

TETANUS VACCINE PLATFORM FOR EMBEDDING COVID-19 VACCINE

CROSS REFERENCE TO RELATED APPLICATION

[0001] The present application claims priority to U.S. Provisional Serial No. 63/032,544 that was filed on May 30, 2020, and is herein incorporated by reference in the entirety.

FIELD OF THE INVENTION

[0002] The present invention relates generally to protein or nucleic acid based COVID-19 vaccines; and more particularly to a pre-used tetanus vaccine vehicle to directly activate memory cells for the processing of the vaccine, without necessarily alerting the entire immune system for any dire immune response.

BACKGROUND OF THE INVENTION

[0003] The corona virus like SARS (Severe Acute Respiratory Syndrome) and MERS (Middle East Respiratory syndrome) causes lethal infections in the humans. The most recent of the Corona viruses is SARS-CoV-2 (Cov-2), also known as 2019-nCoV, that started spreading from Wuhan, China, and has spread across the globe starting from late 2019. Several recent animal studies for SARS-CoV-2 vaccines had identified two potential safety issues: cellular immunopathology and antibody-dependent enhancement (ADE), which have proven that the delivery platforms and adjuvants play very important roles in the host responses to the immunopathology in the host (1, 2). In the 1960s, the formalin-inactivated respiratory syncytial virus (RSV) vaccine caused immune-enhanced disease that may be due to the ADE (3).

[0004] An infection by viruses like SARS-CoV-2 triggers several mechanisms of host immune responses, such as innate, cell-mediated, and adoptive. Depending on the immune status of the host and that of the preponderance of the virions, then these immune responses could mitigate the infection by clearing up the virus, and manage the infection at a suboptimal level to develop long term memory cells for future immune responses to a subsequent infection situation, and/or try to overwhelm the virus by the all out dire immune response to neutralize the virus in plasma, as well as in the infected cells (1, 2). The latter is managed by what is termed a cytokine storm,

which leads to the damage not only the virus infected cells, but also to normal cells, leading complications for the patients aggravating the conditions which often results in death (4-6).

[0005] All the current vaccine efforts either use the whole viral protein, or a corresponding DNA/RNA based vaccine that is to be delivered by a variety of means, such as viral vector, nanoparticles, or lipid vesicles (1). These approaches and vehicles continue to pose the problem of dire immune response mediated by cytokine storm. Part of the problem with the Covid-19 virus is its high virulent nature, which allows it to sneak into cells.

[0006] None of these approaches use a pre-used vaccine vehicle. The advantage of pre-used vaccine vehicles would be to directly activate memory cells for the processing of the vaccine, without necessarily alerting the entire immune system for any dire immune response. At the same time, the antigen presenting cells (APCs) will be able to process the epitopes derived from the S and N proteins of SARS-CoV-2, which would allow more effective and perhaps faster immune response.

Tetanus Vaccines

[0007] Tetanus is often a fatal disease caused by tetanus neurotoxin (TeNT) produced by *Clostridium tetani*, which can infect wounds resulting from general cuts, needle use, and unhygienic birth practices. The tetanus vaccination has become common throughout the world at least during infant ages. One hundred percent tetanus immunization is needed for the world population, as this is a disease, which often turns fatal. In addition, there is a need for booster shots of tetanus every 10 years, which frequently is overlooked. At present there are over 200,000 annual tetanus deaths (mostly maternal and neonatal) worldwide which could be prevented with effective immunization. For all practical purposes the world's entire population is immunized with tetanus vaccine, making a tetanus-based delivery of CoV-2 vaccine a safe proposition.

[0008] The production of tetanus toxoid vaccine by chemical inactivation of tetanus toxin with formaldehyde has remained virtually unchanged since it was first introduced in the 1920, which requires administration of toxoid vaccine injection by trained medical staff. It is required to improve the vaccine in terms of delivering the vaccine without needles. Moreover, injectable vaccines have chances for secondary infection due to needles, and require medically trained person to inject the vaccine. It has also reported that formaldehyde can cause allergic reactions

which includes >8 cm of erythema or induration, sore and swollen arm, and systemic manifestation such as fever and malaise reported in several studies (7-10). The method is inconvenient and frightening for many people, especially children. It has been reported altogether, 24% of parents and 63% of children reported a fear of needles. Needle fear was the primary reason for immunization non-compliance for 7% and 8% of parents and children, respectively (11).

[0009] Moreover, upon intramuscular injection for the patients, local reactions consisting of pain, erythema, tenderness and indurations at the injection site are common, and are usually associated with systemic reactions including transient fever and irritability. The reactions are due to the formaldehyde present in the formulation, which causes adverse effects upon injection.

[0010] What is needed within the vaccine industry is a vaccine composition comprising a detoxified tetanus neurotoxin (TeNT) and epitopes of the targeted pathogen, wherein the vaccine composition does not comprise formaldehyde.

