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Evaluation of the non-toxic mutant of the diphtheria toxin K51E/E148K as carrier protein for meningococcal vaccines

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ABSTRACT

Diphtheria toxin mutant CRM_{197} is a common carrier protein for glycoconjugate vaccines, which has been proven an effective protein vector for, among others, meningococcal carbohydrates. The wide-range use of this protein in massive vaccine production requires constant increase of production yields and adaptability to an ever-growing market. Here we compare CRM_{197} with the alternative diphtheria non-toxic variant DT-K51E/E148K, an inactive mutant that can be produced in the periplasm of *Escherichia coli*. Biophysical characterization of DT-K51E/E148K suggested high similarity with CRM_{197}, with main differences in their alpha-helical content, and a suitable purity for conjugation and vaccine preparation. Meningococcal serogroup A (MenA) glycoconjugates were synthesized using CRM_{197} and DT-K51E/E148K as carrier proteins, obtaining the same conjugation yields and comparable biophysical profiles. Mice were then immunized with these CRM_{197} and DT-K51E/E148K conjugates, and essentially identical immunogenic and protective effects were observed. Overall, our data indicate that DT-K51E/E148K is a readily produced protein that now allows the added flexibility of *E. coli* production in vaccine development and that can be effectively used as protein carrier for a meningococcal conjugate vaccine.

1. Introduction

Diphtheria toxin (DT) is the exotoxin responsible of the toxicity of *Corynebacterium diphtheriae*, the leading cause of diphtheria [1]. It catalyzes the adenosine diphosphate (ADP)-ribosylation of diphtamide, a post-translationally modified histidine residue present on elongation factor-2 (EF-2) [2,3]. Two inactive versions of diphtheria toxin have been used for decades in the production of glycoconjugate vaccines: the diphtheria toxoid, obtained by inactivation of the native toxin with formaldehyde [4,5], and CRM_{197}, an enzymatically inactive and non-toxic mutant of DT [6]. CRM_{197} contains one single point mutation leading to the substitution of a glycine by a glutamic acid in position 52 [7,8]. CRM_{197} has been used as a protein component in vaccines protective against several pathogenic bacteria, such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitidis* [9]. As for other glycoconjugate vaccines, DT variants have potential as carrier proteins by covalent linkage to, for example, the capsular polysaccharide, a T-cell-independent antigen, thus providing T-cell-dependent epitopes that greatly improve the immunogenicity of the saccharide and making it suitable also for infants [10,11].

At present, despite the availability of Pfenex technology in which CRM_{197} is expressed in *Pseudomonas fluorescens* [12,13], for production of licensed conjugate vaccines, CRM_{197} is obtained by fermentation of the *C. diphtheriae* C7(β197)tox- strain, which contains the CRM_{197} gene. The protein is secreted in the supernatant, centrifuged, filtered and concentrated, and finally purified by chromatography, with a final purity of 90% [14]. However, the production of a recombinant protein in a pathogenic bacterial strain such as *C. diphtheriae* imposes regulatory limitations and provides fermentation yields that barely suffice the needs of massive vaccine production.

To overcome the limitations of CRM_{197} production, several methods can be considered: modification of *C. diphteriae* bacterium to increase fermentation yields, new fermentation strategies, or alteration of the inactive diphtheria toxin into a more suitable protein for large scale production. In this manuscript we describe the analysis of a novel recombinant inactive diphtheria toxin variant, DT-K51E/E148K [15–17]. This protein lacks the point mutation of

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CRM_{197} (G52E) and is instead inactivated by two different amino acid substitutions: glutamic acid at position 51 (K51E) and lysine at position 148 (E148K).

We show here that DT-K51E/E148K produced in the periplasm of *Escherichia coli*, a fast growing bacterium capable of easy scale up fermentation, is a suitable protein component for potential vaccines. We performed biophysical analyses on DT-K51E/E148K in comparison with CRM_{197} to evaluate the impact of the mutations on the protein structure. We also used DT-K51E/E148K as carrier protein for a glycoconjugate containing *N. meningitidis* serogroup A polysaccharide (MenA) to directly compare a DT-K51E/E148K-based conjugate vaccine with a CRM_{197} reference vaccine in a mouse model.

