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REVIEW

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Development of an enterotoxigenic *Escherichia coli* vaccine based on the heat-stable toxin

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ABSTRACT

Infection with enterotoxigenic *Escherichia coli* (ETEC) is an important cause of diarrhea-related illness and death among children under 5 years of age in low– and middle-income countries (LMIC). Recent studies have found that it is the ETEC subtypes that produce the heat-stable enterotoxin (ST), irrespective of whether they also secrete the heat-labile enterotoxin (LT), which contribute most importantly to the disease burden in children from LMIC. Therefore, adding an ST toxoid would importantly complement ongoing ETEC vaccine development efforts. The ST's potent toxicity, its structural similarity to the endogenous peptides guanylin and uroguanylin, and its poor immunogenicity have all complicated the advancement of ST-based vaccine development. Recent remarkable progress, however, including the unprecedented screening for optimal ST mutants, mapping of cross-reacting ST epitopes and improved ST-carrier coupling strategies (bioconjugation and genetic fusion), enables the rational design of safe, immunogenic, and well-defined ST-based vaccine candidates.

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Introduction

Infection with enterotoxigenic *Escherichia coli* (ETEC) is one of the major causes of diarrhea-related illness and death among children under 5 years of age in low- and middle-income countries (LMIC).[1–3] These very common infections are estimated to cause some 25,000 child deaths annually and contribute to child malnutrition.[1] Besides, ETEC is the leading cause of diarrhea among tourists[4,5] and military personnel[6,7] staying in ETEC endemic regions. Deaths associated with ETEC infection are declining worldwide; nevertheless, morbidity remains high.[1] Notably, children who survive repeated diarrheal episodes have a higher risk of long-term sequelae, including impaired cognitive development, growth faltering, and obesity.[8] Collectively, these short and long-term negative impacts of ETEC infection underline the need for effective interventions.

Protection against ETEC colonization and diarrhea through the use of effective vaccines appears to be the most promising way forward.[2] There are, however, no effective vaccines against ETEC at present. Currently, multiple efforts, including the development of whole-cell and subunit vaccines, are underway to develop a broadly protective ETEC vaccine.[9] The more advanced candidates include the inactivated whole-cell (ETVAX)[10] and the live-attenuated ACE527[11] vaccines. Recent studies confirm our earlier work[12] that ETEC strains producing the heat-stable enterotoxin (ST), with or without the heat-labile enterotoxin (LT), are strongly associated with diarrhea among children under 5 years of age,[3,13] while the strains that only produce LT contribute only marginally. This underpins the importance of including an ST toxoid in vaccine formulations to achieve a broad protection against ETEC.[9] Herein, we review the recent advances in ETEC subunit vaccine development, with an emphasis on the approaches that target ST.

Mechanisms of human ETEC-mediated diarrhea

Human ETEC is comprised of genetically diverse stains,[14] but they share the ability to colonize the small intestine where they secrete the plasmid-encoded ST and/or LT. Either of these potent toxins can cause diarrhea individually, but when present together, they appear to act synergistically.[15] Following an infection through the fecal-oral route, ETEC utilizes surface exposed adhesins, called colonization factors (CFs), to colonize the small intestine and elaborate the toxins. The CFs consist of repeating protein subunits and can be fimbrial, helical or fibrillary structures.[16]

LT is a heterohexameric protein containing one A subunit (LTA) and five identical B subunits (LTB), and it is structurally and functionally related to the cholera toxin.[17] ST exist in two nearly identical variants, STa I and STa II, which are commonly called STp (18 amino acids) and STh (19 amino acids), where p and h refer to their initial discovery in ETEC isolated from pigs and humans, respectively.[18] While ETEC producing either STh or STp can induce diarrhea in humans,[12,19,20] the STh-producers contribute with the bulk of the diarrheal disease burden in LMIC children.[12,21] ST and LT induce diarrhea by binding to their cognate receptors, the transmembrane guanylate cyclase C (GC-C) and GM1-ganglioside on the epithelial cell surface, respectively,

