Novel universal SARS-CoV DNA vaccine inducing neutralizing antibodies to huCoV-19/WH01, Beta, Delta and Omicron variants and T cells to Bat-CoV

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INTRODUCTORY PARAGRAPH (148 words)

The SARS-CoV-2 pandemic is constantly changing with new variants appearing that are more contagious (Alpha and Delta), evade the neutralising antibody (NAb) response (Beta), or both (Omicron). This is a challenge for vaccine development. We generated a novel universal SARS-CoV-2 DNA vaccine containing the receptor binding domain (RBD) loops from the original huCoV-19/WH01, the Alpha, and the Beta variants, combined with the membrane and nucleoproteins from the huCoV-19/WH01 strain. This vaccine induced high levels of spike antibodies that crossreacted between the huCoV-19/WH01, Beta, and Delta spike proteins, and neutralized the huCoV-19/WH01, Beta, Delta and Omicron virus *in vitro*. The vaccine induced T cells to all vaccine proteins in mice and rabbits that recognized Bat-CoV N sequences. Finally, the vaccine protected K18 mice against lethal SARS-CoV-2 Beta variant infection, whereas only priming N-specific T cells was 60% protective. This universal SARS-CoV vaccine candidate induces a uniquely broad functional immunity.

MAIN TEXT (1428 words)

The SARS-CoV-2 pandemic has completely altered the way the society handles new viral infections, with lockdowns of cities or even entire countries [1]. The extremely rapid development of vaccines by the scientific community and the pharmaceutical industry is an extraordinary achievement [2]. A previous SARS-CoV-2 infection or a vaccination with either adeno- or mRNA-based COVID-19 vaccines induces neutralizing antibodies (NAbs; [3]) and is effective in preventing symptomatic infection, and highly effective in preventing hospitalization or death [4]. However, the ability of the RNA genome of SARS-CoV-2 to undergo mutations and recombination poses continuous challenges [5]. It has recently been shown that among the new mutant strains, especially the newly emerged variants of concern (VOC),

anything from a few to multiple mutations in the receptor-binding domain (RBD) may render the current vaccines less effective against less severe breakthrough infections [6, 7]. Several studies have highlighted that the Beta (B1.351), Delta (B.1.617.2), and Omicron (B.1.1.529) VOC may cause mild to moderate COVID-19 infections in those vaccinated [8, 9, 10, 11]. Recent data suggest that a previous infection with the original huCoV-19/WH01 wildtype strain or the Alpha (B.1.117) variant induces NAbs that retain strong cross-reactivity with the Delta (B.1.617.2) variant, whereas infections with the Beta (B.1.351) and Omicron (B.1.1.529) can escape these NAbs [12]. Thus, the appearance of mild to moderate breakthrough infections are not surprising. Fortunately, greater changes would most likely be needed to completely avoid the host immunity. Mutations in variants that escape NAbs appear in surface-exposed epitopic regions of the virus that are recognized more, or less, uniformly across humans. In contrast, the high diversity of the human leucocyte antigen class I and II makes it much less likely, or maybe even impossible, for T cell-escape variants to appear in viruses such as SARS-CoV-2 that are only causing acute infections [13, 14]. Consistent with this, it has been found that vaccine mediated protection against severe disease from variants such as the Delta (B.1.617.2) variant is maintained, despite a reduced protection against mild and moderate disease [11]. Consequently, although spike- and receptor binding domain (RBD)-specific B cells may lack cross-reactivity, the spike-specific T cells are still cross-reactive towards the different variants [13, 14]. This is further supported by the observation that T cells from SARS-infected patients that were infected with SARS-CoV in 2003 remain for 17 years and ar to a large extent cross-reacting with SARS-CoV-2 [15]. Overall, this strongly supports the notion that T cells may be able to confer a broader cross-reactivity than NAbs both between SARS-CoV-2 mutants and between different SARS viruses. Thus new vaccines that combine sequences from the spike

protein from multiple variants, combined with highly conserved viral protein sequences may overcome these problems.

