SepA Enhances Shigella Invasion of Epithelial Cells by Degrading Alpha-1 Antitrypsin and Producing a Neutrophil Chemoattractant


ABSTRACT Shigella spp. are highly adapted pathogens that cause bacillary dysentery in human and nonhuman primates. An unusual feature of Shigella pathogenesis is that this organism invades the colonic epithelia from the basolateral pole. Therefore, it has evolved the ability to disrupt the intestinal epithelial barrier to reach the basolateral surface. We have shown previously that the secreted serine protease A (SepA), which belongs to the family of serine protease autotransporters of Enterobacteriaceae, is responsible for the initial destabilization of the intestinal epithelial barrier that facilitates Shigella invasion. However, the mechanisms used by SepA to regulate this process remain unknown. To investigate the protein targets cleaved by SepA in the intestinal epithelium, we incubated a sample of homogenized human colon with purified SepA or with a catalytically inactive mutant of this protease. We discovered that SepA targets an array of 18 different proteins, including alpha-1 antitrypsin (AAT), a major circulating serine proteinase inhibitor in humans. In contrast to other serine proteases, SepA cleaved AAT without forming an inhibiting complex, which resulted in the generation of a neutrophil chemoattractant. We demonstrated that the products of the AAT-SepA reaction induce a mild but significant increase in neutrophil transepithelial migration in vitro. Moreover, the presence of AAT during Shigella infection stimulated neutrophil migration and dramatically enhanced the number of bacteria invading the intestinal epithelium in a SepA-dependent manner. We conclude that by cleaving AAT, SepA releases a chemoattractant that promotes neutrophil migration, which in turn disrupts the intestinal epithelial barrier to enable Shigella invasion.

IMPORTANCE Shigella is the second leading cause of diarrheal death globally. In this study, we identified the host protein targets of SepA, Shigella’s major protein secreted in culture. We demonstrated that by cleaving AAT, a serine protease inhibitor important to protect surrounding tissue at inflammatory sites, SepA releases a neutrophil chemoattractant that enhances Shigella invasion. Moreover, SepA degraded AAT without becoming inhibited by the cleaved product, and SepA catalytic activity was enhanced at higher concentrations of AAT. Activation of SepA by an excess of AAT may be physiologically relevant at the early stages of Shigella infection, when the amount of synthesized SepA is very low compared to the concentration of AAT in the intestinal lumen. This observation may also help to explain the adeptness of Shigella infectivity at low dose, despite the requirement of reaching the basolateral side to invade and colonize the colonic epithelium.

KEYWORDS Shigella, alpha-1 antitrypsin, chemotaxis, gut inflammation, neutrophils

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Species from the *Shigella* genus are Gram-negative, highly infective, and primate-restricted pathogens that infect the intestinal colonic epithelium (1). *Shigella* infection, known as shigellosis, results in an acute inflammatory disease of the intestine associated with fever, nausea, anorexia, dehydration, tenesmus, and mucopurulent bloody diarrhea (1). Globally, *Shigella* is responsible for between 80 and 165 million cases of diarrhea, accounting for nearly 600,000 deaths per year (2, 3), constituting the second leading cause of death due to diarrheal disease (4).

Four different *Shigella* species have been defined based on their biochemical, serological, and clinical phenotypes: *S. dysenteriae* (serogroup A), *S. flexneri* (serogroup B), *S. boydii* (serogroup C), and *S. sonnei* (serogroup D) (5). Among them, *S. dysenteriae* is responsible for epidemic disease outbreaks and the most severe form of dysentery, which causes the majority of fatal shigellosis cases (6). On the other hand, *S. flexneri* is the most prevalent worldwide and accounts for most cases of endemic shigellosis in developing countries, affecting mainly children under 5 years of age (1). *Shigella* strains are characterized by possessing a large virulence plasmid containing a 31-kb region (Mxi-Spa locus) shown to be necessary and sufficient for the invasion of epithelial cells and macrophage killing (6). This region harbors genes encoding proteins essential for the assembly and function of a type III secretion system (T3SS), including translocators (IpaB, IpaC, and IpaD), effectors (IpaA, IcsB, IpgB1, and IpgD), and transcriptional activators (VirB and MxiE) (7).

*Shigella* has a distinctive mode of pathogenesis that involves the invasion of colonic epithelial cells through their basolateral pole or submucosa, instead of the apical pole facing the intestinal lumen (8). Therefore, *Shigella* has evolved the ability to become translocated from the intestinal lumen to the submucosa. Two primary mechanisms have been put forward to explain how *Shigella* acquires its basolateral position. The first proposes that *Shigella* undergoes transcytosis of the epithelium via M cells (9). The second mechanism posits that *Shigella* creates a breach in the colonic epithelium to facilitate paracellular transit into the submucosa (10–12). *Shigella* generates this breach directly by disrupting the intercellular junctions (10) or destabilizing the intestinal epithelial barrier (12) or indirectly by stimulating the transepithelial migration of neutrophils from the submucosa to the intestinal lumen, which ultimately opens up intercellular spaces that allow *Shigella* paracellular transit (11).

To more deeply understand the mechanisms underlying *Shigella* pathogenesis, we studied the role of a protease encoded on the virulence plasmid, the secreted serine protease A (SepA), and found that this protein is crucial for the initial destabilization of the epithelial integrity (12). SepA is a member of the superfamily of serine protease autotransporters of Enterobacteriaceae (SPATE), a group of virulence factors widely expressed by enteric pathogens, including *Shigella*, *Escherichia coli*, and *Salmonella* (13). In addition to secreting SepA, *Shigella* secretes two more SPATES encoded on its chromosome: the protein involved in colonization (Pic) and the *Shigella* IgA-like protease homolog (SigA) (13). Uniquely, SepA is the major protein secreted by *S. flexneri* in culture (14) and is also highly secreted during infection (15, 16). We determined that during apical *S. flexneri* infection of colonic epithelium, SepA activates coflin, a major actin polymerization factor (12). Coflin is an actin-binding protein that controls the depolymerization of actin filaments and promotes the opening of tight junctions in the intestinal epithelium, resulting in increased barrier permeability (17, 18). We further showed that SepA-dependent alteration of the epithelial barrier is necessary for *S. flexneri* invasion of epithelial cells and that it is coupled to a strong induction of neutrophil transepithelial migration (12). In this regard, the alteration of the intestinal barrier integrity has also been associated by other groups with the enterotoxin activity of SepA (14, 19).

