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CORONAVIRUS

GRAd-COV2, a gorilla adenovirus-based candidate vaccine against COVID-19, is safe and immunogenic in younger and older adults

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Safe and effective vaccines against coronavirus disease 2019 (COVID-19) are essential for ending the ongoing pandemic. Although impressive progress has been made with several COVID-19 vaccines already approved, it is clear that those developed so far cannot meet the global vaccine demand alone. We describe a COVID-19 vaccine based on a replication-defective gorilla adenovirus expressing the stabilized prefusion severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike protein named GRAd-COV2. We assessed the safety and immunogenicity of a single-dose regimen of this vaccine in healthy younger and older adults to select the appropriate dose for each age group. For this purpose, a phase 1, dose-escalation, open-labeled trial was conducted including 90 healthy participants (45 aged 18 to 55 years old and 45 aged 65 to 85 years old) who received a single intramuscular administration of GRAd-COV2 at three escalating doses. Local and systemic adverse reactions were mostly mild or moderate and of short duration, and no serious adverse events were reported. Four weeks after vaccination, seroconversion to spike protein and receptor binding domain was achieved in 43 of 44 young volunteers and in 45 of 45 older participants. Consistently, neutralizing antibodies were detected in 42 of 44 younger-age and 45 of 45 older-age volunteers. In addition, GRAd-COV2 induced a robust and T helper 1 cell (T_H1)–skewed T cell response against the spike protein in 89 of 90 participants from both age groups. Overall, the safety and immunogenicity data from the phase 1 trial support the further development of this vaccine.

INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic is one of the most major international public health emergencies that have occurred over the last century. COVID-19 is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which emerged in the People's Republic of China at the end of 2019. Since its start, the COVID-19 pandemic has already caused hundreds of millions of infections with several million fatalities worldwide. This unprecedented global health emergency has boosted international efforts to develop a vaccine targeting SARS-CoV-2. A multitude of innovative platforms have been used, including genetic vaccines based on mRNA, DNA, and viral vectors. As of September 2021, there are as many as 315 vaccine candidates still in development. A total of 194 are in preclinical development and 121 in clinical development, of which 22 are either authorized for emergency use or approved by different countries (*1*). However, with most of the world's population yet to be vaccinated and the potential need for booster doses, the COVID-19 vaccines rolled out so far cannot meet the global vaccine demand. Thus, there is an urgent need to develop additional safe and effective vaccines to be included in worldwide vaccination campaigns.

Here, we developed GRAd-COV2, a vaccine candidate based on an adenoviral vector derived from a group C gorilla adenovirus that is made replication defective by deleting the E1 genomic region. To increase cloning space and productivity, the vector is also deleted at the E3 genomic region and includes a modification of the E4 region (*2*). The GRAd-COV2 vaccine encodes for a full-length SARS-COV-2 spike (S) protein. The spike protein is the primary vaccine target for most COVID-19 vaccines, because it is critical for viral cell entry through the human angiotensin-converting enzyme 2 receptor (*3*). The spike gene that was cloned into GRAd-COV2 contains mutations that stabilize the spike protein expressed in the host cell in a prefusion conformation (*4*, *5*). The GRAd vector was selected on the basis of its immunogenicity and manufacturability profile, in

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addition to the low frequency of anti-GRAd neutralizing antibodies in human serum samples as compared with human adenovirus type 5 (*2*). We recently reported that a single dose of GRAd-COV2 elicited strong immune responses, both humoral and cellular, in mice and rhesus macaques (*2*). These preclinical data supported the start of a first-in-human, dose-escalation, phase 1 clinical trial in healthy younger and older adults to evaluate the safety and immunogenicity of GRAd-COV2. Here, we report the trial interim analysis results up to 4 weeks after vaccination.

RESULTS

Volunteer screening and selection

Between 11 August and 20 September 2020, 181 potential volunteers were evaluated for eligibility. Volunteers were screened in excess for speeding up enrollment and to comply with a strict staggering enrollment scheme. Of the 181 participants screened, 91 (45 in the younger adult cohort, aged 18 to 55, and 46 in the older adult cohort, aged 65 to 85) were vaccinated. A total of 55 volunteers (30 in younger age cohort and 25 in the older age cohort) were excluded because they did not meet the inclusion criteria, and 35 (25 younger age cohort and 10 in the older age cohort) were not vaccinated because the time from screening visit to vaccination visit exceeded the window allowed per protocol. Median age, gender distribution, and other characteristics were well balanced within study arms both in the younger adult (Fig. 1A) and in the older adult (Fig. 1B) cohorts.

One vaccinated arm 1 volunteer had detectable anti-nucleoprotein (N protein) and spike protein immunoglobulin G (IgG) starting at week 1 after vaccination. As this finding suggested a natural (asymptomatic) infection with SARS-CoV-2, we excluded this individual from the immunogenicity analysis. One participant who was vaccinated in arm 4 was found to be positive at baseline for anti–spike protein IgG by chemiluminescent immunoassay (CLIA), the primary serology assay per the study protocol for the evaluation of vaccine-induced spike protein–specific antibody responses. The volunteer did not develop antibodies specific to N protein and tested negative by a SARS-CoV-2 virus–based immunofluorescence assay, suggesting a nonspecific reactivity by the CLIA that made the vaccine-induced spike antibody response not reliably evaluable. Thus, the volunteer was replaced with a subsequent one and was included in the safety analysis but excluded from the immunogenicity assessment. Moreover, three additional volunteers (arms 2, 3, and 5) were excluded from interferon-γ (IFN-γ) enzyme-linked immunospot (ELISpot) analysis because of spontaneous IFN-γ production above the acceptability range (Fig. 1).

Vaccine safety

A single intramuscular administration of GRAd-COV2 was well tolerated at all doses and in both cohorts (Fig. 2). Most adverse events (AEs) occurred in the first 2 days after vaccination and were short lived (median time to resolution of 1 day, interquartile range of less than 1 to 2 days). Overall, we observed 328 AEs of any grade; of those, 255 were considered to be related to vaccination. In the younger age cohort, we observed 143 solicited AEs (118 mild and 25 moderate) and eight unsolicited AEs (four mild and four moderate). Among younger volunteers, injection site pain (34), fatigue (30), headache (26), and fever (21) were the most common AEs.

In the older age cohort, we observed 104 solicited AEs (79 mild, 13 moderate, and 2 severe) and 10 unsolicited AEs (5 mild, 3 moderate, and 2 severe). Among older volunteers, chills (18), fever (17), fatigue (15), myalgias (14), and headache (13) were the most common AEs.

Twenty-four volunteers (26%; 7 younger and 17 older) reported no AEs, 44 volunteers (48%; 23 younger and 21 older) reported at least 1 mild AE, and 21 volunteers (48%; 15 younger and 6 older) reported at least 1 moderate AE. Two volunteers (2%; both older-age volunteers) reported at least one severe AE. One of these participants had received a high GRAd-COV2 dose and reported fever (moderate) with severe fatigue, cough, and chills starting a few hours after vaccination and resolving within a few days. The second volunteer was in the intermediate dose group and presented with severe neutropenia without symptoms on day 2 after vaccination (total neutrophil count of 780 cells/mm^3); this individual's neutrophil count was within normal range by day 7 (total neutrophil counts of 1620 cells/mm^3).

No serious AE was reported, and no prespecified trial-halting rules were met. Nineteen volunteers (15 younger and 4 older) received antipyretics to control AEs. One older-age volunteer received inhalator steroids for a cough that started the day after the vaccination that resolved in 9 days. Overall, no clinically meaningful blood count changes were observed. A transient reduction of neutrophils with concomitant increase in monocytes was detected at day 2 in most volunteers in all age and vaccine dose groups that mostly reverted to predose values by week 1 (fig. S1). Other hematological parameters were mostly unaffected (fig. S1).

Binding antibodies to SARS-CoV-2 spike protein were elicited after vaccination

Antibody responses to GRAd-COV2 vaccination were primarily monitored by a clinically validated CLIA, revealing similar kinetics of anti–spike protein IgG induction in all study groups (Fig. 3A and table S1). Seroconversion for spike protein occurred at week 2, when anti–spike protein IgG became detectable in 23 (52%) of 44 younger volunteers and in 15 (33%) of 45 older age participants. At week 4 after vaccination, high concentrations of anti–spike protein IgG were measurable in 40 (91.0%) of the 44 analyzed younger and in 42 (93.3%) of the 45 older age volunteers. In the younger age cohort, three volunteers (one in the intermediate dose arm and two in the high-dose arm) showed a weak increase of anti–spike protein IgG [10.8, 11.5, and 12.8 arbitrary units (AU)/ml, respectively] that were below the diagnostic cutoff applied in the clinical practice (15.0 AU/ml). Only one volunteer in the low-dose arm showed anti–spike protein IgG below the assay limit of detection (LOD; <3.8 AU/ml). Also, in the older age cohort, three volunteers (one in low-dose arm, one in intermediate-dose arm, and one in high-dose arm) showed a weak increase of anti–spike protein IgG (5.0, 8.5, and 9.7 AU/ml, respectively) that were below the diagnostic cutoff.

