Molecular Therapy
Original Article

A first-in-human trial on the safety and immunogenicity of COVID-*e*Vax, a cellular response-skewed DNA vaccine against COVID-19

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The COVID-19 pandemic and the need for additional safe, effective, and affordable vaccines gave new impetus into development of vaccine genetic platforms. Here we report the findings from the phase 1, first-in-human, dose-escalation study of COVID-*e*Vax, a DNA vaccine encoding the receptor binding domain (RBD) of the SARS-CoV-2 spike protein. Sixty-eight healthy adults received two doses of 0.5, 1, or 2 mg 28 days apart, or a single 2-mg dose, via intramuscular injection followed by electroporation, and they were monitored for 6 months. All participants completed the primary safety and immunogenicity assessments after 8 weeks. COVID-*e*Vax was well tolerated, with mainly mild to moderate solicited adverse events (tenderness, pain, bruising, headache, and malaise/fatigue), less frequent after the second dose, and it induced an immune response (binding antibodies and/or T cells) at all prime-boost doses tested in up to 90% of the volunteers at the highest dose. However, the vaccine did not induce neutralizing antibodies, while particularly relevant was the T cell-mediated immunity, with a robust Th1 response. This T cell-skewed immunological response adds significant information to the DNA vaccine platform and should be assessed in further studies for its protective capacity and potential usefulness also in other therapeutic areas, such as oncology.

INTRODUCTION

Coronavirus disease 2019 (COVID-19) is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a highly transmissible agent that has infected more than 600 million people and killed more than 6 million since the start of the pandemic declared in March 2020.[1]

To date over 170 vaccine candidates are being assessed in clinical trials.[2] However, despite the extremely fast authorization for emergency use of the first vaccines already in December 2020 and the aggressive vaccination campaign, most of the world's population remains unvaccinated and susceptible to COVID-19.[3] Thus, additional vaccines that are affordable, scalable, that can be easily distributed/stored, and/or that can improve the performance of already available vaccines are urgently needed. Besides, it is mandatory to understand the characteristics of the different technology platforms that are being studied.

Currently, the latter include inactivated/live attenuated virus, recombinant proteins, or the new genetic technologies, namely based on messenger RNA (mRNA), replicating or non-replicating viral vectors, and DNA vaccines. The mRNA- and DNA-based platforms provide high flexibility in antigen design, rapid development, and easy adaptability to virus mutations. Indeed, mRNA vaccines were the quickest to show strong immune responses in clinical trials, and they have now been administered to hundreds of millions of people. DNA-based vaccines have several potential benefits: they are easy to produce, may provide longer exposure to target epitopes, and the finished products are more stable than mRNA vaccines, which typically require transportation and storage at very low temperatures (−80°C). DNA vaccines are able to activate the innate humoral and cellular immune response,[4] are effective even without adjuvants, and are devoid of possible safety drawbacks of the excipients in

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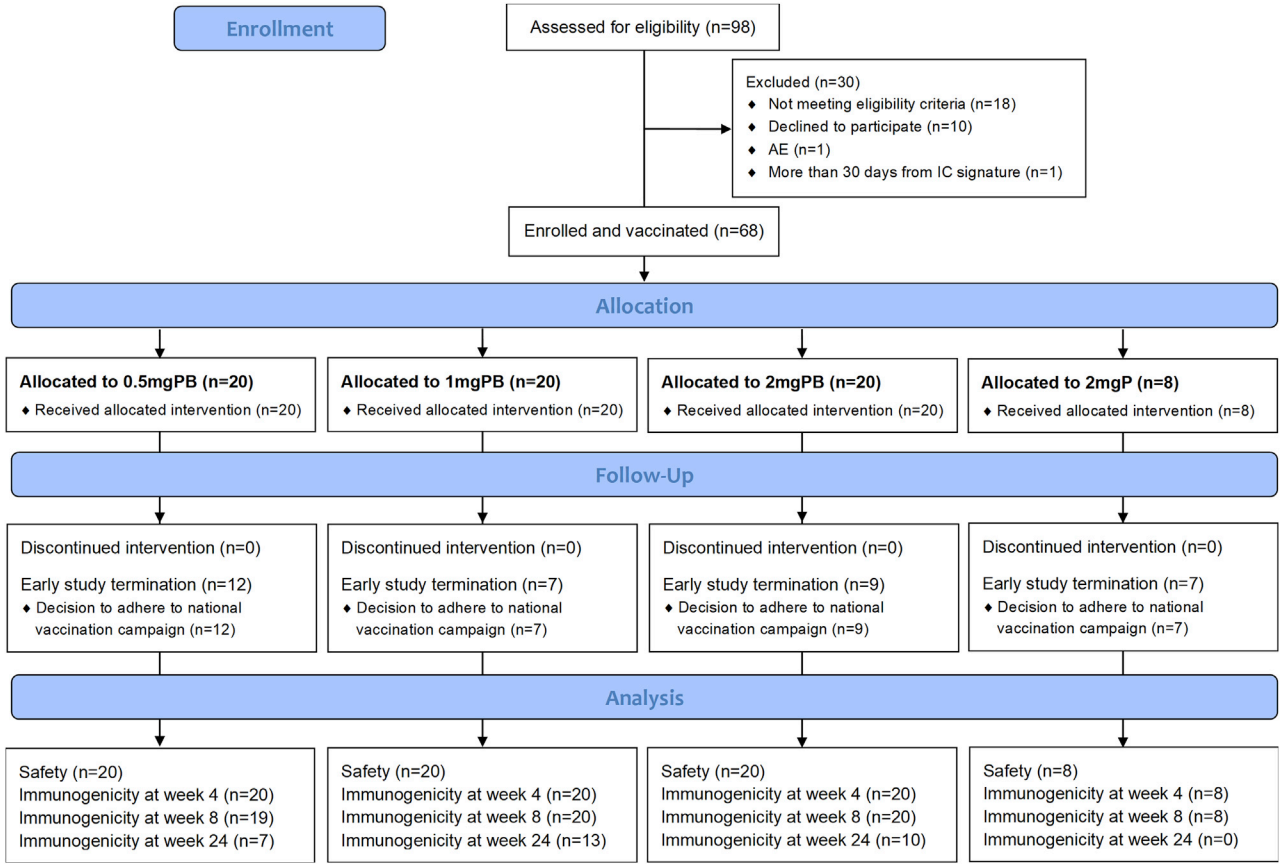

Figure 1. Study flow diagram

The study dates were from February 25, 2021 (start of subjects' eligibility assessments) till December 7, 2021 (last subject last visit). Due to the progress of the COVID-19 vaccination campaign in Italy leading to inability to find eligible subjects, enrollment in the last cohort (2mgP) was halted before reaching the target of 20 participants. One participant in the 0.5mgPB cohort was excluded from immunogenicity analysis at week 8 and at subsequent timepoints because of SARS-CoV-2 infection that occurred a few days after the second vaccination; another participant in the 2mgPB cohort showed seroconversion to N protein at week 24 and was excluded from immunogenicity analysis at that time point; one participant in the 2mgP cohort performed the study completion visit assessments at week 24, but blood samples for immunogenicity were not taken because of adherence to the national campaign close to the visit.

mRNA vaccines. Moreover, when administered as naked DNA and not through viral vectors, they are devoid of the risk of inducing an anti-vector immunity,[5] allowing repeated administrations. Several DNA vaccines are currently licensed for veterinary use in large and small animals[6] and have provided satisfactory immunogenicity responses against different viral diseases in humans.[7–11] Moreover, in August 2021, the first DNA-based vaccine for COVID-19 was authorized in India.[12]

