A third dose of the unmodified COVID-19 mRNA vaccine CVnCoV enhances quality and quantity of immune responses

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A third vaccine dose is often required to achieve potent, long-lasting immune responses. We investigated the effect of three 8-µg doses of CVnCoV, CureVac’s severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccine candidate containing sequence-optimized unmodified mRNA encoding the spike (S) glycoprotein, administered at 0, 4, and 28 weeks, on immune responses in rhesus macaques. After the third dose, S-specific binding and neutralizing antibodies increased 50-fold compared with post-dose 2 levels, with increased responses also evident in the lower airways and against the SARS-CoV-2 B.1.1.7 (Alpha), B.1.351 (Beta), P.1 (Gamma), SARS-CoV-2 B.1.617.2 (Delta) variants. Enhanced binding affinity of serum antibodies after the third dose correlated with higher somatic hypermutation in S-specific B cells, corresponding with improved binding properties of monoclonal antibodies expressed from isolated B cells. Administration of low-dose mRNA led to fewer cells expressing antigen in vivo at the injection site and in the draining lymph nodes compared with a 10-fold higher dose, possibly reducing engagement of precursor cells with the antigen and resulting in the suboptimal response observed after two-dose vaccination schedules in phase IIb/III clinical trials of CVnCoV. However, when immune memory is established, a third dose efficiently boosts the immunological responses and improves antibody affinity and breadth.

INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic resulted in accelerated development of vaccines against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), with 38 approved vaccines in 197 countries and 212 candidates in testing as of June 2022.1 The most notable of these were two nucleoside-modified mRNA vaccines (BNT162b2 [BioNTech/Pfizer] and mRNA-1273 [Moderna]) that were rapidly authorized, manufactured, and distributed, whereas several other sequence-optimized, chemically unmodified mRNA vaccines are still in clinical development.1 CureVac’s vaccine candidate, CVnCoV, was the first unmodified mRNA SARS-CoV-2 vaccine to reach phase III clinical testing.2 In the reported clinical trials of CVnCoV, much lower doses of unmodified mRNA (2–12 µg) were tested than those used in modified mRNA vaccines; e.g., 30 µg in the BioNTech/Pfizer vaccine3 and 100 µg in the Moderna vaccine.4 This was because unmodified mRNA vaccines are considered to induce stronger innate immune activation, which, at high doses, may lead to reactogenicity. A trend of a dose-dependent increase in local and systemic solicited events was observed in the phase 1 dose-escalation study of CVnCoV.5 In the phase IIb/III HERALD trial, the CVnCoV vaccine candidate, containing 12 µg mRNA encapsulated in lipid nanoparticles (LNPs), showed an overall vaccine efficacy (VE) of 48.2% against COVID-19 of any severity and 70.7% against moderate to severe COVID-19, measured in an environment with 15 different circulating SARS-CoV-2 variants.6 CureVac decided to discontinue development of CVnCoV and focus on new-generation candidates.6

To inform the development of better strategies for immunization with more efficacious vaccine candidates, we performed a detailed immunological investigation to understand the magnitude and quality of the immune responses to CVnCoV, including an assessment of the effect of a third 8-µg dose of CVnCoV on the immune response in non-human primates (NHPs). The NHP model allowed us to take multiple samples over time and to collect not only blood samples but also respiratory samples for assessment of mucosal responses and bone marrow for long-lived plasma cell responses. In a two-dose vaccination schedule, 8 µg CVnCoV has been shown to induce seroconversion but with relatively low antibody titers in humans7 and...
NHPs.\textsuperscript{6–8} However, in NHP challenge studies, partial protection against SARS-CoV-2 was achieved, indicating that protective immunity had been established. The NHP model therefore offers an opportunity to perform high-resolution analyses in a physiologically relevant setting.

RESULTS

CVnCoV administration rapidly induced type I interferon-polarized and transient innate immune activation

We measured multiple aspects of the immune response induced by CVnCoV, including immediate responses after administration as well as long-term adaptive responses. Three rhesus macaques were immunized with \(8\mu g\) CVnCoV encapsulated in LNPs at weeks 0 and 4, followed by a booster dose at week 28. Peripheral blood and bronchoalveolar lavage (BAL) samples were collected over the 37-week study period, and bone marrow aspirates were collected after euthanasia at study end (Figure 1A).

Within 24 h of immunization, markers of innate immune activation and toxicity showed no or only minor transient fluctuations that remained within the normal range of the clinical chemistry and complete blood count (CBC) (Figures S1A and S1B). Animals did not show any behavioral changes, increase in body temperature, or long-term weight differences. By combining CBC and phenotyping by flow cytometry (Figure 1B), we were able to detect a transient decrease in circulating lymphocytes, including T cells, B cells, natural killer (NK) cells, and NK T cells 24 h after immunization (Figure 1C), coinciding with an elevated proportion of circulating monocytes (Figure 1C). This increase was mainly represented by CD14^+ CD16^+ intermediate monocytes (Figure 1D), which is consistent with previous reports on intermediate monocyte expansion after administration of TLR7/8-based adjuvants or mRNA vaccines.\textsuperscript{9,11} Most of the 30 plasma analytes measured showed no or very low increases 24 h after CVnCoV administration (Figure 1E). However, in line with the transient increase in intermediate monocytes, elevated levels of monocyte attractant protein 1 (MCP-1; CCL2) were detected at 24 h after immunization (Figures 1E and 1F). Interleukin-1 receptor antagonist (IL-1RA) was induced as well as cytokines associated with a type I interferon (IFN) response, such as IFN-\(\alpha\) and CXCL11 (Figures 1E and 1F). All cytokines had returned to baseline levels by day 14 (Figures 1E and 1F). No detectable levels of classic inflammatory cytokines, such as tumor necrosis factor alpha (TNF-\(\alpha\)) and IL-6, were induced. Principal component analysis (PCA) of the 30 plasma analytes confirmed differences between baseline and 24-h samples, mainly because of the type I IFN-associated cytokines MCP-1 and IL-1RA (Figure 1G). This demonstrated that systemic innate immune activation was induced by CVnCoV with limited and transient adverse events.

