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Short Communication

SHORT COMMUNICATION

Non-typhoidal *Salmonella* Typhimurium ST313 isolates that cause bacteremia in humans stimulate less inflammasome activation than ST19 isolates associated with gastroenteritis

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One sentence summary: Isolates of *Salmonella* Typhimurium that cause bacteremia in humans have evolved to be less inflammatory.

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ABSTRACT

Salmonella is an enteric pathogen that causes a range of diseases in humans. Non-typhoidal *Salmonella* (NTS) serovars such as *Salmonella enterica* serovar Typhimurium generally cause a self-limiting gastroenteritis whereas typhoidal serovars cause a systemic disease, typhoid fever. However, S. Typhimurium isolates within the multi-locus sequence type ST313 have emerged in sub-Saharan Africa as a major cause of bacteremia in humans. The S. Typhimurium ST313 lineage is phylogenetically distinct from classical S. Typhimurium lineages, such as ST19, that cause zoonotic gastroenteritis worldwide. Previous studies have shown that the ST313 lineage has undergone genome degradation when compared to the ST19 lineage, similar to that observed for typhoidal serovars. Currently, little is known about phenotypic differences between ST313 isolates and other NTS isolates. We find that representative ST313 isolates invade non-phagocytic cells less efficiently than the classical ST19 isolates that are more commonly associated with gastroenteritis. In addition, ST313 isolates induce less Caspase-1-dependent macrophage death and IL-1β release than ST19 isolates. ST313 isolates also express relatively lower levels of mRNA of the genes encoding the SPI-1 effector *sopE2* and the flagellin, *fliC*, providing possible explanations for the decrease in invasion and inflammasome activation. The ST313 isolates have invasion and inflammatory phenotypes that are intermediate; more invasive and inflammatory than *Salmonella enterica* serovar Typhi and less than ST19 isolates associated with gastroenteritis. This suggests that both phenotypically and at the genomic level ST313 isolates are evolving signatures that facilitate a systemic lifestyle in humans.

Key words: iNTS; immune evasion; genomic degradation

Salmonella enterica is a Gram-negative, facultative intracellular bacterial species. More than 2300 serovars of *S. enterica* can cause disease in humans (Porwollik *et al.*, 2004). The serovars are classically assigned into two categories based on the human disease they cause: typhoidal and non-typhoidal *Salmonella* (NTS) (de Jong *et al.*, 2012). Typhoidal serovars (e.g. *Salmonella enterica*

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serovar Typhi and Paratyphi A) are human restricted and cause the systemic disease typhoid fever (Selander *et al.*, 1990; Parry *et al.*, 2002; Raffatellu *et al.*, 2008b; Nuccio and Bäumler 2014). The NTS serovars such as *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) generally cause a self-limiting gastroenteritis in humans, which is usually zoonotic in origin (Zhang *et al.*, 2003; de Jong *et al.*, 2012). Occasionally, NTS serovars cause bacteremia, particularly in immunocompromised individuals (de Jong *et al.*, 2012). Beyond this paradigm, certain animal host-adapted *S. enterica* serovars, such as Dublin and Choleraesuis, are still capable of infecting humans and are more often associated with bacteremia and focal extraintestinal infections than gastroenteritis in humans (Fang and Fierer 1991; Werner, Humphrey and Kamei 1979; Nuccio and Bäumler 2014).

After ingestion, *Salmonella* reaches the small intestine, where it crosses the epithelial barrier either through invasion of intestinal epithelial and M cells or is taken up by $CD18^{+}$ immune cells (Vazquez-Torres *et al.*, 1999; Monack *et al.*, 2004). After crossing the epithelium, *Salmonella* interacts with macrophages and other immune cells (Monack *et al.*, 2004). In humans, typhoidal serovars cause limited gastrointestinal inflammation and disseminate throughout the host (de Jong *et al.*, 2012). *Salmonella* Typhi replicates and persists in macrophages at systemic sites, such as the liver and lymph nodes (Raffatellu *et al.*, 2008b). In contrast, infections of humans with NTS predominantly results in substantial gastrointestinal inflammation and the pathogen is largely restricted to the gut and gut-associated lymphoid tissues (Harris 1972; Raffatellu *et al.*, 2008b).

In sub-Saharan Africa, the distinctions between the disease syndromes caused by NTS and typhoidal serovars are less clear. Indeed, NTS serovars, particularly *S.* Typhimurium, are a common cause of systemic disease including febrile bacteremia, septicemia and meningitis (Walsh *et al.*, 2000; Gordon *et al.*, 2008; Kingsley *et al.*, 2009; Morpeth, Ramadhani and Crump 2009; Sigaúque *et al.*, 2009; Gordon 2011; Feasey *et al.*, 2012; Okoro *et al.*, 2012). These systemic NTS infections are often associated with malaria infection, anemia and malnutrition in children and HIV infections in adults, with a mortality rate of 20–45% being reported (Walsh *et al.*, 2000; Gordon *et al.*, 2008; Mackenzie *et al.*, 2010; MacLennan *et al.*, 2010). Phylogenetic analysis of *S.* Typhimurium isolates that cause systemic disease in sub-Saharan Africa revealed that they predominantly belong to a specific multi-locus sequence type, ST313, rarely found outside of sub-Saharan Africa (Kingsley *et al.*, 2009; Okoro *et al.*, 2012). In contrast, many common gastroenteritis-causing isolates of *S. typhimurium* belong to sequence type ST19 (Kingsley *et al.*, 2009; Okoro *et al.*, 2012).