2. Material and methods

2.1. DT-K51E/E148K production

A 20 mL starter culture of LB containing 0.1 mg/mL ampicillin was inoculated with a single colony of *E. coli* BL21(DE3) cells containing pET22(DT-mutant) DNA and was grown by shaking overnight at 37 °C. Eight milliliters of overnight culture were diluted into 800 mL of LB medium containing 0.1 mg/mL ampicillin. The bacteria were grown at 37 °C with shaking to an OD_{600} of 0.6. Protein production was induced with the addition of 1 mM IPTG and then the culture was allowed to continue shaking at 25 °C for 16 h. Cells were harvested by spinning for 20 min at 8000 rpm. Cell pellets were collected and stored at −78 °C until used. The cell pellets from 800 mL culture were treated in 40 mL of buffer (50 mM Tris.HCl, 150 mM NaCl, pH 7.5) containing 1 mg/mL lysozyme and 0.1 mg/mL of DNAse. The pellet and buffer were allowed to incubate on ice for 30 min. Cell pellets were then sonicated, in a plastic beaker on ice, at 20% power in bursts of 30 s with a wait time of 1 min between each burst, for a total of 4 bursts. Cell debris was removed from the lysis mixture by centrifugation at 15,000 rpm for 30 min. Then the cleared lysate was loaded onto the Superdex S200 pre-packed size exclusion column (26/60, GE Healthcare). Protein was eluted using the same buffer and protein-containing fractions (as determined by UV absorbance) were analyzed by SDS PAGE. Major protein fractions were pooled and then concentrated using 10 kDa MWCO membrane spin filter and loaded onto a pre-packed HiTrap Q column (GE Healthcare) and then eluted using the same buffer containing 1 M NaCl. Fractions were analyzed by SDS PAGE and it was found that pure protein was eluted from the binding buffer itself. Fractions were pooled and concentrated using 10,000 MWCO membrane spin filter. Concentrations were analyzed using BCA assay (Pierce) [18]; protein expression levels were found to be typically 15 mg from 1 L of culture.

2.2. DT-K51E/E148K protein characterization

Recombinant diphtheria toxin K51E/E148K obtained by vector expression in *E. coli* underwent diafiltration against 0.01 M sodium phosphate buffer, pH 7.2, and was concentrated using a 30 kDa cut-off Amicon centrifugal filter (Sigma-Aldrich). Protein concentration was determined by BCA assay.

Characterization of DT-K51E/E148K, in comparison with CRM_{197}, was performed through HPLC-Size Exclusion Chromatography (SEC) and ESI-Q-Tof MS. HPLC-SEC analyses were performed on a TSK 3000 Gel SW analytical column with TSK guard column (Tosoh Bioscience) connected to an UltimateTM3000 HPLC system (Dionex-Thermo Fisher Scientific) equipped with a PDA detector. Chromatography was performed in 0.1 M sodium sulfate 0.1 M sodium phosphate 5% CH_3CN pH 7.2 at flow rate of 0.5 mL/min. Data was processed using Chromeleon^{TM} 6.7 software. Exact masses of DT-K51E/E148K and CRM_{197} were measured by Electron Spray Ionization mass spectroscopy, using a Q-Tof *micro* Macromass instrument (Waters).

2.3. Preparation and characterization of meningococcal A oligosaccharide (MenA) conjugates

MenA-protein conjugates were obtained by reacting the carrier protein (CRM_{197} or DT-K51E/E148K) with *N*-hydroxy-succinimido-activated MenA oligosaccharide ester [19] at an active ester to protein molar ratio of 30:1 and a protein concentration of 9 mg/mL (~156 μM), in 0.1 M sodium phosphate buffer pH 7.2. After overnight incubation at room temperature with gentle stirring, the conjugates were purified from the unreacted oligosaccharides by hydrophobic interaction chromatography (HIC) on phenyl sepharose. SDS–PAGE was performed on MenA and protein content was determined by BCA assay.

Total saccharide content of MenA conjugates was determined by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) as described [20,21]. Unconjugated (free) saccharide was separated from the conjugate by Solid Phase Extraction (SPE) (Bioselect, Grace Vydac C4 100 mg/3 mL) and subsequently estimated by HPAEC-PAD analysis.

HPLC-size exclusion chromatography of MenA conjugates was performed as described in the previous paragraph.

