

1 Development of a Synthetic Poxvirus-Based SARS-CoV-2 Vaccine

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14 **One sentence summary:** Chiuppesi *et al.* demonstrate the use of a uniquely designed and fully
15 synthetic poxvirus-based vaccine platform to rapidly develop a SARS-CoV-2 vaccine candidate
16 enabling stimulation of potent humoral and cellular immune responses to multiple antigens.

17 **Abstract**

18 Modified Vaccinia Ankara (MVA) is a highly attenuated poxvirus vector that is widely used to
19 develop vaccines for infectious diseases and cancer. We developed a novel vaccine platform
20 based on a unique three-plasmid system to efficiently generate recombinant MVA vectors from
21 chemically synthesized DNA. In response to the ongoing global pandemic caused by SARS
22 coronavirus-2 (SARS-CoV-2), we used this novel vaccine platform to rapidly produce fully
23 synthetic MVA (sMVA) vectors co-expressing SARS-CoV-2 spike and nucleocapsid antigens,
24 two immunodominant antigens implicated in protective immunity. Mice immunized with these
25 sMVA vectors developed robust SARS-CoV-2 antigen-specific humoral and cellular immune
26 responses, including potent neutralizing antibodies. These results demonstrate the potential of a
27 novel vaccine platform based on synthetic DNA to efficiently generate recombinant MVA vectors
28 and to rapidly develop a multi-antigenic poxvirus-based SARS-CoV-2 vaccine candidate.

29 **Introduction**

30 Modified Vaccinia Ankara (MVA) is a highly attenuated poxvirus vector that is widely used to
31 develop vaccine approaches for infectious diseases and cancer (1-3). As a result of the
32 attenuation process through 570 virus passages on chicken embryo fibroblast (CEF), MVA has
33 acquired multiple major and minor genome alterations (4, 5), leading to severely restricted host
34 cell tropism (6). MVA can efficiently propagate on CEF and a baby hamster kidney (BHK) cell
35 line, while in most mammalian cells, including human cells, MVA replication is limited due to a
36 late block in virus assembly (3, 6). Its excellent safety and immunogenicity profile in addition to
37 its versatile expression system and large capacity to incorporate heterologous DNA make MVA
38 an ideal vector for recombinant vaccine development (1, 7). We developed MVA vaccines for
39 animal models of cytomegalovirus-associated disease in pregnant women while demonstrating
40 vaccine efficacy in several clinical trials in solid tumor and stem cell transplant patients (8-13).

41 Since the outbreak of the novel severe acute respiratory syndrome coronavirus-2 (SARS-CoV-
42 2) in December 2019 (14, 15), the virus has spread to more than 200 countries worldwide,
43 causing a pandemic of global magnitude with over 400,000 deaths. Many vaccine candidates
44 are currently under rapid development to control this global pandemic (16-18), some of which
45 have entered into clinical trials with unprecedented pace (17, 19). Most of these approaches
46 employ antigenic forms of the Spike (S) protein as it is considered the primary target of
47 protective immunity (16, 20-22). The S protein mediates SARS-CoV-2 entry into a host cell
48 through binding to angiotensin-converting enzyme 2 (ACE) and is the major target of
49 neutralizing antibodies (NAb) (23-25). Studies in rhesus macaques show that vaccine strategies
50 based on the S antigen can prevent SARS-CoV-2 infection and disease in this relevant animal
51 model (18), indicating that the S antigen may be sufficient as a vaccine immunogen to elicit
52 SARS-CoV-2 protective immunity. However, a recent study demonstrated that even patients
53 without measurable NAb can recover from SARS-CoV-2 infection, suggesting that protection

54 against SARS-CoV-2 infection is mediated by both humoral and cellular immunity to multiple
55 immunodominant antigens, including S and nucleocapsid (N) antigens (20, 26).

56 We developed a novel vaccine platform based on a uniquely designed three-plasmid system to
57 efficiently generate recombinant MVA vectors from chemically synthesized DNA. In response to
58 the ongoing global pandemic caused by SARS-CoV-2, we used this novel vaccine platform to
59 rapidly produce synthetic MVA (sMVA) vectors co-expressing full-length S and N antigens. We
60 demonstrate that these sMVA vectors stimulate robust SARS-CoV-2 antigen-specific humoral
61 and cellular immunity in mice, including potent NAb. These results emphasize the value of a
62 novel vaccine platform based on synthetic DNA to efficiently produce recombinant poxvirus
63 vectors and warrant further pre-clinical and clinical testing of a multi-antigenic sMVA vaccine
64 candidate to control the ongoing SARS-CoV-2 pandemic and its devastating consequences.

65

66 **Results**

67 **Construction of sMVA**

68 To develop the three-plasmid system of the sMVA vaccine platform, we designed three unique
69 synthetic sub-genomic MVA fragments (sMVA F1-F3) based on the MVA genome sequence
70 published by Antoine *et al.* (4), which is ~178 kbp in length and contains ~9.6 kbp inverted
71 terminal repeats (ITRs) (Figure 1A). The three fragments were designed as follows: sMVA F1
72 comprises ~60 kbp of the left part of the MVA genome, including the left ITR sequences; sMVA
73 F2 contains ~60 kbp of the central part of the MVA genome; and sMVA F3 contains ~60 kbp of
74 the right part of the MVA genome, including the right ITR sequences (Figure 1B). sMVA F1 and
75 F2 as well as sMVA F2 and F3 were designed to share ~3kb overlapping homologous
76 sequences to promote recombination of the three sMVA fragments (Figure 1B). In addition, a
77 duplex copy of the 165-nucleotide long MVA terminal hairpin loop (HL) flanked by concatemeric
78 resolution (CR) sequences was added to both ends of each of the three sMVA fragments
79 (Figure 1C). Such CR/HL/CR sequence arrangements are formed at the genomic junctions in
80 poxvirus DNA replication intermediates and are essential for genome resolution and packaging
81 (27-31). When circular DNA plasmids containing these CR/HL/CR sequence arrangements are
82 transfected into helper virus-infected cells they spontaneously resolve into linear
83 minichromosomes with intact terminal HL sequences (28, 29, 32). Based on these findings, we
84 hypothesized that the three sMVA fragments designed as shown in Figure 1B-C, when co-
85 transfected as circular DNA plasmids into helper virus-infected cells, resolve into linear
86 minichromosomes, recombine with each other via the shared homologous sequences, and are
87 ultimately packaged as full-length genomes into sMVA virus particles. All three sMVA fragments
88 were cloned in *E. coli* as bacterial artificial chromosome (BAC) clones.

89 Using a previously employed procedure to rescue MVA from a BAC (8, 9, 33), sMVA virus was
90 reconstituted with Fowl pox (FPV) as a helper virus upon co-transfection of the three DNA
91 plasmids into BHK cells (Figure 1D), which are non-permissive for FPV(34). Two different FPV
92 strains (HP1.441 and TROVAC) (35, 36) were used to promote sMVA virus reconstitution
93 (Figure 2A). Ultra-purified sMVA virus was produced following virus propagation in CEF, which
94 are commonly used for MVA vaccine production. The virus titers achieved with reconstituted
95 sMVA virus were similar to virus titers achieved with “wild-type” MVA (wtMVA) (Table S1).

96 ***In vitro* characterization of sMVA**

97 To characterize the viral DNA of sMVA, DNA extracts from sMVA and wtMVA-infected CEF
98 were compared for several MVA genome positions by PCR. Similar PCR results were obtained
99 with sMVA and wtMVA for all evaluated genome positions (Figure 1E), including the F1/F2 and
100 F2/F3 recombination sites, indicating efficient recombination of the three sMVA fragments.
101 Additional PCR analysis indicated the absence of any BAC vector sequences in sMVA viral
102 DNA (Figure 1E), suggesting spontaneous and efficient removal of bacterial vector elements
103 upon sMVA virus reconstitution. Comparison of viral DNA from ultra-purified sMVA and wtMVA
104 virus by restriction enzyme digestion revealed similar genome pattern between sMVA and
105 wtMVA (Figure 1F). Sequencing analysis of the sMVA viral DNA confirmed the MVA genome
106 sequence at several positions, including the F1/F2 and F2/F3 recombination sites. Furthermore,
107 whole genome sequencing analysis of one of the sMVA virus isolates reconstituted with FPV
108 TROVAC confirmed the assembly of the reference MVA genome sequence and absence of
109 vector-specific sequences in viral DNA originating from reconstituted sMVA virus.

110 To characterize the replication properties of sMVA, growth kinetics of sMVA and wtMVA were
111 compared on BHK and CEF cells, two cell types known to support productive MVA replication
112 (6). This analysis revealed similar growth kinetics of sMVA and wtMVA on both BHK and CEF

113 cells (Figure 2B). In addition, similar areas of viral foci were determined in BHK and CEF cell
114 monolayers infected with sMVA or wtMVA (Figure 2C), suggesting similar capacity of sMVA and
115 wtMVA to spread in MVA permissive cells. Compared to the productive replication of sMVA and
116 wtMVA in BHK and CEF cells (6), only limited virus production was observed with sMVA or
117 wtMVA following infection of various human cell lines (Figure 2D). These results are consistent
118 with the severely restricted replication properties of MVA and show that the sMVA virus can
119 efficiently propagate in BHK and CEF cells, while it is unable to propagate in human cells.

120 ***In vivo* immunogenicity of sMVA**

121 To characterize sMVA *in vivo*, the immunogenicity of sMVA and wtMVA was compared in
122 C57BL/6 mice following two immunizations at high or low dose. MVA-specific binding antibodies
123 stimulated by sMVA and wtMVA after the first and second immunization were comparable
124 (Figures 3A, S1A). While the antibody levels in the high dose vaccine groups exceeded those of
125 the low dose vaccine groups after the first immunization, similar antibody levels in the high and
126 low dose vaccine groups were observed after the second immunization. In addition, no
127 significant differences were detected in the levels of MVA-specific NAb responses induced by
128 sMVA and wtMVA after the second immunization (Figures 3B, S1B). MVA-specific T cell
129 responses determined after the booster immunization by *ex vivo* antigen stimulation using
130 immunodominant peptides (37) revealed similar MVA-specific T cell levels in mice receiving
131 sMVA or wtMVA (Figures 3C-D and S1C-D). These results indicate that the sMVA virus has a
132 similar capacity to wtMVA in inducing MVA-specific humoral and cellular immunity in mice.