Genomic analysis of ST313 NTS isolates reveals that this lineage possesses a distinct prophage repertoire, as well as a unique Tn21 element encoding multiple resistance genes on the virulence-associated plasmid (Kingsley *et al.*, 2009; Okoro *et al.*, 2012). There is also evidence of a significant level of genome degradation in the ST313 lineage, above that normally seen in ST19 isolates (Kingsley *et al.*, 2009). It is notable that some of the degraded genes are also non-functional in the typhoidal serovars *S.* Typhi and *S.* Paratyphi A (Kingsley *et al.*, 2009). Based on this genomic data, it has been speculated that the ST313 NTS isolates that cause systemic disease are evolving along similar lines to typhoidal serovars to become more host adapted. However, there is currently a lack of phenotypic data to support this hypothesis. Phenotypic characterizations of *S.* Typhimurium ST313 isolates have focused on dissecting host antibody responses and the impact of HIV and malaria coinfections (Gondwe *et al.*, 2010; MacLennan *et al.*, 2010; Schreiber *et al.*, 2011; Rondini *et al.*, 2013; Lokken *et al.*, 2014; Mooney *et al.*, 2014; Siggins *et al.*, 2014). Most studies do not directly compare phenotypic differences between ST313 NTS and other NTS. Indeed, little is known about how ST313 *S.* Typhimurium interact with host cells or if they differ from other *S.* Typhimurium lineages in this regard. In this work, we compare the ability of ST313 isolates to invade epithelial cells and to activate the inflammasome with that of classical gastroenteritis-causing ST19 isolates.

Since ST313 NTS routinely cause disseminated disease, we hypothesized that ST313 NTS are more invasive than ST19 isolates, which would allow them to breach the gut epithelium more efficiently, leading to dissemination to systemic sites. *Salmonella* can actively invade mammalian cells by injecting effector proteins through the *Salmonella* Pathogenicity Island 1 (SPI-1) type III secretion system (T3SS) into host cells (García-del Portillo and Finlay 1994; Mirold *et al.*, 2001; Zhou and Galán 2001; Raffatellu *et al.*, 2004). To test this hypothesis, we compared the abilities of various *Salmonella* isolates to invade the non-phagocytic, epithelial HeLa cell line. Our panel of *Salmonella* isolates included four different NTS ST313 isolates (A130, 5597, 5579 and D23580), two *S.* Typhimurium ST19 isolates that cause gastroenteritis (DT104 ATCC 700408 and SL1344) and a *S.* Typhi isolate (Ty2). The DT104 lineage is associated with a recent global epidemic of gastroenteritis (Threlfall, Ward and Rowe 1998), and SL1344 is a commonly used laboratory strain. In addition, we included an SL1344 *orgA* mutant (BJ66 *orgA::tn5*), which is unable to form a functional SPI-1 T3SS, and therefore cannot actively invade cells (Jones and Falkow 1994; Klein, Fahlen and Jones 2000; Sukhan *et al.*, 2001). We used a gentamicin protection assay to measure invasion of HeLa cells by quantifying intracellular *Salmonella* at 2 h post-infection (Isberg and Falkow 1985). To induce the expression of SPI-1 genes and increase invasion rates, *Salmonella* isolates were subcultured into fresh media and incubated standing at 37°C before infecting cells (Lee and Falkow 1990; Bajaj *et al.*, 1996; Ellermeier, Ellermeier and Slauch 2005). *Salmonella* were centrifuged onto the HeLa cells in order to synchronize infection and overcome any potential differences in motility (Hoffmann *et al.*, 2010). The wild-type ST19 isolates, SL1344 and DT104, exhibit 1.20 ± 0.22 and $3.43 \pm 0.93\%$ invasion, respectively. In contrast the four ST313, *S.* Typhimurium isolates were significantly less invasive (0.32 ± 0.05 to $0.65 \pm 0.20\%$ invasion; Fig. 1a). This represents approximately a 5–10-fold decrease in invasiveness compared to the epidemic isolate DT104. In contrast, the SL1344 *orgA* mutant strain and *S.* Typhi have very low levels of invasion (<0.01% invasion; Fig. 1a and b). Similar results were obtained when the total number of intracellular *Salmonella* colony forming units (CFU) was measured, with significantly lower levels of intracellular ST313 NTS bacteria compared to the ST19 isolates (Fig. 1b). While different culture conditions, *Salmonella* strains and invasion assay procedures cause variability in the actual percent invasion published by different research groups, significantly lower levels of invasion of *S.* Typhi compared to *S.* Typhimurium have been reported in a variety of polarized (T84, Caco-2) and non-polarized (Int-407, Caco-2 and HeLa) epithelial cell lines (Galán and Curtiss 1991; Mills and Finlay 1994; Bishop *et al.*, 2008). Taken together, our results indicate that the ability of ST313 NTS isolates to invade non-phagocytic cells is intermediate between the human-restricted *S.* Typhi and the zoonotic ST19 NTS isolates (Fig. 1).

