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Distinct roles of vaccine-induced SARS-CoV-2-specific neutralizing antibodies and T cells in protection and disease

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-specific neutralizing antibodies (NAbs) lack cross-reactivity between SARS-CoV species and variants and fail to mediate long-term protection against infection. The maintained protection against severe disease and death by vaccination suggests a role for cross-reactive T cells. We generated vaccines containing sequences from the spike or receptor binding domain, the membrane and/or nucleoprotein that induced only T cells, or T cells and NAbs, to understand their individual roles. In three models with homologous or heterologous challenge, high levels of vaccine-induced SARS-CoV-2 NAbs protected against neither infection nor mild histological disease but conferred rapid viral control limiting the histological damage. With no or low levels of NAbs, vaccine-primed T cells, in mice mainly $CD8^+$ T cells, partially controlled viral replication and promoted NAb recall responses. T cells failed to protect against histological damage, presumably because of viral spread and subsequent T cell-mediated killing. Neither vaccine- nor infection-induced NAbs seem to provide long-lasting protective immunity against SARS-CoV-2. Thus, a more realistic approach for universal SARS-CoV-2 vaccines should be to aim for broadly cross-reactive NAbs in combination with long-lasting highly cross-reactive T cells. Long-lived cross-reactive T cells are likely key to prevent severe disease and fatalities during current and future pandemics.

INTRODUCTION

The evolution of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic has constantly surprised with the appearance of new variants that improve replication or evade the host response or both.[1–3] Numerous studies show that previous infection and/or vaccination induces neutralizing antibodies (NAbs) that can be effective in preventing infection for a short period, effective in preventing symptomatic infection for a slightly longer period, and highly effective in preventing hospitalization or death for an extended time.[4,5] However, the ability of SARS-CoV-2 to undergo mutations and recombination poses a continuous challenge. Mutant strains, especially variants of concern (VOCs), render the existing vaccines less effective against breakthrough infections. The Beta (B1.351), Delta (B.1.617.2), Omicron (1.1.529), and XBB variants generally cause mild to moderate infections in those vaccinated.[5,6] A so-called hybrid immunity conferred by the combination of multiple vaccinations and one or more infections, seems to offer the best protection against severe disease.[4,7] However, this highlights the importance of NAbs in protection against infection and a role for T cells in immune maturation and elimination of virally infected cells and thereby protecting against severe disease and death.

Mutations that escape NAbs generally appear in surface-exposed protein domains, and the same epitopic region is recognized among most humans. In contrast, the outbred nature of the major histocompatibility complex limits the risk for complete T cell escape in SARS-CoV-2.[8,9] This is more likely to happen through viral recombination and replacement of larger gene segments. However, extensive mutations in the spike (S) gene may eventually reduce the effectiveness of the T cell response as was recently suggested.[8] In contrast, it has been shown that T cells targeting more conserved genes such as the

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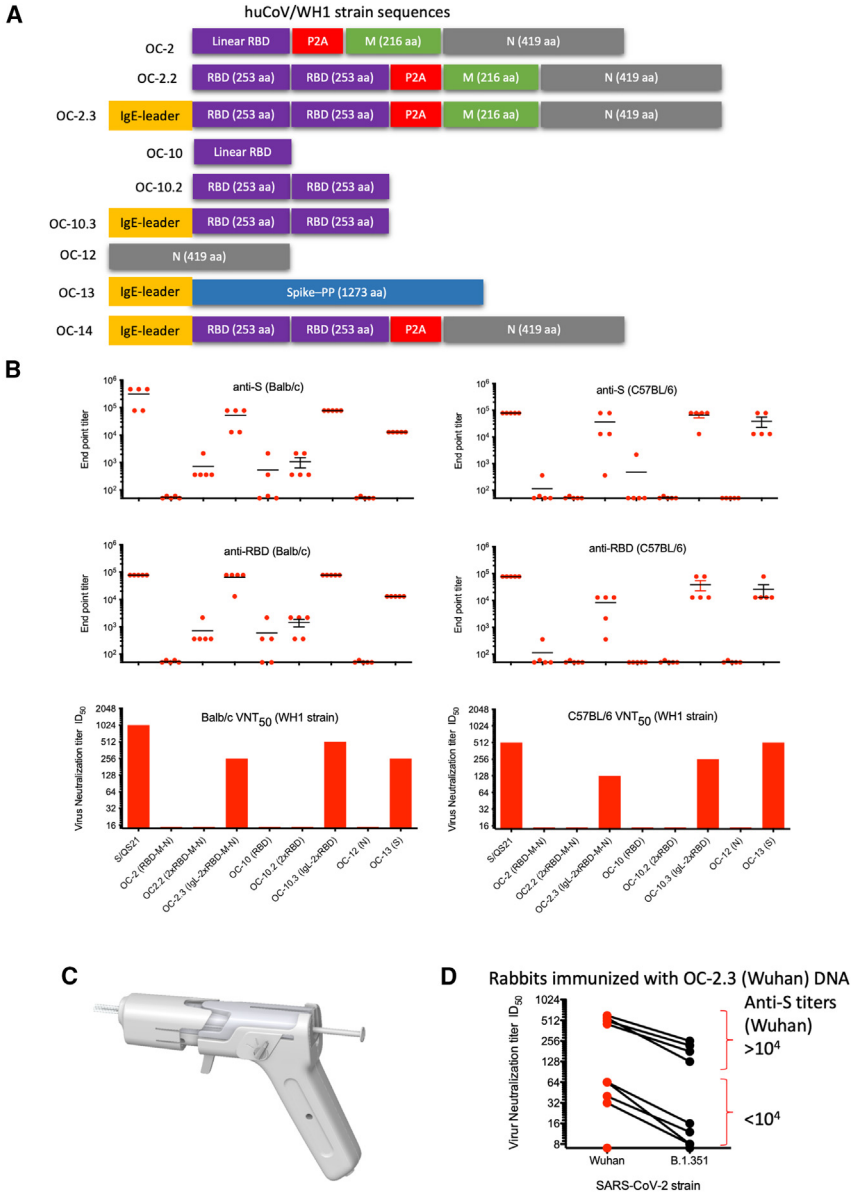


Figure 1. Vaccination strategy.

Schematic representation of the vaccines based on SARS-CoV-2 derived sequences (A). Groups of Balb/c and C57BL/6 mice were immunized as described twice, three weeks apart, and endpoint titers were determined two weeks after the second dose using recombinant receptor binding domain (RBD) or the spike (S) by EIA (upper and middle panels in B). Each red filled circle represent an individual mouse. The black horizontal lines indicates mean ± SD of the group . The level of NAbs to both the WH1 and B.1.351/Beta variants were determined as the antibody titer producing 50% neutralization of virus in a virus neutralization assay (VNT ID_{50}; bottom two panels in B). Also shown is the EPSGun (C) and VNT ID_{50} to WH1 and 1.351/Beta variants in rabbit sera after immunization of rabbits twice with the OC-2.3 plasmid (D).

We therefore designed and evaluated vaccines that induced NAbs, T cells, or both, in different challenge models. We found that T cells indeed can reduce viral replication and that this may explain the partially protective effect against severe disease. However, we also found that T cells may contribute to tissue damage, mainly in the upper airways, in the absence of high levels of NAbs. This supports the role of both B and T cells in the complete protection against severe disease caused by SARS coronaviruses.

RESULTS

Generation of SARS-CoV-2 Vaccines

Many human CoVs originate from bats,[14] and the structural proteins M and N have a higher sequence similarity between SARS-CoV-like viruses than the S protein. The design for a potentially universal DNA vaccine combining sequences of S, M, and N has been shown previously.[11] Building on these achievements, we generated DNA vaccines with unique properties with respect to selectively activating NAbs and/or T cells (Figure 1A).

membrane (M) or nucleoprotein (N) may be maintained over a 17-year period and are cross-reactive with a new SARS coronavirus (CoV).[10] In addition, T cell responses to M and N may even be cross-reactive to animal SARS-like CoVs.[11,12] We are developing a new generation of genetic vaccines that focus on inducing both broadly cross-reactive NAbs and T cells.[11] However, additional studies are needed to fully understand the role of highly cross-reactive T cells. What abilities do the SARS-CoV-2-specific T cells need to have to protect against severe disease? We and others recently showed that N-specific T cells alone may have a partially protective effect against severe disease.[11,13] How these cross-reactive T cells contribute to protection against severe disease, however, is still not fully understood.

