

1 **Single dose immunization with a COVID-19 DNA vaccine encoding a chimeric**
2 **homodimeric protein targeting receptor binding domain (RBD) to antigen-presenting cells**
3 **induces rapid, strong and long-lasting neutralizing IgG, Th1 dominated CD4⁺ T cells and**
4 **strong CD8⁺ T cell responses in mice**

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7 Gunnstein Norheim, Elisabeth Stubrud, Lise Madelene Skullerud, Branislava Stankovic, Stalin
8 Chellappa, Louise Bjerkan, Katarzyna Kuczkowska, Elisabeth Müller, Monika Sekelja and
9 Agnete B. Fredriksen

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12 Affiliations: Vaccibody, Oslo Research Park, Gaustadalléen 21, 0349 Oslo, Norway

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17 Corresponding author: Gunnstein Norheim gnorheim@vaccibody.com

18 **Keywords:** COVID-19, SARS-CoV-2, vaccine, DNA, BALB/c, neutralizing antibodies, CD8+,
19 RBD

20

21 **Abstract**

22 The pandemic caused by the SARS-CoV-2 virus in 2020 has led to a global public health
23 emergency, and non-pharmaceutical interventions required to limit the viral spread are severely
24 affecting health and economies across the world. A vaccine providing rapid and persistent
25 protection across populations is urgently needed to prevent disease and transmission. We here
26 describe the development of novel COVID-19 DNA plasmid vaccines encoding homodimers
27 consisting of a targeting unit that binds chemokine receptors on antigen-presenting cells (human
28 MIP-1 α /LD78 β), a dimerization unit (derived from the hinge and C_H3 exons of human IgG3),
29 and an antigenic unit (Spike or the receptor-binding domain (RBD) from SARS-CoV-2). The
30 candidate encoding the longest RBD variant (VB2060) demonstrated high secretion of a
31 functional protein and induced rapid and dose-dependent RBD IgG antibody responses that
32 persisted up to at least 3 months after a single dose of the vaccine in mice. Neutralizing antibody
33 (nAb) titers against the live virus were detected from day 7 after one dose. All tested dose
34 regimens reached titers that were higher or comparable to those seen in sera from human
35 convalescent COVID-19 patients from day 28. T cell responses were detected already at day 7,
36 and were subsequently characterized to be multifunctional CD8⁺ and Th1 dominated CD4⁺ T
37 cells. Responses remained at sustained high levels until at least 3 months after a single
38 vaccination, being further strongly boosted by a second vaccination at day 89. These findings,
39 together with the simplicity and scalability of plasmid DNA manufacturing, safety data on the
40 vaccine platform in clinical trials, low cost of goods, data indicating potential long term storage at
41 +2° to 8°C and simple administration, suggests the VB2060 candidate is a promising second
42 generation candidate to prevent COVID-19.

43

44 INTRODUCTION

45 In the period from December 2019 to December 2020, over 67 million cases and >1.5 million
46 deaths due to COVID-19 disease have been reported (coronavirus.jhu.edu), and the non-
47 pharmaceutical interventions required to limit the spread are having detrimental negative impacts
48 on humanity. A safe COVID-19 vaccine able to prevent transmission of the SARS-CoV-2 virus
49 and provide persistent protection against disease even in the elderly and immunocompromised is
50 desirable, together with a product that can easily be administered, preferably as one dose regimen,
51 and stored at +2-8°C or above (WHO 2020). This would require it to induce rapid and long-
52 lasting neutralizing antibody levels and preferentially a Th1-biased CD4⁺ plus CD8⁺ T cell
53 response.

54 The SARS-CoV-2 virus is a part of the Coronaviridae family, which apart from SARS-CoV and
55 MERS-CoV mostly consists of human pathogens causing the common cold (Tse et al. 2020).
56 Antibodies against the SARS-CoV-2 Spike (S) surface protein can block the virus from binding
57 to the human cell receptor ACE2 and thus mediate virus neutralization (Barnes et al. 2020), and
58 RBD is the primary target of S-specific neutralizing antibodies in convalescent sera (Robbiani et
59 al. 2020). Neutralizing monoclonal antibodies isolated from convalescent COVID-19 patients
60 have been shown to protect against SARS-CoV2 infection in challenge models in hamsters and
61 non human primates (NHPs) (Baum et al. 2020), and several vaccines have induced nAbs that
62 strongly correlated with reduction of viral load in NHPs (Klasse et al, 2020). This suggests nAbs
63 to serve as a potential correlate of protection for COVID-19 disease, and most vaccine candidates
64 in development focus on inducing neutralizing IgG antibodies against the S protein (Poland et al.
65 2020, Than Le et al. 2020). Studies on human COVID-19 patients also indicate a role of a
66 balanced CD4⁺, CD8⁺ and neutralizing antibody responses in controlling the disease, with the

67 CD8⁺ T cell responses likely required to avoid progression into severe COVID-19 disease in
68 humans (Peng et al. 2020).

69 To address the need for a single-dose SARS-CoV-2 vaccine candidate ensuring persistent
70 protection, on a platform enabling rapid adaptation to antigen changes, easily scalable
71 manufacturing and a product that can be stable at +2-8°C, we developed a SARS-CoV-2 vaccine
72 based on a DNA plasmid vaccine platform encoding a chimeric protein designed to enhance the
73 antigen uptake through targeting the antigen to antigen-presenting cells (APC). The proteins are
74 bivalent homodimers, each chain consisting of a targeting unit, a dimerization unit derived from
75 the hinge and C_H3 exons of human IgG3, and an antigenic unit (Fredriksen et al. 2006, Ruffini et
76 al. 2010). The hinge region provides covalent binding of the two monomers via disulfide bonds
77 while C_H3 contributes to the dimerization through hydrophobic interactions. LD78β is an isoform
78 of the human CC chemokine macrophage inflammatory protein-1α (MIP-1 α), and is suitable as a
79 targeting unit due to its ability to attract APCs and deliver the antigen through chemokine
80 receptors CCR1 and CCR5 (Ruffini et al. 2010). This leads to effective presentation of antigenic
81 epitopes on MHC class I and MHC class II molecules to CD8⁺ and CD4⁺ T cells, respectively.
82 This vaccine format has been shown to induce rapid, strong and dominant CD8⁺ cytotoxic T cell
83 responses (Krauss et al. 2019, Ruffini et al. 2010).

84 We further built on the clinical experience of this vaccine format, where two similar LD78β
85 chimeric vaccine products have been evaluated in clinical trials for the treatment of cancers.
86 VB10.16 is a therapeutic HPV16-specific cancer vaccine which carries HPV16 E6 and HPV16
87 E7 in the antigenic unit and has been tested in a Phase 1/2a trial in patients with high grade
88 cervical intraepithelial neoplasia (NCT02529930) and in an ongoing Phase 2 trial in patients with
89 advanced or recurrent cervical cancer (NCT04405349). VB10.NEO is a fully personalized cancer

90 neoantigen vaccine being tested in a Phase 1/2a trial in patients with multiple locally advanced or
91 metastatic cancers (NCT03548467). Both vaccine candidates are delivered intramuscularly (i.m).
92 using a needle-free jet injector (PharmaJet, U.S.). No significant safety concerns were detected to
93 date and strong antigen specific immune responses were induced after vaccination (Hillemans et
94 al. 2019, Krauss et al. 2019). Preclinical studies have shown that the vaccine platform can achieve
95 both rapid (1 week) and long-lasting (at least 10 months) protection against influenza after a
96 single dose (Grødeland et al. 2013, Lambert et al. 2016, Grødeland et al. 2019). The DNA
97 plasmid vaccine platform exploited in these studies is a safe and highly versatile technology with
98 intrinsic adjuvant effect designed for efficient delivery of antigen and inducing rapid, strong,
99 broad and long-lasting immune responses. We here tailored the vaccine format against the SARS-
100 CoV-2 virus (referred to as VB10.COV2) by including the Spike or RBD antigens into the
101 antigenic unit of the LD78 β chimeric vaccine construct, and evaluated anti-viral antibody and T
102 cell responses in mice.

103

104 **RESULTS**

105 **Design and characterization of VB10.COVID proteins post transfection**

106 Vaccine constructs were designed based on modifications of the antigens Spike and RBD. All
107 synthesized DNA plasmids were evaluated for expression in human cells (HEK293), and
108 subsequently evaluated for immunogenicity in BALB/c mice. VB10.COVID DNA plasmids
109 encoded either a short form of the SARS-CoV-2 RBD (“RBD short”, amino acids 331-524, i.e.
110 193 aa), a longer version (“RBD long”, amino acids 319-542, i.e. 223 aa), or the modified Spike
111 protein (Figure 1a). These constructs were denoted VB2049 (RBD short), VB2060 (RBD long)
112 and VB2065 (Spike), respectively (Figure 1b). The VB10.COVID plasmids (Figure 1c) were
113 transiently transfected into mammalian cells (HEK293), and the presence of the functional
114 VB10.COVID proteins in supernatant were measured by a sandwich ELISA using specific
115 antibodies against the targeting, dimerization and antigenic units of the protein (i.e. LD78 β , hIgG
116 C_H3 domain, the RBD domain or Spike protein). Reactivity confirmed successful expression and
117 secretion, and a conformational integrity of all VB10.COVID protein vaccine candidates (Figure
118 2). The expression level was found to be highest for VB2060 (RBD long), followed by VB2049
119 (RBD short) and VB2065 (Spike); indicating that VB2060 could also be secreted at higher levels
120 from myocytes after i.m. vaccination.

121 **Humoral immune responses to SARS-CoV-2 antigens induced in mice**

122 The constructs were compared for the ability to induce anti-RBD IgG. All three candidates, when
123 administered at a dose of 50 μ g, evoked strong IgG responses against RBD in mice. The
124 antibodies induced by the construct encoding RBD-based antigens (VB2049 and VB2060) were
125 detected already at day 7 (Figure 3), while for candidates encoding S protein (VB2065)

126 antibodies were first detected at day 14. VB2060 seemed to induce higher, and more rapid anti-
127 RBD antibody responses compared to VB2049 and VB2065 (Figure 3). The antibody response
128 induced by VB2060 was further characterized, and demonstrated anti-RBD IgG as early as day 7
129 post single vaccination; even at a low dose (2.5 μg) (Figure 4a), as well as a consistent dose-
130 response (Figure 4b)The antibody levels peaked at day 28 (10^5 endpoint titer) after a single dose
131 and achieved high levels for at least 89 days (Figure 4a). For VB2060, the peak and durability of
132 the response were further increased ($>10^6$ endpoint titer) following a two dose regimen (days 0
133 and 21, at both 50 μg and 25 μg) compared to the single dose group. Limited added benefit was
134 observed at day 99 in mice that received a boost vaccination at day 89 (Figure 4a). A second
135 experiment confirmed a clear tendency of a dose-dependent response in the range of 3, 6, 12.5
136 and 25 μg of VB2060 (Figure 4b), in particular on day 7, with high Ab levels reached for all
137 groups from day 14 until day 28, both with a single and two-dose regime. The responses were
138 significantly different between the groups of mice vaccinated with 3 μg vs. 12 μg , 3 μg vs. 25 μg ,
139 and 6 μg vs. 25 μg . We further tested the kinetics of RBD-specific IgG in bronchoalveolar lavage
140 (BAL) from mice vaccinated once or twice with different doses VB2060 (Figure 4c). RBD-
141 specific IgG was found in BAL at the earliest time point tested (day 14) even with the lowest
142 dose, and the levels increased with dose and over time (Figure 4c).