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thereby triggering a cascade of signaling pathways that disrupt the electrolyte and fluid homeostasis, ultimately leading to secretion of water and electrolytes into the intestinal lumen. While GM1 is the primary receptor for LT, secondary receptors, including blood group antigens, Toll-like receptor 2 and additional gangliosides or ganglioside derivatives have been reported.[22,23] Guanylin and uroguanylin are endogenous ligands that act on GC-C to regulate fluid and electrolyte secretion into the intestinal lumen. Interestingly, ST also appears to modulate the immune system of the host, thereby preventing a strong immune response against the infection.[15] Likewise, exposure to LT has been shown to downregulate expression of human antimicrobial peptides.[24]

Structure and function of the GC-C receptor ligands

The ETEC heat-stable toxins were discovered already in the 1970s[25] and shown to cause increased levels of cGMP in intestinal cells.[26] In 1990, the GC-C receptor was shown to be responsible for the ST-induced increased levels of intracellular cGMP,[27] and a few years later the endogenous peptides guanylin[28] and uroguanylin[29] were discovered.

GC-C is a heavily glycosylated transmembrane protein consisting of an extracellular receptor domain (ECD), a single transmembrane helix, a kinase homology domain, and a catalytic GC domain.[18,30] Although the structure of GC-C has not been solved, homology modeling suggests that the receptor may form oligomers and that the membrane-proximal subdomain of ECD appears to contain the binding site for ST and the endogenous ligands.[18,31,32] An additional receptor for guanylin and uroguanylin has been identified, namely GC-D, which is exclusively expressed in olfactory neurons.[33,34] In kidneys, however, guanylin and uroguanylin act primarily through GC-C independent receptors that are not yet identified.[35]

The structure of a fully toxic truncated synthetic STp analog, where C5 was substituted with β-mercaptopropionic acid, was solved by X-ray crystallography in 1991.[36] The STp analog covers the 13-amino acid *toxic domain*, from the first (C5) to the last (C17) cysteine residues. The solution structures of guanylin[37] and uroguanylin[38] were solved later by NMR, demonstrating striking structural similarities to each other and to STp (Figure 1A). These three experimental structures were used as templates to build structural models for the full-length STp and STh peptides[39] (Figure 1A), which are useful when comparing the structural properties of the four GC-C ligand peptides. The guanylin and uroguanylin peptides have two disulfide bridges that define their structures (1–3/2–4 pattern, Figure 1B). Interestingly, these peptides can form two topological isomers that can dynamically interconvert.[37,38] Isomer A of guanylin and uroguanylin are biologically active and their structures resemble those of the

