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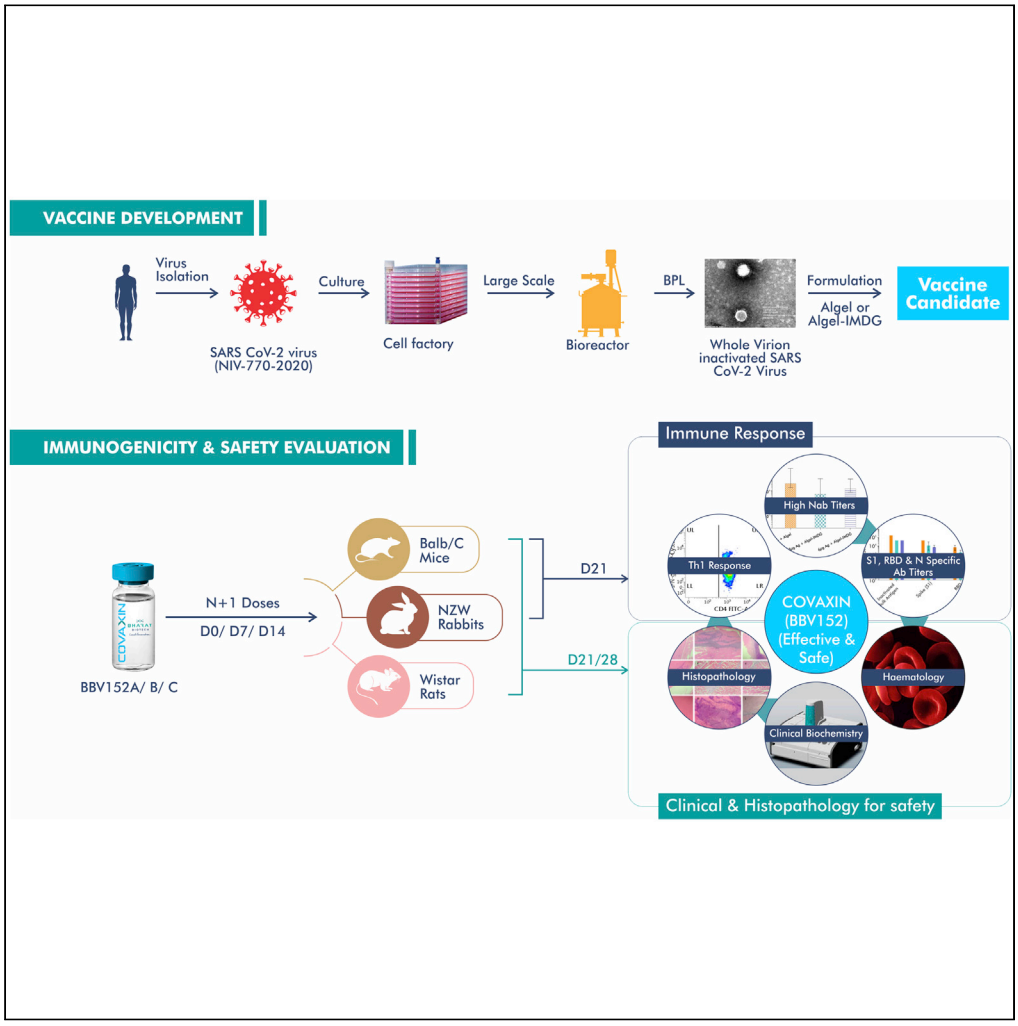

Brunda Ganneru, Harsh Jogdand, Vijaya Kumar Daram, ..., Atanu Basu, Nivedita Gupta, Krishna Mohan Vadrevu

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Highlights

WVI SARS-CoV-2 vaccine (BBV152) was developed using genetically stable strain

Adjuvanted vaccines (BBV152A, B, and C) induced high NAb titers in animal models

Chemisorbed Algel with TLR7/8 agonists vaccine formulations (BBV152A & B) induced robust Th1 biased immunity

Adjuvanted vaccines (BBV152A, B, and C) are found to be safe in animal models

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Th1 skewed immune response of whole virion inactivated SARS CoV 2 vaccine and its safety evaluation

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SUMMARY

We report the development and evaluation of safety and immunogenicity of a whole virion inactivated (WVI) SARS-CoV-2 vaccine (BBV152), adjuvanted with aluminum hydroxide gel (Algel), or TLR7/8 agonist chemisorbed Algel. We used a well-characterized SARS-CoV-2 strain and an established Vero cell platform to produce large-scale GMP-grade highly purified inactivated antigen. Product development and manufacturing process were carried out in a BSL-3 facility. Immunogenicity and safety were determined at two antigen concentrations (3μg and 6μg), with two different adjuvants, in mice, rats, and rabbits. Our results show that BBV152 vaccine formulations generated significantly high antigen-binding and neutralizing antibody titers (NAb), at both concentrations, in all three species with excellent safety profiles. The inactivated vaccine formulation contains TLR7/8 agonist adjuvant-induced Th1-biased antibody responses with elevated IgG2a/IgG1 ratio and increased levels of SARS-CoV-2-specific IFN-γ^+ CD4$^+$ T lymphocyte response. Our results support further development for phase I/II clinical trials in humans.

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a novel human coronavirus, has spread across the world. SARS-CoV-2 belongs to β-genus of Sarbecovirus and is a close relative of SARS-CoV with approximately 80% sequence identity (Zhou et al., 2020). The World Health Organization (WHO) declared the disease caused by SARS-CoV-2 as coronavirus disease-19 (COVID-19), a pandemic in March 2020. So far, SARS-CoV-2 has infected more than 45 million people, causing more than 1.1 million deaths (WHO Coronavirus Disease, n.d.). It is, therefore, imperative to develop effective prophylactic and therapeutic counter measures to prevent and treat COVID-19.

Numerous vaccine candidates such as adenovirus-vectored, nucleic-acid-based, recombinant-protein-based, and inactivated vaccines are at various stages of developmental phase at either preclinical or clinical trials (Draft landscape of COVID-19 candidate vaccines, n.d.; Folegatti et al., 2020; Gao et al., 2020; Jackson et al., 2020; Keech et al., 2020; Wang et al., 2020). However, meeting the global need for billions of doses of COVID-19 vaccines will require collective effort to identify, evaluate, validate, and manufacture effective vaccines. Inactivated vaccines for viral diseases have been licensed for decades with well-established safety profiles (Sanders et al., 2015). The availability of well-characterized Vero cell manufacturing platform with proven safety have aided in rapid vaccine development of inactivated vaccines (Bhandari et al., 2014; "Global Advisory Committee (World Health Organization, 2019); Sampath et al., 2010; Singh et al., 2015; Vadrevu et al., 2020).

It has to be mentioned here that the some of the most advanced developmental stage vaccine candidates such as the inactivated vaccine (PiCoVacc) and the recombinant vaccine (CoV-RBD219N1) are aluminum adjuvant formulations. These vaccines are shown to generate high levels of NAb titers against SARS

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Table 1. Genetic stability of the BBV152 viral strain under specific passages (Vero CCL-81 passage 1 PID-3)

Reference position	Wuhan Hu-1 nucleotide	Current nucleotide	Substitution	Count of reads	Frequency of reads	Region
241	C	T	Synonymous change	10,937	99.7	5′ UTR
3037	C	T	Synonymous change	6,227	99.6	orf1ab
4809	C	T	Nonsynonymous change (S- > F)	11,561	99.91	orf1ab
14408	C	T	Nonsynonymous change (P- > L)	7,562	99.91	orf1ab
23403	A	G	Nonsynonymous change (D- > G)	13,336	99.96	S

CoV 2, which could play an important role in vaccine efficacy. Hence, the development of inactivated vaccines for COVID-19 prevention appears to be a rational approach, while recognizing the fact that such inactivated vaccines with alum adjuvant specifically induce Th2-biased response. For example, inactivated SARS CoV 2 vaccine (CoronaVac, China) formulated with alum-generated Th2 response, but with low levels of Th1 response. Hence, there is no clear indication that CoronaVac induces Th1 response (Zhang et al., 2020). However, recent literature on SARS and SARS CoV 2 showed the importance of Th1-skewed immune response, in providing the protection against infection and its role in reducing the clinical severity toward subsequent infections (Janice Oh et al., 2012; Jeyanathan et al., 2020; Le Bert et al., 2020; Sekine et al., 2020). Further, antigen-specific T cell immune responses live longer than the neutralization antibodies (Ng et al., 2016) and provide long-term immunity (Cañete and Vinuesa, 2020). Hence, SARS CoV 2-specific T cell immunity is found to be critical, while developing a vaccine against SARS CoV 2. Given this, we formulated an inactivated vaccine with an adjuvant (Algel-IMDG) containing TLR7/8 agonists molecule, known to induce Th1-biased immunity (Miller et al., 2020; Shukla et al., 2012; Warshakoon et al., 2009). Here, we report the immunogenicity and safety evaluation of the whole-virion inactivated SARS-CoV-2 vaccine candidate (BBV152) formulated in Algel or Algel-IMDG, in three animal models.

RESULTS

Isolation and selection of SARS-CoV-2 strain for vaccine candidate preparation

During the initial outbreak of SARS-CoV-2 in India, specimens from 12 infected patients were collected and sequenced at the Indian Council of Medical Research-National Institute of Virology (ICMR-NIV), India, a WHO Collaborating Center for Emerging Viral Infections (Sarkale et al., 2020). The SARS-CoV-2 strain (NIV-2020-770) used in developing the BBV152 vaccine candidate was retrieved from tourists who arrived in New Delhi, India (Potdar et al., 2020; Yadav et al., 2020b). The sample propagation and virus isolation were performed in the Vero CCL-81, and strain sequence was deposited in the GISAID (EPI_ISL_420545). The BBV152 vaccine candidate strain is located in the (G clade), also represented as "20A" clade that is the most prevalent strain in India (followed by "19A") as per data represented in the next strain analysis of the Indian analysis (Hadfield et al., 2018). In terms of the overall divergence of SARS-CoV-2, this strain is 99.97% identical to the earliest strain Wuhan Hu-1 (Sardar et al., 2020). The multiple passages done in the Vero CCL-81 demonstrated the genetic stability of the virus. The next-generation sequencing (NGS) reads generated from the nucleotide sequences of the BBV152 vaccine candidate strain and its passage one at PID-3 (post-infection day 3) were found to be comparable with the SARS-CoV-2 Wuhan Hu-1 strain (Table 1). A maximum difference of 0.075% in the nucleotides was observed, indicating negligible changes in the different batches of the samples analyzed. Thus, these results showed genetic stability of the NIV-2020-770 strain, which leads to further vaccine development.

Vaccine candidate preparation

The seed virus was adapted to a highly characterized GMP Vero cell platform, amplified to produce the master and working virus bank. The master virus bank was well characterized based on WHO Technical Report Series guidelines (WHO-TRS_978_Annex_3.pdf, 2013) , including identity, sterility, mycoplasma, virus titration, adventitious agents, hemadsorption etc. GMP production of virus bulk was performed in the bio-safety level-3 (BSL-3) facility using bioreactors.

Growth kinetics analysis revealed that the SARS-CoV-2 virus replicated to 7.0 log10 $TCID_{50}$ between 36 and 72 h (Figure 1A). The β-propiolactone was utilized for the inactivation of the virus by mixing the virus stock at 2-8°C. Inactivation kinetics was performed with varying conditions and concentrations, and

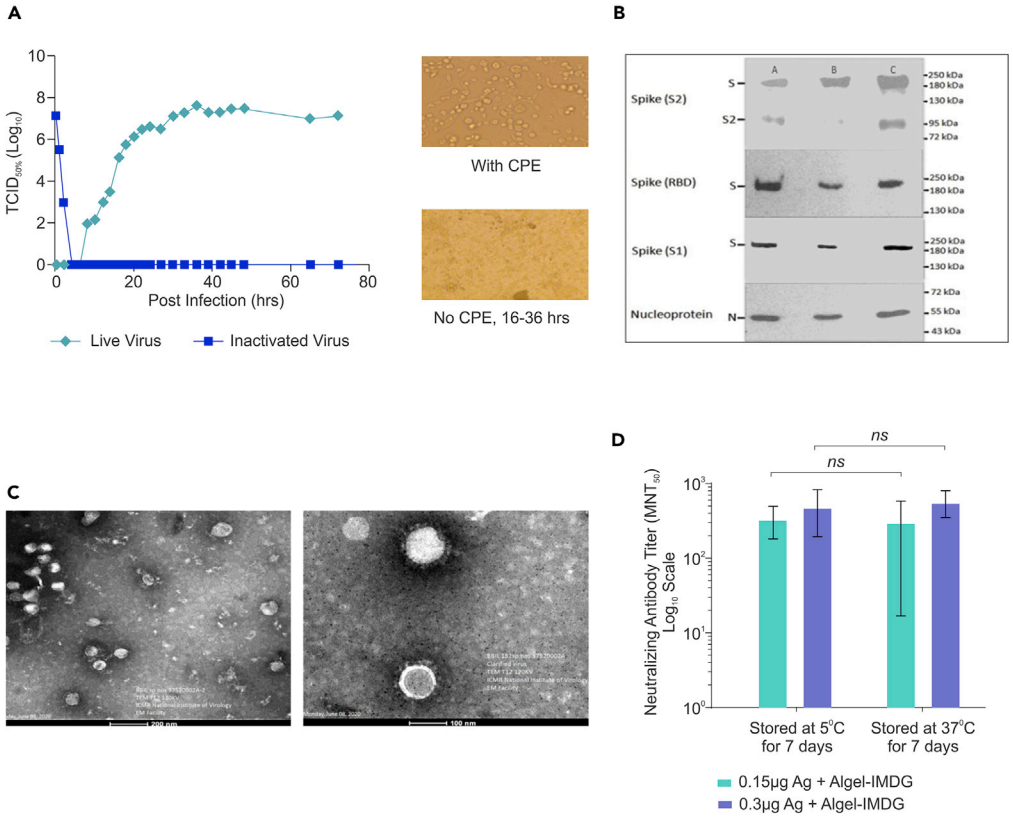

Figure 1. Characterization of inactivated SARS-CoV-2 and evaluation of the stability of BBV152 vaccine formulations

(A) SARS-CoV-2 virus (Strain NIV-770-2020) growth kinetics and its cytopathic effect (CPE) before and after inactivation. (1) Line graph represents virus titer (10^6–10^7) measured by $CCID_{50}$ at every 3 h up to 48 h and after that every 12 h (24, 27, 30, 33, 36, 39, 42), (2) microscopic images represent cells with cytopathic effect (CPE) before inactivation and no CPE after inactivation, (3) image of Vero cell monolayer with no CPE observed from 16 to 36 h;

(B) Western blot analysis of purified inactivated SARS-CoV-2 produced from three production batches; the antibody used for each panel is mentioned on the left side of the image;

(C) Representative electron micrograph of purified inactivated SARS-CoV-2 candidate vaccine (BBV152) at a scale bar: 100 nm (right) and 200 nm (left);

(D) Bar graph represents microneutralization antibody titer of day 14 individual sera (7 days after 2nd dose) collected from mice vaccinated with 1/20th dose of adjuvanted formulations (0.15 µg Ag with Algel-IMDG and 0.3 µg Ag with Algel-IMDG), after subjecting them for stability at 37°C for 7 days and compared with 2–8°C. Error bars represent median with 95% CI and the statistical analysis performed using one-sample T test shown not significant.

samples were collected at various time points (between 0 and 24 h, at 4-h intervals) to evaluate the cytopathic effect. Three consecutive inactivation procedures (each lasting 24 h) were performed to ensure complete viral inactivation without affecting the antigen stability (Figures 1A and 1B). Purified and inactivated whole-virion antigen produced from three production batches was also characterized by western blot for its identity using anti-Spike (S1 & S2), anti-RBD, and anti-N protein (Figure 1B) antibodies. These results demonstrated that the final purified inactivated bulk of the vaccine candidate contains spike and nucleocapsid (N) protein. The uncleaved full-length spike was detected by the spike (S2) (Figure 1B first panel), spike (RBD) (Figure 1B second panel), and spike (S1) (Figure 1B third panel) antibodies. The spike (S2) antibody also detected the S2 subunit (Figure 1B first panel). The full-length spike (S), S2 fragment (S2), and nucleocapsid (N) protein bands were of expected molecular weight (Figure 1B). Transmission electron microscopy (TEM) analysis also showed that the inactivated and purified virus particles were intact, oval-shaped, and were accompanied by a crown-like structure representing the well-defined spike protein on the virus membrane (Figure 1C), and these data corroborate with electron micrograph of the live virus (Prasad et al., 2020).

