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# Immunogenicity of a new gorilla adenovirus vaccine candidate for COVID-19

Stefania Capone,[1,6] Angelo Raggioli,[1,6] Michela Gentile,[1] Simone Battella,[1] Armin Lahm,[1] Andrea Sommella,[1] Alessandra Maria Contino,[1] Richard A. Urbanowicz,[2,3,4,7] Romina Scala,[1] Federica Barra,[1] Adriano Leuzzi,[1] Eleonora Lilli,[1] Giuseppina Miselli,[1] Alessia Noto,[1] Maria Ferraiuolo,[1] Francesco Talotta,[1] Theocharis Tsoleridis,[2,3,4] Concetta Castilletti,[5] Giulia Matusali,[5] Francesca Colavita,[5] Daniele Lapa,[5] Silvia Meschi,[5] Maria Capobianchi,[5] Marco Soriani,[1] Antonella Folgori,[1] Jonathan K. Ball,[2,3,4] Stefano Colloca,[1] and Alessandra Vitelli[1]

[1]ReiThera Srl, Rome, Italy; [2]School of Life Sciences, University of Nottingham, Nottingham, UK; [3]NIHR Nottingham Biomedical Research Centre, University of Nottingham, Nottingham, UK; [4]Wolfson Centre for Emerging Virus Research, University of Nottingham, Nottingham, UK; [5]National Institute for Infectious Diseases "Lazzaro Spallanzani" IRCCS, Rome, Italy

**The coronavirus disease 2019 (COVID-19) pandemic caused by the emergent severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) threatens global public health, and there is an urgent need to develop safe and effective vaccines. Here, we report the generation and the preclinical evaluation of a novel replication-defective gorilla adenovirus-vectored vaccine encoding the pre-fusion stabilized Spike (S) protein of SARS-CoV-2. We show that our vaccine candidate, GRAd-COV2, is highly immunogenic both in mice and macaques, eliciting both functional antibodies that neutralize SARS-CoV-2 infection and block Spike protein binding to the ACE2 receptor, and a robust, T helper (Th)1-dominated cellular response. We show here that the pre-fusion stabilized Spike antigen is superior to the wild type in inducing ACE2-interfering, SARS-CoV-2-neutralizing antibodies. To face the unprecedented need for vaccine manufacturing at a massive scale, different GRAd genome deletions were compared to select the vector backbone showing the highest productivity in stirred tank bioreactors. This preliminary dataset identified GRAd-COV2 as a potential COVID-19 vaccine candidate, supporting the translation of the GRAd-COV2 vaccine in a currently ongoing phase I clinical trial (ClinicalTrials.gov: NCT04528641).**

## INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in December 2019 and is responsible for the coronavirus disease 2019 (COVID-19) pandemic, which has so far caused worldwide 113,076,707 confirmed cases and 2,512,272 deaths as of February 27, 2021. Thanks to an unprecedented global scientific and financial effort, as of February 2021, at least seven different vaccines across three platforms have been rolled out in many countries. Vulnerable populations in all countries are the highest priority for vaccination, but vaccines are a critical tool in the battle against COVID-19, and the dose availability is still extremely limited, considering the need for universal vaccination and a two-dose regimen for most approved vaccines. Therefore, having many additional vaccines in development proving successful is still regarded as a critical global priority. SARS-CoV-2 is the third novel betacoronavirus in the last 20 years to cause substantial human disease; however, unlike its predecessors SARS-associated coronavirus (SARS-CoV) and Middle East respiratory syndrome (MERS)-CoV, SARS-CoV-2 transmits efficiently from person to person.[1] To address the urgent need for a medical countermeasure to prevent the further dissemination of SARS-CoV-2, we have employed a novel replication-defective simian adenoviral vector generated from a gorilla group C isolate: GRAd32. Viral-vectored vaccines are amenable to accelerated developmental timelines due to the ability to quickly insert a foreign antigen into the E1 region of the viral genome and rescue the vaccine vector in an appropriate production cell line to have it ready for preclinical testing. In addition, robust good manufacturing practice (GMP) production processes are already available, which are easy and cheap to scale up to meet the demand for a pandemic vaccine.

Simian adenoviruses derived from the chimpanzee, bonobo, and gorilla, which are not known to infect or cause pathological illness in humans, consequently have low/no seroprevalence (0%–18%) in the human population.[2] Prior studies in thousands of human subjects using simian adenoviral vaccine vectors encoding different antigens (relevant to Ebola, malaria, hepatitis C, human immunodeficiency virus [HIV], and respiratory syncytial virus [RSV]) have shown that this vaccine platform is safe and can generate potent, durable, and high-quality T cell and antibody (Ab) responses.[3–7] Furthermore, the current pandemic is giving a strong boost to the clinical validation

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[6]These authors contributed equally

[7]Present address: Institute of Infection, Veterinary and Ecological Sciences, University of Liverpool, Liverpool, UK

**Correspondence:** Alessandra Vitelli, ReiThera Srl, Via di Castel Romano 100, 00128 Rome, Italy.
**E-mail:** alessandra.vitelli@reithera.com

 

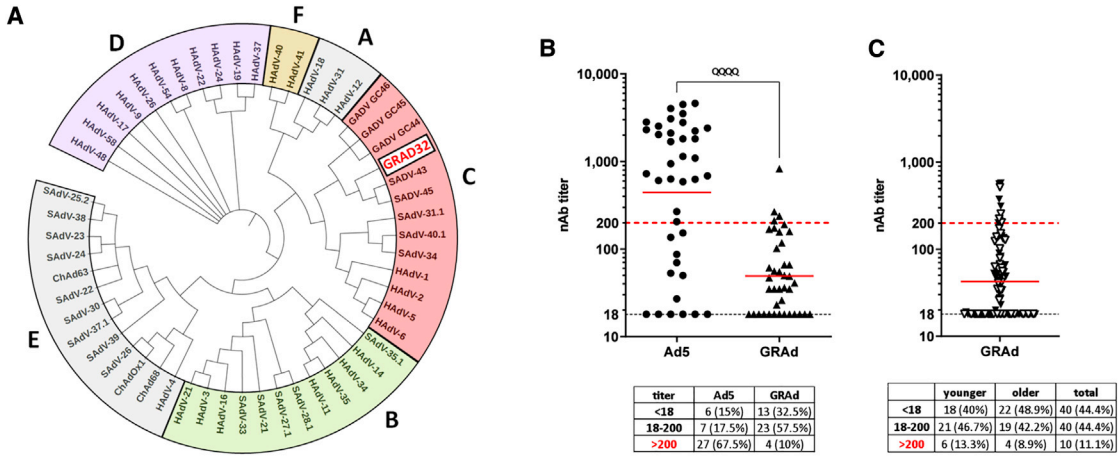


**Figure 1. Phylogenetic analysis of gorilla adenovirus (GRAd)32 and seroprevalence in human sera**

(A) Phylogenetic analysis using adenoviral polymerase sequences identifies GRAd32 as a group C adenovirus. HAdV, human adenovirus; SAdV, simian adenovirus; GAdV, GRAd. (B and C) Neutralizing antibody (NAb) titers measured in sera collected from a cohort of 40 human healthy donors of US origin (B) and in 90 sera from Italian volunteers (C) enrolled in a GRAd-COV2 phase 1 study (C, filled symbols = younger adults 18–55 years; empty symbols = older adults 65–85 years). Data are expressed as the reciprocal of serum dilution resulting in 50% inhibition of SEAP activity. Horizontal black dotted lines indicate assay cut-off (titer of 18). Red dotted lines indicate NAb titer of 200, which is reported to potentially impact vaccine immunogenicity. Red continuous lines indicate geometric mean. Statistical analysis of the datasets was performed by unpaired, two-tailed Mann-Whitney analysis. The tables show the absolute numbers and the percentage of sera with NAb titers to Ad5 or GRAd32 below cut-off (<18), between 18 and 200 (<200), and above 200 (>200).

of simian adeno-vectored vaccine technology, since the AZD1222 vaccine is based on a chimpanzee adenoviral vector, ChAdOx1.[8]

In this study, we generated different genome deletions in the GRAd32 vector backbone and inserted a transgene cassette containing either the SARS-CoV-2 full-length Spike protein (S) or its stabilized form (S-2P)[9] to preserve the neutralization-sensitive epitopes at the apex of the pre-fusion structure and to improve expression levels from transduced cells. The selected COVID-19 candidate vaccine vector, GRAd-COV2, has a genome deleted of the entire E1 and E3 regions and the native E4 region replaced with the E4 orf6 of human adenovirus 5 (hAd5). We show here that this modification of the backbone improved vector productivity using a scalable process in stirred tank bioreactors, which is a relevant feature in the context of a pandemic.

