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REVIEW

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Shigella-mediated immunosuppression in the human gut: subversion extends from innate to adaptive immune responses

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ABSTRACT

The enteropathogen, *Shigella*, is highly virulent and remarkably adjusted to the intestinal environment of its almost exclusive human host. Key for *Shigella* pathogenicity is the injection of virulence effectors into the host cell *via* its type three secretion system (T3SS), initiating disease onset and progression by the vast diversity of the secreted T3SS effectors and their respective cellular targets. The multifaceted modulation of host signaling pathways exerted by *Shigella* T3SS effectors, which include the subversion of host innate immune defenses and the promotion of intracellular bacterial survival and dissemination, have been extensively reviewed in the recent past. This review focuses on the human species specificity of *Shigella* by discussing some possible evasion mechanisms towards the human, but not non-human or rodent gut innate defense barrier, leading to the lack of a relevant animal infection model. In addition, subversion mechanisms of the adaptive immune response are highlighted summarizing research advances of the recent years. In particular, the new paradigm of *Shigella* pathogenicity constituted of invasion-independent T3SS effector-mediated targeting of activated, human lymphocytes is discussed. Along with consequences on vaccine development, these findings offer new directions for future research endeavors towards a better understanding of immunity to *Shigella* infection.

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Exploring Shigella specificity towards the human innate immune barrier in the gut

The striking selectivity of *Shigella* as a human-restricted pathogen relies on its evasion mechanisms of host innate defenses during gut transit and mucosal invasion, which are highly adapted to the human digestive tract. The significance of this species specificity becomes evident when considering that up-to-now there is no experimental animal model available, which convincingly mimics human infection, *i.e.* the onset of dysentery after oral inoculum.[1] As an exception, naturally acquired *Shigella* infection has been reported for some, non-human primates, including Rhesus macaques.[2–4] Nevertheless, under laboratory conditions, the Rhesus macaque model requires an inoculum dose of about 10^8–10^9 bacteria,[5] whereas as few as 10^2–10^3 suffice to cause disease in humans.[6,7] While sharing common features, there is increasing evidence suggesting for important species differences in the innate defense systems – in particular between primates and rodents[8] – which may provide important leads as to understanding susceptibility *versus* resistance to infection observed in these species. For instance, after surviving the acidic environment of the stomach,[9] *Shigella* reaches the small intestine where it has to resist to degradation by bile salts that commonly vary in composition between humans, non-human primates, and rodents.[10] Interestingly, exposure to human-type bile salts was recently shown to increase *Shigella* virulence *via* the upregulation of survival genes as well as the induction of biofilm formation, hence facilitating better resistance to the challenging environmental conditions of the gut.[11] Upon entering the colon, *Shigella* is faced with the great abundance of resident microbes, the gut microbiota, which naturally varies significantly in their composition across species.[12,13] How *Shigella* establishes its niche in such an adverse environment is subject of further investigations but of importance is its binding to the mucus layer, which is at its greatest thickness in the colon in comparison to other sections of the digestive tract.[14,15] Immune properties of the mucus barrier present with marked differences between location and species and are associated with host selectivity. The absence of *Shigella* binding to the small intestinal mucus[16,17] suggests selectivity of the bacterial/mucin interactions which is possibly related to the specific glycan composition present in the colonic mucosa. *Shigella* binds with high affinity to the heavily glycosylated mucins isolated from the human colon, as opposed to those from the guinea pig (less binding) and rat (no binding).[16] In addition, the inner mucus layer contains antimicrobial peptides (AMPs), which are important mediators in keeping the epithelial lining sterile. Interestingly, AMPs also underlie species-specificity presumably having adapted to the diverse ecological niches inhabited by mammals and the multitude of microbial challenges faced within.[18] Here, the rate of similarities and differences across species are also dependent on the type of AMPs. For instance, divergence exists for α-defensins when comparing mouse and human,[18]

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while in contrast Rhesus monkey β-defensins and cathelicidins are close homologs to the human molecules.[19] Considering this homology, the specific down-regulation of cathelicidin and human β-defensin 1 expression by *Shigella* during natural and experimental infection which contributes to bacterial survival in the intestine[20,21] might also happen in Rhesus monkeys, thus contributing to their susceptibility to *Shigella* oral infection.