Table 2. Immunogenicity and safety studies conducted

Study type	Animal model	Test item[a,b,c,d]	Route of administration	No of animals[e]	Key test item result
Immunogenicity	BALB/c mice	PBS, antigen, adjuvanted vaccines, & adjuvants	Intraperitoneal	50 (F)	High spike (S1)-specific Ab titers and NAb titers were observed.
Immunogenicity	BALB/c mice	PBS & adjuvanted vaccines	Intraperitoneal	40 (20M + 20F)	High antigen-specific (S1, RBD, & N) Ab titers and NAb titers along with cell-mediated responses were observed.
Immunogenicity	BALB/c mice	PBS, antigen, & adjuvanted vaccines	Intramuscular	24 (12M + 12F)	Adjuvanted vaccines show high S1-specific titer when compared with antigen.
Long-term immunogenicity	BALB/c mice	PBS, adjuvanted vaccines	Intramuscular	28 (14M + 14F)	Consistent high S1-specific Ab titers & NAb titers were observed upto 98 days.
Repeated dose toxicity studies	Wistar rats	PBS, antigen, adjuvanted vaccines, & adjuvants	Intramuscular	82 (41M + 41F)	All the test items have been demonstrated to be safe from a toxicology perspective[f].
	Swiss albino mice	PBS, adjuvanted vaccines, & adjuvants	Intramuscular	32 (16M + 16 F)	
	New Zealand white rabbits	Adjuvanted vaccines[g]	Intramuscular	10 (5M + 5F)	
Mutagenicity assay (bacterial reverse mutation)	*Salmonella typhimurium*	Algel-IMDG	–	–	
Maximum tolerated dose studies	Swiss albino mice	Algel-IMDG	Intramuscular	10 (5M + 5F)	
Maximum tolerated dose studies	Wistar rats	Algel-IMDG	Intramuscular	10 (5M + 5F)	

[a]Antigen: BBV152 Antigen at 3 (low), 6 (middle), & 9 μg (high).
[b]Adjuvanted vaccines: BBV152A, BBV152B, & BBV152C.
[c]Adjuvants: Algel & Algel-IMDG at 20 & 30 μg agonist.
[d]PBS, phosphate-buffered saline.
[e]M, male; F, female.
[f]Details are given in supplementary section.
[g]Preimmune sera were used as baseline titers for ELISA & $PRNT_{90}$/MNT_{50}.

Vaccine formulations with adjuvants and their stability

Inactivated whole-virion SARS CoV-2 (BBV152) vaccine candidates were formulated with two alum-based adjuvants: Algel (aluminum hydroxide gel) and Algel-IMDG, an imidazoquinoline class molecule (TLR7/TLR8 agonist, abbreviated as IMDG) chemisorbed onto aluminum hydroxide gel. Three vaccine formulations were prepared: first two contain 3 μg and 6 μg antigen with Algel-IMDG (BBV152A and BBV152B, respectively) and the third one contains 6μg antigen with Algel (BBV152C). To determine the stability of these formulations, Algel-IMDG vaccine formulations (BBV152A and BBV152B) were stored at 37°C and 2–8°C temperature for 7 days. These vaccine formulations were diluted 20 times ($1/20^{th}$ human single dose (HSD), equivalent to 0.15 and 0.3 μg antigen) and administered in BALB/c mice intra-peritoneally to evaluate NAb titer by microneutralization test (MNT_{50}) at 7-day post-immunization. Our results demonstrated that the both vaccine formulations are relatively stable at 37°C for 7 days, as shown by equivalent NAb titer compared with formulation stored at real time (2–8°C) temperature (Figure 1D).

Safety evaluation of Algel-IMDG and adjuvanted vaccine formulations (BBV152)

Algel is a well-known adjuvant having been used in a large number of vaccines globally. Hence extensive safety evaluation was performed for the Algel-IMDG adjuvant alone along with three adjuvanted vaccine formulations (BBV152A, B, and C), as per the regulatory guidelines (WHO, 2013; OECD, 2020; CDSCO, 2019). Table 2 summarizes the key toxicology studies/tests performed and the observations thereof.

Safety of Algel-IMDG was evaluated by three experiments: (1) mutagenicity assay (*in-vitro*) to determine mutagenic potential of the Algel-IMDG; (2) maximum tolerated dose test (MTD, *in-vivo*), to ensure the

human intended adjuvant dose is tolerable; and (3) repeated dose toxicity study (RDT, *in-vivo*), to evaluate that repeated administration of Algel-IMDG does not cause any systemic toxicity or mortality. Mutagenicity assay performed with Algel-IMDG at various concentrations revealed that there was no substantial increase in revertant colony numbers in any of the tested strains at any dose level, in both the plate-incorporation and pre-incubation methods in the presence or absence of metabolic activation (S9 mix) (Table S1). Thus, the Algel-IMDG used in the BBV152 A and B-adjuvanted vaccine formulations was found to be non-mutagenic. Further, maximum tolerated dose study performed with single dose of Algel-IMDG also revealed that the Algel-IMDG was tolerated at the tested dose (20 μg agonist/animal) in mice and rats as demonstrated by lack of erythema, edema, or any other macroscopic lesions at the site of injection (Table S2).

Moreover, repeated administration of (N+1 dose regimen) high dose of either Algel-IMDG alone (30μg agonist/animal) in Swiss Albino mice and Wistar rats or high dose of adjuvanted vaccine formulation (9μg Ag with 30μg agonist/animal, which is more than HSD) in Wistar rats did not show any clinical illness, change in body weight (Figure S1), or histopathological changes, except inflammation at the site of injection (Figure S3) and thus established the safety of both Algel-IMDG and adjuvanted vaccine formulations at high dose.

Further, the safety of adjuvanted vaccine formulations (BBV152 A, B, and C) either at full HSD or $1/10^{th}$ or $1/20^{th}$ HSD was also evaluated to be safe in three animal models (BALB/c mice, S. albino mice, and NZW rabbits), as demonstrated by the repeated dose toxicity study with no mortality and with no changes in clinical signs, body weight gain, body temperature, or feed consumption in any of the animals. Representative data of body temperature as a parameter are shown in Table S3.

Clinical pathological parameters such as hematology, clinical biochemistry, coagulation studies, and urinalysis performed in repeated dose toxicity (RDT) studies showed that the animals administered with either adjuvanted vaccine candidates or adjuvants/antigen-alone were comparable with control (Figure S2, Table S4), except increased levels of Alpha 1- acid glycoprotein and neutrophils count on day 2 in adjuvant-alone or adjuvanted vaccine formulation groups. However, these values were comparable with control on day 21. This transient increase may be due to inflammation at the injection site after administration of the first dose. These findings were further correlated with the inflammatory reaction at the injection site observed microscopically, in the animals administered with adjuvant-alone and adjuvanted vaccine with Algel and Algel-IMDG. This inflammation was found to be slightly higher in animals that received Algel-IMDG than in animals that received Algel. However, this inflammation reduced by day 28 (Figures S3 and S4). Other than local reaction at the site of injection, no other treatment-related microscopic findings were observed in any of the animals administered with antigen or adjuvant or adjuvanted vaccine formulations. Histopathological examination of organs such as spleen, lungs, heart, and lymph nodes etc., of all animal models administered with antigen or adjuvant or adjuvanted vaccine formulations was normal (Figures S5 and S6).

Adjuvanted vaccine formulations (BBV152) induced high neutralization antibody titers

We report immunogenicity of three BBV152 formulations in BALB/c mice and New Zealand white rabbits. BALB/c mice were administered either with full or $1/10^{th}$ or $1/20^{th}$ HSD, whereas Rabbits were immunized with intended HSD. Table 2 summarizes the immunogenicity studies performed and the observations thereof.

Immunogenicity in BALB/c mice

Initially, BALB/c mice (n = 5/group, female) were administered with adjuvanted vaccine formulations, antigen or adjuvants alone at $1/20^{th}$of the intended HSD (i.e., $1/20^{th}$ of 3 μg, 6 μg, and 9 μg/mouse or 0.15 μg, 0.3 μg, and 0.45 μg/mouse), to determine the optimal dose. ELISA titers (Figure 2A) and NAb titers (Figure 2B) determined at various time points revealed that immune response elicited against these adjuvanted vaccine formulations tested at three antigen concentrations elicited high levels of binding and NAb titer (Figures 2A and 2B). Antibody response determined on day 7 was found to be less (10^2titer) robust or negligible compared with day 14 and day 21 with a titer of 10^3 and 10^4, respectively. Notably, 3 and 6 μg formulations induced high or similar spike-specific antibody titers compared with 9 μg group. Hence, adjuvanted formulation with high antigen dose (9 μg) was eliminated in further studies of the immunogenicity, whereas safety was evaluated in Wistar rats at this high dose. These results also indicated that adjuvanted vaccine formulations either with Algel or Algel-IMDG elicited high spike (S1)-specific antibody binding titers compared with antigen alone tested at all three concentrations (Figure 2A). This was further evaluated

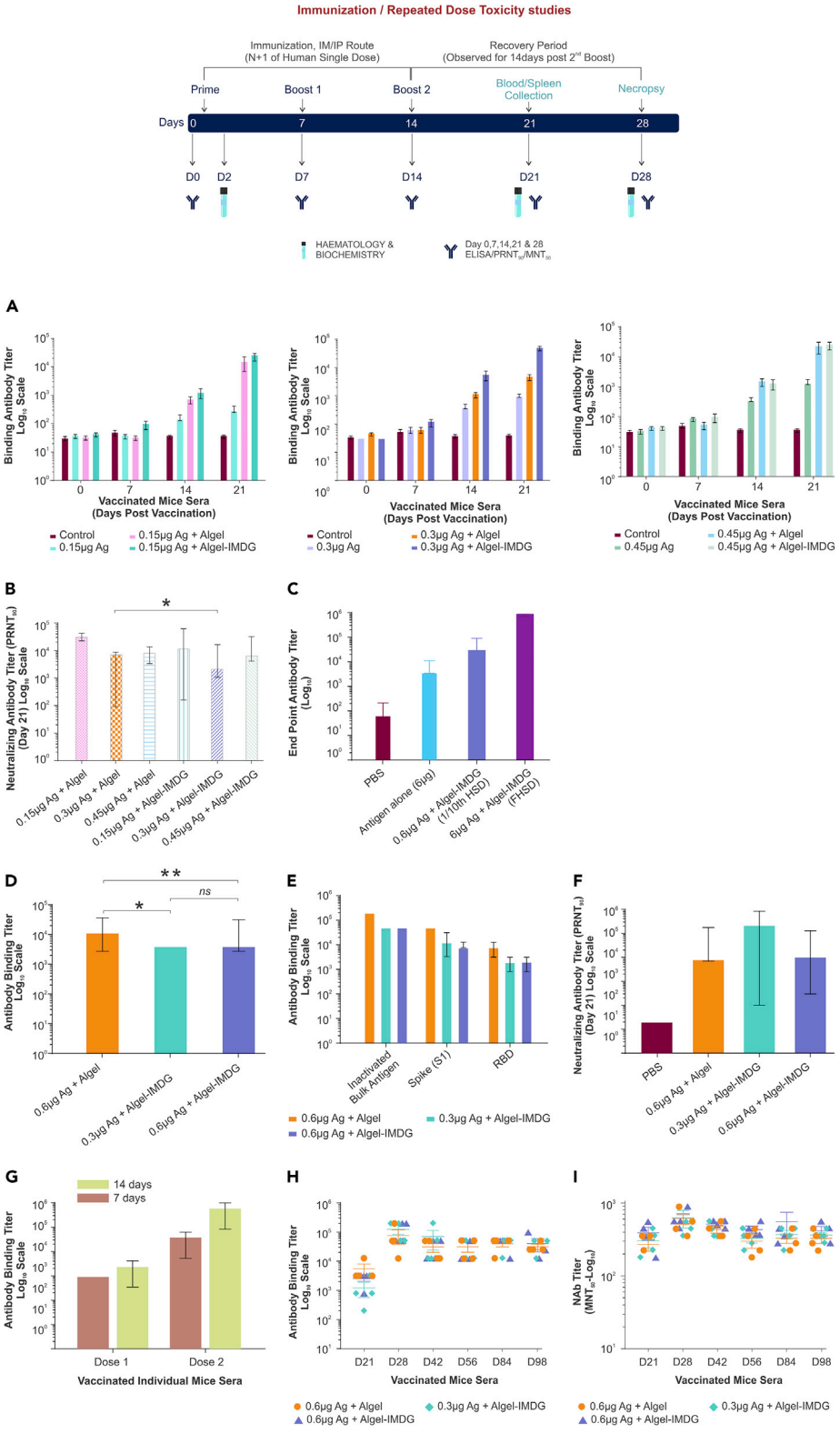
Immunization / Repeated Dose Toxicity studies
Immunization, IM/IP Route
(N+1 of Human Single Dose)
Recovery Period
(Observed for 14days post 2nd Boost)
Prime
Boost 1
Boost 2
Blood/Spleen Collection
Necropsy
Days
0
7
14
21
28
D0
D2
D7
D14
D21
D28
HAEMATOLOGY & BIOCHEMISTRY
Day 0,7,14,21 & 28 ELISA/PRNT50/MNT50
A
Binding Antibody Titer Log10 Scale
Vaccinated Mice Sera (Days Post Vaccination)
Control
0.15μg Ag
0.15μg Ag + Algel
0.15μg Ag + Algel-IMDG
0.3μg Ag
0.3μg Ag + Algel
0.3μg Ag + Algel-IMDG
0.45μg Ag
0.45μg Ag + Algel
0.45μg Ag + Algel-IMDG
B
Neutralizing Antibody Titer (PRNT50) (Day 21) Log10 Scale
0.15μg Ag + Algel
0.3μg Ag + Algel
0.45μg Ag + Algel
0.15μg Ag + Algel-IMDG
0.3μg Ag + Algel-IMDG
0.45μg Ag + Algel-IMDG
C
End Point Antibody Titer (Log10)
PBS
Antigen alone (6μg)
0.6μg Ag + Algel-IMDG (1/10th HSD)
6μg Ag + Algel-IMDG (FHSD)
D
Antibody Binding Titer Log10 Scale
ns
0.6μg Ag + Algel
0.3μg Ag + Algel-IMDG
0.6μg Ag + Algel-IMDG
E
Antibody Binding Titer Log10 Scale
Inactivated Bulk Antigen
Spike (S1)
RBD
0.6μg Ag + Algel
0.3μg Ag + Algel-IMDG
0.6μg Ag + Algel-IMDG
F
Neutralizing Antibody Titer (PRNT50) (Day 21) Log10 Scale
PBS
0.6μg Ag + Algel
0.3μg Ag + Algel-IMDG
0.6μg Ag + Algel-IMDG
G
Antibody Binding Titer Log10 Scale
14 days
7 days
Dose 1
Dose 2
Vaccinated Individual Mice Sera
H
Antibody Binding Titer Log10 Scale
D21
D28
D42
D56
D84
D98
Vaccinated Mice Sera
0.6μg Ag + Algel
0.3μg Ag + Algel-IMDG
0.6μg Ag + Algel-IMDG
I
NAb Titer (MNT50-Log10)
D21
D28
D42
D56
D84
D98
Vaccinated Mice Sera
0.6μg Ag + Algel
0.3μg Ag + Algel-IMDG
0.6μg Ag + Algel-IMDG