Priming of antibodies and T cells to SARS-CoV-2

We first evaluated the vaccines with respect to the activation of antibody responses and NAbs (Figure 1B). As expected, we found that the design of the receptor binding domain (RBD) sequence drastically affected the ability to induce NAbs to RBD and S, consistent with previous observations[15] (Figure 1), but not the ability to induce T cells to RBD, M, and N (Figure 2). As an example, vaccines containing a single linear RBD loop without an Ig leader sequence (OC-2 and OC-10) failed to induce detectable antibodies to recombinant RBD or S (Figure 1B), despite a strong T cell priming (Figure 2A). Constructs containing a double RBD loop (OC-2.2) were also poor in priming antibodies to S and RBD (Figure 1B). As expected, the addition of an immunoglobulin (Ig) leader sequence followed by double or

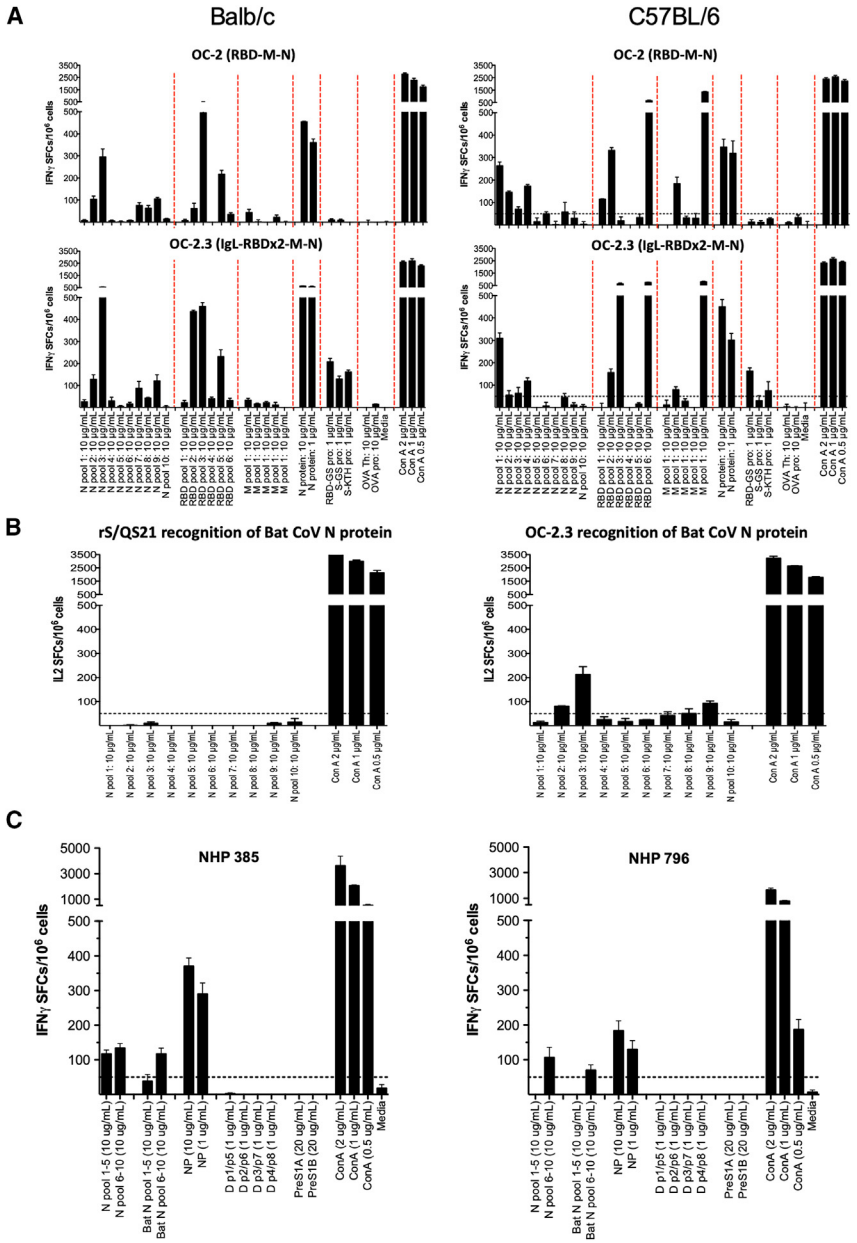


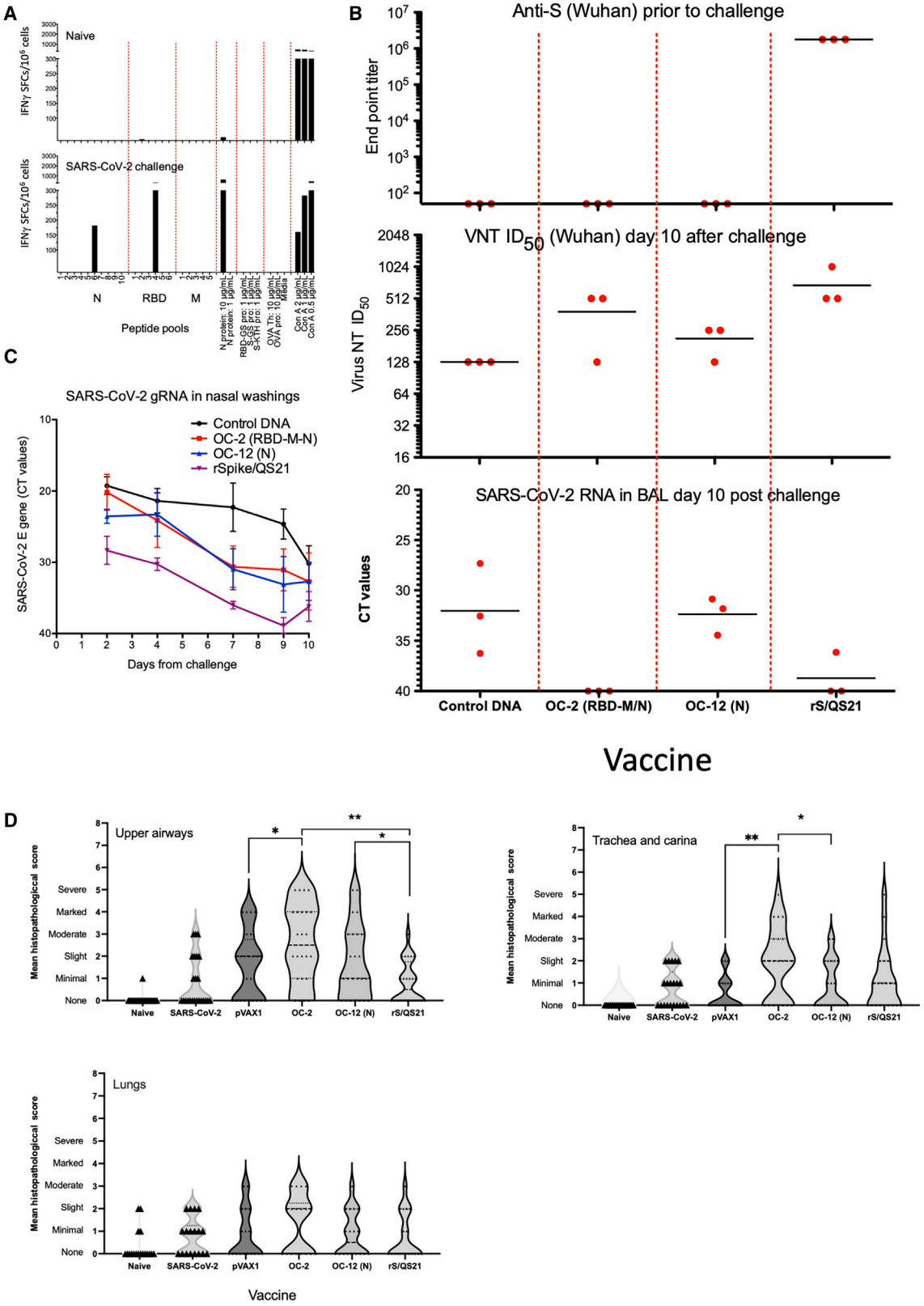
Figure 2. Cross-reactivity of T cell responses.

Priming of SARS-CoV-2-specific T cells in Balb/c and C57BL/6 mice two weeks after the second dose as detected reactivity to peptides and proteins corresponding to the RBD, S, M, and N antigens by an IFN-γ ELISpot (A). Spleens were harvested and pools of mouse splenocytes were analyzed for the presence of IL-2- and IFN-γ-producing T cells using the indicated antigens. Data are expressed as the number of IL-2- or IFN-γ-producing (spot-forming) cells (SFCs) per million splenocytes (+SD). Cross-reactivity of T cells from Balb/c mice primed either by recombinant S in adjuvant or the OC-2.3 DNA vaccine as determined by IL-2 production in ELISpot is shown in (B). Also, the cross-reactivity of T cells from NHPs after two vaccinations with OC-2.3 DNA vaccine as determined by IFN-γ ELISpot is shown in (C).

triple[11] RBD loops was highly effective in inducing antibodies to recombinant RBD or S, either alone or in combination with other SARS-CoV-2 proteins (OC-2.3 and OC-10.3; Figure 1B), as well as T cells to all components of the respective vaccine (Figures 2A–2C). We noted that the antibody responses to RBD and S, but not T cell responses, were weaker in C57BL/6 compared with Balb/c mice (Figures 1B and 2A). The N containing constructs induced antibodies and T cells to N (Figures 1A and 2B). The M containing constructs induced T cells to M (Figure 2A). No antibodies against M could be reproducibly detected regardless of the antigen used, possibly reflecting that the sequences were not exposed to B cells because of the construct design or that the antigens used in the detection simply failed to detect these antibodies.

