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Broad Protection against Avian Influenza Virus by Using a Modified Vaccinia Ankara Virus Expressing a Mosaic Hemagglutinin Gene

Attapon Kamlangdee,[a] Brock Kingstad-Bakke,[a] Tavis K. Anderson,[b] Tony L. Goldberg,[a] Jorge E. Osorio[a]

Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin—Madison, Madison, Wisconsin, USA[a]; Department of Biology, Georgia Southern University, Statesboro, Georgia, USA[b]

ABSTRACT

A critical failure in our preparedness for an influenza pandemic is the lack of a universal vaccine. Influenza virus strains diverge by 1 to 2% per year, and commercially available vaccines often do not elicit protection from one year to the next, necessitating frequent formulation changes. This represents a major challenge to the development of a cross-protective vaccine that can protect against circulating viral antigenic diversity. We have constructed a recombinant modified vaccinia virus Ankara (MVA) that expresses an H5N1 mosaic hemagglutinin (H5M) (MVA-H5M). This mosaic was generated *in silico* using 2,145 field-sourced H5N1 isolates. A single dose of MVA-H5M provided 100% protection in mice against clade 0, 1, and 2 avian influenza viruses and also protected against seasonal H1N1 virus (A/Puerto Rico/8/34). It also provided short-term (10 days) and long-term (6 months) protection postvaccination. Both neutralizing antibodies and antigen-specific $CD4^+$ and $CD8^+$ T cells were still detected at 5 months postvaccination, suggesting that MVA-H5M provides long-lasting immunity.

IMPORTANCE

Influenza viruses infect a billion people and cause up to 500,000 deaths every year. A major problem in combating influenza is the lack of broadly effective vaccines. One solution from the field of human immunodeficiency virus vaccinology involves a novel *in silico* mosaic approach that has been shown to provide broad and robust protection against highly variable viruses. Unlike a consensus algorithm which picks the most frequent residue at each position, the mosaic method chooses the most frequent T-cell epitopes and combines them to form a synthetic antigen. These studies demonstrated that a mosaic influenza virus H5 hemagglutinin expressed by a viral vector can elicit full protection against diverse H5N1 challenges as well as induce broader immunity than a wild-type hemagglutinin.

Influenza viruses are significant health concerns for animals and humans. The World Health Organization estimates that every year influenza viruses infect up to 1 billion people, with 3 million to 5 million cases of severe disease and 300,000 to 500,000 deaths occurring annually (1). Highly pathogenic avian influenza (HPAI) H5N1 viruses have spread as far as Eurasia and Africa since their first emergence in 1996. These viruses infect a range of domestic and wild avian species as well as mammals (2) and pose a pandemic threat (3). Current prevention and treatment strategies for H5N1 virus either are antiviral or vaccine based or involve nonpharmaceutical measures, such as patient isolation or hand sanitation (4, 5). However, these approaches have flaws (5–7), such that a broadly effective strategy for H5N1 control remains elusive.

A powerful tool for preventing future H5N1 pandemics would be a universal H5N1 vaccine. The generation of inactivated vaccines (INVs) has been optimized for seasonal flu but presents several challenges for H5N1 viruses, including the following: (i) the continual evolution of the viruses makes predicting a vaccine strain difficult, (ii) egg propagation of vaccine stock is hindered due to the high lethality of H5N1 viruses to eggs and the poultry that provide them, and (iii) the 6- to 9-month time period required to produce INVs may be too long to protect large populations during a pandemic. In addition, initial studies in mice and ferrets and phase 1 human clinical trials have demonstrated that INVs and other split-virion vaccines may require higher doses of antigen than traditional INVs, with more than one administration being needed to provide protective immunity (8, 9). Live vaccines elicit both humoral and cellular immune responses. However, they are not recommended for use in infants or in elderly or immunocompromised individuals because they can cause pathogenic reactions (10, 11). Moreover, the viruses in live vaccines can revert to wild-type (wt) viruses, potentially leading to vaccine failure and disease outbreaks (12). Thus, the development of new vaccine vectors and novel approaches to antigen expression are urgently needed to generate an effective H5N1 vaccine with broad cross-protective efficacy. The modified vaccinia virus Ankara (MVA) vector offers several advantages, such as (i) safety, (ii) stability, (ii) rapid induction of humoral and cellular responses, and (iv) the ability to be given by multiple routes of inoculation (13–15). In addition, we and others have previously demonstrated the suitability of using MVA as a vaccine vector against H5N1 viruses (14, 16).

Multiple approaches to the development of a universal influenza vaccine that could be applied to H5N1 viruses have been studied. One approach is to use conserved sequences, such as the stalk region of hemagglutinin (HA) (17–19) or the internal nucleoprotein (NP) or M1 protein (20, 21). Another approach involves consensus sequences that combine many H5N1 hemagglu-

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Editor: D. S. Lyles
Address correspondence to Jorge E. Osorio, osorio@svm.vetmed.wisc.edu.

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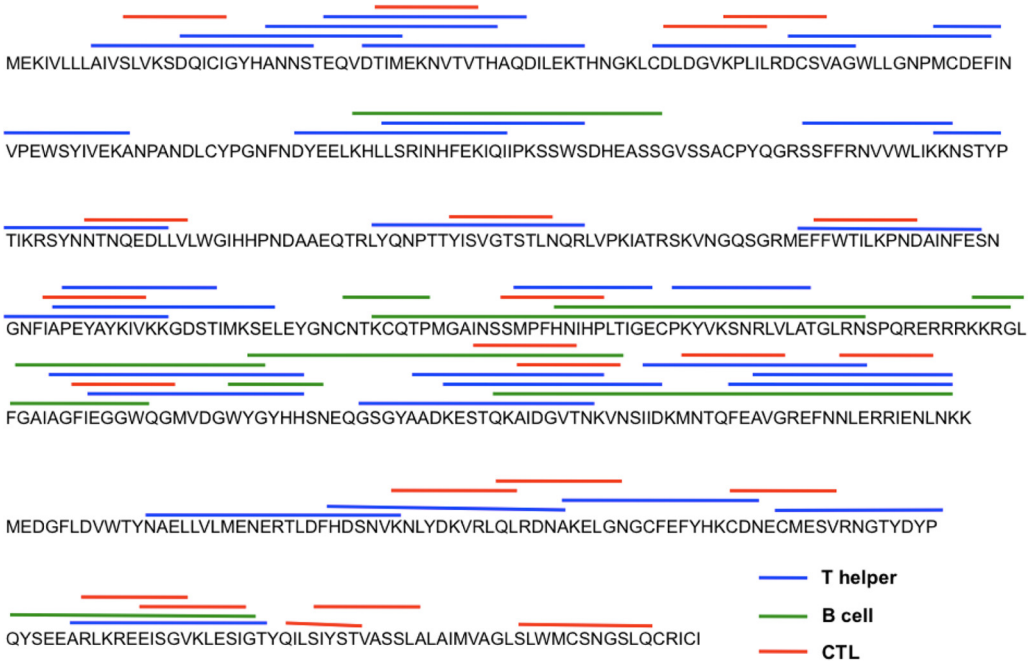

FIG 1 The mosaic H5 hemagglutinin (H5M) sequence. The mosaic H5N1 hemagglutinin (H5M) sequence was deduced from 2,145 HA sequences. The lines above the sequence indicate known T helper cell, B cell, or cytotoxic T lymphocyte (CTL) epitopes that were found in H5M by use of the Influenza Research Database (IRD).

tinin sequences into a single gene. Of these approaches, only the approach with consensus sequences has been shown to provide partial protection against a diverse panel of H5N1 isolates (22).

