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The *Salmonella* SPI2 Effector SseI Mediates Long-Term Systemic Infection by Modulating Host Cell Migration

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Abstract

Host-adapted strains of *Salmonella enterica* cause systemic infections and have the ability to persist systemically for long periods of time despite the presence of a robust immune response. Chronically infected hosts are asymptomatic and transmit disease to naïve hosts via fecal shedding of bacteria, thereby serving as a critical reservoir for disease. We show that the bacterial effector protein SseI (also called SrfH), which is translocated into host cells by the *Salmonella Pathogenicity Island 2 (SPI2)* type III secretion system (T3SS), is required for *Salmonella typhimurium* to maintain a long-term chronic systemic infection in mice. SseI inhibits normal cell migration of primary macrophages and dendritic cells (DC) in vitro, and such inhibition requires the host factor IQ motif containing GTPase activating protein 1 (IQGAP1), an important regulator of cell migration. SseI binds directly to IQGAP1 and co-localizes with this factor at the cell periphery. The C-terminal domain of SseI is similar to PMT/ToxA, a bacterial toxin that contains a cysteine residue (C1165) that is critical for activity. Mutation of the corresponding residue in SseI (C178A) eliminates SseI function in vitro and in vivo, but not binding to IQGAP1. In addition, infection with wild-type (WT) *S. typhimurium* suppressed DC migration to the spleen in vivo in an SseI-dependent manner. Correspondingly, examination of spleens from mice infected with WT *S. typhimurium* revealed fewer DC and CD4+ T lymphocytes compared to mice infected with ΔsseI *S. typhimurium*. Taken together, our results demonstrate that SseI inhibits normal host cell migration, which ultimately counteracts the ability of the host to clear systemic bacteria.

Introduction

*Salmonella enterica* is a pathogenic bacterial species that is an important cause of disease in humans ranging from gastroenteritis to systemic infections. Host-adapted *Salmonella* serovars disseminate from the gastrointestinal tract and colonize systemic sites. For example, *Salmonella enteric serovar Typhi* (*S. typhi*) causes human typhoid fever, whereas *Salmonella enteric serovar Typhimurium* (*S. typhimurium*) has a broad host range, causing disease in a variety of animals. Strains of *S. typhimurium* cause a typhoid-like disease in mice and usually cause a self-limiting gastroenteritis in healthy human adults. However, *S. typhimurium* can cause systemic infections in humans [1–5]. Indeed, recent cases of invasive and recurrent infections in Malawi [3], Mozambique [4], Malaysia [1], and Taiwan [3], were caused by nontyphoidal salmonellae (NTS), which were largely comprised of multidrug-resistant *S. typhimurium* strains [2,3].

*Salmonella*, a facultative intracellular pathogen, enters the host through the gastrointestinal tract where they preferentially enter microfold (M) cells, which are specialized epithelial cells that sample intestinal antigens and transport them to lymphoid cells in the underlying Peyer’s Patches (PP), specialized lymphoid tissue in the small intestine [6,7]. *S. typhimurium* can also translocate through the intestinal epithelia after uptake by CD-18-expressing immune cells [8]. In order for the infection to extend beyond the intestinal mucosa, *Salmonella* must survive and replicate within macrophages, a privileged niche that allows *Salmonella* to elude the adaptive immune response [9–11]. The ability of *Salmonella* bacteria to survive inside of host cells is dependent on the SPI2-encoded T3SS that injects virulence/effector proteins into host cells. Some of the SPI2 T3SS-translocated effector proteins have evolved to allow intracellular bacteria to subvert the bacteriocidal properties of macrophages and to create a specialized *Salmonella*-containing vacuole in which it can replicate [12]. In addition, certain SPI2 secreted effectors can specifically interfere with DC-mediated antigen presentation to CD4+ T cells [13–15], which are required to control bacterial replication within the host during a long-term systemic *Salmonella* infection [16]. Recently, SPI2 was also implicated in early culling of activated CD4+ T cells [17], further illustrating the complex relationship between *Salmonella* and T lymphocytes.

Another important aspect of *Salmonella* pathogenesis is the establishment of an asymptomatic carrier state that serves as a
Reservoir of infection as the bacteria are periodically shed and transmitted to new hosts [18–20]. Indeed, asymptomatic carriers of S. typhi shed the bacilli and are a significant reservoir for this deadly pathogen. To study the basic aspects of host-pathogen interactions during the carrier state, we have characterized a natural model of long-term chronic Salmonella infection in mice [21]. This model utilizes a mouse strain that does not typically succumb to infection. S. typhi can be recovered from systemic sites up to one year after infection and typically these bacteria are sequestered within macrophages in systemic tissues [21,22].

We previously performed a microarray-based screen to identify S. typhi factors required for long-term systemic infection in mice [23]. While most SPI2 genes were required for initial colonization of the spleen, the SPI2 effector SseI did not emerge from the screen until 2 weeks post-infection, indicating that SseI plays a role in long-term infection [23]. SseI is a secreted effector that is expressed by intracellular Salmonella and translocated across the vacuolar membrane into the host cell cytosol via the SPI2-encoded TTSS [24]. SseI has been shown to bind the actin-crosslinking protein filamin and to co-localize with polymerizing actin in the cytoskeleton and with TRIP6 [25,26]. The sseI gene encodes a 322 amino acid polypeptide whose N-terminal domain is highly similar to several other SPI2 effectors, including SspH2, and this domain is important for translocation and subcellular localization in the host [25]. However, no sequence similarities to the SseI C-terminus have been reported.

In this work, we have demonstrated that SseI is required for maintaining a long-term systemic infection and have defined a mechanism for this function. Specifically, we showed that SseI blocks migration of macrophages and DC in vitro, by a mechanism that involves the interaction of SseI with the host factor IQGAP1, an important regulator of the cytoskeleton and cell migration. Salmonella also reduced DC migration in vivo in an SseI-dependent manner, which correlated with a reduction in the number of DC and CD4 \(^+\) T cells in WT Salmonella-infected spleens. This data provides evidence for a novel mechanism by which an intracellular pathogen manipulates host cell migration to dampen the ability of the host to clear systemic bacteria.

