Kent Academic Repository

Full text document (pdf)

Citation for published version

Tully, Claire M and Chinnakannan, Senthil and Mullarkey, Caitlin E and Ulaszewska, Marta and Ferrara, Francesca and Temperton, Nigel J. and Gilbert, Sarah C and Lambe, Teresa (2017) Novel Bivalent Viral-Vectored Vaccines Induce Potent Humoral and Cellular Immune Responses Conferring Protection against Stringent Influenza A Virus Challenge. Journal of immunology,

DOI

https://doi.org/10.4049/jimmunol.1600939

Link to record in KAR

http://kar.kent.ac.uk/62379/

Document Version

Author's Accepted Manuscript

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

Claire M. Tully[*], Senthil Chinnakannan[†], Caitlin E. Mullarkey[‡], Marta Ulaszewska[*], Francesca Ferrara[§], Nigel Temperton[§], Sarah C. Gilbert[*] and Teresa Lambe[*¶]

[*] The Jenner Institute, University of Oxford, ORCRB, Roosevelt Drive, Oxford OX3 7DQ

[†] Experimental Medicine Division, The Peter Medawar Building for Pathogen Research, University of Oxford, South Parks Road, OX1 3SY

[‡] Viral Pseudotype Unit, Department of Microbiology, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, Box 1124, New York, NY 10029

[§] Medway School of Pharmacy, The Universities of Greenwich and Kent at Medway, Anson Building, Central Avenue, Chatham Maritime, Chatham, Kent, ME4 4TB

[¶] Corresponding author: Teresa Lambe

Telephone: + 44 1865 617 621

Fax: +44 1865 617 608

Email: Teresa.lambe@ndm.ox.ac.uk

Keywords: heterosubtypic vaccine, influenza, Viral-vector

Running title: Bivalent Vaccines Protect Against Influenza Challenge

Abstract:

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

Introduction

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

Recent strategies to augment and broaden vaccine efficacy have shifted towards the development of 'universal' vaccines capable of providing heterosubtypic protection against multiple, or possibly all subtypes of IAV. While both humoral and cellular immunity can mediate heterosubtypic responses, inducing antibodies against the more conserved stalk domain of hemagglutinin (HA) has been the recent focus of many vaccine programmes (10, 11). However, multipronged approaches, targeting several antigens inducing both humoral and cellular responses may limit the generation of viral escape mutants compared to vaccines targeting a limited number of protective epitopes on the HA stalk . There are few vaccine technologies that will facilitate the delivery of multiple antigens to generate robust cellular and humoral immunity toward infectious disease antigens.

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

Materials and Methods

Recombinant ChAd and MVA vaccines

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

Immunizations

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

ELISpot

Spleen ELISpot was performed to measure antigen-specific IFN-γ as described previously (14).The immunodominant H2-K^d restricted (BALB/c) epitope $NP_{147-158}$ (TYQRTRALV) was used to measure post-vaccination responses following vaccination regimens with NP+M1 (15). H7HA responses were measured after stimulation with peptide pools. Two peptide pools were generated. The first pool contained peptides unique to A/Netherlands/219/2003. The second pool contained peptides unique to A/Anhui/1/2013 and/or A/Shanghai/1/2013 when compared to A/Netherlands/219/2003

Briefly, peptides that were conserved between A/Netherlands/219/2003 and A/Anhui/1/2013 were pooled from the BEI resource (NR44011). The resultant pool was representative of H7HA from A/Netherlands/219/2003 (H7N7). All peptides that differed between A/Netherlands/219/2003 (H7N7) and divergent strains (A/Anhui/1/2013 (H7N9) and A/Shanghai/1/2013) were pooled to generate a second peptide pool. This H7HA peptide pool is representative of regions of amino acid sequence diversity in the HA of A/Anhui/1/2013 (H7N9) and A/Shanghai/1/2013 (H7N9) when compared to the vaccine insert A/Netherlands/219/2003 (H7N7) and was generated from the BEI (NR44011 and NR-44012). H7 peptide pools were obtained through BEI Resources, NIAID, NIH: Peptide Array, Influenza Virus A/Shanghai/1/2013 (H7N9) Hemagglutinin Protein Diverse Peptides, NR-44012. Medium alone was used as a negative control and pools of overlapping peptides (H7HA) or the $NP_{147-158}$ (TYQRTRALV) were added typically at 2μg/mL.