The lower levels of host cell invasion exhibited by ST313 isolates compared to ST19 isolates is consistent with lower levels of the expression of at least one SPI-1 T3SS effector in ST313 isolates. To test this notion, we measured gene expression of the effectors involved in active invasion in the different isolates.

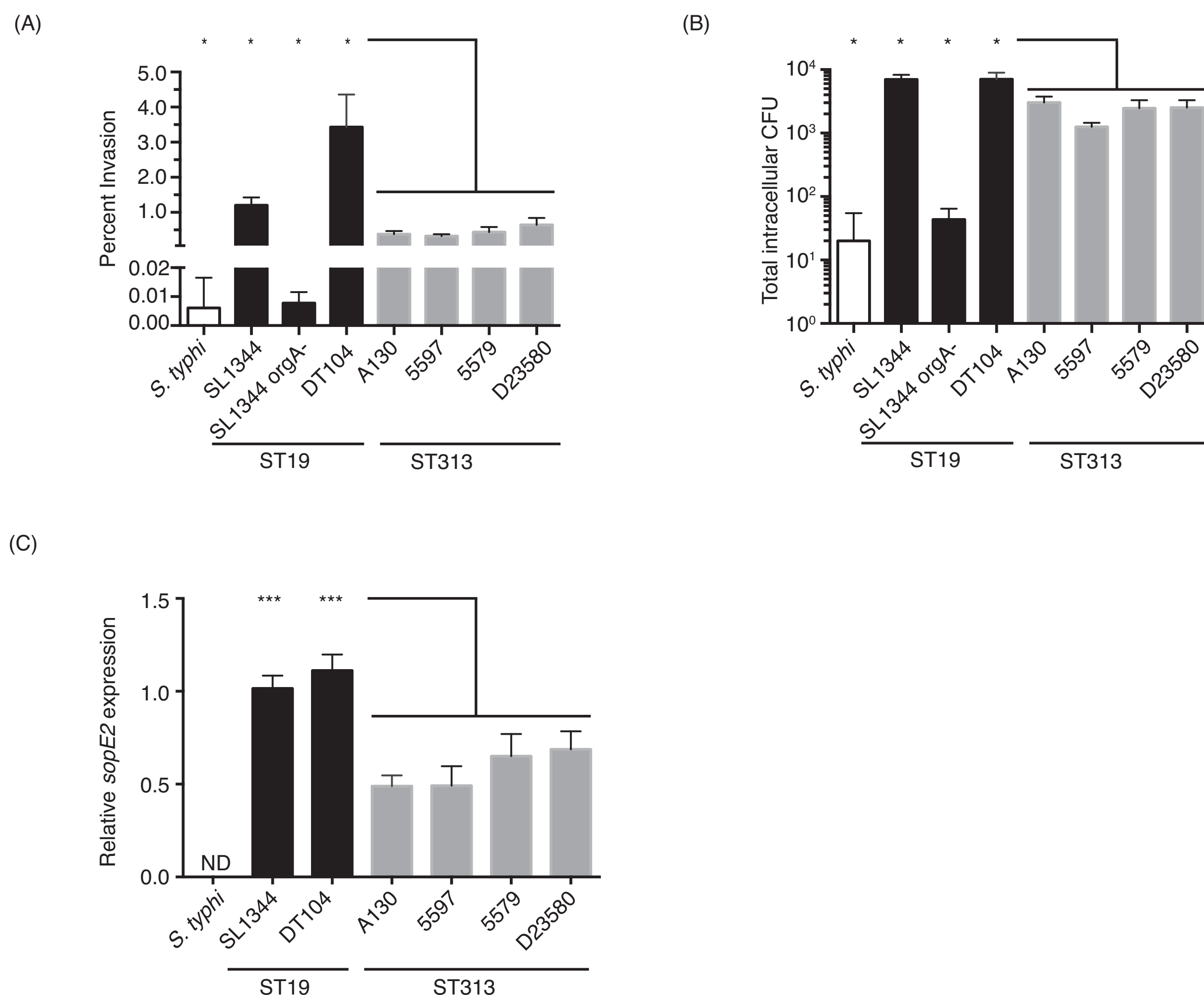

Figure 1. ST313 S. Typhimurium isolates are less invasive than ST19 S. Typhimurium isolates. (**A** and **B**) HeLa cells were seeded into 24-well plates at 200,000 cells per well. Overnight cultures of each *Salmonella* isolate were diluted 1:50 and subcultured standing for 3–4 h. *Salmonella* isolates were centrifuged onto HeLa cells at a multiplicity of infection of 10 and allowed to infect for 30 min. Cells were washed and given media containing 100 ug mL^{-1} gentamicin for 1.5 h. Then HeLa cells were washed, lysed and intracellular bacteria were enumerated by plating. Invasion is quantified as percent invasion over the initial input (A) and total *Salmonella* CFU recovered from the HeLa cells (B). Bars represent the mean and standard deviation for each isolate. Experiments were repeated three times, and data shown is a representative experiment (A and B). (**C**) Overnight cultures were diluted 1:50 in LB broth and subcultured standing for 3–4 h. Culture was placed in RNAprotect (Qiagen) and then RNA was extracted using hot phenol–chloroform and then ethanol precipitated. The RNeasy kit (Qiagen) was used to clean up and on-column Dnase treat the RNA. Then RNA was subjected to a second round of DNase treatment using the Turbo DNA-free kit (Invitrogen) and made into cDNA using Superscript III first-strand synthesis kit (Invitrogen). Primers were designed using Primer3Plus. All qRT-PCR was performed using Applied Biosystems 7300 real-time PCR system and FastStart SYBR Green Master Mix with Rox (Roche). Data presented is fold change over the abundance of *sopE2* mRNA recovered from the SL1344 after standardization to the housekeeping gene gmk. Experiments were repeated three times and bars represent the mean with standard deviation from the data from all three experiments. (A–C) White bar represents S. Typhi strain Ty2, black bars represent ST19 NTS and gray bars represent ST313. Statistics were calculated using student's *t*-test with welch's correction in GraphPad Prism. *P*-values are given as the lowest significance level between the control isolate and the individual four ST313 isolates. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$.