Antibodies measured by CLIA did not show a dose-response relationship in the younger adult cohort (IgG concentrations at week 4 were 59.5, 60.6, and 61.8 AU/ml for low dose, intermediate dose, and high dose, respectively; $P = 0.742$). A dose-response trend was also not observed in the older age cohort (IgG concentrations at week 4 were 31.8, 41.0, and 56.3 AU/ml for low dose, intermediate dose, and high dose, respectively; $P = 0.315$). However, despite the absence of a dose response within the cohort, we observed that low and intermediate vaccine doses were associated with significantly

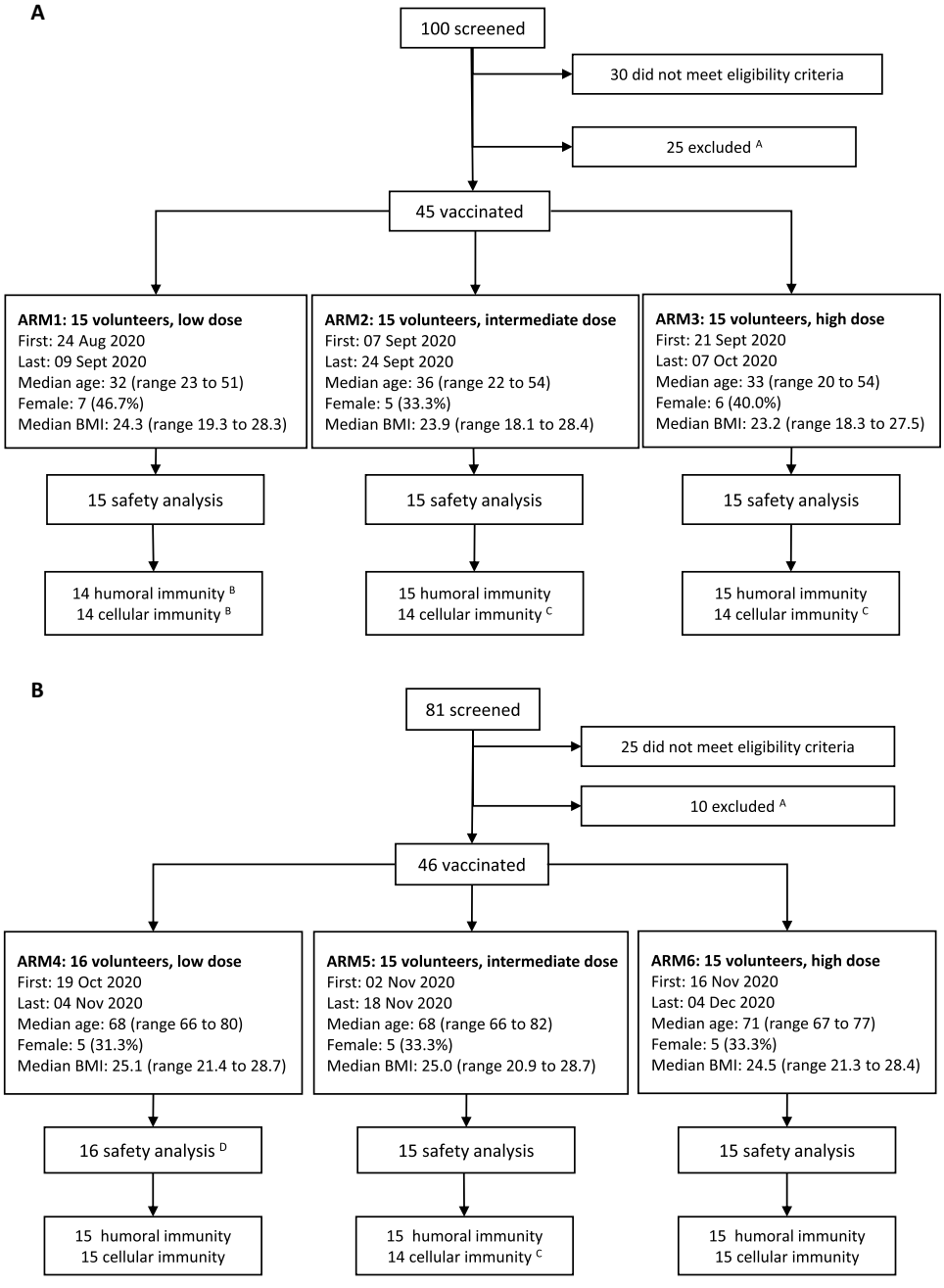


Fig. 1. Study sample selection and analysis. Enrollment, study population characteristics, treatment, and follow-up of the (**A**) 45 volunteers enrolled in younger age cohort (aged 18 to 55 years old) and of the (**B**) 46 volunteers enrolled in older age cohort (aged 65 to 85 years old). Recorded details include age at enrollment in years (median and range) and the body mass index (BMI). [A] indicates that volunteers were not vaccinated because they were lost in the time window between the screening and vaccination visit. [B] indicates that one volunteer was excluded from humoral and cellular immunity analysis because of SARS-CoV-2 infection immediately before or after vaccination. [C] indicates volunteers excluded from ELISpot analysis only because of high nonspecific IFN-γ secretion. [D] indicates that one volunteer was excluded from immunogenicity analysis and replaced because of nonspecific reactivity in an anti–spike protein CLIA assay.

lower IgG responses in older-age volunteers (low-dose arms median IgG was 59.4 in younger and 31.8 in older adults, $P = 0.011$; intermediate-dose arms median IgG was 60.6 in younger and 41.0 in older adults, $P = 0.011$). High vaccine dose elicited similar concentrations of IgG among the two age cohorts (high-dose arms median IgG was 61.8 in younger and 56.3 in older adults, $P = 0.917$).

An enzyme-linked immunosorbent assay (ELISA) showed that 89 (98.8%) of 90 volunteers developed detectable anti–spike protein IgG, including both antibodies against the whole spike protein and against the receptor binding domain (RBD) at 4 weeks after vaccination (Fig. 3, B and C, and table S1). None of the study volunteers showed seroconversion to N protein during the 4 weeks after vaccination, suggesting that no SARS-CoV-2 infection occurred.

Neutralizing antibodies against SARS-CoV-2 were elicited by GRAd-COV2 vaccination

Neutralizing antibodies to SARS-CoV-2 were assessed by two different in vitro assays, both using SARS-CoV-2 live virus. No SARS-CoV-2 neutralizing antibodies were detected at baseline by a microneutralization assay (MNA). Four weeks after vaccination, serum neutralizing antibodies were detected in 25 of 44 (56.8%) younger volunteers and in 33 of 45 (73.3%) older volunteers (Fig. 3D). Anti–SARS-CoV-2 neutralizing antibodies were below the LOD in 8, 5, and 6 younger and in 3, 5, and 4 older volunteers in the low-dose, intermediate-dose, and high-dose arms, respectively.

A more sensitive plaque reduction neutralization test (PRNT) revealed SARS-CoV-2 neutralizing antibodies in 42 of 44 (92.5%) younger and in 45 of 45 (100%) older volunteers (Fig. 3E). The two younger volunteers with undetectable neutralizing antibody were in the low-dose arm. In the older adult cohort, we observed a significant dose-dependent increase in the concentration of neutralizing antibodies (median $PRNT_{50}$ titer 38.0, 35.0, and 61.0 in the low-dose, intermediate-dose, and high-dose arms, respectively; $P = 0.048$). A dose-response trend was not observed across the arms in the younger adult cohort (median $PRNT_{50}$ titer of 23.5, 31.0, and 41.0 in the low-dose, intermediate-dose, and high-dose arms, respectively; $P = 0.197$). Across all arms, titers of binding and neutralizing antibodies elicited by GRAd-COV2 vaccination were in the range of those measured in convalescent individuals who recovered from mild cases of COVID-19 (Fig. 3, A to D, and table S1). We calculated the ratio between geometric mean antibody titers (GMT) in volunteers receiving intermediate or

