Stress-induced host membrane remodeling protects from infection by non-motile bacterial pathogens

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ABSTRACT

While mucosal inflammation is a major source of stress during enteropathogen infection, it remains to be fully elucidated how the host benefits from this environment to clear the pathogen. Here, we show that host stress induced by different stimuli mimicking inflammatory conditions strongly reduces the binding of *Shigella flexneri* to epithelial cells. Mechanistically, stress activates acid sphingomyelinase leading to host membrane remodeling. Consequently, knockdown or pharmacological inhibition of the acid sphingomyelinase blunts the stress-dependent inhibition of *Shigella* binding to host cells. Interestingly, stress caused by intracellular *Shigella* replication also results in remodeling of the host cell membrane, in vitro and in vivo, which precludes re-infection by this and other non-motile pathogens. In contrast, *Salmonella* Typhimurium overcomes the shortage of permissive entry sites by gathering effectively at the remaining platforms through its flagellar motility. Overall, our findings reveal host membrane remodeling as a novel stress-responsive cell-autonomous defense mechanism that protects epithelial cells from infection by non-motile bacterial pathogens.

Keywords: *Shigella* / host stress response / acid sphingomyelinase / *Salmonella* / membrane remodeling

INTRODUCTION

Intestinal epithelial cells (IECs) constitute a physical and biochemical barrier between host and microorganisms. Whilst not considered professional immune cells, IECs are crucial contributors to immune surveillance and the initial inflammatory responses against infection (Peterson & Artis, 2014; Sansonetti, 2004). For example, IECs secrete pro-inflammatory cytokines and chemokines in response to infection by certain enteroinvasive bacterial pathogens such as *Shigella flexneri* and *Salmonella* Typhimurium. This, in turn, leads to a massive infiltration of professional immune cells into the sites of inflammation, from which ensues a local increase in reactive oxygen species and a profound hypoxia (Arena et al, 2017; Colgan & Taylor, 2010; Zeitouni et al, 2016). Such change in environment not only constrains the pathogen, it also dramatically affects the intestinal epithelium. Interestingly, while this inflammation-related stress used to be considered as posing additional harm to the affected tissues, there is recent evidence to suggest that it makes crucial contributions to counteract infection processes at the cellular level (Chovatiya & Medzhitov, 2014).

Bacterial pathogens have evolved sophisticated mechanisms to subvert, and often invade the intestinal epithelium, thereby overcoming host defense mechanisms (Carayol & Tran Van Nhieu, 2013; Pizarro-Cerda & Cossart, 2006; Sansonetti, 2004). *Shigella flexneri*, for example, crosses the intestinal barrier by transcytosis through M-cells (Perdomo et al, 1994; Wassef et al, 1989), before invading IECs from the basolateral side (Mounier et al, 1992). For invasion, *Shigella* uses its type III secretion system (T3SS) to inject effector proteins into target cells to subvert host defense pathways, promoting its own internalization by a trigger mechanism that involves the formation of actin rich membrane ruffles (Ogawa et al, 2008; Parsot, 2009; Schroeder & Hilbi, 2008).

Notwithstanding the diverse active mechanisms used by bacteria to mediate binding and invasion of host cells [e.g. pili, fimbriae, adhesins/invasins, T3SS; (Pizarro-Cerda & Cossart, 2006; Stones & Krachler, 2016)], it has also become clear that efficient bacterial entry requires permissive sites in the host membrane. Membrane rafts, which are highly dynamic membrane domains enriched in sphingolipids and cholesterol that mediate the compartmentalization of signaling proteins and receptors (Lingwood & Simons, 2010; Sezgin et al, 2017), have been shown to be utilized by numerous bacterial pathogens [reviewed in (Bagam et al, 2017; Lafont & van der Goot, 2005)]. For example, *Shigella* uses its IpaB effector protein to bind the host raft-associated CD44 transmembrane receptor (Lafont et al, 2002); entry of *Listeria monocytogenes* into host cells requires the localization of the host receptors E-cadherin and HGF-R/Met in specific lipid domains (Seveau et al, 2004). In addition to receptors, plasma membrane composition itself, specifically cholesterol and sphingolipid membrane content, impacts the binding and

internalization of various bacterial pathogens, including *Shigella* and *Salmonella* species (Garner et al, 2002; Lafont et al, 2002; Misselwitz et al, 2011a; Santos et al, 2013).

Here, we investigated the impact of the general stress response of epithelial cells on the infection by *Shigella flexneri* and *Salmonella* Typhimurium. We found that induction of stress in epithelial cells by inflammatory cues and oxidative insults prevents the binding of *Shigella*, a non-motile pathogen, to host cells. We demonstrate that this inhibition results from extensive remodeling of the host plasma membrane following a stress-induced activation of the acid sphingomyelinase (ASM). By contrast, the related motile pathogen *Salmonella* can overcome this barrier, using flagellar motility to reach and accumulate at the remaining permissive entry sites. Moreover, we show that intracellular replication of *Shigella* activates ASM and subsequent membrane remodeling, thus suppressing re-infection by non-motile pathogens. Collectively, our findings demonstrate a role for the host stress response in protecting cells against *Shigella* infection and demonstrate the involvement of ASM and membrane remodeling in this process.

RESULTS

Host cell response to stress inhibits *Shigella* infection

To investigate whether host cell stress has a deleterious effect on the outcome of *Shigella* infection, we treated HeLa cells, an epithelial cell line commonly used to study *Shigella* infection, with sub-lethal concentrations of sodium arsenite (Fig. 1A). Arsenite is widely used to induce oxidative stress (Bernstam & Nriagu, 2000; Liu et al, 2001). Following arsenite removal, cells were extensively washed and then infected with *Shigella;* infection efficiency was monitored at early, intermediate, and late stages of infection (0.5, 2, and 6 hpi, respectively; Fig. 1A) by: i) fluorescence microscopy, ii) colony forming unit assays and iii) qRT-PCR. Interestingly, pre-treatment of cells with arsenite strongly reduced *Shigella* infection, at all time points tested (4.7- to 8.8-fold compared to control, cfu; Fig. 1B, 1D, EV1A, EV1B). Validating these observations, *Shigella* infection was also inhibited by arsenite in all tested colon epithelial cells, namely HCT-8, HT-29 and Caco-2 cells (Fig. 1C, 1D, EV1B-D).

Response to environmental stress in eukaryotic cells generally dampens bulk protein synthesis due to impaired mRNA translation initiation (Holcik & Sonenberg, 2005). However, inhibition of translation by puromycin and cycloheximide did not affect *Shigella* infection (Fig. EV1E-G), demonstrating that the effect of arsenite on *Shigella* infection is unrelated to translation shutdown.

To understand if inhibition of *Shigella* infection by cellular stress is a broad phenomenon, we tested other stress inducers, namely anisomycin, hydrogen peroxide (H_2O_2), hypoxia or the inflammatory cytokine TNF-α. All the stress inducers have been widely used to mimic conditions encountered by cells during inflammation (arsenite, anisomycin) or are per se stimuli present during inflammation (hypoxia, TNF-α and H_2O_2). These stimuli converge in the production of reactive oxygen species (ROS), key signaling molecules during inflammation. Consistently, pre-treatment with the various stimuli strongly inhibited *Shigella* infection, already at early times post-infection (0.5 hpi; Fig. 1E, 1F, EV1H, EV1I). The various compounds did not affect host cell viability, at the concentrations and incubation periods tested (Fig. EV1J). It should be noted that host cells were extensively washed prior to infection to remove any remaining stressors. Moreover, arsenite or anisomycin treatment did not impair *Shigella* growth (Fig. EV1K), thus excluding direct effects on the bacteria.

Importantly, treatment with the antioxidant N-acetyl-L-cysteine (NAC) reverted the inhibitory effect of arsenite or anisomycin on *Shigella* infection (Fig. 1G, 1H, EV1L), further confirming that the effect of these stressors on *Shigella* infection is mediated by oxidative stress. Overall, these results demonstrate that the response of epithelial cells to oxidative stress limits *Shigella* infection.