COVID-*e*Vax is the first DNA vaccine candidate against COVID-19 to reach clinical development in Europe. It is a DNA plasmid-based vaccine encoding the SARS-CoV-2 receptor binding domain (RBD) of the spike (S) protein, which is known to be a major target of human antibodies deriving from SARS-CoV-2 patients. COVID-*e*Vax is administered through intramuscular injections followed by the delivery of short electrical pulses (i.e., an electroporation procedure). Electroporation enhances DNA transfection inducing transient permeability of biological membranes.[13] In animal models, COVID-*e*Vax was immunogenic, inducing potent neutralizing antibody responses (including against recent variants of concern) and a robust T cell response able to elicit significant protection upon challenge with SARS-CoV-2[14] with good safety.[15]

We report here the safety and immunogenicity results of the COVID-*e*Vax DNA vaccine from a dose-escalation, phase 1 clinical trial evaluating three cohorts (0.5, 1, and 2 mg prime-boost, PB) receiving two doses 28 days apart, and an additional cohort (2 mg prime, P) receiving one dose only.

RESULTS

Subject disposition and demographic characteristics

The study flowchart is presented in Figure 1. Sixty-eight subjects were enrolled and vaccinated, while 30 volunteers were excluded mainly because they did not meet the eligibility criteria.

Table 1. Demographic and baseline characteristics of study participants

	0.5mgPB (n = 20)	1mgPB (n = 20)	2mgPB (n = 20)	2mgP (n = 8)	Overall (n = 68)
Age, years	43.4 (12.9)	40.7 (8.6)	36.6 (10.6)	34.9 (9.0)	39.6 (10.9)
Sex, n (%)					
Female	9 (45.0)	11 (55.0)	11 (55.0)	3 (37.5)	34 (50.0)
Male	11 (55.0)	9 (45.0)	9 (45.0)	5 (62.5)	34 (50.0)
Race, n (%)					
White	20 (100)	20 (100)	20 (100)	8 (100)	68 (100)
BMI, kg/m^2	24.4 (2.8)	25.0 (2.3)	23.2 (2.1)	24.1 (2.2)	24.2 (2.5)
Weight, kg	70.7 (10.2)	72.4 (10.9)	66.0 (10.5)	70.9 (12.1)	69.8 (10.8)

Data are mean (SD), unless otherwise indicated.
BMI, body mass index.

All participants remained in the study up to week 8 and completed the assessment of primary safety and principal immunogenicity outcomes at that time point; afterward, between week 8 and week 24, 35 subjects decided to adhere to the COVID-19 national vaccination campaign and to withdraw from the study.

Demographic and baseline characteristics of the study participants were balanced among the four cohorts (Table 1). All participants were White and 50% were women; overall, the mean (SD) age was 39.6 (10.9) years, and BMI was 24.2 (2.5) kg/m^2.

Safety

No deaths or serious adverse events were reported in the study. No safety issues were identified by the Independent Data and Safety Monitoring Committee (IDSMC) during the dose-escalation steps, and vaccine administration was well tolerated.

In the 7-day period after the first vaccination, the incidence of solicited local adverse events (AEs) (mainly consisting of mild to moderate tenderness, pain, and bruising) ranged between 35% with 0.5 mg and 75%–87% with 1 or 2 mg (Figure 2 and Table S1). Incidence decreased after the second vaccination in all PB cohorts.

Solicited systemic AEs were in the 40%–50% incidence range in all cohorts after the first vaccination and tended to slightly decrease after the second one (Figure 2 and Table S1). They mainly consisted of mild to moderate headache and malaise/fatigue and were severe in four subjects (Table S1). Fever (maximum 37.5°C for 2 days) was reported by one subject only in the 2mgPB cohort. Eleven participants (16%) reported use of pain medications at least once in the 7 days post vaccination, mainly for headache.

Participants experiencing at least one unsolicited AE through 4 weeks after any vaccination were 7–8 (35%–40%) in each PB cohort (Table S2), with no relevant difference between first or second vaccination, although they were less in the small 2mgP cohort. The majority of events were sporadic and mild to moderate, including a few contusions and headache reported after the 7 days post vaccination.

There were three episodes of blood creatine phosphokinase (CPK) increase (laboratory upper limit of 170–190 U/L), observed 1 week after the first or the second vaccination in three participants belonging to the 1mgPB cohort; one episode was severe (CPK value 1909 U/L), one was moderate (651 U/L), and one was mild (376 U/L), and the first two were considered by the investigator as related to the muscle stress induced by the electroporation procedure (to note, according to the investigator, the severe episode was also probably due to a recent intense physical activity). The analysis of unsolicited AEs reported through study completion (i.e., all unsolicited AEs up to 6 months) did not reveal any safety concerns (Table S3). Two participants experienced symptomatic COVID-19 at study day 76 and 141 in the 0.5 and 2mgPB cohorts, respectively, that resolved without complications.

No clinically significant findings were noted at 12-lead ECG 1 h after vaccination, and vital signs remained stable.

Immunogenicity

Binding anti-S antibodies specific for RBD became detectable at week 4 and peaked between weeks 8 and 12 (Figure 3 and Table S4). Geometric mean concentrations were $\geq$4.0 U/mL in the 1mgPB and 2mgPB cohorts at week 8, with an 11- to 12-fold increase relative to baseline (geometric mean fold rise, GMFR). All PB cohorts had an at least 12-fold increase at week 12, peaking at 17 folds in the 2mgPB cohort that still showed a 12-fold GMFR at week 24. Vaccine-induced binding antibody response was lower in the 2mgP cohort. At the week 8 time point, the majority but not all subjects had seroconverted in all cohorts (68% with the 0.5-mg dose and 75% with both 1 and 2 mg PB, and 63% with 2 mg P, respectively: Table S4). Thereafter, the proportion of evaluable participants with a positive binding antibody value was in the 80%–90% range with 2mgPB from week 8 onward and only slightly lower but less sustained in the other PB cohorts (Table S4), while considering all the vaccinated participants, the percentages of volunteers with such a value at least once in the study were 70% in the 0.5mgPB cohort and 75% in the other cohorts.

However, the response in terms of neutralizing antibody titer was poor. Only two subjects in the 2mgPB cohort achieved a significant, although low, neutralizing antibody titer of 10; one of these two was the subject with the highest peak in binding antibodies of 232 U/mL (Figure 3), while the second subject, always in the 2mgPB cohort, had a correspondingly much lower binding antibody concentration of 2.4 U/mL at week 16. In the other participants, neutralizing antibodies were absent at all timepoints.