ELISA

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

IgG Antibody Secreting Cell ELISPOT Assay

Bone marrow IgG Antibody Secreting Cell ELISPOT Assay was performed as described (18, 19) using approximately $1x10^7$ cells/mL in complete Iscove's that had been rested overnight. MultiScreen-IP filter plates were coated with 0.5μg recombinant HA while negative control wells were coated with irrelevant protein (0.5μg ovalbumin).

Pseudotype Neutralisation Assay

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

Challenge

All animal protocols were reviewed and approved by the Mount Sinai Institutional Animal Care and Use Committee (IACUC). To assess the protective efficacy of the prime-boost vaccination regimen, 6-8 week-old female BALB/c ($H-2^d$) mice (Jackson Laboratories Inc) were primed with either ChAdOx1 NP+M1 (1.1×10^7 infectious units (IU), Group 1), ChAdOx1-H7 HA (1×10^8 IU, Group 2), or ChAdOx1-GFP (1×10^8IU, Group 4) (n=10 mice per group). All viral vectors were administered intramuscularly in the musculus tibialis in a final volume of 50µL. One group of animals received both ChAdOx1 NP+M1 and ChAdOx1-H7 HA, where each virus was injected into seperate limbs (n=5 mice per group; Group 3). Animals vaccinated with 10μg of recombinant H7 from A/Anhui/1/13 (H7N9) supplemented with 5 µg of R848 (Invivogen Inc) served as a positive control (n=10, Group 5). Naïve animals remained unvaccinated (n=10, Group 6). Eight weeks following the prime, Groups 1, 2, and 3 were boosted intramuscularly with MVA-NP+M1 (1 x 10^6 IU). Group 4 received MVA-GFP as a boost and Group 5 was administered 10µL of recombinant HA with R848. Blood ELISpots were performed at two weeks post-boost to ensure successful vaccine uptake. Three weeks following boost vaccination all animals (n=55) were anesthetized and challenged with 5 murine 50% lethal doses 5xLD_{50} of a 6:2 reassortment of A/Shanghai/1/13 (H7N9) virus. Weight was monitored daily for 14 days; mice that lost 25% or more of their initial body weight were euthanized.

Statistics

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

Results

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

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

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

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

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

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

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

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

Immunogenicity generated by multi-antigen ChAdOx1-vectored vaccination

BALB/c mice were immunized with ChAdOx1 NP+M1 ($2.2x10^8$IU) or ChAdOx1-H7 ($1x10^8$IU) or both, as described. Two weeks following vaccination, splenocytes were isolated and T cell responses were measured by *ex vivo* IFN-γ ELISpot as before. Mice vaccinated with ChAdOx1 NP+M1 had higher responses compared to mice that received a mixture of ChAdOx1 NP+M1 and ChAdOx1-H7 (**$p \leq 0.01$) (Figure 3A). However, no significant difference in ELISpot responses was observed between mice vaccinated with ChAdOx1 NP+M1 or a combination of ChAdOx1 NP+M1 and ChAdOx1-H7, administered into separate limbs. This approach has previously been shown to augment immune responses and avoid competition between two vaccines administered together (12).

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

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

Adenovirus-MVA prime-boost regimens are currently one of the leading strategies to induce potent immune responses against vaccine antigens (21, 22). BALB/c mice (n=18 (6 per group)) received a priming vaccination of either ChAdOx1-NP+M1, ChAdOx1-H7, or both, administered separately into opposite limbs, as described. At eight weeks post-prime all groups were boosted with MVA-NP+M1-H7

T Cell Responses Following Prime-Boost vaccination

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

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

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

Humoral responses

Serum was collected at 2 and 8 weeks post prime and also post boost vaccinations and the longevity of antibody responses were followed out to 26 weeks following the initial immunzation. Mice primed with ChAdOx1-H7 or ChAdOx1-H7 and ChAdOx-NP+M1 had higher total IgG against H7HA at all time points compared to vaccination with either protein alone or with ChAdOx1 NP+M1 followed by MVA-NP+M1-H7 (Figure 5A). As expected we were unable to detect serum responses to HA in the ChAdOx1-NP+M1 only group, until 2 weeks post-boost with MVA-NP+M1+H7. Peak boost responses for viral vector vaccinations were up to 50 fold higher than the response two weeks following prime immunization (e.g. Group 3 $1.34x10^5$ SFU. (2wk) vs. $7.4x10^6$ SFU (16 wk)) and persisted for at least 26 weeks post vaccination in all groups (Figure 5A). These data suggest that strong humoral immune responses toward H7HA are generated and maintained over time by heterologous ChAd-MVA prime boost regimens.

