

1 **Evaluation of Safety and Immunogenicity of an Adjuvanted, TH-1 Skewed, Whole Virion**  
2 **Inactivated SARS-CoV-2 Vaccine - BBV152**

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## 35 **ABSTRACT**

36 We report the development and evaluation of safety and immunogenicity of a whole virion  
37 inactivated SARS-CoV-2 vaccine (BBV152), adjuvanted with aluminium hydroxide gel (Algel), or a  
38 novel TLR7/8 agonist adsorbed Algel. We used a well-characterized SARS-CoV-2 strain and an  
39 established vero cell platform to produce large-scale GMP grade highly purified inactivated antigen,  
40 BBV152. Product development and manufacturing were carried out in a BSL-3 facility.  
41 Immunogenicity was determined at two antigen concentrations (3 $\mu$ g and 6 $\mu$ g), with two different  
42 adjuvants, in mice, rats, and rabbits. Our results show that BBV152 vaccine formulations generated  
43 significantly high antigen-binding and neutralizing antibody titers, at both concentrations, in all three  
44 species with excellent safety profiles. The inactivated vaccine formulation containing TLR7/8 agonist  
45 adjuvant-induced Th1 biased antibody responses with elevated IgG2a/IgG1 ratio and increased  
46 levels of SARS-CoV-2 specific IFN- $\gamma$ + CD4 T lymphocyte response. Our results support further  
47 development for Phase I/II clinical trials in humans.

48

49 **Keywords:** SARS-CoV-2; covid vaccine; COVID-19; covaxin; BBV152

50

## 51 **1. Introduction**

52 Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), a novel human coronavirus <sup>1</sup>, has  
53 spread to almost every country in the world. SARS-CoV-2 belongs to  $\beta$ -genus of serbecovirus and is a

54 close relative of SARS-CoV with close to 80% sequence identify. The World Health Organization  
55 (WHO) declared the disease caused by SARS-CoV-2, Coronavirus Disease-19 (COVID19), a pandemic  
56 in March 2020. So far, SARS-CoV-2 has infected more than 25 million people causing close to  
57 850,000 deaths. It is, therefore, imperative to develop effective prophylactic and therapeutic  
58 countermeasures to prevent and treat COVID19.

59

60 The development of a safe and effective vaccine has become a top priority globally to prevent the  
61 spread of SARS-CoV-2 infection during the pandemic. Numerous vaccine candidates are in the  
62 preclinical and clinical trial stages. However, meeting the global need for billions of doses of COVID-  
63 19 vaccines will require collective effort to identify, evaluate, validate, and manufacture effective  
64 vaccines. Inactivated vaccines for viral diseases have been licensed for decades with well-established  
65 safety profiles<sup>2</sup>. The availability of well-characterized vero cell manufacturing platform with proven  
66 safety in other licensed, live, and inactivated vaccines have aided in rapid vaccine development<sup>3,4,5</sup>.  
67<sup>6,7</sup>. Prior experience in developing inactivated had given us the confidence to develop a fully  
68 inactivated with an intact virion, imperative for obtaining an antigen that will yield high  
69 immunogenicity. Therefore, to facilitate the development of an effective COVID19 vaccine, we have  
70 used a well-characterized SARS-CoV-2 strain and an established vero cell (CCL-81) platform to  
71 produce large-scale GMP grade highly purified BBV152 vaccine candidate. It has to be mentioned  
72 here that there are several vaccine candidates at different stages of clinical development, such as  
73 adenovirus-vectored vaccines, recombinant protein-based, and inactivated vaccines. The inactivated  
74 vaccine (PiCoVacc) and the recombinant vaccine (CoV-RBD219N1), which are aluminium adjuvant  
75 formulations, have been shown to generate high levels of neutralizing antibodies (NAb) to the S-  
76 protein, which could play an important role in vaccine efficacy. Hence, the development of  
77 inactivated vaccines for COVID-19 disease prevention appears to be a rational approach, while  
78 recognizing the fact that such inactivated vaccines with alum adjuvant specifically induce T helper 2  
79 cells.

80

81 While the development of safe and effective coronavirus vaccines is a priority, vaccine-induced  
82 disease enhancement observed in preclinical animal models due to Th2-like immunity is a concern.  
83 To circumvent the Th-2 bias and to develop a safe vaccine, we formulated a new adjuvant that  
84 contains an imidaquizoquinoline class TLR7/8 agonist adsorbed to Algel. TLR7/8 agonists induce

85 strong type I interferon responses from dendritic cells and monocyte-macrophages that facilitate the  
86 development of Th1 biased immunity instead of a pathogenic Th2-biased immunity<sup>8</sup>.

87 Here, we report the immunogenicity and safety evaluation of the whole virion inactivated SARS-CoV-  
88 2 vaccine candidate BBV152, which was evaluated at three antigen concentrations (3,6, and 9µg)  
89 and two adjuvants in three animal models, i.e., mice, rats, and rabbits. Our results show that these  
90 vaccine formulations induced significantly elevated titers of antigen binding and neutralizing  
91 antibodies in all animal models tested without any safety concerns. We also show that the vaccine  
92 was formulated with Algel-adsorbed TLR7/8 agonist-induced Th1 biased immunity with significantly  
93 elevated SARS-CoV-2 specific IFN $\gamma$ + CD4 T cell response. Collectively these results demonstrate that  
94 the BBV152 vaccine candidate induces protective and durable NAb and T cell responses. As a result,  
95 BBV152 vaccine candidate has been considered for phase I clinical trials.

96

## 97 **2. Results**

### 98 **2.1 Isolation and selection of SARS-CoV-2 strain for vaccine candidate preparation.**

99 During the initial outbreak of SARS-CoV-2 in India, specimens from 12 infected patients were  
100 collected and sequenced at the Indian Council of Medical Research-National Institute of Virology  
101 (ICMR-NIV), India, a WHO Collaborating Center for Emerging Viral Infections<sup>9</sup>. The SARS-CoV-2 strain  
102 (NIV-2020-770) used in developing the BBV152 vaccine candidate was retrieved from tourists who  
103 arrived in New Delhi, India<sup>10, 11</sup>. The sample propagation and virus isolation were performed in the  
104 Vero CCL-81. The SARS-CoV-2 sequence was deposited in the GISAID (EPI\_ISL\_420545). The BBV152  
105 vaccine candidate strain is located in the (G clade), also represented as '20A' clade that is the most  
106 prevalent strain in India (followed by '19A') as per data represented in the Next strain analysis of the  
107 Indian analysis<sup>12</sup>. In terms of the overall divergence of SARS-CoV-2, this strain is 99.97% identical to  
108 the earliest strain Wuhan Hu-1<sup>13</sup>. The multiple passages done in the Vero CCL-81 demonstrated the  
109 genetic stability of the virus. The next-generation sequencing (NGS) reads generated from the  
110 nucleotide sequences of the BBV152 vaccine candidate strain and its passage one at PID-3 was found  
111 to be comparable with the SARS-CoV-2 Wuhan Hu-1 strain (**Table 1**). A maximum difference of  
112 0.075% in the nucleotides was observed, indicating negligible changes in the different batches of the  
113 samples analyzed—these results showed genetic stability of the NIV-2020-770 strain for further  
114 vaccine development. The seed virus (NIV 2020-770 strain) was transferred from ICMR-NIV to Bharat  
115 Biotech, India. Samples from different drug Substance batches of BBIL, along with the original virus,  
116 clustered into a single group and indicate an origin from previous passages.

117

## 118 **2.2 Vaccine candidate preparation**

119 GMP production of virus bulk was standardized in bioreactors. The seed virus was adapted to a  
120 highly characterized GMP vero cell platform, amplified to produce the master and working virus  
121 bank. The master virus bank was characterized based on WHO Technical Report Series guidelines  
122 (identity, sterility, mycoplasma, virus titration, adventitious agents, hemadsorption, virus identity by  
123 Next Generation Sequencing. The viral RNA isolated from the master virus bank (MVB) was  
124 sequenced using NGS the platform at ICMR-NIV and Eurofins Bangalore, India. The sequence  
125 reconfirmed the identity of MVB as the NIV 2020-770 strain of SARS-CoV-2.

126

127 Vero cells and virus were propagated in the bio-safety level-3 (BSL-3) facility using bioreactors.  
128 Growth kinetics analysis showed that the stock replicated to 7.0 log<sub>10</sub> TCID<sub>50</sub> between 36- and 72-  
129 hours protection. β-propiolactone was utilized for the inactivation of the virus by mixing the virus  
130 stock between 2-8°C. During the inactivation kinetics experiments with varying conditions and  
131 concentrations, samples were collected at various time points (between 0 to 24 hours, at 4-hour  
132 intervals) to evaluate the cytopathic effect of live virus. Three consecutive inactivation procedures  
133 were performed to ensure complete viral inactivation without affecting the antigen stability (**Figure**  
134 **1A**). Transmission electron microscopy (TEM) analysis showed that the inactivated and purified virus  
135 particles were intact, oval-shaped, and were accompanied by a crown-like structure representing the  
136 well-defined spike on the virus membrane (**Figure 1B**) Inactivated and purified virus was also  
137 characterized by western blot for its identity with SARS-CoV-2 specific antibodies using various  
138 stages of vaccine candidate development such as cell harvest, clarified supernatant, post-  
139 inactivation, and purification. Western blot analysis showed distinct bands of all proteins. Purified  
140 and inactivated whole virion antigen produced from three production batches were probed with  
141 anti-Spike (S1 & S2), anti-RBD, and anti-N protein (**Figure 1C**). These results showed that the final  
142 purified inactivated bulk of the vaccine candidate is highly pure and contains S (S1, S2), RBD, and N  
143 protein bands with their corresponding equivalent molecular weight.

