Durability and Cross-Reactivity of Immune Responses Induced by an AS03 Adjuvanted Plant-Based Recombinant Virus-Like Particle Vaccine for COVID-19

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Abstract

As the SARS-COV-2 pandemic evolves, what is expected of vaccines extends beyond efficacy and includes evaluations of both durability and cross-reactivity to emerging variants. To complement an on-going Phase 3 efficacy study, this report expands on previously reported immunogenicity results from a Phase 1 trial of an AS03-adjuvanted, plant-based virus-like particle (VLP) displaying the spike glycoprotein of the Wuhan strain of SARS-CoV-2 virus (NCT04450004). Durability of the humoral and cellular responses against the ancestral strain was evaluated 6 months post-second dose (Day 201) at which time ~94% of vaccinated individuals remained seropositive. Interferon gamma (IFN-γ) and interleukin 4 (IL-4) responses remained detectable in ~94% and ~92% of vaccinated individuals respectively. Cross-reactivity of neutralizing antibodies to Alpha (B.1.17), Beta (B.1.351), and Gamma (P.1) variants of concern (VOC) were also measured. Twenty-one days after the second vaccination, detectable neutralizing antibodies were observed to the Alpha variant by both pseudovirion and wild-type assays for all vaccinated individuals, while 94.7% of individuals had detectable antibodies to the Beta variant in both assays. Neutralizing antibodies to the Gamma variant were detected in 100% and 94.7% of individuals using the pseudovirion and live virus neutralization assays, respectively. In all cases, the vaccine-induced neutralizing GMTs to the VOC 3 weeks post-vaccination were greater than the Wuhan-specific neutralization titers seen in individuals recovered from COVID-19.
Introduction

Coronavirus disease 2019 (COVID-19), the disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has spread rapidly across the globe, infecting more than 195 million people and killing 4.18 million people as of July 26, 2021. The disease typically involves the upper and lower respiratory tracts, where it can cause severe clinical features including dyspnea, hypoxemia, tachypnea, lung edema, and acute respiratory failure. Multiple cellular and molecular mediators of immune responses, inflammation, and coagulation appear to be involved in the pathogenesis.

Currently, treatment options are limited and vaccination against SARS-CoV-2 remains the most effective strategy for preventing viral transmission and reducing disease severity, hospitalizations, and deaths. COVID-19 vaccines based on at least six different platforms are currently being pursued, with more than 200 SARS-CoV-2 vaccine candidates in pre-clinical and clinical developmental, 14 of which have minimally received emergency use approval in at least one country.

Vaccine efficacies reported to date range from ~50–95% and are highly correlated with neutralizing antibody (NAb) titers. The inverse correlation between viral load and NAb titers suggests that antibodies are associated with protection against SARS-CoV-2 and its clinical manifestations. This correlation has also been observed in animal challenge models in which vaccine-generated antibody titers were associated with restricted viral loads and greatly reduce lung inflammation. Furthermore, the transfer of antibodies from convalescent animals to naïve animals or between humans in a clinical setting can, in some circumstances, result in clinical
improvement, decline in viral loads, and reduced mortality. Cell-mediated immunity has also been shown to be involved in both protection against disease and in the establishment of long-lasting immunity.\textsuperscript{19-21}

The continued presence of circulating antibodies and cell-mediated immune responses may indicate durable protection. After infection, NAb titers decline gradually after the initial peak and remain detectable for minimally 8 months.\textsuperscript{22,23} Data involving vaccine-induced humoral immunity suggests a similar pattern with half-lives varying depending on the model used.\textsuperscript{24,25} In addition to antibodies, lasting cellular memory responses were observed in the periphery of individuals previously infected with SARS-CoV-2 and are expected to contribute to durability of protection.\textsuperscript{26,27}

Potential reinfection with SARS-CoV-2 depends on both durability of the immune response and cross-reactivity against emerging variants in circulation, which may have several competitive advantages against established strains with respect to enhanced replication, transmission, or escape from immune system responses.\textsuperscript{28,29} As established immunity may provide variable protection against variants of concern (VOC)\textsuperscript{30,31} and existing antibodies provide variable cross-reactivity,\textsuperscript{32} there is a clear need to assess NAbs titers induced by different vaccines and vaccine candidates against new variants.

As many countries move from a primary pandemic response to a sustained endemic response, the durability and breadth of protection induced by vaccines become increasing concerns. This report expands on the previously described phase 1 study evaluating a plant-based virus-like particle displaying SARS-CoV-2 spike glycoprotein...
(CoVLP) adjuvanted with AS03 by investigating the durability of antibody and cell-mediated immune responses as well as the cross-reactivity of vaccine-induced NAbs against Alpha, Beta, and Gamma VOC.

