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1 **Novel Bivalent Viral-Vectored Vaccines Induce Potent Humoral and Cellular**  
2 **Immune Responses Conferring Protection Against Stringent Influenza A**  
3 **Virus Challenge**

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22

23

24

25 **Abstract:**

26 Seasonal influenza viruses (IAV) are a common cause of acute respiratory illness worldwide and  
27 generate a significant socio-economic burden. IAV rapidly mutate, necessitating annual vaccine  
28 reformulation as traditional vaccines do not typically induce broad-spectrum immunity. In  
29 addition to seasonal infections, emerging pandemic influenza viruses present a continued threat  
30 to global public health. Pandemic influenza viruses have consistently higher attack rates and are  
31 typically associated with greater mortality as compared to seasonal strains. Ongoing strategies to  
32 improve vaccine efficacy typically focus on providing broad-spectrum immunity, and while both  
33 B and T cells can mediate heterosubtypic responses, typical vaccine development will augment  
34 either humoral or cellular immunity. However, multipronged approaches, targeting several  
35 antigens, may limit the generation of viral escape mutants. There are few vaccine platforms that  
36 can deliver multiple antigens and generate robust cellular and humoral immunity. In this work,  
37 we describe a novel vaccination strategy, tested pre-clinically in mice, for the delivery of novel  
38 bivalent viral-vectored vaccines. Here, we show this strategy elicits potent T cell responses  
39 toward highly conserved internal antigens, whilst simultaneously inducing high levels of  
40 antibodies towards hemagglutinin (HA). Importantly, these humoral responses generate long-  
41 lived plasma cells and generate antibodies capable of neutralising variant HA-expressing  
42 pseudotyped lentiviruses. Significantly, these novel viral-vectored vaccines induce strong  
43 immune responses capable of conferring protection in a stringent influenza A virus challenge.  
44 Thus, this vaccination regimen induces lasting efficacy toward influenza. Importantly, the  
45 simultaneous delivery of dual antigens may alleviate the selective pressure thought to potentiate  
46 antigenic diversity in avian influenza viruses.

47

## 48 **Introduction**

49 Seasonal influenza A virus (IAV) infections cause significant morbidity and mortality worldwide  
50 and remain a major public health concern. The novel avian-origin influenza A strain (H7N9),  
51 initially identified in 2013, is now circulating with almost annual frequency and accounted for  
52 one-third of all cases in the 2016/2017 influenza season. Most worryingly, the case-fatality rate for  
53 this virus exceeds 40% (1-3). In addition, H7N9 influenza viruses have recently been assessed  
54 as having the highest potential pandemic risk of any novel influenza A viruses; this assessment is  
55 based on recent studies which indicate that H7N9 viruses have increased genetic diversity,  
56 geographical distribution, and in recent outbreaks a significantly higher proportion of H7N9-  
57 infected patients have needed care in an ICU (1-3). For the past 70 years vaccination has been  
58 the mainstay healthcare strategy against influenza infection (4-6). However traditional  
59 inactivated influenza vaccines (IIVs) confer strain-specific protection and do not typically induce  
60 the broad-spectrum immunity needed in the face of a newly emergent IAV (7-9). The possible  
61 threat of a pandemic outbreak has therefore catalysed the development of broadly protective IAV  
62 vaccines.

63

64 Recent strategies to augment and broaden vaccine efficacy have shifted towards the development  
65 of 'universal' vaccines capable of providing heterosubtypic protection against multiple, or  
66 possibly all subtypes of IAV. While both humoral and cellular immunity can mediate  
67 heterosubtypic responses, inducing antibodies against the more conserved stalk domain of  
68 hemagglutinin (HA) has been the recent focus of many vaccine programmes (10, 11). However,  
69 multipronged approaches, targeting several antigens inducing both humoral and cellular  
70 responses may limit the generation of viral escape mutants compared to vaccines targeting a

71 limited number of protective epitopes on the HA stalk . There are few vaccine technologies that  
72 will facilitate the delivery of multiple antigens to generate robust cellular and humoral immunity  
73 toward infectious disease antigens.

74

75 Viral-vectored vaccines have been developed for the induction of strong humoral and potent  
76 cellular immunity toward encoded antigens. An added strength of this platform is that viral  
77 vectors can accommodate more than one antigen (12). For heterologous prime-boost vaccination  
78 strategies, typically, one viral vector (e.g. Chimpanzee Adenovirus (ChAd)) encoding the target  
79 antigen(s) is used for the priming vaccination and a different platform, most often Modified  
80 Vaccinia Ankara (MVA), is used for the boost or repeat vaccination. In the present study we  
81 describe novel ChAd and MVA-vectored vaccines designed to simultaneously induce  
82 heterosubtypic and protective B and T cell responses against three influenza A antigens, HA, NP  
83 and M1. Using a heterologous prime-boost strategy, we induce high levels of heterosubtypic and  
84 homologous immune responses targeting the major virion surface protein, HA and the conserved  
85 internal viral antigens NP and M1. We demonstrate protection, post prime-boost, vaccination in a  
86 stringent challenge model of mouse adapted avian IAV.