Conversely, the vaccine-induced T cells response was pronounced (Figure 4A and Table S5). The week 8 GMFRs showed an approximately 5-fold increase in IFN-γ spot-forming cells (SFC) per million peripheral blood mononuclear cells (PBMCs) in the 1mgPB and 2mgPB cohorts, compared with a 2-fold increase in the 0.5mgPB cohort. All PB increases were significant over baseline ($p < 0.01$ or better; Figure 4A). The T cell response was earlier and numerically higher

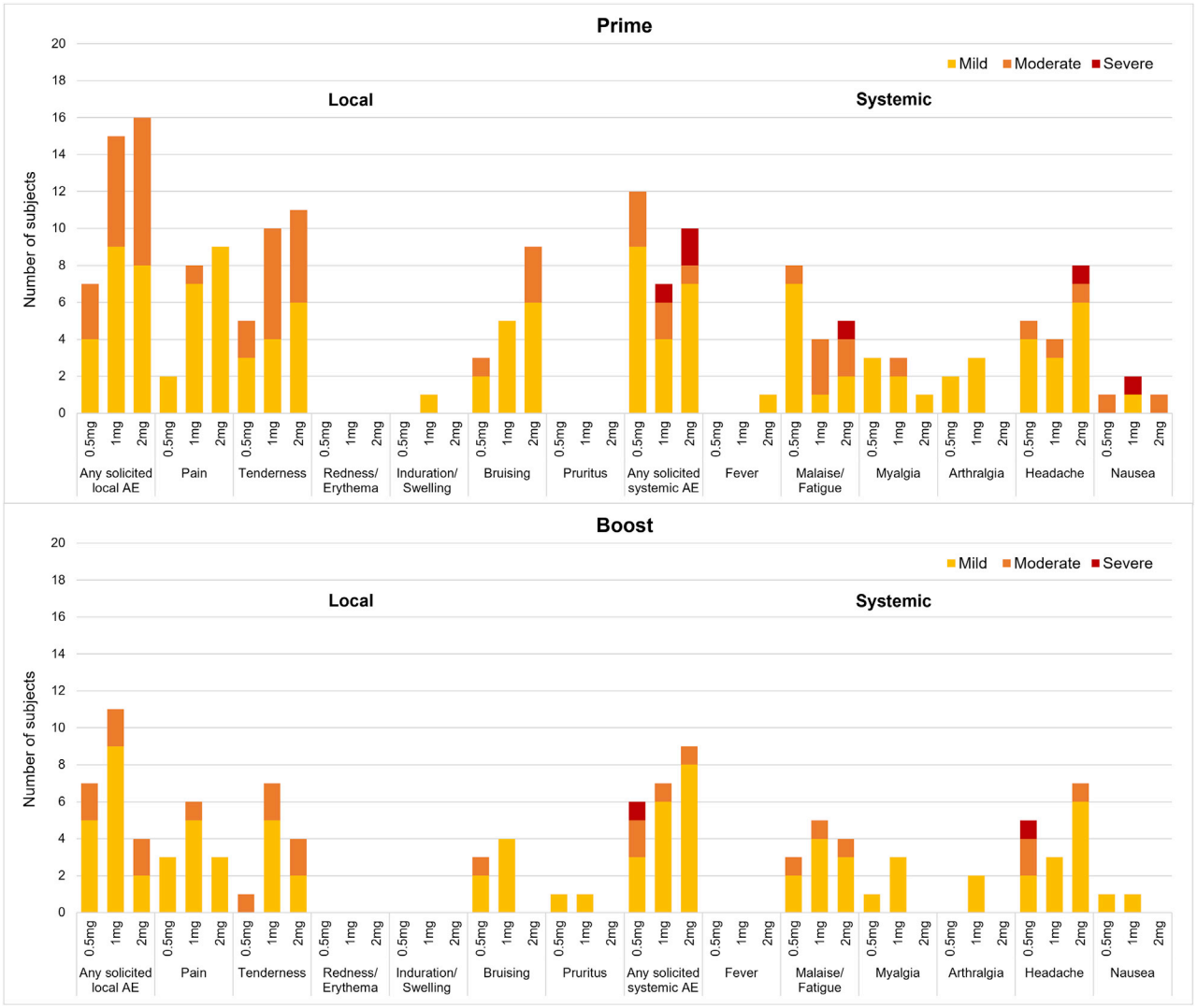

Figure 2. Solicited local and systemic adverse events in PB cohorts study participants

The number of subjects in each prime-boost (PB) cohort with solicited adverse event by maximum severity during the 7 days after the first vaccination (upper panel) and after the second vaccination (lower panel) are shown. Solicited AEs were graded by the participants in the electronic diary according to the toxicity grading scale reported in Table S7. There were no grade 4 (life-threatening) events.

in the 1mgPB cohort and only slightly less sustained than in the 2mgPB cohort at 12 weeks, as shown by the corresponding GMFRs. At week 24, the T cell response tended to return to baseline levels. The response in the 2mgP cohort was lower and not sustained from week 8 onward, compared with the PB cohorts (Figure 4A and Table S5).

To determine whether the T cells generated in response to vaccination could cross-recognize SARS-CoV-2 Omicron variant (B.1.1.529), RBD-specific T cell response against ancestral or Omicron RBD was assessed in a subset of the 1mgPB and 2mgPB cohorts at week 8. No difference was revealed in the elicited T cell response against ancestral RBD and Omicron RBD in both cohorts (Figure 4B).

Further characterization of the T cells response by intracellular cytokine staining (ICS) analysis in a subset of participants in the 2mgPB cohort showed a robust Th1 response at week 8, with a significant increase in both CD4+ and CD8+ T cells secreting IFN-γ and/or IL-2 compared with the negative control (Figure 5). Conversely, no Th2 response was evident, as the fractions of CD4+ and CD8+ T cells secreting IL-4 and/or IL-5 and/or CD40L after RBD peptide pool stimulation were similar to those observed after the negative control stimuli. Expression of each individual cytokine is summarized in Table S6. Contrary to the aspecific ELISpot assay in which response elevation beyond the baseline background could not be observed after 12 weeks, the assessment of T cell phenotype by specific ICS analysis within the 2mgPB cohort showed a persisting percentage

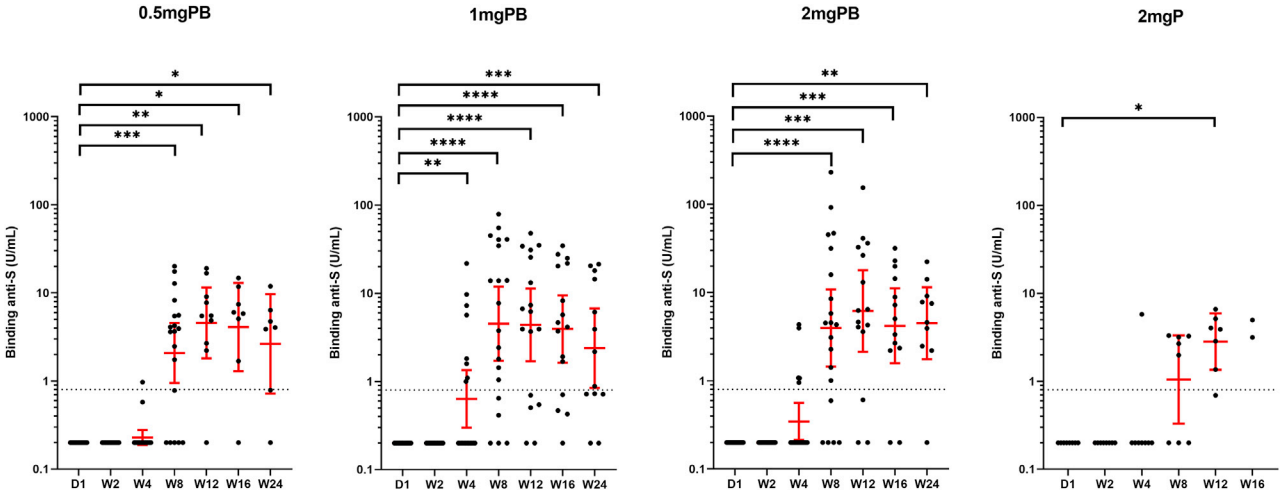

Figure 3. SARS-CoV-2 S protein binding antibodies in study participants

SARS-CoV-2 S protein binding antibodies were measured at baseline (day 1, before vaccination) and at weeks 2, 4, 8, 12, 16, and 24. Individual values of SARS-CoV-2 S protein binding antibodies are shown. Red lines represent geometric means and 95% confidence interval (CI). The dotted line represents the positivity threshold for SARS-CoV-2 RBD-specific antibodies (≥0.80 U/mL). Comparison between post-baseline time point and day 1 was performed by Wilcoxon signed-rank test; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

of CD8+ and CD4+ T cells secreting both IFN-γ and TNFα up to week 24, thus indicating the presence of polyfunctional T cells up to 6 months post vaccination (Figure 6A).