133 **Construction of sMVA SARS-CoV-2 vaccine vectors**

134 Using highly efficient BAC recombination techniques in *E. coli*, full-length SARS-CoV-2 S and N
135 antigen sequences were inserted into commonly used MVA insertion sites located at different
136 positions within the three sMVA fragments. Combinations of modified and unmodified sMVA

137 fragments were subsequently co-transfected into FPV-infected BHK cells to reconstitute sMVA
138 SARS-CoV-2 (sMVA-CoV2) vectors expressing the S and N antigen sequences alone or
139 combined (Figure 4A and 4B). In the single recombinant vectors encoding S or N alone, termed
140 sMVA-S and sMVA-N, the antigen sequences were inserted into the Deletion (Del3) site
141 (Figures 1B and 4B) (5). In the double recombinant vectors encoding both S and N, termed
142 sMVA-N/S and sMVA-S/N, the antigen sequences were inserted into Del3 and the Deletion 2
143 (Del2) site (sMVA-N/S), or they were inserted into Del3 and the intergenic region between 069R
144 and 070L (IGR69/70) (sMVA-S/N) (Figures 1B and 4B) (5, 38). All antigen sequences were
145 inserted into sMVA together with mH5 promoter to promote antigen expression during early and
146 late phase of MVA replication (39, 40). sMVA-CoV-2 vaccine vectors were reconstituted with
147 FPV HP1.441 or TROVAC. Ultra-purified virus of the sMVA-CoV2 vectors produced using CEF
148 reached titers that were similar to those achieved with sMVA or wtMVA (Table S1).

149 ***In vitro* characterization of sMVA-CoV2 vaccine vectors**

150 To characterize S and N antigen expression by the sMVA-CoV2 vectors, BHK cells infected with
151 the sMVA-CoV2 vectors were evaluated by Immunoblot using S and N-specific antibodies. This
152 analysis confirmed the expression of the S or N antigen alone by the single recombinant
153 vaccine vectors sMVA-S and sMVA-N, while the expression of both the S and the N antigen
154 was confirmed for the double recombinant vectors sMVA-N/S and sMVA-S/N (Figure 4C).

155 Further characterization of the antigen expression by the sMVA-CoV2 vectors in HeLa cells
156 using cell surface and intracellular flow cytometry (FC) staining confirmed single and dual S and
157 N antigen expression by the single and double recombinant vaccine vectors. Staining with S-
158 specific antibodies revealed abundant cell surface and intracellular antigen expression by all
159 vectors encoding the S antigen (sMVA-S, sMVA-N/S, sMVA-S/N) (Figure 4D). In contrast,
160 staining with anti-N antibody revealed predominantly intracellular antigen expression by all

161 vectors encoding the N antigen (sMVA-N, sMVA-N/S, sMVA-S/N) (Figure 4D), although cell
162 surface staining was observed to a minor extent. S and N antigen expression by the sMVA-
163 CoV2 vectors was also investigated by immunofluorescence. This analysis confirmed co-
164 expression of the S and N antigens by the double recombinant vaccine vectors and indicated
165 efficient cell surface and intracellular expression of the S antigen, whereas the expression of the
166 N antigen was predominantly observed intracellular (Figure S2A-C). These results demonstrate
167 efficient antigen expression by the single and double recombinant sMVA-CoV2 vectors.

168 ***In vivo* immunogenicity of sMVA-CoV2 vectors**

169 To determine the immunogenicity of the sMVA-vectored S and N antigens alone or combined,
170 SARS-CoV-2-specific humoral and cellular immune responses were evaluated in Balb/c mice by
171 two immunizations with the single or double recombinant vaccine vectors. High-titer antigen-
172 specific binding antibodies were detected in all vaccine groups after the first immunization, and
173 an increase in these responses was observed after the booster immunization (Figure 5A-B and
174 S3A-B). While the single recombinant vectors induced binding antibodies only against the S or
175 N antigen, the double recombinant vectors induced binding antibodies against both the S and N
176 antigens. In addition, all sMVA-CoV2 vectors encoding the S antigen (sMVA-S, sMVA-S/N,
177 sMVA-N/S) stimulated high-titer binding antibodies against the S receptor binding domain
178 (RBD), which is considered the primary target of NAb(22, 24). Antigen-specific binding antibody
179 titers between the single and double recombinant vaccine groups were comparable. Notably,
180 SARS-CoV-2 antigen-specific binding antibody responses stimulated by the sMVA-CoV2
181 vaccine vectors in mice exceeded SARS-CoV-2 S-, RBD-, and N-specific binding antibody
182 responses measured in human convalescent immune sera (Figures 5A-B, and Figure S4).
183 Similar binding antibody responses to those induced by sMVA-CoV2 vectors in Balb/c mice
184 were elicited by the vaccine vectors in C57BL/6 mice (Figure S5). Analysis of the IgG2a/IgG1

185 isotype ratio of the binding antibodies revealed Th-1-biased immune responses skewed toward
186 IgG2a independently of the investigated vaccine group or antigen (Figure 5C and S3C).

187 Potent SARS-CoV-2-specific NAb responses as assayed using pseudovirus were detected after
188 the first immunization in all vaccine groups receiving the vectors encoding the S antigen (sMVA-
189 S, sMVA-S/N, sMVA-N/S), and these NAb responses increased after the booster immunization
190 (Figure 5D-E and S3D-E). Similar potent NAb responses as measured using pseudovirus were
191 also observed in the vaccine groups using infectious SARS-CoV-2 virus (Figure 5F-G and S3F-
192 G). We also evaluated the immune sera for potential antibody-dependent enhancement of
193 infection (ADE) using THP-1 monocytes. These cells do not express the ACE2 receptor, but
194 express Fc γ receptor II, which is considered the predominant mediator of ADE in SARS-CoV
195 infection (41). THP-1 monocyte infection by SARS-CoV-2 pseudovirus was not promoted by the
196 immune sera of any of the vaccine groups even at sub-neutralizing antibody concentrations
197 (Figure S6), suggesting absence of Fc-mediated ADE by the vaccine-antibodies responses.

198 SARS-CoV-2-specific T cells evaluated after the second immunization by *ex vivo* antigen
199 stimulation revealed both S- and N-specific T cell responses in the vaccine groups receiving the
200 double recombinant vectors sMVA-S/N and sMVA-N/S. In contrast, mice receiving the single
201 recombinant vectors sMVA-N or sMVA-S developed T cell responses only against either the N
202 or S antigen (Figure 6A-D, Figures S7-8). High levels of cytokine-secreting (IFN γ , TNF α and IL-
203 4) S-specific CD8 $+$ T cells were measured in all vaccine groups immunized with the S-encoding
204 sMVA-CoV2 vectors (Figure 6A). S-specific CD4 $+$ T-cells mostly produced Th1 cytokines (IFN γ
205 and TNF α), while production of Th2 cytokines (IL-4 and IL-10) did not increase following antigen
206 stimulation (Figure 6C, S8), indicating a Th1-biased response. While activated N-specific CD8 $+$
207 T cells were not detected at significant frequency (Figure 6B), N-specific IFN γ and to some
208 degree TNF α -secreting CD4 $+$ T cells were measured in all animals vaccinated with the single

209 and double recombinant vectors encoding N (Figure 6D and S8). No significant differences were
210 observed in the T cell levels of the single and double recombinant vaccine groups.

211 Stimulation of SARS-CoV-2-specific immune responses by both the S and N antigen was also
212 evaluated in mice by co-immunization using the single recombinant vectors sMVA-S and sMVA-
213 N at different doses. This study revealed similar SARS-CoV-2 antigen-specific humoral and
214 cellular immune responses in vaccine groups receiving sMVA-S and sMVA-N alone or in
215 combination (Figure S9-10). Altogether these results indicate that the sMVA-vectored S and N
216 antigens when expressed alone or combined using a single vector or two separate vectors can
217 stimulate potent SARS-CoV-2-specific humoral and cellular immune responses in mice.

218

219 **Discussion**

220 We developed a novel vaccine platform based on a fully synthetic form of the highly attenuated
221 and widely used MVA vector. In response to the ongoing global SARS-CoV-2 pandemic, we
222 used this novel vaccine platform to rapidly produce sMVA vectors co-expressing SARS-CoV-2 S
223 and N antigens and show that these vectors can induce potent SARS-CoV-2 antigen-specific
224 humoral and cellular immune responses in mice, including potent NAb. These results highlight
225 the feasibility to efficiently produce recombinant MVA vectors from chemically synthesized DNA
226 and to rapidly develop a synthetic poxvirus-based vaccine candidate to prevent SARS-CoV-2
227 infection. We envision that this novel vaccine platform based on synthetic DNA will facilitate the
228 development and clinical use of poxvirus vaccine vectors for infectious diseases and cancer.

229 Our strategy to produce a synthetic form of MVA using chemically synthesized DNA differs from
230 the recently described approach to produce a synthetic horsepox virus vaccine vector (42).
231 While our strategy to generate sMVA involves the use of three large circular DNA fragments
232 (~60 kbp) with intrinsic HL and CR sequences (Figure 1), the approach by Noyce *et al.* to
233 produce a synthetic horsepox vaccine involves the use of multiple smaller linear DNA fragments
234 (~10-30 kbp) and the addition of terminal HL sequences (42). Because the three sMVA
235 fragments can be used in a circular form for the sMVA reconstitution process they are easily
236 maintained in *E. coli* as BACs and transferred to BHK cells for sMVA virus reconstitution without
237 the need for additional purification steps or modifications. This feature greatly facilitates the
238 insertion of heterologous antigen sequences into the sMVA DNA by highly efficient bacterial
239 recombination techniques and to produce recombinant sMVA vaccine vectors. Additionally, the
240 three-plasmid system provides the flexibility for rapid production of recombinant MVA harboring
241 multiple antigens inserted into different MVA insertion sites, which can be particularly laborious
242 when generating recombinant MVA by the conventional transfection/infection procedure (3, 43).
243 Although the precise mechanism and order of events of the sMVA virus reconstitution using

244 circular plasmids was not investigated, we demonstrate that the sMVA fragments efficiently
245 recombine with one another and produce a synthetic form of MVA that is virtually identical to
246 wtMVA in genome content, replication properties, host cell range, and immunogenicity.

247 In contrast to most other currently employed SARS-CoV-2 vaccine approaches that solely rely
248 on the S antigen, our SARS-CoV-2 vaccine approach using sMVA employs immune stimulation
249 by S and N antigens, which both are implicated in protective immunity (20, 26). The observation
250 that the sMVA-CoV2 vectors co-expressing S and N antigens can stimulate potent NAb against
251 SARS-CoV-2 pseudovirus and infectious virions suggests that they can elicit antibodies that are
252 considered effective in preventing SARS-CoV-2 infection and disease (16, 18, 20, 21). We show
253 that the vaccine vectors stimulate a Th1-biased antibody and cellular immune response, which
254 is considered the preferred antiviral adaptive immune response to avoid vaccine-associated
255 enhanced respiratory disease (44, 45). We did not find any evidence for Fc-mediated ADE
256 promoted by the vaccine-induced immune sera, suggesting that antibody responses induced by
257 the vaccine vectors bear minimal risk for ADE-mediated immunopathology, a general concern in
258 SARS-CoV-2 vaccine development (44, 45). In addition, based on findings with other viruses
259 associated with ADE, the stimulation of Th1 immunity with a strong T cell response component
260 appears to be the way forward to develop an effective SARS-CoV-2 vaccine candidate (46).

