

# Modified Vaccinia Virus Ankara Encoding Influenza Virus Hemagglutinin Induces Heterosubtypic Immunity in Macaques

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## ABSTRACT

Current influenza virus vaccines primarily aim to induce neutralizing antibodies (NAbs). Modified vaccinia virus Ankara (MVA) is a safe and well-characterized vector for inducing both antibody and cellular immunity. We evaluated the immunogenicity and protective efficacy of MVA encoding influenza virus hemagglutinin (HA) and/or nucleoprotein (NP) in cynomolgus macaques. Animals were given 2 doses of MVA-based vaccines 4 weeks apart and were challenged with a 2009 pandemic H1N1 isolate (H1N1pdm) 8 weeks after the last vaccination. MVA-based vaccines encoding HA induced potent serum antibody responses against homologous H1 or H5 HAs but did not stimulate strong T cell responses prior to challenge. However, animals that received MVA encoding influenza virus HA and/or NP had high frequencies of virus-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses within the first 7 days of H1N1pdm infection, while animals vaccinated with MVA encoding irrelevant antigens did not. We detected little or no H1N1pdm replication in animals that received vaccines encoding H1 (homologous) HA, while a vaccine encoding NP from an H5N1 isolate afforded no protection. Surprisingly, H1N1pdm viral shedding was reduced in animals vaccinated with MVA encoding HA and NP from an H5N1 isolate. This reduced shedding was associated with cross-reactive antibodies capable of mediating antibody-dependent cellular cytotoxicity (ADCC) effector functions. Our results suggest that ADCC plays a role in cross-protective immunity against influenza. Vaccines optimized to stimulate cross-reactive antibodies with ADCC function may provide an important measure of protection against emerging influenza viruses when NAbs are ineffective.

## IMPORTANCE

Current influenza vaccines are designed to elicit neutralizing antibodies (NAbs). Vaccine-induced NAbs typically are effective but highly specific for particular virus strains. Consequently, current vaccines are poorly suited for preventing the spread of newly emerging pandemic viruses. Therefore, we evaluated a vaccine strategy designed to induce both antibody and T cell responses, which may provide more broadly cross-protective immunity against influenza. Here, we show in a translational primate model that vaccination with a modified vaccinia virus Ankara encoding hemagglutinin from a heterosubtypic H5N1 virus was associated with reduced shedding of a pandemic H1N1 virus challenge, while vaccination with MVA encoding nucleoprotein, an internal viral protein, was not. Unexpectedly, this reduced shedding was associated with nonneutralizing antibodies that bound H1 hemagglutinin and activated natural killer cells. Therefore, antibody-dependent cellular cytotoxicity (ADCC) may play a role in cross-protective immunity to influenza virus. Vaccines that stimulate ADCC antibodies may enhance protection against pandemic influenza virus.

The emergence and spread of pandemic influenza viruses is a major threat to global public health. Effective vaccines could slow the spread of emerging pandemic viruses and/or reduce the severity of associated disease, but for several reasons currently available vaccine modalities are unlikely to be effective during a pandemic. First, current modalities are designed primarily to elicit neutralizing antibodies (NAbs). While NAbs can provide sterilizing immunity, most NAbs, as typically defined in humans by hemagglutination inhibition (HI) assays, are highly strain specific and sensitive to variation in the epitopes they target in the immunogenic globular head region of the hemagglutinin (HA) protein. In recent years, studies have uncovered broadly cross-reactive NAbs in some individuals that target the conserved HA stalk (1–5), but designing vaccine immunogens to reliably stimulate high enough levels of anti-stalk NAbs in humans likely will remain a challenge. Second, because of the narrowly focused immune response vac-

cines elicit, it is necessary to identify and target specific virus strains before vaccine production can begin. As a result, vaccine availability would almost certainly lag several months behind the identification of newly emerging pandemic strains, as was the case in 2009 (6). Underscoring the need for novel, more broadly effective vaccines, a recent meta-analysis showed that available influ-

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enza virus vaccines are less than 70% effective in adults, even when there is a close antigenic match between circulating and vaccine strains (7).

When assessing the immunogenicity of influenza virus vaccines, the induction of Nabs is typically the only parameter measured. Indeed, as nonreplicating immunogens, both the widely used trivalent inactivated vaccine (TIV) and the newly approved recombinant HA vaccine (Flublok) would not be expected to induce potent T cell immunity in most subjects. Live attenuated influenza viruses (LAIV) likely undergo some productive replication in vaccinated individuals, and while they do induce T cell responses in at least some subjects, they often do not elicit strong antibody responses, particularly in adults (8, 9). Vaccines optimized to engage components of the immune response in addition to Nabs could generate cross-reactive immunity against multiple viral subtypes, both ameliorating pandemics and reducing the need for annual immunization. Here, we investigated one such vaccine modality, modified vaccinia virus Ankara (MVA). MVA is a highly attenuated vaccinia virus originally developed as a smallpox vaccine. It has an excellent safety profile, is approved by the United States Food and Drug Administration, and was administered to over 100,000 people during the global smallpox eradication program in the 1970s (10). Because of its safety and immunogenicity, recombinant MVA has been used as a vector for the development of vaccines against multiple pathogens, including influenza virus (11–15). MVA-based vaccines are capable of inducing both antibody and T cell responses against targeted antigens. MVA vaccines have protected mice against H1N1, H3N2, and H5N1 influenza viruses; Nabs were thought to be the primary correlate of protection in these studies (14, 16, 17). Interestingly, MVA expressing only influenza virus nucleoprotein (NP) protected mice and horses against influenza virus challenge, suggesting that Nabs against HA are not essential for protection (18, 19). MVA expressing HA from a clade 1 H5N1 virus has protected mice and nonhuman primates against both clade 1 and clade 2.1 viruses, likely by inducing cross-reactive antibodies (14, 16, 20). These promising results from preclinical trials have led to the evaluation of MVA-based influenza vaccines in humans. MVA vaccines encoding influenza virus NP and matrix protein 1 (M1) expand preexisting influenza-specific T cell responses in human volunteers (21). Such T-cell-based vaccines were ~60% effective at preventing homologous H3N2 virus infection in a human challenge model (15).

Taking into account these data, we reasoned that MVA vaccines encoding both external and internal influenza virus proteins might stimulate both T cell and B cell immunity and provide robust heterosubtypic protection. In particular, we hypothesized that vaccine-elicited T cell responses could confer heterosubtypic immunity. The ability of MVA-based influenza vaccines to induce heterosubtypic immunity against influenza viruses has not been tested previously in nonhuman primates or people. Members of our team have developed a series of MVA constructs expressing HA and/or NP from 2009 pandemic H1N1 (H1N1pdm) or H5N1 influenza viruses. We recently showed that one of these constructs, which encodes HA from an H1N1pdm virus and NP from an H5N1 virus, protected mice against challenge with H1N1pdm and H5N1 viruses and provided partial protection against an H3N2 virus (22). The current study was designed to determine whether MVA-vectored vaccines could induce heterosubtypic immunity in nonhuman primates; we hypothesized that such immu-