Furthermore, the antigen-specific T cells induced by vaccination in the 2mgPB cohort subgroup were characterized, by defining their differentiation status using CCR7 and CD45RA markers, in order to investigate naive (N), central memory (CM), effector memory (EM), and terminally differentiated effector memory (EMRA) populations through 6 months post vaccination. N and CM T cells were rarely found, whereas EM and EMRA T cells were detectable at all time points. Specifically, polyfunctional EM cells (IFN-γ+ and TNFα+) dominated the CD4+ T cell subset up to week 24 post vaccination, whereas CD8+ T cells presented mostly an EMRA phenotype (Figure 6B).

Overall, the percentages of responders with positive binding anti-S antibodies and/or at least 2-fold increase in IFN-γ SFC at week 8 were 89% (17/19), 80% (16/20), and 90% (18/20) in the 0.5, 1, and 2mgPB cohorts, respectively, while they were 62.5% (5/8) in the 2mgP cohort.

There was a significant correlation between humoral (binding antibodies) and cellular response at week 8 in the 2mgPB cohort, but not with 1mgPB (Figure 7). Conversely, the neutralizing antibody response was poor and minimally evident only in the 2mgPB cohort.

DISCUSSION

Different technologies have been deployed in the effort to develop COVID-19 vaccines with optimal efficacy and safety in the current and possible future pandemics, but also to prevent or cure non-transmittable diseases such as cancer.

Among the new genetic platforms, naked DNA vaccines are rapid and cheap to produce or modify,[16] may provide longer exposure to target epitopes, and are temperature-stable compared with mRNA, and they can be repeatedly administered compared with viral vector vaccines. Electroporation is used to let DNA plasmids permeate cell membranes and to enhance immune responses,[17] obtaining good immunogenicity results with vaccines against viral diseases,[10,11] including COVID-19.[9] DNA-based vaccines have intrinsic adjuvant-like properties because plasmids can incorporate cytosine phosphate guanosine nucleotide sequences, which operate through Toll-like receptor 9 and scavenger receptors.[18] In addition, electroporation itself recruits and triggers cells involved in antigen presentation and immune response.[19]

In the present study, we have demonstrated that COVID-*e*Vax—a DNA vaccine construct targeting SARS-CoV-2 delivered intramuscularly by electroporation—is safe, thus satisfying the study primary objective. Reactogenicity was mainly mild and transient, generally lower after the second dose, and included bruising, an expected consequence of electroporation, similarly to transient blood CPK increase possibly due to muscle stress, which was however observed only in a few participants. Unsolicited AEs were sporadic and mild to moderate too.

The assumption that intramuscular electroporation may lead to blood CPK increase due to transient muscle stress and reversible tissue damage has been recently reviewed in rodents[20] and shown in human studies.[21] In animals, CPK leakage is independent of the presence of plasmid DNA, while the latter may promote additional muscle damage.[22] This did not seem to be the case in the present study, since the only three subjects with CPK increase were in the central 1mgPB

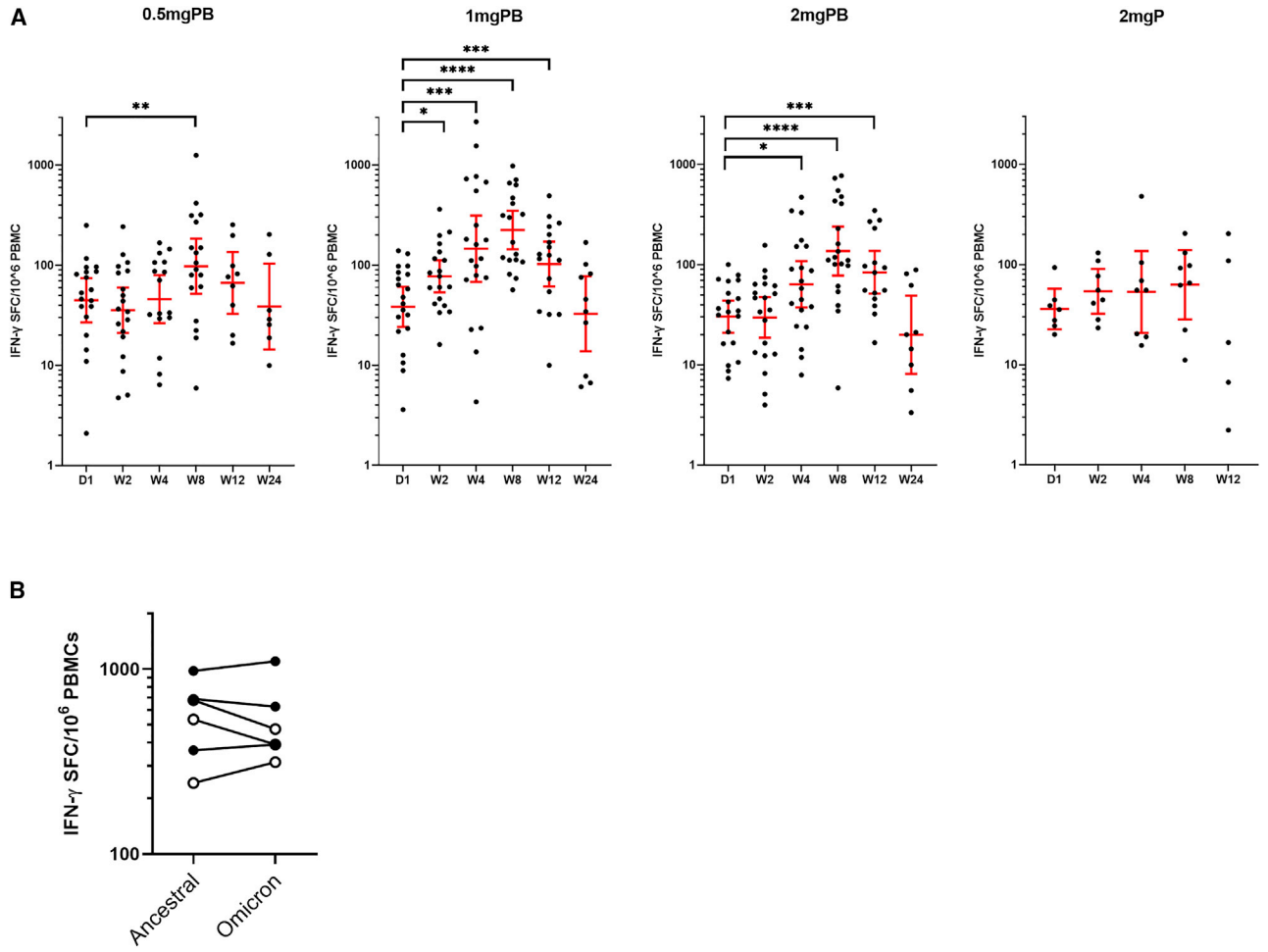

Figure 4. RBD-specific T cell response by IFN-γ ELISpot in study participants and cross recognition with Omicron variant

