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Broad neutralization against SARS-CoV-2 variants induced by ancestral and B.1.351 AS03-Adjuvanted recombinant Plant-Derived Virus-Like particle vaccines

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Since 2019, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection resulting in the coronavirus disease 2019 (COVID-19) has afflicted hundreds of millions of people in a worldwide pandemic. Several safe and effective COVID-19 vaccines are now available. However, the rapid emergence of variants and risk of viral escape from vaccine-induced immunity emphasize the need to develop broadly protective vaccines. A recombinant plant-derived virus-like particle vaccine for the ancestral COVID-19 (CoVLP) recently authorized by Canadian Health Authorities and a modified CoVLP.B1351 targeting the B.1.351 variant (both formulated with the adjuvant AS03) were assessed in homologous and heterologous prime-boost regimen in mice. Both strategies induced strong and broadly cross-reactive neutralizing antibody (NAb) responses against several Variants of Concern (VOCs), including B.1.351/Beta, B.1.1.7/Alpha, P.1/Gamma, B.1.617.2/Delta and B.1.1.529/Omicron strains. The neutralizing antibody (NAb) response was robust with both primary vaccination strategies and tended to be higher for almost all VOCs following the heterologous prime-boost regimen.

1. Introduction

Since the declaration of a pandemic situation caused by the SARS-CoV-2 by the World Health Organisation (WHO), over 410 million cases have been reported and >5.8 million people have died from COVID-19 (WHO Coronavirus Disease (COVID-19) Dashboard, https://covid19.who.int/, 2021). The rapid development and approval of vaccines with efficacy up to 95% led to hope in mid-2021 that the worst of the pandemic was over [1–4]. However, the total number of COVID-19 cases is still growing rapidly worldwide with almost 300 000 reported deaths in just the last month, mostly attributable to highly transmissible SARS-CoV-2 Variants of Concern (VOCs). The most worrisome variants are those with mutations in the Spike (S) protein that not only enhance transmissibility but also increase virulence and evasion of vaccine-induced immunity [5–10] or resistance to neutralization by monoclonal antibodies [8,9,11]. The S protein plays a crucial role in SARS-CoV-2 infection through the interaction of its receptor binding domain (RBD) with the angiotensin-converting enzyme 2 (ACE2) receptor on host respiratory epithelial cells [12–14]. All of the currently approved vaccines target the S protein of the ancestral strain of SARS-CoV-2 identified in Wuhan and a growing number of reports demonstrate that their efficacy against mainly the B.1.351 and the B.1.617.2 variants is reduced [15–18]. Medicago has developed a SARS-CoV-2 vaccine using a platform technology based on transient expression of recombinant proteins in non-transgenic Nicotiana benthamiana plants and a disarmed Agrobacterium tumefaciens as a transfer vector to move targeted DNA constructs into the plant cells [19]. The S protein trimers displayed on the surface of the plant-derived coronavirus-like particles (CoVLP) are in a stabilized, prefusion conformation that resemble native structures on wild-type SARS-CoV-2 virions. Plant-based VLP vaccines are an...
emerging production platform that has many potential advantages such as proper eukaryotic protein modification and assembly, low risk of contamination with adventitious agents, scalability, and rapid production speed [20]. Currently, several plant-based VLP vaccine candidates against pathogens such as Hepatitis B virus [21], Rabies virus [22], Influenza virus [23] and Norwalk virus [24] are under clinical development. At the time of writing, only two plant-based VLP vaccine candidates against SARS-CoV-2 have reached the clinical stage; Medicago's CoVLP has completed its primary vaccine efficacy analyses in Phase 3 (NCT04636697) and has recently been authorized by Canadian Health Authorities [25] and Kentucky Bioprocessing-201 is in Phase 1/2 (NCT04473690).

Herein, we present the preclinical evaluation of a CoVLP candidate targeting the B.1.351 variant compared with the original CoVLP targeting the ancestral SARS-CoV-2 strain, both of which were formulated with AS03, an Adjuvant System containing DL-α-tocopherol and squalene in an oil-in-water emulsion. Both homologous and heterologous primary immunization strategies induced strong neutralizing antibody (NAb) responses with broad cross-reactivity against the B.1.1.7 (Alpha), B.1.351 (Beta), P.1 (Gamma), B.1.617.2 (Delta), and B.1.1.529 (Omicron) VOCI. SARS-CoV-2 variant strains were selected based on the WHO designation for VOCs and degree of global public health concern.

