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Engineering a stable CHO cell line for the expression of a MERS-coronavirus vaccine antigen

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ABSTRACT

Middle East respiratory syndrome coronavirus (MERS-CoV) has infected at least 2040 patients and caused 712 deaths since its first appearance in 2012, yet neither pathogen-specific therapeutics nor approved vaccines are available. To address this need, we are developing a subunit recombinant protein vaccine comprising residues 377–588 of the MERS-CoV spike protein receptor-binding domain (RBD), which, when formulated with the AddaVax adjuvant, it induces a significant neutralizing antibody response and protection against MERS-CoV challenge in vaccinated animals. To prepare for the manufacture and first-in-human testing of the vaccine, we have developed a process to stably produce the recombinant MERS S377–588 protein in Chinese hamster ovary (CHO) cells. To accomplish this, we transfected an adherent dihydrofolate reductase-deficient CHO cell line (adCHO) with a plasmid encoding S377–588 fused with the human IgG Fc fragment (S377–588-Fc). We then demonstrated the interleukin-2 signal peptide-directed secretion of the recombinant protein into extracellular milieu. Using a gradually increasing methotrexate (MTX) concentration to 5 μ M, we increased protein yield by a factor of 40. The adCHO-expressed S377–588-Fc recombinant protein demonstrated functionality and binding specificity identical to those of the protein from transiently transfected HEK293T cells. In addition, hCD26/dipeptidyl peptidase-4 (DPP4) transgenic mice vaccinated with AddaVax-adjuvanted S377–588-Fc could produce neutralizing antibodies against MERS-CoV and survived for at least 21 days after challenge with live MERS-CoV with no evidence of immunological toxicity or eosinophilic immune enhancement. To prepare for large scale-manufacture of the vaccine antigen, we have further developed a high-yield monoclonal suspension CHO cell line.

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1. Introduction

With over 712 deaths and 2040 confirmed cases since its original appearance on the Arabian peninsula in 2012 [1], Middle East Respiratory syndrome (MERS) coronavirus (MERS-CoV) has emerged as an important global pathogen and potential pandemic threat. There remains a critical need for a vaccine targeting MERS-CoV [2], and the newly established Coalition for Epidemic

Preparedness Innovation (CEPI) has now designated research and development for the MERS-CoV vaccine as a global priority [3]. Recently, Phase I studies of DNA-based vaccines against MERS-CoV showed that 98% of vaccinated volunteers generated antibody against MERS CoV [4]. However, to date there is no licensed DNA vaccine for humans due in part to questions about their long-term safety, and their ability to induce high titers of protective or neutralizing antibodies relative to recombinant protein-based vaccines [5,6].

A lead candidate for such a protein-based vaccine is the receptor-binding domain (RBD) of the MERS-CoV spike (S) protein. The MERS-CoV RBD plays an essential role in host receptor binding,

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membrane fusion, and cell entry [7–9], thus making it an ideal vaccine target. Moreover, focusing on the RBD component, rather than the full-length S protein, reduces the likelihood of eosinophilic- or antibody-dependent immune enhancement [10,11]. Expressed and purified as a recombinant fusion protein with the human Fc fragment, the AddaVax(MF59-like)-adjuvanted RBD (residues 377–588) of MERS-CoV has been shown to elicit high neutralizing antibody titers in both mice and rabbits [9,12–16]. These antibodies displayed potent neutralizing activity against almost 20 human and camel MERS-CoV strains, including those with amino acid mutations in the RBD region of their spike proteins [15,17,18]. In a complementary approach, recombinant RBD mutants representing different human and camel virus isolates were all able to elicit broad-spectrum neutralizing antibodies against a wide range of human and camel MERS-CoV strains [15]. Antibodies against the RBD were able to block the binding of the RBD to the MERS-CoV's cellular receptor, DPP4 [17], and thus block the viral entry into permissive human cells [18]. Most importantly, recent studies found that these neutralizing antibodies were indeed associated with protection, when vaccinated Ad5-hDPP4-transduced mice and hDPP4-transgenic mice were found to be immune against lethal MERS-CoV challenge [14,19,20]. Although the vaccine antigen has been produced at small, laboratory scale in a transient HEK293 cell system [17], little effort has been put forth to develop and scale up of MERS-CoV RBD suitable for future vaccine manufacture. Therefore, we have now engineered a stable CHO cell line suitable for producing this MERS-CoV protein vaccine antigen.

2. Materials and methods

2.1. Construction of expression plasmids

The codon-optimized DNA sequence encoding the human IgG Fc-fused S377-588 and the signaling peptide of interleukin-2 (IL2) residing at the N-terminus were synthesized and cloned into pJet2.1 using XbaI and NotI restriction sites (GeneScript) (pJet2.1_IL2_S377-588-Fc). Sequences encoding signal peptides derived from IgK light chain (IgK), human serum albumin (SA), and Azurocidin (Azu) were incorporated into the MERS S377-588-Fc by touchdown PCR with ultramer oligomers (Table 1, Supp. Table 1). The PCR products were gel purified using a QIAquick PCR Purification Kit (Qiagen) and subsequently cloned into the pOptiVEC-TOPO vector (Invitrogen), followed by *Escherichia coli* TOP10 transformation. Ampicillin-resistant transformants were selected on LB agar plates containing 100 µg/mL of ampicillin and subsequently grown in LB broth. Plasmid DNA prepared from isolated colonies were sequenced. To construct the expression plasmid with the IL2 signal peptide, pJet2.1_IL2_S377-588-Fc was digested with XbaI and NotI restriction enzymes, and the gene cassette was gel purified. The expression plasmid pOptiVEC was digested with the same enzymes and gel purified, followed by ligation with T4 DNA ligase.

2.2. Development of an adherent CHO cell line expressing S377-588-Fc

Adherent, dihydrofolate reductase (DHFR)-deficient CHO cells (adCHO) (ATCC® CRL-9096™, CHO-*dhfr*) were cultured in Iscove's

Modified Dulbecco's Medium (IMDM, Gibco) supplemented with 2 mM L-glutamine (Gibco), 5% fetal bovine serum (FBS, Gibco) and 0.1 mM sodium hypoxanthine/0.016 mM thymidine (H/T, Gibco). To establish S377-588-Fc-expressing adCHO cell lines, 1×10^6 cells were transfected with 10 µg plasmid DNA with different signal peptides using Lipofectamine® 2000 (Invitrogen), according to the manufacturer's instructions. All plasmid DNAs were linearized with AhdI prior to transfection. Stable transfectants were selected by culturing in selective medium (same medium as previously described without H/T supplementation). Cells were passaged every 6 days after reaching a maximum cell density of 1.2×10^6 viable cells per mL (split ratio 1:15). To investigate the effect of the different signal peptides on protein expression, conditioned medium from each transfection was collected for quantitative analysis. For gene amplification, transfected adCHO cells were grown to confluence (6 days) in the selective medium supplemented with MTX. The concentration of MTX was increased gradually during each passage (6 days per passage) of adCHO cells (20 nM > 100 nM > 200 nM > 400 nM > 600 nM > 800 nM > 1500 nM > 2000 nM > 3000 nM > 5000 nM, [21]).

