in primary bone marrow derived macrophages from MyD88 deficient mice (Figure 4). The phenotype of these mice was confirmed by diminished IL-6 expression following infection in vivo [27].

To further elucidate the role of TLR-MyD88 signaling in UPR induction by *Brucella*, XBP1 splicing and UPR target gene expression was examined in primary bone marrow derived macrophages from MyD88 deficient mice (Figure 4). The phenotype of these mice was confirmed by diminished IL-6 expression following infection in vivo [27].

**Figure 1. Brucella infection of macrophages induces ER structural reorganization.** RAW 264.7 macrophages were uninfected or infected with YFP expressing *B. melitensis* (green) for 24 h. Arrow indicates condensation and fragmentation of the ER, visualized with anti-calreticulin antibody (red). Results are representative of 5 independent experiments. doi:10.1371/journal.ppat.1003785.g001

**Figure 2. Brucella infection activates the UPR in macrophages in vitro.** RAW 264.7 macrophages were uninfected (NI) or infected with 100 MOI *B. melitensis* (*B. mel*). A) After 24 h, cells were resuspended in TRIzol for RNA processing. Relative expression of reverse transcribed cDNA was determined by quantitative PCR (qPCR) with normalization to 18S rRNA or hprt. Bars are combined mean fold inductions for 4–5 independent experiments (NI = 1) ± sem. *P < 0.05, **P < 0.003. B) Cells were infected with 100 MOI for 24 h and processed for RNA. XBP1 spliced and unspliced mRNA species were resolved by high-density agarose gel or detected by qPCR. %Splicing = spliced/total x 100. Bars represent combined mean ± sem from 2–4 independent experiments. Representative gel is shown below. C) 16 h post infection, RAW cells were lysed and lysates resolved by SDS PAGE. CHOP or β-actin proteins were detected by immunoblot. Results are representative of 3 experiments. doi:10.1371/journal.ppat.1003785.g002

**Figure 3. Brucella induces the UPR in vivo.** BALB/c mice were injected ip with PBS (NI) or 10⁷ *B. melitensis* (*B. mel*). After 24 h, CD11b+ cells were isolated from pooled spleens and cells were resuspended in TRIzol for RNA purification. TNF-α, BiP, CHOP, and ERdj4 gene expression was detected by qPCR with normalization to 18S rRNA. Error bars denote standard deviations between 2 pools (7 mice each). ERdj4 expression is from 1 pool each (NI or *B. mel*) of 4 mice. Results represent 2 independent experiments. *p = 0.01. doi:10.1371/journal.ppat.1003785.g003

**Brucella requirements for UPR target gene induction**

One prediction of these data is that the bacterial surface of *Brucella* (containing LPS) will be sufficient to stimulate XBP1 splicing. However, the induction of other UPR-dependent events (e.g. increased CHOP expression) may involve other surface or intracellular components. To begin testing this premise, RAW macrophages were infected with heat killed *Brucella* (Figure 5A). Heat killed *Brucella* induced XBP1 splicing to a similar extent as living *Brucella*, as predicted, but induced significantly less UPR...
target gene expression. These data suggest other heat-labile or newly produced factors besides bacterial LPS are responsible for activating the host UPR.

The need for living bacteria for optimal UPR target gene induction may reflect the involvement of de novo bacterial protein/factor production following infection. De novo expression of virulence factors directs the distinctive trafficking and replicative events that result in chronic infection. In particular, products encoded in the VirB operon appear to be essential for fusion of BCV with ER membranes and subsequent replication [7]. To test the requirement for VirB, UPR induction was assessed in a VirB4 mutant (Figure 5A) [29]. This mutant displays attenuated virulence, with defects in in vivo persistence. Consistent with above results, XBP1 splicing was intact. Downstream UPR target gene induction following 24 h infection with the VirB deletion mutant (∆VirB) was variable, and not statistically different compared to the wild type control. These data suggested another Brucella factor, besides those encoded by VirB, or utilizing the VirB-dependent type IV secretion system, must be involved in UPR induction.

