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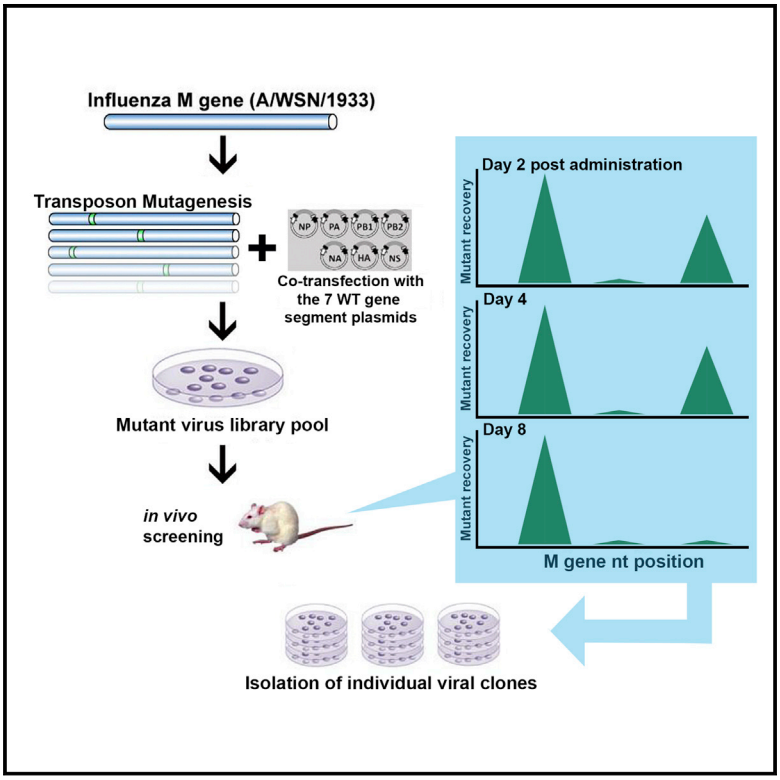
Graphical Abstract



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In Brief

Wang et al. use in vivo screening of a mutant influenza virus library to identify W7-791, an attenuated mutant strain. A single administration of W7-791 provided heterologous protection against lethal virus challenge in mice and ferrets. Adoptive transfer of T cells from W7-791-immunized mice conferred heterologous protection to naive animals.

Highlights

- Attenuated influenza virus mutant W7-791 was identified based on in vivo growth screening
- A single dose of W7-791 fully cross-protected against multiple influenza viruses in mice
- W7-791-elicited cross-protective T cell responses were transferable
- A single dose of W7-791 improved viral clearance in ferrets

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Article

Generation of a Live Attenuated Influenza Vaccine that Elicits Broad Protection in Mice and Ferrets

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SUMMARY

New influenza vaccines that provide effective and broad protection are desperately needed. Live attenuated viruses are attractive vaccine candidates because they can elicit both humoral and cellular immune responses. However, recent formulations of live attenuated influenza vaccines (LAIVs) have not been protective. We combined high-coverage transposon mutagenesis of influenza virus with a rapid high-throughput screening for attenuation to generate W7-791, a live attenuated mutant virus strain. W7-791 produced only a transient asymptomatic infection in adult and neonatal mice even at doses 100-fold higher than the LD_{50} of the parent strain. A single administration of W7-791 conferred full protection to mice against lethal challenge with H1N1, H3N2, and H5N1 strains, and improved viral clearance in ferrets. Adoptive transfer of T cells from W7-791-immunized mice conferred heterologous protection, indicating a role for T cell-mediated immunity. These studies present an LAIV development strategy to rapidly generate and screen entire libraries of viral clones.

INTRODUCTION

Influenza A virus is a major public health problem. In a typical year, influenza infects as many as 500 million people worldwide and leads to more than 500,000 deaths. In the United States, 5%–10% of the population is infected by influenza virus in an average season, resulting in ~220,000 hospitalizations and ~36,000 deaths (WHO, 2003). However, significant mutations in the virus will bypass host immunity to previously exposed strains, leading to considerably greater mortality. The devastating "Spanish flu" pandemic of 1918 was one such example, where the virus itself or complications from secondary infections killed an estimated 40–50 million people worldwide. Vaccination has been the most effective way to prevent the spread of influenza and its complications. Live attenuated influenza vaccines (LAIVs) are known to be more immunogenic than inactivated vaccines, likely because they stimulate both humoral and cell-mediated immune responses (Belshe et al., 2007). Traditionally, attenuated vaccines such as the measles-mumps-rubella and influenza vaccines were made via a forward genetics approach, using random mutagenesis followed by rounds of selection in non-physiological conditions, a time-intensive process that produces few vaccine candidates (Lamb et al., 1981). Moreover, these vaccines need to be reformulated annually due to antigenic drift and poor cross-protection against emerging pandemic strains (Jang and Seong, 2012; Krammer and Palese, 2015). In addition to difficulties in producing and formulating the vaccine, recent findings suggest that the quadrivalent LAIVs used over the last 3 years have not been protective. The CDC has actually recommended against using the LAIV during the 2016-2017 season (Grohskopf et al., 2016). In a proof-of-principle study, we sought to use the reverse genetics system of influenza virus and a transposon mutagenesis system as tools toward the rapid, high-throughput generation and screening of viral clones with attenuated growth in vivo as candidate LAIVs.