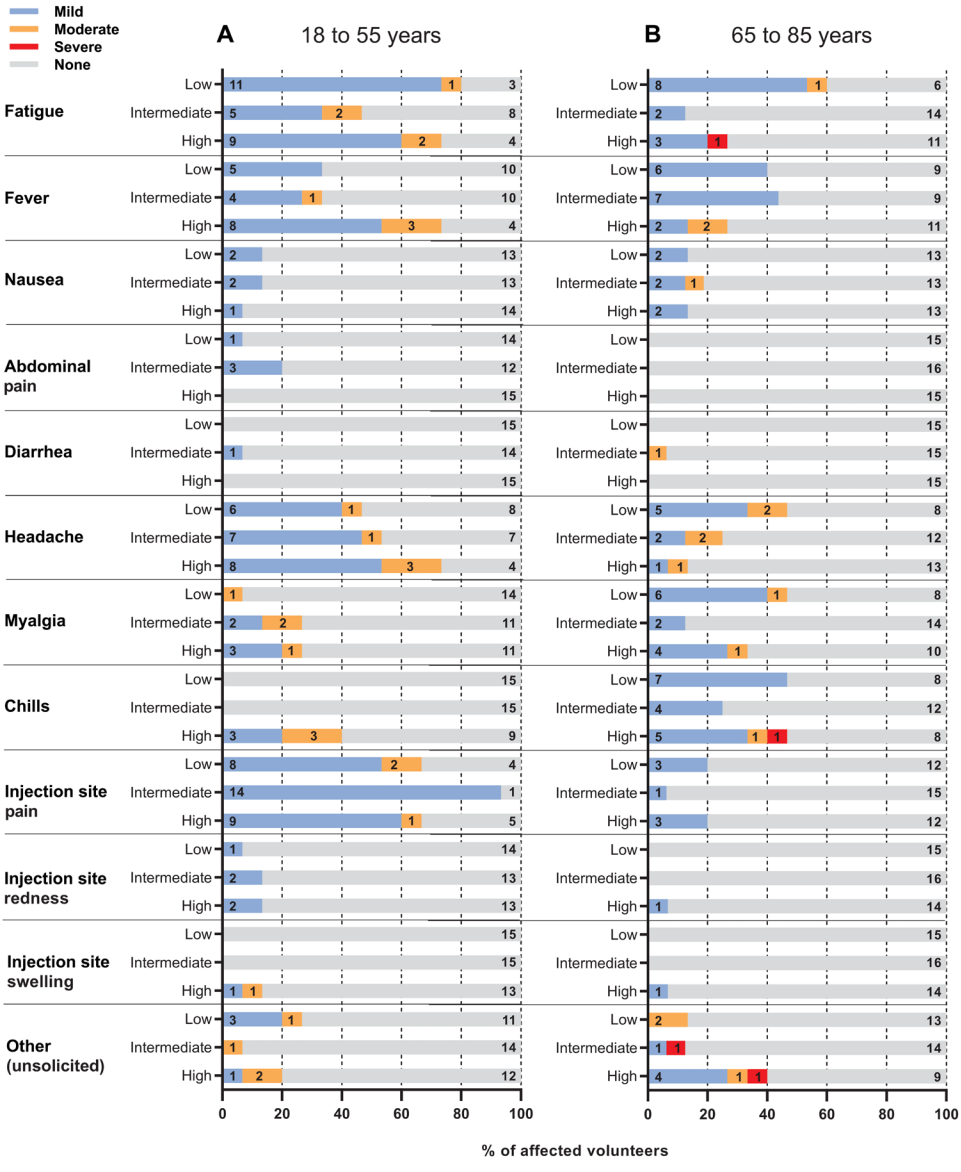


Fig. 2. AEs were recorded within 28 days after vaccination. The proportion of volunteers in each study arm experiencing specific adverse event (AE), and reported at the maximal severity, is shown. The severity of AE is reported according to the intensity scale in the protocol. (**A**) AEs in the younger adult cohort are shown. (**B**) AEs in the older adult cohort are shown. Low indicates the low-dose arm (5×10^{10} viral particles), intermediate indicates the intermediate-dose arm (1×10^{11} viral particles), and high indicates the high-dose arm (2×10^{10} viral particles).

high GRAd-COV2 dose and GMT of convalescent patients with COVID-19 of any severity. The ratios ranged between 0.7 and 1.2 for spike protein and RBD binding antibodies by ELISA and were 0.76 for neutralizing antibodies on the basis of $PRNT_{50}$ values (table S2). These ratios are similar to those reported for other adenovirus-based vaccines whose clinical efficacy has been demonstrated (*6*, *7*).

According to a correlation matrix computed on the main immunological readouts of this study (fig. S2), a strong positive correlation was observed among all serological tests (neutralization, CLIA, anti–spike protein ELISA, and anti-RBD ELISA). Correlation was strongest among assays detecting binding antibodies (CLIA and ELISA to spike protein and RBD; *r* values between 0.89 and 0.94, all $P \leq 0.0001$) and within the two neutralization assays ($r = 0.8$, $P \leq 0.0001$). Correlation was significant but less strong within binding and neutralizing assays (*r* values between 0.5 and 0.66; all $P \leq 0.0001$).

T cell responses were induced by GRAd-COV2 vaccination

A quantitative IFN-γ ELISpot assay was used to assess T cell response on fresh peripheral blood mononuclear cells (PBMCs) isolated from volunteers in both cohorts at week 2 after vaccination. GRAd-COV2 administration at all three doses induced potent spike protein–specific IFN-γ–producing T cell response in both cohorts (Fig. 4A and table S1). Individual responses ranged between 87 and 10,560 IFN-γ spot-forming cells (SFC) per million PBMCs in younger adults and between 283 and 18,877 in older individuals. In total, 80% of individuals evaluated across the two age cohorts showed a response of more than 1000 SFC per million PBMCs. Only one volunteer in arm 1 did not show a detectable spike protein–specific T cell response but still had measurable antibodies to spike protein and RBD (ELISA titer of 1494 and 721, respectively).

A similar T cell response was observed in the younger cohort across all three doses of vaccine (median response of 1162, 2857, and 2272 SFC per million PBMCs in the low-dose, intermediate-dose, and high-dose arms, respectively; $P = 0.154$). Likewise, all three doses elicited a similar T cell response in the older cohort (median response of 1917, 2262, and 3142 SFC per million PBMCs in the low-dose, intermediate-dose, and high-dose arms, respectively; $P = 0.206$). Moreover, we did not observe differences between the younger and older study arms receiving the same vaccine dose (*P* values were 0.116, 0.984, and 0.152 for low dose, intermediate dose, and high dose, respectively).

T cell response induced by vaccination was directed to multiple epitopes, because most of the volunteers in both cohorts had a detectable response against all four spike peptide pools analyzed (fig. S3). Moreover, we found that all regions of the spike protein had a similar degree of immunogenicity in both age cohorts (Fig. 4B). Spike protein–specific T cell response was generally higher in GRAd-COV2–vaccinated individuals than in SARS-CoV-2 convalescent controls that were sampled 1 to 2 months after symptoms onset (Fig. 4A). In addition, T cell immunity moderately correlated with the serological assays ($r = 0.38$ to 0.47, $P = 0.0003$ to <0.0001), suggesting that GRAd-COV2 vaccination induced

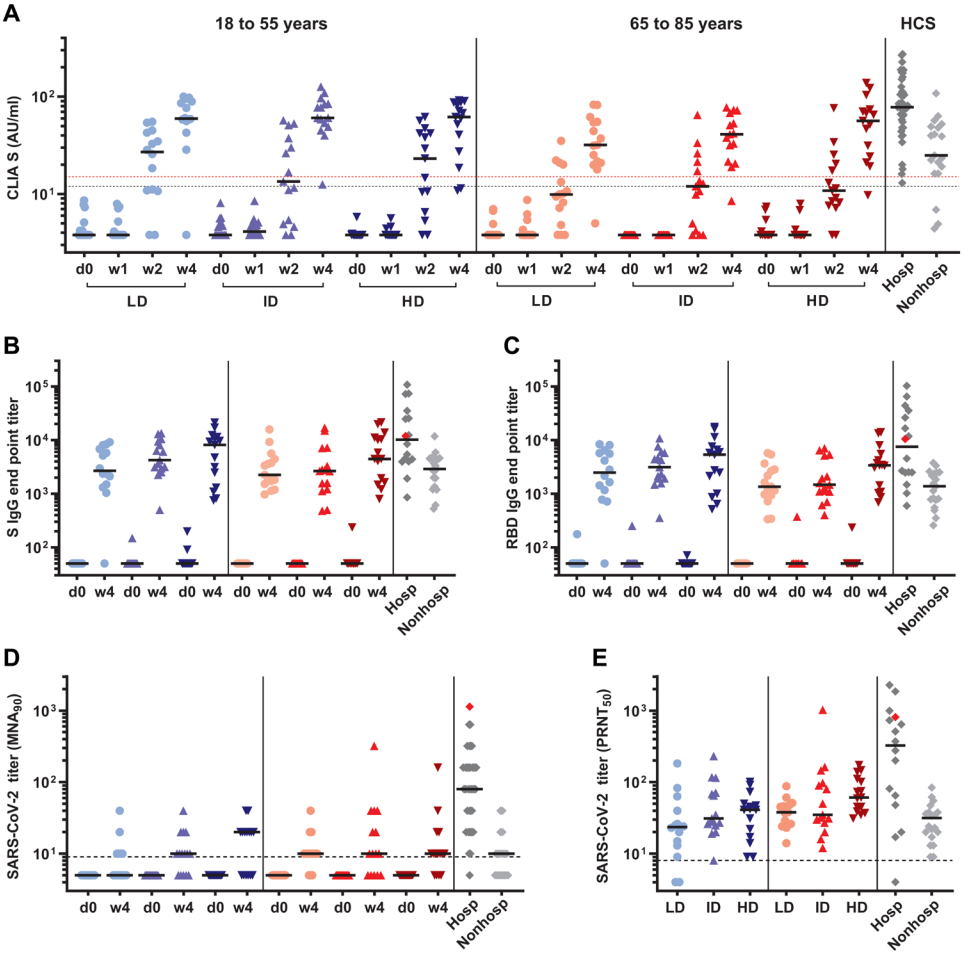


Fig. 3. SARS-CoV-2–specific binding and neutralizing antibody responses were elicited in GRAd-COV2–vaccinated volunteers. Antibody response to SARS-CoV-2 induced by GRAd-COV2 vaccination at low dose (LD; 5×10^{10} viral particles; circles), intermediate dose (ID; 1×10^{11} viral particles; upright triangles), and high dose (HD; 2×10^{11} viral particles; upside-down triangles) are shown. (**A**) IgG binding to S1/S2 was measured by CLIA at the day of vaccination (d0) and 1, 2, and 4 weeks (w1, w2, and w4, respectively) after vaccination. Data are expressed as arbitrary unit (AU)/ml. Blue and red dashed lines are set at 12 and 15 AU/ml. According to the manufacturer, results greater than 15 are clearly positive, between 12 and 15 are equivocal, and less than 12 are negative or may indicate low abundance of IgG antibodies to the pathogen. (**B** and **C**) SARS-CoV-2–specific IgG titers in the serum collected at d0 and w4 after vaccination measured by ELISA on recombinant full-length spike protein (B) or RBD (C). Data are expressed as end point titer, and, for negative serum where a titer cannot be calculated, an arbitrary value of 50 (or one-half of the first serum dilution tested) was assigned. (**D** and **E**) SARS-CoV-2 neutralizing antibodies at w4 after vaccination were detected by SARS-CoV-2 microneutralization assay (MNA) (D) or by plaque reduction neutralization test (PRNT) (E). SARS-CoV-2 neutralization titers are expressed as MNA_{90} and $PRNT_{50}$ or the reciprocal of serum dilution achieving 90 or 50% neutralization, respectively. Dashed lines indicate limit of detection (LOD), and negative serum samples were assigned a value of one-half the LOD. Blue and red color shades identify younger and older age cohorts, respectively. Horizontal black lines are set at median across all panels. HCS indicates human convalescent serum (diamonds), obtained from either previously hospitalized (hosp; dark gray) or from nonhospitalized (nonhosp; light gray) patients with COVID-19. NIBSC 20/130 standard plasma (red diamond) is shown for reference.

coordinated antibody and T cell responses to the encoded spike protein (fig. S2).