144

145

## 146 **2.3 Vaccine formulations with adjuvants.**

147 BBV152 vaccine candidates were formulated with two alum adjuvants: Algel (aluminium hydroxide  
148 gel) and Algel-IMDG, an imidazoquinoline class molecule (TLR7/TLR8 agonist abbreviated as IMDG)  
149 adsorbed on aluminium hydroxide gel. The agonist molecule for Algel-IMDG was licensed from  
150 ViroVax LLC, USA. Three vaccine formulations were prepared with 3μg and 6μg with Algel-IMDG

151 (BBV152A and BBV152B, respectively) and 6 $\mu$ g with Algel (BBV152C). To determine the stability of  
152 the vaccine formulations, inactivated antigen plus adjuvant preparations were stored at 37°C and 2-  
153 8°C temperature for seven days. These vaccine formulations were evaluated in Balb/C mice to  
154 estimate Nab titer by microneutralization test (MNT<sub>50</sub>). Our results demonstrated that the vaccine  
155 formulations are relatively stable at 37°C for 7 days, as shown by equivalent Nab titer compared to  
156 formulation stored at temperature 2-8°C (**Figure 1D**). There is no significant difference between the  
157 two formulations (BBV152A & BBV152B)

158

## 159 **2.4 Safety**

160 All the three BBV152 formulations, the pure antigens at 3 different concentrations, and the two  
161 adjuvants have been evaluated for safety in three animal models (mice, rats, and rabbits) following  
162 the required regulatory guidelines<sup>14, 15, 16, 17</sup>. **Table 2** summarizes the key tests completed and the  
163 observations thereof. Safety has been established in repeat-dose toxicity studies in Balb/C mice  
164 (female, 6-8weeks old) which were vaccinated intraperitoneally (*i.p*) with 1/20<sup>th</sup> of the intended  
165 human single dose (HSD, 3 or 6 or 9  $\mu$ g) of inactivated vaccine candidate with or without adjuvant  
166 on day 0, 7 and 14. In contrast, New Zealand white rabbits, Swiss Albino mice, and Wistar rats were  
167 vaccinated intramuscularly (*i.m*). Algel-IMDG alone was further evaluated for safety by mutagenicity  
168 assay (bacterial reverse mutation). No substantial increase in revertant colony numbers in any of  
169 the tested strains was observed following treatment with Algel-IMDG alone at any dose level, in  
170 both the plate-incorporation and pre-incubation methods in the presence or absence of metabolic  
171 activation (S9 mix). The positive controls (Sodium azide, 4-Nitro-o-phenylenediamine, Methyl  
172 methane sulfonate, and 2-Aminoanthracene) used for various strains showed a distinct increase in  
173 induced revertant colonies in both the methods. (**Figure S1**)

174 In the Maximum Tolerated dose study performed with Algel-IMDG, the test item was tolerated at  
175 the tested dose (200  $\mu$ g/animal) in mice and rats as demonstrated by lack of erythema, edema, or  
176 any other macroscopic lesions at the site of injection. Algel is a well-known adjuvant having been  
177 used in a large number of vaccines globally, we evaluated the safety profile of the novel adjuvant  
178 used in this study. Histopathology examination of the injection site showed active inflammation, as  
179 demonstrated by mononuclear cell infiltration, which is likely a physiological local inflammatory  
180 reaction caused by aluminium salt in the vaccine adjuvant preparation. In any of the studies  
181 conducted, there were no mortality or no changes observed in clinical signs, body weight gain  
182 (**Figure S2**), body temperature, or feed consumption in the treated animals.

183 **2.5 Clinical pathology investigations:**

184 In all the animal models, haematology, clinical biochemistry, coagulation parameters, and urinalysis  
185 treated with adjuvanted vaccine candidates or adjuvants/ antigen alone were comparable to control  
186 (**Figure S3**). The following exceptions were noticed as Alpha 1- acid glycoprotein values were  
187 increased on day 2 with Algel-IMDG in male rats when compared to day 0, which reduced to normal  
188 levels by day 21. Evidence of an acute phase response was indicative of reactogenicity to the vaccine  
189 formulation, and the increase was noticed in the adjuvanted vaccine with the Algel-IMDG group  
190 alone. These findings correlate with the inflammatory reaction at the injection site in this group. The  
191 absolute and relative neutrophil counts were increased in female rats of groups (Antigen 6 µg + Algel  
192 300 µg), and (Antigen 9 µg + Algel-IMDG 300 µg) on day 2 as compared to control. However, these  
193 values were noticed and were comparable to control on Day 21. This transient increase may be due  
194 to inflammation at the injection site after administration of the first dose.

195 **2.5 Necropsy, organ weight, and histopathology:**

196 There were no treatment-related microscopic findings observed in antigen alone by  
197 intramuscular (*i.m*) route. In groups treated with adjuvants alone and adjuvanted vaccine  
198 with Algel & Algel-IMDG, local reaction at the site of injection (quadriceps muscles of the  
199 hindlimb) was observed. In animals treated with Algel alone or adjuvanted vaccine with  
200 Algel, inflammatory changes characterized by mild infiltration of mononuclear cells and the  
201 presence of macrophages containing bluish material (interpreted to be aluminium in Algel)  
202 were observed. On day 21, animals treated with Algel-IMDG alone showed inflammation  
203 around homogeneous bluish material (interpreted to be test item) characterized by the  
204 infiltration of mononuclear cells. Additionally, macrophages containing bluish stained  
205 material understood to be aluminium in the test item (Algel-IMDG) were also observed.  
206 Algel produced a milder reaction when compared to Algel-IMDG. On day 28, reduction of  
207 inflammation was observed in both the adjuvants, and the number of macrophages containing  
208 bluish stained material was also observed less in the recovery groups when compared to day 21  
209 (**Figure S4 & S5**). No microscopic findings were observed in any of the organs examined, including  
210 spleen and lymph nodes, any of the animal models (**Figure S6 & S7**). Organ weights across groups  
211 were comparable.

212 **2.6 Immunogenicity studies:**

213 We assessed the immunogenicity of BBV152 formulations in BALB/c mice and New Zealand white  
214 rabbits]. All immunization studies were conducted based on a three-dose IM regimen conducted on  
215 days 0, 7, and 14. Pooled or individual serum samples collected on days 0, 7, 14, and 21 post-  
216 immunization/boost were evaluated for antibody binding (ELISA) and Nab by plaque reduction  
217 neutralizing titer (PRNT<sub>90</sub>) or MNT<sub>50</sub> against live SARS-CoV-2 strain.

218

#### 219 **Immunogenicity in BALB/c Mice:**

220 To assess the immunogenicity of the candidate vaccines, BALB/c mice (n=10) were injected via *i.p*  
221 route with three concentrations of antigen at 1/20<sup>th</sup> of the intended human single dose (i.e., 3 µg,  
222 6 µg, and 9 µg/mouse). Vaccine formulations adjuvanted tested at three antigen concentrations  
223 elicited high levels of binding and Nab titer (**Figure 2 A & B**). Antigen alone and adjuvants alone were  
224 included in these studies as controls (data not presented for brevity). Further, to assess the  
225 immunogenicity and safety of clinical batch samples, Balb/C mice (n=10/group, 5 Male and 5  
226 Female) were vaccinated via IP route with three adjuvanted formulations with Algel and Algel-IMDG  
227 at 1/10<sup>th</sup> human intended single dose (3, and 6 µg/dose with Algel or Algel-IMDG). All adjuvanted  
228 vaccine formulations elicited antigen-specific binding antibodies (**Figure 2C**). Further, sera collected  
229 on Day 21 were analyzed by ELISA to determine S1, RBD, and N specific binding titer (**Figure 2D**).  
230 Analysis of PRNT<sub>90</sub>, performed with individual mice sera, showed high Nab's in all adjuvanted  
231 vaccines (**Figure 2E**), while **Figure 2F** depicts the effect of dose sparing of Algel-IMDG. **Figure S8**  
232 depicts the 8-fold increase in vaccine potency, when dosing was in day 14 intervals. Additionally,  
233 dosing with antigen alone was found to be immunogenic. However, the responses were significantly  
234 lower than the adjuvanted vaccine (**Figure S9**).

235

#### 236 **Immunogenicity in New Zealand White Rabbits:**

237 Rabbits (n=8) were immunized with antigen concentrations for humans (3 and 6 µg/dose) on days 0,  
238 7, and 14. The groups that received BBV152A & B showed a slightly higher binding antibody  
239 response compared to BBV152C (**Figure 3A**), though not statistically significant. Examination of  
240 neutralizing antibody titers revealed high PRNT<sub>90</sub> titers on day 21 are also reported (**Figure 3B**).  
241 Further, NAB's performed by MNT<sub>50</sub> were compared with Nabs from a panel of human convalescent  
242 sera from recovered symptomatic COVID-19 patients. (**Figure 3C**).



243

244 **BBV152 adjuvanted with TLR7/8 adsorbed algel induces Th1 biased immune response:**

245 **Immunoglobulin Subclasses:** Antibody isotyping (IgG1 & IgG2a) was analyzed on day 21 serum  
246 samples to evaluate the Th1/Th2 polarization by vaccination with the two adjuvants. The average  
247 ratio of IgG2a/IgG1 was higher in all antigen concentrations with Algel-IMDG when compared to  
248 Algel, indicative of Th1 bias (**Figure 4A**). Additionally, antigen immunized with 6 $\mu$ g Algel-IMDG  
249 samples induced significantly higher responses of interferon- $\gamma$ (IFN $\gamma$ ) (**Figure 4B**). These results  
250 suggest that Algel-IMDG adjuvant that contains TLR7/8 agonist induces Th1 biased protective  
251 immunity and thus is a promising adjuvant for further development.

252 To further evaluate whether adjuvanted vaccine formulations (with Algel & Algel-IMDG) induced Th1  
253 response or not, we performed intracellular staining using vaccinated mice splenocytes after  
254 stimulation with inactivated SARS-CoV-2 antigen and determined IFN $\gamma$  producing T lymphocytes.  
255 Interestingly, we found that the adjuvanted formulation with Algel-IMDG (BBV152A & B) showed  
256 elevated levels of IFN $\gamma$  producing CD4 cell population, compared to those with Algel. These results  
257 indicate that antigen formulated with Algel-IMDG skewed towards Th1 mediated response (**Figure**  
258 **4C**) and induced strong T cell immunity.

259

260 **Cytometric Bead Array (CBA)**

261 Expression of TNF- $\alpha$  and interleukins was noticeably expressed in the 6 $\mu$ g Algel-IMDG when  
262 compared to 6 $\mu$ g Algel (**Figure 4D**).

263 **IFN $\alpha$  responses as a function of innate immunity activation** to assess the effect of adjuvants (Algel  
264 or Algel-IMDG) on antigen and understanding the critical role of IFN $\alpha$  in both anti-viral and pro-  
265 inflammatory cytokine functions, and linking innate immunity and adaptive immunity, we used  
266 PBMCs from healthy volunteers to stimulate using the antigen and adjuvanted vaccines for 36-72hrs  
267 at both 3 and 6 $\mu$ g antigen concentration, and measured IFN $\alpha$ . We found that the Inactivated antigen  
268 itself stimulated Anti-viral Cytokine (IFN- $\alpha$ ), an indicator of the first line of defense. Algel-IMDG  
269 containing TLR7/8 agonists also stimulated IFN- $\alpha$  & but not the Algel alone. The addition of Algel and  
270 Algel-IMDG showed a synergistic effect on Antigen, which was demonstrated by the elevated of IFN-  
271  $\alpha$  levels in the cell supernatant (**Figure 4E**); the latter adjuvant being more effective.