Recently, a novel *in silico* mosaic approach has been shown to provide broad and robust protection against highly variable viruses (23). The method uses a genetic algorithm to generate, select, and recombine potential $CD8^+$ T cell epitopes into mosaic proteins that can provide greater coverage of global viral variants than any single wild-type protein. This approach has been able to achieve between 74% and 87% coverage of HIV-1 Gag sequences, whereas a single natural Gag protein achieved only 37% to 67% coverage (23–25). Results in studies with rhesus monkeys showed that mosaic sequences increased both the breadth and the depth of cellular immune responses compared to the breadth and the depth of the responses achieved with consensus and natural sequences (23).

In this study, we used an MVA vector to express a mosaic H5N1 HA gene. In mice, a single dose of an MVA construct that expresses an H5N1 mosaic hemagglutinin (H5M) (MVA-H5M) provided sterilizing immunity (no virus was detectable in lung tissues postchallenge) against H5N1 HPAI clade 0, 1, and 2 viruses. Furthermore, MVA-H5M provided full protection at as early as 10 days postexposure and for as long as 6 months postvaccination. Both neutralizing antibodies and antigen-specific $CD4^+$ and $CD8^+$ T cells were detected at 5 months postvaccination. In addition, MVA-H5M also provided cross-subtype protection against H1N1 virus (A/Puerto Rico/8/34 [PR8]) challenge. Our results indicate that the mosaic vaccine approach has great potential for broadening the efficacy of influenza vaccines, perhaps including protection against multiple influenza virus subtypes.

MATERIALS AND METHODS

Cells and viruses. Chicken embryo fibroblasts (CEFs) and Madin-Darby canine kidney (MDCK) cells were obtained from Charles River Laboratories, Inc. (Wilmington, WA, USA), and the American Type Culture Collection (ATCC; Manassas, VA, USA), respectively. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. CEFs were used for propagating MVA. Highly pathogenic avian influenza (HPAI) H5N1 virus A/Vietnam/1203/04 (A/VN/1203/04) was kindly provided by Yoshihiro Kawaoka (University of Wisconsin—Madison, Madison, WI, USA). Highly pathogenic avian influenza H5N1 viruses A/Hong Kong/483/97 (A/HK/483/97), A/Mongolia/Whooper swan/244/05 (A/MG/244/05), and A/Egypt/1/08 and seasonal influenza viruses, including A/Puerto Rico/8/34 (PR8; H1N1) and A/Aichi/2/1968 (H3N2), were kindly provided by Stacey Schultz-Cherry and Ghazi Kayali (St. Jude Children's Research Hospital, Memphis, TN, USA). All viruses were propagated and titrated in MDCK cells with DMEM that contained 1% bovine serum albumin and 20 mM HEPES. Viruses were stored at −80°C until use. Viral titers were determined and expressed as 50% tissue culture infective doses ($TCID_{50}$s). All experimental studies with HPAI H5N1 viruses were conducted in a biosafety level 3+ (BSL3+) facility in compliance with the University of Wisconsin—Madison Office of Biological Safety.

Plasmid and MVA recombinant vaccine construction. A total of 3,069 HA protein sequences from H5N1 viruses available in the National Center for Biotechnology Information (NCBI) database were downloaded and screened to exclude incomplete and redundant sequences. The resulting 2,145 HA sequences were selected to generate one mosaic protein sequence, as previously described (26). Briefly, all 2,145 HA sequences were uploaded into the Mosaic Vaccine Designer tool webpage (http://www.hiv.lanl.gov/content/sequence/MOSAIC/makeVaccine.html). In the parameter options, the cocktail size was set to 1 in order to generate a single peptide that represented all uploaded sequences. The rare threshold was set to 3 for optimal value, and most importantly, the epitope length parameter was set to an amino acid length of 12-mer in an attempt to match the length of natural T helper cell epitopes (27). The resulting mosaic H5N1 sequence (H5M) was back-translated and codon optimized for mice. The optimized H5M sequence was then synthesized commercially (GenScript USA Inc., Piscataway, NJ, USA). Transfer plasmid PI2-Red encoding *Discosoma* sp. red

fluorescent protein (DsRed) was used to generate recombinant MVA expressing mosaic H5 (MVA-H5M). Briefly, the mosaic H5 was cloned into the multiple-cloning site (MCS) in the PI2-Red vector under the control of the SE/L promoter, and then positive clones were selected. Then, the MVA-H5M construct was generated in CEF cells by cotransfecting the PI2-Red-H5M plasmid and MVA expressing green fluorescent protein (GFP) (MVA-GFP) as described elsewhere (28). MVA expressing wild-type HA from avian influenza virus A/VN/1203/04 (MVA-HA) was constructed as described before (16) and kindly provided by Inviragen, Inc. (Madison, WI, USA).

Analysis of HA expressed by H5M. The hemagglutinin expressed by MVA-H5M was analyzed by Western blot analysis. CEF cells were infected at a multiplicity of infection (MOI) of 1 PFU/cell of the MVA-H5M and MVA-HA constructs and a construct consisting of MVA expressing luciferase (MVA-LUC). Infected cell pellets were harvested at 48 h postinfection and lysed with Laemmli sample buffer (Bio-Rad, Hercules, CA, USA). Protein was fractionated via SDS-PAGE and transferred onto a nitrocellulose membrane for hemagglutinin detection by a specific anti-HA antibody (Ab). 3,3′,5,5′-Tetramethylbenzidine (TMB) was used to visualize the HA protein in the membranes.

Functional analysis of H5M was done by hemagglutination assay (29). CEF cells were infected at an MOI of 1 with MVA-H5M, MVA-HA, and MVA-LUC. At 48 h postinfection, cells were harvested and 2-fold dilutions with phosphate-buffered saline (PBS) were made in round-bottom 96-well plates. Chicken red blood cells (RBCs) were added into each well, and the plates were incubated for 30 min. Lattice formations, which are indicative of the ability of HA to agglutinate RBCs, were observed in positive wells.

Animal studies. All mouse studies were conducted at the University of Wisconsin—Madison animal facilities and were approved by the Interinstitutional Animal Care and Use Committee (IACUC). Challenge experiments involving H5N1 viruses were conducted at animal BSL3+ (ABSL3+) facilities. Challenge studies with seasonal influenza, PR8 (H1N1), and A/Aichi/2/1968 (H3N2) viruses were conducted under BSL2 conditions to facilitate animal monitoring.

Vaccine efficacies. In the first study, groups of 5-week-old BALB/c mice were vaccinated with 1×10^7 PFU of either recombinant MVA-H5M or MVA-LUC via the intradermal (i.d.) route (8 mice per group). Intradermal inoculations were done by injecting 50 μl of PBS containing virus into footpads. At 4 weeks after vaccination, blood samples were collected for serological analysis. At 5 weeks postvaccination, mice were challenged by intranasal (i.n.) instillation, while they were under isoflurane anesthesia, with 100 50% lethal doses (LD_{50}s) of A/Vietnam/1203/04 (1×10^4 $TCID_{50}$s), A/Hong Kong/483/97 (4×10^3 $TCID_{50}$s), A/Mongolia/Whooper swan/244/05 (1×10^3 $TCID_{50}$s), or A/Egypt/1/08 (3.56×10^4 $TCID_{50}$s) contained in 20 μl of PBS. Two mice from each group were euthanized at day 5 postchallenge, and lung tissues were collected for viral titrations and histopathology. For virus isolation, lung tissues were minced in PBS using a mechanical homogenizer (MP Biochemicals, Solon, OH, USA), and the viral titers in the homogenates were quantified by plaque assay on MDCK cells. The remaining lung tissue was fixed in 10% buffered formalin. The remaining animals in each group (6 mice per group) were observed daily for 14 days, and survival and clinical parameters, including clinical score and body weight, were recorded. Mice showing at least a 20% body weight loss were humanely euthanized.