The role of TcpB in UPR gene induction and ER structural reorganization

The B. abortus protein Btp1 (Brucella-TIR-Protein 1) was originally characterized by its ability to inhibit dendritic cell maturation and to antagonize TLR2 signaling [29]. TcpB (Toll/Interleukin 1 like receptor domain containing protein), the correlating protein in B. melitensis also antagonizes TLR signaling and NF-kB activation [30]. We have recently shown that TcpB co-localizes with plasma membrane and microtubules and exerts a microtubule stabilizing effect similar to paclitaxel (Taxol) [31]. Besides co-localizing with cytoskeletal elements, exogenously expressed TcpB also co-localizes by immunofluorescence with the ER protein calreticulin (Figure S1). ER structure is microtubule centrally to form a ring around the nucleus. Thus trafficking may depend upon dramatic ER restructuring. Brefeldin A, which blocks egress from the ER is commonly utilized to induce the UPR [36]. Thus we reasoned that TcpB might contribute to UPR induction through microtubule-related modification of ER structure. As shown in Figure 5A, infection with the TcpB deletion mutant (∆TcpB) resulted in a~60% decreased expression of Bip, CHOP and ERdj4 as compared to wild type Brucella. Note, some CHOP up-regulation by the TcpB mutant with exogenous TcpB recovered UPR gene expression (Figure 5B). These results were consistent with a role for TcpB protein in UPR induction.

We hypothesized that UPR induction and ER restructuring are related events. In this case, the diminished UPR induction by the TcpB mutant should correlate with decreased effect on ER structure. Indeed, infection with the TcpB mutant did not induce the same degree of condensation and vacuolization as observed upon infection with wild type Brucella (Figure 6, Figure S2). The ER remains lacy, reticular and more evenly distributed compared to wild type Brucella. Trafficking of the Brucella within the cell however appears relatively intact, as the Brucella still migrate centrally to form a ring around the nucleus. Thus trafficking may not depend upon dramatic ER restructuring.

To directly test the role of TcpB in UPR induction and ER restructuring, RAW 264.7 macrophages were treated with purified TcpB protein using a concentration previously shown to affect microtubules and NF-kB signaling (Figure 7A) [30,31]. TcpB trafficking [35].
protein was sufficient to upregulate BiP, CHOP, ERdj4 and spliced XBP1. The relative magnitude of effect appeared much greater for BiP and CHOP than for ERdj4 and spliced XBP1. Triggering of UPR events correlated well with effects of TcpB on ER structure as detected by immunofluorescence microscopy (Figure 7B). Compare the diffuse lacy reticular pattern extending throughout the cell in untreated or the MBP treated cells (Figure S3 and 7B) to the circumscribed circular area with large holes and more defined compact structures in TcpB treated cells. The majority of cells examined appeared similarly affected. Overall ER area appears enlarged, particularly at the lower dose of TcpB (Figure S4). ER condensation and fragmentation increases with dose of purified TcpB. Similar effects were observed by 12 h of treatment (not shown). This effect on ER structure was qualitatively similar to that induced by infection of macrophages with wild type Brucella (Figures 1 and 6). Together these results implicate TcpB in both ER fragmentation and UPR induction.

It was unclear how the ER disruption related to the UPR. Were the ER structural changes a result of ER stress or is the UPR downstream of the ER disruption? To begin addressing this question, macrophages (or in some experiments D17 osteosarcoma cells) were treated with the ER stress inducer tunicamycin, a potent N-linked glycosylation inhibitor (Figure 8) [37,38]. Although tunicamycin caused ER vacuolization, most likely related to proteins being retained in the ER, the disposition of ER calreticulin in the cell was different compared to TcpB treatment (or infection, see above): in the tunicamycin treated cells, the ER did not condense in a sphere but remained distributed into the macrophage processes. Thus TcpB induced disruption does not simply reiterate an ER stressor. If TcpB-induced ER restructuring were upstream of the UPR and not dependent on UPR, then blockade of the UPR should have no effect on ER disruption. To address this hypothesis, we inhibited the UPR with tauroursodeoxycholic acid (TUDCA), a chemical chaperone widely utilized in vitro and in vivo to modulate the UPR. The ability of TUDCA to impede BiP and CHOP induction by tunicamycin was confirmed (Figure 8). Inhibition of XBP1 splicing was more variable. TUDCA also inhibited tunicamycin-dependent cytokine induction (Figure S5) as expected. TUDCA treatment mitigated the effect of tunicamycin [less vacuolization and size increase] but had no apparent effect on TcpB-related ER restructuring. These results suggest that ER restructuring is not UPR dependent. If ER structure and UPR are interdependent, ER disruption must occur upstream of UPR induction.