Shigella binding to host cells is inhibited upon cellular stress

The evident inhibition of *Shigella* infection observed at 0.5 hpi strongly indicates that host cell stress affects the early steps of *Shigella* interaction with host cells. Accordingly, pre-treatment of cells with arsenite or anisomycin strongly inhibited *Shigella* binding to HeLa cells (ca. 10.0- and 2.0-fold compared to control, cfu, respectively; Fig. 2A-C, S1A-E). Comparable results were obtained in HCT-8 cells (Fig. 2D, 2E, S1F). A possible effect of the stress inducers on actin cytoskeleton integrity and dynamics was excluded, since normal induction of actin-rich membrane ruffles by wild-type (WT) *Shigella* was observed in cells pre-treated with arsenite or anisomycin (white arrowheads in Fig. 2A and S1C, respectively). Accordingly, the efficiency of ruffle formation, i.e. the percentage of ruffles induced upon bacterial contact, was similar in cells treated with stressors and control cells (Fig. S1G). To uncouple *Shigella* binding to host cells from subsequent steps of invasion, we performed parallel experiments with the *Shigella* Δ*ipaB* mutant strain, which binds efficiently to host cells but is unable to invade (Menard et al, 1993). Treatment of cells with arsenite or anisomycin inhibited binding of *Shigella* Δ*ipaB*, similarly to the WT bacteria (Fig. 2A-E, S1B, S1C, S1E, S1F). Binding of *Shigella* Δ*icsA* mutant strain to host cells was also inhibited by arsenite or anisomycin treatment (Fig. S1H, S1I), demonstrating that the effect of the

stressors is independent of the role of IcsA in *Shigella* adhesion (Brotcke Zumsteg et al, 2014). Overall, these results show that host cell stress has a strong inhibitory effect on *Shigella* binding.

Analysis of *Shigella* cell-to-cell spreading in control and arsenite or anisomycin treated cells, by quantifying the area of *Shigella* infection foci using fluorescence microscopy and automated image analysis (Sunkavalli et al, 2017), excluded an effect of stress in the actin-based spreading of *Shigella* to neighboring cells (Fig. S1J). In these experiments, infections were performed at different MOIs (MOI 10 control, 50 anisomycin and 100 arsenite), to achieve comparable levels of bacterial invasion.

ASM-dependent membrane remodeling upon stress inhibits *Shigella* binding

Considering that binding of *Shigella* to host cells is inhibited by stress and that this occurs in a relatively short timeframe (e.g. 15 min. pre-treatment with TNF-α; Fig. 1E, 1F, EV1H, EV1I), we reasoned that the decreased binding of *Shigella* could result from modifications of the cellular membrane. In line with this possibility, membrane composition, specifically the presence of sphingolipids and cholesterol at the cell surface, has been shown to be required for successful *Shigella* infection (Lafont et al, 2002). The turnover of sphingomyelin, a ubiquitous sphingolipid component of animal cell membranes that is enriched in membrane rafts, is mediated by sphingomyelinases. These enzymes catalyze the breakdown of sphingomyelin to ceramide and phosphocholine (Goni & Alonso, 2002). Interestingly, neutral sphingomyelinase (NSM) and acid sphingomyelinase (ASM) are activated in response to various stress stimuli, including hydrogen peroxide, hypoxia, TNF-α and infection (Grassme et al, 2003; Hannun & Luberto, 2000; Marchesini & Hannun, 2004).

To investigate whether the activation of sphingomyelinases and consequently the disruption of sphingolipid-rich membrane domains could account for the inhibition of *Shigella* binding to host cells observed upon stress, we used specific inhibitors of these enzymes. Inhibition of NSM by GW4869 did not rescue the impairment of *Shigella* infection prompted by arsenite or anisomycin (Fig. EV2A-F). However, treatment with the ASM inhibitor amitriptyline partially reverted the inhibitory effect of arsenite on *Shigella* infection (Fig. 3A-C) and fully reverted that caused by anisomycin (Fig. 3D-F). Corroborating these results, knockdown of ASM blunted the effect of arsenite on *Shigella* infection (Fig. 3G-I). Of note, in the absence of stress, the inhibition of ASM activity by amitriptyline or ASM knockdown did not affect *Shigella* infection (Fig. EV2G-J). The effect of amitriptyline in blunting ASM enzymatic activity and ceramide production upon arsenite or anisomycin treatment was confirmed (Fig. EV2K-M). In addition, the decreased ASM activity, RNA and protein levels upon knockdown were confirmed (Fig. EV2N-P). Taken

together, these results show that, in conditions of cellular stress, ASM activity is responsible for the inhibition of *Shigella* infection.

ASM is usually associated with the lysosomal compartment but, upon activation, it is redistributed to the outer leaflet of the plasma membrane, where it hydrolyzes sphingomyelin giving rise to ceramide-rich platforms (Grassme et al, 2001). To validate if ASM is relocalized as part of the cell response to stress, we used confocal microscopy followed by 3D reconstruction to visualize the accumulation of ASM and ceramide at the membrane. Indeed, treatment of cells with arsenite or anisomycin induced a marked accumulation of ASM at the cell surface, in both HeLa and HCT-8 cells (Fig. 3J, 3K, S2A – HeLa; Fig. S2E – HCT-8). Consistent with these findings, a strong accumulation of ceramide was also observed at the membrane of cells treated with arsenite or anisomycin (Fig. 3L, 3M, S2C – HeLa; S2F – HCT-8). As expected, ASM knockdown diminished ceramide accumulation in response to arsenite (Fig. S2D). The ASM staining specificity was confirmed in cells transfected with ASM siRNA, in which a considerable decrease of ASM foci was observed (Fig. S2B). To further reinforce these observations, we quantified ceramide in live cells, by flow-cytometry. In live cells, the ceramide antibody can only recognize extracellular/exposed epitopes and thus exclusively labels ceramide present at the cellular surface. This analysis confirmed a significant increase of ceramide in cells treated with arsenite and anisomycin, and a reversion of the increase upon amitriptyline treatment (Fig. EV2L, EV2M).

Overall, these results show that the binding of *Shigella* to host cells in conditions of cellular stress is impaired as a consequence of the membrane remodeling induced by ASM activation and translocation to the plasma membrane.

p38 MAPK is required for the inhibition of *Shigella* infection upon stress

Previous studies have shown a strong relation between sphingomyelinases and p38 mitogen-activated protein kinase (MAPK) activity. Oxidative stress leads to the activation of sphingomyelinases and ceramide production, which activates p38 MAPK pathway (Chen et al, 2008). In turn, p38 MAPK was shown to activate ASM in glial cells (Bianco et al, 2009). Thus, we hypothesized that the p38 MAPK pathway could be an important player in the activation of the sphingomyelinases and consequent inhibition of *Shigella* infection, in a context of cellular stress. Firstly, we confirmed that the various stressors (arsenite, anisomycin, TNF-α and H_2O_2) activate the p38 MAPK pathway, by Western-blotting for the active phosphorylated form of p38 (Fig. 4A). Supporting our initial hypothesis, inhibition of p38 MAPK pathway by siRNA knockdown (Mapk14) or treatment with SB203580 reverted the inhibitory effect of arsenite (Fig. 4B-D, EV3A) or anisomycin (Fig. 4E, 4F, EV3B, EV3C) on *Shigella* infection. Importantly, we

verified that inhibition of p38 MAPK pathway, by treatment with SB203580, inhibited the production of ceramide induced by arsenite and anisomycin (Fig. 4G, 4H, EV3D, EV3E), demonstrating that p38 MAPK pathway is required for ASM activation and membrane remodeling upon stress. The effective inhibition of the p38 MAPK pathway by SB203580 upon arsenite or anisomycin treatment, and the knockdown of p38 were confirmed by Western-Blotting (Fig. EV3F-H). p38 knockdown or SB203580 treatment had no effect on *Shigella* infection in the absence of stress (Fig. EV3I, EV3J).

Taken together, these results demonstrate that the inhibition of *Shigella* infection during stress relies on the activation of the p38 MAPK pathway.

