Progress and pitfalls in *Shigella* vaccine research

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Abstract | Renewed awareness of the substantial morbidity and mortality that *Shigella* infection causes among young children in developing countries, combined with technological innovations in vaccinology, has led to the development of novel vaccine strategies in the past 5 years. Along with advancement of classic vaccines in clinical trials and new sophisticated measurements of immunological responses, much new data has been produced, lending promise to the potential for production of safe and effective *Shigella* vaccines. Herein, we review the latest progress in *Shigella* vaccine development within the framework of persistent obstacles.

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Introduction

Shigella diarrhoeal illness remains an important cause of morbidity and mortality globally, particularly among children <5 years of age in developing countries. In 1999, it was estimated that *Shigella* caused ~113 million episodes of diarrhoea and 1.1 million deaths annually.[1] As a pathogen that invades and destroys intestinal mucosa, *Shigella* is less amenable to the salutary effects of oral rehydration than enterotoxigenic pathogens that cause dehydrating diarrhoea. Antibiotics are the standard of care for shigellosis, but therapeutic options are limited by the widespread prevalence of resistant strains, as in Asia where resistance to ciprofloxacin (the standard first-line antibiotic used to treat shigellosis) has become common.[2,3] Resistance has also increased to the three second-line choices, reaching moderate levels (30–50%) for pivmecillinam and azithromycin and has appeared against third-generation cephalosporins, mediated by extended spectrum β-lactamases.[2,4–6] As therapeutic options narrow, the need for a safe and effective *Shigella* vaccine becomes more pressing.

Advances in our understanding of the pathogenesis of *Shigella*, including the identification of new virulence factors and technological advances in vaccine design and manufacture, have led to the development of multiple new innovative vaccine candidates. However, several barriers impede the pace of *Shigella* vaccine development (Box 1). These include some specific knowledge gaps: undefined correlates of immunity; the lack of good small animal models that fully recapitulate the disease in which to test vaccine candidates; the difficulty in providing broad coverage; concerns for the potential of inadvertently inducing reactive arthritis; the perception that other interventions (including water and sanitation) are more appropriate; and insufficient funding to accelerate clinical development. Despite these obstacles, there is substantial progress to report and herein we review advances in *Shigella* vaccine development during the past 5 years, highlighting new vaccine technologies.

Competing interests
The authors declare no competing interests.

Taxonomy and epidemiology of *Shigella*

Shigella is an antigenically diverse pathogen whose taxonomy undergoes periodic modifications. The current official taxonomy encompasses four species (or groups) and 49 serotypes and subtypes that include *S. dysenteriae* (group A, 15 serotypes), *S. flexneri* (group B, 15 serotypes and subtypes including the newly designated 7a and 7b subtypes[7]), *S. boydii* (group C, 20 serotypes) and *S. sonnei* (group D, one serotype). In addition, more than a dozen putative new serotype or subtype strains are being considered for possible official classification.

As the ingestion of a minute inoculum (10 microorganisms) can lead to shigellosis,[8] *Shigella* disseminates easily in settings where there is overcrowding, limited access to water, compromised personal hygiene and inadequate sanitation. *S. flexneri* serotypes are the major agents of endemic shigellosis among children in developing countries, whereas *S. sonnei* is the predominant serotype associated with *Shigella* diarrhoeal illness in subpopulations in industrialized country settings where *Shigella* infections persist and in transitional countries; *S. sonnei* is also an important agent of travellers' diarrhoea.[9] *S. boydii* serotypes are uncommonly associated with diarrhoeal illness. The Global Enteric Multicenter Study (GEMS) of moderate and severe diarrhoea (MSD) among children <60 months of age determined the serotypes of *Shigella* isolates in seven developing countries in sub-Saharan Africa and South Asia.[10,11] Among >1,100 isolates of *Shigella* associated with MSD, 66% were *S. flexneri* and 24% were *S. sonnei*; *S. boydii* and *S. dysenteriae* serotypes collectively accounted for only 10% of the isolates. No isolates of *S. dysenteriae* type 1 were

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Key points

- *Shigella* infection continues to be a leading cause of morbidity and mortality, exerting the greatest burden in children in less-industrialized countries
- Epidemiological studies (for example, Global Enteric Multicenter Study) confirmed the distribution of multiple serotypes in geographical regions as important causes of infection
- Studies on *Shigella* pathogenesis have revealed new virulence factors, which might serve as targets for attenuation in live vaccine strains or as potential vaccine antigens
- Vaccine strategies can be divided into serotype-targeted or conserved protein antigen approaches and multiple candidates are in various stages of development and evaluation
- New immunological measurements are shedding light on important protective responses
- Multiple barriers (such as insufficient funding to accelerate and complete clinical trials) exist that are impeding the pace of *Shigella* vaccine development

Box 1 | Barriers impeding the pace of *Shigella* vaccine development

- Specific knowledge gaps, including a correlate of protective immunity
- Concerns for the ability to provide broad coverage of many serotypes
- The lack of an animal model that adequately recapitulates *Shigella* disease
- Concerns over the potential to inadvertently induce reactive arthritis
- Concerns over possible gastrointestinal adverse effects
- Constraints on manufacture (e.g. for synthetic saccharide components)
- Perceptions that other interventions (including water, sanitation and hygiene) are more appropriate for control of shigellosis
- Insufficient funding to accelerate and complete clinical trials

recorded. Four serotypes, *S. flexneri* 2a, *S. flexneri* 3a, *S. flexneri* 6 and *S. sonnei* comprised 65% of all the isolates identified.[10,11]

S. dysenteriae type 1 (the 'Shiga bacillus'), the only serotype that produces Shiga toxin, can cause severe clinical disease with complications (including haemolytic uraemic syndrome or HUS) and historically has led to devastating epidemics and pandemics with high case fatality in all age groups (reviewed elsewhere[12]). Shiga dysentery typically arises in developing countries experiencing upheaval of civil society or natural disaster.[13] Although Shiga disease has almost disappeared within the past decade, it could reappear at any time.