A third dose increased the levels of neutralizing and cross-reactive antibodies

Using the 8-\(\mu g\) dose of CVnCoV, we found low but detectable antibody titers against SARS-CoV-2 S protein and the receptor-binding domain (RBD) of the spike (S) protein at week 6, 2 weeks after the second dose (Figure 2A). In clinical studies, suboptimal efficacy elicited by immunization with two doses of CVnCoV ultimately led to a halt in clinical development. We therefore investigated the potential of a third dose to increase the responses. The animals received a third dose 24 weeks (6 months) after the second dose, a relevant time frame for a human booster dose. Titers increased significantly after the third (booster) dose, which is in line with the kinetics of antibody responses in the clinical trial testing the booster potential of the third CVnCoV immunization.\textsuperscript{12} Two weeks after the third dose, binding titers for the S protein and RB were 12.8- and 6.4-fold higher, respectively, than peak titers 2 weeks after the second dose (Figure 2A). The boosting effect was even stronger for neutralizing (Figure 2B) and pseudovirus neutralizing titers (Figure 2C). These titers increased by 33.6- and 23.7-fold, respectively, and matched the neutralization capacity of the World Health Organization (WHO) international standard (NIBSC 20/136), which was not the case after the first two immunizations (Figure 2B). This large change in response from the second to the third dose for neutralization of the ancestral (WA-1) SARS-CoV-2 virus has not been reported for the licensed mRNA vaccines mRNA-1273 and BNT162b2, although titers were higher after the first two doses.\textsuperscript{13–16} Although low-dose CVnCoV can elicit high responses with three immunizations, mean responses after three doses were still lower than those reported with the 30–100-\(\mu g\) doses of licensed mRNA vaccines in NHP models\textsuperscript{17,18} or clinical studies,\textsuperscript{19,20} although differences between the assays used have to be considered.

The significant increase in titers after the third CVnCoV dose was also reflected by higher neutralization potency, defined as the ratio between the neutralizing and the binding titers (Figure 2D).\textsuperscript{12} This suggests not only a substantial improvement in titers but also in antibody quality; however, because the group size was small, and one animal persistently showed lower responses, this difference was not significant.

In a recent study, S protein was detected in the serum of BNT162b2 vaccinees after the first but not the second dose because of masking of S epitopes by the serum antibodies.\textsuperscript{16} In our NHP sera, S protein was detectable 24 h after the first dose in all three animals, whereas only the animal with the lowest S-specific titers had systemically detectable S protein 24 h after the third dose (Figure 2E).

In addition to the large increase in antibody response to the ancestral S protein after the third dose, antibody titers against S proteins from
the SARS-CoV-2 B.1.1.7 (Alpha), B.1.351 (Beta), P.1 (Gamma), and B.1.617.2 (Delta) variants were also increased (Figure 2F). The increase after the third dose was also demonstrated by higher ratios between variant and ancestral binding titers (Figure 2G).

Although serum antibodies are often used to assess vaccine responses and predict correlates of protection,\textsuperscript{21–23} it is likely that mucosal immunity to SARS-CoV-2 is necessary to prevent infection and mild disease. Anti-S and RBD-binding immunoglobulin G (IgG) and neutralizing titers in BAL fluid were barely detectable 2 weeks after the second dose, but, as with serum antibodies, they were strongly boosted after the third dose (Figures 2H and 2I). There was a strong correlation between antibody levels in BAL and in plasma ($r = 0.8441, p < 0.001$) (Figure 2J), suggesting that mucosal antibodies may predominantly transudate from serum, as proposed previously.\textsuperscript{21}

Antibody-secreting S and RBD-specific plasmablasts were undetectable after the two priming immunizations but detectable by enzyme-linked immunospot (ELISpot) 4 days after the third dose (Figure 2K). S- and RBD-specific plasma cells in the bone marrow were found at week 37 (study end; Figure 2L), suggesting that the animals had generated vaccine-specific B cell populations critical for establishing longevity of the antibody response. S-specific memory T cells, assayed by an antigen recall assay using stimulation with overlapping S peptides and intracellular cytokine production (Figures S2A and S2B), showed low but detectable CD4\textsuperscript{+} T cell responses after the second dose in the blood and BAL (Figure 2M). These responses waned but were boosted by the third dose, especially in the BAL. The T cell response was Th1 polarized, as shown by IFN-\gamma and IL-2 production, but a proportion of IL-21-secreting, circulating T follicular cells was also detected, especially 2 weeks after the third dose (Figure 2N). Low frequencies of IFN-\gamma producing CD8\textsuperscript{+} T cell responses were detectable in the blood and BAL (Figure 2O).