2.3. Purification of MERS S377-588-Fc

The recombinant MERS S377-588-Fc was loaded onto a HiTrap Protein-A HP column (GE) at a flow rate of 1 mL/min. The column was washed with 1x PBS (pH 7.5, Amesco) for 10 column volumes (CVs) and eluted with 3 CVs of 0–100% elution buffer (100 mM Citric buffer pH 4.0), followed by 7 CVs of 100% elution buffer. The elution fractions (0.5 mL) were collected in tubes containing 0.2 mL of 1 M Tris-HCl (pH 8.0) to elevate the pH of the eluted protein to pH 7.0. SDS-PAGE and Western blotting analysis were performed with Rabbit-anti-MERS-CoV RBD (1:2000, [13]) and Rabbit-anti-Bovine (Fab) 2-Biotin (1:4000, Sigma) antibodies to identify the S377-588-Fc protein in the elution fractions. The peak fractions containing the MERS S377-588-Fc protein were pooled and concentrated to 2 mg/mL with Amicon Ultra-15 centrifugal filter unit (MWCO 10 kDa) and buffer-exchanged into 20 mM Tris-HCl and 20 mM NaCl (pH 7.4).

2.4. Characterization and PTM analysis

The protein secondary structure was predicted from circular dichroism (CD) spectra. Samples for CD experiments were prepared in 20 mM citrate phosphate at a concentration of 0.2 mg/mL. CD spectra were recorded with a Jasco J-1500 s spectrophotometer, scanning from 280 nm to 200 nm at 100 nm/min with a bandwidth of 1 nm and response time of 1 s. Experiments were performed using one quartz cuvette with a path length of 0.1 cm, keeping a constant temperature of 25 °C. The average value was determined after five scans, and the spectrum of the matching 'buffer alone' sample served as the control. The secondary structure was predicted using the CDPro software by comparing with three reference sets (SP43, SDP48 and SMP56) and using two data fitting programs (CONTIN and CDSSTR). A real-time protein melt experiment was performed using Protein Thermal Shift™ Dye (Thermo Fisher Scientific) in an Applied Biosystems ViiA 7 real-time PCR system (Thermo Fisher Scientific), yielding a fluorescence

Table 1
Overview of tested signal peptides in the adCHO expression system.

Construct	Signal peptide	Signal peptide sequence	Ref.
pOpti_IL2_S377-588-Fc	Human Interleukin 2	MYRMLLSIALSLALVTNS	[40]
pOpti_IgK_S377-588-Fc	Mouse Ig kappa light chain precursor	MDMRAPAGIFGFLLVLPFGYRS	[41]
pOpti_SA_S377-588-Fc	Human Serum albumin preproprotein	MKWVTFISLLFLFSSAYS	[27]
pOpti_Azu_S377-588-Fc	Human Azurocidin preproprotein	MTRLTVLALLAGLLASSRA	[27]

profile specific to purified MERS S377-588-Fc. To evaluate PTM, the purified protein was treated with Peptide-N-Glycosidase F (PNGaseF), O-glycosidase and neuraminidase in a non-denatured form and subsequently analyzed by gel electrophoresis.

2.5. Co-immunoprecipitation (Co-IP) assay

A Co-IP assay was carried out to analyze the interactions between adCHO-expressed MERS S377-588-Fc and human DPP4 (hDPP4) receptor in Huh-7 cell lysates, using MERS S377-588-Fc expressed in HEK293T cells [13] as a positive control and HEK293T-expressing SARS-CoV RBD-Fc as a negative control. The Huh-7 cell lysates (5×10^7 /mL in 1 mL lysis buffer containing 0.3% N-decyl-D-maltopyranoside–phosphate-buffered saline [PBS]) were incubated with the aforementioned protein (20 μ g) plus Protein A Sepharose beads (50% [vol/vol], 200 μ L) at 4 °C for 1 h. After being washed with lysis buffer and PBS, beads were subjected to SDS-PAGE and Western blotting analysis for the detection of DPP4 using anti-hDPP4 mAb (1:1000, R&D Systems), MERS-CoV RBD-specific mouse sera (anti-MERS-RBD, 1:1000, [22]) and SARS-CoV RBD-specific 33G4 mAb (anti-SARS-RBD, 1 μ g/mL, [10]).

2.6. Flow cytometry

Flow cytometry analysis was carried out to quantify the binding between MERS-CoV RBD and hDPP4-expressing Huh-7 cells. Cells were incubated with S377-588-Fc (40 μ g/mL), expressed either in adCHO cells or HEK293T cells, for 30 min at room temperature, followed by addition of FITC-labeled anti-human IgG antibody (Abcam) for 30 min. Cells were then analyzed by flow cytometry.

2.7. ELISA

To detect binding between MERS-CoV RBD and hDPP4 protein, 96-well ELISA plates were pre-coated overnight at 4 °C with 50 μ L of 2 μ g/mL purified S377-588-Fc protein, expressed either in HEK293T cells [13] or in adCHO cells, and blocked with 2% fat-free milk at 37 °C for 2 h. Serial dilutions of hDPP4 protein (His-tagged, 50 μ L/well) were then added to the plates and incubated at 37 °C for 1.5 h, followed by four washes with PBS in 0.05% Tween-20 (PBST). Subsequently, the plates were incubated with mouse anti-His primary antibody (1:2000, Sigma) at 37 °C for 1.5 h. After four washes with PBST, horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (1:5000, GE Healthcare) was added to the wells and incubated at 37 °C for 30 min. Finally, plates were washed with PBST, and binding was visualized by adding the colorogenic substrate 3,3',5,5'-Tetramethylbenzidine (TMB, Sigma). The reaction was stopped after 10 min by adding 1 N H₂SO₄, and absorbance at 450 nm (A₄₅₀) was measured on an ELISA plate reader (Tecan).

Detection of the binding between MERS-CoV RBD and RBD-specific neutralizing mAbs was performed following a protocol similar to that described above, except that the plates were pre-coated with 1 μ g/mL of purified S377-588-Fc proteins, followed by sequential incubation with serially diluted mouse mAb (Mers-mab1) or human mAbs (m336-Fab, m337-Fab, and m338-Fab) [23–25] and HRP-conjugated anti-mouse IgG (1:3000, for mouse mAb, GE Healthcare) and anti-human-Fab (1:5000, for human Fab-mAbs, Sigma) antibodies.

The binding between denatured MERS-CoV RBD and the aforementioned RBD-specific neutralizing mAbs or RBD-immunized mouse sera was tested by ELISA as described above, except that the plates were pre-coated with S377-588-Fc (1 μ g/mL) protein treated with dithiothreitol (DTT) (10 mM, Sigma) at 37 °C for 1 h, followed by incubation with iodoacetamide (50 mM, Sigma) at

37 °C for 1 h to stop the reaction [25]. After three washes, the ELISA was carried out as described above.