UPR blockade inhibits Brucella replication

The above data suggests Brucella induces the UPR at least in part via TcpB. However, it was not clear if the host mounts a UPR

Figure 6. TcpB mutation results in less ER structural disruption following infection. RAW 264.7 cells were infected with an YFP-expressing TcpB deletion mutant (ΔTcpB) or wild type (WT) B. melitensis (green) for 24 h. The ER is visualized with anti-calreticulin (red). Arrow indicates ER condensation and fragmentation. Confocal microscopy results are representative of 5 independent experiments. Scale bar is 20 μM. doi:10.1371/journal.ppat.1003785.g006

Figure 7. TcpB protein induces UPR and ER restructuring. A) RAW 264.7 cells were untreated (NT) or stimulated with 50 μg/mL MBP or MBP-TcpB (TcpB). After 24 h, cells were processed for RNA and relative expression of BiP, CHOP, ERdj4, or spliced XBP1 (XBP1s) determined by qPCR, with normalization to 18S rRNA. Bars depict combined means from 2 independent experiments, and are representative of 3 experiments, *p<0.004 vs. MBP. For ERdj4, p value is not significant vs. MBP, but P = 0.03 vs. non-treated cells. B) RAW 264.7 cells were untreated (Control), or stimulated with 10–50 μg/mL purified MBP-TcpB for 24 h. Cells were then fixed, stained for calreticulin, (red) and nuclei counterstained with DAPI (blue). Cells were imaged at 60 x in close up (Zoom, right) or without additional digital zoom (Broad Field, left). doi:10.1371/journal.ppat.1003785.g007
in response to infection, or if the UPR benefits the bacteria (or both). Viral infections manipulate the UPR in a variety of ways, including capitalizing on host protein production and folding machinery to enhance replication. One report utilizing insect cells and mouse embryonic fibroblasts suggests the IRE1 branch of the UPR supports Brucella replication, but the relevance to macrophages was unclear [13].

Brucella may not behave exactly the same in macrophages and non-phagocytic cells [15,39]. TcpB mutant Brucella are defective at spreading systemically early during infection in vivo [27]. However, the other effects of TcpB, in particular inhibition of TLR signaling in the setting of an in vivo immune response, complicate the interpretation. To determine if TcpB plays a role in intracellular replication in macrophages in vitro, RAW 264.7 cells were infected with wild type B. melitensis or the TcpB mutant. Select cultures were also treated with very low dose tunicamycin to enhance the UPR (Figure 9). Initial uptake of the TcpB mutant was greater than wild type (p = 0.008), but the replication growth curve plateaus below the level observed in wild type. This slowed growth resulted in decreased CFU later during the culture period (p ≤ 0.001 after 24 h). Tunicamycin treatment enhanced recoverable TcpB mutant CFU at all time points (p = 0.04 at 4 h and p ≤ 0.006 thereafter). This effect of

Figure 8. TcpB induced ER restructuring is not dependent on the UPR. A) RAW264.7 macrophages were pre-treated with 500 μg/mL TUDCA 30 min., followed by 6 h 10 μg/mL tunicamycin (Tm) as indicated, and then harvested for RNA. Relative UPR gene expression was assessed by qPCR. Results were combined from 2 independent experiments, *p = 0.01 and **p = 0.001 vs. Tm treatment only and NS = not significant vs. untreated cells. B) RAW 264.7 cells were pre-treated with 500 μg/mL TUDCA (TUDCA +) or not pre-treated (TUDCA −) for 60 min prior to stimulation with 50 μg/mL TcpB, 10 μg/mL tunicamycin (Tm), or media (Control). Cells were then fixed, stained for calreticulin (red), and counterstained with DAPI. Images are 100×. doi:10.1371/journal.ppat.1003785.g008
mutant cultures were also pre-treated with tunicamycin at 0.01 µg/mL (dashed lines). At different times following infection, cells were lysed and CFU determined by transfer to dilution plates. Error bars depict standard deviations of quadruplicate determinations. *P≤0.001. Results are representative of 4 independent experiments showing altered late growth of ΔTcpB.

Discussion

Brucella infection mobilizes all three UPR signaling axes in macrophages, stemming from the activation of IRE1, PERK and ATF6. Oxidative stress also strongly activates the PERK pathway, thus the UPR is often referred to as an “integrated stress response” [41]. However robust induction of target genes from the three distinct biochemical signaling pathways is most consistent with the UPR [42]. One report demonstrated IRE1 phosphorylation and PERK pathway activation in M. tuberculosis infected macrophages in vivo. However the direct link between infection and induction of host UPR was not established [19]. Another study implicated the IRE1 pathway in supporting Brucella replication, consistent with the results obtained in this study, however the relevance to macrophages was unclear [13]. Although previous data reported XBPI mRNA splicing by intracellular bacteria such as Francisella [20], this is one of the first reports of more widespread UPR induction resulting directly from intracellular bacterial infection rather than toxin production.