TABLE 1 MVA-vectored vaccine constructs used in this study

Construct name	Source	
	HA	NP
MVA-HA1	A/California/04/2009 (H1N1pdm)	None
MVA-HA1-C13L-NP <sup>a</sup>	A/California/04/2009 (H1N1pdm)	A/Vietnam/1203/2004 (H5N1)
MVA-HA5-C13L-NP	A/Vietnam/1203/2004 (H5N1)	A/Vietnam/1203/2004 (H5N1)
MVA-C13L-NP	None	A/Vietnam/1203/2004 (H5N1)
MVA-gfp	None	None

<sup>a</sup> In each construct, the NP open reading frame is fused at its 5' end with a sequence encoding the vaccinia virus C13L secretory signal.

nity would be mediated largely by T cell responses in these animals. Here, we show that MVAs encoding HA from H1N1pdm or H5N1 viruses induced potent antibody responses in macaques. In contrast, T cell responses to vaccine-encoded antigens were weak or undetectable in blood or lung after 2 immunizations, although vaccination appeared to prime T cell populations that expanded rapidly after infection. MVA encoding only NP derived from an H5N1 virus provided no protection against H1N1pdm challenge. Surprisingly, however, we observed a trend toward reduced viral shedding in animals vaccinated with MVA encoding H5 HA. Furthermore, we found that the vaccine encoding H5 HA induced antibody-dependent cellular cytotoxicity (ADCC)-mediating antibodies capable of activating natural killer (NK) cells in the presence of H1N1pdm HA, while cross-reactive antibodies against H1N1pdm were undetectable by HI or plaque reduction assays. Taken together, our results show that MVA-vectored vaccines can induce heterosubtypic ADCC-mediated antibodies against HA. Such responses may provide cross-protective immunity against influenza.

## MATERIALS AND METHODS

**Animals.** Twenty adult male cynomolgus macaques (*Macaca fascicularis*) were used in this study. The study was conducted according to the guidelines of the U.S. National Research Council (23) and the Weatherall report (24) under a protocol approved by the University of Wisconsin Graduate School Animal Care and Use Committee. All procedures (virus inoculations, blood draws, and bronchoalveolar lavages [BAL]) were performed under ketamine or ketamine/medetomidine anesthesia, and all efforts were made to minimize suffering.

**MVA constructs and immunizations.** Five recombinant MVA constructs were produced for this study (Table 1). MVA-HA1 encoded the full-length HA protein of A/California/04/2009 (CA04; H1N1pdm). MVA-HA1-C13L-NP encoded the CA04 HA protein and NP from A/Vietnam/1203/2004 (VN1203; H5N1). In this construct, the secretory signal encoded by the vaccinia virus C13L gene was inserted immediately 5' of the NP open reading frame; therefore, translation of this region gives an NP protein N-terminally fused to the C13L secretory signal. Similarly, MVA-HA5-C13L-NP encoded the VN1203 HA protein and the C13L-NP fusion protein. MVA-C13L-NP encoded the C13L-NP fusion protein alone. Finally, MVA-gfp encoded green fluorescent protein (GFP) and was used as a negative control. These vectors were constructed as described previously for MVA-HA1-C13L-NP (22); in all constructs, influenza virus gene expression was driven by a synthetic vaccinia virus early/late promoter.

Animals ( $n = 4$  per group) were given 2 doses of  $1 \times 10^8$  PFU of MVA-based vaccine 4 weeks apart. Vaccines were administered intradermally.

**H1N1pdm influenza virus challenge.** Eight weeks after the last MVA immunization, animals were challenged with  $5 \times 10^7$  PFU of A/Norway/3487/2009, an H1N1pdm virus phylogenetically related to A/Lviv/N6/2009 and belonging to the grouping previously designated cluster II (25). A/Norway/3487/2009 was isolated from a fatal human infection, and we had previously shown it to be pathogenic in cynomolgus macaques (26). The total viral inoculum was divided and administered to the trachea, tonsils, and conjunctivae as described previously (27, 28). Virus replication in the upper and lower respiratory tracts was monitored using standard plaque assays and/or quantitative reverse transcription-PCR (qRT-PCR) (29) on nasal wash or BAL samples. The virus stock was prepared and plaque assays performed on Madin-Darby canine kidney (MDCK) cells.

**Hemagglutination inhibition assay.** The hemagglutination inhibition assay is standard for assessing the development of vaccine-induced antibodies with neutralizing activity. This assay was performed according to the protocol described by the World Health Organization ([http://www.wpro.who.int/emerging\\_diseases/documents/manual\\_on\\_animal\\_ai\\_diagnosis\\_and\\_surveillance/en/](http://www.wpro.who.int/emerging_diseases/documents/manual_on_animal_ai_diagnosis_and_surveillance/en/)). Briefly, 1 part serum sample was mixed with 3 parts receptor-destroying enzyme II (RDE; Accurate Chemical & Scientific, Westbury, NY) and incubated at 37°C for 16 h to remove nonspecific inhibitors of hemagglutination. The RDE then was inactivated by incubating the samples at 56°C for 30 min. The samples then were diluted 1:10 with phosphate-buffered saline (PBS), and serial 2-fold dilutions were mixed with 4 HA units of either H1N1 (live A/California/04/2009) or H5N1 (inactivated A/Vietnam/1203/2004) virus in a V-bottomed microtiter plate. The plates were incubated at room temperature for 30 min, after which 0.5% chicken red blood cells were added. The samples were incubated at room temperature for 30 min. The reciprocal of the dilution at which no inhibition was observed was recorded as the HI antibody titer. Wells with 4 HA units of virus and PBS were kept as positive and negative controls for hemagglutination.

**Monitoring T cell responses: intracellular cytokine staining (ICS).** Freshly collected peripheral blood mononuclear cells (PBMCs) were aliquoted into 1.2-ml tubes at  $10^6$  cells per tube. One  $\mu$ l anti-CD28 clone L293 (BD Biosciences, San Jose, CA) and 1  $\mu$ l anti-CD49d clone 9F10 (BD Biosciences, San Jose, CA) then were added together with 2  $\mu$ g brefeldin A, which was added to stop protein transport. Peptide pools representing A/California/04/2009 proteins (11- to 17-mers with 11- to 13-amino-acid overlaps; BEI Resources, Manassas, VA) then were added to each tube to reach a final concentration of 1  $\mu$ M per peptide. Samples then were incubated at 37°C with 5% CO<sub>2</sub> for 6 h. After incubation, a Live/Dead violet viability stain (Invitrogen, Carlsbad, CA) was used according to the manufacturer's instructions, and the following surface-staining antibodies were added: anti-CD3 Alexa Fluor 700 clone SP34-2 (BD Biosciences, San Jose, CA), anti-CD8 allophycocyanin (APC)-H7 clone SK1, and anti-CD4 peridinin chlorophyll protein (PerCP)/Cy5.5 clone OKT4 (BioLegend, San Diego, CA). The cells then were incubated for 30 min in the dark at room temperature. Following incubation, the cells were washed two times with fluorescence-activated cell sorting buffer (FACS) (PBS, 0.1% bovine serum albumin [BSA], 0.1% NaN<sub>3</sub>) and fixed in 1% paraformaldehyde for 15 min. When fixation was complete, the cells were washed once using FACS buffer and stored overnight at 4°C. On the following day, the cells were washed twice with FACS buffer containing 0.1% saponin and stained with anti-gamma interferon (IFN- $\gamma$ ) fluorescein isothiocyanate (FITC) clone 4S.B3 (BD Biosciences, San Jose, CA) for 50 min in the dark at room temperature. Cells then were washed twice with FACS buffer containing 0.1% saponin and stored at 4°C. Events were collected on a BD LSRII flow cytometer and analyzed using FlowJo software (TreeStar, Ashland, OR).