272

273 **3. Discussion**

274 Here, we report the development of a whole virion inactivated SARS-CoV-2 vaccine candidate  
275 (BBV152). The strain used for this candidate is pathogenic in humans and has shown extensive  
276 genetic stability and appropriate growth characteristics for the selection of a vaccine candidate.  
277 Preclinical toxicity and safety evaluation of the three formulations showed minimal to no adverse  
278 events. Our results show that the vaccine formulations induced significantly elevated antigen-  
279 binding antibody and Nab responses in the animals immunized, with a distinct Th1 bias observed  
280 with Algel-IMDG adjuvanted vaccines. Although the neutralizing antibody titers are not statistically  
281 different between the antigen concentration (3 $\mu$ g and 6 $\mu$ g) or the nature of adjuvant, all the  
282 formulations tested have exhibited excellent immunogenicity. Our potency results compare quite  
283 favorably with those reported in the literature for similar COVID-19 vaccines. Inactivated SARS-CoV-2  
284 vaccine candidate (BBIBP-CorV) has been shown to induce high levels of Nab titers in mice and rats  
285 to provide protection against SARS-CoV-2<sup>3</sup>. A purified inactivated SARS-CoV-2 virus vaccine  
286 candidate (PiCoVacc) has also been shown to induce SARS-CoV-2-specific NAb in mice and rats.  
287 These antibodies potentially neutralized 10 representative SARS-CoV-2 strains, indicative of a possible  
288 broader neutralizing ability against SARS-CoV-2 strains circulating worldwide<sup>4</sup>.

289

290 The risk of antibody-dependent enhancement (ADE) is a serious concern for COVID-19 vaccine  
291 development<sup>18, 19, 20, 21</sup>. A few animal studies from animal SARS-CoV-1 and MERS-CoV inactivated or  
292 vectored vaccines adjuvanted with alum have shown correlation to Th2 responses resulting in  
293 eosinophilic infiltration in the lungs<sup>18, 19, 20</sup>. Alum is the most frequently used vaccine adjuvant with  
294 an extensive safety record. It is desired to have a COVID-19 vaccine that can generate both humoral  
295 and cell-mediated immune responses. The response generated from alum is primarily Th2- biased  
296 with the induction of strong humoral responses via neutralizing antibodies<sup>22</sup>. It is not clear if alum  
297 alone can stimulate T-cell responses. Complicating adverse events may be associated with the  
298 induction of weakly or non-neutralizing antibodies that lead to antibody-dependent enhancement  
299 (ADE) or enhanced respiratory disease (ERD), thus warranting COVID-19 vaccines to induce CD4  
300 Th1(interferon- $\gamma$ , interleukin-2, tumor necrosis factor $\alpha$ ) response with minimal Th2 response<sup>23, 24</sup>.  
301 Preclinical studies in mice reported that inactivated vaccine-induced eosinophil immunopathology in  
302 the lungs upon SARS-CoV infection<sup>25</sup> could be avoided using TLR agonist as or in adjuvant  
303 formulations. Although current understanding of the risk of COVID-19 vaccine-associated ADE/ERD is

304 limited, the use of TLR7/8 agonists in an adjuvant in SARS-CoV-2 vaccine formulation will minimize  
305 Th2 response, if any.

306

307 Over many decades it has shown that vaccination is generally a safe and well-tolerated procedure.  
308 Nevertheless, toxic actions of vaccines can result from any of the following, drug substance and drug  
309 product, including excipients used for formulation. The current preclinical studies conducted with  
310 BBV152, adjuvanted with the two adjuvants, did not indicate any undesirable pathological changes  
311 and systemic toxicity. Local reactogenicity to adjuvants used in vaccine formulation were the only  
312 findings noted. Algel (Alum) is the most commonly used agent as an adjuvant. It has been shown to  
313 act by depot formation at the site of injection, allowing for a slow release of antigen. Further, it  
314 converts soluble antigens into particulate forms, which are readily phagocytosed<sup>26</sup>. The microscopic  
315 findings at the site of injection in the present studies showed the infiltration of macrophages and  
316 mononuclear cells. The other adjuvant, Algel-IMDG, contained TLR7/8 in addition to Algel, which was  
317 added to augment innate and adaptive immunity, induced slightly higher reactogenicity. IM injection  
318 induces a depot effect followed by the passive trafficking of algel particles via lymphatic flow from  
319 the interstitial space to the draining lymph nodes, as revealed by IFN- $\beta$ /luciferase reporter mice  
320 (unpublished). The lymph node-targeting of Algel-IMDG ensures high adjuvant activity in the target  
321 organ (lymph nodes) by enabling the induction of a strong, specific, adaptive immune response while  
322 minimizing systemic exposure. The local reaction in the studies conducted was consistent with those  
323 available in the literature for these adjuvants, which is a physiological reaction to injection rather  
324 than any adverse event<sup>26, 27</sup>.

325

326 Collectively, both the adjuvanted vaccines (with Algel and Algel-IMDG), Antigen and Adjuvant alone  
327 did not reveal any treatment-related findings, except local reactions when administered through the  
328 human intended route (intramuscular) on days 0, 7, and 14 (n+1) with full Human single dose (HSD)  
329 or higher than HSD in rodents and non-rodents, thereby establishing the vaccine safety. In our  
330 preclinical studies, we demonstrated that all the three inactivated whole virion SARS-CoV-2 vaccine  
331 candidates showed 100% seroconversion with high titers of antigen binding and neutralizing  
332 antibody responses. Further, the adjuvanted formulation, BBV152B, when immunized in Balb/C  
333 mice, showed 10 times higher dose sparing effect compared to antigen alone (**Figure 2F**). Moreover,  
334 these formulations induced immunity that is biased towards Th1 mediated response, as  
335 demonstrated by the ratio between IgG2a and IgG1 (greater than 1) (**Figure 4A**). Additionally,

336 secretion of anti-viral cytokines such as IL-2, IL-4, IL-6, IL-10, IL-17, TNF-alpha, IFN $\gamma$  was observed on  
337 days 7 and 14(7 days after the 1<sup>st</sup>&2<sup>nd</sup> dose) of vaccination with Algel-IMDG adjuvanted formulations  
338 (**Figure 4D**). Further, the tendency to secrete anti-viral cytokines, IFN-alpha (**Figure 4E**), might  
339 contribute to the activation of the first line of defense mechanisms, which lead to enhanced  
340 activation of antigen-presenting cells, such as dendritic cells or macrophages<sup>28, 29, 30</sup>. It is reported  
341 that TLR recognition in innate cell population drives early type I IFN production, thereby promotes  
342 viral clearance and the early production of proinflammatory cytokines<sup>31, 32</sup>. Though the mechanism  
343 of action is yet to be investigated, we hypothesize that this elevated production of IFN $\alpha$  in the Algel-  
344 IMDG based Adjuvanted vaccine may provide better protection in the Hamster and NHP homologous  
345 challenge study with SARS-CoV-2 virus.

346

347

348 A combination of high neutralizing antibody titers elicited against inactivated antigen alone and the  
349 presence intact spike protein on the surface of the virus confirms that the antigen is in the right  
350 confirmation and can itself may act as a Th1 inducer with its surface glycoproteins, intracellular viral  
351 proteins.

352 A major limitation of this paper is the lack of protective efficacy results conferred from BBV 152.  
353 Additional live challenge studies in hamsters and non-human primates are completed at NIV, India,  
354 and results will be published shortly. With no established correlate of protection, we also evaluated  
355 human convalescent sera from recovered symptomatic SARS-CoV-2 patients. Samples were collected  
356 21 days after virological confirmation (**Figure3C**). Furthermore, two other SARS-CoV-2 inactivated  
357 vaccines (BBIBP-CorV and PiCoVacc) from China have entered late-stage human clinical trials with  
358 published data on the preclinical immune response. Results from these candidates have reported  
359 comparable findings, albeit PRNT<sub>50</sub><sup>33, 34</sup>.

360 Bharat Biotech has developed a promising inactivated whole virion vaccine candidate which has now  
361 entered phase 1/2 clinical development (NCT04471519). The study is designed to evaluate the  
362 safety, reactogenicity, tolerability, and immunogenicity of two intramuscular doses of BBV152 in  
363 healthy volunteers.

364

365 **Tables**

366 **Table 1:** Genetic Stability of the BBV152 viral strain under specific passages (**Vero CCL-81 Passage 1**  
367 **PID-3**)

Reference Position	Wuhan nucleotide	Hu-1 Current nucleotide	Count of reads	Frequency of reads	Region
241	C	T	10937	99.7	5' UTR
3037	C	T	6227	99.6	orf1ab
4809	C	T	11561	99.91	orf1ab
14408	C	T	7562	99.91	orf1ab
23403	A	G	13336	99.96	S

368

369 **Table 2:** Safety studies conducted

Study Type	Test System	Test Item <sup>1-3</sup>	Route of Administration	Key Test Item result
Repeated dose toxicity studies	Wistar Rats	Antigen, Adjuvanted vaccines, & Adjuvants	Intramuscular	All the Test Items have been demonstrated to be safe from a Toxicology perspective <sup>4</sup> .
	Swiss albino Mice	Adjuvanted vaccines & Adjuvants	Intramuscular	
	BALB/c Mice	Antigen, Adjuvanted vaccines, & Adjuvants	Intraperitoneal	
	New Zealand White Rabbits	Adjuvanted Vaccines	Intramuscular	
Mutagenicity assay (Bacterial Reverse Mutation)	<i>Salmonella typhimurium</i>	Algel-IMDG	--	
Maximum Tolerated Dose studies	Swiss albino mice & Wistar Rats	Algel-IMDG	Intramuscular	

370

1. Antigen: BBV152 Antigen at 3, 6 & 9µg.

371

2. Adjuvanted vaccines: BBV152A, BBV152B & BBV152C.

372

3. Adjuvants: Algel & Algel-IMDG at 200 & 300µg.

373

4. Details are given in Supplementary Section.

374

375

## 376 **Methods**

### 377 **1. Cells and Virus**

378 Vero CCL-81 (ATCC# CCL 81) cells were maintained in DMEM supplemented with 10% heat-  
379 inactivated fetal bovine serum. Vero cells were revived from GMP master cell bank, which was  
380 extensively characterized at BioReliance, USA. SARS-CoV-2 (Strain No#NIV-2020-770) was obtained  
381 from the National Institute of Virology, a WHO Collaborating Center for Emerging Viral Infections<sup>9</sup>,  
382 Pune, India. SARS-CoV-2 strain (NIV-2020-770) sequence was deposited in the GISAID  
383 (EPI\_ISL\_420545).

384

385 Specimens from 12 infected patients were collected during the initial outbreak of SARS-CoV-2 at the  
386 National Institute of Virology (NIV), India, a WHO Collaborating Center for Emerging Viral Infections<sup>9</sup>.  
387 SARS-CoV-2 strain (NIV-2020-770) was passaged in vero cell lines (Vero CCL-81) and sequenced, and  
388 the sequence was deposited in the GISAID (EPI\_ISL\_420545).