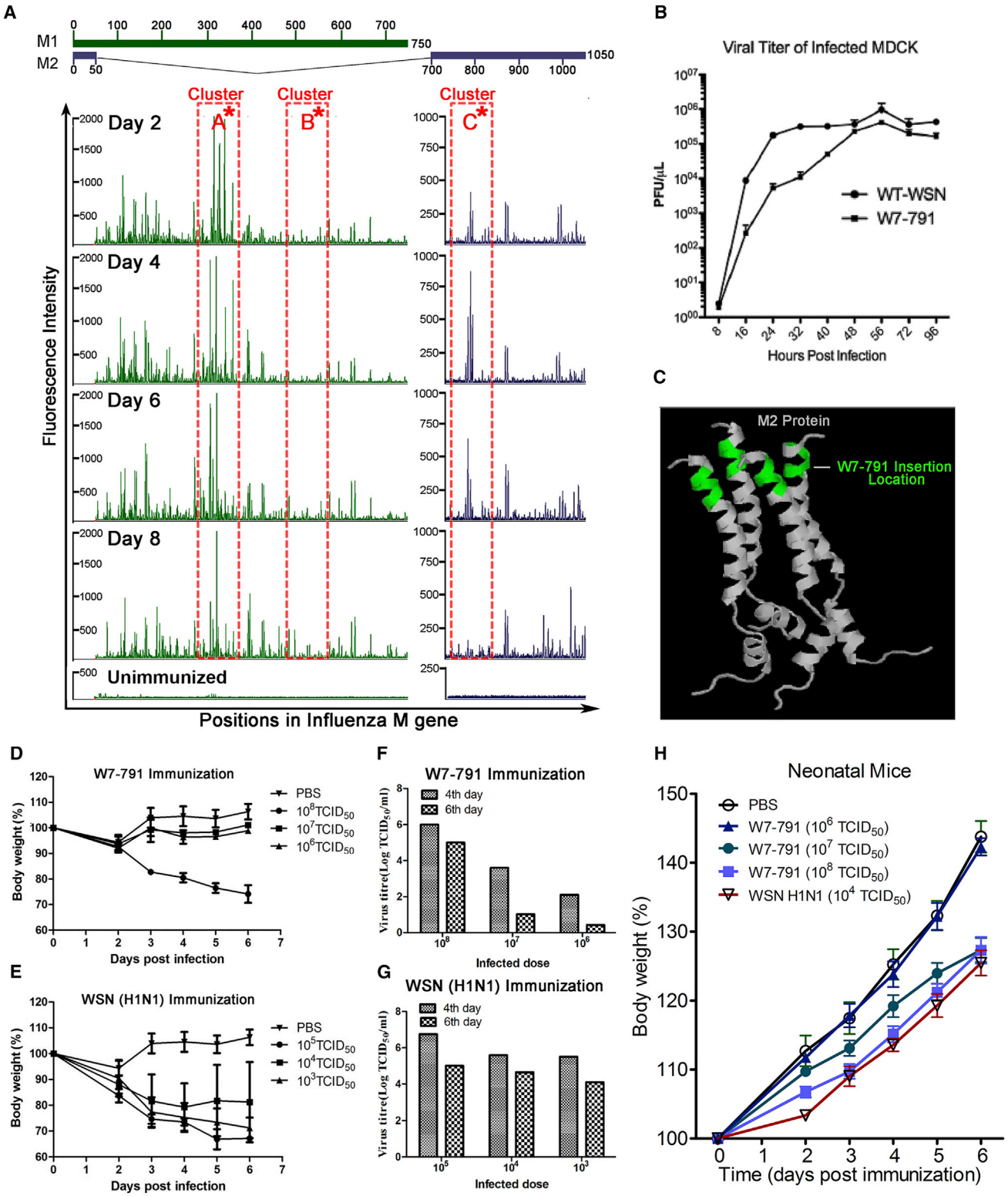

Figure 1. Generation of LAV W7-791 Using Genome-wide Mutagenesis and In Vivo Profiling