B Cell Memory Responses Following ChAdOx1 – MVA Immunisation

Long-lived humoral immunity is principally mediated by two B cell subsets; long-lived plasma cells (LLPCs) and memory B cells (mBCs). LLPCs predominantly reside in the bone marrow (BM) (23, 24) and continuously secrete antibody. In order to further understand the basis of the humoral responses following heterologous prime-boost ChAd-MVA viral-vectored vaccination, LLPCs were enumerated 18 weeks following the boosting vaccination.

Long-Lived Plasma Cells

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

Functionality of Adaptive Immune Responses Following ChAdOx1 – MVA Prime-Boost Vaccination

Pseudotype Virus Neutralisation

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

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

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

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

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

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

Animals vaccinated with ChAdOx1-H7 alone or ChAdOx1-H7 and ChAdOx1 NP+M1 and boosted with MVA-NP+M1-H7 (Groups 2 & 3) all surived lethal challenge. When these studies were repeated, Groups 2 & 3 and Group 1 (primed with ChAdOx1 NP+M1 boosted with MVA-NP+M1-H7), were found to be equally protective as a vaccination regimen that has previously been shown to be protective (Group 5protein and a TLR agonist adjuvant) (25).

Importantly, in the first challenge experiment Groups 2 & 3 (animals vaccinated with ChAdOx1-H7 alone or ChAdOx1-H7 and ChAdOx1 NP+M1 and boosted with MVA-NP+M1-H7) retained starting body weight throughout the monitoring period. Furthermore, in a second challenge experiment Group 1 also retained starting body weight and did not display any weight loss. These results indicate that this vaccination confers protection against both morbitity and mortality. Comparison across the nadir of weight loss (day 4/5 through to 8/9) between ChAdOx1 NP+M1 primed and MVA-NP+M1-H7 boosted animals (Group 1) and those that received a protective regimen (Group 5, positive control) demonstrates no significant difference in weight loss in the first challenge or in a second independent repeat challenge. It is evident that both humoral and cellular immunity can offer improved efficacy in this stringent challenge model when compared to protective vaccination regimens (protein and adjuvant).

Discussion

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

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

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

Advantageously, viral-vectored vaccines can facilitate delivery of multiple disease-specific antigens, which is thought to be key in curbing viral escape mutants when compared to vaccines that target a single antigen. However, delivery of multiple antigens can result in immune competition (38, 39), which can be largely circumvented by administration of the viral-vectored vaccine encoded antigens to separate sites as described here. While the exact mechanisms of antigenic interference following vaccination remains unknown, this phenomenon is thought to be influenced by spatial constraints on T cells (40, 41). The delivery of dual antigens by the bivalent MVA-vectored vaccine is less likely to induce immune interference, as distinct promoters drive antigen expression at different times following infection; the early F11 promoter is expressed before P7.5. In fact very early expression of T cell stimulating antigens by MVA has previously demonstrated higher T cell responses and reversal of immunodominance hierarchies (42). Promisingly, boosting with MVA-NP+M1-H7 significantly enhanced T cell responses against NP+M1 and H7HA, regardless of whether antigenic competition was observed following a priming vaccination.

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

In summary, vaccination against HA, NP and M1 at both prime and boost immunisations delivered by ChAd-MVA viral-vectored vaccines induces potent T and B cell responses. These novel bivalent MVA-vectored vaccines elicit potent T cell responses against NP+M1 whilst simultaneously inducing high levels of antibodies that can recognise different HA subtypes. Furthermore, T cell responses against NP+M1 were significantly higher than responses induced by the first generation of clinically investigated MVA-vectored vaccines, a particularly encouraging result for future clinical work (37, 43). Our data show that these humoral and cellular responses, induced following a prime-boost vaccination, are both heterologous and homologous in nature and can confer protection in a rigorous challenge model. Indeed, the simultaneous delivery of dual antigens (H7HA and NP+M1) outperformed a previously published efficacious vaccination regimen and importantly the dual delivery of antigens may alleviate the selective pressure currently thought to potentiate antigenic diversity in avian influenza vaccination (1, 44).

Acknowledgements

We wish to thank V. Clark, H. Gray, for their animal husbandry; A. Worth and the Jenner Institute Vector Core facility for assistance. The following reagents were obtained through BEI Resources, NIAID, NIH: Peptide Array, Influenza Virus A/Shanghai/1/2013 (H7N9) Hemagglutinin Protein Diverse Peptides, NR-44012.: H5 Hemagglutinin (HA) Protein from Influenza Virus, A/Vietnam/1203/2004 (H5N1), Recombinant from Baculovirus, NR-10510.