A third dose 24 weeks after the two-dose primary series amplified the initial vaccine responses. A strong increase in antibody titers, resulting in improved neutralization of and binding to the ancestral strain as well as variant strains and higher mucosal responses, was accompanied by induction of S-specific, IL-21-secreting, circulating T follicular helper and Th1 memory cells.

The third immunization drives affinity maturation of vaccine-specific B cells

S-specific circulating memory B cells, measured by binding to fluorescently labeled S using flow cytometry (Figures 3A and S2A), showed a clear increase in frequency after the second immunization. Despite the expected waning, S-specific memory B cells remained at detectable levels for 24 weeks (Figure 3B). The third immunization resulted in a clear expansion with readily detectable levels until study end. Of the S-specific memory B cells, only 11.1% (range, 0%–30.3%) were specific to the RBD throughout the study, as reported previously (Figure 3C).\textsuperscript{24–26} Conversely, on average, 64.9% (range, 33%–79%) of the antibodies in plasma were RBD reactive (Figures 3D, S3B, and S3C), similar to the proportions observed in convalescent individuals.\textsuperscript{27} This discrepancy in the memory B cell pool and circulating antibodies may reflect differences between the antibody-producing plasma cells in the bone marrow compartment and memory B cells. Over time and with the third immunization, we observed that, although the proportion of RBD-specific antibodies decreased slightly, they still represented the majority of the response (Figure 3D).

To determine whether there were qualitative differences in affinity maturation and epitope specificities in vaccine-elicited B cell responses, we single-cell-sorted S-specific memory B cells obtained 2 weeks after the second and third immunizations and sequenced the variable regions of the heavy (VH) and light (VL) chains of their B cell receptors. Productive, high-quality sequences were obtained from a total of 444 single memory B cells (155 and 289 after the second and third immunizations, respectively). The level of somatic hypermutation (SHM) in the VH region was calculated after alignment with the largest germline IGHV allele database available, based on multiple rhesus macaques.\textsuperscript{28} Significantly higher SHM was found in the memory B cells after the third immunization compared with the second (Figure 3E). Along with the increase in SHM after the third dose, we found that antibody binding avidity increased significantly at week 30, in agreement with a recent report.\textsuperscript{29} High avidity remained stable until study end, another indication of a qualitative improvement of the humoral response (Figure 3F).

The memory B cell response was highly polyclonal, with the majority of the sequences belonging to independent lineages (defined as the same IGHV and IGK allele, same HCDR3 length, 80% amino acid identity in the HCDR3 and one identical HCDR3 junction) (Figure S3D). However, several lineages were detected at weeks 6 and 30 (labeled in the
same color in Figure S3D), indicating that they were maintained and expanded by the third dose. To investigate maturation of the B cell response after the third dose, we selected 10 sequence pairs from the lineages that were detected at weeks 6 and 30 and expressed them as monoclonal antibodies (mAbs). These selected lineages did not expand within the sampled B cell repertoire with the third dose (Figures S3E and S3F), but they showed significant affinity maturation (Figure 3G). This confirms the increased SHM as found in the memory B cell pool at large. Although 16 of the 20 mAbs we expressed bound S protein, only two (from the same lineage) bound the S1 domain, and none of them bound RBD alone (Figure 3H). Therefore, we predict that most expressed mAbs bind the S2 subunit (Figure 3K), which is rarely a target of neutralizing antibodies. This corroborates our data on expansion of non-RBD-specific memory B cells with the booster immunization.

Figure 3. Maturation of the B cell response after the third immunization
(A) Representative gates used for sorting S-protein-specific IgG+ memory B cells 2 weeks after the second and third immunizations (weeks 6 and 30). (B) Longitudinal assessment of S-protein-specific memory B cells among IgG+ B cells. (C) Proportion of RBD-specific memory B cells among S-protein-specific memory B cells at selected time points. (D) Proportion of RBD-binding antibodies in serum at selected time points. (E) SHM in sorted memory B cells, presented as number of nucleotide substitutions in the heavy-chain V gene segment. (F) Avidity index of a polyclonal plasma antibody response. (G) SHM of expressed mAbs by time point of isolation. (H) Binding of expressed mAbs to ancestral S, the S1 subunit, and RBD. NB, non-binding. (I and J) mAb binding to ancestral S protein (I) and avidity index (J), plotted by time point of isolation, with previously characterized mAbs (Controls (Ctrl): CR3022, B38, hACE2-Fc, C144, S309, S2M11, S2X333, and COVA2-37) for comparison. (K) Heatmap depicting binding and avidity information with predicted subunit binding for isolated and control mAbs. ND, not determined. Statistical analysis was performed using non-parametric Mann-Whitney test (E) or Wilcoxon matched-pairs signed-rank test (F, G, I, and J). *p < 0.05, **p < 0.01. Data are represented as mean ± SEM.
Figure 4. Tracking mRNA vaccines in vivo in NHPs
(A and B) In vitro uptake and translation of 0, 0.5, and 5 μg/mL mCitrine mRNA/LNPs in enriched primary human monocytes at 6, 24, 48, and 72 h. Shown are representative flow cytometry plots (A) and summarized data (B). (C) NHPs were immunized at 0 h with saline, 10 or 100 μg mRNA/LNP. They were sacrificed 24 h later, and draining as well as non-draining tissues were collected and analyzed. (D) Representative flow cytometry plots of CD45+ immune cell infiltration in different tissues. (E) Enumeration of infiltrating cell subsets in different tissues. (F) Monocyte infiltration into the site of injection and draining LNs. (G) Flow cytometry plots of LNP DiD and mCitrine signal in draining LNs by innate cell subsets from saline- and vaccine-injected sites. (H) Proportion of vaccine-positive cells in different tissues by the innate cell subset. (I) Vaccine-positive
isolated from B cells at week 30 showed significantly better binding to S protein than the related sequences isolated at week 6 (Figure 3I). The mAbs from week 30 exhibited a trend toward higher-avidity indices, which were comparable with well-characterized reference mAbs specific for the N-terminal domain (NTD) or RBD of the ancestral S protein (Figure 3I). This shows a clear maturation of the B cell response with the third vaccine dose, demonstrated at the antibody and memory B cell level.