(A) RBD-specific T cell response was assessed by IFN-γ ELISpot on peripheral blood mononuclear cells (PBMCs) collected at baseline (day 1, before vaccination) and at weeks 2, 4, 8, 12, and 24. Individual values of IFN-γ spot forming cells (SFC) per million PBMCs are shown. Red lines represent geometric means and 95% confidence interval (CI). Comparison between post-baseline time point and day 1 was performed by Wilcoxon signed-rank test; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. (B) Comparison of T cell response to ancestral or Omicron RBD in 1mgPB (white dots) and 2mgPB (black dots) vaccinated subjects (three in each cohort: see supplemental methods description) on PBMCs collected at week 8. No significant differences were observed between antigens among vaccinated subjects using a Kruskal-Wallis test with Dunn's multiple comparisons post test.

cohort, i.e., with no apparent DNA concentration or dose response. Moreover, in a previous human study,[21] intramuscular electroporation alone induced at least mild CPK elevation in over 50% subjects, i.e., a much higher proportion than in the present study where lower voltage conditions were coupled with the presence of plasmid DNA. Further studies may better clarify the safety signal linked to the electroporation procedure or other factors.

This first-in-human trial also prompted the opportunity to preliminarily study the immunogenicity of COVID-*e*Vax. The vaccine induced an immune response with all tested PB doses, which was on the other hand limited to the induction of anti-S binding antibodies and of a distinct T cell response. In this respect, the best performance was with the highest 1- and 2-mg doses, inducing an immune response in up to 90% of the volunteers. Surprisingly, however, the vaccine did not induce neutralizing antibodies, in contrast to a particularly relevant cell-mediated response.

Vaccines are believed to protect mainly through neutralizing antibodies.[23] However, the immune system has evolved to be redundant, and emerging evidence points to a key role of T cells for vaccine-induced protection. Indeed, accumulating data suggests that clinical protection by T cells plays an important role against severe COVID-19, particularly against viral variants that partially escape recognition by neutralizing antibodies, by rapid control of virus replication in infected cells. Antigen-specific T cell response after COVID-*e*Vax was at least comparable if not slightly better than what is seen with the most effective mRNA vaccines[24] and was more pronounced

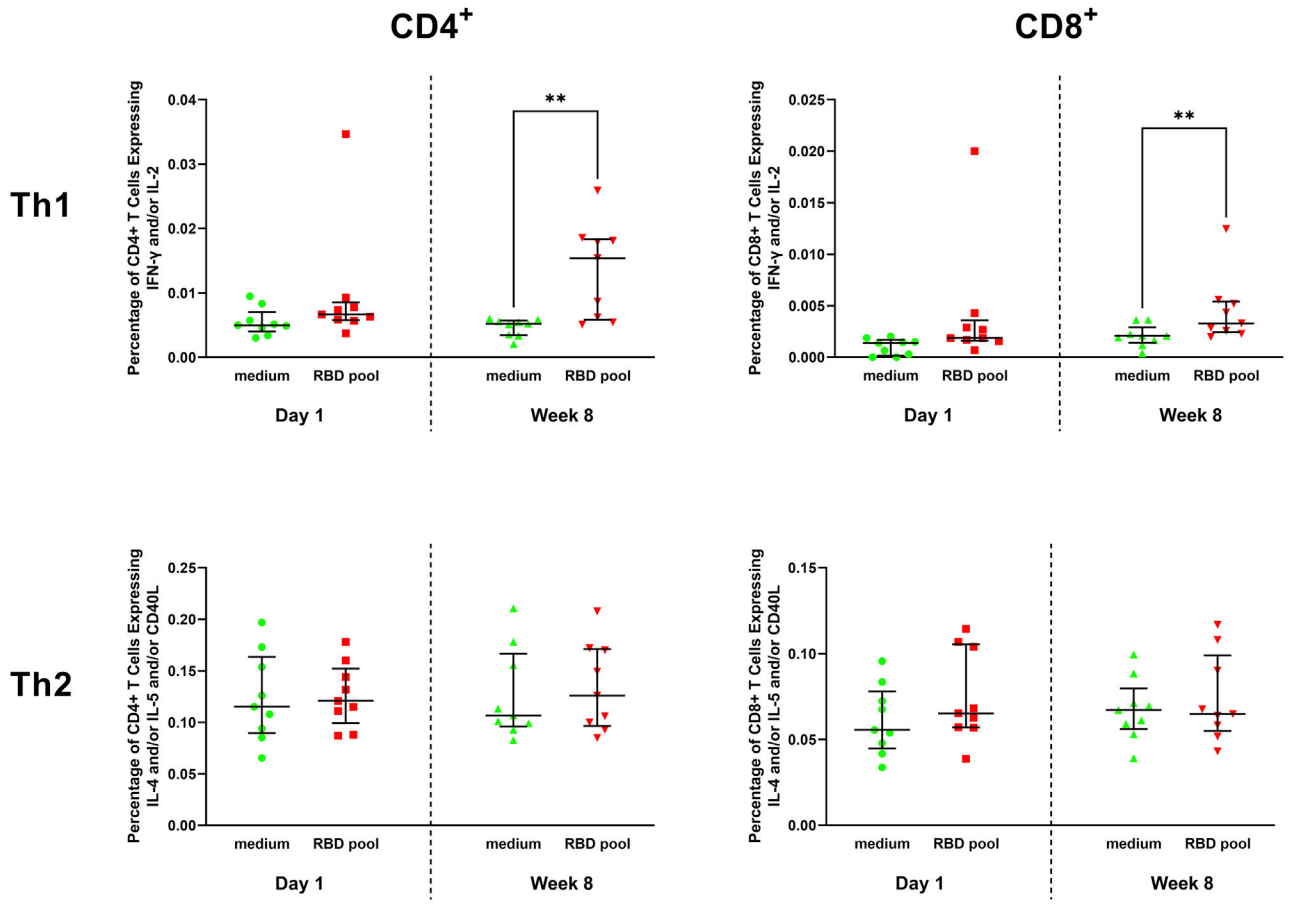

Figure 5. CD4+ and CD8+ T cell response characterization by ICS analysis

CD4+ and CD8+ T cells response characterization was performed by intracellular cytokine staining (ICS) on peripheral blood mononuclear cells (PBMCs) collected at baseline (day 1, before vaccination) and at week 8 from nine subjects belonging to the 2mgPB cohort. Individual values after negative control stimuli (green) and after RBD peptide pool stimulation (red) are shown. Horizontal lines represent medians and bars represent interquartile range. A response in Th1 cells was characterized by the expression of IFN-γ and/or IL-2; a response in Th2 cells was characterized by the expression of IL-4 and/or IL-5 and/or CD40L. Comparison between medium and RBD pool was performed by Wilcoxon signed-rank test; **$p < 0.01$.