(A) Six- to eight-week-old C57BL/6 mice were infected with the M gene segment mutant population by i.t. injection (n = 8). Lungs were harvested at the indicated dpi and homogenized for genotyping. Peaks (M1 gene in green; M2 gene in blue) indicate the amount of viral RNA with insertions at that nucleotide position. Lung homogenates from PBS- or WT WSN-injected mice serve as negative controls. Clusters A*, B*, and C* are highlighted in red, representing the three types of observed growth profiles.

(legend continued on next page)

RESULTS

Generation of LAV W7-791 Using Genome-wide Mutagenesis and In Vivo Profiling

In contrast to the antigenic variability of the influenza hemagglutinin and neuraminidase genes, the matrix (M) gene segment encoding viral capsid protein (M1) and the proton-selective channel protein (M2) has evolved very slowly in all lineages of the virus and has been evaluated as a target for universal vaccine development (De Filette et al., 2005, 2008; Huleatt et al., 2008; Ilyinskii et al., 2008; Krammer and Palese, 2015; Neirynck et al., 1999; Schnell and Chou, 2008; Schotsaert et al., 2009; Tompkins et al., 2007; Wang et al., 2014). We created a mutant library comprised of $>10^5$ random insertions of a 15 nt sequence, 5′-NNNNNTGCGGCCGCA-3′, in the M gene of influenza A/WSN/1933 H1N1 (WSN), using a Mu-transposon mutagenesis method previously described (Arumugaswami et al., 2008). We then used the reverse genetics system of influenza to transfect the M gene mutant library with the seven complementary segments of wild-type (WT) WSN into HEK293T cells, creating a mutant virus library (Figures S1A–S1C). A primer based on the unique sequence contained within the insert was paired with different downstream primers along the length of the gene, generating a genotyping product of a specific length that could be used to extrapolate the position of the insertion. In this way, the mutation coverage of the entire viral pool could be visualized simultaneously. Analysis of this high-throughput genotyping data following in vitro infection of the mutant virus library (Figure S1D) shows that the majority of viable mutants (green peaks) are located in the non-conserved regions of the M1 and M2 proteins (Figures S1E and S1F).

In order to search for a potential LAIV, we monitored the in vivo growth profiles of the entire mutant virus population. Eight mice were given an intratracheal (i.t.) injection of the M gene mutant virus library, and cDNA was synthesized from the RNA recovered from lung homogenates collected 2, 4, 6, and 8 days post-infection (dpi). Through genotyping, we identified the location of each insertion in the pool of surviving mutant viruses at each time point. We observed three distinct growth profiles in these viruses (Figure 1A), possibly related to the mutations' effect on the replication, fitness, or host immune-related properties of the virus. Cluster A* represents an example of the fast-growing and likely disease-causing population. Cluster B* represents a slow-growing population, due either to intrinsically slow growth or suppression due to the host's immune response. By contrast, the viruses in cluster C* grew rapidly during the first 6 days, but were cleared between days 6 and 8. The advantages of this population are that these candidates persist long enough to trigger strong immune responses, but are too attenuated to cause significant disease in the host. We isolated 67 single mutant clones to screen as potential vaccine candidates (Figures S2A and S2B). We amplified three mutants in cluster C* (W7-757, W7-791, and W7-797) in Madin-Darby canine kidney (MDCK) cells and found that W7-791 grew to a high titer (Figure 1B) with a slightly lower cell toxicity (Figure S2C), causing less cell death as measured by LDH viability assay compared to the WT WSN virus. The mutant clone W7-791 had the insertion RHCGRI after the 26th amino acid of the M2 gene (Figure S2D), which is located on the cytoplasmic portion of the proton channel (Figure 1C) (Schnell and Chou, 2008). To ensure that W7-791 does not revert back to the WT WSN strain, we passaged W7-791 in vitro for multiple generations and found that the insertion remains unchanged in the genome (data not shown). To examine the in vivo stability of W7-791, we passaged the virus consecutively to groups of naive mice at 4 dpi (Figures S2E–S2G). We found that the W7-791 titer decreased after each passage, suggesting that the insertion mutation strain remained attenuated. Intranasal immunization of 6- to 8-week-old mice with different titrations of W7-791 showed no signs of weight loss even at 10^7 $TCID_{50}$ (50% tissue culture infection dose) per mouse, while significant weight loss was observed in mice infected with 10^3 $TCID_{50}$ of WSN (Figures 1D and 1E) or 10^4 $TCID_{50}$ of the H3 strain (Figure S3A). Similarly, the viral load in the lungs of W7-791-infected mice measured at 6 dpi was nearly 100-fold lower than in the WSN-infected group (Figures 1F and 1G) or the H3-infected group (Figure S3B). Lung samples collected at 4 dpi in both PBS and W7-791 groups showed no obvious pathology, whereas WT WSN mice, even at a much lower dose, showed severe tissue damage (Figure S3C). We further tested the safety of W7-791 in 15-day-old neonatal BALB/c mice by intranasal injection (10^6, 10^7, or 10^8 $TCID_{50}$) of W7-791 or 10^4 $TCID_{50}$ of WSN. The weight loss (Figure 1H) and lung findings (Figure S3D) suggest that W7-791 also exhibits significantly less pathology than WT WSN in neonatal mice. To test the extent of this particular insertion in causing viral attenuation, we inserted the same mutation into the matrix gene of influenza A/Puerto Rico/8 H1N1 (PR8), another mouse-adapted but heterologous H1 strain. We found that this mutant showed the same level of attenuation compared to its parent strain in vivo (Figure S4A). Taken together, we have established that the mutant W7-791 influenza strain is sufficiently attenuated to cause only a temporary infection and is safe at high titers in both adult and neonatal mice.