It is clear that bats are reservoir for the origin of human coronaviruses [18]. The spike proteins of SARS-CoV and SARS-CoV-2 induce cross-reactive T cells but poorly cross-reactive NAbs [15]. Among the structural proteins of SARS-CoV and SARS-CoV-2 the envelope protein M and the nucleocapsid protein N have a higher genetic similarity to other animal SARS-CoV viruses than the receptor-binding S envelope protein [18, 19]. T cells to these two antigens show a higher cross-reactivity across betacoronaviruses [15]. To take advantage of this, we designed a universal SARS-CoV (OC-2.4) vaccine containing receptor binding domain (RBD) loops of the S protein corresponding to the huCoV-19/WH01, Alpha and Beta variants, combined with the M and N proteins of the huCoV-19/WH01 variant [20]. An autoproteolytic P2A sequence was inserted between the RBD and the M and N proteins to avoid interference with the folding of the RBD. Figure 1a illustrates the design and the concept of inducing both NAbs and broadly cross-reactive T cells. As control vaccines we used either a full length S gene (huCoV-19/WH01) in pVAX1, pVAX plasmid without insert, and a recombinant S protein (huCoV-19/WH01) mixed with QS21 adjuvant. The aim of a universal vaccine is to induce broadly reactive antibodies and T cells. As the S protein differs significantly between SARS-CoV strains and variants, cross-reactive T cells may be of a growing importance.

We first immunized Balb/c mice and found that they developed high levels of antibodies that bound recombinant S proteins of the huCoV-19/WH01, Beta and Delta variants (Figure 1b, d, f, and h) as well as to the N protein (Supplementary Figure 1). The S-specific antibodies effectively neutralized both the huCoV-19/WH01 and Beta variants of SARS-CoV-2 *in vitro* (Figure 1c and e). In addition, the mice developed T cells reactive to all components of the vaccine (depending on the mouse strain; data not shown). Importantly, the T cells to the N

protein were cross-reactive to sequences corresponding to the Bat-CoV N (Supplementary Figure 2).

Next, groups of mice and rabbits were immunized with the universal SARS-CoV DNA vaccine OC-2.4 and were analysed for anti-S levels, the ability to neutralize the Delta and Omicron variants *in vitro*, as well as the cross reactivity of T cells to the Bat CoV N sequences (Supplementary Figures 2 and 3). In mice, priming with recombinant huCoV-19/WH01 S protein in adjuvant and boosting with the universal SARS-CoV DNA vaccine OC-2.4 effectively enhanced anti-S levels by 10-100-fold to huCoV-19/WH01 S protein (Figure 1f). Importantly, heterologous boosting with the universal DNA vaccine OC-2.4 induced higher neutralisation levels than homologous boosting (Figure 1g). Also, homologous boosting with the universal DNA vaccine OC-2.4 seemed superior in inducing NAbs to the Omicron variant (Figure 1g). In female rabbits we found that three doses of the OC-2.4 DNA vaccine induced high levels of anti-S antibody (mean±SD of 20160±28710 endpoint titers) that effectively neutralized both Delta and Omicron variants of SARS-CoV-2 (Figure 1i). Thus, the inclusion of three RBD loops was effective at inducing broadly neutralizing antibodies to the huCoV-19/WH01, Beta, Delta and Omicron variants. As a comparison, three doses of a full length S DNA, or a recombinant spike protein in adjuvant, both corresponding to the huCoV-19/WH01 variant, were generally less effective in priming NAbs to the Beta, Delta and Omicron variants (Figure 1).

Finally, we analysed the ability of the universal DNA vaccine OC-2.4 to induce protective immune responses against a lethal challenge with the SARS-CoV-2 Beta variant in human ACE2 transgenic K18 mice. Groups of mice were immunized three times, three weeks apart, and two weeks after last dose the mice were challenged with 1 x 10^5 pfu of the SARS-CoV-2 Beta variant intranasally (Figure 2a), and then followed closely for symptoms and weight changes for thirteen days. The universal DNA vaccine OC-2.4 fully protected these mice against lethal

infection and showed complete protection against viral replication in the upper airways and in the spleen (Figure 2b and c). The viral replication in the lungs was also significantly reduced as compared to controls (Figure 2c). In contrast, vaccination with recombinant S in QS21 adjuvant also protected against lethal infection, and gave low infection in the lungs, but viral replication could still be detected in the upper airways and in the spleen (Figure 2c). Interestingly, vaccination with the N protein in adjuvant, that only activates antibodies and T helper cell responses to N, showed a 60% protection against lethal disease and viral replication in the upper airways, but less so in the lungs (Figure 2). This strongly support the notion that T cells alone have a key role against protection against severe disease [33], and that the role may differ in the upper and the lower airways. This requires further studies. We found that the universal DNA vaccine effectively primes protective immune responses against severe disease and viral replication caused by a known immune escape variant of SARS-CoV-2.