2.8. Evaluation of immunogenicity and efficacy of MERS S377-588-Fc in CD26/hDPP4 transgenic mice

All *in vitro* and *in vivo* studies required the usage of infectious MERS-CoV (EMC/2012 strain) and were conducted within approved biosafety level 3 (BSL-3) and animal BSL-3 laboratories at the Galveston National Laboratory, strictly following approved notification-of-usage (NOU) and animal protocols and the guidelines and regulations of the National Institutes of Health and AAALAC. For a “proof-of-principal” study to confirm that adCHO-expressed MERS S377-588-Fc is an effective and safe vaccine, two groups of five age-matched CD26/DPP4 transgenic (Tg) mice were immunized twice, four weeks apart, via the intramuscular (i.m.) route, with either 10 μ g of MERS S377-588-Fc formulated with AddaVax (Invivogen) or PBS/AddaVax only (as control). This immunization protocol was selected because it is optimized for MERS-CoV RBD proteins [16]. The AddaVax adjuvant was chosen because it promoted the RBD-Fc protein to generate the highest neutralizing antibodies among several adjuvants tested in our previous studies [14]. Serum specimens were collected at day 28 after the second immunization through the *retro*-orbital bleeding route to determine the prospective capacity to neutralize infectious MERS-CoV by using the standard Vero E6-based micro-neutralization test. Immunized mice were subsequently challenged intranasally (i.n.) with 100x 50% lethal dose (LD₅₀) ($\sim 10^3$ TCID₅₀) of MERS-CoV (EMC/2012 strain), a gift of Heinz Feldmann (NIH, Hamilton, MT) and Ron A. Fouchier (Erasmus Medical Center, Rotterdam, The Netherlands), followed by daily monitoring for the onset of clinical manifestations (e.g., weight loss and other clinical manifestations) and mortality. Three mice from each group were euthanized at day 3 post-infection (p.i.) to assess lung viral loads by Vero E6-based infection assay and quantitative (q) PCR analysis targeting the *upE* gene of MERS-CoV for quantifying infectious virus and viral RNA, respectively. Additionally, de-paraffinized lung tissues were hematoxylin-and-eosin (H&E)-stained for routine histopathologic evaluations, as described. We continued to monitor the remaining two mice in each group for their overall well-being for a total of 3 weeks until terminating the experiment. All methodologies required to assess the immunogenicity (neutralization antibody titers) and efficacy of MERS S377-588-Fc have been previously reported ([19,26], Supplementary Methods).

2.9. Development of serum-free suspension CHO cell line expressing MERS S377-588-Fc

Suspension CHO (CHO DG44, Gibco, hereinafter termed susCHO) cells were cultured in CD DG44 medium (Gibco) supplemented with 8 mM L-glutamine (Gibco) and 0.18% Pluronic® F-68 prior to transfection. Transfection was performed by combining 18 μ g AhdI-linearized plasmid pOpti_IL2_S377-588-Fc and 15 μ L of FreeStyle™ MAX Reagent (Invitrogen) in 1.2 mL OptiPRO SFM and incubated at room temperature for 10 min, followed by dropwise addition to 1.5×10^7 cells in 30 mL of CD DG44 culture medium (non-selective) according to the manufacturer's instructions. After 48 h, cells were transferred to selective medium (CD OptiCHO, Invitrogen), supplemented with 8 mM L-glutamine and 0.18% Pluronic® F-68 (Gibco), and cultivated until cell viability reached 90%. After selection, stably transfected susCHO cells underwent DNA amplification by gradually increasing MTX concentration (20–5000 nM) in selective medium. All suspension culture flasks were maintained in a humidified incubator, 37 °C/8% CO₂ on a shaker, at a constant rotation rate of 135 rpm.

2.10. Clonal cell line selection

The 5 μ M MTX-adapted susCHO cell pools from serum-free medium were used for single-cell cloning by limited dilution at 0.25–2 cells/well. Cloning was performed in 96-well plates (Falcon U-Bottom untreated), utilizing a cloning medium composed of 80% Hybridoma SFM (ClonaCell) and 20% conditioned media supplemented with 0.5X CHO ACF Supplement (ClonaCell) at 37 °C/5% CO₂ for approximately 14 days. Conditioned medium from wells with actively growing single colonies was assayed by ELISA as follows. A mixture of 10 μ L of conditioned medium and 90 μ L of coating buffer were added to a 96-well ELISA plate (Thermo Fisher Scientific) and incubated at 4 °C overnight. After washing the plate with PBST, rabbit anti-human IgG was used to detect S377-588-Fc protein, followed by a biotinylated goat anti-rabbit antibody and Streptavidin HRP. Tetramethylbenzidine (KPL Inc., VWR) was added (100 μ L) before the reaction was stopped with 100 μ L 1 M HCl. ELISA plates were read on an Epoch Microplate Spectrophotometer (BioTek Instruments, Inc) at 450 nm. Clones which gave high absorbance reading were further propagated in 6-well plates, utilizing a CD OptiCHO medium with 8 mM L-glutamine and 0.18% Pluronic® F-68. Conditioned medium from 6-well plates was also screened by ELISA for confirmation, and the highest expressing clone was selected and expanded by passaging in shake flasks (37 °C, 8% CO₂ in air on an orbital shaker platform rotating at 135 rpm). Clonal cell lines and heterogeneous susCHO cell pools were collected daily (up to 10 days) and counted using the Acridine orange (AO) and propidium iodide (PI) nuclear staining dyes (Nexcelom Bioscience), which enter live cells and dead cells, respectively. Conditioned medium from each time point was analyzed by SDS-PAGE, and the protein concentration was

estimated by densitometry, comparing it to protein standards using the ChemiDoc™ Imaging System (BioRad).

2.11. Statistical analysis

Statistical significance was calculated by Student's *t* test using GraphPad Prism statistical software. *** indicates $P < 0.001$.

3. Results

3.1. Recombinant protein expression in CHO cell system

In our previous studies, the human IgG Fc-fused S377-588 fragment of the MERS-CoV S protein (GenBank AFS88936.1) (hereinafter termed MERS S377-588-Fc) had already been expressed in transiently transfected HEK293T cells [17]. However, to establish stably expressing cell clones, we transfected an adherent CHO (adCHO) with a pOptiVec construct containing an Internal Ribosome Entry Site (IRES)-driven *dhfr* gene for selection and copy number amplification, as well as MERS S377-588-Fc gene fusion. Signal peptides were added to the N-terminal end of the S377-588-Fc gene in order to drive secretion into the culture medium (Fig. 1a). Addition of gradually increasing methotrexate (MTX) to the culture medium of transfected cells resulted in binding to and inactivation of dihydrofolate reductase (DHFR) activity. Transfected adCHO cells compensated for this reduced DHFR activity by increasing the DHFR copy number in the genome to overcome inhibition by MTX. Since the MERS S377-588-Fc fusion gene was integrated into the same genetic locus as that of the DHFR gene, the