Salmonella injects four effectors to actively invade mammalian cells: SipA, SopB, SopE2 and SopE (Zhou and Galán 2001; Raffatellu *et al.*, 2004). While SipA and SopB are present in all ST19, ST313 and S. Typhi isolates, the presence of SopE and SopE2 is variable. SL1344 has a functional SopE, while DT104 does not (Mirold *et al.*, 1999; Hopkins and Threlfall 2004). Previous studies indicated that the genomes of ST313 and ST19 isolates contain the gene encoding SopE2, but lack a functional SopE (Kingsley *et al.*, 2009). In contrast, *sopE* is present in the genome of S. Typhi Ty2, but it does not contain an intact *sopE2* gene (Bakshi *et al.*, 2000; Stender *et al.*, 2000). We measured the expression of *sopE2, sipA and sopB* under SPI-1-inducing conditions by qRT-PCR in the NTS isolates used in this study (Fig. 1c, data not shown, Table S2, Supporting Information; Lee and Falkow 1990; Bajaj *et al.*, 1996; Ellermeier *et al.*, 2005). We found no significant differences in the levels of *sipA* and *sopB* mRNA between ST19 and ST313 lineages (data not shown). However, importantly the level of *sopE2* mRNA differed between ST19 and ST313 isolates (Fig. 1c). ST313 NTS isolates have significantly lower levels of *sopE2* mRNA compared to the ST19 NTS isolates (1.6–2.3-fold lower than DT104; Fig. 1c). To investigate possible explanations for the reduced level of *sopE2* gene expression, we analyzed the genomes of our ST313 and ST19 isolates for SNPs in known regulators of *sopE2* gene expression (Table S3, Supporting Information). We did not find any obvious SNPs that could be responsible for this reduction. Interestingly the S. Typhi regulatory protein TviA downregulates the expression of SopE, which is 69% identical to SopE2 and is also a guanine nucleotide exchange factor

(Friebel *et al.,* 2001; Winter *et al.,* 2014). Taken together, our data suggest that lower levels of expression of the invasion-inducing SPI-1 effector protein SopE2 is likely one factor responsible for the decrease in invasion by ST313 isolates.

It is perhaps counterintuitive that *Salmonella* isolates that cause more disseminated disease, such as S. Typhi and ST313 NTS, invade non-phagocytic cells at lower levels and have lower gene expression of key invasion effectors SopE and SopE2, respectively (Winter *et al.,* 2014). A potential explanation for this could be that higher amounts of cellular invasion causes greater activation of the host immune response which limits dissemination of *Salmonella* beyond the gut and gut-associated lymphoid tissue. One of the main distinctions between typical S. Typhimurium and S. Typhi infections is that S. Typhimurium causes substantial gut inflammation while S. Typhi does not (Harris 1972; Raffatellu *et al.,* 2008b; de Jong *et al.,* 2012). Effectors that promote invasion, like SopE2, usually also contribute to gut inflammation in mouse and bovine animal models (Zhang *et al.,* 2002, 2003; Raffatellu *et al.,* 2004). Additionally, intracellular *Salmonella* are detected by immune surveillance systems, such as the nodosome and the inflammasome, leading to increased inflammation (Broz and Monack 2011; Keestra, Winter and Auburger 2013; Keestra and Baumler 2014).Thus, increased invasion could cause more immune activation in a variety of ways and consequently lead to host restriction of bacterial dissemination.