261 Other immune responses besides NAb targeting the S antigen may contribute to protection
262 against SARS-CoV-2 infection, which is highlighted by the finding that even patients without
263 measurable NAb can recover from SARS-CoV-2 infection (20). While antibodies could be
264 particular important to prevent initial SARS-CoV-2 acquisition, T cell responses may impose an
265 additional countermeasure to control sporadic virus spread at local sites of viral infection,
266 thereby limiting virus transmission. Our dual recombinant vaccine approach based on sMVA to
267 induce robust humoral and cellular immune responses to S and N antigens may provide
268 protection against SARS-CoV-2 infection beyond other vaccine approaches that solely employ

269 the S antigen. Our results warrant further preclinical testing of a sMVA vaccine candidate for
270 protective efficacy in animal models towards rapid advancement into phase 1 clinical testing.

271 **Materials and Methods**

272 **Cells and Viruses**

273 BHK-21 (CCL-10), A549 (CCL-185), HeLa (CCL-2), 293T (CRL-1573), 143B (CRL-8303), MRC-
274 5 (CCL-171), HEK293/17 (CRL11268), THP-1 (TIB-202), ARPE-19 (CRL-2302) were purchased
275 from the American Type Culture Collection (ATCC) and grown according to ATCC
276 recommendations. CEF were purchased from Charles River (10100795) and grown in minimum
277 essential medium (MEM) with 10% FBS (MEM10). HEK293T/ACE2 were a kind gift of Pamela
278 J. Bjorkman (47). We acknowledge Bernard Moss (LVD, NIAID, NIH) for the gift of wtMVA (NIH
279 Clone 1) that was used solely as a reference standard. To produce sMVA and wtMVA virus
280 stocks, CEF were seeded in 30x150mm tissue culture dishes, grown to ~70-90% confluence,
281 infected at 0.02 multiplicity of infection (MOI) with sMVA or wtMVA. Two days post infection,
282 ultra-purified virus was prepared by 36% sucrose density ultracentrifugation and virus
283 resuspension in 1 mM Tris-HCl (pH 9) (48). Virus stocks were stored at -80°C. Virus titers were
284 determined on CEF by immunostaining of viral plaques at 16-24 h post infection using
285 polyclonal Vaccinia antibody. FPV stocks were produced following propagation on CEF using
286 FPV strain TROVAC (ATCC VR-2553) (35) or HP1.441 (36), kindly provided by Bernard Moss.
287 FPV titers were evaluated on CEF by virus plaque determination.

288 **Construction of sMVA fragments**

289 The three ~60 kbp sMVA fragments (F1-F3; Figure 1) comprising the complete MVA genome
290 sequence reported by Antoine *et al.* (NCBI Accession# U94848) (4) were constructed as
291 follows: sMVA F1 contained base pairs 191-59743 of the MVA genome sequence; sMVA F2
292 comprised base pairs 56744-119298 of the MVA sequence; and sMVA F3 included base pairs
293 116299-177898 of the reported MVA genome sequence (4). A CR/HL/CR sequence
294 arrangement composed of 5'-TTT TTT TCT AGA CAC TAA ATA AAT AGT AAG ATT AAA TTA

295 *ATT ATA AAA TTA TGT ATA TAA TAT TAA TAA AAT TAT GTA TAT GAT TTA CTA ACT*
296 *TTA GTT AGA TAA ATT AAT AAT ACA TAA ATT TTA GTA TAT TAA TAT TAT AAA TTA ATA*
297 *ATA CAT AAA TTT TAG TAT ATT AAT ATT ATA TTT TAA ATA TTT ATT TAG TGT CTA GAA*
298 AAA AA-3' was added in the same orientation to both ends of each of the sMVA fragments,
299 wherein the italicized letters indicate the duplex copy of the MVA terminal HL sequence and the
300 underlined letters indicate the CR sequences. Notably, the CR/HL/CR sequences incorporated
301 at the ITRs of sMVA F1 and F3 were added in identical arrangement as the CR/HL/CR
302 sequences occur at the ITRs at the genomic junctions of putative MVA replication intermediates
303 (4). The sMVA fragments were produced and assembled by Genscript using chemical
304 synthesis, combined with a yeast recombination system. All sMVA fragments were cloned into a
305 yeast shuttle vector, termed pCCI-Brick, which contains a mini-F replicon for stable propagation
306 of large DNA fragments as low copy BACs in *E. coli*. sMVA F1 and F3 were cloned and
307 maintained in EPI300 *E. coli* (Epicentre), while sMVA F1 was cloned and maintained in DH10B
308 *E. coli* (Invitrogen).

309 Antigen insertion

310 SARS-CoV-2 S and N antigen sequences were inserted into the sMVA fragments by *En*
311 *passant* mutagenesis in GS1783 *E. coli* cells (49, 50). Briefly, transfer constructs were
312 generated that consisted of the S or N antigen sequence with upstream mH5 promoter
313 sequence and downstream Vaccinia transcription termination signal (TTTTTAT), and a
314 kanamycin resistance cassette flanked by a 50 bp gene duplication was introduced into the
315 antigen sequences. The transfer constructs were amplified by PCR with primers providing ~50
316 bp extensions for homologous recombination and the resulting PCR products were used to
317 insert the transfer constructs into the sMVA DNA by a first Red-recombination reaction (49, 50).
318 Primers 5'- AAA AAA TAT ATT ATT TTT ATG TTA TTT TGT TAA AAA TAA TCA TCG AAT
319 ACG AAC TAG TAT AAA AAG GCG CGC C-3' and 5'-GAA GAT ACC AAA ATA GTA AAG ATT

320 TTG CTA TTC AGT GGA CTG GAT GAT TCA AAA ATT GAA AAT AAA TAC AAA GGT TC-3'
321 were used to insert the N antigen sequence into the Del2 site. Primers 5'- ATA TGA ATA TGA
322 TTT CAG ATA CTA TAT TTG TTC CTG TAG ATA ATA ACT AAA AAT TTT TAT CTA GTA TAA
323 AAA GGC GCG CC-3' and 5'-GGA AAA TTT TTC ATC TCT AAA AAA AGA TGT GGT CAT
324 TAG AGT TTG ATT TTT ATA AAA ATT GAA AAT AAA TAC AAA GGT TC-3' were used to
325 insert the S antigen sequence into the IGR69/70 insertion site primers. Primers 5'- TTG GGG
326 AAA TAT GAA CCT GAC ATG ATT AAG ATT GCT CTT TCG GTG GCT GGT AAA AAA TTG
327 AAA ATA AAT ACA AAG GTT C-3' and 5'-ACA AAA TTA TGT ATT TTG TTC TAT CAA CTA
328 CCT ATA AAA CTT TCC AAA TAC TAG TAT AAA AAG GCG CGC C-3' were used to insert the
329 S or N antigen sequence into the Del3 site. Underlined letters indicate the sequences used to
330 produce ~50 bp extensions for homologous recombination. The S and N antigen sequences
331 were based on the SARS-CoV-2 reference strain (NCBI Accession# NC_045512) and codon-
332 optimized for Vaccinia (10, 38). Inserted antigen sequences were verified by PCR, restriction
333 enzyme digestion, and sequencing.

334 sMVA virus reconstitution

335 sMVA virus reconstitution from the three sMVA DNA plasmids in BHK cells using FPV as a
336 helper virus was performed as follows (8-10). The three sMVA DNA plasmids were isolated from
337 *E. coli* by alkaline lysis (51) and co-transfected into 60-70% confluent BHK cells grown in 6-well
338 plate tissue culture plates using Fugene HD transfection reagent (Roche) according to the
339 manufacturer's instructions. At 4 hours post transfection, the cells were infected with
340 approximately 0.1-1 MOI of FPV to initiate the sMVA virus reconstitution. The
341 transfected/infected BHK cells were grown for 2 days and then every other day transferred, re-
342 seeded, and grown for additional two days in larger tissue culture formats over a period of 8-12
343 days until most or all of the cells showed signs of sMVA virus infection. Using this procedure,
344 characteristic MVA viral plaque formation and cytopathic effects (CPEs) indicating sMVA virus

345 reconstitution was usually detected at 4-8 days post transfection/infection. Fully infected BHK
346 cell monolayers were usually visible at 8-12 days post transfection/infection. sMVA virus from
347 infected BHK cell monolayers was prepared by conventional freeze/thaw method and passaged
348 once on BHK cells before producing ultra-purified virus stocks on CEF. sMVA or recombinant
349 sMVA-CoV-2 vectors were reconstituted either with FPV HP1.441 (sMVA hp, sMVA-N/S, sMVA-
350 S/N hp) or TROVAC (sMVA tv1 and tv2, sMVA-S tv, sMVA-N tv, sMVA-N/S tv, sMVA-S/N tv).

351 Host cell range

352 sMVA and wtMVA host cell range using various human cell lines (HeLa, 293T, MRC-5, A549,
353 and 143B) BHK cells, and CEF was determined as follows. The cells were seeded in 6-well
354 plate tissue culture format and at 70-90% confluence infected in duplicates with 0.01 MOI of
355 sMVA or wtMVA using MEM2. At 2 hours post infection, the cells were washed twice with PBS
356 and incubated for two days in normal growth medium (as described under cells and viruses).
357 After the incubation period, virus was prepared by conventional freeze/thaw method and the
358 virus titers of each duplicate infection was determined in duplicate on CEF.

359 Replication kinetics

360 To compare the replication kinetics of sMVA and wtMVA, CEF or BHK cells were seeded in 6
361 well-plate tissue culture format and at 70-90% confluence infected in triplicates at 0.02 MOI with
362 sMVA or wtMVA using MEM2. After 2 hours of incubation, the cells were grown in MEM10. At
363 24 and 48 hours post infection, virus was prepared by freeze/thaw method and the virus titers of
364 each triplicate infection and the inoculum was determined in duplicate on CEF.

365 Plaque size analysis

366 To compare the plaque size of sMVA virus and wtMVA, CEF or BHK cells were seeded in 6-well
367 plate tissue culture format and at 70-90% confluence infected with 0.002 MOI with sMVA or

368 wtMVA using MEM2. After 2 hours of incubation, MEM10 was added and the cells were grown
369 for 16-24 hours. The cell monolayers were stained with Vaccinia virus polyclonal antibody and
370 viral plaques were imaged using Leica DMi8 inverted microscope and measured using LAS X
371 software. The size of 25 viral plaques per sMVA or wtMVA was calculated using the formula
372 $Area = \pi \times a \times b$, where a and b are the major and minor radius of the ellipse, respectively.