87

88

89 **Materials and Methods**

90 **Recombinant ChAd and MVA vaccines**

91 The construction of ChAdOx1 NP+M1 has been described previously (13). Details of the viral  
92 vectored vaccines used in these studies are as described in Table I :

93

94 **Immunizations**

95 Procedures were performed according to the Scientific Procedures act 1986 (U.K.) and were  
96 approved by the University of Oxford Animal Care and Ethical Review Committee. 6-8 week-  
97 old female BALB/c (H-2<sup>d</sup>) mice were obtained from Harlan Laboratories, Oxfordshire and were  
98 housed under specific pathogen free conditions. All vaccines were formulated in endotoxin-free  
99 PBS and administered intramuscularly in a total volume of 50µL. BALB/c mice were immunized  
100 i.m. with either 10µg of HA7 protein or MVA (1x10<sup>6</sup> PFU of MVA-GFP or MVA-NP+M1 or  
101 MVA-NP+M1-H5 or MVA- NP+M1-H7) or ChAdOx1 (2.2x10<sup>6</sup> iu of ChAdOx1 NP+M1 and/or  
102 1x10<sup>8</sup> iu ChAdOx1 H7 or 1x10<sup>8</sup> iu ChAdOx1 GFP). For prime-boost regimens mice were  
103 vaccinated with ChAdOx1 viral vectored vaccines and eight weeks later all mice were boosted  
104 with 1x10<sup>6</sup> PFU of MVA

105

106 **ELISpot**

107 Spleen ELISpot was performed to measure antigen-specific IFN-γ as described previously  
108 (14).The immunodominant H2-K<sup>d</sup> restricted (BALB/c) epitope NP<sub>147-158</sub> (TYQRTRALV) was  
109 used to measure post-vaccination responses following vaccination regimens with NP+M1 (15).  
110 H7HA responses were measured after stimulation with peptide pools. Two peptide pools were  
111 generated. The first pool contained peptides unique to A/Netherlands/219/2003. The second pool

112 contained peptides unique to A/Anhui/1/2013 and/or A/Shanghai/1/2013 when compared to  
113 A/Netherlands/219/2003  
114 Briefly, peptides that were conserved between A/Netherlands/219/2003 and A/Anhui/1/2013  
115 were pooled from the BEI resource (NR44011). The resultant pool was representative of H7HA  
116 from A/Netherlands/219/2003 (H7N7). All peptides that differed between  
117 A/Netherlands/219/2003 (H7N7) and divergent strains (A/Anhui/1/2013 (H7N9) and  
118 A/Shanghai/1/2013) were pooled to generate a second peptide pool. This H7HA peptide pool is  
119 representative of regions of amino acid sequence diversity in the HA of A/Anhui/1/2013 (H7N9)  
120 and A/Shanghai/1/2013 (H7N9) when compared to the vaccine insert A/Netherlands/219/2003  
121 (H7N7) and was generated from the BEI (NR44011 and NR-44012). H7 peptide pools were  
122 obtained through BEI Resources, NIAID, NIH: Peptide Array, Influenza Virus  
123 A/Shanghai/1/2013 (H7N9) Hemagglutinin Protein Diverse Peptides, NR-44012. Medium alone  
124 was used as a negative control and pools of overlapping peptides (H7HA) or the NP<sub>147-158</sub>  
125 (TYQRTRALV) were added typically at 2µg/mL.

126

## 127 **ELISA**

128 ELISA was performed essentially as described (14). Nunc Maxisorp® 96-well plates were  
129 coated with 0.1µg recombinant protein (H7HA protein was produced in-house as described (16))  
130 and recombinant H5HA protein (A/Vietnam/1203/2004 (H5N1), Recombinant from Baculovirus,  
131 NR-10510 from BEI resources) per well and plates were washed and until the 5<sup>th</sup> dilution of the  
132 reference standard (1:1,600 dilution) reached an approximate OD<sub>450</sub> value of 1. This point was  
133 defined as 1 Relative ELISA Unit (REU) and REU of test sera were calculated essentially as  
134 described (14, 17).

135

136 **IgG Antibody Secreting Cell ELISPOT Assay**

137 Bone marrow IgG Antibody Secreting Cell ELISPOT Assay was performed as described (18, 19)  
138 using approximately  $1 \times 10^7$  cells/mL in complete Iscove's that had been rested overnight.  
139 MultiScreen-IP filter plates were coated with 0.5 $\mu$ g recombinant HA while negative control  
140 wells were coated with irrelevant protein (0.5 $\mu$ g ovalbumin).

141

142 **Pseudotype Neutralisation Assay**

143 Starting with an initial 1:40 dilution, test sera was diluted 2-fold in complete DMEM and assayed  
144 as described (14) Results were normalized relative to cell-only and pseudotyped lentivirus-only  
145 wells and expressed as the percentage of inhibition of pseudotyped lentivirus entry  
146 (neutralisation). The half maximal inhibitory concentration ( $IC_{50}$ ) was calculated using GraphPad  
147 Prism 6 software.

148

149 **Challenge**

150 All animal protocols were reviewed and approved by the Mount Sinai Institutional Animal Care  
151 and Use Committee (IACUC). To assess the protective efficacy of the prime-boost vaccination  
152 regimen, 6-8 week-old female BALB/c (H-2<sup>d</sup>) mice (Jackson Laboratories Inc) were primed with  
153 either ChAdOx1 NP+M1 ( $1.1 \times 10^7$  infectious units (IU), Group 1), ChAdOx1-H7 HA ( $1 \times 10^8$   
154 IU, Group 2), or ChAdOx1-GFP ( $1 \times 10^8$  IU, Group 4) (n=10 mice per group). All viral vectors  
155 were administered intramuscularly in the musculus tibialis in a final volume of 50 $\mu$ L. One group  
156 of animals received both ChAdOx1 NP+M1 and ChAdOx1-H7 HA, where each virus was  
157 injected into separate limbs (n=5 mice per group; Group 3). Animals vaccinated with 10 $\mu$ g of



158 recombinant H7 from A/Anhui/1/13 (H7N9) supplemented with 5 µg of R848 (Invivogen Inc)  
159 served as a positive control (n=10, Group 5). Naïve animals remained unvaccinated (n=10,  
160 Group 6). Eight weeks following the prime, Groups 1, 2, and 3 were boosted intramuscularly  
161 with MVA-NP+M1 (1 x 10<sup>6</sup> IU). Group 4 received MVA-GFP as a boost and Group 5 was  
162 administered 10µL of recombinant HA with R848. Blood ELISpots were performed at two  
163 weeks post-boost to ensure successful vaccine uptake. Three weeks following boost vaccination  
164 all animals (n=55) were anesthetized and challenged with 5 murine 50% lethal doses 5xLD<sub>50</sub> of a  
165 6:2 reassortment of A/Shanghai/1/13 (H7N9) virus. Weight was monitored daily for 14 days;  
166 mice that lost 25% or more of their initial body weight were euthanized.