In this study, we demonstrate that by administering a single dose of GRAd-COV2 to non-human primates (NHPs) and mice, we stimulate a T helper (Th)1-skewed T cell response and the production of functional antibodies blocking the interaction between the Spike protein and its receptor hACE2 and neutralizing SARS-CoV-2 infection. These results supported the ongoing clinical development of the GRAd-COV2 vaccine for prevention of COVID-19 (ClinicalTrials.gov: NCT04528641).

## RESULTS

### GRAd32 has low seroprevalence in humans

GRAd32 is a novel simian adenovirus isolated from a captive gorilla. Based on a phylogenetic analysis of aligned adenoviral polymerase sequences, GRAd32 falls into the group C adenoviruses, as the hAd5 and the simian ChAd3 (Figure 1A). The prevalence of pre-existing immunity to the GRAd32 vector and to the common hAd5 was estimated in a set of 40 human sera of US origin by a viral neutralization assay based on secreted embryonic alkaline phosphatase (SEAP) reporter gene activity.[10] Greater than 32% of the serum samples were negative (half-maximal inhibitory concentration [$IC_{50}$] titer < 18) for neutralization of GRAd32. In addition, the $IC_{50}$ titers of the positive sera were low, and only 10% had titers greater than 200 (Figure 1B). Titers less than 200 may not be inhibitory *in vivo*.[11] In comparison, 84% of serum samples were positive for neutralization of hAd5, with 67.5% of the positive samples having endpoint titers greater than 200 (Figure 1B). A second set of sera from Italian healthy younger (18–55 years) and older (65–85 years) volunteers confirmed the initial finding with only 10% of sera with GRAd-COV2 neutralizing titer >200 and similar titer distribution over the two age cohorts (Figure 1C).

### *In vitro* characterization of GRAd32-based COVID-19 vaccine constructs

We explored different GRAd32 vector backbones encoding the wild-type (WT) Spike protein (GenBank: QHD43416.1) or the pre-fusion stabilized Spike protein (S-2P)[9] to find the optimal combination in terms of vaccine immunogenicity and productivity. To this aim, four vaccine variants were generated: GRAd32b-S, GRAd32b-S-2P, GRAd32c-S, and GRAd32c-S-2P, which differed either in the Spike protein (WT or pre-fusion) or in the vector backbone (GRAd32b: ΔE1 and ΔE3, or GRAd32c: ΔE1, ΔE3, and ΔE4::hAd5E4orf6) (Figure S1). In all vectors, the viral E1 and E3 regions were deleted to make the virus replication defective and increase the cloning capacity; the GRAd32c vector backbone was further deleted of the E4 region, which was replaced with the E4 orf6 of hAd5 in an attempt to

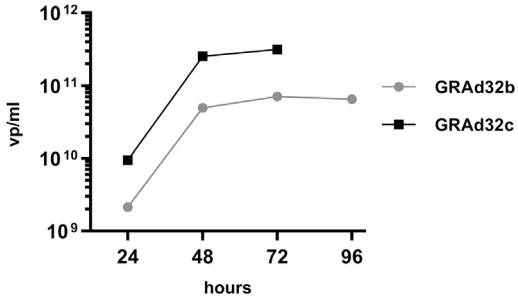


**Figure 2. Productivity of GRAd32 backbone variants in 2 L bioreactor**
Suspension-adapted packaging HEK293 cells were seeded at $5 \times 10^5$ cells/mL and infected at MOI 200. The titer of virus contained in the bulk cell lysates collected at different time points (hours) after infection was measured by qPCR.

optimize growth rate and yield in human cell lines, as previously described.[4,12]

The four vaccine vectors were rescued in a suspension-adapted HEK293 packaging cell line and *in vitro* characterized for productivity, infectivity, genome stability, and potency, as measured by transgene expression. To reflect the growth conditions used for large-scale manufacturing, the productivity of the GRAd32 vector backbones was assessed after infection at controlled MOI (multiplicity of infection; =qPCR viral particles [vps] per cell) in 2 L stirred tank bioreactors, upon collection at 48 h and 72 h post-infection. As shown in Figure 2, substitution of the native E4 region with the E4 orf6 of hAd5, in addition to the deletion of the E3 region, led to an improved yield ($3.15 \times 10^{11}$ vp/mL versus $7.06 \times 10^{10}$ vp/mL) of the GRAD32c vector when harvest was collected at 60−72 h after infection. No difference in terms of genome stability was observed between the two vector backbones, which remained stable over 10 amplification passages, as detected by restriction pattern analysis (Figure S2). However, GRAd32c showed slightly lower infectivity as measured by hexon staining in HEK293 cells with respect to GRAd32b ([vps as detected by genome copies]/[infectious particles] ratio = 178 and 63, respectively). To investigate the impact of the decreased infectivity on transgene expression, we infected the non-permissive HeLa cell line at MOI 50 with GRAd32b and GRAd32c vectors encoding the recombinant antigen S-2P. These data showed that antigen expression is higher in the GRAd32b than in GRAd32c (Figure S3A), consistent with the observed difference in infectivity.

We then investigated the expression potency of the different vaccine vectors by HeLa cell infection and fluorescence-activated cell sorting (FACS) analysis of whole-cell binding to either an anti-S-2 subunit antibody or to the soluble ACE2 receptor and by western blot (WB) analysis of Spike protein expression in total cell lysates (Figures 3 and S3). FACS analysis of anti-S-2 (Figures 3A, 3B, and 3E) or of recombinant hACE2 (Figures 3C, 3D, and 3F) binding to whole cells revealed similar levels of cell surface display of either Spike WT or S-2P, respectively, independently of the encoding GRAd32 backbone. However, in each vector backbone, the displayed S-2P was clearly more abundant than the WT Spike. To distinguish between higher levels of S-2P at the cell surface versus stronger binding of S-2P, we performed WB analysis of total cell lysates using an antibody directed to the receptor-binding domain (RBD) or to the C-terminal hemagglutinin (HA) tag. Such difference in the level of antigen expression, as seen with the FACS analysis, was not confirmed by WB (Figures S3B and S3C), possibly suggesting that increased FACS binding of the stabilized Spike was due to improved trafficking of the Spike to the plasma membrane and/or to better accessibility of the pre-fusion conformation RBD to the binding antibody, rather than to increased expression level. Based on the above-described results of *in vitro* characterization, GRAd32c-S-2P was selected as the candidate COVID-19 vaccine vector backbone given the higher vector productivity and the improved ACE2 binding of the pre-fusion stabilized Spike protein and named GRAd-COV2.

### GRAd-COV2 induces strong humoral and Th1-dominated cellular immune responses in mice

Preclinical characterization of the immunogenicity of GRAd-COV2 was performed in inbred mice. Groups of 8 BALB/c mice were vaccinated intramuscularly (i.m.) with a single dose of GRAd-COV2 at $1 \times 10^9$ vp. Serum samples were taken at weeks 2 and 5 (w2 and w5) post-vaccination, and total immunoglobulin G (IgG) endpoint titers were measured by ELISA on the full-length recombinant SARS-CoV-2 Spike protein and on the Spike RBD. As shown in Figure 4A, anti-Spike IgG titers rose rapidly after vaccination and increased over time, and most of the antibody response was directed against the RBD. Interestingly, an anti-Spike IgG2a/IgG1 ratio > 1 suggested a Th1 polarization of the immune responses, compared to the strongly Th2-dominated response induced by a vaccination regimen based on the recombinant Spike protein in alum (Figures 4B and S4).

Furthermore, the single-dose immunization with GRAd-COV2 elicited functional antibodies that neutralized the cytopathic effect (CPE) of SARS-CoV-2 on Vero E6 cells (50% blocking of CPE = 30−220 reciprocal serum dilution) and inhibited pseudotyped virus infection in the HuH7 cell (90% inhibition of entry = 260−1,233 reciprocal serum dilution) (Figure 4C).

