A single dose, BCG-adjuvanted SARS-CoV-2 vaccine induces Th1-polarized immunity and high-titre neutralizing antibodies in mice

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Abstract

Next-generation vaccines that are safe, effective and with equitable access globally are required to prevent SARS-CoV-2 transmission at a population level. One strategy that has gained significant interest is to 'repurpose' existing licensed vaccines for use against COVID-19. In this report, we have exploited the immunostimulatory properties of bacille Calmette-Guérin (BCG), the vaccine for tuberculosis, to develop a SARS-CoV-2-specific and highly immunogenic vaccine candidate. Combination of BCG with a stabilized, trimeric form of the SARS-CoV-2 spike antigen promoted rapid development of virus-specific IgG antibodies in the sera of vaccinated mice, which could be further augmented by the addition of alum. This vaccine formulation, termed BCG:CoVac, induced a Th1-biased response both in terms of IgG antibody subclass and cytokine release by vaccine-specific $CD4^+$ and $CD8^+$ T cells. A single dose of BCG:CoVac was sufficient to induce high-titre SARS-CoV-2 neutralizing antibodies (NAbs) that were detectable as early as 2 weeks post-vaccination; NAb levels were greater than that seen in the sera of SARS-CoV-2-infected individuals. Boosting of BCG:CoVac-primed mice with a heterologous vaccine combination (spike protein plus alum) could further increase SARS-CoV-2 spike protein-specific antibody response. BCG:CoVac would be broadly applicable for all populations susceptible to SARS-CoV-2 infection and in particular could be readily incorporated into current vaccine schedules in countries where BCG is currently used.

46 **Importance**

47 Effective distribution of vaccine to low- and middle-income countries is critical for the control
48 of the COVID-19 pandemic. To achieve this, vaccines must offer effective protective immunity
49 yet should be cheap to manufacture and meet cold chain management requirements. This study
50 describes a unique COVID-19 vaccine candidate, termed BCG:CoVac, that when delivered as
51 a single dose induces potent SARS-CoV-2 specific immunity in mice, particularly through
52 generation of high-titre, anti-viral neutralising antibodies. BCG:CoVac is built on safe and
53 well-characterised vaccine components: 1) the BCG vaccine, used for control of tuberculosis
54 since 1921 which also has remarkable 'off target' effects, protecting children and the elderly
55 against diverse respiratory viral infections; 2) Alhydrogel adjuvant (Alum), a low cost, globally
56 accessible vaccine adjuvant with an excellent safety record in humans (part of >20 licensed
57 human vaccines and in use >70 years); 3) Stabilized, trimeric SARS-CoV-2 spike protein,
58 which stimulates immune specificity for COVID-19. Further assessment in humans will
59 determine if BCG:CoVac can impart protective immunity against not only SARS-CoV-2, but
60 also other respiratory infections where BCG has known efficacy.

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63

64 **Introduction**

65 The search for a COVID-19 vaccine has progressed at unprecedented speed and magnitude.
66 Greater than 200 COVID-19 vaccine candidates are in development, with a subset of these now
67 in late-phase clinical trials. The impressive number of candidates suggest extensive diversity
68 in the COVID vaccine pipeline, however most of these vaccines fall into a small group of
69 defined classes, which can be represented by the candidates that have reached human trials.
70 The development speed of RNA vaccines has allowed these to move quickly into early trials
71 and results from Phase 3 efficacy results are very encouraging[1]. It will now be critical to
72 determine if these vaccines can induce long-term immunity and be readily distributed to low-
73 and middle-income countries, particularly as these vaccines may have complex logistic
74 requirements (e.g. storage at ultra-low temperature). These vaccines also require multiple
75 doses, a barrier to mass vaccination. Viral-vectored vaccines have been shown to impart some
76 level of SARS-CoV-2 specific immunity in humans, although responses were relatively poor
77 in Phase 2 assessment of the Ad5-nCOV vaccine, particularly in older age groups; this was
78 most apparent when examining neutralising antibodies (NAbs) to live virus[2]. While this
79 vaccine class is generally well tolerated, mild to moderate adverse events are observed in a
80 sizable proportion of vaccinees. For example, testing of the ChAdOx1 nCoV-19 vaccine
81 included administration of paracetamol to reduce side effects[3]. Thus, while RNA and viral-
82 vectored vaccines will be the first to be licensed and could provide some impact on the
83 pandemic, in the long-term vaccines will need to possess a strong safety profile, invoke
84 sustained protective immunity and be cost effective to produce at scale.

85

86 Vaccine approaches with a proven safety and efficacy track record may provide the best
87 solution for the long-term control of COVID-19. Results from the first clinical trial using one
88 of the more established vaccine approaches, recombinant spike protein in adjuvant (NVX-

89 CoV2373), were promising; the vaccine generated strong NAbs and specific $CD4^+$ T cell
90 responses in vaccinees[4]. Two versions of inactivated SARS-CoV-2 formulated in alum have
91 been assessed in Phase 2 trials and showed an excellent safety profile[5,6]. This is consistent with
92 the well-established safety and immunogenicity of alum in humans[7] and the success of other
93 inactivated viral vaccines to control major human pathogens. For one of these vaccines
94 (CoronaVac), NAbs titres were relatively low after immunisation, although higher than that
95 needed for the vaccine to protect non-human primates against disease[8]. None-the-less, the
96 protective capacity of CoronaVac-induced immunity will only be determined after large Phase
97 3 vaccine trials are completed.
98