A Single Dose of W7-791 Can Elicit Protection against Lethal Homologous Influenza Virus Challenge

We next sought to determine whether vaccination with a single dose of W7-791 could protect mice against a lethal influenza virus challenge (Figure 2A). One month after i.t. inoculation with W7-791, mice were given a lethal dose of 4 MLD_{50} (50% mouse lethal dose) of WT WSN virus (Figures 2B and 2C). While the mock-vaccinated group all lost weight and died, all W7-791-vaccinated mice maintained a normal weight and survived. Interestingly, W7-791-vaccinated mice also survived without

(B) MDCK cells were infected at 0.25 MOI of WT WSN and W7-791 to determine the titer of virus at various time points. Data are mean ± SEM.
(C) The insertion position of W7-791 was mapped onto the known M2 crystal structure in green.
(D and E) Mouse body weight was monitored for 7 days post-inoculation with 10^6, 10^7, or 10^8 $TCID_{50}$ of W7-791 (D) or WT WSN (E). Data are mean body weight ± SEM.
(F and G) The viral titers from the W7-791 (F) or WT WSN (G) inoculation were evaluated on day 4 and day 6.
(H) The weight change of BALB/c pup mice (15 days of age) inoculated with the indicated titer of W7-791, WSN, or PBS. Data are mean body weight ± SEM.