167

## 168 **Statistics**

169 Statistical analyses were carried out using GraphPad Prism software version 6 (GraphPad  
170 Software, La Jolla, CA). Data was tested for normal distribution and the appropriate statistical  
171 analysis applied.

172

173

174 **Results**

175 **Immunogenicity of Novel Bivalent Poxviral-Vectored Vaccines Expressing NP+M1 and**  
176 **H5HA**

177 BALB/c mice were immunized intramuscularly (i.m.) with  $1 \times 10^6$  plaque-forming units (PFU) of  
178 MVA-H5, a single antigen vector expressing the Group 1 HA, H5HA (A/Vietnam/1203/2004;  
179 H5N1), or, MVA-NP+M1-H5, a bivalent vaccine expressing the same H5HA antigen in addition  
180 to the T cell fusion antigen, NP+M1.

181

182 These new-generation bivalent constructs express NP+M1 using the early vaccinia promoter  
183 F11, while HA expression is driven by the P7.5 promoter. T cell immunogenicity was assessed 2  
184 weeks after vaccination by *ex vivo* IFN- $\gamma$  ELISpot against the immunodominant BALB/c  
185 epitope, NP<sub>147-158</sub> (TYQRTRALV) (Figure 1). T cell responses to this epitope in mice vaccinated  
186 with the bivalent vaccine, MVA-NP+M1-H5, were higher (median spot forming units  
187 (SFU)=206) ( $p=0.008$ ) when compared to mice vaccinated with MVA-NP+M1 (P7.5) (median  
188 SFU=60) (Figure 1A). It has previously been shown that immunogenicity toward antigens  
189 expressed under the F11 MVA promoter is greater than the response toward P7.5 expressed  
190 antigens (20).

191

192 Two weeks post-vaccination, total serum IgG responses were measured, by ELISA against  
193 recombinant H5HA protein (A/Vietnam/1203/2004; BEI resources) (Figure 1B). No significant  
194 differences were observed between mice receiving MVA-H5 or MVA-NP+M1-H5 (Figure 1B).

195

196 **Immunogenicity of Novel Bivalent Poxviral-Vectored Vaccines Expressing NP+M1 and**  
197 **H7HA**

198 As the immunogenicity of HA can vary greatly depending on subtype, humoral responses  
199 induced by vaccination with MVA-NP+M1-H7, a second bivalent construct expressing the  
200 Group 2 HA, (A/Netherlands/219/2003; H7N7), in addition to NP+M1, was also investigated.  
201 BALB/c mice (n=5-10) were immunized i.m. against H7HA and/or NP+M1.

202

203 As before, T cell responses following vaccination with the bivalent vaccine MVA-NP+M1-H7,  
204 wherein the expression of NP+M1 is driven by the F11 promoter, were significantly higher  
205 compared to vaccination with MVA-NP+M1 (wherein expression was driven under the P7.5  
206 promoter) (\*\*p<0.01). These results indicate that novel bivalent vaccine MVA-NP+M1-H7  
207 elicits potent T cell responses against the NP+M1 fusion protein while also expressing a second  
208 antigen from the same viral vector (Figure 2A).

209

210 Serum was collected at 2 and 8 weeks post vaccination and total IgG responses were measured  
211 against recombinant H7HA protein A/Netherlands/219/2003 (Figure 2B). As a comparator, a  
212 group of mice were vaccinated with 10µg recombinant H7HA protein. Mice immunized with  
213 MVA-NP+M1-H7 had the highest median H7HA-specific IgG antibodies at 2 weeks post  
214 vaccination compared to all other groups (Figure 2B). **Importantly IgG antibody titres were**  
215 **maintained and remained high out to 8 weeks post vaccination. Animals vaccinated with MVA-**  
216 **NP+M1-H7 had the highest median responses at 8 weeks post-vaccination (Figure 2B).**

217

218

## 219 Immunogenicity generated by multi-antigen ChAdOx1-vectored vaccination

220 BALB/c mice were immunized with ChAdOx1 NP+M1 ( $2.2 \times 10^8$  IU) or ChAdOx1-H7 ( $1 \times 10^8$  IU)  
221 or both, as described. Two weeks following vaccination, splenocytes were isolated and T cell  
222 responses were measured by *ex vivo* IFN- $\gamma$  ELISpot as before. Mice vaccinated with ChAdOx1  
223 NP+M1 had higher responses compared to mice that received a mixture of ChAdOx1 NP+M1  
224 and ChAdOx1-H7 (\*\* $p \leq 0.01$ ) (Figure 3A). However, no significant difference in ELISpot  
225 responses was observed between mice vaccinated with ChAdOx1 NP+M1 or a combination of  
226 ChAdOx1 NP+M1 and ChAdOx1-H7, administered into separate limbs. This approach has  
227 previously been shown to augment immune responses and avoid competition between two  
228 vaccines administered together (12).

229  
230 While no significant differences, post viral vector vaccination, were detected between the median  
231 H7HA-specific IgG antibodies, at two weeks (Figure 3B), all responses induced by vaccination  
232 with ChAdOx1-vectored vaccines encoding H7HA were significantly higher compared to  
233 vaccination with 10 $\mu$ g recombinant H7HA (\*\* $p \leq 0.01$ ) (Figure 3 B). These data demonstrate that  
234 vaccination with ChAdOx1-vectored vaccines expressing H7HA elicits superior humoral  
235 immunity compared to protein, and moreover these responses are maintained in multi-antigen  
236 vaccination regimens.

