

# Chapter 14

## Development of a SARS Coronavirus Vaccine from Recombinant Spike Protein Plus Delta Inulin Adjuvant

Clifton McPherson, Richard Chubet, Kathy Holtz, Yoshikazu Honda-Okubo, Dale Barnard, Manon Cox, and Nikolai Petrovsky

### Abstract

Given periodic outbreaks of fatal human infections caused by coronaviruses, development of an optimal coronavirus vaccine platform capable of rapid production is an ongoing priority. This chapter describes the use of an insect cell expression system for rapid production of a recombinant vaccine against severe acute respiratory syndrome coronavirus (SARS). Detailed methods are presented for expression, purification, and release testing of SARS recombinant spike protein antigen, followed by adjuvant formulation and animal testing. The methods herein described for rapid development of a highly protective SARS vaccine are equally suited to rapid development of vaccines against other fatal human coronavirus infections, e.g., the MERS coronavirus.

**Key words** Coronavirus, SARS, MERS, Vaccine, Adjuvant, Delta inulin, Advax adjuvant, Baculovirus, Manufacture, cGMP

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### 1 Introduction

The severe acute respiratory syndrome coronavirus (SARS) was first identified in 2003 after a series of fatal pneumonia cases characterized by an inflammatory cell infiltrate with diffuse alveolar damage [1] started in Hong Kong before spreading to other countries [2]. Before being controlled by quarantine measures, ~8000 humans were infected, with fatality as high as 50 % in the elderly and an overall case fatality rate of ~10 % [2]. More recently, the Middle East respiratory syndrome coronavirus (MERS) has caused a series of serious and, in some cases, fatal human infections [3]. Given the risk of future serious human coronavirus outbreaks, development of a suitable vaccine platform to protect against such viruses is a major priority. These vaccines present several challenges including the rapidity with which these outbreaks develop and hence the need for rapid vaccine manufacture. Hence, a successful coronavirus vaccine platform must overcome multiple challenges.

SARS CoV is a positive-stranded RNA virus 29.7 kb in length with 14 open reading frames [4]. Initial SARS vaccine candidates were produced from inactivated virus. Inactivated whole-virus vaccines provided only modest protection, inducing low-neutralizing antibody titers that did not protect against infection but were associated with faster lung clearance of virus [5]. However, immunization of mice with inactivated vaccines either alone or formulated with alum adjuvant resulted in severe lung eosinophilic pathology in response to virus challenge [6–9], similar to enhanced lung pathology seen with SARS virus reexposure after primary infection [10]. Hence a major challenge when developing a SARS vaccine is to identify strategies to avoid lung eosinophilic pathology.