**SseI-Regulated Cell Migration**

SseI is required for systemic S. typhimurium infection in mice

To measure the contribution of SseI to virulence, mice were infected by the intraperitoneal (IP) route with either WT S. typhimurium or the Asel deletion mutant. The numbers of WT and Asel bacteria in the PP, spleen, and liver were measured at 3, 15, 30 and 45 d post-infection (Fig. 1A–1C). Although both strains colonized the PP equally well at all time points, the level of WT bacteria was significantly higher than the Asel mutant at 30 d post-infection in the spleen (3.9-fold more WT than Asel) and liver (3.7-fold) (Fig. 1A–1C). In addition, the difference between the WT and Asel mutant strains increased between 30 d and 45 d in the spleen (14.2-fold) and liver (30.6-fold), further demonstrating the importance of SseI to maintaining a long-term infection in these tissues (Fig. 1B and 1C). In contrast, an S. typhimurium strain which is deficient for a SPI2 effector that is required for intracellular survival, sseJ (Fig. S1A, S1B and [27]) was attenuated to the same degree at 3 d (CI spleen = 0.24 \(\pm\) 0.07) and 30 d (CI spleen = 0.30 \(\pm\) 0.09) post-infection. To address the possibility that the insertion of a kanamycin resistance gene (kan\(^6\)) into the genome contributed to the attenuation of the Asel mutant at 45 d, we infected mice with another mutant, AcsgDEFG, that contains a kan\(^6\) insertion [23]. The levels of AcsgDEFG mutant bacteria recovered from systemic tissues were not significantly different from the levels of WT bacteria (Fig. 1D). Thus, the attenuation of the Asel mutant in systemic tissues cannot be attributed to minor effects on the growth rate of the bacteria due to the presence of an antibiotic resistance gene over the course of a long-term systemic infection. In addition, a Asel strain expressing WT sseI in trans (Asel+sseeI) was significantly less attenuated at 45 d in both the liver (Fig. 1E) and the spleen (Fig. 1D). We also measured the levels of bacteria in PP, cecum, spleen and liver of orally infected mice 34 d post-infection. The spleen and liver of WT-infected mice contained significantly higher levels of bacteria compared to Asel mutant-infected mice (Fig. 1F), confirming that this SPI2 effector is required to colonize systemic tissues, independent of the route of infection.

SseI binds directly to the cell migration regulator IQGAP1

Previous studies have shown that SPI2 and some of the secreted effector proteins are required for intracellular survival and host cell death [9,28–30]. However, we have shown that SseI is not required for bacterial survival in bone marrow-derived macrophages (BMDM) from 129x1/sv J mice or in RAW264.7 macrophage-like cells (Fig. S1A and S1B). SseI also does not regulate host cell death in BMDM (data not shown) or in bone marrow-derived dendritic cells (BMDC) (Fig. S1C and S1D). To determine the molecular targets of SseI, a GST-SseI fusion protein was incubated with primary macrophage lysates, the cell-type in which Salmonella is commonly found at systemic sites during long-term infection [20–22]. Bound proteins were co-precipitated with GSH-resin, eluted and subjected to SDS-PAGE (Fig. 2A). A band (shown as a doublet in Fig. 2A) migrating at approximately 200 kD was identified by mass spectrometry to be IQGAP1 (21 of 21 tryptic fragments were IQGAP1-specific; no other band was associated with a significant specific protein identity). Immuno-blotting analysis showed that GST-SseI specifically co-precipitated IQGAP1 from whole cell extracts made from either BMDM (naive or activated by pretreatment with 50 ng/ml lipopolysaccharide and 100 U/ml interferon-\(\gamma\)) or BMDC (Fig. 2B).

To determine if SseI can directly bind IQGAP1, co-precipitation studies were conducted with purified proteins (Fig. 2C and...
His-tagged SseI (WT) was incubated with GST-IQGAP1 (Fig. 2C) or free IQGAP1 (Fig. 2D), and in both cases SseI and IQGAP1 specifically co-precipitated with one another, indicating that SseI can directly bind IQGAP1 in vitro. IQGAP1 did not co-precipitate with another His-tagged SPI2 secreted effector, PipB [31], indicating that IQGAP1 co-precipitation was specific to SseI (Fig. 2D). To confirm that SseI interacts with IQGAP1 during infection, BMDM were infected with WT bacteria expressing cya-tagged SseI, and whole cell extracts derived from these infected cells were subjected to co-immunoprecipitation using an anti-CyaA antibody (Fig. 2E). IQGAP1 was specifically co-immunoprecipitated with SseI-cya, confirming that these factors interact.
during *Salmonella* infection. We did not detect an interaction between SseI and TRIP6 in BMDM, in contrast to a previous report using RAW264.7 cells. This may reflect differences in TRIP6 protein levels between these two studies [26].

To characterize the nature of co-localization between SseI and IQGAP1 in primary macrophages, BMDM were transiently transfected with an SseI-GFP fusion construct (Fig. 3A) or GFP alone (Fig. 3B). While expression of GFP alone resulted in green fluorescence that was mostly localized to the nucleus with diffuse fluorescence in the cytosol (Fig. 3B), expression of SseI-GFP resulted in an increased concentration of green fluorescence at the cell periphery (Fig. 3A). When these cells were stained for endogenous IQGAP1 (red), significant co-localization between SseI and IQGAP1 was also detectable at the cell periphery, including the lamella (Fig. 3A, Fig. S2A and S2C). BMDM were also stained with phalloidin (blue) to visualize actin within the cytoskeleton [25], and SseI was confirmed to co-localize with polymerized actin (Fig. 3A).