237

## 238 Prime-Boost Regimen Incorporating Simian Adenoviral Vectors and Poxviral Vectors 239 Expressing NP+M1 and H7HA

240 Adenovirus-MVA prime-boost regimens are currently one of the leading strategies to induce  
241 potent immune responses against vaccine antigens (21, 22). BALB/c mice (n=18 (6 per group))

242 received a priming vaccination of either ChAdOx1-NP+M1, ChAdOx1-H7, or both,  
243 administered separately into opposite limbs, as described. At eight weeks post-prime all groups  
244 were boosted with MVA-NP+M1-H7

245

#### 246 **T Cell Responses Following Prime-Boost vaccination**

247 Splenic cells were isolated 2 weeks post prime and post-boost in order to assess T cell responses  
248 against NP+M1 and H7HA. Consistent with previous data, NP<sub>147-158</sub> specific T cell responses  
249 were slightly higher in mice primed with ChAdOx1 NP+M1 when compared to ChAdOx1  
250 NP+M1 and ChAdOx1-H7 administered into opposite limbs (Figure 4A). However, following  
251 the MVA vaccination, T cell responses against NP+M1 were boosted approximately five-fold  
252 higher in all groups and there were no significant differences, after boost toward NP+M1 antigen  
253 between mice that were primed with either ChAdOx1 NP+M1 or co-administration of ChAdOx1  
254 NP+M1 and ChAdOx1-H7 (Figure 4A). T cell responses against H7HA were also measured by  
255 *ex vivo* IFN- $\gamma$  ELISpot against two different H7HA peptide pools, one representative of H7HA  
256 from A/Netherlands/219/2003 (H7N7) (Figure 4B) and another representative of amino acid  
257 sequence diversity between A/Netherlands/219/2003 (H7N7) and divergent strains  
258 (A/Anhui/1/2013 (H7N9) and A/Shanghai/1/2013 (H7N9)) (Figure 4C). Sequence homology at  
259 the amino acid level for divergent strains (A/Shanghai/1/2013 HA and A/Anhui/1/2013 (H7N9))  
260 and the vaccine insert, A/Netherlands/219/2003 HA was 96%.

261

262 As expected, mice primed only with ChAdOx1 NP+M1 had no detectable H7HA-specific T cell  
263 responses (Figure 4B&C, column 1). There were no significant differences between the H7HA-  
264 specific T cell responses, two weeks post prime or post boost between mice vaccinated with

265 ChAdOx1-H7 or ChAdOx1-H7 co-administered with ChAdOx1 NP+M1 (Figure 4). Collectively  
266 these results demonstrate that vaccination with either ChAdOx1-H7 or co-administration of  
267 ChAdOx1 NP+M1 and ChAdOx1-H7 followed by immunisation with MVA-NP+M1-H7  
268 induces heterosubtypic T cell responses against H7HA.

269

## 270 **Humoral responses**

271 Serum was collected at 2 and 8 weeks post prime and also post boost vaccinations and the  
272 longevity of antibody responses were followed out to 26 weeks following the initial  
273 immunization. Mice primed with ChAdOx1-H7 or ChAdOx1-H7 and ChAdOx-NP+M1 had  
274 higher total IgG against H7HA at all time points compared to vaccination with either protein  
275 alone or with ChAdOx1 NP+M1 followed by MVA-NP+M1-H7 (Figure 5A). As expected we  
276 were unable to detect serum responses to HA in the ChAdOx1-NP+M1 only group, until 2 weeks  
277 post-boost with MVA-NP+M1+H7. Peak boost responses for viral vector vaccinations were up  
278 to 50 fold higher than the response two weeks following prime immunization (e.g. Group 3  
279  $1.34 \times 10^5$  SFU. (2wk) vs.  $7.4 \times 10^6$  SFU (16 wk)) and persisted for at least 26 weeks post  
280 vaccination in all groups (Figure 5A). These data suggest that strong humoral immune responses  
281 toward H7HA are generated and maintained over time by heterologous ChAd-MVA prime boost  
282 regimens.

283

## 284 **B Cell Memory Responses Following ChAdOx1 – MVA Immunisation**

285 Long-lived humoral immunity is principally mediated by two B cell subsets; long-lived plasma  
286 cells (LLPCs) and memory B cells (mBCs). LLPCs predominantly reside in the bone marrow  
287 (BM) (23, 24) and continuously secrete antibody. In order to further understand the basis of the

288 humoral responses following heterologous prime-boost ChAd-MVA viral-vectored vaccination,  
289 LLPCs were enumerated 18 weeks following the boosting vaccination.

290

### 291 **Long-Lived Plasma Cells**

292 Total IgG<sup>+</sup> Antibody secreting cells (ASCs) and H7HA-specific ASCs representative of LLPCs  
293 were measured by IgG ASC ELISpot assay. Elevated numbers of total IgG secreting LLPCs  
294 were detected in the BM of all immunized groups (Figure 5B & 5C). However only elevated  
295 numbers of H7HA-specific LLPC, were detected in mice that had been primed with ChAdOx1-  
296 H7 or ChAdOx1-H7 and ChAdOx-NP+M1 and boosted with MVA-NP+M1+H7, as compared to  
297 naïve BALB/c mice (Figure 5B & 5C; column 2 and 3). There was no significant difference  
298 between the number of H7HA-specific LLPCs detected in mice primed with ChAdOx1-H7 only  
299 (median SFU=541) or ChAdOx1-H7 and ChAdOx1 NP+M1 (median SFU=589). However these  
300 numbers were higher compared to mice primed with ChAdOx1 NP+M1 (median SFU=100) or  
301 protein alone (median SFU=89) (Figure 5B).