To delve more into potential differences in immune activation by the *Salmonella* isolates, we examined their interaction with macrophages. Macrophages are an important systemic niche for *Salmonella* as well as a vital part of host innate immune defenses. A key immune surveillance system for macrophages is the inflammasome, which senses cytosolic pathogen-associated molecular patterns (PAMPs) or danger signals (Broz and Monack 2011; Ng, Kortmann and Monack 2013). Host cytosolic sensors and adaptor proteins detect these signals, then recruit and activate caspase-1 or 11, which ultimately leads to the release of cytokines (IL-1β and IL-18) and sometimes pyroptotic cell death (Broz and Monack 2011; Ng *et al.,* 2013). To characterize the interactions between the *Salmonella* isolates and macrophages, we infected primary bone marrow-derived macrophages (BMDM) from wild-type C57BL/6 mice. There were no significant differences between any of the isolates in total intracellular CFU at 1 h post-infection in BMDM, which is consistent with the primary macrophages being highly phagocytic (Fig. 2a). This allowed us to directly compare the levels of inflammasome activation between the various *Salmonella* isolates.

We detected inflammasome activation induced by each of the *Salmonella* isolates by quantifying cell death and IL-1β release after 1 h of infection of primary murine BMDM. As expected, the wild-type ST19 S. Typhimurium isolates induced high levels of IL-1β secretion in BMDM (Fig. 2b). The ST313 S. Typhimurium isolates induced intermediate levels of IL-1β secretion (Fig. 2b). In contrast, S. Typhi and the SL1344 *orgA* mutant induced relatively little IL-1β secretion (Fig. 2b). The levels of macrophage death following *Salmonella* infection showed similar trends to IL-1β release (Fig. 2c). SL1344 and DT104 induced significantly higher levels of macrophage death compared to infections with the ST313 isolates (Fig. 2c). *Salmonella* Typhi and SL1344 *orgA* mutant caused very little macrophage death (Fig. 2c). Taken together, our results indicate that the ST313 NTS isolates induce intermediate levels of proinflammatory cytokine secretion and macrophage death compared to the zoonotic ST19 NTS isolates and fully human-adapted S. Typhi.

To confirm that the cytokine release and macrophage death were due to inflammasome activation, we infected *Casp1/11*$^{-/-}$ and *Nlrc4*$^{-/-}$ macrophages and measured the amount IL-1β and cell death at 1 h post-infection (Broz and Monack 2011). It has been shown previously that when *Salmonella* are grown under SPI-1-inducing conditions, caspase-1 is rapidly activated and that this activation is dependent on the intracellular sensor NLRC4 (Miao *et al.,* 2006; Broz *et al.,* 2010). The activation of an NLRC4 inflammasome is dependent on the bacteria having an intact SPI-1 T3SS which delivers flagellin and components of the T3SS apparatus (PrgJ and PrgI) into the host cytosol (Miao *et al.,* 2006, 2010; Broz *et al.,* 2010). Indeed, none of the *Salmonella* isolates induced significant levels of IL-1β secretion or cell death in the *Casp1/11*−/− or *Nlrc4*−/− deficient macrophages (Fig. 2b and c). These results indicate that the cytokine release and cell death induced by the isolates of *Salmonella* tested in this study are dependent on the NLRC4 inflammasome, and that ST313 S. Typhimurium induce NLRC4 inflammasome activation at a level that is, once again, between the levels induced by human-adapted S. Typhi and zoonotic ST19 S. Typhimurium.

To determine whether inflammasome activation induced by the different *Salmonella* isolates is similar in human macrophages, we infected differentiated human U937 macrophages. Inflammasome activation in U937 cells can be quantified robustly by measuring IL-1β release (Bryan *et al.,* 2009). Infections of human macrophages with our panel of *Salmonella* isolates yielded a similar pattern of IL-1β release to that observed in murine macrophages. As expected, the ST19 isolates, SL1344 and DT104, elicited high levels of IL-1β secretion while S. Typhi and the SL1344 *orgA* mutant caused very low levels of cytokine release (Fig. 2d). Again, ST313 isolates induced intermediate levels of IL-1β release, which was significantly lower than the amount induced by ST19 isolates, but higher than the levels induced by S. Typhi (Fig. 2d). All together, our results suggest that ST313 S. Typhimurium induce an intermediate level of NLRC4 inflammasome activation in both human and mouse macrophages.

