

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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4

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32 **ABSTRACT**

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

45

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

47

48 **1. Introduction**

49 Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), a novel human coronavirus ¹, has
50 spread to almost every country in the world. SARS-CoV-2 belongs to β -genus of serbecovirus and is a
51 close relative of SARS-CoV with close to 80% sequence identify. The World Health Organization
52 (WHO) declared the disease caused by SARS-CoV-2, Coronavirus Disease-19 (COVID19), a pandemic
53 in March 2020. So far, SARS-CoV-2 has infected more than 25 million people causing close to

54 850,000 deaths. It is, therefore, imperative to develop effective prophylactic and therapeutic
55 countermeasures to prevent and treat COVID19.

56

57 The development of a safe and effective vaccine has become a top priority globally to prevent the
58 spread of SARS-CoV-2 infection during the pandemic. Numerous vaccine candidates are in the
59 preclinical and clinical trial stages. However, meeting the global need for billions of doses of COVID-
60 19 vaccines will require collective effort to identify, evaluate, validate, and manufacture effective
61 vaccines. Inactivated vaccines for viral diseases have been licensed for decades with well-established
62 safety profiles². The availability of well-characterized vero cell manufacturing platform with proven
63 safety in other licensed, live, and inactivated vaccines have aided in rapid vaccine development^{3,4,5,}
64 ^{6,7}. Prior experience in developing inactivated had given us the confidence to develop a fully
65 inactivated with an intact virion, imperative for obtaining an antigen that will yield high
66 immunogenicity. Therefore, to facilitate the development of an effective COVID19 vaccine, we have
67 used a well-characterized SARS-CoV-2 strain and an established vero cell (CCL-81) platform to
68 produce large-scale GMP grade highly purified BBV152 vaccine candidate. It has to be mentioned
69 here that there are several vaccine candidates at different stages of clinical development, such as
70 adenovirus-vectored vaccines, recombinant protein-based, and inactivated vaccines. The inactivated
71 vaccine (PiCoVacc) and the recombinant vaccine (CoV-RBD219N1), which are aluminium adjuvant
72 formulations, have been shown to generate high levels of neutralizing antibodies (NAb) to the S-
73 protein, which could play an important role in vaccine efficacy. Hence, the development of
74 inactivated vaccines for COVID-19 disease prevention appears to be a rational approach, while
75 recognizing the fact that such inactivated vaccines with alum adjuvant specifically induce T helper 2
76 cells.

77

78 While the development of safe and effective coronavirus vaccines is a priority, vaccine-induced
79 disease enhancement observed in preclinical animal models due to Th2-like immunity is a concern.
80 To circumvent the Th-2 bias and to develop a safe vaccine, we formulated a new adjuvant that
81 contains an imidazoquinoline class TLR7/8 agonist adsorbed to Alginate. TLR7/8 agonists induce
82 strong type I interferon responses from dendritic cells and monocyte-macrophages that facilitate the
83 development of Th1 biased immunity instead of a pathogenic Th2-biased immunity⁸.

84 Here, we report the immunogenicity and safety evaluation of the whole virion inactivated SARS-CoV-
85 2 vaccine candidate BBV152, which was evaluated at three antigen concentrations (3,6, and 9µg)
86 and two adjuvants in three animal models, i.e., mice, rats, and rabbits. Our results show that these
87 vaccine formulations induced significantly elevated titers of antigen binding and neutralizing
88 antibodies in all animal models tested without any safety concerns. We also show that the vaccine
89 was formulated with Algel-adsorbed TLR7/8 agonist-induced Th1 biased immunity with significantly
90 elevated SARS-CoV-2 specific IFN γ + CD4 T cell response. Collectively these results demonstrate that
91 the BBV152 vaccine candidate induces protective and durable NAb and T cell responses. As a result,
92 BBV152 vaccine candidate has been considered for phase I clinical trials.

93

94 **2. Results**

95 **2.1 Isolation and selection of SARS-CoV-2 strain for vaccine candidate preparation.**

96 During the initial outbreak of SARS-CoV-2 in India, specimens from 12 infected patients were
97 collected and sequenced at the Indian Council of Medical Research-National Institute of Virology
98 (ICMR-NIV), India, a WHO Collaborating Center for Emerging Viral Infections⁹. The SARS-CoV-2 strain
99 (NIV-2020-770) used in developing the BBV152 vaccine candidate was retrieved from tourists who
100 arrived in New Delhi, India^{10, 11}. The sample propagation and virus isolation were performed in the
101 Vero CCL-81. The SARS-CoV-2 sequence was deposited in the GISAID (EPI_ISL_420545). The BBV152
102 vaccine candidate strain is located in the (G clade), also represented as '20A' clade that is the most
103 prevalent strain in India (followed by '19A') as per data represented in the Next strain analysis of the
104 Indian analysis¹². In terms of the overall divergence of SARS-CoV-2, this strain is 99.97% identical to
105 the earliest strain Wuhan Hu-1¹³. The multiple passages done in the Vero CCL-81 demonstrated the
106 genetic stability of the virus. The next-generation sequencing (NGS) reads generated from the
107 nucleotide sequences of the BBV152 vaccine candidate strain and its passage one at PID-3 was found
108 to be comparable with the SARS-CoV-2 Wuhan Hu-1 strain (**Table 1**). A maximum difference of
109 0.075% in the nucleotides was observed, indicating negligible changes in the different batches of the
110 samples analyzed—these results showed genetic stability of the NIV-2020-770 strain for further
111 vaccine development. The seed virus (NIV 2020-770 strain) was transferred from ICMR-NIV to Bharat
112 Biotech, India. Samples from different drug Substance batches of BBIL, along with the original virus,
113 clustered into a single group and indicate an origin from previous passages.

114

115 **2.2 Vaccine candidate preparation**

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

123

124 Vero cells and virus were propagated in the bio-safety level-3 (BSL-3) facility using bioreactors.
125 Growth kinetics analysis showed that the stock replicated to 7.0 log₁₀ TCID₅₀ between 36- and 72-
126 hours protection. β-propiolactone was utilized for the inactivation of the virus by mixing the virus
127 stock between 2-8°C. During the inactivation kinetics experiments with varying conditions and
128 concentrations, samples were collected at various time points (between 0 to 24 hours, at 4-hour
129 intervals) to evaluate the cytopathic effect of live virus. Three consecutive inactivation procedures
130 were performed to ensure complete viral inactivation without affecting the antigen stability (**Figure**
131 **1A**). Transmission electron microscopy (TEM) analysis showed that the inactivated and purified virus
132 particles were intact, oval-shaped, and were accompanied by a crown-like structure representing the
133 well-defined spike on the virus membrane (**Figure 1B**) Inactivated and purified virus was also
134 characterized by western blot for its identity with SARS-CoV-2 specific antibodies using various
135 stages of vaccine candidate development such as cell harvest, clarified supernatant, post-
136 inactivation, and purification. Western blot analysis showed distinct bands of all proteins. Purified
137 and inactivated whole virion antigen produced from three production batches were probed with
138 anti-Spike (S1 & S2), anti-RBD, and anti-N protein (**Figure 1C**). These results showed that the final
139 purified inactivated bulk of the vaccine candidate is highly pure and contains S (S1, S2), RBD, and N
140 protein bands with their corresponding equivalent molecular weight.