373 PCR analysis

374 To characterize the viral DNA of the sMVA vectors by PCR, CEF were seeded in 6-well plate
375 tissue culture format and at 70-90% confluence infected at 5 MOI with sMVA or wtMVA. DNA
376 was extracted at 16-24 hours post infection by the DNA Easy Blood and Tissue Kit (Qiagen)
377 according to the manufacturer's instructions. All PCR reactions were performed with Phusion
378 polymerase (ThermoFischer Scientific). Primers 5'-TCG TGG TGT GCC TGA ATC G-3' and 5'-
379 AGG TAG CGA CTT CAG GTT TCT T-3' were used to detect MVA ITR sequences; primers 5'-
380 TAT CCA CCA ATC CGA GAC CA-3' and 5'-CCT CTG GAC CGC ATA ATC TG-3' were used
381 to verify the transition from the left ITR into the unique region; primers 5'-AGG TTT GAT CGT
382 TGT CAT TTC TCC-3' and 5'- AGA GGG ATA TTA AGT CGA TAG CCG-3' were used to verify
383 the Del2 site with or without inserted N antigen sequence; primers 5'-TGG AAT GCG TTC CTT
384 GTG C-3' and 5'-CGT TTT TCC CAT TCG ATA CAG-3' with binding sites flanking the F1/F2
385 homologous sequences were used to verify the F1/F2 recombination site; primers 5'-TAT AGT
386 CTT TGT GGC ATC CGT TG-3' and 5'-ACC CAA ACT TTA GTA AGG CCA TG-3' were used to
387 verify the IGR69/70 insertion site with or without inserted S antigen; primers 5'-ATA AGC GTT
388 GTC AAA GCG GG-3' and 5'-AGG AAA TAG AAA TTG TTG GTG CG-3' with binding sites
389 flanking the F2/F3 homologous sequences were used to verify the F2/F3 recombination site;
390 primers 5'-ACA TTG GCG GAC AAT CTA AAA AC-3' and 5'-ATC ATC GGT GGT TGA TTT
391 AGT AGT G-3' were used to verify the Del3 insertion site with and without inserted S or N
392 antigen sequences; primers 5'-TAT CCA CCA ATC CGA GAC CA-3' and 5'-GTC TGT CCG

393 TCT TCT CTA TTG TTT A-3' were used to verify the transition from the unique region into the
394 right ITR; and primers 5'-TTA ACT CAG TTT CAA TAC GGT GCA G-3 and 5'-TGG GGT TTC
395 TTC TCA GGC TAT C-3' were used to detect the *SopA* element of the BAC vector.

396 Restriction pattern analysis

397 BHK cells were seeded in 20x150 mm tissue culture dishes, grown to ~70-90% confluence, and
398 infected at 0.01 MOI with wtMVA, sMVA tv1, or sMVA tv2. Ultra-purified virus was prepared two
399 days post-infection as previously described (48). Viral DNA (vDNA) was phenol/chloroform
400 extracted, followed by ethanol precipitation as previously described(52). DNA concentration
401 and A_{260}/A_{280} ratios were determined using NanoVue (GE Healthcare Bio-sciences Corp). 10 μ g
402 of vDNA were digested with 3 units of either KpnI or Xhol, followed by visualization on 0.5%
403 EtBr-stained agarose gel that was run at 2.4v/cm, overnight.

404 Sequencing of sMVA fragments and genome

405 PacBio Long Read Sequencing analysis confirmed the integrity of the sMVA fragments and
406 sMVA genome, including a single point mutation in a non-coding determining region at 3 base
407 pairs downstream of 021L (4) that was found both in sMVA F1 and in reconstituted sMVA.
408 Briefly, 5 ug of fragmented DNAs were converted to SMRTbell libraries using the SMRTbell
409 Template Prep Kit 1.0 (PacBio). The libraries were size-selected (7-kb size cutoff) with
410 BluePippin (Sage Science). The size-selected libraries were loaded to SMRT cells using
411 MagBeads loading and sequenced on a PacBio RSII with 10 hour movie. Read demultiplexing,
412 mapping to the reference sequence (Vaccinia virus strain Ankara U94848.1), and variants
413 calling were performed using the SMRT Link (v6.0.0.47841). The identified variants were
414 visually inspected in SMRT view Genome Browser for confirmation. De novo assembly was
415 done using either canu v1.7.1 or wtdbg2 v2.5. The 5' start position of the assembled contig was
416 edited by comparing to the U94848.1 reference.

417 Immunblot analysis

418 BHK cells infected at 5 MOI were harvested 24-post infection. Proteins were solubilized in PBS
419 with 0.1% Triton X-100, supplemented with protease inhibitor, then reduced and denatured in
420 Laemmli buffer containing DTT and boiled at 95°C for ~10 minutes. Proteins were resolved on
421 a 4-20% Mini Protean TGX gradient gel (BioRad), and transferred onto PVDF membrane. S
422 protein was probed with anti-SARS-CoV-1 S1 subunit rabbit polyclonal antibody (40150-T62-
423 COV2, Sino Biological); N protein was probed with anti-SARS-CoV1 NP rabbit polyclonal
424 antibody (40413-T62, Sino Biological). Vaccinia BR5 protein was probed as a loading control.
425 Anti-rabbit polyclonal antibody conjugated with horseradish peroxidase (Sigma-Aldrich) was
426 used as a secondary antibody and protein bands were visualized with chemiluminescent
427 substrate (ThermoFisher).

428 Flow cytometry

429 HeLa cells were seeded in a 6-well plate (5x10⁵/well) and infected the following day with sMVA
430 vaccine candidates at an MOI of 5. Following an incubation of 6 hours, cells were detached with
431 non-enzymatic cell dissociation buffer (13151014, GIBCO). Cells were either incubated directly
432 with primary antibody or fixed and permeabilized prior to antibody addition. Anti-SARS-CoV-1
433 S1 mouse (40150-R007, Sino Biological) and S2 rabbit (GTX632604, GeneTex) monoclonal
434 antibodies, anti-SARS-CoV-1 N rabbit monoclonal antibody (40143-R001, Sino Biological), and
435 anti-vaccinia rabbit polyclonal antibody (9503-2057, Bio Rad) were used in dilution 1:2,000. One
436 hour later anti-mouse or anti-rabbit Alexa Fluor 488-conjugated secondary antibodies (A11001,
437 A21206; Invitrogen) were added to the cells at a dilution of 1:4,000. Live cells were ultimately
438 fixed with 1% paraformaldehyde (PFA).

439 Immunofluorescence

440 BHK or HeLa cells were grown on glass coverslips and infected with sMVA or recombinant
441 sMVAs encoding S and/or N proteins at an MOI of 5 for 6 hours at 37°C in a humidified
442 incubator (5% CO₂). After infection, cells were fixed for 15min in 2% PFA and then directly
443 permeabilized by addition of ice cold 1:1 acetone/methanol for 5min on ice. Cells were blocked
444 for 1 hr with 3% BSA at room temperature, incubated with primary antibody mix (1:500) against
445 the S2 subunit or N for 1 hr at 37°C, and then incubated with Alexa-conjugated secondary
446 antibodies (ThermoFischer) (1:2000) for 1 hr at 37°C, with washing (PBS + 0.1% Tween20)
447 between each step. For detection of cell membranes and nuclei, cells were incubated with
448 Alexa-conjugated wheat germ agglutinin at 5 µg/mL (Thermo Fisher) and DAPI for 10 minutes at
449 room temperature. Coverslips were washed and mounted onto slides with Fluoromount-G
450 (SouthernBiotech). Microscopic analysis was performed using a laser-scanning confocal
451 microscope (Zeiss, LSM700). Images were acquired and processed using Zen software (Zeiss).

452 Mouse immunization

453 The Institutional Animal Care and Use Committee (IACUC) of the Beckman Research Institute
454 of City of Hope (COH) approved protocol 20013 assigned for this study. All study procedures
455 were carried out in strict accordance with the recommendations in the Guide for the Care and
456 Use of Laboratory Animals and the Public Health Service Policy on the Humane Care and Use
457 of Laboratory Animals. 6 weeks old C57BL/6 (C57BL/6J, 000664) or Balb/c (BALB/cJ, 000651)
458 were purchased from the Jackson laboratories. C57BL/6 N ramp were bred at the City of Hope
459 animal facility. Mice (N=4-5) were immunized twice in three weeks interval by intraperitoneal
460 route with 5x10⁷ PFU (high dose) or 1x10⁷ PFU (low dose) of sMVA, wtMVA, or sMVA-CoV2
461 vectors. To determine immune stimulation by both the S and N antigen when using separate
462 vectors (Figures S9-10), mice were co-immunized via the same immunization schedule and
463 route with half of the high (2.5x10⁷ PFU) or low dose (0.5x10⁷ PFU) of each of the vaccine
464 vectors. Blood samples for humoral immune analysis were collected by retro-orbital bleeding

465 two weeks post-prime and one-week post booster immunization. Splenocytes for cellular
466 immune analysis were collected at one week post booster immunization and were isolated by
467 standard procedure after animals were humanely euthanized.

468 Binding antibodies

469 Binding antibodies in mice immunized with sMVA, wtMVA, or sMVA-CoV2 vectors were
470 evaluated by ELISA. ELISA plates (3361, Corning) were coated overnight with 1 µg/ml of MVA
471 expressing Venus fluorescent marker (9), S (S1+S2, 40589-V08B1, Sino Biological), RBD
472 (40592-V08H, Sino Biological) or N (40588-V08B, Sino Biological). Plates were blocked with 3%
473 BSA in PBS for 2 hours. Serial dilutions of the mouse sera were prepared in PBS and added to
474 the plates for two hours. After washing the plate, 1:3,000 dilution of HRP-conjugated anti-mouse
475 IgG secondary antibody (W402B, Promega) was added and incubated for one additional hour.
476 Plates were developed using 1-Step Ultra TMB-ELISA (34028, Thermo Fisher) for one to two
477 minutes after which the reaction was stopped with 1M H₂SO₄. Plates were read at 450
478 nanometers wave length using FilterMax F3 microplate reader (Molecular Devices). Binding
479 antibodies endpoint titers were calculated as the latest serum dilution to have an absorbance
480 higher than 0.1 absorbance units (OD) or higher than the average OD in mock immunized mice
481 plus 5 times the standard deviation of the OD in the same group at the same dilution. For
482 evaluation of the IgG2a/IgG1 ratio, mouse sera were diluted 1:10,000 in PBS. The assay was
483 performed as described above except for the secondary antibodies (1:2,000. goat Anti-Mouse
484 IgG2a cross absorbed HRP antibody, Southern biotech, 1083-05; Goat anti-Mouse IgG1 cross
485 absorbed HRP antibody, Thermo Fisher, A10551). The IgG2a/IgG1 ratio was calculated by
486 dividing the absorbance read in the well incubated with the IgG2a secondary antibody divided
487 the absorbance for the same sample incubated with the IgG1 antibody.