Despite the fundamental implication of SepA on *Shigella* invasion (12, 14), particularly in the activation of coflin (12), the direct targets of this protease are still unknown. In this study, we identify alpha-1 antitrypsin (AAT), the archetype member of a family of serine proteinase inhibitors (SERPIN) (20), as the first known target of SepA. Additionally, we describe a mechanism whereby the cleavage products of AAT directly drive the transepithelial migration of neutrophils, a process that also facilitates *Shigella* infection (11).
TABLE 1 SepA targets identified by tandem mass spectrometry

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein name (entry name, UniProt)</th>
<th>Function (reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>2,4-Dienoyl-coenzyme A reductase, mitochondrial (DECRT_HUMAN)</td>
<td>Mitochondrial enzyme required for the $\beta$-oxidation of unsaturated fatty acids (86)</td>
</tr>
<tr>
<td>23</td>
<td>Gamma-actin (ACTG_HUMAN)</td>
<td>Globular multifunctional protein that constitutes the monomeric unit of cytoskeletal microfilaments (87)</td>
</tr>
<tr>
<td>1, 2</td>
<td>Alpha actinin-4 (ACTN4_HUMAN)</td>
<td>Cytoskeletal protein that participates in the organization of cell cytoskeleton and is adjacent to adherent junctions (88)</td>
</tr>
<tr>
<td>5, 6</td>
<td>Alpha-1 antitrypsin (A1AT_HUMAN)</td>
<td>Serine protease inhibitor important for the inhibition of neutrophil elastase at inflammatory sites to abate incidental destruction of surrounding tissue and to facilitate tissue repair (49)</td>
</tr>
<tr>
<td>8, 9</td>
<td>Annexin A2 (ANXA2_HUMAN)</td>
<td>Multifunctional Ca$^{2+}$ and phospholipid-binding protein involved in the activation of plasmin, the regulation of membrane dynamic events, and the regulation of inflammatory processes (89)</td>
</tr>
<tr>
<td>21</td>
<td>Anterior gradient protein 2 homolog (AGR2_HUMAN)</td>
<td>Protein disulfide isomerase involved in protein quality control in the endoplasmic reticulum and essential for the production of intestinal mucus (36, 90)</td>
</tr>
<tr>
<td>10</td>
<td>Calreticulin (CALR_HUMAN)</td>
<td>Multifunctional Ca$^{2+}$-binding protein that operates as a chaperone to assist correct protein folding in the endoplasmic reticulum (91)</td>
</tr>
<tr>
<td>20</td>
<td>Cofilin-1 (COF1_HUMAN)</td>
<td>Actin-binding protein that controls the depolymerization of actin filaments and promotes the opening of tight junctions in the intestinal epithelium (17)</td>
</tr>
<tr>
<td>18</td>
<td>Ferritin light chain (FRIL_HUMAN)</td>
<td>Light subunit of the ferritin protein, important for iron homeostasis (92)</td>
</tr>
<tr>
<td>11, 19</td>
<td>Galectin-4 (LEG4_HUMAN)</td>
<td>Beta-galactoside-binding protein implicated in cell proliferation, apoptosis, differentiation, and intercellular adhesion (93)</td>
</tr>
<tr>
<td>17</td>
<td>Heterogeneous nuclear ribonucleoprotein A2/B1 (ROA2_HUMAN)</td>
<td>RNA-binding protein involved in the transcription, splicing, transport, stability, and translation of a large no. of genes (94)</td>
</tr>
<tr>
<td>13</td>
<td>Heterogeneous nuclear ribonucleoprotein A3 (ROA3_HUMAN)</td>
<td>RNA-binding protein that binds the cis-acting response element A2RE and participates in the cytoplasmic trafficking of RNA (95)</td>
</tr>
<tr>
<td>22</td>
<td>Histidine triad nucleotide-binding protein 1 (HINT1_HUMAN)</td>
<td>Phosphoromimidase that regulates the activities of different transcription factors and participates in the regulation of apoptotic pathways (96, 97)</td>
</tr>
<tr>
<td>3, 4</td>
<td>Ras GTPase-activating-like protein IQGAP1 (IQGA1_HUMAN)</td>
<td>Scaffold protein that participates in the modulation of the cytoskeletal architecture, cytokinesis, intracellular signaling, and intercellular interactions (98)</td>
</tr>
<tr>
<td>7</td>
<td>N-Acetyl-o-glucosamine kinase (NAGK_HUMAN)</td>
<td>Sugar kinase necessary to catalyze the conversion of N-acetylglucosamine (GlcNAc) to GlcNAc-6-phosphate, a component that eventually leads to the synthesis of O-/N-glycans and sialic acid (99)</td>
</tr>
<tr>
<td>16</td>
<td>Peroxiredoxin-2 (PRDX2_HUMAN)</td>
<td>Antioxidant enzyme that hydrolyzes hydrogen peroxide molecules to water and protects cells against oxidative damage from reactive oxygen species (ROS) (100)</td>
</tr>
<tr>
<td>15</td>
<td>Protein/nucleic acid deglycase DJ-1 (PARK7_HUMAN)</td>
<td>Redox-sensitive molecular chaperone that inhibits protein aggregation and helps to regulate oxidative stress (101, 102)</td>
</tr>
<tr>
<td>14</td>
<td>Translationally controlled tumor protein (TCTP_HUMAN)</td>
<td>Multifunctional protein involved in cell growth, development, apoptosis, regulation of protein synthesis, DNA repair, immune response, malignant transformation, and tumor reversion (103, 104)</td>
</tr>
</tbody>
</table>

RESULTS

SepA targets a myriad of proteins involved in intestinal epithelial barrier integrity. We have shown previously that SepA facilitates the bacterial transit of *Shigella flexneri* from the apical to the basolateral surface of the epithelium by disrupting the intestinal epithelial barrier (12). To investigate the protein targets cleaved by SepA in the intestinal epithelium, we incubated a sample of homogenized human colon with purified wild-type (WT) SepA or with a catalytically inactive mutant of this protease, S211A SepA. Cleaved proteins were identified using two-dimensional difference in gel electrophoresis (2D-DIGE) with in-gel trypsin digestion of differentially labeled spots and tandem mass spectrometry (MS/MS) characterization of products.