SUMMARY OF THE INVENTION

[0011] Various embodiments of the present invention comprise a detoxified form of whole tetanus neurotoxin (TeNT) containing at least one mutated light chain and native heavy chain, which can be used alone as a protein or nucleic acid vaccine for subcutaneous or intramuscular administration; or in combination with the neurotoxin associated protein (NAP) for oral/sublingual and intranasal delivery. This recombinant vaccine candidate (detoxified recombinant TeNT or DrTeNT proteins) is a major advancement for the SARS-CoV-2 vaccine, especially as it can be combined with immunogenic recombinant fragments of corona epitopes.

[0012] In an embodiment, the present invention employs the use of neurotoxin associated proteins (NAPs) of botulinum neurotoxin (BoNT) produced by *Clostridium botulinum* as delivery vehicles (e.g. adjuvants and carrier) of the vaccine for oral/sublingual, and/or intranasal administration.

[0013] The present invention further comprises a mutated recombinant vaccine composition, and method of use to immunize, comprising one or more detoxified forms of a whole or part of a tetanus neurotoxin (DrTeNT) proteins, and one or more epitopes derived from a toxin, a pathogen, or a disease, wherein the vaccine composition is able to immunize a human or a non-

human mammal against the toxin, the pathogen, or the disease, which are derived from animals, plants, fungi, bacteria, or viruses. And the epitopes are derived from one or more diseases comprising, by way of non-limiting examples: tuberculosis, malaria, cancer, flu, pneumonia, psoriasis, itch, and lime disease.

[0014] In one or more embodiments, the vaccine composition comprises a detoxified form of the whole or the part of the tetanus neurotoxin (DrTeNT) with at least one mutated light chain and at least one native heavy chain; and/or with the DrTeNT as a carrier protein and one or more epitopes or subunits of the SARS-CoV-2 virus; and/or a hybrid protein linking a receptor-binding domain (RBD) epitope of a SARS-Cov-2 pathogen with a TeNT-Cov2 protein; and/or comprising a S1 subunit (or other subunit) of a SARS-CoV spike (S) protein comprising a receptor-binding domain (RBD) epitope.

[0015] In one or more embodiments, the vaccine compositions of the present invention do not comprise or contain formaldehyde.

[0016] In one or more embodiments, the vaccine compositions of the present invention further comprises an adjuvant comprising one or more neurotoxin associated proteins (NAPs) derived from a botulinum neurotoxin (BoNT) produced by a *Clostridium botulinum*; and/or an adjuvant comprising one or more of: alum, polylactic acid, polyglycolic acid, Vitamin E, and colloidal particle.

[0017] In one or more embodiments, the vaccine compositions of the present invention epitopes with at least 95% sequence identity one or more of SEQ ID NOS: 1-45; and/or at least 95% sequence identity to SEQ ID NOS: 1-6.

[0018] The present invention further comprises a recombinant TeNT or DrTeNT vector comprising a nucleic acid that encodes a polypeptide comprising a DrTeNT protein; and a promoter which is operably linked to said polynucleotide. The vector further comprises a nucleic acid encoding one or more epitopes of a toxin, a pathogen or derived from a disease. In an embodiment, the epitopes are derived from or directed against Covid-19, e.g. encoding a SARS-CoV receptor binding domain (RBD) gene and a Tent-Cov2 gene to express a RBD and Tent-Cov2 hybrid protein (Tent-Cov2 protein).

[0019] The present invention further comprises a cell in which the recombinant vector is introduced; and a vaccine composition or medicament for treating and/or preventing Covid-19 comprising the recombinant vector; and a method of treating by administering the vector encoding a SARS-CoV receptor binding domain (RBD) gene and a Tent-Cov2 gene to express a RBD and Tent-Cov2 hybrid protein (Tent-Cov2 protein) *in vivo*.

[0020] One aspect of the present invention is one or more vaccine vehicles able to directly activate memory cells for the processing of the vaccine, without necessarily alerting the entire immune system for any dire immune response.

[0021] Another aspect is the antigen presenting cells (APCs) are able to process the epitopes derived from the S and N proteins of SARS-CoV-2, which would allow more effective and perhaps faster immune response against the pathogen (e.g., Covid-19).

[0022] Another aspect comprises administering the DrTeNT protein or gene as a hybrid with a pathogen epitope (or SARS-CoV-2), or co-administered as separate proteins or genes.

BRIEF DESCRIPTION OF THE DRAWINGS

[0023] FIG. 1 is a schematic representation of pDrT vector map containing DrTeNT gene (3966 bp include 6-His tag). DrTeNT was cloned in pBN3 vector (3452bp) between EcoRI and PstI site. The new construction of pDrT vector is 7418bp in length.

[0024] FIG. 2A illustrates the active site residues of tetanus toxin. b.

[0025] FIG. 2B shown the Glu 234 Ala and Glu 271 Ala mutated residues in the active site of detoxified recombinant tetanus toxin (drTeNT).

[0026] FIG. 3 is a graph of the immunogenicity of the DrTeNT protein when delivered into rabbits via different routes of administration, and the antibodies produced are estimated using enzyme-linked immunosorbent assay (ELISA).