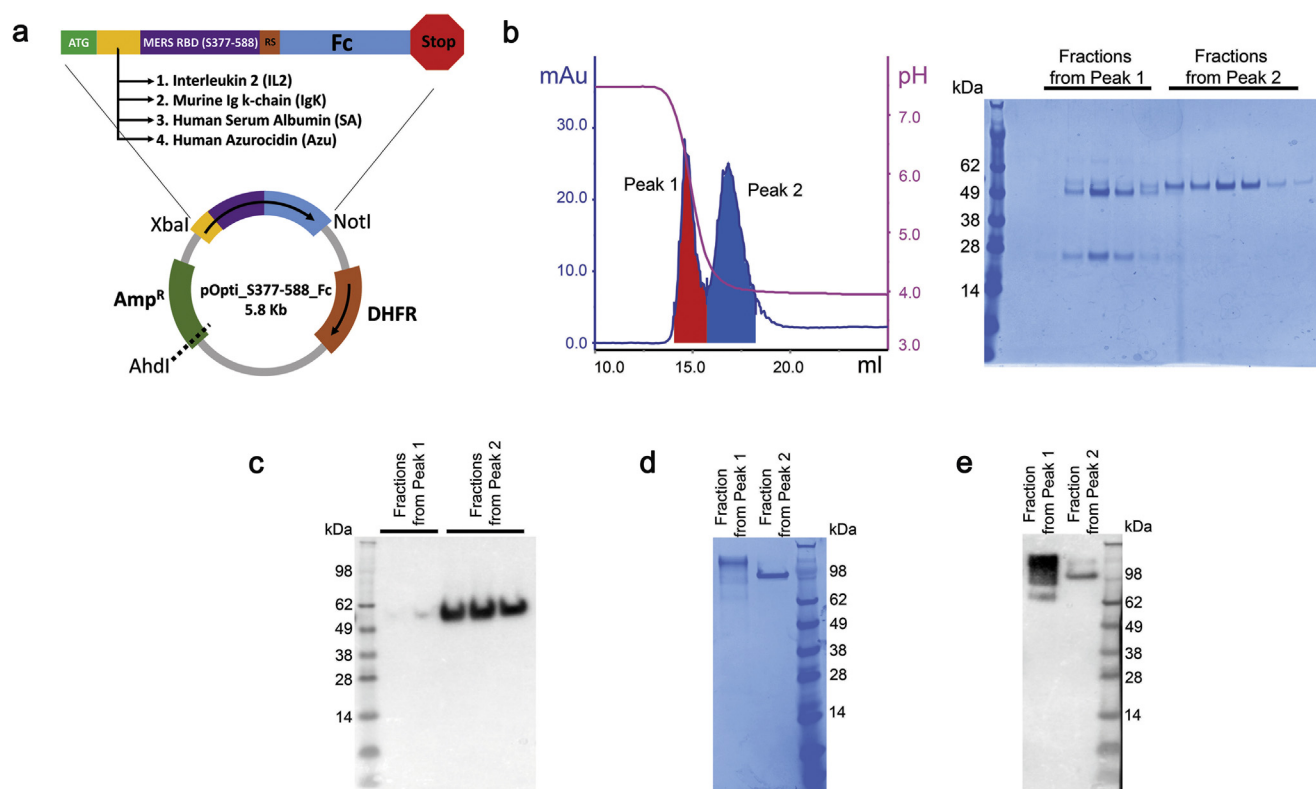


Fig. 1. Expression of MERS S377-588-Fc in adCHO cells. (a) Map of pOpti_S377-588-Fc. (b) Chromatogram of Protein A purification of MERS S377-588 from culture media of adCHO cells. Shown is the UV absorption at 280 nm (blue line) during elution with a pH gradient (purple line). Two peaks were eluted, followed by Western blotting and SDS-PAGE gel analysis. (c) Western blotting of denatured SDS-PAGE using Rb-Anti-MERS RBD antibodies. (d) Non-denatured SDS-PAGE. (e) Western blotting of non-denatured SDS-PAGE using Rb-anti-Bovine (Fab)₂ antibodies. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

S377-588-Fc gene was amplified, as well, leading to increased production of the protein.

In the course of developing the adCHO cell line expressing the S377-588-Fc protein, optimization of a purification protocol using HiTrap Protein-A HP was performed. On the purification chromatogram, two protein peaks were observed in the elution step (Fig. 1b). Denaturing SDS-PAGE and Western blotting analysis with anti-MERS-RBD-specific antibodies confirmed the second peak to be the S377-588-Fc fragment (Fig. 1c). Further analysis using non-denaturing SDS-PAGE and Western blotting with Rb-anti-Bovine (Fab)2 antibodies showed that the first peak was mainly contaminating bovine IgG (Fig. 1d–e) that originated from the fetal bovine serum (FBS) supplemented in the culture medium. We estimated relative productivity of adCHO cells by measuring the ratio of the area of first peak and second peak.

Different signal peptides have been shown to result in different expression levels in CHO cell systems [27]. Therefore, we transfected adCHO cells with linearized MERS S377-588-Fc expression plasmids with four different signal peptides at the N-terminus. Peptides were derived from interleukin 2 (IL2), IgK light chain (IgK), human serum albumin (SA), and azurocidin (Azu). Conditioned media from confluent monolayers of each transfected cell line were collected, followed by protein purification using Protein A to establish yield and estimate relative productivity. In these studies, we found that adCHO cells with the signal peptide derived from IL2 showed 20–50% more secretion of S377-588-Fc than cells with other signal peptides (Fig. 2a).

Elevated expression levels were achieved by gradually increasing MTX concentration during each cell passage from 20 nM to 5 μ M. Conditioned medium from transfected adCHO cells was collected during the DNA amplification process, followed by protein purification using Protein A to estimate relative productivity (Fig. 2b). We observed the expected correlation between the expression of S377-588-Fc and increased resistance to higher levels of MTX. Compared to adCHO cells with 20 nM MTX, expression of S377-588-Fc was increased 39-fold in the presence of 3–5 μ M of MTX (Fig. 2c).

3.2. Characterization and post-translational modification (PTM) analysis of MERS S377-588-Fc

The purified protein was analyzed by circular dichroism (CD) spectroscopy. The MERS-CoV S377-588-Fc consists mainly of beta-sheet (39.4%) and loop structures (53%) with limited helices (7.6%) (Fig. 3a). The secondary structure of the protein starts to unfold at 65 °C. During thermal melt analysis, MERS S377-588-Fc had two endothermic transitions: 52.5 °C and 67.5 °C (Fig. 3b). A comparison between MERS S377-588-Fc expressed from HEK293T cells and adCHO cells was carried out using different PAGE analyses. Both proteins appeared to be identical following reduced and non-reduced SDS-PAGE. On native PAGE and IEF gels, adCHO-expressed MERS S377-588-Fc had a lower isoelectric point (pI 6.6) when compared to the protein expressed in HEK293T cells (pI 7.2) (Fig. 3c). After removal of N-linked glycans, the molecular size of MERS S377-588-Fc was slightly smaller than that of the N-linked glycosylated form (Fig. 3d). No change in molecular weight was observed after O-linked deglycosylation and desialylation (reduced SDS-PAGE). Enzymatic treatment did not affect dimerization of MERS S377-588-Fc, as determined by non-reducing SDS-PAGE. The high pI (>7.4) isomers of S377-588 exhibited a lower shift (between pI 7.4 and pI 6.0) in electrophoretic mobility after N-linked deglycosylation, while no change of band pattern for the protein was seen after treatment with O-glycosidase. After removal of sialic acid through neuraminidase treatment, S377-588-Fc isomers shifted to pI higher than 7.4 (Fig. 3d).