Cy3 and Cy5 were used to label WT SepA-treated and S211A mutant-treated homogenized human colon preparations, respectively (see Fig. S1 in the supplemental material). The image with overlaid Cy3 and Cy5 dyes showed 24 unique spots only in WT SepA-treated homogenized human colon preparations. MS/MS analysis of these unique spots corresponded to 18 different proteins (Table 1), revealing that SepA targets an array of proteins involved in diverse cellular processes, including cytoskeletal organization (gamma-actin, alpha-actinin, and IQGAP1), cell-to-cell interactions (cofilin-1 and galec-4t-4), mucin production (antior gradient protein 2 homolog), iron homeostasis (ferritin light chain), regulation of transcription...
SepA cleaves AAT, escaping the formation of an inhibitory complex. AAT was prominent among the SepA cleavage targets identified (Table 1), and our interest was piqued because AAT is a serpin family serine protease inhibitor that forms complexes with its target protease only after getting cleaved. Thus, we aimed to investigate the potential role of AAT in inhibiting the activity of SepA. We began by validating the result obtained by 2D-DIGE (Table 1) and performed a biochemical analysis in which AAT was incubated with equimolar concentrations of purified WT or S211A SepA for 1 h at 37°C. As shown in Fig. 1A, AAT is cleaved into two fragments of ~50 and 4 kDa only when exposed to WT SepA. The addition of a serine protease inhibitor (phenylmethylsulfonyl fluoride [PMSF]) or incubation with a serine-defective SepA (S211A mutant) protein did not result in AAT cleavage (Fig. 1A). These results suggest that the serine protease activity of SepA specifically accounts for the cleavage of AAT.

Additionally, Edman degradation sequence analysis of the AAT-WT SepA reaction products revealed that SepA proteolytically inactivates AAT by hydrolyzing its Met-358–Ser-359 peptide bond, which results in the production of an ~50-kDa degraded form of the protein and a 36-amino-acid-long (~4.2-kDa) C-terminal peptide. The degradation of AAT is no longer observed when PMSF is added to the reaction mixture or when the proteolytically inactive S211A SepA protein is used instead, demonstrating that this effect is dependent on the proteolytic activity of SepA. (B) SepA can cleave increasing concentrations of AAT without the generation of an inhibitory complex. Arrow, 50-kDa cleaved AAT; arrowhead, 4.2-kDa C-terminal peptide. This image was created with BioRender.

FIG 1 SepA cleaves AAT without generating an inhibitory complex. (A) SDS-PAGE analysis of equimolar reactions between AAT and WT SepA under different conditions. WT SepA proteolytically inactivates AAT by hydrolyzing its Met-358–Ser-359 peptide bond, which results in the production of an ~50-kDa degraded form of the protein and a 36-amino-acid-long (~4.2-kDa) C-terminal peptide. The degradation of AAT is no longer observed when PMSF is added to the reaction mixture or when the proteolytically inactive S211A SepA protein is used instead, demonstrating that this effect is dependent on the proteolytic activity of SepA. (B) SepA can cleave increasing concentrations of AAT without the generation of an inhibitory complex. Arrow, 50-kDa cleaved AAT; arrowhead, 4.2-kDa C-terminal peptide.
Previous reports have demonstrated that inhibitory complexes formed between AAT and serine proteases decay with time and are dissociated in alkaline solutions (25, 26). Thus, we tested WT SepA cleavage of AAT through a range of different pHs (pHs 5 to 9) or incubation times (from 0 to 60 min) but were unable to identify an inhibitory complex between AAT and SepA under any of the conditions tested (Fig. S2). Moreover, maximum cleavage of AAT was determined to be within a pH range of 7.4 and 8.2 (Fig. S2A), and unless otherwise stated, a pH of 7.4 was used for further experiments.

By incubating SepA with increasing amounts of AAT for 1 h, we demonstrated that even a 10-times-higher concentration of AAT in a molar ratio did not promote the formation of an inhibitory complex (Fig. 1B). Instead, this reaction resulted in a higher production of the 4.2-kDa peptide (arrowhead). Besides, the proteolytic activity of SepA was not affected by the presence of an equimolar concentration of AAT (Fig. S3) but was found to be significantly higher when a 5 (5:1, AAT to SepA)- or 10 (10:1, AAT to SepA)-times-higher molar concentration of AAT was used (Fig. S3). Taken together, these results further indicate that AAT behaves as a substrate rather than as an inhibitor of SepA.

**SepA-cleaved AAT induces neutrophil migration.** Prior studies determined that the 4.2-kDa AAT C-terminal peptide (cAAT) generated from the cleavage of AAT by neutrophil elastase (NE) stimulates the migration of neutrophils (24). Since the same 4.2-kDa cAAT peptide was obtained by SepA cleavage of AAT, we reasoned that this product may have the same effect on polymorphonuclear leukocyte (PMN) migration during *Shigella flexneri* infection. To test this notion, we began by assessing the chemotactic activity of the AAT-WT SepA reaction products in our acellular transwell migration model system. Briefly, freshly purified PMNs were applied to the top compartment of acellular collagen-coated transwells, and serial dilutions of the AAT-WT SepA reaction products were added to the bottom of each well. Given that we used the whole product of this reaction, results are presented relative to the initial concentration of AAT in the reaction mixture. As expected, concentrations higher than 0.01 nM AAT induced a significant increase in PMN migration compared to that in untreated media, reaching a maximal migration response at a 100 nM concentration (1.6-fold higher than that of untreated media) (Fig. 2A).