**Infection with *S. typhimurium*** causes an irregular pattern of movement in BMDM that is dependent on SseI

IQGAP1 is a 190 kD scaffolding protein that binds actin and regulates the cytoskeleton and cell migration machinery [32]. Since SseI binds to IQGAP1, we tested the hypothesis that SseI interferes with cell motility. BMDM were seeded onto two-chamber slides, and each chamber was infected with either WT or ΔsseI strains of *S. typhimurium* expressing GFP. Individual infected cells (as well as their uninfected neighbors) were monitored by PLoS Pathogens | www.plospathogens.org 4 November 2009 | Volume 5 | Issue 11 | e1000671
SseI inhibits the directed migration of BMDM and BMDC

Since our results demonstrated that Ssel interferes with normal cell movement (Fig. 4) and that Ssel binds IQGAP1 (Fig. 2), a host protein that promotes cell migration, we next tested whether Ssel influenced the directed migration of primary BMDM and BMDC. These primary cells were seeded onto transwells and infected with WT or mutant bacterial strains at an MOI of 10:1, conditions that resulted in 22±2% of the cells infected. The percentage of host cells that migrated specifically toward an attractant was quantified by confocal microscopy. BMDM infected with WT bacteria did not migrate toward the attractant (Fig. 5A). As Videos S1 and S2 show, BMDM are highly motile cells, and for all samples, there was a low basal level of migration to the bottom of the filter in the absence of attractant. In the case of Ssel-expressing bacteria, this basal level of migration was slightly higher than when the attractant was added, resulting in the negative values (Fig. 5A). However, these negative values were not significantly different from zero when tested in one-sample Student’s t test. In contrast to WT Salmonella, BMDM infected with ssel mutant bacteria readily migrated toward the attractant (Fig. 5A). Furthermore, the ability of the ssel mutant strain to inhibit host cell migration was fully restored by adding back a WT copy of the sseI gene, confirming a specific role for Ssel in the inhibition of directed migration (Fig. 5A and 5B). In tissue, mature DC migrate toward the CCR7 ligand, CCL19, in order to present antigen to T cells in secondary lymphoid tissue [33]. Similar to our results with BMDM, BMDC infected with WT bacteria did not migrate toward CCL19, whereas BMDC infected with the ssel strain did (Fig. 5B). Inhibition of BMDC migration was not due to any alteration in CCR7 surface levels, as WT- and ssel-infected BMDC expressed the same levels of CCR7 (Fig. S5G). Thus, S. typhimurium interferes with the directed migration of host phagocytic cells via a novel mechanism that depends on the secreted bacterial effector Ssel.

Ssel-mediated inhibition of migration is dependent on IQGAP1

While IQGAP1 promotes cell migration [34], it is not absolutely required (Fig. S3). Therefore, we examined the role of IQGAP1 in Ssel-dependent inhibition of directed migration. To test whether IQGAP1 is required for this Ssel-dependent activity, the ability of Ssel to regulate host cell migration was compared in BMDM derived from WT and IQGAP1−/− mice. As expected, WT BMDM infected with the complemented sseI mutant bacterial strain (sseIΔpssel) did not migrate toward heat-killed Salmonella (Fig. 5C). In contrast, IQGAP1−/− BMDM infected with the complemented sseI mutant bacterial strain migrated toward the attractant, and the levels of migration were similar to BMDM infected with the ssel mutant (Fig. 5C), indicating that IQGAP1 is necessary for Ssel-dependent regulation of cell migration. Another possible explanation is that there was decreased bacterial uptake by the IQGAP1−/− BMDM [35,36]; however, the intracellular bacterial loads in IQGAP1−/− BMDM were not less than those in WT BMDM (Fig. 5D). These data confirm that the loss of the ability of S. typhimurium to inhibit migration of IQGAP1−/− BMDM was not due to decreased intracellular bacterial numbers (Fig. 5D). Furthermore, we demonstrated that S. typhimurium infection induced IQGAP1-independent pathways of cell migration, which was dependent on infection with intact bacteria (as infection with heat-killed bacteria did not induce migration, Fig. 5C). Thus, infection with intact bacteria induced migration that was independent of IQGAP1 and Ssel. However, the concomitant presence of both the bacterial effector, Ssel, and the host factor, IQGAP1, resulted in a dominant interference with host cell migration.

**Figure 3. Ssel co-localizes with IQGAP1 and actin at the cell periphery.** BMDM were transiently transfected with pssel-GFP (A) or pEGFP (B) and then fixed and stained for IQGAP1 (red) and actin (phalloidin, blue). Transfected cells were imaged by confocal microscopy (600 x), and the white bars represent 17 microns. Arrows indicate regions of co-localization. doi:10.1371/journal.ppat.1000671.g003

time-lapse microscopy at 24 h p.i. The cells were tracked and analyzed for the number of times each cell reversed its direction of movement (>90° turn between 3 consecutive frames was scored as a reversal) and for their net displacement. Surprisingly, cells infected with WT bacteria made significantly more turns that were >90° compared to cells infected with ssel mutant bacteria (Fig. 4A; p<0.001). In addition, WT-infected cells made significantly more turns compared to their uninfected neighbors (Fig. 4A; p<0.01), indicating that the intracellular bacteria modulated the cells’ normal patterns of movement. These results also demonstrate that the intracellular bacteria exert their influence specifically on the infected cell and that the modulation of cell movement is not due to bystander effects. The cell tracks of two representative movies of WT- (Fig. 4B, Video S1) and ssel- (Fig. 4C, Video S2) infected BMDM are shown. The median net displacement did not change significantly with infection (data not shown). Taken together, our data demonstrate that S. typhimurium alters the movement of infected phagocytic cells in a cell autonomous fashion by an Ssel-dependent mechanism.
SseI plays a distinct role in cell adhesion when expressed in RAW264.7 cells

A previous report published by Worley et al. [26] demonstrated that RAW264.7 cells (a transformed monocytic-like cell line) expressing SseI moved through and detached from transwells at an increased rate, and that this activity was dependent on the host factor TRIP6 [26]. However, in our experiments we were unable to detect an interaction between TRIP6 and SseI in primary macrophages (Fig. 2E). Furthermore, GST-SseI co-precipitation of IQGAP1 in RAW264.7 cells is dramatically reduced even though IQGAP1 is present (Fig. 6A), perhaps indicating that the binding site is blocked. Thus, the fact that SseI interacted with different host factors in RAW264.7 cells as compared to primary BMDM and BMDC suggested that SseI may function differently in the RAW264.7 cell line.