2.4. Differential scanning calorimetry (DSC) analysis

All samples were diluted in 10 mM sodium phosphate buffer, pH 7.2, to a final concentration of 0.5 mg/mL (~8.5 μM) and data were collected with a MicroCal VP-Capillary DSC instrument (GE Healthcare) with integrated autosampler. Sample volumes of 500 μL were transferred to a 96-well plate and loaded into the instrument autosampler thermostatted at 4 °C until analysis. A single DSC scan was recorded for each sample in the temperature range 10–110 °C at a scan rate of 2.5 °C/min and a 5 s filter period. Data were analyzed by subtraction of the reference data for a sample containing only buffer, using the Origin^{TM} 7 software.

2.5. Circular dichroism (CD) analysis

Near-UV CD spectra were recorded at 20 °C in a quartz cuvette with an optical path length of 1 cm, in the range 250–320 nm. Carrier proteins and conjugates samples were diluted to 0.7 mg/mL (~12 mM) in 10 mM sodium phosphate buffer, pH 7.2. Spectra were acquired at 1 nm bandwidth, 8 s response time, 0.2 nm step size and 5 nm/min scan speed. Each spectrum was calculated as the average of 10 accumulations. Far-UV CD spectra were recorded at 20 °C in the range 180–260 nm in a quartz cuvette with an optical path length of 1 mm, at a concentration of 0.35 mg/mL (~6 mM) in 10 mM sodium phosphate buffer, pH 7.2. Spectra were acquired at 1 nm bandwidth, 0.2 nm step size, 4 s response time, with a speed of 10 nm/min and 3 accumulations. All spectra were corrected by subtracting the baseline (buffer) and converted in molar ellipticity ($deg \times cm^2 \times dmol^{-1}$), by using a mean residue molecular mass of 109 Da, in Spectra Manager v2.0 (Jasco).

2.6. Vaccines formulation and animal immunization

All animal protocols were approved by the local animal ethical committee and by the Italian Minister of Health in accordance with Italian law.

Conjugated antigens, 2 μg/dose (saccharide-based), were formulated in 10 mM phosphate buffered saline pH 7.2 (PBS) with aluminum phosphate ($AlPO_4$) as adjuvant (0.12 mg/dose expressed as Al^{3+}). All vaccines were injected subcutaneously in a volume of 200 μL. The immunogenicity of the MenA glycoconjugates was tested in groups of 8 BALB/c (female, four weeks old) mice immunized on days 1, 14 and 28. Serum samplings were performed on day 0 (pre immune), day 13 (Post 1), day 27 (Post 2) and day 42 (Post 3). As control group, 8 mice received at the same time points an equal volume of a PBS/adjuvant formulation.

2.7. Analysis of humoral response by ELISA and rabbit Serum Bactericidal Activity (rSBA) assay

The antibody response induced against the meningococcal polysaccharides was measured by ELISA as previously described [22] (details in Supplementary information). Sera titers were expressed as the reciprocal dilution corresponding to a cut-off at $OD_{405} = 1.0$.

The avidity index (AI) of the anti-MenA IgGs was measured by avidity ELISA [23]. Pools of sera at a pre-determined dilution corresponding to $OD_{405} = 1$ were mixed with TPBS containing increasing amounts of NH_4SCN (0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2.5 M) and then added to a MenA-coated plate, continuing the ELISA analysis as described previously. Linear regression analysis and calculation of affinity index (AI), defined as the molar concentration of thiocyanate required to reduce the initial optical density by 50%, were performed as described and adopting the same acceptance criteria which included a correlation coefficient for the line-fitting >0.88 [24].

Serum bactericidal activity (SBA) against *N. meningitidis* strains was evaluated as described [25], with minor modifications using pooled baby rabbit serum (Pel-Freez) as complement source (rSBA) [26] (details in Supplementary information). Titers were expressed as the reciprocal serum dilution resulting in 50% of bactericidal killing.

3. Results

3.1. DT-K51E/E148K production

Recombinant diphtheria toxin mutant DT-K51E/E148K was produced using *E. coli* BL21(DE3) (sequence details, Fig. S1). The use of a *pelB* leader sequence allowed translocation of protein product via the Sec translocase system [27], and has been previously reported in some cases to allow increased expression yields of certain proteins [28]. After extensive evaluation of expression and purification methods, a protocol using size exclusion chromatography followed by anion exchange chromatography was found to be efficient in delivering reasonable purity. Under laboratory conditions typical yields of ~15 mg of purified DT-K51E/E148K could be achieved from 1 L of culture.