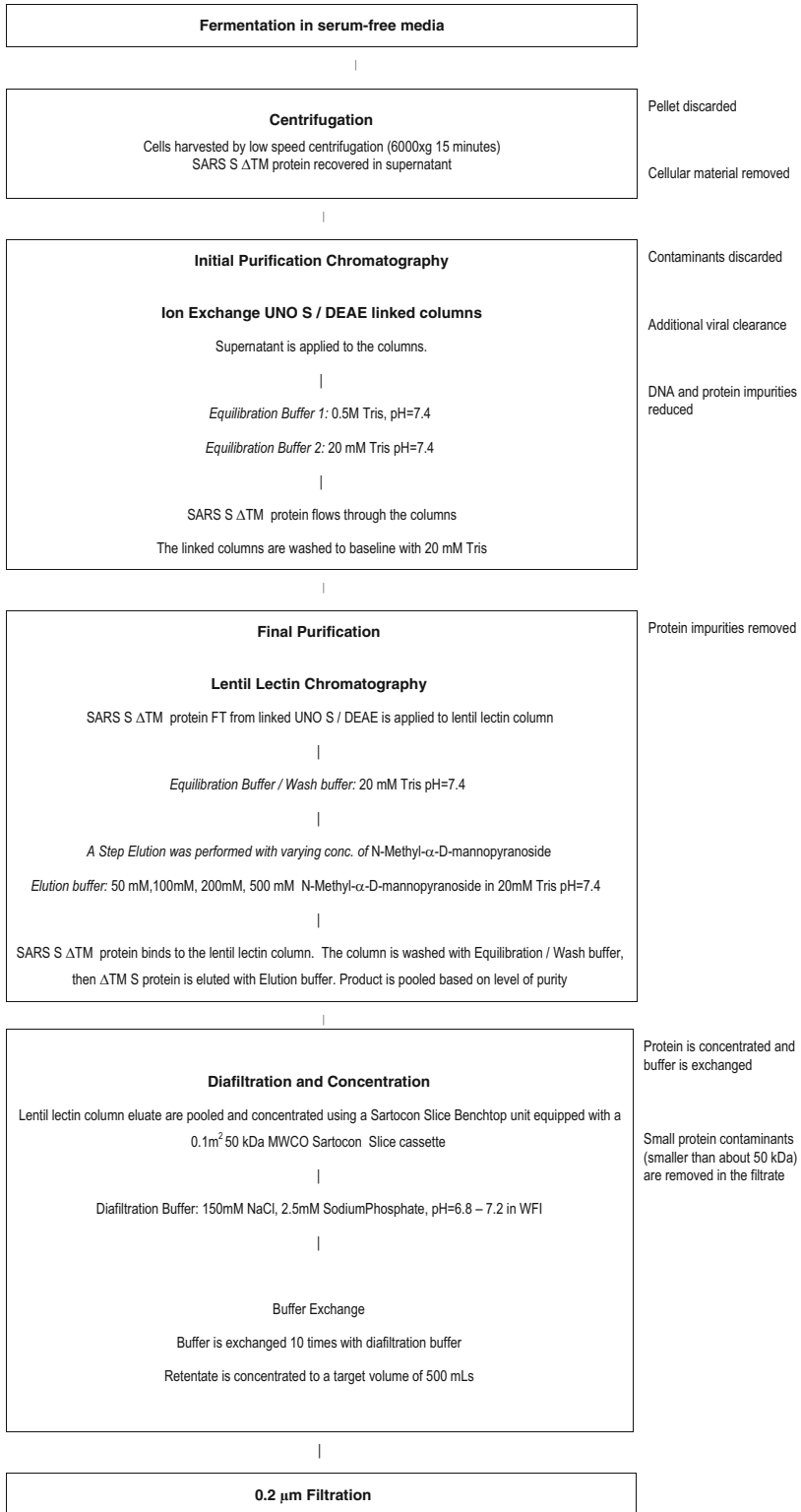
A further challenge when developing vaccines based on inactivated SARS virus is the need for high-containment biosafety level 3 cGMP manufacturing facilities [11]. This makes vaccine manufacture more complex and expensive and restricts the number of vaccine doses that can be rapidly manufactured. To counter these challenges, it would be preferable to produce a subunit vaccine that just like inactivated virus was able to induce neutralizing antibodies against SARS-CoV, but rather than requiring BSL3 manufacture was able to be produced in a regular recombinant protein manufacturing environment.

The potential solution to this problem lies in the coronavirus spike protein (S protein), which in the case of SARS virus binds to angiotensin-converting enzyme 2 and CD209L and induces receptor-mediated virus endocytosis, thereby being critical to virus entry into target cells [12, 13]. S protein could thereby provide an ideal antigen with which to induce neutralizing antibodies against SARS virus [14, 15]. However, while immunization with S protein in animal models provided some protection against SARS virus [16, 17], S protein-immunized animals when subsequently challenged with live virus developed severe lung eosinophilic immunopathology, with this problem exacerbated even further when S protein vaccine was formulated with alum adjuvant [6, 9]. A similar problem of lung eosinophilic immunopathology has been seen with other viruses including respiratory syncytial virus (RSV) vaccines, which prime for an excessive and harmful Th2-dominated lung immune response upon subsequent virus exposure [18]. Lung eosinophilic immunopathology is exacerbated by formulation of such vaccines with any adjuvant that induces excess Th2 immune polarization, e.g., aluminum salt adjuvants [6, 9]. Hence, while S protein would appear to be an ideal SARS vaccine antigen, there is first the need to reduce the risk of lung eosinophilic immunopathology being induced by the vaccine.

Described below are methods used for the development and manufacture of a recombinant subunit vaccine based on an S protein antigen lacking transmembrane and cytoplasmic domains (S  $\Delta$ TM) that was expressed using a baculovirus insect cell expression

platform. As the S  $\Delta$ TM protein antigen itself has low immunogenicity [16, 17], steps are also described for formulation with a safe and effective adjuvant [19]. As aluminum adjuvants are contraindicated for SARS vaccines given they may exacerbate lung eosinophilic pathology; instead methods are described for formulation of the S  $\Delta$ TM protein with Advax<sup>TM</sup>, a safe and effective adjuvant based on delta inulin [20, 21] that has been previously shown in animal models to enhance the immunogenicity of a broad range of viral and bacterial antigens [22–30] and has also been shown safe and effective in preliminary human clinical trials [31, 32]. Notably, Advax adjuvant was recently shown to enhance the immunogenicity and protection conferred by both inactivated and recombinant SARS vaccines, without the excess Th2 bias of alum adjuvants and hence without the risk of inducing lung eosinophilic immunopathology [33].