The second study evaluated the protective efficacies of the MVA-H5M vaccine against seasonal influenza virus, PR8 (H1N1), or A/Aichi/2/1968 (H3N2). Groups of 5-week-old BALB/c mice were vaccinated with MVA-H5M or MVA-LUC as described above (8 mice per group). At 4 weeks after vaccination, blood samples were collected for serological analysis. At 5 weeks postvaccination, the mice were challenged, while they were under isoflurane anesthesia, by i.n. instillation of 50 μl of PBS containing 100 LD_{50}s of PR8 (6.15×10^3 $TCID_{50}$s) or A/Aichi/2/1968 (3.405×10^6 $TCID_{50}$s). Two mice from each group were euthanized at day 3 postchallenge, and lung tissues were collected as described above. The remaining animals in each group (6 mice per group) were observed daily for 14 days as described above.

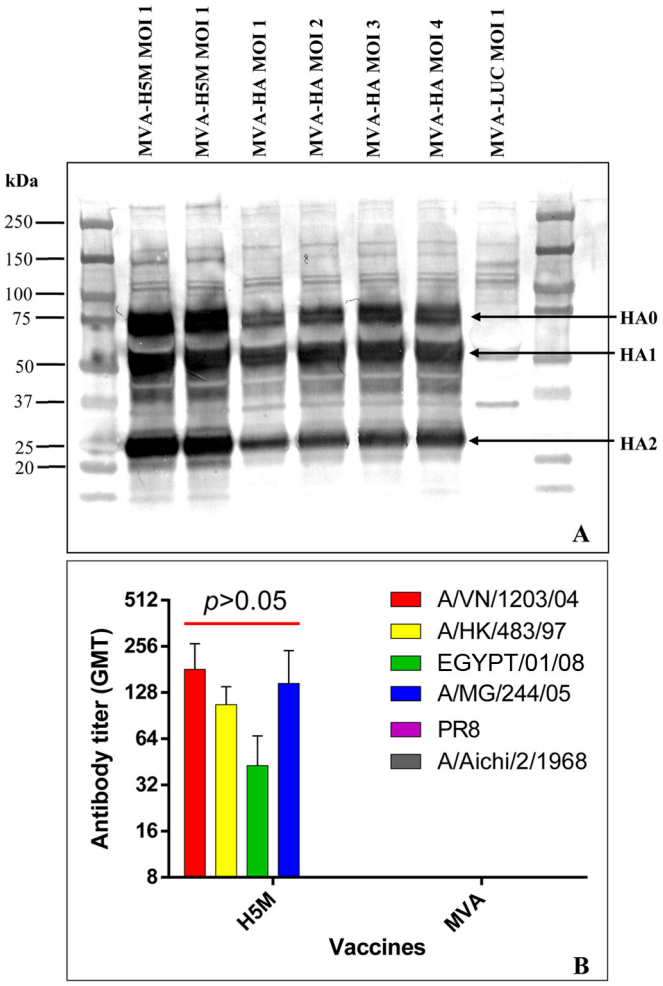

FIG 2 The MVA-H5M vaccine expresses a higher level of protein than MVA expressing wild-type hemagglutinin (MVA-HA) and elicits broad neutralizing antibodies against avian influenza viruses. (A) Western blot analysis of CEF cell lysates infected with MVA-H5M, MVA-HA, and MVA-LUC (negative control). HA from MVA-H5M was expressed as a cleavable protein that was the same as the HA from MVA-HA. The sizes of HA clade 0 (HA0), HA clade 1 (HA1), and HA clade 2 (HA2) are 75 kDa, 50 kDa, and 25 kDa, respectively. (B) The titers of neutralizing antibodies against influenza virus A/VN/1203/04, A/MG/244/05, A/HK/483/97, A/Egypt/1/08 (EGYPT/01/08), PR8 (H1N1), or A/Aichi/2/1968 (H3N2) virus in vaccinated mice ($n = 8$ mice per group) were measured at 4 weeks postvaccination. No statistically significant differences between titers against avian influenza viruses ($P > 0.05$, one-way ANOVA) were found.

In the third study, we evaluated the ability of the MVA-H5M vaccine to confer both short- and long-term protection against avian influenza virus A/Hong Kong/483/97; this strain showed the most virulence from the previous study. Groups of 5-week-old BALB/c mice were vaccinated with MVA-H5M or MVA-LUC as described above (7 mice per group). Blood samples were collected at 10 days and 6 months postvaccination for short- and long-term studies, respectively. At 10 days and 6 months postvaccination, mice were challenged, while they were under isoflurane anesthesia, by i.n. instillation of 100 LD_{50}s of A/Hong Kong/483/97 (4×10^3 $TCID_{50}$s). Two mice from each group were euthanized at day 5 postchallenge, and lung tissues were collected for viral titrations and histopathology. The remaining animals in each group (5 mice per group) were observed daily for 14 days, as described above.