For vaccine developers, preparing a broad-based *Shigella* vaccine based on serotypes, the GEMS and other large survey data suggest that a quadravalent vaccine, containing strains or antigens from *S. sonnei* and *S. flexneri* 2a, 3a and 6, would directly cover ~65% of current circulating strains. With cross protection based on shared *S. flexneri* group antigens, such a quadravalent vaccine could cover >85% of currently circulating *Shigella* strains.[12,14] Many argue for including *S. dysenteriae* 1 coverage in a serotype-based vaccine in the expectation that pandemic Shiga dysentery will return and a vaccine could constitute an important public health tool.

Genomics

Genomic and proteomic technologies have elucidated complete *Shigella* genomes and protein profiles,[15–19] providing information that influences vaccine development. The revelations made have encouraged vaccine development strategies based on the identification of proteins conserved among *Shigella* and related *Escherichia coli* enteropathogens (*vide infra*). Moreover, the genome analyses that document the phylogenetic relatedness of *Shigella* and *E. coli* are prompting a possible reclassification of *Shigella* as a member of the *E. coli* species. Such a taxonomic revision will have to include input from clinicians and epidemiologists to minimize confusion in the clinical and disease control arenas.

Pathogenesis and clinical features

All *Shigella* serotypes have a similar pathogenesis, which involves translocation through ileal and colonic M cells, uptake by macrophages, basolateral invasion of epithelial cells and dissemination within the mucosa (detailed elsewhere[20,21]). After an incubation period of 1–4 days, shigellosis usually begins with systemic symptoms, including fever, headache, malaise, anorexia and occasional vomiting. Watery diarrhoea typically precedes dysentery[22] and can be the only clinical manifestation of mild infection.[23] Watery diarrhoea arises from the action of enterotoxins in the jejunum, whereas bloody diarrhoea results from invasion of the colonic epithelium.[8,24] Frank dysentery manifests as frequent scanty stools containing blood and mucus, accompanied by lower abdominal cramps and rectal tenesmus. Patients with severe infection can pass >20 dysenteric stools daily. Shigellosis in otherwise healthy individuals is generally self-limited and resolves within 5–7 days, without sequelae. However, extraintestinal complications can occur,[25] including generalized convulsions and encephalopathy. HUS can accompany *S. dysenteriae* 1 infection.[26] Acute, life-threatening complications are sometimes seen in malnourished children in developing countries.[27] In the USA, *Shigella* bacteraemia has been reported (rarely) among HIV-infected and other immunocompromised patients.[28]

Whereas the molecular mechanisms that determine *Shigella* invasiveness, rupture of the phagocytic vacuole, movement through the host-cell cytoplasm and modulation of the innate immune response have been intensively studied,[21] less is known about how the organism evokes diarrhoea. Nevertheless, secretogenic proteins elaborated by *Shigella* strains have been identified (Table 1) and serve as targets for attenuating mutations or as new vaccine antigens.

Shigella enterotoxins

In 1995, we identified *Shigella* enterotoxins 1 and 2 (ShET1 and ShET2), by demonstrating their ability to cause fluid accumulation in rabbit ileal loops (ShET1), and greater potential difference and short circuit current (Isc) in Ussing chambers (both measures of electrolytes and water secretion).[29,30] ShET2 secretion requires the type 3 secretion system (T3SS)—a needle-like apparatus used to secrete bacterial proteins—in *S. flexneri* 2a.[31] Functional studies of a ShET2 mutant demonstrated reduced IL-8 secretion following invasion, suggesting that this toxin might also participate in *Shigella*-induced inflammation in epithelial cells.[31] A role for these two toxins in disease was determined in clinical

Table 1 | Factors contributing to diarrhoea in *Shigella*

Protein	Gene(s)	Location (bacteria)	Function(s)	Reference
Enterotoxins				
ShET1	*set1A*, *set1B*	Chromosome, *she* PAI (*S. flexneri* 2a and EAEC)	Enterotoxin (Ussing chamber) Rabbit ileal loops	Fasano *et al.* (1995, 1997);[29,30] Noriega *et al.* (1995)[120]
ShET2	*sen*, *ospD3*	Virulence plasmid (*Shigella*, EIEC)	Enterotoxin (Ussing chamber) T3SS effector	Nataro *et al.* (1995)[53]
SPATEs				
SigA	*sigA*	Chromosome, *she* PAI (*Shigella*, EIEC)	Enterotoxin (rabbit ileal loop) Fodrin degradation	Al-Hasani *et al.* (2000);[37] Boisen *et al.* (2009)[44]
Pic	*pic*	Chromosome, *she* PAI (EAEC 042, UPEC, *S. flexneri* 2a)	Mucinase, enterotoxicity, immunemodulation	Behrens *et al.* (2002);[42] Ruiz-Perez *et al.* (2011);[43] Boisen *et al.* (2009)[44]
SepA	*sepA*	Virulence plasmid (*Shigella*)	Rabbit ileal loop inflammation Enterotoxicity	Benjelloun-Touimi *et al.* (1995, 1998);[39,121] Faherty *et al.* (2012)[40]