In conclusion, with a virus like SARS-CoV-2 that shows an impressive ability to mutate and to spread also among a population with high vaccine coverage, new vaccine designs are needed. Here, we describe a unique universal SARS-CoV DNA vaccine that induces more broadly neutralizing activity against the huCoV-19/WH01, Beta, Delta and Omicron variants than standard spike-based vaccines. Our data supports clinical development of this completely new approach to vaccines against SARS-CoV-2 to complement existing vaccines, and potentially also protect against future SARS-CoV viruses that have replaced, or greatly changed the spike protein of SARS-CoV-2.

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Figure Legends

Figure 1. Schematic representation of the universal SARS-CoV-2 DNA vaccine OC-2.4 gene design has been given (a). Ability of the S-DNA and SARS-CoV-2 DNA vaccine OC-2.4 to induce antibodies against S protein of the huCoV-19/WH01 (b) and Beta variants (d), and the ability to neutralize these viruses *in vitro* (c and e). Also shown is the ability of the universal SARS-CoV-2 DNA vaccine OC-2.4 to prime and boost antibodies to S protein (huCoV-19/WH01) following priming with a recombinant S protein in adjuvant (f) and the ability of these antibodies to neutralize the Delta and Omicron variants *in vitro* (g). Finally, three doses of the universal SARS-CoV-2 DNA vaccine OC-2.4 induce antibodies that cross react with S proteins from the huCoV-19/WH01, Beta, and Delta variants in mice (h), and three doses of the same vaccine induces antibodies that neutralize both the Delta and Omicron variants *in vitro* (i).

Figure 2. Evaluation of different vaccine strategies in the K18-hACE2 mice transgenic for the human ACE2 receptor. The experimental design has been given in (a). Three doses of the respective vaccines fully or partially protected the mice against severe disease as determined by weight loss (b) and viral replication in the nose, lungs and the spleen (c). Values have been given as cycle times (CT), where lower values indicate a higher viral load. Comparisons in the graph are shown as solid lines that indicate a $p<0.01$ (Mann-Whitney), and a dotted line indicate a $p<0.05$ (Mann-Whitney).

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METHODS

Animals

Female BALB/c (H-2^d) mice were obtained from Charles River Laboratories, Sulzfeld, Germany. Female B6.Cg-Tg(K18-ACE2)2Prlmn/J mice (JAX stock #034860) were purchased from Jackson Laboratory, USA. All mice were 8–12 weeks old at the start of the experiments and maintained under standard conditions at Preclinical laboratory (PKL), Karolinska University Hospital Huddinge, Sweden or Astrid Fagraeus Laboratory (AFL), Karolinska Institute, Solna, Sweden. Nine New Zealand White rabbits were purchased from Charles River, France and kept at AFL.

All animal procedures were granted by regional animal ethics committees (approvals Dnr. 03634-2020, 17114-2020 and 16676-2020.

DNA Plasmids and recombinant proteins

Vaccine candidate genes were generated based on the sequence from the huCoV-19/WH01 strain (Figure 1a). The genes contained the full Spike protein or a combination of the RBD, N and M proteins, with a autoproteolytic P2A sequence. All sequences were codon optimized for expression in human cells and were synthesized by a commercial vendor (Genescript). Plasmids were grown in TOP10 *Escherichia coli* cells (Life Technologies) and purified for *in vivo* injections using Qiagen Endofree DNA purification kit (Qiagen) according to the manufacturer's instructions. The correct gene size was confirmed by restriction enzyme digests using BamHI and XbaI (Fast Digest; Thermo Fisher Scientific), and sequencing. Recombinant N protein was designed in-house and produced by Genescript (32878912). Recombinant S, RBD, and M was purchased from Genescript. The spike proteins were produced as full length by transient protein production in mammalian cells (Expi293). To

facilitate trimerization of the full-length spike, a C-terminal T4 fibritin trimerization motif was included according to Wrapp et al [31]. Further a strep-tag, fused to the C-terminal was used for purification [32]. The beta version (B1.351) of the spike was produced with three mutations in the RBD part (K417N E484K N501Y).