**A higher mRNA vaccine dose increases dissemination to more lymph nodes**

We and others have shown in NHPs that mRNA vaccine administration leads to local inflammation in the muscle at the site of injection, consisting of infiltration of immune cells, including antigen-presenting cells; uptake; and translation of the mRNA.32,33 The efficiency of this process is probably affected by the dose of the vaccine, so the low dose used in our study may have limitations regarding the number of cells infiltrating the injection site and becoming available as target cells for the vaccine as well as for disseminating vaccine antigen. This would have consequences for initiation of an adequate vaccine-specific response. We used an mRNA construct based on sequence-optimized unmodified mRNA encoding the fluorescent protein mCitrine that enabled identification of mRNA translation in cells. The mRNA construct was formulated in DiD-labeled LNPs, allowing us to track LNP uptake independent of mRNA translation. By exposing isolated monocytes to 5 μg/mL mRNA/LNP in vitro, we found detectable LNP uptake already after 6-h incubation (Figures 4A and 4B). mCitrine expression was slightly delayed compared with LNP uptake but was detectable at 24 h, and high levels of mCitrine+ cells remained detectable for at least 3 days. Using a 10-fold higher dose of the construct did not result in higher LNP uptake, but we did observe a dose-dependent pattern of mCitrine expression in vitro.

We then investigated whether differences in doses significantly affected biodistribution and antigen expression in vivo, mimicking low-dose CVnCoV administration. Rhesus macaques received intramuscular injections of low (10 μg) or high (100 μg) doses of the labeled LNP/mCitrine mRNA construct (Figure 4C) with intramuscular saline injections as controls. The animals were injected at four different sites simultaneously (left and right deltoid and quadriceps muscles), allowing direct comparison of saline versus mRNA vaccine administration in the same animal. This enabled collection of multiple data points from each animal while limiting the number of animals used. Injection site muscle biopsies were taken after 24 h because we have observed previously that there is a high level of antigen uptake and local innate immune activity at this time point.32,33,35 To determine which lymph nodes (LN) were primarily targeted by mRNA vaccination, several LN clusters were collected and classified as primary (1°; axillary or inguinal), secondary (2°; apical or iliaca), or tertiary/third (3°; supraclavicular or para-aortic) draining LNbs based on their proximity to the injection site (Figure 4C). We analyzed early immune processes critical for initiation of adaptive responses, such as cell infiltration, vaccine uptake and translation, and dissemination to LNbs.

Compared with the control tissues, there was noticeable recruitment of CD45+ immune cells to the LNP/mRNA injection sites as well as into the draining LNs specifically in a dose-dependent manner (Figure 4D). Multiple cell subsets were defined within CD45+ immune cells (Figure S4A). CD66abc+ neutrophils, classic CD14+CD16+ monocytes, and myeloid dendritic cells (mDCs) were the most frequent cell types infiltrating the muscle injection site (Figure 4E), with some infiltration of the muscle by plasmacytoid DCs, T cells, B cells, and NK cells. Of these, monocytes exhibited the most pronounced dose-dependent accumulation in the draining LNs after mRNA vaccine administration (Figures 4E and 4F).

mDCs and monocytes play an essential role in antigen presentation and maintaining adaptive responses. Using mRNA encoding for mCitrine, we determined the target immune cells for the LNP/mRNA vaccine (Figure 4G). No LNP+ or mCitrine+ cells were detected at the saline-injected control sites, demonstrating that uptake and translation are restricted to the vaccination sites and their draining LNs (Figures 4G and 4H). Although we observed that non-immune CD45+ cells in muscle tissue were able to take up LNP and translate mRNA into protein (Figure S4B), production of mCitrine was much less efficient compared with CD45+ immune cells. Monocytes were found to be the most abundant LNP+ mCitrine+ immune cells (Figure 4H), with classic CD14+CD16+ and intermediate CD14+CD16+ subsets infiltrating the site of injection, although classic monocytes were the predominant vaccine+ subset in the draining LNs (Figure 4K). mDCs also showed clear LNP uptake and mCitrine translation (Figures 4G and 4H). We observed that a 10-fold higher dose of vaccine led to broader mRNA dissemination, evidenced by LNP+ mCitrine+ monocytes in more draining LN clusters (Figure 4I).