Next, we used our well-characterized *in vitro* PMN transepithelial migration system with polarized T84 cell monolayers to determine the extent to which the products of the AAT-WT SepA reaction were able to stimulate the transepithelial migration of PMNs (27, 28). Under these conditions, we observed chemotactic activity within the AAT-WT SepA reaction products that resulted in a significant induction of PMN migration (10 to 100 nM AAT) (Fig. 2B). This increase in PMN migration was not due to cytotoxic effects, as lactate dehydrogenase (LDH) assays revealed no significant cellular damage to either PMNs or T84 cell monolayers following treatment with AAT-WT SepA products in the range of concentrations used (Fig. S4). In contrast, different concentrations of AAT (0.001 to 0.1 nM) in the absence of WT SepA showed a significant reduction in PMN migration through T84 cell monolayers compared to that in untreated media (Fig. 2C). Hence, such results indicate that the chemotactic activity of AAT is generated only after it becomes cleaved by SepA.

**AAT enhances PMN migration and bacterial invasion during *Shigella flexneri* infection.** Since the AAT-SepA reaction products promoted the migration of PMN across cell monolayers, we inferred that the degradation of luminal AAT by SepA might facilitate *Shigella* invasion by stimulating the migration of PMN across the intestinal epithelium and, as a consequence, that it perturbs the epithelial barrier, making it more permissive to breaches, as previously suggested (11). To examine this possibility, we standardized an infection system adapted from the model reported by Perdomo et al. (11) that enables simultaneous quantification of both PMN migration and bacterial invasion. For this model, we performed an “inverse” measurement of PMN migration by quantifying those PMNs that remained in the basolateral compartment (and subtracted this number from the initial amount of PMNs added to the system). The inverse measurement of PMN migration was validated by establishing that both the traditional and the inverse detection of migrating PMNs showed similar outcomes when epithelial cells were infected with WT *Shigella* or with the avirulent BS103 strains (Fig. S5; described in Materials and Methods). Thus, implementing the “combined PMN migration and *Shigella* invasion” model, we observed that following apical infection of T84 monolayers with WT *Shigella* flexneri
in the presence of AAT at concentrations between 1 and 100 nM, both PMN transepithelial migration (Fig. 3A) and bacterial invasion (Fig. 3B) significantly increased. The highest level of invading *S. flexneri* bacteria was achieved at a 10 nM concentration of AAT (Fig. 3B), and it was nearly three times higher than the number of bacteria recovered in the absence of AAT.

To control for the effect of PMNs on *S. flexneri* invasion, we also performed experiments in the absence of PMNs. As shown in Fig. 3C, our results corroborated the results of Perdomo et al., showing that bacterial invasion was enhanced when PMNs were
added to the system (WT = 52.93 ± 4.33 versus WT plus PMNs = 100 ± 11.69%, P = 0.0079, as determined by the Mann-Whitney U test [MWUT]). Moreover, when PMNs and a 10 nM concentration of AAT were present during *S. flexneri* infection, we observed a 6.5-fold increase in the number of bacteria invading epithelial cells (WT = 346 ± 25.48% versus WT + PMNs + AAT = 526 ± 43.2%, P = 0.0079, MWUT). On the other hand, when cells were infected in the presence of 100 nM *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), despite the high increase in migration stimulated by this chemoattractant, there was only a

FIG 3  The presence of AAT during *Shigella* infection facilitates PMN migration and enhances the invasion of epithelial cells. (A and B) AAT in concentrations between 1 and 100 nM significantly increased PMN migration (A) and bacterial invasion (B) in comparison to those in cells infected in the absence of AAT. (C) The addition of PMNs and 10 nM AAT during *Shigella* infection led to a 6.5-fold increase in the number of bacteria invading epithelial cells. In comparison, the addition of PMNs alone doubled the number of invading bacteria. The data are expressed as means ± SE from five replicates for all conditions tested. Asterisks denote P values of <0.05 (*) and <0.01 (***) as determined by a 2-tailed nonparametric Mann-Whitney U test. This image was created with BioRender.
mild and not significant increase in bacterial invasion (Fig. S6). Thus, despite the modest level of PMN migration induced by the presence of AAT, it is sufficient and appropriately tuned to facilitate Shigella invasion.

**SepA is sufficient to enhance neutrophil migration and Shigella invasion in the presence of AAT.** To further assess the role of SepA during *S. flexneri* infection in the presence of AAT, we used our combined PMN migration and Shigella invasion model to examine an isogenic mutant strain lacking the gene encoding SepA (ΔsepA), as well as a ΔsepA mutant complemented with an inducible plasmid to express *sepA* (pZK15). Following infection with these strains, we observed that the presence of AAT induced a significant increase in PMN migration and bacterial invasion only in those monolayers infected with *sepA*-harboring strains (Fig. 4A and B). Given that no significant changes were observed when epithelial cells were infected with a ΔsepA mutant in the presence or absence of AAT, these results demonstrate that SepA is essential for the increase in PMN migration and bacterial invasion induced by AAT during *Shigella* infection. Yet, we did note a consistently mild but not significant rise in PMN migration when cell monolayers were infected with the ΔsepA strain in the presence of AAT, leaving open the possibility that other serine proteases secreted by *Shigella* might play a supportive role by degrading AAT and increasing the production of cAAT. Unexpectedly, by using the combined PMN migration and Shigella invasion model in the absence of AAT, we were not able to detect a significant reduction in PMN migration when epithelial monolayers were infected with the *sepA* mutant in comparison to that of cells infected with WT *S. flexneri* (WT = 100 ± 9.84% versus ΔsepA mutant = 58.7 ± 13.3%, *P* = 0.0571, MWUT), as previously reported (12). However, such differences became evident and significant when AAT was present (WT plus AAT = 212.1 ± 6.29% versus ΔsepA mutant plus AAT = 99.98 ± 12.09%, *P* = 0.0286, MWUT). Moreover, infection with the ΔsepA strain resulted in reduced bacterial invasion both in the absence (WT = 100 ± 6.58% versus ΔsepA mutant = 55.56 ± 13.53%, *P* = 0.0286, MWUT) and in the presence (WT = 268.6 ± 49.93% versus ΔsepA mutant = 68.52 ± 10.2%, *P* = 0.0286, MWUT) of AAT.