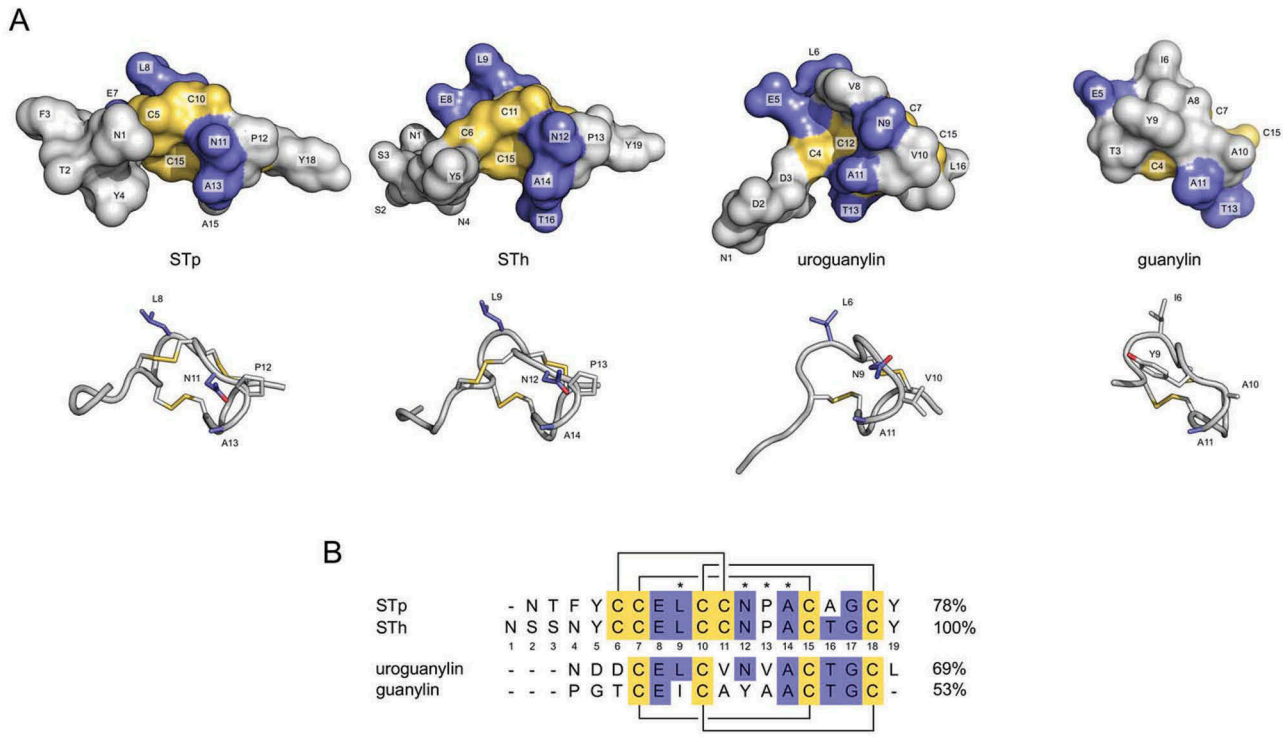

Figure 1. Structural models and sequence alignment of STp, STh, uroguanylin and guanylin. A) Surface (top) and cartoon (bottom) representation of structural models of STp[39] and STh[39] and NMR structures of uroguanylin (PDB accession no. 1UYA) and guanylin (PDB accession no. 1GNA). Each residue is named using the one-letter amino acid code and numbered according to the individual peptide sequence. The first two N-terminal residues in guanylin are largely unstructured in solution, and thus only the structure of the 13 C-terminal residues was solved by NMR.[37] The disulfide bridge-forming cysteines are shown in yellow, and other residues shared between at least three of the peptides are shown in blue. In the cartoon representation, the disulfide bridges are shown as sticks with sulfur atoms in yellow. The receptor-interacting residues of the ST peptides, and the equivalent residues of uroguanylin and guanylin, are also shown as sticks with oxygen atoms in red and nitrogen atoms in dark blue. B) Sequence alignment of STp, STh, uroguanylin, and guanylin. The residues are colored as described for the structures: cysteines in yellow and residues shared between at least three peptides in blue. The disulfide bridge connectivity for STp and STh is shown above the alignment, and that of uroguanylin and guanylin is shown below. Note that all peptides share two of the disulfide bridges. The receptor-interacting residues are marked with asterisks, and the residue numbers of STh are shown below the STh sequence. Sequence identity (percentage) relative to STh is shown to the right of the alignment.

ST peptides (Figure 1A). Isomer B, on the other hand, are unable to activate the GC-C receptor. The ST peptides share the two disulfide bridges of the endogenous peptides (2–5/3–6 pattern, Figure 1B), but has an additional disulfide bridge (1–4 pattern, Figure 1B) which apparently locks the ST structures in the active topological from and may explain why ST has a higher affinity to the GC-C receptor than the endogenous peptides.[18,40,41]

The structural similarities between the four peptides are also reflected in their sequences (Figure 1B). STh and STp are very similar and share 13 of their 19 and 18 residues, respectively (78% sequence identity), and only one residue is different inside the toxic domain (STh: T16; STp: A15). The endogenous peptide which is most similar to the ST peptides is uroguanylin, which shares 11 residues with STh (69% sequence identity). Guanylin is less similar but shares 8 residues with STh (53% sequence similarity).