Brucella induced XBPI mRNA splicing appears to proceed predominantly through the previously described MyD88 (TLR) dependent pathway [20]. The unusual smooth Brucella LPS contains reduced negative charges and unusually long aliphatic hydrocarbon chains in the Lipid A core [22] as compared to C12-16 in enterobacteria [4]. Related to these properties, Brucella smooth LPS displays reduced TLR4 agonist activity [43]. Thus smooth LPS may be a relatively weak inducer of XBPI splicing. Consistent with this prediction, the genomic island 2 deletion Brucella mutant that expresses rough LPS triggers much more robust XBPI splicing (data not shown) [44]. In comparison with XBPI splicing, downstream CHOP and ERdj4 target gene induction was entirely MyD88-independent. BiP induction was not detected in these particular experiments, potentially related to timing (BiP upregulation is an early transient event), mouse strain, or differences in macrophage type [45]. Thus, as noted by others, all signaling pathways encompassed by the UPR are not always coordinately regulated [20]. “UPR” signaling events such asXBPI splicing may be triggered by non-UPR agonists and UPR signaling pathways are not always activated in their entirety. Other examples of XBPI splicing-downstream target disconnection come from the viral literature, and Hepatitis C in particular [46]. The mechanism underlying the dissociation remains unknown. In the present study, it was curious that the canonical XBPI target gene EREDj4 was up regulated in the absence of significantly detectable XBPI splicing in the MyD88−/− bone marrow macrophages. There are several possible explanations. First, sufficient TLR4 TRIF dependent XBPI activity remains to induce EREDj4. Second, only part of XBPI splicing is MyD88 dependent and the assay is not sufficiently sensitive to detect minor differences. The low level XBPI splicing induced by purified TcpB is consistent with this idea, as we would assume this is ER stress rather than MyD88-related. Indeed, TcpB would be expected to antagonize TLR-MyD88-dependent signaling [29,30]. Third, EREDj4 may be induced in an XBPI independent manner [29,47]. Given the evidence that even weak TLR signaling by Brucella still induces XBPI splicing, it will be interesting to determine the role of XBPI in Brucella-induced cytokine production.

Our results reveal a new role for the TcpB protein in regulating host stress responses and ER structure. Indeed, the ability of TcpB to fragment and condense the ER may be the underlying mechanism for the dramatic ER restructuring first reported almost a decade ago [7]. Based on 1) our data correlating ER disruption and UPR induction in response to purified TcpB, 2) the diminished UPR and ER structural impact in the absence of TcpB, and 3) the capacity of an analogous microtubule disrupting drug paclitaxel to induce ER stress, it is highly likely that TcpB induced ER restructuring and UPR are causally linked [33,34]. The comparison with tunicamycin treatment and the lack of TUDCA effect on TcpB induced ER restructuring also suggest...
that TcpB-induced UPR occurs following, or downstream of ER structural disruption. However, it remains possible that ER stress is not directly related to TcpB-induced ER structural changes. Purified TcpB was more effective at upregulating CHOP and BiP than IRE1 dependent events such as XBP1 splicing and ERdj4. Indeed XBP1 splicing was intact in the TcpB mutant infected cells and minimally induced in cells by TcpB protein. The vast majority of XBP1 splicing appears to proceed through the TLR-MyD88 pathway. Thus the readout of “UPR” reflects contributions from multiple bacterial factors. The effect of TUDCA on replication also suggest that the delayed virulence of the TcpB mutant in a susceptible IRF1−/− deficient mouse model may reflect both altered replication and enhanced cytokine production. It is a testament to bacterial efficiency that one protein product may antagonize host immune signaling and induce host stress responses that support bacterial replication.

The ultimate role of TcpB in replication, given the pleiotropic effects of this molecule, remains unclear. Initially, uptake of the mutant is much greater in macrophages, but the growth curve plateaus below the level of the wild type. Growth of the ΔBtp1 B. abortus was not impaired in dendritic cells [29]. This may reflect timing, a difference in macrophages vs. dendritic cells, cell line vs. primary cells on another strain background, or differences in B. abortus vs. B. melitensis. The TcpB mutant is clearly attenuated in dendritic cells [29]. This may reflect both altered replication and enhanced cytokine production. It is a testament to bacterial efficiency that one protein product may both antagonize host immune signaling and induce host stress responses that support bacterial replication.

Although TcpB appears to play a pivotal role in regulating UPR target genes, other virulence factors (e.g. VirB) may contribute. Indeed, CHOP expression was not reduced to the non-infected level in the TcpB mutant infection, consistent with the existence of other UPR inducing molecules [40]. Also, TUDCA inhibited growth of the TcpB mutant (Figure S7B). The experimental variability obtained with the VirB mutants may reflect a timing issue (important earlier or later than our experimental window) or sensitivity. The proportion of cells infected and number of bacteria/cell will affect UPR detection. Since the VirB mutant traffics abnormally and fails to survive inside macrophages, fewer bacteria will be available to produce UPR-inducing factors [7].