488 MVA neutralization assay.

489 ARPE-19 cells were seeded in 96 well plates (1.5×10^4 cells/well). The following day, serial
490 dilutions of mouse sera were incubated for 2 hours with MVA expressing the fluorescent marker
491 Venus (9) (1.5×10^4 PFU/well). The serum-virus mixture was added to the cells in duplicate wells
492 and incubated for 24 hours. After the 24 hours incubation period, the cells were imaged using
493 Leica DMi8 inverted microscope. Pictures from each well were processed using Image-Pro
494 Premier (Media Cybernetics) and the fluorescent area corresponding to the area covered by
495 MVA-Venus infected cells was calculated.

496 SARS-CoV-2 pseudovirus production

497 The day before transfection, HEK293T/17 were seeded in a 15 cm dish at a density of 5×10^6
498 cells in DMEM supplemented with 10% heat inactivated FBS, non-essential amino acids,
499 HEPES, and glutamine (53). Next day, cells were transfected with a mix of packaging vector
500 (pALDI-Lenti System, Aldevron), luciferase reporter vector and a plasmid encoding for the wild
501 type SARS-CoV2 Spike protein (Sino Biological) or vesicular stomatitis virus G (VSV-G,
502 Aldevron), using FuGENE6 (Roche) as a transfection reagent : DNA ratio of 3:1, according to
503 manufacturer's protocol. Sixteen hours post-transfection, the media was replaced and cells
504 were incubated for an additional 24-72 hours. Supernatants were harvested at 24-, 48- and 72
505 hours, clarified by centrifugation at 1,500 RPM for 5 minutes and filtered using a sterile 0.22 μ m
506 pore size filter. Clarified lentiviral particles were concentrated by ultracentrifugation at 20,000
507 RPM for 2 hours at 4°C. The pellet was resuspended in DMEM containing 2% heat inactivated-
508 FBS and stored overnight at 4°C to allow the pellet to completely dissolve. Next day, samples
509 were aliquoted, snap frozen and stored at -80°C for downstream assays.

510 SARS-CoV-2 pseudotype neutralization and ADE assay

511 Levels of p24 antigen in the purified SARS-CoV-2 pseudotype solution was measured by ELISA
512 (Takara). Mouse sera were heat inactivated, pooled and diluted at a linear range of 1:100 to

513 1:50,000 in complete DMEM. For the neutralization assay, diluted serum samples were pre-
514 incubated overnight at 4°C with SARS-CoV-2-Spike pseudotyped luciferase lentiviral vector,
515 normalized to 100 ng/mL of p24 antigen. HEK293T cells overexpressing ACE-2 receptor were
516 seeded the day before transduction at a density of 2×10^5 cells per well in a 96-well plate in
517 complete DMEM (47). Before infection, 5 μ g/mL of polybrene was added to each well.
518 Neutralized serum samples were then added to the wells and the cells were incubated for an
519 additional 48 hours at 37°C and 5% CO₂ atmosphere. Following incubation, cells were lysed
520 using 40 μ L of Luciferase Cell Culture Lysis 5x Reagent per well (Promega). Luciferase activity
521 was quantified using 100 μ L of Luciferase Assay Reagent (Promega) as a substrate. Relative
522 luciferase units (RLU) were measured using a microplate reader (SpectraMax L, Molecular
523 Devices) at a 570 nm wave length. The percent neutralization titer for each dilution was
524 calculated as follows: NT = [1-(mean luminescence with immune sera/mean luminescence
525 without immune sera)] \times 100. The titers that gave 90% neutralization (NT90) were calculated by
526 determining the linear slope of the graph plotting NT versus serum dilution by using the next
527 higher and lower NT. In all the experiments RLU of uninfected cells was measured and was
528 always between 50 and 90.

529 For the ADE assay, THP1 cells were seeded at a confluence of 2×10^6 cells/mL in a 96 well plate
530 and co-incubated for 48 hours with serum samples diluted at 1:5,000 or 1:50,000 in the
531 presence of SARS-CoV-2-Spike pseudotyped or VSV-G luciferase lentiviral vector, normalized
532 to 100 ng/mL of p24 antigen. Following incubation, cells were lysed using 100 μ L of ONE-Glo
533 Luciferase Assay System per well (Promega). RLU were measured as above.

534 SARS-CoV-2 focus reduction neutralization test (FRNT)

535 FRNT assay was performed as described recently (54). Briefly, HeLa-ACE2 cells were seeded
536 in 12 μ L complete DMEM at a density of 2×10^3 cells per well. In a dilution plate, pooled mouse

537 serum was diluted in series with a final volume of 12.5 μ L. Then 12.5 μ L of SARS-CoV-2 was
538 added to the dilution plate at a concentration of 1.2×10^4 pfu/mL.

539 After 1 h incubation, the media remaining on the 384-well plate was removed and 25 μ L of the
540 virus/serum mixture was added to the 384-well plate. The plate was incubated for 20 h after
541 which the plate was fixed for 1h. Each well was then washed three times with 100 μ L of 1xPBS
542 0.05% tween. 12.5 μ L of human polyclonal sera diluted 1:500 in Perm/Wash buffer (BD
543 Biosciences 554723) were added to each well in the plate and incubated at RT for 2 h. Each
544 well was further washed three times and peroxidase goat anti-human Fab (Jackson Scientific)
545 was diluted 1:200 in Perm/Wash buffer, then added to the plate and incubated at RT for 2 h.
546 The plate was then washed three times and 12.5 μ L of Perm/Wash buffer was added to the
547 plate then incubated at RT for 5 min. The Perm/Wash buffer was removed and TrueBlue
548 peroxidase substrate was immediately added (Sera Care 5510-0030). Sera were tested in
549 triplicate wells. Normal human plasma was used as negative controls for serum screening.

550 SARS-CoV-2 convalescent plasma samples

551 IBC Protocol 20004 approved the use of SARS-CoV-2 convalescent plasma. Anonymized
552 plasma samples of SARS-CoV-2 convalescent individuals (N=19) were obtained from UCSD.
553 Individuals were confirmed to be infected in the previous three to ten weeks by PCR and lateral
554 flow assay. All individuals were symptomatic with mild to moderate-severe symptoms. Serum
555 samples (DS-626-G and DS-626-N, Seracare) purchased before SARS-CoV-2 pandemic were
556 used as a negative control. SARS-CoV-2-specific binding antibodies in plasma samples were
557 measured as described above. Cross-adsorbed goat anti-human IgG (H+L) secondary antibody
558 (A18811, Invitrogen) was used at a dilution of 1:3,000.

559 T cell analysis

560 Spleens were harvested and dissociated using a cell mesh following which blood cells were
561 removed using RBC Lysis Buffer (BioLegend). 2.5×10^6 splenocytes were stimulated with S or N
562 peptide libraries (GenScript, 15mers with 11aa overlap, 1 μ g/ml), 0.1% DMSO, or phorbol
563 myristate acetate (PMA)-ionomycin (BD Biosciences) for 1.5 h at 37°C. Anti-mouse CD28 and
564 CD49d antibodies (1 μ g/ml; BioLegend) were added as co-stimulation. Brefeldin A (3 μ g/ml;
565 eBioscience) was added, and the cells were incubated for additional 16 h at 37°C. Cells were
566 fixed using Cytofix buffer (BD Biosciences) and surface staining was performed using
567 fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD3 (Clone 17A2, 555274, BD),
568 BV650 anti-mouse CD8a (Clone 53-6.7, 563234, BD). Following cell permeabilization using
569 Cytoperm buffer (BD Biosciences), ICS was performed using allophycocyanin (APC)-conjugated
570 anti-mouse IFN- γ (Clone XMG1.2, 554413, BD), phycoerythrin (PE)-conjugated anti-mouse
571 TNF- α (Clone MP6-XT22, 554419, BD), and PE-CF594 anti-mouse IL-2 (BD Biosciences (Clone
572 JES6-5H4, 562483, BD). In experiments testing double recombinants SARS-CoV2 vectors IL-2
573 antibody was not included and PE-CF594 anti-mouse IL-4 (clone 11B11, 562450, BD) and
574 BV421 rat anti mouse IL-10 (clone JES5-16E3, 563276, BD) were added. Events were acquired
575 using a BD FACSCelesta flow cytometer (2×10^5 cells/tube). Analysis was performed using
576 FlowJo. Antigen specific T cells were identified by gating on size (FSC vs SSC), doublet
577 negative (FSC-H vs FSC-A), CD3 $^+$, CD8 $^+$ /CD4 $^+$. Cytokine positive responses are presented
578 after subtraction of the background response detected in the corresponding unstimulated
579 sample (media added with Brefeldin A one hour after beginning of mock stimulation) of each
580 individual mouse sample.

581 Cytokines ELISA

582 Splenocytes (1×10^6) from immunized mice were incubated in v-bottom wells in the presence of
583 2 μ g/ml S or N peptide pools, or without stimulus in a volume of 200 μ l. 48 hours later, plates
584 were centrifuged 2000 RPM for 10 minutes and cell supernatant was collected and stored at -

585 80°C. Mouse TNF-alpha (MTA00B), Quantikine ELISA kit (R&D systems) was used according to
586 manufacturer's recommendations.

587 Statistics

588 Statistical evaluation was pursued using GraphPad Prism (v8.3.0). For evaluation of differences
589 in sMVA and wtMVA plaque area in BHK-21 and CEF cells and differences in sMVA and wtMVA
590 host cell range, one-way ANOVA followed by Tukey's and Dunnet's multiple comparison tests
591 were used, respectively. For sMVA and wtMVA growth kinetic analysis, mixed-effects model
592 with the Geisser-Greenhouse correction, followed by Tukey's multiple comparisons test were
593 applied. For ELISAs, one-way ANOVA and Tukey's multiple comparison tests were used to
594 calculate differences in endpoint titers and group means between groups. For IgG2a/IgG1 ratio
595 analysis, one-way ANOVA with Dunnett's multiple comparison test was used to compare the
596 IgG2a/IgG1 ratio measured in each group to a ratio of 1. Pearson correlation analysis was
597 performed to calculate the correlation coefficient r and its significance. For T cell responses
598 analysis, one-way ANOVA followed by Dunnett's multiple comparisons test with a single pooled
599 variance was used to compare the mean of each group.