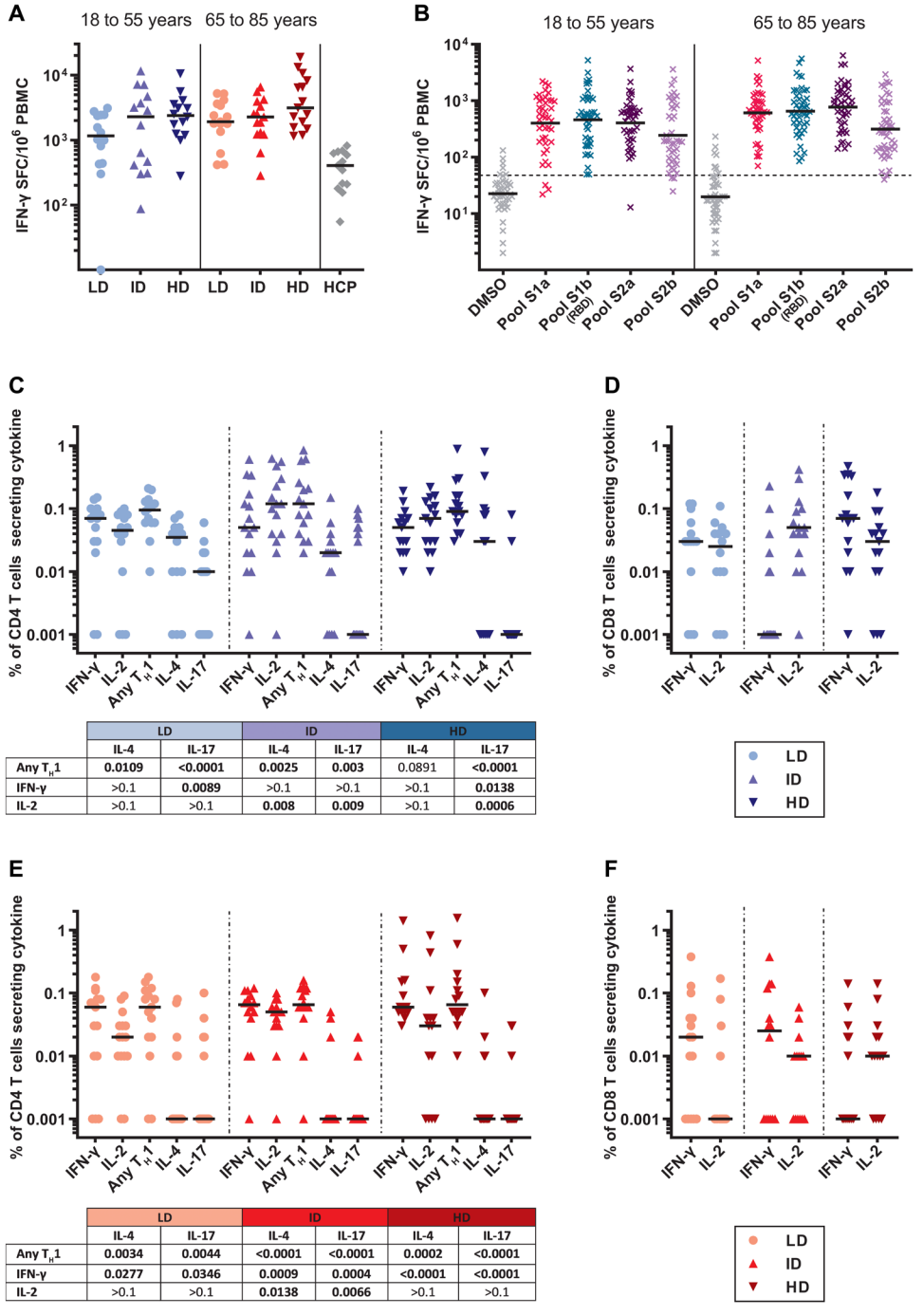
T cell kinetics was analyzed using cryopreserved PBMCs (fig. S4). The amount of baseline T cells cross-recognizing SARS-CoV-2 spike peptides, most likely memory T cells derived from past seasonal coronaviruses infections, increased with age. After GRAd-COV2 vaccination, T cell responses were expanded between a median of 9- and 15-fold in younger-age volunteers, whereas the fold expansion was clear but more limited in older age arms (median of three- to fourfold). The peak varied between weeks 2 and 4 in individual volunteers, but, overall, the T cell response was stable in the first month after vaccination.

Intracellular staining for cytokine production and flow cytometry analysis revealed that vaccine-induced responses involved both spike protein–specific CD4 and CD8 T lymphocytes in younger (Fig. 4, C and D) and older volunteers (Fig. 4, E and F), with higher frequencies of CD4 T cells relative to CD8 T cells. Among GRAd-COV2 vaccine-induced spike protein–specific CD4, IFN-γ production was more prominent than interleukin-4 (IL-4) and IL-17 in both age cohorts, indicating that the vaccine induced a predominantly T helper 1 cell (T_H1) response.

Antivector antibodies were observed at baseline in a subset of individuals and expanded by GRAd-COV2 vaccination

GRAd neutralization was not set as a screening or a randomization parameter but was assessed as an exploratory end point at baseline and 4 weeks after vaccination. Of 90 volunteers, we measured neutralization titers of >200, a value above which an impact on vaccine immunogenicity has been reported, in only 10 volunteers (11%). As expected, vaccination with GRAd-COV2 induced or boosted antivector immunity in most volunteers (Fig. 5A), with no differences attributable to vaccine dose or age.

To assess the impact of anti-GRAd immunity, pooled immunogenicity data at peak after vaccination (T cell responses or binding and neutralizing antibody responses) were stratified according to the baseline GRAd-neutralizing titers (Fig. 5, B to G). Reduced spike protein–binding antibody response to the vaccine antigen was noted in volunteers with GRAd neutralizing antibody titers of >200 at baseline, with significantly lower IgG titers as measured by ELISA ($P = 0.018$ on full-length spike protein and $P = 0.0145$ on RBD). Conversely, vaccine-induced immune responses in individuals with GRAd neutralizing antibody titers in the 18 to 200 range at baseline were undistinguishable or similar to those of volunteers with no antivector immunity. Last, antibody responses to spike and RBD, as measured by ELISA, were inversely correlated with antivector antibody titers at baseline ($r = -0.24$, $P = 0.026$ for spike protein and $r = -0.24$, $P = 0.024$ for RBD; fig. S2).



	LD		ID		HD	
	IL-4	IL-17	IL-4	IL-17	IL-4	IL-17
Any T_H1	0.0109	<0.0001	0.0025	0.003	0.0891	<0.0001
IFN-γ	>0.1	0.0089	>0.1	>0.1	>0.1	0.0138
IL-2	>0.1	>0.1	0.008	0.009	>0.1	0.0006

	LD		ID		HD	
	IL-4	IL-17	IL-4	IL-17	IL-4	IL-17
Any T_H1	0.0034	0.0044	<0.0001	<0.0001	0.0002	<0.0001
IFN-γ	0.0277	0.0346	0.0009	0.0004	<0.0001	<0.0001
IL-2	>0.1	>0.1	0.0138	0.0066	>0.1	>0.1

Fig. 4. SARS-CoV-2 spike protein–specific T cell responses were induced by GRAd-COV2 vaccination. T cell response to spike peptides were induced by GRAd-COV2 vaccination at low dose (LD; 5×10^{10} viral particles; circles), intermediate dose (ID; 1×10^{11} viral particles; upright triangles), and high dose (HD; 2×10^{11} viral particles; upside-down triangles). Blue and red color shades identify younger and older age cohorts, respectively. (**A** and **B**) IFN-γ ELISpots were performed using freshly isolated PBMCs at w2. Data are expressed as IFN-γ spot-forming cells (SFC) per 10^6 PBMCs. In (A), individual data points represent cumulative spike protein–specific T cell response, calculated by summing the response to each S1a, S1b, S2a, and S2b peptide pool stimulation and correcting for background (DMSO stimulation) in each volunteer. HCP indicates freshly isolated human convalescent PBMCs obtained from individuals who recovered from symptomatic SARS-CoV-2 infection. (B) The distribution of IFN-γ ELISpot responses to individual spike peptide pools is shown. Dashed line indicates assay positivity cutoff (48 SFC per million PBMCs). (**C** to **F**) IFN-γ, IL-2, IL-4, and IL-17 cytokine production was evaluated at w2 on fresh PBMCs isolated from younger (C and D) and older volunteers (E and F). Data are expressed as the percentage of spike protein–specific CD4 T cells (C and E) or CD8 T cells (D and F) secreting each cytokine. Any T_H1 indicates the sum of CD4 secreting IFN-γ alone, IL-2 alone, and both IFN-γ and IL-2, obtained by summing responses to each of the four spike peptide pools and corrected for background (DMSO stimulation). The tables below the CD4 T cell plots show *P* values derived by Kruskal-Wallis testing comparing T_H1, T_H2, and T_H17 profiles within each dose group. Horizontal black lines are set at median across all panels.