For this project a recombinant baculovirus was designed to express the ectodomain of the SARS S protein, lacking the transmembrane and cytoplasmic domains [17]. The recombinant protein expressed by this vector was termed SARS S  $\Delta$ TM. This truncated version of the S protein was selected as it contains the receptor-binding domain (RBD) and was able to be expressed in insect cells at a higher level than the full-length membrane-bound version. The SARS S RBD has been shown to induce neutralizing antibodies against the SARS CoV [14, 15]. After infection of *expressSF+*<sup>®</sup> insect cells with the recombinant baculovirus, the S  $\Delta$ TM protein is expressed and secreted into the cell culture medium [17]. The procedure detailed below for purifying the SARS S  $\Delta$ TM protein can be followed after production of the protein in a baculovirus/insect cell system. The process is designed for a 45 L fermentation in a 60 L bioreactor but may be adjusted to other scales as necessary. Following harvest, the SARS S  $\Delta$ TM protein is purified by column chromatography; a schematic is provided in Fig. 1. In the first step, the supernatant is flowed through linked UNOsphere S (UNO S) and DEAE sepharose columns. DNA and protein impurities are removed, and the SARS S  $\Delta$ TM protein remains in the flow through. The flow through from the UNO S/DEAE step is applied to lentil lectin sepharose, SARS S  $\Delta$ TM binds, and impurities are removed. Following elution from the lentil lectin sepharose column, the protein is concentrated, and buffer is exchanged by ultrafiltration. The retentate from the ultrafiltration step is processed through a 0.22  $\mu$ m filter, and the purified protein is stored at  $-20$  °C. Prior to intended use, the purified SARS S  $\Delta$ TM protein is mixed under conditions with a suspension of Advax<sup>TM</sup> delta inulin adjuvant particles and either aseptically filled into single-dose vials or loaded directly into syringes ready for vaccination. Lastly, the adjuvanted SARS vaccine is tested for efficacy and safety in animal immunogenicity and SARS challenge models.



**Fig. 1** Process flow diagram of SARS S ΔTM protein

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## 2 Materials

### 2.1 Fermentation

#### Harvest

1. 1 L Nalgene centrifuge bottles (Thermo Scientific).
2. High-speed centrifuge.
3. Centrifuge rotor to accommodate 1 L bottles.
4. Sterile 50 L Nalgene carboy (Thermo Scientific).
5. 0.22  $\mu\text{m}$  filter (EMD Millipore).

### 2.2 UNO S/DEAE

#### Column

#### Chromatography

1. Two BPG columns (GE Healthcare).
2. UNOsphere S chromatography resin (Bio-Rad).
3. DEAE Sepharose Fast Flow chromatography resin (GE Healthcare).
4. Chromatography system equipped with UV and conductivity monitors.
5. Tris(hydroxymethyl)aminomethane (Trizma; Sigma-Aldrich).
6. Concentrated HCl.
7. Purified water.
8. Sterile 50 L Nalgene carboy (Thermo Scientific).

### 2.3 Lentil Lectin

#### Capture

#### Chromatography

1. XK 50 column (GE Healthcare).
2. Lentil lectin Sepharose 4B (GE Healthcare).
3. Chromatography system equipped with UV and conductivity monitors.
4. Tris(hydroxymethyl)aminomethane (Sigma-Aldrich).
5. Concentrated HCl.
6. Purified water.
7. N-methyl- $\alpha$ -D-mannopyranoside (Sigma-Aldrich).
8. Sterile Nalgene square 250 mL polycarbonate bottles (Thermo Scientific).

### 2.4 Concentration, Ultrafiltration, and 0.22 $\mu\text{m}$ Filtration

1. Sartocoon Slice 200 bench top system (Sartorius).
2. Sartocoon Slice 200 PESU cassette (Sartorius).
3. Pump.
4. Sodium phosphate monobasic monohydrate (Sigma-Aldrich).
5. Sodium phosphate dibasic 12-hydrate (Sigma-Aldrich).
6. Sodium chloride (Sigma-Aldrich).
7. Purified water.
8. Pipettes.
9. Microcentrifuge tubes.
10. Sterile Nalgene square 500 mL polycarbonate bottle (Thermo Scientific).