Results

Samples from a subgroup of 20 healthy individuals with a median age of 36 years (range 19–49 years) who received two doses of vaccine made of 3.75 µg CoVLP adjuvanted with AS03 (CoVLP+AS03) 21 days apart were analyzed 21 days after each vaccination (Days 21, 42) and 6 months after the second vaccination (Day 201) and the data are presented in this report. Demographics are detailed in Table 1. A panel of human convalescent sera/plasma (HCS) from patients recovering from mild, moderate, or severe COVID-19 infection is included for comparison. This report is focusing on the durability of the responses measured at Day 201 as humoral and cell-mediated immune responses elicited 21 and 42 days after immunization were previously reported and discussed.

Durability of Humoral Responses

To evaluate the durability of the humoral response, the anti-spike immunoglobulin G (IgG) enzyme-linked immunosorbent assays (ELISA), pseudovirion neutralization assays (PNA), and live-virus microneutralization assays (MNA) were used, as previously described (Figure 1).
Spike-binding IgG were detected in all participants (18/18; 100%) at Day 201. The percentage of subjects who remained seropositive on Day 201 was the same as that on Day 42 (19/19; 100%; \( p > 0.9999 \); Figure 1a). Similarly, for both the PNA and the MNA assays, Day 201 NAbs were present in almost all participants (17/18; 94%) and were not significantly different from proportions at Day 42 (18/18; 100%; \( p > 0.9999 \) for both assays; Figures 1b and 1c).

The anti-spike IgG GMT (29,518; 95% confidence interval [CI]: 17,937.7–48,574.4) was significantly lower \( (p < 0.0001) \) than the Day 42 GMT (295,240; 95% CI: 137,967.3–631,790.4; Figure 1a) and comparable to that of the HCS (23,659; 95% CI: 10,579-52,909). In order to allow wider context and interpretation of the binding antibody data, World Health Organization (WHO) pooled plasma 20/136 composed of mixed convalescent plasma was included as a reference standard. Based on these results, a normalization factor of 55.18 was applied to transform the GMT values to binding antibody units per milliliter (BAU/mL). The post-vaccination sera had a GMT of 5350 BAU/ml on day 42 and 535 BAU/mL at Day 201.

On Day 201, the GMT values for the PNA and the MNA assays were comparable to HCS GMT values despite a decline from peak Day 42 values (Figures 1b and 1c). The Day 201 GMT in the PNA (190; 95% CI: 96.03- 377.0) was significantly lower \( (p < 0.0001) \) than at Day 42 (2,118; 95% CI: 1,380-3,251) but not significantly different \( (p > 0.9999) \) than the HCS GMT (109; 95% CI: 108.5-364.3; Figure 1b). Similarly, the Day 201 GMT in the MNA (86.4; 95% CI: 44.91-166) was significantly lower \( (p < 0.0001) \) than the Day 42 value (811; 95% CI: 496.0–1,327.0) but not statistically different \( (p = 0.8527) \) to that of HCS (58.3; 95% CI: 35.1-96.8; Figure 1c). WHO mixed
convalescent sera 20/136 was again used as a reference standard to allow reporting of NAb data as International Units per milliliter (IU/mL). Using this standard, a normalization value of 1.872 can be applied to the PNA results to obtain values of 1131 IU/mL at Day 42 and 101 IU/mL at Day 201 post-vaccination. Similarly, a normalization value of 0.91 was applied to the MNA data to obtain values of 896 IU/mL at Day 42 and 95.5 IU/mL at Day 201. Corresponding values for the HCS were 58.2 and 64.1 IU/mL respectively.

The half-lives ($t_{\frac{1}{2}}$) of the vaccine-generated anti-spike IgG binding and NAbs were calculated using the exponential-decay model. The $t_{\frac{1}{2}}$ values obtained for antibodies measured by all three assays were comparable, with overlapping 95% CIs: 55.26 days for anti-spike IgG (n=18; 95% CI: 44.67–65.85), 56.44 (n=17; 95% CI: 44.08–68.80) for the PNA and 59.23 days (n=16; 95% CI: 39.83–78.63) for the MNA.

Overall, these data show that two doses of the CoVLP+AS03 vaccine elicited binding antibodies and NAbs that remained detectable 6 month after the second vaccination in ~95% of individuals. Antibody titers at that late timepoint were comparable to those of patients recovering from natural COVID-19 infection.

**Durability of Cellular Immune Responses**

The cell-mediated immune (CMI) response and associated $T_h1/T_h2$ balance was evaluated by expression of IFN-γ ($T_h1$) and IL-4 ($T_h2$) cytokine ELISpot on PBMC upon ex-vivo antigen recall using a SARS-CoV-2 spike-derived peptide pool (Wuhan strain). At Day 201, almost all participants had a readily detectable IFN-γ response (17/18;
94%) comparable to the proportion of IFN-γ responders at Day 42 (19/19; 100%).