GRAd-COV2–induced immunity is not abrogated against SARS-CoV-2 variants

SARS-CoV-2 variants emerged soon after the pandemic's onset, and assessing the degree of cross-reactivity of vaccine-induced humoral and cellular immune responses to spike proteins from the most relevant variants is of importance. A subset of 12 serum samples were evaluated at 4 weeks after vaccination for their ability to neutralize SARS-CoV-2 variants of concern (VOCs), lineage alpha (B.1.1.7) and gamma (P.1), and compared to the neutralization titers obtained using the reference strain (WA1/2020). Similar neutralization titers were observed when comparing the reference WA1/2020 and alpha, whereas a significantly lower titer (median twofold) was detected against the gamma variant ($P = 0.04$; Fig. 6A). In addition, cryopreserved PBMCs from the same volunteers were analyzed by ELISpot assay using different peptide pools spanning the spike antigens of the WA1/2020 and of the indicated VOCs (Fig. 6B). The results showed a robust T cell response against the reference strain in all the 12 samples analyzed, and T cell responses of similar strength were observed in response to alpha, beta, gamma, and epsilon variant peptide pools.

DISCUSSION

Here, we report first-in-human data on the safety and immunogenicity of a single administration of GRAd-COV2 given at different

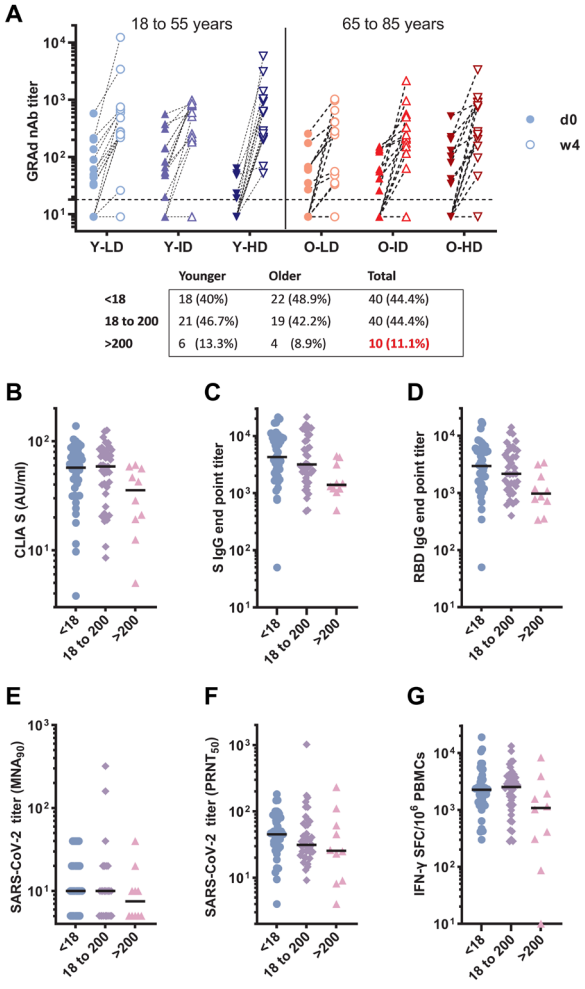


Fig. 5. Neutralizing titers against GRAd were observed in a subset of individuals before and after vaccination. (**A**) Neutralizing titers to the GRAd vector measured in serum from vaccinated volunteers the day of vaccination (d0; filled symbols) and 4 weeks after vaccination (w4; open symbols). The dashed lines set at 18 indicate the assay LOD. The table reports the number (and percentage) of serum samples with GRAd neutralizing antibody (nAb) titer in the indicated range (<18, 18 to 200, or >200) in younger or older individuals and overall across the two age cohorts of volunteers ($N = 90$). (**B** to **D**) Participants were stratified according their GRAd neutralizing antibody serostatus at baseline, irrespective of age cohort and vaccine dose received, and for each stratum, the antibody response is shown at w4 by CLIA for spike protein–specific IgG (B), by ELISA for spike protein–specific antibodies (C), and by ELISA for RBD-specific antibodies (D). (**E** and **F**) The SARS-CoV-2 neutralizing antibody response is shown stratified by GRAd antibody serostatus at w4 by MNA_{90} (E) and $PRNT_{50}$ (F). (**G**) The T cell response is shown stratified by GRAd antibody serostatus at w2 by IFN-γ ELISpot. Horizontal black lines are set at median (B to G).

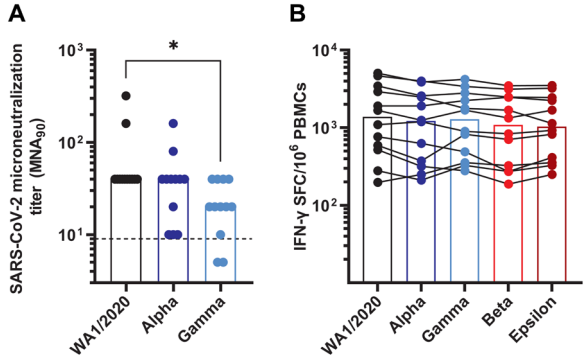


Fig. 6. GRAd-CoV2 elicits immune responses that react to SARS-CoV-2 VOCs. (**A**) Serum samples from 12 volunteers across all study arms 4 weeks after vaccination were analyzed by MNA assay with the reference strain (WA/2020) or the alpha or the gamma variant strains. SARS-CoV-2 neutralization titers are expressed as MNA_{90}. Dashed line indicates the LOD, and negative sera are assigned a value of one-half of the LOD. (**B**) IFN-γ ELISpot was conducted using frozen PBMCs with peptide pools covering the full-length spike protein from the prototype WA1/2020 and the alpha, gamma, beta, and epsilon variant strains. Data are expressed as IFN-γ SFC/10^6 PBMCs. Data points represent cumulative spike protein–specific T cell responses in PBMCs isolated from each individual, summing the response to S1 and S2 peptide pools of each strain and correcting for background (DMSO). Bars in (A) and (B) are set at median. Statistical analysis was done using Friedman's test with Dunn's correction for multiple comparisons (A and B). *$P < 0.05$.

doses in healthy younger adults aged 18 to 55 years old and in healthy older adults aged 65 to 85 years old. GRAd-COV2 is a COVID-19 candidate vaccine based on a replication-defective gorilla adenovirus vector. Our analyses provide evidence that GRAd-COV2 is well tolerated in both younger and older age cohorts at all three doses assessed. Most of the observed AEs were short lived and mild or moderate in intensity. Only two volunteers reported at least one severe AE and none reported serious AEs. Solicited AEs were less common in older-age volunteers, similarly to what was described for other COVID-19 vaccines (*8*–*10*). In general, the AE profile did not differ from those reported in published work for other vector-based vaccines (*10*–*15*), although they were milder than those reported for mRNA formulated in lipid nanoparticles and for adjuvanted vaccines (*8*, *16*, *17*).

The vaccine was immunogenic at all doses, inducing seroconversion in most participants of both age groups. Onset of immune responses was rapid with T cell responses already detectable 14 days after vaccination and antibodies titers increasing to day 28. We used two assays to measure neutralizing antibodies against live SARS-CoV-2 in vaccinated volunteers, which exploit different virus strains and readouts. An MNA based on cytopathic effect (CPE) assessment provided evidence that 65% of the volunteers had measurable MNA_{90} titers, whereas a more sensitive plaque reduction assay showed $PRNT_{50}$ titers in 98% of vaccinated volunteers. In general, titers of neutralizing antibodies to SARS-CoV-2 were lower than those reported in studies of vaccines based on mRNA (*16*, *18*, *19*) and possibly on other viral vectors such as Ad26 and ChAdOx1 (*10*, *12*). However, the lack of standards and the use of different assays complicate the comparison between different COVID-19 vaccines that are currently in use or in development. A comparison with convalescent serum panels stratified according to COVID-19 severity of the donors showed that the GRAd-COV2 vaccine elicited SARS-CoV-2 binding and neutralizing antibody titers similar to nonhospitalized people who had recovered from symptomatic COVID-19.

According to recent publications (*6*, *7*), the GMT ratio of binding and neutralizing antibodies induced in vaccinated individuals and in convalescent patients with COVID-19 may be predictive of vaccine efficacy. This ratio has been proposed as a surrogate correlate of protection, allowing normalization of immunogenicity data against convalescent patients for any vaccine regardless of the specific assay adopted in each trial to generate the data. The calculated ratio for binding and neutralizing antibodies induced in

GRAd-COV2–vaccinated individuals is in the range of that reported for other adenoviral vector vaccines, such as ChAdOx1-nCoV-19 and Ad26.COV2.S.