than that observed with other DNA vaccines.[25,26] The latter encode different DNA constructs, and the administration conditions are different since they either use intradermal rather than intramuscular electroporation[9] or needle-free systems.[12] Indeed, the cellular response mediated by COVID-*e*Vax appeared more efficient than the humoral response, as supported by the correlations between the IFN-γ ELISpot data and the levels of binding antibodies: while the former reached a plateau at 1 mg PB, the humoral response needs a higher dose to be highlighted, being well correlated with the cellular response only at 2 mg PB. This suggests that the class I major histocompatibility complex (MHC) pathway is activated by COVID-*e*Vax at doses lower than those inducing a class II MHC response. This indirectly confirms that COVID-*e*Vax may trigger an immunological response similar to that naturally obtained from exposure to intracellular challenge, such as the viral infection itself, which may allow for effective memory and protection.[27] Furthermore, the cellular response appears to cross-recognize virus variants, even when obtained from the exposure to the original viral clade.[28] Importantly, COVID-*e*Vax is able to elicit a robust Omicron cross-reactive T cell response, thus confirming the preservation of the T cell response to new variants of concern with high degree of escape from neutralization.[29] In addition, CM established by vaccination is in general supposed to be sufficient to confer lasting protection.[30]

COVID-*e*Vax was shown to induce a strong RBD-specific CD4+ and CD8+ T cell Th1 and Tc1 response, and especially CD8+ T cells have demonstrated to provide protective immunity even with suboptimal antibody titers in macaques[31] and to improve survival in COVID-19 patients with hematologic cancers and consequent impaired humoral response,[32] underlying the importance of cellular immunity. In the present study, COVID-*e*Vax induced polyfunctional RBD-specific T cell responses, especially CD8+, with EM phenotype, which were stable throughout the 6-month period of this study. Collectively, these data suggest that COVID-*e*Vax elicits a durable T cell immune

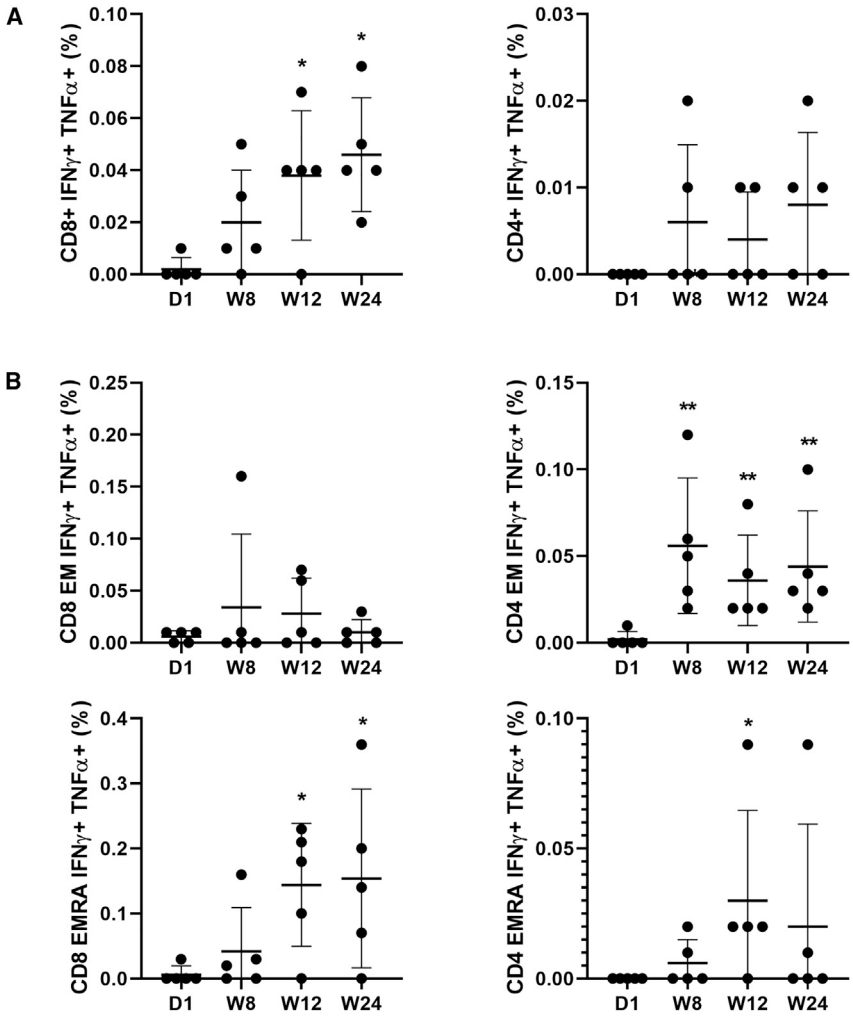

Figure 6. Polyfunctional and memory T cell response

(A) Polyspecific-CD4+ and CD8+ T cells response characterization was performed by intracellular cytokine staining (ICS) for IFN-γ and TNF-α on peripheral blood mononuclear cells (PBMCs) collected in available samples from the 2mgPB group at day 1 and weeks 8, 12, and 24. (B) Analysis of memory T cells in the same 2mgPB cohort samples. Antigen-specific naive (N), central memory (CM), effector memory (EM), and terminally differentiated effector (EMRA) populations were evaluated through 6 months post vaccination. Comparison between measurements at weeks 8, 12, and 24 with day 1 was performed by Wilcoxon signed-rank test; *$p < 0.05$; **$p < 0.01$.

response specific to SARS-CoV-2 RBD, which is particularly relevant in terms of effector memory T cells (EM and EMRA T cells) that are mainly found in the peripheral circulation and in tissues compared with CM T cells that are more commonly found in lymph nodes and were not evident in the present study. In studies of mRNA vaccines too, EM and EMRA T cells were shown to dominate CD4+ and CD8+ T cell subsets,[33] although those studies also found a relevant pool of memory stem cells,[33] which unfortunately could not be investigated in our study. Future studies of COVID-*e*Vax and other DNA vaccines will have to investigate the generation of T memory stem cells, which may better predict durability of the immune response.

Nevertheless, contrary to animal models,[14] the humoral response to COVID-*e*Vax was quantitatively low, with an almost absent neutralizing antibodies response being the main limitation of this clinical study. A first reason might reside in the electroporation conditions adopted. Typically, intramuscular electroporation studies have been affected by low tolerability. Conversely, we have experimentally selected the safest electrical parameters and device configuration in a propaedeutic human study at different low-voltage conditions.[34] In this preparatory study, we have also tested intradermal electroporation conditions, and in our hands, intradermal delivery was always less tolerated in terms of local pain, irrespectively of the electrical parameters chosen.[34] Moreover, the animal studies we performed (unpublished data) suggested that in our conditions the intramuscular route was more efficient than the intradermal one, in terms of both humoral and cellular response. On the other hand, only a proportion of the intramuscularly injected volume may be effectively electroporated in humans under the mild electrical conditions chosen, being adequate as such to stimulate a potent cellular immunogenicity response, but insufficient for a large humoral response in terms of neutralizing antibody titer. Possible solutions to increase intramuscular cell transfection maintaining safety-wise acceptable electrical parameters may be to increase the electroporated volume by increasing the surface between the electrodes by a different electrode geometry and/or to use a longer needle for injection deeper in the deltoid muscle, thus avoiding possible leakage superficially to the muscle itself.