Similarly, at Day 201, the large majority of participants had detectable IL-4 response
(12/13; 92%), again comparable to the proportion of responders at Day 42 (19/19; 100%). Like the humoral response, the magnitude of the cellular response was reduced on Day 201 relative to Day 42. The Day 201 median IFN-γ spot-forming units per million PBMCs (SFU/10⁶) response of 202.5 (95%CI: 62–433) was significantly reduced (p<0.05) relative to the Day 42 value of 628 SFU/10⁶ (95%CI: 403–862). Similarly, at Day 201, the IL-4 median SFU/10⁶ value of 46 (95%CI: 8–151) had also fallen significantly (p<0.05) compared to the Day 42 median SFU/10⁶ value of 445 (95%CI: 339–680). Despite the reduced magnitude of response at Day 201, ongoing spike-specific IFN-γ and IL-4 CMI in the vast majority of participants indicates that two doses of CoVLP+AS03 induces a durable CMI response.

**Cross-Reactivity to SARS-CoV-1, MERS, and Common Cold Coronavirus**

Figure 3 shows the reactivity of serum antibodies from individuals vaccinated with two doses of CoVLP+AS03 compared with HCS for the spike proteins of SARS-CoV-2, SARS-CoV-1, and Middle East respiratory syndrome (MERS) as measured using the fluorescence-based multiplex Vaxarray platform from InDevR.

As expected, pre-vaccination sera were not reactive to the spike proteins of SARS-CoV-2, SARS-CoV-1, or MERS. Sera from subjects vaccinated with CoVLP+AS03 and patients recovering from COVID-19 were highly reactive to the SARS-CoV-2 spike protein in this assay. Antibody binding for vaccinated individuals were approximately
one order of magnitude higher than for individuals in the HCS group ($p<0.0001$).

Although the binding of vaccinated and HCS sera to the SARS-CoV-1 spike protein was lower than to the SARS-CoV-2 spike protein, sera from vaccinated individuals still had significantly higher binding to the SARS-CoV-1 spike protein than HCS ($p<0.0001$).

Neither vaccination nor infection with SARS-CoV-2 induced statistically significant cross-reactive antibodies to the MERS spike protein (Figure 3) or spike proteins from common cold coronaviruses (Supplementary Figure 1).

**Cross-Reactivity to VOCs**

The cross-reactivity of antibodies induced by CoVLP+AS03 at Day 42 (21 days post-second vaccination) was tested in two neutralizing antibody assays to establish the relative reactivity against the ancestral Wuhan SARS-CoV-2 strain and three key VOCs, Alpha (B.1.1.7), Beta (B.1.351) and Gamma (P.1).

PNA assay results are shown in Figure 4a. To provide additional perspective on the relative degree of neutralization, and as previously suggested by Madhi et al. and Wibmer et al. $^{31,35}$, reciprocal titers are presented in three broad bands: <50, 50–400, and >400. Individuals who had been vaccinated with CoVLP+AS03 had high neutralization capacity to the ancestral Wuhan strain: 17/18 (94.4%) had titers >400 and the remaining individual had a titer in the 50-400 band. Vaccinated individuals had an overall GMT of 2118 (95% CI: 1380-3251) against the ancestral Wuhan strain. Against the Alpha variant tested, the GMT was 1544 (95% CI: 908-2626), with 16/19 (84.2%) participants in the >400 band and the remainder (3/19; 15.8%) in the 50-400 band.
None had titers <50. The GMT was 273 (95% CI: 140-535) against the Beta variant tested. Almost half of the participants, 9/19 (47.4%), had titers >400 and 8/19 (42.1%) had titers between 50 and 400. Two of 19 (10.5%) had titers <50, including one (5.3%) who had lost detectable pseudovirion neutralization activity. A GMT of 555 (95% CI: 344-895) was observed against the Gamma variant tested. More than three-quarters of participants (15/19; 78.9%) had titers >400, 3/19 (15.8%) had titers between 50 and 400 and 1/19 (5.3%) had a titer <50, although neutralization remained detectable. For comparison, a GMT of 135 (95% CI: 70.2-260) was obtained for HCS neutralizing ancestral Wuhan pseudovirus, with 3/12 (25.0%) having titers greater than 400, 8/12 (66.7%) with titers between 50 and 400, and 1/12 (8.3%) had a titer less than 50.

Cross-reactivity of Day 42 sera was also tested by live virus neutralization (Figure 4b). Detectable antibody responses were observed in 19/19 (100%) of participants to the ancestral Wuhan strain and Alpha variant, and 18/19 (94.7%) of participants to the Beta and Gamma variants. Among HCS, 30/34 (88.2%) had detectable neutralization response to the ancestral strain. The GMTs at Day 42 post-vaccination to ancestral, Alpha, Beta, and Gamma variants were 812 (95% CI: 497-1327), 391 (95% CI: 227-672), 113 (95% CI: 65.2-196) and 133 (95% CI: 82.0-217), respectively. A GMT of 62.6 against the ancestral strain was observed with HCS.