References

1. Su, S., Y. Bi, G. Wong, G. C. Gray, G. F. Gao, and S. Li. 2015. Epidemiology, Evolution, and Recent Outbreaks of Avian Influenza Virus in China. *J Virol* 89: 8671-8676.
2. Isakova-Sivak, I., and L. Rudenko. 2017. Tackling a novel lethal virus: a focus on H7N9 vaccine development. *Expert Rev Vaccines*: 1-13.
3. Xiang, N., X. Li, R. Ren, D. Wang, S. Zhou, C. M. Greene, Y. Song, L. Zhou, L. Yang, C. T. Davis, Y. Zhang, Y. Wang, J. Zhao, A. D. Iuliano, F. Havers, S. J. Olsen, T. M. Uyeki, E. Azziz-Baumgartner, S. Trock, B. Liu, H. Sui, X. Huang, D. Ni, Z. Feng, Y. Shu, and Q. Li. 2016. Assessing Change in Avian Influenza A(H7N9) Virus Infections During the Fourth Epidemic - China, September 2015-August 2016. *MMWR Morb Mortal Wkly Rep* 65: 1390-1394.
4. Francis, T., Jr. 1953. Vaccination against influenza. *Bulletin of the World Health Organization* 8: 725-741.
5. Davenport, F. M. 1962. Current knowledge of influenza vaccine. *Jama* 182: 11-13.
6. Fiore, A. E., T. M. Uyeki, K. Broder, L. Finelli, G. L. Euler, J. A. Singleton, J. K. Iskander, P. M. Wortley, D. K. Shay, J. S. Bresee, N. J. Cox, C. Centers for Disease, and Prevention. 2010. Prevention and control of influenza with vaccines: recommendations of the Advisory Committee on Immunization Practices (ACIP), 2010. *MMWR. Recommendations and reports : Morbidity and mortality weekly report. Recommendations and reports / Centers for Disease Control* 59: 1-62.
7. Monto, A. S., S. E. Ohmit, J. G. Petrie, E. Johnson, R. Truscon, E. Teich, J. Rotthoff, M. Boulton, and J. C. Victor. 2009. Comparative efficacy of inactivated and live attenuated influenza vaccines. *N Engl J Med* 361: 1260-1267.
8. Ohmit, S. E., J. C. Victor, J. R. Rotthoff, E. R. Teich, R. K. Truscon, L. L. Baum, B. Rangarajan, D. W. Newton, M. L. Boulton, and A. S. Monto. 2006. Prevention of antigenically drifted influenza by inactivated and live attenuated vaccines. *The New England journal of medicine* 355: 2513-2522.
9. Sakaguchi, S., M. Ono, R. Setoguchi, H. Yagi, S. Hori, Z. Fehervari, J. Shimizu, T. Takahashi, and T. Nomura. 2006. Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev* 212: 8-27.
10. Impagliazzo, A., F. Milder, H. Kuipers, M. V. Wagner, X. Zhu, R. M. Hoffman, R. van Meersbergen, J. Huizingh, P. Wanningen, J. Verspuij, M. de Man, Z. Ding, A. Apetri, B. Kukrer, E. Sneekes-Vriese, D. Tomkiewicz, N. S. Laursen, P. S. Lee, A. Zakrzewska, L. Dekking, J. Tolboom, L. Tettero, S. van Meerten, W. Yu, W. Koudstaal, J. Goudsmit, A. B. Ward, W. Meijberg, I. A. Wilson, and K. Radosevic. 2015. A stable trimeric influenza hemagglutinin stem as a broadly protective immunogen. *Science* 349: 1301-1306.
11. Yassine, H. M., J. C. Boyington, P. M. McTamney, C. J. Wei, M. Kanekiyo, W. P. Kong, J. R. Gallagher, L. Wang, Y. Zhang, M. G. Joyce, D. Lingwood, S. M. Moin, H. Andersen, Y. Okuno, S. S. Rao, A. K. Harris, P. D. Kwong, J. R. Mascola, G. J. Nabel, and B. S. Graham. 2015. Hemagglutinin-stem nanoparticles generate heterosubtypic influenza protection. *Nat Med* 21: 1065-1070.
12. Forbes, E. K., S. Biswas, K. A. Collins, S. C. Gilbert, A. V. Hill, and S. J. Draper. 2011. Combining liver- and blood-stage malaria viral-vectored vaccines: investigating mechanisms of CD8+ T cell interference. *J Immunol* 187: 3738-3750.