Figure 2. BBV152 vaccine induces high virus-specific antibody response in mice

(A–I) Immune response elicited against antigens at three concentrations of antigen or adjuvanted vaccine formulations in BALB/c mice (n = 5, female) were represented. Animals were administered via IP route either with 1/20th (Fig A & B) or 1/10th (Fig D, E, & F) human single dose (HSD) or administered via intramuscular route with either full HSD or 1/10th dose (C, G, & H). S1-specific total IgG antibody binding titers measured using individual sera collected (A) at various time points (day 0, 7, 14, & 21) at 1/20th dose; (D) on day 21 with 1/10th dose; (C) on day 21 either with full HSD or 1/10th dose; (G)on post-dose 1 (on day 7 or 14) and 2 (on day 14 or 28), when BALB/c mice were administered with BBV152B via IM route with different immunization schedule with an interval of 7 or 14 days, and (H) at various time points (day 21, 28, 42, 56, 84, and 98) at 1/10th dose via IM route. (E) SARS-CoV-2 specific (S1, RBD, N and total inactivated antigen) antibody binding titers elicited against adjuvant vaccines (BBV152A, B & C) on day 21; neutralizing antibody titers performed by $PRNT_{90,}$ using day 21 sera collected from BALB/c mice, when administered via IP route either with 1/20th (B) or 1/10th dose (F) or collected at various time points day 21, 28, 42, 56, 84, and 98 (I), when administered at 1/10th dose via IM route. Antibody binding titers were performed by ELISA and neutralizing antibody titers by $PRNT_{90}$. Bar graphs representing data represented as mean ± SD (G), mean/mean ± SEM (A, H, & I) derived from individual mice sera data analysis. For the long-term immunogenicity study, sera from four animals per group were tested for ELISA and MNT_{50} analyzed. Statistical analysis performed by (B) Wilcoxon rank test indicates significant difference between 0.3 μg antigen Algel and 0.3 μg antigen Algel-IMDG at p value < 0.05 and error bars indicate median with 95% CI, whereas in figure D, statistical analysis performed by Mann Whitney test showed significant difference at p value < 0.005 (**) and <0.05 (*), respectively. NS indicates not significant.

by administering BALB/c mice intramuscularly with antigen at actual HSD (6 μg Ag) either in the presence or in the absence of adjuvant (Algel-IMDG) and compared with 1/10th HSD of adjuvanted vaccine formulation (0.6 μg Ag and Algel-IMDG). Immune response elicited against adjuvanted vaccine formulation (BBV152B) was significantly (1 log) higher than the antigen alone (6 μg Ag), which is comparable with 1/10th HSD of adjuvanted vaccine formulation (1/10th of BBV152B). These results suggest the dose-sparing effect of Algel-IMDG (Figure 2C).

Further, to assess the immunogenicity and safety of clinical batch samples, BALB/c mice (n = 10/group, 5 male and 5 female) were vaccinated via IP route with three adjuvanted vaccine formulations with Algel and Algel-IMDG at 1/10th human intended single dose (0.3 and 0.6 μg/dose with Algel or Algel-IMDG). All adjuvanted vaccine formulations elicited antigen-specific binding antibodies (Figure 2D). Further, sera collected on day 21 were analyzed by ELISA to determine S1, RBD, and N-specific binding titer (Figure 2E) and showed 100% seroconversion with S1, RBD, and N protein. Analysis of plaque reduction neutralization test ($PRNT_{90}$), performed with individual mice sera, showed high NAbs in all adjuvanted vaccines (Figure 2F).

We also compared different immunization dose schedules (day 7 versus day 14), wherein BALB/c mice were administered intramuscularly with adjuvanted vaccine (full HSD), with one group receiving second dose on day 7 and the other group on day 14 after initial immunization. Our results indicated 8-fold increase in spike-protein-specific antibody titer, when booster dose was given with 14-day interval as compared with that given on day 7 (Figure 2G).

In addition, to demonstrate long-lived immune response, BALB/c mice (n = 8/group, 4 male and 4 female) were vaccinated intramuscularly with three adjuvanted vaccine formulations (1/10th HSD of BBV152A, B, and C) on day 0, 7, and 14 and evaluated antibody titer up to 12 weeks after last dose. These results revealed that the spike-specific antibodies reached peak level on day 28, and the antibody titers were sustained up to day 98, i.e., 12 weeks after last dose (Figure 2H). Similarly, we also found sustained NAb titers up to day 98 (Figure 2I), which indicates the BBV152 vaccine candidates were able to produce long-term immunity.

Immunogenicity in New Zealand white rabbits

To assess the immunogenicity of adjuvanted vaccine formulations at full human single dose (HSD, 3 and 6 μg antigen/dose), rabbits (n = 4) were immunized intramuscularly on days 0, 7, and 14. Similar to mice, immune response in rabbits was also found to be time dependent, and not all animals were seroconverted on day 7 and showed less antibody binding titer ($\geq 10^2$). However, on day 21, we found 100% seroconversion with spike-specific antibody binding titer of greater than or equal to 10^4. All three formulations (BBV152A, B, and C) showed high binding antibody response (Figure 3A), with no statistically significant difference. Similarly, $PRNT_{90}$ results showed high neutralizing antibody titers in all three adjuvanted vaccine formulations on day 21 (Figure 3B). However, there is no significant difference, and similar results were also

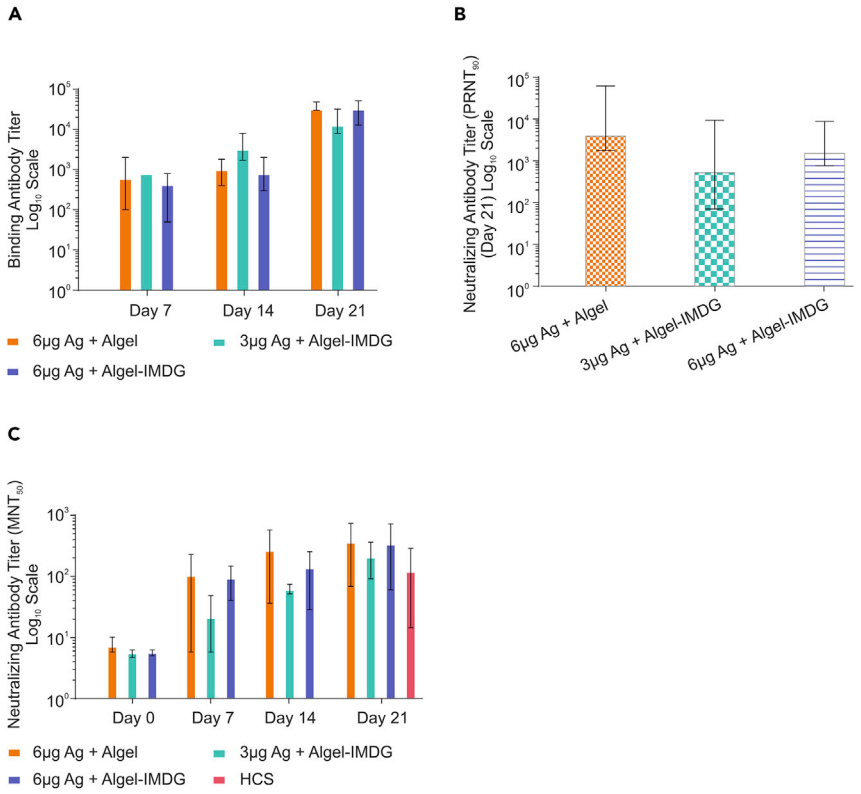

Figure 3. BBV152 induces robust neutralizing antibody response in rabbits

New Zealand white rabbits (n = 4) were administered intramuscularly on days 0, 7, and 14 with full HSD. SARS-CoV-2-specific antibody titers were measured by ELISA. NAb tires were measured by $PRNT_{90}$ and MNT_{50}. Data Points represent median/mean of individual animal data.

(A) S1-specific Ab binding titer of sera collected at various time points (day 0, 7, 14, and 21)

(B) $PRNT_{90}$ neutralizing antibody titers of day 21 sera; error bars indicate median with 95% CI, and statistical analysis performed using Wilcoxon signed rank test found no significant difference among the three adjuvanted vaccine groups.

(C) MNT_{50} neutralizing antibody titers of sera collected at various time points (day 0, 7, 14, and 21) along neutralizing antibody titer (MNT_{50}) with human convalescent sera (HCS) from recovered COVID-19 patients (n = 15). Samples were collected between 21 and 65 days of virological confirmation. Error bars indicate mean with 95% CI.

observed by MNT_{50} titers (Figure 3C). Further, NAb titers determined by MNT_{50} were slightly higher or comparable with NAb titers of human convalescent sera collected from recovered symptomatic COVID-19 patients (Figure 3C).

BBV152 adjuvanted with TLR7/8-adsorbed Algel-induced Th1-biased immune response

Immunoglobulin subclasses (IgG1, IgG2a, and IgG3) were analyzed on day 14 hyperimmunized BALB/c mouse sera samples to evaluate the Th1/Th2 polarization. The average ratio of IgG2a/IgG1 or IgG2a + IgG3/IgG1 was higher in Algel-IMDG groups when compared with Algel, indicative of Th1 bias (Figure 4A). Antigen alone showed Th1-biased response at three tested different concentrations with an average Th1:Th2 index of 3; however, ELISA and $PRNT_{90}$ titers are less compared with adjuvanted vaccine formulations. Similarly, the same sera, when measured for interferon-γ (IFNγ) by ELISA, 6-μg Algel-IMDG samples induced significantly higher responses of IFNγ compared with Algel (Figure 4B). In addition, expression of other cytokines such as interleukin (IL)-2, IL-4, IL-6, tumor necrosis factor (TNF)-α, IL-17A, IL-10, and interferon gamma (IFNγ) were noticeably higher in the 6-μg Algel-IMDG when compared with 6-μg Algel (Figure 4D), especially on day 7 and 14 hyperimmunized sera.

To further evaluate Th1-skewed immune response induced by Algel-IMDG, intracellular staining was performed using vaccinated mice splenocytes after stimulation with inactivated SARS-CoV-2 antigen and determined IFNγ-producing T lymphocytes. Interestingly, we found that the adjuvanted formulation

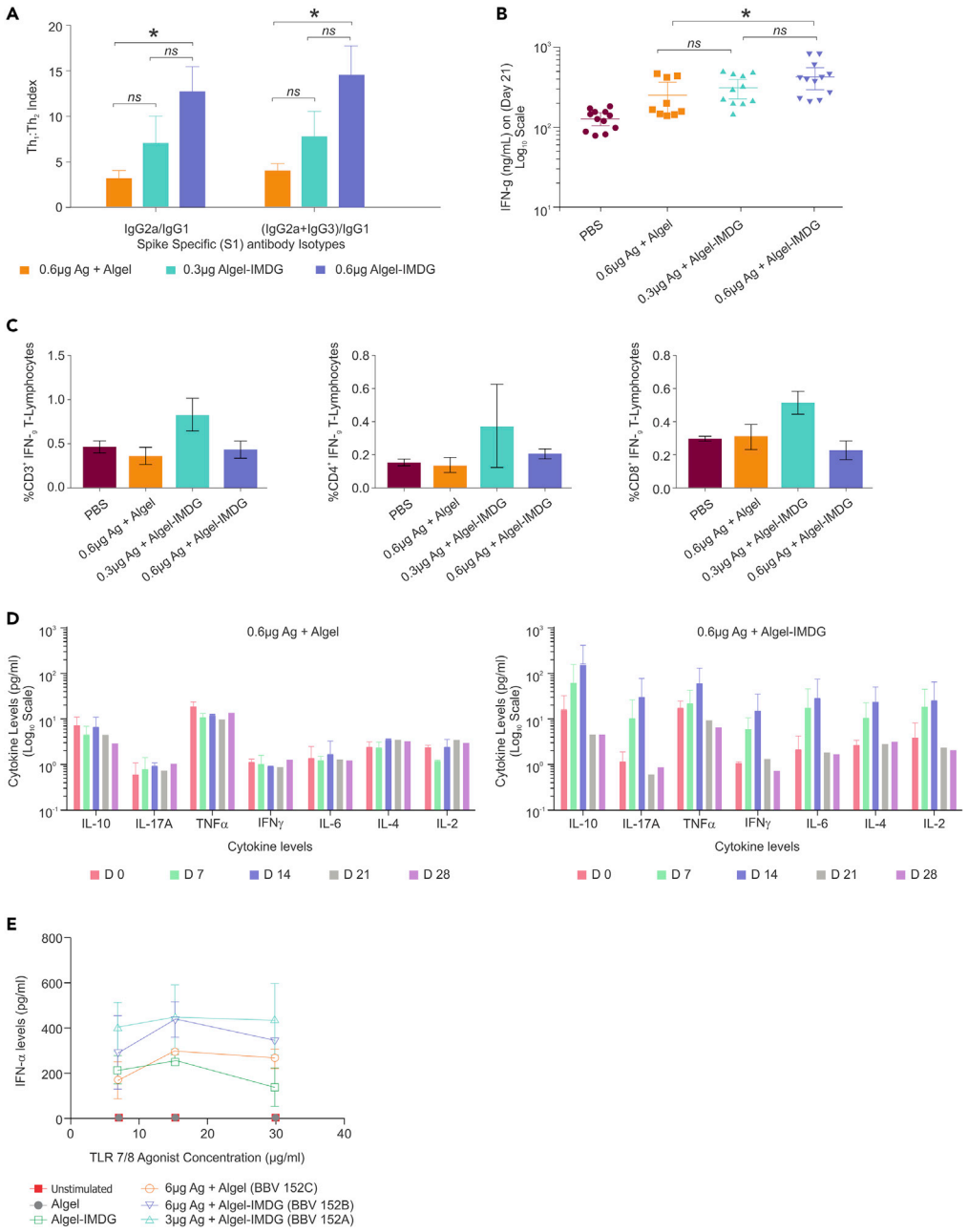

Figure 4. BBV152 induces a robust virus-specific T cell response

Panel of figures represent cell-mediated response data generated either from vaccinated sera or from splenocytes collected from BALB/c mice or cell culture supernatant collected, after stimulation of unvaccinated huPBMCs *(ex-vivo). BALB/c mice (n = 10) were vaccinated with 1/10th HSD of adjuvanted vaccine formulations (BBV 152 A, B, and C) via the IP route.*