There is no direct structural evidence of the interaction between GC-C peptides and the GC-C receptor, but several mutational studies performed in the 1990s and 2000s on STh and STp have resulted in the identification of residues important for function.[42–44] These studies mainly targeted the residues N12, P13, and A14 (STh residue numbering is used here and throughout the review), all of which appeared to be important for the biological activity of the ST peptides, and were labeled *receptor-interacting residues*. We recently published the most comprehensive mutational study of STh to date, where all possible 361 single amino acid substitutions of STh were screened for effects on toxicity and antigenicity.[45] This study confirmed the functional importance of N12, P13, and A14, but additionally identified L9 as a novel receptor-interacting residue (Figure 1A). The A14 residue has the most compelling evidence for being directly involved in the interaction with the receptor, as 17 of 19 possible substitutions in this position leads to dramatically reduced biological activity.-[45] Even the two least dramatic substitutions lead to around 10-fold reduction in biological activity,[43,45] despite entailing the minor changes of either removing the methyl side chain of alanine (glycine mutation), or the addition of a hydroxyl group to the alanine side chain (serine). The importance of the A14 residue for a biological function is further corroborated by the fact that A14 is the only receptor-interacting residue that is conserved in all four GC-C peptides (Figure 1).

With the exception of P13, all the receptor-interacting residues identified in the ST peptides are shared with uroguanylin (Figure 1). Other residues that are shared between STh and uroguanylin are E8, T16, G17, and the four cysteine residues C7, C10, C15, and C18. The extent of shared residues explains the observed immunological cross-reaction between anti-STh antibodies and uroguanylin,[39,45] and underpins the importance of addressing unwanted immunological cross-reaction when constructing and evaluating vaccine candidate ST toxoids.

Recent updates on CF-based ETEC subunit vaccine development

Subunit vaccines have the potential of allowing the development of safe and well-defined vaccines containing only the desired antigens leading to targeted immune responses towards specific epitopes.[46] They further allow exclusion of parts of antigens that may cause unwanted immunological cross-reactions or allergenic and reactogenic responses.[46,47]

In a response to infection with ETEC, the human body normally elicits antibody responses against the CFs and LT.[48,49] Therefore, canonical approaches for ETEC vaccine development have primarily been focused on engendering protection against the CFs and LT.[11,50,51] Unfortunately, CF- and LT-based vaccines have not yet been shown to confer adequate protection, even in travelers, in whom ETEC that express only LT is indeed an important contributor to diarrhea.[51–53] As mentioned above, because such strains do not contribute importantly to the diarrheal disease burden in LMIC children, it is inappropriate to only aim for anti-LT immunity. This challenge could be circumvented by engendering immune responses to important somatic antigens on ETEC strains that express STh. Thus, despite there being no evidence that CFs induce anticolonizing immunity after natural ETEC infections,[54] important efforts are being made to develop a vaccine which induces immune responses against the major CFs, i.e. those on ETEC that account for the bulk of ETEC diarrheal disease burden. However, this vaccine development strategy is complicated by the fact that human ETEC may produce one or more of over 25 immunologically distinct CFs,[16] and it is likely that new CFs will be discovered.[14] Recently, some level of cross-protection was reported.[10]

To overcome the challenges posed by the heterogeneity of CF, recent subunit vaccine approaches have employed multi-epitope fusion antigen (MEFA) technology to integrate epitopes from CFs that are produced by strains most commonly found associated with moderate and severe diarrhea.[55] Accordingly, *in silico* predicted B-cell epitopes from CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS21, and EtpA were inserted into the backbone of the colonization factor antigen I (CFA/I) tip subunit CfaE where they replaced surface exposed, but less antigenic peptides.[56] Notably, mice immunized with the chimeric antigen elicited antibodies against all the nine epitopes, and the sera prevented the attachment of ETEC strains to Caco-2 cells *in vitro*.[56] MEFA CFs were further genetically fused to an ST toxoid and LT antigen to afford even broader protection.[57,58] Immunized mice that were challenged with lethal doses of ETEC (CFA/I^+, LT^+, ST^+) were protected.[58] Collectively, these results are encouraging, and future ETEC challenge studies will determine whether multiepitope fusions may provide broad protection against ETEC diarrhea in humans.

