Protection against SARS-CoV-2 transmission by a parenteral prime—Intranasal boost vaccine strategy

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Summary

Background Licensed vaccines against SARS-CoV-2 effectively protect against severe disease, but display incomplete protection against virus transmission. Mucosal vaccines providing immune responses in the upper airways are one strategy to protect against transmission.

Methods We administered Spike HexaPro trimer formulated in a cationic liposomal adjuvant as a parenteral (subcutaneous – s.c.) prime - intranasal boost regimen to elicit airway mucosal immune responses and evaluated this in a Syrian hamster model of virus transmission.

Findings Parenteral prime - intranasal boost elicited high-magnitude serum neutralizing antibody responses and IgA responses in the upper respiratory tract. The vaccine strategy protected against virus in the lower airways and lung pathology, but virus could still be detected in the upper airways. Despite this, the parenteral prime - intranasal booster vaccine effectively protected against onward SARS-CoV-2 transmission.

Interpretation This study suggests that parenteral-prime mucosal boost is an effective strategy for protecting against SARS-CoV-2 infection and highlights that protection against virus transmission may be obtained despite incomplete clearance of virus from the upper respiratory tract. It should be noted that protection against onward transmission was not compared to standard parenteral prime-boost, which should be a focus for future studies.

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Introduction

Licensed Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) vaccines relying on novel technologies, including messenger RNA vaccines, have proved highly effective against severe COVID-19. However, a major limitation of these vaccines is their lower effectiveness at protecting against virus transmission than against disease. Parenteral vaccines predominantly induce systemic IgG antibody responses, but respiratory viruses with pandemic potential, including coronaviruses, are mainly transmitted from person to person via respiratory droplets and infect the upper respiratory tract, which is not effectively protected by circulating IgG. Failure to elicit sterilizing immunity can lead to local viral replication in respiratory tissues and potentially onward transmission, enabling the development and spread of resistant variants. Mucosal vaccination is an established strategy for induction of secretory IgA

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Research in context

Evidence before this study
Licensed vaccines against SARS-CoV-2 are highly effective at preventing severe disease, but fail to completely protect against virus transmission. Subunit vaccines incorporating an effective mucosal adjuvant can induce mucosal responses in the upper respiratory tract, which have the potential to block respiratory pathogens at the portal of entry. Preclinical models recapitulating natural infection are needed to study the capacity of vaccines to block virus transmission.

Added value of this study
Our study demonstrates that a parenteral prime - mucosal boost vaccine strategy can protect against SARS-CoV-2 infection and pathology in the lower respiratory tract. Furthermore, onward transmission from vaccinated animals was significantly reduced compared to that observed in unvaccinated controls. Our study did not directly assess if parenteral prime - mucosal boost was superior to parenteral only immunization for protecting against onward transmission.

Implications of all the available evidence
The study suggests that a parenteral prime - mucosal booster strategy using protein-based subunit vaccines may be an effective means to protect against transmission of SARS-CoV-2 and potentially other respiratory viruses.

(sIgA) at the mucosal surfaces that, by blocking virus at the portal of entry, may prevent initial viral replication and thus potentially provide sterilizing immunity. Compared to the monomeric IgG, sIgA is multimeric, providing increased avidity and sIgA thus can be better at neutralizing SARS-CoV-2 than IgG.20–22 Intranasal (i.n.) immunization also elicits local tissue resident (TRM) CD8 T cell responses in nasal-associated lymphoid tissue (NALT).23–25 For SARS-CoV-1, an i.n. vaccine induced respiratory CD4 T cells recruiting protective CD8 T cells to NALT via an IFN-γ dependent mechanism.26 The Th17 cell subset has received particular focus in mucosal immune responses27 and Th17-produced IL-17A upregulates polymeric immunoglobulin receptor (pIgR) to promote secretory IgA responses.28–31 One strategy to facilitate both systemic immunity and mucosal immune responses in the upper airways is by a parenteral prime – i.n. boost regimen.13,15–17 We tested this strategy for SARS-CoV-2, using a cationic liposome (CAF01) adjuvanted spike subunit vaccine. Immunization by parenteral prime – i.n. boost induced anti-spike IgG and SARS-CoV-2 neutralizing antibody responses in serum and elicited IgA responses in the upper respiratory tract. In a transmission model in which vaccinated contacts were co-housed with SARS-CoV-2 infected index hamsters, the parenteral prime-mucosal boost strategy lowered virus titres in the upper airways and protected against onward transmission. Overall, a parenteral prime - i.n. boost vaccine strategy may be an effective means to limit virus spread in the population.

Methods

Ethics
Animal studies were conducted in accordance with European Community Directive 2010/63/EU. The experiments have been approved by, and conducted in compliance with, the governmental Animal Experiments Inspectorate under licenses 2017-15-0201-01163 and 2020-15-0201-00544.