143 Sera were also assessed in a live SARS-CoV-2 virus neutralization assayOne dose of 50 μg of
144 VB2060 induced strong and long lasting neutralizing antibodies, and was sufficient to induce
145 detectable neutralizing activity already at day 7 which peaked at day 28 with no signs of decline
146 at day 99 ($\text{ND}_{50} 10^4$) (Figure 5a). Strong neutralizing antibody responses were seen in pools from
147 vaccinated mice with all three candidates VB2049, VB2060 and VB2065 (Figure 5b,
148 Supplemental figure S1). Serum pools from groups mice subjected to various doses and dosing

149 regimens were tested for nAbs at selected time points (day 28, 90 and 99) for VB2060 and
150 VB2049 (Figure 5b). Two doses of 2.5 μg of VB2060 resulted in titers $>10^3$ at day 28 (data not
151 shown). All regimens reached higher or comparable titers to the NIBSC convalescent plasma
152 reference serum 20/130 from day 28 and until the end of the experiment. Independent of the dose,
153 the strongest response was observed at day 99 (after boost at day 89), showing induction of long-
154 lasting, neutralizing antibody responses with VB2060. Taken together, VB2060 was found to be
155 superior to VB2065 and VB2049 in inducing rapid and high levels of neutralizing antibodies.

156

157 **Evaluation of the magnitude and specificity of T cell responses after vaccination**

158 Overall, the VB10.COV2 constructs all induced strong, dose-dependent T cell responses after
159 vaccination, that increased over time. The responses were dominated by CD8^+ T cells and
160 accompanied by significant, but weaker CD4^+ T cell responses. Vaccination with 25 μg of
161 VB2060 induced T cell responses as early as day 7 (~ 550 per 10^6 splenocytes), (Figure 6a). The T
162 cell responses increased until day 28 (Figure 6b) and were still found to persist for at least 90
163 days after vaccination with 50 μg of VB2060 (~ 5000 SFU/ 10^6 splenocytes), with a strong boost
164 effect at day 99; 10 days after a new booster dose was administered at day 89 ($\sim 20\,000$ SFU/ 10^6
165 splenocytes) (Figure 6c). We further sought to characterize the epitopes recognized by the T cells
166 by stimulating with individual 15-mers overlapping with 12 amino acids in splenocytes depleted
167 for either CD4 or CD8 T cell populations. Strong (up to ~ 4000 SFU/ 10^6 cells) CD8^+ T cell
168 responses against 9 peptides were observed. RBD-specific CD4^+ responses were also detected
169 against 7 peptides, but of a lower magnitude (up to ~ 1000 SFU/ 10^6 cells) and fewer epitopes
170 (Figure 7). The amino acid sequence of the overlapping peptides indicated a reactivity against 4

171 distinct MHC class I-restricted epitopes and 3 MHC class II-restricted epitopes (Supplemental
172 figure S2 and table S1) in RBD.

173 Strong T cell responses against the RBD domain of SARS-CoV-2 were detected in spleens from
174 mice vaccinated with one or two doses of both 2.5 μ g or 25 μ g VB2049 (Figure 6d). Depending
175 on dose level and the number of doses, the response ranged from ~1800 to 6000 SFU per 10^6
176 cells in splenocytes sampled 2 weeks after 1st dose or 1st week post-boost-vaccination at day 21
177 and stimulated separately with 6 peptide pools spanning RBD. The response was strong already at
178 14 days post 1st vaccination even with a low dose (2.5 μ g DNA) and was boosted by day 28 in
179 groups receiving a 2nd vaccination at day 21 (in a dose-dependent manner, Figure 6d). When
180 comparing T cell responses induced by two doses of 2.5 μ g of either VB2060 or VB2049,
181 VB2049 induced stronger responses than VB2060 (~3800 versus ~2600 SFU/ 10^6 cells)
182 (Supplemental figure S3). As predicted, VB2065 induced a broader, stronger total T cell response
183 than VB2049 and VB2060 due to the larger antigen with strong, CD8+ dominating T cell
184 responses, accompanied by broad, weaker CD4+ responses. (Figure 6e, Supplemental table S2).

185

186 **Evaluation of polyfunctional T cell responses and Th1/2/17 cytokine profile in splenocytes**

187 Splenocytes from mice vaccinated with 2 doses of 2.5 μ g VB2060 were harvested on day 28 and
188 restimulated with RBD peptide pools and the cell culture supernatants were analyzed for Th1
189 (IFN- γ , TNF- α , IL-12), Th2 (IL-4, IL-5) cytokines and IL-6 (Figure 8). The response was
190 dominated by IFN- γ and TNF- α , and minor quantities of IL-4, IL-5, IL-6 or IL-12 p70 were
191 detected (Figure 8). This indicates that T cell responses showed strong Th1 bias when
192 characterized one month after immunization, while the Th2 responses were minimal.

193 In depth, T cell analysis of splenocytes from mice vaccinated with 1 or 2 doses of VB2060 were
194 performed using flow cytometry on day 90 post prime vaccination. In VB2060 vaccinated mice,
195 RBD specific T cell responses were dose dependent and varied between 2.07% to 6.3% of CD8⁺
196 T cells, and 1.08% to 3.3% of CD4⁺ T cells (Figure 9). CD8⁺ T cell responses were dominated by
197 single or combined production of IFN- γ and TNF- α indicating an effective RBD-specific
198 cytotoxic response (Figure 9B).

199 The CD4⁺ RBD- specific T cells displayed a typical Th1 profile indicated by production of IFN-
200 γ , TNF- α , IL-2 or a combination of these (Figure 9A). The RBD specific multifunctional CD4⁺ T
201 cells (expressing 3 cytokines simultaneously) also showed a dose dependent response, where the
202 highest response was observed in 2 \times 50 μ g VB2060 vaccinated mice (0.13%) 90 days after the
203 initial vaccination. Presence of other markers like IL-4 (Th2 polarization), IL-17 (Th17) and
204 FoxP3 (Treg) was also tested. A minor population of CD4⁺ cells expressed the Th17 cytokine, IL-
205 17 (Figure 9A), while no significant IL-4 or FoxP3 were detected, thus revealing a combination
206 of Th1 and Th17 responses with strong bias towards Th1. Taken together, these data showed that
207 the VB2060 vaccine induced strong Th1 responses which persisted up to at least 3 months (90
208 days).

209

210

211 **DISCUSSION**

212 We here demonstrate that when encoding SARS-Cov-2 RBD in a chimeric DNA vaccine
213 construct, the expressed chimeric fusion protein that binds to chemokine receptors on APC,
214 presents RBD in a conformation able to induce rapid RBD-specific and neutralizing antibody
215 responses. IgG against RBD was shown to correlate with neutralizing antibody activity in humans
216 (Poland et al. 2020), and in non-human primates neutralizing antibodies against Spike was shown
217 to function as a correlate of protection (CoP) (Chandrashekar et al. 2020). The anti-RBD IgG
218 detected in lungs after vaccination with VB2060 may contribute to the local virus neutralization
219 serving the first line of protection against viral respiratory tract infection. In support of this, IgG
220 with neutralizing potential has been shown in BAL samples from COVID-19 patients (Sterlin et
221 al 2020). On the backdrop of observed antibody dependent enhancement (ADE) of disease for
222 Spike based SARS-CoV vaccines in animals, scientific advice recommended avoiding a Th2
223 response, and to limit the induction of non-neutralizing antibodies (Lee et al. 2020, Lamberti et
224 al. 2020). The VB2060 candidate is based on RBD and was here shown to mediate high levels of
225 neutralizing antibodies and a dominant Th1 response, thereby theoretically reducing the risk of
226 inducing vaccine associated enhancement of disease in humans (Halstead et al. 2020).

227 CD8⁺ T cells in VB2060-vaccinated mice were dominated by the presence of IFN- γ and TNF- α
228 and indicate an effective cytotoxic T cell response specific for SARS-CoV-2 infected cells,
229 whereas CD4⁺ T cells showed a predominant polyfunctional Th1 responses (defined by combined
230 IFN- γ /TNF- α /IL-2 production). Recent studies have shown the importance of CD8⁺ T cell
231 responses in controlling SARS-CoV-2 infection, with mild disease associated with CD8⁺ T cell
232 responses in patients (Peng et al. 2020). Another study showed that high levels of SARS-CoV-2
233 responsive T cells were associated with protection from symptomatic SARS-CoV-2 infection

234 among personnel at high risk of infection (i.e. healthcare providers, fire and police services)
235 (Wyllie et al. 2020).

236 With the extensive range of vaccine technology platforms applied to SARS-CoV-2, it is vital to
237 highlight the difference between the vaccine formats in relation to addressing the unmet needs
238 outlined in the WHO TPP for vaccines against COVID-19 (WHO, 2020). The current study has
239 shown that one dose of the VB10.COVID candidate VB2060 induced rapid and high levels of
240 neutralizing antibodies, CD8⁺ and Th1 CD4⁺ T cell responses that lasted for at least 3 months in
241 mice, with a strong boost effect at day 89 indicating effective memory responses. Animal
242 challenge studies are ongoing to inform further clinical development. The findings in this study,
243 together with accrued safety data in humans on the similar vaccines from the same platform
244 (Krauss et al. 2019, Hillemanns et al. 2020), the simplicity and scalability of plasmid DNA
245 manufacturing, low cost of goods, preliminary data indicating long term storage at +2° to 8°C and
246 simple administration, the VB2060 candidate is likely to be a promising future candidate to
247 prevent COVID-19.

248

249 **METHODS**

250 **Plasmid construction and testing of transient transfection in HEK293 cells**

251 The VB10.COVID constructs were designed as shown in Figure 1, and thereafter synthesized,
252 cloned and produced by Genscript. The antigenic unit with either RBD or Spike was synthesized
253 and cloned into a pUMVC4a VB10 master plasmid using SfiI-SfiI restriction enzyme sites. The
254 resulting constructs encoded for homodimeric proteins with LD78 β targeting units (Ruffini et al.
255 2010) and RBD/Spike as an antigenic unit, connected via a homodimerization unit consisting of
256 exons from the hinge h1 and h4 and CH3 of human IgG3 (Figure 1). HEK293 cells (ATCC) were
257 transiently transfected with VB10.COVID DNA plasmids. Briefly, 2×10^5 cells/well were plated in
258 24-well tissue culture plates with growth medium (DMEM, 10% FBS and 1%
259 penicillin/streptomycin) and transfected with 1 μ g VB10.COVID DNA plasmids using
260 Lipofectamine® 2000 reagent under the conditions suggested by the manufacturer (Invitrogen,
261 Thermo Fischer Scientific). The transfected cells were maintained for 3 days at 37°C with 5%
262 CO₂, and the cell supernatant was harvested. An ELISA was performed to verify the amount of
263 VB10.COVID protein produced by the HEK293 cells and secreted into the cell supernatant.
264 Briefly, ELISA plates (MaxiSorp Nunc-immuno plates) were coated with 1 μ g/ml of anti-CH₃
265 (MCA878G, BioRad) in 1x PBS with 100 μ l/well and plates were incubated overnight at 4°C.
266 The microtiter wells were blocked by the addition of 200 μ l/well 4% BSA in 1x PBS. 100 μ l of
267 cell supernatant from transfected HEK293 cells containing VB10.COVID proteins were used. For
268 primary detection antibody, either biotinylated anti-human MIP-1 α (R&D Systems) or SARS-
269 CoV-2/2019-nCoV Spike/RBD Antibody (1:1000) (Sino Biological) was used. Streptavidin-
270 HRP (1:3000) or anti-rabbit IgG-HRP (1:5000) was added as secondary detection antibody. All
271 incubations were carried out at 37°C for 1 hour (h), followed by 3x washing with PBS-Tween,

272 except for the blocking step which was performed at RT for 1h followed by loading of
273 supernatant after discardment of blocking buffer. 100 μ l/well of TMB solution was added, and
274 color development was stopped after 5-15 min adding 100 μ l/well of 1 M HCl. The optical
275 density at 450 nm was determined on an automated plate reader (Thermo Scientific Multiscan
276 GO).