In the migration assay of Worley et al. [26], detection of migration relied on cells migrating through and detaching from the transwell (measuring both migration and loss of adherence simultaneously), whereas in our study migration was scored by counting cells that traversed the transwell without detaching (specifically measuring directed migration). To measure the effect of SseI on cell adherence, we compared the levels of BMDM and RAW264.7 cells that had detached from tissue culture plates when infected with either the complemented sseI mutant bacterial strain (ΔsseI(pFPV25.1)) or the sseI mutant bacterial strain containing the empty vector (ΔsseI(pACYC184)). Since the levels of host cell detachment were very low, we counted the number of bacteria that were released into the supernatant as described by Worley et al. [26]. Infection of RAW264.7 cells with the complemented sseI mutant bacterial strain (Fig. 6B) or WT strain (data not shown) resulted in significantly higher numbers of bacteria released into the supernatant compared to background levels. Thus, we observed an SseI-dependent detachment in RAW264.7 cells, but not in BMDM, suggesting that SseI regulates cell adherence in

Figure 4. SseI causes *S. typhimurium*-infected BMDM to reverse their direction of travel more frequently. A) BMDM were seeded onto two-chamber glass slides and infected with GFP-expressing strains of WT (WT(pFPV25.1)) or ΔsseI (ΔsseI(pFPV25.1)) *S. typhimurium* for 24h. Four locations from each chamber were imaged by time-lapse microscopy (DIC and fluorescence; images were taken every 3min; 45 images were taken in all per movie). The number of times a cell changed its direction of movement more than 90° (per video) are reported for infected cells and their uninfected neighbors (bars represent the median, the data are compiled from 28 total movies (14 movies per bacterial strain) performed in 4 independent experiments, n = 66 for uninfected cells (circles) and n = 82 for infected cells for WT *S. typhimurium*-infected BMDM (filled circles), n = 75 for uninfected cells (triangles) and n = 96 for infected cells for ΔsseI *S. typhimurium*-infected BMDM (filled triangles)). **, p-value<0.01 and ***, p-value<0.001; Mann-Whitney U test. B–C) The frames and cell-tracks of two representative movies are shown (B, WT; C, ΔsseI, tracks of uninfected cells are shown in blue and those of infected cells are orange), and the full videos are available online (Videos S1 and S2).

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RAW264.7 cells but not in primary macrophages. As expected, the percentages of RAW264.7 cells infected with either the WT or sseI mutant bacterial strain were not significantly different at 3h or 24h p.i. (Fig. 6C), demonstrating that SseI-dependent detachment of infected RAW264.7 cells at 24h could not be due to an SseI-dependent difference in the percentage of infected cells. Furthermore, there was not a significant difference in the average number of WT or sseI mutant bacteria per RAW264.7 cell at 24 h (Fig. 6D), which is consistent with our results when comparing intracellular bacterial replication in a gentamicin protection assay (Fig. S1A and S1B). Thus, the SseI-dependent detachment of infected RAW264.7 cells could not be attributed to SseI-dependent differences in intracellular bacterial growth. While the average number of bacteria per infected cell was not significantly different...
between BMDM and RAW264.7 cells, there were more RAW264.7 cells infected with 10 bacteria at 24h than BMDM (Fig. 6D). Thus, it is possible that SseI-dependent detachment is not detectable in BMDM due to the lack of cells with very high numbers of bacteria and is another possible explanation for any differences between results obtained with RAW264.7 cells [26] compared to BMDM.

Cysteine 178 is critical for SseI-dependent inhibition of migration and for colonization of host systemic tissues

To investigate the molecular mechanism of SseI action, the C-terminal domain (159–322) of SseI was subjected to a position iterative (PSI-) BLAST search, which uncovered sequence similarity to several hypothetical proteins, as well as to the bacterial toxin PMT/ToxA (Fig. 7A). Three of the aligned sequences are from known insect and mammalian pathogens (P. askibiota [37], B. dolosa [38], and P. multocida [39]), suggesting that these genes may comprise a family of bacterial virulence factors. PMT/ToxA, recently shown to be a deamidase [40], is required for virulence [39] and has been shown to inhibit DC migration and impair actin reorganization; all these activities have been shown to be dependent on a critical cysteine residue at position 1165 [41].

To test whether the corresponding residue in SseI (C178) was required for its function, the SseIC178A point mutant protein was constructed and compared to WT SseI protein. Co-precipitation studies showed that the SseIC178A mutant protein had similar binding affinity for IQGAP1 as compared to WT SseI (Fig. 2C and S4). However, the S. typhimurium strain expressing SseIC178A (ΔsseI (pSSeIC178A)) did not inhibit BMDM migration, similar to the ΔsseI strain (Fig. 5A). In contrast, mutation of the conserved H and D residues in SseI (H216A and D231A) did not interfere with SseI-dependent regulation of migration (Fig. 5A). To test whether C178 is also critical to SseI function in vivo, the virulence of the ΔsseI (pSSeIC178A) mutant strain was compared to WT in mixed infections where mice were infected with a 1:1 ratio of WT S. typhimurium and ΔsseI S. typhimurium strains transformed with pssel, pSSeIC178A, or pACYC184 (empty vector) (Fig. 7B). The WT strain out-competed the ΔsseI (pSSeIC178A) strain to the same extent as the ΔsseI (pACYC184) strain, demonstrating that C178 is critical for
SseI function in vivo (Fig. 7B). The SseIC178A mutant protein was expressed and translocated through the SPI2 T3SS at levels comparable to WT SseI (Fig. 7C), confirming that loss of activity was due to specific mutation of C178 [42].