**Detection of HA-binding antibodies (ELISA).** We used an enzyme-linked immunosorbent assay (ELISA) to detect antibodies capable of binding HA protein as described previously (28, 30). Briefly, the assay was performed using Immulon 2HB plates (Thermo Fisher Scientific, Waltham, MA) coated with 1  $\mu$ g antigen using purified HA or whole virus in PBS overnight at 4°C. Plates then were washed 6 times in PBS plus 0.05%

Tween 20, and 100  $\mu$ l plasma diluted 1:100 in PBS was added to each well. The plates were incubated at 37°C for 2 h and then washed 6 times with PBS plus 0.05% Tween 20. The detection antibody was mouse anti-human IgG antibody clone G18-145 conjugated to horseradish peroxidase (BD Biosciences, San Jose, CA). This was diluted 1:1,000 in PBS, and 100  $\mu$ l was added to each well, followed by a 1-h incubation at room temperature. The plate then was washed 6 times with the PBS plus 0.05% Tween 20 solution. SureBlue TMB microwell peroxidase substrate (KPL, Gaithersburg, MD) was added as an indicator and incubated at room temperature. Once a blue color change occurred in the control wells, 100  $\mu$ l 1N HCl was added to each well to stop the reactions. The absorbance in each well then was measured at 450 nm.

**ADCC NK cell activation assay.** We measured the ability of serum antibody to activate NK cells in the presence of HA proteins as described previously (30). Briefly, 96-well plates were coated overnight with recombinant HA proteins expressed from mammalian cells (Sinobiologicals, Shanghai, China). Wells then were washed multiple times with PBS to remove unbound proteins. Heat-inactivated EDTA-anticoagulated macaque plasma (56°C for 1 h) then was added to each well and incubated at 37°C for 2 h. Wells again were washed repeatedly with PBS. One million PBMCs freshly isolated from influenza-naïve macaques were added to each well in complete RPMI medium containing 10% fetal calf serum (HyClone, Logan, UT), together with anti-human-CD107a-APC-H7 antibody (H4A3 clone; BD Biosciences, San Jose, CA), 5  $\mu$ g/ml brefeldin A (Sigma, St. Louis, MO), and 5  $\mu$ g/ml monensin (Golgi Stop; BD Biosciences). Plates were incubated for 5 h at 37°C, after which time cells were incubated with the following antibodies for 30 min at room temperature: anti-CD3 Pacific Blue (SP34-2 clone; BD Biosciences), anti-CD14 PE-Cy7 (M5E2 clone; BD Biosciences), and anti-NKG2A APC (clone Z199; Beckman Coulter, Brea, CA). Cells then were fixed with 1% paraformaldehyde (Sigma-Aldrich, St. Louis, MO), permeabilized using 1 $\times$  FACS permeabilizing solution 2 (BD Biosciences), and stained with anti-IFN- $\gamma$  AF700 (B27 clone; BD Biosciences). Finally, cells were fixed with 1% paraformaldehyde and acquired on an LSRII flow cytometer (BD Biosciences). Data were analyzed using FlowJo, version 9.2. NK cells were identified as CD3-negative, CD14-negative, NKG2A-positive lymphocytes as described previously (30).

**Plaque reduction neutralization assay.** We performed the plaque reduction assay as described previously (31). Briefly, we used protein G HP SpinTrap columns (GE Healthcare Life Sciences, Pittsburgh, PA) to purify total IgG from plasma obtained from cynomolgus macaques. We followed the manufacturer's protocol, except that plasma was incubated with protein G Sepharose for 1 h with mild shaking instead of the standard 5 min. Eluted IgG was buffer exchanged in PBS by using an Amicon Ultracel-30K centrifugal filter unit (Millipore, Billerica, MA) with a 30-kDa molecular mass cutoff in a swinging bucket rotor. Protein concentrations were measured using the quick-start Bradford protein assay (Bio-Rad Laboratories, Inc., Hercules, CA). A/Norway/3487/2009 stock virus was diluted to approximately 50 PFU/well and incubated with 3-fold serial dilutions of total IgG for 1 h at room temperature. Twelve-well plates were seeded with MDCK cells and washed twice with PBS. Three hundred  $\mu$ l of antibody-virus mixture was placed over the MDCK monolayer for 45 min at 37°C. The antibody-virus mixture then was aspirated off and washed once with PBS. One ml of agar overlay containing the appropriate antibody concentration and tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin was added to each well. Plates were incubated for 2 days at 37°C and then fixed with 10% formalin at room temperature for 1 h. Plaques were visualized and counted by eye.

**Statistical analyses.** Comparisons of virus titers were performed on log-transformed data using unpaired *t* tests with Welch's correction in GraphPad Prism version 5.

## RESULTS

**MVA-vectored vaccines encoding HA induce potent antibody responses.** A common method for assessing the immunogenicity

of influenza virus vaccines in humans is the hemagglutination inhibition (HI) assay, which detects serum antibodies that prevent the agglutination of red blood cells by HA molecules on influenza virions. MVA vectors encoding H1 HA proteins induced detectable HI antibody responses within 2 weeks of the first immunization; a second administration of the same vaccine boosted H1-specific HI antibody titers 2- to 16-fold (Table 2). Two weeks after this boost, geometric mean HI antibody titer against H1 HA was 1:293 (range, 1:80 to 1:640). Thus, all animals receiving MVA vaccines encoding H1 HA proteins made detectable antibody responses against homologous virus after a single vaccination, and these responses were boosted by a second vaccination. As expected, animals receiving vaccines that encoded H5 HA or no HA did not make antibodies against H1 HA detectable by HI assay (Table 2).

**MVA-vectored vaccines do not stimulate strong T cell responses in naive macaques.** We next assessed the ability of MVA-vectored vaccines to induce influenza-specific T cell responses. We used ICS to detect peptide-specific secretion of IFN- $\gamma$  in PBMC and lung lymphocytes collected by BAL specimens. Cells were stimulated with peptides representing HA and NP from the H1N1pdm strain A/California/04/2009 in order to detect responses that could cross-react with the H1N1pdm challenge. We observed only weak or undetectable peptide-specific CD4<sup>+</sup> or CD8<sup>+</sup> T cell responses in the blood or lungs of MVA-vaccinated animals prior to influenza virus challenge (Fig. 1). These data suggest that MVA-vectored vaccines induced low frequencies of cross-reactive T cells in nonhuman primates, at least when delivered intradermally and in the absence of previous priming.