389

### 390 **2. TCID50**

391 The SARS-CoV-2 virus titer was determined by a cytopathic effect (CPE) method assay. Vero cells  
392 ATCC-81 ( $0.2 \times 10^6$  cells/mL) were seeded in 96 well plates and incubated for 16- 24 hours at 37 °C.  
393 Serial 10-fold dilutions of virus-containing samples were added to 96-well culture plate and cultured  
394 for 5-7 days in 5% CO<sub>2</sub> incubator at 37°C, and cells were observed for cytopathic effect (CPE) under a  
395 microscope. The virus titer was calculated by the Spearman Karber method<sup>35</sup>.

### 396 **3. Virus Inactivation**

397 SARS-CoV-2 Virus (NIV-2020-770) was inactivated with β-propiolactone at a ratio ranging from  
398 1:1500 to 1: 3000 at 2-8°C for 24-32 hours and purified by chromatographic purification method. To  
399 ensure the effectiveness of the virus inactivation procedure inactivated SARS-CoV-2 virus was  
400 inoculated onto vero CCL-81 monolayers and incubated at 37 °C in a 5% CO<sub>2</sub> incubator and  
401 monitored daily for CPE, consecutively for three passages. Further, to reverify the absence of CPE  
402 due to supernatant, neat and 10fold dilution of supernatant was inoculated onto Vero cell  
403 monolayer and cultured in a 37°C incubator for 5-7 days, and cells were observed for CPE under a  
404 microscope.

### 405 **4. Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)**

406 Total RNA was extracted from the virus sample with a QIAamp Viral RNA mini kit (QIAGEN). SARS-  
407 CoV-2 *RdRP-2* gene primer probes sequences are as follows: *RdRP\_SARSr-F2-*  
408 *GTGARATGGTCATGTGTGGCGG,R1-CARATGTTAAASACACTATTAGCATA,P2-FAM*  
409 *CAGGTGGAACCTCATCAGGAGATGC-BHQ1*.The SARS-CoV-2 reaction was set up containing a master  
410 mix of 10  $\mu$ L (Thermo) and RNA template 10  $\mu$ L.qRT-PCR was performed under the following  
411 reaction conditions: RT step- 42°C for 30 min for reverse transcription, Initial Denaturation step:  
412 95°C for 3 min and then 45 cycles of Denaturation95°C for 15 seconds, annealing58°C for 30 seconds  
413 - data acquisition, Extension72°C for 15 seconds. Reactions were set on Biorad-CFX96 as per the  
414 manufacturers' instructions.

#### 415 **5. Western blotting**

416 Protein samples (~30 mg) derived from drug substance estimated by Lowry method<sup>36</sup> and standard  
417 procedures for western blot were adopted. The primary antibodies used were anti-N protein rabbit  
418 monoclonal Ab (1:1000 dilution) and anti- S1 or S2 or RBD protein rabbit polyclonal Ab (1:1000  
419 dilution), either sourced from commercial or in-house and human convalescent sera from patients  
420 (1:500 dilution) at 4°C. The secondary antibodies goat anti-rabbit IgG H&L (HRP) (GE NA934,1:4000)  
421 and HRP-labeled goat anti-human IgG (gamma chain) cross-adsorbed secondary antibody  
422 (Invitrogen, 62-8420) (1:1000). Protein bands were visualized in enhanced chemiluminescence  
423 (Azure biomolecular imager, USA).

#### 424 **6. Formulations Preparation**

425 In the first formulation, BBV152A, 3 $\mu$ g of antigen was mixed with Algel-IMDG, while BBV152B had  
426 6 $\mu$ g of antigen with the same adjuvant (Algel-IMDG), and the third formulation, BBV152C, had 6 $\mu$ g of  
427 antigen adsorbed on alum (Algel). Total protein/unbound protein was estimated by the Lowry  
428 method<sup>36</sup>.

#### 429 **7. Animal husbandry practices**

430 All animal experiments were performed after obtaining necessary approvals from the Institutional  
431 Animal Ethics Committee (IAEC). The experimental protocols adhered to guidelines of the  
432 Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and also  
433 as per the Organization for Economic Co-operation and Development (OECD) Principles of Good  
434 Laboratory Practice (1997) ENV/MC/CHEM (98)17.

#### 435 **8. Immunization:**

436 Three animal models were used to evaluate the immunogenicity and safety of the three inactivated  
437 whole virion vaccine formulations (BBV152 A, B & C).

438 **Mice:** Balb/C or Swiss Albino mice (6-8week old) were vaccinated via an intraperitoneal or  
439 intramuscular route with either 1/10<sup>th</sup> or 1/20<sup>th</sup> of full human single dose (BBV152 A, B or C) of  
440 inactivated vaccine with or without adjuvant on day 0, 7 & 14 days(n+1 (one extra dose compared to  
441 the intended human regimen doses). A formulation with 9µg was also tested.

442 **Rats:** Wistar Rats (6-8weeks old) were vaccinated intramuscularly with 9µg of inactivated whole  
443 virion vaccine with Algel-1 or Algel-2 on days 0, 7 & 14 days (n+1 doses).

444 **Rabbits:** Zealand white rabbits (3-4 months old) were vaccinated via an intramuscular route with full  
445 Human intended single dose (BBV152 A, B or C; n+1 doses). The animals treated were observed up  
446 to 14 days, post third dose.

447 Further, mice and rats were also administered via an intradermal route with full Human intended  
448 single dose (HSD, 1.2µg), and rabbits administered full Human intended single dose (HSD, 2.4µg) of  
449 inactivated whole virion vaccine without any adjuvant via an intradermal route on days 0, 7 & 14  
450 days (n+1 doses).

451 All studies were conducted with an equal number of males and females unless otherwise specified.  
452 The control group was injected with saline. Animals were bled from the retro-orbital plexus, 2hours  
453 before each immunization on 0, 7, 14 & 21 days, and serum was separated and stored at -20°C until  
454 further use.

455 Pooled and individual sera from vaccinated mice and rabbits were used to test the antigen-specific  
456 antibody binding titer and antibody isotyping profile by Enzyme-Linked Immunosorbent Assay  
457 (ELISA). Pooled or Individual sera from all vaccinated species (mice, rabbits & rats) were used to test  
458 neutralization antibody titer by Plaque Reduction Neutralization Test (PRNT<sub>90</sub>) or Micro  
459 Neutralization Test (MNT<sub>50</sub>).

#### 460 **9. Enzyme-linked immunosorbent assay (ELISA)**

461 ELISA tests were performed as per standard protocols specifically for this project. Microtiter plates  
462 were coated with SARS-CoV-2 specific antigens (whole inactivated antigen or spike, S1 /Receptor  
463 Binding Domain (RBD)/ nucleocapsid (N) at a concentration of 1µg/ml, 100µl/well in PBS pH 7.4).  
464 After incubation, wells were added with Goat Anti-mouse IgG HRP(Santa Cruz Biotechnology, USA)  
465 conjugated antibody for mouse sera samples, and Goat anti-rabbit IgG HRP conjugate  
466 antibody(Santa Cruz Biotechnology, USA) (dilution 1:2500) for rabbit sera samples and incubated for



467 1hr at RT. Threshold (Mean + 3SD) was established by taking the absorbance of negative control  
468 (PBS) group, or pre-immune sera and antigen-specific endpoint titers were determined. The  
469 antibody dilution, at which absorbance is above the threshold, was taken as antigen-specific  
470 antibody endpoint titers.

#### 471 **10. Immunoglobulin (IgG) Subclass:**

472 Th1-dependent IgG2a vs. Th2 -dependent IgG1 antibody subclasses were determined from mice  
473 vaccinated sera as previously described<sup>37</sup>. Briefly, 96 well microtiter plates were coated with various  
474 SARS-CoV-2 specific antigens (whole Inactivated antigen, S1, Receptor Binding Domain (RBD),  
475 nucleocapsid (N) at a concentration of 1µg/ml, 100µl/well in PBS pH 7.4) and kept at 2-8°C for  
476 overnight. The next day, plates were washed with washing buffer (PBST) and blocked with a blocking  
477 buffer (PBS with 2% BSA) at RT for one hour. serially diluted (dilutions from 1:50 to 819200 in PBS,  
478 0.1% BSA, 0.05% Tween™20, 0.02% sodium azide) pooled or individual sera from hyperimmunized  
479 animals (mice/rabbits) and incubated at 37°C for 2hrs. After incubation, wells were washed and  
480 added with anti-mouse IgG1 or IgG2a HRP conjugate antibodies at a dilution 1:2500. After  
481 incubation of the plate for 1hr at RT, wells were washed, and 3,3',5,5'-tetramethylbenzidine (TMB)  
482 was added as a substrate to develop color. Absorbance was read at 450 nm. Threshold (Mean + 3SD)  
483 was established by taking the absorbance of negative control (PBS) group, or pre-immune sera and  
484 antigen-specific endpoint titers were determined. The antibody dilution, at which absorbance is  
485 above the threshold, was taken as antigen-specific antibody endpoint titers.

#### 486 **11. Cytokine (IFN $\gamma$ & IFN $\alpha$ ) Estimation by ELISA:**

487 To determine IFN $\gamma$ , Enzyme-Linked Immunosorbent Assay (ELISA) was performed according to the  
488 instruction manual. Briefly, the capture antibody was first diluted in coating buffer and added 100 µL  
489 to each well in 96-well microplate. Plates were incubated overnight at 2-8 °C. Coated plates were  
490 then washed with wash buffer (PBST). After washing, these plates were blocked using 1x assay  
491 diluent for 1hr at room temperature followed by washing with PBST. Serial dilutions of Top Standard  
492 were prepared to make the standard curve. Similarly, 4-fold dilutions (1:4, 1:16 & 1:64) of serum  
493 samples were prepared and added to wells in triplicates, and the plate was incubated at room  
494 temperature for 2hrs. After washing the plate, 100 µL/well of detection antibody diluted in 1X Assay  
495 diluent was added and incubated at room temperature for 1hr. Later, 100 µL/well of Avidin-HRP\*  
496 diluted in 1X Assay diluent was added and incubated at room temperature for 30 minutes. Finally,  
497 after washes, 100 µL of substrate solution was added to each well and incubated at RT for 15

498 minutes. The reaction was stopped by the addition of 50  $\mu$ L of 2N H<sub>2</sub>SO<sub>4</sub> to each well, and the plate  
499 was read at 450 nm.

500 PBMCs cell culture supernatant was used to estimate IFN $\alpha$  using The VeriKine Human Interferon  
501 Alpha ELISA Kit (PBL Assay Science, USA, Cat log# 41100). The assay was performed as per the  
502 manufacturer's instructions. Briefly, Pre-coated plates were incubated with diluted standard (range  
503 500-12.5 pg/ml) or culture supernatant, for 1hr at room temperature. Later, the diluted antibody  
504 and HRP solution were added sequentially. TMB was used as a substrate, followed by the addition of  
505 stop solution. The plate was read at 450nm.