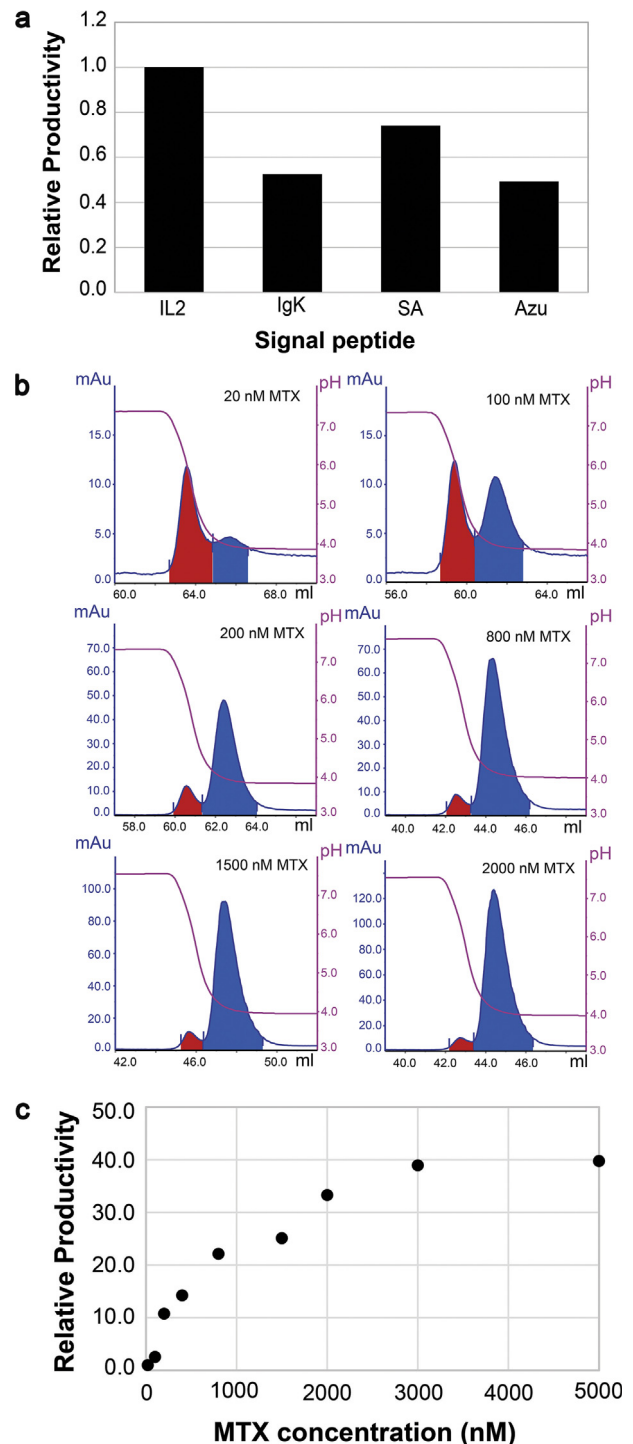


Fig. 2. Selection of signal peptides and gradual stepwise increase of MTX concentration for DNA amplification. (a) Comparison of relative productivity of linearized constructs with different signal peptides derived from interleukin 2 (IL2), IgK light chain (IgK), human serum albumin (SA) and azurocidin (Azu). (b) Area of Peak 1 (IgG from FBS, red area) and Peak 2 (S377-588-Fc, blue area) were measured to estimate relative productivity. (c) Relative productivity of S377-588-Fc increased in the presence of MTX. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Functionality and antigenicity studies of S377-588-Fc

Three assays, including co-immunoprecipitation (Co-IP), flow cytometry, and ELISA, were performed to detect the binding of MERS-CoV RBD to its receptor, hDPP4. Co-IP demonstrated that similar to the HEK293T-expressed MERS S377-588-Fc protein,

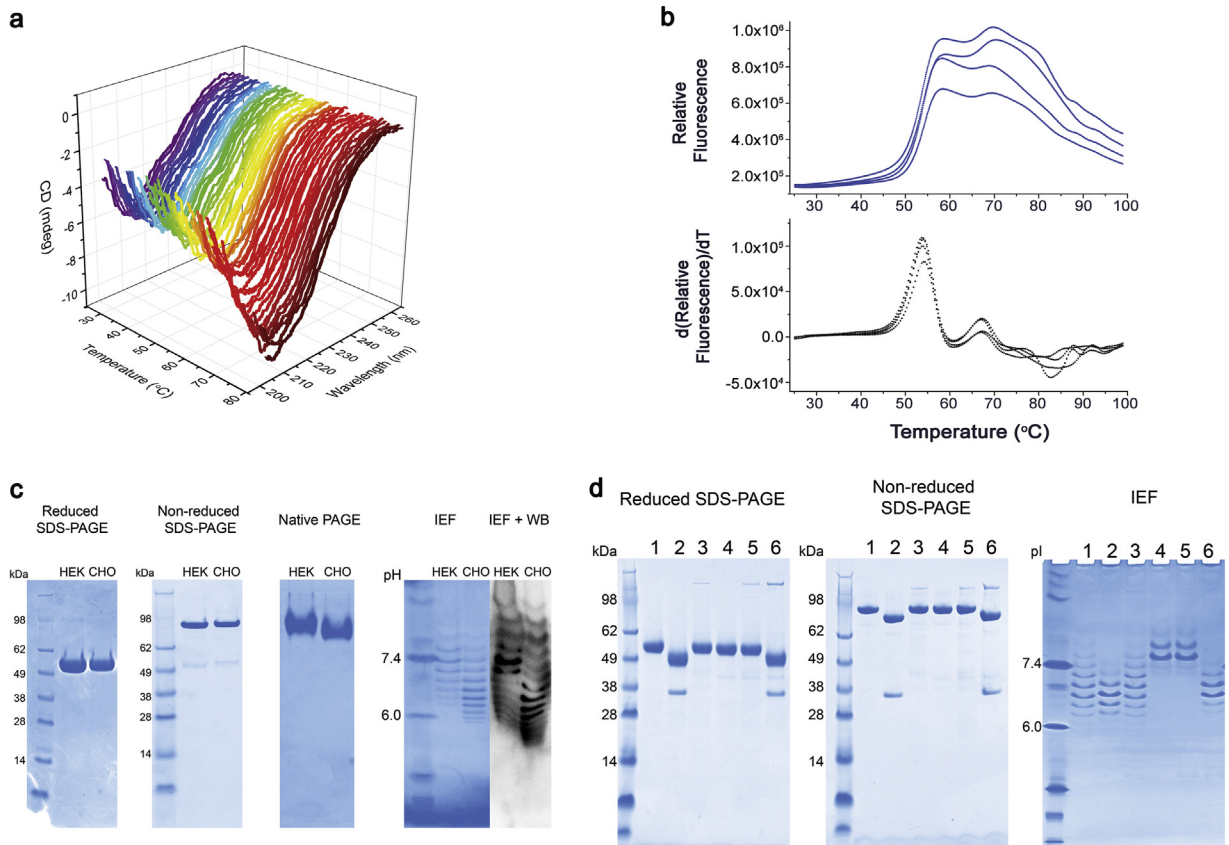


Fig. 3. Biophysical characterization and PTM analysis of MERS S377-588-Fc protein. (a) CD spectrum. (b) Melting curve. (c) Comparison of S377-588-Fc expressed in adCHO and HEK293T cells. (d) PTM analysis using PNGaseF, O-glycosidase and neuraminidase (1: control, 2: PNGaseF, 3: O-glycosidase, 4: neuraminidase, lane 5: O-glycosidase + neuraminidase, 6: PNGaseF + O-glycosidase + neuraminidase).