Antigens and adjuvants
Recombinant SARS-CoV-2 prefusion-stabilized spike ectodomain (S-2P19 and HexaPro trimer30), and the RBD domain (RVQ-VNF) from the Wuhan-Hu-1 strain were produced by transient expression in freestyle 293-F cells, as reported previously.20,21 CAF01 (250 μg DDA/50 μg TDB) in 10mM TRIS buffer with 2.2% glycerol (pH 7.0) was produced as described previously.22

Characterization of formulations
A compatibility study of the spike HexaPro trimer in CAF01 was performed at room temperature. Formulations were analysed visually for potential flocculation and then characterized for particle size and polydispersity index (PDI) by dynamic light scattering, using the photon correlation spectroscopy technique. The zeta potential was measured by laser-Doppler electrophoresis. For the size measurements, the samples were diluted 10 times, whereas for the zeta potential measurements, the samples were diluted 100 times in milli-Q water. The measurements were performed at 25 °C, using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) with a 633 nm laser and 173° detection optics. Malvern Zetasizer software v.8.01 was used for analysis.

Animals
Female C57Bl/6 (C57BL/6JolaHsd) wild type mice, 7–9 weeks old, were obtained Envigo (The Netherlands). Male Syrian golden hamsters (Mesocricetus auratus), nine weeks old, were obtained from Janvier. Both species were housed in the animal facilities at Statens Serum Institut, Denmark during the studies and maintained in rooms with controlled environment (20–23 °C; relative humidity 52 ± 10%; 12/12 h light/dark cycle). The mice were randomly allocated to cages (type III polycarbonate cages (820 cm3)) with up to eight
mice per cage and the hamsters were housed in polycarbonate cages type IV (1820 cm²) with high lid (in total app. 30 cm. high) with up to four animals/cage. A total of 28 mice and 51 hamsters were used for the studies. All animals were offered Aspen bedding and bricks (Tapvei), Sizzelnest (Datesand) and tunnels or houses made of polycarbonate. Besides, mice were offered DesRes paper houses (LBS) while hamsters had twisted paper rolls (“Diamond Twist” Envigo Teclad) hanging in their cage lids. Irradiated sunflower seeds, corn grains and peanuts or bits of carrots were offered all the animals once a week.

Pelle diet (Envigo Teclad 2016) and tap water was provided ad libitum.

Immunizations
Mice and hamsters were administered with two immunizations 21 days apart, using 5 µg (mice) or 20 µg (hamsters) of recombinant SARS-CoV-2 prefusion-stabilized spike ectodomain formulated in CAF²⁰⁻⁰¹ as described previously. The spike S-2P variant was used in initial mouse studies, whilst the HexaPro variant was used in all hamster studies and in mouse studies where indicated. The vaccine was either given as two subcutaneous (s.c.) immunizations (s.c./s.c.) or as s.c. immunization followed by intranasal (i.n.) boost (s.c./i.n.). The s.c. immunizations were given at the base of tail (mice) or in the scruff of the neck (hamsters) at a final volume of 200 µl per immunization. The i.n. immunizations were performed under isofluorane anaesthesia. Mice were given a final volume of 20 µl (10 µl per nostril). Hamsters were placed in supine position and given 50 µl (25 µl per nostril). The number of animals in each group is stated in the figure legend. Power calculations were based on levels of antigen-specific IgA (against another protein antigen) in s.c./i.n. immunized compared to unvaccinated controls in a previous mouse study with effect size (OD₄₅₀) of 0.58 and SD of 0.297. An analysis plan was prepared for each study as part of the study protocol.

Sampling and organ preparation from mice
Mice were euthanized by CO₂ (80%)/O₂ (20%), after anaesthetization with Zoletil-mix (Zolazepam, Tiletamin, Xylazin and Butorphanol). Lungs and spleens were filtered through a 70 µm nylon mesh (BD Biosciences). The cells were washed and re-suspended in PBS with 1% pyruvate, 1% HEPES, 1% (v/v) pre-mixed penicillin-streptomycin solution (Invitrogen Life Sciences). The cells were added in 200 µl of cell culture medium into 96-well cell culture plates at a density of 2 x 10⁶ cells/well. Nasal washes were sampled by flushing with 350 µl phosphate buffered saline (PBS) +0.05% bovine serum albumin.

ELISA for spike-ACE2 binding
ACE2 binding ELISA was performed to assess if spike-ACE2 binding was intact after formulation of spike in CAF²⁰⁻⁰¹ liposomes. Maxisorb Plates (Nunc, Denmark) were coated overnight with recombinant human ACE2 (ACE-HM101, Kactus Biosystems) at 2 µg/ml in carbonate buffer pH 9.6. Plates were washed with PBS containing 0.2% Tween 20 and blocked with 2% skimmed-milk powder (SM). After blocking, spike protein alone or spike protein formulated in CAF²⁰⁻⁰¹ were added serially diluted in PBS with 1% SM, followed by incubation with hamster-anti-spike protein serum (generated in-house by immunizing hamsters with recombinant spike protein) as primary antibody, HRP-conjugated goat anti-hamster IgG antibody (Invitrogen, AB-2356752) was used as secondary antibody, TMB-PLUS (Kem-En-TEC, Denmark) was used as substrate, and the absorbance was recorded at 450 nm with subtraction of the absorbance value measured at 620 nm.