600

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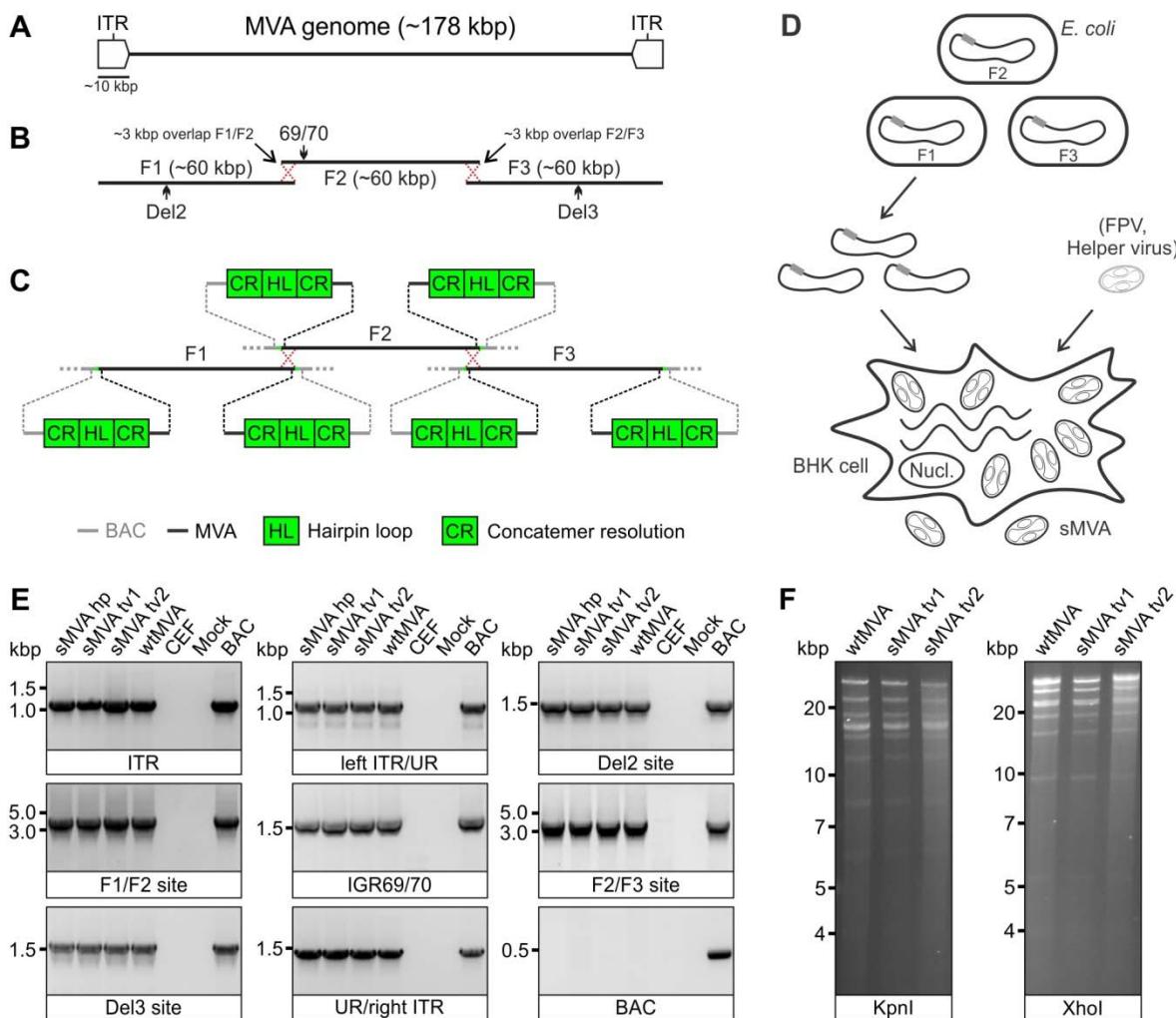
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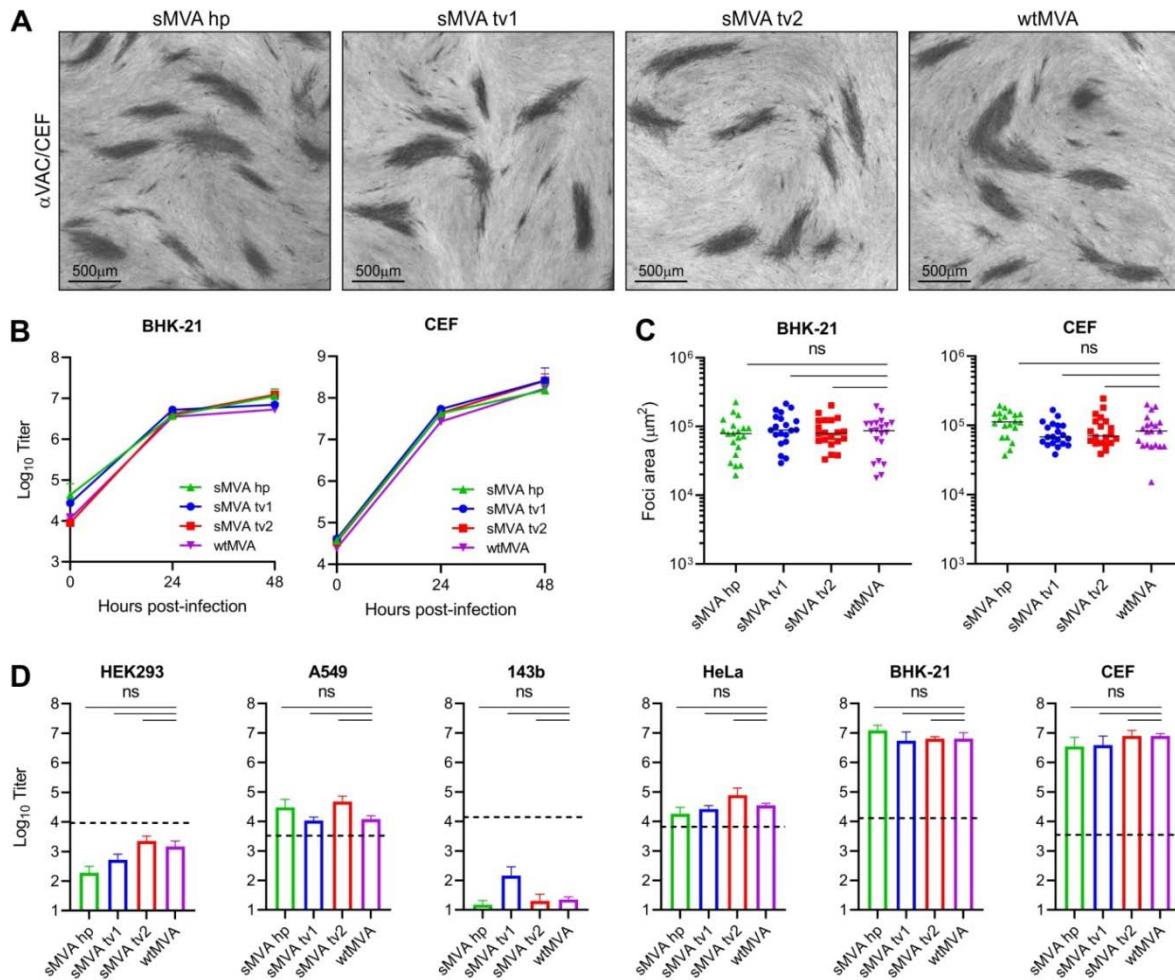
810 **Figures**



811

812 **Figure 1. sMVA construction and characterization.** **A)** Schematic of MVA genome. The MVA genome is ~178 kbp in length and
 813 contains ~9.6 kbp inverted terminal repeat (ITR) sequences. **B)** sMVA fragments. The three sub-genomic sMVA fragments (F1-F3)
 814 comprise ~60 kbp of the left, central, and right part of the MVA genome as indicated. sMVA F1/F2 and F2/F3 share ~3 kbp
 815 overlapping homologous sequences for recombination (red dotted crossed lines). Approximate genome positions of commonly used
 816 MVA insertion (Del2, IGR69/70, Del3) are indicated **C)** Terminal CR/HL/CR sequences. Each of the sMVA fragments contains at
 817 both ends a sequence composition comprising a duplex copy of the MVA terminal hairpin loop (HL) flanked by concatemeric
 818 resolution (CR) sequences. BAC = bacterial artificial chromosome vector. **D)** sMVA reconstitution. The sMVA fragments are isolated
 819 from the *E. coli* and co-transfected into BHK cells, which are subsequently infected with FPV as a helper virus to initiate sMVA virus
 820 reconstitution. **E)** PCR analysis. CEF infected with sMVA, derived with FPV HP1.441 (sMVA hp) or TROVAC from two independent
 821 virus reconstitutions (sMVA tv1 and sMVA tv2), were investigated by PCR for several MVA genome positions (ITR sequences,
 822 transition left or right ITR into internal unique region (left ITR/UR; UR/right ITR), Del2, IGR69/70 and Del3 insertion sites, and F1/F2
 823 and F2/F3 recombination sites) and absence of BAC vector sequences. PCR reactions with wtMVA-infected and uninfected cells,
 824 without sample (mock), or with MVA BAC were performed as controls. **F)** Restriction fragment length analysis. Viral DNA isolated
 825 from ultra-purified sMVA (sMVA tv1 and sMVA tv2) or wtMVA virus was compared by KpnI and Xhol restriction enzyme digestion.