141

142

143 **2.3 Vaccine formulations with adjuvants.**

144 BBV152 vaccine candidates were formulated with two alum adjuvants: Algel (aluminium hydroxide
145 gel) and Algel-IMDG, an imidazoquinoline class molecule (TLR7/TLR8 agonist abbreviated as IMDG)
146 adsorbed on aluminium hydroxide gel. The agonist molecule for Algel-IMDG was licensed from
147 ViroVax LLC, USA. Three vaccine formulations were prepared with 3µg and 6µg with Algel-IMDG
148 (BBV152A and BBV152B, respectively) and 6µg with Algel (BBV152C). To determine the stability of
149 the vaccine formulations, inactivated antigen plus adjuvant preparations were stored at 37°C and 2-

150 8°C temperature for seven days. These vaccine formulations were evaluated in Balb/C mice to
151 estimate Nab titer by microneutralization test (MNT₅₀). Our results demonstrated that the vaccine
152 formulations are relatively stable at 37°C for 7 days, as shown by equivalent Nab titer compared to
153 formulation stored at temperature 2-8°C (**Figure 1D**). There is no significant difference between the
154 two formulations (BBV152A & BBV152B)

155

156 **2.4 Safety**

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

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

180 **2.5 Clinical pathology investigations:**

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

192 **2.5 Necropsy, organ weight, and histopathology:**

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

209 **2.6 Immunogenicity studies:**

210 We assessed the immunogenicity of BBV152 formulations in BALB/c mice and New Zealand white
211 rabbits]. All immunization studies were conducted based on a three-dose IM regimen conducted on

212 days 0, 7, and 14. Pooled or individual serum samples collected on days 0, 7, 14, and 21 post-
213 immunization/boost were evaluated for antibody binding (ELISA) and Nab by plaque reduction
214 neutralizing titer (PRNT₉₀) or MNT₅₀ against live SARS-CoV-2 strain.

215

216 **Immunogenicity in BALB/c Mice:**

217 To assess the immunogenicity of the candidate vaccines, BALB/c mice (n=10) were injected via *i.p.*
218 route with three concentrations of antigen at 1/20th of the intended human single dose (i.e., 3 µg,
219 6 µg, and 9 µg/mouse). Vaccine formulations adjuvanted tested at three antigen concentrations
220 elicited high levels of binding and Nab titer (**Figure 2 A & B**). Antigen alone and adjuvants alone were
221 included in these studies as controls (data not presented for brevity). Further, to assess the
222 immunogenicity and safety of clinical batch samples, Balb/C mice (n=10/group, 5 Male and 5
223 Female) were vaccinated via IP route with three adjuvanted formulations with Algel and Algel-IMDG
224 at 1/10th human intended single dose (3, and 6 µg/dose with Algel or Algel-IMDG). All adjuvanted
225 vaccine formulations elicited antigen-specific binding antibodies (**Figure 2C**). Further, sera collected
226 on Day 21 were analyzed by ELISA to determine S1, RBD, and N specific binding titer (**Figure 2D**).
227 Analysis of PRNT₉₀, performed with individual mice sera, showed high Nab's in all adjuvanted
228 vaccines (**Figure 2E**), while **Figure 2F** depicts the effect of dose sparing of Algel-IMDG. **Figure S8**
229 depicts the 8-fold increase in vaccine potency, when dosing was in day 14 intervals. Additionally,
230 dosing with antigen alone was found to be immunogenic. However, the responses were significantly
231 lower than the adjuvanted vaccine (**Figure S9**).

232

233 **Immunogenicity in New Zealand White Rabbits:**

234 Rabbits (n=8) were immunized with antigen concentrations for humans (3 and 6 µg/dose) on days 0,
235 7, and 14. The groups that received BBV152A & B showed a slightly higher binding antibody
236 response compared to BBV152C (**Figure 3A**), though not statistically significant. Examination of
237 neutralizing antibody titers revealed high PRNT₉₀ titers on day 21 are also reported (**Figure 3B**).
238 Further, Nab's performed by MNT₅₀ were compared with Nabs from a panel of human convalescent
239 sera from recovered symptomatic COVID-19 patients. (**Figure 3C**).

240

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

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

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

256

257 **Cytometric Bead Array (CBA)**

258 Expression of TNF- α and interleukins was noticeably expressed in the 6 μ g Algel-IMDG when
259 compared to 6 μ g Algel (**Figure 4D**).

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

269

270 **3. Discussion**

271 Here, we report the development of a whole virion inactivated SARS-CoV-2 vaccine candidate
272 (BBV152). The strain used for this candidate is pathogenic in humans and has shown extensive
273 genetic stability and appropriate growth characteristics for the selection of a vaccine candidate.
274 Preclinical toxicity and safety evaluation of the three formulations showed minimal to no adverse
275 events. Our results show that the vaccine formulations induced significantly elevated antigen-
276 binding antibody and Nab responses in the animals immunized, with a distinct Th1 bias observed
277 with Algel-IMDG adjuvanted vaccines. Although the neutralizing antibody titers are not statistically
278 different between the antigen concentration (3 μ g and 6 μ g) or the nature of adjuvant, all the
279 formulations tested have exhibited excellent immunogenicity. Our potency results compare quite
280 favorably with those reported in the literature for similar COVID-19 vaccines. Inactivated SARS-CoV-2
281 vaccine candidate (BBIBP-CorV) has been shown to induce high levels of Nab titers in mice and rats
282 to provide protection against SARS-CoV-2³. A purified inactivated SARS-CoV-2 virus vaccine
283 candidate (PiCoVacc) has also been shown to induce SARS-CoV-2-specific NAb in mice and rats.
284 These antibodies potently neutralized 10 representative SARS-CoV-2 strains, indicative of a possible
285 broader neutralizing ability against SARS-CoV-2 strains circulating worldwide⁴.

286

287 The risk of antibody-dependent enhancement (ADE) is a serious concern for COVID-19 vaccine
288 development^{18, 19, 20, 21}. A few animal studies from animal SARS-CoV-1 and MERS-CoV inactivated or
289 vectored vaccines adjuvanted with alum have shown correlation to Th2 responses resulting in
290 eosinophilic infiltration in the lungs^{18, 19, 20}. Alum is the most frequently used vaccine adjuvant with
291 an extensive safety record. It is desired to have a COVID-19 vaccine that can generate both humoral
292 and cell-mediated immune responses. The response generated from alum is primarily Th2- biased
293 with the induction of strong humoral responses via neutralizing antibodies²². It is not clear if alum
294 alone can stimulate T-cell responses. Complicating adverse events may be associated with the
295 induction of weakly or non-neutralizing antibodies that lead to antibody-dependent enhancement
296 (ADE) or enhanced respiratory disease (ERD), thus warranting COVID-19 vaccines to induce CD4
297 Th1(interferon- γ , interleukin-2, tumor necrosis factor α) response with minimal Th2 response^{23, 24}.
298 Preclinical studies in mice reported that inactivated vaccine-induced eosinophil immunopathology in
299 the lungs upon SARS-CoV infection²⁵ could be avoided using TLR agonist as or in adjuvant
300 formulations. Although current understanding of the risk of COVID-19 vaccine-associated ADE/ERD is
301 limited, the use of TLR7/8 agonists in an adjuvant in SARS-CoV-2 vaccine formulation will minimize
302 Th2 response, if any.