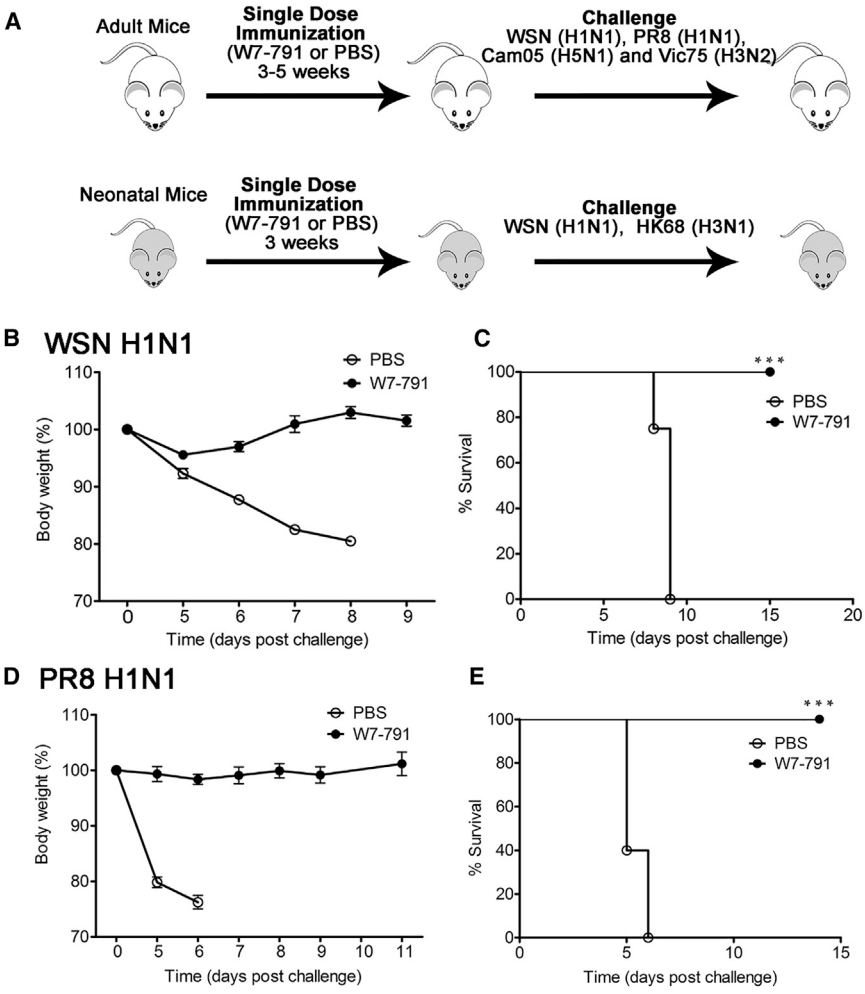

Figure 2. A Single Dose of W7-791 Can Elicit Protection against Lethal Homologous Influenza Virus Challenge

(A) Schematics of strategies used for mouse immunization and challenge.

(B and C) Mice were immunized intratracheally with 10^5 PFU of W7-791 (n = 5) or PBS (n = 5). One month after vaccination, mice were challenged with 4 MLD_{50} of WSN. Mouse weight (B) and survival (C) were measured at the indicated days after lethal challenge. Data are mean body weight ± SEM.

(D and E) Mice were immunized intratracheally with 10^5 PFU of W7-791 (n = 5) or PBS (n = 5). One month after vaccination, mice were challenged with 4 MLD_{50} of PR8. Mouse weight (D) and survival (E) were measured at the indicated days after lethal challenge. Data are mean body weight ± SEM. ***$p < 0.001$.

showing signs of illness or weight loss after challenge with 4 MLD_{50} of PR8 virus (Figures 2D and 2E).

A Single Dose of W7-791 Can Elicit Robust Cross-Protection against Lethal Heterologous Influenza Virus Challenge

We further explored whether W7-791 could cross-protect against heterologous highly pathogenic avian influenza (HPAI) A/Cambodia/P0322095/05 H5N1 (Cam/H5) (Figures 3A and 3B). Groups of BALB/c mice were inoculated intranasally (i.n.) with 10^6 plaque-forming units (PFU) of W7-791 and challenged 3 weeks later with 2 MLD_{50} of Cam/H5. All of the unvaccinated mice died, but the W7-791-vaccinated mice resisted the challenge without exhibiting significant weight loss. We extended our study to another phylogenetic influenza group, A/Victoria/3/75 H3N2 (Vic/H3) (Figures 3C and 3D). Mice were vaccinated with 10^5 PFU of W7-791, and then lethally challenged with 2 MLD_{50} after 4 weeks. Interestingly, W7-791-vaccinated mice only lost about 10% of their initial weight at 3–5 dpi with Vic/H3 before fully recovering, whereas the mock-vaccinated group succumbed to the H3N2 infection. Furthermore, we challenged W7-791-immunized mice against an escalated lethal dose of 5 MLD_{50} of WSN or HK68/H3 (Figure S4B). As before, all of the mice that were vaccinated with W7-791 survived the challenge, whereas PBS-immunized groups succumbed. Lastly, we tested whether W7-791 could cross-protect neonatal mice against lethal homologous and heterologous infections (Figures 3E and 3F). Fifteen-day-old BALB/c mice were immunized i.n. with 10^6 $TCID_{50}$ of W7-791 or PBS and then challenged with a lethal dose (10^5 or 10^6 $TCID_{50}$/mice) of WSN, or a lethal dose (10^6 or 10^7 $TCID_{50}$/mice) of A/Hong Kong/68 H3N1 (HK68/H3). Again, all vaccinated mice cleared the virus and survived the infection. Lastly, we compared our W7-791 strain with a quadrivalent LAIV used over the 2015-2016 season, FluMist. That LAIV contains attenuated viruses carrying antigens of two influenza B viruses, an H3N2 virus (Switzerland/9715293/2013), and an H1N1 strain (California/7/2009 pandemic virus). In vivo immunization and challenge with the same $TCID_{50}$ showed that W7-791 confers greater protection to mice against HK68/H3 (Figure S4C). All together, these results illustrate the capacity of our minimally modified mutant influenza strain to generate heterosubtypic protection against lethal virus challenge with a single immunization.