Abbreviations: EAEC, enteroaggregative *Escherichia coli*; EIEC, enteroinvasive *E. coli*; PAI, pathogenicity island; SPATE, serine protease autotransporters of Enterobacteriaceae; T3SS, type 3 secretion system; UPEC, uropathogenic *E. coli*.

trials in which reduced reactogenicity in live attenuated *S. flexneri* 2a vaccine strains containing mutations in these two toxins was demonstrated.[32] ShET1 and ShET2 continue to serve as targets for attenuating mutations in multiple vaccine candidates.[33–35]

SPATEs

The most common secretion mechanism in the Gram-negative bacterial envelope is the autotransporter system, in which full-length protein is passed across the inner membrane by virtue of the Sec apparatus, which then uses its own C-terminus to enable translocation across the outer membrane. A large and growing family of serine protease autotransporters of Enterobacteriaceae (SPATEs) has been identified, produced almost exclusively by pathogenic *E. coli*, *Shigella* and *Salmonella* strains.[36] Three SPATEs have been identified as potential contributors to the enterotoxic activity of *Shigella*—SigA, SepA and Pic (Table 1).

SigA is a chromosomally encoded class I SPATE (cytotoxic to epithelial cells) in *S. flexneri* 2a that exerts a cytopathic effect in HEp-2 cells,[37] suggesting that it might be a cell-altering toxin with a role in pathogenesis. SigA was demonstrated to be partly responsible for the ability of *S. flexneri* to stimulate fluid accumulation in ligated rabbit ileal loops.[37] A fragment of SigA is the basis for one conserved protein vaccine strategy.[38]

SepA is a class II noncytotoxic SPATE with a potential role in pathogenesis in the rabbit ligated ileal loop assay, in which a mutation in *sepA* caused markedly less inflammation and tissue damage than the wild-type parent strain.[39] Faherty and colleagues[40] have demonstrated in Ussing chamber studies that SepA exerts enterotoxic activity.

Pic is a SPATE encoded on the chromosomes of enteraggregative *E. coli* EAEC 042 uropathogenic *E. coli* and *Shigella flexneri* 2a.[41,42] Functional analyses of the Pic protein implicate this factor in mucinase activity, serum resistance, haemagglutination, enterotoxicity and immune modulation in targeting a broad range of human leukocyte adhesion proteins.[43] The gene encoding Pic is located as overlapping DNA on the opposite strand from *set1AB*, encoding ShET1.[42] Thus, live attenuated strains containing mutations in *set1AB* also contain mutations in *pic*.

Whereas most commensal and diarrhoeagenic *E. coli* encode few SPATEs, most *Shigella* strains harbour one or more SPATE-encoding gene,[44] suggesting that class I SPATEs are important in *Shigella* pathogenesis. Moreover, the most common *Shigella* serotypes (for example, *S. flexneri* 2a, 2b) generally carry the greatest number of SPATE-encoding genes. The class II Pic protease is largely found in *S. flexneri* 2a, which is globally the most important *Shigella* serotype. As more is learned about the precise roles of the SPATEs, their inclusion in vaccine strategies could be expanded.

Animal models of shigellosis

No small animal model replicates all aspects of *Shigella* pathogenesis as seen in humans. Nonhuman primates constitute one useful model, as they exhibit diarrhoea and dysentery after oral infection with virulent *Shigella* strains and have aided the evaluation of efficacy of vaccine candidates.[45–47] However, the cost and availability of nonhuman primates, combined with the fact that enormous inocula (>10^9 colony-forming units [CFUs]) are required to consistently induce shigellosis, are recognized drawbacks. Rabbit models have been investigated, but they require either extensive pretreatment or surgical procedures for induction of symptoms upon *Shigella* infection.[48,49] The guinea pig keratoconjunctivitis model described by Sereny[50]—which correlates with the ability of the organism to invade cells, spread through a single cell layer and induce inflammation—is helpful for evaluating the safety of live vaccines, as well as for testing the efficacy of many types of vaccines. The mouse lung model is used for the same purposes; that is, to reveal the inflammatory potential of live vaccines and its mutants and to demonstrate protection conferred by *Shigella* vaccines.[51] A model involving intrarectal inoculation of guinea pigs,[52] which leads to severe acute rectocolitis and a robust inflammatory response, might also be useful in vaccine evaluation.