SseI-dependent suppression of DC migration in vivo correlates with lower numbers of DC and CD4\(^+\) T cells in infected spleens

We found that WT \textit{S. typhimurium} inhibits migration of infected BMDC in vitro by a mechanism that depends on SseI. Therefore, we investigated the potential role of SseI in inhibiting migration of \textit{Salmonella}-infected DC in vivo (Fig. 8A). BMDC stained with the vital dye PKH26 were infected with GFP-expressing WT(p\textit{FPV25.1}) or \textit{D}sseI(p\textit{ACYC184}) strains of \textit{S. typhimurium}, and approximately 5\times10^6 labeled BMDC (\~50% GFP\(^+\)) were injected into 129x1/sv J mice by the IP route. The migration of the infected BMDC to the spleen was measured at 6h post-infection by flow cytometry. To control for heterogeneity in the exact numbers of migrating BMDM between mice, we calculated an in vivo migration index for each injected animal. We defined the in vivo migration index as the ratio of infected to uninfected BMDC (GFP\(^+\)PKH26\(^+\) cells/GFP\(^-\)PKH26\(^-\) cells) used for injection (input) (details in Materials and Methods). An in vivo migration index value of less than 1 would indicate that infection with \textit{S. typhimurium} attenuates the migration of BMDC to the spleen. By comparing the migration indices for WT- and \textit{D}sseI \textit{S. typhimurium} strains (just 6h post-injection) also is consistent with the gradual attenuation of the \textit{D}sseI mutant in systemic tissues over a period of 1.5 months.

In addition, the cellular composition of WT(p\textit{ACYC184})-, \textit{A}ss\textit{eI}(p\textit{ACYC184})-, or \textit{A}ssel\textit{p}ss\textit{eI}-infected spleens were compared at 12d post-infection when the numbers of WT and mutant strains of bacteria in the spleens were not significantly different (Fig. 8B). While the numbers of GR-1\(^+\) cells were not significantly different between the WT and mutant strains (Fig. 8A), the numbers of DC and CD4\(^+\) T cells in the spleens of \textit{A}ssel\textit{p}ss\textit{eI}-infected mice were significantly higher than those of WT(p\textit{ACYC184})- and \textit{A}ssel\textit{p}ss\textit{eI}-infected mice (Fig. 8C and 8D), suggesting a more pronounced T cell response in the \textit{A}ssel-immunized mice. These data are in accordance with previous results showing that \textit{Salmonella}...
interferes with T cell proliferation in vivo and inhibits DC-mediated antigen presentation by a SPI2-dependent mechanism [13,15]. However, we (Fig. S5B, S5C, S5D) and others [14] have shown that SseI does not directly interfere with DC-antigen presentation to T cells in vitro. Furthermore, surface upregulation of MHC-II and B7.2 in Salmonella-infected DC, which is not altered in a SPI2-dependent manner [13,15], were the same in WT and DsseI infections in vivo and in vitro (Fig. S5E, S5F, S5H).

While it is unlikely that SseI directly modulates the CD4+ T cell response, our data demonstrated that SseI suppressed DC migration in vivo, which correlated with the ability of Salmonella to continuously maintain a systemic infection for at least 45d.

Discussion

SseI is continuously required for S. typhimurium to colonize the spleen and liver and to maintain a long-term systemic infection, as the attenuation of Assel mutant strains significantly increased over the duration of infection (Fig. 1B and 1C). In contrast, SseI does not contribute to colonization of Peyer’s patches and cecum within the GI tract (Fig. 1A and 1F). This tissue-specificity may reflect differences in S. typhimurium localization (i.e. extracellular vs. intracellular), host cell interactions or host immune clearance mechanisms. This is a question that we are currently investigating.

SseI specifically binds the cell migration regulator IQGAP1 (Fig. 2 and Fig. 3) and inhibits migration of BMDM and BMDC toward known attractants (Fig. 5A and 5B). IQGAP1 is a large scaffolding protein that binds actin and several small G proteins, including Cdc42 and Rac1, but does not bind RhoA itself [34,43,44]. However, all of these Rho family GTPases play important roles in cell migration [45]. IQGAP1 binding inhibits the intrinsic GTPase activity of Cdc42 and Rac1 and prolongs G protein signaling [44,46]. IQGAP1 also captures microtubules (via CLIP-170 and APC), thereby regulating the directionality of cell migration [47,48]. Cdc42 and Rac1 are important regulators of IQGAP1 activity, because overexpression of IQGAP1 mutants that cannot bind Cdc42 or Rac1 induce the formation of multiple leading edges and

![Image](image-url)
inhibit cell migration in a dominant manner [34,47]. We have shown that Salmonella-infected macrophages exhibit a higher frequency of reversals in their direction of movement and that this change in movement behavior is dependent on SseI (Fig. 4A). SseI directly binds IQGAP1 (Fig. 2C and 2D) in primary macrophage and DC lysates (Fig. 2B) and during Salmonella-infection of primary macrophages (Fig. 2E). We have shown that while infection with live bacteria that are lacking sseI induced macrophage migration, infection with SseI-expressing bacteria blocked directed migration in an IQGAP1-dependent manner (Fig. 5C), demonstrating a functional interaction between SseI and IQGAP1. Whether SseI interferes with the regulation of IQGAP1 or causes IQGAP1 to adopt an aberrant activity remains to be determined. Ultimately however, this interaction between SseI and IQGAP1 leads to the interference in the host cell’s ability to efficiently migrate toward an attractant. The determination of the role of IQGAP1 in the ability of S. typhimurium to cause long-term systemic infection awaits the generation of IQGAP1−/− 129s1/sv J mice.

The C-terminal sequence of SseI is similar to several hypothetical proteins, two of which are from pathogenic bacteria species that are able to cause disease in humans [37,38]. Similarity was also found to PMT/ToxA, a P. multocida toxin that inhibits DC migration [41], and alignment of all these sequences revealed several conserved amino acids, including C178 (Fig. 7A). PMT/ToxA was recently shown to be a deamidase that acts on heterotrimeric G proteins [40], and its activity as a toxin is dependent on a catalytic triad formed by the conserved residues, C1165, H1205, and D1220 (Fig. 7A) [49,50]. We demonstrated that the substitution of C178 for an A in SseI impairs the ability of S. typhimurium to colonize host systemic sites and to inhibit directed host cell migration (Fig. 7B, 5A and 5B). However, these results also indicate that while IQGAP1 is required for SseI function in the host, binding of SseI to IQGAP1 is not sufficient because SseIC178A also binds IQGAP1 (Fig. 2C and S4). Thus, part of SseI-function also must be attributed to a specific activity that is dependent on C178. However, due to the fact that the conserved H216 and D231 were not essential for SseI-function (Fig. 5A), it is less apparent what this activity might be. Although a structural role for C178 cannot be ruled out, we have shown that the SseIC178A mutant protein is efficiently translocated into host cells and is stable (Fig. 7C and S4). Therefore, we hypothesize that SseI possesses a distinct biochemical activity that could act on IQGAP1. An alternative hypothesis is that SseI could be taking advantage of IQGAP1’s role as a scaffolding protein [51] in order to be brought into contact with other host cell proteins (e.g. heterotrimeric G proteins, similar to PMT) that are altered by SseI, leading to a disruption in normal host cell migration. Characterization of SseI’s associated biochemical activity is under active investigation.