[0027] FIG. 4 is a schematic representation of a pET-11a vector used to insert the receptor binding domain (RBD) gene and the Tent-Cov2 gene to produce a RBD and Tent-Cov-2 protein (Tent-Cov-2 protein).

[0028] FIG. 5 is gel showing the protein purification profile of the RBD protein of Covid using the SDS-PAGE electrophoresis.

[0029] FIG. 6 shows the purification profile of the TeNT-CoV2 proteins.

[0030] FIG. 7 illustrates the chemical between linking of the two proteins using EDC reaction.

[0031] FIG. 8 is an ELISA titer of RBD, TeNT-CoV2 and hybrid proteins titers before and after the drTeNT priming in the rabbit.

DETAILED DESCRIPTION

[0032] Before the present compositions and formulations of the presently claimed invention are described, it is to be understood that this invention is not limited to particular compositions and formulations described, since such compositions and formulation may, of course, vary. It is also to be understood that the terminology used herein is not intended to be limiting, since the scope of the presently claimed invention will be limited only by the appended claims.

[0033] Furthermore, the particular features, structures or characteristics may be combined in any suitable manner, as would be apparent to a person skilled in the art from this disclosure, in one or more embodiments. Furthermore, while some embodiments described herein include some, but not other features included in other embodiments, combinations of features of different embodiments are meant to be within the scope of the presently claimed invention, and form different embodiments, as would be understood by those in the art. For example, in the appended claims, any of the claimed embodiments can be used in any combination.

Oral or Intranasal Vaccine Delivery

[0034] Availability of an oral delivery system (Singh, “Composition for Oral or Nasal Delivery of Tetanus, Diphtheria, and Pertussis Vaccine alone or in combination using Neurotoxin Associated Proteins”, United States Patent application number 20190076518 A1 that published March 14, 2019, which is incorporated herein by reference on its entirety) would facilitate the vaccination schemes for pregnant women, infants and the world population in general. The same technology can be incorporated to deliver DrTeNT-CoV vaccine or any other vaccine on the DrTeNT platform to be implemented under this invention. Currently no such vaccine delivery system is available, nor is any effort known to be underway in this direction against Covid-19.

[0035] The oral/sublingual or intranasal delivery of DrTeNT or DrTeNT-CoV is proposed to be facilitated by neurotoxin associated proteins or NAPs produced by *Clostridium botulinum*, an organism that is related to *C. tetani*. Using NAPs as adjuvants to protect the recombinant protein vaccines can solve the problem of digestive condition in the oral cavity harsh digestive conditions in the stomach, like very low pH and bile acid, and proteases. Further, with the present invention, NAPs protect DrTeNT and SARS-CoV from intestinal enzymes and facilitate the vaccine to evoke the immune response. Additionally, NAPs are well known to help proteins cross the epithelial barrier (12-15), which allows for the delivery of the vaccine

intranasally. This newly developed vaccine also solves the problem of safety concerns related to the injection of vaccines by not requiring an injection.

[0036] Further, the present invention solves the problem of introduction of formaldehyde. The present invention will both eliminate the possibility of the introduction of formaldehyde to the patient due to the preparation of the vaccine and delivery system.

[0037] The present invention employs the use of neurotoxin associated proteins (NAPs) of botulinum neurotoxin (BoNT) produced by *Clostridium botulinum* as delivery vehicles of tetanus vaccine. BoNT is a food poison produced in the form of a complex with NAPs, which protect it from the low pH and proteases of the gastro-intestinal tract. In addition, NAPs are known to help translocate the BoNT across the mucosal layer of intestine as well as the nasal passage. Given the similarity of structure of BoNT and TeNT and their common mode of action, NAPs bind and protect and translocate a tetanus vaccine across the mucosal layer.

[0038] An oral/sublingual delivery system for the COVID-19 vaccine will facilitate convenient immunization regime especially for infants, particularly in countries where access to clinics is limited. An oral/sublingual delivery method for administration of COVID-19 vaccine will be less painful, and safer. There will be no issue of contaminated needles. Currently many vaccines cannot be delivered by oral route because the harsh digestive conditions in stomach like very low pH, bile acid, and proteases. Moreover, several safety concerns related to the current practice injectable vaccines, e.g., allergic reaction and/or secondary infection will be alleviated.

[0039] Thus, the present strategy of using NAPs of botulinum neurotoxin for oral/sublingual delivery of SARS-CoV-2 vaccine is quite innovative and practical. Oral/sublingual delivery offers a number of significant advantages over other routes of administration. Oral vaccines are cheaper to administer since they can be delivered outside of a formal clinical setting, without the need for trained personnel. Elimination of needles from the vaccination process can alleviate concerns regarding the reuse and disposal of needles, which has been shown to be responsible for a number of vaccination-related infections. However, oral vaccination has been regarded historically as likely to be less effective, as vaccine antigens undergo digestion in the GI tract prior to induction of an immune response. NAPs can be employed to address some of these concerns, as these are well known to resist digestion in the GI tract, and are in fact known to help translocate the cargo across the epithelial layer of the gut (13). In fact, certain isolated components of the NAPs have been demonstrated to play a critical role in the

protection and translocation process (16, 17), which can be exploited in the future to develop a cleaner oral delivery system using only those isolated components. At present, there are a limited number of oral vaccines approved for human use, but more are in the late stages of clinical development (oral cholera vaccines, typhoid vaccine, tuberculosis, etc.) (18-21). Majority of vaccines are acid labile and need to be administered in a formulation which protects them from acids of GI tract. In the case of cholera oral vaccine, it is formulated in a bicarbonate buffer to protect the vaccine. But it has been reported that such buffers are not well tolerated by some of those taking such vaccine formulation, as it causes adverse GI symptoms such as bloating, gas, cramps, and diarrhea (22).