**2.5 Adjuvant Formulation**

1. Delta inulin adjuvant suspended in bicarbonate buffer (Vaxine Pty Ltd).
2. CpG oligonucleotide powder (Oligo Factory, USA).
3. Water for injection (Baxter).

**2.6 Mouse Immunogenicity Testing**

1. Female 6–8-week-old BALB/c mice weighing 18–20 g.
2. 0.5 mL Insulin syringes (BD).
3. 5 mL Syringes.
4. 25G 5/8 needles.
5. Animal lancet, 4 mm (Medipoint Inc., USA).
6. 96-Well ELISA plates (Greiner Bio-One).
7. 24-Well culture plates (Greiner Bio-One).
8. 0.1 M Sodium carbonate buffer, pH 9.6.
9. 1 % BSA/PBS.
10. Biotinylated anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG3, or IgM antibodies (Abcam).
11. Streptavidin-HRP (BD Biosciences).
12. TMB substrate (KPL, USA).
13. 1 M Phosphoric Acid.
14. Cell strainers, 70  $\mu$ m Nylon (Falcon).
15. RPMI complete medium with 10 % heat-inactivated FBS (Invitrogen Life Technologies).
16. Red blood cell (RBC) lysis buffer (155 mM  $\text{NH}_4\text{Cl}$ ; 10 mM  $\text{KHCO}_3$ ; 0.1 mM EDTA, pH 7.3).
17. Carboxy-fluorescein diacetate succinimidyl ester (CFSE) (Invitrogen Life Technologies).
18. MultiScreen HTS, 96-well filtration plate (Merck Millipore).
19. Anti-mouse CD16/CD32 (BD Biosciences).
20. Anti-mouse CD4-APC (BD Biosciences).
21. Anti-mouse CD8a-PE-Cy7 (BD Biosciences).
22. Anti-mouse IFN- $\gamma$ , IL-2, IL-4 antibody pairs (BD Biosciences).
23. LEAF anti-mouse IL-17A and biotin-anti-mouse IL-17A antibody (BioLegend, USA).

**2.7 Animal Challenge Studies**

1. Female 4–8-week-old BALB/c mice weighing 18–20 g.
2. SARS-CoV virus strain Urbani (200300592), Centers for Disease Control and Prevention, Atlanta, GA, USA.
3. Vero 76 cell line (American Type Culture Collection, Manassas, VA, USA).
4. Hematoxylin and eosin stain.

5. Rat monoclonal antibody (Clone MT-14.7) to eosinophil major basic protein MBP (Lee Laboratory, Mayo Clinic, Arizona).
6. DAB chromogen.

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### 3 Methods

#### 3.1 Fermentation Harvest

1. Separate cells and culture supernatant by centrifugation at  $5900 \times g$  at 2–8 °C for 15 min.
2. Transfer culture supernatant to a sterile 50 L carboy.
3. After all culture supernatant is collected, filter through 0.22  $\mu\text{m}$  filter into a second sterile 50 L carboy (*see Note 1*).
4. Store at 2–8 °C.

#### 3.2 UNO S/DEAE Column Chromatography

1. Pack a BPG column with 1.9 L of UNO S resin (*see Note 2*).
2. Pack a BPG column with 1.5 L of DEAE Sepharose Fast Flow resin.
3. pH equilibrate the UNO S column with 0.5 M Tris pH 7.4 at a flow rate of 200–400 mL/min until outflow pH is 7.0–7.7. This step typically requires 3 column volumes of buffer (*see Note 3*).
4. Continue equilibration of the UNO S column with 20 mM Tris pH 7.4 at a flow rate of 200–400 mL/min until outflow is pH 7.2–7.5 and conductivity is  $\leq 500$  mS. This step typically requires 5 column volumes.
5. pH equilibrate the DEAE column with 0.5 M Tris pH 7.4 at a flow rate of 200–400 mL/min until outflow pH is 6.9–7.6. This step typically requires 3 column volumes of buffer.
6. Continue equilibration of the DEAE column with 20 mM Tris pH 7.4 at a flow rate of 200–400 mL/min until outflow is pH 7.0–7.5 and conductivity is  $\leq 500$  mS. This step typically requires 5 column volumes.
7. Connect the DEAE column to the outflow of the UNO S column.
8. Equilibrate the linked UNO S and DEAE columns with 20 mM Tris pH 7.4 at a flow rate of 200–400 mL/min until outflow is pH 6.9–7.6 and conductivity is  $\leq 500$  mS.
9. Apply the culture supernatant from Subheading 2.1 to the linked UNO S/DEAE columns at a flow rate of not more than 300 mL/min (*see Note 4*).
10. Begin collecting the flow through from the linked columns into a 50 L carboy when the UV trace begins to rise.
11. Wash the linked columns with 20 mM Tris pH 7.4 and collect the wash in the carboy with the column flow through.