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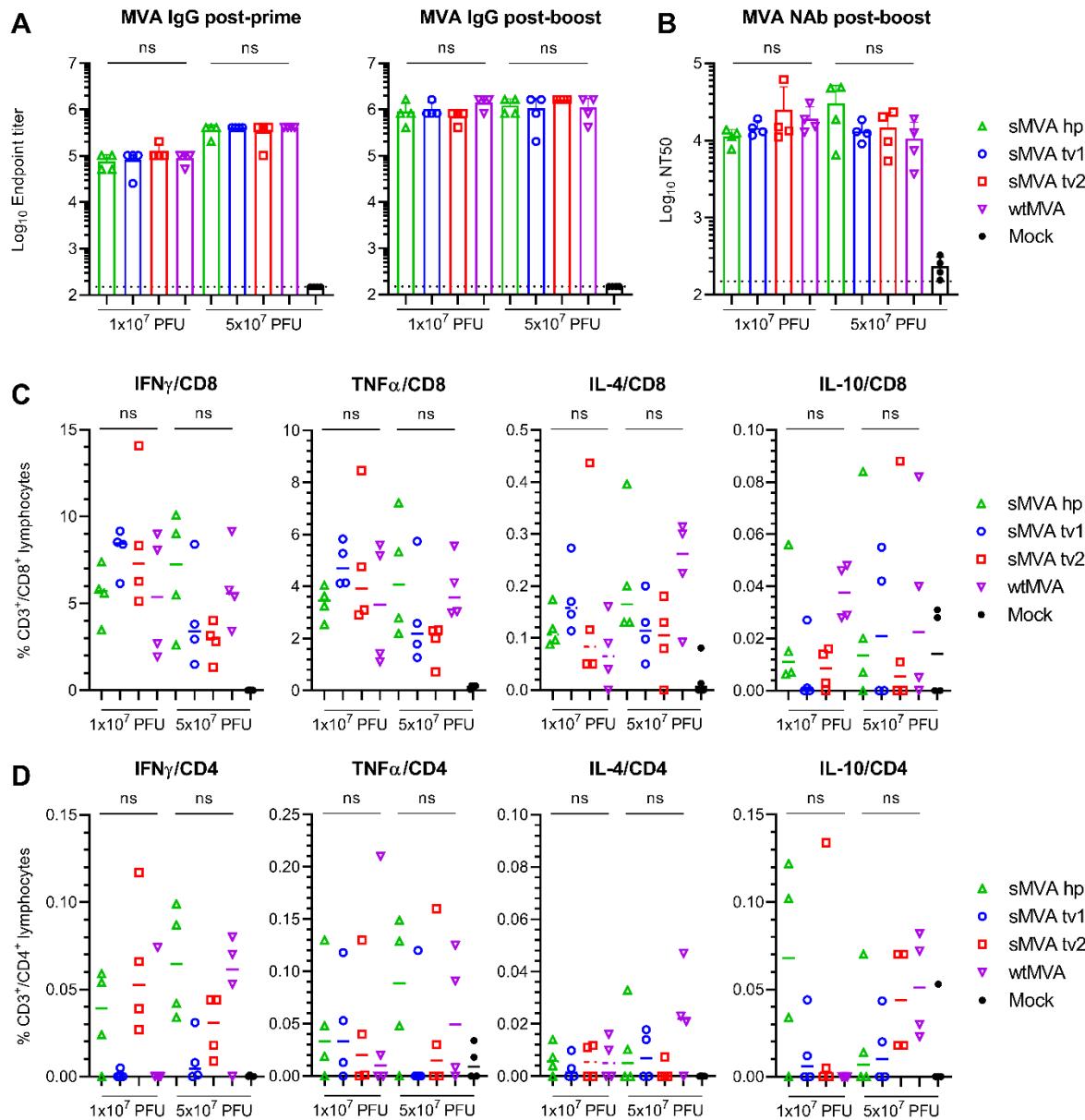


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828 **Figure 2. sMVA replication properties.** The replication properties of sMVA derived with FPV HP1.441 (sMVA hp) or TROVAC
 829 from two independent sMVA virus reconstitution (sMVA tv1 and sMVA tv2) were compared with wtMVA. **A**) Viral foci. CEF infected
 830 at low multiplicity of infection (MOI) with the reconstituted sMVA virus or wtMVA were immunostained using anti-Vaccinia polyclonal
 831 antibody (αVAC). **B**) Replication kinetics. BHK or CEF cells were infected at 0.02 MOI with sMVA or wtMVA and viral titers of the
 832 inoculum and infected cells at 24 and 48 hours post infection were determined on CEF. Mixed-effects model with the Geisser-
 833 Greenhouse correction was applied; at 24 and 48 hours post-infection differences between groups were not significant. **C**) Viral foci
 834 size analysis. BHK or CEF cell monolayers were infected at 0.002 MOI with sMVA or wtMVA and areas of viral foci were determined
 835 at 24 hours post infection following immunostaining with αVAC antibody. **D**) Host cell range analysis. Various human cell lines
 836 (HEK293, A549, 143b, and HeLa), CEF or BHK cells were infected at 0.01 MOI with sMVA or wtMVA and virus titers were
 837 determined at 48 hours post infection on CEF. Dotted lines indicate the calculated virus titer of the inoculum based on 0.01 MOI.
 838 Differences between groups in C-D were calculated using one-way ANOVA followed by Tukey's (C) or Dunnett's (D) multiple
 839 comparison tests. ns = not significant.

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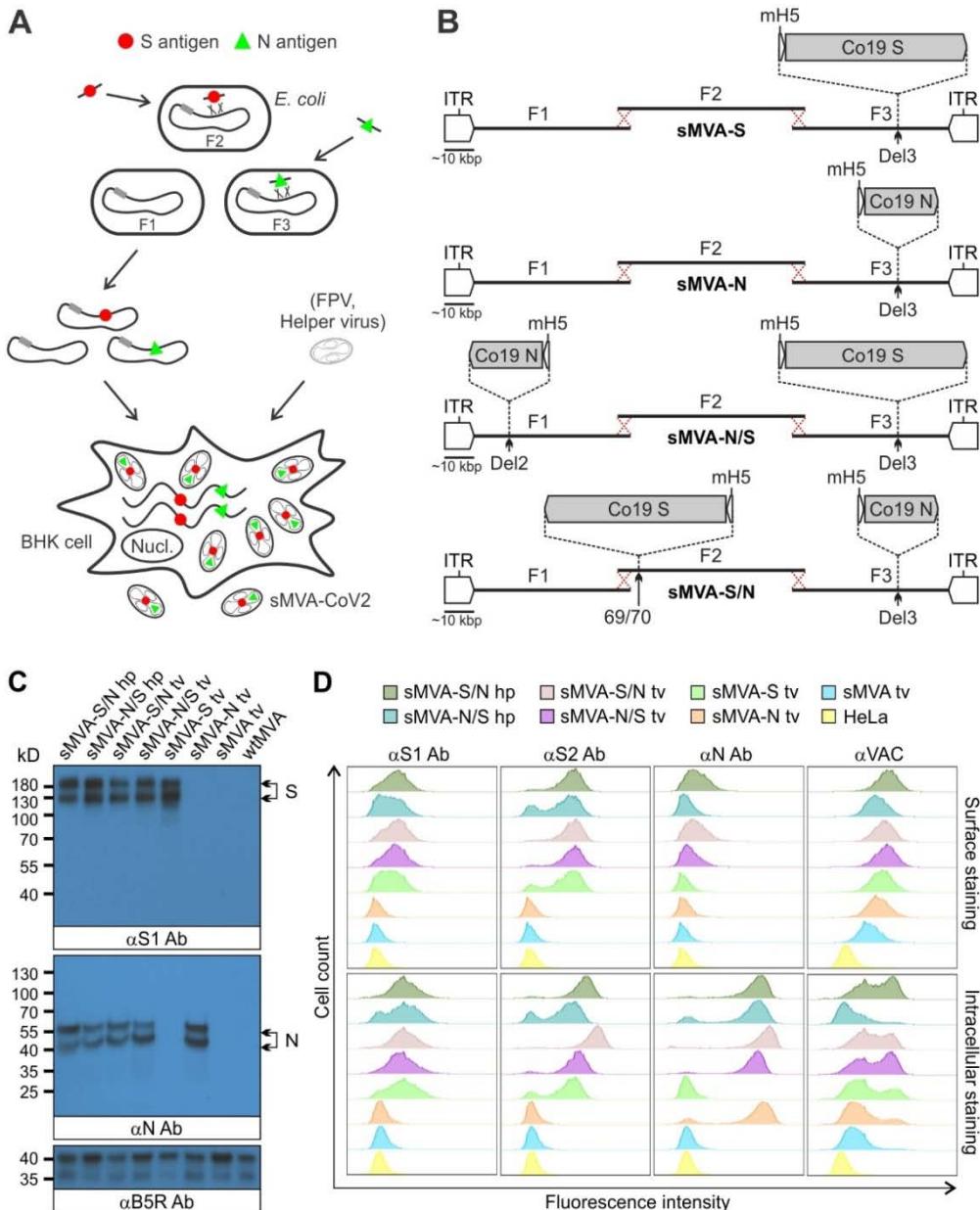


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Figure 3. sMVA *in vivo* immunogenicity. sMVA derived either with FPV HP1.441 (sMVA hp) or TROVAC from two independent virus reconstitution (sMVA tv1 and sMVA tv2) was compared by *in vitro* analysis with wtMVA. C57BL/6 mice were immunized twice at three week interval with low (1×10^7 PFU) or high (5×10^7 PFU) dose of sMVA or wtMVA. Mock-immunized mice were used as controls. **A**) Binding antibodies. MVA-specific binding antibodies (IgG titer) stimulated by sMVA or wtMVA were measured after the first and second immunization by ELISA. **B**) NAb responses. MVA-specific NAb titers induced by sMVA or wtMVA were measured after the booster immunization against recombinant wtMVA expressing a GFP marker. **C-D**) T cell responses. MVA-specific IFN γ , TNF α , IL-4, and IL-10-secreting CD8+ (C) and CD4+ (D) T cell responses induced by sMVA or wtMVA after two immunizations were measured by flow cytometry following *ex vivo* antigen stimulation using B8R immunodominant peptides. Differences between groups were evaluated using one-way ANOVA with Tukey's multiple comparison test. ns = not significant.