2. Materials and methods

2.1. AS03-Adjuvanted CoVLP vaccine and CoVLP.B1351 vaccine candidate

The full-length S glycoprotein of SARS-CoV-2 from the GISAID database ([https://www.gisaid.org/]), strain hCoV-19/USA/CA2/2020 (nucleotides sequence 21,563 to 25,384 from EPI_ISL_406036) corresponding to the ancestral Wuhan strain for CoVLP or hCoV-19/Belgium/AZDelta05413-2105-2021 (nucleotides sequence 21,521 to 25,342 from EPI_ISL_961189) for CoVLP.B1351 were expressed in Nicotiana benthamiana plants as previously described [19]. The S protein was modified at the S1/S2 cleavage site (CoVLP: R667G, R668S and R670S substitutions; CoVLP.B1351: R682G, R683S and R685S substitutions; relative to native S protein from original B strain from EPI_ISL_406036) to increase stability and to stabilize the protein prefusion conformation (CoVLP: and K971P and V972P substitutions; CoVLP.B1351: K986P and V987P: relative to native S protein from original B strain from EPI_ISL_406036). The signal peptide was replaced with a plant gene signal peptide and the transmembrane domain (TM) and cytoplasmic tail (CT) of S protein were also replaced with TM/CT from Influenza H5 A/Indonesia/5/2005 to increase VLP assembly and budding. The self-assembled VLPs bearing S protein trimers were isolated from the plant matrix and subsequently purified using a process similar to that described for Medicago's plant-derived influenza VLP vaccine candidates [26]. The AS03 Adjuvant System, an oil-in-water emulsion containing 11.86 mg DL-α-tocopherol, 10.69 mg squalene and 4.86 mg Polysorbate 80 per adult human dose, was supplied by GSK, (Rixensart, Belgium) and was used as recommended by the manufacturer.

The control article was phosphate buffered saline (PBS) solution with Polysorbate 80. On each dosing day, CoVLP and CoVLP.B1351 were diluted with PBS to achieve the appropriate concentration and then mixed in a 1:1 (volume:volume) ratio with adjuvant prior to administration.

2.2. Animals, immunizations and In-Life/Post-Mortem observations

Female specific pathogen free BALB/c mice (8 weeks old) were supplied from Charles River (St-Constant, Québec, Canada) and the study was conducted at ITR Laboratories Canada Inc (Baie d'Urfe, Quebec, Canada). The study protocol was approved by ITR’s internal Animal Care Committee (ACC) and all animals used were cared for in accordance with the principles outlined in the current “Guide to the Care and Use of Experimental Animals” published by the Canadian Council on Animal Care, the NIH’s “Guide for the Care and Use of Laboratory Animals” and the Animal Research Reporting In Vivo Experiments guidance. In summary, animals were maintained under standard laboratory conditions (lighting: 12 / 12 h, temperature: 21 ± 3 °C, relative humidity: 50 ± 20%) with certified rodents pellet feed and drinking water ad libitum. The mice (8/- group, except for no vaccine control; 5/group) were immunized intramuscularly (IM) with 3.75 μg AS03-adjuvanted CoVLP or CoVLP.B1351 or the PBS control on Days 0 and 21 (final volume 0.1 mL; 0.05 mL per injection site). The administered dose was calculated based on the total protein content (measured by the BCA method) and adjusted for the purity of the CoVLP content. The purity is based on the relative abundance of the S protein measured by reduced SDS-PAGE and densitometry analyses. The purity of CoVLP and CoVLP.B1351 were 81% and 80% respectively. The AS03-adjuvanted vaccines were administered as either a homologous (CoVLP-CoVLP or CoVLP.B1351-CoVLP.B1351) or heterologous (CoVLP-CoVLP.B1351) prime-boost during primary vaccination (Fig. 1). Mortality, clinical signs, body weight, food consumption and injection site observations were evaluated throughout the study. Macroscopic observations were performed at euthanasia on Day 35. Blood was collected on Days 0 (pre-immune), 21 and 35 to measure serum NAb levels.