A single administration of GRAd-COV2 elicits a robust and T_H1-skewed immune response within 2 weeks that is broadly directed to multiple spike protein epitopes, similar to that induced by a single dose of other vectored COVID-19 vaccines such as ChAdOx1 nCoV-19 (*12*), Ad5-nCoV (*14*, *15*), and Ad26.COV2.S (*10*) and by two doses of mRNA-1273 (*16*, *18*). A robust T cell response, such as that elicited by GRAd-COV2, may determine the duration of anti–COVID-19 immunity (*20*, *21*) and prevent unintended immune reactions such as vaccine-associated enhanced respiratory disease (*22*, *23*). Moreover, spike protein–specific T cell responses directly correlated with binding and neutralizing antibodies, suggesting that the GRAd-COV2 vaccine was able to shape a well-balanced and coordinated cellular- and humoral-specific immune response, similar to what naturally occurs in mild COVID-19 (*24*, *25*).

We detected spike protein–reactive T cells before vaccination in many otherwise SARS-CoV-2 seronegative volunteers, suggesting that these individuals had memory T cells from previous seasonal coronaviruses infections. Further supporting this, the frequency of spike protein–reactive T cells at baseline increased accordingly, increasing with the volunteers' age. Similar findings have been reported for SARS-CoV-2–unexposed individuals in both immunological studies (*26*, *27*) and in COVID-19 vaccine clinical trials (*12*). Although the spike protein–specific T cell response was expanded more vigorously in younger-age compared to older-age volunteers vaccinated with GRAd-COV2, the frequency of virus-specific T cells reached similar frequencies in the two cohorts.

Emerging SARS-CoV-2 variants with amino acid substitutions in the spike protein are becoming predominant, leading to growing concerns over increased transmissibility and decreased vaccine coverage because of immune evasion. Our analysis of a limited set of serum samples from vaccinated individuals suggests that GRAd-COV2–induced neutralizing antibody titers were not reduced against the alpha variant, although were lower against the gamma variant. Several reports have shown the partial resistance of SARS-CoV-2 VOCs to vaccine-induced antibodies (*28*–*34*). Vaccination with Ad26.COV2.S resulted in five- to sevenfold decreases in median inhibitory concentration titers against the beta variant (*35*, *36*), resulting in mean neutralizing antibody titers of 33; this, according to mathematical modeling, could result in decreased protection against infection (*7*). However, published phase 3 trial data showed that a single dose of Ad26.COV2.S provided 64.0% protection against moderate to severe disease and 81.7% against severe to critical COVID-19 in a country where 95% of circulating SARS-CoV-2 was the beta variant (*37*). This real-world evidence suggests a role for the T cell response or for non-neutralizing antibody Fc-mediated effector functions in maintaining protection against the partially neutralizing antibody–resistant beta variant.

As expected, evidence is emerging that SARS-CoV-2 may evade immune pressure by mutating epitopes recognized by CD8 T cells at the individual level (*38*). However, these mutations are not often fixed at the population level, possibly because of selective advantage for the variant virus limited to individuals with the relevant allele(s) only. The cellular response induced by natural SARS-CoV-2 infection and vaccination, as reported here for GRAd-COV2, targets many epitopes (*27*) and is composed of both CD4 and CD8 T cells. CD4 T cell epitopes are more promiscuous in terms of major histocompatibility complex class II restriction and are less affected by single amino acid mutations. Our observation of a cross-reactive T cell response to spike at baseline in most of our SARS-CoV-2 spike and N seronegative volunteers also suggests that a subset of the T cells are memory T cells developed after prior exposure to seasonal coronaviruses. This also suggests that T cell responses are induced toward conserved epitopes. A study on COVID-19 convalescent individuals or recipients of mRNA vaccines (*39*), as well as a report on INOVIO INO-4800 DNA vaccine recipients (*40*), shows that spike protein–specific T cell responses are only marginally affected by mutations present in the current VOCs. Similarly, we show here that GRAd-COV2–induced T cell responses were largely cross-reactive against all the tested VOCs. A single dose of GRAd-COV2 induced higher median T cell frequencies specific for both WA1/2020 and VOCs than those measured in convalescent individuals with COVID-19 and in recipients of mRNA or DNA vaccines after the second dose (about 1000 SFC for GRAd-COV2 recipients versus about 100 SFC for samples from convalescent individuals and other vaccine recipients).

The description of a rare but serious and potentially lethal complication of vaccine-induced thrombotic thrombocytopenia (VITT) among recipients of ChAdOx1 and Ad26.COV2.S vaccination gave rise to the question of whether this is a potential class effect of all adenoviral vector vaccines (*41*). No corresponding signals have emerged for Sputnik 5 or CanSino vaccines, but, so far, there is lack of detailed information on safety data. VITT resembles heparin-induced thrombocytopenia, because it is associated with the induction of platelet-activating autoantibodies against platelet factor 4 (PF4) (*42*). However, a recent study revealed that PF4 autoantibodies detectable by an ELISA are occasionally elicited by vaccination with both mRNA- and adenoviral vector–based COVID-19 vaccines in the absence of clinical manifestations (*43*). Therefore, because a positive anti-PF4/polyanion ELISA result alone is not sufficient to diagnose VITT, we have not included this analysis in the present study.

Different mechanisms have been evoked for the adenovirus vector–induced VITT, which are currently under active investigation (*44*–*46*). However, it would seem unlikely that VITT constitutes a class effect for all adenoviral vector vaccines, because differences among vaccines regarding the adenovirus strain, the spike protein inserts, the manufacturing process, and the formulation should theoretically affect the risk of VITT manifestation (*47*). Different adenovirus strains may bind to distinct cellular receptors and, hence, may infect a different spectrum of host cells (*47*).

Adenoviral vector vaccines still constitute the bulk of the vaccination program in many countries globally, because of the low costs of a full vaccination course and the considerably simpler storage and transport conditions than those of their mRNA counterparts (*48*). Furthermore, a single-dose vaccine has provided a viable solution for the rapid achievement of adequate coverage rates of geographically remote areas. Until the complication of VITT is understood, the risk-benefit ratio of continued use of adenoviral vector vaccines or their restriction to specific age groups or complete withdrawal will depend on the status of viral shedding in the community, and thus should be constantly and dynamically evaluated for their benefits and risks.

As for other candidate vaccines using viral vectors, preexisting immune responses against the vector can compromise the induction of an immune response against the target antigen (*49*, *50*), and

this was also shown for a candidate COVID-19 vaccine based on the common human Ad5 serotype (*15*). For simian adenoviruses, preexisting immunity in humans is mostly because of cross-reactive antibodies originated upon human adenovirus exposure, as suggested by lower frequency of occurrence and lower neutralizing antibody titers compared to those measured against common human adenoviruses, such as Ad5 (*2*). In our study, high titers of neutralizing antibodies against GRAd-COV2 were associated with a reduced median T cell response and reduced concentrations of antibodies specific to the spike protein. However, the presence of preexisting anti-GRAd antibodies did not abrogate the immune response in most of the volunteers. These findings highlight the potential utility of vectors such as GRAd, for which preexisting immunity is infrequent in the human population.

So far, preexisting immunity to GRAd was only assessed in serum samples from the United States (*2*) and Italy. However, it is well known and documented for the adenovirus of both simian and human origin (*51*–*53*) that the prevalence of preexisting immunity is higher in geographical regions such as sub-Saharan Africa or Southeast Asia, possibly related to poorer sanitary conditions. The frequencies of preexisting immunity to Ad5 and other human adenoviruses can vary depending on the serotype and the study population, including its age and place of residency; for example, about 35% of adults living in the United States have neutralizing antibodies of any titer to Ad5, whereas prevalence rates increase to more than 90% in individuals living in Cote d'Ivoire (*53*, *54*). Antibody prevalence to the rare Ad26 serotype is less than 20% in the United States and Europe, but more than 90% in South Africa (*55*, *56*). It will be important therefore to extend GRAd seroprevalence studies to world regions outside Europe or the United States that are more likely to benefit from adenovirus-based COVID vaccines in the near future.

Our interim analysis indicates that vaccine candidate GRAd-COV2 is safe and immunogenic in both younger and older adults. This finding has supported and guided our decision to proceed with an ongoing phase 2/3 trial (NCT04791423) to evaluate the efficacy of either a single-dose or of a two-dose regimen of GRAd-COV2. Experience from other ongoing clinical trials on adenovirus-based vaccines provided evidence that a second administration of vaccine is safe and can improve the production of SARS-CoV-2 neutralizing antibodies (*12*, *13*). The observed increase of anti-GRAd neutralizing antibody titers after vaccination is in line with that observed with other adenovirus-based COVID-19 vaccines (*9*, *10*), which did not prevent boosting of anti–spike protein antibodies upon a second administration. Note that most of the COVID-19 vaccines require a prime-boost scheme, including nonreplicating viral vectored vaccines, mRNA, and adjuvanted subunit vaccines.

This study has some limitations because of the low number of volunteers per arm ($n = 15$) and the lack of participant randomization among study arms on the basis of GRAd neutralizing titer at baseline. Both these aspects could have had an impact in the poor vaccine dose-response effect observed. However, the explored dose range (fourfold between low and high dose) was limited, and no major dose effect was expected. Nevertheless, a high-dose vaccine (2×10^{11} viral particles) was selected for an ongoing phase 2/3 single-dose trial, because of higher and more consistent immunogenicity especially in the elderly cohort. As for studies evaluating a two-dose regimen, the intermediate dose of 1×10^{11} viral particles was selected, because it represents the best compromise between tolerability and immunogenicity. Another limitation lies in the choice of immunological assays that, at the present stage, do not allow for easy comparison with immunogenicity of other vaccines. Last, data beyond week 4 after vaccination on persistence of humoral and cellular responses will be part of a future report once the study follow-up is completed.