3.2. DT-K51E/E148K protein characterization

The amino acid sequence of DT-K51E/E148K and alignment with CRM_{197} is provided (Fig. S2).

Recombinant diphtheria toxin K51E/E148K (DT-K51E/E148K) from *E. coli* showed a comparable HPLC-SEC profile to CRM_{197} from *C. diphtheriae* with only a slight reduction in retention time and a slightly broader curve, indicating a purity of the sample of >90% (Fig. 1b and c; Table 1). These fragments were not clearly observed using HPLC-SEC methods but were visible at low levels using Coomassie Blue staining in SDS–PAGE (Fig. S3); this was taken as suitably pure (>90%) for conjugation. The mass obtained

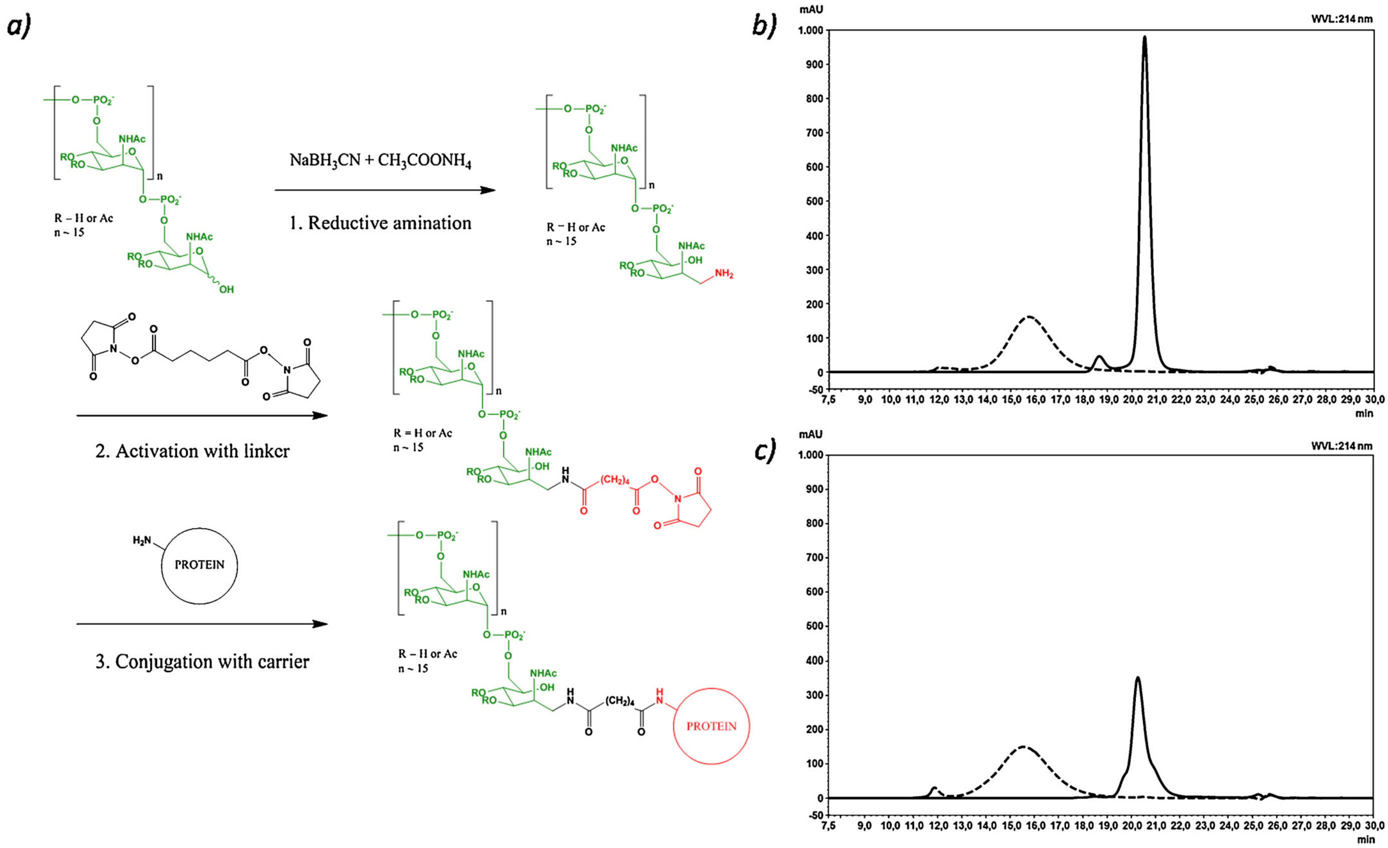

Fig. 1. Synthesis and characterization of MenA conjugates. (a) Schematic representation of conjugation method and MenA conjugate structure. HPLC-SEC profiles of (b) CRM_{197} and MenA-CRM_{197} conjugate, and (c) DT-K51E/E148K and MenA-DT-K51E/E148K conjugate; protein (solid line), conjugate (dashed line).

Table 1
Characterization of proteins and MenA glycoconjugates. K_d = distribution coefficient; T_m = melting temperature.

	CRM_{197}	MenA-CRM_{197}	DT-K51E/E148K	MenA-DT-K51E/E148K
MALDI-TOF MW (Da)	58,412	–	59,284	–
HPLC-SEC K_d	0.61	0.27	0.60	0.26
Saccharide/protein (w/w)	–	0.32	–	0.40
Free saccharide (%)	–	3.50	–	2.70
DSC T_m (°C)	45.9	30.5	51.2	54.2

(59,284 Da) was consistent with the calculated (59,284 Da) (Fig. S4; Table 1).

Cross-reactivity of DT-K51E/E148K to CRM_{197}-specific polyclonal serum (obtained by mice immunization) was observed by Western blot (Fig. S5)

3.3. *Preparation and characterization of meningococcal A oligosaccharide (MenA) conjugates*

N-hydroxy-succinimido-activated MenA oligosaccharide was reacted with carrier proteins CRM_{197} and DT-K51E/E148K (Fig. 1a). Resulting MenA-protein conjugates were purified by hydrophobic interaction chromatography on phenyl sepharose.

Purified MenA conjugates were obtained with conjugation yields of 64% for both, in terms of protein recovery, and a saccharide-to-protein ratio ('glycosylation degree') of 0.32 and 0.40 weight/weight for MenA-CRM_{197} and MenA-DT-K51E/E148K, respectively, with free saccharide of 3.5% for MenA-CRM_{197} and 2.7% for MenA-DT-K51E/E148K (Table 1).