2.3. Pseudovirus neutralization assay (PNA)

The PNA was performed by Nexelis (Laval, Quebec, Canada) using a pseudovirus based on SARS-CoV-2 ancestral Wuhan strain (reference MN908947) as previously described [27]. Analyses were performed in duplicate and included appropriate controls. The assay was qualified for the ancestral pseudovirus strain. Cross-reactivity was evaluated using modified pseudovirions expressing SARS-CoV-2 S glycoproteins from representative B.1.351 (L18F, D80A, D215G, del242-244, R246I, K417N, N501Y, E484K, D614G, A701V, plus Δ19aa C-terminal for the PP processing), B.1.1.7 (del69-70, del144, N501Y, A570D, D614G, T716I, S982A, D1118H, plus Δ19aa C-terminal for the PP processing), B.1.1.7 (del69-70, del144, N501Y, A570D, D614G, T716I, S982A, D1118H, plus Δ19aa C-terminal for the PP processing), P.1 (L18F, T20N, P26S, D138Y, R190S, K417T, E484K, N501Y, D614G, H655Y, T1027I, V1176F, plus Δ19aa C-terminal for the PP processing), B.1.617.2 (T19R, G414D, D1516G, D1517G, R158G, L452R, T478K, D614G, P681R, D950N) and B.1.5.2.9 (L6V7, A143-145, Δ211/L212I, ins214EPE, C339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493K, G496S, Q498R, N501Y, Y505H, T547K, D614G, H655Y, N679K, P681H, N764K, D950N, Q969K, N969K, L981F) prime-boost pseudovirus based on ancestral Wuhan strain sequences. In brief, serum samples were heat-inactivated at 56 ± 2 °C for 30 min and diluted in duplicates in cell growth media at a starting dilution of 1/25 or 1/250, followed by a serial dilution (2-fold dilutions, 5 times). A previously pre-determined concentration of pseudovirus was then added to diluted sera samples and pre-incubated for 1 h at 37 °C with CO2. The mixture was then added to pre-seeded confluent Vero E6 cells expressing the ACE2 receptor (ATCC CRL-1586) and incubated for 18–24 h at 37 °C with 5% CO2. Following incubation and removal of media, ONE-Glo EX Luciferase Assay Substrate (Promega, Madison, WI) was added to cells and incubated for 3 min at room temperature with shaking. Luminescence was measured using a SpectraMax i3x microplate reader (Molecular Devices, San Jose, CA). A titration curve was generated based on a 4-parameter logistic regression (4PL) using Microsoft Excel. The NAb titer was defined as the reciprocal of the sample dilution for which the luminescence was equal to a
pre-determined cut-point value corresponding to 50% neutralization. Responders were considered positive if the NAb titer was \( \geq 25 \). NAb results presented in the current study were obtained using pseudotyped SARS-CoV-2 virus. Note that results obtained with PNA generally correlates with live virus based microneutralization assay [28].

### 2.4. Statistical analyses

The descriptive statistics and statistical comparisons were performed using GraphPad Prism software (Version 8.4.2; GraphPad Prism Software, La Jolla, CA, USA). The geometric mean titers (GMT) of NAb titers with 95% confidence intervals (CI) and percentage of positive responders were calculated for each group of mice. A titer value of 12.5 was attributed to titers lower than the minimum required dilution (MRD) (i.e., 1/25). Statistical comparisons to evaluate differences between groups were performed using either a one-way ANOVA followed by a Tukey post hoc test, or a two-way ANOVA followed by a Bonferroni post hoc test on \( \log_{10} \)-transformed antibody titers. Wilcoxon matched-pairs signed rank was used to assess differences between the various SARS-CoV-2 pseudovirus strains. The threshold for statistical significance was set to \( p < 0.05 \).

