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How Do the Virulence Factors of Shigella Work Together to Cause Disease?

Emily Mattock and Ariel J. Blocker*

Faculty of Biomedical Sciences, Schools of Cellular and Molecular Medicine and Biochemistry, University of Bristol, Bristol, UK

Shigella is the major cause of bacillary dysentery worldwide. It is divided into four species, named S. flexneri, S. sonnei, S. dysenteriae, and S. boydii, which are distinct genomically and in their ability to cause disease. Shigellosis, the clinical presentation of Shigella infection, is characterized by watery diarrhea, abdominal cramps, and fever. Shigella’s ability to cause disease has been attributed to virulence factors, which are encoded on chromosomal pathogenicity islands and the virulence plasmid. However, information on these virulence factors is not often brought together to create a detailed picture of infection, and how this translates into shigellosis symptoms. Firstly, Shigella secretes virulence factors that induce severe inflammation and mediate enterotoxic effects on the colon, producing the classic watery diarrhea seen early in infection. Secondly, Shigella injects virulence effectors into epithelial cells via its Type III Secretion System to subvert the host cell structure and function. This allows invasion of epithelial cells, establishing a replicative niche, and causes erratic destruction of the colonic epithelium. Thirdly, Shigella produces effectors to down-regulate inflammation and the innate immune response. This promotes infection and limits the adaptive immune response, causing the host to remain partially susceptible to re-infection. Combinations of these virulence factors may contribute to the different symptoms and infection capabilities of the diverse Shigella species, in addition to distinct transmission patterns. Further investigation of the dominant species causing disease, using whole-genome sequencing and genotyping, will allow comparison and identification of crucial virulence factors and may contribute to the production of a pan-Shigella vaccine.

Keywords: Shigellosis, Shigella, bacterial pathogenesis, type III secretion system, virulence effectors

INTRODUCTION

Shigella was recognized as the causative agent of bacillary dysentery in 1897 by Kiyoshi Shiga. He determined that it was a Gram negative bacillus, which was capable of fermenting dextrose, but was indole-reaction negative and incapable of producing acid from mannitol (Trofa et al., 1999). Shigella is a non-sporulating, facultative anaerobe. Shigella is also a primate-restricted pathogen, which differentiates it from the other members of the Enterobacteriaceae family in which it is classified.

The Shigella genus is divided into four species: Shigella dysenteriae (serogroup A, 15 serotypes), Shigella flexneri (serogroup B, 19 serotypes), Shigella boydii (serogroup C, 20 serotypes), and
**Shigella sonnei** (serogroup D, 1 serotype). These are divided into multiple serotypes dependent on O-antigen and biochemical differences. Different species are linked to disease in varying geographical locations. *S. dysenteriae* causes severe epidemic disease in less developed countries, *S. flexneri* causes disease in developing countries, *S. boydii* is confined to the Indian subcontinent, and *S. sonnei* occurs in both transitional and developed countries (Levine et al., 2013).

Shigellosis is the clinical presentation of *Shigella* infection. Disease is transmitted through the fecal-oral route, with an infectious dose of only 10–100 organisms (Levine et al., 2013). After 1–4 days, infection is acute, non-systemic and enterically invasive, leading to destruction of the colonic epithelium (detailed in Figure 1). Damage along the colonic epithelium is dramatic but erratic, and leads to the main clinical symptom of diarrhea, containing blood and sometimes mucus, which may be accompanied by abdominal cramps and fever. Further complications, depending on the infecting *Shigella* species and host HLA subtype, include Haemolytic-Uremic Syndrome (HUS) and Post-Reactive Arthritis (WHO, 2005). HUS occurs in 2–7% of *S. dysenteriae* type 1 infections, whereby the Shiga toxin harbored by this species attaches to the endothelium and activates platelets, which adhere to the endothelium and occlude blood vessels leading microangiopathic haemolysis of red blood cells as they squeeze through the restricted blood vessel lumen (O’Loughlin and Robins-Browne, 2001). Symptoms include acute renal failure, thrombocytopenia, micro-angiopathic haemolytic anemia, with a 35% fatality rate (Mayer et al., 2012). Post-reactive arthritis is another complication of *Shigella* infection, occurring in 2% of cases, and is characterized by painful joints, painful urination, and irritation of eyes, with chronic arthritis lasting from months to years.

Comparison of the main subtypes of these species by Yang et al. (2005) indicates that each *Shigella* species contains a single circular chromosome and a virulence plasmid. The virulence plasmid has been thoroughly researched in relation to pathogenesis, and the majority of the important virulence factors involved in the *Shigella* life-cycle are localized to a 30 kb region termed the “entry region” (Figure 2). This region contains the *mxi-spa* locus, which encodes the Type III Secretion System (T3SS), and *ipa* and *ipg* genes, which are essential for invasion of epithelial cells and initiation of *Shigella* infection. In addition to the virulence plasmid, distinct regions within the *Shigella* chromosome have also been shown to contribute to infection. These are termed “pathogenicity islands” (PAI) (Table 1), which are unstable transferable elements that can be found in a variety of combinations depending on the *Shigella* species and subtype (Yang et al., 2005). A combination of both chromosomal virulence factors and plasmid virulence factors mediate the *Shigella* life cycle that leads to destruction of the colonic epithelium and disease symptoms.

The T3SS harbored by *Shigella* is pivotal to infection, delivering from the bacterial cytoplasm into the host cell effectors that play a role in cellular invasion, manipulation, and apoptosis (Parrot, 2009). At 37°C the T3SS components are assembled (Figure 3) but secretion of effectors is prevented until the T3SS is activated by contact with the host cell (Veenendaal et al., 2007). These effectors can be classified dependent on the timing of gene expression as either early, middle, or late effectors, and unless stated otherwise, all of the effectors discussed below are encoded on the virulence plasmid and secreted in a T3SS-dependent manner (Table 2).

Dysentery, the main clinical symptom of shigellosis, is due to the infectious cycle of *Shigella* and its ability to penetrate and colonize the colonic epithelium, leading to loss of barrier function and inflammation (Jennison and Verma, 2004). This initial inflammation (Figure 1) is paramount for efficient infection. However, *Shigella* must also overcome this innate immune response and dampen inflammation in order to establish infection, especially in the epithelial cell niche. *Shigella* diminishes the inflammatory response by delivering effectors to inhibit the NFκB and MAPK signaling pathways and epigenetically regulate the repression of pro-inflammatory cytokines such as IL-8 (Ashida et al., 2011). In addition, *Shigella* is capable of downregulating production of antimicrobial...
peptides, including human β-defensin hBD-3, and chemokines, such as CCL20, leading to defective dendritic cell recruitment (Sperandio et al., 2008). This allows for increased replication, efficient infection of neighboring cells, and evasion of the immune response (Figure 1). Eventually, however, the initial inflammatory response which allows for efficient infection consequently leads to Shigella clearance. Polymorphonuclear leukocytes (PMN), such as neutrophils, eliminate the infection within 5–7 days in healthy individuals (Figure 1).

Shigella has evolved to successfully re-infect its host and probably subverts the production of efficient immunological memory to do so. After infection, seroconversion produces protective antibodies against Shigella lipopolysaccharide (LPS), however the antibodies produced are serogroup specific. The diversity of Shigella LPS serotypes means that protection against re-infection is limited to homologous disease, and these LPS-specific antibodies are also short lasting (Cohen et al.,

![FIGURE 2 | Genomic organization of the entry region on plasmid pWR100 (Roerich-Doenitz, 2013). Genes are clustered in two operons, the ipa/ipg and the mxi/spa operon. They are colored in the legend according to their protein class, some of which, such as T3SS effectors, are detailed in the text. Secretion machinery refers to the components that build the T3SS. Translocators are components of the translocon, a pore inserted into the host membrane to allow effector translocation and chaperones are components that stabilize individual effectors prior to secretion from the bacterium. Regulators modulate T3SS expression and function but they are largely beyond the scope of this review. This figure was modified from the virulence plasmid map by Buchrieser et al. (2000).](image)

![FIGURE 3 | Schematic drawing of the Shigella Type III Secretion System (Roerich-Doenitz, 2013). The architecture of Shigella T3SS is based on the virulence plasmid mxi-spa locus (Figure 2), which encodes proteins that produce the T3SS apparatus, a structure composed of a 60 nm hollow extracellular needle, a transmembrane domain, and a cytoplasmic bulb (Blocker et al., 1999).](image)

### TABLE 1 | Genes and protein functions involved in virulence on the Shigella chromosome.

| PAI | Gene(s) | Protein function | References |
|-----|---------|------------------|------------|
| SHI-1 | sigA | Putative enterotoxin | Al-Hasani et al., 2009 |
|      | pic | Intestinal colonization | Navarro-Garcia et al., 2010 |
|      | set1A, set1B | SheET1 enterotoxin | Fasano et al., 1997 |
| SHI-2 | iucA–D | Siderophore, complexes with iron | Vokes et al., 1999 |
|      | iutA | Bacterial receptor for iron-siderophore complex | Vokes et al., 1999 |
|      | shiA–G | Novel ORFS, Shia involved in reduction in host inflammatory response | Ingersoll et al., 2003 |
| SHI-3 | iucA–D | Siderophore, complexes with iron | Purdy and Payne, 2001 |
|      | iutA | Bacterial receptor for iron-siderophore complex | Purdy and Payne, 2001 |
| SHI-O | gtrA, gtrB, gtr | Serotype conversion and O-antigen modification | Allison and Verma, 2000 |
| Sbx-phage P27 | stxB | Shiga toxin | Yang et al., 2005 |
TABLE 2 | Effectors secreted in a T3SS-dependent manner.