(A) Th1:Th2 index generated using the formulas IgG2a/IgG1 or (IgG2a + IgG3)/IgG1, from endpoint antibody (immunoglobulin subclasses: IgG1, IgG2a, and IgG3) titer analysis measured by ELISA, using sera collected from day 21 (7 days post-third dose), from BALB/c mice, administered with 1/20th HSD of adjuvanted vaccine formulations (BBV 152 A, B, and C) via the IP route. Endpoint titer of respective immunoglobulin sub classes obtained from PBS/Algel/Algel-IMDG were taken as baseline. Error bars represent mean ± SEM.

(B) Secreted IFN gamma levels estimated by ELISA, on day 21 sera (7 days post-third dose). Statistical analysis done by Mann Whitney test showed significant difference at p value < 0.05, between 0.6 µg antigen Algel and 0.6 µg Algel-IMDG.

Figure 4. *Continued*

(C) Percent of $CD3^+$ (Left) or $CD4^+$ (Middle) or $CD8^+$ (right) T lymphocytes producing IFN gamma measured by intracellular staining assay using the splenocytes of individual BALB/c mice administered with $1/10^{th}$ HSD. Error bars indicate mean ± SEM.

(D) Cytokine profile measured on various time points using vaccinated BALB/c mice sera, when administered with adjuvanted vaccine formulations ($1/20^{th}$ HSD via IP route). Left—BBV152C-antigen 6μg + Algel. Right—BBV152B-antigen 6μg + Algel-IMDG. Error bars indicate mean ± SDE. IFNα levels measured by ELISA from culture supernatant, when stimulated healthy PBMCs with Algel or Algel-IMDG or adjuvanted vaccine formulations (BBV152A, B, and C)). Two-fold serial dilutions of the human intended dose of adjuvanted vaccine formulations were used. Corresponding antigen or adjuvant alone concentration were also maintained simultaneously as controls. Error bars indicate mean ± SD of triplicate values.

BBV152A (0.3μg Ag + Algel-IMDG) showed elevated levels of IFNγ producing CD4 cell population, whereas, BBV152B formulation (0.6μg Ag + Algel-IMDG) induced comparable levels of IFNγ producing $CD4^+$ T cells to the BBV152C (0.6Ag + Algel) (Figure 4C). However, there is no significant difference among the three groups. Similarly, BBV152A formulation (0.3μg Ag + Algel-IMDG) also induced higher IFNγ production in $CD3^+$ and $CD8^+$ T cells compared with other two formulations.

To assess the effect of adjuvants (Algel or Algel-IMDG) on immune response and understanding the critical role of IMDG in eliciting IFNα (which induces robust antibody and Th1 response), we stimulated PBMCs from healthy volunteers with adjuvants alone and adjuvanted vaccines for 36–72 h at 2-fold dilutions of BBV152A, B, and C and measured IFNα in the cell supernatant. We found that Algel-IMDG containing TLR7/8 agonists alone stimulated IFN-α but not the Algel. In addition, adjuvanted vaccine formulations with Algel-IMDG induced elevated levels of IFN-α compared with Algel formulation, demonstrating the enhanced activation of immune system by the Algel-IMDG group compared with Algel (Figure 4E).

DISCUSSION

Here, we report the development of a whole-virion inactivated SARS-CoV-2 vaccine candidate (BBV152A-C). The strain (NIV-2020-770), which belongs to G clade, used for this vaccine candidate is pathogenic in humans, which is the current predominant circulating strain all over the world. It is also observed that the D614G mutation increases viral neutralization (Sarkale et al., 2020). This strain also showed extensive genetic stability and appropriate growth characteristics and thus we chose NIV-2020-770 strain for further vaccine development.

Preclinical toxicity or safety evaluation of either adjuvant-alone (Algel-IMDG) or three formulations did not indicate any undesirable pathological changes and systemic toxicity, except local reactogenicity at the site of injection (Figures S3 and S4), which was attributed to the use of adjuvants in the vaccine formulation. Algel (Alum) is the most commonly used adjuvant, known to show depot formation at the site of injection, which helps the antigen for slow release (Gupta and Gupta, 2020). The microscopic findings at the site of injection in the present study showed the infiltration of macrophages and mononuclear cells indicates the activation of innate immunity. The other adjuvant namely Algel-IMDG, containing TLR7/8 agonist, induced slightly higher reactogenicity. Intra-muscular injection induces a depot effect followed by the passive trafficking of Algel particles via lymphatic flow from the interstitial space to the draining lymph nodes, as revealed by IFN-β/luciferase reporter mice (unpublished data from Dr. Sunil David, ViroVax, LLC, KS, USA). The lymph node targeting of Algel-IMDG ensures high adjuvant activity in the target organ (lymph nodes) by enabling the induction of a strong, specific, adaptive immune response while minimizing systemic exposure. Further, Algel-IMDG did not show mutagenicity in the five strains of *Salmonella typhimurium* tested. The local reaction in the studies conducted was consistent with those available in the literature for these adjuvants, which is a physiological reaction to activate immune system rather than any adverse event (Gupta and Gupta, 2020; Sellers et al., 2020).

Our results show that the vaccine formulations induced significantly elevated antigen-binding antibody and NAb responses in the immunized animals, with a distinct Th1 bias observed with Algel-IMDG adjuvanted vaccines. Although the neutralizing antibody titers are not statistically different between the antigen concentration (3 μg and 6 μg) or the nature of adjuvant, all the formulations tested have exhibited excellent immunogenicity. Recently developed, two other inactivated SARS-CoV-2 vaccine candidates (BBIBP-CorV and PiCoVacc) have been shown to induce high levels of NAb titers in mice and rats and showed protection in rhesus macaques against SARS-CoV-2 (Wang et al., 2020). Reportedly, antibodies

raised against PiCoVacc also neutralized 10 representative SARS-CoV-2 strains and indicate possible broader neutralizing ability toward multiple SARS-CoV-2 strains circulating worldwide (Gao et al., 2020). Our potency results are quite favorably comparable with those reported in the literature for similar COVID-19 vaccines (Gao et al., 2020; Wang et al., 2020).

Further, in our preclinical studies, we demonstrated that all the three inactivated whole-virion SARS-CoV-2 vaccine candidates showed 100% seroconversion with high titers of antigen binding and neutralizing antibody responses. Further, the adjuvanted IMDG formulation (BBV152B) showed more than 10 times higher antibody response, compared with antigen-alone (Figure 2C), thus Algel-IMDG formulation providing dose-sparing effect. Moreover, these formulations induced immunity that is biased toward Th1-mediated response, as demonstrated by the ratio between IgG2a and IgG1 (greater than 1) (Figure 4A). In addition, secretion of anti-viral cytokines such as IL-2, IL-4, IL-6, IL-10, IL-17, TNF-alpha, and IFNγ observed on days 7 and 14 (7 days after the 1st and 2nd dose) (Figure 4D) and higher induction of IFN-alpha (Figure 4E) in Algel-IMDG adjuvanted formulations might have contributed to enhance activation of antigen-presenting cells, such as dendritic cells or macrophages. However, mechanism of action of Algel-IMDG in the induction of Th1-biased response is yet to be investigated. These results were further supported by our Hamster and non-human primate animal challenge study, wherein Algel-IMDG adjuvanted formulations provided early protection compared with Algel formulation, with the significant reduction in the viral load (Mohandas et al., 2021; Yadav et al., 2020a). It is also reported earlier that TLR recognition in innate cell population drives early type I IFN production, thereby promoting viral clearance and the early production of proinflammatory cytokines (Hijano et al., 2019; Stephens and Varga, 2020).

Although major research is focused on spike as the target protein for SARS CoV-2 vaccine development, there is some attention being paid toward nucleocapsid protein as a target protein, due its 90% amino acid homology and stability with fewer mutations over time (Dutta et al., 2020; Grifoni et al., 2020). Thus, it is predicted that vaccine strategies with conserved epitope regions could generate cross-protective immunity across betacoronaviruses. Le Bert et al., 2020 showed the presence of T cell responses against the structural (nucleocapsid (N) protein) and non-structural (NSP7 and NSP13 of ORF1) regions of SARS-CoV-2 in individuals convalescing from coronavirus disease 2019 (COVID-19) (Duan et al., 2020; Le Bert et al., 2020).

Further recent research findings based on bioinformatic analysis of epitope mapping revealed that nucleocapsid protein is composed of both T and B cell immunodominant epitopes (Chen et al., 2020; Sarkar et al., 2020). Earlier, animal studies conducted using DNA vaccine against SARS CoV showed that the nucleocapsid is able to produce enhanced antigen-specific humoral and cellular immune responses (Chunling et al., 2006; Zhao et al., 2005). It is also to be noted that, though, the earlier immunization studies performed in animal models against nucleocapsid protein reported to cause pneumonia (Deming et al., 2006; Yasui et al., 2008), yet, there is not much established research evidence so far on the pathogenicity of nucleoprotein in humans.

In conclusion, we believe that the ability to induce Th1-skewed immune response and the presence of conserved S and N protein in inactivated vaccine candidate formulated in Algel-IMDG would help to combat other SARS CoV-2 variants.

In our findings, we also observed high binding titers with a 100% seroconversion toward S1, RBD, and N protein. Further, high neutralization titers and protective effectiveness of COVAXIN in hamster and non-human primate models might be attributed toward the structural integrity of the inactivated whole-virion vaccine composed of target proteins, both spike and N proteins.

Bharat Biotech has developed a promising inactivated whole-virion vaccine candidate, which has now entered phase 3 clinical development (NCT04641481). The study is designed to evaluate the safety and immunogenicity of two intramuscular doses of BBV152 in healthy volunteers.

Limitations of the study

Long-term protective efficacy of these vaccine candidates, cross-neutralization with other SARS CoV 2 variants, and mechanism of action of Algel-IMDG in inducing cell-mediated responses need to be evaluated further.

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Raches Ella (ellar@bharatbiotech.com).

Materials availability

The materials used in this study are available upon request. This study did not generate any unique reagents.

Data and code availability

SARS-CoV-2 strain (NIV-2020-770) sequence was deposited in the GISAID (GenBank: EPI_ISL_420545). Primary data will be available upon request. This study did not generate any new software code.

METHODS

All methods can be found in the accompanying transparent methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102298.

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AUTHORS CONTRIBUTION

All listed authors meet the criteria for authorship set forth by the International Committee for Medical Editors and have no conflicts to disclose. BG., R.R., and J.J. led the immunogenicity and safety preclinical experiments. H.J., V.D., N.M., V.K.S., S.P., and K.M.V. led the manufacturing and quality control efforts. P.P.R. and D.K. provided technical support for inactivated virus characterization. K.M.V., P.S., and E.R. provided technical assistance with design, analysis, and manuscript preparation. Y.P., S.G., S.A., M.S., A.B., A.P., B.B., and N.G. of ICMR-NIV, Pune conducted electron microscopy and neutralizing antibody assays. A.A. conducted cell-mediated response-related assays at THSTI.

DECLARATION OF INTERESTS

This work was supported and funded by Bharat Biotech International Limited and the Indian Council of Medical Research. All authors are employees of either organization. Authors from RCC Laboratories Private Ltd were utilized for contract research purposes. All authors have no personal financial or non-financial interests to disclose. KE is the Chairman and Managing Director of Bharat Biotech.

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Supplemental information

Th1 skewed immune response of whole virion inactivated SARS CoV 2 vaccine and its safety evaluation

Brunda Ganneru, Harsh Jogdand, Vijaya Kumar Daram, Dipankar Das, Narasimha Reddy Molugu, Sai D. Prasad, Srinivas V. Kannappa, Krishna M. Ella, Rajaram Ravikrishnan, Amit Awasthi, Jomy Jose, Panduranga Rao, Deepak Kumar, Raches Ella, Priya Abraham, Pragya D. Yadav, Gajanan N. Sapkal, Anita Shete-Aich, Gururaj Desphande, Sreelekshmy Mohandas, Atanu Basu, Nivedita Gupta, and Krishna Mohan Vadrevu

Supplemental Information

Supplementary figures and legends

Figure S1: Percent Body Weight Gain in Wistar Rats.