### 3. Results

#### 3.1. Neutralizing and Cross-Reactive antibodies induced by AS03-Adjuvanted CoVLP and CoVLP.B1351 following homologous Prime-Boost primary vaccination strategies

In this study, mice were immunized following either a homologous or heterologous prime-boost regimen with AS03-adjuvanted CoVLP and/or CoVLP.B1351 (Fig. 1). A single dose of either AS03-adjuvanted CoVLP or CoVLP.B1351 induced a significant NAb response against the homologous strain (CoVLP versus ancestral strain: GMT 661 [95% CI: 454–963]; CoVLP.B1351 versus the B.1.351 strain: GMT 6 066 [95% CI: 4 628–7 952] (both \( \geq 0.05 \)). The heterologous, AS03-adjuvanted CoVLP-CoVLP.B1351 vaccination also successfully induced high titers of NAbs against both strains included in the regimen as well as the other VOCs tested (Fig. 3), with the exception of the B.1.1.529 variant, for which NAb levels were 12–15 folds lower compared to the ancestral and the B.1.351 strains. Compared to the AS03-adjuvanted CoVLP-CoVLP group, the heterologous prime-boost group induced a significantly greater cross-reactive response for the B.1.351 (Fig. 3B) and P.1 (Fig. 3D) VOCs (\( p < 0.05 \)). Compared to the CoVLP.B1351-CoVLP.B1351 homologous regimen, only the response against the ancestral strain was higher in the heterolo-

#### 3.2. High levels of Cross-Reactive response against other VOCs

Both homologous prime-boost strategies (CoVLP-CoVLP or CoVLP.B1351-CoVLP.B1351) induced high levels of cross-reactive NAbs against several other VOCs including B.1.1.7 and P.1 (Fig. 2C). A significant decrease in NAbs was observed against the B.1.617.2 and B.1.1.529 variants (Fig. 2C). The degree of cross-reactive neutralization induced by AS03-adjuvanted CoVLP-CoVLP was similar to that elicited by AS03-adjuvanted CoVLP.B1351-CoVLP.B1351 (\( p > 0.05 \)) (Fig. 3) except for the P.1 and B.1.1.529 strains, for which the latter strategy generated significantly higher titers (P.1: GMT 22 380 [95% CI: 14 529–34 473] versus 9 929 [95% CI: 5 843–1 016]; B.1.1.529: GMT 3 218 [95% CI: 1 547–6 693] versus 470 [95% CI: 218–1 016]; \( p < 0.05 \) (Fig. 3D and 3F). As previously shown in Fig. 2C, the degree of cross-neutralization for the AS03-adjuvanted CoVLP-CoVLP homologous prime-boost regimen varied across the tested VOCs as follows: P.1 > B.1.1.7 > B.1.351 > B.1.617.2 > B.1.1.529 and for the CoVLP.B1351-CoVLP.B1351 regimen (Fig. 2C): P.1 > ancestral/B.1.1.7 > B.1.1.529 > B.1.617.2.

#### 3.3. Heterologous Prime-Boost vaccination also induced a strong and Cross-Reactive antibody response to VOCs

The heterologous, AS03-adjuvanted CoVLP-CoVLP.B1351 vaccination also successfully induced high titers of NAbs against both strains included in the regimen as well as the other VOCs tested (Fig. 3), with the exception of the B.1.1.529 variant, for which NAb levels were 12–15 folds lower compared to the ancestral and the B.1.351 strains. Compared to the AS03-adjuvanted CoVLP-CoVLP group, the heterologous prime-boost group induced a significantly greater cross-reactive response for the B.1.351 (Fig. 3B) and P.1 (Fig. 3D) VOCs (\( p < 0.05 \)). Compared to the CoVLP.B1351-CoVLP.B1351 homologous regimen, only the response against the ancestral strain was higher in the heterolo-
A. Neutralizing Antibody Response