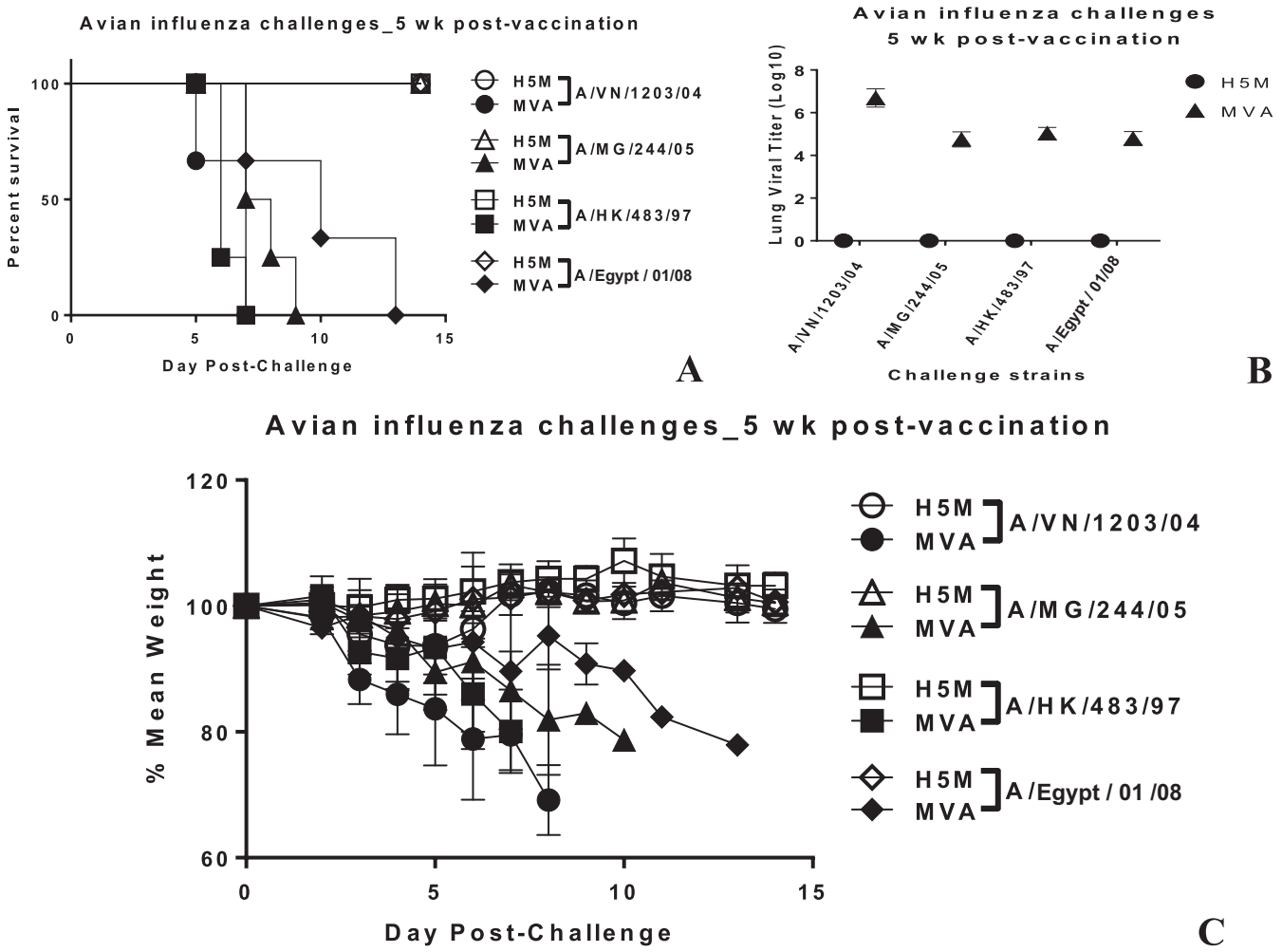

FIG 3 MVA-H5M provides broad protection against multiple clades of avian influenza virus. (A) Efficacies of a single dose of the MVA-H5M or MVA-LUC vaccine against highly pathogenic avian influenza viruses: clade 0 influenza virus A/HK/483/97 virus, clade 1 influenza virus A/VN/1203/04, clade 2.2 influenza virus A/MG/244/05, and clade 2.2.1 influenza virus A/Egypt/1/08. Mice vaccinated with MVA-H5M showed 100% survival, and MVA-H5M prevented morbidity during the studies. (B and C) No viral titers were detectable in the lung at day 5 postinfection (B), and MVA-H5M-vaccinated mice maintained their weight throughout the studies (C). Vaccinated mice were challenged at 5 weeks postvaccination, 2 mice per group were sacrificed at day 5 postinfection, and survival data were monitored for 14 days ($n = 6$ mice per group).

Serology. Serum antibody titers were determined by microneutralization assay. Briefly, serum was incubated at 56°C for 30 min to inactivate complement and then serially diluted 2-fold in microtiter plates. Two hundred $TCID_{50}$s of virus were added to each well, and the plates were incubated at 37°C for 1 h. The virus-serum mixture was added to duplicate wells of MDCK cells in 96-well plates, the plates were incubated at 37°C for 72 h, and then the cells were fixed and stained with 10% (wt/vol) crystal violet in 10% (vol/vol) formalin to determine the $TCID_{50}$. The titer was determined as the serum dilution resulting in the complete neutralization of the virus.

Histopathology and immunohistochemistry. Lung tissue samples for histological analysis were processed by the histopathology laboratory at the School of Veterinary Medicine, University of Wisconsin—Madison (Madison, WI, USA), and stained with hematoxylin and eosin. For immunohistochemistry, tissue sections were deparaffinized and rehydrated as previously described (30). Slides were treated with antigen retrieval buffer, followed up with treatment with 3% H_2O_2. The slides were placed in blocking solution and incubated in goat anti-HA (1/300 dilution; Biodefense and Emerging Infections Research Resources Repository number NR-2705) avian influenza virus polyclonal antibody for 24 h. Secondary horseradish peroxidase-conjugated antigoat antibody at a 1/5,000 dilution was added onto the slides, and the slides were incubated for 1 h. Then, the slides were stained with 0.05% 3,3′-diaminobenzidine (DAB) substrate to visualize the presence of avian influenza virus antigens.

T cell responses. At 6 weeks and 5 months postvaccination, MVA-H5M-vaccinated mice were euthanized (3 mice per group) and spleens were aseptically removed. Splenocytes from individual animals were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.14 mM β-mercaptoethanol. Red blood cells were lysed with 1× BD Pharm Lyse buffer (BD Biosciences, San Jose, CA, USA). Following washing with RPMI 1640 medium, cells were resuspended in the same medium and 1×10^6 splenocytes were surface stained with anti-mouse CD4 fluorescein isothiocyanate (RM4-5) and anti-mouse CD8a peridinin chlorophyll protein (53-6.7) monoclonal antibodies (BD Biosciences, San Jose, CA, USA).

In order to study intracellular cytokine responses, 1×10^6 splenocytes were plated onto a 96-well flat-bottom plate and stimulated with diverse H5N1 HA peptide pools (5 μg/ml) in a 200-μl total volume for 16 h. Brefeldin A (BD GolgiPlug; BD Biosciences, San Jose, CA, USA) was

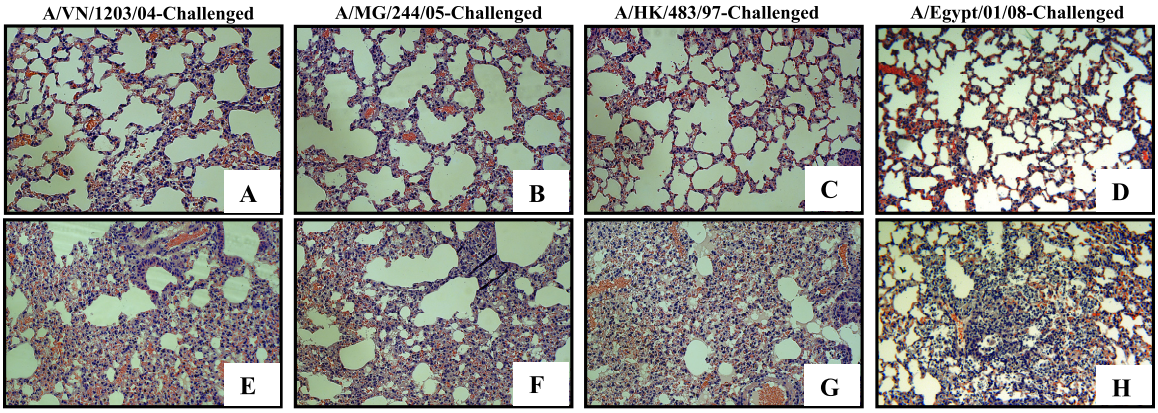

FIG 4 MVA-H5M reduces lung pathology after challenge with avian influenza viruses. Tissue sections of the lungs of mice vaccinated with MVA-H5M (A to D) or MVA-LUC (E to H) and challenged with A/VN/1203/04 (A and E), A/MG/244/05 (B and F), A/HK/483/97 (C and G), or A/Egypt/1/08 (D and H) virus are shown. MVA-H5M-vaccinated mice showed normal to mild lung lesions, whereas MVA-LUC-vaccinated mice showed severe lung lesions, including lung consolidation, leukocyte infiltration, thickening of the alveolar septa, and alveolar edema.

added at a final concentration of 1 μg/ml for the last 5 h of incubation to block protein transport. Cells were stained intracellularly for gamma interferon (IFN-γ) allophycocyanin (XMG1.2) and interleukin-2 (IL-2) phycoerythrin (JES6-5H4) after surface staining for CD4 and CD8a. All antibodies were from BD Biosciences (San Jose, CA, USA), except where noted. The samples were acquired on a BD FACSCalibur flow cytometer and analyzed with FlowJo (v10.0.6) software (TreeStar Inc., Ashland, OR, USA). The background cytokine level from medium-treated groups was subtracted from the cytokine level for each treated sample. The frequency of cytokine-positive T cells was presented as the percentage of gated $CD4^+$ or $CD8^+$ T cells.