Strong antigen-specific T cell responses were induced by the vaccination and measured by enzyme-linked immune absorbent spot (ELISpot) and intracellular cytokine staining (ICS) from splenocytes isolated 5 weeks after immunization and stimulated with overlapping 15-mer peptides. As shown in Figure 5A, interferon (IFN)-γ-secreting T cells recognizing epitopes in both S-1 and S-2 domains of the Spike protein were detected (mean 1,390 and 390 spot-forming cells (SFCs) per million splenocytes, respectively). In-depth characterization of the responding T cell subset ($CD8^+/CD4^+$) and Th polarization by ICS and FACS analysis revealed a 10% mean frequency of IFN-γ-secreting, Spike-specific $CD8^+$ T cells and a lower but clearly measurable Th1-dominated $CD4^+$ T cell response (Figure 5B). Type 2 cytokines interleukin (IL)-4 and IL-13 and IL-17 secretion were also investigated by ICS and ELISpot but were undetectable or below the limit of detection of our assays.

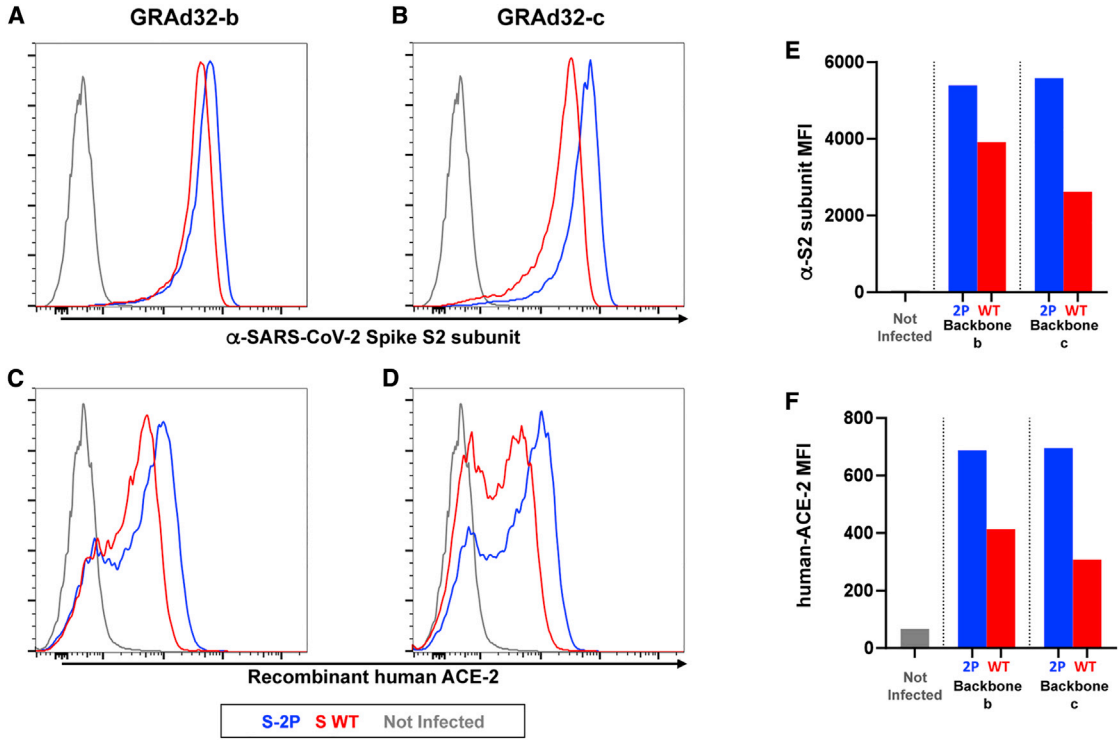


**Figure 3. Expression of Spike antigens in GRAd32 vectors**

(A–D) Whole cell FACS analysis of HeLa cells not infected or infected with 150 MOI of GRAd32b (A and C) or GRAd32c (B and D). 48 h after infection, cells were stained with anti-S-2 antibody (A and B) or with human soluble his-tagged ACE2 receptor and anti-His antibody (C and D), both then detected with goat anti-mouse IgG mAb conjugated with Alexa Fluor 647. The histograms of gated live cells corresponding to cells infected with the vector-encoded wild-type (WT) Spike protein are labeled in red and with the stabilized S-2P in blue; the gray line represents non-infected cells. (E and F) Quantification of expression levels as detected by anti-S-2 (E) or ACE2 (F) binding, expressed as the mean fluorescence intensity (MFI).

To benchmark the potency of the novel GRAd32 vector in comparison with other described simian adenoviral vectors,[4] we have performed a dose-response immunization exploring a range of doses from $1 \times 10^9$ to $1 \times 10^5$ vp, measuring IFN-γ-secreting cells by ELISpot in the spleen 3 weeks after vaccination (Figure S5). Detectable immune responses in all animals were already observed at the dose $1 \times 10^6$ vp, revealing the strong immunological potency of the new gorilla vector. As a control for assay specificity, BALB/c mice were immunized with a GRAd32 vector encoding the HIV-1 gag antigen. No humoral or cellular responses to the Spike protein or to the Spike peptide pools were detected in the immunized animals, whereas as expected, both antibody and T cell responses to gag were measured (Figure S6).

We then compared the humoral responses induced by the WT Spike protein versus the S-2P. Sera were taken 5 weeks after immunization with a single administration at $1 \times 10^9$ vp of GRAd32 vectors encoding the WT Spike or S-2P. We observed similar induction of total anti-S IgG in spite of significantly higher neutralization titers for the S-2P antigen (Figure 6A) measured by pseudotyped virus neutralization. Consistently, the same sera assayed for the capacity to interfere with the binding of the soluble ACE2 receptor to the immobilized RBD showed that the S-2P antigen elicited antibodies with greater interference capacity (Figure 6B).

### GRAd-COV2 immunogenicity in NHPs

Having determined that GRAd-COV2 elicits neutralizing antibodies (NAbs) and promotes the generation of multifunctional Th1-biased antigen-specific T cells in mice, we next evaluated the immunogenicity of the vaccine in a more relevant animal species: NHPs. Four cynomolgus macaques were immunized i.m. with a single administration of $5 \times 10^{10}$ vp GRAd-COV2 or GRAd32c-S. Blood was drawn 1 week before vaccination and at w2, w4, w6, and w10 after vaccination. Before vaccination, all animals showed different degrees of IgG titers cross-reacting with the SARS-CoV-2 Spike protein and RBD, presumably deriving from previous contact with other coronaviruses (Figures 7A and 7B). However, serum IgG titers against the Spike protein and RBD increased rapidly after vaccination, peaking between w2 and w4, and remained stable up to w10 (Figures 7A and 7B). Functional antibodies, as measured by inhibition of SARS-CoV-2-pseudotyped virus infection in Vero E6 cells and by SARS-CoV-2 microneutralization (MN) assay, were also rapidly induced in all animals and peaked with different kinetics (Figures 7C and 7D). Peak NAb titers ranged between 340−1,070 (50% CPE in MN assays) and

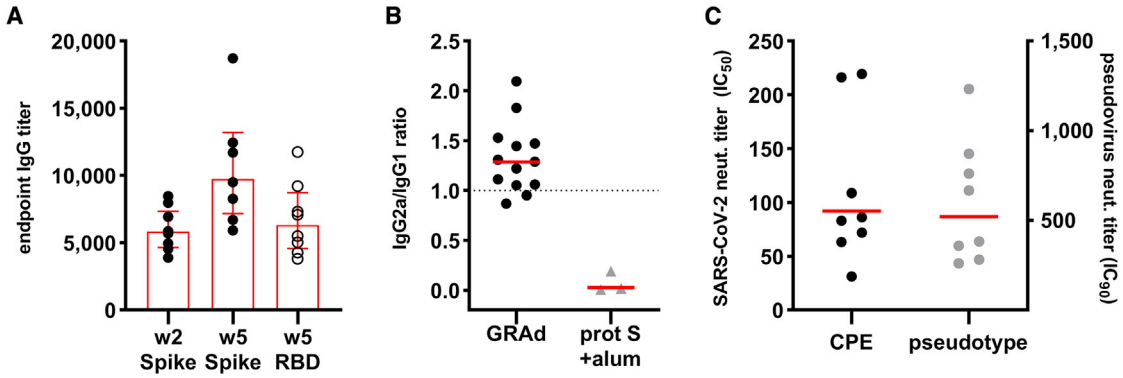


**Figure 4. Humoral response to SARS-CoV-2 Spike induced in BALB/c mice 2 and 5 weeks (w2 and w5) after immunization with GRAd-COV2**