W7-791 Activates Both Humoral and Cell-Mediated Immune Responses

Live attenuated vaccines are thought to confer broad cross-protection against heterologous strains through activation of both humoral and cell-mediated immune responses. To determine the mechanisms responsible for the protective effects of W7-791, we immunized mice i.n. with W7-791, then challenged with WSN or PR8 viruses. We found that W7-791-vaccinated mice had significantly improved viral clearance against both viruses (Figure 4A). Surprisingly, W7-791-vaccinated mouse serum only showed hemagglutination inhibition (HAI) against WSN and not against heterologous strains PR8, HK68/H3,

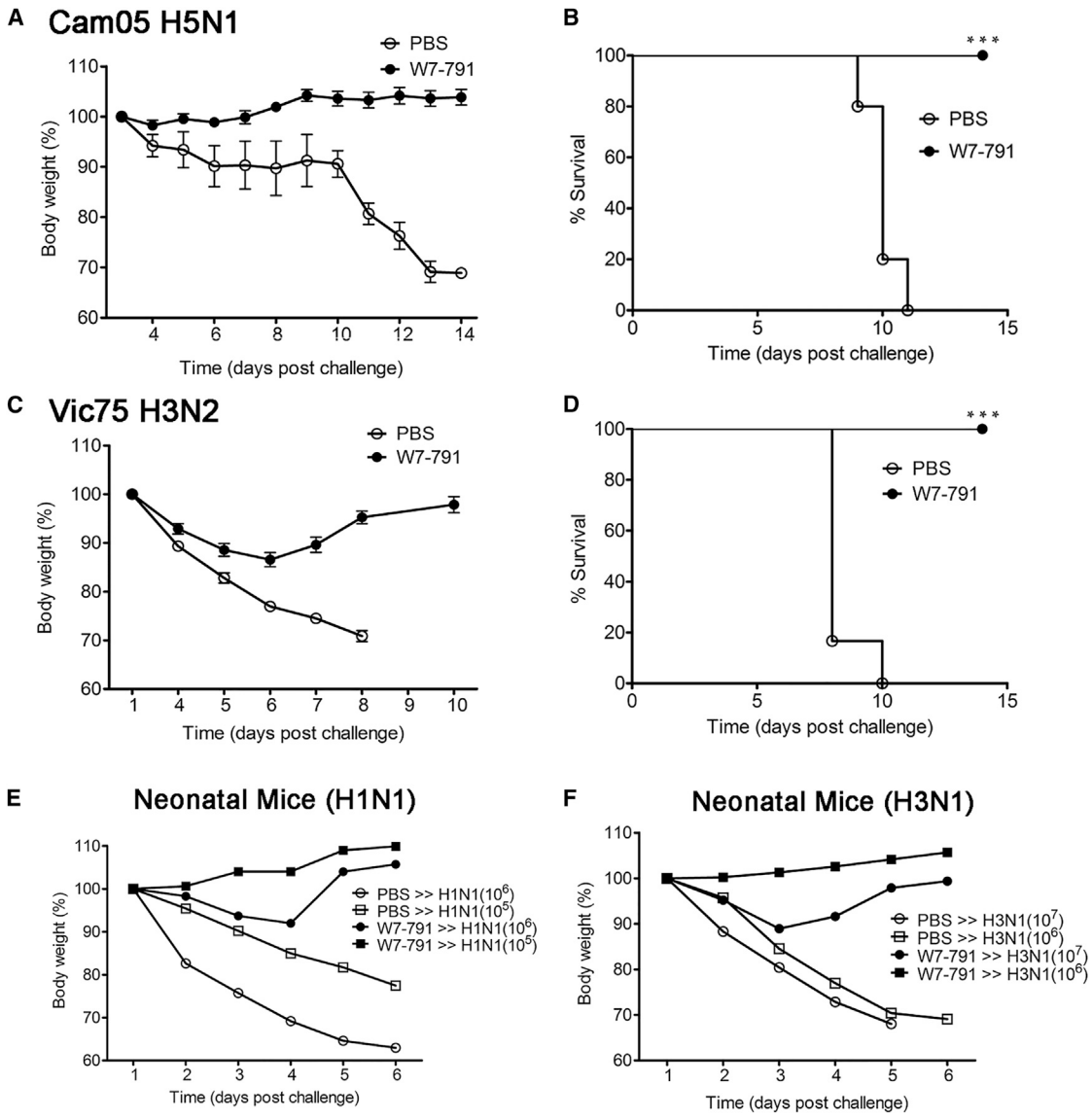

Figure 3. A Single Dose of W7-791 Can Elicit Robust Cross-Protection against Lethal Heterologous Influenza Virus Challenge

(A and B) Mice were immunized i.n. with 10^6 PFU of W7-791 (n = 6) or PBS (n = 5). Three weeks after vaccination, mice were challenged with 2 MLD_{50} of Cam/H5. Mouse weight (A) and survival (B) were measured at the indicated days after lethal challenge. Data are mean body weight ± SEM.