HPLC-SEC analysis of proteins and conjugates showed comparable profiles for both MenA conjugates (Fig. 1b and c; Table 1). Absence of unreacted protein was observed by SDS–PAGE (Fig. S3).

3.4. *Differential Scanning Calorimetry (DSC) analysis*

Thermal stability and denaturation transition profiles were tested by DSC (Fig. 2). The thermogram of unconjugated CRM_{197} showed a neat transition curve at 45.9 °C, while unconjugated DT-K51E/E148K showed a shift to higher temperatures with a melting temperature (T_m) of 51.2 °C (Table 1).

Conjugation of both scaffolds with MenA induced a profound change in the thermal curves of both CRM_{197} and DT-K51E/E148K: broadening of denaturation interval, decrease in transition enthalpy and shifts in T_m occurred, possibly, as already observed, due to a loss of protein tertiary structure and/or the very different structural nature of the resulting conjugates (Table 1) [29].

3.5. *Circular dichroism (CD) analysis*

Spectra of CRM_{197} and DT-K51E/E148K showed differences between these two proteins, both in the near-UV and far-UV regions (Fig. 3). CRM_{197} showed higher signals in the near-UV regions of tryptophan, phenylalanine and tyrosine emission, indicating a well-structured conformation; this was confirmed by the far-UV spectrum, which gave a typical alpha-helix pattern. DT-K51E/E148K showed lower signals in the same regions of the near-UV spectrum, and a less marked (but observable) pattern in the far-UV spectrum, potentially indicating less alpha-helical content.

Covalent attachment of MenA oligosaccharide induced marked changes in the CD profiles of both CRM_{197} and DT-K51E/E148K, confirming the observations from DSC analysis and indicating a loss of tertiary and secondary structure associated with chemical modification due to oligosaccharide conjugation. However, a less marked modification was observed for the MenA-DT-K51E/E148K conjugate CD spectrum, thus suggesting that this protein may be less susceptible to this conjugation-induced modification.