A central question regarding DNA vaccines is the ability to retain immunogenicity in larger animals. A newly developed device intended for *in vivo* electroporation (EP) in humans (EPSGun, IGEA Spa; Figure 1C) was therefore used to immunize rabbits with a SARS-CoV DNA vaccine (OC-2.3). The device allows a single-step delivery of DNA together with *in vivo* EP. Rabbits receiving two DNA doses of 250, 500, or 750 μg developed anti-S levels between 10^3 and 10^5 (WH1 strain). The antisera were evaluated for the ability to neutralize WH1 and Beta/B.1.351 SARS-CoV-2 strains *in vitro* (Figure 1D). As expected, a priming of high levels of anti-S to WH1 virus was required for cross-neutralization of the Beta/1.351 variant.

We compared the ability to induce T cells between a DNA vaccine that induced anti-S and NAbs (OC-2.3) to a one that failed to induce such antibodies (OC-2; Figure 2A). This showed that T cells were equally well primed by these two vaccines despite the difference in the ability to prime anti-S and NAbs. The T cell recognition of the different vaccine components differed slightly between Balb/c and C57BL/6 mice, with Balb/c mice recognizing mainly RBD and N, whereas C57BL/6 also showed a strong response to M (Figure 2A). Thus, multiple SARS-CoV-2 proteins in a vaccine can prime broadly reactive T cell responses in genetically diverse hosts, consistent with previous observations.[11] We found that the SARS-CoV-2-specific T cells primed by the multicomponent vaccine cross-reacted with peptides corresponding to N protein sequence from bat CoV (Figure 2B). This is consistent with the observation that murine N-specific T cells recognize regions that have 100% similarity with Pangolin-derived SARS-like CoV



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sequences[12] and supports the concept of multicomponent SARS-CoV vaccines. In addition, two of three macaques immunized twice with the OC-2.3 vaccine (described in detail later in the text), developed SARS-CoV-2 N-specific T cells cross-reactive with bat CoV N sequences (Figure 2C). This further supports the notion that addition of M and N sequences ensures cross-reactivity to animal SARS-like CoVs regardless of the species.

To better understand characteristics of the vaccine-primed SARS-CoV-2-specific T cells Balb/c mice immunized with the OC-2.3 DNA twice and analyzed anti-S and anti-N antibody levels, T cell responses using IFN-γ ELISpot and by fluorescence-activated cell sorting (FACS) analysis of antigen stimulated of splenocytes (Figure S1). We found that the OC-2.3 induced high levels of anti-S (WH1 strain) and high levels of anti-N (Figure S1A). We also found that the vaccination induced IFN-γ-producing T cells specific for RBD, S, M, and N (Figure S1B). Importantly, we showed an induction of both $CD4^+$ and $CD8^+$ T cells specific for RBD and N by intracellular staining (ICS) analysis of peptide and antigen stimulated cells (Figure S1C). The were no obvious differences in the cytokines primed by the RBD or N components of the vaccine. The ICS analysis also revealed that the major source of the IFN-γ stems from RBD- and N-specific $CD8^+$ T cells. This suggests that the major T cells detected by the IFN-γ ELISpot in mice are $CD8^+$ T cells. Overall, the OC-2.3 DNA vaccine induces SARS-CoV-2-specific $CD4^+$ and $CD8^+$ T cells, and the major producers of IFN-γ are the $CD8^+$ T cells.

Role of antibodies and T cells in the protection against SARS-CoV-2 infection in ferrets

The role of T cells in the protection or control of SARS-CoV-2 infection is poorly understood. We used the ferret model to take advantage of our DNA vaccines that induced SARS-CoV-2-specific T cells, but not NAbs or memory B cells to S, to better separate the role of these responses in the protection against SARS-CoV-2. In a pilot experiment, in which three ferrets were left naive and three were infected with 10^6 plaque-forming units (pfu) of SARS-CoV-2 WH1 strain, the infected animals developed SARS-CoV-2-specific antibodies and T cells at 10 days post-infection (PI) (Figure 3A). Next, groups of ferrets were immunized with a control DNA vaccine,[16,17] the linear OC-2 construct (RBD-M-N; Figure 1), the OC-12 (N; Figure 1A) construct, or a recombinant spike protein in adjuvant QS21 (rS/QS21; WH1 strain), at weeks 0 and 3 (Figures 3 and 4). Two weeks after the last vaccination, all ferrets were challenged with 10^6 pfu SARS-CoV-2 WH1 strain. Consistent with the murine data (Figure 1B), the groups vaccinated with OC-2 (RBD/M/N) or OC-12 (N) DNA vaccines did not develop antibodies to S but to N prior to challenge (Figures 3B and 4). In contrast, vaccination with rS/QS21 induced high levels of S antibodies prior to challenge (Figures 3B and 4). All ferrets except two (one in the OC-2 group and one in the OC-12 group) displayed no or mild clinical symptoms such as slight drowsiness at days 2–4. The remaining two showed symptoms at days 2–4, with increased body temperature, thick mucus in the nose, loss of appetite, and drowsiness, and both recovered by days 4–5. Overall, the two groups vaccinated with the T cells vaccines had more symptoms than the control and S/QS21 vaccinated groups. The control vaccine group had high levels of SARS-CoV-2 RNA in nasal washings at days 2–9, and all had RNA in bronchoalveolar lavage (BAL) at day 10 (Figures 3C and 4). In contrast, the group vaccinated with rS/QS21 had rapidly dropping levels of SARS-CoV-2 RNA in nasal washings (Figures 3C and 4), and all but one had cleared virus in BAL by day 10 (Figure 3B). Interestingly, the two groups with only vaccine-induced SARS-CoV-2-specific T cells prior to challenge had mean nasal SARS-CoV-2 RNA levels in between the control DNA group and the rS/QS21 vaccine group (Figure 3C). The two animals vaccinated with OC-2 or OC-12 that developed most pronounced clinical symptoms (Figure 4, panels 4 and 7) had nasal SARS-CoV-2 RNA levels comparable with those in the control DNA group (Figure 4, panels 1–3). Finally, only the OC-2 (RBD-M-N) DNA vaccinated group was completely negative for SARS-CoV-2 RNA in BAL at day 10 (Figures 3B and 4, panels 4–6), supporting a role for T cells in the control of viral replication.

The control DNA group had a slow increase S and N antibodies including NAbs, reflecting the kinetics of the natural infection (Figures 3B and 4, panels 1–3). In contrast, the animals with vaccine-induced SARS-CoV-2-specific T cells (Figure 3A) had N antibodies prior to challenge, and developed S antibodies and NAbs after challenge (Figures 3B and 4, panels 4–9). Both NAbs and N antibodies showed anamnestic responses suggestive of a helper function of the primed T cells (Figures 3B and 4). In addition, four of six animals (Figure 4, panels 5, 6, 8, and 9), had a rapid drop in nasal SARS-CoV-2 RNA levels, like that of the rS/QS21 group, despite the absence of S antibodies and NAbs at challenge. This lends further support to the suggestion that that T cells help control viral replication. As expected, the group vaccinated with rS/QS21 had high levels of S antibodies and NAbs prior to challenge and consistently displayed a rapid control of viral replication (Figure 4, panels 10–12). Importantly, despite high levels of S antibodies and NAbs at challenge in the rS/QS21 group, all animals became infected. However, the absence of N antibodies after challenge suggest that the infection was rapidly

Figure 3. The role of antibodies and T cells in the protection against SARS-CoV-2 challenge in ferrets

In a pilot experiment, groups of three ferrets were left uninfected or infected by 10^6 pfu SARS-CoV-2 WH1 strain, and T cell responses were determined as reactivity to peptides and proteins corresponding to the RBD, S, M, and N antigens by an IFN-γ ELISpot (A). In the next experiment, four groups of three ferrets each were vaccinated with a control DNA plasmid, the OC-2 or OC-12 plasmids that do not induce NAbs, and rS/QS21. Two weeks after the last dose, all animals were challenged with by 10^6 pfu SARS-CoV-2 WH1 strain and followed for 10 days. Antibody levels to S were determined prior to challenge, and NAbs and SARS-CoV-2 levels in BAL were determined 10 days after challenge. The black horizontal line indicates the mean of the group (B). SARS-CoV-2 RNA levels were determined in nasal washings at days 2, 4, 7, 9, and 10 after challenge and are expressed as group mean cycle times (CT) (C). Also shown is the histological summary of the analysis of upper respiratory tract (top left), trachea, and carina (top right), and lung (bottom) tissues with a statistical comparison using the Mann-Whitney U test. *$p < 0.05$ and **$p < 0.01$ (D).