Another key point in the inter-species variation of innate immune system components includes the sensing of microbial associated molecular patterns (MAMPs) by host pattern recognition receptors (PRRs). PRR activation triggers the transcription of IL-8 (also known as CXCL-8), which serves as a potent chemoattractant responsible for the recruitment of polymorphonuclear neutrophils (PMNs), a hallmark of *Shigella* infection in humans. In contrast, mice, which show resistance to oral infection by *Shigella* fail to elicit PMN recruitment to the intestinal mucosa. However, if infection is performed together with recombinant human IL-8, PMN infiltration and subsequent mucosal inflammation and invasion occur.[22] This suggests that murine resistance to *Shigella* infection might be related to a defective pathogen sensing, needed for the initiation of an inflammatory response, which promotes the disruption of the epithelial integrity and further bacterial translocation and epithelial invasion.[23–25] In other words, one might speculate that suboptimal MAMP/PRR interactions are involved in the lack of inflammation observed in response to *Shigella* infection in the mouse. Of note, in guinea pigs on the contrary, intrarectal administration of a dose of 10^9 *Shigella* induces rectocolitis accompanied by diarrhea similarly to human disease, suggesting for further differences between mice and guinea pigs in triggering gut inflammation.[26] Important is the notion of organ-specificity for such a differential bacterial sensing, since mice infected intranasally with *Shigella* develop an acute pulmonary infection characterized by a massive PMN infiltration with extensive tissular destruction.[27,28]

In summary, inter-species differences in key components of the human innate defense barrier present throughout the gastrointestinal tract including the colon are likely to reflect the discrepancies in the human susceptibility to *Shigella* infection *versus* the reduced sensitivity or resistance observed in non-human primates, guinea pig or mouse infection models.

T3SS-mediated subversion of innate immunity: a brief reminder

The main weaponry of *Shigella* to dampen host defenses, *i.e.* the type three secretion system (T3SS), has been extensively studied and its composition and structure, as well as regulation and mode of action have been recently reviewed.[29–31] In brief, the T3SS mediates the delivery of virulence effectors from the bacterial cytoplasm into the targeted host cell. It is composed of a type three secretion apparatus (T3SA), the secreted effectors, translocators, chaperones and transcription regulators, which are all encoded on the *Shigella* virulence plasmid.[32] The syringe-like cylindrical T3SA spans both bacterial membranes and is composed of the protruding needle complex supported by the basal body.[33] The assembly of the T3SA is temperature-dependent and only triggered at 37°C,[34] the human body temperature, in order to keep the energetic costs related to its expression to a minimum. After its assembly, the T3SA remains inactive, *i.e.* translocators and several effectors remain associated to chaperons in the bacterial cytoplasm, until sensing of the host cell membrane occurs at the needle tip complex.[35] This acts as activation signal[36] and initiates the secretion of the stored translocators and effectors into the host cell cytoplasm[37-39] and additionally triggers the transcription of genes encoding for a second set of bacterial effectors. After host cell invasion and vacuolar escape, cytosolic *Shigella* down-regulate the T3SA activity[40,41] in order to replenish the bacterial effector pool required for its subsequent dissemination into neighboring cells.[41–43]

It is now well-established that *Shigella* T3SS effectors, similarly to those of other T3SS-carrying enteropathogens, target key cellular pathways of enterocytes and gut resident macrophages, leading to the modulation of important host cell functions.[44] Numerous *Shigella* T3SS effectors, listed in recent reviews,[44–46] have been shown to affect signaling pathways involved in host cell actin cytoskeleton dynamics, trafficking, cell viability, and NF-κB-mediated inflammatory pathways. The system is amazingly efficient. On one hand, one given effector can ensure a diversity of actions towards different cell types while infection proceeds. On the other hand, different effectors can target multiple host proteins in a given pathway. In addition, effectors with antagonistic effects towards a given pathway ensure a timely efficient modulation of host innate immune responses as infection proceeds.[44–46] In a nutshell, as depicted in Figure 1, upon crossing the colonic mucosa, *Shigella* exerts a multitude of deregulatory mechanisms leading to the initiation of a pro-inflammatory milieu, accompanied by massive immune cell death, including macrophage pyroptosis, as well as facilitating its intra-epithelial survival and spreading along the colonic epithelium.