B. Cross- Reactive Comparisons after the First Dose

C. Cross-Reactive Comparisons after the Second Dose Following Homologous Regimen

Fig. 2. Serum Neutralizing Antibody Response and Cross-Reactivity Comparisons of AS03-Adjuvanted CoVLP or CoVLP.B1351 Following Homologous Prime-Boost Regimen. BALB/c mice (n = 8) were immunized IM on Days 0 and 21 with 3.75 μg of CoVLP or CoVLP.B1351 formulated with AS03 adjuvant. NAb titers were measured against SARS-CoV-2 pseudoparticles in serum samples using a cell-based PNA targeting the ancestral or B.1.351 strains. Half of the minimum required dilution (MRD) of the method was assigned to non-responders (i.e. 12.5). (A) GMT with 95% CI measured 21 days after the 1st immunization (Day 21) and 14 days after the 2nd immunization (Day 35). Statistical comparisons were performed using a two-way ANOVA followed by a Bonferroni post hoc test on log10-transformed NAb titers. (B-C) Results from individual mouse serum samples (n = 8 per antigen) are represented as dots on each figure with lines connecting ancestral of B.1.351 to the B.1.351, B.1.1.7, P.1, B.1.617.2 or B.1.1.529 neutralization titers. Statistical comparisons were performed using Wilcoxon matched pairs signed rank test. p-values are indicated on the graphs. ns: Not significant (p > 0.05).

guous prime-boost group (Fig. 3A; \( p < 0.05 \)). Again, a significantly lower response against the B.1.1.529 strain was observed (Fig. 3F; \( p < 0.05 \)). The amplitude of the cross-reactive neutralizing antibody response after heterologous prime-boost vaccination varied across the strains tested (Fig. 4): P.1 > B.1.351 > ancestral/B.1.1.7 > B.1.617.2 > B.1.1.529.

3.4. Safety of CoVLP and CoVLP.B1351 vaccines in animals

Overall, no safety concerns were raised following homologous prime-boost strategies or the heterologous strategy. The post-immunization variations observed for body weight and food consumption were transient and/or within the normal variations (Figures S1 and S2). An unexpected increase in food consumption was observed in the CoVLP + AS03 group between Days 7–14 that was likely attributable to eating-like behavior (i.e. stashing of food pellets at the bottom of the cage) in a small number of the animals in this group. Transient signs of discomfort (Table S1) and inflammation at the dosing sites (edema and erythema) were reported in all treated groups following the prime (Figure S3). All observations generally subsided within 10–14 days and were no longer seen prior to the second administration. After
the second administration, no signs of reactogenicity or discomfort were reported at the injection site. No macroscopic anomalies were reported following euthanasia and collection of organs and tissues.

4. Discussion and conclusions

The first anti-COVID-19 vaccines were approved for emergency use within a year of the start of the pandemic with reported

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Fig. 3. Cross- Reactive Neutralization against the ancestral, Beta (B.1.351) Alpha (B.1.1.7), Gamma (P.1), Delta (B.1.617.2), and Omicron (B.1.1.529) strains Following Homologous or Heterologous Prime-Boost Regimen. BALB/c mice (n = 8) were immunized IM on Days 0 and 21 with 3.75 μg of CoVLP.B1351 or CoVLP (both formulated with AS03 adjuvant). NAb titers were measured against SARS-CoV-2 pseudoparticles in serum samples using a cell-based PNA targeting the ancestral, B.1.351, B.1.1.7, P.1, B.1.617.2 or B.1.1.529 strains. Half of the minimum required dilution (MRD) of the method was assigned to non-responders (i.e. 12.5). GMT with 95% CI obtained 14 days after the boost (Day 35) for the (A) ancestral, (B) B.1.351, (C) B.1.1.7, (D) P.1, (E) B.1.617.2 or (F) B.1.1.529 strains. Statistical comparisons between the CoVLP-treated groups were performed using a One-way ANOVA followed by a Tukey post hoc test (Day 35) on log₁₀-transformed NAb titers. Significant differences are indicated with p-values on the graphs.
B.1.351, B.1.617.2, and B.1.1.529 strains raising in vitro, body neutralization some of these mutations can confer significant resistance to anti-pared with B.1.1.7 variants. It is very clear however that variant is associated with a higher risk of emergency care consul-

The rapid worldwide spread of some VOCs can be attributed to enhanced transmissibility. For example, transmission of the ancestral Wuhan-like strain with one or a sequence of these variants of concern "VOCs". The different waves of variants has complicated diagnostic efforts in some cases and generally frustrated efforts to control the spread and impact of the pandemic. Among the most important VOCs that have emerged over the last year include the B.1.1.7, B.1.351, P.1/B.1.1.248, B.1.617.2 and B.1.1.529 strains.