99 One unique strategy is to 'repurpose' existing licensed vaccines for use against COVID-19.
100 Much interest has focussed on *Mycobacterium bovis* bacille Calmette-Guerin (BCG), the
101 tuberculosis (TB) vaccine. A large amount of data has been accumulated to show that BCG has
102 beneficial, non-specific effects on immunity that affords protection against other pathogens,
103 particularly respiratory infections[9]. Most recently, BCG vaccination was shown to protect
104 against viral respiratory tract infections in the elderly (greater than 65 years old) with no
105 significant adverse events[10]. This non-specific protective effect is attributed to the ability of
106 BCG to induce 'trained immunity' i.e. reprogramming of innate immune responses to provide
107 heterologous protection against disease. For these reasons, a Phase 3, randomised controlled
108 trial in healthcare workers has commenced, in order to determine if BCG vaccination can
109 reduce the incidence and severity of COVID-19 (The BRACE Trial)[9]. While the BRACE trial
110 will determine if BCG can reduce the impact on COVID-19 during the current pandemic, BCG
111 does not express SARS-CoV-2 specific antigens and thus would not be expected to induce
112 long-term SARS-CoV-2-specific immune memory.
113

114 In this report, we have exploited the immunostimulatory properties of BCG to develop a SARS-
115 CoV-2 vaccine, termed BCG:CoVac, that combines a stabilized, trimeric form of the spike
116 protein with the alum adjuvant. BCG:CoVac resulted in the stimulation of SARS-CoV-2-
117 specific antibody and T cell responses in mice after a single vaccination, including the
118 elicitation of high-titre NAbs, suggesting that BCG can synergise with spike antigen and alum
119 to result in a highly immunogenic and promising vaccine candidate.
120
121
122

123 **Materials and Methods**

124 **Bacterial culture**

125 *M. bovis* BCG (strain Pasteur) was grown at 37°C in Middlebrook 7H9 medium (Becton
126 Dickinson, BD, New Jersey, USA) supplemented with 0.5% glycerol, 0.02% Tyloxapol, and
127 10% albumin-dextrose-catalase (ADC) or on solid Middlebrook 7H11 medium (BD)
128 supplemented with oleic acid–ADC. To prepare single cell suspensions, cultures in exponential
129 phase (OD_{600}=0.6) were washed in PBS, passaged 10 times through a 27G syringe, briefly
130 sonicated and centrifuged at low speed (800 rpm) for 10 min to remove residual bacterial
131 clumps. BCG suspensions were frozen at -80° C in PBS 20% glycerol and colony forming units
132 (CFU) for vaccination enumerated on supplemented Middlebrook 7H11 agar plates.

133

134 **Ethics statement and patient samples**

135 All mouse experiments were performed according to ethical guidelines as set out by the Sydney
136 Local Health District (SLHD) Animal Ethics and Welfare Committee, which adhere to the
137 Australian Code for the Care and Use of Animals for Scientific Purposes (2013) as set out by
138 the National Health and Medical Research Council of Australia. All experiments within this
139 manuscript were approved under protocol number 2020/019 by the SLHD Animal Ethics and
140 Welfare Committee. COVID-19 patients were recruited through RPA Virtual Hospital, a
141 virtual care system enabling remote monitoring of patients[11]. Serum specimens (n=22) were
142 collected from patients with PCR-confirmed COVID-19 (mean time from diagnosis to blood
143 sampling of 16 days). The study protocol was approved by the RPA ethics committee (Human
144 ethics number X20-0117 and 2020/ETH00770) and by the participants' verbal consent. All
145 associated procedures were performed in accordance with approved guidelines.

146

147

148 **Immunization and blood collection**

149 Female C57BL/6 (6-8 weeks of age) were purchased from Australian BioResources (Moss
150 Vale, Australia), and housed at the Centenary Institute in specific pathogen-free conditions.
151 SARS-CoV-2 full-length spike stabilized, trimeric protein (SpK) was expressed in
152 EXPI293F™ cells and purified as described previously[12]. Mice were vaccinated
153 subcutaneously (s.c) with $5x10^5$ CFU of BCG alone, 5 μg of SpK combined with either BCG
154 (BCG^{SpK}) or 100 μg of Alhydrogel (Alum) (Invivogen, California, USA, $Alum^{SpK}$), or a
155 combination of BCG ($5x10^5$ CFU), SpK (5 μg) and Alyhydrogel (100 μg) (BCG:CoVac).
156 When required mice were boosted three weeks after the first vaccination with 5 μg of SpK
157 combined with 100 μg of Alhyhdrogel. Mice were bled twice weekly after the first
158 immunization (collected in 10 μl of Heparin 50 U/mL). Plasma was collected after
159 centrifugation at 300 g for 10 min and remaining blood was resuspended in 1 mL of PBS
160 Heparin 20 U / mL, stratified on top of Histopaque 10831 (Sigma-Aldrich, Missouri, USA) and
161 PBMCs layer collected after gradient centrifugation.