In contrast to the clear expression of mCitrine in monocytes and mDCs, neutrophils were efficient at internalizing LNP but not at translating the mRNA (Figures 4G and 4H), in accordance with our earlier data.30 Other immune cells, such as T cells, B cells, and plasmacytoid DCs, showed low signals for LNP and mCitrine in comparison with monocytes and mDCs (Figure S4C). There was a correlation between the number of infiltrating monocytes in the muscle and the number of mCitrine+ monocytes in 1° and 2° draining LNs (Figure 4J). This demonstrates that a sequence-optimized unmodified mRNA vaccine has a similar pattern of biodistribution and cell-specific targeting as that reported for modified mRNA vaccines, but a low dose of mRNA results in restricted dissemination to the 2° lymphoid organs compared with a higher dose.

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monocytes in 1°, 2°, and 3° draining LNs 24 h after immunization. (J) Correlation between the number of infiltrating monocytes in the muscle and the number of vaccine-positive monocytes in 1° and 2° draining LNs. (K) Vaccine signal in different monocyte subsets and tissues at 24 h. Statistical analyses were performed using a non-parametric Kruskal-Wallis test or Spearman correlation. *p < 0.05, **p < 0.01. ns, not significant. Data are represented as mean ± SEM.
DISCUSSION
The 48.7% efficacy against COVID-19 of any severity observed in the phase IIb/III clinical trial of CVnCoV was followed by the decision to reorient the development of this vaccine candidate. However, the results from this NHP study showed that a third CVnCoV dose significantly enhanced the magnitude and quality of the immune response compared with two doses. Although the average neutralizing antibody responses after three 8-μg doses of CVnCoV were numerically lower than those reported with the 30 to 100-μg doses of the licensed mRNA vaccines in NHPs or humans, the marked fold increase in neutralizing activity against ancestral SARS-CoV-2 after the third dose, compared with after the second dose, was substantially higher than that reported for three doses of the licensed mRNA vaccines mRNA-1273 and BNT162b2. Establishment of vaccine-specific plasma cells in the bone marrow after the third dose, combined with neutralizing serum antibody titers in the range of the WHO international standard 2.5 months after the last dose suggests that three CVnCoV doses elicit durable immune responses beyond the investigated study period. Similar to immunization with BNT162b2, we detected S protein in sera of CVnCoV-vaccinated NHPs 24 h after administration but at a concentration 10-fold lower than that observed in BNT162b2 vaccinees. This may reflect the overall antigen load and affect development of the vaccine responses, although the physiological differences in size and weight between humans and NHPs need to be considered. Our study results suggest that a third CVnCoV dose provides efficient boosting of the immune responses when SARS-CoV-2-specific memory has been established, and it provides mechanistic information about how this boosting effect is brought about. In recent clinical studies, CVnCoV performed well as a booster vaccine in previously vaccinated individuals, eliciting superior antibody titers compared with Valneva’s alum/CpG-adjuncted inactivated vaccine candidate VLA2001 but inferior responses to licensed mRNA vaccines. We found clear evidence of qualitative enhancement of the responses in plasma antibodies and the B cell repertoire, manifested by higher binding avidity and SHM, respectively. We also showed that the third immunization enhanced cross-reactivity of plasma antibodies to several variant S proteins, which is relevant in the light of continuing emergence of new variants with divergent mutations in the S protein.

The suboptimal responses to CVnCoV in clinical trials spurred development of the next-generation vaccine candidate CV2CoV. This updated version of CVnCoV contains unmodified nucleosides but with optimized non-coding regions and has been reported to induce higher titers of binding and neutralizing antibodies, memory B cell responses, and T cell responses as well as more robust protection compared with CVnCoV when two doses were administered 4 weeks apart in NHPs. The S-specific antibody and B and T cell responses after the third dose of CVnCoV found in our study were in a range similar to that found for CV2CoV after two doses. Although our study did not include virus challenge, the serum neutralizing titers after the third dose were consistent with titers reported in other studies that provided significant protection in challenge experiments. Based on these published data, protection from infection would presumably be higher after three doses of CVnCoV compared with two and in the range observed with CV2CoV. However, CV2CoV also induced higher levels of type I IFN responses and MIP-1α 24 h after immunization compared with CVnCoV. This needs to be evaluated further because undesired side effects as a result of innate immune activation have been a concern with unmodified mRNA vaccines.

The first unmodified mRNA vaccine tested in humans was against rabies virus; about 78% of study participants reported transient mild to moderate systemic side effects after administration of protamine-complexed mRNA. More recently, in a phase I clinical trial, considerable side effects were reported with 5 μg unmodified rabies mRNA in LNPs, whereas 1- or 2-μg doses were well tolerated and elicited immune responses comparable with those of a licensed rabies vaccine. These data on dosing contributed to the design of the doses selected for CVnCoV, which also displayed dose-dependent increases in reactogenicity up to the maximum dose of 12 μg tested.

The innate immune activation characterized by type I IFN responses after mRNA vaccination likely plays an important role in the immunogenicity of the mRNA platform and its Th1 polarized profile of adaptive responses. Type I IFN responses have been shown to directly support B cell differentiation and survival, resulting in enhanced antibody responses. Increased antibody half-life and durability of humoral responses have been shown with type I IFN-inducing adjuvants, such as TLR3, TLR7/8, and TLR9 ligands (poly(IC:LC), R848, CpG). In the current study, we observed that CVnCoV induced a strong, transient type I IFN response (IFNg, CXCL11) as well as monocyte activation, evidenced by MCP-1 induction and intermediate monocyte differentiation within 24 h of administration. A similar activation profile after administration of nucleoside-modified mRNA vaccines has been reported previously. Intermediate monocytes have been shown to be important for antigen presentation to CD4+ T cells and support differentiation of naive B cells into antibody-secreting plasmablasts.