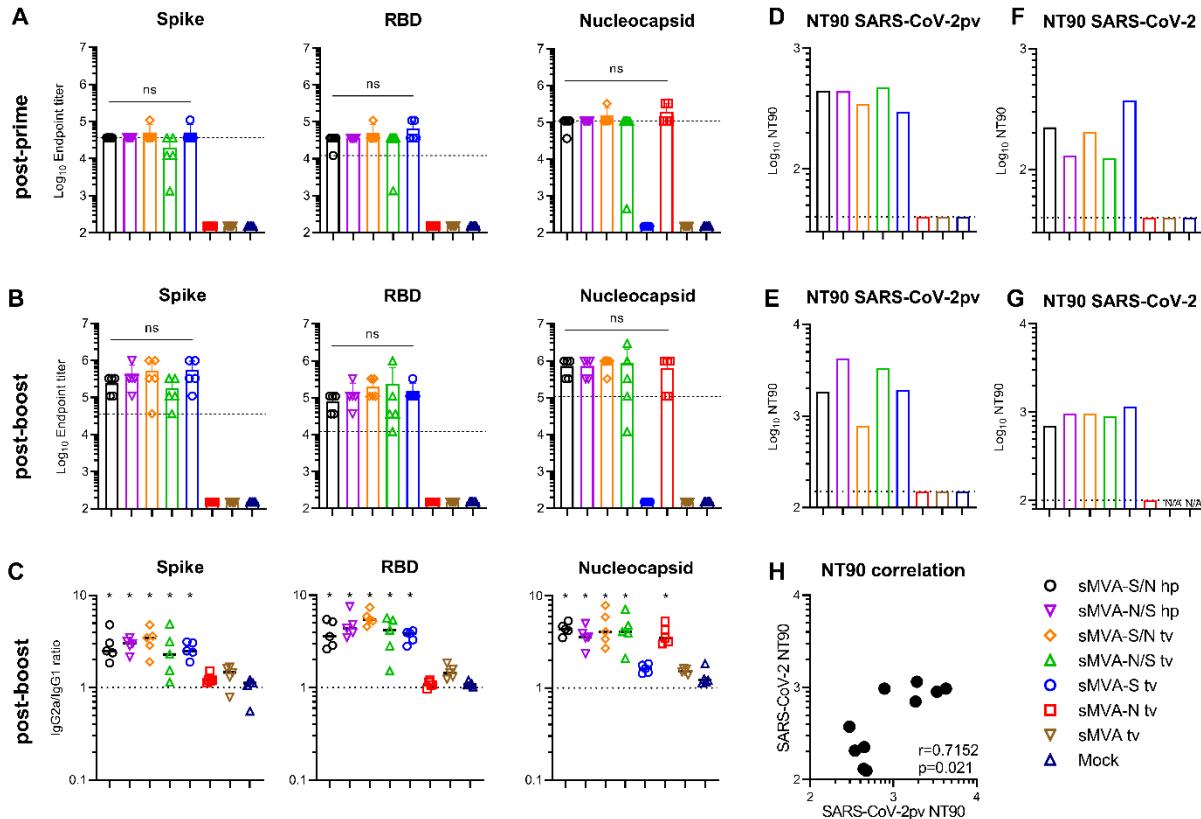
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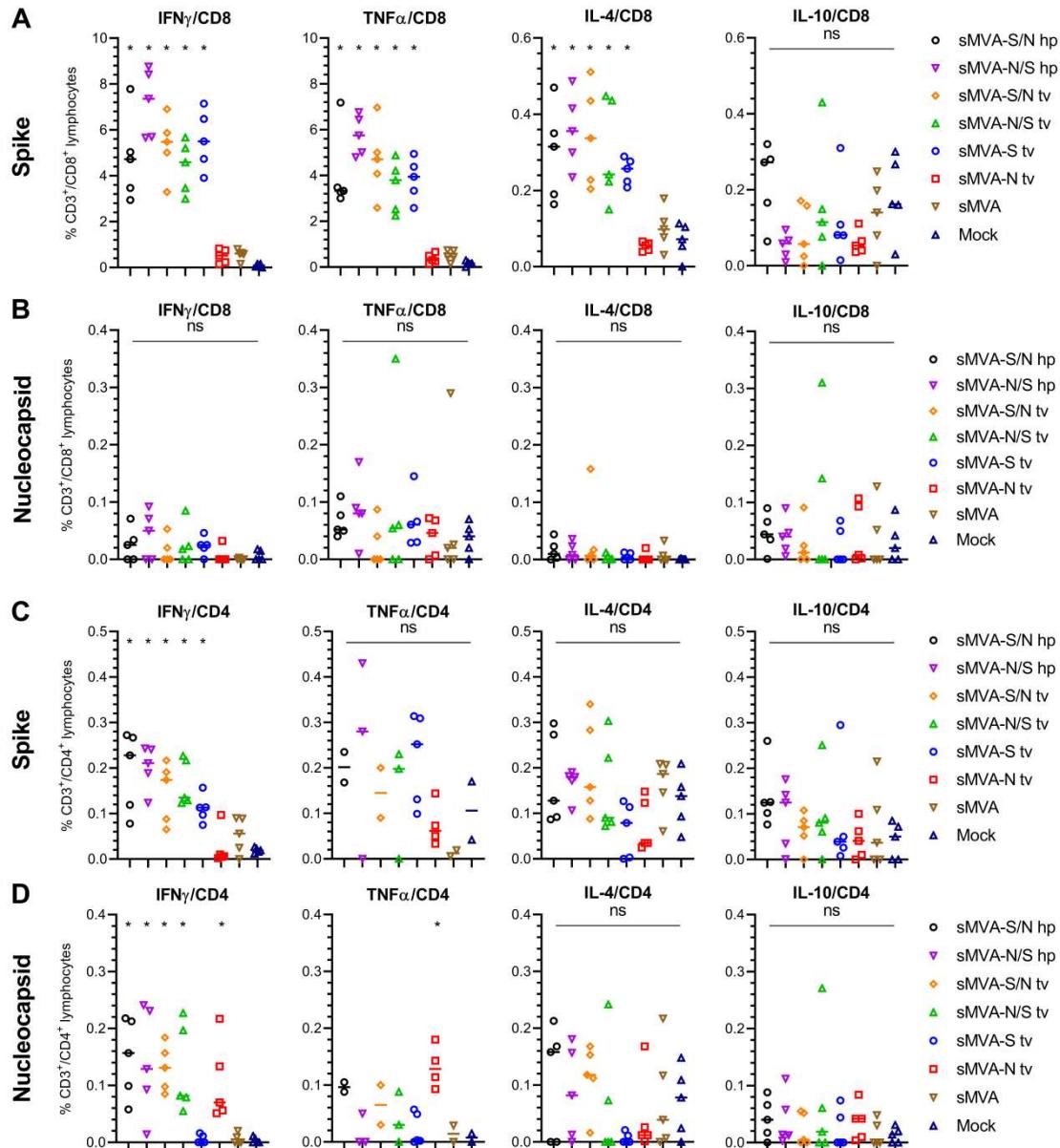
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855 **Figure 4. Construction and characterization of sMVA-CoV2 vectors. A)** Schematic representation of vector construction. S and
 856 N antigen sequences (red spheres and green triangles) were inserted into sMVA fragments F2 and F3 by bacterial recombination
 857 methods in *E. coli*. The modified sMVA fragments of F1 and F2 with inserted antigen sequences and the unmodified sMVA fragment
 858 F1 were isolated from *E. coli* and co-transfected into FPV-infected BHK cells to initiate virus reconstitution. **B)** Schematics of single
 859 (sMVA-S, sMVA-N) and double (sMVA-N/S, sMVA-S/N) recombinant sMVA-CoV2 vectors with S and N antigen sequences inserted
 860 into commonly used MVA insertion sites (Del2, IGR69/70, Del3). All antigens were expressed via the Vaccinia mH5 promoter. **C)**
 861 Western Blot. BHK cells infected with the single and double recombinant sMVA-CoV2 vectors derived with FPV HP1.441 (sMVA-
 862 S/N hp, sMVA-N/S hp) or TROVAC (sMVA-S/N tv, sMVA-N/S tv, sMVA-S tv, sMVA-N tv) were evaluated for antigen expression by
 863 Western Blot using anti-S1 and N antibodies (αS1 and αN Ab). Vaccinia B5R protein was verified as infection control. Higher and
 864 lower molecular weight bands may represent mature and immature protein species. **D)** Flow cytometry staining. HeLa cells infected
 865 with the vaccine vectors were evaluated by cell surface and intracellular flow staining using anti-S1, S2, and N antibodies (αS1, αS2,
 866 and αN Ab). Live cells were used to evaluate cell surface antigen expression. Anti-Vaccinia virus antibody (αVAC) was used as staining control to verify MVA protein expression. Cells infected with sMVA or wtMVA or uninfected cells were used as controls for experiments in C and D as indicated.



869

870 **Figure 5. Humoral immune responses stimulated by sMVA-CoV2 vectors.** Balb/c mice immunized twice in a three week interval
 871 with 5×10^7 PFU of the single and double recombinant sMVA-CoV2 vectors derived with FPV HP1.441 (sMVA-S/N hp and sMVA-N/S
 872 hp) or TROVAC (sMVA-S/N tv, sMVA-N/S tv, sMVA-S tv, sMVA-N tv) were evaluated for SARS-CoV-2-specific humoral immune
 873 responses **A-B**) Binding antibodies. S-, RBD-, and N-specific binding antibodies induced by the vaccine vectors were evaluated after
 874 the first (A) and second (B) immunization by ELISA. Dashed lines in A and B indicate median binding antibody endpoint titers
 875 measured in convalescent human sera (Figure S4). One-way ANOVA with Tukey's multiple comparison test was used to evaluate
 876 differences between binding antibody end-point titers. **C**) IgG2a/IgG1 isotype ratio. S-, RBD-, and N-specific binding antibodies of
 877 the IgG2a and IgG1 isotype were measured after the second immunization using 1:10,000 serum dilution, and absorbance reading
 878 was used to calculate IgG2a/IgG1 antibody ratio. One-way ANOVA with Dunnett's multiple comparison test was used to compare
 879 each group mean IgG2a/IgG1 ratio to a ratio of 1 (balanced Th1/Th2 response). **D-G**) NAb responses. SARS-CoV-2-specific NAb
 880 (NT90 titer) induced by the vaccine vectors were measured after the first (D, F) and second (E, G) immunization against SARS-
 881 CoV-2 pseudovirus (pv) (D-E) or infectious SARS-CoV-2 virus (F-G) in pooled sera of immunized mice. Shown is the average NT90
 882 measured in duplicate (D-E) or triplicate (F-G) infection. N/A=failed quality control of the samples. Dotted lines indicate lowest
 883 antibody dilution included in the analysis. **H**) SARS-CoV-2/SARS-CoV-2pv correlation analysis. Correlation analysis of NT90
 884 measured in mouse sera after one and two immunizations using infectious SARS-CoV-2 virus and SARS-CoV-2pv. Pearson
 885 correlation coefficient (r) was calculated in H. * $p < 0.05$. ns= not significant.



886

887 **Figure 6. Cellular immune responses stimulated by sMVA-CoV2 vectors.** Balb/c mice immunized twice in a three week interval
888 with 5×10^7 PFU of the single or double recombinant sMVA-CoV2 vectors derived with FPV HP1.441 (sMVA-S/N hp and sMVA-N/S
889 hp) or TROVAC (sMVA-S/N tv, sMVA-N/S tv, sMVA-S tv, sMVA-N tv) were evaluated for SARS-CoV-2-specific cellular immune
890 responses. Antigen-specific CD8+ (A and B) and CD4+ (C and D) T cell responses induced by the vaccine vectors after two
891 immunizations were evaluated by flow cytometry for IFN γ , TNF α , IL-4 and IL-10 secretion following ex vivo antigen stimulation using
892 SARS-CoV-2 S and N-specific peptide libraries. Due to technical issues, 1-3 animals/group were not included in the CD4/TNF α
893 analysis in C and D. One-way ANOVA with Tukey's multiple comparison test was used to compare differences in % of cytokine-
894 specific T-cells between groups. *p<0.05. ns=not significant.

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