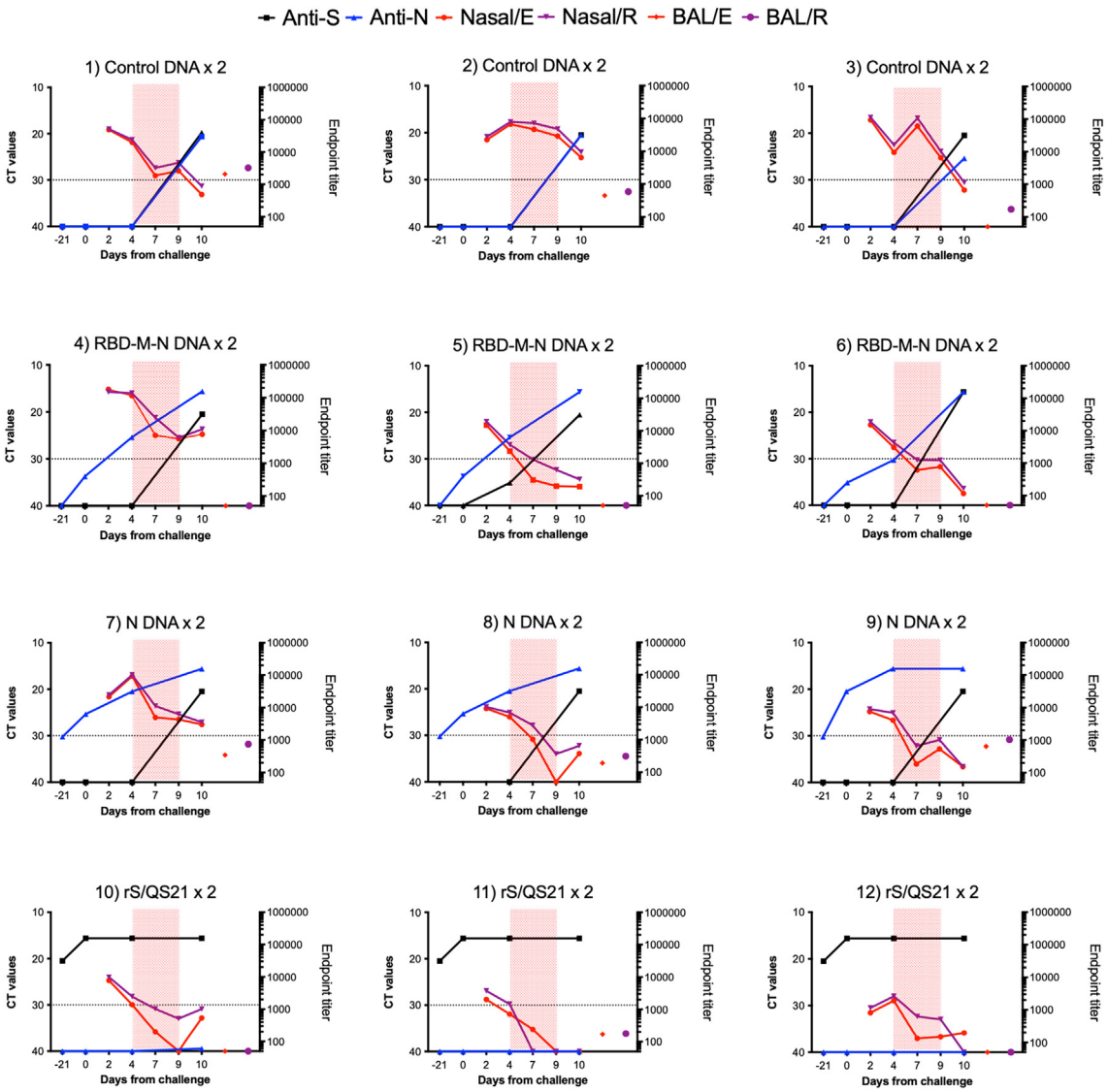


Figure 4. Kinetics of anti-S, anti-N, nasal SARS-CoV-2 RNA, and SARS-CoV-2 RNA in BAL from individual ferrets immunized twice with the indicated immunogens

Data are expressed as cycle threshold (CT; left y axis) in qPCR and endpoint titers (anti-S and anti-N; right y axis).

cleared (Figure 4). This observation may have implications for detecting a past SARS-COV-2 in human vaccinees.

All animals had developed histological changes in airway tissues at necropsy after challenge, regardless of the immunogen (Figure 3D). The histological damage was significantly more pronounced in the upper respiratory tract of the two groups immunized with the T cell vaccines, compared with both the control vaccine group and the group vaccinated with rS/QS21 (Figure 3D, upper left). This is consistent with the more pronounced clinical symptoms seen in these groups. In two respiratory regions severe changes were recorded. In the nasal turbinates the intraluminal cellular debris was severe in one animal in the OC-2 and one animal in the OC-12 vaccinated group. The diffuse inflammatory cell infiltration of the carina was severe in two animals on the OC-2 vaccinated group, and one animal in the S/QS21 vaccinated group (Figure 3D, upper right; Table S2). However, this was not the case in the lungs (Figure 3D, lower panel). The most prominent changes were multifocal intraluminal cellular debris in the trachea and carina in all animals vaccinated with OC-2 (RBD-M-N) or OC-12 (N) DNA and in one in the group vaccinated with rS/QS21 (see Figure 3D and Tables S1 and S2). In nasal turbinates varying degrees of inflammation were noted in all groups. In the trachea and carina there seemed to be more inflammation in the OC-2 (RBD-M-N) or OC-12 (N)

DNA vaccinated groups, than in the other groups (see Figure 3D and Tables S1 and S2).

In conclusion, vaccine-primed T cells can partially control SARS-CoV-2 replication, as evidenced by the reduced SARS-CoV-2 RNA levels in the nasal cavity and lungs in animals with vaccine-primed T cells prior to challenge. However, the histological examinations suggest that this control was, at least in part, exerted by a T cell-mediated elimination of SARS-CoV-2-infected cells resulting in a more pronounced histological damage in the upper airways. The histological findings showed that none of the immunogens completely protected against infection or histological signs of disease despite the presence of high levels of S antibodies and NAbs (Figures 3B and 3D). This may be explained partly by the 2- to 10-fold higher dose used in this study for challenge than often used in infection experiments.[18] This study supports a role for SARS-CoV-2-specific T cells in controlling SARS-CoV-2 replication, either directly by eliminating infected cells, or indirectly by promoting anamnestic antibody responses, or a combination of both. The increased clinical symptoms and pathology in the upper respiratory tissues seen in the absence of NAbs prior to a high-dose challenge, suggest that the elimination of infected cells by T cells, presumably $CD8^+$, may contribute to both viral control and the pathology. Thus, this supports a central role of NAbs in limiting the spread of the SARS-CoV-2 and suggests a coordinated action of B cells and T cells in control and clearance of SARS-CoV-2.

Role of antibodies and T cells in the protection against severe SARS-CoV-2 infection in mice transgenic for human ACE2

To further evaluate the role of NAbs and T cells in a severe disease model, we immunized K18-hACE2 mice twice with six different immunogens followed by a homologous challenge with 10^5 pfu SARS-CoV-2 WH1 strain. As expected, the group vaccinated with rS/QS21, which induce NT titers of 1:512 in wild-type (WT) C57BL/6 mice (Figure 1B), was fully protected against lethal disease (Figure 5). In contrast, the groups lacking anti-S and in which only T cells were primed, like the ferrets, were only partially protected against lethal disease (20–25%; Figure 5). However, the weight loss was significantly delayed in the T cell vaccine groups compared with controls (>10% weight loss at day 4; 5 of 5 in controls vs. 2 of 9 in the OC-2 and OC-12 groups combined; $p < 0.05$, Fisher's exact test). In the two groups immunized with OC-2.3 and OC-10.3, which induced lower levels of NAbs in WT C57BL/6 mice (Figure 1B), the protection was 60% and 80%, respectively. Of note, the OC-2.3 group had about 40-fold lower anti-S antibody levels in this experiment than expected, for which we do not have an explanation. By comparison with Figure 1B, in which C57BL/6 mice immunized with OC-2.3 had an anti-S mean titer of 1:36,360 and NT levels of 128, the K18 mice immunized with OC-2.3 had mean anti-S levels of 1:810 and presumably much lower NT titers (Figure 5 and not tested). Also, when looking at individual mice immunized with OC-2.3 or OC-10.3, the lowest anti-S levels were found in the mice with most severe weight loss (Figure 5). Overall, this experiment, albeit with its obvious limitations, suggests that the level of NAbs is proportional to protection against lethal disease. To conclude, in this model, the priming of T cells only provides delayed disease and 20%–25% survival, priming of T cells and low-level anti-S from 10^2 to 10^3 increase survival to 60%, and with anti-S levels of $>10^3$–10^4, survival reaches 80%–100%. Thus, this clearly confirms a key role of antibodies for full protection against severe disease in this model but also provides additional support for a partially protective role of T cells against severe disease.

To further explore the role of antibodies and T cells in protection against homologous and heterologous viral challenge, additional experiments were performed in WT and K18-hACE2 mice using vaccines containing mutations in the RBD sequences from different viral variants. In WT Balb/c mice three instead of two vaccinations increased anti-S levels and neutralization of homologous (WH1) and heterologous (B1.351/Beta VOC) virus (Figure 6A vs. Figure 1B). In addition, the OC-2.3 DNA effectively boosted anti-S and NAb responses to both WH1 and Beta VOC after priming once with rS/QS21 (Figure 6A), like our previous report.[11] The OC-2.3 variant containing the four mutations related to the 1.351/Beta variant OC-2.3 (501Y-484K-439K-417N) effectively neutralized the 1.351/Beta variant but had a reduced neutralization of the WH1 variant, suggesting poor cross-neutralization (Figure 6A).