(A) Spike-binding total IgG titers in sera from mice immunized with $1 \times 10^9$ vp of GRAd-COV2. IgG titers were measured in sera collected 2 or 5 weeks post-immunization by ELISA on recombinant full-length Spike or RBD. Data are expressed as endpoint titer. The main and error bars indicate geometric mean and 95% confidence interval (CI). (B) The ratio between IgG2a and IgG1 titers measured in week 5 sera by ELISA on full-length Spike in mice vaccinated with either $1 \times 10^9$ vp of GRAd-COV2 (n = 13) or with two injections 2 weeks apart of 2.5 μg Spike protein formulated in alum adjuvant (n = 3). (C) SARS-CoV-2 NAbs induced by GRAd-COV2 and detected in sera at week 5 post-immunization by SARS-CoV-2 (2019-nCoV/Italy-INMI) microneutralization assay on VERO E6 cells (CPE, black symbols plotted on left y axis) or by SARS-CoV-2-pseudotyped virus neutralization assay (gray symbols plotted on right axis). Neutralizing titers are expressed as $IC_{50}$ or $IC_{90}$, or the reciprocal of serum dilution achieving 50% or 90% neutralization, respectively. Horizontal lines in (B) and (C) represent geometric mean.

1,580−4,635 ($IC_{50}$-pseudotyped virus entry inhibition). Importantly, vaccine-induced neutralization titers remained quite stable throughout the observation period, and NAb titers measured at w10 were comparable or higher than those measured in serum obtained from COVID-19 convalescent patients, using the same MN assay (Figure 7E).

Peripheral blood mononuclear cells (PBMCs) were collected 2 and 8 weeks after immunization, and T cell response was measured by IFN-γ and IL-4 ELISpot assay (Figure 8A). Strong IFN-γ-secreting T cell responses were detected in all animals at w2 (700−3,500 SFC/$10^6$ PBMCs), which somewhat contracted at w8 but remained at a high level (400−2,500 SFC/$10^6$ PBMCs). Instead, IL-4 levels were too low to be detected in macaques by ELISpot analysis (Figure 8A). The animals immunized with S-2P showed the highest number of IFN-γ-secreting T cells (Figure 8B), and the S-1 region of the Spike protein was most immunogenic, even in this outbred animal model. ICS performed on frozen PBMC showed that vaccination primed both $CD8^+$ and $CD4^+$ T cells (Figure 8C).

Overall, the immunogenicity of the GRAd-COV2 vaccine in non-human primates is consistent with the mouse immunogenicity results and further supports the efficacy of the vaccine in promoting the generation of NAbs and Th1-biased cellular responses.

## DISCUSSION

In this report, we describe the development of a COVID-19 vaccine candidate based on a novel simian adenovirus, GRAd32, isolated from a captive gorilla and belonging to species C adenoviruses. We show here that a single immunization with the GRAd-COV2 vector encoding a pre-fusion stabilized S immunogen (S-2P) in mice and in NHP induced robust NAb responses and cellular immunity. The GRAd-COV2-encoded S-2P antigen consists of the full-length membrane-bound Spike protein with the WT leader sequence and with two proline mutations that were described to stabilize the trimer in the pre-fusion conformation.[9] The stabilization of the pre-fusion conformation of class I fusion proteins has successfully improved immunogenicity of these important vaccine targets for RSV,[13] parainfluenza virus,[14] Nipah virus,[15] MERS-CoV,[9] and HIV.[16] This improvement is based on preserving neutralization-sensitive epitopes at the apex of pre-fusion structures, and it was mainly shown to improve immunogenicity of secreted fusion proteins. Our data of comparison of the WT versus the stabilized, full-length, membrane-anchored Spike protein extend recent preclinical studies of a vectored vaccines for SARS-CoV-2, Ad26.COV2-S, in NHPs,[17] wherein a WT full-length Spike protein with proline-stabilizing mutations (S.PP) represented the best antigen in terms of immunogenicity and protective efficacy in comparison with several other Spike protein antigen forms. Here, we demonstrate that the GRAd vector encoding S-2P shows higher expression of membrane-anchored antigen *in vitro* and elicits higher titers of NAbs in mice in comparison to the WT antigen. Similar conclusions cannot be drawn for NHPs due to the very low number of animals used for this study. A more extended immunization and challenge study in NHPs is ongoing to confirm these observations and, more importantly, to evaluate the protective efficacy of the vaccine. However, all of the vaccinated monkeys responded rapidly to the single vaccine shot inducing a robust antibody response, which remained sustained up to 10 weeks. A single-shot SARS-CoV-2 vaccine would have important logistic and practical advantages compared with a two-dose vaccine for mass vaccination campaigns and pandemic control. We speculate here that a species C adenoviral vector could reach this major objective based on recent reports that hAd5 and ChAd3 (both species C) vectors, which in mice induced the strongest T cell responses, showed high and persistent

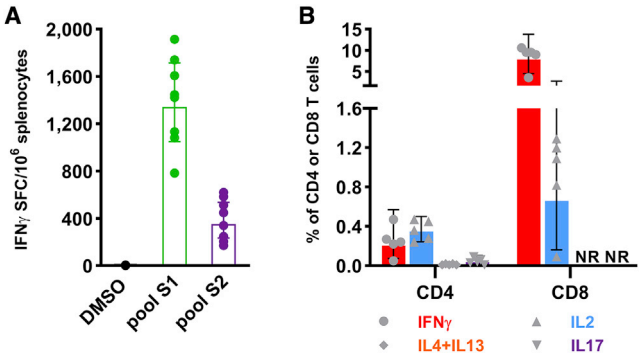


**Figure 5. T cell response to SARS-CoV-2 Spike induced in BALB/c mice 5 weeks after GRAd-COV2 immunization**

(A) IFN-γ ELISpot on splenocytes. Data are expressed as IFN-γ spot-forming cells (SFCs)/$10^6$ splenocytes. Individual data points represent response to S-1 and S-2 pools' stimulation compared to mock stimulation (DMSO) in each animal. (B) IFN-γ/IL-2/IL-4 + IL-13/IL-17 intracellular staining and FACS analysis on splenocytes. Data are expressed as the percentage of cytokine-secreting CD8 or CD4 T cells in response to S-1 and S-2 Spike peptide pools' stimulation, obtained by summing reactivity to each of the 2 Spike peptide pools and subtracting 2 times the DMSO background. Main and error bars indicate geometric mean and 95% CI in both (A) and (B).

antigen expression in the draining lymph nodes (dLNs), with low innate immunity gene activation.[18] Differently, less potent vaccine vectors, like the ones based on hAdV-28 (species D), hAdV-35 (species B), and ChAd63 (species E), induced lower antigen expression associated with robust induction of innate immunity genes as determined by expression profiling in dLNs that were primarily associated with IFN signaling.[18] However, these preclinical observations will soon receive a feedback from the clinical testing and the deployment of several COVID-19 vaccine candidates based on different adenoviral vectors. In the actual landscape of COVID-19 vaccines based on adenoviral vectors there was ample heterogeneity in the clinical development plans: the group E simian adenovirus vaccine candidate ChAdOx1 nCoV-19 (AZD1222) showed that re-administering the same vector 28 days after the first dose increased antibody responses leading to detectable NAb responses in all vaccinated subjects, and therefore, this was the regimen selected for the phase III efficacy study.[8] On the contrary, Janssen's COVID-19 vaccine candidate, the group D Ad26.COV2.S (also known as JNJ-78436735), has shown satisfactory immunogenicity data in a phase I/II trial[19] and was explored in phase III studies either as a single-dose regimen or as homologous prime/boost (ClinicalTrials.gov: NCT04505722 and NCT04614948). A single-dose regimen of the COVID-19 vaccine candidate Ad5-nCoV was evaluated in phase III by the Chinese CanSino (ClinicalTrials.gov: NCT04526990), whereas phase III (ClinicalTrials.gov: NCT04642339) of the Russian COVID-19 vaccine candidate by the Gamaleya Institute tested a heterologous prime/boost with two recombinant adenovirus vectors: Ad26 and Ad5. All of these vaccines showed different but satisfactory degrees of protective efficacy and therefore, were authorized for emergency use. However, a more accurate comparison of the different platforms in terms of elicited immune responses and levels of protective efficacy is still awaiting standardized assays and the establishment of immune correlates of protection resulting from larger studies.