the RBD protein expressed in adCHO bound strongly to hDPP4-expressing Huh-7 cells. Two clear bands were identified from immunoprecipitated mixture of S377-588-Fc and Huh-7 cell lysates, and these bands were recognized by both anti-hDPP4 antibody and anti-MERS-RBD antibody. In contrast, only one band was identified in Huh-7 cells only and S377-588-Fc protein only samples, and it was reactive with either anti-hDPP4 antibody or anti-MERS-RBD antibody, but not with both antibodies. As expected, SARS-CoV RBD-Fc protein was only recognized by SARS-CoV RBD-specific mAb 33G4 (Fig. 4a). Flow cytometry analysis further quantified the binding between MERS-CoV RBD protein and hDPP4 receptor in Huh-7 cells. Results showed a similar strong binding for all MERS S377-588-Fc proteins from adCHO and HEK293T, but not for SARS-CoV RBD-Fc control (Fig. 4b). The ELISA analysis demonstrated a dose-dependent binding between these MERS-CoV RBD proteins and hDPP4 protein, while no binding was observed between hFc control and hDPP4 (Fig. 4c). The antigenicity of MERS-CoV RBD proteins was carried out using ELISA to test their binding with RBD-specific neutralizing antibodies (Mersmab1, m336, m337, and m338), which recognize epitopes at RBD residues F506, D510, R511, W535, D539, Y540, R542, or W553, and demonstrate strong activity to block RBD-hDPP4 receptor binding and neutralize MERS-CoV infection [23–25,28]. Similar to the S377-588-Fc protein expressed in HEK293T, the RBD proteins expressed in adCHO bound strongly to mouse mAb Mersmab1 and human mAbs m336, m337, and m338 in a dose-dependent manner (Fig. 4d), confirming their antigenicity. While these mAbs bound strongly to the non-denatured (no DTT) S377-588-Fc proteins expressed in both adCHO and HEK293T, they had significantly

reduced affinity to RBD proteins treated with DTT, a reducing agent cleaving disulfide bonds of RBDs and thus disrupting a protein's native conformation (Fig. 4e). These results demonstrate that the neutralizing mAbs recognize conformational structures of MERS-CoV RBD [25].

3.4. Immunogenicity and efficacy of MERS S377-588-Fc

Transgenic mice expressing the human CD26/DPP4 receptor (hDPP4-Tg) are a well-characterized animal model with which to evaluate the efficacy of vaccine candidates against MERS-CoV infection and disease [19,20]. The immunogenicity of the MERS S377-588-Fc-based subunit vaccine was verified in hDPP4-Tg mice. Immune sera obtained from immunized mice were tested by ELISA for the binding with denatured and non-denatured S377-588-Fc protein or subjected to the Vero E6-based micro-neutralization assay to quantify their capacity to neutralize infectious MERS-CoV. To evaluate protective efficacy against viral infection, viral loads and histopathology of the lungs of three mice in immunized and control groups were measured at day 3 after lethal challenge with 100x LD₅₀ of MERS-CoV. The remaining two mice in each group were monitored for morbidity (weight loss) and mortality to determine if this vaccine formulation would sufficiently protect against the disease and lethality caused by MERS-CoV infection. MERS S377-588-Fc induced high titers of RBD-specific IgG antibodies, which reacted strongly with non-denatured S377-588-Fc protein. Nevertheless, these RBD-specific antibodies had significantly reduced activity with S377-588-Fc treated by DTT (Fig. 5). This suggests that the RBD vaccine-induced antibody response was

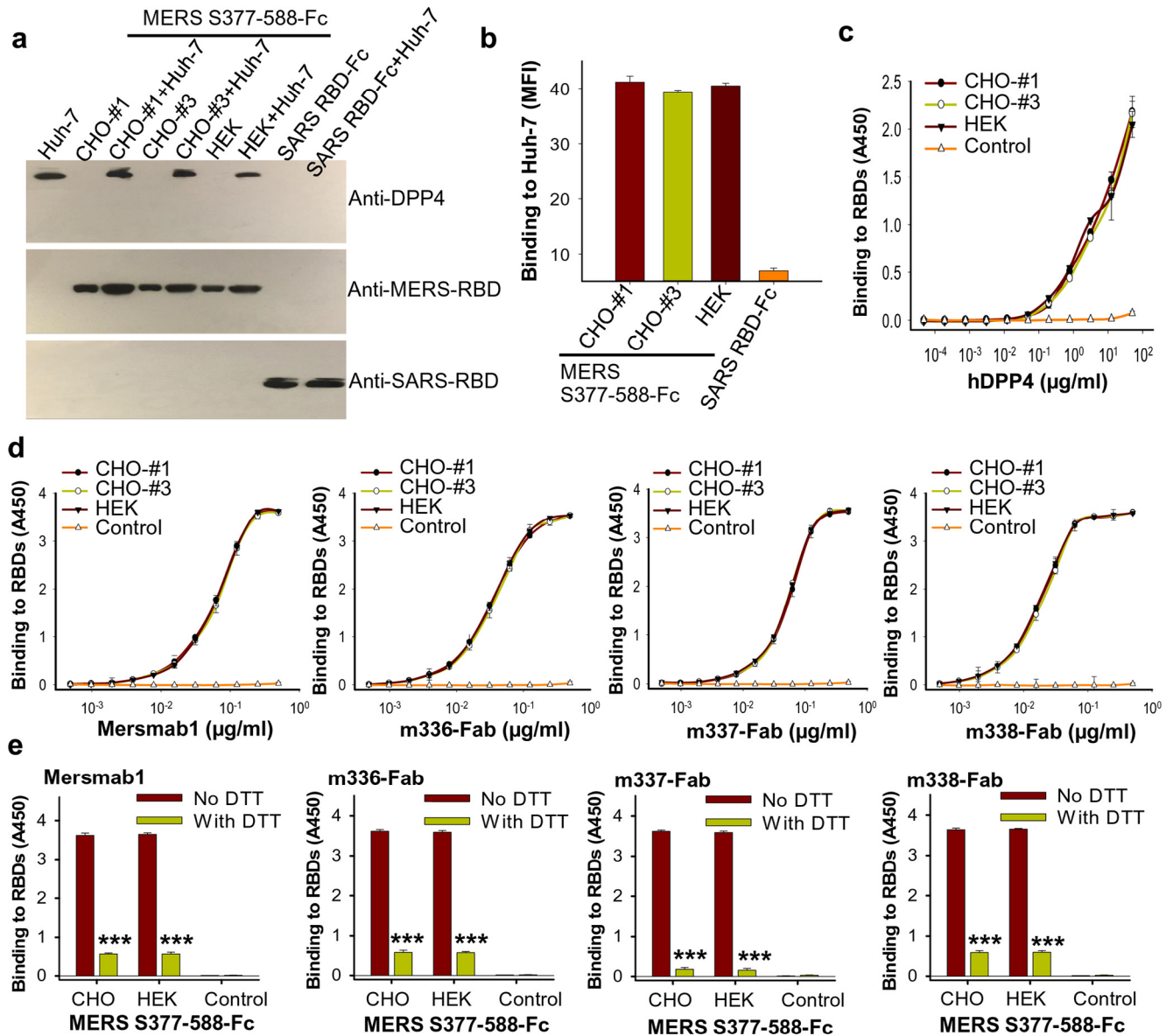


Fig. 4. Functionality and antigenicity of MERS S377-588-Fc proteins. CHO-#1 and CHO-#3 represent two different batches of the S377-588-Fc proteins purified from adCHO cells, and HEK indicates RBD protein purified from HEK293T. (a) Co-IP analysis of interactions between S377-588-Fc expressed from adCHO and HEK293T. The interactions between S377-588-Fc and hDPP4 in the Huh-7 cell lysates were detected by Co-IP analysis, using anti-hDPP4 and anti-MERS-RBD antibodies. Anti-SARS-RBD: SARS-CoV RBD-specific mAb 33G4. (b) Quantification of the interaction between S377-588-Fc (40 µg/mL) and hDPP4 by flow cytometry analysis in hDPP4-expressing Huh-7 cells. MFI: median fluorescence intensity. For (a) and (b), a HEK293T-expressed SARS-CoV RBD protein (e.g., SARS RBD-Fc) was used as a control, and this protein did not interact with hDPP4. (c) ELISA was performed to detect the binding between MERS S377-588-Fc (2 µg/mL) and recombinant hDPP4. Binding affinity was characterized as OD450 value. (d) Similar to S377-588-Fc expressed in HEK293T cells, S377-588-Fc proteins expressed in adCHO cells maintained good antigenicity in binding MERS-CoV RBD-specific neutralizing antibodies (including Mersmab1, m336, m337, and m338). (e) ELISA was performed to compare the binding between the above neutralizing mAbs and denatured (with DTT) or non-denatured (no DTT) MERS S377-588-Fc proteins expressed in both adCHO and HEK293T. ***, $P < 0.001$. Control in (c)–(e): human IgG Fc (hFc) protein. Error bars indicate standard error.