303

304 Over many decades it has shown that vaccination is generally a safe and well-tolerated procedure.
305 Nevertheless, toxic actions of vaccines can result from any of the following, drug substance and drug
306 product, including excipients used for formulation. The current preclinical studies conducted with
307 BBV152, adjuvanted with the two adjuvants, did not indicate any undesirable pathological changes
308 and systemic toxicity. Local reactogenicity to adjuvants used in vaccine formulation were the only
309 findings noted. Algel (Alum) is the most commonly used agent as an adjuvant. It has been shown to
310 act by depot formation at the site of injection, allowing for a slow release of antigen. Further, it
311 converts soluble antigens into particulate forms, which are readily phagocytosed²⁶. The microscopic
312 findings at the site of injection in the present studies showed the infiltration of macrophages and
313 mononuclear cells. The other adjuvant, Algel-IMDG, contained TLR7/8 in addition to Algel, which was
314 added to augment innate and adaptive immunity, induced slightly higher reactogenicity. IM injection
315 induces a depot effect followed by the passive trafficking of algel particles via lymphatic flow from
316 the interstitial space to the draining lymph nodes, as revealed by IFN- β /luciferase reporter mice
317 (unpublished). The lymph node-targeting of Algel-IMDG ensures high adjuvant activity in the target
318 organ (lymph nodes) by enabling the induction of a strong, specific, adaptive immune response while
319 minimizing systemic exposure. The local reaction in the studies conducted was consistent with those
320 available in the literature for these adjuvants, which is a physiological reaction to injection rather
321 than any adverse event^{26, 27}.

322

323 Collectively, both the adjuvanted vaccines (with Algel and Algel-IMDG), Antigen and Adjuvant alone
324 did not reveal any treatment-related findings, except local reactions when administered through the
325 human intended route (intramuscular) on days 0, 7, and 14 (n+1) with full Human single dose (HSD)
326 or higher than HSD in rodents and non-rodents, thereby establishing the vaccine safety. In our
327 preclinical studies, we demonstrated that all the three inactivated whole virion SARS-CoV-2 vaccine
328 candidates showed 100% seroconversion with high titers of antigen binding and neutralizing
329 antibody responses. Further, the adjuvanted formulation, BBV152B, when immunized in Balb/C
330 mice, showed 10 times higher dose sparing effect compared to antigen alone (**Figure 2F**). Moreover,
331 these formulations induced immunity that is biased towards Th1 mediated response, as
332 demonstrated by the ratio between IgG2a and IgG1 (greater than 1) (**Figure 4A**). Additionally,
333 secretion of anti-viral cytokines such as IL-2, IL-4, IL-6, IL-10, IL-17, TNF-alpha, IFN γ was observed on
334 days 7 and 14(7 days after the 1st&2nd dose) of vaccination with Algel-IMDG adjuvanted formulations

335 **(Figure 4D)**. Further, the tendency to secrete anti-viral cytokines, IFN-alpha **(Figure 4E)**, might
336 contribute to the activation of the first line of defense mechanisms, which lead to enhanced
337 activation of antigen-presenting cells, such as dendritic cells or macrophages^{28, 29, 30}. It is reported
338 that TLR recognition in innate cell population drives early type I IFN production, thereby promotes
339 viral clearance and the early production of proinflammatory cytokines^{31, 32}. Though the mechanism
340 of action is yet to be investigated, we hypothesize that this elevated production of IFN α in the Algel-
341 IMDG based Adjuvanted vaccine may provide better protection in the Hamster and NHP homologous
342 challenge study with SARS-CoV-2 virus.

343

344

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

349 A major limitation of this paper is the lack of protective efficacy results conferred from BBV 152.
350 Additional live challenge studies in hamsters and non-human primates are completed at NIV, India,
351 and results will be published shortly. With no established correlate of protection, we also evaluated
352 human convalescent sera from recovered symptomatic SARS-CoV-2 patients. Samples were collected
353 21 days after virological confirmation **(Figure3C)**. Furthermore, two other SARS-CoV-2 inactivated
354 vaccines (BBIBP-CorV and PiCoVacc) from China have entered late-stage human clinical trials with
355 published data on the preclinical immune response. Results from these candidates have reported
356 comparable findings, albeit PRNT₅₀^{33, 34}.

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

361

362 **Tables**

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

Reference Position	Wuhan nucleotide	Hu-1 nucleotide	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

365

366 **Table 2:** Safety studies conducted

Study Type	Test System	Test Item ¹⁻³	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 ⁴ .
	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	

367

368

369

370

1. Antigen: BBV152 Antigen at 3, 6 & 9µg.
2. Adjuvanted vaccines: BBV152A, BBV152B & BBV152C.
3. Adjuvants: Algel & Algel-IMDG at 200 & 300µg.
4. Details are given in Supplementary Section.

371

372

373 **Methods**

374 **1. Cells and Virus**

375 Vero CCL-81 (ATCC# CCL 81) cells were maintained in DMEM supplemented with 10% heat-
376 inactivated fetal bovine serum. Vero cells were revived from GMP master cell bank, which was
377 extensively characterized at BioReliance, USA. SARS-CoV-2 (Strain No#NIV-2020-770) was obtained
378 from the National Institute of Virology, a WHO Collaborating Center for Emerging Viral Infections⁹,
379 Pune, India. SARS-CoV-2 strain (NIV-2020-770) sequence was deposited in the GISAID
380 (EPI_ISL_420545).

381

382 Specimens from 12 infected patients were collected during the initial outbreak of SARS-CoV-2 at the
383 National Institute of Virology (NIV), India, a WHO Collaborating Center for Emerging Viral Infections⁹.
384 SARS-CoV-2 strain (NIV-2020-770) was passaged in vero cell lines (Vero CCL-81) and sequenced, and
385 the sequence was deposited in the GISAID (EPI_ISL_420545).

386

387 **2. TCID50**

388 The SARS-CoV-2 virus titer was determined by a cytopathic effect (CPE) method assay. Vero cells
389 ATCC-81 (0.2×10^6 cells/mL) were seeded in 96 well plates and incubated for 16- 24 hours at 37 °C.
390 Serial 10-fold dilutions of virus-containing samples were added to 96-well culture plate and cultured
391 for 5-7 days in 5% CO₂ incubator at 37°C, and cells were observed for cytopathic effect (CPE) under a
392 microscope. The virus titer was calculated by the Spearman Karber method³⁵.

393 **3. Virus Inactivation**

394 SARS-CoV-2 Virus (NIV-2020-770) was inactivated with β-propiolactone at a ratio ranging from
395 1:1500 to 1: 3000 at 2-8°C for 24-32 hours and purified by chromatographic purification method. To
396 ensure the effectiveness of the virus inactivation procedure inactivated SARS-CoV-2 virus was
397 inoculated onto vero CCL-81 monolayers and incubated at 37 °C in a 5% CO₂ incubator and
398 monitored daily for CPE, consecutively for three passages. Further, to reverify the absence of CPE
399 due to supernatant, neat and 10fold dilution of supernatant was inoculated onto Vero cell
400 monolayer and cultured in a 37°C incubator for 5-7 days, and cells were observed for CPE under a
401 microscope.

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

403 Total RNA was extracted from the virus sample with a QIAamp Viral RNA mini kit (QIAGEN). SARS-
404 CoV-2 *RdRP-2* gene primer probes sequences are as follows: *RdRP_SARSr-F2-*
405 *GTGARATGGTCATGTGTGGCGG*, *R1-CARATGTAAASACACTATTAGCATA*, *P2-FAM*
406 *CAGGTGGAACCTCATCAGGAGATGC-BHQ1*. The SARS-CoV-2 reaction was set up containing a master

407 mix of 10 μ L (Thermo) and RNA template 10 μ L.qRT-PCR was performed under the following
408 reaction conditions: RT step- 42°C for 30 min for reverse transcription, Initial Denaturation step:
409 95°C for 3 min and then 45 cycles of Denaturation95°C for 15 seconds, annealing58°C for 30 seconds
410 - data acquisition, Extension72°C for 15 seconds. Reactions were set on Biorad-CFX96 as per the
411 manufacturers' instructions.