Peptides

A total of 42 20-mer peptides with 10 aa overlap, corresponding to the huCoV-19/WH01 RBD (25 peptides), M (22 peptides) and N (41 peptides) and Bat N (42 peptides) were purchased from Sigma-Aldrich (St. Louis, MO). The peptides were divided in pools of 4-5 or 8-10 peptides/pool depending on experimental setup.

Immunization and infection schedules in mice and rabbits.

BALB/c (H-2^d) (n=5) mice were immunized up to three times with three weeks intervals, and sacrificed 2 weeks after the second immunization for spleen and blood collection. Twenty K18-ACE2 mice were divided into 4 groups (n=5) and immunized with indicated vaccines. Each K18-hACE mouse received three immunizations with three weeks between each injection. Two weeks after the last immunization, the K18-hACE2 mice were infected with SARS-CoV-2 Beta. Immunization method in brief, BALB/c or K18-hACE2 mice (5 per group) were immunized intramuscularly in the *Tibialis cranialis anterior* muscle with 50 µg plasmid DNA in a volume of 50 µL sterile phosphate-buffered saline (PBS) by regular needle (27G) injection followed by *in vivo* electroporation using a Cliniporator2 device (IGEA) using 2 needle electrodes. Prior to vaccine injections, mice were given analgesic and kept under isoflurane anesthesia during the vaccinations. During *in vivo* electroporation (in both mice and rabbits) a 1-ms 600-V/cm pulse followed by a 400-ms 60-V/cm pulse pattern was used to facilitate better uptake of the DNA.

In addition, groups of mice were injected subcutaneously at the base of the tail with recombinant SARS-CoV-2 spike (S) or nucleo (N) protein mixed (1:1) with QS21 adjuvant (GMP grade, Alpha diagnostics).

Each New Zealand White rabbits were immunized with 840 µg OC-2.4 DNA vaccine or only formulation buffer (Tris-EDTA, pH 7.4). Injection were administered in the right *Tibialis anterior* muscle in 500 µL followed by *in vivo* electroporation under anaesthesia using the GeneDriVe (IGEA) device and GeneGun electrode (IGEA) with a 4 needle electrode array at a depth of 21 mm.

Mice challenge model

Two weeks post the last immunization the K18-hACE2 mice were challenged with $1x10^5$ pfu SARS-CoV-2 Beta via intranasal administration in a volume of 40 µl. The health of the animals was assessed daily for up to 13 days and evaluated based on several parameters, including body weight, general condition, piloerection, as well as movement and posture. At the time of euthanization, blood, nasal lavage, lungs and spleen was collected.

Detection of IgG specific antibodies

Serum from mice and rabbits were used for detection of immunoglobulins against S or N protein. In brief, plates were coated with 1 µg/ml recombinant S or N protein (Genescript) in 50 mM Sodium Carbonate buffer pH 9.6 overnight at 4 °C. Plates were blocked by incubation with dilution buffer (phosphate-buffered saline, 2% goat serum, 1% BSA) for 1 hour at 37°C. Serum was added in serial dilutions with a starting dilution of 1:60 and then in serial dilution

of 1:6. Serum antibodies were detected by an alkaline phosphatase conjugated goat anti-mouse IgG (Sigma A1047) 1:1 000 or goat anti-rabbit IgG (Sigma A2556) 1:1 000 and visualized using p-nitrophenyl phosphate substrate solution. Optical density (OD) was read at 405 nm with a 620 nm background. Antibody titers were determined as endpoint serum dilutions at which the OD value was at least three times the OD of the negative control (nonimmunized or control animal serum) at the same dilution.