The NLRC4 inflammasome recognizes components of the SPI-1 T3SS (PrgJ and PrgI) and flagellin (Miao *et al.,* 2006, 2010). During the first hour post-infection with S. Typhimurium SL1344, flagellin causes the majority of NLRC4 inflammasome activation (Franchi *et al.,* 2006; Miao *et al.,* 2006; Broz *et al.,* 2010). The reduced levels of proinflammatory cytokine secretion and macrophage death induced by ST313 isolates compared to ST19 isolates could potentially be due to lower levels of flagellin in ST313 isolates. To test this, we measured expression of the gene that encodes the flagellin monomer, *fliC*, under SPI-1-inducing conditions by the *Salmonella* isolates (Lee and Falkow 1990; Bajaj *et al.,* 1996; Ellermeier *et al.,* 2005).We found that *fliC* expression was significantly lower in ST313 S. Typhimurium compared to ST19 S. Typhimurium (1.8–2.6-fold lower than DT104; Fig. 2e). *FliC* mRNA expression by S. Typhi was significantly lower than that of ST313 isolates (Fig. 2e). Thus, ST313 isolates express levels of *fliC* mRNA that is intermediate between ST19 isolates and S.Typhi. We next examined the genomes of ST19 and ST313 isolates for SNPs in known *fliC* regulators, but did not detect any SNPs that could explain the difference (Table S3, Supporting Information). Lower levels of *fliC* gene expression in ST313 compared to ST19 isolates suggest that the ST313 isolates produce less flagellin. Indeed, FliC protein levels during SPI-1-inducing conditions showed similar trends to the qRT-PCR data, with protein levels in ST313 isolates ranging from 30 to 70% of those seen in SL1344 (Fig. 2f). No FliC was detected in a control SL1344 mutant lacking *fliC* (SL1344 Δ*fljAB*Δ*fliC*),