indeed directed towards one or more conformational epitopes. Mice immunized with PBS/AddaVax uniformly failed to elicit any detectable neutralizing antibodies; however, those immunized with MERS S377-588-Fc/AddaVax consistently produced readily detectable titers of neutralizing antibody, ranging from 10–160 and 20–320 of neutralizing titer (NT)-100 (NT₁₀₀) and NT-50 (NT₅₀), respectively (Table 2). Consistent with the readily detectable neutralization antibodies, MERS S377-588-Fc/AddaVax-immunized mice were fully protected against viral infection and disease, as evidenced by the absence of recoverable infectious virus and negligible focal inflammatory responses, if any (Supp. Fig. 3), within the infected lungs at 3 days post-infection (dpi).

Importantly, the remaining two immunized mice did not suffer any significant morbidity (weight loss) and survived until 21 dpi when the experiment was terminated. In contrast, pulmonary infectious viruses, albeit low in titers, were detected from all three unimmunized controls, with an average of 2.7 ± 0.1 TCID₅₀/g of MERS-CoV recovered at 3 dpi, accompanied by mild inflammatory responses. The remaining two control mice suffered profound weight loss prior to succumbing to infection by 8 dpi. Taken together, results of this “proof-of-principle” pilot study indicated not only immunogenicity of MERS-CoV S377-588-Fc, but also its efficacy and safety in the protection of hDPP4-Tg mice against lethal challenge with MERS-CoV.

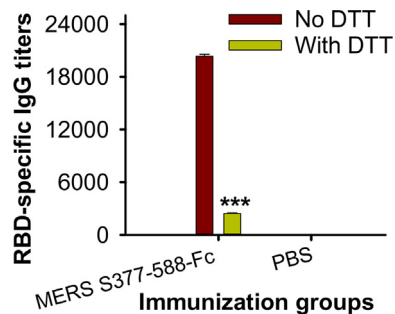


Fig. 5. Immunogenicity of MERS S377-588-Fc protein expressed in adCHO cells. Sera from RBD-immunized hDPP4-Tg mice were tested by ELISA for RBD-specific IgG antibodies. Plates were coated with S377-588-Fc (1 $\mu\text{g}/\text{mL}$), and treated with or without DTT, followed by incubation with serially diluted mouse sera ($n = 4$). Error bars indicate standard error. ***, $P < 0.001$.

3.5. Development of serum-free CHO cell line expressing S377-588-Fc

Similar to the adCHO cell development described earlier, the DNA copy number of stably transfected suspension CHO DG44 *dhfr*-cells was amplified by gradual exposure to increased MTX

concentrations of up to 5 μM . The resulting heterogeneous susCHO cell pools were subsequently cloned by limited dilution to obtain a monoclonal cell population. ELISA analysis was performed on the supernatants from individual clones, leading to the identification of clone 1B11 as the highest expressing clone. By comparing the growth curves of clone 1B11 to the heterogeneous (non-clonal) susCHO cells, we discovered that clone 1B11 and heterogeneous susCHO cells reached their maximum growth on the seventh day with viable cell counts of 8×10^6 cells/mL and 7×10^6 cells/mL, respectively (Fig. 6). Through quantitative analysis using SDS-PAGE, we determined that the supernatant of clone 1B11 expressed approximately 97 mg/L of MERS S377-588-Fc, while the heterogeneous non-clonal cell pools expressed about 65 mg/L (Table 3, Supp. Fig. 4) on the seventh day.

4. Discussion

The MERS-CoV RBD subunit fragment S377-588 has been identified to be a critical neutralizing receptor-binding fragment and an ideal candidate for the development of an effective MERS-CoV recombinant protein vaccine [13]. Our aim here was to optimize

Table 2
Immunogenicity and Efficacy of the MERS S377-588-Fc subunit vaccine in hDPP4-Tg mice.

Group #	Immunized w/	Animal IDs	50% or 100% Neutralizing titers (NT)		Protective efficacy against infection and disease				
			NT ₅₀	NT ₁₀₀	Viral loads (Log ₁₀ TCID ₅₀ /g)	Lung pathology	Lung eosinophilic infiltration	Morbidity (weight loss)	Survived or died
1	MERS-CoV RBD-Fc/AddaVax	1	320	160	ND ^a	0	0	NA	NA
		2	20	10	ND	0	0	NA	NA
		3	40	20	ND	0	0	NA	NA
		4	40	20	NA ^b	NA	NA	<3%	Survived ^d
		5	320	160	NA	NA	NA	<3%	Survived
			148 \pm 70 ^c	74 \pm 35	ND	0	0	<3%	
2	PBS/AddaVax	6	<10	–	2.7	1	0	NA	NA
		7	<10	–	2.9	1	0	NA	NA
		8	<10	–	2.5	0–1 (two small foci)	0	NA	NA
		9	<10	–	NA	NA	NA	$\geq 20\%$	Died ^e
		10	<10	–	NA	NA	NA	$\geq 20\%$	Died
			<10	–	2.7 \pm 0.1	≤ 1	0	$\geq 20\%$	

^a Not detectable (the limit of detection is 1.5 log TCID₅₀/g).

^b Not applicable.

^c Mean \pm SE.

^d Survived through 21 dpi at which time the experiment was terminated.

^e Died at 7–8 dpi.

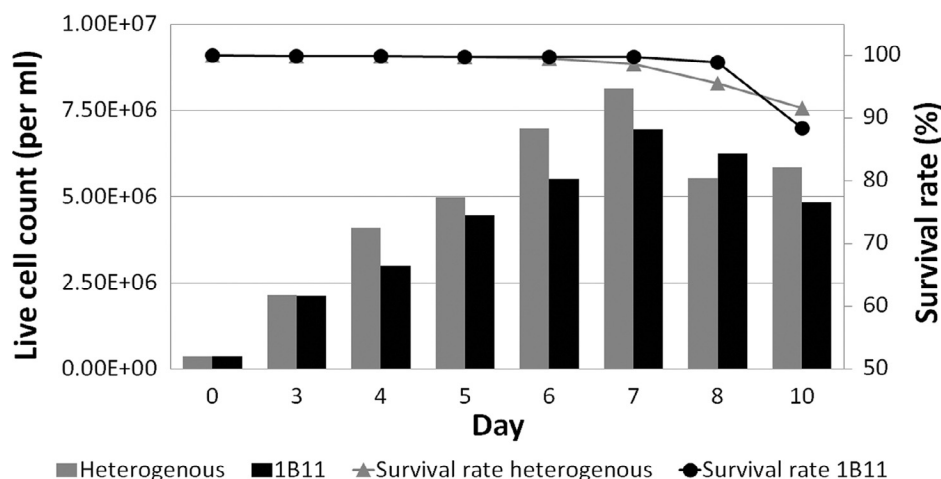


Fig. 6. Growth curve comparison of heterogeneous susCHO cell pools and clone 1B11. The high-producing clone 1B11 has a lower growth rates than the heterogeneous non-clonal cell pools.