| Effectors   | Early/Middle/Late | Enzyme activity | Function Description                                                                 | References                                                                                           |
|-------------|-------------------|-----------------|--------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------|
| IcsB        | Early             |                 | Inhibits autophagy                                                                    | Allaoui et al., 1992; Ambrosi et al., 2014; Baxt and Goldberg, 2014; Huang and Brunelli, 2014; Campbell-Valois et al., 2015 |
| IpaA        | Early             |                 | Actin depolymerization                                                               | Tran Van Nhieu et al., 1997; Bourdet-Sicard et al., 1999; Izard et al., 2006; Ramarao et al., 2007   |
| IpaB        | Early             |                 | Translocon pore formation                                                            | High et al., 1992; Thirumalai et al., 1997; Page et al., 1999; Skoudy et al., 2000; Lafont et al., 2002; Yang et al., 2013; Suzuki et al., 2014b |
| IpaC        | Early             |                 | Translocon pore formation; Actin polymerization; Docking and effector induction.     | Bárzu et al., 1997; Tran Van Nhieu et al., 1999; Osiecki et al., 2001; Terry et al., 2008; Mounier et al., 2009; Du et al., 2016; Russo et al., 2016 |
| IpaD        | Early             |                 | Activation of T3SS                                                                   | Blocker et al., 1999; Arizmendi et al., 2016                                                       |
| IpaH07/22   | Early             | E3 ubiquitin ligase | Ubiquitinates TRAF2                                                                  | Ashida et al., 2013                                                                                    |
| IpaH7.8     | Late              | E3 ubiquitin ligase | Ubiquitinates glomulin                                                               | Suzuki et al., 2014a                                                                                  |
| IpaH9.8     | Late              | E3 ubiquitin ligase | Ubiquitinates U2AF35 and NEMO/IκKγ                                                  | Okuda et al., 2005; Ashida et al., 2010                                                              |
| IpaJ        |                  | Cysteine protease | Cleave ARF1-GTP                                                                     | Burnaevsky et al., 2015; Dobbs et al., 2015                                                          |
| IpgB1       | Early             | GEF             | Activates Rac1 and Cdc42                                                             | Chya et al., 2005; Hachani et al., 2007; Huang et al., 2009                                          |
| IpgB2       | Early             | GEF             | Activates RhoA                                                                       | Hachani et al., 2007; Huang et al., 2009; Klink et al., 2010                                      |
| IpgD        | Early             | Inositol 4-phosphatase | Converts PtdIns(4,5)P2 to PtdIns(5)P                                              | Niebuhr et al., 2000; Mayo and Donner, 2001; Melloux et al., 2014; Garza-Mayers et al., 2015    |
| OspB        | Middle            |                 | Phosphorylation of ERK and p38 MAPK                                                  | Zurawski et al., 2009; Ambrosi et al., 2014; Lu et al., 2015                                       |
| OspC1       | Middle            |                 | Phosphorylation of ERK                                                               | Zurawski et al., 2006                                                                                  |
| OspC3       | Middle            |                 | Binds p19                                                                            | Kobayashi et al., 2013                                                                                |
| OspD3       | Late              |                 | Enterotoxic activity                                                                  | Farfán et al., 2011; Faherty et al., 2016                                                           |
| OspE1/2     | Late              |                 | Adhesive Interacts with ILK                                                           | Miura et al., 2006; Kim et al., 2009; Faherty et al., 2012a                                         |
| OspF        | Middle            | Kinase           | Dephosphorylates MAPKs                                                               | Aribe et al., 2007; Li et al., 2007; Zhu et al., 2007                                               |
| OspG        | Late              |                 | Inhibits SCF-β-TrCP, ubiquitinating IκBα                                            | Kim et al., 2005; Zhou et al., 2013; Grishin et al., 2014; Pruneda et al., 2014                     |
| OspI        | Middle            | Glutamine deamidase | Deamidates UBC13                                                                     | Sanada et al., 2012; Nishide et al., 2013                                                           |
| OspZ1–188   |                   |                 | Phosphorylation of ERK                                                               | Newton et al., 2010                                                                                  |
| OspZ        |                   |                 | Blocks p65 nuclear translocation                                                      | Zurawski et al., 2008; Nadler et al., 2010                                                          |
| VirA        | Middle            | GAP             | Inactivates Rab proteins                                                              | Germane et al., 2008; Dong et al., 2012; Campbell-Valois et al., 2015                              |

The individual virulence factors of Shigella have been compiled and reviewed previously. Here we aim to understand how they collaborate to cause acute enteric destruction, leading to the clinical manifestation of shigellosis. We will analyse the available primary data for the function of Shigella effector proteins and their effect on host cells, and then discuss how these are co-ordinated in time and space to create a detailed picture of Shigella infection, how it leads to disease and manipulates the immune response.

**ANALYSIS**

**Epithelial Barrier Destabilization and Inflammation**

**OspB: Promotes PMN Migration, Inflammation, and Cell Proliferation**

Like most effectors, OspB is found in the four Shigella species, and has homolog in Salmonella species (Zurawski et al., 2009). Although its biochemical function is unknown,
it is thought that OspB plays a role in the activation of extracellular-signal-regulated kinases (ERK) and p38 MAPK pathways, resulting in phosphorylation of phospholipase A2 and the generation of eicosanoids. OspB is capable of nuclear localization for activation of MAPK signaling pathways. This contributes to inflammation and PMN migration, possibly inducing hepxolin A3, an arachidonic acid derivative, and apical secretion of IL-8, a PMN chemoattractant (Ambrosi et al., 2014). An ospB- mutant had a 60% decrease in PMN migration and a 30% decrease in ERK1/2 activation 90 min post-infection when compared to wild-type Shigella (Zurawski et al., 2009). Furthermore, Ambrosi et al. (2014) showed that an ospB- knockout displayed significantly reduced onset and severity of symptoms in the guinea pig keratoconjunctivitis model of infection (Sereny test). However, OspB also activates the master regulator of cell growth mTOR via a direct interaction with the cellular scaffold protein IQGAP1, which also interacts with mTOR activators ERK1/2. This seems to restrict the spread of S. flexneri in cell monolayers, possibly by enhancing cell proliferation in infected foci (Lu et al., 2015).

**OspC1: Promotes PMN Migration and Inflammation**

OspC1 is part of the ospC family. There is 96% identity between ospC2, ospC3, and ospC4, but only 74% identity between these three ospC genes and ospC1 (Buchrieser et al., 2000). This level of similarity may indicate redundancy. However, ospC4 is a pseudogene and different functions have been identified for OspC1 and OspC3 (discussed later). Tagged OspC1 is found throughout the host cytoplasm, localizing primarily to the nucleus (Zurawski et al., 2006). An ospC1- knockout showed a significant decrease in the amount of neutrophil recruitment to the epithelial cells in PMN migration assays, which was restored to wild-type levels on complementation with a plasmid expressing ospC1. Zurawski et al. (2006) showed that this increase in PMN migration correlated with increase in the phosphorylation of ERK1/2 pathways mediated by OspC1. An ospC1- knockout showed a decrease in phosphorylation of ERK1/2 compared to wild-type levels but no reduction in IL-8 secretion. OspC1 plays a role in Shigella virulence in vivo as an ospC1- knockout had reduced amounts of swelling and inflammation in the Sereny test, with clearance of infection after 2 days (Zurawski et al., 2006).

**OspZ: Promotes PMN Migration and Inflammation**

In S. flexneri 2a, an ospZ- knockout has no effect on the Sereny test. However, an ospZ- knockout caused a significant decrease in PMN migration. The knockout also had 63 and 53% ERK1/2 phosphorylation and NFκB activation, respectively, when compared to wild-type S. flexneri (Zurawski et al., 2008). OspZ therefore plays a role in the migration of PMN leukocytes across the epithelial barrier. However, Newton et al. (2010) discovered that S. flexneri species, excluding S. flexneri serotype 6, contain a stop codon at amino acid 188, forming a truncated protein lacking an IDSYMK motif at position 209. The full length OspZ proteins in the remaining Shigella species were found to have an immunosuppressive function through prevention of NFκB activation. Finally, an OspZ homolog, NleE, is found in enteropathogenic Escherichia coli (EPEC), and both NleE and OspZ can substitute for each other (Zurawski et al., 2008).

**Serine Protease Autotransporters of Enterobacteriaceae**

Serine Protease Autotransporters of Enterobacteriaceae (SPATEs) are a family of proteases which catalyse their own secretion via the Type V secretion pathway. Shigella has three known SPATEs, not all of which are found in each species. Their secretion is thermoregulated (37°C) and pH-dependent (Dautin, 2010). They have different proposed activities relevant to intestinal penetration: induction of mucin secretion and cleavage (Pit), destabilization of focal adhesions via cleavage of fodrin (SigA), and, through unknown targets, enterotoxicity, fluid accumulation and epithelial desquamation (SigA and SepA) (Table 3).

**Shigella Enterotoxin 1 and Shigella Enterotoxin 2: Enterotoxic Activity in the Jejunum**

Shigella enterotoxin 1 (ShET1) and Shigella enterotoxin 2 (ShET2) are virulence determinants proposed to mediate early fluid secretion in the jejunum to establish infection in the colon and produce to the characteristic watery diarrhea seen early in shigellosis. The shared name is due to their similar properties as enterotoxins, as there is no homology between ShET1 and ShET2.

ShET1 is encoded by set1A and set1B genes on the Shigella chromosome as part of the SHI-1 PAI, and only present in S. flexneri 2a isolates (Vargas et al., 1999) (Yavorz et al., 2002). The two subunits are proposed to form a holo-AB-type toxin complex in an A1-B5 configuration, producing a 55 kDa complex (Fasano et al., 1995). The holotoxin may follow a secretion mechanism similar to that of the cholera holotoxin, via the Sec pathway and Type II secretion. When ion transport across a cultured epithelium was measured in an Using chamber, a set1AB- knockout had 60% lower $I_{sc}$ (short circuit current) in comparison to wild-type strains (Faherty et al., 2012b). The effect on $I_{sc}$ of ShET1 was also dose-dependent, and washout of ShET1 produced no change in $I_{sc}$, indicating irreversible binding of ShET1 to epithelial receptors (Fasano et al., 1997). However, the set1A and set1B genes overlap with the pic gene but are divergently transcribed. Therefore, the additional pic- knockout may have caused these effects. Faherty et al. (2012b) complemented the pic/set1AB mutant with pic and set1AB individually, showing that pic has a more significant contribution to restoring $I_{sc}$ levels to wild-type, although set1AB complementation also produced a significant increase in $I_{sc}$.

ShET2 is a 63 kDa protein encoded by ospD3 (sen). It is found in all serotypes and one of the three ospD genes found on the virulence plasmid. Sequence alignments between ospD2 and ospD3 show a high degree homology while ospD1 is more divergent. OspD1 has a unique role in regulating type III secretion not shared with OspD2 and OspD3 (Parisot et al., 2005), but redundancy in their effector function(s) is unknown. Unlike ShET1, ShET2 secretion is dependent on the T3SS (Farfán et al., 2011) but how this is regulated unclear (Faherty et al., 2016). The ospD3- (ShET2) knockout has similar...
effects in Using chamber experiments to a set1AB− (ShET1) knockout, with reduced Ic− increase in comparison to the wild-type strain (Nataro et al., 1995). An ospD3− mutant also had a reduction in IL-8 secretion, which could be restored to wild-type levels by ospD3 plasmid complementation, indicating a possible role in IL-8 secretion by epithelial cells (Farfán et al., 2011).

### Adhesion to the Colonic Epithelium at the Basolateral Surface

**Lipopolysaccharide: Glucosylation for T3SS Accessibility**

The lipopolysaccharide (LPS) is a common feature of Gram negative pathogens, triggering the host immune response and inflammatory reactions during infection. LPS modification by glucosylation is thought to contribute to Shigella adhesion and invasion by revealing the T3SS for efficient activation upon contact with the host cell. Guan et al. (1999) showed that glycosyltransferase gtrA− and gtrB− mutants had only a partial conversion of the O-antigen serotype, and a gtrX− mutant had no conversion at all. A mutation in the gtr operon leads to a reduced ability to invade, and this invasion is restored when the gtr operon is reintroduced (West et al., 2005). The reduction in O-antigen length by glucosylation enhances accessibility of the T3SS for contact with the host epithelial cell to initiate invasion.