In the present study, we do not address the safety or the possibility of vaccine-associated enhanced respiratory disease or antibody-dependent enhancement of infection.[20] However, it is worth noting that the GRAd-COV2 vaccine elicited strong antibody and Th1-biased rather than Th2-biased T cell responses in both mice and NHPs.

Another big challenge posed by the current pandemic is the unprecedented need for scaling up vaccine manufacturing at a massive scale and at record speed. To face this challenge, we generated two backbone mutants of our GRAd: one deleted in E3 and retaining the native entire GRAd E4 region and the other deleted in region E3 and with region E4 substituted by the E4 orf6 of hAd5. With the comparison of productivity in bioreactors and stability upon many amplification passages, an optimal vector backbone (ΔE1, ΔE3, ΔE4::hAd5E4orf6), termed GRAd-COV2, has been chosen as the most promising candidate, and the results of the interim analysis of a phase 1 study (ClinicalTrials.gov: NCT04528641) in healthy volunteers have been submitted for publication.

## MATERIALS AND METHODS

### GRAd isolation, amplification, and classification

Stool specimens from healthy gorilla housed in the zoo of Bristol (UK) were collected and processed for inoculation into cell cultures as previously described,[4] with minor modifications. Briefly, frozen stool specimens were thawed in excess of chilled DMEM and then clarified by centrifugation, followed by 0.8 μm and 0.22 μm syringe filtration. The filtered material was inoculated into monolayers of A549 cultivated in DMEM completed with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Pen-Strep), and the cultures were visually monitored for CPE for at least 28 days after inoculation. Cell monolayers showing clear sign of CPE were detached and subjected to 3 cycles of freeze/thaw (−80°C/37°C) to release the vps. Lysates were then clarified by centrifugation and applied onto fresh A549 to perform a large-scale preparation. Purified vps were obtained by ion exchange (VivaPure AdenoPACK 20; Sartorius, Göttingen, Germany) and the DNA extracted and subjected to next-generation sequencing. Group C adenovirus assignment for GRAd32 was based on a phylogenetic analysis of aligned adenoviral polymerase sequences using a bootstrap-confirmed maximum likelihood tree (500 replicates) calculated with MEGA 10.1.7.[21] Tree display was performed with iTol.[22] Polymerase sequences were aligned with MUSCLE.[23] DMEM, FBS, and Pen-Strep were purchased from Gibco, Thermo Fisher Scientific (Waltham, MA, USA).

### Vector construction

A pBeloBAC11-based GRAd-specific shuttle bacterial artificial chromosome (BAC) containing the adenoviral genome ends, a ΔE1 cassette and the adenovirus pIX gene, was generated as previously described.[4] Purified virus genome was isolated by proteinase K (Merck KGaA, St. Louis, MO, USA) digestion followed by phenol/chloroform (Merck KGaA) extraction, and homologous

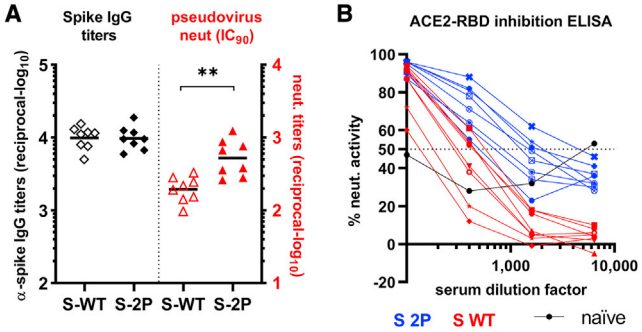


**Figure 6. Humoral response to SARS-CoV-2 Spike induced in BALB/c mice by GRAd encoding for pre-fusion stabilized versus WT Spike**

(A) Full-length Spike-binding total IgG titers (black symbols, plotted on left y axis) and neutralizing titers on pseudotyped viruses (red symbols, plotted on right y axis) in week 5 sera from mice immunized with $1 \times 10^9$ vp of GRAd encoding either WT Spike (open symbols) or S-2P (filled symbols). Data are expressed either as endpoint titer or as $IC_{90}$ neutralizing titer. Horizontal lines represent geometric mean. Two-tailed unpaired t test was used (**p = 0.007, t = 3.16, degree of freedom [df] = 14). (B) Inhibition of binding between immobilized RBD and recombinant human ACE2 by dilution curves of week 5 sera from mice immunized with $1x10^9$ vp of GRAd encoding either WT Spike (red curves) or S-2P (blue curves). Data are expressed as percentage of neutralization activity relative to binding in the absence of serum. Inhibition curve from a naive, non-immunized mouse as control is shown in black.

recombination occurring between the right inverted terminal repeat (ITR) and pIX present on both the shuttle BAC and the purified viral genome allowed for the insertion of the GRAd genome in the shuttle plasmid with the deletion of the E1 region from bp 445 to 3,403. This plasmid was designated as pGRAd32 ΔE1. SEAP coding sequence was inserted under the control of the cytomegalovirus (CMV) promoter (including the CMV enhancer) and the bovine growth hormone polyadenylation (bGHpA) signal in the E1 locus to generate GRAd32 ΔE1 SEAP.

The pGRAd32 ΔE1 vector was further modified by recombineering to generate the ΔE1ΔE3 (named "GRAd32b," with deletion of the E3 region from bp 28,479 to bp 32,001, encompassing part of the E3 12.5K and then the entire E3 CR1-alpha, E3 gp19K, E3 CR1-beta, E3 CR1-gamma, E3 RID-alpha, E3 RID-beta, and E3 14.7K of the GRAd32 WT genome) and the ΔE1ΔE3ΔE4 (named "GRAd32c," with an additional deletion of the entire E4 region from bp 34,144 to 36,821 and its substitution with hAd5 E4 orf6[4]) backbones.

### Construction, amplification, and purification of GRAd32 vectors expressing SARS-CoV-2 Spike gene

A human codon-optimized version of the SARS-CoV-2 Spike coding sequence was synthesized by Doulix (Venice, Italy) and subcloned into a shuttle plasmid between the AscI and PacI restriction sites, between the tetO-hCMV promoter and the woodchuck hepatitis virus (WHP) post-transcriptional regulatory element (WPRE)-bGHpA sequence to generate a functional expression cassette. Two mutations were introduced to convert amino acid (aa) 986–987 KV into 2P to stabilize the protein in its pre-fusion state.[9] An HA tag, derived from the human influenza HA protein and composed of a 9-aa peptide sequence, Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala, was fused downstream of the last SARS-CoV-2 Spike protein aa (Thr1273) flanked at its 5′ and 3′ side by a Gly and a Ser, respectively, to facilitate antigen expression detection by the widely commercially available HA antibodies (Abcam, Cambridge, UK). In addition, a minimal Kozak sequence (5′-CCACC-3′) was placed immediately upstream of the start codon to enable efficient initiation of translation. The cassette encoding for either SARS-CoV-2 S or SARS-CoV-2 S-2P was inserted by homologous recombination in the E1 locus of the GRAd32b and GRAd32c vectors, thus generating the GRAd32b-S, GRAd32b-S-2P, GRAd32c-S, and GRAd32c-S-2P vectors.

All cloning PCR amplifications were performed using the Q5 High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) according to standard procedures.

GRAd32 preAd plasmids were digested with PmeI (New England Biolabs) to release the viral ITRs and transfected in a suspension-adapted, HEK293-based cell line to rescue the virus vector. Vectors were then expanded up to a production in 2 L Bioreactor (Biostat B DCU; Sartorius), whereby suspension cells were seeded at $5 \times 10^5$ cells/mL, grown in CD293 medium (Gibco) complemented with L-glutamine 6 mM (Gibco) and Pluronic F68 0.05% (Gibco), and infected at MOI 200. The titer of virus contained in the bulk P2 cell lysates collected at 60–72 h after the infection was measured by qPCR. Productivity was defined as the ratio between the number of vps quantified by qPCR in the P2 bulk lysate over the number of cells counted at the time of infection. Finally, P2 purification was performed by VivaPure AdenoPACK 20 (Sartorius) and purified vps used for the infectivity assay (see below).