277 **Immunization of animals**

278 6-week-old, female BALB/c mice were obtained from Janvier Labs (France). All animals were
279 housed in the animal facility at the Radium Hospital (Oslo, Norway). All animal protocols were
280 approved by the Norwegian Food Safety Authority (Oslo, Norway). Mice were given either one
281 dose (day 0) or two doses (days 0 and 21) or three doses (day 0, 21 and 89), of DNA plasmid
282 vaccine administrated to each *tibialis anterior* (TA) muscle by needle injection followed by
283 AgilePulse *in vivo* electroporation (EP) (BTX, U.S.). Dose-response levels explored were 2.5 μ g,
284 25 μ g and 50 μ g, or 3, 6, 12.5 and 25 μ g of VB10.COVID constructs. Blood sampling was
285 performed on days 0, 7, 14, 20, 28, 42, 56, 70, 90 and 99, and spleens were collected on days 7,
286 14, 28, 90 and 99. Bronchoalveolar lavage (BAL) samples were collected on days 14, 21 and 28
287 by injection of one mL of sterile PBS into the lungs via the trachea, followed by three rounds of
288 flushing.

289 **Anti-RBD IgG ELISA**

290 The humoral immune response was evaluated in sera and bronchoalveolar lavages (BAL)
291 collected at different time points (day 7, 14, 20, 28, 42, 56, 70, 90 or 99) after vaccination by an
292 ELISA assay detecting total IgG specific for RBD from SARS-CoV2. ELISA plates (MaxiSorp
293 Nunc- Immuno plates) were coated with 1 μ g/ml recombinant RBD-His protein antigen (Cat. No.

294 40592-V08H, Sino Biological) in 1x D-PBS overnight at 4°C. Plates were blocked with 4% BSA
295 in 1x D-PBS for 1 h at RT. Plates were then incubated with serial dilutions of sera or undiluted
296 BAL samples for 2 h at 37°C. Plates were washed 3x and incubated with a 1:50,000 dilution of
297 anti-mouse total IgG-HRP antibody (Southern Biotech) and incubated for 1h at 37°C. After final
298 wash, plates were developed using TMB substrate (Merck, cat. CL07-1000). Plates were read at
299 450 nm within 30 min using a Multiscan GO (Thermo Fischer Scientific). Binding antibody
300 endpoint titers were calculated as the reciprocal of the highest dilution resulting in a signal above
301 the cutoff. For BAL, responses were reported as OD₄₅₀ values.

302 **SARS-COV-2 live neutralization assay**

303 Live virus microneutralization assays (MNA) were performed at Public Health England (Porton
304 Down, UK) as described (Folegatti et al. 2020). Neutralising virus titres were measured in heat-
305 inactivated (56°C for 30 min) serum samples. Diluted SARS-CoV-2 (Australia/VIC01/20202)
306 (Caly et al. 2020) was mixed 50:50 in 1% FCS/MEM with doubling serum dilutions in a 96-well
307 V-bottomed plate and incubated at 37°C in a humidified box for 1 hour. The virus/serum
308 mixtures were then transferred to washed Vero E6 (ECACC 85020206) cell monolayers in 96-
309 well flat-bottomed plates, allowed to adsorb at 37°C for a further hour, before removal of the
310 virus inoculum and replacement with overlay (1% w/v CMC in complete media). The box was
311 resealed and incubated for 24 hours prior to fixing with 8% (w/v) formaldehyde solution in PBS.
312 Microplaques were detected using a SARS-CoV-2 antibody specific for the SARS-CoV-2 RBD
313 Spike protein and a rabbit HRP conjugate, infected foci were detected using TrueBlue™
314 substrate. Stained microplaques were counted using ImmunoSpot® S6 Ultra-V Analyzer and
315 resulting counts analysed in SoftMax Pro v7.0 software. International Standard 20/130 (human

316 anti-SARS-CoV-2 antibody from human convalescent plasma, NIBSC, UK) was used for
317 comparison.

318

319

320 **IFN- γ ELISpot assay**

321 Splenocytes from vaccinated mice were analyzed in IFN- γ ELISpot assay detecting RBD-/Spike-
322 specific T cell responses. Briefly, the animals were sacrificed at day 7, 14, 28, 90 or 99, and the
323 spleens were harvested aseptically. The spleens were homogenized, single-cell suspensions were
324 incubated with $1 \times$ ACK buffer to remove erythrocytes, washed and re-suspended to a cell
325 concentration of 6×10^6 cells. CD4 or CD8 T-cell populations were depleted from the total
326 splenocyte population using the Dynabead (catalog no. 11447D or 11445D, Thermo Fischer
327 Scientific) magnetic bead system according to the manufacturer's recommended procedures.
328 Cells were then re-suspended at 6×10^6 cells/ml for the ELISpot assay. Depletion was confirmed
329 by flow cytometry. The cells were plated in triplicates (6×10^5 cells/well) and stimulated with 2
330 μ g/ml of RBD/Spike peptide pools (RBD: 6 pools consisting of 10-11 x of 15 mers overlapping
331 with 12 aa and Spike: 24 pools consisting of 12 x 15 mers overlapping with 11 aa) or individual
332 peptides for 24h. Cells without peptide stimulation was used as negative controls. The stimulated
333 splenocytes were analyzed for IFN- γ responses using the mouse IFN- γ ELISpot Plus kit (Mabtech
334 AB, Sweden). Spot-forming cells (SFU) were measured in a IRIS™ ELISpot reader using the
335 APEX™ software from Mabtech AB, Sweden. Results are shown as the mean number of IFN- γ +
336 spots/ 10^6 splenocytes with subtracted background.

337

338 **Cell stimulation and staining for flow cytometry**

339 The animals were sacrificed 28 days post the first dose and spleens were removed aseptically.
340 The spleens were mashed to obtain single-cell suspensions, and 1x ACK buffer was used to
341 remove erythrocytes. The splenocytes were then washed, plated (2.0×10^6 cells/well in 24 well
342 plates) and stimulated for 16 h with 6 $\mu\text{g/ml}$ of RBD peptide pools. For detection of cytokines
343 with flow cytometry, 1x monensin and 1x brefeldin were added to the wells 1h post incubation
344 start. Following the stimulation with RBD peptide pools, the cells were harvested, and
345 centrifuged twice with PBS to wash away the medium. The cells were incubated with fixable
346 viability dye (eFluor780) in the dark for 10 min at RT. Cells were further stained with the
347 extracellular antibodies (anti-CD3, anti-CD4, anti-CD8 and $\gamma\delta\text{TCR}$), fixed and permeabilized,
348 and stained for detection of cytokines (anti-TNF α , anti-IFN- γ , anti-IL-4, anti-IL-17 antibodies)
349 and a transcription factor (anti-FoxP3 antibody). Detailed description of antibodies used for flow
350 cytometry are shown in Supplemental Table S3. The stained cells were run in BD FACSymphony
351 A5 (BD Biosciences, U.S.) and analyzed using FlowJo software.

352

353 **Multiparameter flow cytometry analysis**

354 The RBD-stimulated mice splenocyte T cells were defined through the exclusion of dead cells,
355 doublets and CD3 $^-$ non-T cells (Figure S4A-D). CD3 $^+$ T cells were then analyzed for the presence
356 of $\gamma\delta\text{TCR}$ T cells and these cells were further removed from the analysis (Figure S4E). The
357 remaining T cells were then examined for CD4 $^+$ and CD8 $^+$ markers, and the majority expressed
358 either CD4 $^+$ or CD8 $^+$ T cell populations (Figure S4F). Both populations were examined for
359 individual expression of IFN- γ , TNF- α , IL-4, IL-17 or FoxP3 and gates were set to define
360 positive cells. These positive cells were further analyzed using Boolean gating algorithm in

361 FlowJo software. The algorithm calculated all possible combinations of cytokines produced by
362 each cell, thus allowing analysis of multifunctional T cells on a single-cell level.

363 **Cytokine release testing**

364 Cell culture supernatant from splenocytes stimulated with RBD peptides for 16 h was harvested
365 and analyzed for cytokine presence. In short, 50 μ l of the cell culture supernatant was used as
366 described in the supplier's protocol for Essential Th1/Th2 Cytokine 6-Plex Mouse ProcartaPlex™
367 Immunoassay (Thermo Fisher Scientific). Presence of IFN- γ , TNF- α and IL-12p70 in the
368 supernatant defined Th1 response. The Th2 response was defined through the production of IL-4
369 and IL-5.

370

371 **Statistical analysis**

372 Statistical analyzes of antibody responses in sera to compare groups were performed by two-
373 tailed Mann-Whitney test was performed (GraphPad Software). A value of $p < 0.05$ was
374 considered significant.

375

376 **Data availability**

377 The data that support the findings of this study are available from the corresponding author, GN,
378 upon reasonable request.

379

380

381 **REFERENCES**

- 382 Chandrashekar, A. et al. SARS-CoV-2 infection protects against rechallenge in rhesus macaques.
383 *Science* **369**, 812-817 (2020).
- 384 Barnes C.O. et al. SARS-CoV-2 neutralizing antibody structures inform therapeutic strategies.
385 *Nature* (2020). <https://doi.org/10.1038/s41586-020-2852-1>
- 386 Baum, A. et al. REGN-COV2 antibodies prevent and treat SARS-CoV-2 infection in rhesus
387 macaques and hamsters. *Science* **370**, 1110-1115 (2020).
- 388 Caly, L. et al. Isolation and rapid sharing of the 2019 novel coronavirus (SARS-CoV-2) from the
389 first patient diagnosed with COVID-19 in Australia. *Med J Aust* **212**, 459-462 (2020).
- 390 Folegatti, P.M. et al. Safety and immunogenicity of the ChAdOx1 nCoV-19 vaccine against
391 SARS-CoV-2: a preliminary report of a phase 1/2, single-blind, randomised controlled trial.
392 *Lancet* **396**, 467-478 (2020).
- 393 Fredriksen, A.B. et al. DNA vaccines increase immunogenicity of idiotypic tumor antigen by
394 targeting novel fusion proteins to antigen-presenting cells. *Mol Ther* **13**, 776-85 (2006).
- 395 Grødeland, G. et al. The specificity of targeted vaccines for APC surface molecules influences the
396 immune response phenotype. *PLoS One* **8**, e80008 (2013).
- 397 Grødeland, G. et al. Targeting of HA to chemokine receptors induces strong and cross-reactive T
398 cell responses after DNA vaccination in pigs. *Vaccine* **38**, 1280-1285 (2020).
- 399 Halstead et al. COVID-19 Vaccines: Should We Fear ADE? *J Infect Dis* **13**, 1946-1950 (2020).

400 Hillemanns et al. P22. An exploratory safety and immunogenicity study of human papillomavirus
401 (HPV16+) immunotherapy VB10.16 in women with high grade cervical intraepithelial neoplasia
402 (HSIL; CIN 2/3). *Int J Gynecol Cancer* **29**, A64.2-A65 (2019).

403 Krauss J. et al. Preliminary safety, efficacy and immunogenicity results from a phase 1/2a study
404 (DIRECT-01) of cancer neoantigen DNA vaccine VB10.NEO in patients with locally advanced
405 or metastatic solid tumors. ID: P424 at 34th Annual Meeting & Pre-Conference Programs of the
406 Society for Immunotherapy of Cancer (SITC 2019): part 1. *J ImmunoTher Cancer* **7**, 282 (2019).

407 Lambert, P. H., et al. Consensus summary report for CEPI/BC March 12-13, 2020 meeting:
408 Assessment of risk of disease enhancement with COVID-19 vaccines. *Vaccine*, **38**, 4783–4791
409 (2020).

410 Le TT et al. Evolution of the COVID-19 vaccine development landscape. *Nat Rev Drug Discov*
411 **19**, 667-668 (2020).

412 Lee, W.S., et al.. Antibody-dependent enhancement and SARS-CoV-2 vaccines and therapies.
413 *Nat Microbiol* **5**, 1185–1191 (2020).

414 Peng, Y. et al. Broad and strong memory CD4+ and CD8+ T cells induced by SARS-CoV-2 in
415 UK convalescent individuals following COVID-19. *Nat Immunol* **21**, 1336-1345 (2020).

416 Poland, G.A. et al. SARS-CoV-2 immunity: review and applications to phase 3 vaccine
417 candidates. *Lancet* **396**, 1595-1606 (2020).