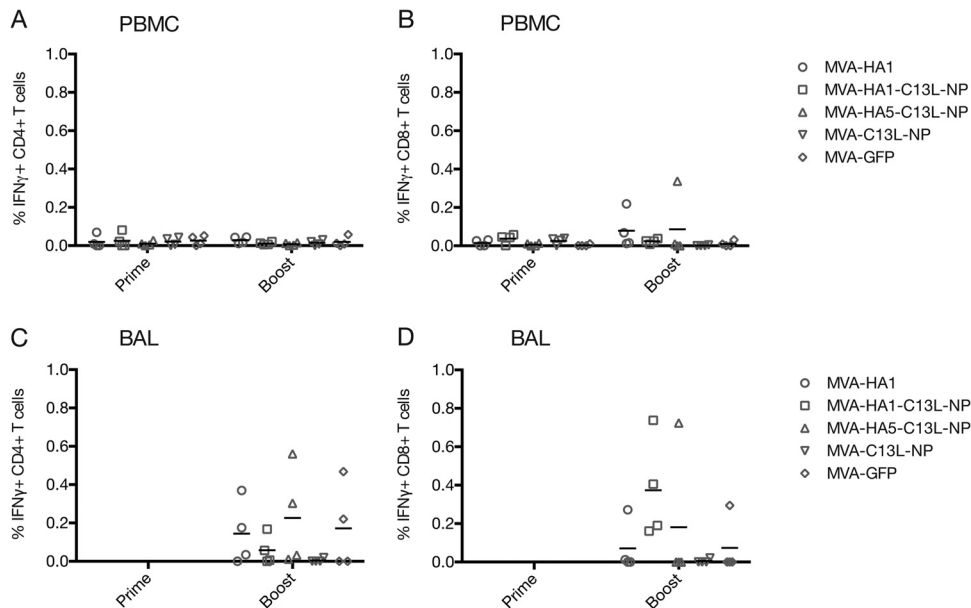
**Homosubtypic and partial heterosubtypic protection by MVA-vectored vaccines encoding HA.** To assess the protective efficacy of MVA-vectored vaccines, we challenged macaques with 50 million PFU of the H1N1pdm isolate A/Norway/3487/2009.

TABLE 2 H1-HA-specific Ab titers measured by HI assay

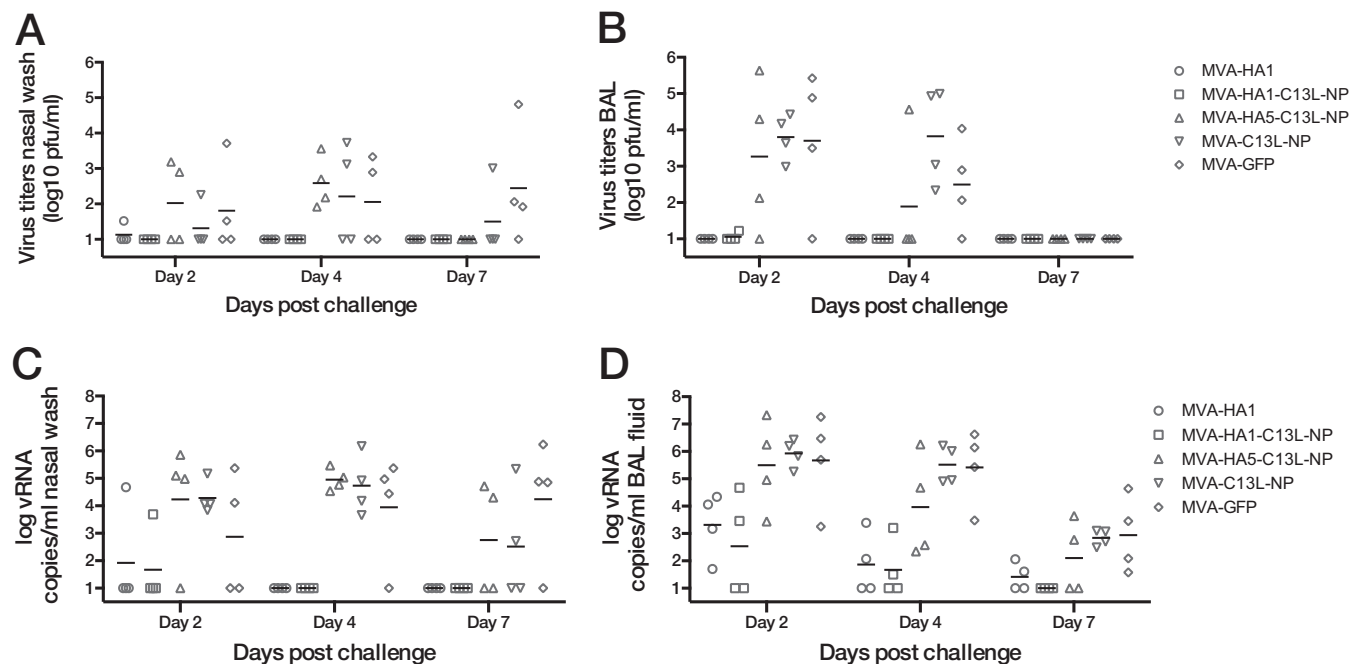
Vaccine and animal ID	Ab titer at:			
	MVA 1 <sup>a</sup>	MVA 2 <sup>b</sup>	Day of challenge	14 days postchallenge
<b>MVA-HA1</b>				
cy0476	40	640	320	2,560
cy0477	40	640	320	320
cy0478	20	160	40	2,560
cy0480	80	640	320	1,280
<b>MVA-HA1-C13L-NP</b>				
cy0489	80	160	80	1,280
cy0503	40	160	80	1,280
cy0504	40	80	40	2,560
cy0507	320	640	320	2,560
<b>MVA-HA5-C13L-NP</b>				
cy0483	5	5	5	640
cy0484	5	5	5	320
cy0485	5	5	5	640
cy0487	5	5	5	320
<b>MVA-C13L-NP</b>				
cy0493	5	5	5	320
cy0494	5	5	5	320
cy0496	5	5	5	320
cy0500	5	5	5	80
<b>MVA-gfp</b>				
cy0501	5	5	5	320
cy0508	5	5	5	320
cy0510	5	5	5	320
cy0513	5	5	5	1,280

<sup>a</sup> Measured 2 weeks after the first MVA administration.

<sup>b</sup> Measured 2 weeks after the second MVA administration.



**FIG 1** Low-frequency HA- and NP-specific T cell responses in the blood and lungs of macaques after MVA vaccination. We determined the frequency of CD4<sup>+</sup> and CD8<sup>+</sup> T cells responding to synthetic peptides representing H1N1pdm A/California/04/2009 HA and NP after each MVA inoculation using IFN- $\gamma$  ICS. Summed frequencies of CD4<sup>+</sup> and CD8<sup>+</sup> cells responding to HA and NP peptides are shown for both blood (A and B) and bronchoalveolar lavage specimens (BAL; C and D). Data points indicate individual monkeys, with the mean indicated by a line.



**FIG 2** Replication of H1N1pdm virus A/Norway/3487/2009 in MVA-vaccinated cynomolgus macaques. Groups ( $n = 4$ ) vaccinated with different MVA constructs were challenged with  $5 \times 10^7$  PFU of A/Norway/3487/2009 via multiple routes, as described in Materials and Methods. Viral titers in the upper respiratory tract (nasal wash) (A) and lower respiratory tract (bronchoalveolar lavage) (B) were measured using standard plaque assays on MDCK cells. Titers below the detection limit of the plaque assay are assigned a titer of  $1 \times 10^1$  for the purposes of plotting and geometric mean calculation. Replication was measured using qRT-PCR in the upper respiratory tract (nasal wash) (C) and lower respiratory tract (bronchoalveolar lavage) (D). Samples with a viral RNA content below the qRT-PCR assay's detection limit are assigned a value of 10 copies/ml for the purposes of plotting and geometric mean calculation. Bars represent geometric means for each group. Lines indicate geometric means for each group.