Table 3

S377-588-Fc expressed from heterogeneous non-clonal susCHO cell pools and from the monoclonal clone 1B11. The protein concentration (mg protein per liter of culture supernatant) was determined by SDS-PAGE gel analysis (Supp. Fig. 3).

Conditioned media @	Heterogeneous cell pool/[mg/L]	1B11 clone/[mg/L]
Day 4	27.16	40.95
Day 5	51.03	58.26
Day 6	57.64	85.64
Day 7	64.89	96.80
Day 8	68.61	111.00

expression and purification conditions suitable for pilot scale production of this RBD vaccine candidate. Initially, both *Escherichia coli* (bacteria) and *Pichia pastoris* (yeast) expression systems proved well suited for recombinant vaccine production because of low production costs. However, our data showed that *E. coli* could not produce soluble MERS S377-588, and yeast cells could not overexpress MERS S377-588. Thus, these two systems were not considered suitable to advance the MERS vaccine candidate into process development and scale up production (see [supplementary information](#) for more detail). In previous reports, MERS Fc-fused S377-588 had been expressed in transiently transfected HEK293T cells. However, transiently transfected cell lines may give low protein yield and potentially lose their production ability over time in continuously expressing recombinant proteins [29,30]. Unlike transient transfection, DNA is integrated into cells long-term through stable transfection. Despite the fact that the development of stably expressing cell lines is laborious and time-consuming, production with stable cell lines can be scaled up easily and would be suitable for use in manufacturing processes. Hence, we generated a stably transfected adCHO cell line by transfecting MTX-driven pOptiVEC into cells to produce recombinant MERS S377-588-Fc protein.

Fc-fused gp120 protein was first constructed in 1989 as a potential candidate for AIDS therapy [31]. While there is no FDA approved Fc-fusion vaccine, vaccine development using Fc-fusion proteins is active and ongoing. A number of studies have been initiated on the development of vaccines against Ebola, HIV, influenza, as well as tuberculosis [32–35]. It is noted that adverse side effects with vaccines are likely limited as biotherapeutic Fc fusions have been repeatedly shown to be safe and biocompatible in humans. Currently, all commercial therapeutics use the Fc domain from human IgG1, although other options, such as IgG3, IgA, and IgM are also currently being explored [36]. Furthermore, the Fc domain is known to increase plasma half-life and simplify the purification process [36]. Purification of S377-588-Fc was performed using a Protein A Sepharose column, removing most of the impurities from the culture medium. Bovine IgG originates from FBS in the culture medium in constant amount and was used as a standard to gauge expression levels of the S377-588-Fc protein. This estimation method allowed us to select the most suitable signal peptide and to evaluate the effect of the MTX-induced DNA amplification process.

Since the pOptiVEC plasmid has no signal peptide, four different signal peptides were subsequently tested at the N-terminus of the S377-588-Fc sequence to drive secretion of the protein, leading to the identification of IL2 signal peptide as the most suitable signal sequence. Although signal peptides derived from human SA and human Azu have been reported to improve production rates in other adCHO systems [27], the yield in our hands was lower than that with the IL2 signal peptide. Stable and highly productive cell pools were then isolated through a gradual increase in the MTX concentration in the culture [21]. Analysis of the relative productivity of adCHO cells indicated an increase in S377-588-Fc in media proportional to the MTX concentration in the medium, reaching a plateau at 3 μ M MTX.

The biophysical and biochemical characterization of the MERS S377-588-Fc protein revealed that it was stable up to a temperature of 52.5 °C. After the first major unfolding event at 52.5 °C, another unfolding event occurred between 65 and 67.5 °C, which could have resulted from destabilization of the CH2-CH2 bond of the Fc domain of the recombinant protein [37]. No change in molecular weight was observed after removal of O-linked glycan on MERS S377-588-Fc, which suggested no O-linked glycosylation in the protein. After neuraminidase treatment, the recombinant protein band pattern showed fewer bands and a pI shift, suggesting that sialic acids might contribute to the charge of the protein. Interestingly, although the complexities of the multiple protein bands were greatly reduced after glycosidase and sialidase treatments, the pattern representing charge heterogeneity remained, suggesting the existence of additional PTMs, such as mannose-6-phosphate, of the recombinant MERS S377-588-Fc protein.

Due to the charge differences between adCHO- and HEK293T-expressed S377-588-Fc proteins, we evaluated the functionality and antigenicity of the target protein. Functionality studies, including Co-IP assays, flow cytometry analyses, and ELISA binding assay, confirmed that the adCHO-expressed MERS S377-588-Fc protein maintained functionality equal to that expressed in HEK293T cells in binding the DPP4 receptor of MERS-CoV, both of which showed dose-dependent binding with the soluble hDPP4 protein. In addition, MERS S377-588-Fc expressed in either adCHO or HEK293T demonstrated similar dose-dependent binding to RBD-specific neutralizing antibodies, an indicator that both S377-588-Fc proteins could maintain sufficient antigenicity. We further investigated the protective efficacy of adCHO-expressed S377-588-Fc protein vaccine in protecting against MERS-CoV infection in the established transgenic mouse model expressing hDPP4 (hDPP4-Tg). By formulating this S377-588-Fc protein with AddaVax all vaccinated animals could produce neutralizing antibodies and survive a live viral challenge for 21 days. Taken together, we confirmed the absence of functional, antigenic and immunogenic differences between adCHO- and HEK293T-expressed MERS S377-588-Fc proteins. Moreover, mouse vaccinations with the RBD subunit vaccines did not appear to elicit eosinophilic or antibody-dependent immune enhancement.

Although we verified adCHO-expressed MERS S377-588-Fc protein as an effective vaccine against MERS-CoV infection, the use of FBS in the growth medium proved unsuitable for a human vaccine antigen [38]. For both safety and compliance with future regulatory requirements, we therefore developed a stably transfected suspension CHO cell line in serum-free medium. The adCHO cell development process described here became the foundation for the establishment of the serum-free suspension CHO cell line. Subsequently, we transfected the pOptiVEC expression plasmid with the IL2 signal peptide into susCHO cells and carried out the DNA amplification as before. From the heterogeneous cell pools adapted to 5 μ M MTX, we isolated clone 1B11, which was the highest expressing clone from a two-cycle screening process. In shake flasks, the growth of clone 1B11 was slightly slower than that from the heterogeneous cell pools, but 1B11 expressed 50% more MERS S377-588-Fc protein than the heterogeneous cell pool. Typically, highly productive cell clones have lower growth rates since a significant portion of resources are used for expression of the recombinant protein [39]. Additional experiments in the transgenic mice and non-human primate models will be needed to further determine the immunogenicity of MERS S377-588-Fc protein that produced by susCHO.

We envision that with a proper production process, the recombinant protein can be scaled up, manufactured, formulated and stockpiled as an efficient countermeasure against future MERS-CoV outbreaks.

Conflicts of interest

The authors are involved in the development of a vaccine against MERS coronavirus.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.vaccine.2018.02.065>.

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