**IpaB: Binds CD44 at the Basolateral Surface**

IpaB mediates adhesion to the basolateral membrane via interactions with the ubiquitous glycoprotein CD44 (Figure 1, step 7). CD44 is located within lipid microdomain rafts, and is involved in binding of ezrin, radixin, and moesin (ERM) proteins to produce rearrangements of the actin cytoskeleton. IpaB binds the CD44 N-terminal domain with weak affinity but up-regulation of CD44 expression to levels found on lipid microdomains increases binding and internalization of Shigella (Skoudy et al., 2000). Lipid microdomain rafts are found at the basolateral surface and the IpaB-CD44 adhesion interaction may contribute to the polarity of Shigella invasion of epithelial cells (Lafont et al., 2002). Although increased adherence mediated by IpaB-CD44 binding may improve invasion efficiency, this binding alone is not sufficient to induce Shigella entry, as both ipaC− and ipaD− mutants are unable to mediate invasion (Skoudy et al., 2000).

**IcsA (VirG): Polar Adhesion**

IcsA, also referred to as VirG, is a 120 kDa outer membrane protein. IcsA is not dependent on the T3SS for its secretion as it is an autotransporter, with an atypical N-terminal signal sequence mediating secretion via the Sec pathway (Brandon et al., 2003). It is most well-known for its involvement in actin based motility however a more recent function has been described, whereby IcsA is involved in polar adhesion of Shigella to epithelial cells (Brotcke Zumsteg et al., 2014). The adhesion function can be separated from actin-based motility, as an icsA− mutant complemented with a plasmid encoding an adhesion-defective icsA formed plaques similar to wild-type Shigella (Brotcke Zumsteg et al., 2014). The adhesion-defective icsA also produced an attenuated infection phenotype in the Sereny test, indicating the importance of IcsA as an adhesin in Shigella pathogenesis. IcsA-mediated adhesion was present in an ipaBCDA−mxiE− mutant, but not in an ipaD−spa33− strain, indicating that the assembled T3SS, but not the secretion of T3SS effectors, is required for adhesion activity. This initial observation was then linked to T3SS-dependent activation of IcsA to mediate this adhesive phenotype, as the application of the bile salt deoxycholate (DOC) led to an increase in IcsA-dependent adhesion (Brotcke Zumsteg et al., 2014). Deoxycholate has previously been described to bind to IpaD at the T3SS needle tip and induce IpaB recruitment for T3SS activation (Barta et al., 2012). However, DOC is also known to effect LPS molecules, causing them to disperse within the membrane (Shands and Chun, 1980). Assessment of protease accessibility using proteinase K with an ipaD− mutant and after DOC treatment showed that hyperadhesive IcsA was more resistant to degradation, and this was proposed to be due to an alternate conformation of IcsA induced by T3SS activation (Figures 4C, 5D—see below). DOC was proposed to induce Shigella entry, as both ipaC− and ipaD− (see below) mutants are unable to mediate invasion (Skoudy et al., 2000).

### TABLE 3 | SPATEs harbored by Shigella and their function in infection.

| SPATE | Gene location | Putative function | Role in infection | References |
|-------|---------------|-------------------|------------------|------------|
| Pic   | ShI-1 (opposite set1AB) | Cleavage of mucin Mucin secretagogue | Penetrate colonic mucus layer to access epithelium Mucus-containing dysentery in shigellosis | Gutierrez-Jimenez et al., 2008 Navarro-Garcia et al., 2010 |
| SigA  | ShI-1 | Cytopathic activity | Cleavage of fodrin to destabilize links between actin cytoskeleton and membrane proteins, detachment of focal adhesions | Canizalez-Roman and Navarro-Garcia, 2003; Al-Hasani et al., 2009 |
| SepA  | Virulence plasmid | Enterotoxic activity | Fluid accumulation | Benjelloun-Tourni et al., 1995 |

### Lipopolysaccharide: Glucosylation for T3SS Accessibility

- **IpaD:** Produces different cleavage patterns. Changes seen in the ipaD− mutant could occur via an independent regulatory pathway.
whereby activation of the T3SS leads to modulation of LPS structure.

**OpsE1/E2: Bile Salts-Dependent Adhesion**

Faherty et al. (2012a) also noticed that subculture in media containing bile salts significantly enhanced ability of *Shigella* to adhere to the apical surface of polarized epithelial cells. However, microarray expression analysis indicated that the *ospE1/ospE2* genes were induced in the presence of bile, and bile-induced adherence was lost in a Δ*ospE1/ΔospE2* mutant. The OspE1/OspE2 proteins, which are effectors secreted by the T3SS, were also shown to remain localized to the bacterial outer membrane following exposure to bile salts, where they may therefore serve as adhesins.

**Macropinocytic Uptake into Colonic Epithelial Cell**

*IpaD* and *IpaB: Activation of the T3SS*

*IpaD* is part of the *Ipa* family required for *Shigella* invasion, and polymerizes at the T3SS needle tip (Espina et al., 2006). The identification of Class I *ipaD−* mutants, which had premature secretion of effectors, and Class II *ipaD−* mutants, which were non-inducible, shows that *IpaD* has a dual role in the activation of the T3SS (Roehrich-Doenitz et al., 2013). *IpaD* acts as the scaffold protein at the tip of the T3SS needle acting as the display support for *IpaB*, located at the needle tip along with *IpaD* (Veenendaal et al., 2007), and the delivery mechanism of the hydrophobic *IpaB-IpaC* translocation pore (translocon) to host cell membranes (Blocker et al., 1999). From there *IpaD* acts as a signal transducer to activate effector secretion (Figure 4) (Roehrich-Doenitz et al., 2013). *IpaD*, in conjunction with *MxiC*, is also part of the cytoplasmic signal transduction pathway required for full activation of the T3SS and secretion of remaining T3SS effectors (Martinez-Argudo and Blocker, 2010). *IpaB* initially senses the host cell membrane in a manner that is not yet understood and, with *IpaD*, co-transduces this signal down the T3SS needle to activate secretion (Murillo et al., 2016).

*IpaC: Actin Polymerization and Induction of Effector Translocation*

*IpaC* belongs to the group of *Ipa* proteins crucial for translocon formation and cell invasion. The structure of *IpaC* includes an N-terminal signal sequence, a region for association with *IpgC* (cytoplasmic chaperone), a central hydrophobic region for penetration of membranes, and a C-terminal domain for oligomerization (Terry et al., 2008). The hydrophobic region allows *IpaC* to interact with *IpaB* and insert into host membranes, however its topology in the membrane is disputed. The use of anti-*IpaC* monoclonal antibodies has shown that both the N- and C-terminal regions face the host cell cytoplasm (Tran Van Nhieu et al., 1999) although other experiments have found that the central loop is on the cytoplasmic face, with the N- and C-terminal regions being extracellular (Kuwae et al., 2001). *IpaC* insertion into the epithelial cell membrane triggers cytoskeletal rearrangements for the macropinocytic uptake of *Shigella* (Figure 4). Menard et al. (1996) describe how an *IpaB-IpaC* complex on the surface of latex beads is sufficient for engulfment by non-phagocytic cells. However, this has not been reproduced and therefore may not applicable in vivo. It is likely that the *IpaC* C-terminus faces the host cytoplasm because *IpaC* has been attributed the ability to polymerize actin indirectly at its C-terminus, via interactions with Cdc42 and Rac GTPases (Tran Van Nhieu et al., 1999) and activation of Src tyrosine kinase pathway (Mounier et al., 2009). However, the *Salmonella* homolog, *SipC*, can polymerize actin at its C-terminal domain, independently of any host cell factor (Hayward and Koronakis, 1999). *IpaC* displays sequence similarity to *SipC* within its C-terminal activator regulation domain, and both *IpaC* and *SipC* use this domain to oligomerize. *IpaC* may therefore also be capable of polymerizing actin independently. In pull down assays, the C-terminal domain of *IpaC* was incapable of binding to Cdc42 and Rac1, indicating that actin polymerization does not occur through direct interactions with these GTPases (Terry et al., 2008). Finally, the C-terminus of *IpaC* has also been shown to bind vimentin and the intestinal epithelial intermediate filament keratin 18. This interaction is required for stable docking of the bacteria to cells and a prerequisite for induction of secretion of the other effectors (Russo et al., 2016).

**IpgB1 and IpgB2: Actin Remodeling**

*IpgB1* and *IpgB2* share 25% amino acid identity and both require Spa15 as a chaperone for secretion, with an additional requirement for stability by *IpgB1* (Hachani et al., 2007). They contain a WxxxE motif which is common in guanine nucleotide exchange factors (GEFs) involved in the activation of Rho GTPases. These GTPases are required for the induction of actin filament structures to produce membrane ruffles for bacterial entry into non-phagocytic cells. An *ipgB1−* knockout has a 50% reduction in epithelial cell invasion compared to the wild type strain, which was restored when complemented by plasmid expression of *ipgB1* (Ohy et al., 2005). Membrane ruffle size is also affected, as wild-type strains achieved ruffle size of 60 μm², an *ipgB1−* mutant achieved 16 μm², and an *ipgB1−* hyperproducing strain achieved 138 μm² (Ohy et al., 2005). This indicates that *IpgB1* is involved in the production of membrane ruffles in a dose-dependent manner. It was disputed as to whether *IpgB1* activated Rac1 directly (Alto et al., 2006) or whether *IpgB1* mimicked RhoG for activation of Rac1 indirectly via the ELMO-Dock180 pathway (Handa et al., 2007). However, crystal structures of *IpgB1* confirmed that it acts as a GEF, specifically recognizing the β2-3 residues of Cdc42 and Rac1 GTPases to catalyse the GDP-GTP exchange for activation (Huang et al., 2009). Crystal structures and functional studies also confirmed that *IpgB2* is a GEF capable of directly binding and activating RhoA (Huang et al., 2009; Klink et al., 2010). Activation of Rac1 and RhoA by *IpgB1* and *IpgB2* contributes to formation of lamellipodia and actin stress fibers, respectively. In the Sereny test, an *ipgB2−* mutant produced the same disease phenotype as the wild-type strain, and a negative result only occurred in an *ipgB1−* *ipgB2−* mutant, indicating redundancy (Hachani et al., 2007). However, an *ipgB1−* mutant alone produced a more severe
inflammatory phenotype than the wild-type strain, which was unexpected.