Taken together, these data show that 2 doses of the CoVLP+AS03 vaccine given 3 weeks apart induced an antibody response that retained detectable neutralizing cross-reactivity to the Alpha, Beta, and Gamma variants in the vast majority (~95%) of subjects and which remained comparable to or better than levels seen with HCS against the ancestral strain.
Discussion

This report expands on a Phase 1 immunogenicity and tolerability study in which immunogenicity of CoVLP without adjuvant was compared to CoVLP+AS03 or CoVLP adjuvanted with CpG1018 and CoVLP doses of 3.75, 7.5 or 15.0 µg were compared. Herein, durability of humoral and cellular responses measured 6 month after the second vaccination (Day 201) and the cross-reactivity to three VOCs measured 3 weeks after the second vaccination (Day 42) are reported for final formulation (VLP antigen dose and adjuvant) selected for the Phase 2/3 clinical trial.

Overall, the humoral immune response induced by to CoVLP+AS03 was demonstrated to be durable with IgG binding and NAb titers at Day 201 that were comparable to those seen in sera and plasma from patients recovered from COVID-19. Based on the GMT at Day 42 and Day 201, $t_{1/2}$ of the vaccine-induced anti-spike IgG binding and NAbs were determined using the exponential-decay model. The calculated $t_{1/2}$ are comparable to results reported for antibody decay after natural disease and similar to those calculated by Doria-Rose et al. for other SARS-CoV-2 vaccines using an exponential-decay model. Doria-Rose et al. calculated a longer $t_{1/2}$ when they applied the power law model, which assumes that decay rates decrease over time. Even though a dedicated kinetics analysis with multiple sampling timepoints would be required to select the most appropriate model and more precisely define the $t_{1/2}$ of the antibodies induced by CoVLP+AS03, the current analysis nevertheless provides confidence regarding the durability of the humoral response and illustrates that, even 6 months after the second vaccination, the vaccine-induced antibody response is comparable to that seen after natural COVID-19 infection.
Consistent with previous reports, subjects with the highest Day 42 antibody titers still had greater titers at Day 201 than those with lower Day 42 titers, but these subjects also experienced a greater fold drop over time. For example, subjects with the nine lowest Day 42 ELISA titers had a mean 14.0-fold reduction in titer by Day 201 while the nine subjects with the highest titers experienced a mean 23.6-fold reduction. Similarly, the nine lowest live virus NAb titers at Day 42 had a mean 10.6-fold reduction at Day 201 while the nine with highest live virus NAb titers saw a mean 20.8-fold reduction. In view of this analysis and in recognition of the immune system's capacity to mount anamnestic responses upon repeat exposure to the same antigen, it is perhaps more physiologically relevant to consider the persistence of detectable antibodies after vaccination rather than the absolute titers of these antibodies. By ELISA, binding IgG remained detectable in 100% of CoVLP+AS03 vaccinees while, in both NAb assays, 94% of vaccinated individuals still had readily detectable neutralizing antibodies at Day 201. While factors other than circulating antibodies likely contribute to long-term vaccine efficacy (memory B and T-cells for example), the continued presence of serum neutralizing antibodies at Day 201 suggests that the protection after CoVLP+AS03 vaccination may be quite durable. In support of this possibility, Feng et al have recently used the WHO pooled reference standard 20/136 to correlate binding antibody titers with efficacy and found a titer of 264 (95% CI: 108-806) BAU/mL was predictive of 80% vaccine efficacy against primary symptomatic COVID-19. In the current study, the observed binding antibody value at Day 201 after CoVLP+AS03 vaccination was 535 BAU/mL; this inspires confidence in the potential longer-term efficacy of CoVLP+AS03.
Beyond humoral immunity, the durability of cellular responses is likely to be important for longer-term protection, particularly as antibody titers decay over time. T cells specific for SARS-COV-2 antigens have been shown to support humoral responses during convalescence\(^{27,43}\) and to persist for up to 8 months post-infection\(^{26}\).

CoVLP+AS03 vaccination induced Th1 and Th2 responses persisted until Day 201. Spike-induced IFN-\(\gamma\) and IL-4 production in response to a SARS-CoV-2 peptide pool remained detectable in all but one vaccine recipient at Day 201. Although we observed a significant decrease in CMI responses between Days 42 and 201, both spike-induced IFN-\(\gamma\) and IL-4 responses at this later timepoint remained significantly higher than levels measured at Day 0.

While the role of IFN-\(\gamma\) and Th1-type response play a critical role in protection against viral infection including coronaviruses\(^{44-48}\), IL-4 is associated with a Th2-type response which in turn has been associated with both protective immunity and concerns involving vaccine-associated enhanced disease. This was a concern during early coronavirus vaccine development\(^{49,50}\), and it is considered an important potential risk for SARS-CoV-2 vaccines. Assessment of the \(T_h1/T_h2\) polarization of immune responses in the context of VAED was therefore considered critical\(^{51,52}\). To date however, no VAED has been observed with any SARS-CoV-2 vaccine, including the CoVLP vaccine candidate\(^{33,53}\). In the development program of CoVLP vaccine candidate specifically, cumulative clinical data up to phase 2 and experimental non-human primate data have not revealed any sign of VAED associated with CoVLP with or without an adjuvant despite the induction of both Th1- and Th2-type cytokines\(^{15,33,37}\). In fact, the IL-4 response elicited by CoVLP+AS03 may play an important role in supporting strong antibody responses,
potentially by supporting follicular T-cell involvement and germinal center development \(^{40,54-56}\). Indeed, AS03 administered with other antigens has been shown to promote durable humoral response \(^{57,58}\).