3.6. *Analysis of humoral response by ELISA and rabbit Serum Bactericidal Activity (rSBA) assay*

Serum samples from three time points (Post 1, Post 2, and Post 3 immunizations) were collected, and the sera were analyzed by ELISA and rabbit Serum Bactericidal Activity (rSBA). Evaluation was performed in pre-clinical murine model, commonly used for meningococcal conjugate vaccines [30–34].

ELISA was used to determine the anti-MenA IgG titers and, at every time point (Post 1, Post 2, and Post 3), no significant difference ($p > 0.1$) was observed between the two groups immunized with MenA-CRM_{197} or MenA-DT-K51E/E148K (Fig. 4). These two immunization groups showed comparable immunogenicity when compared to the reference group that received only PBS/adjuvant, both after one, two, or three doses ($p < 0.001$). Carrier-specific IgG

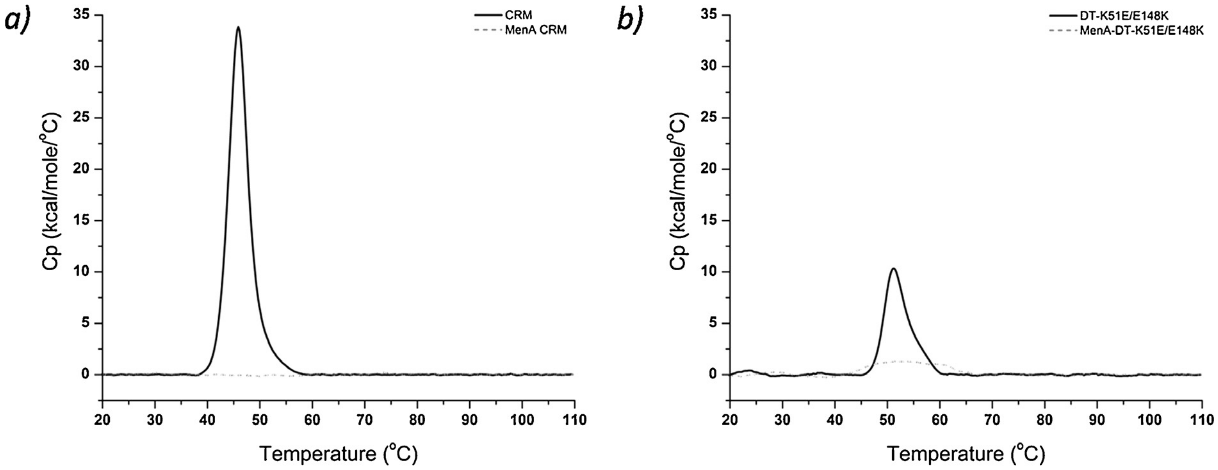

Fig. 2. DSC analysis of proteins (solid line) and conjugates (dashed lines); (a) CRM_{197} and MenA-CRM_{197}, (b) DT-K51E/E148K and MenA-DT-K51E/E148K.