**IpaA: Actin Depolymerization**

IpaA is involved in regulating actin protrusions at the epithelial membrane and depolymerization of actin filaments in the host cell during *Shigella* entry. This is postulated to be achieved through its interactions with vinculin, a host protein that links the cytoskeleton to the extracellular matrix and is involved in focal adhesion structures. There are three vinculin binding sites, which are arranged tandemly at the IpaA C-terminus, each of which can bind one vinculin head. Binding of IpaA to the vinculin head induces a conformational change in vinculin, revealing an F-actin binding site in the vinculin tail (Izard et al., 2006). IpaA is important for cell entry, as an *ipaA* mutant has a 10-fold decrease in invasion capacity, and requires vinculin to mediate its effects, as vinculin-deficient cells had a similar invasion defect (Tran Van Nhieu et al., 1997). Pelleting assays showed that no actin depolymerization occurred in the presence of IpaA or vinculin alone, with actin principally in the pellet, but when the IpaA:vinculin complex was added, actin was found mostly in the supernatant, with the amount of depolymerization correlating with vinculin concentration (Bourdet-Sicard et al., 1999). The IpaA:vinculin complex has a 3-fold increased affinity for F-actin compared to vinculin alone and acts as a “leaky cap” on the barbed end of the F-actin filaments to prevent addition of further monomers and cause depolymerization (Bourdet-Sicard et al., 1999; Ramarao et al., 2007). IpaA therefore prevents the uncontrolled formation of IpaC-induced microspike structures at the site of bacterial contact, which would repel *Shigella* from the epithelial cell surface (Tran Van Nhieu et al., 1997).
**IpgD: Membrane Ruffles**

IpgD plays an important role in the formation of bacterial entry structures on contact with host epithelial cells. An *ipgD* mutant induces a less efficient entry structure than the wild type, due to smaller membrane ruffles and a reduction in actin rearrangements (Niebuhr et al., 2000). IpgD functions as an inositol (phosphoinositide) 4-phosphatase, and has sequence motifs similar to mammalian phosphoinositide phosphatases and *Salmonella* homolog SigD with similar inositol phosphate phosphatase activity (Niebuhr et al., 2002). Its main substrate in the host cell is phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂/PtdIP2] which it dephosphorylates to produce phosphatidylinositol 5-monophosphate [PtdIns(5)P/PtdIP5P]. An *ipgD* mutant, or IpgD with a cysteine to serine (C438S) substitution in its active site, has no affect on PtdIns(4,5)P₂ levels (Niebuhr et al., 2002). The dephosphorylation activity of IpgD on PtdIns(4,5)P₂ correlates with a decrease in membrane tether force, as PtdIns(4,5)P₂ controls the adhesion force between the plasma membrane of the epithelial cell and the actin cytoskeleton (Niebuhr et al., 2002). Combined with actin cytoskeleton rearrangements mediated by other *Shigella* effectors, the reduction in membrane tether allows the extension of filopodia and membrane ruffles characteristic of the trigger mechanism seen in *Shigella* entry into non-phagocytic cells (Figure 1, step 7) (Niebuhr et al., 2002). IpgD inositol-4-phosphatase activity has also been implicated in a positive feedback loop involving ARF6 GTPase (Garza-Mayers et al., 2015), which stimulates actin remodeling and membrane ruffles through Rac1 activation. The production of PtdIns(5)P by IpgD activates phosphoinositide 3-kinase (PI3K), which generates PtdIns(3,4,5)P₃. This recruits ARF nucleotide binding site opener (ARNO), a GEF that activates ARF6 GTPase. Active ARF6-GTP promotes actin remodeling through Rac1-dependent pathways, further contributing to membrane ruffles for *Shigella* entry.

**Replication and Spread within Colonic Epithelium**

**IpaB and IpaC: Lysis of Single Membrane Entry Vacuole**

Due to the pre-requisite for invasion of epithelial cells and time coupling between entry and vacuolar lysis (15 min), disruption of components required for entry can seem to have pleotropic effects on downstream infection events. This creates intrinsic limitations in studying their role in intracellular pathogenesis (Guichon et al., 2001). Nonetheless, an "entry region" encoding only the T3SS and IpaD, IpaB, and IpaC has been demonstrated to be sufficient for vacuolar lysis (Figure 1, step 8) (Sansonetti et al., 1986; Du et al., 2016). High et al. (1992) used macrophages, which are naturally phagocytic, to overcome the non-invasive *ipaB* phenotype, and described how IpaB plays a role in lysis of the phagocytic vacuole. It has been postulated that the insertion of the IpaB-IpaC translocon into the vacuolar membrane is the cause of membrane lysis (High et al., 1992). This would occur through pore formation, which could lead to vacuolar destabilization, or through translocation of unidentified membranolytic effector(s) across the membrane (Page et al., 1999). Senerovic et al. (2012) found that purified IpaB internalized into cells oligomerized in endocytic membranes to form ion channels which affected their integrity. However, the IpaB used was purified recombinantly using a detergent, which naturally lysed membranes, and this effect was not controlled for. Moreover, when within the naturally inserted translocon IpaB is connected to the T3SS via the needle tip, which - along with low osmolarity in the vacuole - would prevent the influx of water into the vacuole to cause lysis. Furthermore, by exploring the functional interchangeability of translocon components from *Shigella* and *Salmonella*, which remains in its invasion vacuole, IpaC was shown to be directly involved in lysis of the single membrane vacuole (Osiecki et al., 2001; Du et al., 2016). Yet, any environmental cue for the translocon to switch between possible invasion, translocation, and lysis conformations remains unknown.

**IpgD: Lysis of Single Membrane Entry Vacuole?**

IpgD may be involved in the modulation of the *Shigella*-induced entry vacuole by recruiting Rab11 to macropinosomes. An siRNA screen and Rab11-depleted cells showed that the absence of Rab11, a small GTPase involved in endocytic recycling, leads to decelerated *Shigella*-induced vacuolar rupture. Like the Rab11-depleted cells, vacuolar rupture was delayed in an *ipgD* mutant, taking twice as long as for the wild-type strain (Mellouk et al., 2014). Functionally impaired Rab11 and a GDP-locked dominant negative Rab11 showed that it is the absence of Rab11 activity that causes this delay in vacuolar rupture (Weiner et al., 2016). Immunofluorescence staining co-localized Rab11-positive vesicles at the *Shigella* invasion site and *Shigella*-containing vacuoles, however this accumulation did not occur if an *ipgD* mutant or IpgD lacking its inositol-4-phosphatase activity was present in the vacuole (Mellouk et al., 2014). Using C-FIB/SET, Weiner et al. (2016) then showed that the Rab11-positive vesicles are macropinosomes, which are formed during membrane ruffling induced by *Shigella*. IpgD is involved in macropinosome formation, through its stimulation of ruffling, and hence in making these organelles available to the *Shigella* entry vacuole (Weiner et al., 2016). Macropinosomes are required for efficient entry vacuole rupture and have been visualized close to the entry vacuole, making direct contact just prior to vacuolar rupture (Weiner et al., 2016). This suggests that the phosphoinositide phosphatase activity of IpgD is required to regulate Rab11 recruitment to macropinosomes for attachment to the *Shigella*-containing entry vacuole (Figure 6). How macropinosome attachment, which does not lead to fusion with the vacuole, leads to rapid vacuolar rupture is unknown.

**IcsA (VirG): Actin-Based Motility**

IcsA is an autotransporter and is composed of three domains: An N-terminal signal sequence, a C-terminal β barrel core which forms a pore in the outer membrane, and a central α-domain which is translocated through the β core membrane pore and present at the *Shigella* surface (Suzuki et al., 1995). Surface
exposed IcsA is sometimes cleaved, however this is not required for IcsA function (Fukuda et al., 1995). The importance of IcsA in Shigella intercellular spread was identified early, as an icsA− mutant was unable to spread within an epithelial monolayer (as measured by plaque formation) and had a negative Sereny test (Bernardini et al., 1989). IcsA-mediated actin based motility is sufficient for membrane protrusion formation (Figure 1, step 10) and entry into neighboring cells (Figure 5). IcsA acts as a mimic of Cdc42 to activate N-WASP, which allows the N-WASP C-terminus to recruit Arp2/3 (Egile et al., 1999; Shibata et al., 2002). This promotes rapid F-actin assembly and filament growth at the N-terminus of N-WASP, providing a propulsive force for Shigella to move through the cell. When IcsA is expressed in E. coli, these bacteria are capable of forming membrane protrusions with similar morphology to Shigella-induced protrusions, indicating that no other Shigella factors are required for this process (Monack and Theriot, 2001). Shigella factors, such as IcsP (SopA), are required for correct localization and cleavage of IcsA at the Shigella surface, contributing to efficient motility (Egile et al., 1999). On addition of icsP to icsA-expressing E. coli there was an increase in actin polymerization and increase protrusion frequency. Furthermore, in E. coli LPS O-antigen mutants there was a decrease in formation of actin tails compared to the wild-type. A galU− mutant, which normally encodes a UDP-glucose pyrophosphorylase involved in O-antigen biosynthesis, produces a diffuse circumferential pattern of IcsA on the Shigella surface, which is still capable of polymerizing actin but forms no membrane protrusions (Sandlin et al., 1995). However, it is unknown how the LPS modulates IcsA localization. Therefore, both IcsP and LPS are required for the unipolar localization of IcsA to produce efficient unidirectional movement, which is strongly correlated with frequency of membrane protrusions (Monack and Theriot, 2001).

**IcsB: Inhibition of Autophagy**

IcsB requires the LpgA chaperone for both its stability and its secretion (Ogawa et al., 2003). An icsB− mutant produced plaques with a smaller diameter than the wild type and a negative result in the Sereny test, suggesting a role for IcsB post-invasion. This role is the prevention of autophagy (Figure 5). IcsB prevents autophagic recognition by masking the Atg5 binding site on IcsA, preventing Atg5 from binding and initiating autophagosome formation. IcsB is also capable of recruiting Toca-I to prevent LC3-mediated phagocytosis (Baxt and Goldberg, 2014). IcsB prevents the formation of septin cages, which in turn may prevent the recruitment of ubiquitin (Ub), p62 and NDP52 (Huang and Brumell, 2014). In an icsB− knockout, autophagic double membranes were visualized around the Shigella bacilli, with asymmetric distribution similar to IcsA placement (Ogawa et al., 2005). IcsB, in conjunction with VirA, has also been implicated in lysis of the double membrane entry vacuole after intercellular spread (Figure 1, step 11). Electron microscopy (EM) 3 h after cell infection visualized icsB− mutants remaining trapped in a double membrane, with several bacteria in one vacuole (Allaoui et al., 1992). Galectin-3 has then been used to show that an icsB− mutant has only a 53% disruption of the double membrane vacuole, compared to 70% disruption mediated by the wild type (Campbell-Valois et al., 2015). However, on closer inspection, we think the EM images (Allaoui et al., 1992, Figure 8B) were misinterpreted, and the double membrane interpreted as the secondary entry vacuole was actually a starting autophagosome wrapping around the icsB− mutant unable to inhibit autophagy. Furthermore, galectin-3 can be used to label endomembranes or autophagic membranes, as it interacts with β-galactos-containing glycoconjugates which are present in both endocytic and secretory compartments (Maejima et al., 2013). Therefore, instead of rupturing the secondary entry vacuole IcsB inhibits autophagy of Shigella.