Generally, a multivalent subunit vaccine that targets different stages of the ETEC infection process, including mucin degradation, adhesion and toxin neutralization, may lead to a broadly protecting ETEC vaccine. In this regard, the recently identified novel and fairly conserved protective antigens, including YghJ, EtpA, EatA, EaeH, and flagellin[59] could be exploited for vaccine development, whilst evaluating the risk that YghJ, EaeH and flagellin-based vaccines may pose to the *E. coli* commensals, since these antigens are often also produced by commensal *E. coli*.

Strategies for designing ST-based subunit vaccines

The ST amino acid sequences are highly conserved (Figure 1B)[60] and STh-producing ETEC, whether they also produce LT or not, contribute to the bulk of diarrhea in LMIC children.[3,13,21] In addition, ETEC that produce STp and/or LT contribute to diarrhea in travelers and may cause outbreaks.[19] Taken together, this makes it important to also target ST when developing a broadly protective ETEC vaccine. Nonetheless, the *poor immunogenicity* of ST, its *potent toxicity*, and its potential for *immunological cross-reactivity* with the human gastrointestinal peptides (guanylin and uroguanylin) have so far hampered the advancement of an ST-based vaccine.[39,42] The development of an ST-toxoid for inclusion in a broadly protective ETEC vaccine thus needs to overcome these three challenges, while ensuring that the ST toxoid engenders antibodies that effectively neutralize STh, and preferably also STp.

Making ST immunogenic

Natural ETEC infections do not appear to induce an immune response against ST. This is presumably due to the small size of ST (~ 2 kDa) as small molecules called haptens (incomplete antigens) are generally poor immunogens.[61] To elicit anti-ST antibodies, ST has commonly been coupled to an immunogenic protein (carrier protein) through chemical crosslinking (bioconjugation)[62,63] or genetic fusion.[64] Both approaches have been effective in eliciting antibodies against ST. Efforts have also been made to develop ST-based vaccines without using carrier proteins. Encouraging results have emerged from experiments with ST polymerization using glutaraldehyde,[65,66] ST coupled to a poly lactic-co-glycolic acid (PLGA) nanoparticle,[67] and ST coupled to a lipopeptide module (T-helper cell epitope and S-[2,3-bis (palmitoyloxy) propyl] cysteine).[68]

Abolishing ST toxicity

ST's toxicity is significantly reduced or abolished when conjugated[63] or genetically fused[69] to carrier proteins. However, the reversibility of some conjugation reactions[70] or proteolytic activity[71] in the vaccine recipients after oral delivery could potentially release the native toxin. Therefore, detoxifying mutations are required to ensure a safe ST-based vaccine. But due to its small size, ST has a limited repertoire of epitopes, and mutations must be carefully chosen to avoid disrupting protective epitopes or forming neo-epitopes.

Minimizing immunological cross-reactivity with guanylin and uroguanylin

The endogenous peptides guanylin and uroguanylin play key roles in regulating electrolytes and fluid homeostasis in the human intestine via GC-C, which is the primary receptor for guanylin and uroguanylin.[27,72] Although GC-C is predominantly expressed in the intestines,[73] transcripts have also been detected in kidneys, adrenal gland, brain, lung and reproductive organs,[33,34,74,75] suggesting crucial roles played by the GC-C ligands in the human physiology. Recently, there is a renewed interest in these peptide hormones as potential therapeutic targets for obesity, ulcerative colitis, and metabolic diseases.[75–77]

Given their key roles in human physiology, their resemblance to ST (Figure 1A) raises a safety concern that anti-ST antibodies elicited by ST-based vaccines may cross-react with these endogenous peptides. Indeed, it has already been shown that anti-STh antibodies can cross-react with uroguanylin, albeit only partially and with lower affinity.[39] Hence, one or more mutations may also be needed to minimize the risk of engendering antibodies that cross-react with the endogenous ligands.