Little is known about the mechanisms of action by which LNP/mRNA-based vaccines generate strong vaccine responses. Using an in vitro experimental approach, we showed that lower doses of mRNA led to detectable protein production in fewer monocytes compared with higher doses. Antigen availability is an important determinant of the outcome of the germinal center reaction, and a low protein translation rate could result in less protein antigen being available to support the germinal center reaction, limiting the B cell response. Another major finding of our study is that sequence-optimized unmodified and modified mRNA/LNP vaccine formulations appear to have a similar biodistribution pattern and cell-specific targeting. Our in vivo biodistribution data showed that a lower dose resulted in a limited spread of vaccine-positive immune cells and fewer targeted LN, demonstrating restricted vaccine dissemination. This restricted dissemination would have a greater effect in a primary immunization setting, where induction of vaccine-specific immune responses relies on encounters between the vaccine and sparse
antigen-specific naive lymphocytes, than in a booster setting, when memory B and T cell pools would already have been established.

Although this study has obvious limitations because of having only three animals per group, we followed the animals with multiple samplings over 8 months, and we were able to analyze numerous aspects of the immune responses. Therefore, we were able to study the evolution of the vaccine responses over a significant time period with control measurements from the same individual. We mapped antibody responses in detail and monitored the emergence of S-specific memory B cells over time. After two doses, most serum antibodies were RBD-specific. The third immunization had the particular effect of expanding the immune response against non-RBD epitopes, proportions of which remained stable until study end. This is important because SARS-CoV-2 vaccination has been shown to induce responses that are dominated by non-neutralizing antibodies, and although animal studies have shown that non-neutralizing antibodies can contribute to protection, clinical studies have shown that serum neutralizing antibody titer strongly correlates with vaccine-induced efficacy against symptomatic COVID-19. In this study, NHPs developed high titers of neutralizing antibody after the third dose. However, none of the mAbs we expressed from the clones in the B cell repertoire, as reported elsewhere, and warrants further investigation of immunization strategies to expand the RBD-specific B cell repertoire.

Our study in NHPs adds important mechanistic information on CVnCoV, including use of a three-dose immunization regimen that has been reported in a clinical trial. Our data will complement those observed in human volunteers to elucidate the mechanism of action of CVnCoV and inform development of an improved version for future use.

MATERIALS AND METHODS

Vaccines

The CVnCoV vaccine candidate is based on the RNActive platform. It has a 5' cap structure, 3' UTR, a GC-enriched open reading frame, 3' UTR, and poly(A) tail and no chemically modified nucleosides. The mRNA was encapsulated using the LNP technology of Acuitas Therapeutics (Vancouver, BC, Canada). The LNPs used in this study are particles of ionizable amino lipid, phospholipid, cholesterol, and a PEGylated lipid. The mRNA-encoded protein is based on the S-protein of the SARS-CoV-2 NCBI reference sequence (NCBI: NC_045512.2, GenBank: YP_009724390.1) and encodes for the full-length S-protein featuring K986P and V987P mutations. The mRNA-encoded protein for mCitrine is based on the description by Griesbeck et al.

Immune response flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated by standard gradient density centrifugation from heparinized blood using Ficoll-Paque (GE Healthcare). PBMCs were used immediately for downstream applications or cryopreserved in 10% dimethyl sulfoxide (DMSO)/fetal calf serum (FCS) until use. BAL cells were separated from the supernatant by centrifugation. Cells were passed through a 70-μm cell strainer and used fresh in a T cell stimulation assay. Supernatants were stored separately and concentrated 10-fold using Amicon-Ultra centrifugal filter units with 30-kDa cutoff (Millipore) before downstream analysis.

Plasma cytokine and chemokine quantification by Luminex

Plasma cytokine and chemokine analyses at baseline and 24 h and 14 days after the first dose were performed using the ProcartaPlex NHP Cytokine & Chemokine Panel 30plex (Thermo Fisher Scientific) according to the manufacturer’s instructions. Samples were analyzed using a MagPix (Luminex) instrument, and the data were analyzed with Belysa Immunoassay Curve Fitting software (Millipore). Standard curves were generated using 5-parameter logistic (5-PL) curve fit.

NHP plasma ELISAs

Recombinant proteins were acquired through the Global Health-Vaccine Accelerator Platform (GH-VAP) funded by the Bill & Melinda Gates Foundation (Seattle, WA, USA). Polyclonal antibody
responses elicited in blood and BAL were analyzed using 96-well half-area ELISA plates coated with recombinant antigens (SARS-CoV-2 prefusion [S-2P] stabilized S protein, RBD, and variant S proteins [HexaPro S backbone]) in PBS at 1 µg/mL overnight at 4°C. Plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T) and blocked with blocking buffer (PBS + 5% skim milk powder) for 1 h at room temperature (RT). Samples were serially diluted in blocking buffer, added to the ELISA plate, and incubated for 2 h at RT. For the RBD competition ELISA, samples were pre-incubated with or without 20 µg/mL RBD in blocking buffer for 30 min before being added to S-coated ELISA plates. For the chaotropic wash ELISA, plates were treated with 1.5 M NaSCN or PBS for 10 min at RT after sample incubation. Plates were washed three times, and goat anti-monkey IgG-horseradish peroxidase (HRP) (Nordic Mueller) was used to assess the strength of the binding interaction. After sample incubation for 2 h, plates were washed, and goat anti-human IgG-HRP, Fc specific (Jackson ImmunoResearch), in blocking buffer was added for 1-h incubation. Plate development and data analysis were performed as described above.