In summary, we present here favorable interim safety and immunogenicity results of GRAd-COV2 phase 1 trial. GRAd-COV2 was well tolerated and induced humoral and cellular responses to SARS-CoV-2 spike antigen similarly in younger and older adults. Thus, GRAd-COV2 merits further consideration as a SARS-CoV-2 vaccine candidate.

MATERIALS AND METHODS

Study design

This study is a phase 1, dose-escalation, open-labeled clinical trial designed to determine the safety and immunogenicity of GRAd-COV2. The study included two age cohorts, of either younger (18 to 55) or older (65 to 85) adults. No formal sample size calculation was carried out. Each cohort consisted of three arms of 15 volunteers each for assessing a single administration at three different doses of GRAd-COV2: low dose (5×10^{10} viral particles), intermediate dose (1×10^{11} viral particles), and high dose (2×10^{11} viral particles). We report here the safety and immunogenicity end points collected in the first 4 weeks after vaccination for the volunteers enrolled in both age cohorts, as foreseen in the study protocol (interim analyses 1 and 2). The full study protocol is provided in the Supplementary Materials. Eligible participants were healthy adults aged 18 to 55 years old with no history of COVID-19, no laboratory findings suggestive of current or previous infection with SARS-COV-2, and who have attended the screening visit no more than 21 days before vaccination.

GRAd-COV2 (ΔE1, ΔE3, ΔE4) was manufactured by ReiThera under good manufacturing practice conditions in the proprietary cell line ReiCell35S [a suspension-adapted packaging cell line derivative of human embryonic kidney–293 (HEK293) 293] and purified by an extensive downstream process including host cell DNA precipitation, depth filtration, two chromatographic purification steps followed by nuclease digestion, and ultrafiltration. The clinical material was finally formulated in formulation buffer at a concentration of 2×10^{11} viral particles/ml. The volunteers received a single intramuscular injection in the deltoid. For administration of the high dose, 1 ml of GRAd-COV2 was injected without dilution. For low-dose and intermediate-dose arms, the vaccine was diluted in sterile saline solution to reach a final 1-ml injection volume. Volunteers were expected to attend several visits, at day 2 and weeks 1, 2, 4, 8, 12, and 24 after vaccination. During the visit, the volunteers underwent blood sampling and medical evaluation. Participants also discussed with a doctor any potential AEs.

The dose-escalation staggered enrollment included three sentinel volunteers in the low-dose arm followed by enrollment of the full low-dose arm. Intermediate- and high-dose sentinel volunteers were enrolled 7 days after that safety data of low-dose or intermediate-dose arms were available, respectively. All the enrollment stages were supervised by an independent data safety monitoring board. Volunteers recorded local and systemic reactions on a diary card for 28 days. The severity and relatedness with vaccination or AEs were assessed by the medical team in each center.

Safety assessment

Solicited AEs are foreseeable AEs after vaccination with GRAd-COV2 and include injection site pain, erythema, swelling, myalgia, headache, fatigue, fever, chills, nausea, vomiting, diarrhea, ulceration, and abdominal pain. All these events were recorded on the diary card by volunteers and investigated by a doctor at each visit. Unsolicited AEs are all those events that were directly reported by the volunteers or emerged in any other way during visits. Severity of AEs has been assessed according to the Common Terminology Criteria for Adverse Events Version 5 into mild, moderate, and severe. A serious AE is an event that results either in death, life-threatening condition, persistent disability/incapacity, hospitalization, or congenital anomaly/birth defect (specific definitions are reported in the supplementary materials).

As comparator for immunogenicity analysis, we used three independent sets of anonymized specimens (serum and PBMCs) from patients with COVID-19 either hospitalized or recovering from mild symptomatic disease, collected 20 to 60 days after symptom onset. A reference anti–SARS-CoV-2 plasma sample from National Institute for Biological Standards and Control (NIBSC; code 20/130), acquired from a donor who recovered from COVID-19, was included as a positive control.

Ethical statement

All participants provided written informed consent before enrollment. The trial was conducted at the National Institute for Infectious Diseases Lazzaro Spallanzani (INMI) in Rome and at Centro Ricerche Cliniche in Verona. The study was conducted according to the Declaration of Helsinki and was approved by the Italian Regulatory Drug Agency (AIFA) and the Italian National Ethical Committee for COVID-19 clinical studies (ClinicalTrials.gov, NCT04528641; European Union Drug Regulating Authorities Clinical Trials Database, 2020-002835-31). Serum samples from convalescent patients who resolved SARS-CoV-2 infections came from residual specimens used for diagnostic purposes and were used according to INMI protocols for observational studies approved form internal ethical committee.

SARS-CoV-2 anti–spike protein and anti–N protein IgG high-throughput chemiluminescence immunoassay

Two commercial assays were used according to the manufacturer's protocols. DiaSorin LIAISON SARS-CoV-2 S1/S2 IgG test is a CLIA detecting anti-S1/S2 IgG on LIAISON XL analyzers. IgG antibody concentrations are expressed as AU per ml, with AU/ml $\geq$ 15 considered as positive. The Abbott SARS-CoV-2 assay is a chemiluminescence microparticle assay detecting anti–N protein IgG and is run on an Abbott ARCHITECT i2000SR. An index value sample cutoff $\geq$ 1.4 was considered positive. Assays were performed according to the manufacturer's instructions.

SARS-CoV-2 spike and RBD ELISA

SARS-CoV-2 ELISA was developed using either SARS-CoV-2 full-length soluble prefusion stabilized spike protein (expressed in Expi293 cells at ReiThera) or a recombinant RBD (expressed in HEK293 cells; ACROBiosystems) as coated antigens. Proteins were coated on Nunc MaxiSorp plates (Thermo Fisher Scientific) at an optimized concentration (5 μg/ml for R121 and 2.5 μg/ml for RBD) in phosphate-buffered saline (PBS) overnight at 4°C. The following day, the plates were washed with PBS 0.05% Tween 20 (PBS-T) and then blocked with PBS-T + 3% nonfat dry milk for 1.5 hours at 25°C with shaking. After wash, serum dilution curves (six threefold serial dilution from 1:100 to 1:24,300) prepared in PBS-T + 1% nonfat dried milk were plated and incubated for 2 hours at 25°C with shaking. Plates were then washed and incubated with 1:2000 diluted anti-human IgG (Fc-specific; Sigma-Aldrich) for 1 hour at 25°C with shaking. Plates were then washed one last time and incubated with an alkaline phosphatase substrate SIGMAFAST (Sigma-Aldrich) at 25°C. Absorbance was read at 405 and 620 nm using an EnSight multiple plate reader (PerkinElmer) at 10, 20, and 30 min; the read with the best *R*-squared value was used to calculate end point titers. The end point titer was defined as the highest serum dilution that resulted in an absorbance value [OD (optical density)] just above the calculated background of fourfold the OD from a secondary antibody alone. A COVID-19 convalescent patient's serum at 1:200 and 1:6400 dilution was included as positive control in each plate to ensure interassay reproducibility. Anti–SARS-CoV-2 plasma (NIBSC, code 20/130) was used as a standard reference in each experiment.

SARS-CoV-2 MNA

Serum samples collected from vaccinated volunteers or convalescent patients with COVID-19 were heat-inactivated at 56°C for 30 min and titrated in duplicate in seven twofold serial dilutions (starting dilution of 1:10). Equal volumes of 100 $TCID_{50}$ (median tissue culture infectious dose) of SARS-CoV-2 (strain 2019-nCoV/Italy-INMI1; GISAID accession ID: EPI_ISL_412974) and serum dilutions were mixed and incubated at 37°C 5% CO_2 for 30 min. Subsequently, 96-well tissue culture plates with subconfluent VeroE6 cell [American Type Culture Collection (ATCC)] monolayers were infected with 100 μl per well of virus-serum mixtures and incubated at 37°C and 5% CO_2. To standardize interassay procedures, positive control samples showing high (1:160) and low (1:40) neutralizing activity were included in each MNA assay. After 48 hours, microplates were observed using a light microscope for the presence of CPE. The supernatant of each plate was carefully discarded and 120 μl of a crystal violet solution containing 2% formaldehyde was added to each well. After 30 min, the fixing solution was removed by washing with tap water, and cell viability was measured using a photometer at 595 nm (Synergy HTX Multi-Mode Microplate Reader, BioTek). The highest serum dilution inhibiting at least 90% of the CPE was indicated as the neutralization titer and expressed as the reciprocal of serum dilution (MNA_{90}). Serum from the NIBSC with known neutralization titer was used as a reference in MNA (NIBSC, code 20/130). Where indicated, two alternative viral strains were used to test serum sample neutralizing activity against SARS-CoV-2 VOCs (hCoV-19/Italy/LAZ-INMI-129/2020 lineage B.1.1.7, GISAID accession ID: EPI_ISL_765567 and hCoV-19/Italy/LAZ-INMI-216isl/2021 lineage P.1, GISAID accession ID: EPI_ISL_1023524).