This virus was isolated from a fatal human case during the 2009 pandemic and encoded an aspartate-to-glycine substitution at HA amino acid position 222 (D222G; D225G in H3 amino acid numbering), which has been associated with increased pathogenic potential in mice, macaques, and humans (26, 32–34). A hallmark of H1N1pdm is its ability to replicate robustly in both the upper and lower respiratory tracts; therefore, we assessed virus titers in both nasal wash, representing the upper respiratory tract, and BAL specimens, representing the lower tract.

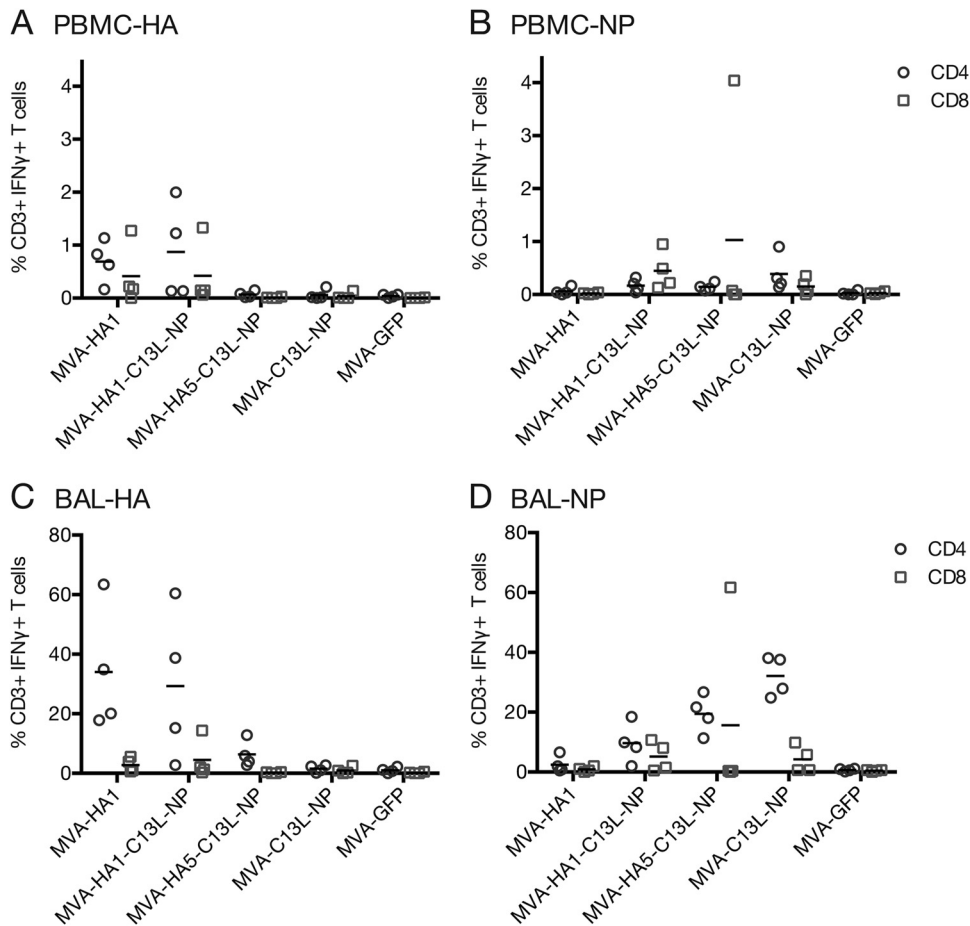
H1N1pdm replicated to high titer in both the upper and lower respiratory tracts of animals vaccinated with MVA-gfp (Fig. 2), consistent with our previous observations in macaques lacking preexisting virus-specific immunity (26, 28, 35). We observed almost no replication of the challenge virus in either compartment in animals vaccinated with MVA encoding H1 HA; the only exceptions were single time points at which infectious virus just exceeded the detection limit of plaque assays in one animal vaccinated with MVA-HA1 and one vaccinated with MVA-HA1-C13L-NP (Fig. 2). These data suggest that vaccine-induced Abs provided strong protection against homologous H1N1pdm challenge in macaques.

In a previous study, we found evidence that T cell responses could limit H1N1pdm replication in macaques in the absence of virus-specific NAbs (28). Therefore, we originally anticipated that animals vaccinated with MVA-HA5-C13L-NP and MVA-C13L-NP would clear H1N1pdm more rapidly than animals vaccinated with the control immunogen MVA-gfp. However, perhaps consistent with the lack of T cell responses during the vaccine phase, we observed no evidence of protection in animals vaccinated with MVA-C13L-NP: virus titers in

the upper and lower respiratory tract in these animals were not significantly different at any time point after challenge from those observed in animals that received MVA-gfp (Fig. 2).

Interestingly, animals vaccinated with MVA-HA5-C13L-NP appeared to clear H1N1pdm infection from the upper and lower respiratory tracts more rapidly than animals that received vaccines encoding NP alone or the irrelevant antigen GFP. Infectious virus was undetectable in the nasal washes of MVA-HA5-C13L-NP-vaccinated animals by day 7 postinoculation, while virus was still detectable in 1 MVA-C13L-NP-vaccinated animal and 3 of 4 animals that received MVA-gfp, although differences between groups did not reach statistical significance (Fig. 2A). On day 4 postinoculation, virus was undetectable in the lungs of 3 of 4 MVA-HA5-C13L-NP-vaccinated animals, while 3 of 4 MVA-gfp-vaccinated animals and all 4 MVA-C13L-NP-vaccinated animals still were shedding infectious virus (Fig. 2B), although again these differences did not reach statistical significance. qRT-PCR to detect viral RNA in nasal swabs and BAL fluid showed similar results (Fig. 2C and D). Together, these data raise the possibility that vaccination with the construct encoding H5 HA allowed animals to control H1N1pdm replication more effectively than animals receiving MVA encoding NP alone or GFP, although this trend did not reach statistical significance.

**Rapid expansion of T cell responses after challenge in animals vaccinated with influenza antigens.** Although influenza virus-specific T cell responses were very low or undetectable after 2 administrations of MVA vaccines, we reasoned that vaccination might have primed low frequencies of T cell populations capable of rapid expansion after influenza virus challenge. Therefore, we



**FIG 3** Peripheral and mucosal T cell responses after H1N1pdm challenge. We used ICS for IFN- $\gamma$  to measure the response of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes to synthetic peptides representing H1N1pdm HA (A and C) or NP (B and D); shown are background-subtracted response frequencies for each monkey, with averages indicated by a line at day 7 postchallenge. Peptide-specific T cell frequencies in peripheral blood (A and B) and peptide-specific cell frequencies in bronchoalveolar lavage specimens (C and D) are shown.

used ICS to measure responses in BAL fluid and PBMC lymphocytes to pools of synthetic peptides representing H1N1pdm HA and NP protein sequences. T cells recognizing these peptide pools would be expected to recognize the H1N1pdm challenge virus regardless of whether H1N1pdm sequences were present in the vaccine. No responses to these peptides were detected in any animal on the day of influenza virus challenge (data not shown). However, by day 7 postchallenge, mean frequencies of H1 HA-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells ranged between 0.4% and 0.8% of all T cells in the peripheral blood of animals vaccinated with MVA expressing H1 HA, while responses to H1 HA remained low or undetectable in other animals (Fig. 3A). Similarly, peripheral CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses to NP were detectable at day 7 postchallenge in all groups of animals that received MVA constructs encoding this protein, while such responses were undetectable in animals receiving vaccines encoding HA alone or GFP (Fig. 3B).