Table 2 | *Shigella* vaccine candidates

Vaccine candidate	Gene mutations/description	Development stage	Route	Reference
O-antigen directed (live attenuated)				
S. flexneri 2a CVD 1204	*guaBA*	Phase I	Oral	Kotloff *et al.* (2004)[32]
S. flexneri 2a CVD 1208S	*guaBA*, *set*, *sen*	Phase II	Oral	Kotloff *et al.* (2007)[33]
S. dysenteriae 1 CVD 1256	*guaBA*, *sen*, *stxA*, *virG*	Preclinical	Oral	Wu *et al.* (2011)[122]
S. sonnei WRSs1	*virG*	Phase I	Oral	Kotloff *et al.* (2002)[123]
S. sonnei WRSs2, 3	*virG*, *senA*, *senB*, *msbB2*	Preclinical NHP	Oral	Bedford *et al.* (2011);[35] Barnoy *et al.* (2010, 2011);[75,124] Collins *et al.* (2008)[125]
S. flexneri 2a SC602	*virG*, *iuc*	Phase I–II	Oral	Coster *et al.* (1999);[71] Katz *et al.* (2004);[77] Rahman *et al.* (2011)[78]
S. flexneri 2a WRSf2G11, 12, 15	*virG*, *senA*, *senB*, *msbB2*	Preclinical	Oral	Ranallo *et al.* (2007, 2010, 2012)[34,76,126]
S. dysenteriae 1WRSd1	*virG*, *stxAB*	Phase I	Oral	McKenzie *et al.* (2008);[127] Venkatesan *et al.* (2002)[128]
Conjugate				
Chemical conjugate	*S. flexneri* 2a LPS-rEPA	Phase I–III	Parenteral	Cohen *et al.* (1996);[61] Passwell *et al.* (2010);[62] Cohen *et al.* (1996)[129]
	S. sonnei LPS-rEPA	Phase I–III	Parenteral	Cohen *et al.* (1996);[61] Passwell *et al.* (2010);[62] Cohen *et al.* (1996)[129]
	S. dysenteriae 1 LPS-rEPA	Preclinical	Parenteral	Chu *et al.* (1991)[130]
GlycoVaxyn bioconjugate	*S. dysenteriae* 1 LPS-exoA	Phase I	Parenteral	Dro & Sinclair (2010)[65]
Synthetic oligosaccharide	O-antigen mimic–tetanus toxoid	Preclinical	Parenteral	Phalipon *et al.* (2009);[66] Theillet *et al.* (2011)[131]
Common protein directed				
Purified Ipa proteins	*S. flexneri* 2a IpaB plus IpaD	Preclinical	Intranasal	Martinez-Beccera *et al.* (2012)[90]
GMMA vesicles	Outer membrane and periplasmic proteins	Preclinical	Intranasal	Berlanda *et al.* (2012)[95]
Conserved proteins IcsP, SigA	Protein fragments	Preclinical	Mucosal	Czerkinsky & Kim (2010)[38]
Combined O-antigen specific plus common protein				
Invaplex	LPS plus IpaB, IpaC and IpaD	Phase I	Intranasal	Riddle *et al.* (2011);[88] Tribble *et al.* (2010)[89]

Abbreviations: GMMA, generalized modules of membrane antigen; Ipa, invasion plasmid antigen; LPS, lipopolysaccharide; NHP, nonhuman primate; rEPA, recombinant *Pseudomonas* exoprotein A.

We have shown that *Shigella* strains and their extracellular enterotoxins induce ion flux (correlating with a secretory state) in rabbit tissue mounted in Ussing chambers.[29,53] Ussing chambers provide a far more quantitative readout than rabbit ileal loops of pathophysiological effects on mucosal epithelium. We have also used mouse small intestine to study *Shigella* enterotoxic activity in *ex vivo* models.[40]

Vaccine candidates

The lack of an ideal small animal model of *Shigella* infection represents one hurdle in vaccine development. Despite this obstacle, multiple vaccine strategies have been advanced in the past 5 years, buoyed by increased knowledge of *Shigella* epidemiology and pathogenesis and of human immune responses to the pathogen, as well as by new vaccinology technologies. These efforts can be categorized into two broad approaches—serotype-based vaccines or conserved antigen vaccines (Table 2). Serotype-specific strategies extend the demonstration that an initial clinical infection stimulates acquired immunity and serotype-specific protection against shigellosis. This phenomenon has been well documented in challenge studies in nonhuman primates,[54] in adult volunteers experimentally infected with virulent strains[55,56] and in epidemiological studies in endemic regions.[57]

Serotype-targeted vaccines

Serotype-specific candidates include conjugate vaccines composed of purified *Shigella* O polysaccharides (or O antigen, a component of lipopolysaccharide [LPS]) conjugated to a protein carrier, genetically engineered O polysaccharide protein fusions, live attenuated strains and killed whole-cell formulations.

Conjugate vaccines

The most advanced conjugate vaccines, developed by investigators at the National Institute of Child Health and Human Development, include *S. flexneri* 2a LPS conjugated to recombinant *Pseudomonas* exoprotein A (rEPA) and *S. sonnei* LPS conjugated to rEPA. These conjugates were shown to be safe and immunogenic in adults and young children.[58] Furthermore, the *S. sonnei* conjugate was efficacious against disease when tested in Israeli soldiers in field trials.[59–61] *S. sonnei* and *S. flexneri* 2a conjugate vaccines were tested for efficacy in Israeli children aged 1–4 years; however, there was not enough baseline levels of disease attributable to *S. flexneri* in this population to calculate efficacy for the *S. flexneri* conjugate.[62] The *S. sonnei* conjugate did not meet the primary aim of the study in providing statistically significant efficacy overall in children <4 years. However, when subgroup analyses by age were undertaken it was revealed that 71%

efficacy was observed against *S. sonnei* infection in the 3–4 year age group, 35.5% efficacy in 2–3 year group, but no efficacy was found in children aged 1–2 years at the time of vaccination.[62] Efficacy paralleled the age-related immune responses induced by the vaccine. One interpretation of these data is that the *Shigella* conjugate vaccine boosted children old enough to have had likely previous exposure, but was unable to prime and protect immunologically naive young children under 2 years.