Statistical analysis. Student's *t* tests and one-way analysis of variance (ANOVA) were used to evaluate viral lung titers, antibody titers, and T cell responses between groups. Survival analyses were performed to assess vaccine effectiveness against challenge viruses. Probability values of <0.05 were considered significant. GraphPad Prism (v6) software (La Jolla, CA, USA) was used for all statistical analyses.

RESULTS

Construction of pox-based H5N1 mosaic hemagglutinin vaccine. A mosaic vaccine that targets the hemagglutinin protein of influenza H5N1 virus was constructed (Fig. 1). The HA mosaic was generated using an input of 2,145 HA sequences from H5N1 influenza viruses available in GenBank. To maximize T helper cell epitope coverage, the *in silico* algorithm was set to an amino acid length of a 12-mer (27). The mosaic H5 (H5M) sequence was back-translated into DNA and cloned into modified vaccinia virus Ankara (MVA) to generate MVA-H5M. Recombinant H5M was expressed as cleavable HA that resembled wild-type (wt) HA from avian influenza virus A/VN/1203/04, as shown in Fig. 2A. Interestingly, the level of protein expression from MVA-H5M-infected cell pellets was higher than that from MVA expressing wild-type hemagglutinin from A/VN/1203/04 (MVA-HA) (16) (Fig. 2A). Additionally, *in vitro* functional analysis of H5M resulted in hemagglutination of RBCs at levels similar to those for wt HA (A. Kamlangdee, B. Kingstad-Bakke, and J. E. Osorio, unpublished data). These data thus demonstrate the successful generation of an MVA vector expressing a mosaic H5N1 HA gene.

Efficacy of MVA-H5M vaccine against influenza viruses. To determine whether antibodies to MVA-H5M had virus-neutralizing activity, mice were intradermally inoculated with MVA-H5M, MVA-LUC, or PBS; and the titers of antibodies against A/VN/1203/04, A/MG/244/05, A/HK/483/97, and A/Egypt/1/08 challenge viruses in immunized mice were measured. At 4 weeks postimmunization, MVA-H5M elicited significant neutralizing antibody (Ab) titers and protected against all four H5N1 strains after challenge (Fig. 2B and 3A to C). The geometric mean titers (GMTs) of Abs against A/VN/1203/04, A/MG/244/05, A/HK/483/97, and A/Egypt/1/08 did not differ significantly ($n = 8$ per group, $P > 0.05$) (Fig. 2B). None of animals injected with the MVA-LUC control survived the challenge (Fig. 3A to C). Notably, no virus replication was detected in the lungs of any of the MVA-H5M-vaccinated mice challenged with any of the four H5N1 viruses (Fig. 3B). Furthermore, vaccination with MVA-H5M reduced the lung pathology after challenge with avian influenza viruses (Fig. 4A to D). MVA-H5M-vaccinated mice showed no to mild lung lesions, whereas the MVA-LUC-vaccinated groups did show lung lesions (Fig. 4E to H). Lesions included thickening of the alveolar wall, lung consolidation with white blood cell infiltration, necrosis of alveolar walls, and pulmonary edema. Immunohistochemistry staining revealed large quantities of viral antigen in the MVA-LUC control group (Fig. 5E to H). In contrast, viral antigen was not detected in the lungs of mice that received MVA-H5M (Fig. 5A to D). These data demonstrate the ability of MVA-H5M to confer both broad and strong protection against multiple clades of avian influenza viruses.

We also evaluated the ability of the MVA-H5M construct to protect mice against seasonal influenza viruses. Vaccination followed the same protocol used with H5N1 viruses, except that mice were challenged with A/Puerto Rico/8/1934 (PR8; H1N1) and A/Aichi/2/1968 (H3N2). At 4 weeks postvaccination, the titers of neutralizing Abs against both seasonal influenza viruses ($n = 8$ per group) were below detectable levels (Fig. 2B). Despite the lack of detectable neutralizing Ab, MVA-H5M conferred complete protection against PR8 (H1N1), with no significant weight loss being observed (Fig. 6A and C). In contrast, no protection against A/Aichi/2/68 (H3N2) challenge was observed (Fig. 6A and C). Regardless of the strain tested, viral replication was observed in the lungs of mice vaccinated with MVA-H5M and challenged with seasonal influenza viruses; however, mice challenged with PR8 had significantly lower viral lung titers than mice vaccinated with the MVA-LUC control (Fig. 6B). In contrast, vaccination with MVA-H5M

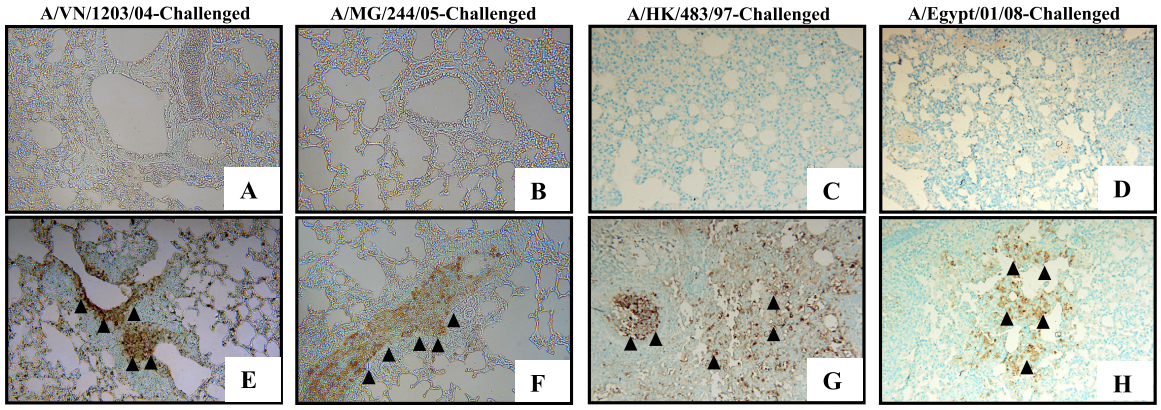

FIG 5 MVA-H5M prevents viral replication in the lung after challenge with avian influenza viruses. Tissue sections of the lungs of mice vaccinated with MVA-H5M (A to D) or MVA-LUC (E to H) and challenged with A/VN/1203/04 (A and E), A/MG/244/05 (B and F), A/HK/483/97 (C and G), or A/Egypt/1/08 (D and H) virus are shown. Lungs from mice were processed by immunohistochemistry with H5N1-specific antibody. Brown staining for viral antigen is indicated with arrowheads.

had no effect on viral replication in the lungs of mice challenged with A/Aichi/2/68 (Fig. 6B).