Next, we reasoned that besides generating a PMN chemoattractant, the degradation of AAT might hinder the inhibition of other serine proteases important for *Shigella* pathogenesis. Thus, we analyzed the effect of AAT on the proteolytic activities of Pic and SigA, as well as its potential to form inhibitory complexes with both proteases. For Pic, we did not detect the formation of an inhibitory complex with AAT (Fig. 5A). However, when AAT was exposed to a 10-fold molar excess of Pic (1:10 AAT to Pic, mol/mol) we observed a small amount of cleaved protein (arrowhead) (Fig. 5A). Of note, a band of ~100 kDa (β) was detected at high concentrations of Pic, which corresponds to a breakdown product of Pic, an observation that has been reported for other SPATES (29–31). Moreover, no significant inhibition of
Pic's proteolytic activity was detected in the presence of increasing concentrations of AAT (Fig. S7A). These results suggest that as with SepA, Pic can cleave AAT and escape the formation of an inhibitory complex. However, unlike with SepA, Pic-mediated cleavage of AAT appears to be a rather inefficient process. Concerning SigA, we observed the formation of an ~150-kDa inhibitory complex when the protease was incubated with an equimolar concentration of AAT (Fig. 5B, arrowhead), which became more evident by increasing the concentration of SigA. Similarly to what was observed for Pic, an ~75-kDa breakdown product of SigA (δ) was also detected. What is more, an equimolar concentration of AAT was sufficient to completely inhibit SigA's proteolytic activity (Fig. S7B). All together, these results indicate that SigA cleaves AAT at its P1–P1′ peptide bond and forms a stable complex with the inhibitor; thus, besides SepA, SigA may participate to a minor extent in the generation of cAAT.

Therefore, we evaluated the effect of AAT during an infection with a double mutant of Shigella lacking the genes encoding SepA and SigA (ΔsepA ΔsigA mutant), both of which are relevant for AAT cleavage. By using the ΔsepA ΔsigA strain, we observed that the increases in both PMN migration (Fig. 5C) and bacterial invasion (Fig. 5D) induced by AAT were completely abrogated. Moreover, in the absence of AAT, infection with the S. flexneri ΔsepA ΔsigA double mutant resulted in a significant reduction in PMN migration and bacterial invasion compared to that of monolayers infected with WT S. flexneri. This finding indicates that both serine proteases, SepA and SigA, also participate in those two crucial events of S. flexneri pathogenesis in an AAT-independent manner.

**DISCUSSION**

Previously, we reported that SepA is crucial for Shigella pathogenesis through its ability to disrupt the intestinal epithelial barrier, which facilitates subsequent bacterial transit to the basolateral pole (12). While we demonstrated that this effect was orchestrated by the activation of coflin via LIMK1 downregulation, SepA was not able to directly degrade...
The degradation of SepA targets may play an important role in the colonization of the colonic epithelium by facilitating different stages of Shigella pathogenesis. For instance, the cleavage of galectin-4, a protein suggested to help maintain the integrity of the epithelial barrier (32, 33), may facilitate the opening of intercellular junctions and promote the basolateral translocation of Shigella. The degradation of proteins involved in cytoskeleton dynamics (such as IQGAP1, gamma-actin, and alpha-actinin), may be of relevance for the basolateral invasion of epithelial cells and the invasion of neighboring cells (34, 35). Moreover, the degradation of an anterior gradient protein 2 homolog may result in a reduction of intestinal mucin production (36). Of note, even though we found cofilin to be a cleavage target of SepA in this work (spot 20 in Table 1), we failed to observe such a cleavage event, as described in our prior study (12).

A possible explanation for this apparent discrepancy may be the distinct nature of the experimental setups; the current study employed an extract from homogenized human colon and purified SepA, whereas our prior study used human intestinal epithelial cell monolayers infected with various S. flexneri strains. While beyond the scope of the current report, future studies to address this difference are planned.

Regardless, the central finding from this study is that we identified AAT to be a key target of SepA. AAT is a major circulating serine proteinase inhibitor in humans (37). The inhibitory function of AAT depends on its RCL, which acts as a bait for serine proteases (23). After binding to the RCL of AAT, serine proteases hydrolyze the P1–P1’ peptide bond (M358–S359), forming an acyl-intermediate bond with the backbone carbon of the P1 residue (38). The cleavage of the AAT RCL gives rise to a large-scale structural rearrangement from a stressed to a relaxed conformation, which distorts the protease’s catalytic center, leading to formation of the classic tightly bound serpin inhibitory complex (39).

We found that SepA was able to cleave AAT without the formation of an inhibitory complex, thus evading the potent inhibitory activity characteristic of serpins. This activity is unusual but not unprecedented. The V8 serine protease secreted by Staphylococcus aureus (40) is able to cleave AAT without forming an inhibitory complex. However, while SepA hydrolyzes the P1–P1’ peptide bond in the RCL of AAT, the V8 protease cleaves the Glu354–Ala355 peptide bond, similar to what has been described for cysteine proteases (40, 41). An analogous effect has been described for the degradation of another serpin, alpha-1 antichymotrypsin (ACT), by neutrophil elastase, showing that ACT behaves as a substrate rather than an inhibitor of the enzyme (42, 43). Finally, the SPATE EspPx from enterohemorrhagic E. coli also has been shown to cleave and inactivate AAT without the formation of an inhibitory complex (31). As with SepA, cleavage occurs in the AAT RCL (31).

We observed that high concentrations of AAT induced a significant increase in the proteolytic activity of SepA. A similar phenomenon, termed “activation by an excess of the substrate,” has been reported for other serine proteases, and is thought to result from the binding of a second substrate molecule to a regulatory site of the enzyme (44–48). We, therefore, speculate that the activation of SepA by an excess of AAT may be physiologically relevant during the earlier stages of S. flexneri infection when the amount of synthesized SepA would be very low compared to the concentration of AAT in the intestinal lumen.