302

### 303 **Functionality of Adaptive Immune Responses Following ChAdOx1 – MVA Prime-Boost** 304 **Vaccination**

#### 305 **Pseudotype Virus Neutralisation**

306 In order to assess the breadth of anti-HA antibody functionality, sera collected 8 weeks after  
307 boosting with MVA-NP+M1-H7 was assayed against a number of pseudotyped lentiviruses.  
308 Two strains of H7HA pseudotypes were tested, A/chicken/Italy/1082/1999 (H7N1), a low  
309 pathogenic avian influenza (LPAI) strain closely (98% at the amino acid level) related to the  
310 vaccine immunogen, and A/Shanghai/2/2013 (H7N9) (96% at the amino acid level), the novel

311 H7N9 first identified in humans in 2013. A third group 2 HA lentivirus, expressing a different  
312 subtype, H3HA from A/Udorn/307/1972 (H3N2) (48% at the amino acid level), was also tested.

313

314 Pooled sera from mice primed with ChAdOx1-H7 or ChAdOx1-H7 and ChAdOx1 NP+M1  
315 completely neutralized both H7 pseudotypes at all serum dilutions tested (Table II). In addition,  
316 IC<sub>50</sub> values from mice primed with ChAdOx1 NP+M1 and boosted with MVA-NP+M1-H7 were  
317 higher compared to mice vaccinated with protein alone. IC<sub>50</sub> values against the H3N2  
318 pseudotype lentivirus were comparable between all groups vaccinated with viral vectors but  
319 lower in the control group vaccinated with protein alone (Table II).

320

### 321 **Prime-boost vaccinated mice intranasally challenged with divergent pandemic H7N9 IAV**

322 To assess heterosubtypic protective efficacy, mice were vaccinated, as described, and challenged  
323 with a lethal dose (5xLD<sub>50</sub>) of A/Shanghai/1/13. Amino acid sequence homology for  
324 A/Shanghai/1/13 HA7 (EPI439486) and A/Netherlands/219/2003 (AY340089.1) HA7 is 96%.  
325 While sequence homology, at the amino acid level, for the challenge strain NP and M1 and viral  
326 vector encoded NP and M1 is 97% and 93% respectively.

327

328 Negative controls (n=10) were naïve animals or animals that received an ChAdOx1 and MVA  
329 prime (both encoding an irrelevant antigen GFP) boost vaccination. Three weeks after the last  
330 immunization, animals were challenged with 5 murine 50% lethal doses (LD<sub>50</sub>) of SH1  
331 (A/Shanghai/1/13) virus. Weight loss was monitored over a period of 14 days, and mice that lost  
332 more than 25% of their initial body weight were euthanized.

333



334 Animals vaccinated with ChAdOx1-H7 alone or ChAdOx1-H7 and ChAdOx1 NP+M1 and  
335 boosted with MVA-NP+M1-H7 (Groups 2 & 3) all survived lethal challenge. When these studies  
336 were repeated, Groups 2 & 3 and Group 1 (primed with ChAdOx1 NP+M1 boosted with MVA-  
337 NP+M1-H7), were found to be equally protective as a vaccination regimen that has previously  
338 been shown to be protective (Group 5 protein and a TLR agonist adjuvant) (25).  
339 Importantly, in the first challenge experiment Groups 2 & 3 (animals vaccinated with ChAdOx1-  
340 H7 alone or ChAdOx1-H7 and ChAdOx1 NP+M1 and boosted with MVA-NP+M1-H7) retained  
341 starting body weight throughout the monitoring period. Furthermore, in a second challenge  
342 experiment Group 1 also retained starting body weight and did not display any weight loss.  
343 These results indicate that this vaccination confers protection against both morbidity and  
344 mortality. Comparison across the nadir of weight loss (day 4/5 through to 8/9) between  
345 ChAdOx1 NP+M1 primed and MVA-NP+M1-H7 boosted animals (Group 1) and those that  
346 received a protective regimen (Group 5, positive control) demonstrates no significant difference  
347 in weight loss in the first challenge or in a second independent repeat challenge. It is evident that  
348 both humoral and cellular immunity can offer improved efficacy in this stringent challenge  
349 model when compared to protective vaccination regimens (protein and adjuvant).

350

351

352 **Discussion**

353 In 2013, avian influenza A (H7N9) first caused an outbreak of severe respiratory illness in  
354 China. It subsequently re-emerged during winter 2013–2014 with at least 630 laboratory-  
355 confirmed infections documented by April, 2015, and an associated mortality greater than 30%  
356 (1). However most recently more than 600 new cases have been reported during the fifth wave of  
357 H7N9 (start of 2017), which is now the biggest wave since human infection was first detected  
358 with a worryingly, high the case-fatality rate (40% (1-3)). Consequently, there is an ongoing and  
359 pressing need for vaccines that can protect against avian derived influenza viruses, especially  
360 given that H7N9 viruses now exhibit a seasonal pattern of circulation World Health Organization  
361 2015).

362

363 Clinical development of influenza vaccines is ongoing, however split virus or subunit vaccines  
364 for avian influenza are known to be poorly immunogenic (26, 27) and often require multiple  
365 doses and/or formulation with potent adjuvants to achieve seroconversion (28). Although live  
366 attenuated vaccines (LAIVs) can induce of both humoral and cellular immunogenicity, in adults  
367 these vaccines have previously been associated with lower seroconversion rates and higher rates  
368 of laboratory-confirmed influenza when compared to trivalent influenza vaccine. These  
369 phenomena may possibly due to pre-existing immunogenicity at mucosal sites (7, 29, 30). Less  
370 than half of the vaccinees in a recent phase I clinical trial assessing safety and immunogenicity of  
371 a H7N9 LAIVs seroconverted (48%, (95% CI 29·4–67·5)) after one vaccination (31).