Short- and long-term immunity. To assess the ability of the MVA-H5M construct to confer both short- and long-term immunities, groups of mice were vaccinated with a single dose of MVA-H5M and then challenged at either 10 days or 6 months postvaccination. MVA-H5M provided full protection against a lethal dose of A/HK/483/97 at both tested time points (Fig. 7A and B). Neu-

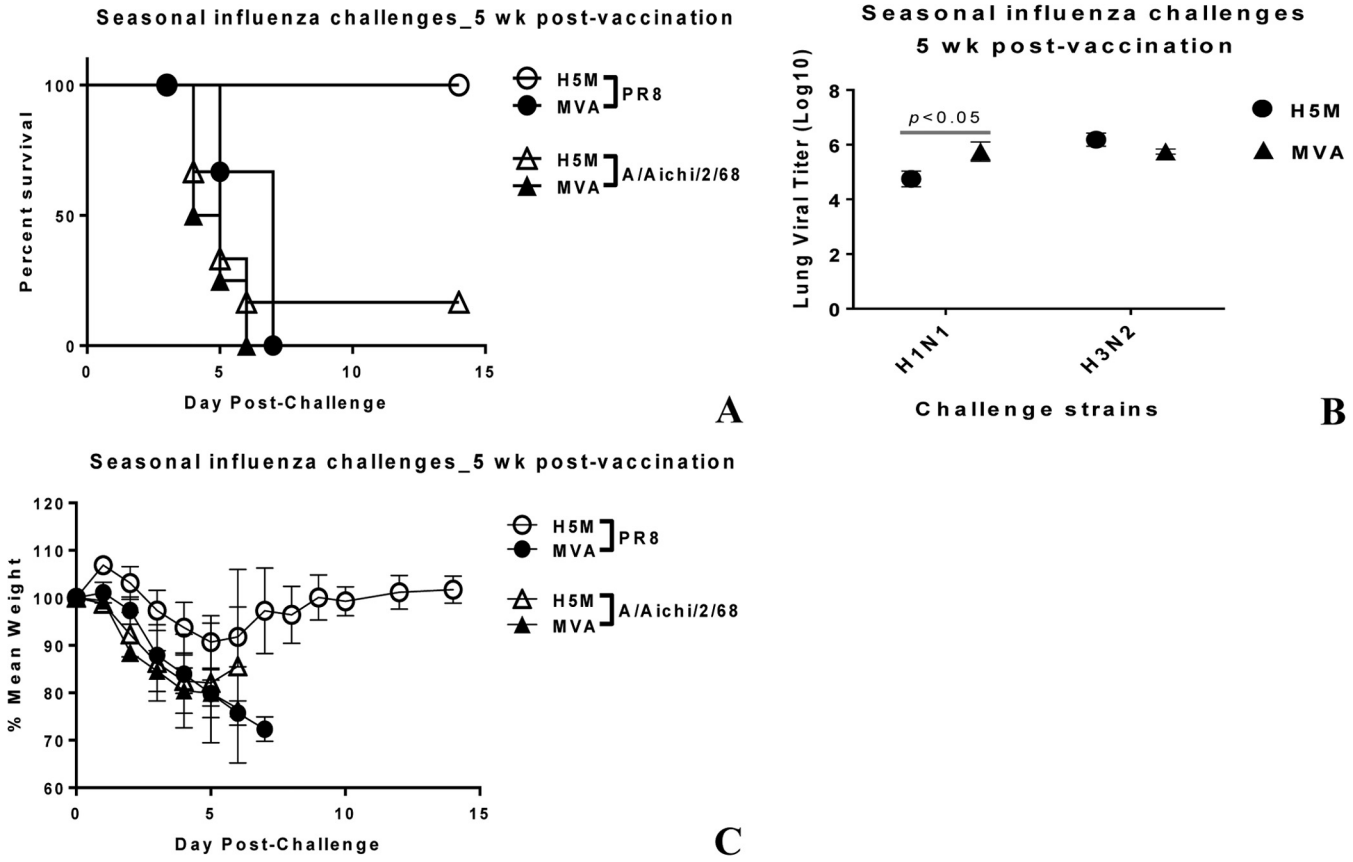

FIG 6 MVA-H5M provides heterosubtypic protection against H1N1 virus. (A) Efficacies of a single dose of MVA-H5M or MVA-LUC vaccine against seasonal H1N1 and H3N2 influenza viruses. MVA-H5M provided 100% protection with low weight loss against H1N1 virus but failed to prevent H3N2 virus infection. (B) MVA-H5M significantly lowered H1N1 viral replication in the lung compared to that in groups of mice that were challenged with H3N2 virus. (C) MVA-H5M prevented infection of vaccinated mice upon challenge with H1N1 virus, and the mice showed no to mild clinical signs and slight weight loss, but the vaccine failed to prevent infection upon challenge with H3N2 virus. Vaccinated mice were challenged at 5 weeks postvaccination, 2 mice per group were sacrificed at day 3 postinfection, and survival data were monitored for 14 days ($n = 6$ mice per group).

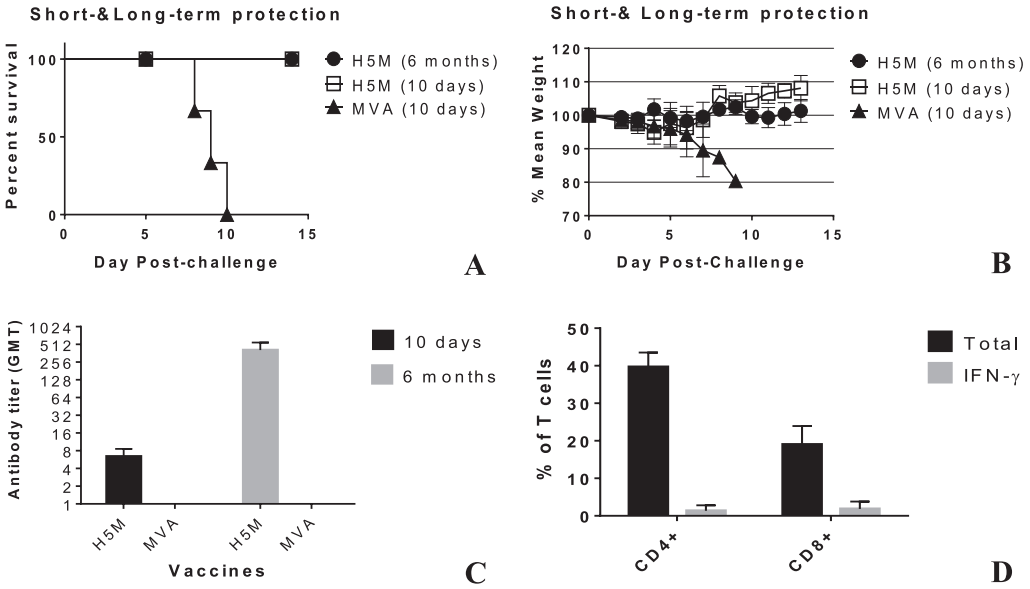

FIG 7 MVA-H5M provides short- and long-term immunities. (A and B) BALB/c mice were immunized with a single dose of MVA-H5M or MVA-LUC ($n = 7$ mice per group). At 10 days or 6 months postvaccination, mice were challenged with a lethal dose of influenza virus A/HK/483/97. Survival data (A) and percent weight loss (B) were monitored for 14 days. (C) Neutralization titers from vaccinated mice at 10 days and 6 months postvaccination ($n = 7$ mice per group). (D) $CD4^+$ and $CD8^+$ T cells responses at 5 months postvaccination.

tralizing antibodies against A/HK/183/97 were also detected at both 10 days and 6 months postvaccination (Fig. 7C). Microneutralization assays and a challenge study were conducted using A/HK/183/97 virus because it was the most virulent strain among the four H5N1 strains used in the present study. Surprisingly, the GMTs of Abs detected at 6 months postvaccination were substantially higher than those detected at 4 weeks postvaccination (Fig. 2B and 7C). In these animals, H5N1-specific IFN-γ-releasing $CD4^+$ and $CD8^+$ T cell responses were detected using flow cytometry. IFN-γ-releasing $CD4^+$ and $CD8^+$ T cells were found in MVA-H5M-vaccinated mice 5 months after dosing (2 weeks before challenge in the long-term protection study), indicating a long-term memory response (Fig. 7D).