In the following, we review recent efforts to address the three challenges, focusing on two strategies for making ST immunogenic by coupling it to a protein carrier, and on abolishing toxicity and reducing the risk for unwanted immunological cross-reaction by mutation.

Making ST immunogenic by coupling it to a protein carrier

Protein carriers provide the epitopes needed for recognition by T-helper cells in genetic fusions or chemical conjugates, thereby enabling the induction of high-affinity antibodies and immune memory also to peptide haptens.[46,78] Carrier proteins to which ST has been coupled include the porcine immunoglobulin G, bovine serum albumin (BSA), cholera toxin B (CT-B), outer membrane OmpC, major subunit ClpG of *E. coli* CS31A fimbriae, *Salmonella* flagellin, and green fluorescent protein.[42,79,80] In addition, LT and LT derivatives have been widely used with the rationale that broad protection can be achieved against ETEC strains producing either or both enterotoxins.[62,63,69,81,82] To provide a more comprehensive protection, an ST toxoid-LTB fusion has further been genetically fused to ETEC CF antigens or epitopes that elicit anti-adhesin antibodies.[57,58] The non-toxic double mutant LT (dmLT; $LT_{R192G/L211A}$) has also been used as a carrier, offering the additional benefit of engendering neutralizing anti-LT antibodies.[83–85] Additionally, an LT-STh_{P13F} fusion was incorporated into a live attenuated oral *E. coli* vaccine (ZCR533) that constitutively express the antigen on its surface or as a secretion.[86]

ST-carrier genetic fusion

The finding that native ST genetically fused with a carrier protein such as LT could elicit anti-ST neutralizing antibodies was first made in the 1980s.[87] A genetic fusion of an ST toxoid and LTB coupled to a gelatin nanoparticle was shown to protect mice when experimentally infected with ETEC.[88] Genetic fusion approaches to ST-based vaccine development has, therefore, been an important strategy for developing ST toxoid-based ETEC vaccines.[57,81,89–92] Three copies of an STh-N12S mutant genetically fused with dmLT, 3xSTh_{N12S}-dmLT, was identified as a promising vaccine candidate that may engender neutralizing antibodies against both ST and LT.[84,85,92] Notably, piglets born from 3xSTh_{N12S}-dmLT immunized pregnant gilts seemed to be passively protected

from ETEC diarrhea during challenge.[85] The 3xSTh$_{N12S}$-dmLT construct was further genetically fused to seven putative immunodominant colonization factor epitopes from prevalent ETEC strains (CFA/I/II/IV).[57] Interestingly, the expressed single polypeptide immunogen elicited anti-adhesin antibodies against all the seven CFA in addition to anti-ST and anti-LT antibodies in mice.[57,93] The related adhesin-toxoid MEFA (CFA/I/II/IV-3xSTh$_{N12S}$-mnLT$_{G192G/L211A}$) containing epitopes of seven CF adhesins, three copies of STh toxoid and a monomeric LT mutant is currently among the leading ETEC subunit candidates in the preclinical phases,[93] and it remains to be seen whether it can elicit an active protective response in animal and human challenge studies.

The abovementioned recently developed ST fusion constructs were expressed as inclusion bodies in the cytoplasm of *E. coli* and subjected to a simple refolding protocol (Novagen). To our knowledge, the refolded immunogens have not been characterized structurally, and hence, it remains unclear to what extent the individual ST copies have correctly formed disulfide bridges and intact structures. Despite the fact that these fusion immunogens seem to elicit neutralizing anti-ST antibodies, one concern is to what extent that different immunogen batches will have consistent biophysical properties.

ST-carrier bioconjugation

Making ST immunogenic by chemically conjugating it to a protein carrier has two advantages over genetic fusion. The first is that the ST peptides (both native and mutants) can be made either recombinantly or by chemical synthesis,[94] and subsequently be subjected to thorough biophysical characterizations to ensure that protective epitopes are intact. This is important because ST contains conformational epitopes.[95,96] The second advantage is that one can achieve much higher hapten-to-carrier ratios with bioconjugation than genetic fusions. This may be essential for eliciting strong and consistent immune responses. The drawback compared to genetic fusion is that the production procedure is more elaborate.