A novel bioconjugate vaccine technology has been advanced by investigators at GlycoVaxyn (Switzerland) that utilizes recombinant DNA technology to catalyze the *in vivo* synthesis of conjugate vaccines. The glycosylation machinery from *Campylobacter* was cloned in an *E. coli* production strain to generate a protein carrier glycosylated with the O-antigen-specific *Shigella* LPS, which was then purified as a conjugate vaccine.[63,64] A *S. dysenteriae* 1 O-antigen–rEPA conjugate vaccine was produced in this system and tested in a phase I trial in which it was found to be safe and immunogenic after two doses.[65]

Carbohydrate vaccines

Investigators at the Institut Pasteur, France, have formulated carbohydrate vaccines encompassing synthetic oligosaccharides mimicking the protective determinants carried by the *Shigella* O antigen. The synthetic oligosaccharides fused to tetanus toxoid resulted in a functional O-antigen mimic recognized by human serum *in vitro*.[66,67] The use of synthetic technology might enable great flexibility in the production of vaccine antigens.

Live attenuated or killed whole-cell vaccines

Orally administered live attenuated or killed whole-cell vaccines represent another strategy based on the serotype specificity of the human protective response and include the advantage of presenting much more of the antigenic repertoire of the bacteria to the host immune system. Inactivated whole-cell vaccines have been shown to be protective in animal models[68] and to be safe and immunogenic in volunteers ingesting three or five doses of 10^{10} CFU killed *S. sonnei* organisms.[69] Measurement of protective efficacy awaits challenge studies.

Live attenuated vaccines have induced protective responses against virulent challenge in volunteer studies and have protected adult and paediatric populations against disease in controlled field trials.[70–72] Although sophisticated genetic techniques enable the introduction of specifically targeted modifications into vaccine strains, achieving the correct balance of safety and immunogenicity has been a formidable challenge.[73,74] Two attenuating strategies continue to progress through clinical trials. We have engineered a series of *Shigella* strains containing mutations in *guaBA* (encoding critical enzymes for bacterial metabolism) and in the *sen* and *set* loci (encoding ShET1 and ShET2). *S. flexneri* 2a strain CVD 1208S seemed safe and immunogenic in phase I studies[12,33] and has advanced through process development, current Good Manufacturing Practice guideline and to phase II clinical studies. The Center for Vaccine Development, University of Maryland, USA, is advancing an overall vaccine strategy utilizing five attenuated strains including *S. dysenteriae* 1, *S. sonnei*, *S. flexneri* 2a, *S. flexneri* 3a and *S. flexneri* 6 to encompass the most important strains and the type-specific and group-specific antigens found on all *Shigella* isolates.[12,14]

Investigators at the Walter Reed Army Institute of Research have developed a series of live attenuated vaccine candidates containing a fundamental mutation in *virG* (also known as *icsA*), which is required for actin-based motility and cell-to-cell spread of the bacteria. Additional mutations in some strains include genes encoding ShET1 and ShET2 as well as MsbB, which is thought to detoxify lipid A of LPS and render the strain less reactogenic (causing few symptoms and adverse effects),[34,35,75,76] *S. flexneri* 2a vaccine SC602, containing mutations in *virG* and *iuc* (encoding aerobactin), was previously demonstrated to be immunogenic and protective against challenge in North American volunteers, although reactogenic at moderate and high doses.[71,77] This vaccine was subsequently tested in healthy adults and school-age children (8–10 years) in a *Shigella*-endemic region of Bangladesh; single oral doses of 10^4, 10^5 or 10^6 CFU resulted in minimal vaccine shedding, minimal reactogenicity, no transmission and low immune stimulation in both populations tested.[78] The dietary deficiency of iron in Bangladeshi volunteers has been suggested as one reason why this particular vaccine, with a mutation in iron uptake, might have induced different responses in the two populations. This study underscores a critical point surrounding the use of orally administered live attenuated strains in *Shigella*-endemic regions where the nutritional and immune status as well as the microbiota of individuals could affect vaccine performance.[79] Other oral live attenuated vaccines (including polio, cholera and rotavirus) have engendered reduced immune responses among vaccinees in developing countries compared with those in industrialized countries.[80–83] Nonetheless, some of these vaccines have provided protection against severe illness in these populations[83,84] and it is expected that the optimal formulation (possibly including mucosal adjuvants to boost the immune response) and vaccination regimen (including multiple doses with a potential booster) of a live attenuated *Shigella* vaccine will be equally successful.[80,85,86]

Conserved antigen vaccines

Ipa proteins

The concept of using an antigen conserved among *Shigella* strains as an immunogen to provide broad protection is the basis for several new vaccine candidates. The most advanced vaccine that contains components of conserved proteins plus serotype-specific O antigen has been established by investigators at Walter Reed Army Institute of Research. The *Shigella* Invaplex (for *Shigella* invasion complex) vaccine was initially formulated from a bacterial extract composed of invasion plasmid antigen (Ipa) proteins, which are highly conserved among all *Shigella* serotypes and have an essential role in *Shigella*

pathogenesis, and LPS. In animals, serotype-specific protection has been demonstrated, mediated by the LPS component.[87] Invaplex has been shown to be safe and immunogenic after intranasal delivery in healthy volunteers.[88,89] Current studies are underway to optimize formulation and delivery.