(C and D) Mice were immunized i.n. with 10^5 PFU of W7-791 (n = 9) or PBS (n = 6). One month after vaccination, mice were challenged with 2 MLD_{50} of Vic/H3. Mouse weight (C) and survival (D) were measured at the indicated days after lethal challenge. Data are mean body weight ± SEM.

(E and F) Weight change of neonatal mice lethally challenged with WSN (10^5 or 10^6 $TCID_{50}$) (E) or HK68/H3 (10^6 or 10^7 $TCID_{50}$) (F) 3 weeks after challenge. ***$p < 0.001$.

A/Wisconsin/2005 H3N2 (Wis/H3), or Cam/H5 pseudotype virus (Figures 4B and 4C). We further tested the serum using a micro-neutralization assay against WSN, PR8, and HK68/H3. Mice vaccinated with W7-791 showed a high neutralization titer against WSN and a lower level against PR8 and HK68/H3 (Figure 4D). These findings led us to believe that antibodies may not be the only source of protection mediated by W7-791.

To differentiate the cross-protective effect of cell-mediated and humoral responses, we immunized mice i.n. with 10^6 PFU of W7-791 or PBS, and collected serum and total T cells from spleens and lymph nodes 1 month later. We transferred these separately to naive mice and challenged them i.n. with a lethal dose of WSN or HK68/H3. The directly vaccinated (not transferred) group was used as a positive control and showed 100% protection. Adoptive transfer of serum from W7-791-immunized mice gave full protection against WSN, but not against HK68/H3 (Figure 4E). However, adoptive transfer of T cells from W7-791-immunized mice was able to partially or fully protect naive mice from HK68/H3 or WSN, respectively (Figure 4F). This demonstrates that W7-791 vaccination can generate a T cell-mediated immune response with a heterosubtypic protective ability. Furthermore, we evaluated CD8 T cell