**VirA: Inhibition of Autophagy and Promotion of Golgi Fragmentation**

VirA was initially thought to play a role in Shigella invasion, as a virA− mutant had a 5-fold reduced capacity for invasion (Uchiya et al., 1995). This was linked to its apparent cysteine protease activity and capability for microtubule degradation (Yoshida et al., 2002). However, structural analysis showed that VirA lacks the suggested papain-like protease activity for tubulin cleavage, and instead exhibits homology with EspG, an EPEC effector that fragments the Golgi (Germane et al., 2008). VirA belongs to a family of GTPase-Activating Proteins, which share
the conserved Rab GTPase catalytic Tre-2/Bub2/Cdc16 domain to mediate Rab1 GTP hydrolysis (Dong et al., 2012). Rab1 GTPase is involved in ER-to-Golgi vesicular transport and is crucial in the formation of autophagosomes (Zoppino et al., 2010). VirA stabilizes Rab1 in the inactive GDP state, thereby directly interfering with autophagy induction and ER-to-Golgi trafficking (Figure 6) (Dong et al., 2012; Huang and Brunell, 2014). A virA− mutant leads to reduced Shigella intercellular persistence, but does not greatly reduce Golgi fragmentation, as IpaJ is more potent in fragmenting the Golgi (Figure 6) (Dong et al., 2012; Burnaevskiy et al., 2013). VirA, similarly to IcsB, has been implicated in the disruption of the secondary vacuole after intercellular spread following membrane protrusion formation (Figure 1, step 10) (Campbell-Valois et al., 2015). However, like for IcsB, this may have been misinterpreted. In our view, the evidence indicates that VirA is involved in lysis of the single membrane entry vacuole (Figure 1, step 8). Indeed, Lysosomal Associated Membrane Protein 2 (LAMP2), a marker for lysosomal fusion with the entry vacuole, has been localized by confocal microscopy to entry vacuoles containing a single virA− mutant and a double icsB− virA− mutant (Campbell-Valois et al., 2015, Figures 2B, 3C) (Dong et al., 2012, Figure S3). A single icsB− mutant is capable of escaping the entry vacuole, visualized by actin comet tail formation (Allouei et al., 1992, Figure 8A). Therefore, the lack of escape from a single membrane entry vacuole by the double icsB− virA− mutant (Campbell-Valois et al., 2015, Figure 4) can be attributed to loss of VirA function. To confirm this, EM analysis of a single virA− mutant is required. VirA may mediate vacuolar lysis through an indirect mechanism, whereby its inhibition of endosomal trafficking prevents membrane vesicle fusion to the entry vacuole (Figure 6). This could occur through interactions with multiple Rab GTPases, as in vitro assays have indicated that VirA can bind many Rab proteins involved in ER-to-Golgi traffic and recycling (Dong et al., 2012). The vacuole may consequently lyse as it cannot grow to accommodate Shigella replication. Passive lysis of the vacuole has been shown for Salmonella, whereby a sfa− knockout prevents the recruitment of membrane to the entry vacuole. The vacuole cannot sustain Salmonella replication and subsequently lyases, releasing Salmonella into the epithelial cytoplasm (Beuzón et al., 2000).

**IpaJ: Golgi Fragmentation**

*ipaJ* is encoded downstream of the *ipaBCDA* operon and transcribed divergently to it. But the original *ipaJ*− mutant showed no defect in plaque formation and was Sereny test positive. Therefore, it did not seem to play a crucial role in epithelial invasion or cell-to-cell spread (Buyssse et al., 1997). Structural bioinformatics analysis indicated that IpaJ harbored catalytic residues required for peptide bond hydrolysis, and further experiments identified that IpaJ is a cysteine protease which preferentially cleaves N-myristoylated proteins (Burnaevskiy et al., 2013). Although in vitro studies indicated that IpaJ has a large spectrum of N-myristoylated targets (Burnaevskiy et al., 2015, Figure 1D), in vivo it specifically targets ADP-ribosylation factors (ARF), particularly ARF1 (Burnaevskiy et al., 2015, Figure 4D). ARF1 GTPase is localized to the Golgi membrane and the plasma membrane as it plays a role in ER-to-Golgi transport, including vesicle formation for cargo transport and maintenance of the Golgi (D’Souza-Schorey and Chavrier, 2006). Removal of the myristoyl group from GTP-active ARF1 by IpaJ causes its irreversible release from the Golgi, inhibiting vesicular trafficking (Figure 6). An *ipaJ*− mutant has no effect on the ARFI intracellular pool, but the wild-type strain decreases the amount of ARF1 GTPase bound to the Golgi (Burnaevskiy et al., 2015). Mounier et al. (2012) suggested that IpaB mediates Golgi fragmentation via modulation of the membrane cholesterol concentration, and state that VirA has no obvious effect on disruption. However, the effects of IpaJ were not accounted for, and as the dominant effector in this semi-redundant pair, it is likely that effects attributed to IpaB were actually mediated by IpaJ. The consequences of Golgi disruption by IpaJ are not
fully understood but inhibition of STING relocalization from the endoplasmic reticulum (ER) to the intermediate compartment between ER and Golgi (ERGIC) may be one them (Dobbs et al., 2015). STING is a major sensor of cytoplasmic pathogens through detection of DNA and cyclic dinucleotides, where upon it translocates from the ER to ERGIC and activates of the IFN-I pathway.

OspE1 and OspE2: Promotion of Host Cell Adherence to Basement Membrane

OspE1 and OspE2 are 99% identical, which suggests they may have arisen from a gene duplication event (Buchrieser et al., 2000). They are capable of functioning redundantly, however in *S. sonnei*, ospE1 is a pseudogene (Miura et al., 2006). An ospE2− knockout caused cellular rounding that was not as a result of apoptosis or necrosis, and wild-type phenotype was restored when ospE2 knockouts were complemented with functioning ospE2 encoded on a plasmid (Miura et al., 2006). OspEs are capable of interacting with integrin-linked kinase (ILK), which is found in the membrane of host cells where it reinforces focal adhesions (Kim et al., 2009). The interaction between OspE and ILK reinforces adhesion contacts between the epithelial cell and the basement membrane (Miura et al., 2006). The OspE-ILK complex interferes with focal adhesion disassembly, reducing focal adhesion kinase phosphorylation and increasing surface β1 integrins (Kim et al., 2009). Tagged OspE was visualized at focal adhesions, however it was diffuse in ILK−/− cells, indicating that ILK is required for OspE membrane localization, and this in turn increases the amount of ILK in the host membrane (Miura et al., 2006). An in vivo inoculation model in the distal colon of guinea pigs showed no *Shigella*-induced symptoms when a dual ospE knockout was used, however dual complementation of ospE restored the wild-type phenotype, with both inflammation and hemorrhaging (Kim et al., 2009).

**IpaB: Cell Cycle Arrest**

IpaB has been linked to cell cycle arrest through interactions with Mad2L2, an anaphase promoting complex inhibitor (Iwai et al., 2007). Mad2L2 is involved in promoting entry of epithelial cells into mitosis during G2/M phase by interacting with the Cdh1, an anaphase promoting complex (APC) associated factor involved in preventing mitosis. After mitosis has occurred, Mad2L2 and Cdh1 dissociate and Cdh1 is activated to suppress mitotic cyclins. IpaB interferes with Mad2L2-Cdh1 binding, causing Cdh1 to be constitutively activated (Iwai et al., 2007). Permanent mitotic cyclin suppression by Cdh1 prevents epithelial turnover during *Shigella* infection, promoting more efficient bacterial replication by keeping the cells better attached to the adjacent cells and to the lamina. Cell cycle arrest thereby prevents epithelial cell turnover and allows *Shigella* to establish a better niche for replication. Interactions with Mad2L2 allow IpaB to be translocated into the epithelial nucleus (Iwai et al., 2007). IpaB binds Mad2L2, and this occurs at the same location on IpaB where the IpgC chaperone binds. Introduction of a single amino acid substitution conferring weaker binding of IpaB to Mad2L2 leads to a reduction in colonization of rabbit ileal loops suggesting that IpaB and its interaction with Mad2L2 contributes to more efficient *Shigella* colonization of the epithelium (Iwai et al., 2007, Figure 6B). However, the point mutation could instead have pleotropic effects on IpaB function (see IpaB and IpaC: Lysis of single membrane entry vacuole?) rather than directly affecting Mad2L2 binding.

**Lysis of the Double Membrane Vacuole**

**Vps and VacJ: Proposed ABC Transporter**

The vpsABC operon is found on the *Shigella* chromosome, and consists of VpsA, a possible ATP-Binding Cassette (ABC) transporter protein, and VspB and VspC, proposed transmembrane proteins. Both vpsC− and vspA− knockouts had a defect in plaque formation but were similar to wild-type strains in their capability to invade, indicating that they play a role in intercellular spread (Hong et al., 1998). VacJ is also encoded on the chromosome, and a vacJ− knockout is incapable of escaping into the recipient cell cytoplasm, suggesting that VacJ also plays a role in intercellular spread (Suzuki et al., 1994). Carpenter et al. (2014) describe a Vps/VacJ ABC transporter, which maintains asymmetry of lipids in the outer membrane and in the context of *Shigella* infection is required for lysis of the double membrane vacuole. Transformation of vps/vacJ knockouts with a plasmid expressing pldA, a phospholipase in other Gram negative bacteria, was able to restore the maintenance of outer membrane lipid asymmetry but was unable to lyse the double membrane vacuole, indicating that these two functions of the proposed Vps/VacJ ABC transporter are separate (Carpenter et al., 2014). Another substrate may therefore be transported across the membrane to induce vacuolar lysis, however this is yet to be discovered.

**IpaB and IpaC: Translocon Formation and Lysis of the Double Membrane Vacuole**

Studying intracellular roles of IpaB and IpaC is difficult as their non-invasive mutants have pleotropic effects. However, Page et al. (1999) overcame this issue using a recombinant plasmid with IPTG-inducible lac promoter to regulate expression of IpaB and IpaC. After initial entry, IPTG was removed from the medium, producing *ipaB−* and *ipaC−* phenotypes which have a 3-fold decrease in plaque diameter compared to the wild-type. The inducible *ipaB−* or *ipaC−* mutants both exhibited a defect in lysis of the double membrane vacuole (Figure 1, step 11), with abolished membrane contact formation (Campbell-Valois et al., 2014). Several bacteria were visualized within such vacuoles, indicating that enough time was spent in the vacuole for replication to occur (Page et al., 1999). IpaB is located at the needle tip along with IpaD, from where it senses the host cell surface. *IpaB* and *IpaC* knockouts were complemented with functioning ospE1 and ospE2, respectively, in vivo inoculation model in the distal colon of *Shigella*-infected rabbit ileal loops suggesting that IpaB and its interaction with Mad2L2 contributes to more efficient *Shigella* colonization of the epithelium (Iwai et al., 2007, Figure 6B). However, the point mutation could instead have pleotropic effects on IpaB function (see IpaB and IpaC: Lysis of single membrane entry vacuole?) rather than directly affecting Mad2L2 binding.

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**Modulation of Innate Immune System**

We cover here effectors directly involved in suppressing the innate immune response in epithelial cells and/or macrophages, rather than events up-stream of it discussed above, such as autophagy.