162

163 **PBMC restimulation and intracellular staining**

164 To assess SpK-specific cytokine production by T cells, murine PBMCs were stimulated for 4
165 hours with SpK (5 μg/mL) and then supplemented with Protein Transport Inhibitor cocktail
166 (Life Technologies, California, USA) for a further 10-12 hours. Cells were surface stained with
167 Fixable Blue Dead Cell Stain (Life Technologies) and the marker-specific fluorochrome-
168 labelled antibodies rat anti-mouse CD4-AF700 (clone RM414, 1:200, BD cat#557956), rat
169 anti-mouse CD8-APCy7 (clone 53-6.7, 1:200, BD cat#557654), rat anti-mouse CD44-FITC
170 (clone IM7, 1:300, BD cat#561859). Cells were then fixed and permeabilized using the BD
171 Cytofix/Cytoperm™ kit according to the manufacturer's protocol. Intracellular staining was
172 performed using rat anti-mouse IFN-γ-PECy7 (clone XMG1-2, 1:300, BD cat#557649), rat

173 anti-mouse IL-2-PE (clone JES6-5H4, 1:200, BD cat#554428), rat anti-mouse IL-17-PB (clone
174 TC11-18H10.1, 1:200, cat#506918, BioLegend California, USA), rat anti-mouse TNF-
175 PErCPCy5.5 (clone MP6-XT22, 1:200, BD cat#560659). Samples were acquired on a BD
176 LSR-Fortessa (BD), and analyzed using FlowJo^{TM} analysis software (Treestar, USA).
177
178 **Antibody ELISA**
179 Microtitration plates (Corning, New York, USA) were incubated overnight with 1 µg/mL SpK
180 at room temperature, blocked with 3% BSA and serially diluted plasma samples were added to
181 for 1 hour at 37˚C. Plates were washed and biotinylated polyclonal goat anti-mouse IgG1
182 (1:50,000, abcam Cambridge, UK, cat#ab97238), polyclonal goat anti-mouse IgG2c (1:10,000,
183 abcam, cat# ab97253), or polyclonal goat anti-mouse IgG (1:350,000, clone abcam
184 cat#ab6788) added for 1 hour at RT. After incubation for with streptavidin-HRP (1:30,000,
185 abcam, cat#405210) for 30 minutes at RT, binding was visualized by addition of tetramethyl
186 benzene (Sigma-Aldrich). The reaction was stopped with the addition of 2N H_2SO_4 and
187 absorbances were measured at 450 nm by the M1000 pro plate reader (Tecan, Männedorf,
188 Switzerland). End point titres were calculated as the dilution of the sample that reached the
189 average of the control serum ± 3 standard deviations.
190
191 **Live virus neutralization assay**
192 Two-fold dilutions of patient plasma samples were mixed with an equal volume of virus
193 solution (8 x 10^3 TCID50/ml) and incubated at 37°C for 1 hour. After the virus-plasma
194 incubation, 40 µl virus/plasma mixture was added to Vero E6 cells seeded in 384-well plates
195 at 5 x 10^3 cells per well in a final volume of 40 µl. Plates were then incubated for 72 hours at
196 37°C, 5% CO_2. Cell nuclei were stained and each well was imaged by a high-content
197 fluorescence microscopy system (IN Cell Analyser 2500HS, Cytiva Life Sciences, Parramatta,

198 Australia). The number of cells per well was determined with the automated InCarta image
199 analysis software (Cytiva). The percentage of virus neutralisation for each well was calculated
200 using the following formula: Neutralisation (%) = (D-(1-Q)) x 100/D, where "Q" represents a
201 well's nuclei count divided by the nuclei count of the untreated control (i.e. cells and media
202 only), and "D" represents 1 minus the Q value for the positive infection control (i.e. cells +
203 virus, without plasma).
204
205 **Statistical analysis**
206 The significance of differences between experimental groups was evaluated by one-way
207 analysis of variance (ANOVA), with pairwise comparison of multi-grouped data sets achieved
208 using the Dunnett's post-hoc test. Differences were considered statistically significant when p
209 ≤ 0.05.
210
211

212 **Results and Discussion**

213 **BCG promotes SARS-CoV-2 spike protein-specific antibody and T cell responses in**
214 **vaccinated mice**

215 The immunostimulatory properties of BCG[13] led to us to test if the vaccine could serve as the
216 backbone of a unique vaccine platform. To examine this, mice were vaccinated s.c with a single
217 dose of BCG Pasteur strain formulated with a stabilized, trimeric form of the SARS-CoV-2
218 spike protein[14] (BCG^{Spk}) and the titre of IgG2c or IgG1 anti-SpK antibodies (Abs) determined
219 at various timepoints post-immunization (Fig. 1A). While BCG-vaccination resulted in
220 background levels of anti-SpK Abs, titres were approximately 100-fold higher for both isotypes
221 after BCG^{Spk} vaccination, and similar to Ab levels achieved with SpK formulated in
222 Alyhydrogel/alum (Alm^{SpK}) (Fig. 1B, 1C). Addition of alum to BCG^{Spk} (BCG:CoVac) further
223 increased Ab titres, particularly IgG2c, which were significantly greater after BCG:CoVac
224 vaccination compared with mice immunised with either BCG or Alm^{SpK}, at all timepoints
225 examined (Fig. 1B, 1C). Thus BCG could serve to promote early and pronounced anti-SARS-
226 CoV-2 humoral response when co-delivered with the trimeric SpK antigen, which could be
227 further enhanced with the addition of alum.