12. Collect  $\leq 10$  L of wash, and stop collection of the wash when the UV trace returns to baseline.
13. Proceed to lentil lectin capture chromatography.

### **3.3 Lentil Lectin Capture Chromatography**

1. Pack XK-50 column with 250 mL of lentil lectin sepharose (*see Note 2*).
2. Equilibrate lentil lectin column with 20 mM Tris pH 7.4 at a flow rate of 50–100 mL/min using 10 column volumes of buffer. pH should be 7.2–7.5.
3. Load UNO S/DEAE flow through to the lentil lectin column at a flow rate of 50–100 mL/min (*see Note 5*).
4. Wash column with 20 mM Tris pH 7.4 at a flow rate of 50–100 mL/min for 5 column volumes or until UV trace returns to baseline.
5. Collect fractions in sterile Nalgene square 250 mL polycarbonate bottles.
6. Elute with 2 column volumes of 50 mM N-methyl- $\alpha$ -D-mannopyranoside and 20 mM Tris pH 7.4 at a flow rate of 50–100 mL/min. Collect two 250 mL fractions.
7. Elute with 2 column volumes of 100 mM N-methyl- $\alpha$ -D-mannopyranoside and 20 mM Tris pH 7.4 at a flow rate of 50–100 mL/min. Collect two 250 mL fractions.
8. Elute with 2 column volumes of 200 mM N-methyl- $\alpha$ -D-mannopyranoside and 20 mM Tris pH 7.4 at a flow rate of 50–100 mL/min. Collect two 250 mL fractions.
9. Elute with 5 column volumes of 500 mM N-methyl- $\alpha$ -D-mannopyranoside and 20 mM Tris pH 7.4 at a flow rate of 50–100 mL/min. Collect 250 mL fractions until UV trace is flat and stable.
10. Store fractions at 2–8 °C.
11. Analyze all fractions by SDS-PAGE and Western blot to determine which fractions contain SARS S  $\Delta$ TM protein.
12. Pool fractions containing detectable SARS S  $\Delta$ TM protein.

### **3.4 Concentration, Ultrafiltration, and 0.22 $\mu$ m Filtration**

1. Assemble Sartocon Slice 200 bench top ultrafiltration system with a 0.1 m<sup>2</sup> 50 kDa molecular weight cutoff Sartocon Slice cassette according to the manufacturer's instructions.
2. Attach a process tank according to the manufacturer's instructions.
3. Fill process tank with water for injection (WFI).
4. Close permeate valve and circulate WFI through the system at 200 mL/min for 3–4 min.
5. Open permeate valve.
6. Increase circulation rate to 600–800 mL/min.



7. Adjust retentate valve to obtain a transmembrane pressure (TMP) of 8.0–17.0 psi.
8. Continue rinsing for 5–10 min.
9. Repeat **steps 3–8** using diafiltration buffer (150 mM NaCl, 2.5 mM NaPO<sub>4</sub> pH 6.8–7.2 in WFI).
10. Fill process tank with pooled SARS S ΔTM protein.
11. Retentate line should be connected to process tank, and valve should be open.
12. Allow S ΔTM to recirculate through the system at a flow rate of ≤200 mL/min for 3–4 min.
13. Increase recirculation flow rate to 600–800 mL/min.
14. Ensure a TMP of 13.0–17.0 psi.
15. Monitor volume in process tank.
16. Stop concentration when volume in process tank is approximately 400 mL. This is the initial concentration retentate.
17. Set up a 2 L vessel to siphon into process tank.
18. Fill container with 2 L of diafiltration buffer (150 mM NaCl, 2.5 mM NaPO<sub>4</sub> pH 6.8–7.2 in WFI).
19. Retentate valve should be open.
20. Start pump and circulate at a flow rate of ≤200 mL/min for 3–4 min.
21. Increase flow rate to 600–800 mL/min.
22. Adjust retentate valve to maintain a TMP of 13.0–17.0 psi.
23. Monitor volume of diafiltration buffer in vessel.
24. Continue until a total volume of diafiltration buffer equal to 10 times the volume of initial concentration retentate has been used.
25. Volume in process tank should be approximately equal to initial concentration retentate volume. This is the diafiltration retentate.
26. Process the diafiltration retentate through a 0.22 μm filter into a sterile Nalgene polycarbonate bottle. This is the S ΔTM bulk drug substance.
27. Remove aliquots for testing.
28. Store bulk drug substance at –20 °C.