Interestingly, in the IRF1−/− mouse model, patterns of in vivo virulence differed between the VirB and TcpB mutants consistent with roles in different parts of the bacterial life cycle. TcpB appears to regulate early spread of infection whereas VirB contributes more to bacterial persistence [28,30]. The requirement for living bacteria to optimize UPR target gene induction suggests the UPR is an active process supported by new protein(s) or other factor(s) produced following infection; it is not just a host response to components present in dead bacteria. The residual UPR induction by heat-killed bacteria may reflect TcpB, or an unidentified factor produced by the bacteria during growth in broth.

The UPR may support the intracellular life cycle of Brucella in a number of ways. First, the UPR mobilizes amino acid transport and supports lipid biogenesis. Second, the UPR also initiates autophagy, thus providing more nutrients. As described by Starr et al, the UPR regulated autophagy may participate in completing the Brucella intracellular life cycle, allowing spread to neighboring cells [17]. Third, the UPR enhances protein-folding capacity through induction of chaperones and other folding machinery. Fourth, the UPR allows cells to cope with oxidative stresses. Finally, as a means of physiological adaptation, the UPR may enable host cells to survive the disruption of ER structure and function. The UPR encompasses anti-apoptotic mechanisms and only promotes apoptosis when stress is severe or prolonged. In the viral literature, Dengue activates all three UPR pathways, yet suppresses downstream apoptosis [48]. It will be interesting to determine if some of the same apoptosis modulating mechanisms apply to Brucella. Another possibility is that Brucella LPS may sufficiently temper CHOP induction to avert apoptosis [20].

In this study, TUDCA pre-treatment exerted a dramatic effect, decreasing recoverable CFU in culture. The simplest interpretation is that the host UPR plays an absolutely critical role in supporting Brucella replication. This hypothesis is consistent with the work from Qin et al. showing decreased Brucella CFU following IRE-1 knockdown. We also have preliminary data suggesting this UPR axis supports replication in macrophages, most likely through the IRE1-kinase-JNK signaling pathway rather than through XBP1 (data not shown). The contrasting effect of...
TUDCA on *Brucella* replication and XBP1 splicing/ERdj4 expression is consistent with our preliminary XBP1 RNAi data showing no effect on replication. The XBP1 variability in response to TUDCA may reflect multiple mechanisms of XBP1 splicing induction. However, the apparent effect of TUDCA on Brucella-induced BiP and CHOP expression may also result from greatly diminished numbers of bacteria. Although TUDCA is widely utilized to assess the role of the UPR in vivo, and is approved for use in humans, the drug may affect other cellular processes besides the UPR [49]. The non-specificity of TUDCA is one limitation of this study. However, these results supply strong rationale to further investigate which specific UPR-related molecules might be involved in supporting *Brucella* replication in macrophages. Also, despite non-specificity, TUDCA may be useful therapeutically, particularly in view of safety and cost. It may prove important to inhibit multiple arms of the UPR, as inhibition of one specific signaling axis may not be sufficient. Successful inhibition of *Brucella* virulence in vivo by TUDCA or other more selective UPR modulation would open a new avenue of drug development.

TUDCA has an excellent safety profile and is being studied in humans to counteract UPR-related metabolic syndromes [49,50]. It will be essential to determine whether TUDCA mediated inhibition of replication outweighs the effect of UPR blockade on inflammatory cytokine production in vivo. We, along with other researchers, have described dramatic augmentation of interferon and inflammatory cytokine production by the UPR [20,51]. Indeed, the UPR has been implicated in numerous inflammatory and autoimmune diseases [52,53]. Currently, little is known about the role of the UPR in immune responses to *Brucella*, and the formation of immune memory [54].

The concept that subverting the host UPR enables bacterial replication in macrophages, thus promoting infectious success represents a paradigm shift for the field that merits further investigation. The results from this study have broad implications for other bacteria that establish an intracellular replicative niche, particularly those that interact with the ER [55].

**Materials and Methods**

**Cells, bacterial strains, and reagents**

The RAW264.7 murine macrophage and D17 canine osteogenic sarcoma cell lines (both ATCC) were maintained in RPMI 1640/high glucose with 4 mM L-glutamine, sodium pyruvate (Hyclone Laboratories) and supplemented with 10% FBS (Hyclone), 100 U/mL penicillin, and 100 μg/mL streptomycin.