Bacterial motility overcomes the restriction posed by stress-induced host cell membrane remodeling

Membrane composition has been shown to play critical roles for the adhesion and invasion of various bacterial pathogens in addition to *Shigella*, including the closely related pathogen *Salmonella* Typhimurium (Garner et al, 2002; Santos et al, 2013). We thus reasoned that the stress-induced membrane remodeling could likewise affect *Salmonella* infection. Surprisingly, however, *Salmonella* infection at 0.5 hpi was normal in cells pre-treated with arsenite or anisomycin (Fig. 5A-C), suggesting that *Salmonella* is able to cope with the remodeled host cell membrane. Interestingly, the pattern of infection was altered in cells treated with the stress inducers, showing fewer infected cells, but more bacteria per infected cell (Fig. 5A). This observation was further strengthened by the results of *Salmonella* binding assays, in which a significant increase in the number of bacteria per infected cell was observed in cells pre-exposed to arsenite or anisomycin, compared to control cells (Fig. 5D, 5E, S3A). To confirm that this phenotype was related to bacterial binding, we used an invasion-deficient but binding proficient *Salmonella* mutant strain (*Salmonella* Δ4), which lacks four effector proteins (SopE, SopE2, SopA, and SipB) essential for invasion (Lara-Tejero & Galan, 2009; Misselwitz et al, 2011b; Schlumberger et al, 2005). Similarly to WT *Salmonella*, in conditions of cellular stress the Δ4 mutant strain infected a lower number of cells than in the absence of stress, but also accumulated in specific sites, resulting in a higher number of bacteria per infected cell (Fig. 5D, 5F, S3A). Moreover, a direct effect of stress on *Salmonella* intracellular replication was excluded, given that arsenite treatment post-invasion did not affect infection (Fig. S3B, S3C). These results indicate that, although the total bacterial load is similar, in cells subjected to stress there are fewer *Salmonella* infected cells, though exhibiting a higher number of bacteria bound per cell.

The patchy pattern of *Salmonella* binding to cells exposed to stress suggests that the bacteria is able to accumulate in the remaining permissive binding sites after stress-induced membrane remodeling

has occurred. In contrast to *Shigella*, *Salmonella* is a motile pathogen and therefore a possible explanation for this phenomenon is that *Salmonella* can compensate for the lack of permissive sites by scanning the cellular surface through a process of near cellular surface swimming, enabled by its flagellar motility (Misselwitz et al, 2012). Most *Salmonella* serovars, including Typhimurium, have two flagellin genes, *fliC* and *fljB*, allowing antigen switches between two alternative forms of its flagellin filament protein (Andrewes, 1922). To test the relevance of bacterial motility in conditions of host cell stress, we used the *Salmonella* motility-deficient mutant strain Δ*fliC*, which lacks the *fliC* gene but retains expression of FljB. However, the frequency of flagellin switching is low [approximately 1/5,000 per cell per generation, biased towards the $fljB^{OFF}$ orientation (Gillen & Hughes, 1991)], and thus the *Salmonella* Δ*fliC* mutant strain is essentially non-motile. Additionally, we used a completely non-motile *Salmonella* mutant (Δ*flhC*), due to an insertion of the MudJ transposon into the flagella master regulator *flhC* gene. Remarkably, arsenite and anisomycin pre-treatment inhibited very efficiently the binding of the non-motile *Salmonella* Δ*fliC* and Δ*flhC* mutants (Fig. 5G, 5H, S3D, S3E). Complementation of the Δ*fliC* mutant, by ectopic expression of FliC from a native promoter, restored the ability of *Salmonella* to bind to cells subjected to stress, at levels comparable to that of control cells (Fig. 5G, 5H, S3D, S3E), demonstrating causality between bacterial motility and the ability to overcome membrane remodeling. Based on these observations, we next addressed the effect of host stress on the infection by two additional pathogens, *Listeria monocytogenes* and *Yersinia pseudotuberculosis*, which repress flagella at 37°C and are thus non-motile when grown in these conditions (Kapatral & Minnich, 1995; Peel et al, 1988). In agreement with the results reported above for *Shigella* and *Salmonella*, arsenite treatment inhibited the infection by *Listeria* and *Yersinia* (Fig. 5I-L), demonstrating that membrane remodeling generally impairs infection by non-motile bacterial pathogens.

Overall, these results show that motility is a strong determinant of binding of bacteria to cells exposed to stress. Under these conditions, while binding of non-motile bacteria to host cells is strongly compromised, motile bacteria are able to efficiently sample the host cellular surface and accumulate at the remaining permissive sites for efficient bacterial invasion.

Shigella intracellular replication inhibits re-infection by non-motile bacteria

Given that *Shigella* intracellular replication causes stress, entailing oxidative stress, cytokine secretion and MAPKs activation (Carneiro et al, 2009; Kasper et al, 2010; Pedron et al, 2003), and that the host cell response to stress ultimately inhibits *Shigella* binding, we hypothesized that cells infected with *Shigella* could become refractory to re-infection. To test this possibility we designed a re-infection assay

in which mock-treated or cells infected with *Shigella*-mCherry for 3 hours (primary infection) were re-infected with *Shigella*-GFP (secondary infection; Fig. 6A). Interestingly, the secondary infection with *Shigella*-GFP was significantly decreased in cells previously infected with *Shigella*-mCherry, when compared to mock-treated cells (Fig. 6B, 6C, EV4A). To test if the internalization of a replication deficient *Shigella* mutant strain is sufficient to block re-infection, we performed parallel experiments using the *Shigella*-mCherry Δ*ipaB*/Inv strain for the primary infection. The *Shigella* Δ*ipaB*/Inv strain has a compromised T3SS secretion, though it is internalized due to the expression of the *Yersinia* invasin protein (Isberg et al, 1987). Once inside cells, this mutant is unable to escape the internalization vacuole and does not replicate (Suzuki et al, 2006). Interestingly, we observe that primary infection with this mutant strain did not compromise re-infection, which was comparable to that observed in mock-treated cells (Fig. 6B, 6C, EV4A). Taken together, these observations demonstrate that *Shigella* intracellular replication and/or presence of the bacteria in the host cytoplasm is required for inhibition of re-infection.

In agreement with the results described above showing that host response to stress does not restrict infection by motile bacteria, infection with *Salmonella* WT-GFP (secondary infection) was not inhibited in cells previously infected with *Shigella*-mCherry (Fig. 6D, 6E, EV4B). Similar results were obtained in HCT-8 cells, specifically we observed that primary infection with *Shigella*-mCherry inhibits re-infection by *Shigella*-GFP (Fig. EV4E-G), but not by *Salmonella*-GFP (Fig. EV4H-J). Of note, the primary infection with *Shigella*-mCherry WT or Δ*ipaB*/Inv mutant strain was comparable in the different experimental conditions, both in HeLa and HCT-8 cells (Fig. EV4C, EV4D, EV4K, EV4L). In agreement with the lack of membrane remodeling observed upon *Salmonella* infection (Fig. S3F), the primary infection with *Salmonella*-mCherry did not affect re-infection by *Shigella*-GFP (Fig. EV4M, EV4N).

To evaluate if the motility of *Salmonella* is related to its ability to circumvent the restriction to infection caused by *Shigella* intracellular replication, we performed re-infection assays using the *Salmonella* motility mutants described above. Infection with *Salmonella* Δ*fliC* and Δ*flhC* was inhibited in cells containing replicating *Shigella*-mCherry (Fig. 6F, 6G), whereas infection with the Δ*fliC*/pFliC complemented strain was not affected (Fig. 6F, 6G). This clearly reinforces the notion that the motility of *Salmonella* is the decisive feature to overcome the inhibition posed by the cellular response to *Shigella* infection. Of note, we confirmed that the levels of the primary *Shigella*-mCherry infection were similar in the experiments with the different *Salmonella* strains, excluding any indirect effect on the primary infection (Fig. EV4O).

Collectively, these results demonstrate that *Shigella* intracellular replication in epithelial cells inhibits subsequent binding and re-infection by *Shigella* and, presumably, of other non-motile bacteria.

Shigella* intracellular replication induces host cell membrane remodeling *in vitro* and *in vivo

Next, we pondered whether *Shigella* infection could cause membrane remodeling, and thus explain the inhibition of re-infection. To address this question, we either performed the re-infection experiment in ASM knockdown cells or used amitriptyline to inhibit ASM activity in cells at 2 hpi with *Shigella*, proceeding with re-infection with *Shigella*-GFP after 1 h of amitriptyline treatment (Fig. 6H). Under these conditions, secondary infection by *Shigella*-GFP was not inhibited by the primary infection (Fig. 6I-K, EV5A-C). Similarly, inhibition of the p38 MAPK pathway achieved by p38 knockdown or treatment with SB203580 (Fig. 6L, 6M, EV5D, EV5E), as well as oxidative stress mitigation by NAC treatment (Fig. EV5F, EV5G), dampened the inhibitory effect of the primary infection. Of note, treatment with amitriptyline, SB203580, NAC, as well as ASM or p38 knockdown, had no effect on the primary infection by *Shigella*-mCherry (Fig. EV5H-N). Overall, these results imply that ASM-mediated membrane remodeling, induced by oxidative stress activated p38 MAPK pathway, is responsible for the inhibition observed in the re-infection experiments reported above.