Of particular note, similar trends were observed in recently reported results on neutralization of VOCs with human serum samples collected in Medicago’s ongoing clinical development program. Multiple viral variants have emerged in different geographic regions with varied transmissibility, virulence and resistance to vaccine-induced immunity. Many parts of the world have experienced rapid replacement of the ancestral Wuhan-like strain with one or a sequence of these variants of concern "VOCs". These observations highlight the need both to evaluate the ability of vaccines already deployed or in advanced development to neutralize the VOCs and to develop next generation vaccines with broader cross-reactivity. In this study, the cross-reactive neutralizing antibody responses elicited by AS03-adjuvanted CoVLP (targeting the ancestral SARS-CoV-2 strain) were generally promising. Despite slight (1-2x) reductions in neutralization, AS03 still elicited high levels of serum cross-neutralizing antibodies, particularly for the B.1.351 strain. Although the relationship between different mutations and disease severity is not yet fully understood, recent evidence suggests that the B.1.1.7 variant is associated with a higher risk of emergency care consultation and hospital admission for unvaccinated individuals compared with B.1.1.7 variants. It is very clear however that some of these mutations can confer significant resistance to antibody neutralization in vitro, particularly those present in the B.1.351, B.1.617.2, and B.1.1.529 strains raising important concerns about the countermeasures available to overcome the COVID-19 pandemic.

Fig. 4. Cross-Reactivity Comparisons against Alpha (B.1.1.7), Gamma (P.1), Delta (B.1.617.2), and Omicron (B.1.1.529) strains Following Heterologous Prime-Boost Regimen. BALB/c mice (n = 8) were immunized IM on Days 0 with 3.75 µg CoVLP and 21 with 3.75 µg of CoVLP.B1351 (both formulated with AS03 adjuvant). NAB titers were measured against SARS-CoV-2 pseudoparticles in serum samples using a cell-based PNA targeting the ancestral, B.1.351, B.1.1.7, P.1, B.1.617.2 or B.1.1.529 strains. Half of the minimum required dilution (MRD) of the method was assigned to non-responders (i.e. 12.5). Results from individual mouse sera (n = 8 per antigen) are represented as dots on each figure with lines connecting the ancestral or B.1.351 variant to the B.1.351, B.1.1.7, P.1, B.1.617.2 or B.1.1.529 neutralization titers. Statistical comparisons were performed using Wilcoxon matched pairs signed rank test. p-values are indicated on the graphs. ns: Not significant (p > 0.05).
Currently known if these differences between high and very high neutralization activity were pronounced following the administration of the candidate B.1.351 vaccine compared to the vaccine based on the ancestral strain, possibly due to the closer phylogenetic relationship between B.1.351 and the Omicron variants [68].

Despite these promising data, it is possible that one or more VOCs will eventually emerge that is/are no longer effectively neutralized by vaccine-induced immunity. It is in this context that Medicago and others have chosen to develop next-generation vaccine candidates targeting the B.1.351 strain since this strain is one of the most antigenically distant VOC to emerge to date. The B.1.351 variant has consistently proved to be difficult to neutralize in vitro [69,70] and has caused large decrements in vaccine efficacy in several randomized controlled trials [15–17,61]. In the current study, animals that received two doses of either AS03-adjuvanted CoVLP or CoVLP.B1351 mounted neutralizing antibody responses that were comparable for both homologous and heterologous strains while reports for other candidate B.1.351 vaccines in mice have shown either strong homologous (ie: B.1.351-specific) responses only [71] or the requirement for three doses to achieve high levels of NABS [64]. The pattern of the NAB response was consistent across multiple VOCs in the current study with the CoVLP.B1351 candidate generally eliciting higher titers than the ancestral CoVLP and this difference reached significance for the P.1 (2.3x) and the B.1.1.529 (6.8x) strains. Although the level of cross-neutralization in the animals that received CoVLP.B1351 was lower for the B.1.1.7 (~1.6x) and B.1.617.2 (~4.0x) variants compared to the homologous response, such differences are expected given the genetic and antigen ‘distance’ between these VOCs [43]. Furthermore, while these relative decreases were observed, the absolute titers of cross-reactive antibodies induced by two doses of CoVLP.B1351 with AS03 against the VOCs tested was still substantial. These findings are consistent with observations of others [64,71,72] and suggest that vaccines targeting the original Wuhan-like strain may be eventually become suboptimal in the next stages of the pandemic, opening the door to less conventional vaccination approaches including heterologous prime-boost strategies.