418 Robbiani D.F. et al. Convergent antibody responses to SARS-CoV-2 in convalescent individuals.
419 *Nature* **584**, 437-442 (2020).

420 Ruffini, P.A. et al. Human chemokine MIP1 α increases efficiency of targeted DNA fusion
421 vaccines. *Vaccine* **29**, 191-9 (2010).

422 Ryan, K.A. et al. Dose-dependent response to infection with SARS-CoV-2 in the ferret model:
423 evidence of protection to re-challenge. *bioRxiv* doi:10.1101/2020.05.29.123810 (2020).

424 Smith, T.R.F., et al. Immunogenicity of a DNA vaccine candidate for COVID-19. *Nat Commun*
425 **11**, 2601 (2020).

426 Sterlin, D. et al. IgA dominates the early neutralizing antibody response to SARS-CoV-2. *bioRxiv*
427 doi:10.1101/2020.06.10.20126532 (2020).

428 Tse L.V. et al. The Current and Future State of Vaccines, Antivirals and Gene Therapies Against
429 Emerging Coronaviruses. *Front Microbiol* **11**, 658 (2020).

430 World Health Organization. WHO Target Product Profiles for COVID-19 Vaccines. Version 3 -
431 29 April 2020. Accessed 05.12.2020 at [https://www.who.int/publications/m/item/who-target-](https://www.who.int/publications/m/item/who-target-product-profiles-for-covid-19-vaccines)
432 [product-profiles-for-covid-19-vaccines](https://www.who.int/publications/m/item/who-target-product-profiles-for-covid-19-vaccines) (2020).

433 Wrapp, D. Et al. Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation.
434 *Science* **367**, 1260-1263 (2020).

435 Wyllie, D. et al. SARS-CoV-2 responsive T cell numbers are associated with protection from
436 COVID-19: A prospective cohort study in keyworkers. *medRxiv*
437 doi:10.1101/2020.11.02.20222778 (2020).

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440

441 **ACKNOWLEDGEMENTS**

442 Audun Bersaas and Renate Skarshaug are thanked for excellent work in supporting and
443 performing laboratory analyses, and the Vaccibody team for project support. We also want to
444 thank the staff at the animal research facility at the Oslo Radium hospital. We also thank the staff
445 at Oslo Radium Hospital Flow Cytometry Core Facility for the technical support. We thank PHE,
446 Porton Down, U.K., for neutralization assay testing performed as a service in collaboration with
447 Nexelis, Canada.

448 **AUTHOR CONTRIBUTIONS**

449 ABF, MS, GN, ES, LMS, BS conceptualized experiments. ES, LMS, BS, SC, RS, LB, KK, AB
450 and EM performed the experiments. GN, ES and ABF wrote the manuscript. All authors
451 supported the review of the manuscript.

452 **COMPETING INTERESTS**

453 All authors listed with an affiliation of Vaccibody, Oslo, Norway, are employees of Vaccibody; a
454 biopharmaceutical company dedicated to the discovery and development of novel
455 immunotherapies for cancer and infectious diseases. All authors may hold shares or stock options
456 in the company. ABF, ES, MS and GN are inventors on one or more patents on DNA vaccines
457 and use of these.

458 **ADDITIONAL INFORMATION**

459 Supplementary information is available for this paper (Figures and Tables denoted “S”). The
460 research is funded by Vaccibody. Correspondence and requests for materials should be addressed
461 to gnorheim@vaccibody.com

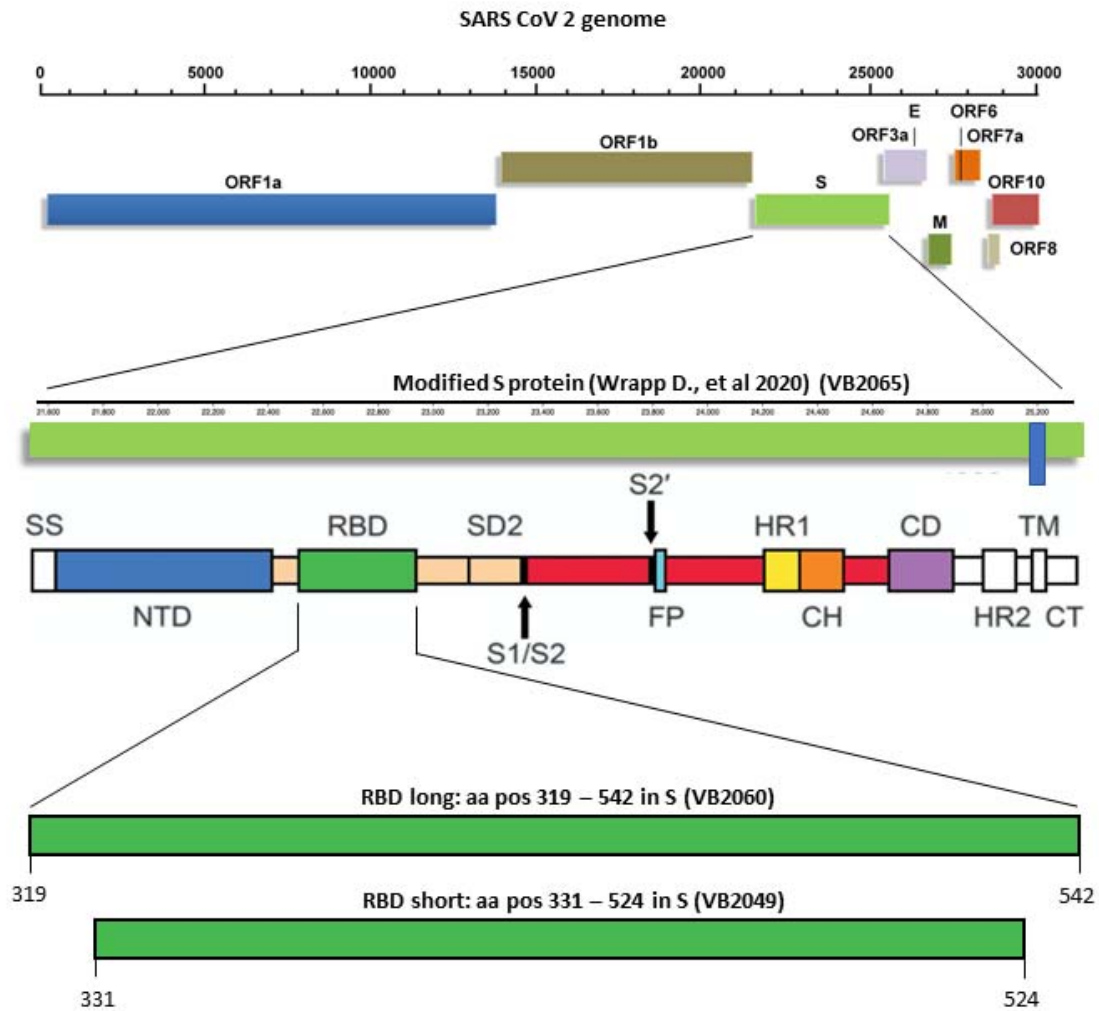
1 **Figures and captions**

2

3 **Figure 1. Alternative vaccine construct designs explored and characterized for**
4 **immunogenicity in mice. a** Schematic presentation of the SARS-CoV-2 genome, with the
5 modified S protein (introduction of two prolines in the S2 subunit to stabilize S in prefusion
6 conformation, and mutation of the furin cleavage site between S1 and S2 (Wrapp et al. 2020))
7 and amino acid positions of the “RBD long” and “RBD short” used in vaccine candidates
8 indicated. **b** VB10.COV2 homodimeric proteins. Each chain of the dimer contains a N-terminal
9 LD78 β targeting unit (turquoise), a dimerization unit (yellow) composed of a shortened IgG
10 hinge and C_H3 domain from human γ 3 chains, and a C-terminal antigen unit genetically linked to
11 the dimerization unit. LD78 is an isoform of the human CC chemokine macrophage inflammatory
12 protein-1 α (MIP-1 α). Antigens encoded by the different constructs: VB2065; codon-optimized
13 stabilized S protein without furin cleavage. VB2060; RBD (long, aa 319 – 542). VB2049; RBD
14 (short, aa 331 – 524). **c** Structure of plasmid construct encoding VB2060, based on the expression
15 vector pUMVC4a and with the homodimeric RBD protein insert shown.

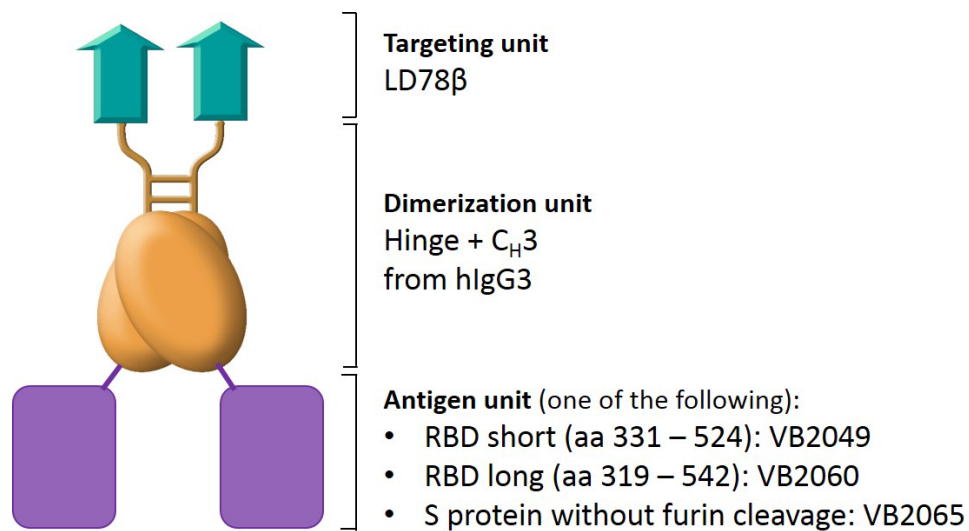
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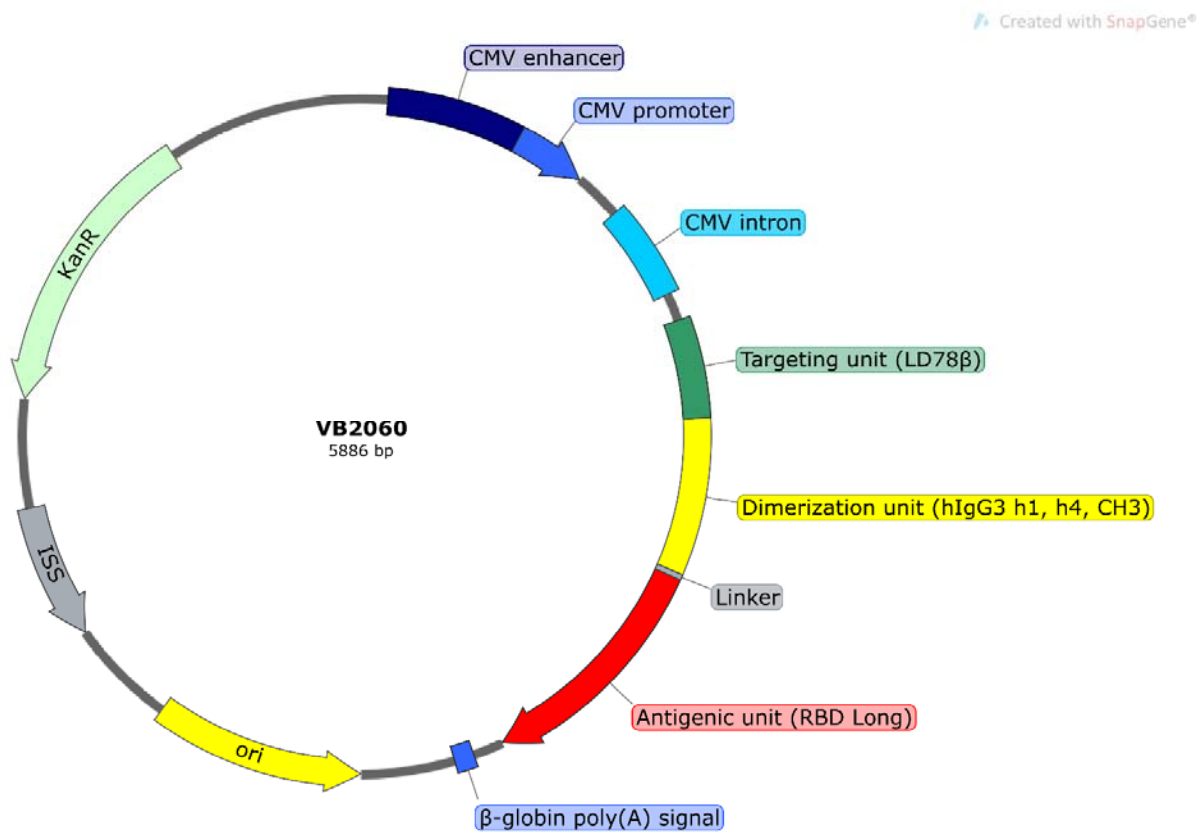
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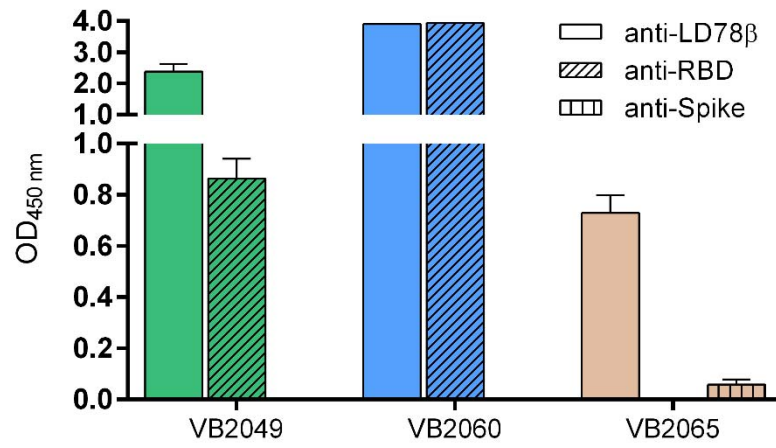
23 **Figure 2. VB10.COVID2 construct proteins (VB2049, VB2060 and VB2065) produced and**