372

373 Vaccines that target conserved antigens, such as internal proteins of influenza A viruses, may  
374 provide greater cross-protective responses toward diverse influenza strains including newly

375 emergent pandemic variants. We demonstrate that while mice primed with ChAdOx1 NP+M1  
376 and boosted with MVA-NP+M1-H7 had significantly less HA-specific antibodies (Figure 5)  
377 there was no difference in morbidity or mortality, compared to a protein and adjuvant only  
378 regimen (Figure 6). These data, highlight and confirm that T cells confer a degree of protection  
379 against the clinical symptoms of influenza A virus infection. Most commercially available  
380 influenza vaccines primarily induce strain-specific antibodies, however it has been demonstrated  
381 that heterosubtypic T cells can confer broad-spectrum protection (32-34). A correlation between  
382 IAV-directed T cells and reduced viral shedding with less severe illness in humans has been  
383 demonstrated in a number of clinical studies (32, 33, 35). It has also been demonstrated that  
384 protective levels of NP-specific T cell responses are found in 43% of the adult population (36).  
385 Importantly, if a vaccine can boost the numbers of pre-existing influenza-specific T cells into  
386 this protective range, these vaccinees would be conferred a degree of protection toward newly  
387 pandemic influenza viruses. This level of boosting is achievable with clinical vaccination with  
388 MVA-NP+M1 in humans (37). In the event of a virulent pandemic outbreak, vaccination with  
389 MVA-NP+M1 could curb disease symptoms while the strain specific HA protein or vaccine  
390 modality encoding the outbreak HA antigen could be manufactured. Follow-on vaccination with  
391 a strain specific HA could then provide neutralising antibodies toward emergent viruses and curb  
392 disease transmission.

393

394 Advantageously, viral-vectored vaccines can facilitate delivery of multiple disease-specific  
395 antigens, which is thought to be key in curbing viral escape mutants when compared to vaccines  
396 that target a single antigen. However, delivery of multiple antigens can result in immune  
397 competition (38, 39), which can be largely circumvented by administration of the viral-vectored

398 vaccine encoded antigens to separate sites as described here. While the exact mechanisms of  
399 antigenic interference following vaccination remains unknown, this phenomenon is thought to be  
400 influenced by spatial constraints on T cells (40, 41). The delivery of dual antigens by the bivalent  
401 MVA-vectored vaccine is less likely to induce immune interference, as distinct promoters drive  
402 antigen expression at different times following infection; the early F11 promoter is expressed  
403 before P7.5. In fact very early expression of T cell stimulating antigens by MVA has previously  
404 demonstrated higher T cell responses and reversal of immunodominance hierarchies (42).  
405 Promisingly, boosting with MVA-NP+M1-H7 significantly enhanced T cell responses against  
406 NP+M1 and H7HA, regardless of whether antigenic competition was observed following a  
407 priming vaccination.

408

409 In a stringent challenge model, inclusion of a viral-vector encoded HA at the prime and boost  
410 significantly outperformed all regimens in both challenges across the nadir of infection (day 4/5  
411 to 8/9) and indeed all animals in these groups retained their starting body weight throughout the  
412 heterologous challenge. Encouragingly, the two strains of H7-pseudotyped viruses used to assess  
413 responses were neutralized at all serum dilutions tested in mice primed with both HA7 and  
414 NP+M1 antigens, these humoral responses were maintained for up to 18 weeks post vaccination.  
415 This is an important finding in light of the pandemic threat posed by currently circulating avian  
416 H7 viruses.

417

418 In summary, vaccination against HA, NP and M1 at both prime and boost immunisations  
419 delivered by ChAd-MVA viral-vectored vaccines induces potent T and B cell responses. These  
420 novel bivalent MVA-vectored vaccines elicit potent T cell responses against NP+M1 whilst

421 simultaneously inducing high levels of antibodies that can recognise different HA subtypes.  
422 Furthermore, T cell responses against NP+M1 were significantly higher than responses induced  
423 by the first generation of clinically investigated MVA-vectored vaccines, a particularly  
424 encouraging result for future clinical work (37, 43). Our data show that these humoral and  
425 cellular responses, induced following a prime-boost vaccination, are both heterologous and  
426 homologous in nature and can confer protection in a rigorous challenge model. Indeed, the  
427 simultaneous delivery of dual antigens (H7HA and NP+M1) outperformed a previously  
428 published efficacious vaccination regimen and importantly the dual delivery of antigens may  
429 alleviate the selective pressure currently thought to potentiate antigenic diversity in avian  
430 influenza vaccination (1, 44).

431

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437 Influenza Virus, A/Vietnam/1203/2004 (H5N1), Recombinant from Baculovirus, NR-10510.

438

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- 608

609 **Footnotes**

610 **Footnote 1:** This work has been funded by grants from the Wellcome Trust (097113/Z/11/

611 BVRVBZO)

612 **Footnote 2: Abbreviations**

613 Hemagglutinin (HA)

614 Influenza A viruses (IAV)

615 Inactivated influenza vaccines (IIVs)

616 Chimpanzee Adenovirus (ChAd)

617 Modified Vaccinia Ankara (MVA)

618 Intramuscularly (i.m.)

619 Long-lived plasma cells (LLPCs)

620 Memory B cells (mBCs)

621 Antibody secreting cells (ASCs)

622 Low pathogenic avian influenza (LPAI)

623 50% lethal doses (LD50)

624 Live attenuated influenza vaccines (LAIVs)

625 Spot forming units (SFU)

626

627 **Figure legends & Tables**

628 **Figure 1: Influenza-specific immune responses generated by multi-antigen MVA-vectored**  
629 **vaccination**