In related efforts, a vaccine composed of purified IpaB plus IpaD has been demonstrated to confer homologous as well as heterologous protection in a mouse model of *Shigella* infection when delivered with adjuvant.[90] Antibodies against Ipa proteins are produced after natural and experimental human infection and are believed to contribute to protection.[91,92] A vaccine that could induce protective anti-Ipa responses could provide protection against all *Shigella* strains expressing these highly conserved antigens.

Outer membrane proteins

The use of outer membrane protein preparations as vaccine formulations has also been explored.[93,94] A novel protein vesicle technology named generalized modules of membrane antigens (or GMMA) is an industrial, high-yielding production process for genetically derived outer membrane particles composed of predicted *Shigella* outer membrane and periplasmic proteins without LPS.[95] In preclinical mouse studies, immunization with GMMA provided 65–100% protection against lethal challenge.[95]

Taking advantage of genomic and proteomic data, investigators at the International Vaccine Institute, South Korea, have identified two conserved protein candidates: IcsP2 (an outer membrane protease that cleaves VirG from the surface and which is present on all *Shigella* species and enteroinvasive *E. coli*) and SigA2 (a SPATE present on all *S. flexneri* 2a, *S. boydii* and *S. sonnei*). These antigens have demonstrated protection in animal models.[38]

Immune responses

The evaluation of vaccine candidates relies on an understanding of which immune responses are critical for protection. Studies of humans and nonhuman primates following natural infection and vaccination provide the most relevant data and suggest that a complex series of responses engaging multiple arms of the immune system are involved in immunity to disease caused by *Shigella*.

Natural infection and vaccination

Humans develop an array of immune responses following *Shigella* infection, including humoral and cell-mediated immune responses. Of particular importance are the high levels of serum IgG (mainly IgG1 and IgG2, depending on the serotype) and IgA antibodies against *Shigella* O antigen, which appear 1–2 weeks after primary exposure.[96] Results from multiple epidemiological[57] and seroepidemiological studies[97–99] suggest that O-antigen-specific antibodies have a critical role in protection. Antibodies against Ipa proteins are also produced after natural and experimental infection and are believed to contribute to protection.[91,92,100] Antibodies derived from natural infection have complement-mediated bactericidal activity[101] and promote opsonophagocytic killing by mononuclear cells.[102]

In addition to systemic immunity, strong mucosal immune responses are induced.[71,103] Gut-derived O-specific IgA antibody secreting cells (ASCs) are believed to play a critical part in protection against *Shigella*. These cells are detected in peripheral blood 7–10 days after exposure to the organism or vaccine and are believed to represent a pool of transiently migrating antigen-specific B cells with the capacity to home to mucosal effector sites where they will participate in host defence by producing local antibodies. IgA O-antigen ASCs represent a measure of oral priming that has been associated with efficacy of live attenuated vaccines.[71,104] The number of O-specific IgA ASCs and the levels of O-specific serum IgG are commonly used as primary readouts of immunogenicity in clinical trials of attenuated live and nonliving whole-cell oral vaccines,[32,98] whereas serum IgG O antibody is typically used to assess immune responses induced by parenteral O polysaccharide-protein conjugate vaccines;[60,98] these parameters are generally considered to be predictors of the efficacy of these vaccines. Secretory IgA (sIgA) also seems to have a major role in limiting the duration of illness.[105,106] However, measurements of sIgA in mucosal secretions (for example, stool and saliva) can be variable and no consensus exists in the literature showing a clear association with resistance to infection.[104]

The efficacy of conjugate vaccines has been correlated with high levels of IgG O-antibody.[62,107] Speculation exists that *Shigella* might be inactivated by parenterally induced IgG leaked into the intestinal lumen, possibly through complement-mediated lysis in the epithelial cell surface.[59] It is reasonable to assume that the presence of a critical level of protective IgG antibodies implies the presence of strong underlying T-helper (T_H) immunity, yet T-cell measurements were not reported in *Shigella*-conjugate vaccine studies.

Shigella infection has also been shown to induce cell-mediated immune responses, including upregulation of IFN-γ receptor expression and production of pro-inflammatory cytokines, such as IFN-γ.[108,109] Moreover, an expansion of T cells, particularly $CD8^+$ and T-cell receptor (TCR) $\gamma\delta^+$ T-cell subsets in the gut mucosa,[110] has been described in the rectal mucosa of patients with shigellosis. Of note, increased levels of activated and memory $CD4^+$ and $CD8^+$ T cells and expansion of defined TCR Vβ families have been reported in peripheral blood of patients with shigellosis.[111,112] However, a direct association between cell-mediated immune responses and protection has not been demonstrated and the extent to which these responses contribute to clearing infection and to the pathogenesis of shigellosis remains unknown.