228

229 IgG2c Ab isotype correlates with Th1-like immunity in C57BL/6 mice[15], and such responses
230 are considered necessary for effective protection against SARS-CoV-2 infection[16]. We
231 therefore examined the frequency of IFN-γ-secreting T cells after a single dose BCG:CoVac
232 at 2 weeks post-vaccination. BCG^{SpK} and BCG:CoVac induced the generation of SpK-specific
233 $CD4^+$ and $CD8^+$ T cells secreting IFN-γ (Fig. 2A, 2B), consistent with Th1 immunity observed
234 after BCG vaccination[17]. The greatest response was observed after vaccination with
235 BCG:CoVac, with the number IFN-γ-secreting T cells significantly increased compared to
236 responses of mice vaccinated with either BCG or $Alum^{SpK}$.

237

238 The relative paucity of IFN-γ-secreting T cells observed after Alum^{SpK} vaccination corresponds
239 with that previously seen with alum-precipitated vaccines using the spike protein[18] and is
240 consistent with studies that show preferential priming of Th2-type immunity by aluminum
241 hydroxide-based adjuvants[19]. The ability of BCG:CoVac to induce Th1-like immunity
242 correlates to the known adjuvant effect of BCG components to induce such responses[20]. This
243 has clear importance, as T cell responses in recovering COVID-19 patients are predominately
244 Th1[21], expression of IFN-γ is lower in severe COVID-19 cases compared to mild ones[22] and
245 the induction of Th2 immunity is correlated with vaccine-associated enhanced respiratory
246 disease (VAERD)[23].We also observed background levels of the inflammatory cytokines IL-17
247 and TNF after BCG:CoVac delivery, suggesting reduced levels of potentially deleterious,
248 circulating inflammatory cytokines (Fig. 2B). Heightened expression of IL-17 correlates with
249 severe COVID-19 disease[24], while blocking IL-17 has been suggested as a possible therapy to
250 treat acute respiratory distress syndrome in SARS-CoV-2-infected individuals[25]. In addition,
251 the development of VAERD is also associated with Th17 immunity[26].

252

253 **High-titre SARS-CoV-2 neutralizing antibodies after a single immunization with**
254 **BCG:CoVac**

255 We next determined if a single dose of BCG:CoVac could induce neutralizing antibodies
256 (NAbs) to block live SARS-CoV-2 entry into host cells, a critical determinant for protection
257 against infection[23]. No NAbs were detected in the sera of vaccinated mice with BCG (Fig 3A).
258 Surprisingly, NAb titres were at near background levels for mice vaccinated with BCG^{SpK} (Fig.
259 3A), despite the high levels of IgG Ab isotypes detected in these same animals (Fig. 1).
260 Encouragingly, high level of NAbs were detected as early as 2 weeks post-immunization upon
261 vaccination of mice with BCG:CoVac, and titres were significantly increased compared to

262 vaccination with Alum^{SpK} (approximate 10 fold). The mean NAbs titres in the sera of
263 BCG:CoVac-vaccinated mice were approximately 10-fold greater than that seen in sera from
264 SARS-CoV-2 infected individuals (Fig 3A). Although the levels of NAbs peaked at 2 weeks
265 post-vaccination with BCG:CoVac, they remained significantly elevated up to day 42 post-
266 immunization compared to the other immunized groups.
267
268 As previous work suggests that IgG antibody levels correlates with NAbs titres after SARS-
269 CoV-2 infection[27], we examined if a similar phenomenon was observed after vaccination with
270 BCG:CoVac. Strong corelation ($r > 0.9$) was observed between IgG2c isotype and NAbs in
271 groups vaccinated with BCG:CoVac or Alum^{SpK} (Fig. 3B), with a significant yet less robust
272 correlation between IgG1 and NAbs for these groups (Fig. 3C). There was no correlation
273 between NAbs and either IgG1 or IgG2c Ab for mice vaccinated with BCG^{SpK} alone (Fig. 3D,
274 3E). These data suggests alum is required for the optimal generation of NAbs after BCG:CoV
275 vaccination; this is a significant advantage for implementation of the vaccine, due to the low
276 cost and long standing safety record of alum[7,28]. Importantly, the potential risk of VAERD due
277 to the selective induction of Th2 by alum is offset by the strong Th1immunity driven by BCG
278 (Fig. 2B).
279
280 **Augmentation of BCG:CoVac antibody response by heterologous vaccine boosting**
281 COVID-19 subunit vaccines typically display poor immunity after a single dose and require a
282 booster to allow sufficient generation of NAbs[29]. Whilst we observed high-titre NAbs as early
283 as two weeks post-BCG:CoVac vaccination (Fig. 3), we sought to determine if responses could
284 be further augmented by boosting with a prototype subunit vaccine (Alum^{SpK}) (Fig. 4A). At 7
285 days post-boost, IgG2c titres in sera from mice primed with BCG^{SpK} or BCG:CoVac remained
286 elevated by day 42 after the prime (Fig. 4B). A corresponding augmentation of NAbs was also