412 **5. Western blotting**

413 Protein samples (~30 mg) derived from drug substance estimated by Lowry method³⁶ and standard
414 procedures for western blot were adopted. The primary antibodies used were anti-N protein rabbit
415 monoclonal Ab (1:1000 dilution) and anti- S1 or S2 or RBD protein rabbit polyclonal Ab (1:1000
416 dilution), either sourced from commercial or in-house and human convalescent sera from patients
417 (1:500 dilution) at 4°C. The secondary antibodies goat anti-rabbit IgG H&L (HRP) (GE NA934,1:4000)
418 and HRP-labeled goat anti-human IgG (gamma chain) cross-adsorbed secondary antibody
419 (Invitrogen, 62-8420) (1:1000). Protein bands were visualized in enhanced chemiluminescence
420 (Azure biomolecular imager, USA).

421 **6. Formulations Preparation**

422 In the first formulation, BBV152A, 3 μ g of antigen was mixed with Algel-IMDG, while BBV152B had
423 6 μ g of antigen with the same adjuvant (Algel-IMDG), and the third formulation, BBV152C, had 6 μ g of
424 antigen adsorbed on alum (Algel). Total protein/unbound protein was estimated by the Lowry
425 method³⁶.

426 **7. Animal husbandry practices**

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

432 **8. Immunization:**

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

435 **Mice:** Balb/C or Swiss Albino mice (6-8week old) were vaccinated via an intraperitoneal or
436 intramuscular route with either 1/10th or 1/20th of full human single dose (BBV152 A, B or C) of

437 inactivated vaccine with or without adjuvant on day 0, 7 & 14 days(n+1 (one extra dose compared to
438 the intended human regimen doses). A formulation with 9µg was also tested.

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

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

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

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

452 Pooled and individual sera from vaccinated mice and rabbits were used to test the antigen-specific
453 antibody binding titer and antibody isotyping profile by Enzyme-Linked Immunosorbent Assay
454 (ELISA). Pooled or Individual sera from all vaccinated species (mice, rabbits & rats) were used to test
455 neutralization antibody titer by Plaque Reduction Neutralization Test (PRNT₉₀) or Micro
456 Neutralization Test (MNT₅₀).

457 **9. Enzyme-linked immunosorbent assay (ELISA)**

458 ELISA tests were performed as per standard protocols specifically for this project. Microtiter plates
459 were coated with SARS-CoV-2 specific antigens (whole inactivated antigen or spike, S1 /Receptor
460 Binding Domain (RBD)/ nucleocapsid (N) at a concentration of 1µg/ml, 100µl/well in PBS pH 7.4).
461 After incubation, wells were added with Goat Anti-mouse IgG HRP(Santa Cruz Biotechnology, USA)
462 conjugated antibody for mouse sera samples, and Goat anti-rabbit IgG HRP conjugate
463 antibody(Santa Cruz Biotechnology, USA) (dilution 1:2500) for rabbit sera samples and incubated for
464 1hr at RT. Threshold (Mean + 3SD) was established by taking the absorbance of negative control
465 (PBS) group, or pre-immune sera and antigen-specific endpoint titers were determined. The
466 antibody dilution, at which absorbance is above the threshold, was taken as antigen-specific
467 antibody endpoint titers.

468 **10. Immunoglobulin (IgG) Subclass:**

469 Th1-dependent IgG2a vs. Th2 -dependent IgG1 antibody subclasses were determined from mice
470 vaccinated sera as previously described³⁷. Briefly, 96 well microtiter plates were coated with various
471 SARS-CoV-2 specific antigens (whole Inactivated antigen, S1, Receptor Binding Domain (RBD),
472 nucleocapsid (N) at a concentration of 1µg/ml, 100µl/well in PBS pH 7.4) and kept at 2-8°C for
473 overnight. The next day, plates were washed with washing buffer (PBST) and blocked with a blocking
474 buffer (PBS with 2% BSA) at RT for one hour. serially diluted (dilutions from 1:50 to 819200 in PBS,
475 0.1% BSA, 0.05% Tween™20, 0.02% sodium azide) pooled or individual sera from hyperimmunized
476 animals (mice/rabbits) and incubated at 37°C for 2hrs. After incubation, wells were washed and
477 added with anti-mouse IgG1 or IgG2a HRP conjugate antibodies at a dilution 1:2500. After
478 incubation of the plate for 1hr at RT, wells were washed, and 3,3',5,5'-tetramethylbenzidine (TMB)
479 was added as a substrate to develop color. Absorbance was read at 450 nm. Threshold (Mean + 3SD)
480 was established by taking the absorbance of negative control (PBS) group, or pre-immune sera and
481 antigen-specific endpoint titers were determined. The antibody dilution, at which absorbance is
482 above the threshold, was taken as antigen-specific antibody endpoint titers.

483 **11. Cytokine (IFN γ & IFN α) Estimation by ELISA:**

484 To determine IFN γ , Enzyme-Linked Immunosorbent Assay (ELISA) was performed according to the
485 instruction manual. Briefly, the capture antibody was first diluted in coating buffer and added 100 µL
486 to each well in 96-well microplate. Plates were incubated overnight at 2-8 °C. Coated plates were
487 then washed with wash buffer (PBST). After washing, these plates were blocked using 1x assay
488 diluent for 1hr at room temperature followed by washing with PBST. Serial dilutions of Top Standard
489 were prepared to make the standard curve. Similarly, 4-fold dilutions (1:4, 1:16 & 1:64) of serum
490 samples were e prepared and added to wells in triplicates, and the plate was incubated at room
491 temperature for 2hrs. After washing the plate, 100 µL/well of detection antibody diluted in 1X Assay
492 diluent was added and incubated at room temperature for 1hr. Later, 100 µL/well of Avidin-HRP*
493 diluted in 1X Assay diluent was added and incubated at room temperature for 30 minutes. Finally,
494 after washes, 100 µL of substrate solution was added to each well and incubated at RT for 15
495 minutes. The reaction was stopped by the addition of 50 µL of 2N H₂SO₄ to each well, and the plate
496 was read at 450 nm.

497 PBMCs cell culture supernatant was used to estimate IFN α using The VeriKine Human Interferon
498 Alpha ELISA Kit (PBL Assay Science, USA, Cat log# 41100). The assay was performed as per the
499 manufacturer's instructions. Briefly, Pre-coated plates were incubated with diluted standard (range

500 500-12.5 pg/ml) or culture supernatant, for 1hr at room temperature. Later, the diluted antibody
501 and HRP solution were added sequentially. TMB was used as a substrate, followed by the addition of
502 stop solution. The plate was read at 450nm.