To provide direct evidence that membrane remodeling occurs as a consequence of the host cell response to *Shigella* infection, we measured ASM activity and analyzed the accumulation of ASM and ceramide at the membrane of *Shigella* infected cells. In agreement with an involvement of ASM in this process, we observed a clear accumulation of ASM in the membrane of *Shigella* WT infected cells (Fig. 7A, EV5O), alongside a clear increase of ceramide (Fig. 7B, EV5P); this phenotype was not observed in *Shigella* Δ*ipaB*/Inv (Fig. EV5R, EV5S), which is in perfect agreement with the observation that primary infection with this mutant strain does not compromise re-infection. Interestingly, both ASM and ceramide accumulated to a higher extent in bystander cells, compared to cells with intracellular bacteria (Fig. 7A, 7B, EV5O-Q). Enzymatic assays on membrane fractions from *Shigella* infected cells (3 hpi) showed a modest but significant increase in the activity of ASM (Fig. 7C), correlating with a clear increase in ceramide levels (Fig. 7D).

Collectively, our results demonstrate that *Shigella* intracellular replication in epithelial cells leads to the activation of ASM, which results in formation of ceramide domains, which impairs re-infection by *Shigella*.

Having shown that *Shigella* infection induces major changes in membrane composition in vitro, we wondered whether this process could be observed in vivo. For this purpose, we used a guinea pig model, a well established animal model for the study of *Shigella* infection (Arena et al, 2015), and compared the ceramide accumulation in colon tissue samples from non-infected and *Shigella* infected

animals. In complete agreement with the in vitro data, we observed a clear accumulation of ceramide in colon tissue samples from *Shigella* infected animals (4 hpi of challenge with *Shigella* WT) compared to non-infected controls (Fig. 7E-I). Interestingly, the enhanced ceramide signal in infected samples was clearly more noticeable in bystander cells than in cells with intracellular bacteria.

Overall, these results strongly corroborate the notion that *Shigella* intracellular replication, both in vitro and in an animal model of *Shigella* infection, leads to the activation of ASM and consequent membrane remodeling.

DISCUSSION

Although intestinal epithelial cells are now considered crucial players in the defense against infection by bacterial pathogens, exactly how their general stress response helps to fend off potential bacterial invaders is incompletely understood. In this study, we reveal that epithelial cell stress generated by inflammatory cues and oxidative insults, namely arsenite, anisomycin, hypoxia, hydrogen peroxide and cytokines, cause a strong inhibition of *Shigella* binding to host cells. Mechanistically, we determined that this inhibition results from an extensive remodeling of the host plasma membrane due to stress-induced activation of acid sphingomyelinase that is enforced by p38 MAPK activation. This depletes permissive binding sites on the plasma membrane drastically and so hinders *Shigella*'s attempts to bind and, ultimately, invade host cells (Fig. 7J).

The relevance of ASM activity and ensuing membrane remodeling in the context of infection by diverse bacterial pathogens has been studied before. ASM is required for efficient infection by *Neisseria gonorrhoeae* and *Neisseria meningitidis* (Faulstich et al, 2015; Grassme et al, 1997; Simonis et al, 2014), whereas for other pathogens ASM is involved in the host defense against infection. For example, ASM KO mice are much more susceptible to *Listeria monocytogenes* infection than wild-type animals (Utermohlen et al, 2003), a phenotype that was linked with lysosomal dysfunction in macrophages (Schramm et al, 2008; Utermohlen et al, 2008; Utermohlen et al, 2003). Based on the results described herein, we suggest that the higher susceptibility of ASM KO mice to *Listeria* infection can also be linked with the role of ASM in the remodeling of epithelial cell membrane to restrict adhesion and entry of non-motile pathogens. It is conceivable that the absence of ASM precludes membrane remodeling events that would normally occur, effectively increasing the number of permissive sites on the cell surface. In agreement with this hypothesis, the entry of *Listeria* into host cells was shown to require the localization of the host receptors E-cadherin and HGF-R/Met in specific membrane domains (Seveau et al, 2004). Of note, *Listeria* should

be considered a non-motile pathogen in the human host due to the temperature-dependent expression of flagella, which is strongly reduced at mammalian physiological temperature [37°C; (Peel et al, 1988)], and therefore likely to be strongly affected by host plasma membrane remodeling (see below).

Noticeably, ASM KO mice are also more susceptible to *Salmonella* infection than wild-type animals, albeit less so than to *Listeria* (Utermohlen et al, 2003). The apparent discrepancy with our results (showing that *Salmonella* binding to epithelial cells is not affected by ASM activation) can likely be explained by the fact that the reported increased susceptibility of ASM KO mice to *Salmonella* derives from an impaired ability of ASM KO macrophages to kill *Salmonella* (McCollister et al, 2007). Nonetheless, we cannot exclude the possibility that *in vivo* the inflammatory conditions associated with *Salmonella* infection can induce a stronger effect at the level of membrane remodeling leading to inhibition of *Salmonella* invasion of epithelial cells.

Our results reinforce the relevance of the flagellar motility as a crucial mechanism used by bacteria to reach preferred sites of infection, particularly when these are scarcely available, such as under stress conditions. This is well illustrated by the ability of *Salmonella* – a motile pathogen – to overcome host defenses based on host membrane remodeling, which are thus particularly detrimental to non-motile pathogens. Here, we show that global binding and invasion of *Salmonella* is unaffected by stress, since flagellar motility enables this bacterium to scan the host cellular surface and accumulate at the remaining permissive entry sites. Conversely, non-motile *Salmonella* mutants show impaired binding to host cells in stress conditions. These in vitro observations are in agreement with previous reports showing that mutations affecting *Salmonella* motility impair bacterial colonization of the inflamed mouse intestine, but not of the normal gut (Stecher et al, 2008; Stecher et al, 2004). This phenotype is, at least in part, attributed to flagella facilitated chemotactic movement, which facilitates access to the nutrient-rich molecules released as part of the mucosal defense during inflammation (Stecher et al, 2008). However, our findings showing that host membrane remodeling in response to stress protects epithelial cells from infection by non-motile pathogens might help to explain the reported impaired in vivo fitness of the non-motile mutant *Salmonella* strains. The relevance of motility is further reinforced by the demonstration that *Listeria monocytogenes* and *Yersinia pseudotuberculosis*, which repress flagella at 37°C and are thus non-motile when grown at this temperature (Kapatral & Minnich, 1995; Peel et al, 1988), also exhibit impaired binding/invasion in stress conditions.

Unlike *Salmonella*, *Shigella* has lost flagellar motility (Ewing, 1949) despite motility being an important trait for pathogenicity in the intestinal environment at various stages of infection, including reaching the sites of infection, adhesion to and invasion of host cells, and biofilm formation [reviewed in

(Erhardt, 2016; Rossez et al, 2015)]. However, flagellin, the major component of the bacterial flagellum, is a potent inducer of the innate immune response and it is therefore deleterious for bacterial survival within the host, with the ensuing greater clearance of flagellated versus non-flagellated strains (Dons et al, 2004; Lai et al, 2013; Lockman & Curtiss, 1990; Olsen et al, 2013). This trade-off between higher motility / higher immunogenicity and lower motility / lower immunogenicity is particularly interesting in light of the results presented here.

Importantly, ASM activation and subsequent membrane remodeling under stress has propagative properties linked to the inducing stimuli. For instance, most stress conditions occurring during colonic inflammation, such as TNF-α stimulation, hypoxia or oxidative stress, elicit responses at the level of cell populations rather than just individual cells. Moreover, a common denominator of the response to stress is the ability of cells to produce signals or activate pathways to alert neighboring cells to the presence of the stress elicitor, and orchestrate a common response (Chovatiya & Medzhitov, 2014). Along this line, our results show that, even in the context of *Shigella* infection, the remodeling of the host membrane is not restricted to infected cells, but is in fact more evident in the yet non-infected adjacent cells. Specifically, we show that *Shigella* infection induces ASM activation and strong ceramide accumulation in the bystander cells. Paracrine signaling, for example, TNF-α release upon *Shigella* infection, can explain the host membrane remodeling in the bystander cells. In addition, direct cell-cell communication can be involved, given that the p38 MAPK pathway activation has been shown to be propagated from *Shigella* infected to bystander uninfected cells via gap-junctions (Kasper et al, 2010). These mechanisms are further supported by our results showing that stress conditions, namely TNF-α, arsenite or anisomycin treatment, activate the p38 MAPK pathway and that this stimulation is required for the inhibition of *Shigella* infection upon stress. P38-dependent ASM activation upon infection is also in agreement with the observed stronger accumulation of ceramide in bystander cells, when compared with cells with replicating bacteria. Indeed, p38 activation in cells with internalized bacteria is modest due to the phosphothreonine-lyase activity of the *Shigella* OspF effector protein (Arbibe et al, 2007; Li et al, 2007).