### **3.5 Antigen Release Testing**

1. Testing and acceptance criteria for SARS S ΔTM are listed in Table 1.

### **3.6 Vaccine Adjuvant Formulation**

1. Advax™ is a preservative-free sterile suspension of delta inulin microparticles at 50 mg/mL in a bicarbonate buffer, which when combined with vaccine antigen enhances both Th1 and Th2 immunity in a balanced fashion.

**Table 1**  
**SARS S ΔTM analytical tests and acceptance criteria**

Parameter	Method	Acceptance criterion
Identity	SDS-PAGE/Western blot	Detection of approx. 150 kDa protein with SARS S antiserum
DNA content	PicoGreen	≤15 ng/dose
pH	USP <791>	7.0 ± 0.4
Osmolality	USP <785>	≤330 mOsm/hg
Bacterial endotoxin	USP <85>	<10 EU/dose
Purity	SDS-PAGE/Western blot	≥90 %
Lentil lectin content	SDS-PAGE/Western blot	<10 ng lentil lectin/μg SARS S ΔTM
Microbial limits	USP <61>	<10 CFU/mL
Total protein	Bicinchoninic acid assay	Perform and report
Potency	ELISA	≥60 % of total protein content

2. To further enhance Th1 and reduce Th2 immune bias, 10 μg CpG oligonucleotide per 1 mg delta inulin is added to the Advax™ adjuvant, as a simple admixture.
3. Advax™ adjuvant formulations are administered to mice at a standardized dose of 1 mg delta inulin per mouse, irrespective of the antigen dose.
4. Advax™ adjuvant is formulated with S ΔTM bulk drug substance in a laminar flow hood by aseptic simple admixture of the Advax™ suspension with the S ΔTM bulk drug substance and drawing up the combined milky white suspension into a 0.5 mL insulin syringe immediately prior to immunization.

### **3.7 Animal Immunogenicity Testing**

1. Vaccine immunogenicity studies can be conveniently performed on adult female BALB/c mice at 6–8 weeks of age but can also be performed on other strains such as C57BL/6 (*see Note 6*).
2. Mice are immunized twice 3 weeks apart by an intramuscular injection into the thigh, in order to mimic the most common route of human vaccine administration. The maximum volume that can be injected into an adult mouse thigh muscle is 50 μl. If the vaccine cannot be reduced to this volume, then providing the vaccine is not reactogenic and then larger vaccine volumes can be administered by injection of 50 μl amounts into multiple legs.
3. Starting at 1 week post-immunization, at intervals of 1–4 weeks mice are bled using cheek vein bleeding using a lancet in order to obtain ~25–50 μl of blood from which serum is

obtained by centrifugation and then stored at  $-20^{\circ}\text{C}$  for later use in antibody assays.

4. SARS-specific antibodies are conveniently determined in mouse serum by ELISA. S  $\Delta$ TM protein is adsorbed to ELISA plates in 0.1 M sodium carbonate buffer, pH 9.6 and incubated overnight at  $4^{\circ}\text{C}$ . After blocking with 1 % BSA/PBS for 1 h, serum samples diluted in 1 % BSA/PBS are incubated for 2 h at room temperature (RT) and then washed. Subsequently, 100  $\mu\text{l}$  biotinylated anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG3, or IgM antibodies (Abcam) plus streptavidin-HRP (BD Biosciences) are added and incubated for 1 h at RT. After washing, wells are incubated with 100  $\mu\text{l}$  of TMB substrate for 10 min and then stopped by 1 M phosphoric acid. The optical density is measured at 450 nm (OD<sub>450</sub> nm) with a VersaMax ELISA microplate reader (Molecular Devices, CA, USA) and analyzed using SoftMax Pro Software.
5. At the termination of immunogenicity studies, mice are killed by cervical dislocation and bones and spleens are collected to enable measurement of SARS-specific memory T and B cells. Bone marrow is isolated from femurs by flushing with 3 % FBS/PBS. Splenocytes are released by pressing against a cell strainer with a rubber syringe plunger and RBCs are removed by osmotic shock. Cells are washed with 3 % FBS/PBS and then resuspended in RPMI complete medium with 10 % heat-inactivated FBS.
6. For T cell proliferation assays, splenocytes are labeled with 5  $\mu\text{M}$  CFSE (Invitrogen Life Technologies) for 8 min at RT. CFSE starts to react when exposed to aqueous solutions. It is, therefore, important to avoid dilution of CFSE until immediately before cell labeling.
7. Labeled cells are cultured in 24-well plates at  $10^6$  cells/mL/well with or without S  $\Delta$ TM protein 1  $\mu\text{g}/\text{mL}$ . After 5-day incubation at  $37^{\circ}\text{C}$  and 5 %  $\text{CO}_2$ , cells are washed with 0.1 % BSA/PBS, treated with anti-mouse CD16/CD32 (BD Biosciences) for 5 min at  $4^{\circ}\text{C}$  and then stained with anti-mouse CD4-APC and anti-mouse CD8a-PE-Cy7 (BD) for 30 min at  $4^{\circ}\text{C}$ . Cells are washed with 0.1 % BSA/PBS and then analyzed by FACS (FACSCanto II, BD Biosciences) with FACSDiva software. For each lymphocyte subset, proliferation is expressed as the percentage of divided cells (CFSE low) compared to undivided cells (CFSE high). Dot plots representing analysis of  $10^5$  cells are generated by FlowJo software. It is important to have CFSE-labeled, unstimulated cells as a nondividing cell control.
8. The frequency of antigen-specific antibody- or cytokine-secreting cells is analyzed using biotinylated anti-mouse IgG,