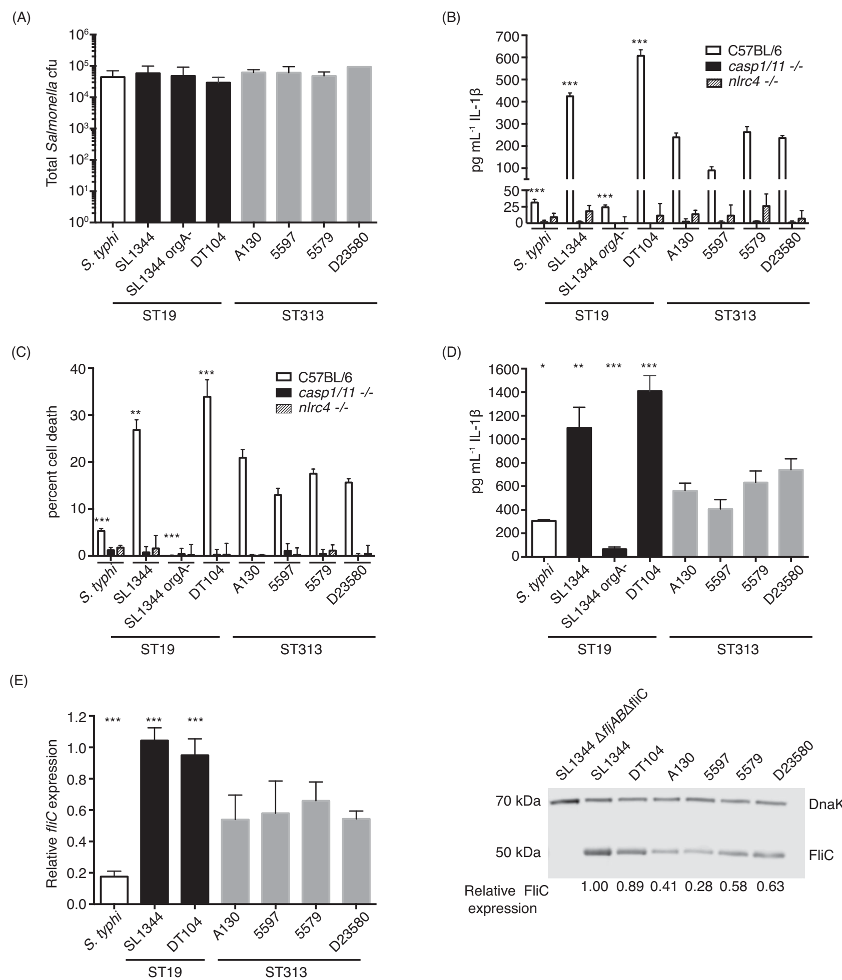

Figure 2. ST313 isolates induce less NLRC4 inflammasome activation in macrophages than ST19 isolates. Bone marrow-derived C57BL/6 macrophages were differentiated for 5 days in DMEM (invitrogen) with 10% FBS (Thermo Fisher Scientific), 20% MCSF (L929 cell supernatant) and 10 mm HEPES (Invitrogen) (A–C). (A) One day before infection macrophages were seeded into 24 well at densities of 2.5×10^5 cells well^{-1} in DMEM with 10% FBS, 10% MSCF and 10 mM HEPES and prestimulated with 0.1 μg mL^{-1} LPS (invitrogen) for 16 h. Overnight cultures of each *Salmonella* isolate were diluted 1:50 and subcultured standing for 3–4 h. Salmonella isolates were centrifuged onto macrophages at a multiplicity of infection of 10 and allowed to infect for 1 h. Macrophages were washed, lysed and intracellular *Salmonella* were enumerated by plating. Total intracellular CFU of *Salmonella* is presented in (A). Bars represent the mean and standard deviation for each isolate. White bar represents *S. typhi* strain Ty2, black bars represent ST19 NTS isolates and gray bars represent ST313 NTS isolates. (B and C) C57BL/6, *Casp1/11−/−* or *Nlrc4−/−* bone marrow-derived macrophages were differentiated as above. One day before infection macrophages were seeded into 96-well plates at densities of 5×10^4 cells well^{-1} in DMEM with 10% FBS, 10% MSCF and 10 mM HEPES and prestimulated with 0.1 μg mL^{-1} LPS (invitrogen) for 16 h. Overnight cultures of each *Salmonella* isolate were diluted 1:50 and subcultured standing for 3–4 h. *Salmonella* isolates were centrifuged onto macrophages at a multiplicity of infection of 10 (B) or 20 (C). One hour post-infection, supernatants were collected and release of IL-1β (B) or lactose dehydrogenase (C) was quantified. (B) Pg ml^{-1} IL-1β released into the supernatant in response to infection by C57BL/6J, *Casp1/11−/−* or *Nlrc4−/−* macrophages was quantified by IL-1β ELISA (R&D Biosystems). (C) The amount of cell death in response to infection was measured using the Cytox96 non-radioactive cytoxicity kit (Promega) measuring LDH release into the supernatant compared to an uninfected total lysis control for by C57BL/6J, *Casp1/11−/−* or Nlrc4−/− macrophages. (B and C) Bars represent the mean and standard deviation for each isolate. White, black and hatched bars represent infection of C57BL/6, *Casp1/11−/−* or Nlrc4−/−, respectively. For panels (B) and (C), differences between all isolates infecting WT C57BL/6J macrophages and *Casp1/11−/−* or *Nlrc4−/−* macrophages were significant at $P < 0.001$, except for infection with SL1344 orgA$^-$ in C which was not significantly different. **D**. Human monocytic cell line U937 cells were differentiated into macrophages for 2 days with 100 nM PMA (Sigma) in RPMI supplemented with 10% FBS (Thermo Fisher Scientific). One day before infection U937 macrophages were seeded into 96 well at densities of 5.0×10^4 cells well^{-1} in DMEM with 10% FBS, 1 0% MSCF and 10 mM HEPES and prestimulated with 0.1 ug ml^{-1} LPS (invitrogen) for 16 h. Overnight cultures of each *Salmonella* isolate were diluted 1:50 and subcultured standing for 3–4 h. Salmonella isolates were centrifuged onto macrophages at a multiplicity of infection of 10 and allowed to infect for 1 h. One hour post-infection, supernatants were collected and release of IL-1β was quantified by ELISA (eBioscience). (A–D) Experiments were repeated at least three times, and data shown are from a representative experiment. (**E**) Overnight cultures were diluted 1:50 in LB broth and subcultured standing for 3–4 h. Culture was placed in RNAprotect (Qiagen) and then RNA was extracted using hot phenol–chloroform and then ethanol precipitated. The RNeasy kit (Qiagen) was used to clean-up and on-column Dnase treat the RNA. Then RNA was subjected to a second round of DNase treatment using the Turbo DNA-free kit (Invitrogen) and made into cDNA using Superscript III first-strand synthesis kit (Invitrogen). Primers were designed using Primer3Plus. All qRT-PCR was performed using Applied Biosystems 7300 real-time PCR system and FastStart SYBR Green Master Mix with Rox (Roche). Data presented is fold change over the abundance of *fliC* mRNA recovered from the SL1344 after standardization to the housekeeping gene gmk. Experiments were repeated three times and bars represent the mean with standard deviation from the data from all three experiments. White bar represents *S. typhi* strain TY2, black bars represent ST19 NTS isolates and gray bars represent ST313 NTS isolates. (A–E) Statistics were calculated using student's *t*-test with welch's correction in GraphPad Prism. *P*-values are given as the lowest significance level between the control isolate and the individual four ST313 isolates. $^*P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$. (**F**) Overnight cultures were diluted 1:50 in LB broth and subcultured standing for 3–4 h. Cells were lysed and proteins were separated by SDS-PAGE. Western blots were performed using anti-S. Typhimurium Flagellin FliC antibody (Invivogen) diluted 1:3750 and anti-DnaK antibody clone 8E2/2 (Enzo Life Sciences) at 1:5000. Protein levels were visualized and quantified using Licor Odyssey system. FliC levels are presented as fold change relative to SL1344 protein levels after normalization to the housekeeping protein DnaK expression. The experiment was repeated twice and data shown are from a representative experiment.

confirming that our staining was specific to FliC (Fig. 2f). *Salmonella* Typhi FliC levels are not shown because the antibody did not recognize the S. Typhi flagellin. Since flagellin causes the majority of early inflammasome activation in macrophages (Franchi *et al.,* 2006; Miao *et al.,* 2006; Broz *et al.,* 2010), lower levels of FliC protein in ST313 isolates likely accounts for the decrease in NLRC4 inflammasome activation by those isolates.