24 **secreted as functional homodimers 3 days after transfection of HEK293 cells.**

25 Conformational integrity of the epitopes expressed in the constructs was confirmed by binding to

26 antibodies detecting the human LD78 β , human IgG C_H3 domain, the RBD domain or Spike

27 protein in ELISA.

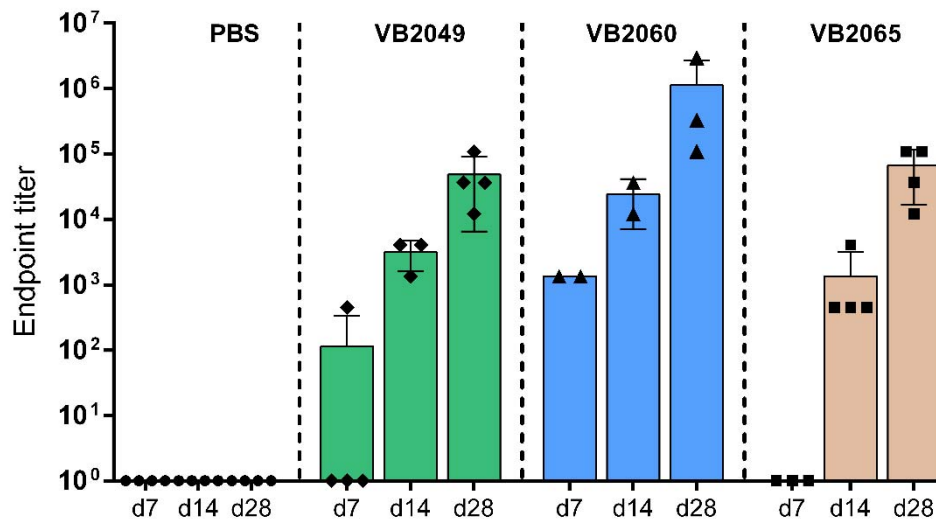


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30 **Figure 3. Anti-RBD IgG responses in mice vaccinated with 50 µg of VB10.COV2 candidates**
31 **(VB2049, VB2060 or VB2065).** Mice were vaccinated by i.m. administration of DNA
32 immediately followed by electroporation of the injection site at day 0 and day 21. Sera obtained
33 at day 7, 14 and 28 post first vaccination with VB2049, VB2060 or VB2065 were tested for anti-
34 RBD IgG antibodies binding the RBD protein. Data are shown as mean ± SEM with individual
35 values ($n = 2-4$).

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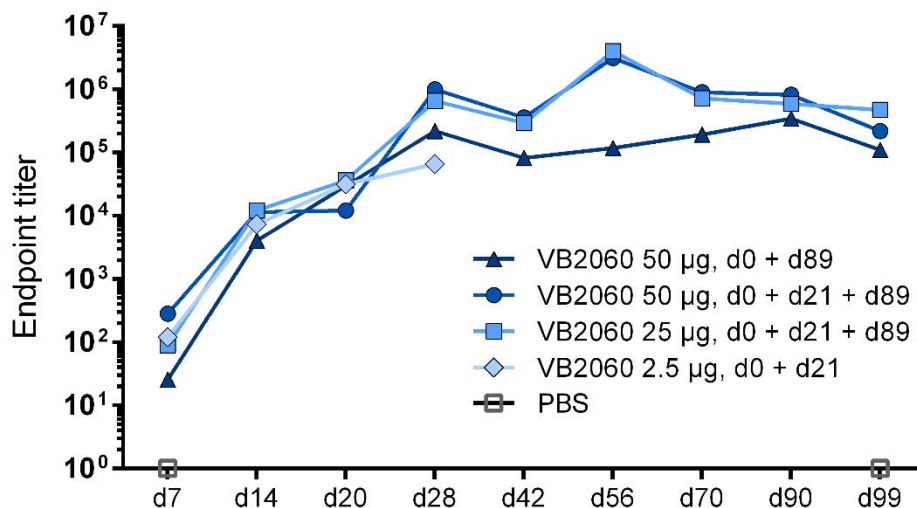
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45 **Figure 4. Kinetics of anti-RBD IgG response in mice vaccinated with VB10.COV2 VB2060.**

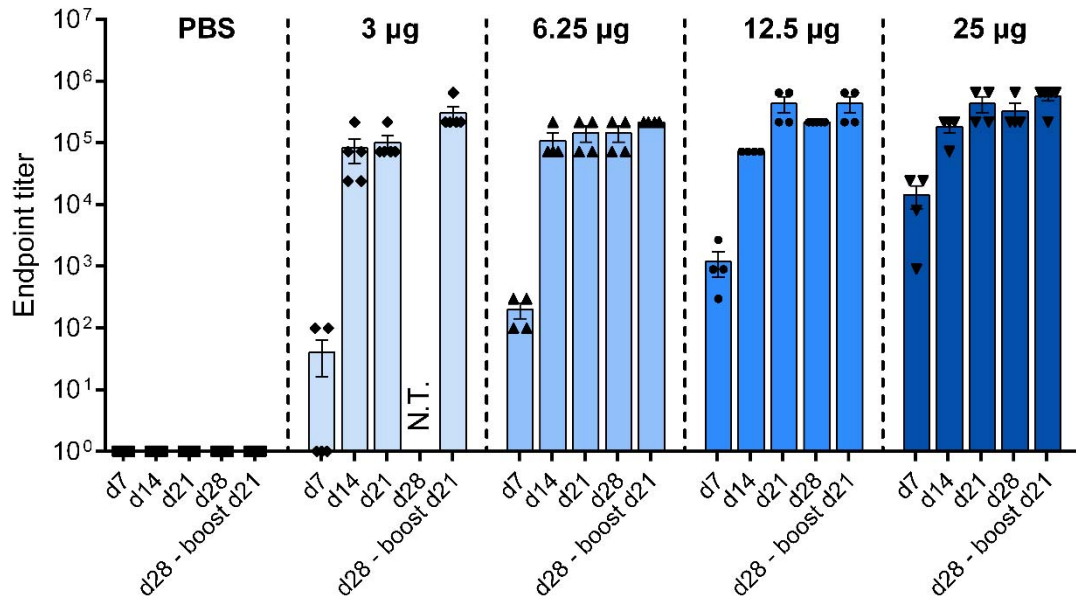
46 Mice were vaccinated by i.m. administration of VB2060 immediately followed by electroporation
47 of the injection site. Dose time points and dose levels are indicated. **a** Serum anti-RBD IgG
48 assessed until day 99 post first vaccination. The results are shown as a mean of two independent
49 ELISA experiment. Group size is $n = 5$ until day 70, $n = 2-3$ at the two last time points (days 90
50 and 99). **b** Serum anti-RBD IgG responses in mice vaccinated with 1 (day 0) or 2 doses (days 0
51 and 21) of either 3, 6, 12.5 or 25 μg of VB2060, measured weekly for up to 4 weeks ($n = 4-5$
52 mice per group, data shown as mean \pm SEM with individual values, NT; not tested). **c** Anti-RBD
53 IgG responses in bronchoalveolar lavage (BAL) from mice immunized with 1 (day 0) or 2 doses
54 (days 0 and 21) of either 3, 6, 12.5 or 25 μg of VB2060, measured at 14 and 21 and 28 days after
55 first vaccination and 7 days post boost. Data are shown as mean \pm SEM with individual values (n
56 = 3-4).