Recombinant hACE2-human Fc fusion protein and previously characterized S protein-specific mAbs were used as references: CR3022, B38, C144, S309, SM211, S2X333, and COVA2-37.

Neutralization assays
The live virus neutralization assay was performed at Vismederi (Siena, Italy) using SARS-CoV-2 2019-2019-nCoV strain 2019-nCoV/Italy-INMI1 clinical isolate as described previously. Briefly, serial 2-fold dilutions of heat-inactivated serum samples, starting at 1:10, were mixed with an equal volume of viral solution with 100 TCID_{50} of SARS-CoV-2 and incubated for 1 h before being transferred in duplicate to plates containing semi-confluent Vero E6 monolayers. The plates were incubated for 4 days at 37°C and 5% CO_{2}. After 4 days, the plates were inspected using an inverted optical microscope. The highest serum dilution that protected more than 50% of cells from cytopathic effects was designated as the NT_{50}. The first WHO international standard for anti-SARS-CoV-2 IgG (NIBSC 20/136) was analyzed in parallel to NHP samples for comparison.

Pseudovirus particle neutralization assays
The pseudovirus particle neutralization assay (PNA) was performed at Nextelis (Laval, QC, Canada) using vesicular stomatitis virus (VSV) G S pseudotyped virus with a luciferase reporter as described previously. Briefly, serial 2-fold dilution series of heat-inactivated serum were incubated with a constant amount of pseudotyped virus particles and then transferred onto Vero E6 cells in 96-well plates. Test plates were incubated at 37°C and 5% CO_{2} overnight. The next day, luciferase substrate was added to the plates, which were read using a luminomicroscope microplate reader equipped with SoftMax Pro GxP software (v.6.5.1. or higher). The assay was run in duplicate, and the serum dilution that neutralizes 50% of the pseudovirus particles (PNT_{50}) was interpolated by linear regression of the two serum dilutions flanking 50% of the control luminescence signal.

Electrochemiluminescence-based S antigen detection
SARS-CoV-2 S antigen was quantified using the S-PLEX SARS-CoV-2 Spike Kit (K150ADJS, Meso Scale Diagnostics, MD, USA) according to the manufacturer’s instructions at the SciLifeLab Affinity Proteomics Unit (Uppsala, Sweden). For the analysis, a 25-µL sample was used, and the plates were read using a Meso QuickPlex SQ 120 instrument. An 8-point calibration standard curve, based on the recombinant SARS-CoV-2 protein included in the kit, was used to convert raw signals into data expressed in femtograms per milliliter.

T cell stimulation
To assess the frequency of S-protein-specific memory T cells in the blood and BAL, T cell re-stimulation with overlapping peptides was performed as described previously. Briefly, 1.5 × 10^6 PBMCs or BAL cells were cultured in 0.2 mL complete medium (RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine) in a 96-well plate at 37°C, 5% CO_{2}. The cells were stimulated with 2 µg/mL PepMix SARS-CoV-2 overlapping peptide library in DMSO (15mers with 11-amino-acid overlap, JPT Peptide Technologies), spanning the whole S protein, or an equal volume of DMSO only in the presence of 10 µg/mL brefeldin A (Life Technologies). After overnight stimulation, cells were stained with Live/Dead Fixable Blue Dye (Life Technologies), surface stained, fixed and permeabilized with the Cytofix/Cytoperm Kit (BD Biosciences), and stained intracellularly with the panel of antibodies listed in Table S2. Cells were washed after staining and fixed with 1% PFA. Samples were acquired on a BD LSRSFortessa cell analyzer, and the data were analyzed using FlowJo software v.10.7.1 (FlowJo). DMSO-stimulated cells were used for background subtraction.

S-protein-specific memory B cell quantification and sorting
The recombinant SARS-CoV-2 prefusion stabilized S protein and RBD were biotinylated using an EZ-Link Micro Sulfo-NHS-LC Biotinylation Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Probes were generated by coupling biotinylated...
proteins to fluorophore-conjugated streptavidin (SA) molecules for
detection by flow cytometry (SA-BV421, SA-APC, and SA-PE,
BioLegend). Isolated PBMCs were stained by a panel of antibodies
listed in Table S3 as well as S protein and RBD probes (S-APC,
S-PE, and RBD-BV421, 100 ng each). The samples were analyzed
on a BD Aria III Fusion cell sorter (weeks 6 and 30) or a BD
LSRFortessa flow cytometer (all other time points). At weeks 6 and
30, memory B cells (CD3–CD11c–CD14–CD16–CD123–HLA-
DR+CD20+IgM–IgG+) double-positive for S protein binding were
single cell sorted into 96-well plates and frozen immediately on dry
ice for subsequent B cell receptor (BCR) amplification. Data were
analyzed using FlowJo v.10.7.1 (FlowJo).