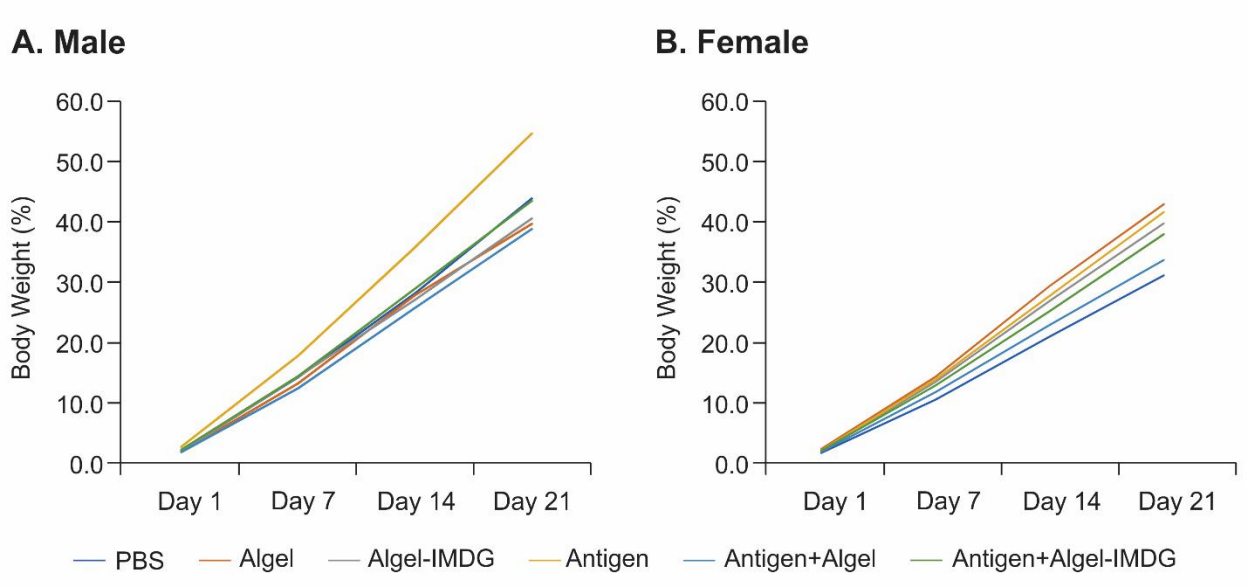

Figure S1:*Percent body weight gain recordedin (A) Male; (B) Female Rats during the experimental period (Day 1, 7, 14, and 21), when administered withN+1 dose high dose of either antigen (9 μg) or Algel or Algel-IMDG (30 μg agonist) or adjuvanted vaccines (Antigen+Algel or Antigen+Algel-IMDG). PBS denotes Phosphate buffered saline. Line graph represents Mean percent body weight gains. Related to Table 2*

Figure S2: Safety evaluation of Differential leucocyte counts in vaccinated Wistar Rats.

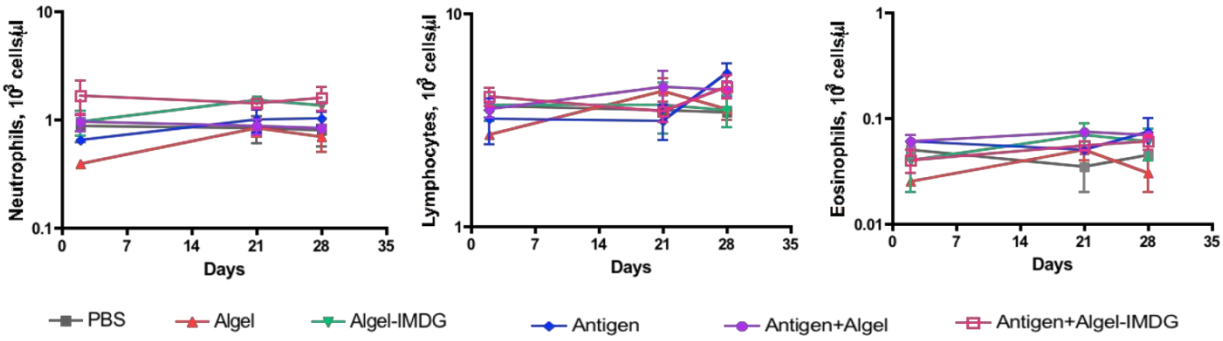

Figure S2: *Representative haematology parameters such as neutrophils, lymphocytes, eosinophils measured on day 2, 21, and 28 were shown from the Wistar rats administered with phosphate buffer saline (PBS) or antigen (9µg) or Algel or Algel-IMDG (30 µg agonist) alone and adjuvanted vaccine formulations with Algel or Algel-IMDG (antigen+ Algel or Algel-IMDG (30µg agonist). Error bars signify Means ± S.D. Related to Table 2*

Figure S3: Algel-IMDG induces more inflammatory reaction at the site of injection (quadriceps muscles).

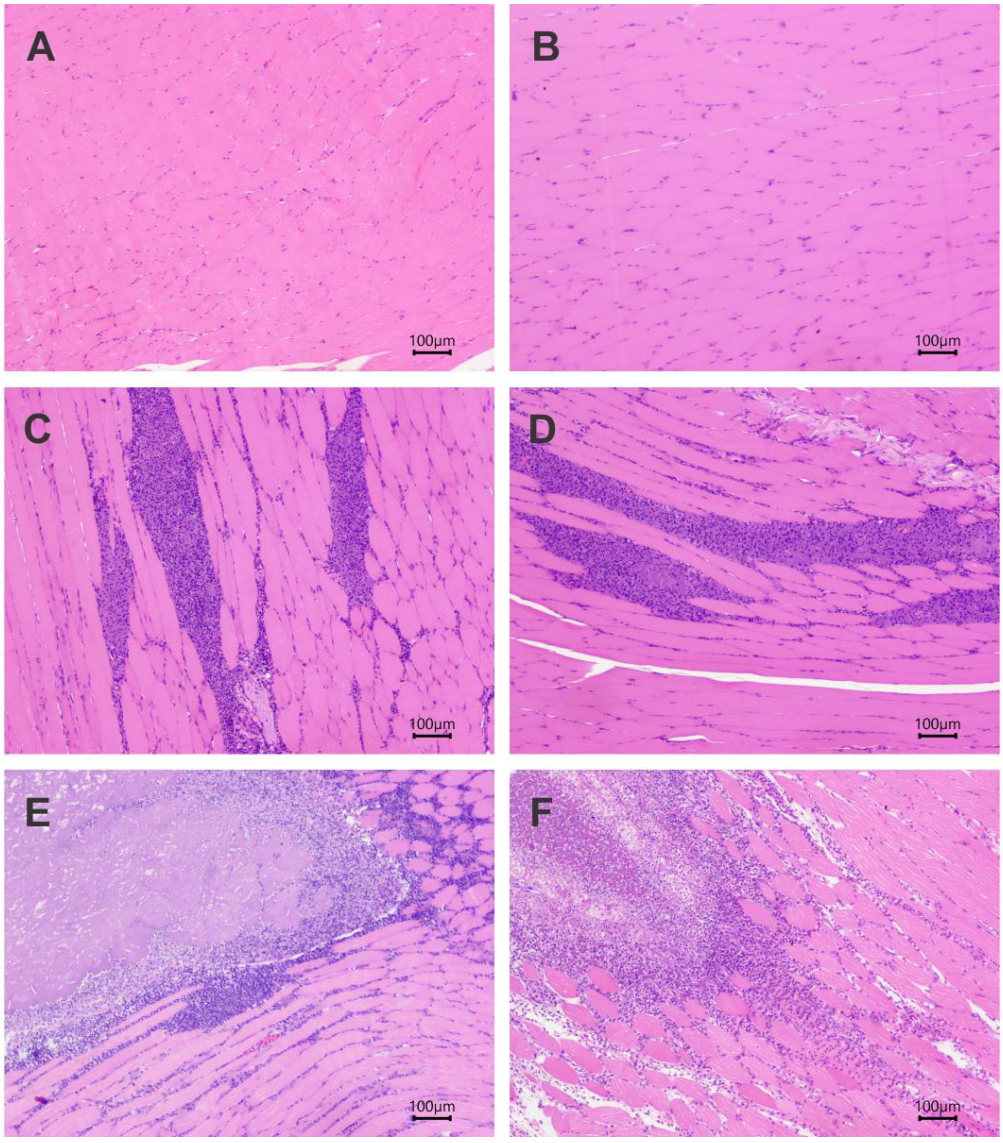

Figure S3: *Representative photomicrographs of the site of injection (quadriceps muscles) from Swiss Albino mice (Left Column) and Wistar rats (Right Column) showing macrophage infiltration containing bluish stained material in Algel (C and D) and Algel-IMDG showing chronic inflammation around test item deposits (E & F, 30 µg agonist). Injection site from PBS group(A and B) used as control for comparison. PBS denotes Phosphate buffered saline. Site of injection tissue stained with hematoxylin and eosin at magnification 4X. Related to Table 2*

Figure S4: Reduction of Macrophage Infiltration at The Site of Injection during the recovery phase

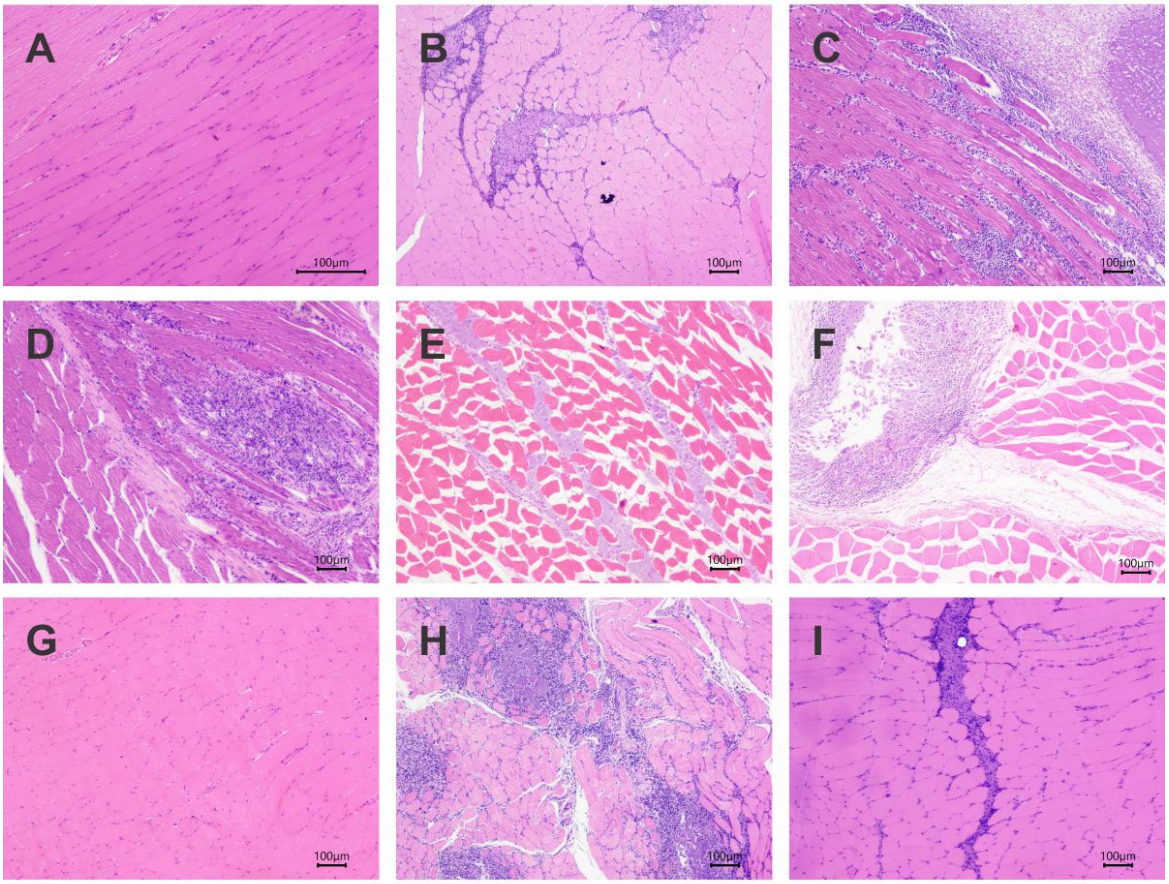

Figure S4: ***Representative photomicrographs of the site of injection (quadriceps muscles) from Wistar rats ().*** *Animals vaccinated with (A) Phosphate buffer Saline, (B) Antigen (9µg) + Algel and (C) Antigen (9µg) + Algel-IMDG (30 µg agonist). B&C shows inflammation around the site of injection, indicates infiltration of inflammatory cells and (D) Recovery group i.e., Antigen (9µg) + Algel-IMDG (30µg agonist) showing the reduction in theinflammatory reaction.* ***New Zealand White Rabbits:*** *Animals administered with Full HSD (E) Antigen (6 µg) + Algel, (F) Antigen (6 µg) + Algel-IMDG (15µg agonist) showed macrophage infiltration (hematoxylin and eosin, original magnification was 4X).****Swiss Albino mice.*** *Animals vaccinated with Full HSD (G) Phosphate buffer Saline, (H) Antigen (6 µg) + Algel-IMDG (15µg agonist) group showing chronic inflammation around test item deposits, (I) Recovery group Antigen (6 µg) + Algel-IMDG (15µg agonist) showing thereduction in the inflammatory reaction. (hematoxylin and eosin, original magnification was 4X). Related to Table 2*