We show that intracellular replication of *Shigella* induces host membrane remodeling and inhibition of re-infection by extracellular non-motile bacteria. Importantly, we have also observed membrane remodeling, visualized by a strong ceramide accumulation, in colon samples of guinea pigs infected with *Shigella*. Accordingly, we propose that membrane remodeling constitutes part of the host response to the stress elicited by *Shigella* infection, acting as a protective mechanism against further pathogen invasion. In addition to bacterial pathogen infection, this mechanism might also be relevant to restrict commensal bacteria translocation through the intestinal epithelium. Given that the mucus layer

is reduced during inflammation (Swidsinski et al, 2007) and thus more bacteria are probing the epithelial surface, it is conceivable that the depletion of membrane rafts limits the docking of such bacteria and their potential translocation through the intestinal barrier. Indeed, commensal bacteria have been shown to exploit membrane raft mediated transcellular pathways to cross the intestinal epithelium, in mechanisms dependent (Kalischuk et al, 2009) or independent (Clark et al, 2005) of co-infection with bacterial pathogens.

In summary, we describe a novel mechanism by which host epithelial cell response to stress limits *Shigella* binding and invasion via reinforcement of the physical barrier formed by epithelial cells, through the activation of acid sphingomyelinase and resulting depletion of permissive bacterial binding sites. Moreover, we provide evidence that global binding of motile pathogens to host cells is not affected in stress conditions, indicating that motility is an important feature to overcome host defenses based on host membrane remodeling.

MATERIALS AND METHODS

Bacterial infections

For bacterial infections, overnight cultures were diluted 1:100 in LB and grown aerobically at 37°C with shaking until OD_{600} 0.4 (*Shigella*) or OD_{600} 2.0 (*Salmonella*). Bacteria were harvested by centrifugation (12,000 g, 2 min) and resuspended in complete medium. Bacterial infections were performed at the multiplicity of infection (MOI) indicated in the Figure legends. Following addition of bacteria, cells were centrifuged at 2,000 g for 15 min (*Shigella*) or 250 g for 10 min (*Salmonella*) at RT. Cells were then incubated at 37°C in a 5% CO_2 humidified atmosphere for 15 or 20 min, respectively, followed by replacement with fresh medium supplemented with 50 μg/ml gentamycin (defined as time point 0 of infection) and incubated for 30 min to kill extracellular bacteria. The medium was then replaced and cells were maintained in medium supplemented with 10 μg/ml gentamycin, until analysis.

For binding experiments, bacteria were added to the host cells as above, and processed immediately after the centrifugation step (*Salmonella*) or after an additional 10 min incubation at 37°C (*Shigella*). Cells were then extensively washed with PBS to remove non-bound bacteria and processed for microscopy, colony forming units (cfu) assays, or RNA extraction. For *Listeria* infections, overnight cultures were diluted 1:50 in BHI medium and grown at 37°C with shaking until OD_{600} 0.7. *Listeria* was added to the cells after dilution of bacterial cultures in complete medium. Cells were then centrifuged at 250 g for 10 min at RT and

incubated for 10 min at 37°C. For *Yersinia* infection, overnight culture was performed at 28°C. The culture was then diluted to OD_{600} 0.05 and grown with shaking at 28°C for 1 h, followed by 2 h growth at 37°C reaching approx. OD_{600} 2.0. Dilution of bacteria and infections was performed as described for *Shigella*, except that following addition of bacteria, cells were centrifuged at 400 g for 10 min, followed by 1 h incubation at 37°C in a 5% CO_2 humidified atmosphere.

To quantify intracellular bacterial replication by cfu assays, cells were washed three times with PBS and lysed in PBS containing 0.1% Triton-X-100. The lysates were then serially diluted in PBS and plated on LB agar plates.

For re-infection assays, the cells were washed with PBS at 3 hpi (primary infection) and re-infected as described above. For treatment with compounds, these were added directly to the medium containing 10 μg/ml of gentamycin at the appropriate time prior to the secondary infection. Cells were then washed with PBS and re-infected. Discrimination of the colonies derived from primary and secondary infections was possible since the strains used for the re-infection experiments have different antibiotic resistance cassettes, as follows: *Shigella*-mCherry, kanamycin resistance and *Salmonella*-mCherry, ampicillin resistance (primary infection); *Shigella*-GFP and *Salmonella*-GFP (secondary infections), chloramphenicol resistance.

Animals, Infections and Sample Preparation

Shigella infection of guinea pigs (120–250 g; Charles River Laboratories) and sample preparation were performed as described previously (Arena et al, 2015). Infections were performed in accordance with Institut Pasteur animal protocols (no. 2013-0113). Two animals per condition (non-infected and *Shigella* infected) were sacrificed at 4 hpi, and the distal 10 cm of colon were harvested and processed for immunofluorescence analysis.

Immunofluorescence, fluorescence microscopy and analysis

Cells seeded on glass coverslips were fixed with 4% paraformaldehyde (PFA) for 15 min at RT, followed by permeabilization with 0.5% Triton-X-100 in PBS for 10 min. Blocking was performed in 1% Bovine Serum Albumin (BSA) in PBS for 30 min. Incubation with primary antibodies was performed in blocking solution. The following primary antibodies were used: *Salmonella* LPS (1:1,000, 2 h RT; Abcam, ab8274), *Listeria* monocytogenes (1:750, 2 h RT; antibodies-online, ABIN237765), human acid sphingomyelinase [1:250, overnight at 4˚C; Santacruz, sc-293189 (4H2)], ceramide (1:50, 1 h RT; Enzo life sciences, ALX-804-196-

T050). After washing with PBS, cells were incubated with the corresponding secondary antibodies conjugated with Alexa Fluor 488 or 594 (1:400, 1 h RT; Life Technologies).

For F-actin staining, cells were incubated with Alexa Fluor 594 Phalloidin (1:50, 1 h RT; Life Technologies, A12381), after the blocking step.

When indicated, cells were stained with HCS CellMask Deep Red stain (1:10,000, 1 h RT; Life Technologies, H32721). Nuclei were counterstained with Hoechst 33342 (1:5,000, 15 min RT; Life Technologies, 62249). Slides were mounted in Vectashield (VectorLabs).

Confocal microscopy images were acquired with a Leica SP5 laser scanning confocal microscope (Leica Microsystems). For the analysis of the number of bacteria per cell, at least 50 infected cells per condition and independent experiment (total of at least 250 cells) were counted manually from maximum-projected Z-stack confocal images.

The 3D reconstruction of Z-stack confocal images was performed using the Imaris software (Bitplane). An average of 20-25 Z-stacks were acquired for each image; the various channels were surface rendered by voxel distance.

For quantification of *Shigella* infection and spreading, image acquisition was performed using Operetta automated high-content screening fluorescence microscope (PerkinElmer) at a 20× and 10× magnification, respectively; a total of 9 (infection) or 1 (spreading) images were acquired per coverslip/well, corresponding to approximately 2,500 cells analyzed. Image analysis was performed using Columbus image analysis software (PerkinElmer) as described previously (Sunkavalli et al, 2017).

For the guinea pig colon tissue sections (10 µm-thick transversal sections), samples were permeabilized with 0.5% Triton-X-100 in PBS for 2 h and blocked in 1% BSA in PBS for 1 h. Sections were then incubated overnight at 4°C with the primary antibody against ceramide (1:50; Enzo life sciences, ALX-804-196-T050), a rabbit polyclonal serum specific for *Shigella*-LPS (1:300; P.S. lab) and Alexa Fluor 647 Phalloidin (1:200; Life Technologies, A22287), in 0.1% Triton-X-100/1% BSA solution. After 2 washes with PBS, samples were incubated for 1 h at RT with the goat-anti mouse and goat-anti rabbit secondary antibodies conjugated with Alexa 568 and Alexa 488 (Life Technologies), followed by DAPI staining (1:1,000; Life Technologies, 62247) for 5 minutes at RT. The sections were then washed with PBS and mounted with ProLong Gold (Life Technologies, P36930).