287 seen in these boosted groups, with significantly elevated response in BCG:CoVac primed mice
288 boosted with Alum^{SpK} (Fig. 4C). Boosting Alum^{SpK} vaccination with a second dose led to a
289 greater than 10-fold increase in NAbs in boosted mice, however responses were significantly
290 lower than that observed with the superior BCG:CoVac-prime, Alum^{SpK-}boost combination
291 (Fig. 4C). Taken together, these data indicate that the strong antigen-specific immunity
292 imparted by BCG:CoVac can be further enhanced by heterologous boosting with a second
293 SARS-CoV-2 vaccine.
294
295 **Conclusion**
296 In this report we demonstrate a single dose of the BCG:CoVac vaccine candidate can induce
297 rapid and pronounced development of SARS-CoV-2-specific cellular and humoral immune
298 responses in mice. Encouragingly, the level of immunity observed (particularly the generation
299 of neutralizing antibodies) is equivalent to or exceeds responses elicited by vaccines in late
300 stage humans trials, when these candidates were testing in the murine model[29-31]. BCG:CoVac
301 may have the additional advantage of inducing protection against other respiratory infection
302 where BCG is known to induce some level of protective immunity[13]. In addition, the possibility
303 that prior BCG exposure may impart protection against severe COVID-19[32], which is currently
304 under evaluation through randomised control studies[9], raises the possibility that a BCG-based
305 vaccine could afford protection against SARS-CoV-2 escape mutants or new pandemic
306 coronavirus that may emerge. BCG:CoVac could also provide additional benefit in countries
307 where BCG is part of immunization programs for the control of TB, based on recent findings
308 that a second BCG vaccination significantly reduced rates of *M. tuberculosis* infection[33].
309 Overall, the strong anti-SARS-CoV-2 immunity afforded by BCG:CoVac, together with the
310 excellent safety profile of both BCG and alum, supports the progression of BCG:CoVac to
311 human trials.

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414 **Figure Legends**

415 **Figure 1. A single immunisation with BCG:CoVac vaccination induces rapid**
416 **development of anti-SARS-CoV-2 spike antibodies.** C57BL/6 mice were vaccinated
417 subcutaneously with PBS, BCG, BCG^{SpK}, $Alum^{SpK}$ or BCG:CoVac and whole blood collected
418 at day 14, 28 and 42 (**A**). Spike-specific IgG2c (**B**) and IgG1 (**C**) titres in plasma were
419 determined by ELISA with endpoint titres estimated by the sigmoidal curve of each sample
420 interpolated with the threshold of the negative sample± 3 standard deviations. The dotted line
421 shows the limit of detection. The significance of differences between groups compared to
422 BCG^{Spk} (*$p<0.05$, **$p<0.01$, ***$p<0.01$) or $Alum^{Spk}$ (†$p<0.05$, ††$p<0.01$, †††$p<0.001$) was
423 determined by one-way ANOVA with Dunnett's post hoc test for multiple comparisons.

424

425 **Figure 2. BCG:CoVac induces the generation of IFN-γ-secreting T cells with minimal**
426 **release of inflammatory cytokines.** Mice (n=3-4) were vaccinated as in Figure 1 and two
427 weeks post-vaccination PBMCs were restimulated *ex vivo* with 5 μg/mL of SARS-CoV-2
428 spike protein for 4 hrs and cytokine production was determined by flow cytometry. A.
429 Representative dot plots of $CD44^+$ $CD4^+$ T cells and $CD44^+$ $CD8^+$ T cells expressing IFN-γ. B.
430 Number of circulating $CD4^+$ and $CD8^+$ T cells expressing IFN-γ or $CD4^+$ T cells expressing
431 IL-17 or TNF. The significant differences between groups compared to BCG^{Spk} (*$p<0.05$) or
432 $Alum^{Spk}$ (†$p<0.05$) was determined by one-way ANOVA with Dunnett's post hoc test for
433 multiple comparisons.

434

435 **Figure 3. BCG:CoVac induces high titre neutralizing antibodies against live SARS-CoV-**
436 **2 which correlates with production of antigen-specific IgG2c.** Plasma from mice vaccinated
437 as in Figure 1 tested for neutralizing activity against live SARS-CoV-2 infection of Vero E6
438 cells. Neutralizing antibody (NAb) titres (IC_{50}) were calculated as the highest dilution of