ELISA for antibody responses
Maxisorb Plates (Nuncs) were coated overnight with 0.05 µg/well SARS-CoV-2 trimer or RBD (4 °C). For antigen-specific IgG, plates were blocked with 2% BSA and serum was added diluted in PBS with 1% BSA. Polyclonal HRP-conjugated secondary rabbit anti-mouse IgG (Thermofisher, RRID: AB_138451) or goat anti-hamster IgG antibody (Invitrogen, AB-2356752), was diluted in PBS with 1% BSA. For antigen-specific IgA, plates were blocked with 2% milk, and serum and nasal washes were added in PBS with 1% milk. Biotin-coupled goat anti-mouse IgA (Southern Biotech, AB-2794374) diluted in 1% milk was used as primary antibody, followed by streptavidin-HRP (BD, AB-2868972) diluted in PBS containing 0.2% Tween 20.

After incubation of secondary antibodies, Spike- or RBD-specific antibodies were detected using TMB substrate as described by the manufacturer (Kem-En-Tec Diagnostics), and the reaction was stopped with H₂SO₄. The absorbance was measured at 450 nm with subtraction of the absorbance value measured at 620 nm.

Cytokine profiling
The Mouse U-plex (IFN-γ, IL-17A, IL-5, IL-13 and IL-10) was performed according to the manufacturer’s instructions (Meso Scale Discovery) to measure CD4 T cell profiles after ex vivo re-stimulation of splenocytes with SARS-CoV-2 trimer antigen (2 µg/ml cell culture medium incubated for 72 h at 37 °C and 5% CO₂). The plates were analysed on a Sector Imager 2400 system (Meso Scale Discovery) and calculation of cytokine
concentrations was performed by 4-parameter logistic non-linear regression analysis of the standard curve.

**Neutralization assay**
Neutralization of SARS-CoV-2 pseudo-particles was evaluated using a pseudotyped lentivirus neutralization assay with SARS-CoV-2 spike (Wu-Hu-1) and HEK293T cells engineered to express human ACE2, as previously described. This isolate is closely related to the Wu-Hu-1 sequence (with V367F and D614G substitutions in the spike protein). For cross-neutralization studies, a B.1.617.2 delta-variant (DK-AHH3 isolate) and a B.1.1.529 omicron-variant (DK-AHH4 isolate) were used. In brief, SARS-CoV-2 at a multiplicity of infection (MOI) of 0.01 to 0.15 was incubated for 1 h at room temperature with serially diluted heat inactivated (at 56 °C for 30 min) plasma (1/100 to 1/51200 dilution) at a 1:1 ratio. Following incubation, plasma/virus mixtures were added to naive Vero E6 cells in quadruplicates in 96-well plates. After incubation for 48 h at 37 °C and 5% CO2, cells were fixed and stained as described, but using SARS-CoV-2 spike chimeric monoclonal primary antibody (Sino Biological #40592-MM57, RRID: AB_2857935) and F(ab’2)-goat anti-human IgG Fc cross-adsorbed secondary antibody conjugated to HRP (Abcam) and goat anti-(H+L) serum samples (diluted x25, x250 and x2500) as positive controls for each plate, as previously described. This isolate is closely related to the Wu-Hu-1 sequence (with V367F and D614G substitutions in the spike protein and a G232V substitution in ORF3a). In the transmission model each infected hamster was isolated for six hours and then placed in a cage containing one hamster immunized s.c./i.n. and one unvaccinated control (six cages in total). In the onwards transmission model, index hamsters were isolated for 24 h and then housed with s.c./i.n. vaccinated or unvaccinated contacts. 24 h later the contacts were co-housed with another set of naive hamsters for the rest of the study (five days) to assess onwards transmission. Weight and signs of disease (lethargy, ruffled fur, hunched posture) were monitored until the study was terminated at seven days post infection of the index hamsters. Humane endpoints included weight loss >20% of none of the animals reached humane endpoints. Virus titers were determined in nasal washes at days two and seven post challenge and in lungs at the time of termination.

**RT-qPCR for SARS-CoV-2 detection**
Lungs were dissociated by GentleMACS (M-tubes, Miltenyi). Nasal washes were collected by flushing with 350 µl of PBS. Total RNA extractions were done by MagNa Pure Kit (Roche Molecular Biochemicals, Indianapolis, Indiana, United States (US)), using MagNA Pure LC DNA Isolation Kit I lysis buffer. All oligonucleotides were synthesized by Eurofins Genomics. RT-qPCR was performed using 5 µl of resuspended RNA in a 25 µl reaction volume using the Luna® Universal Probe One-Step RT-qPCR with Luna WarmStart® RT Enzyme Mix (New England Biolabs) with 400 nM concentrations primers and 200 nM of probe. Primers and probes for the SARS-CoV-2 E gene target (diagnostic PCR) were as previously described. Cycling conditions were 55 °C for 10 min, denaturation at 95 °C for 3 min, and 45 cycles of 95 °C (15 s) and 58 °C (30 s). To generate standard curves, synthetic SARS-CoV-2 RNA controls (MT007544.1) (Twist Bioscience) of a known copy number was serially diluted. A stabilized RNA for 2019-nCoV E gene (EVA) was used as positive control. RT-qPCR for detection of subgenomic RNA for the SARS-CoV-2 E-gene was performed as previously described using 50 °C for 10 min, 95 °C for 3 min, and 45 cycles

**Meso Scale discovery ACE2 competition assay**
The V-PLEX SARS-CoV-2 Panel 19 (ACE2) Kit was run according to the manufacturer’s instructions. Briefly, serum samples (diluted x25, x250 and x2500) and nasal washes (diluted x5) were added to pre-coated V-plex plates followed by ACE2 binding detection using a Sergeant Imager 2400 system (Meso Scale Discovery).
of 95 °C (10 s), 56 °C (15 s) and 72 °C (5 s). Reactions were carried out using a Lightcycler-480 Real-Time PCR System (Roche). Results were expressed as log_{10} genome equivalent copies per ml (nasal washes) or gram (lungs).