AAT is known to exert dual effects depending on its structure (49). In its native form, AAT has anti-inflammatory effects, as it has been observed in the lungs of patients with AAT deficiency after AAT replenishment or in patients with cystic fibrosis and pneumonia after exogenous supplementation with AAT (50–52). Furthermore, in vitro AAT can reduce the production of proinflammatory cytokines in monocytes and inhibit the migration of PMNs (53–58).
However, when AAT is hydrolyzed by the action of serine proteases, a 36-amino-acid peptide is generated from its C-terminal domain (cAAT). This product can stimulate PMN chemotaxis, adhesion, degranulation, and superoxide generation (24, 59, 60). In agreement with these observations, we demonstrate that the degradation products of AAT (generated by the proteolytic activity of SepA) induce a dose-dependent increase in PMN migration through acellular collagen-coated transwells. We also observed that the AAT-SepA reaction products stimulated the transmigration of PMNs from the basolateral to the apical side of epithelial cell monolayers. Maximal induction of neutrophil transepithelial migration was detected at a 10 nM concentration of AAT (initially added to the reaction mixture), which is comparable to the concentration of AAT necessary to promote a maximal chemotactic effect following its degradation by human neutrophil or mouse macrophage elastase (24, 59).

AAT is also an acute-phase protein that is commonly used to determine the leaking of serum proteins into the gut in patients suffering gastrointestinal disorders, including inflammatory bowel diseases or intestinal infections (61). In line with this observation, shigellosis is associated with high concentrations of AAT in the stool (average of 10.9 mg/g [dry weight] of stool) in comparison to what has been reported for healthy subjects (<2 mg/g [dry weight]) (62, 63). This rise in stool AAT is associated with the presence of erythrocytes and is inversely correlated with serum protein concentrations, demonstrating the leakage of plasma proteins in the gut (63–65). Since one of the clinical features of shigellosis is the presence of high numbers of PMNs in the colonic lamina propria and surface epithelium, AAT may be important to protect the intestinal mucosa from the action of PMN serine proteases during *Shigella* infection (66–69).

Despite their being the first line of defense against bacterial infections, the recruitment of PMNs to the intestinal lumen can be advantageous for *Shigella* pathogenesis at early stages of infection by disrupting the intestinal epithelial barrier and loosening the intercellular junctions (10, 11), thus allowing *Shigella* to travel to the basolateral pole, from which it can efficiently invade the epithelium. On the balance of our results, we posit that this effect is further facilitated by the degradation of luminal AAT due to SepA’s proteolytic activity, the sum of which produces a cAAT peptide chemoattractant that enhances PMN migration and the subsequent invasion of epithelial cells at the basolateral surface.

A potential limitation of our *in vitro* migration invasion system is that it does not allow us to investigate whether the degradation products of the AAT-SepA reaction are sufficient to induce the initial recruitment of PMNs from the bloodstream to the submucosa. Nevertheless, our data support the concept that cAAT is involved in the generation of a PMN chemoattractant gradient that ultimately directs PMNs to cross the intestinal epithelium from the submucosa to the intestinal lumen, similar to what has been described for another PMN chemoattractant, hepxilin A₁ (28, 70, 71). In this context, we posit that there is a temporal cascade of chemoattractants that support the movement of neutrophils across the intestinal epithelium. Therefore, the participation of cAAT would be preceded by the basolateral secretion of interleukin-8 (IL-8), which drives the recruitment of neutrophils into the submucosa. There is a significant increase in the synthesis and secretion of IL-8 in the intestinal epithelium during infection with *S. flexneri*, and it is also documented that this occurs early after infection (72, 73). In addition, human biopsy specimens collected from patients with shigellosis showed that IL-8 remains high in the acute and convalescent stages of the disease (74). It also appears that multiple T3SS effectors contribute to this process by activating the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway and promoting the secretion of IL-8, including OspB, OspC₁, and OspZ (75–78).

Curiously, in comparing the effect of AAT during *S. flexneri* infection to the action of the prototypical PMN chemoattractant, fMLP, we observed that despite the higher induction of PMN migration over AAT, there was an insignificant increase in bacterial invasion. This result implies that while the induction of PMN migration contributes to pathogenesis, the recruitment of high numbers of PMNs may affect the progression of the infection. Hence, we favor the hypothesis that the more modest chemotactic effect exhibited by the AAT-SepA reaction products better enables bacterial invasion. In other words, a less potent PMN chemoattractant
may dampen inflammation sufficiently to allow for *S. flexneri* invasion of the epithelium, which would be most critical at early stages of infection. Eventually, however, a more robust inflammatory response presumably induced by HXAl (70) likely leads to *Shigella* clearance, since PMNs eliminate the infection within 5 to 7 days in healthy individuals (67). Of note, at this later stage of the infection, the concentration of stool AAT also shows a significant reduction (2.2 mg/g), most likely due to healing of the epithelial barrier (63).

In a previous study, we demonstrated that SepA facilitates the migration of PMNs and the invasion of epithelial cells by *S. flexneri* in the absence of AAT (12). In the current work, we also detected a pronounced trend in the reduction (P = 0.0571) of PMN migration after infecting T84 cells in the absence of AAT with the Δ*sepA* strain compared to WT *Shigella*. We believe that this trend approached but did not reach significance because of the modifications made to the infection model. For instance, since PMNs were present at the time of the infection in the combined PMN migration and *Shigella* invasion model, an inverse measurement of PMN migration was performed to avoid the interference of bacteria in the solution used for quantification. Unlike with the PMN migration results, we did observe a significant reduction in bacterial invasion when T84 cell monolayers were infected with the Δ*sepA* strain in comparison to that with WT *Shigella*. However, further optimization of the model system is required to rule out the possibility that SepA has AAT-dependent and AAT-independent effects in PMN transmigration and bacterial invasion.

By cleaving AAT without forming an inhibitory complex, SepA may also impact other serine proteases essential for *Shigella* pathogenesis. In this regard, our results revealed that SigA is inhibited by an equimolar concentration of AAT. This serine protease has been described to mediate the accumulation of fluid in rabbit ileal loops and to elicit a cytopathic effect in nonconfluent epithelial cells by degrading fodrin (79, 80). These effects are similar to those reported for Pet and EspC, two other SPATES secreted by enteropathogenic *Escherichia coli* strains, respectively (13). Despite the presence of high anti-SigA titers detected following *Shigella* infection, the precise role of this protease remains unknown (80). Still, it is possible that in addition to SigA, other serine proteases crucial for *Shigella* pathogenesis may become inactivated by AAT, further underscoring the importance of SepA, and future studies will investigate this notion.