630 **A:** BALB/c mice (n=5) were immunized i.m. with  $1 \times 10^6$  PFU of MVA GFP(expression driven  
631 by the F11 promoter) or MVA-NP+M1 (P7.5; expression driven by the P7.5 promoter) or MVA-  
632 NP+M1-H5 (F11; expression driven by the F11 promoter). Splenocytes were isolated 2 weeks  
633 post vaccination and T cell responses were measured by *ex vivo* IFN- $\gamma$  ELISpot against the  
634 immunodominant BALB/c epitope in NP, NP<sub>147-158</sub> (TYQRTRALV). Mann Whitney analysis of  
635 MVA-NP+M1 (P7.5) and MVA-NP+M1 (F11) showed a significant difference of p=0.0079.  
636 **B:** BALB/c mice (n=5) were immunized i.m. with  $1 \times 10^6$  PFU of MVA-H5, expressing, H5HA  
637 (A/Vietnam/1203/2004), or, MVA-NP+M1-H5, a bivalent vaccine expressing the same H5HA  
638 antigen and the T cell fusion antigen, NP+M1. Serum was collected at 2 weeks post-vaccination  
639 and total serum IgG responses were measured by ELISA against recombinant H5HA protein  
640 (A/Vietnam/1203/2004; BEI resources). No significant differences were observed.

641

642 **Figure 2: Influenza-specific immune responses generated by multi-antigen MVA-vectored**  
643 **vaccination**

644 **A:** BALB/c mice (n=5) were immunized i.m. with  $1 \times 10^6$  PFU of MVA NP+M1 (P7.5;  
645 expression driven by the P7.5 promoter) or MVA-NP+M1 (F11; expression driven by the F11  
646 promoter) or MVA-NP+M1(p7.5)-H7 (F11). Splenocytes were isolated 2 weeks post-vaccination  
647 and T cell responses were measured by *ex vivo* IFN- $\gamma$  ELISpot against the BALB/c epitope in  
648 NP, NP<sub>147-158</sub> (TYQRTRALV). Responses post bivalent viral-vectored vaccine were significantly  
649 higher than post MVA NP+M1 (p7.5) as assessed (\* P  $\leq$  0.05) by Kruskal-Wallis one-way

650 ANOVA, with Dunn's multiple comparisons test. **B:** BALB/c mice (n=5) were immunized i.m.  
651 with  $1 \times 10^6$  PFU of MVA-H7 (p7.5), expressing, H7HA (A/Netherlands/219/2003; H7N7),  
652 MVA-NP+M1-H7, a bivalent vaccine expressing the same H7HA antigen and the T cell fusion  
653 antigen, NP+M1. Serum was collected at 2 and 8 weeks post vaccination and total serum IgG  
654 responses were measured by ELISA against recombinant H7HA (A/Netherlands/219/2003;  
655 H7N7).

656

657 **Figure 3: Influenza-specific immune responses generated by multi-antigen MVA-vectored**  
658 **vaccination**

659 **A:** BALB/c mice were immunized with ChAdOx1 viral vector vaccines encoding NP+M1 or  
660 H7HA or both; either as a mixture or by administration into separate limbs. Doses administered  
661 were  $2.2 \times 10^6$  iu of ChAdOx1 NP+M1 and/or  $1 \times 10^8$  iu ChAdOx1-H7. Splenocytes were isolated  
662 2 weeks post vaccination and T cell responses were measured by *ex vivo* IFN- $\gamma$  ELISpot against  
663 the BALB/c epitope in NP, NP147-158 (TYQRTRALV). Responses post a mixture of viral  
664 vectored vaccines were lower than post ChAdOx1 NP+M1 as assessed (\*\* $P \leq 0.01$ ) by Kruskal-  
665 Wallis one-way ANOVA, with Dunn's multiple comparisons test. No significant difference was  
666 observed with the response post ChAdOx1 NP+M1 and when the response when viral vectored  
667 vaccines were administered, singly, into separate limbs. **Data representative of two experiments.**

668 **B:** BALB/c mice were immunized with 10 $\mu$ g of H7 protein or ChAdOx1 viral vector vaccines  
669 encoding NP+M1 or H7HA or both; either as a mixture or by administration into separate limbs.  
670 Doses administered were  $2.2 \times 10^6$  IU of ChAdOx1 NP+M1 and/or  $1 \times 10^8$  IU ChAdOx1-H7. Total  
671 serum IgG responses at all time points against recombinant H7HA protein  
672 A/Netherlands/219/2003 are shown. **Data representative of two experiments.**

673

674 **Figure 4: Influenza-specific T cell responses following prime-boost viral-vector**

675 **vaccination**

676 BALB/c mice were immunized with ChAdOx1 viral vector vaccines encoding NP+M1 or H7HA  
677 or both by administration into separate limbs. Doses administered were  $2.2 \times 10^6$  IU of ChAdOx1  
678 NP+M1 and/or  $1 \times 10^8$  IU ChAdOx1-H7. Eight weeks later all mice were boosted with  $1 \times 10^6$  PFU  
679 MVA-NP+M1-H7. Splenocytes were isolated 2 weeks post vaccination and T cell responses  
680 were measured by *ex vivo* IFN- $\gamma$  ELISpot against

681 **A:** the BALB/c epitope in NP, NP<sub>147-158</sub> (TYQRTRALV). Post-boost, T cell responses were  
682 greater when ChAdOx1 NP+M1 was used as a prime as compared to a prime with ChAdOx1  
683 HA7 (\*\*p  $\leq$  0.01) by Kruskal-Wallis one-way ANOVA, with Dunn's multiple comparisons  
684 test). No other significant differences were measured. **Data representative of two experiments.**

685 **B:** H7HA peptide pools, representative of H7HA from A/Netherlands/219/2003 (H7N7). Post  
686 boost, T cell responses were greater when ChAdOx1 HA7 was used as a prime as compared to  
687 priming with ChAdOx1 NP+M1 (\*\*\*P  $\leq$  0.001) by Kruskal-Wallis one-way ANOVA, with  
688 Dunn's multiple comparisons test). No other significant differences were measured. **Data**  
689 **representative of two experiments.**