Pathology
From animals euthanized on day seven post challenge, the right diaphragmatic lung lobe was pseudo-perfused fixed in 10% neutral buffered formalin and then placed in histo-cassettes for immersion fixation in 10% neutral buffered formalin for 24 h before being transferred to ethanol. Paraffin embedded tissues were sectioned (4 μm) and stained with haematoxylin and eosin (H&E) for histopathological examination. Sections were evaluated for infiltration of inflammatory cells (neutrophils and macrophages). In all sections the inflammatory reaction was scored as 0: absent, 1: few sporadic inflammatory cells present, and 2: numerous accumulated inflammatory cells. The inflammatory cells were identified from their characteristic morphology. The presence of type-II pneumocyte hyperplasia was determined from the presence of this cell type laying the alveolar lumen in a row morphology. Type-II pneumocyte hyperplasia was examined by H&E staining and confirmed by cytokeratin staining using a mouse anti-cytokeratin antibody (clone AE1/AE3, DAKO M3515). The investigators performing the histological examination were blinded to the experimental groups.

Statistics
Differences between groups were analysed by one-way ANOVA comparing the mean of each column with each other column. Differences between naïve and s.c./i.n. immunized groups in the onward transmission model were analysed by unpaired student’s t test (GraphPad v8.2.1). Sample size determination is described under immunizations.

Role of funders
The funding source had no role in study design, collection, analysis or interpretation of data or in the writing of the publication.

Results
Parenteral prime – intranasal boost with adjuvanted SARS-CoV-2 spike trimer induces serum neutralizing antibodies and nasal IgA responses in mice
Systemic neutralizing antibodies effectively protect the lower airways against SARS-CoV-2 pathology.29 We investigated if parenteral priming - i.n. boosting would induce similar or lower neutralizing antibody responses compared to administering the vaccine as a standard two-dose parenteral regimen. Prefusion-stabilized spike trimers containing two stabilizing proline mutations (S2P)18 were formulated in a cationic liposome adjuvant (CAF01) and characterised as described previously.25 The vaccine was tested in mice, administered as two s.c. immunizations (s.c./s.c.) or as one s.c. immunization followed by i.n. boost (s.c./i.n.) (Figure 1a). Serum anti-spike and anti-RBD IgG antibody levels at 21 days after the second immunization were similar between the two groups (Figure 1b), whilst spike-specific IgA responses were only detected in the s.c./i.n. group (Figure 1c). To investigate if the vaccine-elicited antibodies were capable of neutralizing SARS-CoV-2, we performed a homotypic SARS-CoV-2 pseudovirus neutralization assay and found similar neutralizing capacity of the sera (ID50 of 1/7222 for s.c./s.c. versus 1/4672 for s.c./i.n immunization) (Figure 1d). We also measured T cell responses in the spleen and found that s.c./i.n. immunization elicited similar systemic Th1 and Th17 responses as s.c./s.c. immunization (Figure 1e).

To investigate if i.n. boosting could facilitate a mucosal antibody response in the upper respiratory tract, we compared spike-specific IgA responses in nasal washes of s.c./i.n and s.c./s.c. immunized mice. For these studies we used the further stabilized Spike HexaPro trimer variant.19 Following formulation in CAF01, the liposomes maintained a net positive charge and an intact binding of HexaPro spike to ACE2 was confirmed (Figure 2). s.c./i.n immunization elicited significantly higher spike-specific IgA responses both in serum and in nasal washes compared to the s.c./s.c. regimen (p<0.001, one-way ANOVA) (Figure 1f). Thus, administering the booster immunization i.n. did not compromise systemic vaccine-elicited immunity, but facilitated IgA responses both systemically and in the upper airways.