All four tested vaccines provided 100% protection in the K18-hACE2 model against heterologous challenge with 1.351/Beta variant following three immunizations (Figure 6). Thus, improving the immunogenicity by adding one more vaccination increased anti-S levels and improved protection against severe disease (Figure 6). All mice in the DNA control group were sacrificed because of severe weight loss on day 6, as reported previously.[11] With respect to viral load in the nasal cavity, lungs, and the spleen, all four vaccines reduced nasal excretion of virus, OC-2.3 and OC-10.3 had reduced replication in lungs, whereas OC-10.3 and OC-14 showed reduced replication in the spleen (Figure 6D; control group data have been reported previously[11]). In addition, we noted lower histological damage in the vaccinated groups compared with the control (control group data were reported previously[11]) (Figure 6E). The increased immunity provided by an additional DNA vaccine dose improved responses that protected against severe disease after heterologous challenge.

To summarize the murine experiments, the priming of T cells alone offers a partial protection against severe disease in the K18-hACE2 model. By increasing anti-S and NAb levels, however, the protection against severe disease is improved. This is consistent with the observation from the high-dose challenge in ferrets.

Role of antibodies and T cells in the protection against SARS-CoV-2 in non-human primates

We evaluated the two-dose regimen of one SARS-CoV DNA (OC-2.3) in a pilot experiment in cynomolgus macaques to further understand the role of anti-S and T cell responses. Groups of three non-human primates (NHPs) were immunized with a control DNA encoding a hepatitis B virus (HBV) vaccine[17] or the universal SARS-CoV DNA vaccine OC-2.3 at days −35 and −14 and then challenged on day 0 with a total dose of 5×10^6 pfu SARS-CoV-2 (WH1 strain) in the

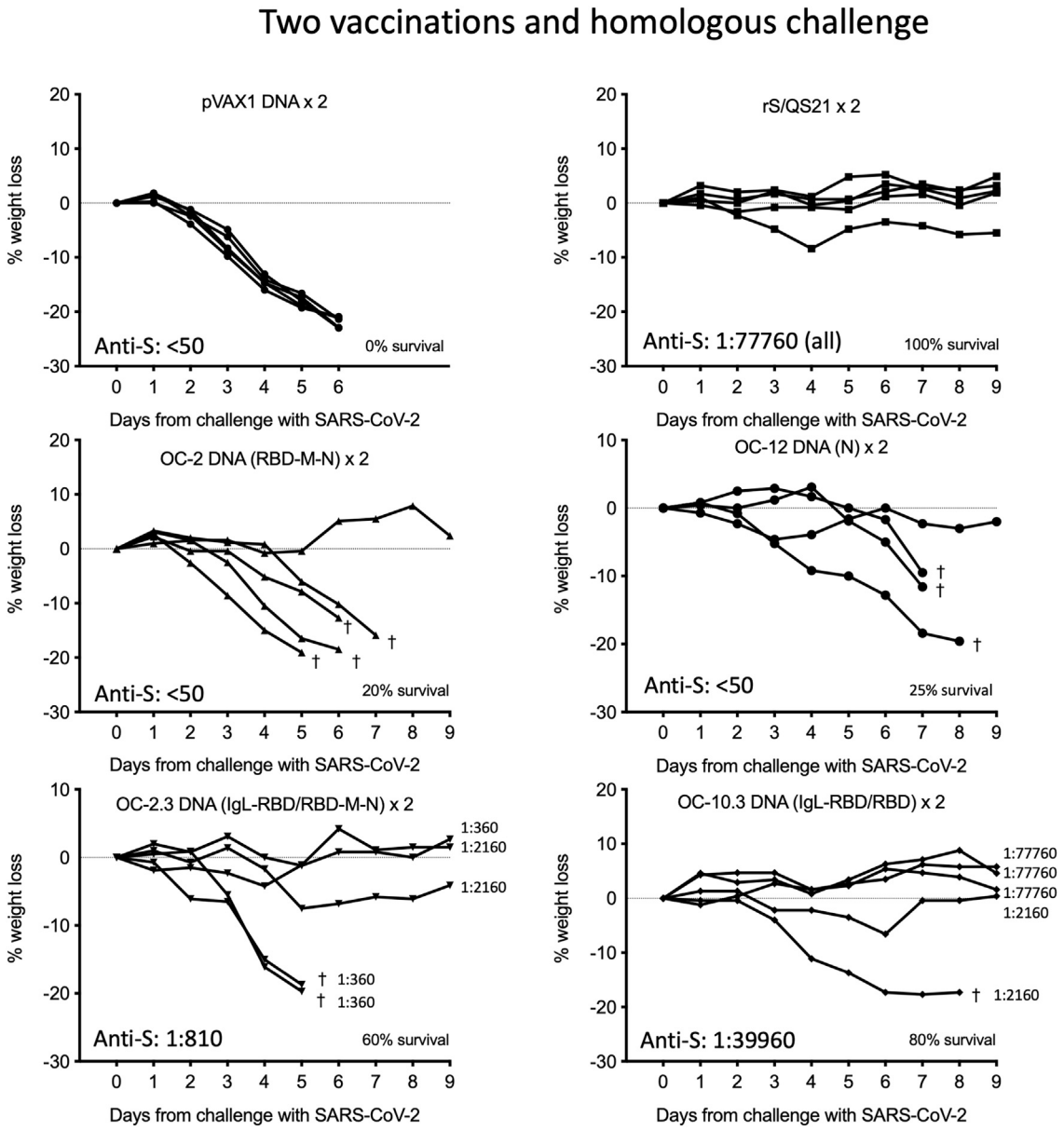
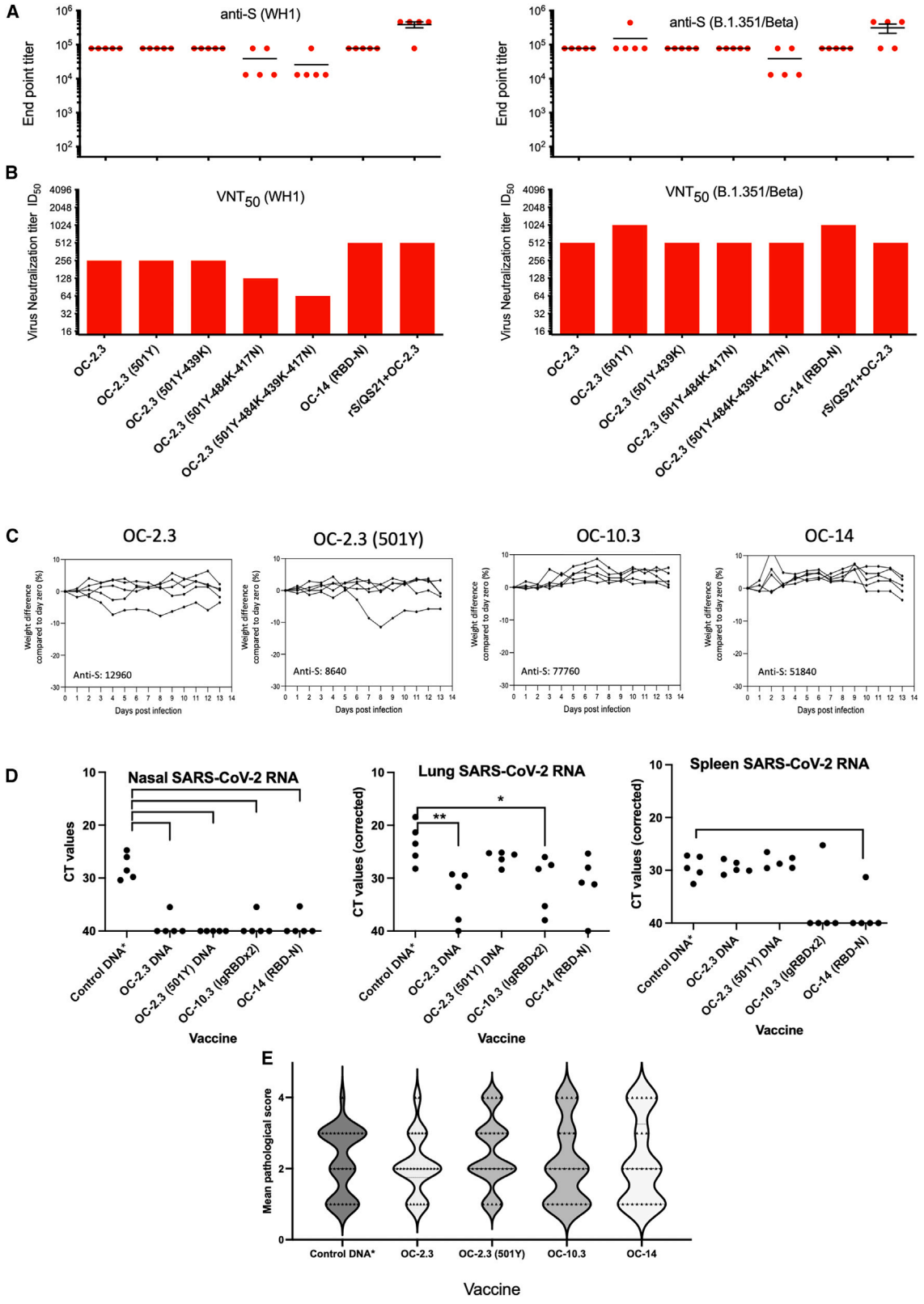


Figure 5. Percentage change in weight of individual mice after challenge with 10^5 pfu SARS-CoV-2 WH1 strain of K18-hACE2 mice vaccinated twice with the indicated immunogen

Also given is the mean anti-S titer in each group at the bottom left of each graph, as well as individual anti-S titers.

right nostril and intratracheally, to ensure a robust challenge. This resulted in SARS-CoV-2 infection in all six animals (Figure 7). One of the control vaccine animals (#969) died under anesthesia during BAL sampling at day 4, possibly as a reaction to anesthesia, or a combination of a reaction to the anesthesia and the infection (Figure 7). All control animals had RNA in BAL at day 4, and one (#636) of two had virus in BAL at day 20 (Figures 7A and 7D). The histological lesions in the six lung lobes from day 20 of five animals were scored by an independent pathologist as minimal, mild, pronounced, severe, and very severe (range 1–5 for statistical comparison). The animal that died at day 4 had a severe to very severe histological lesions in the lungs at autopsy when one lobe from each lung was analyzed. The remaining two control animals lacked antibodies, NAbs, and T cells prior to challenge and showed a range from minimal to severe histological lesions at day 20 (Figures 7A–7E). Both animals developed weak but detectable T cell responses to M, N, and S proteins after challenge (Figure 7C). The antibody responses to S were not detectable until day 10 after challenge (Figure 7B).