13. Antrobus, R. D., L. Coughlan, T. K. Berthoud, M. D. Dicks, A. V. Hill, T. Lambe, and S. C. Gilbert. 2013. Clinical Assessment of a Novel Recombinant Simian Adenovirus ChAdOx1 as a Vectored Vaccine Expressing Conserved Influenza A Antigens. *Mol Ther*.

14. Mullarkey, C. E., A. Boyd, A. van Laarhoven, E. A. Lefevre, B. Veronica Carr, M. Baratelli, E. Molesti, N. J. Temperton, C. Butter, B. Charleston, T. Lambe, and S. C. Gilbert. 2013. Improved adjuvanting of seasonal influenza vaccines: preclinical studies of MVA-NP+M1 coadministration with inactivated influenza vaccine. *Eur J Immunol* 43: 1940-1952.

15. Perkins, D. L., M. Z. Lai, J. A. Smith, and M. L. Gefter. 1989. Identical peptides recognized by MHC class I- and II-restricted T cells. *J Exp Med* 170: 279-289.

16. Kong, W. P., C. Hood, Z. Y. Yang, C. J. Wei, L. Xu, A. Garcia-Sastre, T. M. Tumpey, and G. J. Nabel. 2006. Protective immunity to lethal challenge of the 1918 pandemic influenza virus by vaccination. *Proc Natl Acad Sci U S A* 103: 15987-15991.

17. Miura, K., A. C. Orcutt, O. V. Muratova, L. H. Miller, A. Saul, and C. A. Long. 2008. Development and characterization of a standardized ELISA including a reference serum on each plate to detect antibodies induced by experimental malaria vaccines. *Vaccine* 26: 193-200.

18. de Cassan, S. C., E. K. Forbes, A. D. Douglas, A. Milicic, B. Singh, P. Gupta, V. S. Chauhan, C. E. Chitnis, S. C. Gilbert, A. V. Hill, and S. J. Draper. 2011. The requirement for potent adjuvants to enhance the immunogenicity and protective efficacy of protein vaccines can be overcome by prior immunization with a recombinant adenovirus. *Journal of immunology* 187: 2602-2616.

19. Douglas, A. D., S. C. de Cassan, M. D. Dicks, S. C. Gilbert, A. V. Hill, and S. J. Draper. 2010. Tailoring subunit vaccine immunogenicity: maximizing antibody and T cell responses by using combinations of adenovirus, poxvirus and protein-adjuvant vaccines against Plasmodium falciparum MSP1. *Vaccine* 28: 7167-7178.

20. Alharbi, N. K., A. J. Spencer, A. M. Salman, C. M. Tully, S. K. Chinnakannan, T. Lambe, Y. Yamaguchi, S. J. Morris, T. Orubu, S. J. Draper, A. V. Hill, and S. C. Gilbert. 2016. Enhancing cellular immunogenicity of MVA-vectored vaccines by utilizing the F11L endogenous promoter. *Vaccine* 34: 49-55.

21. Hill, A. V., A. Reyes-Sandoval, G. O'Hara, K. Ewer, A. Lawrie, A. Goodman, A. Nicosia, A. Folgori, S. Colloca, R. Cortese, S. C. Gilbert, and S. J. Draper. 2010. Prime-boost vectored malaria vaccines: progress and prospects. *Human vaccines* 6: 78-83.

22. Rampling, T., K. Ewer, G. Bowyer, D. Wright, E. B. Imoukhuede, R. Payne, F. Hartnell, M. Gibani, C. Bliss, A. Minhinnick, M. Wilkie, N. Venkatraman, I. Poulton, N. Lella, R. Roberts, K. Sierra-Davidson, V. Krahling, E. Berrie, F. Roman, I. De Ryck, A. Nicosia, N. J. Sullivan, D. A. Stanley, J. E. Ledgerwood, R. M. Schwartz, L. Siani, S. Colloca, A. Folgori, S. Di Marco, R. Cortese, S. Becker, B. S. Graham, R. A. Koup, M. M. Levine, V. Moorthy, A. J. Pollard, S. J. Draper, W. R. Ballou, A. Lawrie, S. C. Gilbert, and A. V. Hill. 2015. A Monovalent Chimpanzee Adenovirus Ebola Vaccine - Preliminary Report. *N Engl J Med*.

23. Manz, R. A., A. Thiel, and A. Radbruch. 1997. Lifetime of plasma cells in the bone marrow. *Nature* 388: 133-134.

24. Slifka, M. K., R. Antia, J. K. Whitmire, and R. Ahmed. 1998. Humoral immunity due to long-lived plasma cells. *Immunity* 8: 363-372.