Chemical conjugation offers a plethora of options for making conjugates. The choice of coupling chemistry will govern the overall structure of the conjugate vaccine as well as how ST epitopes are presented to the immune system. It is important that the chosen coupling method leads to a well-defined conjugate that elicits ST-specific neutralizing antibodies with a high and consistent titer.

The most commonly used chemical crosslinkers for coupling ST to carrier proteins have been the carbodiimides[62,79,97] and glutaraldehyde.[39,63,65] However, these crosslinkers promote polymerization, leading to poorly defined and higher molecular weight conjugates that may eventually precipitate.[98] An improved protocol that involves introducing carboxyl groups on BSA and coupling ST through its N-terminus using dicyclohexylcarbodiimide (DCC) crosslinker has been reported.[80] Alternatively, heterobifunctional crosslinkers (containing different reactive groups on either end) used in a two-step conjugation will allow one to tailor the molecular orientation of ST on the carrier.[98] Additional factors that need to be considered for optimization include the solubility of the resulting conjugate, spacer arm (affects flexibility and steric hindrance), the potential for immunogenicity of the crosslinker, and *in vivo* toxicity.[99]

The way ST is oriented on the carrier may have a profound effect on the immune response to the conjugate. For example, ST coupled to a lipopeptide module via N-terminus elicited better neutralizing antibodies than when coupled via the C-terminus.[68] Likewise, a 19-amino acid peptide (verotoxin 2e) conjugated to ovalbumin through a central amino acid gave higher antibody titers than when the peptide was coupled via terminal residues.[100] ST has three reactive groups that can be used for conventional conjugation: the amino group of the N-terminus, the carboxyl groups of the C-terminus and the glutamic acid residue (E8), and the thiol groups of the cysteines. However, the thiol groups should be excluded to avoid disruption of the disulfide bridges. When producing ST by chemical synthesis it is possible to block certain reactive groups or to introduce non-natural amino acids to enable conjugation through a targeted residue.[101] Interestingly, linking ST with a lipopeptide module via an oxime linkage generated antibodies with a better neutralizing capacity than when thioether linkage was used[68] suggesting that the chemical nature of the linkage could also affect the quality of the immune response to the toxoid.

As mentioned, bioconjugation allows for higher hapten-to-carrier ratios than genetic fusion. However, although higher ratios usually lead to higher antibody titers, it does not guarantee that the antibodies produced will have a higher affinity to ST or be more capable of neutralizing ST. For instance, an increasing ratio of verotoxin 2e to ovalbumin was found to correlate with an increased antibody titer, but the affinity of the antibodies became poorer.[100] Therefore, the ST-to-carrier ratio will require optimization for each vaccine design.

Mutating ST to abolish toxicity and reduce the risk of unwanted immunological cross-reactions

Two safety concerns require that the native ST toxin is modified by mutation, namely to abolish toxicity and to reduce the risk of eliciting antibodies that cross-react with the endogenous peptides. As mentioned, the small size of ST implies that it has a limited repertoire of epitopes, and that mutations must be carefully chosen to avoid disrupting protective epitopes.

In a recent screen of 14 single amino acid ST mutants fused to a dmLT, STh-N12S, STh-N12T and STh-A14H were identified as promising ST toxoids.[92] The STh-N12S seems to be the most promising one, and is the current lead candidate mutation for the LT-based genetic fusions reviewed above.[84,85]

In our mutational study of all possible 361 single-amino acid substitutions of STh, where we screened for effects on toxicity and antigenicity, the top 30 toxoid candidates (based on their antigenicity-toxicity ratios) had mutations either in L9, N12 or A14.[45] Notably, 17 A14 mutation toxoids had undetectable toxicities in the T84 cell assay,[45] suggesting that A14 is the prime residue to target for abolishing toxicity.