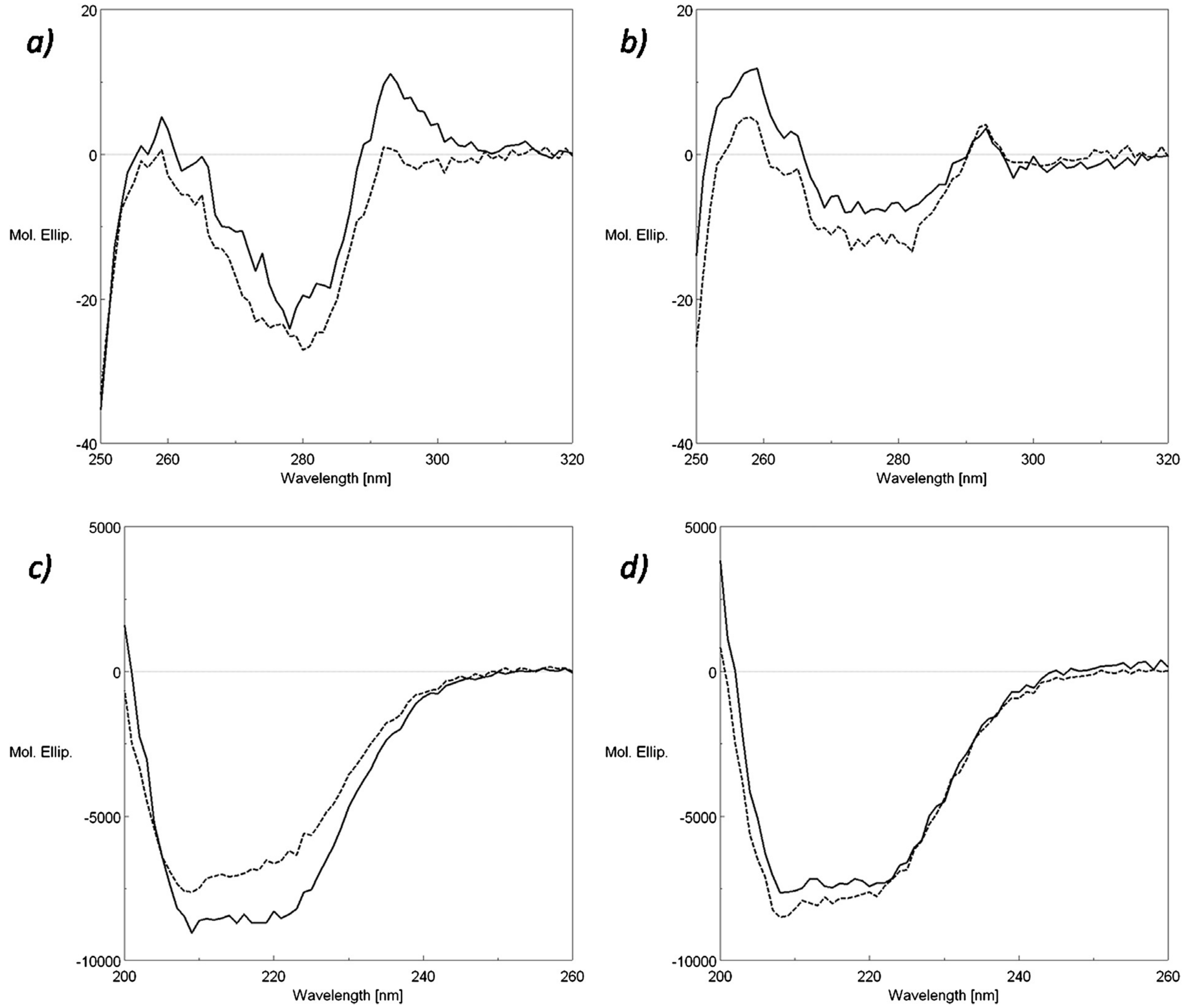

Fig. 3. CD spectra of carrier proteins (solid line) and their glycosylated MenA conjugate (dashed line); (a) near-UV and (c) far-UV spectra of CRM_{197} conjugates; (b) near-UV and (d) far-UV spectra of DT-K51E/E148K conjugates. The buffer blank was subtracted from the baseline of all spectra. Mol. Ellip. = molar ellipticity, expressed in degree × cm^2 × $dmol^{-1}$.

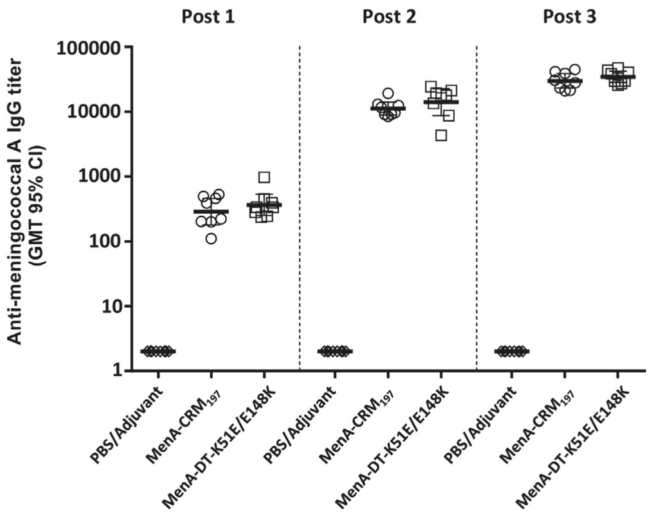

Fig. 4. Anti-MenA IgG response induced in mice by one, two, and three doses of $MenA\text{-}CRM_{197}$, MenA-DT-K51E/E148K conjugates, or only PBS/adjuvant (negative control), GMT ELISA (95% Confidence Interval).