In summary, ST313 isolates of S. Typhimurium are less invasive and less inflammatory compared to other S. Typhimurium isolates. However, the ST313 isolates invade non-phagocytic cells more efficiently and stimulate more inflammasome activation than S. Typhi Ty2. Lower levels of cell invasion and inflammasome activation are likely due to lower levels of mRNA expression of the genes-encoding *sopE2* and flagellin (*fliC*), respectively. Interestingly, it is known that SopE2 and flagellin both induce the release of IL-8, an important neutrophil chemoattractant, in epithelial cells (Gewirtz *et al.,* 2001; Huang *et al.,* 2004). Classically, during human infections NTS induce massive influx of neutrophils into the gut lumen, while in contrast S. Typhi induces very little neutrophil transmigration (Gal-Mor, Boyle and Grassl 2014). The lower levels of *sopE2* and *fliC* in ST313 S. Typhimurium compared to ST19 S. Typhimurium isolates could result in lower amounts of IL-8 and neutrophil influx during ST313 infection and represent another intermediate phenotype of ST313 isolates between ST19 isolates and S. Typhi. Immune evasion by S. Typhi reduces host inflammatory responses, which may allow it to effectively disseminate to systemic tissues, establish a persistent infection within a host and eventually transmit to a new host (Wangdi, Winter and Baumler 2012). We have shown here that at a cellular level, the ST313 NTS isolates are causing an infection that also displays aspects of immune evasion by downregulating *fliC* and *sopE2* expression compared to ST19 NTS isolates.

This study adds to a growing body of work indicating that dampening flagellin expression and inflammation helps *Salmonella* disseminate from the gut. Previous studies have shown that the S. Typhi regulator TviA downregulates the expression of SPI-1 genes and flagellin (Winter *et al.,*, 2009, 2014). Introduction of a deletion or TviA repression of flagellin in S. Typhimurium causes an increase in dissemination to the spleen but does not increase the bacterial load in gut tissues in mice and chicks (Winter *et al.,* 2010; Atif *et al.,* 2014). In addition, T cell responses against flagellin help restrict dissemination of *Salmonella* expressing flagella in the mouse model (Atif *et al.,* 2014). This indicates that SPI-1 and flagellin repression by TviA can help *Salmonella* bypass host restriction of dissemination to systemic sites (Winter *et al.,* 2010; Atif *et al.,* 2014). Although ST313 NTS isolates lack the gene that encodes TviA, it appears that these isolates elicit less of an immune response by expressing lower levels of genes that are recognized by mammalian cytosolic innate immune receptors (Miao *et al.,* 2006; Müller *et al.,* 2009; Broz *et al.,* 2010; Hoffmann *et al.,* 2010). This common theme of flagellin repression or deletion has been described in other host-adapted serovars, such as S. *enterica* serovar Gallinarum in chickens and S. *enterica* serovar Dublin in cattle (Freitas Neto *et al.,* 2013; Yim *et al.,* 2014). Similarly, the S. Typhimurium DT2 clade, which is restricted to feral pigeons and causes a typhoid-like disease in these animals, downregulates the expression of flagellar and SPI-1 genes at 42°C, which is the approximate body temperature of pigeons (Kingsley *et al.,* 2013). Thus, repressing flagella gene expression to reduce inflammation seems to be a common theme in *Salmonella* serovars that cause invasive disease and is potentially an important step for host adaptation (Winter *et al.,* 2009, 2010, 2014; Freitas Neto *et al.,* 2013; Kingsley *et al.,* 2013; Yim *et al.,* 2014).

It is worth noting that in sub-Saharan Africa a significant proportion of the population have comorbidities such as malnutrition, malarial infection and HIV that contribute to the amount of disseminated ST313 infections (Walsh *et al.,* 2000; Raffatellu *et al.,* 2008a; Schreiber *et al.,* 2011; Lokken *et al.,* 2014; Mooney *et al.,* 2014). For example, SIV infection blunts the T_H17 response causing defects in the intestinal barrier and increasing *Salmonella* dissemination in rhesus macaques (Raffatellu *et al.,* 2008a). ST313 isolates may be adapting to cause systemic disease specifically in humans with these comorbidities or these comorbidities might form a more permissive niche that ST313 NTS that allows adaptation to cause disseminated disease in immunocompetent humans.

Epidemiologically, the ST313 NTS isolates also seem to be intermediate between NTS isolates that cause gastroenteritis and S. Typhi. Kariuki *et al.,* found that NTS isolates that cause gastroenteritis have similar pulsed field gel electrophoresis (PFGE) types as isolates that cause bacteremia in Kenya, indicating that the same PFGE type commonly cause both forms of the disease (Kariuki *et al.,* 2006). Although ST313 routinely cause systemic disease in humans, they are not host restricted and can infect chickens (Parsons *et al.,* 2013). Interestingly, another study in Kenya showed a lack of clonal relationship between S. Typhimurium strain types from humans (symptomatic and asymptomatic) and those isolated from animals living in close contact (Kariuki 2006). Although ST313 NTS isolates are not restricted to humans, this suggests that they can persist and possibly even transmit between humans (Kariuki 2006). Studying how ST313 NTS differ from other NTS allows us the unique opportunity to see how these isolates of this lineage are able to cause disseminated human disease and potentially adapt to new niches.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSPD online.

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