[0040] The SARS-CoV-2 virus carries genetic material as RNA, and is one of the longest (27 to 32kb) known viral RNA genome. The structural proteins of SARS-CoV include the surface spike glycoprotein (S), the membrane protein (M), the small envelope glycoprotein (E), and the nucleocapsid protein (N). These four structural proteins are essential for virion assembly (23) .

[0041] The spike (S) protein projects through the viral envelope and forms the characteristic spikes in the coronavirus. Under cryo-electron microscope, it looks like "crown" (PDB- 6VSB) (24). It is heavily glycosylated. The spike (S) glycoprotein of coronaviruses is known to be essential in the binding of the virus to the host cell at the advent of the infection process. The "S" protein is a homotrimer; and it is the sole viral membrane protein responsible for cell entry. It binds to the receptor on the target cell and mediates subsequent virus-cell fusion; hence it is the key target for vaccine design. Because of the dual role (i.e. host receptor recognition, and virus attachment and entry) of S protein, it is the target protein for the development of the antidote or the vaccine against the SARS-CoV-2.

Tetanus Based Vaccine

[0042] The SARS-CoV spike (S) protein is composed of two subunits S1 and S2 proteins; the S1 subunit contains a receptor-binding domain (RBD) that engages with the host cell receptor angiotensin-converting enzyme 2 (ACE2); and the S2 subunit mediates fusion of the viral and host cell membranes. Human proteases cut the spike proteins at specific sites, which help SARS-CoV-2 proteins to change its conformation and bind to the ACE2 receptor (24). The protease-mediated entry facilitated a 100- to 1,000-fold higher efficient infection than the endosomal pathway used in the absence of protease (25). Proteolytic cleavage of the SARS-CoV-2 spike protein is important for cell-cell fusion and/or virus entry. The different proteases

are involved in the cleavage of SARS-CoV-2 spike protein and their roles in activating cell-cell fusion and/or virus entry. There are many studies carried out identifying the various epitopes present on the different proteins on the SARS-CoV-2 (26-29). We believe the N terminus- FSQILPDPSKGPSKRSFIEDLLFNKVTLACD – C (SEQ ID NO: 1) terminus would be a better target on the surface of S protein.

[0043] Recently crystal structural (PDB- 6VW1) of RBD (S1) domain binding directly to the ACE2 receptor has published (30, 31). When the SARS-CoV-2 protein binds to ACE2 receptor, all three RBD shifts in the less stable “up” conformation (30). Looking closely at the binding of RBD sequence 481-505 NGVEGFNCYFPLQSYGFQPTNGVGY (SEQ ID NO: 2) and Sequence TESNKKFLPFQQFGRDIAG (SEQ ID NO: 3). The final epitopes would be created by linking one or more of these sequences together.

[0044] SEQ ID NO: 2 and 3 have been selected as the possible epitope to design the vaccine against the SARS-CoV-2. Most of the studies identified only 20 or so amino acid residues. The small peptide alone could not elicit the enough immune response when inserted alone. Synthetic small peptide-based vaccines containing immunogenic epitope are able to exceed the antibody titers when combined with the adjuvant like DrTeNT. We predicted multiple epitopes from all the SARS-CoV-2 proteins (Table 3) using SVMTriP software developed by University of Nebraska – Lincoln (32) that can also be used as epitopes. The concept of exceeding the immune response using DrTeNT as a carrier protein (or vector) is not limited to the selected epitope (Table 2) but can be used with almost any epitopes of SARS-CoV-2 virus, e.g. in Table 3.

[0045] The DrTeNT along with epitope for SARS-CoV-2 virus can also be inserted in the host by the intramuscular or by the subQ injection route in the absence of the NAPs proteins of *C.botulinum*. As shown in the FIG. 3, when DrTeNT is administered through subQ route it elicits more immune response as compared to the oral route.

Recombinant TeNT or DrTeNT:

[0046] Our group has developed a detoxified form of whole tetanus neurotoxin containing mutated light chain and native heavy chain (FIG. 1) which can be used alone as a vaccine for subcutaneous administration or in combination with the neurotoxin associated protein for oral/sublingual and intranasal delivery. Therefore, creating just the recombinant vaccine candidate (detoxified recombinant TeNT or DrTeNT) already accomplished in our lab is a

major advancement for the SARS-CoV-2 vaccine, especially as it can be combined with immunogenic recombinant fragments of corona epitopes.