**OspC3: Inhibits Caspase-4-Mediated Inflammatory Cell Death**

OspC3 is part of the osp gene family. An ospC3− mutant has an increased inflammatory cell death when compared to wild-type *Shigella* and ospC1−/ospC2− mutants (Kobayashi et al., 2013). This indicates that OspC3 plays a role in the down-regulation of acute inflammatory cell death, and suggests a lack of redundancy in the ospC family as OspC1 has pro-inflammatory effects. Inflammatory cell death was not abolished with a cytochrome c or caspase-3/caspase-7 inhibitor. However, a caspase-1/caspase-4/caspase-5 inhibitor did reduce cytotoxicity and their activity increased during ospC3− infection, suggesting that OspC3 mediates its activity via one of these caspase pathways (Kobayashi et al., 2013). Tagged OspC3 bound to the caspase-4 p19 subunit in a pull-down assay, and in-frame deletions showed that the terminal 190–484 residues of OspC3 were involved in p19 binding, specifically at a conserved consensus sequence, X1-Y-X2-D-X3 (Kobayashi et al., 2013). Substitution of all five residues with alanine, in addition to substitution of the conserved 450–478 residues in the C-terminal ankyrin region, impaired OspC3 binding to p19 and increased epithelial cytotoxicity (Kobayashi et al., 2013). Incubation of the p19 subunit with increasing concentrations of OspC3 correlated with increasingly impaired p19–p10 binding (Kobayashi et al., 2013). The biochemical function of OspC3 may therefore be interacting with p19 to inhibit caspase-4 activation and prevent inflammatory cell death (Figure 7).

**OspF: Inactivates MAPKs Which Prevents Phosphorylation of Histone H3**

OspF was first described as a dual specific phosphatase (Arbibe et al., 2007), desphosphorylating threonine and tyrosine residues in the MAPK signaling pathway (Figure 7). However, it was determined by Li et al. (2007) through tandem mass spectrometry that OspF instead displays phosphothreonine lyase activity in that it irreversibly desphosphorylates threonine, but not tyrosine, through beta elimination (Zhu et al., 2007). An ospF− mutant has increased PMN recruitment and severity of epithelial destruction in the rabbit ileal loop model when compared to the wild-type strain, indicating a role in down-regulating the immune response to *Shigella* infection (Arbibe et al., 2007). Phosphothreonine lyase activity is also seen in SpvC, a *Salmonella* homolog with 63% amino acid identity with OspF. Antibodies against phospho-amino acids confirmed specific removal of phosphate from

![FIGURE 7 | Shigella modulates the innate immune response in epithelial cells.](image)
threonine which inactivates MAPKs (Mazurkiewicz et al., 2008). OspF has also been attributed pro-inflammatory roles (Zurawski et al., 2006; Reiterer et al., 2011). The identification of accurate in vivo substrates may explain the apparent pro- and anti-inflammatory roles mediated by OspF.

**OspG: Inhibits NFκB Activation**

OspG plays a role in dampening the host immune response, shown by an ospG− mutant exhibiting increased inflammation and destruction of the mucosa in comparison to wild-type Shigella in the rabbit ileal loop model (Kim et al., 2005). OspG has a minimal kinase domain, and its kinase activity requires binding of an E2 ubiquitin conjugating enzyme in conjunction with ubiquitin (Pruneda et al., 2014). Binding of UbcH7−Ub stabilizes OspG and confers an active kinase conformation, increasing kinase activity 20-fold (Grishin et al., 2014). OspG is also capable of binding UbcH5b−Ub, which is a component of the E3 ligating enzyme SCβ−TrCP (Kim et al., 2005). An ospG− mutant exhibits IkBα degradation 20 min post-invasion, whereas in wild-type Shigella this degradation occurs after 60 min (Kim et al., 2005). The exact mechanism of how OspG prevents SCβ−TrCP from ubiquitinating phospho-IκBα is unknown, but the OspG kinase activity is postulated to be involved in the attenuation of NFκB activation (Figure 7) (Zhou et al., 2013).

**OspI: Inhibits NFκB Activation**

OspI functions as a glutamine deamidase, and has been shown to interfere with NFκB activation via the TNF-receptor-associated-factor (TRAF) 6 pathway (Sanada et al., 2012). An ospI− mutant, when compared to wild-type Shigella, leads to increased levels of cytokine mRNA transcripts, increased phosphorylation of IkBα and a 4-fold increase in nuclear translocation of the p65 subunit of NFκB (Sanada et al., 2012). All of these lead to increased NFκB activation and consequently an increase in the host inflammatory response. OspI has a cysteine-histidine-aspartic acid catalytic triad which is crucial for deamidation, as activity was abolished by a cysteine-to-serine substitution (Sanada et al., 2012). A substrate of OspI is UBC13, an E2 ubiquitin conjugating enzyme required for TRAF6-induced NFκB activation, shown by binding of OspI to His-UBC13 during pull-down assays (Nishide et al., 2013). Hydrophobic interactions are important for this binding, and a crystal structure shows a glutamine residue at position 100 on UBC13 is positioned in the OspI active site (Nishide et al., 2013). OspI specifically deamidates Gln100, converting it to glutamic acid and abolishing the E2 ubiquitin conjugating function of UBC13 to prevent activation of the TRAF6-NFκB pathway (Figure 7) (Sanada et al., 2012).

**OspZ: Inhibits NFκB Activation**

As previously described, OspZ has a pro-inflammatory role in some S. flexneri species. In the remaining Shigella species the full length OspZ has an anti-inflammatory role, similar to that of its NleE homolog in EPEC (Newton et al., 2010). Both NleE and OspZ have been shown to block the nuclear translocation of p65, a subunit of NFκB, in response to TNFα and IL-1β (Figure 7). This leads to a reduction in transcription of pro-inflammatory cytokine genes, such as il-8, thereby reducing inflammation during Shigella infection. OspZ and NleE are also capable of inhibiting IκB degradation, further suppressing NFκB activity. Newton et al. (2010) determined that the crucial region for the anti-inflammatory effect of OspZ and NleE was between the amino acids 208–214, and has the sequence IDSYMK. Deletion of this region or single amino acid substitutions for alanine in NleE led to an increase in NFκB-dependent transcription (Newton et al., 2010, Figure 6D). NFκB activity was not abolished, indicating that this region is a binding site rather than an enzyme active site. The precise mechanism of how OspZ inhibits IκB degradation is unknown. However, Nadler et al. (2010) propose that NleE inhibits IKKβ, which is normally responsible for IκB phosphorylation and degradation in response to pro-inflammatory stimuli. OspZ may therefore work by a similar mechanism to prevent NFκB activation.

**IpaH9.8: Inhibits NFκB Response**

IpaH9.8 is a member of the ipaH gene family, and one of four found on the virulence plasmid, which includes ipaH1.4, ipaH2.5, ipaH4.5, and ipaH9.8. IpaH proteins are characterized by an N-terminal leucine-rich repeat (LRR) region and a highly conserved C-terminal region (CTR) which contains a cysteine residue (Suzuki et al., 2014a). The LRR motif is thought to play a role in protein-protein interactions, such as cell adhesion and signaling, while the cysteine residue in the CTR is required for enzyme 3 (E3) ubiquitin ligase activity. An ipaH9.8− mutant has an increased inflammatory phenotype in comparison to wild-type Shigella in the murine lung model, indicating that IpaH9.8 has a role in attenuating inflammation during Shigella infection (Okuda et al., 2005). This is achieved through E3 ubiquitin ligase activity via the CTR of IpaH9.8 (Rohde et al., 2007). The substrates of IpaH9.8 include U2AF35 and NEMO/IKKγ (Okuda et al., 2005; Ashida et al., 2010). Pull down assays confirmed IpaH9.8 and U2AF35 binding, which specifically occurs at the C-terminus of IpaH9.8 and 107–197 residues on U2AF35 (Okuda et al., 2005). Binding of IpaH9.8 to NEMO/IKKγ requires an ABIN-1 (A20 Binding Inhibitor) adaptor, as ABIN-1 knockdown leads to lack of IpaH9.8-induced effect on NEMO levels (Ashida et al., 2010). Ubiquitination of both U2AF35 and NEMO/IKKγ mediated by IpaH9.8 leads to their degradation in a proteasome-dependent manner (Figure 7) (Ashida et al., 2010; Perrett et al., 2011). Seyedarabi et al. (2010) describe how IpaH9.8 domain swapping occurs in response to host cell damage and this leads to dimerization and inactivation of its E3 ubiquitin ligase activity. Shigella may therefore sense the host cell conditions to maintain a suitable environment for its continued proliferation and survival.

**IpaH0722: Inhibits NFκB Activation**

IpaH0722 is encoded on the Shigella chromosome. In an ipaH−null mutant, whereby all seven of the chromosomal ipaH family genes were deleted, there was an increase in the severity of inflammation in the murine lung model in comparison to wild-type Shigella infection (Ashida et al., 2007). When individual ipaH knockouts were examined, it was found that IpaH0722 plays a role in dampening the inflammatory response, as an ipaH0722− knockout had increased levels of IκBα degradation leading to NFκB activation (Ashida et al., 2013). IpaH0722

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is also an E3 ubiquitin ligase, with the conserved CTR and crucial cysteine residue that is found in other IpaH proteins. A cysteine-to-alanine substitution increased NFκB activation, indicating that the E3 ubiquitin ligase activity is crucial for downregulation of NFκB (Ashida et al., 2013). Pull down assays showed that IpaH0722 could bind to TRAF2, however a CTR-truncation was unable to interact with TRAF2, further indicating the importance of IpaH0722 ubiquitin ligase activity (Ashida et al., 2013). IpaH0722 causes increased TRAF2 degradation leading to a reduction in NFκB activity, thereby dampening the host inflammatory response (Figure 7).

**IpaD: Activates Akt/PI3K Signaling Pathway**

IpaD-mediated increase of PtdIns(5)P has been shown to induce Akt phosphorylation through activation of phosphatidylinositol 3-kinase (PI3K) (Mayo and Donner, 2001). An ipdD− knockout has abolished Akt phosphorylation, which also occurs if PtdIns(5)P is sequestered or phosphorylated to PtdIns(4,5)P2 (Pendaries et al., 2006). Reduction in Akt phosphorylation was correlated with an increase in apoptosis and decreased phosphorylation of Mdm2, the negative regulator of p53, by the Akt serine-threonine kinase (Mayo and Donner, 2001). Bergouinioux et al. (2012) showed that an ipdD− mutant had a reduction in early phase Mdm2 phosphorylation, causing a delay in p53 degradation and increased apoptotic phenotype. The *Salmonella* homolog SopB and SigD also have pro-survival functions through interactions with Akt and activation of the PI3K-Akt survival pathway (Steele-mortimer et al., 2000; Knodler et al., 2005).