Immune responses of mosaic and natural sequence vaccines. We compared the immune responses to the mosaic vaccine (MVA-H5M) to those to the wild-type or natural sequence vaccine (MVA-HA). Flow cytometry analyses of cytokine-producing cells demonstrated that MVA-H5M-vaccinated mice had broader and higher IFN-γ-producing $CD4^+$ responses as well as broader and higher IL-2-producing $CD8^+$ T cell responses than mice vaccinated with MVA-HA (Fig. 8A and B) after stimulation with diverse H5 HA peptide pools. Furthermore, the T cell responses against H1N1 (PR8) peptides were analyzed. The results showed that MVA-H5M elicited $CD8^+$ T cells responses to HA peptides from H1N1 (PR8) virus, and the response was higher than that to the MVA-HA wild-type vaccine (Fig. 8C). While MVA-H5M elicited neutralizing antibodies against all viruses tested, MVA-HA failed to elicit detectable responses against A/Egypt/1/08 virus (Fig. 8D).

DISCUSSION

The rapid evolution of influenza viruses poses global health challenges necessitating the development of vaccines with broad cross-protective immunity. Herein, we describe the development of a broadly protective vaccine, MVA-H5M, based on a mosaic epitope approach. The mosaic epitope approach minimizes genetic differences between selected vaccine antigenic sequences and circulating influenza virus strains, while it maximizes the overall breadth of cross-protective immune responses. Our results demonstrated that a single dose of MVA expressing a mosaic H5 hemagglutinin (MVA-H5M) provided broad protection against multiple H5N1 viruses, including the highly pathogenic Egyptian strains, and also an H1 subtype virus (PR8). The MVA-H5M vaccine provided robust and prolonged protection against a lethal dose of a highly pathogenic avian influenza virus at as early as 10 days and as long as 6 months postvaccination.

In the past few years, commercially available vaccines have failed to induce the expected level of protection against the currently circulating clade 2.2.1 in Egypt (31, 32). It is very important that an H5N1 influenza virus vaccine provide broad cross-clade protection against these clade 2.2.1 viruses, particularly the A/Egypt/1/08 strain, because this strain possess one of the four mutations that are necessary to sustain human-to-human transmission (33). The MVA-H5M vaccine provided complete protection against this H5N1 strain in mice. The ability to provide complete protection against H5N1 viruses with a single dose is also important for implementation; societal acceptance of a single-dose vaccine would likely be higher than that of a multidose vaccine, especially during a pandemic.

On the basis of our data, there are several plausible hypotheses to explain how our mosaic vaccine confers broad protection against influenza virus challenge. One possible explanation is that the 12-mer mosaic sequence captures more T helper cell epitopes, in which case the broad protective ability of MVA-H5M likely results from greater epitope coverage for the mosaic approach than for previous approaches (23). This could translate to a higher level of $CD4^+$ T cells and antibody responses broader than those induced by the wild-type sequence (MVA-HA). This hypothesis is supported by the fact that MVA-H5M showed broader IFN-γ-