CD4<sup>+</sup> and/or CD8<sup>+</sup> T cells specific for H1N1pdm HA were detectable in BAL lymphocytes at day 7 postchallenge in each group of animals, although they reached substantially higher frequencies in animals vaccinated with constructs encoding H1 HA (Fig. 3C). Consistent with our previous observations (28), influ-

enza virus-specific memory T cells had expanded dramatically at day 7 postchallenge in animals vaccinated against H1-HA: CD4<sup>+</sup> T cells responding to HA reached mean frequencies of approximately 30% of all BAL fluid CD4<sup>+</sup> T cells in these groups. Surprisingly, however, HA-specific CD8<sup>+</sup> T cells reached comparatively lower frequencies (mean of 3% to 5% of all BAL fluid T cells) in these same animals. We also detected relatively robust HA-specific CD4<sup>+</sup> T cell responses at day 7 in animals vaccinated with MVA-HA5-C13L-NP (Fig. 3C). NP-specific T cells similarly were detectable in the lungs of animals in most groups at day 7, with the exception of the MVA-gfp group (Fig. 3D). As for NP-specific responses, CD4<sup>+</sup> T cell responses generally were higher in magnitude than CD8<sup>+</sup> T cell responses, and the frequency of NP-specific T cells of either subset was higher in animals that received vaccines encoding NP. Together, our results suggest that MVA vaccines primed low-frequency T cell responses during the vaccine phase that could be effectively recalled upon challenge with influenza virus, even if they were not detectable prior to challenge.

**MVA vaccines elicit cross-reactive nonneutralizing HA-specific antibodies.** In previous studies, we showed that the infection of macaques with a seasonal H1N1 influenza virus induced antibodies capable of binding HA from H1N1pdm viruses, although

TABLE 3 HA-binding Ab titers measured by ELISA

Vaccine and animal ID	Ab titer for <sup>a</sup> :	
	A/California/04/2009 HA	A/Vietnam/1203/04 HA
MVA-HA1		
cy0476	2,560	NT
cy0477	1,280	NT
cy0478	320	NT
cy0480	2,560	NT
MVA-HA1-C13L-NP		
cy0489	640	NT
cy0503	640	NT
cy0504	320	NT
cy0507	5,120	NT
MVA-HA5-C13L-NP		
cy0483	40	2,560
cy0484	20	2,560
cy0485	20	2,560
cy0487	20	1,280
MVA-C13L-NP		
cy0493	<5	NT
cy0494	<5	NT
cy0496	5	NT
cy0500	<5	NT
MVA-gfp		
cy0501	5	NT
cy0508	<5	NT
cy0510	5	NT
cy0513	<5	NT

<sup>a</sup> NT, not tested.

no NAb were detectable (28). We subsequently found that some of these nonneutralizing antibodies were able to mediate ADCC (30). ADCC (36) is a multistep process in which virus-specific antibodies first bind antigens expressed on the surface of infected cells. Cytotoxic effector cells, primarily NK cells, then can recognize membrane-bound antibodies via their Fc receptor (CD16). CD16 binding of antibody triggers NK cells to release cytotoxic

granules containing perforin and granzymes, leading to apoptosis of the target cell. We reasoned that ADCC plays a role in the relatively early clearance of H1N1pdm we observed in animals vaccinated with MVA-HA5-C13L-NP; therefore, we next determined whether this vaccine elicited antibodies capable of binding H1N1pdm HA proteins.

To detect HA-binding antibodies, we used plate-bound HA proteins to capture serum antibodies in an ELISA. Vaccination with constructs encoding HA induced high-titer antibodies capable of binding homologous HA proteins (the geometric mean titer of H1-binding antibodies was 2,153 in animals vaccinated with MVA-HA1 or MVA-HA1-C13L-NP; the geometric mean titer for H5-binding antibodies also was 2,153 in animals vaccinated with MVA-HA5-C13L-NP) (Table 3). However, the H5 HA-encoding vaccine also induced antibodies capable of binding the heterologous HA of H1N1pdm (geometric mean titer of 23.8 in MVA-HA5-C13L-NP vaccinees) (Table 3). These data show that MVA vaccines encoding HA induce antibodies capable of binding heterosubtypic HA proteins, even in the absence of cross-reactive HI antibodies.

To determine the neutralizing capacity of MVA vaccine-elicited HA-binding antibodies, we performed a plaque reduction neutralization assay (31). Unlike HI assays, the plaque reduction assay is capable of detecting neutralization mediated by broadly cross-reactive NAb targeting the HA stalk domain, which typically act after virus attachment to prevent membrane fusion (37). Vaccination with MVA-HA1 and MVA-HA1-C13L-NP induced high-titer antibodies capable of neutralizing the homologous challenge virus. These NAb were detectable as early as 21 days postvaccination and also were present as early as 4 days after challenge (Fig. 4). Conversely, IgG from animals vaccinated with MVA-HA5-C13L-NP and MVA-C13L-NP showed no neutralization capacity until 10 days after H1N1pdm challenge (Fig. 4). These data suggest that MVA-vectored vaccination induced antibodies capable of neutralizing homologous virus. However, although vaccination with H5 HA induced antibodies capable of binding heterosubtypic H1N1pdm HA, these antibodies lacked neutralizing activity.

**MVA vaccines elicit cross-reactive ADCC antibodies against influenza.** The presence of cross-reactive HA-specific antibodies in vaccinated animals suggested that MVA-HA5-C13L-NP had induced cross-reactive ADCC antibodies. To explore this possibil-