503

504 **12. Intracellular Staining:**

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

517 **13. Cytokine Estimation:**

518 To assess the secretion of Th1 or Th2 mediated cytokines, if any, and to differentiate between Algel1
519 and Algel2, we used vaccinated mice sera samples collected at various time points (Day 0, 7, 14, 21
520 & 28, 7 days post-vaccination) and measured Cytokines using the BD CBA Mouse Th1/Th2/Th17
521 Cytokine Kit (BD Bioscience, San Jose, CA, USA). Sera samples were processed as per the
522 manufacturer's instructions. Briefly, the kit was used for the simultaneous detection of mouse IL-2,
523 IL-4, IL-6, IFN- γ , TNF, IL-17A, and IL-10 in a single sample. For each sample, 50 μ L of the mixed
524 captured beads, 50 μ L of the unknown serum sample or standard dilutions, and 50 μ L of
525 phycoerythrin (PE) detection reagent were added consecutively to each assay tube and incubated
526 for 2 h at room temperature in the dark. The samples were washed with 1 mL of wash buffer for 5
527 min and centrifuged. The bead pellet was resuspended in 300 μ L buffer after discarding the
528 supernatant. Samples were measured on the BD FACS Verso and analyzed by FCAP Array Software
529 (BD Bioscience).

530 **14. Plaque Reduction Neutralization Test (PRNT₉₀):**

531 The Plaque reduction neutralization test was performed in a biosafety level 3 facility. To perform
532 PRNT₉₀, Vero CCL-81 cell suspension (1.0×10^5 /mL/well) was added in duplicates in 24-well tissue
533 culture plates and cultured in a CO₂ incubator at 37°C for 16-24 hrs. Vaccinated serum samples were
534 inactivated by keeping in a 56°C-water bath for 30 min. Serial dilutions (4 fold) of vaccinated serum
535 samples were mixed with the virus, which can form 50 plaque-forming units and then incubated for
536 1 h at 37°C. The virus-serum mixtures were added onto the preformed Vero CCL-81 cell monolayers
537 and incubated 1 h at 37°C in a 5% CO₂ incubator. The number of plaques was counted, and the
538 Neutralizing antibody titer was determined based on the 90% reduction in the number of plaque
539 count, which was further analyzed using 50% Probit Analysis³⁸. A neutralization antibody titer < 1:20
540 considered negative, while that of > 1:20 considered as positive.

541 **15. Micro Neutralization assay (MNT)**

542 The serum of the animal to be tested was inactivated in a 56°C -water bath for 30 min. Serum was
543 successively diluted 1:8 to the required concentration by a 2-fold series, and an equal volume of
544 challenge virus solution containing 100 CCID₅₀ viruses was added. After neutralization in a 37°C
545 incubator for two hours, a 1.0×10^5 /mL cell suspension was added to the wells (0.1 mL/well) and
546 cultured in a CO₂ incubator at 37°C for 3-5 days. The Karber method³⁵ by observing the CPE was used
547 to calculate the neutralization endpoint (convert the serum dilution to logarithm), which means that
548 the highest dilution of serum that can protect 50% of cells from infection by challenge with 100
549 CCID₅₀ virus is the antibody potency of the serum. A neutralization antibody potency < 1:20 is
550 negative, while that R 1:20 is positive.

551

552 **16. Mutagenicity Assay (Bacterial Reverse Mutation)**

553 The mutagenic potential of the Adjuvant, Algel-IMDG, was evaluated by Bacterial Reverse Mutation
554 assay through plate incorporation and pre-incubation methods using *Salmonella typhimurium* strains
555 TA 1535, TA 1537, TA 98, TA 100, and TA 102 following OECD Guidelines for Testing of Chemical¹⁴,
556 with and without S9. Toxicity was apparent either as a reduction in the number of His⁺ revertants or
557 as an alteration in the auxotrophic background (*i.e.*, background lawn).

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

559 Two animals (Swiss Albino mice and Wistar Rats) species were tested with Algel-IMDG with a single
560 maximum dose (containing 200µg Algel and 20µg TLR7/8 agonist molecule). Animals (Swiss Albino
561 mice and Wistar Rats) were administered via an intramuscular route with Algel-IMDG on day 0 and

562 observed for clinical signs, mortality, and changes in body weight if any up to 14 days. The site of
563 injection was also observed for erythema and edema at 24, 48, and 72 hours after dosing to detect
564 the local tolerance (local reactogenicity) of Algel-IMDG. All animals were necropsied and examined
565 macroscopically. Histopathology was performed for the site of injection.

566 **18. Repeated dose toxicity:**

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

575

576 **Test system**

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

583 **Treatment regimen**

584 The adjuvanted vaccines or adjuvants alone were administered intramuscularly (IM) in quadriceps
585 muscles of the hindlimb on days 0, 7, and 14 (n+1) with full Human single dose (HSD) to NZW rabbits
586 and SA mice and higher dose than HSD to Wistar rats and full HSD to. In BALB/c Mice, 1/20th HSD
587 was administered intraperitoneally. The animals were observed up to 14 days, post last dose.

588 **Experimental Design - Adjuvant alone**

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

596

597 **Experimental Design - Adjuvanted Vaccines**

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

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

603 **In-life Observations**

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

613 **Clinical Pathology Investigations**

614 Detailed clinical pathology was performed using automated equipment as per referred guidelines
615 following validated procedures^{145, 37-39}. Blood and urine samples were collected for clinical
616 evaluations (hematology, coagulation parameters, acute phase proteins, serum chemistry, and
617 urinalysis) from all the groups.

618 **Necropsy, Organ Weight and Histopathology**

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

625 **18. Statistical Methods**

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

630

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639 **Author Contributions**

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

647 **Competing Interests**

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653 4. References

- 654 1. WHO Coronavirus Disease (COVID-19) Dashboard. Available at <https://covid19.who.int/>.
- 655
- 656 2. Sanders B, Koldijk M, Schuitemaker H. Inactivated Viral Vaccines. *Vaccine Analysis: Strategies, Principles, and Control*, 45-80 (2014).
- 657
- 658
- 659 3. Bhandari N, *et al.* Efficacy of a monovalent human-bovine (116E) rotavirus vaccine in Indian
660 infants: a randomised, double-blind, placebo-controlled trial. *Lancet* **383**, 2136-2143 (2014).
- 661
- 662 4. World Health Organization WER, 24 JANUARY 2020, 95th YEAR / 24 JANVIER 2020, 95e,
663 ANNÉE No 4, 95, 25–3624 January 2020. Accessed on 17 August, 2020. Available at
664 [https://extranet.who.int/iris/restricted/bitstream/handle/10665/330607/WER9504-eng-
665 fre.pdf?ua=1](https://extranet.who.int/iris/restricted/bitstream/handle/10665/330607/WER9504-eng-fre.pdf?ua=1).
- 666
- 667 5. Vadrevu KM, Potula V, Khalatkar V, Mahantshetty NS, Shah A, Ella R. Persistence of Immune
668 Responses With an Inactivated Japanese Encephalitis Single-Dose Vaccine, JENVAC and
669 Interchangeability With a Live-Attenuated Vaccine. *The Journal of Infectious Diseases*,
670 (2019).
- 671
- 672 6. Singh A, *et al.* A Japanese Encephalitis Vaccine From India Induces Durable and Cross-
673 protective Immunity Against Temporally and Spatially Wide-ranging Global Field Strains. *The
674 Journal of Infectious Diseases* **212**, 715-725 (2015).
- 675
- 676 7. Sampath G, *et al.* Immunogenicity and safety study of Indirab: A Vero cell based
677 chromatographically purified human rabies vaccine. *Vaccine* **28**, 4086-4090 (2010).

678

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

682

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

685

686 10. Potdar V, *et al.* Genomic analysis of SARS-CoV-2 strains among Indians returning from Italy,
687 Iran & China, & Italian tourists in India. *Indian Journal of Medical Research* **151**, 255-
688 260 (2020).