Another reason for the poor humoral response may concern the nature of the RBD target.[35,36] COVID-*e*Vax encodes a secreted monomeric form of RBD, chosen at the beginning of the coronavirus pandemic, to minimize possible antibody-dependent enhancement (ADE) effects.[37] However, monomeric RBD may exhibit hapten-like properties and thus lower humoral immunogenicity capability in humans,[38] leading to the speculation that a close third dose might be necessary for COVID-*e*Vax to exploit its full immunogenicity profile. Other DNA-based vaccines have been indeed approved with a close three-dose cycling.[25] Data obtained so far from clinical trials with mRNA vaccines indicated that individuals that received three doses were highly protected from the more serious consequences of the infection. Interestingly, the third dose is generally accompanied

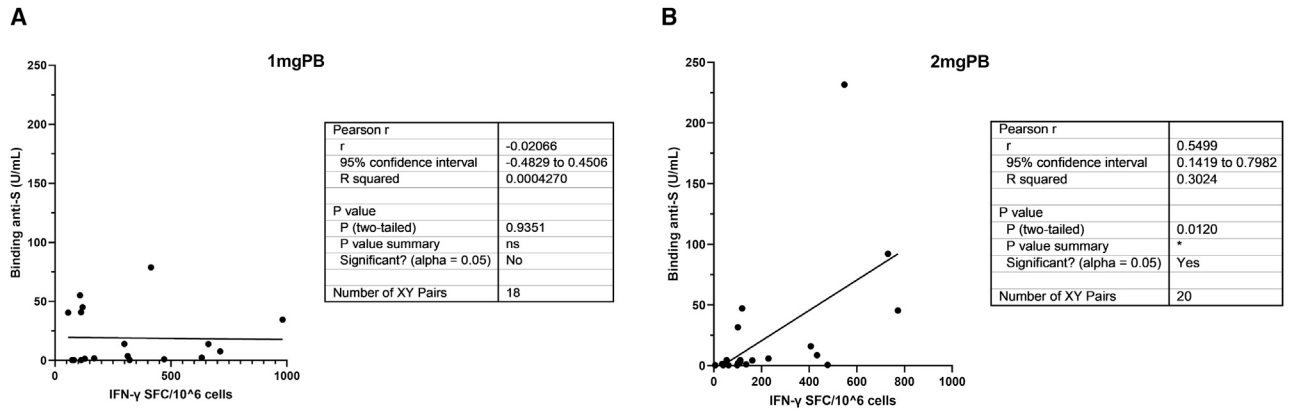

Figure 7. Correlation between humoral and cellular response

The correlation between humoral response (binding anti-S antibodies) and cellular response (IFN-γ SFC per million PBMCs) at week 8 was assessed in the 1mgPB cohort (A) and 2mgPB cohort (B) by Pearson's correlation coefficient.

by an increase in anti-RBD-specific and expanded memory B cell clones.[39] Future studies of DNA vaccines in general, and of COVID-*e*Vax in particular, should explore the potential benefit of a third vaccine dose.

Whether a close third booster dose would be necessary or if the good cellular response observed in the present study would be sufficient to confer a clinically relevant protection is unknown. The correlation between antibodies titers, cellular response, and protection is not clear in SARS-CoV-2 infection yet.[40] However, it is hypothesized that even low levels of neutralizing antibodies are able to slow viral growth rates and reduce the severity of infection,[39,41] especially in the presence of a pronounced cellular response as in the present case.

The potential risk of ADE of anti-SARS-CoV-2 antibodies, frequently associated with low levels of neutralizing, sub-neutralizing, and cross-reactive immunoglobulins is not currently confirmed by the recent data available about SARS-CoV-2 reinfections or infections after vaccination.[42] It is still difficult to make a prediction on those antibodies that have the potential to induce ADE because clinical data have not clarified their role in COVID-19. However, the disease of vaccinated individuals after SARS-CoV-2 infection or reinfection by different variants of concern is less severe despite the reduction in neutralization capacity of the antibody response, thus confirming that a broad and lower neutralizing activity of antibodies does not directly support their role in the disease severity,[43] but certainly they have, together with cellular immunity, a role in protection.[44]

All these considerations and hypotheses should be tested in future studies.

This first-in-human study of COVID-*e*Vax has several other limitations. First, this open-label, phase 1 trial did not include a placebo group, which makes it particularly difficult, e.g., to decipher the actual durability of the cellular response over baseline background beyond 12 weeks, as suggested by the specific ICS assay compared with the ELISpot analysis. Second, the dose-escalation design of the study, in order to preserve safety, did not allow to randomize cohorts. Third, enrollment in the last cohort (2mgP) was halted before reaching the target, due to the success of the national vaccination campaign: however, data from the few volunteers receiving that regimen seem to indicate that at least a second close booster dose is necessary to obtain an immunogenicity response. Fourth, about half of the study participants adhered to the national vaccination campaign and withdrew from the trial after the 8-week primary assessments, thus preventing the possibility to collect long-term safety and immunogenicity data in all study participants after the primary observation period.

In conclusion, this phase 1 clinical trial showed a tolerable safety profile and a preliminary immunogenicity response especially in terms of cellular immunity of COVID-*e*Vax, a SARS-CoV-2 DNA-based vaccine candidate, with instead an almost absent neutralizing antibodies response. Additional phase 2 and 3 investigations are needed to understand if a protective immunity is induced by COVID-*e*Vax. Moreover, it would be of great interest to explore COVID-*e*Vax as a booster of pre-existing immunity induced by other technology platform vaccines. The T cell-skewed immunological response observed adds significant information to the DNA vaccine platform and can be potentially useful also in other therapeutic areas, such as oncology, in which a cell-mediated immune response is essential.

MATERIALS AND METHODS

Trial design and participants

This was a phase 1, first-in-human, dose-escalation, open-label clinical trial conducted at three sites to assess the safety and immunogenicity of COVID-*e*Vax. Three escalating cohorts (0.5, 1, and 2 mg) receiving two vaccinations 28 days apart (PB) and an additional cohort (2 mg) receiving one vaccination only (P) were planned, each consisting of 20 subjects with a 1:1 sex ratio.

Healthy men and nonpregnant women, aged 18 to 65 years, negative for COVID-19 history or SARS-CoV-2 infection at screening were eligible. Inclusion/exclusion criteria are provided in the supplemental information.

Enrollment within each cohort was staggered: one, two, and three sentinel subjects were dosed 3 days apart, followed by full cohort enrollment. The next cohort was opened 7 days after vaccination of all participants in the previous cohort. Cohort progression and dose escalation proceeded only after safety data were reviewed by an IDSMC.

All participants provided written informed consent before undergoing screening for study eligibility. The study was conducted in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki, and it was approved by the Italian Medicines Agency and the National Ethical Committee for COVID-19 clinical studies. The trial has been registered on ClinicalTrials.gov with the identifier NCT04788459 and in EudraCT with the number 2020-003734-20.

Trial vaccine

COVID-*e*Vax (Takis Biotech, Rome, Italy, and Rottapharm Biotech, Monza, Italy) target plasmid antigen is the RBD portion of the SARS-CoV-2 S protein (residues 319–541, Wuhan strain NC_045512.2) fused with a tissue plasminogen activator (tPA) leader sequence that allows proper secretion of the antigen into the extracellular space. The synthetic RBD gene sequence was engineered to contain exclusively codons preferred by highly expressed human genes. The RBD sequence was cloned into vector pTK1A under the control of the human CMV/intA promoter. The vector includes intron A from hCMV upstream of the coding region to enhance the expression and stimulation of an immune response, an optimal translation initiation (Kozak) sequence at 5′ to the ATG, and two consecutive stop codons inserted downstream of the coding sequence followed by the bovine growth hormone polyadenylation signal to increase the level of transcription.