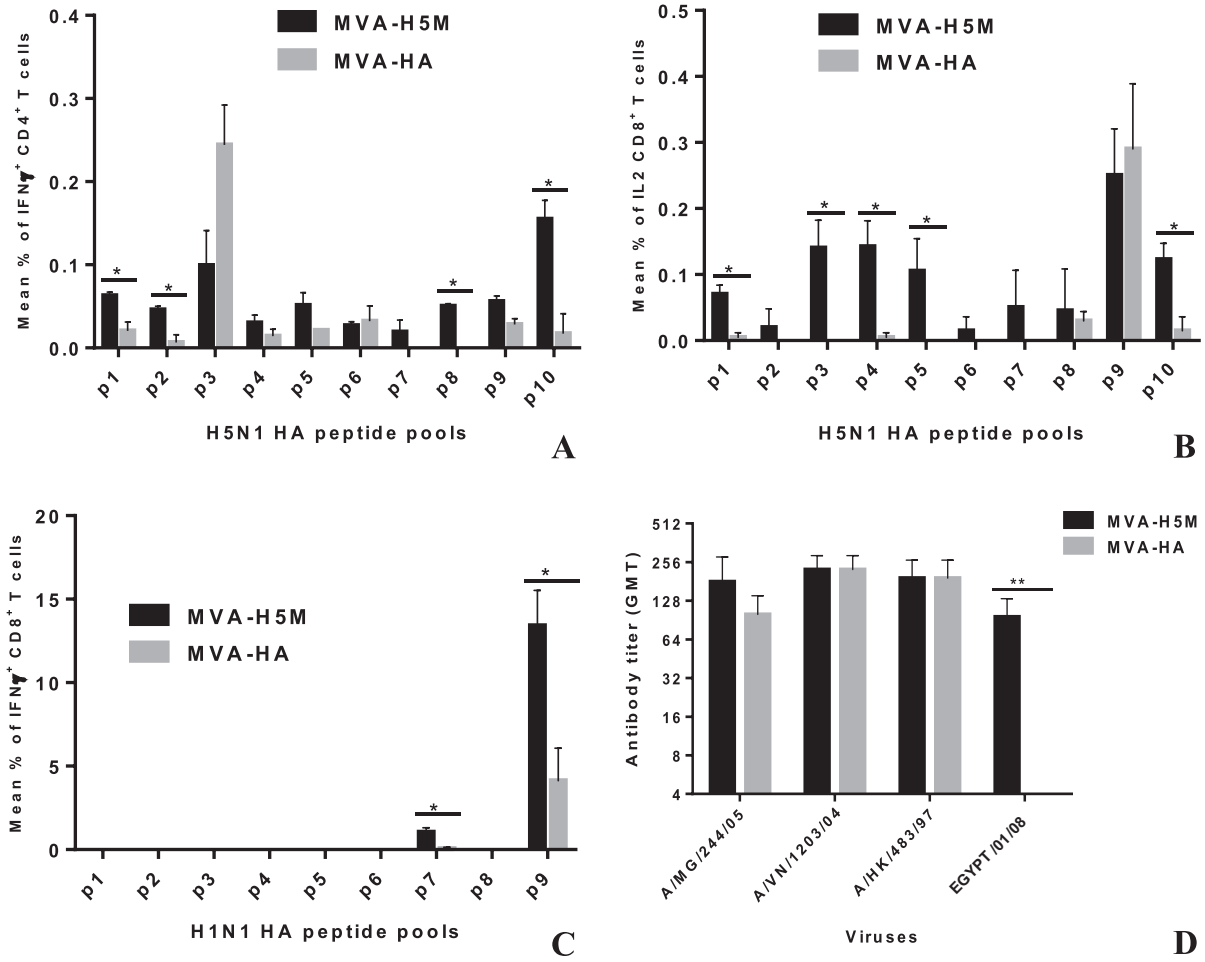

FIG 8 MVA-H5M elicits broader T cell epitope coverage, higher T cell responses, and broader neutralizing antibody responses than the wild-type hemagglutinin-based vaccine. (A and B) Percentages of IFN-γ-producing $CD4^+$ T cells (A) and IL-2-producing $CD8^+$ T cells (B) from mice that were vaccinated with MVA-H5M or MVA-HA ($n = 3$ mice per group). Splenocytes from vaccinated mice were collected and stimulated with HA peptide pools (indicated by p1 to p10) from H5N1 viruses. (C) IFN-γ-producing $CD8^+$ T cells from mice that were vaccinated with MVA-H5M or MVA-HA ($n = 3$ mice per group). Splenocytes from vaccinated mice were collected and stimulated with HA peptide pools (indicated by p1 to p9) from H1N1 (PR8) virus. (D) Titers of neutralizing antibodies against H5N1 viruses elicited by the MVA-H5M and MVA-HA vaccines ($n = 7$ mice per group). *, $P < 0.05$, Student's *t* test; **, $P < 0.01$, Student's *t* test.

producing $CD4^+$ T cell epitope coverage and elicited broader cross-clade neutralizing antibody responses (Fig. 8A, B, and D). This MVA-H5M mosaic vaccine also elicited broader and higher $CD8^+$ T cell responses than the wild-type (MVA-HA) vaccine (Fig. 8B). However, other immunological aspects of the MVA-H5M vaccine still need to be further characterized. For example, cytokine profiles, antibody epitope coverage, and mapping would all be necessary to fully understand the mechanism responsible for protection. Also, challenge studies comparing the mosaic (MVA-H5M) vaccine and the wild-type vaccine or a natural sequence (MVA-HA) vaccine will be conducted in the future. However, the *in vitro* data from T cell and neutralizing antibody responses strongly indicate the broader immunogenicity of the mosaic H5 than the wild-type H5; i.e., the mosaic H5 elicited broader epitope responses for both $CD4^+$ and $CD8^+$ against H5N1 peptides (Fig. 8A and B). It also elicited higher $CD8^+$ T cell responses against PR8 peptides (Fig. 8C) than wild-type H5 (MVA-HA). Furthermore, the mosaic H5 elicited broad neutralizing antibodies against all 4 clades of avian influenza viruses that we tested, including clade 0, clade 1, clade 2.2, and clade 2.2.1, whereas the wild-type vaccine failed to elicit neutralizing antibodies against clade 2.2.1 influenza virus A/Egypt/1/08, which is considered a mutant strain (31, 32, 34). Commercially available vaccines also failed to protect against this mutant Egyptian strain in Egypt in recent years (32).

A second mechanism that may explain the breadth of protection conferred by the MVA-H5M vaccine is that the mosaic approach maintains an intact antigenic structure and, presumably, physiological function (35). It has previously been reported that most universal neutralizing antibodies are elicited by peptides in the stalk regions (36, 37). Our MVA-H5M vaccine has a normal hemagglutination function and is also expressed as a cleavable protein. Furthermore, our mosaic H5 might provide a higher level of accessibility to the stalk region and stimulate a more robust neutralizing antibody response against epitopes in the stalk region. Crystallography of the expressed mosaic H5 would likely be required to reveal the actual structure of this protein and compare it to the known structure of H5 hemagglutinin.

The MVA-H5M construct provided sterilizing lung protection against H5N1 viruses, and no mortality or morbidity was detected

(Fig. 3). Moreover, no viral antigens were detected in the lung after challenge (Fig. 5). These results are likely due to the high concentrations of neutralizing antibodies (endpoint titer, at least 1:32) (Fig. 2). A previous study demonstrated that a minimum neutralizing antibody concentration with a 1:16 endpoint titer is sufficient to provide complete protection against H5N1 viruses (38). Although antibody-mediated protection is suggested to be the main contributor of protection in our vaccine, T cells may also play a role.

It is currently unclear whether the use of a live viral vector such as MVA contributed to the increased cross protection described herein. It is possible that H5M expression by MVA induced high levels of cross-reactive neutralizing antibodies as well as $CD4^+$ and $CD8^+$ T cells via innate immune activations (39). In future studies, we will evaluate the protective efficacy of the H5M antigen as a recombinant protein without the MVA vector. This approach will likely require the use of adjuvants and multiple doses to achieve the desired protection.

The mosaic approach has previously been used for developing vaccines against the highly variable HIV strains, capturing potential $CD8^+$ T cell epitopes with a length of 9 amino acids (23, 26) while still maintaining a normal protein structure. Because complete protection against influenza viruses is based primarily on humoral immunity (40), we modified the algorithm for epitopes of 12 amino acids in order to capture potential T helper cell epitopes (27) in order to target antibody-producing plasma cell via T helper cell activation. We speculate that this strategy may have facilitated achievement of a high neutralizing antibody titer with a single dose of MVA-H5M (Fig. 2B).

The vaccine elicited strong humoral responses against multiple H5N1 viruses but no cross-neutralizing antibodies against seasonal influenza viruses (H1N1 and H3N2). Despite the lack of neutralizing antibodies against H1N1 PR8 virus, the MVA-H5M vaccine provided 100% protection against the PR8 virus. This suggests a substantial role of cellular immune responses against the PR8 virus, as shown in Fig. 8C, likely because the H5M protein possesses some cytotoxic T lymphocyte epitopes of the PR8 virus (35). This protection can also be explained by the genetic relationship between the H5 and H1 hemagglutinin subtypes, as both belong to group 1 influenza A virus hemagglutinin (41), and it elicits a large amount of nonneutralizing antibody which then targets and destroys H1N1 virus via an antibody-dependent cellular cytotoxicity (ADCC) mechanism (42). However, the MVA-H5M vaccine did not protect immunized mice against influenza virus A/Aichi/2/68, which is likely due to antigenic differences, as H3 belongs to group 2 influenza A virus hemagglutinin. Because the MVA vector can be designed to contain multiple inserts, future constructs will contain mosaics from several hemagglutinin subtypes, including important seasonal (e.g., H3) and emerging (e.g., H7) pathogens. Since this vaccine provides broad protection and a long duration of immunity, utilizing an MVA vector expressing seasonal mosaics might diminish the need for annual vaccination.

The ability of the MVA-H5M vaccine to confer broad protective immunity against various heterologous strains as well as heterosubtypic strains makes the mosaic approach a very promising strategy to combat the antigenic diversity of influenza viruses. Taken together with codon optimization of HA for a high level of protein expression and the use of an MVA vector as a backbone for cellular immunity activation, this approach promises to increase the broad efficacy of influenza vaccines substantially. Should this and similar approaches prove effective for other viruses in other animal models, it could help reduce or eliminate the need for annual seasonal influenza vaccine updates, as well as provide a framework for a pandemic preparedness vaccine.

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A.K., B.K.-B., and J.E.O. conceived and designed the experiments. A.K. and B.K.-B. performed the experiments. A.K. analyzed the data. T.K.A., T.L.G., and J.E.O. contributed reagents, materials, and analysis tools. A.K., B.K.-B., and J.E.O. wrote the paper.

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