*B. melitensis* 16M, the engineered bioluminescent strain GR019 (VirB mutant), or the TcpB deletion mutant were grown in *B. melitensis* broth (BB, Difco) supplemented with 50 μg/mL kanamycin [28,30]. To heat kill bacteria, *B. melitensis* in BB was incubated at 65°C for 60 min. The purification of TcpB protein has been described [30]. MBP-TcpB was used at a concentration of 50 μg/mL, with maltose binding protein (MBP) as a control [30].

**Mice and bone marrow derivation**

Mice were kept in facilities at the University of Wisconsin-Madison that are accredited by the American Association of Laboratory Animal Care. Mouse experiments were performed with oversight and approval of the University of Wisconsin-Madison School of Medicine and Public Health and School of Veterinary Medicine Animal Care and Use Committee (NIH assurance number: A3368-01), in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. MyD88−/− femurs were a gift from Laura Knoll, University of Wisconsin-Madison. Bone marrow cells from C57BL/6 wild type or MyD88−/− femurs were isolated on Histopaque 1073 (Sigma-Aldrich, St. Louis, MO) and differentiated for 7 days in RPMI 1640 with 10% FBS and 50 ng/mL recombinant murine M-CSF (Peprotech). For *in vitro* infections, 4–7 mice/group of 6–8 week old BALB/c mice were injected i.p. with PBS or 10^7 GR023 (bioluminescent *B. melitensis*). After 24 h, spleens were pooled within groups, homogenized, and splenic macrophages were isolated using CD11b+ magnetic cell separation (Miltenyi) according to manufacturer’s protocol. Cells were immediately resuspended in Trizol for further processing.

**In vitro infections**

RAW 264.7 or bone marrow derived macrophages (BMDM) were cultured in 6-well dishes (unless otherwise indicated) overnight prior to infection. Macrophages were infected with either 10:1 or 100:1 multiplicity of infection (MOI) with late log or stationary phase *Brucella* for times indicated and then harvested for RNA analysis or CFU evaluation. Cultures were incubated at 37°C with 5% CO2. Select cultures were treated with 0.01 μg/mL tunicamycin (Sigma) 30 minutes prior to infection. Note: although gentamycin is routinely used during *Brucella* infections, it decreases detection of UPR induction (particularly CHOP).

**Complementation assay**

The coding sequence of Bmel 1674 encoding TcpB1 was inserted into the *Brucella* plasmid pNstrD [56]. The plasmid was electroporated into *B. melitensis* ΔTcpB1 by standard methods. RAW 264.7 or J774A.1 (both from ATCC) mouse macrophage cell lines were seeded in 6 well tissue culture plates at 3×10^6 per well 1 day prior to infection. Cultures of *B. melitensis*, *B. melitensis* ΔTcpB1, and *B. melitensis* Δ TcpB1+pNstrD/Bm1674 (3 ml each in BHI media with appropriate antibiotics) were seeded 2–3 days before infection to be in late log phase at the time of infection. Macrophage cells were infected at 100 MOI and cultured for 24 h. Cells were then washed 1× in PBS, lysed and harvested in 1 ml/well of Trizol (Invitrogen) for RNA processing.

**UPR detection (PCR)**

Real time PCR: Following culture, superantigen was removed and samples were resuspended in TRIzol (Invitrogen). RNA was purified according to manufacturer’s instructions and treated with DNAseI (Invitrogen) to remove genomic DNA. RNA was reverse transcribed using random primers (Promega). Relative cDNA was quantified using SYBR Green (Bio-Rad) and detection in MyQ, or CFX96 real time PCR machines (both Bio-Rad). Primers were designed using Beacon Design software (Premier Biosoft) and are as follows: 18S rRNA: forward, 5'-GGACACGGAGACAGTATGAGC-3' and reverse, 5'-ATCGCTCCACAACTACTGAAGACG-3'. Hprt1: forward, 5'-GTTAAGACGATACGCCCTCA-3' and reverse, 5'-AGGGCATAATCCACACCAAACACCTT. BiP: forward, 5'-AGGATGGCGGACATTGAGAC-3' and reverse, 5'-AGGCTGGTATCTTGGCTTC-3'. CHOP: forward, 5'-CATCACCCTCTCTGTGTGTCT-3' and reverse, 5'-AGCCCTCTGCCTGCTACAG-3'. Erdj4: forward, 5'-AGGGAAGATGGAGAATTGCA-3' and reverse, 5'-ACTGGTGCATTGCCTTGGG-3'. IL-6: forward, 5'-AGCAGATGATGACATTCAG-3' and reverse, 5'-GTAAGCATTGGTAGCAGAACAG-3'. XBP1 splicing was assessed through 3 assays: 1) Agarose gel assay: XBP-1 primers for splicing (Invitrogen). % XBP unspliced (OD) quantified using Image Quant (GE Healthcare). % XBP splicing was spotted on 3% gel and optical density (OD) quantified using Image Quant (GE Healthcare). % XBP splicing was normalized to total (spliced+unspliced) OD×100. 2) Quantification of separate species by Agilent. 3) qPCR assay:
XBPI(ψ): forward, 5'-TCCGACGAGCTCAAGATATGCATGT-3' and reverse, 5'-ATGCCCAAAAGGATATCGTCAGTC-3'. XBPI(α): forward, 5'-TGGTTCGGCAAGCGGTG-3' and reverse, 5'-GGTGTCAGGCTAGAGGAA-3'. % splicing = XBPI(ψ)/XBPI(α)×100 [57].