Detection of specific IFN-γ producing T cells and antibodies

Two weeks post last vaccination, splenocytes from each group of wildtype mice or peripherial blood mononuclear cells (PBMCs) were harvested and tested for their ability to induce specific T cells based on IFN-γ secretion after peptide or protein stimulation for 48h essentially as described [16, 17] using a commercially available enzyme-linked immunospot (ELISpot) assay (Mabtech).

Virus propagation

The SARS-CoV-2 huCoV-19/WH01, Beta, and Omicron strains were isolated from patient samples at the Public Health Agency of Sweden and confirmed by sequencing. The SARS-CoV-2 Delta variant was provided by Dr. Charlotta Polacek Strandh, Statens Serum Institute, Copenhagen, Denmark. All variants were propagated on Vero-E6 cells and titered using a plaque assay as previously described [3],with fixation after 72 hrs. The huCoV-19/WH01, Delta and Omicron strains used in this study was passaged 3 times and the Beta strain 2 times.

Neutralization of SARS-CoV-2 *in vitro*

Titer of neutralizing antibodies in serum from mice and rabbits were determined by CPE based microneutralization assay. For mice, serum from each vaccination group was pooled, while for rabbits serum from each individual was tested. Briefly, serum was heat inactivated at 56°C for 30 min before serial diluted 2-fold. Each dilution was conducted in quadruplets and mixed with 500 pfu of SARS-CoV-2 huCoV-19/WH01, Beta, Delta or Omicron in a 1:1 dilution. After 1 hour of incubation at 37°C, 5% CO_2 100 uL of serum-virus mix was added to Vero E6 cells on a 96-well plate ($20x10^4$ cells/well) and incubated for 72 hrs at 37°C, 5% CO_2. CPE for each well was determined using a Nicon Eclipse TE300 microscope. As controls, wells with medium only, diluted serum only, virus only and serum known to contain SARS-CoV-2 neutralizing antibodies mixed with virus was included in each experiment.

PCR/Viral RNA

Trizol (Sigma-Aldrich) in a ratio of 1:3 was used to inactivate potential virus in nasal lavage samples (50 µL) from SARS-CoV-2 infected K18-hACE2 mice . For lung and spleen, PBS was added to each sample (1g/ml) and pestles were used to crush the organs. Thereafter, the samples were centrifuged (5 min at 7000 rpm) and 50 µL of each lung or spleen sample was added to Trizol (1:3). Total RNA was extracted using the Direct-zol RNA Miniprep kit (Zymo Research) according to the manufacturer's instructions. Viral RNA were thereafter measured by quantitative real-time polymerase chain reaction (qRT-PCR) using TagMan Fast Virus 1-Step master mix (Thermo Fisher Scientific) with primers and probe for the SARS-CoV-2 E gene.

Forward: 5'- ACAGGTACGTTAATAGTTAATAGCGT-3'

Reverese: 5'- ATATTGCAGCAGTACGCACACA -3'

Probe: FAM- ACACTA GCC ATC CTT ACT GCG CTT CG MGB

For lung and spleen samples, mouse ACTB mix (Thermo Fisher Scientific) was used as endogenous control. The PCR reaction was performed using a capillary Roche LightCycler 2.0 system.

Statistical analysis

Data was analyzed with use of on GraphPad Prism V.5 software and Microsoft Excel V.16.13.1.