Herein, the capacity of the vaccine-induced antibodies to cross-react to spike proteins from SARS-CoV-2, MERS, and common human coronaviruses was tested. Antibodies induced by natural infection may cross-react with spike conformational epitopes \(^{59}\). CoVLP+AS03 induced antibodies with cross-reactivity to viral spike proteins of SARS-CoV-1 but not MERS or common cold coronaviruses.

For the purposes of examining the cross-reactivity to Alpha, Beta, and Gamma VOC, this report has adopted the methodology proposed by others \(^{31,35}\) who divided peak humoral responses post-vaccination into 3 broad ranges of concentrations in order to present the proportion of the population that fall into each neutralizing antibody category. Like both convalescent sera and widely used vaccines with good variant-specific efficacy, CoVLP+AS03 induced antibodies that had excellent cross-reactivity with an Alpha variant but reduced cross-reactivity to Beta and Gamma variants \(^{60-62}\).

Nonetheless, cross-reactive titers >50 were still observed at Day 42 in almost 90% of participants against the Beta variant and ~95% of participants against the Gamma variant suggesting possible cross-reactive protection against these more immune-evasive variants. Data from the live virus antibody neutralization assay strongly support this hypothesis with all participants retaining measurable cross-reactivity to the Alpha variant and about 95% retaining measurable cross-reactive neutralizing antibody to the Beta and Gamma variants. All variant-specific titers at Day 42 were comparable to or greater than the GMT of HCS to the ancestral Wuhan strain. Finally, to provide a
broader perspective across the vaccine development landscape, it is worth considering the work of Oliver who recently presented a meta-analysis of vaccine-induced antibody neutralization of variants \(^3\)\(^\text{2} \). Based on this analysis, differential responses (i.e., fold-change versus the Wuhan response) to variants are relatively consistent across vaccine platform and differences in cross-reactivity between specific vaccines are largely attributable to the scale of absolute neutralizing antibody titers elicited. Comparing to CoVLP+AS03, the fold-change in antibody neutralization across the VOC tested was overall comparable to what has been reported for other vaccines \(^3\)\(^\text{2} \). These observations reinforce the importance of considering cross-reactivity based on a tiered approach or as a proportion of vaccinees with detectable neutralizing antibodies. Importantly, the analysis presented here does not include data for the Delta or Lambda variants of increasing global concern. Investigating the cross-reactivity of antibodies generated by CoVLP+AS03 to these variants is of significant interest and is underway.

Vaccines inducing immune responses with strong variant cross-reactivity and long durability have the potential to provide protection against infection when deployed either as part of a primary pandemic response or as part of an ongoing vaccination strategy to eventually address endemic COVID-19. The data for CoVLP+AS03 presented herein demonstrate that this vaccine can induce a durable immune response against SARS-CoV-2 with broad cross-reactivity to several of the emerging VOCs. While the durability and cross-reactivity data presented herein are promising, a large Phase 3 trial ongoing in several countries with active circulation of multiple VOCs will likely provide insight into the implications of these observations for the efficacy of CoVLP+AS03.
Methods

CoVLP Vaccine Candidate and Adjuvant

The CoVLP vaccine candidate has previously been described in detail \(^{33}\). Briefly, full-length spike protein from SARS-CoV-2 (strain hCoV-19/USA/CA2/2020) incorporating the modifications R667G, R668S, R670S, K971P, and V972P is expressed in *Nicotiana benthamiana* by transient transfection, resulting in spontaneous trimer formation, VLP assembly and budding. The purified CoVLP is mixed with AS03 immediately prior to injection. The AS03 adjuvant is an oil-in-water emulsion containing DL-\(\alpha\)-tocopherol (11.69 mg/dose) and squalene (10.86 mg/dose) and was supplied by GlaxoSmithKline.

Study Design

The Phase 1 study design investigating tolerability and immunogenicity of CoVLP with and without adjuvants was previously described \(^{33}\). Ethical approved was provided by the Advvarra Institutional Review Board as well as the Health Products and Food Branch of Health Canada and the study was carried out in accordance with the Declaration of Helsinki and the principles of Good Clinical Practices. Participants were recruited from existing databases of volunteers, and written informed consent was obtained from all study participants before any study procedure. Participants were offered modest compensation for their participation in this study (that is, time off work and displacement costs).
SARS-CoV-2 Spike Protein ELISA

This ELISA measured binding to SARS-CoV2 S protein in its prefusion configuration (SARS-CoV2/Wuhan/2019, Immune Technology Corp.: amino acids 1-1208 with the furin site removed and no transmembrane region) as previously described 33.