25. Krammer, F., R. A. Albrecht, G. S. Tan, I. Margine, R. Hai, M. Schmolke, J. Runstadler, S. F. Andrews, P. C. Wilson, R. J. Cox, J. J. Treanor, A. Garcia-Sastre, and P. Palese. 2014. Divergent H7 immunogens offer protection from H7N9 virus challenge. *J Virol* 88: 3976-3985.

26. Cox, R. J., A. S. Madhun, S. Hauge, H. Sjursen, D. Major, M. Kuhne, K. Hoschler, M. Saville, F. R. Vogel, W. Barclay, I. Donatelli, M. Zambon, J. Wood, and L. R. Haaheim. 2009. A phase I clinical trial of a PER.C6 cell grown influenza H7 virus vaccine. *Vaccine* 27: 1889-1897.

27. Couch, R. B., S. M. Patel, C. L. Wade-Bowers, and D. Nino. 2012. A randomized clinical trial of an inactivated avian influenza A (H7N7) vaccine. *PLoS One* 7: e49704.

28. Bart, S. A., M. Hohenboken, G. Della Cioppa, V. Narasimhan, P. R. Dormitzer, and N. Kanesa-Thasan. 2014. A cell culture-derived MF59-adjuvanted pandemic A/H7N9 vaccine is immunogenic in adults. *Sci Transl Med* 6: 234ra255.

29. Treanor, J. J., K. Kotloff, R. F. Betts, R. Belshe, F. Newman, D. Iacuzio, J. Wittes, and M. Bryant. 1999. Evaluation of trivalent, live, cold-adapted (CAIV-T) and inactivated (TIV) influenza vaccines in prevention of virus infection and illness following challenge of adults with wild-type influenza A (H1N1), A (H3N2), and B viruses. *Vaccine* 18: 899-906.

30. Nichol, K. L., P. M. Mendelman, K. P. Mallon, L. A. Jackson, G. J. Gorse, R. B. Belshe, W. P. Glezen, and J. Wittes. 1999. Effectiveness of live, attenuated intranasal influenza virus vaccine in healthy, working adults: a randomized controlled trial. *JAMA* 282: 137-144.

31. Rudenko, L., I. Isakova-Sivak, A. Naykhin, I. Kiseleva, M. Stukova, M. Erofeeva, D. Korenkov, V. Matyushenko, E. Sparrow, and M. P. Kieny. 2015. H7N9 live attenuated influenza vaccine in healthy adults: a randomised, double-blind, placebo-controlled, phase 1 trial. *Lancet Infect Dis*.

32. McMichael, A. J., F. M. Gotch, G. R. Noble, and P. A. Beare. 1983. Cytotoxic T-cell immunity to influenza. *N Engl J Med* 309: 13-17.

33. Sridhar, S., S. Begom, A. Bermingham, K. Hoschler, W. Adamson, W. Carman, T. Bean, W. Barclay, J. J. Deeks, and A. Lalvani. 2013. Cellular immune correlates of protection against symptomatic pandemic influenza. *Nature medicine*.

34. Price, G. E., M. R. Soboleski, C. Y. Lo, J. A. Misplon, C. Pappas, K. V. Houser, T. M. Tumpey, and S. L. Epstein. 2009. Vaccination focusing immunity on conserved antigens protects mice and ferrets against virulent H1N1 and H5N1 influenza A viruses. *Vaccine* 27: 6512-6521.

35. Wilkinson, T. M., C. K. Li, C. S. Chui, A. K. Huang, M. Perkins, J. C. Liebner, R. Lambkin-Williams, A. Gilbert, J. Oxford, B. Nicholas, K. J. Staples, T. Dong, D. C. Douek, A. J. McMichael, and X. N. Xu. 2012. Preexisting influenza-specific CD4(+) T cells correlate with disease protection against influenza challenge in humans. *Nature medicine* 18: 274-280.

36. Hayward, A. C., L. Wang, N. Goonetilleke, E. B. Fragaszy, A. Bermingham, A. Copas, O. Dukes, E. R. Millett, I. Nazareth, J. S. Nguyen-Van-Tam, J. M. Watson, M. Zambon, G. Flu Watch, A. M. Johnson, and A. J. McMichael. 2015. Natural T Cell-mediated Protection against Seasonal and Pandemic Influenza. Results of the Flu Watch Cohort Study. *Am J Respir Crit Care Med* 191: 1422-1431.