Concern over the ability of any single S protein antigen to elicit a broad enough response to neutralize all of the known and possibly future VOCs prompted us to evaluate the possible benefits of a heterologous prime-boost strategy with the Wuhan-like CoVLP as the prime and CoVLP.B1351 as the boost; both adjuvanted with AS03. Heterologous vaccination strategies that use two distinct platforms and/or deliver two slightly different antigens have shown considerable promises for a wide range of viral pathogens that rapidly mutate such as HIV [73], hepatitis C virus [74] or influenza to both broaden the immune response and focus the response on conserved epitopes [75]. This approach was largely confirmed in the current study since the neutralizing antibody titers were consistently higher in the animals that had received the AS03-adjuvanted CoVLP-CoVLP.B1351 regimen, reaching statistical significance over the AS03-adjuvanted CoVLP-CoVLP.B1351 regimen for B.1.351 and P.1 strains and over the AS03-adjuvanted CoVLP.B1351-CoVLP.B1351 regimen for the ancestral strain. It is not currently known if these differences between high and very high neutralizing antibody responses will have any clinical significance. However, induction of very high initial titers is likely desirable since it is well-documented that antibody titers wane substantially with time after both natural disease and vaccination [76]. These observations are similar to the results recently released by others [72,77] [Pfizer, Novavax] but distinct from those reported by Moderna [71] in that no evidence of original antigenic sin was noted [78]. Since these animals only received two doses, it is currently unknown how humoral response against VOCs would be influenced by a third (booster) dose but others have reported very high and cross-protective neutralizing antibody responses both in animals [64,71] and human trials [65,79,80] after this additional dose.

Finally, it is worth noting that these observations focus entirely on vaccine-induced antibody responses and particularly on the induction of antibodies capable of neutralizing SARS-CoV-2 variants in vitro. Although many consider NAB levels to be a good candidate for a correlate of protection [81], this is a fairly limited evaluation of vaccine-induced immunity and it is very likely that non-neutralizing but functional antibodies and cellular responses also contribute to vaccine-induced protection [82]. Data from a large non-human primate study [83] as well as ongoing clinical trials [27,65,84] demonstrate that AS03-adjuvanted CoVLP stimulates multiple arms of the adaptive response to SARS-CoV-2. Results from Medicago’s ongoing pivotal Phase 3 efficacy study [25] (NCT04636697), performed in different regions of the world where several VOCs have been circulating, demonstrated a good protection of the CoVLP vaccine (targeting the ancestral strain) against several VOCs such as B.1.617.2 and P.1. These results are in line with the non-clinical cross-neutralization data presented in this study. Based on these Phase 3 results, it is unclear what immediate benefit might be gained by switching to a heterologous prime-boost strategy for primary vaccination. However, both the magnitude and the breadth of response need to be considered as SARS-CoV-2 continues to mutate under increasing immune pressure including the most recent example of the B.1.1.529 variant. The data presented herein suggest two doses of AS03-adjuvanted CoVLP or CoVLP.B1351 can induce a strong immune response against a broad range of VOCs. Moreover, recently published preclinical data also highlight the added value of a third dose [64,71]. These observations provide further support for the growing body of data suggesting that the use of heterologous antigens, whether B.1.351 of some new VOC yet to emerge, in either primary or third-dose booster strategies may have advantages over traditional homologous antigen vaccination approaches and further clinical trials will be needed to confirm the efficacy of such vaccination strategies.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CD, SPR, GA, MAD, BJW and ST are either employees of Medicago Inc or receive salary support from Medicago Inc.

CG is an employee of the GSK group of companies and reports ownership of GSK shares.