A previous report by Worley et al. showed that SseI (SsH) stimulated macrophage movement through and detachment from transwells and caused early escape of S. typhimurium from the GI tract into the blood stream [26]. This SseI-dependent activity also was shown to be dependent on the host protein, TRIP6, a factor required for normal cell adhesion [52]. Our results show that in RAW264.7 cells, SseI specifically regulates cell adherence (Fig. 6B), whereas in primary BMDM and BMDC, SseI blocks cell migration (Fig. 5A–5C). Taken together, these results provide evidence that SseI plays at least two different roles, one of which is to regulate cell adherence in order to cause early escape of S. typhimurium out of the GI tract and into the blood stream as reported previously [8,26]. This role could explain the slight attenuation of the AsseI mutant at 3d in the liver, a highly perfused organ that also filters blood from the GI tract (Fig. 1C). However, our results clearly demonstrate that SseI also plays an important inhibitory role in the regulation of host cell migration. This role becomes critical during later stages of infection and allows S. typhimurium to maintain a long-term systemic infection of the host, as demonstrated by the striking increase in attenuation of the AsseI mutant that occurs between 30d and 45d post-infection in the spleen and liver (Fig. 1B and 1C).

We have demonstrated an SseI-dependent decrease of DC migration in vivo (Fig. 8A) which correlates with a decrease in DC and CD4+T cell numbers by suppressing DC migration and limiting their ability to effectively prime naive T cells. However, this global effect of sseI would not entirely account for the competitive advantage of WT bacteria over the AsseI mutant in mixed infections (Fig. 7B) and suggests that the SseI-mediated decrease in host cell migration also may reduce the accessibility of infected cells to local immune cell-mediated killing mechanisms. Nevertheless, the effects of SseI on host cell migration, through its interactions with the host molecule IQGAP1, correlate with a reduced capacity of the host to clear S. typhimurium from systemic sites of infection.

Materials and Methods

Ethics statement

All animal experiments were performed in accordance to NIH guidelines, the Animal Welfare Act, and US federal law. Such experiments were carried out under the supervision of Stanford University’s Administrative Panel on Laboratory Animal Care (A-PLAC) which has been accredited by the Association of Assessment and Accreditation of Laboratory Animal Care International (AAALAC). All animals were housed in a centralized and AAALAC-accredited research animal facility that is fully staffed with trained husbandry, technical, and veterinary personnel.

Mouse strains and mammalian cell culture

Female 129s1/sv J mice (6–8 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME). Bone marrow was harvested from femurs of IQGAP1−/− mice and WT littermate controls [55]. Marrow was differentiated into primary macrophages (BMDM) or dendritic cells (BMDC) as described previously [56,57]. RAW264.7 cells were cultured in DMEM with 10% heat-inactivated FBS. Cell cultures were incubated in a humidified chamber at 37°C in 5% CO2.

Bacterial strains and plasmid constructs

S. typhimurium SL1344 (2) was used as the parent strain for all experiments presented here, and the AsseI strain was created by replacing the sseI coding sequence with that of a kanamycin-resistance gene [58]. To complement, the sseI gene plus the 476 bp upstream sequence was cloned into the low copy number plasmid pACYC184 (pseI), which also contains a chloramphenicol-resistance marker. The C178A mutation was generated by site-directed mutagenesis via the QuickChange II mutagenesis kit.
in vivo binding

SseI fusion proteins were purified using standard methods utilizing GSH conjugated- or nickel-charged resin, and purity was checked by SDS-PAGE and Coomassie stain [60]. Briefly, overnight cultures of *E. coli* BL21 transformed with *pHis-sseI*, *pHis-sseIC178A*, *pHis-pipB*, *pGST-sseI* or *pGST-sseIC178A* were diluted 50× and grown at 37°C with shaking until reaching an optical density between 0.7–0.9 at 600 nm. Cultures were heat-shocked at 42°C for 10 min, cooled to room temperature, and incubated with 1 mM IPTG for 1 h. Cells were lysed by resuspending in BugBuster lysis buffer (EMD Chemicals, Inc., Gibbstown, NJ) with 50 mM AEBSF using a needle and syringe, and the lysate was cleared by spinning at 20,000g for 15 min at 4°C. The lysate was then combined with either GST-agarose resin (incubated overnight at 4°C, for GST-tagged proteins), nickel-charged resin (incubated for 5 min at room temperature, for His-tagged proteins), or protein G plus resin (incubated for 3 h at 4°C, EMD Chemicals Inc., Gibbstown, NJ). In the case of protein G plus resin, the lysate was first pre-incubated with anti-His tag antibody (R&D Systems, Minneapolis, MN) for 30 min on ice. The resins were then washed: 5 times in PBS for GST-agarose resin, 3 times with 1× binding buffer (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 5 mM imidazole) and 3 times with 1× wash buffer (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 60 mM imidazole) for nickel-charged resin, or twice with lysis/binding buffer (1% NP-40, 150 mM NaCl, 50 mM Tris-HCl pH 7.2, 2 mM EDTA, Na Vanadate 400 µM, 50 µM NaF and 1mM AEBSP) for protein G resin. The resin-bound SseI proteins were then used for in vitro binding assays, or nickel-charged resin-bound His-SseI proteins were eluted using 20 mM Tris-HCl pH 7.9, 500 mM NaCl, 1 M imidazole. Free His-SseI proteins were then dialysed against a buffer containing 20 mM Tris-HCl pH 7.9, 100 mM NaCl, and 0.3mM DTT before being used for in vitro binding assays as well. Purified GST-IQGAP1 was prepared as previously described by Ho et al. [61]. Approximately 5×10^6 BMDM or BMDC were lysed in 800 µl lysis(binding) buffer. Cleared cell lysates were pre-incubated with GST-resin for 2 h at 4°C before combining with a given GST fusion protein pre-bound to GST-resin and incubated overnight at 4°C. The resin was washed, and bound proteins were eluted by boiling in 1× SDS-sample buffer: 125 mM Tris-HCl pH 6.8, 1.8% SDS, 5% glycerol, 0.1 M dithiothreitol, and 0.002% bromphenol blue. Free IQGAP1 was generated by cleaving the GST tag with His-tagged Tobacco Etch Virus (TEV) protease and combined with resin-bound His-SseI or His-PipB in lysis(binding) buffer for overnight incubation at 4°C, or resin-bound GST-IQGAP1 was incubated with free His-SseI and incubated overnight at 4°C. The resin was washed 5 times with lysis(binding) buffer and bound proteins were eluted as before. All eluates were subjected to SDS-PAGE and Coomassie staining or immunoblotting. The membranes were stained with antibodies reactive against IQGAP1 (Santa Cruz Biotechnology, Santa Cruz, CA), 6X-His tag (R&D Systems, Minneapolis, Mn), rabbit IgG-660 (Molecular Probes, Carlsbad, CA), and mouse IgG-800 (Rockland Immunochemicals, Gilbertsville, PA) and detected using the Odyssey system (Li-Cor Biosciences, Lincoln, NE). To detect SseI-IQGAP1 binding in the context of an infection, BMDM (caspase-1−/−) were infected (MOI of 25) with *S. typhimurium* (with or without *pssI-cya*) grown standing in Luria Broth to induce SPI1 expression. At 6h, harvested proteins were subjected to co-immunoprecipitation with anti-CyaA antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and immunoblotted for IQGAP1 or TRIP6 (antibody was a generous gift from Dr. M. Beckerle, University of Utah).