Mucosal immunological priming

Our current understanding of the processes involved in *Shigella* pathogenesis offers some insights into how the organism might interact with the immune cells in the gastrointestinal mucosa and trigger immune

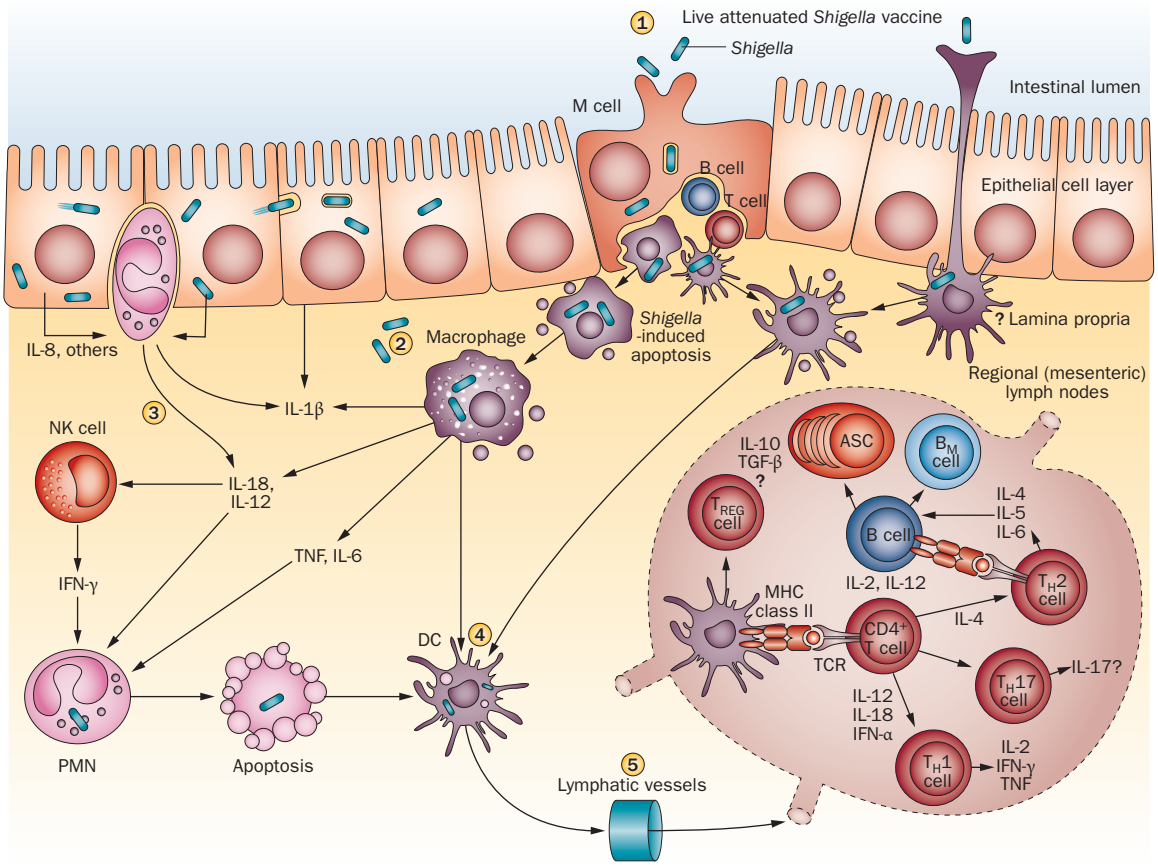

Figure 1 | Live *Shigella* mucosal priming. *Shigella* crosses the intestinal epithelial barrier through M cells and is endocytosed by macrophages and DCs in the M cell pocket (1). Conceivably, the organism could also be sampled by DCs within the epithelial layer.[132] The bacteria escape the phagocytic vacuole and induce apoptosis of the infected cells. Live bacteria are released and invade epithelial cells from the basolateral side, spreading from cell-to-cell via actin-based motility (2). Infected epithelial cells secrete IL-8 and other chemotactic factors that will recruit PMNs, macrophages and NK cells (3). Activated macrophages and PMNs produce proinflammatory molecules (for example, IL-18, IL-1β, TNF, IL-6, and IL-12) further attracting phagocytic cells. Apoptotic macrophages, PMNs and antigens released from infected cells may be taken up by DCs (4).[133] These antigen-loaded DCs are transported to adjacent interfollicular T-cell zones of mucosal lymphoid follicles or regional lymph nodes where they activate $CD4^+$ T_H2 cells, which contribute to B-cell differentiation into ASCs or B_M and T_H1 cells, a process that will facilitate inflammatory responses (5). Mucosally primed B cells and T cells acquire homing receptors that will enable them to migrate to mucosal effector sites. Abbreviations: ASC, antibody secreting cell; B_M cell, B memory cell; DC, dendritic cell; PMN, polymorphonuclear neutrophil; NK cell; natural killer cell; TNF, tumour necrosis factor; T_H cell, T-helper cell; T_{REG}, T-regulatory cell.