Figure S5: Photomicrographs of tissues of Wistar Rats

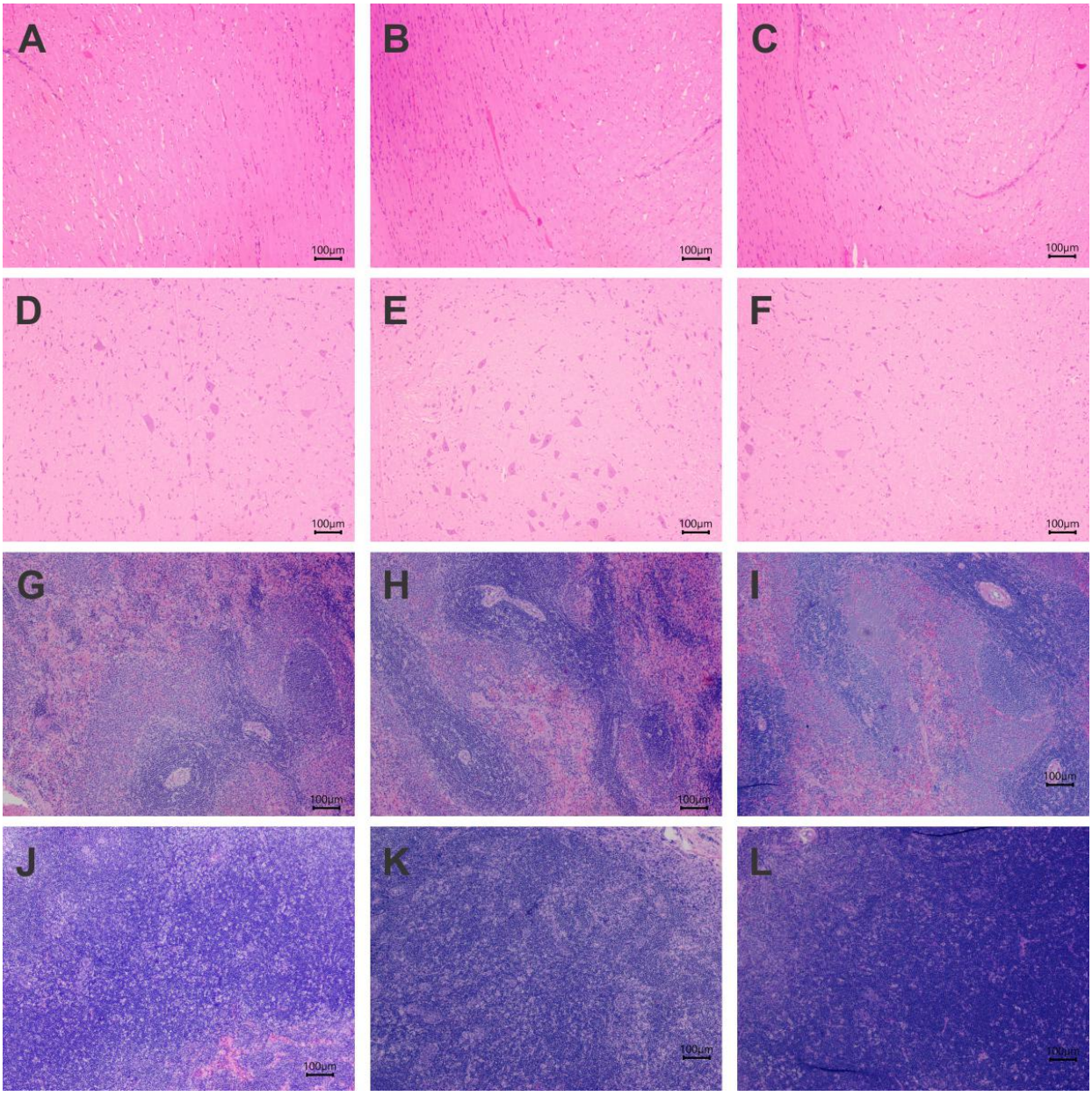

Figure S5:*Representative photomicrographs of Heart (A,B & C), Brain (D, E & F), Spleen (G, H & I) and inguinal lymph nodes (J, K & L) from Wistar Rats, when administered with PBS (control, Left Column) or high dose of adjuvanted vaccine formulations (Antigen 9µg + Algel, Middle Column) or Algel-IMDGat 30µg agonist (Right Column).These organs were within normal histological limits, when stained with hematoxylin and eosin shown under 4X original magnification. Related to Table 2*

Figure S6: Representative photomicrographs of Liver, Kidney, and Lungs from New Zealand White Rabbits.

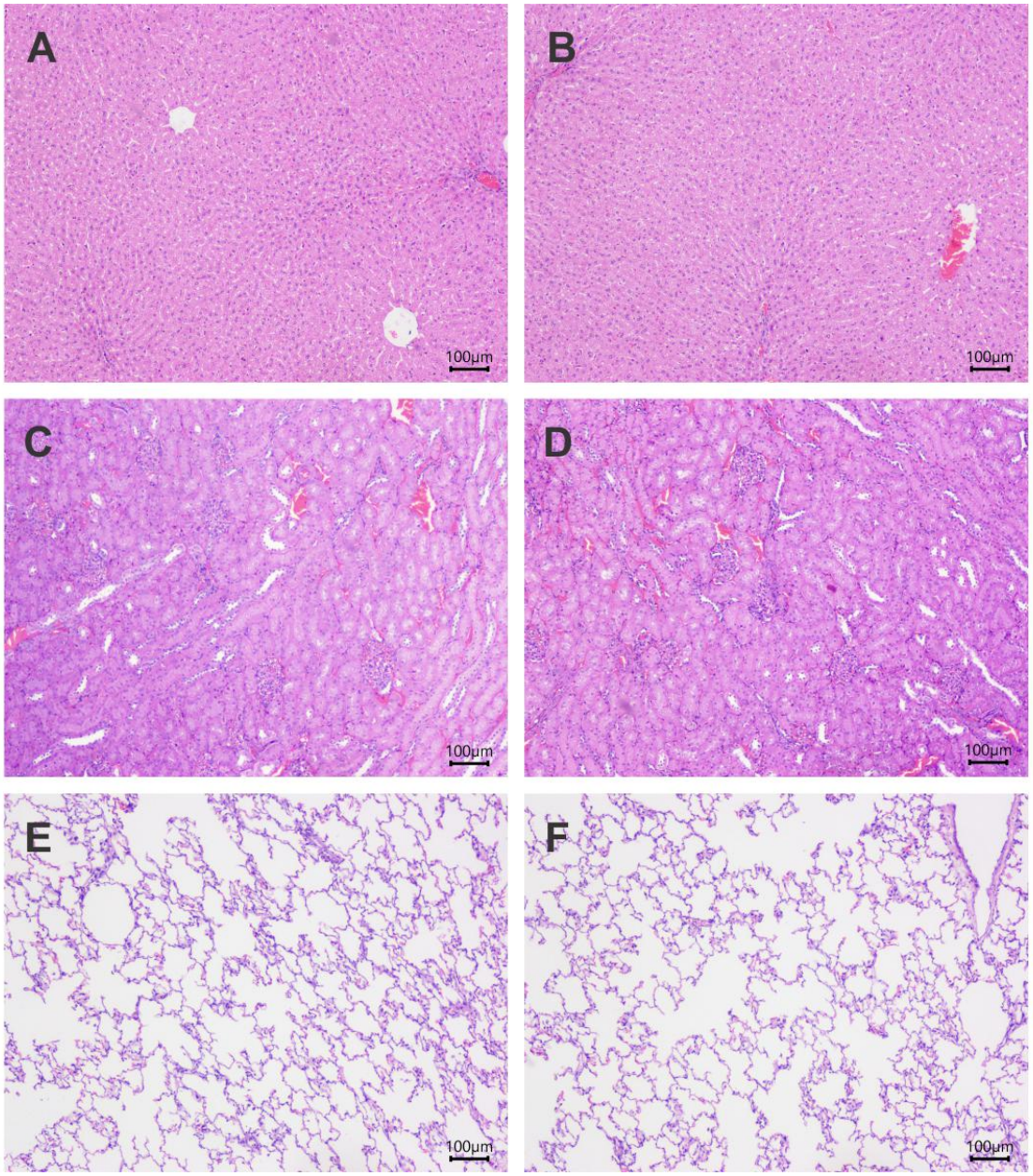

Figure S6: *Representative photomicrographs of Liver (A & B), Kidneys (C & D) and Lungs (E & F) from NZW rabbits, when administered with adjuvanted vaccines,6µg Ag + Algel (Left Column) and 6µg Ag + Algel-IMDG (Right Column) at Full HSD. Histopathological sections of all organs were within normal limits.when stained with hematoxylin and eosin shown under 4X original magnification. Related to Table 2*

Supplementary Tables

Table S1: Mutagenicity Assay

Plate Incorporation Method							Pre-Incubation Method				
Algel-IMDG (agonist Concentration (µg/plate)	**TA1537**	**TA1535**	**TA98**	**TA100**	**TA102**		**TA1537**	**TA1535**	**TA98**	**TA100**	**TA102**
	In the Presence of Metabolic Activation (+S9) (No. of Revertant Colony count/plate)						**In the Presence of Metabolic Activation (+S9) (No. of Revertant Colony count/plate)**				
0.00	5.00±1.41	13.50±0.71	22.00±2.83	122.00±5.66	259.00±15.56		5.50±0.71	13.00±1.41	23.00±2.83	124.00±5.66	257.00±9.90
0.3	7.50±0.71	16.00±1.41	26.50±2.12	123.00±4.24	265.00±9.90		8.00±0.00	16.00±2.83	26.50±2.12	130.00±8.49	266.00±5.66
0.95	6.00±1.41	14.00±2.83	24.00±2.83	122.00±2.83	261.00±9.90		6.00±1.41	14.50±2.12	23.50±3.54	126.50±4.95	259.00±4.24
3.0	6.50±0.71	14.50±0.71	26.00±1.41	123.50±3.54	263.00±9.90		6.50±0.71	15.00±2.83	24.50±2.12	128.00±8.49	264.00±8.49
9.5	5.50±0.71	15.00±2.83	23.50±2.12	121.50±4.95	260.00±8.49		7.50±0.71	14.00±2.83	25.50±2.12	125.50±6.36	261.00±9.90
30	7.00±1.41	15.50±2.12	25.50±2.12	122.50±4.95	262.00±8.49		7.00±1.41	15.50±2.12	25.00±2.83	127.50±6.36	263.00±9.90
PC	178.00±19.80	408.00±22.63	1032.0±56.57	1428.00±118.79	1542.00±161.22		134.00±8.49	354.00±25.46	1116.00±50.91	1584.00±101.82	1722.00±93.34
	Absence of Metabolic Activation (-S9)						**Absence of Metabolic Activation (-S9)**				
0.00	4.50±0.71	12.00±2.83	21.00±4.24	119.00±1.41	255.00±18.38		4.50±0.71	12.50±2.12	21.50±2.12	122.00±5.66	253.00±15.56
0.3	7.00±1.41	15.00±1.41	25.00±2.83	120.50±3.54	261.00±12.73		7.00±1.41	15.50±2.12	25.00±2.83	127.50±6.36	261.00±9.90
0.95	5.50±0.71	13.00±2.83	23.50±2.12	119.50±4.95	257.00±12.73		5.50±0.71	13.00±2.83	23.00±2.83	123.50±4.95	255.00±9.90
3.0	6.50±0.71	14.50±2.12	24.50±2.12	121.00±7.07	259.00±12.73		6.00±0.00	14.50±2.12	24.50±0.71	126.00±5.66	259.50±6.36
9.5	5.00±0.00	13.50±2.12	23.00±2.83	118.00±4.24	256.00±11.31		5.00±1.41	13.50±2.12	23.50±2.12	124.00±5.66	257.00±7.07
30	6.00±1.41	14.00±2.83	24.00±1.41	120.00±5.66	260.00±8.49		6.50±0.71	15.00±2.83	24.00±2.83	125.00±5.66	258.00±11.31
PC	184.00±16.97	1066.00±189.50	820.00±39.60	1398.00±76.37	1746.00±195.16		184.00±16.97	996.00±175.36	716.00±73.54	1314.00±110.31	1818.00±59.40

PC: Positive Control; Values represented are in Mean±SD; TA1537, TA1535, TA98, TA100 and TA102 are five *Salmonella typhimurium* strains used to perform mutagenicity assay.
Related to Table 2

Table S2: Skin Reactions observed in Maximum Tolerated Dose Study in Wistar Rats

Animal Nos.	Sex	Erythema (Hours)			Edema (Hours)		
		24	48	72	24	48	72
01	Male	0	0	0	0	0	0
02	Male	0	0	0	0	0	0
03	Male	0	0	0	0	0	0
04	Male	0	0	0	0	0	0
05	Male	0	0	0	0	0	0
06	Female	0	0	0	0	0	0
07	Female	0	0	0	0	0	0
08	Female	0	0	0	0	0	0
09	Female	0	0	0	0	0	0
10	Female	0	0	0	0	0	0

Related to Table 2

Table S3: Summary of Body Temperature recorded in Repeated Dose Toxicity study in NZW Rabbits, after the administration of Adjuvanted vaccine

Group	Sex	Body Temperature (°C)									
		Day0 (Dose-1)			Day 7 (Dose-2)			Day14 (Dose-3)			Day21
		P.T	3 Hour	24 Hour	P.T	3 Hour	24 Hour	P.T	3 Hour	24 Hour	
6µgAg+ Algel	M	38.4 ± 0.07	38.4 ± 0.14	38.4 ± 0.07	38.3 ± 0.00	38.4 ± 0.07	38.5 ± 0.07	38.5 ± 0.00	38.7 ± 0.07	38.6 ± 0.07	38.6 ± 0.07
	F	38.4 ± 0.07	38.5 ± 0.07	38.4 ± 0.14	38.4 ± 0.21	38.6 ± 0.07	38.5 ± 0.07	38.6 ± 0.07	38.7 ± 0.07	38.6 ± 0.07	38.7 ± 0.07
6µgAg+ Algel-IMDG	M	38.3 ± 0.14	38.4 ± 0.14	38.4 ± 0.14	38.4 ± 0.21	38.4 ± 0.00	38.5 ± 0.00	38.4 ± 0.07	38.6 ± 0.07	38.5 ± 0.07	38.6 ± 0.07
	F	38.4 ± 0.14	38.5 ± 0.07	38.5 ± 0.07	38.3 ± 0.14	38.5 ± 0.00	38.5 ± 0.14	38.3 ± 0.14	38.6 ± 0.07	38.6 ± 0.07	38.6 ± 0.07

Key: °C = Degree Centigrade; A. No. = Animal Number; P.T = Pre-treatment; Values represented in Mean ± SD. *Related to Table 2*