Single-cell VDJ amplification and Sanger sequencing
RNA extraction and reverse transcription using random hexamers
from single-cell-sorted S protein-specific memory B cells were
performed using the Superscript III reverse transcriptase (Invitrogen)
according to the manufacturer’s instructions. Nested PCR was
performed using reagents and procedures as reported previously for
heavy and light Ig chains.26 PCR products were Sanger sequenced
by Genewiz (Leipzig, Germany), the chromatograms were prepro-
cessed using scifer (v 0.99.3, DOI: 10.18129/B9.bioc.scifer) and the re-
sulting sequences were aligned to the Karolinska macaque database
KIMDB (1.0)28 using IgDiscover.71

Sequence data analysis and mAb selection
Clonally related sequences were identified using heavy chain se-
quences and the Clonotypes module of IgDiscover,71,72 applying es-
blished criteria to de

Antigen-specific antibody-secreting cell detection in blood
Antigen-specific plasmablasts were enumerated by ELISpot on the
day of the booster dose and 4 days thereafter. Multiscreen IP filter
ELISpot 96-well plates (Millipore) were activated with 35% ethanol
for 1 min and washed three times with PBS. Plates were coated over-
night with 1 μg/mL Affinity Pure goat anti-human IgG Fc fragment-
specific antibody (Jackson ImmunoResearch). The next day, plates
were washed three times with PBS and blocked with complete me-
dium for 1.5 h. Serially diluted, freshly isolated PBMCs were incu-
bated overnight at 37°C, 5% CO2. After incubation, plates were
washed six times with PBS-T (0.05%) and incubated with biotinylated
probes for 1.5 h (0.25 μg/mL goat anti-human IgG Fc fragment-spe-
cific antibody [Jackson ImmunoResearch], 1 μg/mL mCitrine-encoding mRNA in DiD-labeled LNP. Sites of injection as well as vaccine-draining and non-draining tissues were
sampled at necropsy 24 h after immunization and stored in RPMI
1640 medium on ice as described previously.35,73

Biodistribution experiment sample processing
Muscle biopsies were dissected and weighed before digestion into sin-
gle-cell suspensions as described previously.35,73 Brieﬂy, muscle tissue
was incubated at 37°C for 2 h without agitation in the presence of
0.25 mg/mL Liberase TL (Roche) and 0.5 mg/mL DNase I (Sigma).
Enzyme activity was quenched by addition of complete medium,
the mixtures were ﬁltered through 70-μm cell strainers twice, and
single-cell suspensions were washed before proceeding to the next step.
Liver and bone marrow samples were processed by standard gradient
density centrifugation in the same manner as blood samples.
Lymphoid tissues were mechanically disrupted using a plunger and
a 70-μm cell strainer and washed with complete medium. When sam-
ple processing was complete, single-cell suspensions were immedi-
ately stained for flow cytometry analyses.

Biodistribution experiment flow cytometry
To quantify the mCitrine vaccine signal in different immune cell
populations, cell suspensions corresponding to approximately 2 g of
muscle tissue or 5 million LN cells were stained for flow cytometry.
First, cells were stained with Live/Dead Fixable Blue Dye (Life
Technologies) and FcR blocking reagent (Miltenyi Biotec),

In vitro uptake and translation of mCitrine LNP/mRNA
Primary human monocytes were isolated from buffy coats using
RosetteSep Human Monocyte Enrichment Cocktail (STEMCELL
Technologies) and gradient density centrifugation using Ficoll-
Paque (GE Healthcare) according to the manufacturer’s instructions.
After isolation, 1.5 × 107 monocytes were cultured in 0.5 mL com-
plete medium (RPMI 1640 medium supplemented with 10% heat-in-
activated FCS, 100 U/mL penicillin, 100 μg/mL streptomycin,
and 2 mM L-glutamine) at 37°C and 5% CO2 in the presence of mCi-
trine-encoding mRNA in DiD-labeled LNPs at different mRNA con-
centrations (0.0–5.0 μg/mL) for the indicated amount of time (6–72 h).
After culture, cells were washed with PBS, stained with Live/
Dead Fixable Blue Dye (Life Technologies) and FcR blocking reagent
(Miltenyi Biotec), followed by surface staining with the panel of anti-
bodies listed in Table S4. Cells were then washed with PBS and ﬁxed
with 1% PFA. Samples were acquired on a BD LSRFortessa cell
analyzer, and the data were analyzed using FlowJo software v.10.7.1
(FlowJo).
followed by a panel of antibodies listed in Table S4. Samples were then washed and fixed with 1% PFA. Before acquisition, AccuCount beads (Spherotech) were added to each sample for quantification according to the manufacturer’s instructions. Samples were acquired on a BD LSRS Fortessa flow cytometer cell analyzer, and the data were analyzed using FlowJo v.10.7.1 (FlowJo).

Statistics
No statistical methods were used to predetermine sample size. The results were considered statistically significant when p < 0.05. For comparison of two groups of paired and unpaired samples, non-parametric Wilcoxon matched-pairs signed-rank test and Mann-Whitney U test were used, respectively. For comparison of three of more groups, non-parametric Kruskal-Wallis test with Dunn’s multiple comparison test was used. Correlations were assessed using non-parametric Spearman’s correlation. Analyses were performed in GraphPad Prism 9.

Data availability statement
BCR sequencing data have been deposited to GenBank: OP575232–OP573208.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2022.10.001.

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