### Infectivity assay

$2 \times 10^5$ HEK293 were seeded onto 24-well plates previously precoated with poly-L-lysine (Merck KGaA) and infected with $2 \times 10^5$, $1 \times 10^5$, or $0.5 \times 10^5$ vps. Infected cells were blocked at the indicated time points with ice-cold methanol, incubated with anti-hexon primary antibody (Abcam, UK), followed by detection with secondary anti-mouse IgG peroxidase (Merck KGaA,) and Vector NovaRED Substrate Kit for peroxidase (Vector Laboratories, Burlingame, CA, USA). The infectious unit ratio was determined as the ratio between the number of stained cells over the number of physical particles used in the assay.

### WB

$1 \times 10^6$ HeLa cells, cultivated in DMEM completed with 10% FBS + 1% Pen-Strep, were infected at MOI 150 and collected at the indicated time points, and cell pellets were lysed in the appropriate amount of radioimmunoprecipitation assay (RIPA) buffer (Merck KGaA) completed with protease inhibitor (Merck KGaA) for 30 min on ice. Cell lysates were cleared by centrifugation for 30 min at 15,000 rpm 4°C and the supernatant quantified by Bradford (Thermo Fisher Scientific, Waltham, MA, USA). 50 μg of clarified lysates was loaded onto 4%–12% acrylamide gel (Thermo Fisher Scientific), transferred onto iBlot 2 nitrocellulose (NC) mini stacks membranes

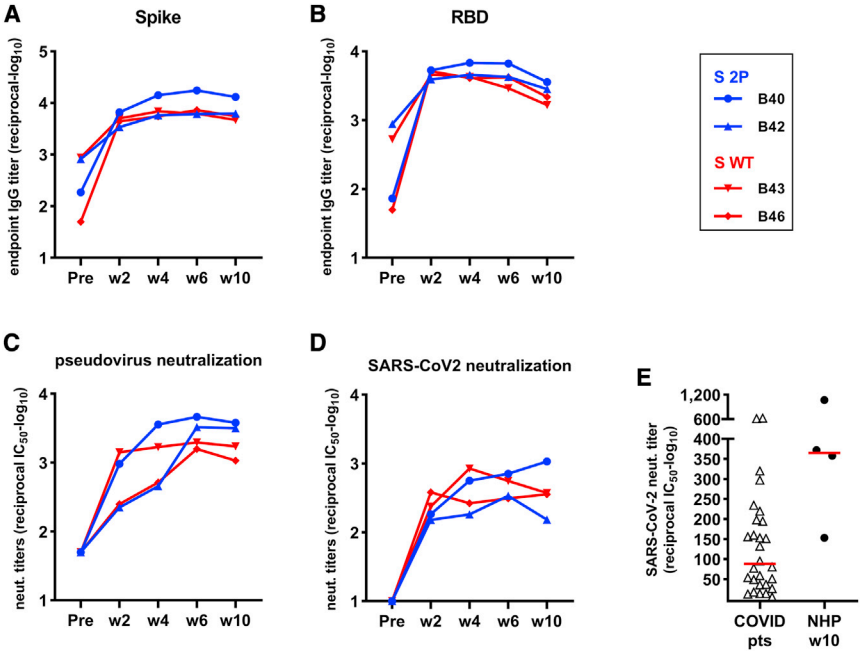


**Figure 7. Humoral response to SARS-CoV-2 Spike induced in cynomolgus macaques by GRAd-COV2 immunization**

Four cynomolgus macaques received a single $5 \times 10^{10}$ vp intramuscular injection of either GRAd-COV2 (blue curves) or GRAd32c-S (red curves). (A–D) Serum samples obtained before (Pre) and 2, 4, 6, and 10 weeks post-immunization were tested by (A) full-length Spike-binding ELISA (total IgG endpoint titers), (B) RBD-binding ELISA (total IgG endpoint titers), (C) pseudotyped virus neutralization assay (neutralizing $IC_{50}$ titers), or (D) SARS-CoV-2 microneutralization assay (neutralizing $IC_{50}$ titer). (E) $IC_{50}$ titers measured by the SARS-CoV-2 microneutralization assay in a panel of COVID-19 convalescent human sera and in GRAd-COV2-immunized macaques 10 weeks post-immunization. Horizontal lines represent geometric mean.

(Thermo Fisher Scientific), and incubated with the anti-HA (Abcam) or anti-RBD (Sino Biological, Wayne, PA, USA) primary antibodies. Detection of the SARS-CoV-2 Spike protein was achieved by incubation with an anti-rabbit (Merck KGaA) secondary antibody, followed by enhanced chemiluminescence (ECL; Thermo Fisher Scientific) incubation, and images were taken using a ChemiDoc (Thermo Fisher Scientific).

### NAb assay

Human sera of US origin (n = 40, 20 male and 20 female) were purchased from TCS Biosciences (Botolph Claydon, Buckingham, UK), and its supplier is registered with the US Food and Drug Administration (FDA) Blood Establishment Registration. Samples have been collected by donations and permission obtained from the donors for the material to be used for commercial use. Data protection of donors is assured through data integrity and de-linking donors. Sera from 90 Italian healthy volunteers (45, aged 18−55, and 45 aged 65−85) were collected the day of vaccination (baseline) in the context of the RT-CoV-2 phase I clinical trial. All subjects provided written, informed consent before being enrolled. The RT-CoV-2 clinical trial was approved by the Italian Regulatory Drug Agency (AIFA) and the Italian National Ethical Committee for COVID-19 clinical studies (ClinicalTrials.gov: NCT04283461; EudraCT: 2020-002835-31). NAb titers in human sera were assayed as previously described.[10] Briefly, $8 \times 10^4$ HEK293 cells per well were seeded in 96-well plates the day before the assay. Each adenoviral vector (Ad5 or GRAd32) encoding for SEAP was preincubated for 1 h at 37°C alone or with serial dilutions of control or test serum samples and then added to the 80%–90% confluent 293 cells. After incubation for 1 h at 37°C, supernatant was then removed and replaced with 10% FBS in DMEM. SEAP expression was measured 24 h later with the chemiluminescent substrate from the Phospha-Light kit (Applied Biosystems, Foster City, CA, USA). Neutralization titers were defined as the dilution at which a 50% reduction of SEAP activity from serum sample was observed relative to SEAP activity from virus alone.

### HeLa cell infection and S-2/ACE2 staining

HeLa cells were plated at $5 \times 10^5$ cells/well in 6-well plates 2 days before infection to allow adhesion and then infected with GRAd vectors at MOI of 150 for 48 h. Cells were then harvested by pipetting with cold PBS + 5 mM EDTA and aliquoted in 5 mL polystyrene FACS tubes (BD Biosciences, San Jose, CA, USA). Cells were then stained with Live/Dead fixable dye (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA); recombinant human His-tagged ACE2 protein (RayBiotech, Peachtree Corners, GA, USA) was incubated on cells before adding any antibodies and then detected with anti-His antibody (Sigma, Thermo Fisher Scientific, St. Louis, MO, USA). Spike protein expression was detected with anti-SARS-CoV-2 Spike protein S-2 subunit mouse monoclonal antibody (mAb) unconjugated (GeneTex, Irvine, CA, USA). Antibody binding was detected with goat anti-mouse IgG mAb conjugated with Alexa Fluor 647 fluorochrome (Sigma). Sample acquisition was performed on a LSRFortessa X-20 cytofluorimeter (BD Biosciences), and sample analysis was performed with FlowJo (Tree Star, Ashland, OR, USA).