UPR detection (biochemistry)

Non-infected and B. melitensis infected RAW cells were harvested and resuspended in a buffer containing 20 mM Tris-HCl [5,0] and 0.5% SDS. Samples were boiled for 20 min. and mixed with equal amount of sample buffer. Cell lysates were resolved on a 4–20% SDS PAGE and transferred to immunoblot PVDF membrane (Millipore). The membrane was blocked with Tris-buffered saline containing 0.1% Tween 20 (TTBS) and 5% nonfat milk for 1 h at room temperature followed by three washes with TTBS. The membrane was incubated with anti-CHOP antibody (Cell Signaling Technology) in blocking buffer over night at 4°C. After washing three times with TTBS, the membrane was incubated with HRP-conjugated anti-mouse IgG (Pierce) in blocking buffer for 1 h at room temperature. After three washes with TTBS, protein bands were detected using SuperSignal West Pico Chemiluminescent Substrate according to manufacturer’s instructions (Pierce). The membrane was re-probed with anti-actin (Santa Cruz Biotechnology). Chemiluminescence was detected by CL-XPosure Film (Thermo Scientific).

Immunofluorescence microscopy

RAW264.7 cells, D17 cells or bone marrow derived macrophages were seeded into chamber slides (Lab Tek and Ibidi) and allowed to adhere 16–24 h. For infections, macrophages were infected (1000 MOI) with either wild-type B. melitensis or B. melitensis containing a TcpB gene deletion for 24 h. Both strains express YFP under control of the trcD promoter. TUDCA (500 μg/mL) pre-treatments were 30–60 min. For purified protein treatments, the medium was then replaced with fresh medium (1 ml) containing purified maltose binding protein (MBP, MBP-TcpB protein (10 or 50 μg/mL), or tunicamycin (1 or 10 μg/mL) and the plates were incubated over night (12–24 h). The cells were washed 3X with PBS, fixed with 4% paraformaldehyde for 10 min, followed by permeabilization with 0.1% Triton X100 for 10 min. Cells were treated with blocking buffer containing 5% normal serum and 50 mM NH4CI in 1X PBS for 30 min, then washed and incubated with 1:100 dilution of anti-calreticulin antibody (Thermo Scientific) in PBS containing 0.1% Triton X100 for 1 h. Cells were treated with blocking buffer containing 5% normal serum and 50 mM NH4CI in 1X PBS for 30 min, then washed and incubated with 1:100 dilution of anti-calreticulin antibody (Thermo Scientific) in PBS containing 0.1% Triton X100 for 1 h. Cells were washed 3X with PBS and incubated with 1:1000 dilution of Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen) or secondary conjugated to DyLight 550 (Thermo Scientific) for 1 h, or anti-rabbit 550 (Cell Signaling) overnight, washed 3X with PBS, and mounted in ProLong Gold antifade reagent with DAPI (Cell Signaling). Images were collected using either a Radiance 2100 MP Rainbow confocal/multiphoton microscope (Bio-Rad) or Nikon A1R confocal laser microscope.