506

## 507 **12. Intracellular Staining:**

508 Vaccinated splenocytes (2x10<sup>6</sup>/ml) were cultured in 24 well plates and stimulated with inactivated  
509 SARS-COV-2 antigen (1.2  $\mu$ g/ml) or PMA (25 ng/ml, cat # P8139; Sigma) and Ionomycin (1  $\mu$ g/ml, cat  
510 # I0634, Sigma) along with Protein transport inhibitor (Monensin, 1.3 $\mu$ l/ml cat # 554724, BD  
511 biosciences). Cells were washed and centrifuged at 1000rpm for 5-10min and stained with APC-Cy<sup>™</sup>7  
512 Rat Anti-Mouse CD3 (clone: 17A2, Cat # 560590, BD Biosciences), FITC Rat Anti-Mouse CD4 (Clone:  
513 H129.19, Cat # 553650, BD Biosciences), and PE-Cy<sup>™</sup>7 Rat Anti-Mouse CD8a (Clone: 53-6.7, Cat #  
514 552877, BD Biosciences) for 30 minutes at 4°C. Cells were again washed twice with PBS and fixed  
515 using fixation/Permeabilize solution (Cat # 554722, BD Biosciences) for 20 mins at 4°C. Following  
516 fixation/permeabilization, cells were washed with 1x permeabilization buffer and stained with  
517 intracellular cytokines (IFN- $\gamma$  (BV421 Rat Anti-Mouse IFN- $\gamma$ , Clone: XMG1.2, cat # 560660, BD  
518 Biosciences) for 30 mins at 4°C. Cells were washed and resuspended in 500 $\mu$ l FACS buffer (Cat #  
519 554657, BD Biosciences). All samples were acquired using BD FACSVerser (BD Biosciences).

## 520 **13. Cytokine Estimation:**

521 To assess the secretion of Th1 or Th2 mediated cytokines, if any, and to differentiate between Algel1  
522 and Algel2, we used vaccinated mice sera samples collected at various time points (Day 0, 7, 14, 21  
523 & 28, 7 days post-vaccination) and measured Cytokines using the BD CBA Mouse Th1/Th2/Th17  
524 Cytokine Kit (BD Bioscience, San Jose, CA, USA). Sera samples were processed as per the  
525 manufacturer's instructions. Briefly, the kit was used for the simultaneous detection of mouse IL-2,  
526 IL-4, IL-6, IFN- $\gamma$ , TNF, IL-17A, and IL-10 in a single sample. For each sample, 50  $\mu$ L of the mixed  
527 captured beads, 50  $\mu$ L of the unknown serum sample or standard dilutions, and 50  $\mu$ L of  
528 phycoerythrin (PE) detection reagent were added consecutively to each assay tube and incubated

529 for 2 h at room temperature in the dark. The samples were washed with 1 mL of wash buffer for 5  
530 min and centrifuged. The bead pellet was resuspended in 300  $\mu$ L buffer after discarding the  
531 supernatant. Samples were measured on the BD FACS Verso and analyzed by FCAP Array Software  
532 (BD Bioscience).

#### 533 **14. Plaque Reduction Neutralization Test (PRNT<sub>90</sub>):**

534 The Plaque reduction neutralization test was performed in a biosafety level 3 facility. To perform  
535 PRNT<sub>90</sub>, Vero CCL-81 cell suspension ( $1.0 \times 10^5$  /mL/well) was added in duplicates in 24-well tissue  
536 culture plates and cultured in a CO<sub>2</sub> incubator at 37°C for 16-24 hrs. Vaccinated serum samples were  
537 inactivated by keeping in a 56°C-water bath for 30 min. Serial dilutions (4 fold) of vaccinated serum  
538 samples were mixed with the virus, which can form 50 plaque-forming units and then incubated for  
539 1 h at 37°C. The virus-serum mixtures were added onto the preformed Vero CCL-81 cell monolayers  
540 and incubated 1 h at 37°C in a 5% CO<sub>2</sub> incubator. The number of plaques was counted, and the  
541 Neutralizing antibody titer was determined based on the 90% reduction in the number of plaque  
542 count, which was further analyzed using 50% Probit Analysis<sup>38</sup>. A neutralization antibody titer < 1:20  
543 considered negative, while that of > 1:20 considered as positive.

#### 544 **15. Micro Neutralization assay (MNT)**

545 The serum of the animal to be tested was inactivated in a 56°C -water bath for 30 min. Serum was  
546 successively diluted 1:8 to the required concentration by a 2-fold series, and an equal volume of  
547 challenge virus solution containing 100 CCID<sub>50</sub> viruses was added. After neutralization in a 37°C  
548 incubator for two hours, a  $1.0 \times 10^5$  /mL cell suspension was added to the wells (0.1 mL/well) and  
549 cultured in a CO<sub>2</sub> incubator at 37°C for 3-5 days. The Karber method<sup>35</sup> by observing the CPE was used  
550 to calculate the neutralization endpoint (convert the serum dilution to logarithm), which means that  
551 the highest dilution of serum that can protect 50% of cells from infection by challenge with 100  
552 CCID<sub>50</sub> virus is the antibody potency of the serum. A neutralization antibody potency < 1:20 is  
553 negative, while that R 1:20 is positive.

554

#### 555 **16. Mutagenicity Assay (Bacterial Reverse Mutation)**

556 The mutagenic potential of the Adjuvant, Algel-IMDG, was evaluated by Bacterial Reverse Mutation  
557 assay through plate incorporation and pre-incubation methods using *Salmonella typhimurium* strains  
558 TA 1535, TA 1537, TA 98, TA 100, and TA 102 following OECD Guidelines for Testing of Chemical<sup>14</sup>,

559 with and without S9. Toxicity was apparent either as a reduction in the number of His<sup>+</sup> revertants or  
560 as an alteration in the auxotrophic background (*i.e.*, background lawn).

#### 561 **17. Maximum Tolerated Dose Test or Single Dose Toxicity Study:**

562 Two animals (Swiss Albino mice and Wistar Rats) species were tested with Algel-IMDG with a single  
563 maximum dose (containing 200µg Algel and 20µg TLR7/8 agonist molecule). Animals (Swiss Albino  
564 mice and Wistar Rats) were administered via an intramuscular route with Algel-IMDG on day 0 and  
565 observed for clinical signs, mortality, and changes in body weight if any up to 14 days. The site of  
566 injection was also observed for erythema and edema at 24, 48, and 72 hours after dosing to detect  
567 the local tolerance (local reactogenicity) of Algel-IMDG. All animals were necropsied and examined  
568 macroscopically. Histopathology was performed for the site of injection.

#### 569 **18. Repeated dose toxicity:**

570 Studies were performed following both national and international guidelines in compliance with  
571 OECD principles of GLP<sup>14, 15, 37-39</sup>. Three animal models (Mice, Rats & Rabbits) were administered via  
572 an intramuscular or intraperitoneal with three doses (N+1) of antigen or adjuvanted vaccine at  
573 different concentrations. All animals were observed for mortality during the experimental period.  
574 Blood collected on day 2 and 21 from the main groups and day 28 from the recovery group were  
575 analyzed for detailed clinical pathology investigations. Animals were euthanized either on day 21  
576 (main groups) or on day 28 (recovery groups) and necropsied, and organs were evaluated for  
577 macroscopic and microscopic findings.

578

#### 579 **Test system**

580 The test system *viz.*, Swiss albino mice (SA), BALB/c mice, Wistar rats, and New Zealand White (NZW)  
581 rabbits (*in vivo* models) were sourced from CPCSEA approved vendor and strains of *Salmonella*  
582 *typhimurium* (Moltox, Switzerland) for *in vitro* assay, and these test systems were selected as per the  
583 recommendations of WHO guidelines<sup>16, 39</sup> and Schedule Y (2019)<sup>15</sup>. The studies were conducted in  
584 an equal number of adult males and females except in the BALB/c mice study, where only females  
585 were used. The control group was administered with PBS.

#### 586 **Treatment regimen**

587 The adjuvanted vaccines or adjuvants alone were administered intramuscularly (IM) in quadriceps  
588 muscles of the hindlimb on days 0, 7, and 14 (n+1) with full Human single dose (HSD) to NZW rabbits

589 and SA mice and higher dose than HSD to Wistar rats and full HSD to. In BALB/c Mice, 1/20<sup>th</sup> HSD  
590 was administered intraperitoneally. The animals were observed up to 14 days, post last dose.

#### 591 **Experimental Design - Adjuvant alone**

592 Maximum Tolerated Dose (MTD) studies were conducted using Wistar rats and Swiss Albino mice  
593 with ten animals in each study. The animals were treated with a single dose of Algel-IMDG at the  
594 dose of 200 µg /animal and observed for 14 days. Two repeated dose toxicity studies with Algel and  
595 Algel-IMDG in Wistar rats and Swiss Albino mice were performed. Control and reversal groups were  
596 maintained. The site of injection was observed for erythema and edema at 24, 48, and 72 hours after  
597 dosing to detect the local tolerance (local reactogenicity) of Algel-IMDG. All animals were necropsied  
598 and examined macroscopically. Histopathology was performed for the site of injection.

599

#### 600 **Experimental Design - Adjuvanted Vaccines**

601 Four repeated dose toxicity studies were performed with Adjuvanted vaccines in Wistar Rats, New  
602 Zealand White Rabbits, BALB/c Mice, and Swiss Albino Mice.

603 Algel alone, Antigen alone, Adjuvanted Vaccine with Algel, and adjuvanted vaccine with Algel-IMDG  
604 along with control and recovery groups were assigned. We have tested adjuvants in the highest  
605 concentration of 300ug and antigen at the concentration of 9ug, to evaluate safety.

#### 606 **In-life Observations**

607 All animals were observed twice daily for mortality. Clinical signs were recorded twice a day from  
608 day 0 to 2 and once daily thereafter. The cage side observations included changes in the skin, fur,  
609 eyes, and mucous membranes and clinical signs observed for edema, erythema, alopecia, irritation,  
610 necrosis, locomotor activity, lacrimation, hyperthermia, and hypothermia, etc. The body weight of  
611 each animal was recorded once daily after the first dose for a week and weekly once thereafter.  
612 Mean body weights and mean body weight gain was calculated for the corresponding intervals. The  
613 amount of feed consumed by each cage of animals was recorded once daily after the first dose for a  
614 week and weekly once thereafter. Body temperature was recorded for rats and rabbits on day 0, 3  
615 hours, and 24 hours after each dose, and on the day of sacrifice

#### 616 **Clinical Pathology Investigations**

617 Detailed clinical pathology was performed using automated equipment as per referred guidelines  
618 following validated procedures<sup>145, 37-39</sup>. Blood and urine samples were collected for clinical

619 evaluations (hematology, coagulation parameters, acute phase proteins, serum chemistry, and  
620 urinalysis) from all the groups.