In sum, we described for the first time that AAT is a key target of SepA. Functionally, by cleaving AAT, SepA releases a 42-kDa peptide that promotes neutrophil migration, which in turn disrupts the intestinal epithelial barrier to enable *Shigella* invasion (Fig. 6). These results are consistent with the hypothesis that PMN migration facilitates *Shigella* invasion by loosening the intercellular junctions of intestinal epithelial cells, which allows the bacteria to travel to the basolateral pole (11).  

**MATERIALS AND METHODS**

**Bacterial strains.** A total of four plasmids and 10 *S. flexneri* strains were used in this study (see Table S1 in the supplemental material). Both the 2457T Δ*sepA* and 2457T Δ*sepA* plus p2ZK15 strains were obtained from the Maurelli laboratory (14). Plasmid pS211A, encoding a catalytically inactive serine protease, SepA, with a serine 211-to-alanine point mutation (S211A), was previously described (12). The lambda red recombination system was used as previously described (81) to generate Δ*sepA* and BS103 derivative mutants lacking the gene encoding SigA (sigA), without affecting any upstream or downstream genes. The BS103 ΔsigA strain was further modified to delete the gene encoding Pic (pic) to generate the double mutant strain BS103 Δ*pic* Δ*sigA*. PCR primers used to amplify the kanamycin resistance cassette for lambda red recombination and to identify each *S. flexneri* derivative are shown in Table S2. Bacterial cultures were stored in 20% glycerol at −80°C. Bacteria were streaked onto tryptic soy agar plates with 0.2% Congo red, and plates were incubated at 37°C for 18 h. For infections, one colony was selected from a fresh plate and was incubated overnight in tryptic soy broth, with ampicillin (100 μg/ml) and IPTG (isopropyl-β-d-thiogalactopyranoside; 0.5 mM) when required. Approximately 14 h later, bacterial cultures were diluted 1:100 in fresh tryptic soy broth and incubated for 2 1/2 h, again, with ampicillin and IPTG when required. Subsequently, bacteria were prepared by washing them once with Hank’s balanced salt solution (HBSS; Thermo Fisher Scientific) and resuspending them in the same buffer. It should be noted that we detected the expression of SepA in the Δ*sepA* plus p2ZK15 strain even in the absence of IPTG. SepA expression might be driven by the *sepA* promoter located within the 259-nucleotide sequence upstream of *sepA* start codon, which was included in the p2ZK15 plasmid when *sepA* was originally cloned.

**Pic, SepA, and SigA protein purification.** Genes encoding full-length Pic and SigA were amplified using the Expand long-template PCR system from Roche (catalog no. 11681842001). PCR amplicons
containing pic and sigA were cloned into pUC19 to generate pPic1 and pSigA3, respectively. BS103 Δpic ΔsigA was transformed with plasmids encoding SepA (pZK15), inactive SepA (pS211A), Pic (pPic1), or SigA (pSigA3). Each strain was grown in tryptic soy broth at 37°C with shaking (200 rpm) to an optical density at 600 nm (OD600) of 0.6. Then, bacteria were induced with IPTG (0.5 mM) for 30°C with shaking. Bacterial cultures were centrifuged at 5,000 g for 20 min at 4°C. Supernatants were collected, filtered through a 0.2-μm polyethersulfone filter (ThermoFisher; 569-0020), and concentrated 1,000-fold using an Amicon stirred-cell 400-ml filter unit (Millipore; UFSC40001) with a 30-kDa-cutoff ultrafiltration disc (Millipore; PLTK07610). The concentrated supernatant was then loaded onto a HiLoad 16/600 Superdex 200 pg gel filtration column (Cytiva; 28989335) previously equilibrated with phosphate-buffered saline (PBS). Fractions corresponding to the passenger domain of each protein were concentrated to 10 mg/ml.

Tissue homogenization and 2D-DIGE. In order to discover SepA protein cleavage targets, a human colon tissue homogenate was prepared and incubated with SepA and cleavage products were detected by two-dimensional difference gel electrophoresis (2D-DIGE) and tandem mass spectrometry (MS/MS). Specifically, a section of fresh healthy human colon measuring approximately 2 cm by 2 cm was obtained as a discarded surgical by-product from the University of Massachusetts Medical School Cancer Center Tissue Bank (internal review board [IRB] docket no. H1173). This tissue was subjected to homogenization by 20 strokes using a Dounce tissue homogenizer, on ice. The sheared tissue was centrifuged at 15,000 g for 30 min at 4°C, and the supernatant was subsequently incubated either with purified wild-type (WT) SepA or with the protease-inactive S211A SepA-treated homogenized human colon preparations, respectively, and differences in migration were sought using 2-dimensional gel electrophoresis.

AAT incubation with SepA, Pic, and SigA. AAT (Sigma-Aldrich; A9024) was incubated with SepA, Pic, or SigA in PBS at 37°C for different periods of time, and the cleavage reactions were stopped by adding a 10-fold molar excess of PMSF. Reaction products were separated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 4 to 20% precast polyacrylamide gels (Bio-Rad; 4561093) and silver stained (Bio-Rad; 1610449).

Edman degradation. Edman degradation analysis was performed at the Molecular Structure Facility (via Science Exchange) by following standard procedures.

SepA, Pic, and SigA proteolytic activity assays. As described previously (82), the synthetic substrate N-succinyl-Ala-Ala-Pro-Phε-p-nitroanilide (Suc-AAPF-pNA; S7388; Sigma-Aldrich) was used to evaluate the proteolytic activity of SepA. N-Succinyl-Ala-Ala-Pro-Abu-p-nitroanilide (Suc-AAP-Abu-pNA, SAP-3667-Pi; Peptides International), a synthetic substrate previously shown to be efficiently cleaved by other SPATES (29), was used to analyze the proteolytic activities of Pic and SigA.