SARS-CoV-2 Pseudovirus Neutralization Assay

Pseudovirion neutralizing antibody analysis was performed using a genetically modified Vesicular Stomatitis Virus (VSV) engineered to express the SARS-CoV-2 S glycoprotein (NXL137-1 in POG2 containing 2019-nCOV Wuhan-Hu-1; Genebank: MN908947) from which the last nineteen amino acids of the cytoplasmic tail were removed (rVSVΔG-Luc-Spike ΔCT) (Nexelis, Quebec, Canada) as previously described 33.

Cross-reactivity to variants was tested using modified pseudovirion expressing SARS-CoV-2 S glycoprotein from Alpha (Nexelis lot #: NL2102M-N; del69-70, del144, N501Y, A570D, D614G, P681H, T716I, S982A, D1118H, plus Δ19aa C-terminal for the PP processing), Beta (Nexelis lot #: NL2103K-N; L18F, D80A, D215G, del242-244, R246I, K417N, N501Y, E484K, D614G, A701V, plus Δ19aa C-terminal for the PP processing), and Gamma (Nexelis lot #: NL-2102O-N; L18F, T20N, P26S, D138Y, R190S, K417T, E484K, N501Y, D614G, H655Y, T1027I, V1176F, plus Δ19aa C-terminal for the PP processing) variants.

SARS-CoV-2 Microneutralization CPE-based assay (MNA)
Neutralizing antibody analysis was performed using a cell-based cytopathic effect assay (VisMederi, Sienna, Italy) based on ancestral SARS-CoV-2 virus (2019 nCOV ITALY/INMI1, provided by EVAg; Genebank: MT066156) as previously described 33.

For cross-reactivity against variants, the assay was conducted with Alpha (swab isolate 14484; mutations: N501Y, A570D, D614G, P678H, T716I, S982A, T572I, S735L, D69/70, D144Y), Beta (hCoV-19/Netherlands/NoordHolland_10159/2021), and Gamma (human isolate PG_253 Clade Nexstrain 20J/501Y.V3; Mutations: L18F, T20N, P26S, D138Y, R190T, K417T, E484K, N501Y, D614G, H655Y) variants.

**Standardization of Antibody Titers with the WHO 20/136 Pooled Sera**

As previously described 37, WHO International Standard anti-SARS-CoV-2 immunoglobulin (human; NIBSC code: 20/136) was included in antibody binding and neutralization assays for the purpose of facilitating comparison of results with other studies. This standard material is pooled plasma from eleven individuals who recovered from SARS-CoV-2 infection with very high NAb responses 41.

For the ELISA, a reference titer of 55,175 was observed; hence a normalization factor of 55.18 was used to allow expression of the ELISA results in binding antibody units per milliliter (BAU/mL).

For the PNA assay, a reference GMT value of 1872 was observed, hence a normalization factor of 1.872 was used when expressing PNA titers in international units per milliliter (IU/mL). Similarly, for the MNA assay, 20/136 generated a titer of 905.1
hence a normalization factor of 0.91 was applied to the MNA titers to allow expression in IU/mL.

**Calculation of Antibody Half-Lives**

Antibody $t_{1/2}$ were calculated by exponential decay model based on values observed at Day 42 and Day 201. The mean of the individually calculated $t_{1/2}$ values were reported along with 95% confidence intervals (CI). GraphPad Prism software was used to calculate means and 95% CIs.

**Cross reactivity to SARS-CoV-1, MERS and Common Cold Coronaviruses**

Cross-reactivity to SARS, MERS and common cold coronaviruses was quantified using the VaxArray platform and the Coronavirus SeroAssay at InDevR, Inc. (Boulder, CO). Spike protein antigens representing full-length spike, receptor binding domain (RBD), and the S2 extracellular domain of SARS-CoV-2, and the spike proteins from SARS, MERS, HKU1, OC43, NL63, and 229E were printed on the microplates.

Prior to use, the microarray slides were equilibrated to room temperature for 30 minutes. All serum samples were diluted at 100-fold and a predetermined subset of 20 samples were diluted at 1000-fold in Protein Blocking Buffer (PBB) and applied to the microarray and allowed to incubate in a humidity chamber on an orbital shaker at 80 rpm for 60 minutes. After incubation, the samples were removed using an 8-channel pipette and the slides were subsequently washed by applying 50uL of Wash Buffer 1.
Slides were washed for 5 minutes on an orbital shaker at 80 RPM after which the wash solution was removed. Anti-human IgG Label (VXCV-7623) was prepared by diluting the label to 1:10 in PBB after which 50uL of label mixture was added to each array. Detection label was incubated on the slides in the humidity chamber for 30 minutes before subsequent, sequential washing in Wash Buffer 1, Wash Buffer 2, 70% Ethanol, and finally ultrapure water. Slides were dried using a compressed air pump system and imaged using the VaxArray Imaging System (VX-6000).