Intranasal booster vaccine protects against disease and reduces viral replication in the upper airways in a SARS-CoV-2 hamster transmission model
SARS-CoVs mainly transmit via aerosol or large droplet transmission. To investigate if i.n. booster vaccination could protect against virus transmission, we developed a model in Syrian hamsters reflecting natural SARS-CoV-2 transmission, where index hamsters were infected i.n. and co-housed with animals vaccinated either s.c./s.c. or s.c./i.n. or left unvaccinated. Hamsters were immunized with an identical regimen as in the mouse studies, using pre-fusion stabilized Spike HexaPro trimer (Figure 2a). Similarly to what we observed in mice, s.c./i.n. vaccination in hamsters elicited comparable serum IgG responses (Figure 2b) and neutralizing antibody responses against the homologous strain (Wuhan-1) as s.c./s.c. immunization, when measured in a replication competent virus neutralization assay (Figure 2c). Similar cross-neutralizing responses were also found against the delta (B.1.617.2) variant...
whilst only one of six animals in the s.c./s.c. immunized group and three of six in the s.c./i.n. immunized group had antibodies cross-neutralizing the omicron (B.1.1.529) variant (Figure 2e). To further probe neutralizing antibody responses against other SARS-CoV-2 variants, we used an ACE-2 binding competition assay. Both s.c./s.c and s.c./i.n. immunized animals had antibodies competing for ACE-2 binding of the beta (B.1.351), gamma (P.1), lambda (C37) and Mu (B.1.621) variants (Figure 2f). To test the capability of the two vaccine strategies to elicit antibody responses in the upper respiratory tract, nasal washes were isolated 21 days after s.c. or i.n. booster immunization and tested in the ACE-2 competition assay. Although responses were low in all groups, s.c./i.n. immunized hamsters had significantly higher responses than naive animals against the homologous Wu-Hu-1 and the B.1.351, B.1.621 and C37 strains (*p<0.05, one-way ANOVA) (Figure 2g). s.c./i.n. immunized hamsters also had higher responses than their s.c./s.c. immunized counterparts against the B.1.351, P.1 and C37 strains (*p<0.05, one way ANOVA).

The hamsters were challenged with SARS-CoV-2 at 21 days after the 2nd immunization. Animals were housed in six cages each containing one s.c./s.c and one s.c./i.n. immunized animal together with two unvaccinated animals. One of the latter was placed in a separate cage and challenged i.n. with $1.8 \times 10^7$ TCID$_{50}$ of the SARS-CoV-2/Hu/DK/SS1-H5 isolate (index hamster) (Figure 3a). Six hours later, the index hamsters were returned to their original cages. Index hamsters and contacts were then followed for seven days to measure virus transmission and signs of disease. Only the infected index animals had marked weight loss (Figure 3b). However, at day seven post challenge, viral RNA was detected in the lungs of both index animals and unvaccinated contacts by a diagnostic PCR for the E-gene (Figure 3c). In contrast, no viral RNA was detected in the lungs of vaccinated animals (both after s.c./s.c. and s.c./i.n. immunization). In the upper airways, viral RNA was detected in all groups, although there was a tendency towards lower levels in nasal washes from contacts vaccinated s.c./i.n. compared to unvaccinated controls at two days post challenge (not significant, *p=0.06, one-way ANOVA) and significantly lower levels at day 7 post challenge (*p<0.05) (Figure 3d). There was no statistically significant differences in viral load between s.c./s.c. and s.c./i.n. immunized animals. Infection with the Wu-hu-1 strain further boosted neutralizing antibody titres in both the s.c./s.c. and s.c./i.n. immunized groups against both the homologous strain and the delta and omicron variants (sFigure 3). Infection per se only elicited detectable neutralizing antibodies against the Wu-hu-1 strain and the delta variant, but not the omicron variant (sFigure 3).
Figure 2. Syrian Hamsters were immunized with two doses of spike HexaPro trimer protein formulated in cationic liposomes (CAF/C210). The vaccine was either administered as a subcutaneous two dose regimen (s.c./s.c.) or as subcutaneous priming followed by intranasal boosting (s.c./i.n.). a) Experimental setup. b) Serum IgG antibody responses against spike protein (mean+95% CI). Serum neutralization of SARS-CoV-2 was tested in a culture derived SARS-CoV-2 assay against c) the homologous Wu-hu-1 strain and d) the delta variant (B.1.617.2). A SARS-CoV-2 spike neutralizing monoclonal antibody (40592-MM57) was used as positive control at 1/80 dilution, which gave an average of 81% neutralization for the homologous Wu-Hu-1 variant and 96% for the B.1.617.2 variant. e) Neutralization of the omicron variant (B.1.1.529). Plasma from a COVID-19 vaccinated individual (1/80 dilution) was used as positive control giving 95% neutralization. The stippled line indicates neutralization below the limit of detection and is plotted as IDso=100. f) ACE2 competition assay measuring serum antibodies towards the Wu-Hu-1, B.1.351, P.1, B.1.621 and C37 strains. g) ACE2 competition assay measuring nasal washes antibodies towards the Wu-Hu-1, B.1.351, P.1, B.1.621 and C37 strains. Bars indicate...
Overall, these results demonstrated that the s.c./s.c. and s.c./i.n. vaccine elicited similar systemic neutralizing antibody responses and both immunization strategies effectively protected against SARS-CoV-2 in the lower airways. In addition, s.c./i.n. immunization elicited antibody responses in the nasal cavity and partially protected against virus in the upper airways.