Images were acquired in an Opterra swept-field confocal microscope (Bruker) with a 100× objective. The quantification of the mean fluorescent intensity (MFI) of ceramide staining in the sections was performed using Fiji (Schindelin et al, 2012). Briefly, maximal intensity projections of 20 z-stacks were generated and

specific region of interests (ROIs) were manually drawn on the surface epithelial layer using the phalloidin staining as reference, and MFI was calculated.

Plasma membrane acid sphingomyelinase activity assays

For determining membrane sphingomyelinase activity, HeLa cells (3.5×10^5 cells/well) were seeded in 6-well plates 2 days prior to the assay. Acid sphingomyelinase activity was measured in the membrane fractions, as described previously (Tonnetti et al, 1999), using the Amplex Red Sphingomyelinase Assay Kit (Invitrogen, A12220).

To obtain the membrane fractions, following treatment with the various compounds or after infection, the cells were washed with PBS, and harvested in PBS with 5 mM EDTA and washed 3 times in ice-cold PBS with 100 µM sodium orthovanadate at 4˚C. Cells were then resuspended in 100 µl of lysis buffer (20 mM Tris-HCl pH 7.5, 2 mM EDTA, 5 mM EGTA, 1 mM sodium orthovanadate, 10 mM β-glycerol phosphate, 1 mM PMSF, 1× protease inhibitors cocktail) and disrupted by five cycles of freezing and thawing in a methanol/dry-ice bath. The lysate was centrifuged for 10 min at 1,000 g at 4˚C, and the supernatant (post-nuclear homogenate) was transferred to a new tube. The supernatant was centrifuged for 1 h at 100,000 g at 4˚C. The resulting pellet (membrane fraction) was resuspended in 40 µl 1× reaction buffer provided with the kit. For each treatment/infection, a replicate well was used for cell counting; 1.2×10^6 cells were used on average as input for the extraction of the membrane fractions.

10 µl of the membrane fractions (i.e. corresponding to 3.0×10^5 cells per condition) were used for the measurement of acid sphingomyelinase activity, which was performed according to the manufacturer's instructions. A standard curve was established for each experiment, based on 1:4 serial dilutions of the provided commercial sphingomyelinase (10U/ml; sphingomyelinase from *Bacillus cereus;* 1U corresponds to the amount of enzyme required to hydrolyze 1 µmole substrate in 1 min at 37°C), starting from 40 mU to 0.01 mU in a total volume of 100 µl in 1× reaction buffer. The reactions were incubated at 37°C for 1 hour and fluorescence was measured with a microplate reader using excitation at 530 nm and emission at 590 nm. For each experiment, the fluorescence of the samples was converted to enzyme units using a standard curve.

Ceramide quantification by flow-cytometry

After treatment with stressors or infection, cells were collected in PBS+5mM EDTA and washed 2 times in PBS. Cells were then resuspended in 1% BSA in PBS containing the ceramide antibody (1:30, 1 h RT, with agitation; Enzo life sciences, ALX-804-196-T050). Cells were washed three times in PBS, and again

resuspended in 1% BSA in PBS containing the anti-mouse secondary antibody conjugated with Alexa Fluor 488 (1:300, 1 h RT, with agitation; Life Technologies). Finally, cells were washed and resuspended in cold PBS before measurement. Cells incubated with only the secondary antibody were used as a control. Flow cytometry was performed on a BD Accuri C6 or a FACSAria III flow cytometer (BD Biosciences). FlowJo software (Tree Star Inc) was used for data analysis

Cell viability

Analysis of cell viability was performed using 7-Amino-Actinomycin (7-AAD; BD Biosciences, 51-68981E), a viability dye excluded from cells with intact membranes (viable cells), as described previously (Maudet et al, 2014). Flow cytometry was performed on a BD Accuri C6 flow cytometer (BD Biosciences); a minimum of 10,000 cells were analyzed per sample. FlowJo software (Tree Star Inc) was used for data analysis.

Statistical analysis

All data are presented as mean ± standard error of the mean (s.e.m.), with the exact number of experiments performed indicated in the respective Figure legend. Statistical analysis was performed using Prism Software (GraphPad). For statistical comparison of datasets from two groups/conditions, two-tailed Student's t-test was used; for data from three or more groups/conditions, one-way ANOVA with Tukey's or Dunnet's post-hoc test was used or two-way ANOVA with Sidak's multiple comparisons test. Mann-Whitney test was used for the comparison of medians from two groups. A P-value < 0.05 was considered significant.

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AUTHOR CONTRIBUTIONS

C.T. and A.E. designed the research; C.T, G.N., I.L., C.A. and C.L. performed the experiments; C.T., G.N., I.L., C.A., C.L., M.M., P.S., J.V. and A.E. analyzed the data and discussed the results; and C.T and A.E. wrote the manuscript with input from all the authors.

FIGURE LEGENDS

Figure 1. *Shigella* infection is inhibited by host cell stress

A. Schematic representation of the experimental design.

B and C. Representative images of HeLa (B) or HCT-8 (C) cells infected with *Shigella* WT pre-treated or not with arsenite, analyzed at the indicated times post-infection.

D. Cfu quantification of intracellular bacteria in HeLa and HCT-8 cells pre-treated or not with arsenite and infected with *Shigella.*

E and F. Representative images (E) and cfu quantification (F) of intracellular bacteria in HeLa cells infected with *Shigella* WT after pre-treatment with TNF-α, H_2O_2, anisomycin, hypoxia and corresponding controls, analyzed at 0.5 hpi.

G and H. Representative images (G) and cfu quantification (H) of intracellular *Shigella* in HeLa cells pre-treated with arsenite, anisomycin, stressors plus NAC, and corresponding controls.

Shigella infection was performed at MOI 10. Results are shown as mean ± s.e.m. from 5 independent experiments; **p<0.01, ***p<0.001 (t-test adjusted for multiple comparison for D – HeLa; paired t-test for D – HCT-8 and F; one-away ANOVA for H). Scale bar, 50 μm.

Figure 2. Host cellular stress inhibits *Shigella* binding to host cells

A and B. Representative images (A) and cfu quantification (B) of *Shigella* WT or Δ*ipaB* mutant strain bound to HeLa cells pre-treated or not with arsenite. Ruffle formation induced by *Shigella* WT in panel A is indicated by white arrowheads.

C. Cfu quantification of *Shigella* WT or Δ*ipaB* mutant strain bound to HeLa cells pre-treated with anisomycin or DMSO (control).

D and E. Representative images (D) and cfu quantification (E) of *Shigella* WT or Δ*ipaB* mutant bound to HCT-8 cells pre-treated or not with arsenite.

Shigella infection was performed at MOI 10 for *Shigella* WT or at MOI 50 for the Δ*ipaB* mutant strain; cells were incubated with the bacteria for 25 min. Results are shown as mean ± s.e.m. from 5 (C) or 6 (B, E) independent experiments; **p>0.01, ***p<0.001 (paired t-test). Scale bar, 25 μm.

Figure 3. Inhibition of *Shigella* infection upon host cell stress is a consequence of ASM activation

A - F. Representative images (A, D), cfu (B, E) and qRT-PCR (C, F) quantification of intracellular *Shigella* in HeLa cells pre-treated with arsenite or anisomycin in the presence or not of amitriptyline, and corresponding controls.

G - I. Representative images (G), cfu (H) and qRT-PCR (I) quantification of intracellular *Shigella* in HeLa cells transfected with ASM siRNA or control siRNA and pre-treated or not with arsenite prior to infection.

J - M. 3D reconstruction of representative images of ASM (J, K) or ceramide (L, M) staining in HeLa cells treated with arsenite or anisomycin and corresponding controls. ASM, ceramide and Hoechst staining were surface converted by voxel distance.

Infection was performed with *Shigella* WT at MOI 10 and analyzed at 0.5 hpi. Results are shown as mean ± s.e.m. from 5 (H, I) or 7 (B-F) independent experiments; *p<0.05, **p<0.01, ***p<0.001 (one-way ANOVA). Scale bar, 50 µm (A, D, G) and 5 µm (J-M).

Figure 4. MAPK p38 inhibition restores *Shigella* infection of host cells exposed to stress

A. Western blot analysis of p38 MAPK phosphorylation in HeLa cells treated with arsenite, TNF-α, H_2O_2 or anisomycin; β-actin was used as loading control.

B, C and E. Representative images (B) and cfu quantification (C, E) of intracellular *Shigella* in HeLa cells pre-treated with arsenite or anysomycin in the absence or presence of the p38 inhibitor SB203580, and corresponding controls.

D and F. Cfu quantification of intracellular *Shigella* in HeLa cells transfected with p38 siRNA or control siRNA and pre-treated or not with arsenite (D) or anisomycin (F) prior to infection.