UPR blockade

To determine the effect of chemical chaperones on Brucella viability, Brucella were plated in 96 well plates at 5×106 cells/well in RPMI containing serial dilutions of tauroursodeoxycholic acid (TUDCA, Sigma). BacTiter-Glo assay (Promega) was performed to determine ATP content (viability) as assessed by luminescence. To determine the effect on RAW cell viability, cells were plated in 96 well plates at 104 cells/well one day prior to challenge. The medium was then replaced with fresh medium containing serial dilutions of chemical chaperones. CellTiter-Glo assay (Promega) was performed to determine ATP content (viability) as detected by luminescence. Effect of TUDCA on Brucella viability in broth was also determined utilizing this assay. To confirm TUDCA inhibition of UPR, RAW cells were pre-treated 30 min. with TUDCA, then stimulated with 10 μg/mL tunicamycin (Sigma) for 6 h or B. melitensis for 24 h prior to processing in TRIzol. Inhibition of replication: RAW 264.7 macrophages were plated in 24 well dishes at 5×103/well the day prior to infection. Cells were pre-treated with 500 μg/ml TUDCA (4 experiments) or 4 mg/ml TUDCA (1 experiment, no significant RAW cell viability effect) for 30 min. prior to infection with either 10 or 100 MOI of stationary phase B. melitensis. After 30 min., cells were washed 4X with warm PBS and fresh media with 50 μg/ml gentamycin added with or without TUDCA. To evaluate colony-forming units (CFU), cells were washed 3X with PBS and then lysed in 1% Triton-X 100 in water. CFU were determined by serial dilution plating on agar after 3–4 days. In parallel, samples were lysed in TRIzol to determine effect of TUDCA on UPR target gene induction at 24 h.

Statistical analysis

Differences between data were evaluated using Students T-test with p<0.05 considered significant.

Supporting Information

Figure S1 TcpB co-localizes with ER calreticulin. D17 cells were transfected with pCMV-TcpB-HA plasmid and fixed 24 h later [31]. Cells were stained with anti-HA (red), anti-calreticulin (green) and DAPI (blue), and imaged by fluorescence microscopy (50X). Co-localization of TcpB-HA and calreticulin appears yellow. Similar results were obtained in RAW 264.7 cells. Images are 50X. (TIF)

Figure S2 TcpB mutant Brucella infection induces less ER structural disruption. RAW 264.7 cells were infected with an YFP-expressing TcpB deletion mutant (ΔTcpB) or wild type (WT) B. melitensis (green) for 24 h (as in Figure 6). The ER is visualized with anti-calreticulin (red). Broad field images from 2 experiments are shown. (TIF)

Figure S3 Comparison of untreated, MBP-treated and TcpB treated cell ER structure. RAW cells were treated with 50 μg/ml purified MBP or MBP-TcpB for 12 h. The ER is visualized with anti-calreticulin (red). Arrow indicates ER condensation and fragmentation. Bar is 20 μM. (TIF)

Figure S4 TcpB increases vacuole diameter and ER size. RAW 264.7 cells were cultured in the presence of 50 μg/ml purified TcpB for 24 hours then washed and fixed for staining as shown in Figures 7–8. Five non-dividing cells in each frame were randomly selected for quantification. Measurements were taken using the Ruler Tool within Adobe Photoshop, with the scale set at 1 pixel = 62.15 nm for 100× magnified microscopy images. A) Vacuole diameters were measured in control (29 vacuoles) and TcpB treated (47 vacuoles) cells. TcpB treatment increased vacuole diameter significantly (**P=0.0003). B) Calreticulin staining area was assessed by measuring the entire anti-calreticulin labeled fluorescent area and then subtracting out the area of the nuclei. Calreticulin area was significantly increased in TcpB treated cells (*P=0.03), while nuclear size was equivalent to the control cells (data not shown). (TIF)
Figure S5 TUDCA inhibits cytokine induction by the ER stressor tunicamycin. RAW264.7 macrophages were pre-treated with 500 μg/mL TUDCA 30 min., followed by 6 h 10 μg/mL tunicamycin (Tm) as indicated, and then harvested for RNA. Relative cytokine gene expression was assessed by qPCR. Results are combined from 2 independent experiments. (TIF)

Figure S6 Minimal effects of TUDCA on host cell and pathogen viability. A) left panel: RAW 264.7 macrophages were treated with 500 μg/mL for the times indicated. Viability (ATP content) was determined by Cell-titer glo assay. Error bars represent standard deviation of triplicate determinations. *p = 0.04. Right panel: Bars depict average content) was determined by Cell-titer glo assay. Error bars represent standard deviation of triplicate determinations. *P = 0.02. B) RAW cells were untreated (black circles) or pre-treated with 500 μg/mL TUDCA as above (gray squares), and then infected with 10 MOI of the ΔTcpB mutant Brucella. CFU were determined as in (A). Error bars represent standard deviation of triplicate determinations. *p<0.04, **p<0.006. (TIF)

Author Contributions
Conceived and designed the experiments: GAS JAS DDM. Performed the experiments: YPL JSH MK MD GKR. Analyzed the data: GAS JAS DDM YPL JSH MK MD GKR DDM. Wrote the paper: JAS GAS.

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