Figure 3. Syrian hamsters were vaccinated subcutaneously (s.c./s.c.) or via subcutaneous priming / intranasal boost (s.c./i.n) with spike HexaPro trimer protein formulated in cationic liposomes (CAF®/C210). The hamsters were housed with index animals, which had been challenged intranasally with $1.8 \times 10^5$ TCID$_{50}$ of SARS-CoV-2. a) Schematic of the study setup. b) Percent weight change (mean±SEM). c) Viral load in lungs at 7 dpi and d) nasal washes at 2 dpi and 7 measured by a diagnostic qPCR against the E-gene (bars indicate mean). e) Following fixation the right diaphragmatic lung lobe were fixed in 10% formalin, cut and stained with H&E to examine for pulmonary pathology at 7 dpi. Plots display the numbers of hamsters in each group having influx of the indicated inflammatory cells, type II pneumocyte hyperplasia, formation of syncytial cells and necrosis. ($n=6$ hamsters per group). Statistically significant differences between contact animals are indicated by * or *** (one-way ANOVA, comparing the mean of each column with each other column $p<0.05$ or 0.001, respectively). There was no statistically significance among groups if not otherwise indicated. Figures represent $n=6$ hamsters per group. Created with BioRender.com.
To examine if the vaccines could protect against pulmonary pathology, hamsters were examined for lung pathology at seven days post infection. Index animals and unvaccinated contacts had influx of neutrophils and macrophages into the alveolar tissue (Figure 3e and sFig4a-b). This was accompanied by marked type II pneumocyte hyperplasia and syncytial cell formation (Figure 3e and sFigures 4c-d). Four of six animals in the unvaccinated contact group also had necrosis of alveolar tissues. Of vaccinated animals, one in the s.c/s.c. that had moderate pulmonary inflammation with macrophage and neutrophil influx and presence of syncytial cells, whilst another animal had mild inflammation with neutrophil influx. In the s.c/i.n. group, only one hamster had mild pulmonary inflammation with neutrophil influx but no syncytial cells observed (Figure 3e and Figure 4). Thus, both s.c./i.n. and s.c./s.c. immunization with CAF01-adjuvanted spike HexaPro vaccine effectively protected against lung pathology in the hamster model.

**Parenteral prime - intranasal booster vaccine protects against onward transmission**

Apart from protecting the individual, a major goal of vaccination is to prevent virus transmission, thereby protecting against virus spread in the population. To study the impact of vaccination on transmissibility of SARS-CoV-2, we refined the hamster transmission model to assess onward virus transmission from vaccinated animals (Figure 4a). Previous studies in Syrian golden hamsters found that the highest frequency of
transmission to contacts occurred at 16–48 h post infection, which was the time of peak viral load in the donor animals.\(^{39}\) Index hamsters were therefore infected with 1.8 x 10^5 TCID50 of SARS-CoV-2 and isolated for 24 h and were then co-housed with either vaccinated animals, having received parenteral prime – i.n. booster vaccine, or naïve animals for another 24 h to allow virus transmission. The index hamsters had confirmed SARS-CoV-2 by a diagnostic qPCR at day 2 post infection (Figure 4b). To study if the parenteral prime – mucosal boost vaccine protected against onward transmission, the vaccinated or naïve contacts were subsequently co-housed with another set of naïve hamsters. qPCR of nasal washes sampled 24 h later (Day 3), revealed virus in all s.c./i.n. vaccinated hamsters, although virus titres were significantly lower than in naïve (unvaccinated) controls (p<0.001, unpaired t-test) (Figure 4d). In contrast, both of the onward transmission groups had virus loads at the detection limit at this time point. At 48 h later (day 5), extensive onward transmission had occurred from naïve contacts, whilst naïve animals co-housed with the vaccinated group had significantly lower virus levels, still approaching the limit of detection (p<0.001) (Figure 4f). Onward transmission was also evident in the lungs of all naïve hamsters co-housed with the unvaccinated controls, whilst only one of eight animals co-housed with the s.c./i.n. immunized hamsters had detectable viral RNA in the lungs (significantly lower than in the naïve contacts, p<0.001, unpaired t-test) (Figure 4e). Another qPCR for subgenomic RNA indicated that the virus detected in the upper airways of s.c./i.n. immunized was replication-competent at days 3 and 5 into the study, but levels were significantly lower than in the unvaccinated group (p<0.05, unpaired t-test) (Figure 5). Thus, although s.c./i.n. immunization did not provide sterilizing immunity in the upper respiratory tract, the vaccine effectively protected against onward transmission.

**Discussion**

SARS-CoV-2 has caused more than 6 million deaths to date. Safe and effective vaccines against SARS-CoV-2 have been produced and distributed at unprecedented speed and have completely changed the course of the pandemic. Vaccines have proven extremely effective at date to reducing morbidity and mortality. However, with continued onward transmission, there is still a risk that novel variants of concern (VOC) emerge, necessitating continued development of novel vaccines. Furthermore, improved vaccination strategies must be explored to prepare for future pandemics caused by respiratory viruses. One major drawback of licensed SARS-CoV-2 vaccines is the relatively low protection induced in the upper airways,\(^{39,35}\) which may allow for virus replication and onward transmission. To protect against emerging viruses with pandemic potential, a vaccine approach that can effectively prevent virus transmission is desired. One key strategy to this aim is via induction of mucosal immune responses in the upper airways. Whilst parenteral vaccines provide only low immunity at this site, vaccines applied in the nasal cavity can induce a number of immune mediators that can block viral entry or virus replication, including IgA\(^{30}\) and local nasal-associated lymphoid tissue (NALT) CD4 and CD8 T cell responses.\(^{34}\) In the present study we tested a combination of parenteral priming with mucosal boosting to elicit both systemic and local airway immunity and evaluated this in a Syrian hamster transmission model.