[0047] FIG. 2A illustrates the active site residues of tetanus toxin. b. The mutated residues Glu 234 Ala and Glu 271 Ala are shown in the tetanus toxin active site. FIG. 2B represents the active site of drTeNT protein. The 2 amino acid residues of the tetanus protein have mutated to create detoxified recombinant tetanus toxin protein. The introduced mutation created less toxic protein.

[0048] FIG. 3 illustrates the immunogenicity of DrTeNT – with and without complex with NAPs when administered subcutaneous and oral administration. The adjuvant protects the protein from the harsh GI tract condition hence it can be used as an oral vaccine. As per the data shown, oral mix (DrTeNT and NAPs) generate similar immune responses when injected through subcutaneous and oral route. The ELISA titer for the TeNT antibody production in the rabbit when DrTeNT with and without NAPs administered through subcutaneous (subQ) administration and oral route.

Development of titer of DrTeNT

[0049] For subQ administration of DrTeNT, monthly dose of 10 µg was sufficient to produce steady titer of 1:128,000, whereas for oral administration 3 doses of 10 µg/month were given for six months to obtain 1:5,000 titer. For the subQ and oral administration of DrTeNT and NAPs mixtures to obtain a titer of 1:25,000.

TABLE 1

Sample Dosing		Sub DrTeNT	Q	Oral DrTeNT	SubQ DrTeNT+NAPs	Oral DrTeNT+NAPs	SubQ NAPs
Monthly	Protein	10 µg		30 µg (3x10 µg)	~ 0.05 µg	~30 µg (3x10 µg)	~ 0.05 µg
Load							

DrTeNT clone with inserted SARS-CoV-2 epitopes

[0050] Seven different sites of the drTeNT proteins have been selected using software SVMTrip™ : A tool to predict linear antigenic epitopes, produced by the University of

Nebraska-Lincoln. It was used to select possible immunogenic sites. Seven different sites have been used to insert the combined epitopes from S1 and S2 subunit of the SARS-CoV-2 proteins. These epitopes were attached with the poly-glycine linker for the additional flexibility. The created drTent protein with epitopes added from the SARS-CoV-2 protein is called **Tent-CoV-2**. The epitopes are selected from S1 and S2 subunits for the SARS-CoV-2 proteins were mentioned as below in **Table 2** and in **Table 3**.

TABLE 2

His – N^{ter} – DrTent- G-PSKRSFIEDLLFNKVTLA-GG-TESNKKFLPFQQFGRDIAG- C^{ter} (SEQ ID NO: 4)
 His – N^{ter} – DrTent - linker- GG - FSQILPDPSKGPSKRSFIEDLLFNKVTLACD – C^{ter} (SEQ ID NO: 5)
 His – N^{ter} – DrTent - linker- GG - TESNKKFLPFQQFGRDIAG – C^{ter}His – N^{ter} – DrTent – linker- SARS-CoV-2 epitope from Table 2 - C^{ter} (SEQ ID NO: 6)
 His – N^{ter} – DrTent-has 7 different sites selected which can produce the most immune response.

Table 3: List of the predicted epitopes of the SARS-CoV-2 virus.

SEQ ID NO:	SARS-CoV-2 proteins	Location	Epitope	Score
7	S	1186 - 1205	LNEVAKNLNESLIDLQELGK	1
8	S	961 - 980	TLVKQLSSNFGAISSVLNDI	0.987
9	S	1010 - 1029	QQLIRAAEIRASANLAATKM	0.963
10	S	1149 - 1168	KEELDKYFKNHTSPDVLGD	0.961
11	S	1228 - 1247	VMVTIMLCCMTSCCCLKGC	0.827
12	S	740 - 759	MYICGDSTECNLLQYGSF	0.701
13	S	882 - 901	ITSGWTFGAGAALQIPFAMQ	0.681
14	S	690 - 709	QSIIAYTMSLGAENSVAYSN	0.509
15	S	571 - 590	DTTDAVRDPQTLEILDITPC	0.466
16	S	595 - 614	VSVITPGTNTSNQVAVLYQD	0.457
17	N	239 - 258	QQQQGQTVTKKSAEASKKP	1
18	N	276 - 295	RRGPEQTQGNFGDQELIRQG	0.865
19	N	217 - 236	AALALLLDRLNQLESKMSG	0.676
20	N	186 - 205	SSRSRNRNTPGSSRG	0.663
21	N	359 - 378	AYKTFPTEPKDKKKKADE	0.638
22	N	333 - 352	YTGAIKLDDKDPNFKDQVIL	0.471