T3SS-mediated subversion of adaptive immunity: an as of yet underestimated implication

Upon crossing of the epithelial barrier, *Shigella* encounters *lamina propria* cells responsible for the priming of the adaptive immunity, which include dendritic cells (DCs), B and T lymphocytes. In addition, interactions with these cells may also occur in the lymphoid structures that are associated with the intestinal mucosa. In experimental infection, *Shigella* has been shown to reach the mesenteric lymph nodes (P. J. Sansonetti, personal communication), which constitutes the end of its journey since systemic dissemination is usually not observed, except for rare cases of immunocompromized, malnourished infants in endemic settings.[47]

Immunity to *Shigella* is characterized by a humoral response mediated by mucosal sIgAs and systemic IgGs directed against the LPS *O*-antigen and some other bacterial molecules such as the Invasion plasmid Antigens (Ipa) proteins.[48–50] Antibodies directed towards the LPS *O*-antigen are associated with protection in cohort studies.[50] The composition of the *O*-antigens differs greatly across strains and serotypes, accounting for the serotype-specificity observed in antibody-mediated protection.[28,51]

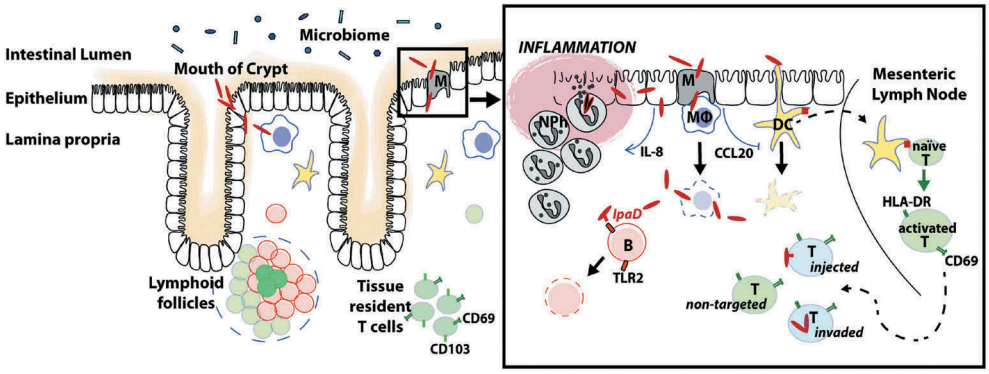

Figure 1. Model of *Shigella* pathogenicity.

Note. After its successful journey through the gut, passing a diversity of luminal innate immune components, *Shigella* eventually reaches the colonic epithelium. The apical pole of colonocytes is commonly regarded as resistant to invasion.[102] Instead, *Shigella* crosses the epithelial barrier *via* M cells[103] or *via* colonocytes located at the mouth of the intestinal crypts, which has recently been described as alternative route of entry.[104] In case of the latter, it was hypothesized that physical forces employed by the gut peristalsis facilitate invasion by entrapping bacteria at the crypt opening and thus enforce entry of the colonocytes in close proximity. Independently of its mode of invasion, *Shigella* subsequently arrives in the subjacent *lamina propria*, which is densely populated with immune cells involved in the priming of both innate and adaptive immunity. The pro-inflammatory environment induced by *Shigella* in the colon is correlated with a massive cell death as observed in biopsies obtained from infected individuals.[49,55,105] *Shigella* evades from macrophage-induced killing *via* the induction of pyroptosis, a caspase-1 dependent cell death associated with the release of the pro-inflammatory cytokines IL-1β and IL-18.[106–108] Escaping from the dying macrophage, *Shigella* invades enterocytes from their basolateral side and spreads throughout the epithelium.[42,109–111] Intracellular bacteria and their T3SS effectors, influence the innate inflammatory response by mobilizing a cascade of immunomodulatory processes in a time-dependent manner (reviewed in Ref[44]). A few hours post-infection, mucosal invasion progresses and is further exacerbated due to successive epithelial damage mediated by the arrival of PMN[25] following the chemoattractant, IL-8.[57,112–114] While PMN-induced tissue damage is thought to facilitate bacterial colonization, invasion and multiplication during the early phases of bacterial infection, *Shigella* is unable to counteract the antimicrobial activity of these professional phagocytes and is ultimately eliminated.[115,116] *Shigella* impairs adaptive immune cell priming by hampering epithelial CCL20 secretion and the subsequent recruitment of dendritic cells (DCs).[21] Antigen-presenting DCs migrate to the mesenteric lymph nodes where they prime naïve T cells. These activated T lymphocytes then home back to the *lamina propria* where they render targetable by *Shigella via* injection-only or invasion mechanisms.[65] Finally, *Shigella* also inhibits B lymphocyte function by the induction of apoptosis *via* the T3SS needle-tip effector IpaD.[66]