439 plasma that still retained at least 50% inhibition of infection compared to controls (**A**). NAb
440 titres from PCR confirmed SARS-CoV-2-infected individuals (COVID) were determined
441 using the same method. Spearman correlations of spike-specific IgG2c (**B**) or IgG1 (**C**) titres
442 and NAbs after $Alum^{SpK}$ or BCG:CoVac vaccination are shown, as well as correlation of IgG2c
443 (**D**) or IgG1 (**E**) titres and NAbs after vaccination with BCG^{SpK}. The dotted line shows the limit
444 of detection. The significance of differences between groups compared to BCG^{Spk} (**p<0.01,
445 ***p<0.01) or $Alum^{Spk}$ (†p<0.05, †††p<0.001) was determined by one-way ANOVA with
446 Dunnett's post hoc test for multiple comparisons.
447
448 **Figure 4. Heterologous boosting of BCG:CoVac-primed mice results in augmented**
449 **SARS-CoV-2-specific IgG2c titres and neutralizing antibodies.** C57BL/6 mice were
450 vaccinated subcutaneously (s.c.) with PBS, BCG, BCG^{SpK}, $Alum^{SpK}$ or BCG:CoVac. At day
451 21 they received a s.c boost with $Alum^{Spk}$ (**A**). Spike-specific IgG2c titres in plasma were
452 determined by ELISA with endpoint titres estimated by the sigmoidal curve of each sample
453 interpolated with the threshold of the negative sample± 3 standard deviations (**B**). Neutralizing
454 antibody titres (IC_{50}) were calculated as the highest dilution of plasma that still retained at least
455 50% inhibition of infection compared to controls (**C**). The dotted line shows the limit of
456 detection. The significance of differences between groups compared to BCG^{Spk} (*p<0.05,
457 **p<0.01, ***p<0.01) or $Alum^{Spk}$ (†p<0.05, ††p<0.01, †††p<0.001) was determined by one-
458 way ANOVA with Dunnett's post hoc test for multiple comparisons.
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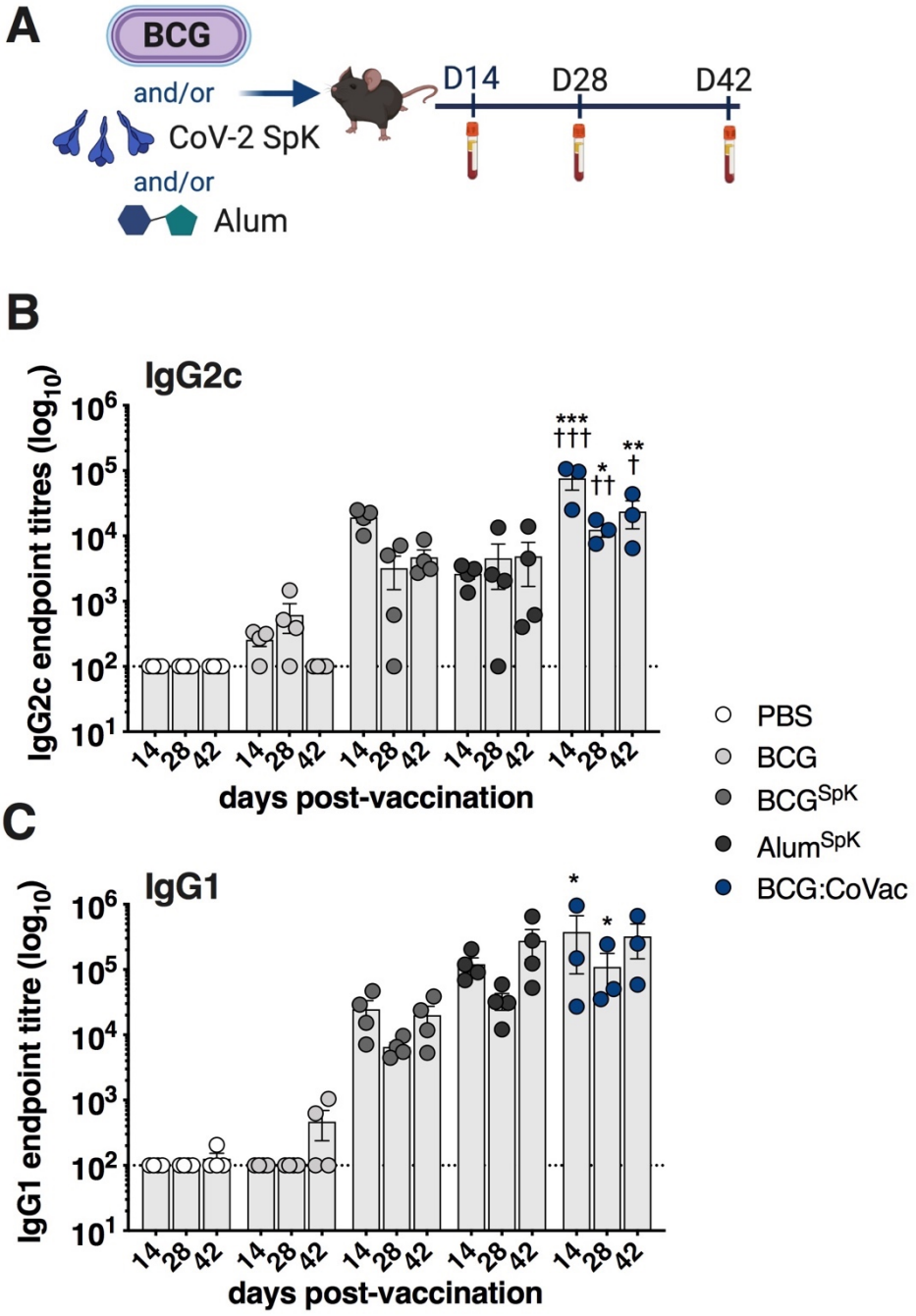
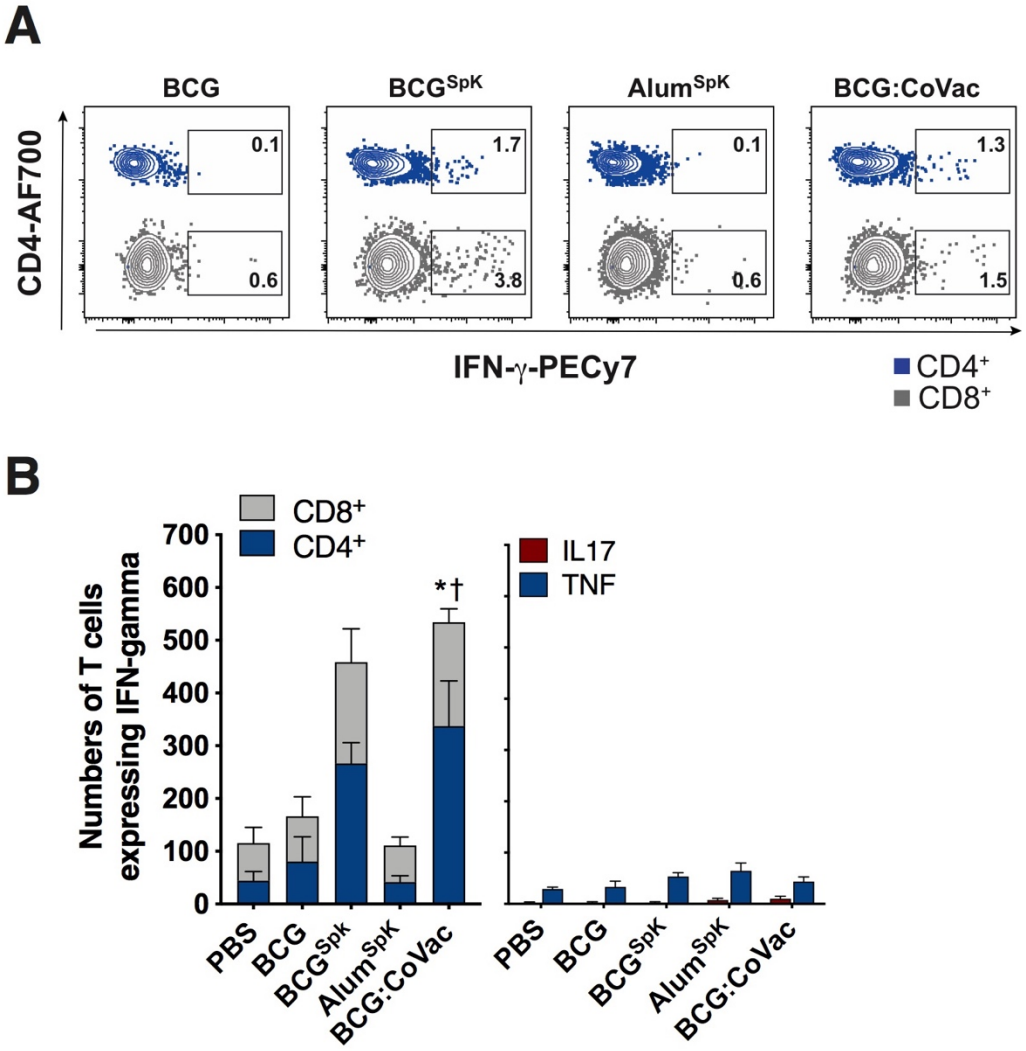
Figure 1