IgG1, IgG2a, or IgM antibodies (Abcam) or anti-mouse IFN- $\gamma$ , IL-2, and IL-4 antibody pairs (BD) or LEAF anti-mouse IL-17A and biotin-anti-mouse IL-17A antibody (BioLegend, USA) with streptavidin-HRP (BD Biosciences), according to the manufacturer's instruction. Briefly, single-cell suspensions are prepared from bone marrow and spleens of mice at indicated time points, plated at  $2 \times 10^5$  cells/well in 96-well filtration plates pre-coated with S  $\Delta$ TM protein (for antibody detection) or anti-mouse cytokine mAb (for cytokine detection) overnight at 4 °C, and then blocked with RPMI/10 % FBS. For cytokine assays, the cells are incubated with S  $\Delta$ TM protein (10  $\mu$ g/mL) at 37 °C and 5 % CO<sub>2</sub> for 2 days. Wells are washed and incubated with biotinylated anti-mouse Ig or anti-mouse cytokine mAb at RT for 2 h, and washed, and then streptavidin-HRP is added for 1 h before washing and addition of AEC substrate solutions (BD Biosciences). Spots are counted by ImmunoSpot S6 ELISPOT analyzer (CTL, USA) and analyzed using ImmunoSpot Software. Spots in negative control wells are subtracted from the number of spots in S protein wells and the results are expressed as antibody-secreting cells (ASC) per 10<sup>6</sup> BM cells or spots per 10<sup>6</sup> splenocytes.

9. For statistical analysis, group comparisons for antibody and ELISPOT tests are done by Mann–Whitney test.

### **3.8 SARS CoV Mouse Challenge Studies**

1. Female 4–6-week-old BALB/c mice weighing 18–20 g are obtained from Charles River Laboratories (Wilmington, MA), maintained on Wayne Lab Blox, and fed with standard mouse chow and tap water ad libitum (*see Note 6*).
2. To generate a mouse-adapted SARS-CoV, the SARS-CoV strain Urbani (200300592) was obtained from Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA). This strain was propagated and titrated in Vero 76 cells obtained from American Type Culture Collection (ATCC, Manassas, VA), and grown in minimal essential medium (MEM) supplemented with 10 % heat-inactivated fetal bovine serum (FBS, Thermo Fisher Scientific Co., Logan, UT). For in vitro antiviral assays, the serum was reduced to 2 % FBS and gentamicin was added to the medium up to a final concentration of 50  $\mu$ g/mL. BALB/c mice were infected with the Urbani strain. Three or five days after infection, the lungs were removed and homogenized and then used to reinfect a subsequent group of mice. This infection step was continued 25 times through BALB/c mice lungs. The virus was then plaque-purified three times and yielded a virus causing severe lung disease and mortality in infected mice. The virus was verified as SARS-CoV by enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR). All experiments involving

infectious SARS-CoV viruses need to be conducted in an approved biosafety level 3+ laboratory.