689

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

692

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

694

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

699

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

703

704 15. Schedule Y (Amended version of 2019) of the Drugs and Cosmetics Act 1940 and Rules 1945
705 of the Government of India. Accessed on July 30, 2020. Available at
706 [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)
707 [documents/acts_rules/2016DrugsandCosmeticsAct1940Rules1945.pdf](https://cdsco.gov.in/opencms/export/sites/CDSCO_WEB/Pdf-documents/acts_rules/2016DrugsandCosmeticsAct1940Rules1945.pdf).

708

709 16. Guidelines on the nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines.
710 World Health Organization 2013, Adopted by the 64th meeting of the WHO Expert

- 711 Committee on Biological Standardization, 21–25 October 2013. Accessed on July 30, 2020.
712 Available at
713 https://www.who.int/biologicals/areas/vaccines/ADJUVANTS_Post_ECBS_edited_clean_Guidelines_NCE_Adjuvant_Final_17122013_WEB.pdf?ua=1. .
714
715
- 716 17. WHO guidelines on nonclinical evaluation of vaccines, WHO Technical Report Series, No.
717 927, 2005 ([https://www.who.int](https://www.who.int/biologicals/publications/trs/areas/vaccines/nonclinical_evaluation/ANNEX%20Nonclinical.P31-63.pdf)
718 [/biologicals/publications/trs/areas/vaccines/nonclinical_evaluation/ANNEX%20Nonclinical.P31-63.pdf](https://www.who.int/biologicals/publications/trs/areas/vaccines/nonclinical_evaluation/ANNEX%20Nonclinical.P31-63.pdf)), refer to section 4.2.2 (Developmental toxicity studies) pg 49-50. Accessed on
719 July 30, 2020. Available at
720 https://www.who.int/biologicals/publications/trs/areas/vaccines/nonclinical_evaluation/ANNEX%20Nonclinical.P31-63.pdf?ua=
721
722
723
- 724 18. Diamond MS, Pierson TC. The Challenges of Vaccine Development against a New Virus
725 during a Pandemic. *Cell Host & Microbe* **27**, 699-703 (2020).
726
- 727 19. Graepel KW, Kochhar S, Clayton EW, Edwards KE. Balancing Expediency and Scientific Rigor
728 in Severe Acute Respiratory Syndrome Coronavirus 2 Vaccine Development. *The Journal of Infectious Diseases* **222**, 180-182 (2020).
729
730
- 731 20. Hotez PJ, Corry DB, Bottazzi ME. COVID-19 vaccine design: the Janus face of immune
732 enhancement. *Nature Reviews Immunology* **20**, 347-348 (2020).
733
- 734 21. Graham BS. Rapid COVID-19 vaccine development. *Science* **368**, 945-946 (2020).
735
- 736 22. He P, Zou Y, Hu Z. Advances in aluminum hydroxide-based adjuvant research and its
737 mechanism. *Human vaccines & immunotherapeutics* **11**, 477-488 (2015).
738
- 739 23. Grifoni A, *et al.* Targets of T Cell Responses to SARS-CoV-2 Coronavirus in Humans with
740 COVID-19 Disease and Unexposed Individuals. *Cell* **181**, 1489-1501.e1415 (2020).
741
- 742 24. Weiskopf D, *et al.* Phenotype and kinetics of SARS-CoV-2-specific T cells in COVID-19 patients
743 with acute respiratory distress syndrome. *Sci Immunol* **5**, eabd2071 (2020).

744

745 25. Iwata-Yoshikawa N, *et al.* Effects of Toll-Like Receptor Stimulation on Eosinophilic Infiltration
746 in Lungs of BALB/c Mice Immunized with UV-Inactivated Severe Acute Respiratory
747 Syndrome-Related Coronavirus Vaccine. *Journal of Virology* **88**, 8597-8614 (2014).

748

749 26. Gupta T, Gupta SK. Potential adjuvants for the development of a SARS-CoV-2 vaccine based
750 on experimental results from similar coronaviruses. *International immunopharmacology* **86**,
751 106717 (2020).

752

753 27. Sellers RS, *et al.* Scientific and Regulatory Policy Committee Points to Consider*: Approaches
754 to the Conduct and Interpretation of Vaccine Safety Studies for Clinical and Anatomic
755 Pathologists. *Toxicologic Pathology* **48**, 257-276 (2020).

756

757 28. Masopust D, Soerens AG. Tissue-Resident T Cells and Other Resident Leukocytes. *Annu Rev*
758 *Immunol* **37**, 521-546 (2019).

759

760 29. Kayraklioglu N, Horuluoglu B, Elango M, Klinman DM. Critical Role of B Cells in TLR7-
761 Mediated Protection Against *L. Monocytogenes* Infection. *Infection and*
762 *immunity*, IAI.00742-00719 (2019).

763

764 30. Clingan JM, Matloubian M. B Cell–Intrinsic TLR7 Signaling Is Required for Optimal B Cell
765 Responses during Chronic Viral Infection. *The Journal of Immunology* **191**, 810-818 (2013).

766

767 31. Hijano DR, Vu LD, Kauvar LM, Tripp RA, Polack FP, Cormier SA. Role of Type I Interferon (IFN)
768 in the Respiratory Syncytial Virus (RSV) Immune Response and Disease Severity. *Frontiers in*
769 *Immunology* **10**, (2019).

770

771 32. Stephens LM VSFaMoTIIIdRSVIV.

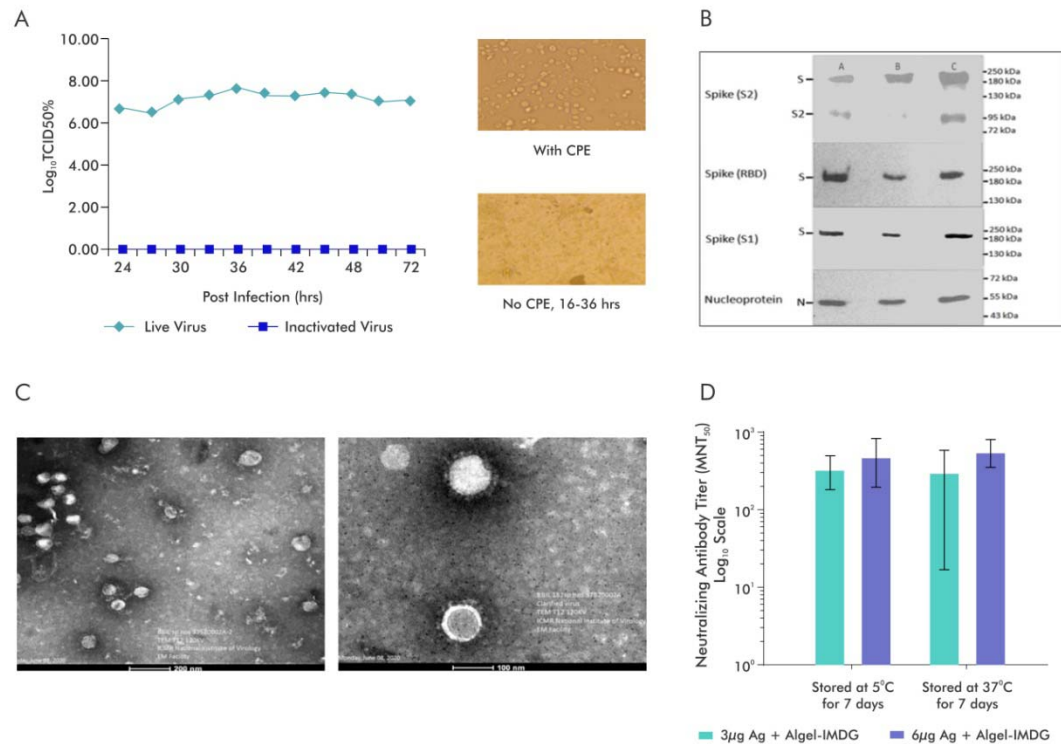
772

773 33. Wang H, *et al.* Development of an Inactivated Vaccine Candidate, BBIBP-CorV, with Potent
774 Protection against SARS-CoV-2. *Cell*, (2020).