Shigella antigen-specific B memory cells can be primed and are associated with decreased disease severity in subjects challenged with *Shigella*. [52]Nevertheless, the establishment of a protective adaptive immunity requires several rounds of infection and is only of short-term duration.[50,53,54] Interference with the innate and adaptive immune response during primary infection has been associated with the higher susceptibility of children to shigellosis, as compared to adults.[55,56] Evidence indicates that both the induced pro-inflammatory response and a direct targeting of DCs, B and T lymphocytes might contribute to an inefficient priming of the adaptive immune response upon infection. *Shigella* reprogramming of gene expression in infected enterocytes towards a pro-inflammatory profile, includes the down-regulation of CCL20 production, a chemokine mediating DCs recruitment.[57] Accordingly, in an experimental model of infection, a reduced recruitment of DCs is observed towards the *lamina propria* of animals infected with invasive *Shigella*, as compared to those infected with non-invasive *Shigella* harboring a non-functional T3SS.[21] In addition, DC death occurs within a couple of hours of *Shigella* infection *in vitro* as a response to host cell caspase activation.[58] Whether recruited and gut resident DCs are susceptible to bacterial killing during the natural infection remains to be established, however the massive cell death observed in patient biopsies are supportive of this notion.[55] Another link between the host innate and adaptive immune responses is provided by the induction of *Shigella*-specific Th17 cells,[59] as a result of the pro-inflammatory environment induced upon infection in the mouse pulmonary model of infection.[60,61] This might reflect what happens in the gut, since Th17 cells are a lineage of T helper cells abundantly present in the gut mucosa and defined by the secretion of IL-17, an important cytokine in the host immune response towards bacterial pathogens.[62] IL-6, which is significantly induced upon *Shigella* infection, contributes to the induction of Th17 cells while blocking the development and function of regulatory T cells (T_{reg}).[63] Noteworthy, antigen-specific $CD8^+$ T lymphocytes fail to be primed during *Shigella* infection *in vivo*,[59,64] suggesting that specific $CD8^+$ T cell-mediated killing of invaded enterocytes is likely to be impaired.

Evidence for the direct targeting of human lymphocytes by *Shigella* has been provided in the last years, predominantly conducted by our laboratory. Historically, *Shigella* is regarded as primarily intracellular bacterium able to invade a large diversity of cells following the injection of T3SS effectors *in vitro*. We recently showed that the injection of T3SS effectors does not inevitably result in cell invasion.[65] Indeed, using an optimized T3SS injection reporter, we demonstrated that effector injection without subsequent cell invasion, termed the "injection-only" mechanism, is the main route of lymphocyte targeting utilized by *Shigella*.[65] *In vitro*-activated human peripheral blood B, $CD4^+$ T, and $CD8^+$ T lymphocytes, as well as switched memory B cells, are primarily targeted by the injection-only mechanism, as are B and T lymphocytes extracted from human colonic tissue, *i.e.* representing the *lamina propria*-residing cells encountered by *Shigella* in the gut. These findings reveal that through this mechanism, *Shigella* targeting can be extended to a large diversity of host cells, including those refractory to invasion.[65] Furthermore, B cells were shown to undergo apoptosis after the activation of a TLR2-dependent signaling pathway triggered by the T3SS needle-tip protein, revealing a *Shigella* targeting mechanism that is independent of both invasion and T3SS effector injection.[66] Consequently, we propose an additional mode of *Shigella* pathogenicity, termed the "kiss-and-run" strategy, describing the ability of *Shigella* to target cells *via* a T3SS-dependent contact, which results in either effector interaction

with cell-surface receptors or effector delivery into the host cells not followed by cell invasion. These findings highlight an as of yet underestimated facet of *Shigella*'s extracellular mode of action.

Another interesting aspect is the observation that only activated human $CD4^+$ T lymphocytes are found targeted by *Shigella*, as opposed to non-activated cells. Investigating the underlying molecular mechanism, we demonstrated that non-activated human $CD4^+$ T lymphocytes, which are refractory to *Shigella* invasion and effector-injection, become susceptible to targeting upon loading of their plasma membrane with sialylated glycosphingolipids (gangliosides) that are abundantly present in activated cells.[67] Interactions between the sugar polar part of gangliosides and the polysaccharide moiety of *Shigella* lipopolysaccharide (LPS) promote bacterial binding resulting in the injection of T3SS effectors. While interactions of surface glycans has been previously reported,[68] these findings suggest that bacterial-host glycan interactions are important for *Shigella* pathogenesis by driving selective interactions with host immune cells. This study also provides a new cell adherence mechanism available to *Shigella* and maybe other pathogens, which are devoid of adhesins and therefore rely on alternative strategies to effectively bind to the host cells. In this context, various molecules promoting *Shigella* binding to enterocytes have been identified including the human enteric α-defensin 5 (HD5),[69] the virulence T3SS factors OspE1/E2 and IcsA acting as adhesin-like molecule following bile salt exposure.[70,71] Therefore, beyond its potential importance in driving host specificity, *Shigella* binding to specific cell surface glycans is likely to direct selective targeting of the different T lymphocyte subsets and, more globally, the wide-range of mucosal immune cells with their specific membrane glycosylation patterns.[72]