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The group immunized with the SARS-CoV-2 DNA OC-2.3 all had anti-S and anti-RBD prior to challenge (Figures 7A and 7B). The two animals with anti-S/RBD levels of $>10^4$ both had low levels of NAbs to SARS-CoV-2 WH1 strain but not to 1.351/Beta variant prior to challenge (Figure 7A). All vaccinated animals showed strong anamnestic responses and had by day 10 anti-S titers of $>10^5$ to WH1 strain S protein and $>10^4$ to the 1.351/Beta variant S protein (Figure 7B). All vaccinated animals had varying levels of detectable SARS-CoV-2-specific T cell responses prior to challenge, and the strength of these seemed to correlate with the anti-S levels (Figures 7B and 7C). There was no difference in SARS-CoV-2 RNA levels in nasal washings between the control and vaccine groups (Figure 7D). In contrast, two of three vaccinated animals cleared SARS-CoV-2 RNA in BAL at day 4, and two were RNA negative at day 20 (Figures 7A and 7D). Interestingly, the mean body temperatures were somewhat higher by 0.13°C–0.87°C in the control group (range, 38.8°C–40.0°C) every day after challenge, compared with the SARS-CoV-2 vaccinated group (range, 37.7°C–39.6°C) (data not shown).

All SARS-CoV-2 DNA-vaccinated animals developed rapid strong multi-specific anamnestic T cell response after challenge (Figure 7C). There was no statistical difference in histological changes between the two control animals and the three animals vaccinated with the SARS-CoV-2 DNA (Figure 7E; $p = 0.26404$, Mann-Whitney U test), showing that the vaccination regimen did not prevent histological damage caused by the infection. It was noted that the animal with the lowest anti-S and T cell responses prior to challenge had a more pronounced histological changes compared with the two animals with the strongest vaccine-induced response.

In conclusion, this pilot study of a two dose SARS-CoV-2 DNA vaccine regimen showed variable levels of priming of both anti-S and T cells to SARS-CoV-2. The animal (#796; Figure 7) with anti-S levels $>10^4$ and the strongest T cell response ($>10^3$ spot-forming cells [SFCs]/10^6 peripheral blood mononuclear cell [PBMC]) prior to challenge rapidly and durably cleared viral replication in the lungs and had a rapid drop in nasal SARS-CoV-2 RNA levels. In contrast, the animal with the lowest anti-S response ($<10^4$) and the lowest T cell response ($<10^2$ SFCs/10^6 PBMC) prior to challenge (#576; Figure 7) had detectable virus in BAL at day 20, presence of nasal RNA at day 12, and the most obvious signs of histological disease. Overall, this very limited pilot study supports the notion that a coordinated potent activation of NAbs and T cells is required for a rapid clearance of SARS-CoV-2 RNA and control of infection in the airways and lends further support for a role of T cells in control of SARS-CoV-2 replication.

DISCUSSION

There are numerous vaccines against SARS-CoV-2 in clinical development, and more than 10 have already been approved for human use in several countries.[19–21] All these, except three killed whole-virus vaccines, are based solely on the S protein of SARS-CoV-2, with the primary aim of inducing NAbs. An early observation was the ability of SARS-CoV-2 variants carrying mutations in position 484 to reduce the effectivity of S-based vaccines in clinical trials.[22] This strongly suggest a high dependence of NAbs in protection against mild and moderate disease but possibly less so against severe disease and death. It was subsequently shown that the enrichment of mutations in the S severely impaired the protection against infection with the Omicron variants.[4,7,23] However, the protection against severe disease and death was better maintained by all vaccines and following a previous infection, suggesting a role for broadly reactive T cells in controlling SARS-CoV-2 replication.[4,7,23] In fact, recent studies suggest that WH1-primed T cells indeed are often cross-reactive with Omicron variants.[9] However, the role of T cells outside the S protein have been much less well characterized with respect to protection against severe disease. We recently showed that vaccination with a recombinant N protein in adjuvant had a 60% protective effect against lethal disease in the K18-hACE2 model.[11] Similarly, it has been shown that peptides that prime T cells alone can have a partially protective effect in the same model.[13]

To further our understanding of NAbs and T cells, we herein generated DNA vaccines encoding RBD, M protein, and/or N that effectively induced T cells but not NAbs, or both, to elucidate how T cells can help control COVID-19. These SARS-CoV vaccines effectively induced NAbs and/or primed broadly reactive T cells. When high levels of NAbs were reached, then these antibodies neutralized both WH1 and 1.351/Beta variants *in vitro*. However, when using vaccines that induced only T cells, we showed that pre-existing T cells resulted in anamnestic antibody responses and a better control of SARS-CoV-2 replication as evidenced by both lower nasal SARS-CoV-2 levels and clearance in the lungs in the ferret model. Thus, broadly cross-reactive vaccine-induced SARS-CoV-specific T cells promote control and clearance of SARS-CoV-2, which may be relevant for both infection and vaccination. However, it is important to note that in the absence of NAbs, viral control may come at the cost of more pronounced clinical signs and histological damage possibly caused by the elimination of infected cells by the vaccine-primed $CD8^+$ T cells.

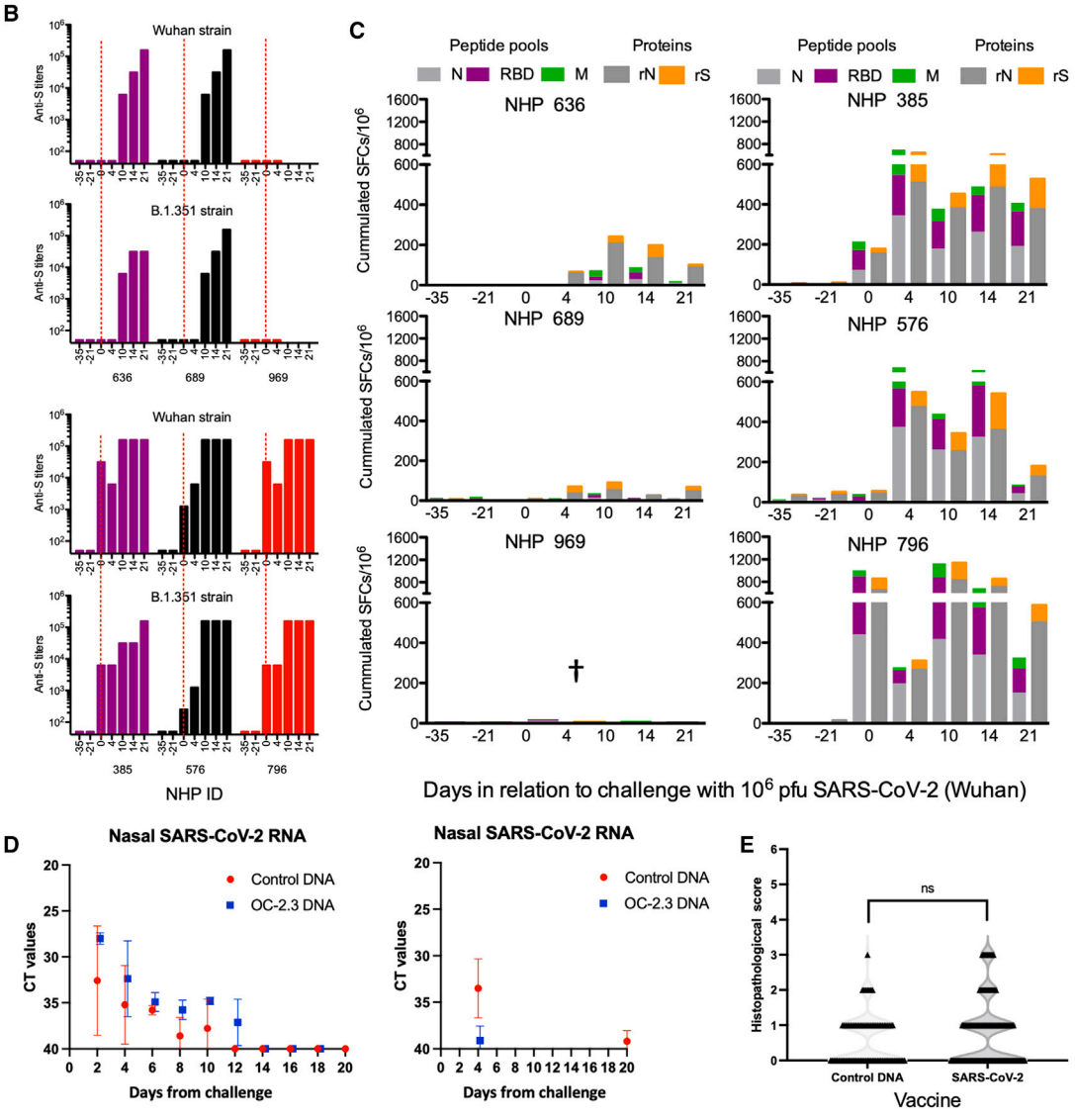
We showed in the mouse model that $CD4^+$ and $CD8^+$ T cells were induced to the RBD and the N components by the DNA vaccine. Also, the major cell type responsible for IL-2 was $CD4^+$ T cells and

Figure 6. T cells partially protect against severe disease.