The slides were imaged at a 100 ms exposure time. The raw signal was converted to signal to background ratio and reported as arbitrary relative binding units.

**Interferon-γ and Interleukin-4 ELISpot:**

PBMC samples from study subjects were analyzed by IFN-γ or IL-4 ELISpot (Caprion, Quebec, Canada) using a pool of 15-mer peptides with 11aa overlaps from SARS-CoV-2 S protein (USA-CA2/2020, Genbank: MN994468.1, Genscript, purity >90%). Full details of the methodology are detailed elsewhere.

**Convalescent Samples:**

Sera/plasma from COVID-19 convalescent patients were collected from a total of 35 individuals with confirmed disease diagnosis. Time between the onset of the symptoms and sample collection varied between 27 and 105 days. Four serum samples were collected by Solomon Park (Burien, WA, USA) and 20 sera samples by Sanguine.
BioSciences (Sherman Oaks, CA, USA); all were from non-hospitalized individuals. Eleven plasma samples were collected from previously hospitalized patients at the McGill University Health Centre. Disease severity were ranked as mild (COVID-19 symptoms without shortness of breath), moderate (shortness of breath reported), and severe (hospitalized). These samples were analyzed in parallel with clinical study samples, using the assays described above. Demographic characteristics are presented in the Table 1.

For the pseudovirion antibody neutralization assay, only 12 of the 35 convalescent sera samples remained and could be included.

**Statistical Analysis:**

Humoral assays compared data across timepoints (Figure 1) used one-way analysis of variance using a mix-effect model of log-transformed data. Comparisons of the proportion of individuals with detectable antibodies or not were conducted using Fisher’s exact test. Comparisons of cell-mediated immune response durability (Figure 2) across timepoints were conducted using Friedman’s test follow by Dunn’s comparisons test. Analysis of antibody binding to coronavirus spike protein (Figure 3) used one-way analysis of variance on log-transformed data.
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Author contributions

All authors contributed significantly to the submitted work. BJ Ward and N Landry contributed to all aspects of the clinical study from conception to completion. P Gobeil, S Pillet, I Boulay, A Lorin, N Charland, and MA D’Aoust contributed to design and execution of the study as well as analysis and presentation of the data. P. Boutet, F. Roman, R. Van Der Most, and M. de los Angeles Ceregido Perez contributed to analysis and presentation of data. MP Cheng provided access to reagents and consulted on study design and execution. DC Vinh provided access to reagents. All authors contributed to critical review of the data and the writing of the manuscript. All Medicago authors had full access to the data. BJW made the final decision to submit the manuscript.
Competing interest statement

P. Gobeil, I. Boulay, N. Charland, A. Lorin, S. Pillet, B Ward, M-A D'Aoust, and N Landry are were either employees of Medicago Inc. or received salary support from Medicago Inc. P. Boutet, F. Roman, R. Van Der Most, and M. de los Angeles Ceregido Perez are employees of GlaxoSmithKline.

Data Availability

Medicago Inc. is committed to providing access to anonymized data collected during the trial that underlie the results reported in this article, at the end of the clinical trial, which is currently scheduled to be 1 year after the last participant is enrolled, unless granted an extension. Medicago Inc. will collaborate with its partners (GlaxoSmithKline, Rixensart, Belgium) on such requests before disclosure. Proposals should be directed to wardb@medicago.com or daoustma@medicago.com. To gain access, data requestors will need to sign a data access agreement and access will be granted for non-commercial research purposes only.
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Figure legends

**Figure 1:** Serum antibodies collected at Day 0, 21, 42 and 201 from subjects vaccinated with 3.75 µg CoVLP adjuvanted with AS03 to protein S were measured by ELISA (panel A). Serum antibody neutralization were measured using Wuhan-strain derived vesicular stomatitis virus pseudovirus (panel B) or live virus (panel C) -based assays. Values from convalescent sera or plasma (HCS) collected at least 14 days after a positive diagnosis of COVID-19 (RT-PCR) from individuals whose illness was classified as mild, moderate, or severe/critical (n=35) are shown in the right-hand panels. Individual data at baseline (Day 0), and 21 days after one immunization (Day 21), or two immunizations (Day 42), and Day 201 post-vaccination are indicated with red lines; geometric means are indicated with horizontal black lines and numerical values. Error bars indicate 95% CI. Black triangles indicate immunization. Significant differences between timepoints are indicated by asterisk(s) (*p<0.05; ****p<0.0001. One-way analysis of variance using a mixed-effect model on log-transformed data GraphPad Prism, v9.0).

**Figure 2:** Antigen-specific IFN-γ and IL-4 responses were quantified by ELISpot. Frequencies of antigen-specific cells producing IFN-γ and IL-4 per million PBMCs at baseline (Day 0) and 21 days after one immunization (Day 21), or two immunizations (Day 42), and Day 201 post-immunization with 3.75 µg CoVLP adjuvanted with AS03 were measured after ex vivo restimulation with a peptide pool consisting of S protein-derived 15-mer peptides overlapping by 11 amino acids. Individual values are indicated by red lines; medians are indicated by black lines and numerical values. Error bars
indicate 95% CI. Significant differences between timepoints are indicated by asterisk(s)
(*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 Friedman test followed by Dunn’s
comparisons test, GraphPad Prism, v9.0.1).