The so far only example of the outcomes of direct targeting of human T lymphocytes by *Shigella* is the demonstration of the impairment of activated $CD4^+$ T cell dynamics and migration, both *in vitro*[73] and *in vivo*,[74] which is mediated by the T3SS effector IpgD.[73] IpgD is a phosphoinositide 4-phosphatase mediating hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2). In T lymphocytes, the IpgD-mediated reduction of the pool of PIP_2 at the plasma membrane leads to dephosphorylation of the ERM proteins and their inability to relocalize at one T cell pole upon chemokine stimulus, likely affecting the formation of the polarized edge required for cell migration.[73] The impact on other key T cell functions such as synapse formation and subsequent activation are currently under investigation. An updated model of *Shigella* physiopathology comprising the recent findings on B and T lymphocyte targeting is schematized in Figure 1.

Future perspectives

The molecular mechanisms downstream of *Shigella* lymphocyte targeting and the global role of the injected T3SS effectors on adaptive immunity remain to be deciphered. While the impact of specific immune priming on infection can be accomplished by *in vitro* studies on human *lamina propria* cells, further in-depth investigations of the effector phase and *in vivo* implications are compromized by the previously mentioned lack of a suitable animal model. The use of the controlled human infection model (CHIM) can present an important alternative, as recently exemplified by studying human immune response to *Salmonella* infection.[75–77] However, so far CHIM for *Shigella* are restricted to efficacy tests for vaccine candidates but may present an opportunity to be further exploited.[78,79] Another important point lays in the establishment of adult and infant cohort studies in endemic regions of shigellosis, which especially in the light of now available high throughput analysis tools would certainly provide important insights into human immune responses to natural infection.

In the context of the adaptive immunomodulation and recurrent infections frequently observed in *Shigella* infected individuals, further insights into the underlying mechanisms are needed which include a better understanding of the adaptive immune cell subsets that are targeted as well as the specific T3SS effectors involved and the thereby affected host signaling pathways. Of particular interest here is the analysis of the vast diversity of immune cells present in the human colonic mucosa at a single-cell level. It is important to note that most experiments conducted so far are based on either T cell-lines or peripheral blood lymphocytes, which do not represent the entire spectrum of lymphocyte subsets encountered by *Shigella* in the colonic *lamina propria*. Based on human peripheral blood mononuclear cells (PBMCs), T lymphocytes are classified into $CD45RA^+$ naïve and $CD45RO^+$ memory T cells, the latter being further subdivided into central memory T cells (T_{CM}) or effector memory T cells (T_{EM}) according to the presence or absence of the lymphoid homing factor CCR7 and CD62L on the cell surface, respectively.[80] However, more recent publications highlight the existence of a non-circulating, tissue resident memory T cell (T_{RM}) population that constitutively express the integrin CD103, while being devoid of lymphoid homing factors.[80,81] Analysis of various tissue samples obtained from healthy individuals confirmed the presence of these specific T_{RM} cells in all mucosal sites and in particular in the colon, while being completely absent in circulating PBMCs.[81] Colonic T_{RM} cells are predominantly of the $CD8^+$ resident effector type as identified by the constitutive expression of $CD69^+$ in the absence of CCR7, and play an important role in the local protective immunity to infection.[80,82] Of particular interest in the light of our previous findings highlighting *Shigella* preferential targeting of activated T cells, is the "active state" of colonic T_{RM} cells, as judged by the constitutive surface expression of the activation marker CD69.[80] Therefore, it would be meaningful to investigate *Shigella* interactions with this specific non-circulating T_{RM} subset and to assess its implication on the local immunity. Another interesting point to be considered for further investigation is the observed effect of *Shigella* on the host cell metabolism by rerouting the metabolic output of intestinal epithelial cells after invasion to facilitate its energetic demands.[83] Interestingly, metabolic programming is also implicated in the switch from effector to memory T cell phenotype and therefore play an important role in the establishment of long-term immunity.[84]