37. Berthoud, T. K., M. Hamill, P. J. Lillie, L. Hwenda, K. A. Collins, K. J. Ewer, A. Milicic, H. C. Poyntz, T. Lambe, H. A. Fletcher, A. V. Hill, and S. C. Gilbert. 2011. Potent CD8+ T-cell immunogenicity in humans of a novel heterosubtypic influenza A vaccine, MVA-NP+M1. *Clin Infect Dis* 52: 1-7.

38. Elias, S. C., K. A. Collins, F. D. Halstead, P. Choudhary, C. M. Bliss, K. J. Ewer, S. H. Sheehy, C. J. Duncan, S. Biswas, A. V. Hill, and S. J. Draper. 2013. Assessment of immune interference, antagonism, and diversion following human immunization with biallelic blood-stage malaria viral-vectored vaccines and controlled malaria infection. *J Immunol* 190: 1135-1147.

39. Forbes, E. K., S. Biswas, K. A. Collins, S. C. Gilbert, A. V. Hill, and S. J. Draper. 2011. Combining liver- and blood-stage malaria viral-vectored vaccines: investigating mechanisms of CD8+ T cell interference. *Journal of immunology* 187: 3738-3750.

40. Kastenmuller, W., G. Gasteiger, J. H. Gronau, R. Baier, R. Ljapoci, D. H. Busch, and I. Drexler. 2007. Cross-competition of CD8+ T cells shapes the immunodominance hierarchy during boost vaccination. *The Journal of experimental medicine* 204: 2187-2198.

41. Kedl, R. M., W. A. Rees, D. A. Hildeman, B. Schaefer, T. Mitchell, J. Kappler, and P. Marrack. 2000. T cells compete for access to antigen-bearing antigen-presenting cells. *The Journal of experimental medicine* 192: 1105-1113.

42. Baur, K., K. Brinkmann, M. Schweneker, J. Patzold, C. Meisinger-Henschel, J. Hermann, R. Steigerwald, P. Chaplin, M. Suter, and J. Hausmann. 2010. Immediate-early expression of a recombinant antigen by modified vaccinia virus ankara breaks the immunodominance of strong vector-specific B8R antigen in acute and memory CD8 T-cell responses. *Journal of virology* 84: 8743-8752.

43. Antrobus, R. D., T. K. Berthoud, C. E. Mullarkey, K. Hoschler, L. Coughlan, M. Zambon, A. V. Hill, and S. C. Gilbert. 2014. Coadministration of seasonal influenza vaccine and MVA-NP+M1 simultaneously achieves potent humoral and cell-mediated responses. *Molecular therapy : the journal of the American Society of Gene Therapy* 22: 233-238.

44. Pu, J., S. Wang, Y. Yin, G. Zhang, R. A. Carter, J. Wang, G. Xu, H. Sun, M. Wang, C. Wen, Y. Wei, D. Wang, B. Zhu, G. Lemmon, Y. Jiao, S. Duan, Q. Wang, Q. Du, M. Sun, J. Bao, Y. Sun, J. Zhao, H. Zhang, G. Wu, J. Liu, and R. G. Webster. 2015. Evolution of the H9N2 influenza genotype that facilitated the genesis of the novel H7N9 virus. *Proc Natl Acad Sci U S A* 112: 548-553.

Footnotes

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

Footnote 2: Abbreviations

Hemagglutinin (HA)
Influenza A viruses (IAV)
Inactivated influenza vaccines (IIVs)
Chimpanzee Adenovirus (ChAd)
Modified Vaccinia Ankara (MVA)
Intramuscularly (i.m.)
Long-lived plasma cells (LLPCs)
Memory B cells (mBCs)
Antibody secreting cells (ASCs)
Low pathogenic avian influenza (LPAI)
50% lethal doses (LD50)
Live attenuated influenza vaccines (LAIVs)
Spot forming units (SFU)

Figure legends & Tables

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

A: BALB/c mice (n=5) were immunized i.m. with $1x10^6$ PFU of MVA GFP(expression driven by the F11 promoter) or MVA-NP+M1 (P7.5; expression driven by the P7.5 promoter) or MVA-NP+M1-H5 (F11; expression driven by the F11 promoter). Splenocytes were isolated 2 weeks post vaccination and T cell responses were measured by *ex vivo* IFN-γ ELISpot against the immunodominant BALB/c epitope in NP, $NP_{147\text{-}158}$ (TYQRTRALV). Mann Whitney analysis of MVA-NP+M1 (P7.5) and MVA-NP+M1 (F11) showed a significant difference of p=0.0079.