Table S4: Summary of Clinical Biochemistry parameters observed in NZW Rabbits

GROUP		GLU	UREA	CREA	CHOL	TRIGL	AST	ALT	ALP	BIL	Na	K	Cl	TPO	ALB	GLB	A/G
		mmol/L	mmol/L	µmol/L	mmol/L	mmol/L	U/L	U/L	U/L	µmol/L	mmol/L	mmol/L	mmol/L	g/L	g/L	g/L	–
Day 0																	
6µgAg+ Algel (*n=4*)	**M**	8.43 ±1.22	9.40 ±0.04	89.4 ±15.20	1.15 ±0.01	1.39 ±0.27	41.5 ±16.69	50.25 ±11.53	166.3 ±24.25	0.4 ±0.28	146.3 ±2.97	4.72 ±0.73	101.4 ±4.53	63.4 ±1.48	56.96 ±4.89	6.4 ±3.39	10.6 ±6.36
	F	7.99 ±0.24	11.39 ±1.26	92.9 ±12.37	1.53 ±0.64	0.96 ±0.06	45.4 ±28.28	79.15 ±32.74	114.6 ±10.61	0.4 ±0.28	147.9 ±2.76	5.00 ±0.38	102.1 ±1.20	63.2 ±5.16	56.31 ±0.55	6.8 ±5.66	12.9 ±10.82
6µgAg+ Algel-IMDG (*n=4*)	**M**	9.05 ±0.99	13.74 ±2.33	109.0 ±8.34	1.20 ±0.95	0.92 ±0.11	36.9 ±16.40	86.20 ±23.19	110.2 ±73.82	0.2 ±0.00	148.3 ±0.21	6.13 ±0.45	102.8 ±3.11	74.7 ±11.03	64.59 ±8.05	10.1 ±2.97	6.6 ±1.13
	F	9.80 ±0.54	7.64 ±0.82	84.7 ±15.20	1.53 ±0.50	0.63 ±0.31	32.8 ±15.41	52.15 ±10.82	178.1 ±53.81	0.2 ±0.00	147.3 ±2.05	5.26 ±0.76	103.3 ±2.19	66.2 ±8.70	58.89 ±13.82	7.3 ±5.09	11.6 ±9.97
Day 2																	
6µgAg+ Algel (*n=4*)	**M**	7.08 ±0.311	9.77 ±0.47	79.2 ±17.54	1.21 ±0.21	0.97 ±0.66	19.8 ±1.27	52.30 ±15.98	123.7 ±31.40	0.6 ±0.57	137.1 ±3.39	4.18 ±0.34	98.5 ±2.12	59.5 ±2.12	53.64 ±6.06	5.9 ±4.03	12.4 ±9.48
	F	8.33 ±0.523	12.60 ±0.50	101.5 ±12.94	2.03 ±0.58	1.21 ±0.75	25.2 ±0.78	68.65 ±15.34	92.7 ±1.77	0.2 ±0.00	141.3 ±0.07	3.99 ±0.23	101.0 ±1.34	59.7 ±5.09	53.68 ±0.16	6.0 ±4.95	13.6 ±11.17
6µgAg+ Algel-IMDG (*n=4*)	**M**	8.84 ±0.03	10.48 ±3.81	111. ±27.58	0.97 ±0.81	0.85 ±0.02	29.4 ±9.40	67.25 ±17.89	97.5 ±71.98	0.4 ±0.28	141.8 ±0.92	4.37 ±0.14	100.7 ±3.89	65.3 ±8.06	55.40 ±5.95	9.9 ±2.12	5.7 ±0.64
	F	8.28 ±0.04	6.20 ±0.62	70.9 ±25.60	1.90 ±0.05	0.74 ±0.09	27.9 ±11.17	45.6 ±7.00	145.8 ±12.73	0.4 ±0.28	142.4 ±0.71	5.01 ±0.22	105.5 ±2.40	60.9 ±4.67	52.74 ±7.26	8.2 ±2.55	7.0 ±3.04
Day 23																	
6µgAg+ Algel (*n=4*)	**M**	5.34 ±0.53	7.77 ±0.59	80.0 ±4.03	0.83 ±0.13	0.68 ±0.07	19.2 ±2.47	38.40 ±13.72	110.4 ±36.35	0.0 ±0.28	140.6 ±0.00	3.75 ±0.91	97.5 ±3.04	56.2 ±1.91	51.54 ±1.61	4.6 ±0.28	11.2 ±0.28
	F	6.42 ±0.40	8.33 ±0.60	78.8 ±16.48	1.35 ±0.38	0.69 ±0.03	23.6 ±3.89	52.75 ±12.80	120.5 ±34.01	0.0 ±0.28	139.8 ±0.92	4.53 ±1.33	99.3 ±0.85	53.7 ±7.35	49.74 ±4.93	4.0 ±2.40	15.0 ±7.85
6µgAg+ Algel-IMDG (*n=4*)	**M**	5.31 ±0.74	9.22 ±0.06	96.5 ±10.68	0.74 ±0.33	0.86 ±0.25	49.0 ±6.43	81.35 ±7.57	100.4 ±85.21	-0.6 ±1.98	141.2 ±5.16	5.82 ±1.62	98.8 ±1.13	62.4 ±2.26	53.33 ±3.78	9.1 ±6.01	7.7 ±5.52
	F	6.37 ±0.93	7.25 ±0.26	77.2 ±2.69	1.33 ±0.47	0.54 ±0.05	30.5 ±10.54	34.3 ±8.91	117.8 ±39.74	-2.8 ±3.32	141.2 ±3.04	5.54 ±3.22	103.8 ±0.21	57.8 ±0.14	50.54 ±0.19	7.3 ±0.28	7.0 ±0.35

Abbreviations: GLU- Glucose, CREA- Creatinine, CHOL- Cholesterol, total, TRIGL- Triglycerides ,AST- Aspartate aminotransferase ,ALT-Alanine aminotransferase, ALP- Alkaline phosphatase, BIL- Bilirubin, Na- Sodium, K- Potassium, Cl- Chloride, TPO- Protein, total, ALB -Albumin, GLB- Globulin, A/G - Albumin-globulin ratio. *Related to Table 2*

Transparent Methods

Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti- S1 protein rabbit polyclonal Antibody	Ella Foundation, Hyderabad, India	N/A
Rabbit polyclonal Anti-SARS-CoV-2 spike (S2) protein	Abgenex Private Limited, Bhubaneshwar, Odissa, India	Cat# 11-2002
Anti-Spike (RBD) protein rabbit polyclonal Ab	Ella Foundation, Hyderabad, India	N/A
Rabbit polyclonalAnti-SARS-CoV-2 Nucleocapsid protein	Abgenex Private Limited, Bhubaneshwar, Odissa, India	Cat# 11-2003
Anti-rabbit IgG whole molecule peroxidase	Sigma-Aldrich, USA	Cat# A6154
HRP-labeled goat anti-human IgG (gamma chain)	Invitrogen, USA	Cat# 62-8420
Goat Anti-mouse IgG HRP Conjugate	Sigma-Aldrich, USA	Cat#4416
Goat Anti-Rabbit IgG HRP Conjugate	R&D Systems, Thermo Fisher Scientific	Cat#HAF008; 65-6120
Immunoglobulin subclass Goat anti-mouse (IgG1, IgG2a & IgG3) – HRP Conjugate	Santa Cruz Biotechnology, USA	Sc-2060, Sc-2061, Sc-2972
APC-Cy™7 Rat Anti-Mouse CD3, clone: 17A2,	BD biosciences, CA, USA	Cat # 560590
FITC Rat Anti-Mouse CD4 (Clone: H129.19,	BD biosciences, CA, USA	Cat # 553650
PE-Cy™7 Rat Anti-Mouse CD8a (Clone: 53-6.7,	BD biosciences, CA, USA	Cat # 552877
BV421 Rat Anti-Mouse IFN-γ, Clone: XMG1.2	BD biosciences, CA, USA	cat # 560660
Bacterial and Virus Strains		
SARS CoV-2	National Institute of Virology (WHO Collaborating Center for Emerging Viral Infections), Pune, India	NIV-2020-770, Global Initiative on Sharing All Influenza Data (GISAID): EPI_ISL_420545
Salmonella typhimurium strains TA 1535, TA 1537, TA 100, TA 98, TA 102	Moltox, USA	LO No#: 5241D (PART No: 71-1535L); LO No#: 5246D, PART No: 71-1537L; LO No#: 5251D, PART No: 71-100L; 5317D, PART No: 71-1598L; 5260D, PART No: 71-102L

Biological Samples		
Bovine Serum Albumin	Sigma-Aldrich	Cat#A3059
Human Convalescent Sera	Gandhi Medical Hospital, Hyderabad, Telangana.	N/A
Chemical, Peptides and Recombinant Proteins		
β-propiolactone	Ferak	N/A
Aluminium hydroxide (Algel)	CRODA, Denmark	CAS No. 21645-51-2 Batch No. 0001606459
Imidazoquinoline class molecule (TLR7/8 agonist)	ViroVax LLC, KS, USA	N/A
Algel-IMDG	Bharat Biotech International Limited	N/A
Phorbolmyristate acetate (PMA)	Sigma-Aldrich, USA	Cat # P8139
Ionomycin	Sigma-Aldrich, USA	Cat # I0634
Golgistop (monensin)	BD biosciences, CA, USA	Cat # 554724
SARS CoV2 Spike 1 (S1) protein-His tag	Syngene, Bangalore, India	Batch No. PRB026913
SPIKE RBD PROTEIN, HIS TAG (ACTIVE)	GeneTex, California, USA	Cat# GTX01546-PRO
SPIKE S1 PROTEIN, HIS & AVL TAG (ACTIVE)	GeneTex, California, USA	Cat# GTX01548-PRO
NUCLEOCAPSID PROTEIN	GeneTex, California, USA	Cat# GTX135592-PRO
3,3',5,5'-Tetramethylbenzidine (TMB)	DenovaBiolabs, Bangalore, India	Cat# AR1002
Critical Commercial Assays		
Cytokine Bead Array Kit	BD biosciences, CA, USA	Cat. # 560485
Cytoperm/permeabilization kit	BD biosciences, CA, USA	Cat. # 554722
Protein transport inhibitor	BD biosciences, CA, USA	cat # 554724
QIAamp Viral RNA mini kit	QIAGEN, Germany	Cat No.ID: 52904
VeriKine Human Interferon Alpha ELISA Kit	PBL Assay Science, USA	Cat log# 41100
Mouse IFNγ ELISA Kit	R&D systems, Minnesota, USA	Cat# DY485-05
Rat Alpha-1-AGP	Life Diagnostics, USA	Cat# AGP-2
Rabbit C-Reactive Protein	Life Diagnostics, USA	Cat# CRP-10
Mouse Alpha-1-AGP	Life Diagnostics, USA	Cat# AGP-1
Deposited Data		
SARS CoV-2 Strain	National Institute of Virology (WHO Collaborating Center for Emerging Viral Infections), Pune, India	Strain No# NIV-2020-770, Global Initiative on Sharing All Influenza Data (GISAID): EPI_ISL_420545
Experimental Models: Cell Lines		
Vero Cell Lines	ATCC, Manassas, USA	Cat. # Vero ATCC CCL-81
Experimental Models: Organisms/Strains		

BALB/c mice, Swiss Albino Mice, Wistar Rats, New Zealand White Rabbits	RCC Laboratories India Private Limited, Hyderabad, India	N/A
Oligonucleotides		
*RT-PCR Forward Primer: RdRP*_SARSr-F2-GTGARATGGTCATGTGTGGCGG	Eurofins Analytical Services India Private Limited, Bangalore	N/A
RT-PCR Reverse Primer: *RdRP*_SARSr-R1-CARATGTTAAASACACTATTAGCATA	Eurofins Analytical Services India Private Limited, Bangalore	N/A
P2-FAM CAGGTGGAACCTCATCAGGAGATGC-BHQ1	Eurofins Analytical Services India Private Limited, Bangalore	N/A
Master mix	Thermo Fisher Scientific, USA	catalogue no: EP0441, #R0181
Software and Algorithms		
Prism	GraphPad Prism version 8	N/A
R software	R version 4.0.1	N/A
Flow Cytometer	FACS Verse, BD Biosciences, CA, USA	N/A

Experimental Model and Subject Detail

Ethics statements

The studies were conducted post approval from Institutional Animal Ethics Committee (IAEC) and Institutional Biosafety Committee (IBSC) following all ethical practices as laid down in the guidelines for animal care. All studies were performed following both national and international guidelines (CDSCO, 2019).

Experimental animals

Experimental animals *viz*., Wistar rats, Swiss albino mice (SA), BALB/c mice and New Zealand White (NZW) rabbits (*in vivo* models) were sourced from CPCSEA approved vendor. Young adult animals were used and the average body weight ranged from 150 - 200 g in males and 145 - 195 g in females for Wistar rats, 21 - 25 g in males and 17 - 20 g in females for Swiss albino mice, 14 - 16 g in females for BALB/c mice and ~ 2 kg in both males and females for New Zealand White (NZW) rabbits. The studies were conducted in equal number of males and females except in BALB/c mice study where only females were used (**Table 2**).

Animal Husbandry Practices

Maintenance (housing and care) of all animals (mice, rats & rabbits) involved in the study was adhered to guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, 2003). The animal rooms were air-conditioned with adequate (12 - 15) air changes per hour and provided

with a light cycle of 12 hours light and 12 hours dark. The room temperature was maintained in the range of 22 ± 3°C (rat and mice), 20 ± 3°C (rabbits) and relative humidity between 30 and 70% and it was continuously monitored. Animals were housed in cages with dimensions adhering to CPCSEA guidance. Maintenance Diet and UV Purified water were provided *ad libitum*.

Bacterial Strains:

Salmonella typhimurium strains obtained from Moltox, USA, were used in the Mutagenicity assay, as per the recommendations of OECD guidelines (OECD, 2020)).

Convalescent Serum Samples:

Serum samples were collected from healthy adult volunteers, who were recovered from symptomatic COVID-19 infection i.e., between 21-65 days after virological confirmation. Serum was separated by centrifugation, aliquoted and stored at −80°C until further use. The sera were heat-inactivated at 56°C for 30 min and stored at 4°C prior to analysis.

Cells and Virus

Vero CCL-81 (ATCC# CCL 81) cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum. Vero cells were revived from GMP master cell bank, which were extensively characterized at BioReliance, USA. SARS-CoV-2 (NIV-2020-770) was obtained from the National Institute of Virology, a WHO Collaborating Center for Emerging Viral Infections(Sarkale et al., 2020), Pune, India and the strain sequence was deposited in the GISAID (EPI_ISL_420545).

Method Details:

1. TCID50

The SARS-CoV-2 virus titer was determined by a cytopathic effect (CPE) method assay. Vero cells ATCC-81 (0.2 x 10^6 cells/mL) were seeded in 96 well plates and incubated for 16- 24 hours at 37 °C. Serial 10-fold dilutions of virus-containing samples were added to 96-well culture plate and cultured for 5-7 days in 5% CO_2 incubator at 37℃, and cells were observed for cytopathic effect (CPE) under a microscope. The virus titer was calculated by the Spearman Karber method (Ramakrishnan, 2016).

2. Virus Inactivation

SARS-CoV-2 Virus (NIV-2020-770) was inactivated with β-propiolactone at a ratio ranging from 1:1500 to 1: 3000 at 2-8 ° C for 24-32 hours and purified by chromatographic purification method. To ensure the effectiveness of the virus inactivation procedure inactivated SARS-CoV-2 virus was inoculated onto veroCCL-

81 monolayers and incubated at 37 °C in a 5% CO_2 incubator and monitored daily for CPE, consecutively for three passages. Further, to reverify the absence of CPE due to supernatant, neat and 10fold dilution of supernatant was inoculated onto Vero cell monolayer and cultured in a 37°C incubator for 5-7 days, and cells were observed for CPE under a microscope.

3. Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from the virus sample with a QIAamp Viral RNA mini kit (QIAGEN). SARS-CoV-2 *RdRP-2* gene primer probes sequences are as follows: *RdRP*_SARSr-F2-GTGARATGGTCATGTGTGGCGG,R1-CARATGTTAAASACACTATTAGCATA,P2-FAM CAGGTGGAACCTCATCAGGAGATGC-BHQ1.The SARS-CoV-2 reaction was set up containing a master mix of 10 µL (Thermo) and RNA template 10 µL. qRT-PCR was performed under the following reaction conditions: RT step- 42°C for 30 min for reverse transcription, Initial Denaturation step: 95°C for 3 min and then 45 cycles of Denaturation 95°C for 15 seconds, annealing 58°C for 30 seconds - data acquisition, Extension72°C for 15 seconds. Reactions were set on Biorad-CFX96 as per the manufacturers' instructions.