Implications for vaccine development

The new paradigm of *Shigella* pathogenicity including the novel targeting mechanisms of human lymphocytes has

implications on the different *Shigella* vaccine strategies that are currently under investigation. One strategy relies on the use of orally-administered, live, rationally attenuated vaccine candidates for which T3SS activity toward B and T lymphocytes has been so-far-underestimated. The latest developed candidates of this type[85] have yet been rationally attenuated on the bases of virulence knowledge (i.e. alteration of metabolic functions essential for in vivo growth, of dissemination capacities in invaded tissues) that preceded recent deciphering of *Shigella* immunosuppressive capacities. In consequence, these candidates are likely to express a functional T3SS and secrete its dedicated effectors, thus retaining their ability to subvert human lymphocyte functions. As a matter of fact, although some of these candidates have shown decent mucosal and systemic immunogenicity in phase I-II studies, including significant protection against dysentery in a challenge study carried out in Western volunteers,[86] the performance of these candidates were far less promising in phase I-II studies carried out in endemic areas.[87] These latter studies pointed to the issue that colonization by these candidates was insufficient in level and duration in order to achieve sufficient and reproducible protective mucosal immunogenicity. This poor colonization capacity in endemic zones may be linked to several factors including a colonization barrier effect of the resident intestinal microbiota keeping attenuated candidates at bay and leading to minimal engagement with the epithelial surface and its associated immune system. Hence the joined action of persisting immunosuppressive properties and deficient colonization performance possibly prevents efficacy of current live-attenuated candidates. Trying to address these combined issues and rationally design a new generation of candidates is not impossible but sets substantial challenges and must address two main questions: (i) the mutation, both individually and collectively, of several immunosuppressive effectors and their global assessment, (ii) the yet poorly identified parameters involved in colonization and interaction with the microbiota barrier. All this has to be achieved in the context of a human-specific disease and under consideration of the low predictive value of animal models. Considering the limited resources available for research of neglected infectious diseases such as shigellosis, the time and funding scale to achieve the goal of a tetravalent vaccine on such basis seems rather challenging.

Besides orally administered, live, rationally attenuated vaccine strains, a different approach relies on the development of parenterally delivered subunit vaccines. This strategy is based on the notion that specific bacterial surface polysaccharides, *i.e.* the *O*-antigen of membrane lipopolysaccharide (LPS), are the primary targets of the antibody response associated to protection against homologous re-infection.[50] Among the different vaccine candidates,[85] only the glycoconjugate vaccine approach incorporating detoxified LPS, *i.e.* the *O*-antigen conjugated to a carrier protein, has reached phase III clinical trial testing. In particular, along with safety, protective efficacy was shown with a *S. sonnei* glycoconjugate in adults and children above, but not below three years of age.[88] Based on these promising results, several candidates are now under development. Bioconjugate vaccines, sourced from gene-edited *E. coli*, have the benefit of an easy and large-scale production and have been shown to be safe and immunogenic.[89,90] On the other hand, synthetic molecular glycovaccines also bear advantages for they are rationally designed and fully defined. Such a glycoconjugate vaccine incorporating a synthetic oligosaccharide mimicking the *O*-antigen of *S. flexneri* 2a has been shown to be safe, well tolerated and immunogenic in healthy adults[91] (Cohen D. et al., in preparation). Along with the GMMA (Generalized Module for Membrane Antigens) approach based on the use of bacterial outer membrane particles,[92,93] these three strategies are currently supported for further clinical development by the Bill and Melinda Gates Foundation[94] and the Wellcome Trust.[95] The aim is to develop a tetravalent vaccine against *S. flexneri* 2a, 3a and 6, and *S. sonnei* to cover, with some expected cross-protection, about 90–95% of the circulating *Shigella* strains. Hopefully, with the current support from funding agencies in advancing clinical studies on the already available tetravalent bioconjugates and GMMA candidates,[94–97] a tetravalent *Shigella* vaccine might be available at the horizon 2028. Preventing shigellosis remains more than ever a public health priority.[98–100] Indeed, as recently reviewed,[101] there is a growing appreciation for the role of vaccines in confronting the problem of antimicrobial resistance, which includes the alarming emergence of multi-drug resistant *Shigella* strains.[98]

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed

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