SARS-CoV-2 PRNT

As an exploratory end point, the SARS-CoV-2 $PRNT_{50}$ titers of vaccinated volunteers' serum samples were determined by means of a PRNT developed and run at Viroclinics Biosciences. Briefly, a standard number of SARS-CoV-2 (Bav/Pat1/2020 strain) infectious units were incubated with eight twofold serial dilutions of heat-inactivated serum, starting from 1:8 and up to 1:1024. After a 1-hour preincubation, the virus/serum mixtures were inoculated on VeroE6

cells (ATCC) for 1 hour, then washed, and replaced with infection medium, and the cells were left overnight. After 16 to 24 hours, the cells were formalin-fixed, permeabilized with ethanol, and incubated with primary anti–SARS-CoV-2 nucleocapsid monoclonal antibody (Sino Biological) followed by a secondary anti-mouse IgG horseradish peroxidase conjugate (Life Technologies) and TrueBlue substrate, which forms a blue precipitate on positive cells. Images of all wells were acquired by an ImmunoSpot analyzer [Cellular Technology Limited (CTL)], equipped with software capable to accurately count the virus-positive cells. The 50% neutralization titers were calculated according to a method described earlier (*57*). The NIBSC standard 20/130 was used as a reference in the same assay as the RT-COV-2 trial serum samples, with resulting $PRNT_{50}$ of 697 and 934 for younger and older adult assay runs, respectively.

GRAd neutralizing antibody assay

Neutralizing antibody titers in human serum samples were assayed as previously described (*58*). Briefly, 8×10^4 HEK293 cells per well were seeded in 96-well plates the day before the assay. GRAd vector encoding the reporter gene that secreted alkaline phosphatase (SEAP) at a preoptimized multiplicity of infection was preincubated for 1 hour at 37°C alone or with serial dilutions of control or test serum samples. Samples were then added to the 80 to 90% confluent HEK293 cells. After incubation for 1 hour at 37°C, the serum/infection mix was removed and replaced with 10% fetal bovine serum (FBS; Cytiva HyClone) in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific). SEAP expression was measured 24 hours later in cell supernatant by means of the chemiluminescent substrate from the Phospha-Light Kit (Applied Biosystems). Neutralization titers were defined as the dilution at which a 50% reduction of SEAP activity from the serum sample was observed relative to SEAP activity from virus alone.

PBMC isolation and stimulation

Peripheral venous blood (40 ml) was collected in 7 ml of lithium heparin Vacutest blood collection tubes (Kima). PBMCs were isolated from peripheral blood by density gradient centrifugation (Histopaque 1077, Sigma-Aldrich). After separation, PBMCs were suspended in RPMI 1640 (Sigma-Aldrich) supplemented with 10% heat-inactivated highly defined FBS (Cytiva HyClone), 2 mM L-glutamine, 10 mM Hepes buffer (*N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid, Sigma-Aldrich), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Gibco), hereafter termed R10. PBMC count and viability were performed by using Guava Muse (Luminex). PBMCs (12×10^6) were immediately used in ELISpot and intracellular staining experiments (at week 2 visit only). The remaining PBMCs were immediately frozen in FBS plus 10% dimethyl sulfoxide (DMSO).

PBMCs were thawed quickly in 37°C water bath with thawing medium [CTL wash supplemented medium in RPMI 1640 (Sigma-Aldrich) and supplemented with L-glutamine (Gibco) and Benzonase (Merck) (50 U/ml)]. After one wash, PBMCs were resuspended into 50 ml of polypropylene vented cap tubes with prewarm R10 medium, counted, and rested at 2×10^6 cells/ml at 37°C and 5% of CO_2 for at least 16 hours. PBMCs were then counted, resuspended at 4×10^6 cell/ml in R10 medium, and used in ELISpot assays.

A set of 316 15-mer peptides overlapping by 11 amino acids (synthetized by Elabscience Biotech Inc. and distributed by TEMA RICERCA), designed to cover the full-length spike protein, was arranged into four pools (S1a, S1b-including RBD domain, S2a, and S2b).

Freshly isolated PBMCs (2×10^6/ml) were stimulated with the four spike peptides pools (S1a, S1b, S2a, and S2b) for 18 hours (3 μg/ml each peptide final concentration). Thawed PBMCs (2×10^6/ml) were stimulated with two spike peptide pools (S1a + S1b and S2a + S2b) for 18 hours (3 μg/ml each peptide final concentration). For flow cytometry experiments, brefeldin A (10 μg/ml; Sigma-Aldrich) was added. Different 15-mer S1 and S2 peptide pools spanning the spike protein of the Wuhan reference strain and of the lineages B.1.1.7 (alpha), B.1.351 (beta), P.1 (gamma), and B.1.429 (epsilon) (1 μg/ml each peptide final concentration; JPT Peptide Technologies, Germany) were also used in a sample of 12 volunteers to analyze the reactivity of T cells against SARS-CoV-2 variants.

IFN-γ ELISpot assay

The frequency of IFN-γ–producing T cells was assessed by ELISpot after specific stimulation. Freshly isolated or thawed PBMCs were resuspended in R10; stimulated with peptides pools, as described above; and plated at 2×10^5 cells per well in ELISpot plates (human IFN-γ ELISpot plus kit, Mabtech). PBMCs were incubated for 18 to 20 hours with 5% CO_2. At the end of incubation, the ELISpot assay was developed according to the manufacturer's instructions. Spontaneous cytokine production (background) was assessed by incubating PBMCs with DMSO (Sigma-Aldrich), which was used to dilute peptides. Results are expressed as SFC per 10^6 PBMCs in stimulating cultures after subtracting spontaneous background. A result was considered positive if matching two criteria: (i) higher than >48 SFC/10^6 PBMCs and (ii) higher than three times the background value. Data from three volunteers (in experiments with freshly isolated PBMCs) and from nine volunteers (in experiments with thawed PBMCs) were excluded from all ELISpot analyses because their spontaneous IFN-γ secretion in DMSO wells was above the mean + 3 SD of the study population (mean = 42 SFC, SD = 72.27, mean + 3 SD = 258 SFC per million PBMCs), leading to unreliable quantitative analysis.

Intracellular staining and flow cytometry

Intracellular flow cytometry was performed by using ViaKrome 808 fixable viability dye, anti–IL-17A Alexa Fluor 700, anti-CD45 Krome Orange (all three from Beckman Coulter), anti-CD3 BUV661, anti-CD8 peridinin-chlorophyll-protein, anti-CD4 V450, anti–IL-2 fluorescein isothiocyanate, anti–IL-4 phycoerythrin (PE) (all five from BD Biosciences), and anti–IFN-γ PE-Vio770 (from Miltenyi Biotec). Briefly, PBMCs were washed in Dulbecco's PBS (Corning) and stained with 1:20 diluted ViaKrome 808 Fixable Viability dye, anti-CD45, anti-CD3, anti-CD8, and anti-CD4 for 15 min at 4°C. After washing, cells were fixed with 1% paraformaldehyde (Electron Microscopy Sciences) and then stained with 1:20 diluted anti–IL-17A, anti–IL-2, anti–IL-4, and anti–IFN-γ in PBS (Corning) plus 0.5% bovine serum albumin (Sigma-Aldrich), 0.01% sodium azide (SERVA Serving Scientists), and 0.5% saponin (Sigma-Aldrich) for 30 min at room temperature. Acquisition of 200,000 events was performed in the $CD3^+$ gated population on a CytoFLEX LX flow cytometer and analyzed with CytExpert software (Beckman Coulter). Data were analyzed with CytExpert software [Beckman Coulter; doublets were excluded in the forward scatter height (FSC-H)/forward scatter area (FSC-A) dot plot]. Live cells were selected as ViaKrome-negative cells. Next, $CD45^+$ were gated, followed by gating of $CD3^+$ lymphocytes. Among the $CD3^+$ cells, $CD4^+$ or $CD8^+$ cells were selected, and the percentage of CD4 or CD8 T cells producing cytokines was

evaluated. Spontaneous cytokine production (DMSO stimulation) was subtracted. The gating strategy is shown in the Supplementary Materials (fig. S5).

Statistical analysis

Categorical variables, including occurrence of AEs and detection of binding and neutralizing antibodies against SARS-CoV-2 and GRAd, were reported as proportions. Continuous variables, including results of CLIA anti–spike protein IgG, MNA_{90} titers, $PNRT_{50}$ titers, and ELISpot values for IFN-γ secretion, were reported as the median and interquartile range. Comparison of medians across arms and the impact of preexisting immunity against GRAd were evaluated by a two-tailed Kruskal-Wallis one-way variance analysis. Associations between categorical variables were carried out using Fisher's exact test. Correlations between immunogenicity assays were assessed by nonparametric Spearman's rank tests. Data in Fig. 6 were analyzed by Friedman's test with Dunn's multiple comparisons correction. Raw, individual-level data are presented in data file S1.

SUPPLEMENTARY MATERIALS

www.science.org/doi/10.1126/scitranslmed.abj1996
Figs. S1 to S5
Tables S1 and S2
RT-CoV-2 study protocol
Data file S1

View/request a protocol for this paper from *Bio-protocol*.

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GRAd-COV2, a gorilla adenovirus-based candidate vaccine against COVID-19, is safe and immunogenic in younger and older adults

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A gorilla adenovirus vaccine for COVID-19

Multiple COVID-19 vaccines currently in-use use adenoviral vectors for delivery of the SARS-CoV-2 spike protein. However, as these vaccines use human or closely related chimpanzee adenoviruses, there is increased likelihood of preexisting antivector immunity, which may dampen the response to the vaccine. To avoid this, Lanini *et al.* developed GRAd-COV2, a gorilla adenovirus-based SARS-CoV-2 vaccine. Here, the authors report the results of a phase 1, dose-escalation, open-labeled clinical trial showing that GRAd-COV2 was safe and immunogenic in both younger (aged 18 to 55 years old) and older (aged 65 to 85 years old) adults. In addition, participants had little to no preexisting immunity to the gorilla adenoviral vector. Together, these results support the further development of GRAd-COV2 as a COVID-19 vaccine.