4. Western blotting

Thirty microliter of samples were mixed with 10 microliters of 4 x sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, boiled for 10 minutes, and the samples were separated on 10% denaturing SDS polyacrylamide gels by applying constant current. Proteins were transferred from the polyacryamide gel onto a polyvinylidene fluoride (PVDF) membrane for 90 min at 250 mA using semi-dry transblot apparatus. The membrane was blocked with 5% skimmed milk powder (SMP) in PBS overnight at 4°C to reduce non-specific binding. The membrane was incubated with primary antibodies [Rabbit Spike (S1, S2 and RBD) and nucleoprotein antibodies) in 3% SMP (in PBS) at room temperature for 1 h, washed three times with PBS containing 0.05% Tween-20 (PBST), followed by incubation with secondary anti-Rabbit IgG conjugated with horseradish peroxidase in 3% SMP at 37°C for 1 h. The PVDF membranes were then washed three times with PBST, and once with PBS. The luminol-based enhanced chemiluminescence reagent (SignalFire™ ECL Reagent, Cell Signaling Technology, USA) was added to the membrane and X-ray films were exposed to capture the signals obtained on the membrane. The exposed X-ray films were developed and fixed.

5. Experimental Design for both Immunogenicity and safety Evaluation:

Three animal models were used to evaluate both immunogenicity and safety of the three inactivated whole virion adjuvanted vaccine formulations (BBV152 A, B & C) with N+1 dose regimen (1 extra dose than human intended two doses), administered *via* intraperitoneal or intramuscular route. Two animal species (BALB/c

mice & NZW Rabbits) were used to combine both immunogenicity and safety as mentioned under the immunization section. Apart from these studies, repeated dose toxicity studies were also performed with adjuvant alone (Algel and Algel-IMDG) at high dose (30 μg TLR7/8 agonist molecule) in two animal species (Wistar rats and Swiss Albino mice), whereas adjuvanted vaccine formulations containing high dose of antigen (9μg) was tested in Wistar rats. Further, adjuvanted vaccine formulations with actual single human dose (3μg & 6μg) were tested in two animal species (Swiss Albino Mice & Rabbits). All animal studies were conducted with an equal number of adult males and females, unless otherwise stated. Appropriate antigen or adjuvant controls or vehicle control were also maintained, wherever specified.

6. Immunization:

BALB/c Mice: BALB/c mice (inbred mice, 6-8week old) were vaccinated via an intraperitoneal or intramuscular route with either full or $1/10^{th}$ or $1/20^{th}$ of human intended single dose of adjuvanted vaccine formulations containing various antigen concentrations (3, 6 & 9 μg). Schematic representation of dosing regimen and dosing schedule is as represented in **Figure 2**.

New Zealand White Rabbits: NZW rabbits (3-4 months old) were vaccinated via an intramuscular route with full Human intended single dose of adjuvanted vaccine formulation/s (BBV152 A, B or C).

Animals were bled from the retro-orbital plexus, and serum was separated and stored at -20°C until further use. Individual sera from all vaccinated species (mice and rabbits) were used to test the antigen-specific antibody binding titer and antibody isotyping profile by Enzyme-Linked Immunosorbent Assay (ELISA) and neutralization antibody titer by Plaque Reduction Neutralization Test ($PRNT_{90}$) or Micro Neutralization Test (MNT_{50}).

7. Enzyme-linked immunosorbent assay (ELISA)

ELISA tests were performed as per standard protocols designed for SARS CoV-2. Microtiter plates were coated with SARS-CoV-2 specific antigens (whole inactivated antigen or spike, S1 /Receptor Binding Domain (RBD)/nucleocapsid (N) at a concentration of 1μg/ml, 100μl/well in PBS pH 7.4). Serially diluted pooled or individual sera from vaccinated animals were added followed by the secondary antibody Goat Anti-mouse IgG HRP (Sigma, USA) conjugated antibody and Goat anti-rabbit IgG HRP conjugate antibody (R&D systems, USA) (dilution 1:2500) for mice and rabbit sera samples respectively. Tetra (3,3',5,5') methyl benzidine was used as a substrate. In ELISAs, Threshold (Mean + 3SD) was established by taking the absorbance of negative control (PBS) group, or pre-immune sera and antigen-specific end point titers were determined.

8. Immunoglobulin (IgG) Subclass:

Th1-dependent IgG2a vs. Th2 -dependent IgG1 antibody subclasses were determined by ELISA from mice vaccinated sera as previously described. Briefly, 96 well microtiter plates were coated with spike (S1) protein, at a concentration of 1µg/ml, in PBS pH 7.4) and blocked. Serially diluted individual sera from hyper immunized mice were added followed by the addition of anti-mouse IgG1 or IgG2a or IgG3 HRP conjugate antibodies (dilution 1:2500) After incubation of the plate for 1hr at RT, wells were washed, and 3,3',5,5'-tetramethylbenzidine (TMB) was added as a substrate to develop color. Threshold (Mean + 3SD) was established by taking the absorbance of negative control (PBS) group, or pre-immune sera and antigen-specific IgG1, IgG2a and IgG3 end point titers were determined.

9. Cytokine (IFNγ & IFNα) Estimation by ELISA:

To determine IFNγ, Enzyme-Linked Immunosorbent Assay (ELISA) was performed according to the instruction manual. Briefly, the capture antibody was coated to plates and blocked. Serial dilutions of Standard or sera samples were prepared and added to wells in triplicates. The plate was further added with detection antibody followed by Avidin-HRP was added and incubated as per described in the manual. Finally, substrate solution was added and 2N $H_2SO_4$was used as stopping solution. The plate was read at 450 nm.

PBMCs cell culture supernatant was used to estimate IFNα using The VeriKine Human Interferon Alpha ELISA Kit (PBL Assay Science, USA). The assay was performed as per the manufacturer's instructions. Briefly, Pre-coated plates were incubated with diluted standard (range 500-12.5 pg/ml) or culture supernatant, for 1hr at room temperature. Later, the diluted antibody and HRP solution were added sequentially. TMB was used as a substrate, followed by the addition of stop solution. The plate was read at 450nm.

10. Intracellular Staining:

Vaccinated splenocytes ($2x10^6$/ml) were cultured in 24 well plates and stimulated with inactivated SARS-COV-2 antigen (1.2 µg/ml) or PMA (25 ng/ml, Sigma) and Ionomycin (1 µg/ml, Sigma) along with Protein transport inhibitor (BD biosciences, CA, USA). Cells were washed and centrifuged at 1000rpm for 5-10min and stained with APC-Cy™7 Rat Anti-Mouse CD3 (BD Biosciences, CA, USA), FITC Rat Anti-Mouse CD4 (BD Biosciences), and PE-Cy™7 Rat Anti-Mouse CD8a (BD Biosciences, CA, USA) for 30 minutes at 4°C. Cells were again washed twice with PBS and fixed using fixation/Permeabilize solution (BD Biosciences, CA, USA) for 20 mins at 4°C. Following fixation/permeabilization, cells were washed with 1x permeabilization buffer and stained with intracellular cytokines (IFN-ɣ (BV421 Rat Anti-Mouse IFN-γ, BD Biosciences) for 30 mins at 4°C. Cells were washed and resuspended in 500µl FACS buffer (BD Biosciences). All samples were acquired using BD FACSVerse (BD Biosciences, CA, USA).

11. Cytokine Estimation by Cytokine Bead Array (CBA) assay:

To assess the secretion of multiple Th1 or Th2 mediated cytokines, if any, and to differentiate between Algel1 and Algel2, we used vaccinated mice sera samples collected at various time points (Day 0, 7, 14, 21 & 28, 7 days post-vaccination) and measured Cytokines using the BD CBA Mouse Th1/Th2/Th17 Cytokine Kit (BD Bioscience, San Jose, CA, USA). Sera samples were processed as per the manufacturer's instructions. Samples were measured on the BD FACS Verso and analyzed by FCAP Array Software (BD Bioscience).

12. Plaque Reduction Neutralization Test ($PRNT_{90}$):

The Plaque reduction neutralization test was performed in a biosafety level 3 facility as described earlier (Deshpande et al., 2020). Briefly, Serial dilutions (4 fold) of vaccinated serum samples were mixed with the virus, which can form 50 plaque-forming units and then incubated for 1 h at 37°C. The virus–serum mixtures were added onto the preformed Vero CCL-81 cell monolayers and incubated for 1 h at 37°C in a 5% CO_2 incubator and overlay medium (2% carboxymethyl cellulose with 2% FBS in 2X MEM)was added to cell monolayer, which was further incubated for 4-5days. The number of plaques were counted, and $PRNT_{90}$ were further analyzed using 50% Probit Analysis (Deshpande et al., 2020). A neutralization antibody titer of < 1:20 was considered negative, while that of > 1:20 was considered as positive.

13. Micro Neutralization Test assay (MNT)

The serum of the animal to be tested was inactivated in a 56 ° C -water bath for 30 min. Serum was successively diluted 1:8 to the required concentration by a 2-fold series, and an equal volume of challenge virus solution containing 100 (Cell Culture Infectious Dose 50) $CCID_{50}$viruses was added. After neutralization in a 37°C incubator for two hours, a 1.0 x 10^5 /mL cell suspension was added to the wells (0.1 mL/well) and cultured in a CO_2 incubator at 37℃ for 3-5 days. The Karber method (Ramakrishnan, 2016) by observing the CPE was used to calculate the neutralization endpoint (convert the serum dilution to logarithm), which means that the highest dilution of serum that can protect 50% of cells from infection by challenge with 100 $CCID_{50}$ virus is the antibody potency of the serum. A neutralization antibody potency < 1:20 is negative, while that of > 1:20 is positive.

14. Mutagenicity Assay (Bacterial Reverse Mutation)

The mutagenic potential of the Adjuvant, Algel-IMDG, was evaluated by Bacterial Reverse Mutation assay through plate incorporation and pre-incubation methods using *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and TA 102 following OECD Guidelines (OECD 2020) either in the presence or absence of S9. Mutagenicitywas assessed either as a reduction in the number of His+ revertants or as an alteration in the auxotrophic background (*i.e*., background lawn).

15. Maximum Tolerated Dose Test or Single Dose Toxicity Study:

Maximum Tolerated Dose (MTD) study was performed in two animal species (Swiss Albino mice and Wistar Rats) species with Algel-IMDG alone with a single maximum dose (20μg TLR7/8 agonist molecule). Animals (Swiss Albino mice and Wistar Rats) were administered via an intramuscular route with Algel-IMDG on day 0 and observed for clinical signs, mortality, and changes in body weight, if any up to 14 days.

16. Repeated dose toxicity:

Repeated dose toxicity studies were performed following both national and international guidelines "Guidelines on the nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines-WHO 2013," n.d.; "OECD Guidelines for Testing of Chemicals, Section 4, No. 471: 'Bacterial Reverse Mutation Test', adopted July 21st, 1997," n.d.; "Schedule Y (Amended version of 2019)of the Drugs and Cosmetics Act 1940 and Rules 1945 of the Government of India," n.d.in compliance with OECD Principles of GLP (). Animals were administered with Adjuvanted vaccine or antigen or adjuvant alone or PBS *via* an intramuscular route with three dose (N+1) regimen with an interval of 7 days (Day 0, 7, and 14).. Recovery groups were maintained, wherever specified, otherwise animals were observed upto 14 days, after the third dose to evaluate recovery effects, if any as a part of safety assessment. All animals were observed for mortality and clinical signs during the experimental period. Animals were bled on Day 2 and also on the day of necropsy (either on day 21, for main groups, or on day 28 for recovery groups) and analyzed for detailed clinical pathology investigations. Organs collected on day 21 (main groups) and/or on day 28 (recovery groups) were evaluated for macroscopic and microscopic findings. See also schematic representation of dosing regimen, dosing schedule and blood collection is as outlined in the **Figure 2**.

Clinical Biochemistry

Blood and urine samples were collected under light isoflurane anesthesia (E-Z anesthesia, Euthanex, USA) and performed clinical evaluations such as hematology (Advia 2120, Siemens), serum chemistry (CobasC111 Analyser, Roche), coagulation parameters (STA Compact®, DiagnosticaStago, France), urinalysis (Urisys® 1800, Roche) and acute phase proteins using the validated ELISA method (Life Diagnostics, USA).

Samples for hematology and clinical biochemistry were collected on day 2 and 21 for main groups and on day 28 for the recovery groups. Urinalysis and Coagulation was performed in rat and rabbit on day 21 and 28. Acute phase protein [Alpha 1-acid glycoprotein (α1-AGP) in rats and mice and C-reactive protein in rabbit] analysis were evaluated in plasma samples collected on days 0 (before dosing), 2 and 21.

Histopathology

Animals were euthanized either on Day 21 (main groups) and/or on day 28 (recovery groups), by carbon dioxide asphyxiation (Smart Box, Euthanex, USA) for rats and mice and using thiopentone for rabbits, and necropsied and observed macroscopically. Organs such as the brain, thymus, spleen, ovaries, uterus, heart, kidneys, testes, liver, adrenals, lungs, epididymides, and prostate with seminal vesicles and coagulating glands were weighed and all organs as per the WHO guidelines were collected for microscopic examinations. Organs for microscopic examination were preserved in 10% neutral buffered formalin (NBF). Tissues were processed (Leica Biosystems, Germany) and stained with hematoxylin and eosin.

17. Statistical Methods

Statistical Analysis was performed in Graph Pad Prism 7.01 by applying two-sided one sample t-test, two sample t-test, MannWhitney and Wilcoxon signed rank test with 5% level of significance for continuous variables

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List of abbreviations and their full form

Abbreviations	Full form
SARS-CoV-2	Severe acute respiratory syndrome coronavirus-2
WHO	World Health Organization
COVID-19	Coronavirus disease-19
SARS	Severe acute respiratory syndrome
MERS	Middle East Respiratory Syndrome
BPL	BPL β-Propiolactone
TCID50	Tissue Culture Infectious Dose 50
NAb	Neutralizing antibody
SD	Standard deviation
CCID50	Cell Culture Infectious Dose 50
MEM	Minimum Essential Media
FBS	Fetal bovine serum
CPCSEA	Committee for the Purpose of Control And Supervision of Experiments on Animals.
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline with Tween 20
TMB	3’,3’5,5’-tetramethylbenzidine
PRNT90	Plaque Reduction Neutralization Test 90
ELISA	Enzyme Linked Immunosorbent Assay
HRP	Horseradish peroxidase
CPE	Cytopathic effect
PCR	Polymerase Chain Reaction
HRP	Horseradish peroxidase
BBIL	Bharat Biotech International Limited
ICMR-NIV	Indian Council of Medical Research-National Institute of Virology