### Animals and *in vivo* procedures

Animal husbandry and all experimental procedures were approved by the local Animal Ethics Council and were performed in accordance with national and international laws and policies on the protection of animals used for scientific purposes (EU Directive 2010/63/EU; Italian Legislative Decree 26/2014). Mouse and macaque studies were conducted under research projects authorized by the Italian

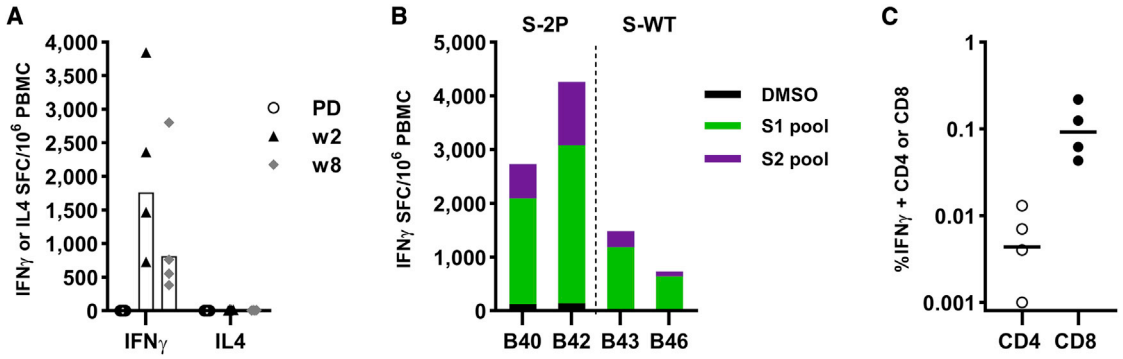


**Figure 8. T cell response to SARS-CoV-2 Spike induced in cynomolgus macaques upon GRAd-COV2 or GRAd32c-S immunization**

(A) IFN-γ or IL4 ELISpot on freshly isolated PBMCs 2 and 8 weeks post-immunization. Data are expressed as IFN-γ or IL4 SFCs/$10^6$ PBMC. Individual data points represent a cumulative Spike T cell response, calculated by summing S-1 and S-2 peptide pools' stimulation response corrected for the DMSO background of each animal. Bar represents geometric mean. (B) IFN-γ ELISpot response to individual S-1 and S-2 peptide pools in PBMC 2 weeks post-immunization. (C) Intracellular staining and FACS analysis on PBMC. Data are expressed as the percentage of either CD8- or CD4-secreting cytokine in response to S-1 and S-2 Spike peptide pools' stimulation, obtained by summing reactivity to each of the 2 Spike peptide pools and subtracting 2 times the DMSO background. Horizontal lines represent geometric mean.

Ministry of Health (authorization numbers 1065/2015-PR mouse and 984/2015-PR macaques)

### *Mice*

6-week-old female BALB/c mice purchased from Envigo (Indianapolis, IN, USA) were acclimatized and housed in individually vented cages at the Plaisant animal facility (Castel Romano, Rome, Italy). Animal handling procedures (immunization and bleed) were performed under anesthesia. Animals were divided into experimental groups of 6 or 8 mice each and immunized with a single administration of different doses of GRAd-COV-2 (from $1 \times 10^9$ vp to $1 \times 10^5$ vp) or with two administrations 2 weeks apart of 2.5 μg Spike protein (2019-nCoV Spike protein S-1 + S-2 extracellular domain [ECD], His tag [Sino Biological, Wayne, PA, USA] formulated in final 20 mg/mL Imject Alum Adjuvant [Thermo Fisher Scientific]). A control group of 5 mice received $1 \times 10^9$ vp of a GRAd32 vector encoding for HIV-1 gag antigen. All immunizations were performed via bilateral i.m. injections in the quadriceps (50 μL per side). After euthanasia, spleens were collected and processed into single-cell suspensions. Splenocytes were isolated by mechanical disruption and resuspended in culture medium after red blood cell lysis by treatment with ACK buffer (Invitrogen). Isolated cells were then used for ICS after *in vitro* re-stimulation and/or tested for IFN-γ ELISpot assay. Mice sera were obtained by standard 30 min blood coagulation at room temperature and centrifugation.

### *Macaques*

The study was conducted at Aptuit Srl (Verona, Italy) in accordance with national legislation, under approval of the internal Aptuit Committee on Animal Research and Ethics. General procedures for animal care and housing are in accordance with the current Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International recommendations. Four male cynomolgus monkeys (*Macaca fascicularis*) originating from Mauritius of approximately 3−4 years of age and 4 to 5 kg of body weight at study start were housed in groups of 2 in stainless-steel cages, with constant access to environmental enrichment devices within each cage. Conscious animals were vaccinated in deltoid muscle with a single dose of $5 \times 10^{10}$ vp GRAd-COV2 (group 1, animal ID B40/B42) and GRAd32c-S (group 2, animal ID B43/B46). Bleedings were performed from the femoral vein. Serum samples were harvested at w1 (pre-dose [PD]), w2, w4, w6, and w10 post-injection, obtained by 30 min coagulation at room temperature, and centrifugation and used for ELISA and *in vitro* neutralization assays. PBMCs were obtained from blood collected in lithium heparin tubes and isolated by separation on Ficoll gradient (GE Healthcare Life Sciences); PBMCs were harvested at w1 (PD), w2, and w8 and used fresh the same day for *ex vivo* IFN-γ/IL-4 ELISpot or frozen for ICS.

### SARS-CoV-2 Spike and RBD ELISA

SARS-CoV-2 ELISA was developed in-house using His-tagged proteins bound on Ni-NTA HisSorb strips or plates (QIAGEN, Hilden, Germany). ELISA assay on mouse sera was performed with His-tagged SARS-CoV-2 full-length Spike protein (produced in baculovirus [Sino Biological]) or with a commercial RBD (ACROBiosystems, Newark, DE, USA), whereas ELISA with monkey sera was performed with a soluble his-tagged stabilized SARS-CoV-2 Spike protein produced in-house in Expi293 cells or with a commercial RBD (ACROBiosystems, Newark, DE, USA). In brief, an optimized amount of proteins in 100 μL vol was bound to Ni-NTA plates/strips for 1 h at 25°C in shaking (1 μg/mL for full-length Spike, 2 μg/mL for R121, 0.5 μg/mL for RBD); plates were then incubated with serial dilutions of sera for 2 h at 25°C in shaking, and then binding was detected with specific alkaline phosphatase-conjugated secondary antibodies (anti-mouse total IgG and anti-monkey total IgG from Sigma) and anti-mouse IgG1 and IgG2a from BD Biosciences. Alkaline phosphatase substrate was prepared by dissolving SigmaFast (Sigma) tablets in sterile distilled $H_2O$ (Gibco). Absorbance was read at 405 and 620 nm using EnSight multiple plate reader (PerkinElmer, Waltham, MA, USA). The endpoint titer was defined as

the highest serum dilution that resulted in an absorbance value (OD [optical density]) just above calculated background: for mice experiments, 3-fold the OD of 1:100 serum dilution from naive mice; for monkey experiments, 4-fold the OD from secondary antibody alone. Serum from naive BALB/c animals is routinely tested at 1:100 dilution as negative control in ELISA, and the resulting OD is nearly at the level of secondary antibody alone; therefore, for mouse experiments, baseline titers cannot be detected, calculated, and plotted.

#### HIV-1 gag ELISA

Briefly, HIV-1 gag p24 protein (Austral Biologicals, San Ramon, CA, USA) was diluted to 1 μg/mL in PBS and 100 μL (or 100 ng gag protein) per well dispensed on Nunc MaxiSorp 96-well flat-bottom plates (Thermo Fisher Scientific, Waltham, MA, USA) for coating overnight at 4°C. During blocking of the plates in milk buffer, serial dilutions of sera were prepared in the same buffer and then incubated for 2 h at 25°C. Binding was then detected with specific alkaline phosphatase-conjugated secondary antibody (anti-mouse total IgG from Sigma). Alkaline phosphatase substrate was prepared, plates were read, and endpoint titer was calculated in the same way mentioned for SARS-CoV-2 ELISA.

#### RBD/ACE2 neutralization ELISA

RBD/ACE2 neutralization ELISA (ACROBiosystems) was performed according to the manufacturer's instruction. In brief, MaxiSorp ELISA plates (Nunc, Thermo Fisher Scientific, Waltham, MA, USA) were coated with 50 ng/well (100 μL at 0.5 μg/mL) of SARS-CoV-2 RBD protein for 16 h at 4°C, blocked for 1.5 h at 37°C, and incubated 1 h at 37°C with 50 μL of 0.12 μg/mL biotinylated recombinant hACE2 protein alone or with serial dilutions of mice sera. ACE2 binding was detected with horseradish peroxidase (HRP)-conjugated streptavidin (BD Pharmingen, Franklin Lakes, NJ, USA) and developed with TMB substrate (Sigma). Absorbance was read at 450 nm using the EnSight multiple plate reader (PerkinElmer). Data are expressed as neutralization curves calculating the percentage of ACE2/RBD-binding inhibition at the different serum dilutions relative to the control without inhibitory antibodies.