Isolation of PMNs. PMNs were isolated from whole blood obtained from normal healthy human volunteers by venipuncture as described previously (12, 83).
Tissue culture. T84 human intestinal epithelial cells were maintained in Dulbecco’s modified Eagle’s medium-F-12 supplemented with HEPES (15 mM; pH 7.5) and 10% fetal bovine serum. To obtain polarized monolayers, cells were grown in an inverted fashion on collagen-coated permeable polycarbonate transwells (Corning; 3388) and incubated at 37°C with 5% CO₂, as described previously (12, 27, 28). Monolayers were used when cellular confluence and differentiation were achieved, usually after 7 to 8 days of inoculation and with the transepithelial electrical resistance of ≥1,200 Ω/cm². All tissue culture reagents were obtained from Invitrogen.

In vitro PMN migration assay. A reaction mixture containing AAT (10 μM) and SepA (1 μM) was incubated in PBS at 37°C for 4 h and stopped by adding a 10-fold molar excess of PMSF. Logarithmic dilutions of this reaction mixture, corresponding to concentrations between 0.001 and 1,000 nM AAT initially added to the solution, were made and placed in plastic wells to evaluate their chemotactic activity. As described before, acellular collagen-coated transwells (84) or confluent T84 cell monolayers grown on transwells in an inverted fashion (12, 27, 28) were placed in wells containing different dilutions of the AAT-SepA reaction products. Freshly isolated human PMNs were then added to the upper compartment of each transwell. For acellular or transepithelial assays, samples were incubated at 37°C for 2 or 3 h, respectively. The number of migrated PMNs was determined via a colorimetric peroxidase assay, as previously described (12, 84). A 100 nM solution of fMLP and untreated medium (HBSS⁺) were also included as positive and negative controls of PMN migration, respectively.

Combined PMN migration and in vitro Shigella invasion experiments. Confluent T84 cell monolayers grown on transwells were apically infected with 5 × 10⁸ bacteria and incubated at 4°C for 30 min. Thereafter, the monolayers were transferred to wells with different concentrations of AAT in the lower (apical) compartment. Freshly isolated human PMNs were next added to the upper (basolateral) surface, and the transwells were incubated for 4.5 h at 37°C. PMNs remaining in the upper compartment of the transwells were recovered and counted via a colorimetric peroxidase assay. Transwells were further incubated in gentamicin (100 μg/ml) in both compartments for 3 h before the cells were washed with fresh HBSS⁺ and lysed with Triton X-100 (1%), as previously described (12). Gentamicin is an antibiotic that does not permeate eukaryotic plasma membranes and is therefore used to maintain only invading viable bacteria while removing extracellular populations of bacteria. To determine the number of invading bacteria, serial dilutions of each infected epithelial cell monolayer were performed and plated onto tryptic soy agar plates with 0.2% Congo red (27). Similarly, serial dilutions from the original bacterial inoculum used to infect the monolayers were also carried out. The number of CFU per condition was standardized by dividing the number of CFU obtained from the monolayers by the number of CFU in the inoculum. Results were normalized to values obtained from monolayers infected with WT S. flexneri (100%). Due to the high number of bacteria in the apical side, we performed an inverse measurement of PMN migration by subtracting the number of PMNs that remained in the upper compartment of the transwells from the initial amount added to the system. This method was validated by performing migration experiments with epithelial cell monolayers infected for 3 h at 37°C with WT S. flexneri or the avirulent, noninvasive BS103 strain. Following apical infection, epithelial cell monolayers were washed three times with fresh medium, PMNs were added to the basolateral surface, and they were incubated at 37°C for 3 more hours. Thereafter, the number of PMNs detected in both the apical and basolateral compartments were quantified, and the results were used to compare the traditional and inverse measurements of PMN migration (Fig. S3).

Cytotoxicity assays. The cytotoxic effects of AAT or the products of the AAT-SepA reaction were determined by the release of lactate dehydrogenase (LDH) from T84 cell monolayers or PMNs. T84 cell monolayers grown on 24-well plates or freshly isolated PMNs were incubated for 3 h in media containing different concentrations of AAT or the products of the AAT-SepA reaction. The supernatants were collected and used to evaluate the amounts of LDH released by using the LDH-Glo cytotoxicity assay (Promega; J2381) according to the manufacturer’s instructions.

Statistical analysis. Data are shown as means ± standard errors (SE). All statistical analyses were performed using GraphPad Prism 5 software. A 2-tailed nonparametric Mann-Whitney U test (MWUT) was used to calculate significant differences between treatment conditions. Asterisks are used to denote P values as follows: *, <0.05, and **, <0.01.

Study approval. Human colon collection was approved by the IRB of the University of Massachusetts Medical School Cancer Center Tissue Bank (IRB docket no. H1173). All neutrophil-based studies were approved by the IRB of the University of Massachusetts Medical School (protocol 13006). All subjects provided informed consent prior to participation in these studies.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 1.1 MB.
FIG S2, TIF file, 0.7 MB.
FIG S3, TIF file, 0.2 MB.
FIG S4, TIF file, 0.3 MB.
FIG S5, TIF file, 0.2 MB.
FIG S6, TIF file, 0.2 MB.
FIG S7, TIF file, 0.3 MB.
TABLE S1, DOCX file, 0.01 MB.
TABLE S2, DOCX file, 0.01 MB.
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B.A.M., J.R.B., M.M.-S., and A.M.-C. conceived the original idea. B.A.M., M.M.-S., J.R.B., A.M.-C., C.M., and L.J.S. contributed to study design. M.M.-S. and J.R.B. performed all the experiments. B.A.M., M.M.-S., A.M.-C., L.J.S., and J.R.B. wrote the manuscript. All the authors discussed the results, reviewed the final manuscript, and approved the submitted version. B.A.M. supervised the project.

Beth A. McCormick is a coinventor on a patent application (85). She, along with her academic institution, stand to gain financially through potential commercialization outcomes resulting from activities associated with the licensing of that intellectual property. The remaining authors do not have any form of interest to disclose.

REFERENCES


SepA Cleaves AAT and Facilitates Shigella Invasion