**Transient transfections and fluorescence microscopy**

5×10^6 BMDM were combined with 5 µg of pSseI-GFP or pEGFP and electroporated using the Amaxa Nucleofector device (Lonna, Cologne, Germany). Immediately afterward, BMDM were seeded onto coverslips and later fixed in 2% paraformaldehyde phosphate buffer. BMDM were stained with anti-IQGAP1 antibody (1:50, Santa Cruz, CA) and Alexa fluor 647 phalloidin (1:50, Molecular Probes, Carlsbad, CA), and z-stack...
images were taken by confocal microscopy at 600× and analyzed using Velocity software (Improvision Inc., Waltham, MA).

BMDM and BMDC in vitro and in vivo migration and motility assays

One day prior to infection, BMDM or BMDC were seeded onto transwell inserts for 24-well plates (5 µm pore size, Corning, Corning, NY) at 1×10^4 or 2.5×10^4 cells/well, respectively. *Salmonella* strains were opsonized in a 1:1 solution of normal mouse serum and cellular medium solution, and then used to infect BMDM or BMDC at a multiplicity of infection (MOI) of 10:1. Extracellular bacteria were killed by adding 100 µg/ml gentamicin after 30 minutes, and 1.5h later reduced to 10 µg/ml gentamicin. Each infection was done in duplicate wells. An attractant was added to the bottom chamber of one well from each infection at 24h. An equivalent of 12.5 million cfu of heat-killed *Salmonella* (WT, boiled 10min in PBS) was used as the attractant for BMDM, and 100ng/ml CCL19 (PeproTech, Rocky Hill, NJ) was used for BMDC. Five hours later, the cells were fixed to the membrane, stained with DAPI, and the percentage of cells migrating to the bottom side of the filter was counted by confocal microscopy (at least 300 cells were counted per sample). The percent directed migration was reported as the difference: (% migration toward the added attractant) – (% migration without added attractant). For migration assays independent of *S. typhimurium* infection, murine macrophages (WT and IQGAP1Δ-/+ ) seeded on to transwell filters (8 µm pore size, coated on the underside with fibronectin) were placed over chambers, each containing 600 µl medium with macrophage-colony stimulating factor (100 ng/ml). After 5h of incubation at 37°C, the migrated cells attached to the bottom surface of the transwell filters were stained with Dif-Quick, and the numbers of migrated cells per filter were counted in 10 random fields with an inverted microscope.

In vivo DC migration was measured by staining BMDM with PKH26, and then infecting these cells with *S. typhimurium* SL1344 strains (WT or *AseI*) transformed with the GFP expression vector pFPV25.1 [62]. Infections were carried out as above, except with an MOI of 50:1 such that 49±1.6 % of the cells were infected. Infected cells were incubated in 100µg/ml gentamicin to kill extracellular bacteria, washed and resuspended in PBS at 25 million cells/ml. 129x1/sv J mice were injected IP with 0.2ml of infected WT S. typhimurium (as in the migration assay). The infected BMDM were lysed 2h and 24h after initiating the infection and plated for cfu. The number of intracellular bacteria was recorded as a percent of the input, and each experiment was performed in triplicate.

To measure the loss of cell adherence of infected macrophages, BMDM and RAW264.7 cells were seeded into 6-well plates (5×10^5 cells/well) and infected at an MOI of 10:1 as in the cell migration assay (2 wells/replicate, 3 replicates per sample). Immediately after changing the cell medium to 10µg/ml gentamicin, heat-killed *Salmonella* was added to one half of the wells, and 24h later detached cells were harvested, lysed gently in 1% triton-X100, and plated for cfu. The cfu recovered from detached cells was recorded as the number of cfu in detached cells per well, and the results are reported as the difference: (# cfu in detached cells treated with heat-killed *Salmonella*) – (# cfu in detached cells without heat-killed *Salmonella*).