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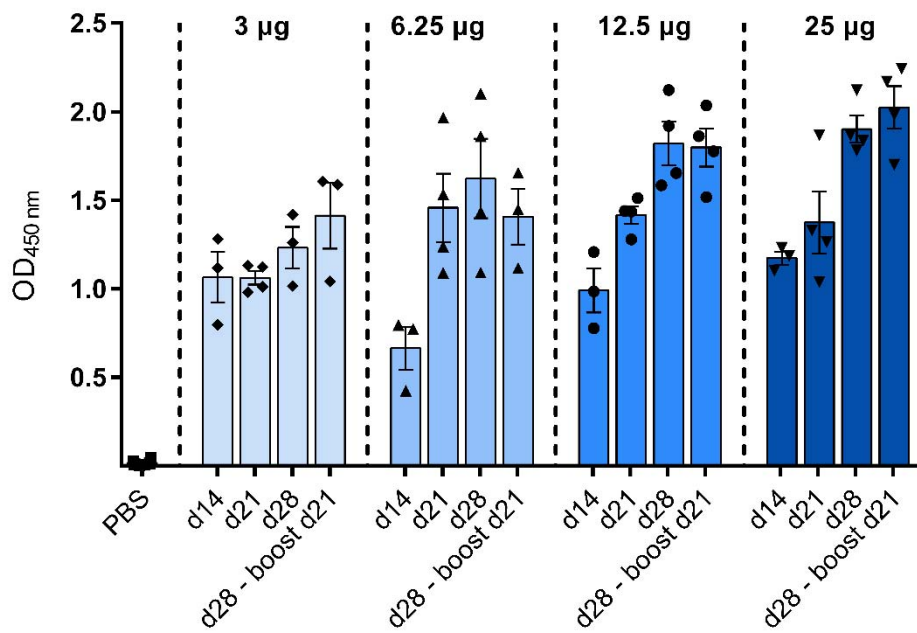
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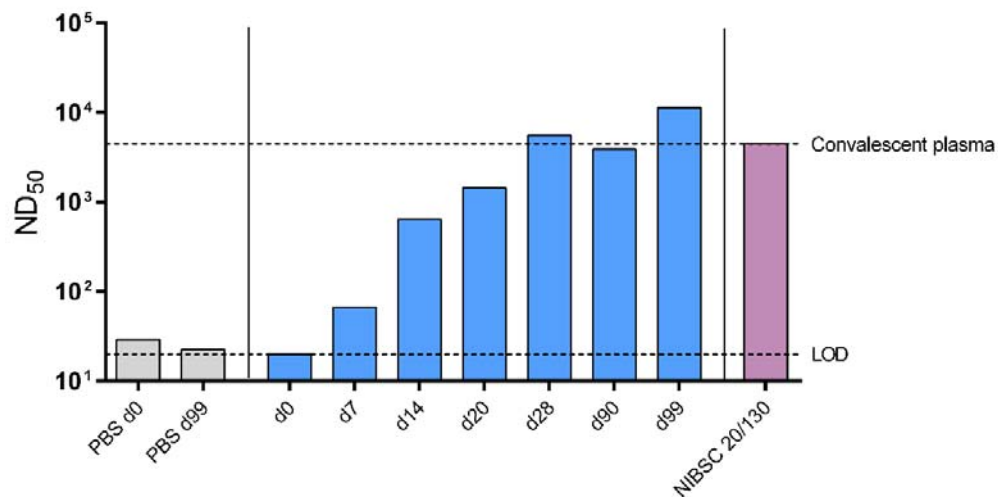
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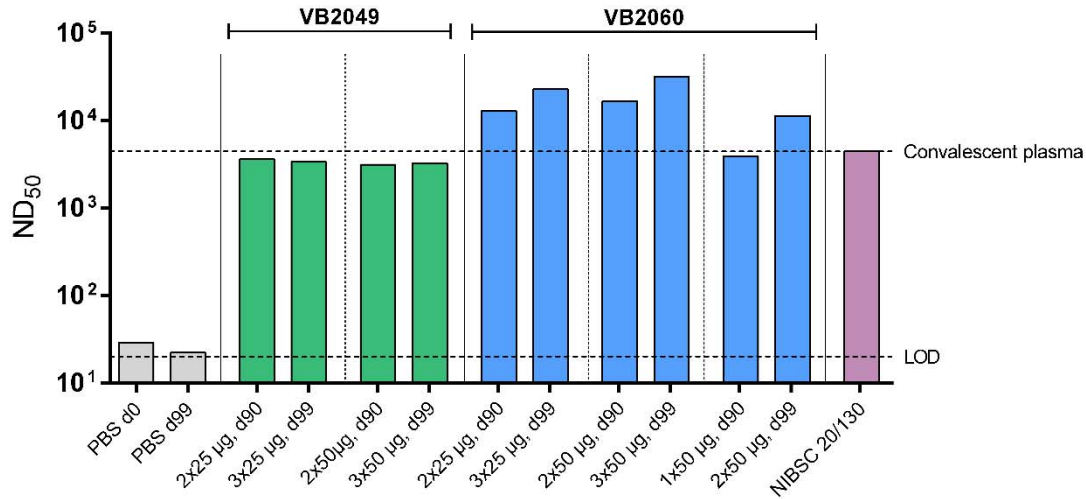
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61 **Figure 5. Neutralizing antibody responses in sera after immunization with VB2060.** Bars
62 represent endpoint titers for pooled sera assessed in live virus neutralization against homotypic
63 SARS-CoV-2 live virus strain Australia/VIC01/2020. Lower dashed line indicate the limit of
64 detection (LOD) of the assay, and the upper dashed line the titer value for the convalescent serum
65 reference NIBSC 20/130 (ND_{50} endpoint titer 4443). **a** Neutralizing antibody responses in sera
66 after immunization with one 50 μ g dose of VB2060. **b** Neutralizing antibody responses in sera
67 from mice immunized with 25 μ g or 50 μ g of VB2060 or VB2049 vaccines either as a one dose
68 regimen (day 0) or two dose regimen (days 0 and 21) and boosted at day 89.

A



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71 **Figure 6. T cell responses induced with different doses and number of doses of VB10.COV2**

72 **DNA vaccines VB2049, VB2060 or VB2065.** Splenocytes were harvested at day 7, day 14, day

73 28, day 90 or day 99 post first immunization and/or at day 7 or day 10 post boost vaccination at

74 day 21 or day 89, respectively, and the total number of IFN- γ positive spots/ 1×10^6 splenocytes

75 after restimulation with overlapping RBD or Spike peptide pools or individual RBD peptides

76 were determined by IFN- γ ELISpot. **a** Mice ($n = 5$) were i.m. vaccinated once with 25 μ g

77 VB2060 plasmid and spleens harvested at day 7. **b** Mice ($n = 5$) were i.m. vaccinated at day 0

78 and day 21 with 2.5 μ g VB2060 DNA plasmid and spleens harvested at day 28. **c** Persistence of

79 RBD-specific T cell responses after vaccination with VB2060 plasmid, measured by IFN- γ +

80 ELISpot assay tested for 61 individual RBD peptides. Responses at different dose levels (25 μ g

81 or 50 μ g); for the 25 μ g dose level, responses were measured at day 90 after a two dose regimen

82 (days 0 and 21) and 10 days post boost (i.e. day 99). For the 50 μ g dose level, responses were

83 measured at day 90 after a two dose regimen (days 0 and 21) and 10 days post boost (i.e. day 99),

84 as well as at day 90 after a one dose regimen (day 0) and 10 days post boost (i.e. day 99). **d** Mice

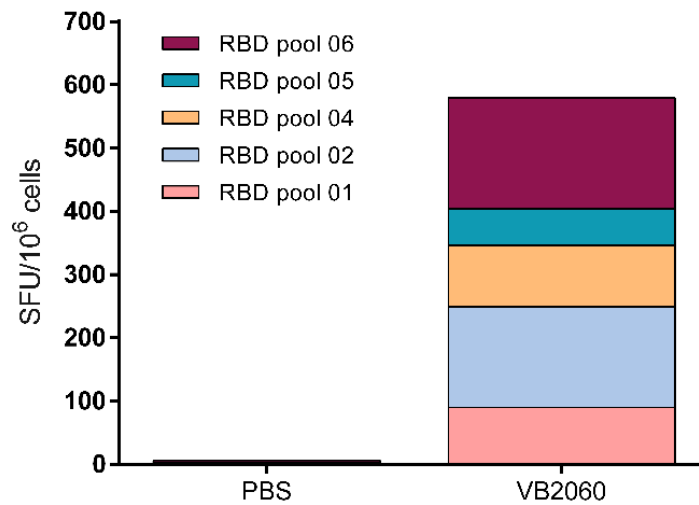
85 ($n = 5$) were i.m. vaccinated at day 0 and day 21 with 2.5 μ g or 25 μ g VB2049 DNA plasmid and

86 spleens harvested at day 14 (for mice vaccinated at day 0) or at day 28 (for mice vaccinated at

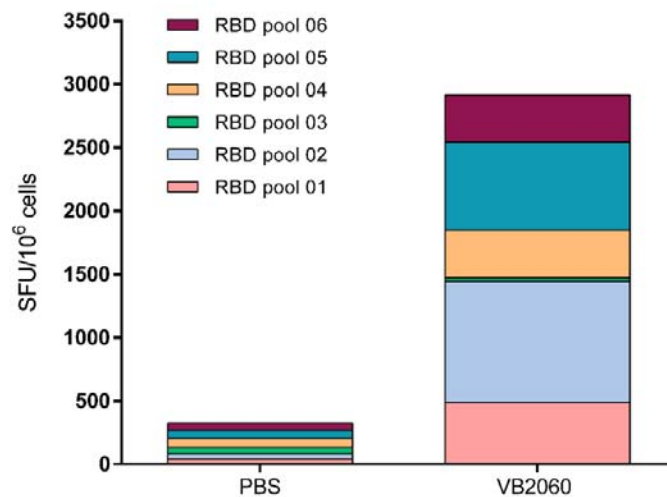
87 day 0 and day 21). **e** Mice ($n = 8$) were i.m. vaccinated at day 0 and day 21 with 50 μg VB2065
88 DNA plasmid and spleens harvested at day 28. CD4^+ and CD8^+ depletion of splenocytes was
89 performed to elucidate the epitope specific distribution of responses among CD4^+ or CD8^+ T cell
90 populations.

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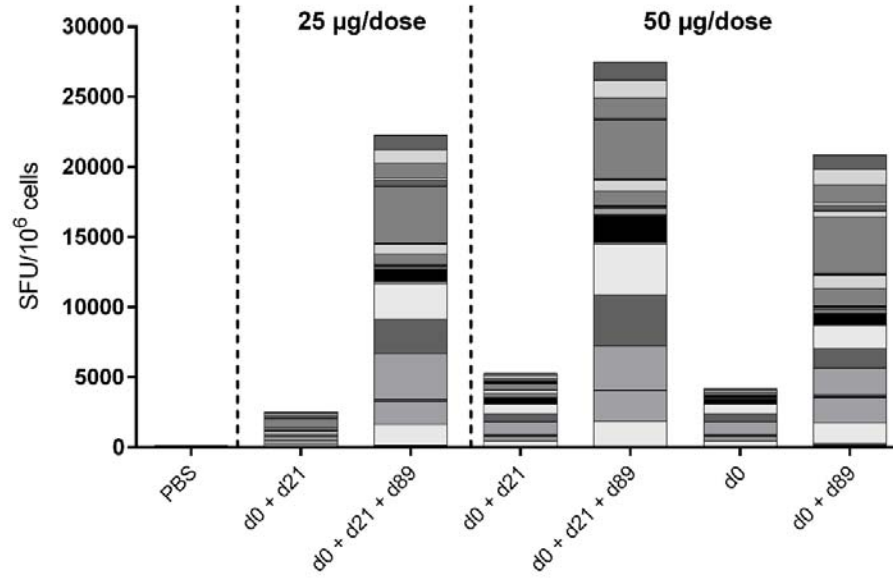