Syrian hamsters are a highly relevant model for human SARS-CoV-2 infection, displaying similar viral kinetics, pathological signs and disease course as seen in patients with COVID-19.\(^{31,35}\) Previous studies have demonstrated that hamsters immunized i.n. with chimpanzee adenovirus encoding prefusion-stabilized spike protein had reduced virus titres in the upper respiratory tract compared to unvaccinated controls.\(^{36}\) Similarly, hamsters immunized i.n. with the adenovirus-vectored vaccine ChAdOx1 nCoV-19/AZD1222 had significantly lower virus titres in the upper airways than unvaccinated controls, after being co-housed with SARS-CoV-2 infected animals.\(^{37}\) A recombinant spike-based protein vaccine linked to outer membrane vesicles (OMV) was also found to protect hamsters against pathology after i.n. administration, but did not reduce virus titres in nasal turbinates.\(^{38}\) Here we have demonstrated that the prefusion stabilized (HexaPro) SARS-CoV-2 spike protein,\(^{39}\) adjuvanted with CAFA\(^{30}\) and given as parenteral prime – i.n. boost protects against lower respiratory tract infection and pathology upon SARS-CoV-2 challenge in Syrian hamsters. Furthermore, the vaccine significantly reduced viral load in the upper airways. It is possible that other vaccine strategies, such as parental prime followed by a mucosal boost with a heterologous vaccine,\(^{39}\) would be even more beneficial at inducing mucosal immunity. As application of the mucosal booster vaccine in humans would require special devices, e.g. nasal spray syringe or nebulizer, the methods of delivery could also influence the effectiveness of mucosal booster strategies.

A major goal of vaccination in a pandemic scenario is to protect against onwards transmission, thus limiting spread of virus in the population. We therefore developed a model in Syrian hamsters designed to investigate onwards transmission from vaccinated animals. Interestingly, we observed little onward transmission from hamsters vaccinated via parenteral prime – i.n. boost, although all the vaccinated animals had detectable virus in the upper airways. Even though we did not directly measure infectious titres in the current study, it is possible that a certain viral load in the upper airways is required for effective transmission, which would be surprising since previous studies have demonstrated that hamsters are highly susceptible to SARS-CoV-2,
immune responses in a number of disease settings11,39,44 demonstrated to elicit strong and protective mucosal against influenza. Parenteral prime only vaccines, e.g. the licensed NVX-CoV237343 or vaccines. Future studies should therefore include paren-

giment head-to-head with parenterally administered vaccines against onward transmission, we did not compare this regimen head-to-head with parenterally administered vaccines. Future studies should therefore include paren-
teral only vaccines, e.g. the licensed NVX-CoV237343 or Comirnaty®4. in the onward transmission model to gauge the benefit of parenteral prime-intranasal boost over standard parenteral vaccines.

Despite the promise of i.n. vaccines to offer localized mucosal immunity in the upper airways, only few i.n. vaccines are registered, including the Flumist® vaccine against influenza. Parenteral prime – i.n. boost protected against onward transmission, we did not compare this regimen head-to-head with parenterally administered vaccines. Future studies should therefore include parenterally only vaccines, e.g. the licensed NVX-CoV237343 or Comirnaty®4, in the onward transmission model to gauge the benefit of parenteral prime-intranasal boost over standard parenteral vaccines.

Although unadjuvanted protein given i.n. can boost parenteral primed responses (e.g. after mRNA-LNP or protein in adjuvant immunization),13,45 a more feasible strategy, particularly in a future pandemic mass-vaccination setting, would be to use similar vaccine formulations for both the systemic priming and mucosal boosting. We demonstrate here that spike protein formulated in CAF®01 and given as a parenteral prime - i.n. boost regimen completely protected hamsters against lung pathology following SARS-CoV-2 infection. Furthermore, the vaccine reduced virus loads in the upper airways and protected against onward transmission, thus highlighting the promise of this approach for vaccination against respiratory viruses of pandemic potential.

Contributors
D.C., C.P., G.M., B.M., H.E.J., G.J., K.I., L.K.I., I.R., F. F., J.B. and G.K.P. designed research D.J.S., L.H., A.M., M., K.T.H., R.F.J., J.S.H., C.F., S.R. performed experiments D.C., C.P., H.E.J., K.T.H., R.F.J., J. S. H., C.F., S. R. and G.K.P. analyzed data. D.C., J.B., G.K.P. wrote the paper. All authors read and approved the final version of the manuscript, and have had access to the raw data. D.C and G.K.P. can verify the accuracy of the raw data for the study.

Data sharing statement
Study protocol and all data collected for the study, including raw data and data analysis will be made available to others upon request. All data will be available upon publication of the manuscript, by contacting the corresponding author. Data will be made available after approval of a proposal and with a signed data access agreement.