690 **C:** H7HA peptide pools representative of regions of amino acid sequence diversity in the HA of  
691 A/Anhui/1/2013 (H7N9) and A/Shanghai/1/2013 (H7N9) when compared to the vaccine insert  
692 A/Netherlands/219/2003 (H7N7). Post boost, T cell responses were greater when ChAdOx1  
693 HA7 was used as a prime as compared to priming with ChAdOx1 NP+M1 (\*\*p  $\leq$  0.01) by  
694 Kruskal-Wallis one-way Anova, with Dunn's multiple comparisons test). No other significant  
695 differences were measured. **Data representative of two experiments.**

696

697 **Figure 5: Influenza-specific B cell responses following prime-boost viral-vectored**  
698 **vaccination.** BALB/c mice were immunized with ChAdOx1 viral vector vaccines encoding  
699 NP+M1 or H7HA or both by administration into separate limbs. Doses administered were  
700  $2.2 \times 10^6$  IU of ChAdOx1 NP+M1 and/or  $1 \times 10^8$  IU ChAdOx1-H7. Eight weeks later all mice  
701 were boosted with  $1 \times 10^6$  PFU MVA-NP+M1-H7. **A:** Total serum IgG responses at indicated  
702 time points post-boost against recombinant H7HA protein A/Netherlands/219/2003 are shown.  
703 Serum responses were higher in mice that were primed with ChAdOx1 HA7, either alone or in  
704 combination with ChAdOx1 NP+M1. **Data representative of two experiments.** **B:** IgG H7HA-  
705 specific ASC ELISpot *ex vivo* responses to H7HA (A/Netherlands/219/2003) in BALB/c mice  
706 (n=4-6) 18 weeks post boost vaccination with MVA-NP+M1-H7 are shown. A greater number of  
707 IgG SFUs were observed from mice primed with ChAdOx1 HA7 and boosted with MVA-  
708 NP+M1-H7 as compared to mice vaccinated with protein alone as assessed ( $*p \leq 0.05$ ) by  
709 Kruskal-Wallis one-way ANOVA, with Dunn's multiple comparisons test. **C:** Total IgG ASC  
710 ELISpot *ex vivo* responses in BALB/c mice (n=4-6) 18 weeks post boost vaccination with MVA-  
711 NP+M1-H7. Post vaccination, there were a greater number of IgG SFU isolated from mice  
712 primed and boosted (ChAdOx1 NP+M1 followed by MVA-NP+M1-H7) as compared to naïve  
713 mice as assessed ( $*P \leq 0.05$ ) by Kruskal-Wallis one-way ANOVA, with Dunn's multiple  
714 comparisons test.

715

716 **Figure 6: Bivalent viral vectors provide *in vivo* protection against influenza viral challenge**

717 Balb/c mice were unvaccinated or received an irrelevant ChAdOx1 prime and MVA boost  
718 vaccination. All other groups were vaccinated as described and **three** weeks after the last

719 immunization, animals were challenged with SH1 (A/Shanghai/1/13) virus. Weight loss was  
720 monitored over a period of 14 days, as depicted. 2way ANOVA analysis assuming a non-  
721 Gaussian distribution and Dunnetts multiple comparison test comparing Group5 (H7+Adjuvant)  
722 to  
723 A: Group1 (ChAdOx1 NP+M1 Prime, MVA-NP+M1-H7 boost) was not different at day 4, 5, 6,  
724 7 or 8. Comparing Group5 (H7+Adjuvant) to Group 2 (ChAdOx1-H7 Prime, MVA-NP+M1-H7  
725 boost) demonstrated that the latter was significantly different at day 4 (\*\*\*\*P  $\leq$  0.0001), day 5  
726 (\*\*\*\*p  $\leq$  0.0001), day6 (\*\*p  $\leq$  0.01), day 7 (\*\*p  $\leq$  0.01), but not at day 8 (N.S.). Comparing  
727 Group5 (H7+Adjuvant) to Group 3 (ChAdOx1 NP+M1 and ChAdOx1-H7 prime, MVA-  
728 NP+M1-H7 boost) demonstrated that the latter was significantly different at day 4 (\*p  $\leq$  0.05),  
729 day 5 (\*\* p  $\leq$  0.01), day6 (\*\* p  $\leq$  0.01), day 7 (\*p  $\leq$  0.05), and day 8 (\*p  $\leq$  0.05).

730 **B:** Group 1 (ChAdOx1 NP+M1 Prime, MVA-NP+M1-H7 boost) demonstrated that the latter  
731 was significantly different at day 5 (\*\*p  $\leq$  0.01), day 6, 7 (\*\*\*\*p  $\leq$  0.0001), day 8 (\*\*\*p  $\leq$  0.001)  
732 and day 9 (\*\*p  $\leq$  0.01). Comparing Group5 (H7+Adjuvant) to Group 2 (ChAdOx1-H7 Prime,  
733 MVA-NP+M1-H7 boost) demonstrated that the latter was significantly different at day 5 (\*p  $\leq$   
734 0.05), day 6, 7, 8 (\*\*\*\*p  $\leq$  0.0001) and day 9 (\*\*p  $\leq$  0.01). Comparing Group5 (H7+Adjuvant)  
735 to Group 3 (ChAdOx1 NP+M1 and ChAdOx1-H7 prime, MVA-NP+M1-H7 boost)  
736 demonstrated that the latter was significantly different at day 5 (\*\* p  $\leq$  0.01), day 6, 7 (\*\*\*\*p  $\leq$   
737 0.0001), day 8 (\*\*\*p  $\leq$  0.001) and day 9 (\*p  $\leq$  0.05).