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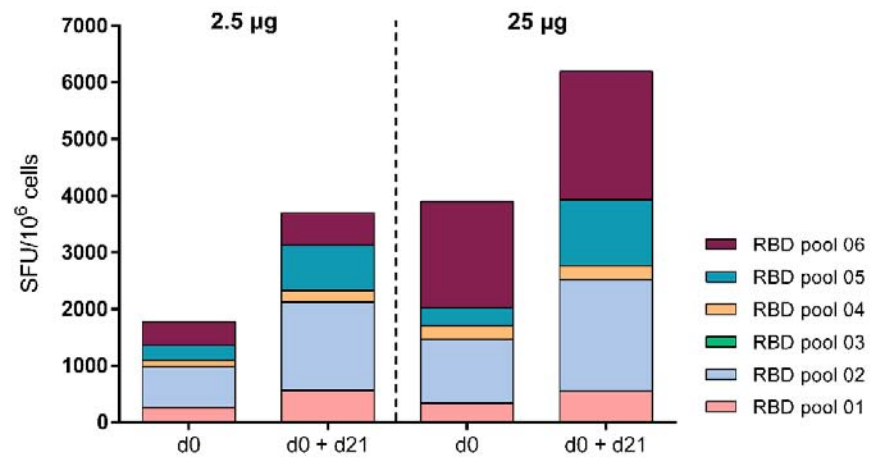
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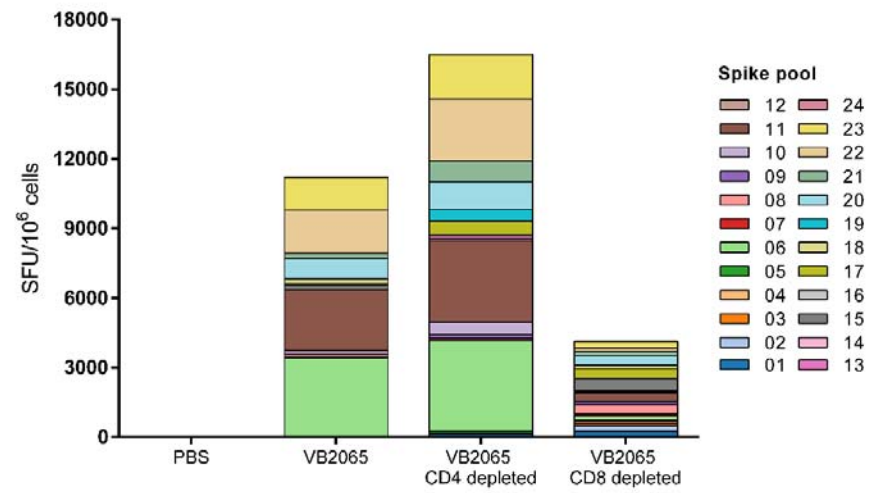
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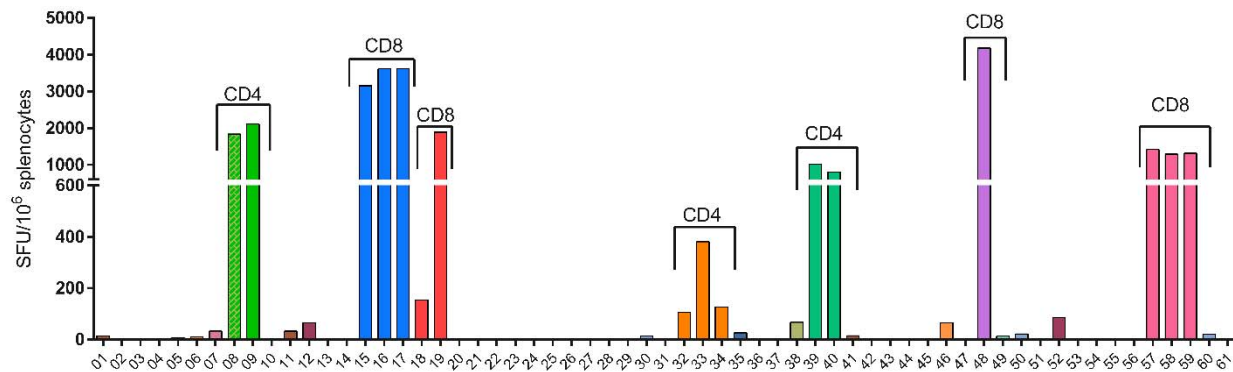
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97 **Figure 7. Identification of CD4⁺ and CD8⁺ RBD-specific T cell epitopes at day 99 post first**
98 **vaccinations of VB2060 (*n* = 2).** Splenocytes were harvested at day 99 from mice vaccinated at
99 day 0, 21 and 89 and stimulated for 24 hours with 61 individual RBD peptides (15-mer peptides
100 overlapping by 12 aa from SARS-CoV2 RBD domain) and the number of IFN- γ positive
101 spots/ 1×10^6 splenocytes were detected in an ELISpot assay. Indications of CD4⁺ and CD8⁺
102 specific responses are derived from an CD4 and CD8 depletion experiment with VB2049 with the
103 same 61 peptides (Supplemental Figure S2).



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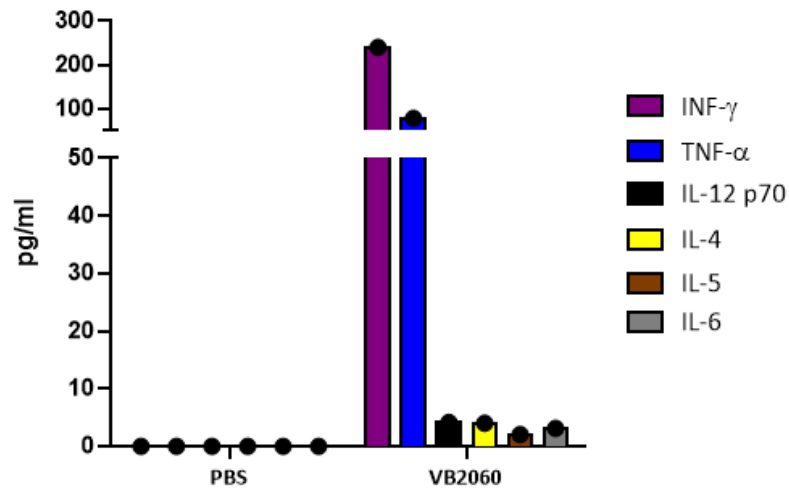
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108 **Figure 8. Characterization of the Th1 (IFN- γ , TNF- α , IL-12p70) and Th2 (IL-4, IL-5)**
109 **cytokines in cell culture supernatant.** Splenocytes from mice vaccinated with 2.5 μ g VB2060 at
110 day 0 and day 21 were restimulated for 16 h with RBD peptide pools (1-6) on day 28. Cytokine
111 concentrations were measured using bead based immunoassay (ProcartaPlex).

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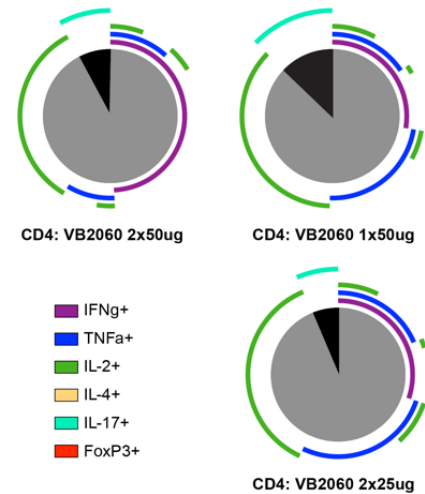
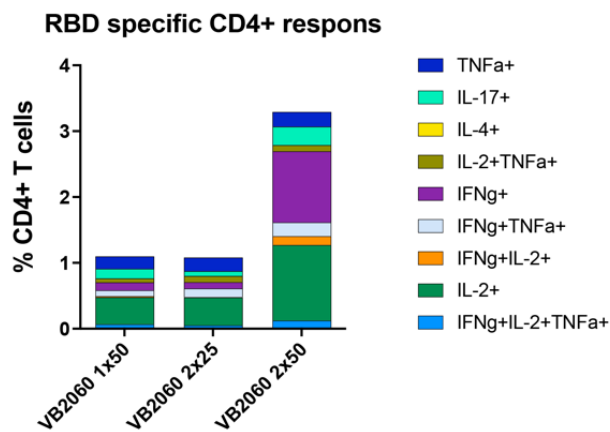
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116 **Fig. 9.** RBD specific multifunctional T cell responses in mice vaccinated with VB2060. Mice
 117 were vaccinated on day 0 and 21 and T cell responses were analyzed on day 90 using
 118 multiparameter flow cytometry. **a** Percent of CD4⁺ and **b** CD8⁺ T cells responding to RBD
 119 stimulation. Percent of RBD specific cells is shown in bar graphs. Cells expressing one marker,
 120 or combinations of multiple markers are shown as percent of parent population. Pie charts show
 121 cytokine profile. CD4⁺ T cell responses presented with Th1 (grey) / Th17 (black) bias. Pie charts

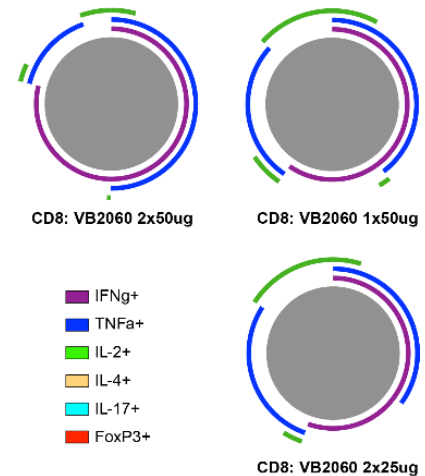
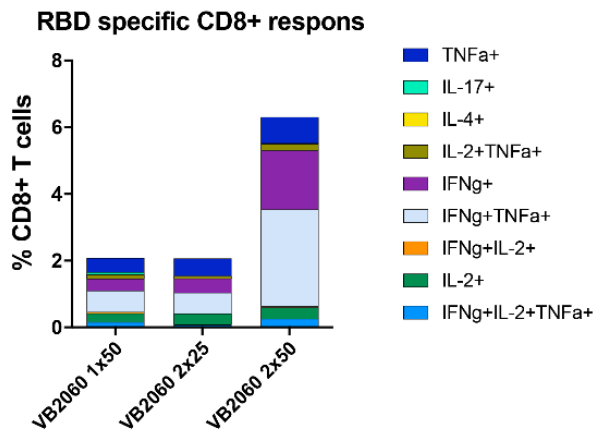
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122 were made using Spice software.

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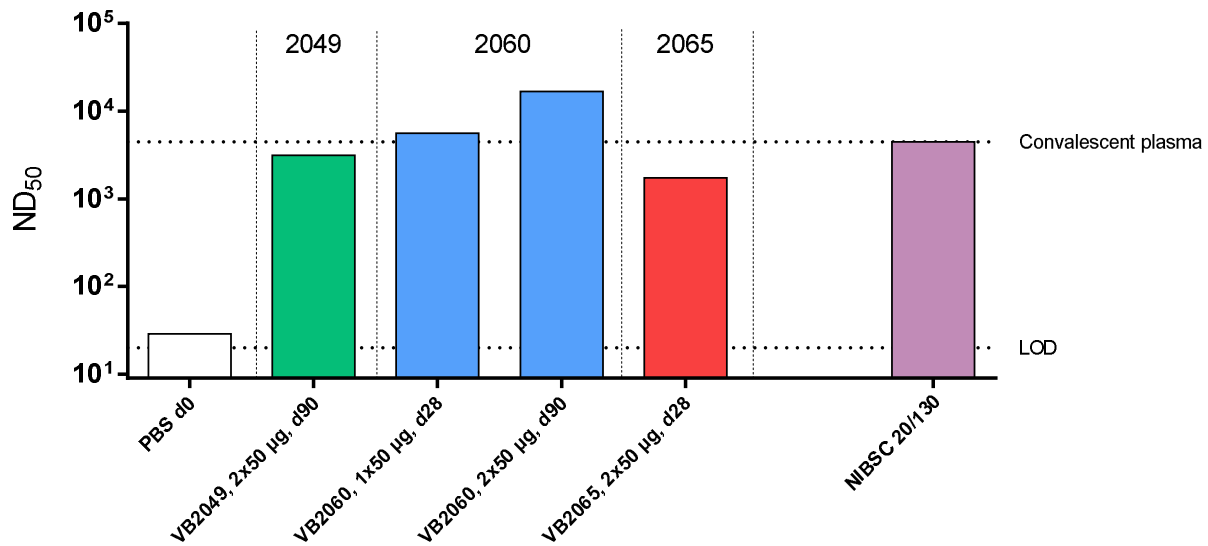


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126 **Supplemental Materials**

127 **Supplemental figure S1. Neutralizing antibody responses in sera after immunization with**
128 **VB2049, VB2060 and VB2065.** Bars represent endpoint titers for pooled sera assessed in live
129 virus neutralization against homotypic SARS-CoV-2 live virus strain Australia/VIC01/2020.
130 Lower dashed line indicate the limit of detection (LOD) of the assay, and the upper dashed line
131 the titer value for the convalescent serum reference NIBSC 20/130 (ND50 endpoint titer 4443).
132 Due to limited serum volume available, only pools from selected timepoints could be tested.