Figures

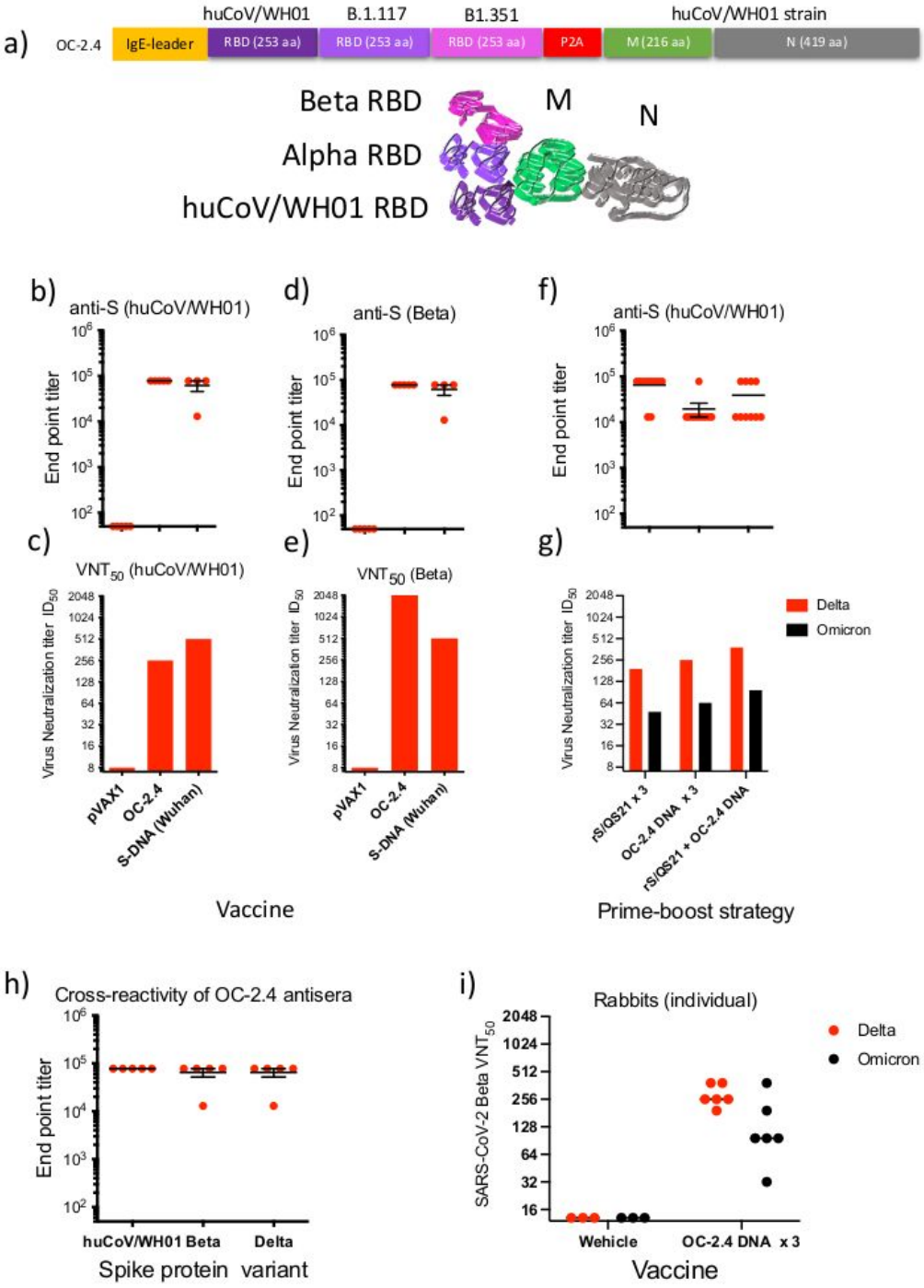

Figure 1

Figure 1

Schematic representation of the universal SARS-CoV-2 DNA vaccine OC-2.4 gene design has been given (a). Ability of the S-DNA and SARS-CoV-2 DNA vaccine OC-2.4 to induce antibodies against S protein of the huCoV-19/WH01 (b) and Beta variants (d), and the ability to neutralize these viruses in vitro (c and e).

Also shown is the ability of the universal SARSCoV-2 DNA vaccine OC-2.4 to prime and boost antibodies to S protein (huCoV-19/WH01) following priming with a recombinant S protein in adjuvant (f) and the ability of these antibodies to neutralize the Delta and Omicron variants in vitro (g). Finally, three doses of the universal SARS-CoV-2 DNA vaccine OC-2.4 induce antibodies that cross react with S proteins from the huCoV-19/WH01, Beta, and Delta variants in mice (h), and three doses of the same vaccine induces antibodies that neutralize both the Delta and Omicron variants in vitro (i).