23	N	74 - 93	INTNSSPDDQIGYYRRATTR	0.317
24	N	128 - 147	DGIHWVATEGALNTPKDHIG	0.282
25	N	29 - 48	NGERSGARSQRRPQGLPNN	0.221
26	N	303 - 322	QIAQFAPSASAFFGMSRIGM	0.216
27	M	96 - 115	FIASFRLFARTRSMWSFNPE	1
28	M	151 - 170	IAGHHLGRCDIKDLPEITV	0.255
29	Orf1ab	5921 - 5940	VATLQAENVGTGLFKDCSKVI	1
30	Orf1ab	2793 - 2812	VHVMSKHTDFSSEIIGYKAI	0.712
31	Orf1ab	3967 - 3986	AVANGDSEVVLKLLKSLNV	0.691
32	Orf1ab	571 - 590	DGISQYSLRLIDAMMFTSDL	0.651
33	Orf1ab	288 - 307	VEKKKLDGFMGRIRSVYPVA	0.639
34	Orf1ab	5278 - 5297	QYIRKLHDELTGHMLDMYSV	0.618
35	Orf1ab	4023 - 4042	AKVTSAMQTMLFTMLRKLDN	0.612
36	Orf1ab	5703 - 5722	ATNYDLSVVNARLRAKHVYV	0.589
37	Orf1ab	6619 - 6638	LIGEAVKTQFNYYKKVDGVV	0.566
38	Orf1ab	5018 - 5037	MPNMLRIMASLVLARKHTTC	0.565
39	Orf3a	184 - 203	YQIGGYTEKWESGVKDCVVL	1
40	Orf3a	24 - 43	TPSDFVRATATIPQASLPF	0.563
41	Orf3a	139 - 158	LLYDANYFLCWHTNICYDYCI	0.51
42	Orf7a	70 - 89	GVKHVYQLRARSVSPKLFIR	1
43	Orf7a	14 - 33	TCELYHYQECVRGTTVLLKE	0.921
44	Orf8	41 - 60	FYSKWYIRVGARKSAPLIEL	1
45	Orf8	98 - 117	LVVRCSFYEDFLEYHDVRVV	0.7

FIGS. 4, 5, 6: The purification of the TeNT-Cov-2 and RBD (319-541 amino acid) of SARS-Cov-2 protein.

[0051] TeNT-Cov2 gene and RBD gene were inserted in the pET-11a vector between Nde I and Xba I sites. Using previously prepared LB Agar plates with 0.05 mg/mL Carbenicillin, newly transformed TeNT-Covid-2 (+) (TC2) plasmid or RBD (+) plasmid BL21DE3 competent cells were spread and incubated at 37°C overnight. After 14+ hours of growth individual colonies were observed on the plates. These plates were placed at 4°C and used for growing overnight cultures.

[0052] Overnight starter cultures were sterilized 2XYT media with 0.05 mg / mL Carbenicillin added after cooled to room temperature. Several colonies of either TC2 or RBD were grown and after about 12-15 hours, 37°C, 250 rpm. 20mL of overnight culture was added to 1 Liter of 2XYT media in a 3L flask, 37°C, 200rpm. Turbidity was monitored at 600nm, when the cultures had an absorbance of 0.6-0.8, 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added. Then incubated at room temperature, 75rpm for overnight 15 to 17 hours. Cells

were pelleted by centrifugation, 4°C, 13,000 x g, 20 minutes. Pellets were then kept and frozen at -20°C.

[0053] Purification of the newly grown cells were done in 50mM Tris HCl, pH8 with 150mM Sodium Chloride. This was kept cold throughout the procedure. Lysis buffer was prepared with this buffer, along with Protease Inhibitor tablet, trace amounts of lysozyme and DNase. The frozen pellet of TC2 or RBD was then suspended in the lysis buffer. This was mechanically sonicated with 3 minutes of processing on cold beads. It was cycled 1 second on / 5 seconds off, 18 to 22 Watts of peak power. The lysate was centrifuged at 13,000 x g, 45 minutes at 4°C.

[0054] While centrifuging, 2mL of fresh nickel agarose beads were equilibrated with the Tris Buffer in cold chamber. The clarified lysate was then applied to the beads at about 8-14 s / drop in a 1.5cm x 20cm column. After loading, 10mL of additional fresh Tris was used to wash the beads. Then using imidazole added to the buffer the protein was collected in fractions. TC2 was eluted with varying 20 to 100mM Imidazole and 250mM imidazole containing buffer, pH8, where the majority of the pure product was in the 250mM eluent. RBD was eluted with several 5mM imidazole and 10mM imidazole to capture the purest product. Higher imidazole did elute protein but there was a greater amount of non-target material mixed with the RBD.

[0055] Samples were measured by UV/VIS Spectrophotography and protein was estimated by using absorbance data as follows: $(\text{Abs}_{235\text{nm}} - \text{Abs}_{280\text{nm}})/2.51 = \sim 0.66 \text{ mg/ml}$ of TeNT-Cov-2 and 0.3 mg/ml of RBD. Proteins were stored with 20% added Glycerol and flash frozen and stored at least -20°C. Sample purity was monitored by SDS-PAGE electrophoresis.

[0056] FIG. 5: The lanes in the FIG. 5 represent the purification of RBD protein from SARS-CoV-2 as follows: Lane 1. Molecular weight marker; lane 2. Lysate from cells; lane 3. Flow through from column; lane 4. 5mM imidazole; Lane 5. 20mM imidazole; Lane 6-7. 50mM imidazole; Lane 8-10. 100mM imidazole; lane 11-12. 200mM imidazole.