### 621 **Necropsy, Organ Weight and Histopathology**

622 Animals were euthanized by carbon dioxide asphyxiation and necropsied. Organs, as per WHO  
623 guidelines, which included spleen, thymus, and draining lymph nodes (inguinal for IM), were  
624 collected from all terminally sacrificed animals, and macroscopic abnormalities were recorded. Wet  
625 weights for organs such as brain, thymus, spleen, ovaries, uterus, heart, kidneys, testes, liver,  
626 adrenals, lungs, epididymides, and prostate with seminal vesicles and coagulating glands were  
627 recorded.

### 628 **18. Statistical Methods**

629 Statistical Analysis was performed in R 4.0.1. We used two-sided one sample t-test with 5% level of  
630 significance for continuous variables which followed a normal distribution. To test the significance of  
631 the sample, mean and for the variables that do not satisfy the normality assumption, we used the  
632 Mann-Whitney test with 5% level of significance to test the significance of median.

633

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### 642 **Author Contributions**

643 All listed authors meet the criteria for authorship set forth by the International Committee for  
644 Medical Editors and have no conflicts to disclose. BG., J.H., S.R., J.J., led the immunogenicity and  
645 safety preclinical experiments. H.J., V.D., N.M., V.K.S., S.P., K.M.V the manufacturing and quality  
646 control efforts. K.M.V, P.S., and E.R. provided technical assistance with design, analysis, and  
647 manuscript preparation. Y.P., S.G., S.A., M.S., A.B., A.P., B.B., N.G of ICMR-NIV, Pune conducted

648 electron microscopy and neutralizing antibody assays. A.A conducted cell-mediated response related  
649 assay activities at THSTI. J.J., R.R., led the safety assessments in animals.

#### 650 **Competing Interests**

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654 interests to disclose.

655

#### 656 **4. References**

657 1. WHO Coronavirus Disease (COVID-19) Dashboard. Available at <https://covid19.who.int/>.

658

659 2. Sanders B, Koldijk M, Schuitemaker H. Inactivated Viral Vaccines. *Vaccine Analysis: Strategies, Principles, and Control*, 45-80 (2014).

661

662 3. Bhandari N, *et al.* Efficacy of a monovalent human-bovine (116E) rotavirus vaccine in Indian  
663 infants: a randomised, double-blind, placebo-controlled trial. *Lancet* **383**, 2136-2143 (2014).

664

665 4. World Health Organization WER, 24 JANUARY 2020, 95th YEAR / 24 JANVIER 2020, 95e,  
666 ANNÉE No 4, 95, 25–3624 January 2020. Accessed on 17 August, 2020. Available at  
667 [https://extranet.who.int/iris/restricted/bitstream/handle/10665/330607/WER9504-eng-  
668 fre.pdf?ua=1](https://extranet.who.int/iris/restricted/bitstream/handle/10665/330607/WER9504-eng-fre.pdf?ua=1).

669

670 5. Vadrevu KM, Potula V, Khalatkar V, Mahantshetty NS, Shah A, Ella R. Persistence of Immune  
671 Responses With an Inactivated Japanese Encephalitis Single-Dose Vaccine, JENVAC and  
672 Interchangeability With a Live-Attenuated Vaccine. *The Journal of Infectious Diseases*,  
673 (2019).

674

675 6. Singh A, *et al.* A Japanese Encephalitis Vaccine From India Induces Durable and Cross-  
676 protective Immunity Against Temporally and Spatially Wide-ranging Global Field Strains. *The  
677 Journal of Infectious Diseases* **212**, 715-725 (2015).

678

679 7. Sampath G, *et al.* Immunogenicity and safety study of Indirab: A Vero cell based  
680 chromatographically purified human rabies vaccine. *Vaccine* **28**, 4086-4090 (2010).

681

682 8. Shukla NM, Mutz CA, Malladi SS, Warshakoon HJ, Balakrishna R, David SA. Toll-Like Receptor  
683 (TLR)-7 and -8 Modulatory Activities of Dimeric Imidazoquinolines. *Journal of Medicinal*  
684 *Chemistry* **55**, 1106-1116 (2012).

685

686 9. Sarkale P, *et al.* First isolation of SARS-CoV-2 from clinical samples in India. *Indian Journal of*  
687 *Medical Research* **151**, 244-250 (2020).

688

689 10. Potdar V, *et al.* Genomic analysis of SARS-CoV-2 strains among Indians returning from Italy,  
690 Iran &#38; China, &#38; Italian tourists in India. *Indian Journal of Medical Research* **151**, 255-  
691 260 (2020).

692

693 11. Yadav PD, *et al.* Full-genome sequences of the first two SARS-CoV-2 viruses from India.  
694 *Indian J Med Res* **151**, 200-209 (2020).

695

696 12. Nextstrain / ncov / global. Available from: <https://nextstrain.org/ncov/global> aoJ, 2020.

697

698 13. Sardar R, Satish D, Birla S, Gupta D. Comparative analyses of SAR-CoV2 genomes from  
699 different geographical locations and other coronavirus family genomes reveals unique  
700 features potentially consequential to host-virus interaction and pathogenesis. *bioRxiv*,  
701 2020.2003.2021.001586 (2020).

702

703 14. OECD Guidelines for Testing of Chemicals, Section 4, No. 471: "Bacterial Reverse Mutation  
704 Test", adopted July 21st, 1997. Accessed on July 30, 2020. Avaiable at  
705 <https://www.oecd.org/chemicalsafety/risk-assessment/1948418.pdf>.

706

707 15. Schedule Y (Amended version of 2019) of the Drugs and Cosmetics Act 1940 and Rules 1945  
708 of the Government of India. Accessed on July 30, 2020. Available at  
709 [https://cdsco.gov.in/opencms/export/sites/CDSCO\\_WEB/Pdf-](https://cdsco.gov.in/opencms/export/sites/CDSCO_WEB/Pdf-documents/acts_rules/2016DrugsandCosmeticsAct1940Rules1945.pdf)  
710 [documents/acts\\_rules/2016DrugsandCosmeticsAct1940Rules1945.pdf](https://cdsco.gov.in/opencms/export/sites/CDSCO_WEB/Pdf-documents/acts_rules/2016DrugsandCosmeticsAct1940Rules1945.pdf).



711

712 16. Guidelines on the nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines.  
713 World Health Organization 2013, Adopted by the 64th meeting of the WHO Expert  
714 Committee on Biological Standardization, 21–25 October 2013. Accessed on July 30, 2020.  
715 Available at  
716 [https://www.who.int/biologicals/areas/vaccines/ADJUVANTS\\_Post\\_ECBS\\_edited\\_clean\\_Gui](https://www.who.int/biologicals/areas/vaccines/ADJUVANTS_Post_ECBS_edited_clean_Guidelines_NCE_Adjuvant_Final_17122013_WEB.pdf?ua=1)  
717 [delines\\_NCE\\_Adjuvant\\_Final\\_17122013\\_WEB.pdf?ua=1](https://www.who.int/biologicals/areas/vaccines/ADJUVANTS_Post_ECBS_edited_clean_Guidelines_NCE_Adjuvant_Final_17122013_WEB.pdf?ua=1).

718

719 17. WHO guidelines on nonclinical evaluation of vaccines, WHO Technical Report Series, No.  
720 927, 2005 ([https://www.who.int](https://www.who.int/biologicals/publications/trs/areas/vaccines/nonclinical_evaluation/ANNEX%201Nonclinical.P31-63.pdf)  
721 [/biologicals/publications/trs/areas/vaccines/nonclinical\\_evaluation/ANNEX%201Nonclinical.](https://www.who.int/biologicals/publications/trs/areas/vaccines/nonclinical_evaluation/ANNEX%201Nonclinical.P31-63.pdf)  
722 [P31-63.pdf](https://www.who.int/biologicals/publications/trs/areas/vaccines/nonclinical_evaluation/ANNEX%201Nonclinical.P31-63.pdf)), refer to section 4.2.2 (Developmental toxicity studies) pg 49-50. Accessed on  
723 July 30, 2020. Available at  
724 [https://www.who.int/biologicals/publications/trs/areas/vaccines/nonclinical\\_evaluation/AN](https://www.who.int/biologicals/publications/trs/areas/vaccines/nonclinical_evaluation/ANNEX%201Nonclinical.P31-63.pdf?ua=)  
725 [NEX%201Nonclinical.P31-63.pdf?ua=](https://www.who.int/biologicals/publications/trs/areas/vaccines/nonclinical_evaluation/ANNEX%201Nonclinical.P31-63.pdf?ua=)

726

727 18. Diamond MS, Pierson TC. The Challenges of Vaccine Development against a New Virus  
728 during a Pandemic. *Cell Host & Microbe* **27**, 699-703 (2020).

729

730 19. Graepel KW, Kochhar S, Clayton EW, Edwards KE. Balancing Expediency and Scientific Rigor  
731 in Severe Acute Respiratory Syndrome Coronavirus 2 Vaccine Development. *The Journal of*  
732 *Infectious Diseases* **222**, 180-182 (2020).

733

734 20. Hotez PJ, Corry DB, Bottazzi ME. COVID-19 vaccine design: the Janus face of immune  
735 enhancement. *Nature Reviews Immunology* **20**, 347-348 (2020).

736

737 21. Graham BS. Rapid COVID-19 vaccine development. *Science* **368**, 945-946 (2020).

738

739 22. He P, Zou Y, Hu Z. Advances in aluminum hydroxide-based adjuvant research and its  
740 mechanism. *Human vaccines & immunotherapeutics* **11**, 477-488 (2015).