Declaration of interests
D.C. is co-inventor on patents on the cationic adjuvant formulations (CAF). All rights have been turned over to Statens Serum Institut, which is a non-profit govern-
ment research facility. The rest of the authors declare that there are no competing interests.

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Supplementary materials
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References
1. Jackson LA, Anderson EJ, Roushael NG, et al. An mRNA vaccine against SARS-CoV-2 - preliminary report. N Engl J Med. 2020;383 (20):1920–1931.
2. Erasmus HJ, Khandhar AP, O’Connor MA, et al. An alphavirus-derived replicon RNA vaccine induces SARS-CoV-2 neutralizing antibody and T cell responses in mice and nonhuman primates. Sci Transl Med. 2020;12(555).
3. Polack FP, Thomas SJ, Kitchin N, et al. Safety and efficacy of the BNT162b2 mRNA Covid-19 vaccine. N Engl J Med. 2020;383 (27):2551–2615.
4. Krammer F. The human antibody response to influenza A virus infection and vaccination. Nat Rev Immunol. 2019;19(6):353–357.
5. Hou YJ, Okuda K, Edwards CE, et al. SARS-CoV-2 reverse genetics reveals a variable infection gradient in the respiratory tract. Cell. 2020;182(2):429–446.e14.
6. Sterling D, Mathian A, Miyara M, et al. IgA dominates the early neutralizing antibody response to SARS-CoV-2. Sci Transl Med. 2021;13 (577).
7 Wang Z, Lorenzo JG, Muecksch F, et al. Enhanced SARS-CoV-2 neutralization by dynamic IgA. Sci Transl Med. 2021;13(657):eabd223.
8 Pizzolla A, Nguyen TH, Smith JM, et al. Resident memory CD8 (+) T cells in the upper respiratory tract prevent pulmonary influenza virus infection. JCI Insight. 2016;1(10):e85832.
9 Zhao J, Zhao J, Mangalam AK, et al. Airway memory CD4(+) T cells mediate protective immunity against emerging respiratory coronaviruses. Immunity. 2020;54(4):637–651.
10 Zens KD, Chen JK, Farber DL. Vaccine-generated lung tissue-resident memory CD8(+) T cells provide heterosubtypic protection to influenza infection. JCI Insight. 2016;1(10):e85832.
11 Christensen D, Mortensen R, Rosenkranz I, Dietrich J, Andersen P. Vaccine-induced Th17 cells are established as resident memory cells in the lung and promote local IgA responses. Mucosal Immunol. 2017;10(1):260–270.
12 Jaffar Z, Ferrini M, Herritt LA, et al. Cutting edge: lung mucosal Th17-mediated responses after a single immunization in mice. J Immunol. 2013;190(4):313–315.
13 Hirota K, Turner JR, Veiga M, et al. Plasticity of Th17 cells in Peyers patches is responsible for the induction of T cell dependent IgA responses. Nat Immunol. 2011;12(4):372–379.
14 Abrahim S, Joel HB, Bang P, et al. Safety and immunogenicity of the chlamydia vaccine candidate CTH522 adjuvanted with CAPOI liposomes or aluminum hydroxide: a first-in-human, randomized, double-blind, placebo-controlled, phase 1 trial. Lancet Infect Dis. 2021;21(9):1095–1106.
15 Ozberk A, Reynolds S, Huo Y, et al. Prime-pull immunization with a bivalent M-protein and spray-CEP peptide vaccine adjuvanted with CAPOI liposomes induces both mucosal and peripheral protection from covid/S Mutant Streptococcus pyogenes. mBio. 2021;12(1):e00379–20.
16 Sui Y, Li J, Zhang R, et al. Protection against SARS-CoV-2 infection by a mucosal vaccine in rhesus macaques. JCI Insight. 2021;6(10):e144564.
17 Wrapp D, Wang J, Corbett KS, et al. Cryo-EM structure of the SARS-CoV-2 spike in the prefusion conformation. Science. 2020;367(6483):1260–1263.
18 Hanke L, Vidakovics Perez L, Sheward DJ, et al. An alpaca nanobody neutralizes SARS-CoV-2 by blocking receptor interaction. Nat Commun. 2020;11(1):4420.
19 Davidsen J, Rosenkranz I, Christensen D, et al. Characterization of cationic liposomes based on dimethyloldodecylammonium and synthetic cord factor from M. tuberculosis (trehalose 6,6-dibehenate)-a novel adjuvant inducing both strong CM1 and antibody responses. Biochim Biophys Acta. 2005;1718(1-2):22–31.
20 Wierzner K, Sheward DJ, Schmidt ST, et al. Adjuvanted SARS-CoV-2 spike protein elicits neutralizing antibodies and CD4 T cell responses after a single immunization in mice. EBiomedicine. 2021;63:103577.
21 Ramirez S, Fernandez-Antunez C, Galli A, et al. Overcoming culture restriction for SARS-CoV-2 in human cells facilitates the screening of compounds inhibiting viral replication. Antimicrob Agents Chemother. 2021;65(7):e00097-21.
22 Underwood AP, Solund C, Fernandez-Antunez C, et al. Neutralization titres against SARS-CoV-2 are sustained 6 months after onset of symptoms in individuals with mild COVID-19. EBiomedicine. 2021;71:103519.