**Acknowledgements**

The study was sponsored by Medicago Inc. The authors would like to acknowledge Philippe Boutet, Margherita Coccia, Marie-Ange Demoitié, Ulrike Krause and Eric Destexe from GSK for critical review of the manuscript. The authors also wish to acknowledge all the Medicago employees and their contractors (ITR Laboratories Canada Inc and Nexelix) for their exceptional dedication and professionalism.
[52] Diamond M, Chen R, Xie X, Case J, Zhang X, VanBlargan L, et al. SARS-CoV-2 variants show resistance to neutralization by many monoclonal and serum-derived polyclonal antibodies. Research square. 2021:rs;3:rs-228079.

[53] Planas D, Bruel T, Grzelak L, Guivel-Benhassine F, Staropoli I, Porrot F, et al. Sensitivity of infectious SARS-CoV-2 B.1.1.7 and B.1.351 variants to neutralizing antibodies. Nat Med. 2021;27:917–24.

[54] Rees-Spear C, Mui L, Griffith SA, Heaney J, Aldon Y, Snitselaar JL, et al. The effect of spike mutations on SARS-CoV-2 neutralization. Cell Rep. 2021;34:108890.

[55] Liu C, Ginn HM, Dejnirattisai W, Supasa P, Wang B, Tuelpkraphon A, Nutalai R, Zhou D, Mentzer AJ, Zhao Y, Duyvesteyn HM. Reduced neutralization of SARS-CoV-2 B.1.617 by vaccine and convalescent serum. Cell. 2021;184 (16):4220–36.

[56] Cao Y, Wang J, Jian F, Xiao T, Song W, Yismayi A, et al. Omicron escapes the majority of existing SARS-CoV-2 neutralizing antibodies. Nature 2021.

[57] Lazariević I, Pravica V, Miljanović D, Cuić M. Immune Evasion of SARS-CoV-2 Emerging Variants: what Have We Learnt So Far? Viruses. 2021:13.

[58] Wu K, Werner AP, Koch M, Choi A, Narayanan E, Stewart-Jones GBE, et al. Serum Neutralizing Activity Elicited by mRNA-1273 Vaccine. N Engl J Med. 2021;384:1468–70.

[59] Camereni E, Saliba C, Bowen JE, Rosen LE, Culap K, Pinto D, et al. Broadly neutralizing antibodies overcome SARS-CoV-2 Omicron antigenic shift. bioRxiv. 2021.

[60] Novavax. Novavax COVID-19 Vaccine Demonstrates 89.3% Efficacy in UK Phase 3 Trial. Novavax. Novavax Confirms High Levels of Efficacy Against Original and Variant COVID-19 Strains in United Kingdom and South Africa Trials. March 11, 2021.

[61] van Dorp L, Acman M, Richard D, Shaw LP, Ford CE, Ormond L, et al. Emergence of genomic diversity and recurrent mutations in SARS-CoV-2. Infect Genet Evol. 2020;83:104351.

[62] Zhou HY, Ji CY, Fan H, Han N, Li XF, Wu A, et al. Convergent evolution of SARS-CoV-2 in human and animals. Protein. Cell 2021:1–4.

[63] Su D, Li X, He C, Huang X, Chen M, Wang Q, et al. Broad neutralization against SARS-CoV-2 variants induced by a modified B.1.351 protein-based COVID-19 vaccine candidate. bioRxiv. 2021.

[64] Gobeil PA, Pillet S, Boulay I, Charland N, Lorin A, Cheng M, et al. Durability and Cross-Reactivity of Immune Responses Induced by an AS03 Adjuvanted Plant-Based Recombinant Virus-Like Particle Vaccine for COVID-19. MedRxiv. 2021.

[65] Gagne M, Moliva JI, Foulds KE, Andrew SF, Flynn BJ, Werner AP, et al. mRNA-1273 or mRNA-Omicron boost in vaccinated macaques elicits comparable B cell expansion, neutralizing antibodies and protection against Omicron. bioRxiv. 2022;2022.02.03.479037.

[66] Chandrashekhar A, Yu J, McMahan K, Jacob-Dolan C, Liu J, He X, et al. Vaccine Protection Against the SARS-CoV-2 Omicron Variant in Macaques. bioRxiv. 2022;2022.02.06.479285.

[67] Kandeel M, Mohamed MEM, Abd El-Lateef HM, Venugopala KN, El-Beltagi HS. Omicron variant genome evolution and phylogenetics. J Med Virol. 2021.