[0057] FIG. 6: The purification of the TeNT-Cov-2 (FIG. 6) protein represents as follows: Lane 1. Molecular weight marker; Lane 2. Flow through from column; Lane 3. Lysate from cells; Lane 4. Buffer wash; Lanes 5-6. 20mM imidazole; Lane 7. 50mM imidazole; Lanes 8-10. 100mM imidazole; lanes 11-12. 250mM imidazole. The Major band for TC2 was observed at about 97 kDa and the major bands for RBD were observed at 28 kDa and 75 kDa (trimer). This material was further investigated by ELISA.

FIG. 7: Creation of the Hybrid protein by combining TC2 and RBD by EDC crosslinking to the Sulfo-NHS using two Step linking.

[0058] EDC is the cross linker used for the linking RBD of SARS-Cov-2 with the TeNT-Cov2 to create the hybrid protein. EDC and Sulfo-NHS allows, 2-step coupling of the both the proteins without affecting the carboxyls of the second proteins. EDC activates carboxyl groups and spontaneously reacts with primary amines to form an amide bond. EDC couples NHS to carboxyls, which forms an NHS ester and allows for efficient conjugation to primary amines at physiologic pH. The EDC and NHS were equilibrated at room temperature. 1 mg/ml of TeNT-Cov2 solution in conjugation buffer 1. 0.4 mg EDC and 1.1 mg sulfo-NHS were mixed and incubated for 15 min at room temperature. 1.2µl 2-mercaptoethanol was added to the mixture to quench the EDC reaction. RBD protein was added to in the reaction mixture and incubated for 2 hours at room temperature. The reaction was quenched by adding hydroxylamine. Thus, the Hybrid protein was created by attaching TeNT-Cov2 to RBD proteins.

FIG. 8: Immune response in rabbit

[0059] Rabbits were exposed to Tetanus toxoid and drTeNT three months prior to the exposure them with the TC2, Hybrid and RBD proteins. The primed and unprimed rabbit were exposed to same 7µg of the all three proteins TC2, RBD and hybrid. The serum from the rabbit was collected after 3 months to check the immune response using ELISA.

[0060] Antigen was prepared for coating the plates. 5µg/well of RBD antigen was used in Phosphate Buffered Saline (PBS). Similar plates were also prepared in this manner with TeNT-Covid Hybrid antigen using 5µg/well in PBS. This was incubated at 4°C for 15 hours. The wells were blocked using 3% Bovine Serum Albumin (BSA) in PBS, 0.2mL / well. This was blocked at 37°C for 1 hour. The plates were washed 3 times using PBS + 0.05% Tween 20 (PBST), 0.3mL / well / wash for 3 to 5 minutes.

[0061] After washing, the plates were blotted onto clean paper towels until wells were dry. Next 3%BSA in PBS, 0.1mL was added to all the wells. A series of dilutions were done with the sera from the 6-15-21 bleeds.

[0062] Rabbits NR9, NR10, P198 and P200 were tested. Similar dilutions were done against the TeNT-Covid Hybrid antigen protein; the rabbits used for that ELISA were NR9, NR10, P198 and P199. The sera dilutions were allowed to incubate 1 to 3 hours at 37°C. The dilutions

started at 1:200 and were double diluted to ~1:6 million. The plates were washed with PBST, as before.

[0063] Secondary antibody was prepared by adding commercially purchased goat anti-rabbit peroxidase conjugated IgG antibodies. 1: 10,000 dilution into 3% BSA in PBS. 0.1mL / well, 1 hour, 37°C. Plates were washed again, as before and blotted to dryness on clean paper towels.

[0064] The Plates were then developed by peroxidase reactive developer that was monitored by Thermomax plate reader. Reactions were then read at 450nm to obtain readings and results were plotted to give results. The results suggest that the rabbits primed with DrTent showed highest immune response against Covid epitopes imbedded in DrTent. This was nearly 32-fold higher than unprimed vaccination and 16-fold higher than the RBD vaccination.

Conclusion

[0065] It will be appreciated that the methods and compositions of the present disclosure can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will also be apparent for the expert skilled in the field that other embodiments exist and do not depart from the spirit of the invention. Thus, the described embodiments are illustrative and should not be construed as restrictive.

[0066] The transitional term "comprising", which is synonymous with "including," "containing," or "characterized by," is inclusive or open-ended and does not exclude additional, unrecited elements or method steps. The transitional phrase "consisting of" excludes any element, step, or ingredient not specified in the claim. The transitional phrase "consisting essentially of" limits the scope of a claim to the specified materials or steps "and those that do not materially affect the basic and novel characteristic(s)" of the claimed invention.

[0067] Or, the technology illustratively described herein suitably may be practiced in the absence of any element(s) not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising," "consisting essentially of," and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and use of such terms and expressions do not exclude any equivalents of the features shown and described or portions thereof, and various modifications are possible within the scope of the technology claimed.