Groups of Balb/c mice were immunized with the indicated immunogen three times three weeks apart, and endpoint titers were determined two weeks after the third dose using recombinant S corresponding to the WH1 (A, left) or 1.351/Beta variants by EIA (A, right). The black horizontal line indicates the mean +SD of the group. The level of NAbs to both the WH1 (B, left) and B.1.351/Beta (B, right) variants were determined as the antibody titer producing VNT ID_{50}. Also shown is protection against weight loss after challenge with the SARS-CoV-2 1.351/Beta variant in K18-hACE2 mice after three vaccinations with the indicated four vaccines (C); the levels of SARS-CoV-2 RNA in nasal lavage, lungs, and spleen (D); and the summary scores of histological damages in lungs at termination (E). The data from the control group have been reported previously[11] and are shown here again for comparative purposes.

A

Marker	Control DNA (HBV)			SARS-CoV-2 OC-2.3 DNA (IgL-2xRBD-M-N)		
	636	689	969	385	576	796
Anti- S titer (w5; Wuhan)	<50	<50	<50	31250	1250	31250
Anti- S titer (w5; B.1.351)	<50	<50	<50	6250	250	6250
Anti-RBD titer (w5; Wuhan)	<50	<50	<50	31250	6250	31250
Anti-HBV PreS1 titer (w5)	50	6250	31250	<50	<50	<50
VNT_{50} (Wuhan strain)	<8	<8	<8	32	<8	32
SARS-CoV-2 gRNA BAL d4 (CT value)	31,52	31,86	37,14	37,34	>40	>40
SARS-CoV-2 gRNA BAL d20 (CT value)	38,39	>40	†d4	>40	37,04	>40



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for IFN-γ was $CD8^+$ T cells. Thus, a strong activation of $CD8^+$ T cells would help explain both the partial viral control and tissue damage seen in the upper airways of DNA-vaccinated ferrets. This suggest that in the absence of NAbs, the virus is allowed to spread to more cells, while the infected cells are eliminated by the $CD8^+$ T cell response. Recent studies suggest that T cells to either human CoVs or to non-structural proteins of SARS-CoV-2 has a protective effect against disease.[24,25] This is fully consistent with our approach to generate broadly cross-reactive T cell responses to genetically stable viral proteins. Interestingly, neither antibodies nor T cells seem to be related to the symptoms seen in long COVID-19.[26]

Different vaccine technologies differ in how effectively they induce different types of T cells. It was recently shown that vaccination with mRNA induce antibodies and both $CD4^+$ and $CD8^+$ T cells to the S.[27,28] In contrast, an inactivated vaccine induces mainly antibodies and $CD4^+$ T cells, with maintained strong cross-reactivity against Omicron variants.[27] This supports our concept of including M and N sequences in a genetic vaccine, as these responses are maintained and highly cross-reactive, but also inducing $CD8^+$ T cells. Vaccination with an S protein delivered by adenoviral vectors was highly effective in inducing $CD8^+$ T cells.[27,28] In contrast, protein and inactivated vaccines were highly effective in inducing $CD4^+$ T cells.[27,28] Overall, this differential activation of $CD4^+$ and $CD8^+$ T cells by different vaccine modalities support the use of all these different vaccines as well as heterologous prime-boost strategies. In particular, the inclusion of multiple viral proteins in different vaccine platforms should be of importance.

It was equally clear that NAbs were essential for a rapid control and containment of the infection in the ferret model. These data were reiterated in the K18hACE2 challenge model in which T cells alone offered a limited protection, whereas control improved with increasing antibody levels to S and RBD. Finally, when vaccination of NHPs with a SARS-CoV-2 DNA vaccine generated high levels of anti-S, low levels of NAbs, but strong and broadly reactive T cells, this resulted in the rapid elimination of virus in the lungs and minimal pathology. Accordingly, the study suggests an association among strong T cell responses, high anti-S responses, and better protection against SARS-CoV-2 challenge. Interestingly, the recall responses after challenge were strong with respect to both S/RBD antibodies and broadly reactive T cells in all SARS-CoV-2-vaccinated animals. Even in the NHP with the lowest vaccine-primed T cell response prior to challenge, a strong recall response was rapidly developed, suggesting effective T cell priming by the vaccination. The observation that a lower anti-S response protects viral replication in the lungs but not as effectively in the upper airways is fully consistent with other vaccine studies in NHPs.[29] In addition, the changing pattern of the COVID-19 pandemic from mainly moderate and severe disease caused by the WH1 variant (91.3%[30]) to a predominantly asymptomatic or mild lung disease (91.2%[30]), combined with no or mild upper respiratory tract symptoms caused by the Omicron variant, is consistent with better vaccine-induced protection in the lungs, possibly by systemic antibodies, B cells, and T cells, combined with T cell-mediated clearance in the upper airways characterized by mild symptoms. This deserves further studies.

The strength of the study is the use and testing of multiple vaccines in four different animal species with consistent results. The limitations are mainly the limited sizes of some studies, in particular the NHP study. However, the consistent data show the pros and cons of T cells in their ability to control viral replication but also contribute to pathology.

In conclusion, we show in this study for the first time in several independent animal models that vaccine-induced SARS-CoV-2-specific T cells alone can promote control and clearance of SARS-CoV-2. This T cell-mediated protection was more evident in the lower respiratory tract compared with the upper airways. However, we also showed that good protection against disease required both NAbs and T cells. Future generations of SARS-CoV vaccines should have the ability to induce T cell memory that is cross-reactive to diverse SARS-CoVs. These T cells may, in the absence of cross-reactive Nabs, help control infection from future SARS-CoV outbreaks by rapidly inducing recall antibody and T cell responses and minimize the burden on health care. In addition, the presence of vaccine-primed S-specific T cells that partially control viral replication and provide T cell assistance helps explain why variants carrying mutations that escape NAbs and cause breakthrough infections still cause mild to moderate disease, whereas prevention from hospitalization or death is upheld.

MATERIALS AND METHODS

Animals

Female C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were obtained from Charles River Laboratories (Sulzfeld, Germany). Female B6.Cg-Tg(K18-ACE2)2Prlmn/J (K18-hACE2) mice were purchased from The Jackson Laboratory. All mice were 8–12 weeks old at the start of the experiments and maintained under standard conditions at the Preclinical Laboratory (PKL), Karolinska University Hospital Huddinge. Nine New Zealand white rabbits were purchased from commercial vendors and kept at the Comparative Medicine Fagraeus (KMF) Facility at Karolinska Institutet. A total of 18 ferrets were purchased from Marshall Bioresources and acclimatized for 21 days.

Figure 7. In a pilot experiment, six NHPs were vaccinated twice three weeks apart with either a control DNA vaccine or the OC-2.3 DNA vaccine, followed by challenge with 5×10^6 pfu SARS-CoV-2 WH1 strain

Data are expressed as the anti-S and anti-RBD titers before challenge, VNT_{ID50}, and SARS-CoV-2 RNA in BAL at day 4 and 20 after challenge (A). The kinetics of the anti-S levels to WH1 and 1.351/Beta S proteins are shown in (B). The kinetics of the cumulative T cell responses determined as IFN-γ ELISpot are shown in (C). The kinetics of the SARS-CoV-2 RNA in nasal washings and BAL are shown in (D). Finally, the summary of histological changes in the lungs of the two animals in the control group and the three animals in the vaccine groups at day 20 after challenge are shown in (E). No statistical difference was noted.

They were housed three animals per two-level cage at the Biosafety Level 3 (BSL-3) facility at KMF at Karolinska Institutet according to the guidelines for ferrets. A total of six NHPs (*Macaca fascicularis*) were purchased through KMF at Karolinska Institutet. The animals were housed in pairs at the BSL-3 facility at KMF at Karolinska Institutet according to guidelines for NHPs.

All animal procedures were approved by regional animal ethics committees (03634-2020, 16171-2020, 7602-2020, 4566-2020, and 16676-2020).