A
BCG
and/or
CoV-2 SpK
and/or
Alum
D14
D28
D42
B
IgG2c
IgG2c endpoint titres (log10)
days post-vaccination
C
IgG1
IgG1 endpoint titre (log10)
days post-vaccination
PBS
BCG
BCGSpK
AlumSpK
BCG:CoVac

464

Figure 2


A
BCG
BCGSpK
AlumSpK
BCG:CoVac
CD4-AF700
0.1
0.6
1.7
3.8
0.1
0.6
1.3
1.5
IFN-γ-PECy7
CD4+
CD8+
B
CD8+
CD4+
IL17
TNF
Numbers of T cells expressing IFN-gamma
700
600
500
400
300
200
100
0
*†
PBS
BCG
BCGSpk
AlumSpK
BCG:CoVac
PBS
BCG
BCGSpK
AlumSpK
BCG:CoVac

465

Figure 3

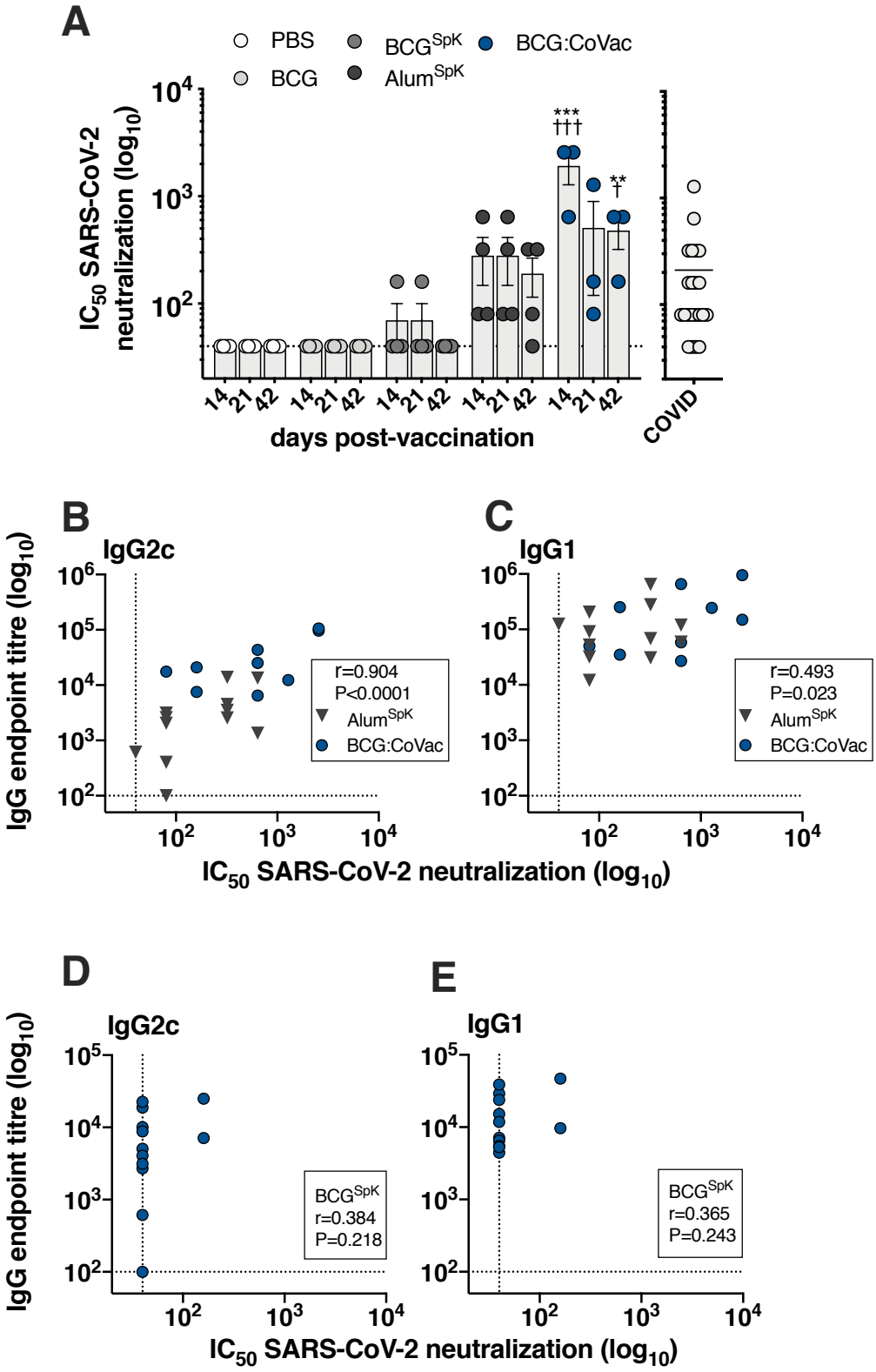


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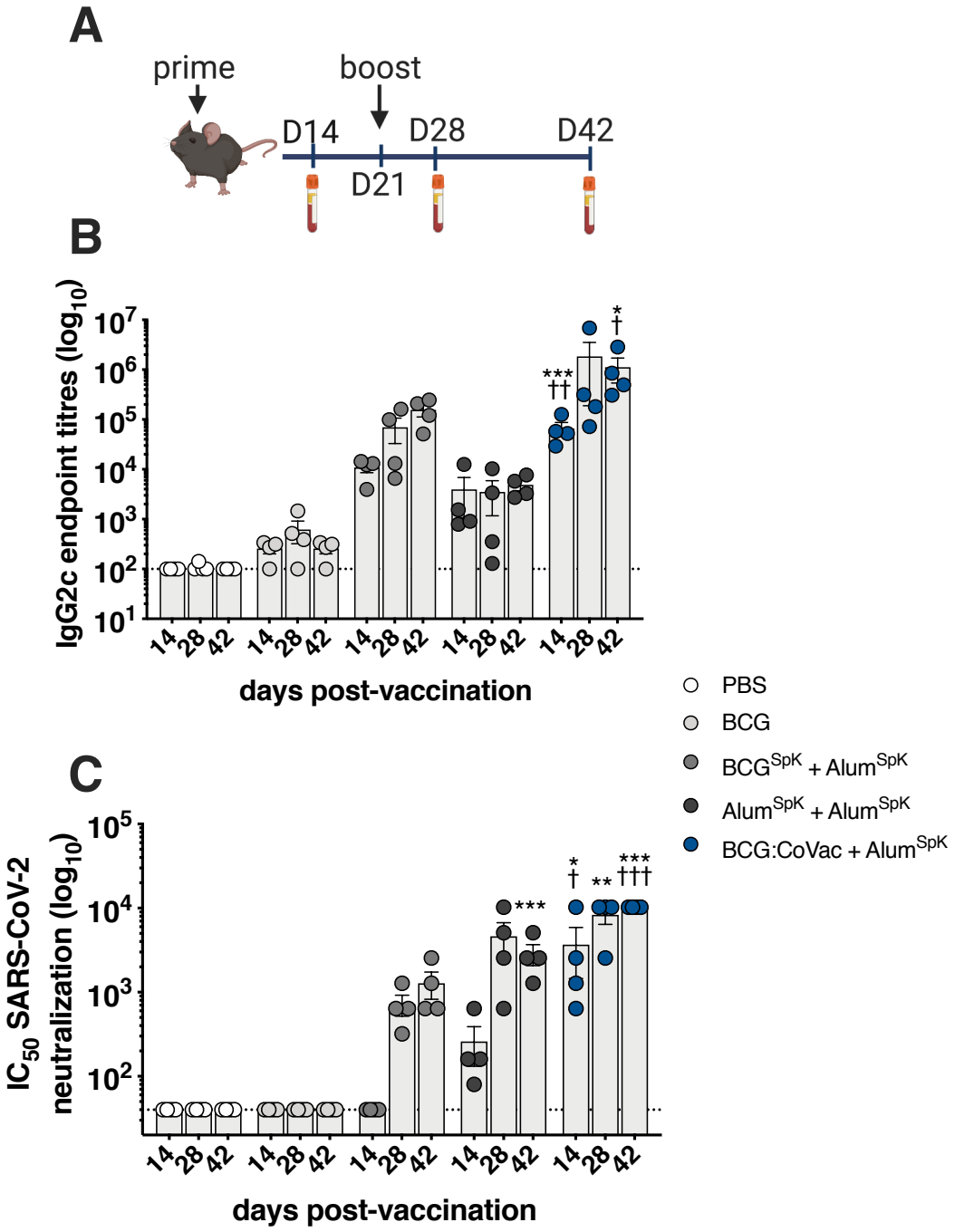
Figure 4



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