741

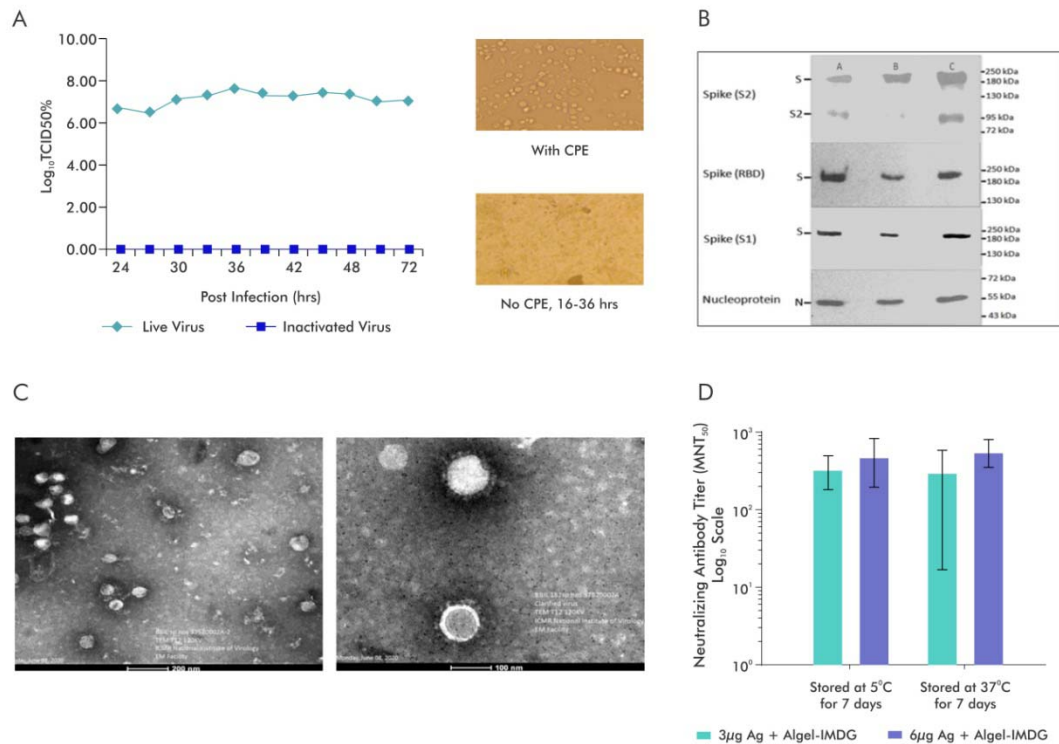
742 23. Grifoni A, *et al.* Targets of T Cell Responses to SARS-CoV-2 Coronavirus in Humans with  
743 COVID-19 Disease and Unexposed Individuals. *Cell* **181**, 1489-1501.e1415 (2020).

744

- 745 24. Weiskopf D, *et al.* Phenotype and kinetics of SARS-CoV-2-specific T cells in COVID-19 patients  
746 with acute respiratory distress syndrome. *Sci Immunol* **5**, eabd2071 (2020).
- 747
- 748 25. Iwata-Yoshikawa N, *et al.* Effects of Toll-Like Receptor Stimulation on Eosinophilic Infiltration  
749 in Lungs of BALB/c Mice Immunized with UV-Inactivated Severe Acute Respiratory  
750 Syndrome-Related Coronavirus Vaccine. *Journal of Virology* **88**, 8597-8614 (2014).
- 751
- 752 26. Gupta T, Gupta SK. Potential adjuvants for the development of a SARS-CoV-2 vaccine based  
753 on experimental results from similar coronaviruses. *International immunopharmacology* **86**,  
754 106717 (2020).
- 755
- 756 27. Sellers RS, *et al.* Scientific and Regulatory Policy Committee Points to Consider\*: Approaches  
757 to the Conduct and Interpretation of Vaccine Safety Studies for Clinical and Anatomic  
758 Pathologists. *Toxicologic Pathology* **48**, 257-276 (2020).
- 759
- 760 28. Masopust D, Soerens AG. Tissue-Resident T Cells and Other Resident Leukocytes. *Annu Rev*  
761 *Immunol* **37**, 521-546 (2019).
- 762
- 763 29. Kayraklioglu N, Horuluoglu B, Elango M, Klinman DM. Critical Role of B Cells in TLR7-  
764 Mediated Protection Against *L. Monocytogenes* Infection. *Infection and*  
765 *immunity*, IAI.00742-00719 (2019).
- 766
- 767 30. Clingan JM, Matloubian M. B Cell–Intrinsic TLR7 Signaling Is Required for Optimal B Cell  
768 Responses during Chronic Viral Infection. *The Journal of Immunology* **191**, 810-818 (2013).
- 769
- 770 31. Hijano DR, Vu LD, Kauvar LM, Tripp RA, Polack FP, Cormier SA. Role of Type I Interferon (IFN)  
771 in the Respiratory Syncytial Virus (RSV) Immune Response and Disease Severity. *Frontiers in*  
772 *Immunology* **10**, (2019).
- 773
- 774 32. Stephens LM VSFaMoTIIIdRSVIV.
- 775

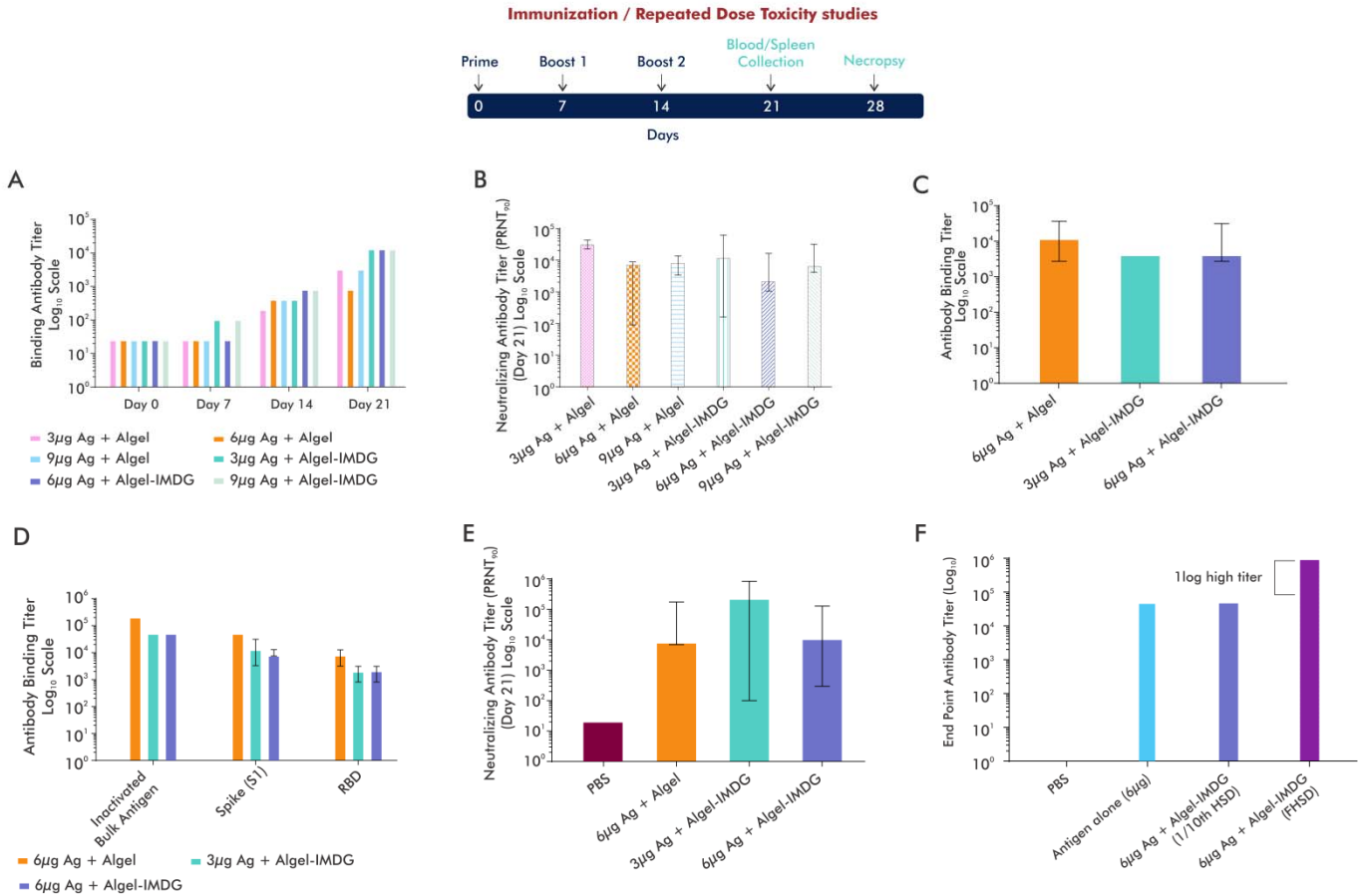
- 776 33. Wang H, *et al.* Development of an Inactivated Vaccine Candidate, BBIBP-CorV, with Potent  
777 Protection against SARS-CoV-2. *Cell*, (2020).
- 778
- 779 34. Gao Q, *et al.* Development of an inactivated vaccine candidate for SARS-CoV-2. *Science* **369**,  
780 77-81 (2020).
- 781
- 782 35. Ramakrishnan MA. Determination of 50% endpoint titer using a simple formula. *World J*  
783 *Virology* **5**, 85-86 (2016).
- 784
- 785 36. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol  
786 reagent. *J Biol Chem* **193**, 265-275 (1951).
- 787
- 788 37. Hovden AO, Cox RJ, Madhun A, Haaheim LR. Two doses of parenterally administered split  
789 influenza virus vaccine elicited high serum IgG concentrations which effectively limited viral  
790 shedding upon challenge in mice. *Scand J Immunol* **62**, 342-352 (2005).
- 791
- 792 38. 10.4103/ijmr.IJMR\_2382\_20 DeaNartS-C-iC-pIJMREaopD.
- 793
- 794 39. Guidelines on the nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines.  
795 Available at  
796 [https://www.who.int/biologicals/areas/vaccines/ADJUVANTS Post ECBS edited clean Gui](https://www.who.int/biologicals/areas/vaccines/ADJUVANTS_Post_ECBS_edited_clean_Guidelines_NCE_Adjuvant_Final_17122013_WEB.pdf?ua=1)  
797 [delines NCE Adjuvant Final 17122013 WEB.pdf?ua=1](https://www.who.int/biologicals/areas/vaccines/ADJUVANTS_Post_ECBS_edited_clean_Guidelines_NCE_Adjuvant_Final_17122013_WEB.pdf?ua=1).
- 798
- 799
- 800