**Figure 3:** Binding of serum antibodies from Day 42 of subjects vaccinated with 3.75 µg CoVLP adjuvanted with AS03 to protein S from SARS-CoV-2, SARS-CoV-1, and MERS were quantified using the VaxArray platform from InDevR, Inc. Convalescent sera or plasma collected at least 14 days after a positive diagnosis of COVID-19 (RT-PCR) from individuals whose illness was classified as mild, moderate, or severe/critical (n=35) were analyzed concurrently. Horizontal lines indicate geometric means. Dotted line indicates mean background control values. Significant differences between sera are indicated by asterisks (**p<0.01; ****p<0.0001. One-way analysis of variance on log-transformed data, GraphPad Prism, v9.0).

**Figure 4:** To evaluate cross-reactivity, Day 42 NAbs of subjects vaccinated with 3.75 µg CoVLP adjuvanted with AS03 were quantified in a (A) VSV pseudovirion neutralization assay or (B) live virus neutralization assay with the ancestral Wuhan strain and the Alpha, Beta, and Gamma variants. Convalescent sera or plasma (HCS) samples were collected at least 14 days after a positive diagnosis of COVID-19 (RT-PCR) from individuals whose illness was classified as mild, moderate, or severe/critical (Panel A: n=35, Panel B: n=12). Individual values are indicated with red lines; geometric
means are indicated above each variant. In panel A, background colors indicate dilution titers; pie charts provide proportions per dilution titers for each variant tested.

**Supplemental Figure:** Binding of serum antibodies from Day 42 of subjects vaccinated with 3.75 µg CoVLP adjuvanted with AS03 to protein S to the four common cold coronaviruses was quantified using the VaxArray platform from InDevR, Inc. Convalescent sera or plasma collected at least 14 days after a positive diagnosis of COVID-19 (RT-pCR) from individuals whose illness was classified as mild, moderate, or severe/critical (n=35) were analyzed concurrently. Horizontal lines indicate geometric means. Dotted line indicates mean background control values. Significant differences between sera are indicated by asterisks (***p<0.001; ****p<0.0001. One-way analysis of variance on log-transformed data. GraphPad Prism, v9.0).
Table

Table 1: Summary demographics and baseline characteristics of the trial subgroup of participants who received 3.75 µg CoVLP adjuvanted with AS03 (NCT04450004) and patients convalescing from COVID-19.

| Comparisons                  | Healthy individuals | Convalescent individuals |
|------------------------------|---------------------|--------------------------|
|                              | 3.75 µg CoVLP with AS03 adjuvant | Mild | Moderate | Severe |
| Subjects, n                  | 20                  | 16 | 8 | 11 |
| Sex, n (%)                   |                     |               |       |       |
| Male                         | 5 (25.0)            | 10 (62.5)     | 2 (25.0) | 8 (72.7) |
| Female                       | 15 (75.0)           | 5 (31.3)      | 6 (75.0) | 3 (27.3) |
| Race, n (%)                  |                     |               |       |       |
| White                        | 20 (100.0)          | 7 (43.8)      | 6 (75.0) | 5 (45.5) |
| Black or African American    | 0 (0.0)             | 1 (6.3)       | 0 (0.0) | 5 (45.5) |
| Asian                        | 0 (0.0)             | 4 (25.0)      | 2 (25.0) | 0 (0.0) |
| Ethnicity, n (%)             |                     |               |       |       |
| Hispanic/Latinx              | 0 (0.0)             | 2 (12.5)      | 1 (12.5) | 1 (9.1) |
| Age, years                   |                     |               |       |       |
| Mean ± SD                    | 34.7 ± 9.1          | 42.7 ± 13.6   | 37.8 ± 13.0 | 51.9 ± 16.0 |
| Median (range)               | 36 (19–49)          | 39 (20–66)    | 40.5 (19–58) | 50.0 (28–82) |

CoVLP, plant-produced virus-like particle; SD, standard deviation.
Figures

Figure 1

A. Anti-Spike IgG Titers

B. Pseudovirus Neutralizing Antibody Titers

C. Live Virus Neutralizing Antibody Titers

△ CoVLP (3.75 μg) + AS03
◇ Mildly III ◇ Moderately III ◇ Severely III
Figure 2

IFN-γ

IL-4
Figure 3

Coronavirus Spike Binding

- Pre-Vaccination
- CoVLP (3.75 μg) + AS03
- Mildly III
- Moderately III
- Severely III

Relative Binding (arbitrary units)

SARS-CoV-2  SARS-CoV-1  MERS

Virus Target
Figure 4

A) Pseudovirion Neutralizing Antibody Titers

B) Live Virus Neutralizing Antibody Titers