Table 2
Rabbit serum bactericidal (rSBA) titers after one, two, and three doses of MenA conjugates and avidity indexes (AI) after three doses of MenA conjugates (SD = standard deviation of 3 replicates).

	rSBA titer			Avidity index (Post 3)	
Conjugate	Post 1	Post 2	Post 3	Average AI	±SD
$MenA\text{-}CRM_{197}$	128	8192	16,384	0.28	0.01
MenA-DT-K51E/E148K	128	4096	16,384	0.29	0.02

titers of Post 3 sera were also determined, using CRM_{197} or diphtheria toxoid as coating agents (Fig. S6), evidencing comparable IgG titers for coated CRM_{197} ($p < 0.05$) and similarly low cross-reactivity against diphtheria toxoid ($p > 0.05$).

Avidity analyses using ammonium thiocyanate revealed an almost identical avidity index for $MenA\text{-}CRM_{197}$ and MenA-DT-K51E/E148K post-immunization sera (Post 3) of 0.28 ± 0.01 and 0.29 ± 0.02, respectively (Table 2).

Rabbit Serum Bactericidal Activity assay (rSBA) confirmed the comparable protective effect of the two conjugates (Table 2). After one, two, or three doses, the rSBA titers for both vaccination groups was identical (rSBA titer 128 at Post 1 and 16,384 at Post 3), or closely related (rSBA titer 8192 for $MenA\text{-}CRM_{197}$ and 4096 for

MenA-DT-K51E/E148K immunization groups at Post 2), indicating comparable bactericidal activity for the two conjugates.

4. Discussion

CRM$_{197}$ has proven to be a valuable protein scaffold in conjugate vaccine design and has achieved great popularity in the field of vaccine design as a useful T-epitope-dependent scaffold onto which suitable immunogens can be attached. In the field of glycoconjugate vaccine development it has arguably become one of the most popular scaffolds [35]. However, disadvantages associated with production have hampered a more wide-ranging use of CRM$_{197}$. Despite the availability of highly productive bacterial platforms for recombinant proteins, as the Pfenex technology [12,13], so far the CRM$_{197}$ protein has been produced by fermentation of the *C. diphtheriae* C7(β197)tox- strain and not much effort has been put on the expression of inactive mutants in a more flexible expression system, such as *E. coli* fermentation.

Here we have shown that the alternative, inactive DT mutant DT-K51E/E148K can not only be produced and purified from *E. coli*, when using a suitable vector and sequence, but that this protein scaffold is highly suitable as a scaffold for glycoconjugate vaccine design. Applying a variety of physicochemical methods, which we recently used to compare CRM$_{197}$ and diphtheria toxoid meningococcal conjugates [29], we observed that the DT-K51E/E148K protein scaffold showed biophysical properties consistent with CRM$_{197}$. As for CRM$_{197}$, conjugation-induced denaturation was also observed. Moreover, and most importantly, DT-K51E/E148K showed essentially identical immunogenic and protective effects in vaccinated mice, with clearly positive implications for a putative MenA glycoconjugate vaccine. Although further work will be needed, based on these evidences, and on the structural similarity between these two proteins, which differ only for three mutations on a flexible domain [1], we expect a comparable T-helper response for CRM$_{197}$ and DT-K51E/E148K.

As well as now importantly confirming the utility of an inactive DT scaffold that might be readily produced by laboratories around the world, this study will also allow new directions in vaccine design that will combine the much greater flexibility of *E. coli* in producing protein substrates. We have previously proposed that a coordinated strategy using convergent and precise protein assembly could allow the ready creation of homogeneous glycoconjugate vaccines [36]. This method used unnatural amino acids at predefined positions to control the attachment of glycans in a precise way. With a production system for a suitable DT variant now available (disclosed here) then various methods for inserting unnatural amino acids into DT to allow the application of a 'tag-and-modify' strategy may now be considered [37]. The precise positioning of glycans in this way has only rarely been considered previously in the past [36,38,39], and this method now opens the door to many possible strategies in the valuable DT protein scaffold. Experiments creating and using such ad hoc designed DT-based vaccines will be reported in due course.

Conflict of interest statement

The authors Proietti D., Lo Surdo P., Balocchi C., Mori E. and Berti F. are GSK Vaccines employees. Pecetta S. has been recipient of a GSK Vaccines Fellowship from the PhD program in Cellular Biology of the University of Rome "La Sapienza", Italy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2016.01.040.

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