responses following infection (Figure 1). Upon re-exposure to the organism, the host displays a plethora of immunological effector mechanisms to resist the infection (Figure 1). Of particular importance are antibodies and immune cells in the gut (for example, O-specific ASCs and plasma cells, sIgA, memory B [B_M] cells, T_H1 and T_H2 T cells), which will provide the first line of defence to prevent the microorganism from invading the epithelial barrier. These responses are finely regulated, with mucosal dendritic cells having a central role in imprinting mucosal homing receptors on T cells and B cells and inducing differentiation of IgA-producing plasma cells and T-regulatory cells.[113,114]

Mucosally primed ASCs will produce IgA, which is secreted through the epithelial cells, or IgG, which can diffuse into the intestinal lumen or be actively transported through the FcRn receptor. Both antibodies could block cell attachment in the intestinal lumen or prevent further invasion by bacteria that have breached the intestinal epithelial barrier. IgG could mediate bacterial killing through opsonophagocytosis or lysis in the presence of complement. B cells primed in the mucosa can also differentiate into memory cells.

Anamnestic humoral immune responses, largely dependent on the presence of B_M cells, are generally faster and higher in magnitude than primary responses and are crucial for protection from subsequent infections.[115] Direct evidence of the presence of B_M cells specific to *Shigella* antigens in volunteers immunized with a single oral dose of *S. flexneri* 2a CVD 1204 or CVD 1208 attenuated vaccine candidates has now been provided.[116,117] Using a modified B_M ELISPOT assay, the researchers found that volunteers developed IgA and IgG B_M cells to *S. flexneri* 2a LPS and IpaB antigens, which correlated with serum antibody levels. Moreover, raised frequencies of IgA^+, but not IgG^+, $CD19^+CD27^+CD20^+$

and $CD19^+CD27^+CD20^+CD20^{-/dim}$ B-cell subsets expressing the gut-homing receptor integrin α4β7 were detected 28 days after oral vaccination, suggesting an increase in the circulating pool of *Shigella*-specific IgA B_M cells and plasmablasts with gut-homing potential in individuals that developed humoral responses to *Shigella*. These findings are important for the development of effective vaccines as the presence of B_M cells is likely to contribute to the persistence of systemic and mucosal antibodies and the ability to mount an anamnestic response when circulating antibody levels have already declined.[118] We therefore suggest including measurements of specific B_M cells to relevant antigens in future vaccine studies as they might represent an important immunological correlate of protection and indicate long-term immunity.

Although antibodies against the O-polysaccharide and *Shigella* Ipa proteins seem to be critical for protection, other immunological effectors are probably necessary to clear an infection. T_H1 cells could limit bacterial dissemination through induction of intraepithelial lymphocytes with cytotoxic capacity. Moreover, being an intracellular pathogen, *Shigella* is expected to activate cytotoxic $CD8^+$ T cells (CTLs) that could eventually kill infected cells and secrete IFN-γ and other cytokines to further enhance T_H1 cell-mediated immune response.

Conclusions

Technological advancements—including genomic technologies, synthetic carbohydrate chemistry, *in vivo* conjugation techniques and sophisticated assays to measure immune responses—have facilitated the development and evaluation of a new generation of *Shigella* vaccines. In parallel, the progression of clinical trials assessing classic conjugate and live attenuated vaccines is revealing new information about relevant immune responses associated with protection. Despite these advances, no consensus exists on what constitutes critical immunological correlates of protection. This issue remains an obstacle impeding the development of safe and efficacious vaccines and is further complicated by the lack of an optimal small animal model for studying the disease and the effects of vaccines, leading to reliance, ultimately, on clinical trials for relevant data. Other barriers impeding the pace of vaccine development include the concern for the potential of inadvertently eliciting reactive arthritis. This concern was addressed by a panel of rheumatologists and vaccinologists, who concluded that vaccination was unlikely to induce these sequelae.[119] Additional concerns have been raised about manufacturing constraints for complex vaccines. Emerging vaccine companies in developing countries have become increasingly sophisticated in the products that they can manufacture. Through full transfer-of-technology partnerships for some vaccines or finish and fill (of transported complex components) partnership arrangements, there are multiple options for assuring the consistent manufacture of *Shigella* vaccines and for creating supply to match anticipated global demand. Moreover, the depth and breadth of clinical investigators and teams in developing countries experienced in Good Clinical Practice has markedly expanded in the past 15 years including several sites in Africa and Asia that have performed phase I, II and even III clinical trials of both oral (for example, rotavirus and cholera) and parenteral vaccines (for example, RTS,S malaria vaccine and meningococcal A conjugate vaccine).

Notwithstanding some difficulties, now is a time for optimism as in the past few years there has been a renewed recognition of the excessive morbidity and mortality that *Shigella* causes among young children in developing countries and a commitment has been made to diminish this burden. This recognition has translated to new funding partners joining the efforts of other traditional supporters in providing resources for *Shigella* vaccine development. As a consequence, consortia of scientists have been contributing their expertise toward the common goal of developing safe and efficacious *Shigella* vaccines that might in the future become widely utilized public health tools to diminish the burden of shigellosis in populations most in need.

Review criteria

We searched for articles focusing on original research on *Shigella* vaccine development. A PubMed search was performed using the search terms "*Shigella*" and "vaccines". All papers identified were English-language, full-text papers. We also reviewed the reference lists of identified articles for further relevant papers.

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Author contributions

All authors contributed equally to all aspects of this manuscript.