3. At days 3 and 6 post-virus challenge, five mice from each immunized and control group are sacrificed and the lungs harvested for gross pathology (lung score), lung weights, lung virus titers, and measurement of anti-SARS IgG in lung homogenate.
4. For lung scoring, samples from each mouse lung lobe are weighed and placed in a petri dish. Lungs are scored based on surface appearance of lungs. Lungs are then assigned a score ranging from 0 to 4, with 0 indicating that the lungs looked normal and 4 denoting that the entire surface area of the lungs was inflamed and exhibited plum-colored lung discoloration. Significant differences in lung scores are determined by Kruskal-Wallis test followed by Dunn's pairwise comparison post tests. Analysis of variance (ANOVA) is used to determine significant differences in lung weights. Pairwise comparisons are made by Newman-Keuls posttests.
5. Lung virus titers are analyzed from mice sacrificed on days 3 and 6 post-virus exposure. A lobe from each mouse lung is homogenized in MEM supplemented with 10 % FBS and the tissue fragments are allowed to settle. The varying dilutions of the supernatant fluids are assayed in triplicate for infectious virus in Vero 76 cells by cytopathic effect (CPE) assay. The titers (50 % tissue culture infectious dose, CCID<sub>50</sub> values) are calculated using the Reed-Muench method. Significant differences are detected by ANOVA. Pairwise comparisons are made by Newman-Keuls posttests.
6. For SARS-CoV neutralizing antibody assay sera are harvested by submandibular bleeding from surviving mice at days 7 and 14 after virus challenge. 7  $\mu$ l aliquot of each serum sample is added to approximately 63  $\mu$ l of MEM, mixed, and then serially diluted by  $\frac{1}{2}$  to achieve 1/40 to 1/8192 dilutions in 96-well plates. Virus stock is diluted in MEM to approximately 200 CCID<sub>50</sub> per 60  $\mu$ l. Next 60  $\mu$ l of virus is added to each well, and the plates are vibrated for approximately 1 min, and then incubated for 1 h at 37 °C for neutralization. 100  $\mu$ l of the liquid from each well is then transferred to 96-well plates containing sub-confluent monolayers of Vero 76 cells, and 100  $\mu$ l of MEM+4 % FBS added to each well. Plates are sealed with tape, incubated for 5 days at 37 °C with 5 % CO<sub>2</sub>, and scored for the presence or absence of virus CPE. Uninfected wells serve as a negative cell control, and a serum sample with known anti-SARS antibody as a positive control. Results are reported as the inverse of the greatest dilution where virus CPE is not detected.
7. To assess for effects of vaccines on lung eosinophilic immunopathology, immunized and vehicle-treated mice are sacrificed at days 3 and 6 post-virus challenge, and lungs removed.

Formalin-fixed lungs are mounted in paraffin blocks. Paraffin-embedded lung sections are stained with hematoxylin and eosin (H&E) and a rat monoclonal antibody (Clone MT-14.7) to eosinophil major basic protein MBP (Lee Laboratory, Mayo Clinic, Arizona) following a standard IHC procedure. DAB chromogen identifies eosinophils as brown-stained cells. Eosinophil infiltration is scored without knowing animal identity using H&E-stained slides. An overall infiltration score, 0–3, is assigned to each section according to the amounts of eosinophils in the parenchyma and their distributions through the lung. Score 0=no to a few eosinophils; score 1=mild eosinophil infiltration; score 2=moderate infiltration; score 3=severe infiltration. For confirmation, immunohistochemistry to the eosinophil major basic protein is performed in sections with the highest score of each treatment group.

8. The remainder of the mice are followed for 2 weeks post-challenge to assess survival. Survival analysis is done using the Kaplan-Meier method and a Logrank test. If that analysis reveals statistically significant differences among the treatment groups, pairwise comparison of survivor curves is analyzed by the Mantel-Cox Logrank test, and the relative significance adjusted to a Bonferroni-corrected significance threshold for the number of treatment comparisons done. All group comparisons of virus titers are done using analysis of variance to determine experimental significance followed by Newman-Keuls pairwise comparison tests.

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## 4 Notes

1. Because the S  $\Delta$ TM protein the culture supernatant is in a rich culture medium, all practical precautions should be taken to prevent microbial growth. All containers should be sterile, buffers should be 0.22  $\mu$ m filtered, columns and chromatography systems should be thoroughly sanitized after use, and process intermediates should be processed immediately or stored at 2–8 °C.
2. Columns should be packed ahead of time and stored according to the resin manufacturer's recommendations.
3. Columns may be equilibrated during harvest of the culture.
4. Culture supernatant containing S  $\Delta$ TM may be loaded onto the linked UNO S/DEAE columns immediately after harvest.
5. The lentil lectin sepharose column may be loaded immediately after completion of the UNO S/DEAE step, and the load may be run overnight.
6. BALB/c mice are typically used for vaccine studies as they have a Th2 immune bias and hence are good at making antibody responses, whereas C57BL/6 mice have a Th1 bias.

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