The vaccine is a sterile, endotoxin-free solution (manufactured at Biomay, Vienna, Austria) for intramuscular injection followed by electroporation. It was formulated in Dulbecco's phosphate-buffered saline at the concentration of 4 mg/mL, supplied frozen, and stored at −20°C. The injection volume was 0.5 mL, undiluted (2-mg dose), or diluted in saline solution (0.5- and 1-mg doses).

Trial procedures

On days 1 and 29 ± 1 or only on day 1 (PB or P cohorts, respectively), subjects received the intramuscular injection in the deltoid muscle of the right arm, immediately followed by electroporation via Cliniporator (IGEA, Carpi, Italy), a mobile electrical pulse generator (details are available in the supplemental information). The low-voltage amplitude of 40 V (corresponding to an electric field strength of 100 V/cm) was used, with four pulses of 5 msec separated by 5-msec intervals, delivered by four single-use, sterile intramuscular needle electrodes included in a newly designed electroporation system gun (supplemental information) connected to the Cliniporator.

Participants remained at the clinical site for 4 h and were then contacted by telephone in the evening and 24 h after vaccination for inquiry of early reactions. Follow-up visits were scheduled on day 3 and at weeks 1, 2, 4 (i.e., day 29, boost administration), 5 (PB cohorts only), 6, 8, 12, 16, and 24.

Safety assessments

Safety assessments included electronic diary self-reporting of solicited local and systemic AEs and the use of antipyretic and/or pain medications for 7 days after vaccination. The list of solicited AEs and their severity grading are reported in Table S7. Safety assessments also included unsolicited AEs, 12-lead ECG evaluation 1 h after vaccination, laboratory (hematology, chemistry, and urinalysis), and vital signs throughout the study. Routine blood chemistry included plasma total CPK assay, since electroporation was previously described to induce minor injury to muscle tissue and consequent CPK leakage in animals[20] and shown in human studies.[21]

Immunogenicity assessments

They were performed by VisMederi (Siena, Italy) and at Takis laboratories (Rome, Italy) and are detailed in the supplemental methods. Briefly, serum antibodies against the SARS-CoV-2 S protein RBD were quantified by the Elecsys Anti-SARS-CoV-2-S immunoassay (Roche Diagnostics) in U/mL (positive test cutoff: 0.80 U/mL) on day 1 and weeks 2, 4, 8, 12, 16, and 24. Antibodies against the SARS-CoV-2 nucleocapsid (N) protein to monitor the occurrence of natural infection were qualitatively assayed by the Elecsys Anti-SARS-CoV-2 immunoassay (Roche Diagnostics). All samples were processed according to the manufacturer's instructions.

The vaccine-induced neutralizing activity was assessed at day 1 and weeks 4, 8, 16, and 24 by the Micro Neutralization test.[45]

The cell-mediated immune response was assessed by the IFN-γ ELISpot assay (Human-IFN-γ Single Color Enzymatic ELISPOT Immunospot kit, CTL Europe, Bonn, Germany) on PBMCs collected at day 1 and weeks 2, 4, 8, 12, and 24 and frozen for shipment and storage. In addition, to assess the functionality and polarization of RBD-specific T cells (CD4+ and CD8+), ICS analysis for IFN-γ, TNFα, IL-2, IL-4, IL-5, and CD40L was performed at baseline and at week 8, focusing on samples from those subjects in the 2mgPB cohort having available and adequate (cell viability) PBMC material (a total of nine subjects). A response in type 1 helper T (Th1) cells was characterized by the expression of IFN-γ and/or IL-2; a response in type 2 helper T (Th2) cells was characterized by the expression of IL-4 and/or IL-5 and/or CD40L.[46] To characterize memory T cell phenotype, CD4+ and CD8+ T cells were stained for CD45RA and CCR7, at day 1 (before vaccination) and weeks 8, 12, and 24 in available and adequate samples from the 2mgPB cohort.

Outcomes

As evident from the study design, this first-in-human study was primarily a safety trial. The primary safety outcomes were the incidence of solicited local and systemic AEs through 7 days and of unsolicited

AEs and changes in safety laboratory parameters through 4 weeks after each vaccination, respectively. Secondary safety outcomes included the incidence of unsolicited AEs through study completion.

On the other hand, the design of the study allowed us to preliminarily assess the immunogenicity of COVID-*e*Vax as a secondary objective. The principal immunogenicity outcomes were binding anti-S (RBD) antibodies levels, including the proportion of participants with a positive test, neutralizing antibodies titers, and cellular immune responses by IFN-γ ELISpot through 4 weeks after each vaccination. Other immunogenicity outcomes included the above through study completion and CD4+ and CD8+ T cell response by ICS analysis. A combined humoral and cellular responder criterion considered positive binding anti-S antibody ($\geq$0.80 U/mL) and/or at least 2-fold increase in IFN-γ SFC per 10^6 PBMCs over baseline. For all immunogenicity assessments, data were excluded upon occurrence of SARS-CoV-2 natural infection or COVID-19 vaccination from the national campaign.

Statistics

The sample size was based on clinical and practical considerations, not on a formal statistical power calculation. Categorical variables were reported as proportions and continuous immunogenicity variables as geometric means and 95% confidence intervals based on the t distribution of the log-transformed values. Fold increases vs. baseline were also calculated. IFN-γ values were normalized to express the results as SFC per 10^6 cells. Comparisons within and between groups were performed by the Wilcoxon signed-rank and Kruskal-Wallis tests, respectively. Correlation between humoral and cellular response was analyzed using Pearson's correlation coefficient.

DATA AVAILABILITY

Data are available in the main text or in the supplemental information. All individual data from this study are available from the corresponding author upon request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.ymthe.2022.12.017.

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AUTHOR CONTRIBUTIONS

L.C.R., L.A., F.G., N.B., G.G., C.V., G.C., and M.V. contributed to the study design. M.E.C., P.B., S.M., and P.A.A. enrolled subjects and collected data as study investigators; S.C. was involved as sub-investigator; M.E.C., P.B., and P.A.A. also provided feedback on the study design. N.B. was the study project manager. L.A., L.C.R., F.G., E.Marra, F.P., G.R., A.C., M.Compagnone, L.L., E.S., E.P., A.Muzi, G.C., M.V., F.F., M.G., N.B., G.G., and C.V. were involved in the overall COVID-*e*Vax development. M.Cadossi directed the team that designed the dedicated EPS gun used in this study. E.Montomoli and A.Manenti are the representatives of VisMederi, a Coalition for Epidemic Preparedness Innovations (CEPI) reference laboratory for COVID-19 vaccines assessment, who collaborated to COVID-*e*Vax development supporting and executing the immunogenicity analyses of this study. C.V., G.G., and G.C. contributed to the statistical analysis of data. C.V., G.C., M.V., G.G., and F.G. drafted the initial version of the manuscript. All authors reviewed and edited the manuscript and approved the final version.

DECLARATION OF INTERESTS

L.A., E.Marra, F.P., G.R., A.C., M.Compagnone, L.L., E.S., E.P., and A.Muzi are Takis employees. L.C.R., F.G., N.B., G.G., G.C., M.V., F.F., M.G., and C.V. are Rottapharm Biotech employees. Takis and Rottapharm Biotech are jointly developing COVID-*e*Vax. M.E.C., P.B., S.M., P.A.A., and S.C. are investigators in this study, and their institutions received fees for participation.

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