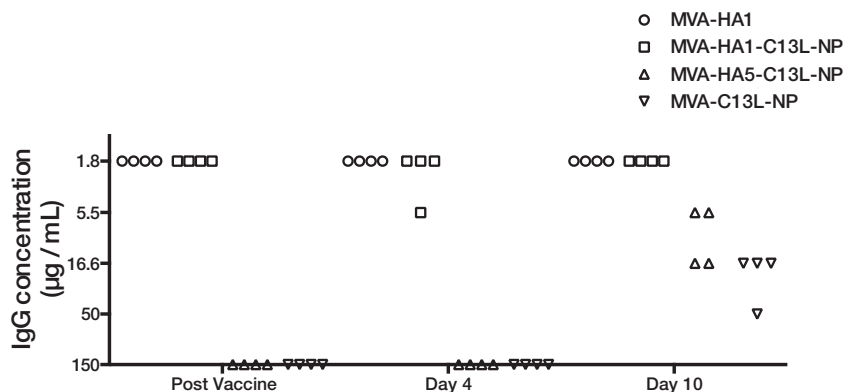
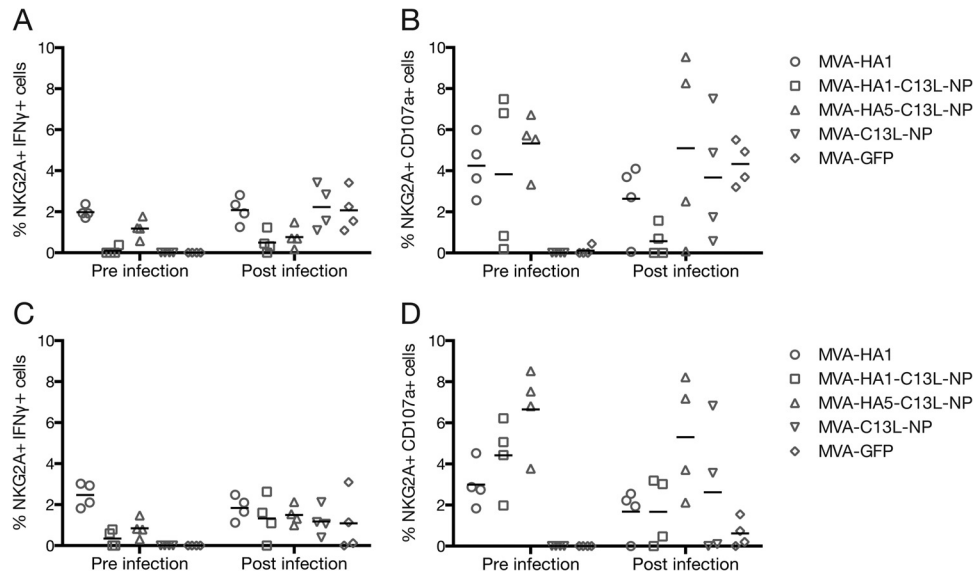


FIG 4 MVA encoding HA elicits homosubtypic, but not heterosubtypic, NAb. The capacity of MVA-vectored vaccines to elicit neutralizing antibodies was assessed by plaque reduction assay. Total IgG collected from plasma was incubated with A/Norway/3487/2009 in a series of 3-fold dilutions. Three time points were observed, including 21 days postvaccination, 4 days postinfection, and 10 days postinfection. Data are reported as the IgG concentration at which at least 50% inhibition of plaque formation was observed.



**FIG 5** MVA encoding HA induces heterosubtypic ADCC antibodies. We assessed the ability of antibodies in plasma collected prior to challenge to induce NK cell effector functions in the presence of seasonal or pandemic virus HA proteins. Plates were coated with HA from H1N1pdm A/California/04/2009 (A and B) or seasonal A/New Caledonia/20/1999 (C and D). NK cell effector activity was measured by the expression of IFN- $\gamma$  (A and C) and the degranulation marker CD107a (B and D). Data are presented as individual monkeys, with the mean indicated by a line.

ity, we determined whether plasma antibodies from MVA-vaccinated macaques were capable of activating NK cells in the presence of HA proteins from seasonal and pandemic H1N1 viruses, using an assay we recently described (30). In this assay, HA proteins are immobilized on a 96-well plate and incubated with test plasma, and then lymphocytes from influenza-naïve macaques are added. If the plasma contains ADCC antibodies that bind the HA protein, NK cells from the donor animals will be activated, as detected by the secretion of antiviral cytokines such as IFN- $\gamma$  and the release of cytotoxic granules. Activated NK cells are enumerated using flow cytometry that detects intracellular IFN- $\gamma$  and/or cell surface CD107a, a marker of degranulation.

Plasma sampled after vaccination but before challenge harbored ADCC antibodies capable of activating NK cells in the presence of either seasonal or pandemic H1 HA proteins in animals vaccinated with MVA encoding HA but not in animals vaccinated with other MVAs (Fig. 5A and C). The group mean frequency of activated NK cells detected in these assays ranged from 0.1% to 2.5%, with the strongest NK responses detected in animals vaccinated with MVA-HA1. We found higher frequencies of NK cells expressing the degranulation marker CD107a when they were stimulated by HA-bound antibodies, but again these ADCC antibodies were detectable prior to challenge only in animals that had received vaccines encoding HA (range, 3% to 7% of NK cells) (Fig. 5B and D). Interestingly, the strongest overall response detected prior to challenge was in animals vaccinated with MVA-HA5-C13L-NP, whose antibodies stimulated  $\sim$ 7% of NK cells to degranulate in the presence of HA from a seasonal H1N1 virus (Fig. 5D). By 2 weeks after infection, plasma from animals in each group contained cross-reactive ADCC antibodies capable of activating NK cells in the presence of either seasonal or pandemic H1N1 virus HA protein (Fig. 5). Together, these data show that vaccination with MVA encoding HA proteins elicits an ADCC antibody response, and that ADCC antibodies raised by vaccina-

tion with MVA encoding an H5 HA protein can cross-react with HA proteins from both seasonal and pandemic H1N1 viruses.

## DISCUSSION

The need for pandemic influenza virus vaccines is underscored by the recent outbreak of H7N9 viruses in China. Human infection with viruses of this subtype had not been documented previously. Accordingly, studies have shown that preexisting NAbs against H7 HAs are absent or at low frequency in human populations (38–40). Current vaccines that focus on inducing NAbs do not elicit strong cross-reactive immunity and take months for development and approval. Broadly reactive NAbs that target conserved domains, such as the M2 ectodomain or the HA stalk, show promise in animal models but face obstacles in translation to the clinic (41). Meanwhile, vaccines capable of stimulating a broader range of potentially cross-reactive immune responses could limit the incidence of severe disease and death in the case of a pandemic.

Here, we evaluated MVA-vectored vaccines designed to elicit both cellular and humoral immunity in a translational primate model, testing the ability of vaccine-induced immune responses to limit replication of an H1N1pdm challenge. Vaccines encoding the H1 HA from an H1N1pdm isolate induced strong antibody responses detectable by HI assays. Accordingly, animals challenged with homologous H1N1pdm were robustly protected.

A recent study reported that MVA vectors expressing only NP from A/Vietnam/1203/2004 protected mice against H5N1, H9N2, and H7N1 influenza viruses; cross-reactive CD4<sup>+</sup> and CD8<sup>+</sup> T cells were thought to be the main correlate of protection (42). While MVA alone can induce strong T cell responses in mice, previous studies have suggested that MVA vectors alone, even with multiple booster immunizations, may not be strongly immunogenic for CD8<sup>+</sup> T cells in macaques (43, 44). In agreement with these results, we did not detect robust cellular immune responses in the blood or lungs of monkeys even after 2 doses; the frequency



of influenza-specific IFN- $\gamma$ -secreting CD4<sup>+</sup> and CD8<sup>+</sup> T cells in blood remained at or below about 0.2%. These values in influenza-naïve macaques are slightly lower than those observed in recent immunogenicity trials of MVA-vectored vaccines in humans, who likely have some preexisting immunity to influenza (13, 45). The reasons for the differential immunogenicity of MVA vaccines in various species likely are multifactorial and cannot be directly addressed with the data presented here. We note, however, that humans and macaques share a close phylogenetic relationship and show broadly similar (and modest) magnitudes of T cell responses to MVA vaccines, while MVA alone elicits potent T cell immunity in mice. Therefore, we suggest that macaques represent a more representative model of human immunity for the purposes of translational/preclinical evaluations of vaccine immunogenicity.