#### SARS-CoV-2 MN assay

Human sera were obtained from post-convalescent COVID-19 patients after signing an informed consent, in the frame of a project aimed at following up patients at the Spallanzani hospital.

Sera collected from patients and vaccinated animals were heat inactivated at 56°C for 30 min and titrated in duplicate in two-fold serial dilutions. Equal volumes of 100 TCID50 SARS-CoV-2 (strain 2019-nCoV/Italy-INMI, GSAID accession number: EPI_ISL_410546) and serum dilutions were mixed and incubated at 37°C for 30 min. Subsequently, 96-well tissue-culture plates with sub-confluent Vero E6 cell (ATCC CRL-1586; LGC Standards Srl, Milan, Italy) monolayers were infected with 100 μL/well of virus-serum mixtures and incubated at 37°C and 5% $CO_2$. After 48 h, microplates were observed at the microscope for the presence of CPE. To standardize inter-assay procedures, positive control samples showing high and low neutralizing activity were used for each MN assay. The supernatant of each plate was carefully discarded, and 120 μL of a Crystal Violet solution (Diapath S.p.A., Martinengo, Bergamo, Italy) containing 2% formaldehyde (AppliChem, Darmstadt, Germany) was added to each well. After 30 min fixation, the fixing solution was removed and cell viability measured by photometer at 595 nm (Synergy HTX; BioTek Instruments, Winooski, VT, USA). The serum dilution inhibiting 50% of the CPE ($IC_{50}$) was calculated using Prism 7 (GraphPad Software, San Diego, CA, USA) as described in Ferrara and Temperton.[24]

#### SARS-CoV-2-pseudotyped virus neutralization assay

Pseudotyped viruses were produced as previously described.[25] Briefly, $1.5 \times 10^6$ HEK293T cells were seeded overnight in a 10-cm-diameter Primaria-coated dish (Corning, Flintshire, UK). Transfections were performed using 2 μg each of the murine leukemia virus (MLV) Gag-Pol packaging vector (phCMV-5349), luciferase encoding reporter plasmid (pTG126), and plasmid encoding the same human codon-optimized version of the SARS-CoV-2 Spike coding sequence as detailed above, minus the HA tag. These were mixed with 24 μL cationic polymer transfection reagent (polyethylenimine; Polysciences, Warrington, PA, USA) in the presence of Opti-MEM (Gibco) and the media replaced with 10 mL complete DMEM after 6 h. A no-envelope control (empty pseudotype) was used as a negative control in all experiments. Supernatants containing SARS-CoV-2 pseudotypes were harvested at 72 h post-transfection and filtered through 0.45 μm pore-size membranes. For infectivity and neutralization assays, either $1.5 \times 10^4$ HuH7 cells or $2 \times 10^4$ VeroE6 cells/well were plated in white 96-well tissue-culture plates (Corning) and incubated overnight at 37°C. The following day, SARS-CoV-2 pseudotypes were incubated with heat-inactivated sera for 1 h at room temperature before being added to cells for 4 h. Following this, sera and media were discarded, and 200 μL DMEM was added to the cells. After 72 h, media were discarded, and cells were lysed with cell lysis buffer (Promega, Southampton, UK) and placed on a rocker for 15 min. Luciferase activity was then measured in relative light units (RLUs) using a FLUOstar Omega plate reader (BMG Labtech, Aylesbury, UK) with MARS software. Each sample was tested in triplicate.

#### ELISpot

For ELISpot assays, MSIP 96-well plates were from Merck Millipore (multiscreen filter plates; Burlington, MA, USA), and anti-mouse or anti-monkey IFN-γ or IL-4 capture and detection antibodies were all from U-CyTech (Utrecht, Netherlands). Lymphocytes (from mouse spleens/lungs or macaque PBMC) were plated in duplicate at appropriate cell densities and cultured for 18–20 h at 37°C, 5% $CO_2$ with 15-mer peptide pools covering the entire SARS-CoV-2 Spike protein referred to as S-1 or S-2 (JPT PepMix; JPT, Berlin, Germany) or covering HIV-1 gag protein (Elabscience, distributed by Tema Ricerca Srl, Italy). Stimulation conditions were 1 (for mouse lymphocytes) or 3 μg/mL (for monkey's PBMCs) final single peptide concentration or the equivalent amount of DMSO (Sigma), the peptide diluent, as negative control. Cytokine production and spot formation were detected with alkaline phosphatase-conjugated

streptavidin and NBT/BCIP alkaline phosphatase substrate (Thermo Scientific). The number of spots per well, directly related to the precursor frequency of antigen-specific T cells, was counted and analyzed with a CTL ImmunoSpot reader (ImmunoSpot, Shaker Heights, OH, USA). Data are expressed as cytokine SFCs per million splenocytes, lung lymphocytes, or PBMC and are shown upon subtraction of DMSO background.

#### ICS and FACS analysis

Mouse splenocytes were used freshly isolated, while monkey PBMCs were stimulated after thawing and overnight resting in culture medium. Cells were plated at $1 \times 10^6$ per well in a 96-well round-bottom plate and stimulated with either SARS-CoV-2 peptide pool S-1 or S-2 or with DMSO (Sigma) as a negative control, all in the presence of anti-CD28 antibody (BD Pharmingen), for 18 h (mouse cells) or 5 h (monkey cells) in the presence of brefeldin A (Sigma). Cells were then washed to stop the stimulation, stained with Live/Dead fixable dye (Invitrogen), fixed and permeabilized with BD Cytofix/Cytoperm Kit (BD Biosciences) following the manufacturer's instructions, and then stained with specific fluorochrome-conjugated antibodies. Sample acquisition was performed with the LSRFortessa X-20 cytofluorimeter (BD Biosciences), and sample analysis was performed with FlowJo (Tree Star). Mouse antibodies included the following: CD3 Alexa Fluor 647, CD4 BV421, CD8 BUV395, IL-17a PerCP-Cy 5.5, IFN-γ BV650, IL-2 APC-R700 (BD Biosciences), IL-4 phycoerythrin (PE), and IL-13 PE (Invitrogen); NHP antibodies included the following: CD3 AF700, CD4 PerCP-Cy 5.5, CD8 PE (BD Biosciences), and IFN-γ fluorescein isothiocyanate (FITC; U-CyTech).

#### Statistics

GraphPad Prism version 8 for Windows (GraphPad Software, San Diego, CA, USA) was used for graphs and statistical analysis. The presented studies are mostly descriptive in nature, and no comparisons between groups were presented, or numerosity was too small (i.e., for macaque study) to allow statistical analysis. In Figure 6, since data showed Gaussian distribution, as per all normality tests run, a two-tailed unpaired Student's t test was used. Only statistically significant results were reported in the figures: $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$.

## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.ymthe.2021.04.022.

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## AUTHOR CONTRIBUTIONS

Conceptualization, S. Colloca, S. Capone, A.F., A.R., and A.V.; methodology, A.R., S. Capone, J.K.B., C.C., and A. Lahm; investigation, M.G., S.B., A.S., A.M.C., R.S., R.A.U., F.B., A. Leuzzi, E.L., G. Miselli, A.N., M.F., F.T., T.T., G. Matusali, F.C., D.L., and S.M.; formal analysis, S. Capone, C.C., and R.A.U.; writing – original draft, S. Capone, A.V., and A.R.; writing – review & editing, J.K.B., M.G., S.B., A.S., A.M.C., A. Lahm, S. Colloca, A.F., and F.B.; supervision, M.C., M.S., J.K.B., A.V., and S. Capone; funding acquisition, A.V., S. Colloca, and A.F.

## DECLARATION OF INTERESTS

S. Capone, A.R., M.G., S.B., A.S., A.M.C., R.S., F.B., A. Leuzzi, E.L., G. Miselli, A.N., M.F., F.T., M.S., A.F., S. Colloca, and A.V. are employees of ReiThera Srl. A.F. and S. Colloca are also shareholders of Keires AG. A. Lahm is a consultant for ReiThera Srl. S. Colloca, A. Lahm, A.R., and A.V. are inventors of the patent application number 20183515.4, titled "Gorilla Adenovirus Nucleic Acid- and Amino Acid-Sequences, Vectors Containing Same, and Uses Thereof." The other authors declare no competing interests.

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