DNA plasmids and recombinant proteins

A total of nine genetic vaccines were generated based on the sequence from the Wuhan strain (Figure 1A). They contained a combination of, or alone, the S, the RBD, N, and M proteins, with or without autoproteolytic P2A sequences. All sequences were codon optimized for expression in human cells and were synthesized as DNA by a commercial source (GenScript). Plasmids were grown in TOP10 *Escherichia coli* cells (Life Technologies) and purified for *in vivo* injections using the Qiagen Endofree DNA purification kit following the manufacturer's instructions. The correct gene size was confirmed by restriction enzyme digests using BamHI and XbaI (Fast Digest; Thermo Fisher Scientific). Recombinant N protein was designed in-house and produced by GenScript (32878912). Recombinant S, RBD, and M were purchased from GenScript. The S proteins were produced as full length by transient protein production in mammalian cells (Expi293). To facilitate trimerization of the full-length S, a C-terminal T4 fibritin trimerization motif was included according to Wrapp et al.[31] Furthermore, a strep tag fused to the C terminus was used for purification.[32] The Beta version (B1.351) of the S was produced with three mutations in the RBD part (K417N E484K N501Y).

Peptides

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

Immunization schedules in mice, ferrets, and NHPs

Mice were immunized two to three times with three week intervals and sacrificed 2 weeks after the last immunization, and spleens and blood were collected as previously described.[11,12,17] In brief, female C57BL/6, 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 in sterile PBS by regular needle (27G) injection followed by *in vivo* EP using the Genedrive device (IGEA) using a 2-needle electrode. Prior to vaccine injections, mice were given analgesic and kept under isoflurane anesthesia during the vaccinations. During *in vivo* EP (mice, rabbit, ferret, and NHP) a single 1 ms 600 V/cm pulse followed by a single 400 ms 60 V/cm pulse was used to promote plasmid uptake.[11] In some experiments, groups of mice were injected subcutaneously (s.c.) at the base of the tail with recombinant protein mixed (1:1) with QS21 adjuvant (Good Manufacturing Practice [GMP] grade; Alpha Diagnostics).

For studies in rabbits, 3 New Zealand white rabbits per group were immunized with 250, 500, or 750 μg OC-2.3 DNA vaccine. DNA vaccines were administered in right quadriceps muscle in 125, 250, or 500 μL sterile PBS followed by *in vivo* EP using the Genedrive device and a 4-needle electrode (EPSGun; IGEA) at a depth of 1 cm.

Ferrets, in groups of 3, were immunized in the tibialis anterior muscle with 300 μg DNA vaccine followed by *in vivo* EP using the Genedrive. One group of ferrets was immunized s.c. with recombinant protein in QS-21.

Six NHP were immunized intramuscularly in right quadriceps muscle with 1 mg SARS-CoV-2 OC-2.3 DNA or control DNA (HBV) in 500 μL followed by *in vivo* EP using the Genedrive and EPSGun.

Mouse challenge model

Two or three weeks after the last immunization, K18-hACE2 mice were infected, under anesthesia, with 1×10^5 pfu SARS-CoV-2 Wuhan or Beta strain via intranasal administration in a total volume of 40 μL. After infection, the mice were monitored daily and assessed on the basis of their weight and general health status. Mice that reached the pre-determined humane endpoint were humanely euthanized before the study endpoint, while the remaining mice were observed up to 13 days before euthanasia. Nasal lavage (NAL), blood, lungs, and spleen were collected at the time of death. In addition, blood was collected before immunization started and 2 weeks after each immunization.

Ferret challenge model

Ferrets were challenged with 1×10^6 pfu SARS-CoV-2 (Wuhan) via intranasal administration in a volume of 0.5 mL. Animals were assessed with regard to weight, general health status, and body temperature daily following infection. NAL was collected 2, 4, 7, and 9 days PI. Ten days PI, animals were euthanized, after which BAL was performed, and trachea and lungs were excised for histopathological analysis.

NHP challenge model

NHPs were challenged with 5×10^6 pfu SARS-CoV-2 via intranasal administration in a volume of 0.5 mL and intrathecally in a volume of 4.5 mL. Animals were assessed with regard to health status daily following infection. Blood samples were collected −35, −21, and 0 days prior to infection and 4, 10, 14, and 21 days PI. BAL was performed days 4 and 20 PI for detection of virus.

Detection of IgG-specific antibodies

Detection of mouse, rabbit, ferret, and NHP Igs against S, RBD, or N was performed according to previously described protocol.[11,33,34]

Serum antibodies were detected by an alkaline phosphatase conjugated goat anti-mouse IgG (A1047; Sigma-Aldrich) 1:1,000 or mouse anti-rabbit IgG (A2556; Sigma-Aldrich) 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 by ELISpot and detection of CD4$^+$ and CD8$^+$ T cells by ICS

Two weeks post last vaccination, or at different time points after viral challenge, splenocytes or PBMCs from each group of immunized mice, ferrets, or NHPs were harvested and tested for their ability to induce specific T cells on the basis of IFN-γ, and IL-2 (mouse) secretion after peptide stimulation for 36–48 h, essentially as previously described.[11,16,17] Detection of SARS-CoV-2-specific CD4$^+$ and CD8$^+$ T cells by flow cytometry was performed as described.[35] In brief, 5×10^5 splenocytes were restimulated for 12 h in the presence of GolgiPlug (BD Biosciences, San Jose, CA) with N/RBD peptide (10 μg/mL), N recombinant protein (10 μg/mL), and RBD recombinant protein (1 μg/mL). Staining was done using BV421 rat anti-mouse CD3, Alexa Fluor 700 rat anti-mouse CD8a, BV711 rat-anti-mouse CD4, APC rat anti-mouse IL-2, fluorescein isothiocyanate (FITC) rat anti-mouse IFN-γ, PE rat anti-mouse CD107a, and PE-Cy7 rat anti-mouse TNF-α antibodies (BD Biosciences).

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 h. The huCoV-19/WH01, Delta, and Omicron strains used in this study were passaged 3 times and the Beta strain 2 times.

Neutralization of SARS-CoV-2 *in vitro*

Titer of NAbs in serum from mice and rabbits were determined by cytopathogenic effect (CPE)-based microneutralization assay. For mice, sera from each vaccinated group were pooled, while rabbit serum samples were tested individually. Briefly, serum was heat inactivated at 56°C for 30 min before serial dilution 2-fold. Each dilution was conducted in quadruplicate and mixed with 500 pfu SARS-CoV-2 huCoV-19/WH01, Beta, Delta, or Omicron in a 1:1 dilution. After 1 h of incubation at 37°C and 5% CO_2, 100 μL of serum-virus mix was added to Vero E6 cells on a 96-well plate (20×10^4 cells/well) and incubated for 72 h at 37°C and 5% CO_2. CPE for each well was determined using a Nikon Eclipse TE300 microscope as described.[11] As controls, wells with medium only, diluted serum only, virus only, and serum known to contain SARS-CoV-2 NAbs mixed with virus were included in each experiment.

Real-time qPCR/viral RNA

TRIzol (Sigma-Aldrich) in a ratio of 1:3 was used to inactivate potential virus in NAL samples (50 μL) from SARS-CoV-2 infected K18-hACE2 mice.[11] For lung and spleen, PBS was added to each sample (1 g/mL), and pestles were used to crush the organs. Thereafter, the samples were centrifuged (5 min at 7,000 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 was thereafter measured using real-time qPCR using TagMan Fast Virus 1-Step master mix (Thermo Fisher Scientific) with primers and probe for the SARS-CoV-2 E gene: forward: 5′-ACAGGTACGTTAATAGT TAATAGCGT-3′; reverse: 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. PCR was performed using a capillary Roche LightCycler 2.0 system.

Histological analysis

The lungs (mice, ferret, and NHP) were formalin fixed, embedded in paraffin, and sectioned for H&E staining. The sections were analyzed by independent pathologists blinded to the treatment groups. All sections were scored according to bronchial and/or alveolar signs of inflammation and tissue damage.

Statistical analysis

Data were analyzed using GraphPad Prism version 5 and Microsoft Excel version 16.13.1.

DATA AND CODE AVAILABILITY

All raw data will be available upon request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.ymthe.2024.01.007.

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AUTHOR CONTRIBUTIONS

M.S., A.M., L.F., and G.A. designed the study, participated in experiments, and wrote the manuscript. J.Y., C.R.B., N.N., S. Appelberg., D.N.S., H.Y., A.P., S.W., and O.L. performed experiments, analyzed data, and edited the manuscript. F.W., U.H., G.B., M.G., S. Aleman., O.T., and E.-K.G. discussed experimental design, analyzed data, and edited the manuscript. M.C., S.S., H.T., and S.H. contributed with

apparatus and/or reagents and edited the manuscript. L.S. and A.S. analyzed the pathology of macaques and edited the manuscript.

DECLARATION OF INTERESTS

M.S. is a founder of and has shares in SVF Vaccines AB, which holds patents for vaccines. L.F. is a founder of and has shares in SVF Vaccines AB, which holds patents for vaccines. G.A. is a consultant to SVF Vaccines AB, which holds patents for vaccines.

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