775

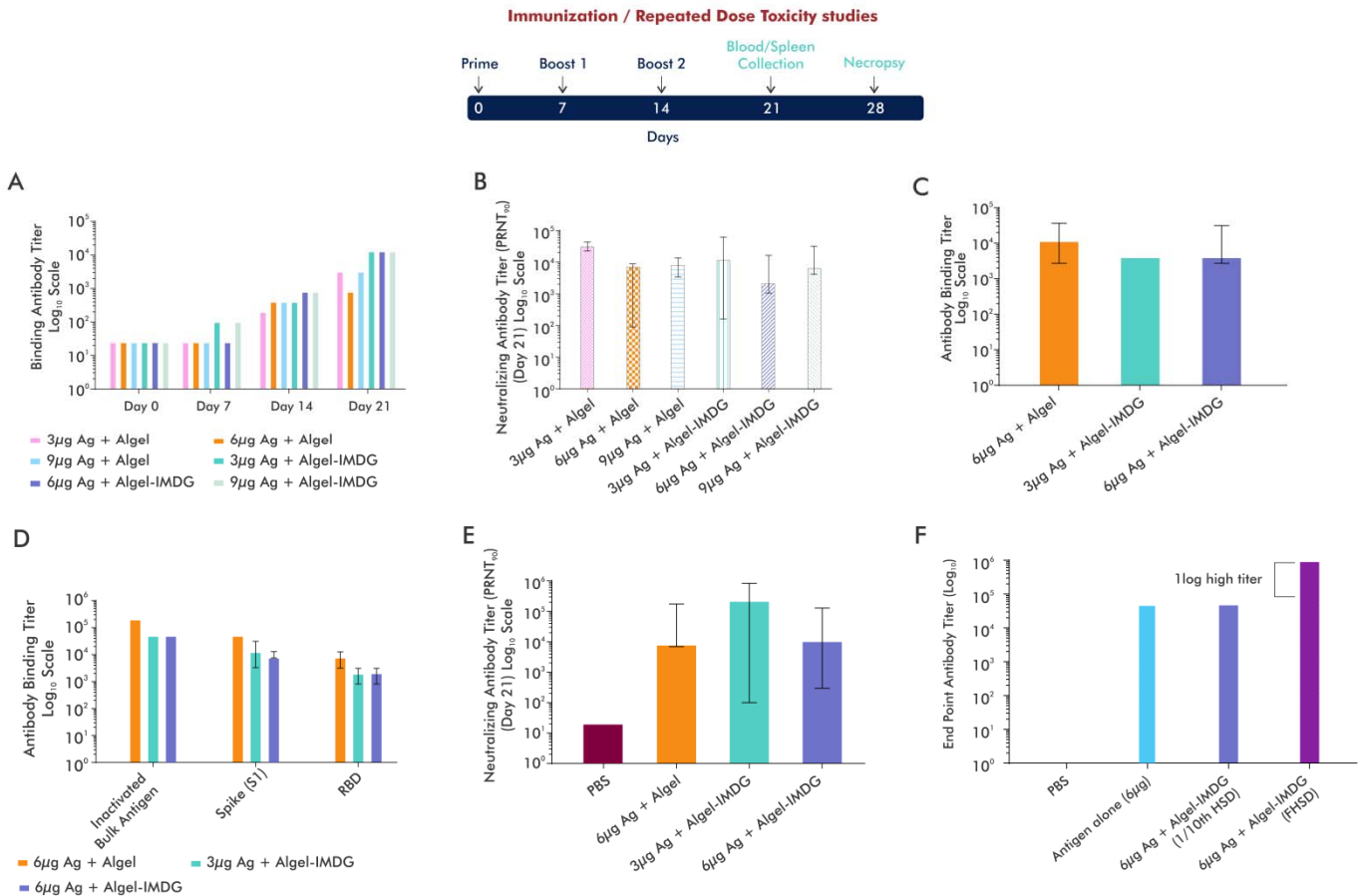
- 776 34. Gao Q, *et al.* Development of an inactivated vaccine candidate for SARS-CoV-2. *Science* **369**,
777 77-81 (2020).
778
- 779 35. Ramakrishnan MA. Determination of 50% endpoint titer using a simple formula. *World J*
780 *Viro* **5**, 85-86 (2016).
781
- 782 36. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol
783 reagent. *J Biol Chem* **193**, 265-275 (1951).
784
- 785 37. Hovden AO, Cox RJ, Madhun A, Haaheim LR. Two doses of parenterally administered split
786 influenza virus vaccine elicited high serum IgG concentrations which effectively limited viral
787 shedding upon challenge in mice. *Scand J Immunol* **62**, 342-352 (2005).
788
- 789 38. 10.4103/ijmr.IJMR_2382_20 DeaNartS-C-iC-pIJMREaopD.
790
- 791 39. Guidelines on the nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines.
792 Available at
793 [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)
794 [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).
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Figure 1: Characterization of inactivated SARS-CoV-2 and evaluation of the stability of BBV152 vaccine formulations.