The screen further demonstrated that mutations of the structurally important cysteine residues had a profound

impact on both toxicity and antigenicity,[45,102] and that mutations of P13 also, but to a lesser extent, affected both toxicity and antigenicity.[45] Hence, to ensure the formation of ST mutants with a correct structure and intact epitopes, these residues should not be targeted for mutation.

The STh mutant library was also used to map the epitopes of three neutralizing monoclonal antibodies.[45] This led to two key observations: that the L9 residue which is shared with uroguanylin is the main epitope residue of a cross-reacting anti-STh monoclonal antibody and that the main epitope residue of two non-cross-reacting anti-STp monoclonal antibodies was Y19. This implies that L9 is an attractive residue to target for mutation, resulting in mutant variants with both reduced toxicity and risk for cross-reactions to the endogenous peptides. It also implies that Y19 constitutes an ST-specific and safe epitope, which should be left intact and exposed in a vaccine candidate toxoid. This may also explain why ST coupled to a lipopeptide module via the N-terminus elicited better neutralizing antibodies than when coupled via the C-terminus.[68]

The single STh mutant, N12S, and two double mutants, L9A/N12S, and N12S/A14T, were recently reported to elicit neutralizing antibodies in mice that showed little immunological cross-reactions to the endogenous peptides, when genetically fused in triplicate to monomeric-$LT_{R192G/L211A}$.[91] However, when the authors assessed the immunological cross-reactions using a competitive ST ELISA, the ratio between free and bound peptide competing for binding to antibody was kept constant, in contrast to our previously published approach where we varied the ratio between free and bound peptides.[39] In our opinion, a proper dose-response experiment must be conducted in order to convincingly describe possible immunological cross-reactions or the lack thereof. In addition, sera were pooled from six individual mice prior to testing for both neutralization and cross-reactions, which is unfortunate, as it may mask inter-individual variations, which we have recently observed (Diaz et al., manuscript in preparation).

In conclusion, over the recent years considerable progress has been made on making ST immunogenic, and on identifying mutations that will reduce or abolish toxicity. But the important challenge of demonstrating the reduction of unwanted immunological cross-reactions to guanylin and uroguanylin remains.

Concluding remarks and future perspectives

The lack of well-conserved antigens to formulate a broadly protective vaccine has been the main challenge in ETEC vaccine development. The heterogeneity of the CFs has so far posed a practical challenge in formulating a CF-based ETEC vaccine that targets the ETEC strains responsible for the bulk of ETEC diarrhea. Recent ST toxoid-based ETEC subunit strategies outlined here are showing encouraging results that we believe may importantly complement the CF- and LT-based approaches. We expect that these efforts will soon result in ST toxoid candidates that can be evaluated in clinical trials.

Meanwhile, the lack of a suitable animal model that recapitulates ETEC mediated infection and diarrhea hinders the screening of potential vaccine candidates at an early stage of vaccine development. So far, mouse models are restricted to studying the protective efficacy of antigens in ETEC colonization[103,104] and protection against lethal ETEC doses.[58] Furthermore, human challenge models for evaluating the protective efficacy of ETEC vaccines have so far focused on strains that also produce LT.[105,106] There is a need to develop a human challenge model for ETEC that only express STh, and important progress towards establishing such a model has recently been made.[107]

Finally, since ETEC is an enteric pathogen, the ETEC vaccine needs to primarily induce mucosal IgA responses. In this respect, dmLT has shown promise as an adjuvant that redirects parenterally administered vaccines to an immune response in the gut.[108]

It is our hope that ongoing efforts to develop a safe and immunogenic ST toxoid capable of inducing strong mucosal immune responses in the human gut can importantly complement ETEC vaccine candidates currently in the pipeline to produce a broadly protective ETEC vaccine that can substantially reduce the diarrheal disease burden in LMIC children.

Disclosure of potential conflicts of interest

The research team has submitted a patent application (application no. 61/766,958) for ST toxoid which is intended for use in ETEC vaccines.

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