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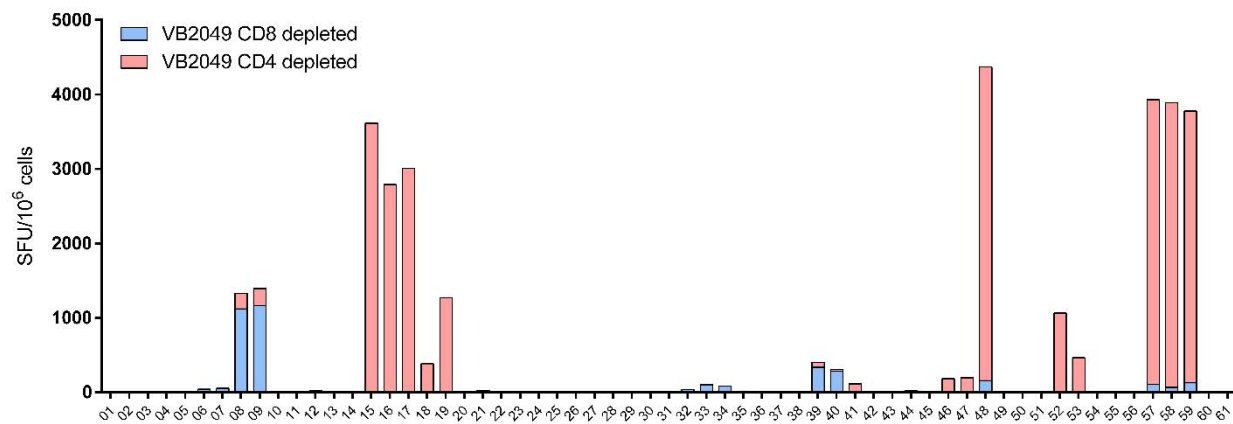
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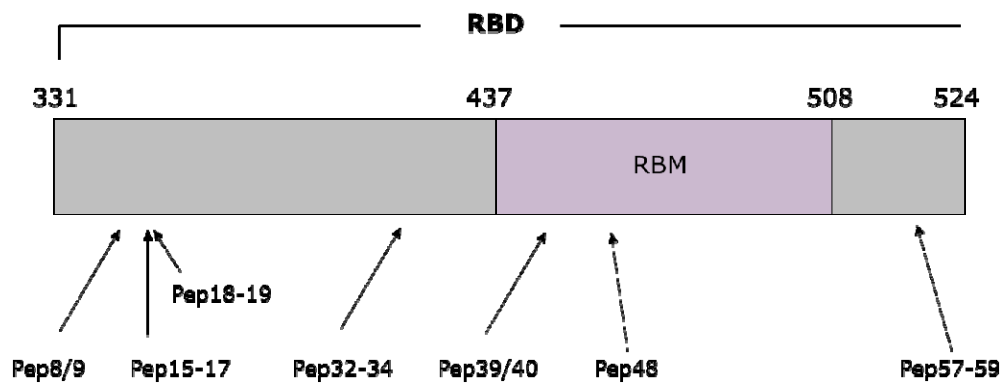
137 **Supplemental figure S2. Identification of CD4⁺ and CD8⁺ RBD-specific T cell epitopes**
138 **following i.m. vaccination with 25 µg VB2049 at day 0 and day 21 in mice (*n* = 5). a** CD4⁺
139 **and CD8⁺ T cell populations were stimulated for 24 h with 61 individual RBD peptides (15-mer**
140 **peptides overlapping by 12 aa from SARS-CoV2 RBD domain) and the number of IFN-γ positive**
141 **spots/1x10⁶ splenocytes were detected in an ELISpot assay 1 week post boost vaccination. b** Map
142 **of the SARS-CoV2 RBD domain and identification of immunodominant peptides in mice. RBM;**
143 **receptor-binding motif.**

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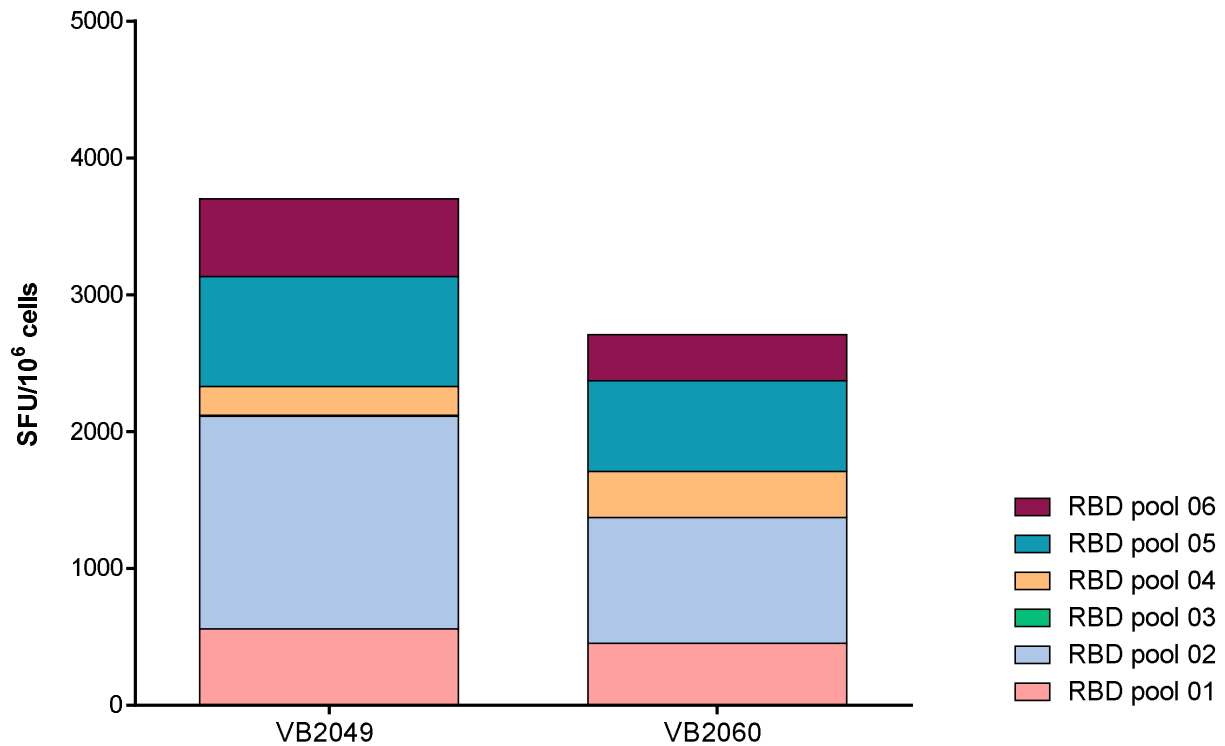


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148 **Supplemental figure S3. Comparative T cell responses induced by VB10.COV2 DNA**
149 **vaccines VB2049 and VB2060.** Two doses of 2.5 μg vaccine administered i.m. at days 0 and 21,
150 and splenocytes were harvested at day 28 post first immunization. The total number of IFN- γ
151 positive spots/ 1×10^6 splenocytes after restimulation with overlapping RBD peptide pools were
152 determined by IFN- γ ELISpot.

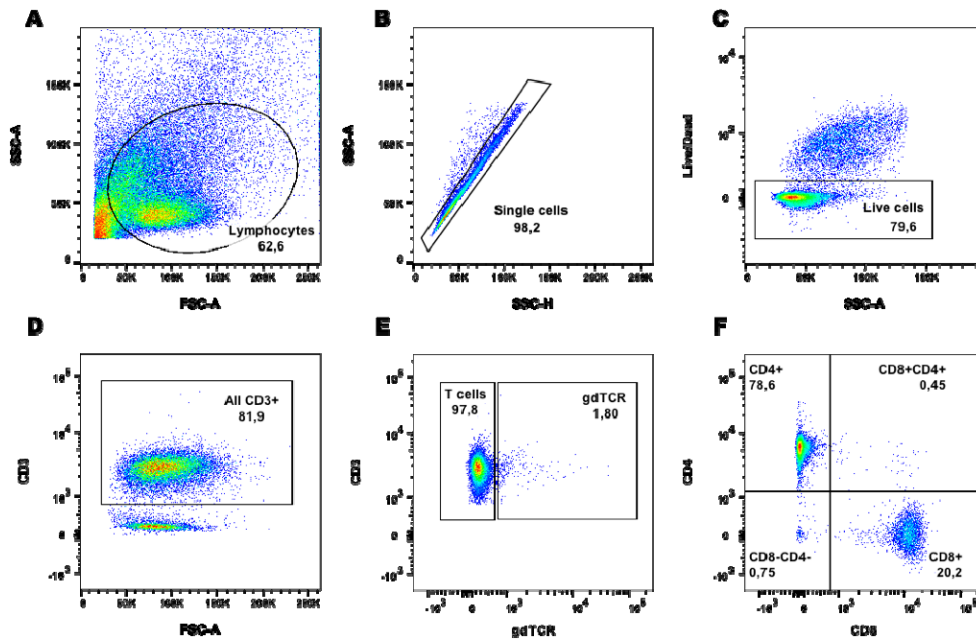
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156 **Supplemental figure S4. Gating strategy for the identification of T cells.** **a** All cells were
157 examined using side scatter (SSC) and forward scatter (FSC) parameters. Lymphocyte gate was
158 set based on the relative size (FSC) of the cells. **b** Lymphocytes were analyzed for presence of
159 doublets, and a gate was set to include only single cells in further analysis. **c** Dead cells were
160 identified using viability dye and a gate was set to include live cells in further analysis. **d** In the
161 population of live cells, all CD3⁺ cells were gated for future analysis. **e** T cells were defined as
162 CD3⁺ and $\gamma\delta$ TCR T cells were excluded from the analysis. **f** All T cells were analyzed for
163 expression of CD4 and CD8 markers.



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168 **Supplemental table S1. Individual peptides tested for induction of CD4+ and CD8+ RBD**
 169 **specific immune responses and T cell epitope mapping after i.m. vaccination with 25 µg of**
 170 **VB2049 in mice.** Sequences that were immunodominant in mice (i.e. strong CD4⁺ or CD8⁺
 171 responses) are shown in shaded columns. *; epitopes verified in a separate study mapping T cell
 172 epitopes induced by a Spike based DNA vaccine candidate in mice (Smith et al. 2020).

Dominant peptides #	Sequence	Amino acid	CD4 or CD8 dominated response
08	AWNRRKRISNCVADYS	355-369	CD4 (medium)
09	RKRISNCVADYSVLY	358-370	CD4 (medium)
15	SFSTFKCYGVSP TKL	374-388	CD8 (strong)
16	TFKCYGVSP TKLNDL	377-391	CD8 (strong)
17	CYGVSP TKLNDLCFT	380-394	CD8 (strong)
18	VSPTKLNDLCFTNVY	383-397	CD8 (weak)
19	TKLNDLCFTNVYADS	386-400	CD8 (medium)
32	KLPDDFTGCVIAWNS	424-438	CD4 (weak)
33*	DDFTGCVIAWNSNNL	427-441	CD4 (weak)
34	TGCVIAWNSNNLDSK	430-444	CD4 (weak)
39*	VGGNYNYLYRLFRKS	445-459	CD4 (weak)
40	NYNYLYRLFRKSNLK	448-462	CD4 (weak)
48	IYQAGSTPCNGVEGF	471-485	CD8 (strong)
57	PTNGVGYQP YRVVVL	499-511	CD8 (medium)
58	GVGYQP YRVVLSFE	502-516	CD8 (medium)
59*	YQP YRVVLSFELLH	505-519	CD8 (medium)

173

174 **Supplemental table S2.** Specificity of peptides eliciting significant T cell responses by VB2065
175 (Spike).

Spike pool	Peptides	aa	Position in Spike
Pool 06	62-73	244-297	NTD (N-terminal domain)
Pool 11	123-134	488-541	RBD (receptor binding domain)
Pool 20	231-238,240-242,244,245	920-986	HR1 (heptapeptide repeat sequence 1)
Pool 22	257-269	1024-1081	
Pool 23	270-276,278,280-283	1076-1137	

176

177 **Supplemental table S3. Description of antibodies used for flow cytometry.**

Target	Fluorochrome	Vendor	Catalogue No.
CD3	Brilliant ultraviolet 395	BD Bioscience	740268
CD4	Brilliant violet 785	Biolegend	100453
CD8	PE/Cyanine 7	Biolegend	100721
$\gamma\delta$ TCR	PerCP/Cyanine5.5	Biolegend	118117
TNF- α	Brilliant violet 605	Biolegend	506329
IFN- γ	APC	Biolegend	505809
IL-4	PE	Biolegend	504103
IL-2	Brilliant violet 421	Biolegend	503825
IL-17	Alexa Fluor 488	Biolegend	506909
FoxP3	Alexa Fluor 700	Biolegend	126421

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