Adenylate cyclase assay

The coding sequence of the first 399 residues of CyaA (adenylate cyclase domain) from *B. pertussis* was cloned in frame onto the 3’ end of *sst* constructs (omitting the *sst* stop codon), creating *pssl-cya* and *psslC178A-cya*. WT and *AseI* (SPI2 *T3SS* deficient) *S. typhimurium* strains were transformed with *pssl-cya* or *psslC178A-cya* and used to infect RAW264.7 cells seeded in 6-well plates. Infected cells were lysed 6h later by sonication (10 sec at 40%, 6 times, at 4°C) and cleared by centrifugation. After reserving an aliquot of the lysate for protein determination by Bradford assay (Bio-Rad, Hercules, CA), the following reaction buffer was added to the lysates (final concentration: 2mM ATP, 6mM MgCl2, 100µg/mL bovine serum albumin, 0.12 mM CaCl2, and 0.1 µM calmodulin) and immediately assayed for cAMP content using the cAMP EIA kit from Cayman Chemical (Ann Arbor, Mi).

Sequence alignment and statistics

A position iterative (PSI) algorithm of the Blast search engine (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) was used with default BLOSUM62 parameters and a 0.005 threshold to search the
non-redundant protein database for homology to SseI (159–322 aa). Hypothetical proteins from *B. dolosa* and *P. asymbiotica* were identified by scanning unfinished microbial genome databases at NCBI and Sanger Center, respectively. The alignment was prepared using VectorNTI 9 (Invitrogen, Carlsbad, CA) and GeneDoc (ref at http://www.nrbsc.org/gfx/genedoc/gledoc.htm). Error bars represent standard errors of the mean, all results are representative of at least 3 experiments (unless stated otherwise), and all statistics were calculated using either Microsoft Excel or Graphpad Prism 4.

**Supporting Information**

**Figure S1** SseI is not required for intracellular bacterial survival or for *S. typhimurium*-induced host cell death. A and B) WT BMDM (A) or RAW264.7 macrophages (B) were infected with WT (black squares), *Assel* (grey triangles), or *Assel* (white upside down triangles) strains of *S. typhimurium*, and the amount of intracellular bacteria was measured by plating on cFU at the indicated times. C and D) BMDC were infected as in Fig. 4B with the indicated strains (C) or with these strains grown under SPN-inducing conditions (D). Host cell death was measured at 24h (C) or 6h (D) by measuring the leakage of lactate dehydrogenase (LDH).

**Figure S2** Analysis of SseI and IQGAP1 co-localization. A and B) These images were taken directly from Fig. 3A and 3B, respectively; green staining denotes SseI-GFP (A) or GFP (B) and red staining denotes endogenous IQGAP1. Lines were drawn through the lamella and the red and green pixel intensities were measured along these lines from top to bottom using ImageJ. C and D) The plot profiles from each image are shown on the right; green line represents GFP intensities and red line represents IQGAP1-staining intensities (SseI-GFP; C, GFP; D).

**Figure S3** IQGAP1<sup>−/−</sup> BMDC are less motile than WT BMDC. WT and IQGAP1<sup>−/−</sup>- murine macrophages were similarly seeded onto transwells and M-CSF (100 ng/ml) was added to the baso-lateral compartment for 5h. The number of cells that migrated through the filter was counted (cells/field) and is presented as the percent of WT. Ten fields were counted per sample, and the results are presented as the average ± standard deviation of two independent experiments.

**Figure S4** SseI and SseIC178A proteins both can bind IQGAP1. A) Increasing amounts of *E. coli* extracts over-expressing His-tagged SseI proteins (WT and C178A) were incubated with GST or GST-IQGAP1 and then co-precipitated with GSH-agarose resin. Bound proteins were immunoblotted using anti-His antibody as in Fig. 2C.

**References**

2. Gordon MA, Graham SM, Walsh AL, Wilson L, Phiri A, et al. (2008) Epidemics of invasive *Salmonella* enterica serovar enteritidis and *S. enterica* Serovar *S. typhimurium* in *S. typhimurium* induced host cell death. *Assel* (D) BMDC were infected as in Fig. 4B with the indicated strains of *S. typhimurium* or *RAW264.7* macrophages (B) were infected with WT (black squares), *Assel* (grey triangles), or *Assel* (white upside down triangles) strains of *S. typhimurium*, and the amount of intracellular bacteria was measured by plating on cFU at the indicated times. C and D) BMDC were infected as in Fig. 4B with the indicated strains (C) or with these strains grown under SPN-inducing conditions (D). Host cell death was measured at 24h (C) or 6h (D) by measuring the leakage of lactate dehydrogenase (LDH).

**Figure S5** SseI-regulation of cellular composition of the spleen in vivo, DC-mediated T cell proliferation in vitro, and DC surface marker expression. A) The spleens mice were infected as described in Fig. 8C and 8D, and the number of GR-1<sup>+</sup> cells were determined. B-D) The effect of BMDC infected with WT (C), *Assel* (D), or *Assel* (E). *S. typhimurium* on T cell proliferation was measured by co-culturing with *C. jejuni* (moth cytochrome C-reactive) T cells with infected BMDC and 10µg/ml cytochrome C. To detect proliferation, T cells were pre-stained with Carbboxylfluorescein succinimidyl ester (CFSE), and staining was measured by FACS. Each peak indicates one round of cell division; representative histograms are shown, n = 2. E and F) Surface expression of *MHC-II* (E) and B7.2 (F) on DC isolated from the spleens of infected mice was assessed by staining with specific antibodies. G and H) BMDC were infected with the indicated strains of *S. typhimurium* (UVK = ultraviolet radiation-killed *S. typhimurium*), and one day later, the cell surface expression of *CCR7* (G) and *MHC-II* (H) was analyzed by flow cytometry. Representative histograms are shown.

**Acknowledgments**

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**Author Contributions**

Conceived and designed the experiments: LMM GRG CG SG HJ DBS. Performed the experiments: LMM GRG CG SG KP GL HJ ZL. Analyzed the data: LMM GRG CG SG KP GL YC HJ DBS DMM. Contributed reagents/materials/analysis tools: YhC ZL MB DBS. Wrote the paper: LMM DMM.

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**Author Contributions**

Conceived and designed the experiments: LMM GRG CG SG HJ DBS DMM. Performed the experiments: LMM GRG CG SG KP GL HJ ZL. Analyzed the data: LMM GRG CG SG KP GL YC HJ DBS DMM. Contributed reagents/materials/analysis tools: YhC ZL MB DBS. Wrote the paper: LMM DMM.