G and H. 3D reconstruction of representative images of ceramide staining in HeLa cells treated with arsenite (G) or anisomycin (H), in the presence or absence of SB203580, and corresponding controls. Ceramide and Hoechst staining were surface converted by voxel distance.

Infection was performed with *Shigella* WT at MOI 10 and analyzed at 0.5 hpi. Results are shown as mean ± s.e.m. from 5 (E), 6 (D and F) or 7 (C) independent experiments; *p<0.05, **p<0.01, ***p<0.001 (one-way ANOVA). Scale bar, 50 µm (B) and 5 µm (G, H).

Figure 5. *Salmonella* motility compensates for membrane remodeling in cells exposed to stress

A - C. Representative images (A), cfu (B) and qRT-PCR (C) quantification of intracellular *Salmonella* in HeLa cells pre-treated with arsenite or anisomycin and corresponding controls. Infection was performed with *Salmonella* WT and analyzed at 0.5 hpi.

D. Representative images of bacteria bound to HeLa cells pre-treated or not with arsenite followed by incubation with *Salmonella* WT or Δ4 mutant strain for 10 min.

E and F. Violin plots showing the distribution of the number of bacteria bound per infected cell in HeLa cells pre-treated or not with arsenite followed by incubation with *Salmonella* WT (E) or Δ4 mutant strain (F) for 10 min. Results are shown for 50 infected cells per condition and independent experiment (total 250 cells); the median for each group is shown (white dot).

G and H. Representative images (G) and cfu quantification (H) of bacteria bound to HeLa cells pre-treated or not with arsenite followed by incubation with *Salmonella* mutant strains Δ*fliC*, Δ*fliC*/pFliC or Δ*flhC* for 10 min.

I and J. Representative images (I) and cfu quantification (J) of *Listeria* bound to HeLa cells pre-treated or not with arsenite followed by incubation with the bacteria for 20 min.

K and L. Representative images (K) and cfu quantification (L) of *Yersinia* bound/internalized by HeLa cells pre-treated or not with arsenite followed by 70 min incubation with the bacteria.

Infection was performed at MOI 25 (A-C) or MOI 50 (D-H) for *Salmonella*, MOI 50 for *Listeria* (I, J) and *Yersinia* (K, L). Results are shown as mean ± s.e.m. from 5 (C, E, F, H), 6 (B – arsenite and L) or 7 (B – anisomycin and J) independent experiments; ***p<0.001 (paired t-test for B, C, J, L; Mann–Whitney U test for E, F; two-way ANOVA for H). Scale bar, 50 µm (A) and 25 µm (D, G).

Figure 6. Re-infection by non-motile bacteria is inhibited by *Shigella* intracellular replication

A. Schematic representation of the experimental design for reinfection experiments. Cells were infected with *Shigella*-mCherry (primary infection) or mock-treated. At 3 hpi, cells were re-infected with *Shigella* or *Salmonella* expressing GFP (secondary infection). Secondary infection was analyzed at 0.5 hpi (i.e. 1 h after addition of bacteria to cells).

B - E. Representative images (B, D) and cfu quantification (C, E) of re-infection assays with *Shigella* WT (B, C) or *Salmonella* WT (D, E) of HeLa cells primarily infected with *Shigella* WT or Δ*ipaB*/Inv mutant strain, or mock-treated. Secondary infection is shown in bottom images.

F and G. Representative images (F) and cfu quantification (G) of the re-infection assays with *Salmonella* Δ*fliC*, Δ*fliC*/pFliC or Δ*flhC* mutant strains in HeLa cells primarily infected with *Shigella* WT or mock-treated. Secondary infection is shown in bottom images.

H. Schematic representation of the experimental design for reinfections upon knockdown or inhibitor treatment.

I and J. Representative images (I) and cfu quantification (J) of the secondary infection with *Shigella* WT, in cells transfected with ASM siRNA or control siRNA.

K - M. Cfu quantification of the secondary infection with *Shigella* WT in HeLa cells following amitriptyline (K), transfection with MAPK p38 siRNA (L) or SB203580 treatment (M).

Infection was performed at MOI 10 for *Shigella* WT, MOI 350 for *Shigella* Δ*ipaB*/Inv, MOI 25 for *Salmonella* WT (D, E, G), or MOI 50 for *Salmonella* WT, Δ*fliC*, Δ*fliC*/pFliC or Δ*flhC* mutant strains (F, G). Results are shown as mean ± s.e.m. from 5 independent experiments; *p<0.05, **p<0.01, ***p<0.001 (one-way ANOVA for C, E, J-M, two-way ANOVA for G). Scale bar, 50 µm.

Figure 7. Intracellular *Shigella* replication induces remodeling of the host cell plasma membrane in vitro and in vivo

A and B. 3D reconstruction of representative images of ASM (A) or ceramide (B) staining in HeLa cells infected with *Shigella* WTor mock-treated, analyzed at 3 hpi. ASM, ceramide, Hoechst and *Shigella* staining were surface converted by voxel distance.

C and D. ASM enzymatic activity (C) and ceramide (D) measurement in HeLa cells infected with *Shigella*-WT or mock-treated, analyzed at 3 hpi. The ASM enzymatic activity was determined in the membrane fraction corresponding to 3.0×10^5 cells per condition. Ceramide is shown normalized to mock-treated cells.

E - H. Representative spinning-disk confocal images of colon tissue sections of non-infected (E and F) and *Shigella* infected (G and H) guinea pigs, collected at 4 hpi. Dashed boxes in E and G are shown enlarged in F and H, respectively.

I. Mean fluorescence intensity of ceramide signal in surface epithelial cells (outermost tissue layer) of non-infected or *Shigella*-infected guinea pig colon tissue sections.

Shigella infection was performed at MOI 100 (A, B). Results are shown as mean ± s.e.m. from 9 (C) or 6 (D) independent experiments; whiskers in the box-plot (I) correspond to min/max from 10 (non-infected) or 13 (*Shigella*) tissue sections. *p<0.05, ***p<0.001 (one-way ANOVA for C, D; unpaired t-test for I). Scale bar, 5 µm (A, B), 50 µm (D, F) and 12.5 µm (E, G).

J. Model depicting the effect of stress-induced host plasma membrane remodeling on infection by bacterial pathogens. Different stressors, including TNF-α stimulation and *Shigella* intracellular replication, induce the relocalization and activation of acid sphingomyelinase (ASM) to the host plasma membrane, leading to the conversion of sphingomyelin into ceramide. As a consequence of membrane remodeling, the binding of non-motile bacteria, such as *Shigella*, to host cells is inhibited. Motile bacteria, such as

Salmonella, are able to scan the host membrane for the remaining permissive binding sites, and thus circumvent this defense mechanism.

EXPANDED VIEW FIGURE LEGENDS

Figure EV1. *Shigella* infection is inhibited by host cell stress

A. Percentage of HeLa cells infected with *Shigella* after pre-treatment with arsenite or control, analyzed at 0.5, 2 and 6 hpi.

B. qRT-PCR quantification of intracellular bacteria in HeLa and HCT-8 cells pre-treated or not with arsenite and infected with *Shigella* WT. Analysis was performed at 0.5, 2 and 6 hpi for HeLa cells and at 0.5 hpi for HCT-8 cells. Results are shown normalized to the control at 0.5 hpi.

C - D. Representative images (C) and cfu quantification (D) of HT-29 or Caco-2 cells pre-treated or not with arsenite and infected with *Shigella* WT, analyzed at 0.5 hpi.

E - G. Representative images (E), cfu (F) and qRT-PCR (G) quantification of intracellular bacteria in HeLa cells pre-treated with puromycin or cycloheximide, or control, and infected with *Shigella*. Analysis was performed at 0.5 hpi.

H. Percentage HeLa cells infected with *Shigella* WT after pre-treatment with TNF-α, H_2O_2, anisomycin, hypoxia and corresponding controls, analyzed at 0.5 hpi.

I. qRT-PCR quantification of intracellular bacteria in HeLa cells infected with *Shigella* WT after pre-treatment with TNF-α, H_2O_2, anisomycin, hypoxia and corresponding controls, analyzed at 0.5 hpi.

J. Percentage of 7-AAD positive cells following treatment with arsenite, TNF-α, H_2O_2, amitriptyline, anisomycin and corresponding controls.

K. Growth curve of *Shigella* WT or *Salmonella* WT (OD_{600}) in LB medium (10 hours) in the presence of arsenite, anisomycin or corresponding controls.