[0068] The term "a" or "an" can refer to one of or a plurality of the elements it modifies (e.g., "a reagent" can mean one or more reagents) unless it is contextually clear either one of the elements or more than one of the elements is described.

[0069] The term “about” as used herein refers to a value within 10% of the underlying parameter (i.e., plus or minus 5%), and use of the term “about” at the beginning of a string of values modifies each of the values. As used herein, the term “substantially” refers to approximately the same shape as stated.

[0070] While several embodiments of the disclosure have been described, it is not intended that the disclosure be limited thereto, as it is intended that the disclosure be as broad in scope as the art will allow and that the specification be read likewise. Therefore, the above description should not be construed as limiting, but merely as exemplifications of embodiments.

[0071] Trademarks: the product names used in this document are for identification purposes only; and are the property of their respective owners.

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33. United States Patent application number 20190076518 A1 that published March 14, 2019

What is claimed is:

1. A mutated recombinant vaccine composition comprising one or more detoxified forms of a whole or part of a tetanus neurotoxin (DrTeNT) proteins, and one or more epitopes derived from a toxin, a pathogen, or a disease, wherein the vaccine composition is able to immunize a human or a non-human mammal against the toxin, the pathogen, or the disease.
2. The vaccine composition of claim 1, wherein the toxin, pathogen, or disease is derived from animals, plants, fungi, bacteria, or viruses.
3. The vaccine composition of claim 1, wherein the epitopes are derived from one or more diseases comprising: tuberculosis, malaria, cancer, flu, pneumonia, psoriasis, itch, and lime disease.
4. The vaccine composition of claim 1, wherein the detoxified form of the whole or the part of the tetanus neurotoxin (DrTeNT) comprises at least one mutated light chain and at least one native heavy chain.
5. The vaccine composition of claim 1, comprising the DrTeNT as a carrier protein and one or more epitopes or subunits of the SARS-CoV-2 virus.
6. The vaccine composition of claim 5, comprising a hybrid protein linking a receptor-binding domain (RBD) epitope of a SARS-Cov-2 pathogen with a TeNT-Cov2 protein.
7. The vaccine composition of claim 5, comprising a S1 subunit of a SARS-CoV spike (S) protein comprising a receptor-binding domain (RBD) epitope.
8. The vaccine composition of claim 1, wherein formaldehyde is not present.
9. The vaccine composition of claim 1, further comprising an adjuvant comprising one or more neurotoxin associated proteins (NAPs) derived from a botulinum neurotoxin (BoNT) produced by a *Clostridium botulinum*.

10. The vaccine composition of claim 1, further comprising an adjuvant comprising one or more of: alum, polylactic acid, polyglycolic acid, Vitamin E, and colloidal particle.
11. The vaccine composition of claim 1, wherein the epitopes with at least 95% sequence identity one or more of SEQ ID NOS: 1-45.
12. The vaccine composition of claim 11, comprising epitopes with at least 95% sequence identity to SEQ ID NOS: 1-6.
13. A recombinant TeNT or DrTeNT vector comprising a nucleic acid that encodes a polypeptide comprising a DrTeNT protein; and a promoter which is operably linked to said polynucleotide.
14. The recombinant vector of claim 13, further comprising the nucleic acid encoding one or more epitopes of a toxin, a pathogen or derived from a disease.
15. The recombinant vector of claim 14, wherein the epitopes are derived from Covid-19, and/or comprises at least one SARS-CoV epitope or subunit.
16. The recombinant vector of claim 15, encoding a SARS-CoV receptor binding domain (RBD) gene and a Tent-Cov2 gene to express a RBD and Tent-Cov2 hybrid protein (Tent-Cov2 protein).
17. A cell in which the recombinant vector according any one or claims 13-16 is introduced.
18. A vaccine or medicament for treating and/or preventing Covid-19 comprising the recombinant vector of claim 16.
19. An isolated non-naturally occurring polypeptide sequence encoding the DrTeNT protein.

20. A method of immunizing a mammal against Covid-19 by administering a vector comprising a nucleic acid encoding a SARS-CoV epitope or subunit gene and a Tent or DrTeNT gene.
21. The method of immunizing of claim 20, wherein the vector comprises a nucleic acid encoding a SARS-CoV receptor binding domain (RBD) gene and a Tent-Cov2 gene to express *in vivo* a RBD and Tent-Cov2 hybrid protein (Tent-Cov2 protein).
22. The method of claim 20, wherein the vector is administered subcutaneously or intramuscularly.
23. The method of claim 20, wherein the vaccine composition further comprises an adjuvant and/or a carrier to protect the vector from degradation.
24. The method of claim 23, wherein the carrier is a lipid molecule or a nanocarrier comprising one or more synthetic polymers or biopolymers under a suitable pH and salt conditions for biological systems.
25. The method of claim 20, wherein the vaccine composition further comprises an adjuvant comprising one or more neurotoxin associated proteins (NAPs) derived from a botulinum neurotoxin (BoNT) produced by a *Clostridium botulinum*.
26. The method of claim 25, further comprising administering the vaccine composition orally, sublingually or intranasally.