B:BALB/c mice (n=5) were immunized i.m. with $1x10^6$ PFU of MVA-H5, expressing, H5HA (A/Vietnam/1203/2004), or, MVA-NP+M1-H5, a bivalent vaccine expressing the same H5HA antigen and the T cell fusion antigen, NP+M1. Serum was collected at 2 weeks post-vaccination and total serum IgG responses were measured by ELISA against recombinant H5HA protein (A/Vietnam/1203/2004; BEI resources). No significant differences were observed.

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

A: BALB/c mice (n=5) were immunized i.m. with $1x10^6$ PFU of MVA NP+M1 (P7.5; expression driven by the P7.5 promoter) or MVA-NP+M1 (F11; expression driven by the F11 promoter)or MVA-NP+M1(p7.5)-H7 (F11). Splenocytes were isolated 2 weeks post-vaccination and T cell responses were measured by *ex vivo* IFN-γ ELISpot against the BALB/c epitope in NP, $NP_{147\text{-}158}$ (TYQRTRALV). Responses post bivalent viral-vectored vaccine were significantly higher than post MVA NP+M1 (p7.5) as assessed (* $P \leq 0.05$) by Kruskal-Wallis one-way

ANOVA, with Dunn's multiple comparisons test. **B:** BALB/c mice (n=5) were immunized i.m. with 1x10^6 PFU of MVA-H7 (p7.5), expressing, H7HA (A/Netherlands/219/2003; H7N7), MVA-NP+M1-H7, a bivalent vaccine expressing the same H7HA antigen and the T cell fusion antigen, NP+M1. Serum was collected at 2 and 8 weeks post vaccination and total serum IgG responses were measured by ELISA against recombinant H7HA (A/Netherlands/219/2003; H7N7).

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

A: : BALB/c mice were immunized with ChAdOx1 viral vector vaccines encoding NP+M1 or H7HA or both; either as a mixture or by administration into separate limbs. Doses administered were 2.2x10^6 iu of ChAdOx1 NP+M1 and/or 1x10^8 iu ChAdOx1-H7. Splenocytes were isolated 2 weeks post vaccination and T cell responses were measured by *ex vivo* IFN-γ ELISpot against the BALB/c epitope in NP, NP147-158 (TYQRTRALV). Responses post a mixture of viral vectored vaccines were lower than post ChAdOx1 NP+M1 as assessed (**$P \leq 0.01$) by Kruskal-Wallis one-way ANOVA, with Dunn's multiple comparisons test. No significant difference was observed with the response post ChAdOx1 NP+M1 and when the response when viral vectored vaccines were administered, singly, into separate limbs. Data representative of two experiments.

B: BALB/c mice were immunized with 10μg of H7 protein or ChAdOx1 viral vector vaccines encoding NP+M1 or H7HA or both; either as a mixture or by administration into separate limbs. Doses adminstered were 2.2x10^6 IU of ChAdOx1 NP+M1 and/or 1x10^8 IU ChAdOx1-H7. Total serum IgG responses at all time points against recombinant H7HA protein A/Netherlands/219/2003 are shown. Data representative of two experiments.

Figure 4: **Influenza-specific T cell responses following prime-boost viral-vectored vaccination**

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

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

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

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

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

Figure 6: Bivalent viral vectors provide *in vivo* protection against influenza viral challenge Balb/c mice were unvaccinated or received an irrelevant ChAdOx1 prime and MVA boost vaccination. All other groups were vaccinated as described and three weeks after the last

immunization, animals were challenged with SH1 (A/Shanghai/1/13) virus. Weight loss was monitored over a period of 14 days, as depicted. 2way ANOVA analysis assuming a non-Gaussian distribution and Dunnetts multiple comparison test comparing Group5 (H7+Adjuvant) to

A: Group1 (ChAdOx1 NP+M1 Prime, MVA-NP+M1-H7 boost) was not different at day 4, 5, 6, 7 or 8. Comparing Group5 (H7+Adjuvant) to Group 2 (ChAdOx1-H7 Prime, MVA-NP+M1-H7 boost) demonstrated that the latter was significantly different at day 4 (****$P \leq 0.0001$), day 5 (****$p \leq 0.0001$), day6 (**$p \leq 0.01$), day 7 (**$p \leq 0.01$), but not at day 8 (N.S.). Comparing Group5 (H7+Adjuvant) to Group 3 (ChAdOx1 NP+M1 and ChAdOx1-H7 prime, MVA-NP+M1-H7 boost) demonstrated that the latter was significantly different at day 4 (*$p \leq 0.05$), day 5 (** $p \leq 0.01$), day6 (** $p \leq 0.01$), day 7 (*$p \leq 0.05$), and day 8 (*$p \leq 0.05$).

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