L. Cfu quantification of intracellular bacteria in HeLa cells treated or not with amitriptyline and infected with *Shigella* WT, analyzed at 0.5 hpi.

Shigella infection was performed at MOI 10. Results are shown as mean ± s.e.m. from 5 (panels A, B – HCT-8, D, F, I, J) or 6 (panels B – HeLa, G, H) independent experiments; *p<0.05, **p<0.01, ***p<0.001 (t-test adjusted for multiple comparison for panels A and B – HeLa; paired t-test for panels B – HCT-8, D, I and L; one-away ANOVA for panel F-H and J). Scale-bar, 50 μm.

Figure EV2. NSM inhibition does not affect *Shigella* infection upon host cell stress

A - C. Representative images (A), cfu (B) and qRT-PCR (C) quantification of intracellular *Shigella* in HeLa cells, pre-treated with arsenite, with arsenite plus the NSM inhibitor GW4869, or control.

D - F. Representative images (D), cfu (E) and qRT-PCR (F) quantification of intracellular *Shigella* in HeLa cells, pre-treated with anisomycin, with anisomycin plus GW4869, or DMSO (control).

G and H. Cfu (G) and qRT-PCR (H) quantification of intracellular bacteria in HeLa cells treated or not with amitriptyline and infected with *Shigella* WT.

I and J. Cfu (I) and qRT-PCR (J) quantification of intracellular bacteria in HeLa cells transfected with ASM or control siRNA.

K. ASM enzymatic activity quantification in HeLa cells, treated with arsenite, with arsenite plus the ASM inhibitor amitriptyline, or control. The ASM enzymatic activity was determined in the membrane fraction corresponding to 3.0×10^5 cells per condition.

L and M. Ceramide quantification in HeLa cells, treated with arsenite (L) or anisomycin (M), with the stressors plus amitriptyline, or control. Ceramide levels are shown normalized to mock-treated cells.

N. Analysis of *smpd1* (ASM) expression determined by qRT-PCR in HeLa cells transfected with ASM or control siRNA. Results are shown normalized to cells transfected with control siRNA.

O. Western blot analysis of ASM levels in HeLa cells transfected with ASM siRNA or control siRNA; β-actin was used as loading control.

P. ASM enzymatic activity quantification in HeLa cells transfected with ASM siRNA or control siRNA, and treated or not with arsenite. The ASM enzymatic activity was determined in the membrane fraction corresponding to 3.0×10^5 cells per condition.

Shigella infection was performed at MOI 10 and analyzed at 0.5 hpi. Results are shown as mean ± s.e.m. from 5 (panels J, K-M and P), 6 (panel I) or 7 (panels B, C, E-H and N) independent experiments; **p<0.05, **p<0.01, ***p<0.001 (one-way ANOVA for panels B, C, E, F, K-M; paired t-test for panels G-J and N; two-way ANOVA for panel P). Scale bar, 50 µm.

Figure EV3. MAPK p38 inhibition restores *Shigella* infection of host cells exposed to stress

A and C. Representative images of *Shigella* infection in HeLa cells transfected with p38 siRNA or control siRNA and pre-treated or not with arsenite (A) or anisomycin (C) prior to infection.

B. Representative images of *Shigella* infection in HeLa cells pre-treated with anisomycin, with anisomycin plus SB203580, or control.

D and E. Representative images of HeLa cells treated with arsenite (D) or anisomycin (E) and the corresponding controls in the presence or not of SB203580, stained for ceramide.

F and G. Western blot analysis of p38 MAPK phosphorylation in HeLa cells treated with arsenite (F) or anisomycin (G) in the presence or not of SB203580. Phosphorylated and total p38 protein are shown; β-actin was used as loading control.

H. Western blot analysis of p38 levels in HeLa cells transfected with p38 siRNA or control siRNA; β-actin was used as loading control.

I. Cfu quantification of intracellular bacteria in HeLa cells transfected with p38 or control siRNA.

J. Cfu quantification of intracellular bacteria in HeLa cells treated or not with SB203580 and infected with *Shigella* WT.

Shigella infection was performed at MOI 10 and analyzed at 0.5 hpi. Results are shown as mean ± s.e.m. from 6 (panel I) or 7 (panel J) independent experiments; Scale bar, 50 µm (panels A - C) and 10 µm (panels D and E).

Figure EV4. *Shigella* intracellular replication inhibits re-infection by non-motile bacteria

A and B. qRT-PCR quantification of the secondary infection with *Shigella* WT (A) or *Salmonella* WT (B) of HeLa cells primarily infected with *Shigella* WT or Δ*ipaB*/Inv mutant strain, or mock-treated. Results are shown normalized to mock-treated cells.

C and D. Cfu (C) and qRT-PCR (D) quantification of the primary infection by *Shigella* WT or Δ*ipaB*/Inv mutant strain of HeLa cells (corresponding secondary infection shown in Fig. 5B-E).

E and H. Representative images of re-infection assays with *Shigella* WT (E) or *Salmonella* WT (H) of HCT-8 cells primarily infected with *Shigella* WT or Δ*ipaB*/Inv mutant strain, or mock-treated, as depicted in Fig 5A. Top panels shows merge image with primary infection (red, mCherry expressing *Shigella* strains) and secondary infection (green, GFP expressing bacteria); bottom panels show secondary infection exclusively.

F, G, I and J. Cfu (F and I) and qRT-PCR (G and J) quantification of the secondary infection with *Shigella* WT (F and G) or *Salmonella* WT (I and J) of HCT-8 cells primarily infected with *Shigella* WT or Δ*ipaB*/Inv mutant strain, or mock-treated.

K and L. Cfu (K) and qRT-PCR (L) quantification of the primary infection by *Shigella* WT or Δ*ipaB*/Inv mutant strain of HCT-8 cells (corresponding secondary infection shown in Fig. S6E-J).

M. Representative images of the re-infection assays with *Shigella* WT in HeLa cells primarily infected with *Salmonella* WT or mock-treated. Top panels show merge image with primary infection (red, *Salmonella* WT) and secondary infection (green, *Shigella*); secondary infection is shown in bottom panels.

N. Cfu quantification of the secondary infection with *Shigella* in HeLa cells primarily infected with *Salmonella* WT or mock-treated.

O. Cfu quantification of the primary infection by *Shigella* WT in HeLa cells re-infected with *Salmonella* Δ*fliC*, Δ*fliC*/pFliC or Δ*flhC* mutant strains.

Infection was performed at MOI 10 for *Shigella* WT or MOI 350 for *Shigella* Δ*ipaB*/Inv and MOI 25 for *Salmonella* WT or MOI 50 for the *Salmonella* Δ*fliC*, Δ*fliC*/pFliC or Δ*flhC* mutant strains. Results are shown as mean ± s.e.m. from 5 independent experiments; *p<0.05, ***p<0.001 (paired t-test for panel N; one-way ANOVA for all others). Scale bar, 50 µm.

Figure EV5. *Shigella* replication induces ASM activation

A, D, E and F. Representative images of re-infection assays with *Shigella* WT of HeLa cells primarily infected with *Shigella* WT or mock-treated, following amitriptyline (A), p38 knockdown with siRNA (D), SB203580 (E) or NAC (F). Top panels show merge image with primary infection (red, mCherry) and secondary infection (green, GFP); secondary infection is shown in bottom panels.

B and C. qRT-PCR quantification of the secondary infection with *Shigella* WT, following the ASM siRNA (B) or amitriptyline (C) treatment as described in panel 6H.

G. Cfu quantification of the secondary infection with *Shigella* WT following NAC treatment.

H-N. Cfu (H, J, L-N) and qRT-PCR (I, K) quantification of the primary infection by *Shigella* WT in HeLa cells (corresponding secondary infection shown in Fig. 6I-M and EV5A-G).

O-R. Representative images of ASM (O and R) or ceramide (P, Q and S) staining in HeLa cells infected with *Shigella* WT (O, P and Q) or Δ*ipaB*/Inv mutant strain (R and S), analyzed at 3 hpi.

Shigella infection was performed at MOI 10 (panels A-N), MOI 100 (panels O-Q) or MOI 350 (panels R and S). Results are shown as mean ± s.e.m. from 5 (panels B, C, G, I, L-N) or 6 (panels H and K) independent experiments; *p<0.05 (one-way ANOVA for panels B, C and G; paired t-test for all others). Scale bar, 50 µm (panels A, D-F), 10 µm (panels O, P, R and S) and 25 µm (panel Q).

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