**Macrophage Vacuolar Rupture and Pyroptosis**

**IpaC: Rupture of Phagocytic Vacuole**

IpaC interacts with IpaB at the tip of the T3SS needle to form a translocon that inserts into lipid membranes (Blocker et al., 1999). An ipaC− mutant has no haemolytic activity and is unable to escape the phagocytic vacuole (Bârzu et al., 1997). The acquisition of the SHI-O PAI means that *Shigella* is capable of modifying its LPS (Figure 8). As humoral immunity is serotype specific, and protection against re-infection is often unsuccessful because of the variety of *Shigella* serotypes. Furthermore, O-antigen of LPS is a carbohydrate, thymus-independent type 1 (TI-1) antigen, which activates B-cells in the absence of helper T-cells (Murphy, 2012). The lack of helper T-cell involvement means that these activated B-cells cannot undergo class switching or develop a memory B-cell response to protect against re-infection. At a low concentration of TI-1 antigens, such as when LPS molecules are released from damaged bacteria, the naïve B-cells are activated due to specific binding of their B-cell receptors to the antigen (i.e., O-antigen of LPS). This induces the production of O-antigen-specific antibodies, which are protective against *Shigella* infection, however they are not long-lasting and are overcome by serotype conversion. A high concentration of TI-1 antigens, like the O-antigen on LPS at the bacterial surface, leads to non-specific polyclonal activation of B cells and the production of non-specific and hence likely non-protective antibodies (Murphy, 2012). Thus, O-antigen behaves as an "immunological decoy" at more than one level, making it a particularly poor choice as a vaccine antigen.

IpaB is localized at the bacterial surface and in discrete aggregates in the macrophage cytoplasm, which suggests that IpaB interacts with caspase-1 after vacuolar lysis rather than being injected into the cytoplasm from the vacuole (Thirimulalai et al., 1997). The IpaB-ICE complex cleaves the precursors of pro-inflammatory cytokines IL-1β and IL-18 to produce mature IL-1β and IL-18, which are released in parallel to the induced pyroptosis (Figure 1, steps 4, 6). IpaB may also promote macrophage pyroptosis by allowing delivery of the T3SS the needle and rod proteins, MxiI and MxiH, into the cytosol. These bind the NAIP family of inflammasome receptors that trigger activation of caspase-1 (Yang et al., 2013; Suzuki et al., 2014). Recent work also suggests that IpaD promotes macrophage apoptosis independent of caspase-1 but via host caspases accompanied by mitochondrial disruption (Arizmendi et al., 2016).

**IpaH7.8: Promotes Macrophage Pyroptosis**

IpaH7.8 is part of the ipaH gene family found on the *Shigella* virulence plasmid. It was suggested that IpaH7.8 had a role in vacuolar lysis as an ipaH7.8− mutant show reduced from escape the phagocytic vacuole (Fernandez-Prada et al., 2000). However, how this mutant strain (PWR700) was made is unclear, and its complementation was poor. Paetzold et al. (2007) then described how an ipaH7.8− knockout was able to escape the phagosome. Therefore, IpaH7.8 has no role in vacuolar escape. Instead, the IpaH7.8 E3 ubiquitin ligase targets glomulin, an inhibitor of inflammasome activation, for ubiquitination leading to glomulin degradation. Macrophage-specific cell death (Figure 1, step 6) is then triggered through activated inflammasomes (Suzuki et al., 2014).

**Modulation of Adaptive Immune System**

LPS—Serotype Conversion and Thymus-Independent T-cell Activation

The production of the SHI-O PAI means that *Shigella* is capable of modifying its LPS (Figure 8). As humoral immunity is serotype specific, and protection against re-infection is often unsuccessful because of the variety of *Shigella* serotypes. Furthermore, O-antigen of LPS is a carbohydrate, thymus-independent type 1 (TI-1) antigen, which activates B-cells in the absence of helper T-cells (Murphy, 2012). The lack of helper T-cell involvement means that these activated B-cells cannot undergo class switching or develop a memory B-cell response to protect against re-infection. At a low concentration of TI-1 antigens, such as when LPS molecules are released from damaged bacteria, the naïve B-cells are activated due to specific binding of their B-cell receptors to the antigen (i.e., O-antigen of LPS). This induces the production of O-antigen-specific antibodies, which are protective against *Shigella* infection, however they are not long-lasting and are overcome by serotype conversion. A high concentration of TI-1 antigens, like the O-antigen on LPS at the bacterial surface, leads to non-specific polyclonal activation of B cells and the production of non-specific and hence likely non-protective antibodies (Murphy, 2012). Thus, O-antigen behaves as an “immunological decoy” at more than one level, making it a particularly poor choice as a vaccine antigen.
IpgD: Interferes with T-Lymphocyte Migration

IpgD is capable of interfering with T-cell migration by dephosphorylating PtdIns(4,5)P₂ (Figure 9). Wild-type Shigella causes a 50% decrease in T-cell migration toward a chemokine, such as CXCL12 (Konradt et al., 2011). Cells transfected with IpgD-GFP and incubated with CXCL12 had no ERM protein localization at the pole, preventing T-cell polarization and migration (Konradt et al., 2011). Mean velocity of T-cells was measured in vivo, with uninfected T-cells exhibiting 9 and 4 µm/min for wild-type Shigella-infected T-cells (Salgado-Pabón et al., 2013). This indicates that Shigella is capable of affecting T-cells in the context of infection, and that this ability is dependent on IpgD. Shigella may prevent T-cell migration and recruitment to areas of infection as they could be primed by CD1 antigen-presenting cells, which are involved in the presentation of lipid antigens, including LPS (Murphy, 2012). This could induce a specific antigen-response as priming of T-cells leads to stimulation of B-cells for isotype switching and immunological memory, therefore it is advantageous to prevent T-cell involvement in the adaptive immune response. Reducing migration of CD8⁺ cytotoxic T-cell may also slow clearance of infected cells from the epithelium.

IpaD: Promotes B-Lymphocyte Apoptosis

To prevent an antibody response to the LPS, specific or non-specific, and production of immunological memory, Shigella also induces apoptosis in B-cells. This is mediated by IpaD in a manner independent of Shigella invasion and effector injection (Nothelfer et al., 2014). Incubation of B-cells with IpaD alone does not induce cell death, and it was deduced that bacterial co-signals work in conjunction with IpaD to mediate B-cell apoptosis (Figure 9) (Nothelfer et al., 2014). When anti-IpaD antibodies are applied to rectal biopsies of shigellosis patients, they are visualized within isolated lymphoid follicles and are contacting B-cells, suggesting that IpaD-induced B-cell apoptosis occurs in vivo (Nothelfer et al., 2014).

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**FIGURE 8 | S. flexneri serotype composition and conversion.** LPS O-antigen modifications include glucosylation (the addition of glucosyl groups), mediated by the gtr operon and the addition of O-acetyl groups, achieved by the O-acetyltransferase protein encoded by the oac gene. This is the basis of serotype conversion, as Shigella begins with serotype Y and the basic O-antigen structure, and further modification produces different serotypes (Allison and Verma, 2000).

**FIGURE 9 | IpgD inhibits T-cell migration and IpaD induces B-cell apoptosis.** (A) The PIP2 concentration at the plasma membrane is responsible for the dynamic interchange between active and inactive conformations of ezrin, radixin, and moesin (ERM) proteins (Konradt et al., 2011). ERM proteins are involved in cell-cortex organization of T-cells, and their active conformations localize at the membrane in response to chemokine stimulation to allow T-cell migration toward chemoattractants (Konradt et al., 2011). In the subcapsular sinus of the lymph node (Salgado-Pabón et al., 2013) Shigella can invade T-cells or inject IpgD via the T3SS into T-cells to reduce PtdIns(4,5)P₂ concentration. This prevents cell polarisation induced by active ERM proteins and T-cell migration to sites of infection (Konradt et al., 2011). (B) Bacterial co-signals increase pro-apoptotic proteins, induce loss of mitochondrial membrane potential (MMP), and also upregulate tlr2 mRNA, leading to increased TLR2 expression on the surface of the cell. IpaD signaling via the TLR2-1 heterodimer leads to up-regulation of FAS-associated death domain (FADD) protein, which ultimately induces B-cell apoptosis (Nothelfer et al., 2014).
DISCUSSION
How Do the Virulence Factors Work Together?
Epithelial Barrier Destabilization and Fluid Secretion Causes Severe Intestinal Inflammation and Diarrhoea

Once ingested, \textit{S. flexneri} 2a delivers ShET1 in the jejunum to elicit fluid secretion (Fasano et al., 1997). Other \textit{Shigella} species that harbor ShET2 require activation of the T3SS for its secretion and this most likely occurs later after contact with the colonic epithelium (Farfán et al., 2011). Pic and SigA, also harbored by \textit{S. flexneri} 2a species, act within the lumen of the colon, on the apical side of the epithelium. Pic degrades the thick mucus layer by its mucinolytic activity to give \textit{Shigella} better access to the epithelium (Gutiérrez-Jimenez et al., 2008). SigA mediates enterotoxic activity similar to ShET1 (Al-Hasani et al., 2000) and may have cytopathic effects to initially destabilize the epithelial barrier (Al-Hasani et al., 2009). SepA causes fluid secretion in \textit{S. flexneri} 5a (Benjelloun-Touimi et al., 1995). \textit{Shigella} traverses the epithelium via M cells, and reaches the basolateral surface of the epithelium. Here the T3SS is activated, and \textit{Shigella} can produce ShET2 to induce further fluid secretion (Nataro et al., 1995). Other T3SS effectors, including OspB (Zurawski et al., 2009; Ambrosi et al., 2014), OspC1 (Zurawski et al., 2006), and OspZ (Zurawski et al., 2008) (in \textit{S. flexneri}) are secreted to phosphorylate and activate MAPK pathways. Consequently, increased apical secretion of the chemotactrant IL-8 causes PMN leukocytes to migrate across the epithelium in a basolateral to apical direction. This destabilizes the epithelial barrier, and the remaining bacteria at the apical surface can access the basolateral surface. \textit{Shigella} that are phagocytosed by macrophages mediate pyroptosis via IpaB, T3SS components and probably LPS, which bind and activates caspase-1 (Thirumalai et al., 1997), and IpaH7.8 E3 ubiquitin ligase, which targets gloulin, an inflammasome inhibitor, for degradation (Suzuki et al., 2014a). Pyroptosis leads to release of pro-inflammatory cytokines IL-1β and IL-18, which further recruit PMN leukocytes and increase inflammation. All of these virulence determinants and effectors are therefore involved in the characteristic inflammation seen in shigellosis, including the watery diarrhea seen in the early stages. In \textit{S. flexneri} 2a infection, mucus and blood may also be present due to the mucin secretagogue activity of Pic, and the cytopathic effects of SigA. Shiga toxin, found only in \textit{S. dysenteriae}, may cause severe bloody dysentery by damaging the vascular endothelium of the colon (Fontaine et al., 1988).