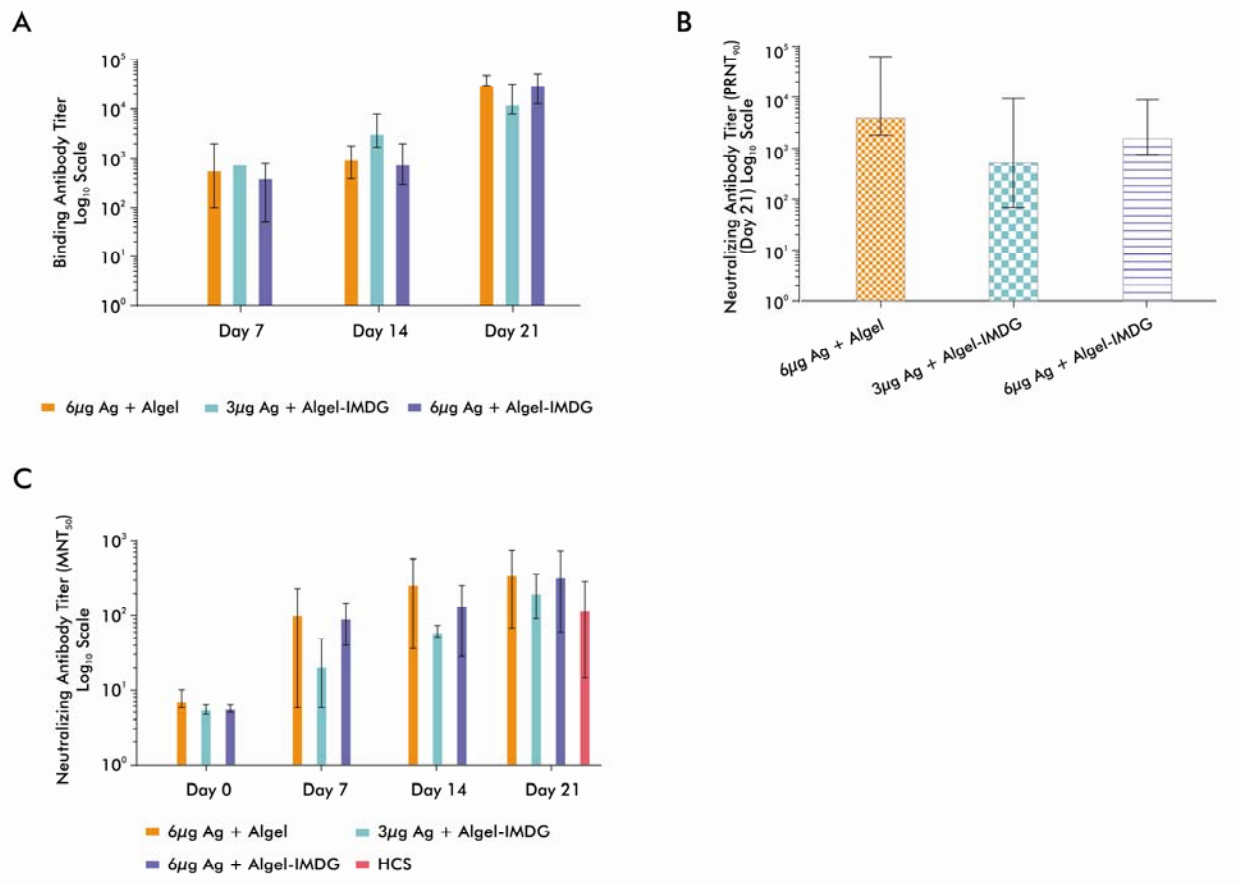
A. SARS-CoV-2 Virus (Strain NIV-770-2020) Growth Kinetics & Cytopathic effect (CPE) of virus before and after Inactivation (i) Virus titer (10^6 - 10^7) measured by CCID₅₀ at every 3 hours up to 48 and after that every 12hrs various time points (24, 27, 30, 33, 36, 39, 42), (ii) Cells with Cytopathic 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



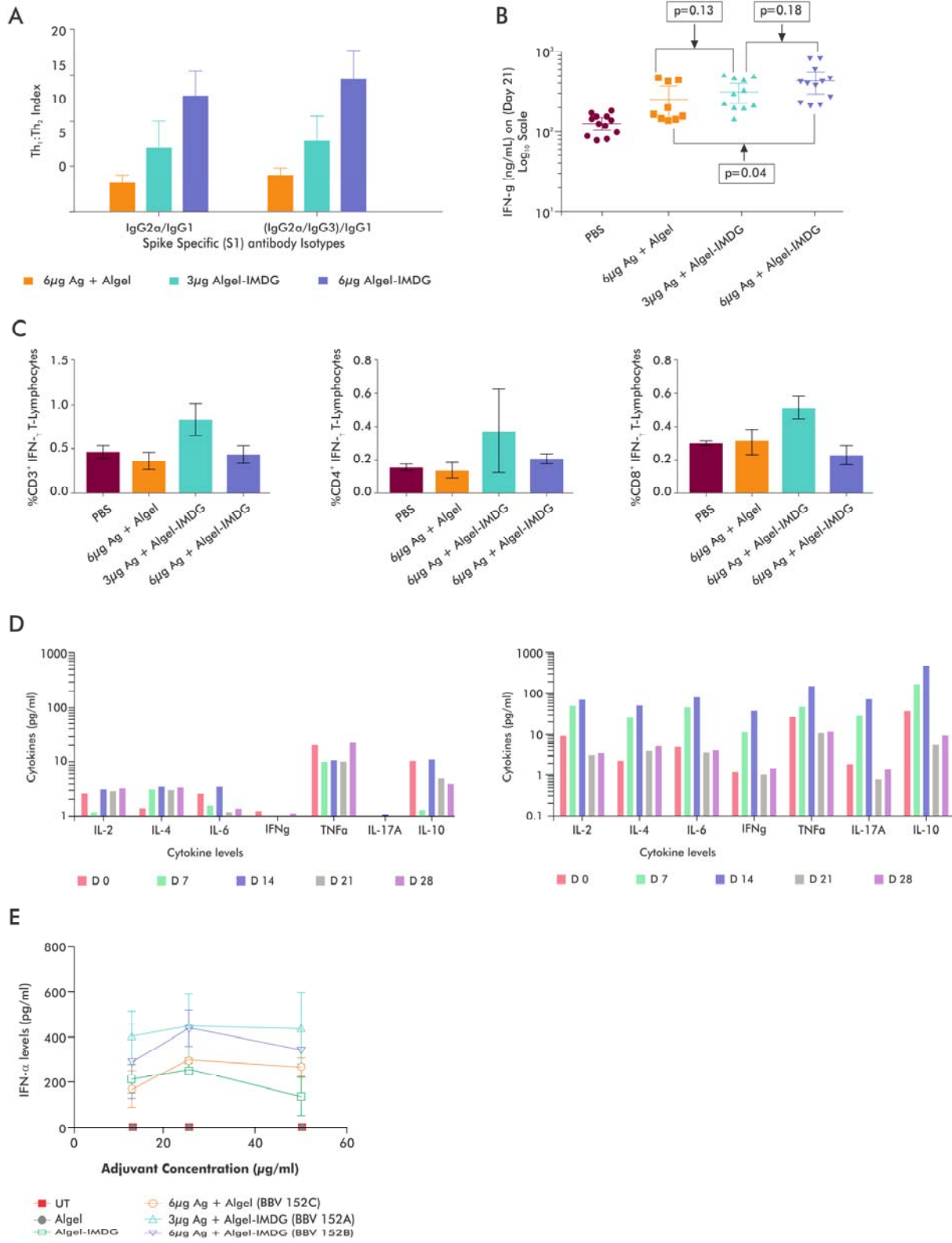
Balb/C mice (n=10) were administered with adjuvanted vaccine formulations via IP route either with 1/20th (Fig A&B) or 1/10th (Fig C, D & E) Human Single Dose (HSD): A. S1 specific Total IgG antibody binding titer performed by ELISA, using sera collected at various time points (Day 0, 7, 14 & 21); B. Neutralizing antibody titers performed by PRNT₉₀, using day 21 sera, when administered with 1/20th HSD respectively; C. S1 specific Total IgG antibody binding titer performed by ELISA, using Day 21 sera when administered with 1/10th 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 1/10th 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 PRNT₉₀ and MNT₅₀. 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. PRNT₉₀ neutralizing antibody titers of Day 21 sera; C. MNT₅₀ neutralizing antibody titers of sera collected at various time points (Day 0, 7, 14 & 21) along Neutralizing antibody titer (MNT₅₀) 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 γ 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⁺ or CD4⁺ or CD8⁺ T lymphocytes producing IFN γ from the respective group of animals (i) CD3⁺ T lymphocytes population, (ii) CD4⁺ T lymphocytes population, (iii) CD8⁺ T lymphocyte population. Error bars indicate Mean \pm SD. Vaccinated mice splenocytes from Balb/C mice (n=10), administered with 1/10th 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 (1/20th HSD via IP route) Left - BBV152C- Antigen 6 μ g+Algel); Right – BBV152B- Antigen 6 μ g+Algel-IMDG, E. IFN α 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.