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



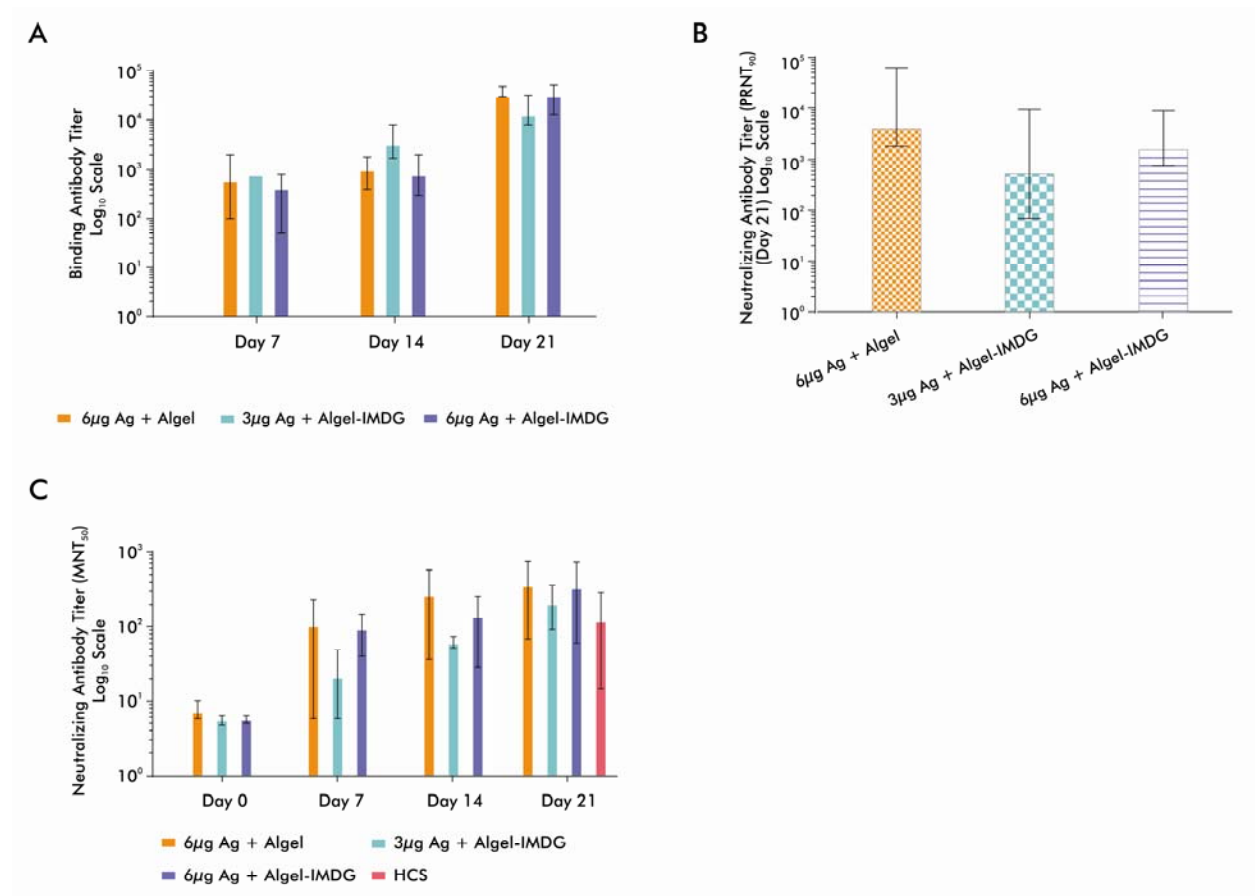
**A.** SARS-CoV-2 Virus (Strain NIV-770-2020) Growth Kinetics & Cytopathic effect (CPE) of virus before and after Inactivation (i) Virus titer ( $10^6$  -  $10^7$ ) measured by CCID<sub>50</sub> at every 3 hours up to 48 and after that every 12hrs various time points (24, 27, 30, 33, 36, 39, 42), (ii) Cells with Cytopathic Effect (CPE) before inactivation and No CPE after Inactivation, (iii) Image of Vero cell monolayer with no CPE observed from 16-36hrs; **B.** Representative electron micrograph of purified inactivated SARS-CoV-2 candidate vaccine (BBV152) at a scale bar: 100 nm (right) and 200 nm (left); **C.** Western blot analysis of Purified Inactivated SARS-CoV-2 produced from three production batches; **D.** Microneutralization antibody titer of Day 14 sera collected from mice vaccinated with Adjuvanted formulations (3μg Ag with Algel-IMDG and 6μg Ag with Algel-IMDG), after subjecting them for stability at 37°C for 7 days and compared with 2-8°C

**Figure 2: BBV152 Vaccines Induces High Virus-specific Antibody Response in Mice and Rats**



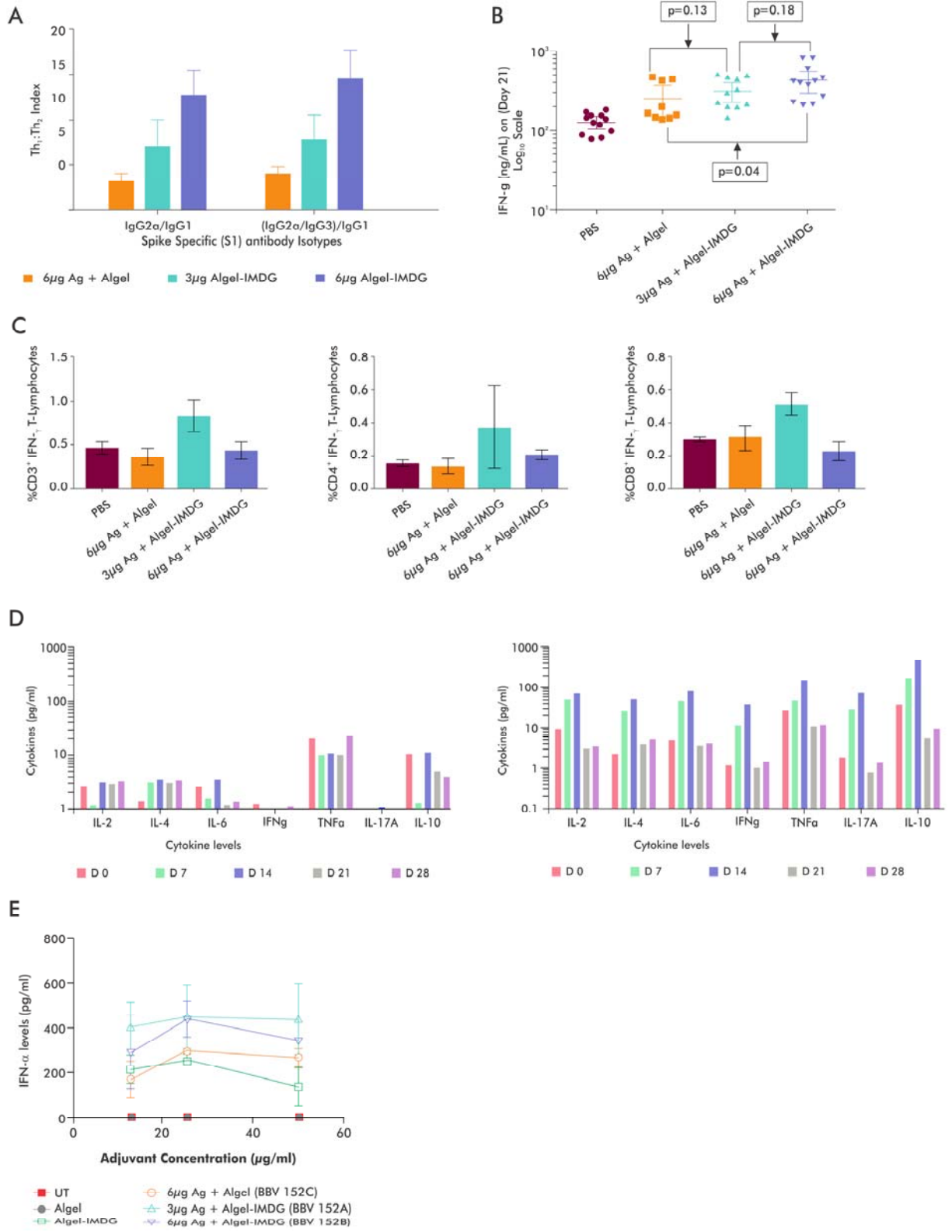
*Balb/C mice (n=10) were administered with adjuvanted vaccine formulations via IP route either with 1/20<sup>th</sup> (Fig A&B) or 1/10<sup>th</sup> (Fig C, D & E) Human Single Dose (HSD): A. S1 specific Total IgG antibody binding titer performed by ELISA, using sera collected at various time points (Day 0, 7, 14 & 21); B. Neutralizing antibody titers performed by PRNT<sub>90</sub>, using day 21 sera, when administered with 1/20<sup>th</sup> HSD respectively; C. S1 specific Total IgG antibody binding titer performed by ELISA, using Day 21 sera when administered with 1/10<sup>th</sup> HSD; D. SARS-CoV-2 specific (S1, RBD, N and total inactivated antigen) antibody binding titers elicited against adjuvant vaccines (BBV152A, B & C); E. neutralizing antibody titers performed by PRNT90, using day 21 sera, when administered with 1/10<sup>th</sup> HSD respectively; F. Balb/C mice were administered with Antigen & BBV152B via IM route at the specified doses on day 0 & 14. Sera were collected on Day 28 (post 2nd dose) and determined S1 specific antibody titer by ELISA.*

**Figure 3: BBV152 Induces Robust Neutralizing Antibody Response in Rabbits**



New Zealand white rabbits (n=8) were vaccinated intramuscularly on days 0, 7, and 14 with Full HSD via IM route. SARS-CoV-2 specific Antibody titers were measured by ELISA. Nab tires were measured by PRNT<sub>90</sub> and MNT<sub>50</sub>. Data Points represent mean ± SEM of individual animal data. A. S1 specific Ab binding titer of sera collected at various time points (Day 0, 7, 14 & 21); B. PRNT<sub>90</sub> neutralizing antibody titers of Day 21 sera; C. MNT<sub>50</sub> neutralizing antibody titers of sera collected at various time points (Day 0, 7, 14 & 21) along Neutralizing antibody titer (MNT<sub>50</sub>) with Human convalescent sera (HCS) from recovered COVID-19 patients (n=15). Samples were collected between 21-65 days of virological confirmation.

**Figure 4: BBV152 Induces A Robust Virus-specific T Cell Response.**



*Balb/C mice (n=10) were vaccinated with 1/10th HSD of adjuvanted vaccine formulations (BBV 152 A, B & C) via the IP route. A. Immunoglobulin subclasses (IgG1, IgG2a & IgG3) were measured by ELISA. Th1:Th2 index was measured using the formulas IgG2a/IgG1 or (IgG2a+IgG3)/IgG1. B. IFN $\gamma$  estimation by ELISA, on Day 21 sera (7 days post 3rd Dose). Statistical analysis was done Graph Pad Prism version. 7.0; C. Bar graph represents mean data of percent CD3<sup>+</sup> or CD4<sup>+</sup> or CD8<sup>+</sup> T lymphocytes producing IFN $\gamma$  from the respective group of animals (i) CD3<sup>+</sup> T lymphocytes population, (ii) CD4<sup>+</sup> T lymphocytes population, (iii) CD8<sup>+</sup> T lymphocyte population. Error bars indicate Mean $\pm$ SD. Vaccinated mice splenocytes from Balb/C mice (n=10), administered with 1/10<sup>th</sup> HSD via IM route were used for the analysis; D. Cytokine profile measured on various time points using vaccinated Balb/C mice sera, when administered with Adjuvanted vaccine formulations (1/20<sup>th</sup> HSD via IP route) Left - BBV152C- Antigen 6 $\mu$ g+Algel); Right – BBV152B- Antigen 6 $\mu$ g+Algel-IMDG, E. IFN $\alpha$  levels measured by ELISA from culture supernatant, when treated healthy PBMCs with Algel or Algel-IMDG or adjuvanted vaccine formulations (BBV152A, B & 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 $\pm$ SD of triplicate values.*