Adhesion and Entry to Epithelium Causes an Epithelial Genotoxic Stress Response

At the basolateral surface, glucosylation of the LPS is induced by an unknown trigger, and allows the T3SS to access the epithelium for activation (West et al., 2005). IpaB at the tip of the T3SS needle complex may bind to CD44 lipid microdomains to maintain contact of the T3SS with the epithelial cell (Skoudy et al., 2000), and IcsA increases polar adhesion (Brotcke Zumsteg et al., 2014). IpaB is inserted into the epithelial membrane, activating IpaD and leading to recruitment of IpaC to the host membrane to form the translocon pore (Blocker et al., 1999). IpaD activation also signals for the T3SS to facilitate early effector translocation through the T3SS needle and into the epithelial cell (Roehrich-Doenitz et al., 2013). IpaC, as one of the immediate effectors accessing the epithelial cell, mediates actin polymerization, either independently or indirectly via Cdc42 and Rac1 (Tran Van Nhieu et al., 1999; Mounier et al., 2009). This initiates membrane ruffles and entry foci, further promoted by IpgB1 and IpgB2, which remodel actin and the host cytoskeleton via their GEF activity (Huang et al., 2009). IpaA, in conjunction with host proteins, facilitates actin depolymerization, interfering with focal adhesions and preventing uncontrolled actin polymerization by IpaC (Tran Van Nhieu et al., 1997; Bourdet-Sicard et al., 1999). IpgD converts PtdIns(4,5)P$_2$ to PtdIns(5)P, which reduces the membrane tether force and further stimulates membrane ruffle formation (Niebuhr et al., 2002). Together these effectors mediate the trigger mechanism for \textit{Shigella} uptake into non-phagocytic epithelial cells. Upon enclosure in the primary entry vacuole, \textit{Shigella} mediates membrane lysis. This involves primarily IpaB and/or IpaC in the translocon pore (Osiecki et al., 2001; Du et al., 2016), and as accessory processes IpgD-mediated recruitment of Rab11 to macropinosomes which make contact with the entry vacuole (Weiner et al., 2016), and/or VirA preventing growth of the vacuole membrane (Mellouk et al., 2014).

Intercellular Spread and Immune Evasion Causes Erratic Epithelial Destruction and Prevention of Immunological Memory

Once inside the epithelial cytoplasm, \textit{Shigella} uses VirA and IpaJ to inactivate mainly Rab1 and ARF6, respectively. This halts ER-to-Golgi traffic, preventing autophagic membrane formation and inducing Golgi fragmentation (Dong et al., 2012; Burnaevskiy et al., 2013, 2015). \textit{Shigella} further prevents autophagy using IcsB to mask the Atg5 binding site on IcsA (Ogawa et al., 2005). Unipolar localization of IcsA at the \textit{Shigella} old pole, possibly achieved by LPS and IcsP, is crucial for efficient unidirectional movement (Monack and Theriot, 2001) and allows \textit{Shigella} to move through the epithelial cytoplasm until it makes contact with the inner surface of the plasma membrane. It then protrudes into the adjacent epithelial cell, forming a secondary entry vacuole consisting of a double membrane. This is subsequently lysed by an unknown mechanism to allow access into the adjacent epithelial cell cytoplasm. Remaining within the epithelial layer is important for renewing the \textit{Shigella} replication niche and also evading immune detection. Evasion of the immune response is achieved by T3SS effectors that inhibit activation pathways of NfkB, including OspG (Kim et al., 2005), OspI (Sanada et al., 2012), OspZ (Newton et al., 2010), IpaH9.8 (Ashida et al., 2010), and IpaH0722 (Ashida et al., 2013). Degradation of the proapoptotic factor p53 is achieved by IpgD (Mayo and Donner, 2001). Inhibition of the innate immune response also prevents development of an adaptive immune response. The movement of \textit{Shigella} through the epithelium and the subsequent necrotic epithelial cell death is the cause of the colonic destruction and abdominal pain in shigellosis patients, contributing to prevention of fluid absorption and dysentery containing blood.
Virulence Determinants in Disease-Causing Species

*S. flexneri* has a relatively stable genome, and acquired the virulence plasmid early in its evolution. *S. flexneri* is capable of persisting in water for several months, similarly to *Vibrio cholera* (Faruque et al., 2002). This may explain its epidemiological prevalence, as access to human hosts for months at a time could facilitate the endemics seen in countries with poor water sanitation. Speculatively, it may cause the most disease as *S. flexneri* 2a harbors the SHI-1 PAI, which encodes Pic, SigA, and ShET1. Pic may confer an advantage for scavenging nutrients, therefore other strains may have a metabolic disadvantage when compared to *S. flexneri* (Henderson et al., 1999; Harrington et al., 2009). The importance of Pic and ShET1 in pathogenesis has also been highlighted by Kotloff et al. (2004). The deletion of *pic* and set1AB, in addition to *ospD3*, in a guanine autotrophic (guaaAB−) background produced an increasingly attenuated vaccine, with none of the 14 volunteers developing diarrhea (Kotloff et al., 2004). The truncated OspZ found in *S. flexneri* has a pro-inflammatory role, compared to its anti-inflammatory in the remaining sub-species, which may confer an inflammatory advantage for initial establishment of infection (Zurawski et al., 2008).

*S. sonnei* causes endemics in industrialized countries. Its emergence was defined by its acquisition of the pINVb plasmid, which harbors genes for the *Plesiomonas shigelloides*-related serotype 17 O-antigen (Shepherd et al., 2000). *P. shigelloides*, similarly to *S. flexneri*, can contaminate and persist in water sources. Infection with *P. shigelloides* from contaminated water may protect human hosts from established *S. sonnei* infection due to immunological memory and cross-reactive antibodies to the identical O-antigen (Sack et al., 1994). This hypothesis could also explain why an improvement in water sanitation causes a decrease in *S. flexneri* infection but an increase in *S. sonnei* infections, as there is no previous infection with *P. shigelloides* from contaminated water to induce a protective immunological memory response. Direct transmission of *S. sonnei* in schools and care settings is also capable of maintaining endemics in communities, without the requirement of an environmental reservoir.

*S. dysenteriae* infection is the most severe, which has been linked to the effects of the Shiga toxin harbored by *S. dysenteriae* type I. There is no known natural reservoir for *S. dysenteriae*, and it causes sporadic epidemics linked to poor hygiene and overcrowding. The Shiga toxin does not play a role in the intracellular infection, but can stimulate the recruitment of PMN leukocytes and damages the colonic vascular endothelium, leading to the characteristic blood-containing dysentery (Fontaine et al., 1988). It has been suggested that, like *Salmonella enterica* serovar Typhi, *S. dysenteriae* can be maintained and transmitted within a community by an asymptomatic carrier. Long term carriers with attenuated symptoms have been previously reported (Levine et al., 1973; Clements et al., 1988), and this may explain the epidemiology of *S. dysenteriae* epidemics, which disappear to then reappear years later, and are also transferred intercontinentally (Rohmer et al., 2014).

*S. boydii* causes the least burden of disease worldwide. It represents 1–2% of *Shigella* isolated, and is mostly confined to the Indian subcontinent. In 2000, *S. boydii* serotype 20 was discovered. Its transmission was linked to travel in Mexico (Kalluir et al., 2004) and it was the most frequent agent of *S. boydii* infection in Canada (Woodward et al., 2005). However, *S. boydii* still only caused 1% of *Shigella* infections in the United States compared to the 77% of infections caused by *S. sonnei* (Kalluir et al., 2004). Due to the diversity of *S. boydii* serotypes, and its lack of clinical relevance there is little known about its virulence determinants and why it causes less disease than *S. flexneri*, *S. sonnei*, and *S. dysenteriae*.

Effectors Interplay

Multiple Effectors for One Target Pathway

Effector-Triggered Immune Pathology (ETIP) is an advanced model of both the Guard Hypothesis and Effector-Triggered Immunity (ETI). If the host is “resistant” and encodes the guardee protein, it is capable of recognizing the damage mediated by *Shigella* effectors and this induces an efficient innate immune response. *Shigella* can use this to its advantage, increasing intestinal inflammation which is required for infection establishment and transmission by diarrhea (Stuart et al., 2013). However, this inflammation eventually leads to the clearance of infection, therefore *Shigella* harbors a large repertoire of anti-inflammatory mechanisms for down-regulation of ETIP. The host is not able to “guard” all the steps/signaling pathways involved in NFκB activation, therefore multiple effectors with different biochemical functions are capable of efficiently interfering with these pathways and dampening the host immune response. Similarly, VirA and IpaJ act to fragment the Golgi via exertion of differed biochemical activities on different small GTP binding proteins involved in its maintenance.

More Than One Effector with a Similar Biochemical Function

IpgB1 and IpgB2 are similar in their WxxE motif and GEF activity, but they have non-overlapping substrates. Only a dual *ipgB1−ipgB2−* knockout had a negative Sereny test, indicating that they also have similar but non-overlapping functions (Hachani et al., 2007). Lack of redundancy was also confirmed, as an *ipgB2−* knockout has the same phenotype as the wild-type strain, however the *ipgB1−* knockout had an increase in inflammation compared to the wild-type strain. This may link to the guard hypothesis, whereby IpgB1 prevents detection of IpgB2 by the host guardee proteins, perhaps via its own non-overlapping enzyme activity, to prevent an excessive inflammatory response.

One Effector with Multiple Functions?

An effector characterized with multiple roles in pathogenesis is normally due to a single function which mediates several effects. For example, the only characterized activity of IpgD is that it dephosphorylates phosphoinositides, decreasing levels of PtdIns(4,5)P₂ and increasing levels of PtdIns(5)P. Changes in the intracellular concentrations of these phosphoinositides then produces effects directly, such as membrane ruffles (Niebuhr et al., 2002).
et al., 2002), or indirectly, such as vacuolar lysis (Mellouk et al., 2014), activation of Akt/PI3K signaling pathway (Pendaries et al., 2006), and interference with T-lymphocyte migration (Konradt et al., 2011). Therefore, IpgD has one biochemical activity, which mediates multiple effects on the host cell. This is also probably the case for IpaJ in Golgi fragmentation and STING activation and for OspB in modulating cell-to-cell spread via mTOR and ERK/MAPK signaling. Some factors, such as IcsA involved in adhesion and actin-tail formation, may genuinely have two very different biochemical functions, used at different points in the infectious cycle. OspE1/E2 seem involved in adhesion to host cells and stability of tight junctions. However, others may not. At its position at the T3SS needle tip, IpaB is involved in CD44 interactions (Skoudy et al., 2000), host cell sensing, translocon pore formation (Blocker et al., 1999), and directly or indirectly, and lysis of the single and double membrane entry vacuoles (High et al., 1992; Page et al., 1999). Once secreted, IpaB plays a role in macrophage pyroptosis (Guichon et al., 2001) and cell cycle arrest (Iwai et al., 2007). It is difficult to comprehend how IpaB could exert all these functions without possessing multiple biological activities, which remain unclear. However, some of these functions may have been misattributed to IpaB due to pleotropic effects stemming from its involvement in translocon formation, which is crucial for Shigella entry.

CONCLUDING REMARKS

The vast majority of the functional work presented here has been done on S. flexneri. Comparative analysis of the dominant Shigella genomes causing disease is required for further analysis of virulence determinants, such as any involved lysis of the single and double membrane entry vacuoles, which remain completely unclear, and of their epidemiological consequences. The importance of such genomic investigations has recently been highlighted (The et al., 2016). Identification of important and conserved effectors and other virulence determinants that cause disease will contribute to an overall understanding of infection, further illuminating species tropism and transmission and helping to create a pan-Shigella vaccine, which so far has been unsuccessful.

AUTHOR CONTRIBUTIONS

EM planned, researched, wrote, and subsequently also formatted the manuscript for publication, assisted by discussions, analysis of published works and editing with AB. EM also generated Figures 5–9.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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