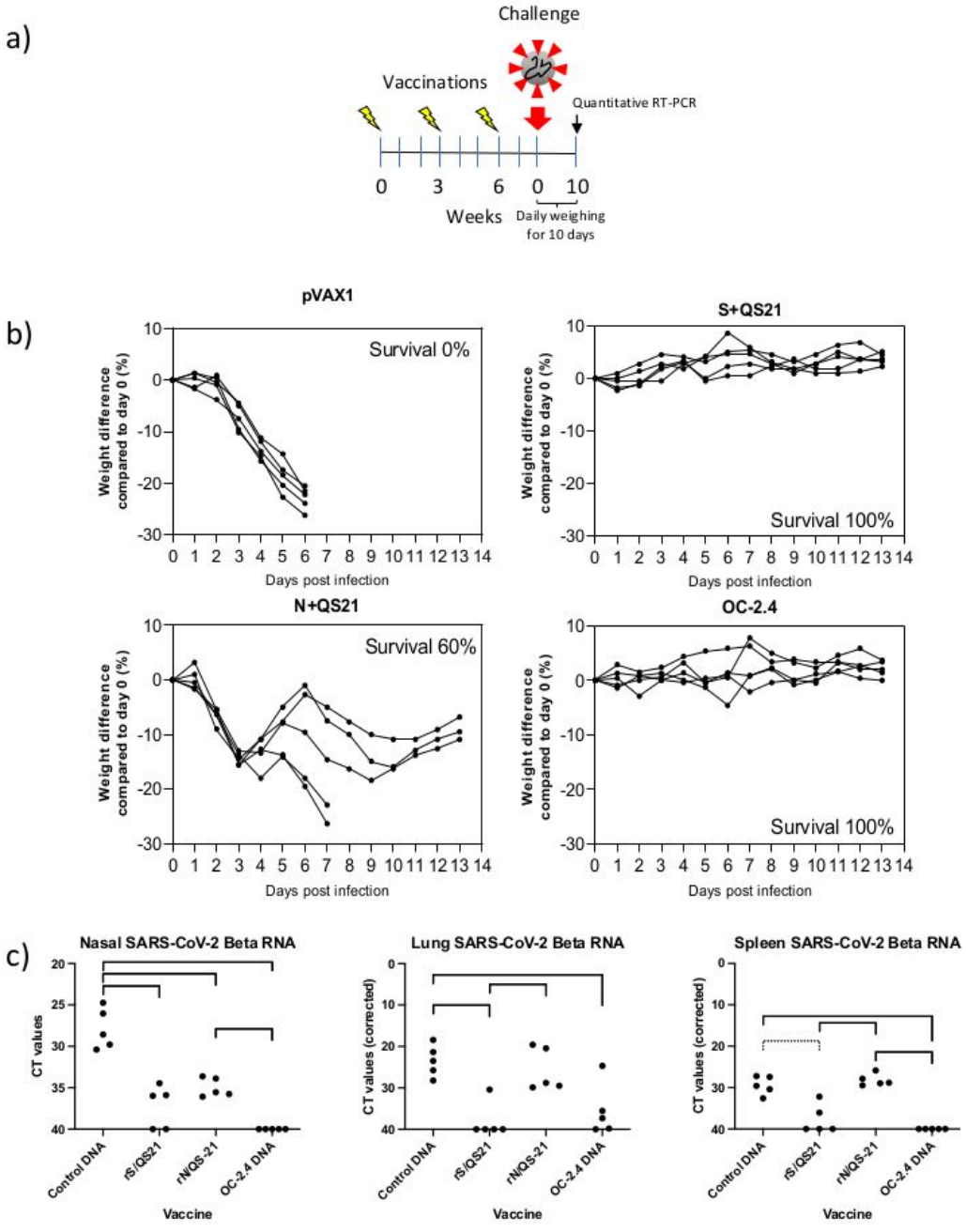

Figure 2

Figure 2

Evaluation of different vaccine strategies in the K18-hACE2 mice transgenic for the human ACE2 receptor. The experimental design has been given in (a). Three doses of the respective vaccines fully or partially protected the mice against severe disease as determined by weight loss (b) and viral replication in the nose, lungs and the spleen (c). Values have been given as cycle times (CT), where lower values indicate a higher viral load. Comparisons in the graph are shown as solid lines that indicate a $p<0.01$ (Mann-Whitney), and a dotted line indicate a $p<0.05$ (Mann-Whitney).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- suppfig3.jpg
- suppfig2.jpg
- suppfig1.jpg