After H1N1pdm challenge, we detected rapid expansion of CD4<sup>+</sup> and CD8<sup>+</sup> T cell populations against vaccine-encoded antigens. These T cell responses were absent from animals vaccinated with MVA-gfp, suggesting that vaccination did indeed prime low-frequency cell-mediated immunity. However, despite this rapid recall of cellular responses, there was no evidence for protection in animals vaccinated with NP alone. These data suggest that vaccine-induced T cells did not play an important role in reducing virus replication after infection, in contrast to our previous observations with macaques serially infected by seasonal and pandemic H1N1 (28).

There are multiple potential explanations for this result. First, it is possible that there was greater conservation of key T cell epitopes between the seasonal and pandemic H1N1 viruses in our previous study than between the H5N1-derived vaccine immunogens and H1N1pdm challenge in this study. The NP sequences of the vaccine strain A/Vietnam/1203/2004 (H5N1) and the challenge H1N1pdm strain A/Norway/3487/2009 in this study were 93.3% identical at the amino acid level, while NP amino acid identity between the seasonal and pandemic H1N1 viruses used in our previous study was slightly lower, at 89.8% (28). Therefore, it is possible that there was actually greater T cell epitope conservation in NP between the vaccine and challenge in the present study than in our previous serial infection experiment. However, we have not comprehensively mapped minimal optimal T cell epitopes recognized by either the Indian rhesus macaques used in our previous study or the cynomolgus macaques used here. Moreover, these two macaque species have different major histocompatibility complex genetics and would be expected to recognize distinct sets of T cell epitopes. Furthermore, our previous study used infection with a seasonal influenza virus to prime immune responses. Thus, the animals in that study were exposed to the entire influenza virus proteome, rather than only to HA and/or NP, and made T cell responses against a broader range of viral antigens prior to challenge than animals in this study. In addition, seasonal H1N1 virus infection elicited potent cross-reactive T cell responses, whereas T cell responses to MVA vaccination in this study were modest. In sum, we speculate that cross-reactive T cells played a more important role in heterosubtypic immunity in our previous study than they did here. We note that cross-reactive T cells and ADCC antibodies are not mutually exclusive potential correlates of heterosubtypic immunity to influenza virus and may play different roles in different contexts.

Animals vaccinated with MVA-HA5-C13L-NP appeared to clear H1N1pdm challenge more rapidly than animals vaccinated with MVA encoding NP alone or GFP. The small number of animals in this study makes it difficult to draw firm conclusions, and

differences in group mean virus titers did not reach statistical significance. Interestingly, however, we found that MVA-HA5-C13L-NP vaccination induced nonneutralizing antibodies that bound H1 subtype HA proteins. These antibodies were capable of activating macaque NK cells to secrete IFN- $\gamma$  and to release cytotoxic granules in the presence of HA proteins from both seasonal and pandemic H1N1 viruses. Combining the data reported here with our previous observations in serially infected macaques (30), we speculate that vaccine-induced ADCC antibodies play a role in heterosubtypic immunity to influenza virus. Importantly, our previous study showed that the titers of ADCC antibodies decline dramatically within a few months of seasonal H1N1 infection. Here, we challenged the animals 8 weeks after the end of the vaccination phase, so antibody titers had little time to wane. Interestingly, recent studies suggest that the protective effects of HA stem-specific antibodies *in vivo* can be attributed mostly to ADCC activity (3, 46). Therefore, antibodies binding the HA stem may mediate multiple effector functions, including both ADCC and virus neutralization, that could contribute to broad protection. It is important to note that neutralization and ADCC are effector functions that may or may not overlap those of antibodies of a given specificity.

H5 and H1 subtype HAs are phylogenetically related and both belong to group 1, which might explain why we observe cross-reactive nonneutralizing antibodies in these studies (47). A previous study showed that DNA vaccines based on synthetic consensus H5N1 sequences provided better protection in macaques against the H5N1 virus A/Vietnam/1203/2004 when the vaccines encoded both HA and NP rather than NP alone (48). These results are not directly comparable with ours, since most animals in the previous study made at least low-level NAb against the challenge virus, and HA-binding, nonneutralizing antibodies were not measured. In another experiment, the immunization of macaques with 2 doses of MVA encoding the HA from A/Vietnam/1194/2004 provided robust protection against both the completely homologous isolate A/Vietnam/1194/2004, a clade 1 H5N1 virus, and against the clade 2.1 H5N1 virus A/Indonesia/5/2005 (20). Low levels of NAb against the clade 2.1 virus were detected in 4 of 12 animals in that study; neither T cell responses nor nonneutralizing HA-binding antibodies were measured. The vaccine strain A/Vietnam/1194/2004 and the challenge virus A/Indonesia/5/2005 share 96% amino acid identity, which may explain the presence of cross-reactive NAb in some animals in that study. A/Vietnam/1203/2004 was also the source of the HA sequence in our vaccine construct MVA-HA5-C13L-NP, but its amino acid identity with H1N1pdm viruses is only 63%, consistent with the lack of heterosubtypic antibodies detected by HI or plaque reduction assays in our animals vaccinated with this construct.

The ability of vaccination to induce cross-reactive ADCC-mediated antibodies is unclear. Recently we showed that the trivalent inactivated vaccine was not able to induce either neutralizing or ADCC-mediated antibodies in pigtail macaques given two doses of vaccine (49). Accordingly, a previous study showed that whole-killed virus formulations were more effective than the standard split virion formulations at inducing IgG2a antibodies, which are the principal mediators of ADCC (50). Thus, vaccine formulations are likely to differ in their ability to stimulate ADCC antibodies, in part due to differences in the efficiency of inducing IgG2a responses. Here, vaccination with MVA-vectored vaccines appeared to induce robust levels of heterosubtypic ADCC anti-

bodies. As a replicating vector, MVA may induce a cytokine profile that more closely mimics that of influenza virus infection than does vaccination with inactivated whole viruses or split virions (50). Interestingly, after vaccination, antibodies from animals receiving MVA encoding both HA and NP appeared to induce lower frequencies of IFN- $\gamma$ -secreting NK cells than animals receiving MVA encoding H1 HA alone (Fig. 5A and C), while antibodies from all three groups efficiently induced CD107a expression by NK cells in the presence of HA (Fig. 5B and D). With our present data, however, we cannot determine whether this represents a significant and/or functionally important difference.

Taken together, our study and others show that MVA-vectored vaccines against influenza virus are immunogenic in human and animal models, and that they are capable of stimulating multiple immune effectors in addition to NAbs. Even if immunity provided by mechanisms other than NAbs is not sterilizing, limiting virus replication could mean the difference between death and survival, between severe and mild disease, or between efficient and poor onward transmission. Our study shows that MVA vaccines can induce heterosubtypic ADCC antibodies in a relevant translational model. The small number of animals in the present study prevents us from drawing conclusions about the protective efficacy of these vaccine-induced heterosubtypic antibodies, but previous observations suggest that ADCC can play an important role in heterosubtypic immunity to influenza virus. Therefore, vaccines optimized to induce ADCC antibodies may provide a measure of heterosubtypic protection against emerging influenza viruses.

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