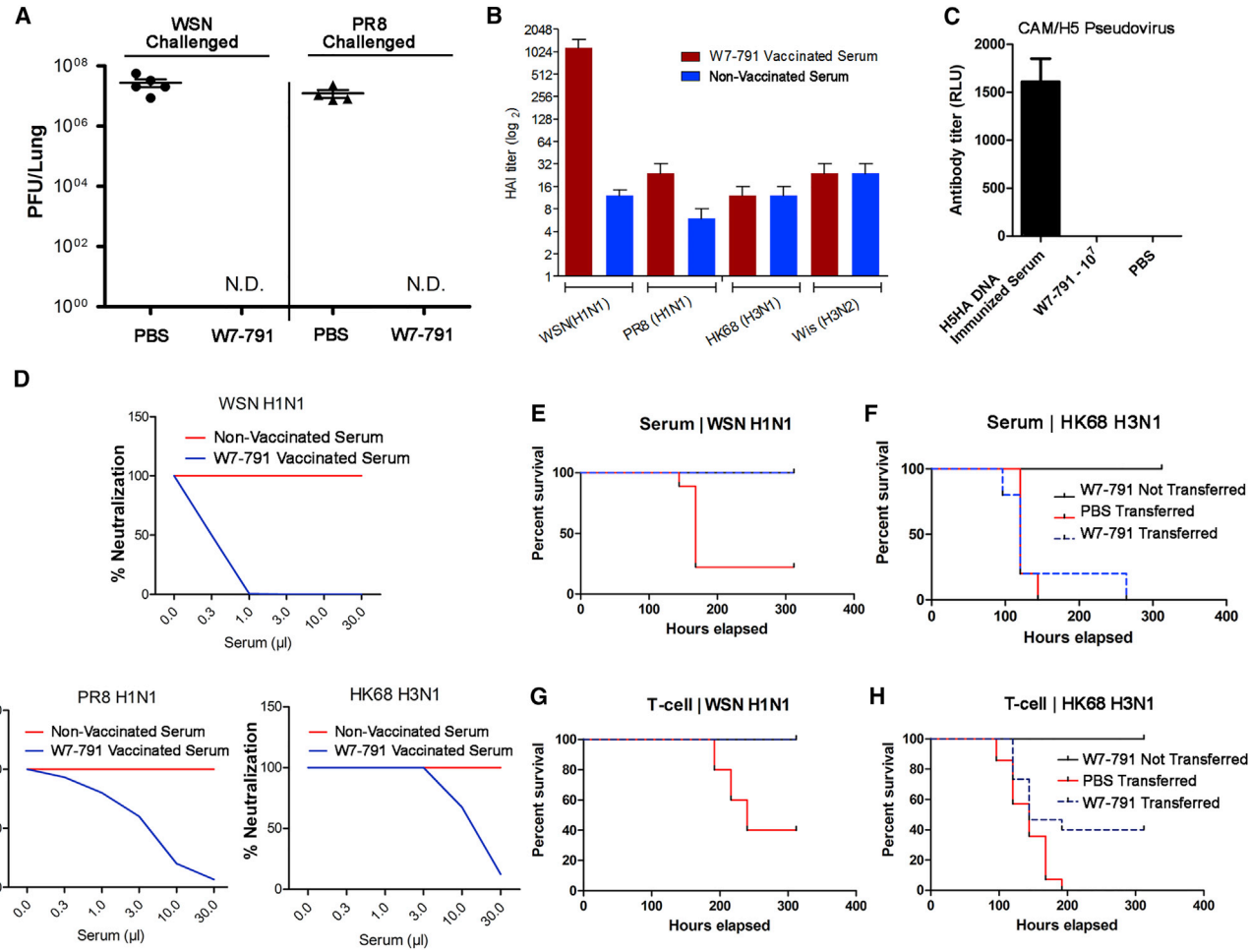

Figure 4. W7-791 Activates Both Humoral and Cell-Mediated Immune Responses

(A) Viral titers in lung homogenates of vaccinated and unvaccinated mice, challenged with lethal WSN or PR8, were quantified by plaque assay at the time of death or euthanasia.

(B) HAI assay of vaccinated and unvaccinated mouse serum against WSN, PR8, HK68/H3, and A/Wisconsin/05 H3N2. Mean ± SEM.

(C) The level of antibody in vaccinated and unvaccinated mouse serum was measured using Cam/H5 pseudovirus neutralization. Measurements are in relative light units (RLU). Mean ± SEM.

(D) Microneutralization assay of W7-791 vaccinated or unvaccinated mouse serum against WSN, PR8, and HK68/H3.

(E and F) Survival of mice receiving serum from W7-791- (n = 14) or PBS-vaccinated mice (n = 10). Mice were challenged 24 hr later with a lethal dose of WSN (E) or HK68/H3 (F).

(G and H) Survival curve of mice receiving T cells from W7-791- (n = 20) or PBS-immunized mice (n = 19), subsequently challenged with a lethal dose of WSN (G) or HK68/H3 (H). W7-791-vaccinated, but not transferred, mice were used as a control (n = 10).

epitopes in the W7-791-vaccinated mice (data not shown). We found that a large proportion of lung CD8+ T cells in W7-791-immunized mice were specific to H-2^{Db}-ASNENMETM (NP) 7 days after an H3N1 challenge. These data, combined with our adoptive T cell transfer experiment (shown in Figures 3F and 3H), has led us to believe that W7-791 immunization elicits cross-protective CD8+ T cell responses in our mouse model.

A Single Dose of W7-791 Elicits Heterologous Protection in the Ferret Model

To further explore the efficacy of W7-791 as an LAIV strain, we selected the ferret model, which resembles the pathophysiology of human influenza infection more closely than the mouse (Maher and DeStefano, 2004; Matsuoka et al., 2009). To evaluate the clinical response, we inoculated ferrets with 10^6, 10^7, and 10^8 $TCID_{50}$ of W7-791. We observed no significant changes in temperature (Figure 5A) or clinical symptoms (Figure 5B) after inoculation with 10^8 $TCID_{50}$ of W7-791. W7-791-vaccinated ferrets showed a significant increase in serum antibody titer (Figure 5C), but HAI assay confirmed that these antibodies bind to only the HA from WSN, and not HK68/H3 (Figures 5D) or H5N1 (Figure S5A). To examine protection against heterologous viruses, we challenged the ferrets 4 weeks post-immunization with either 10^6 $TCID_{50}$ of WSN, 10^6 $TCID_{50}$ of HK68/H3, or PBS. Ferrets that had been immunized with 10^3 and $10^{4.7}$ $TCID_{50}$ of W7-791 showed significant reduction in shedding of the challenge virus

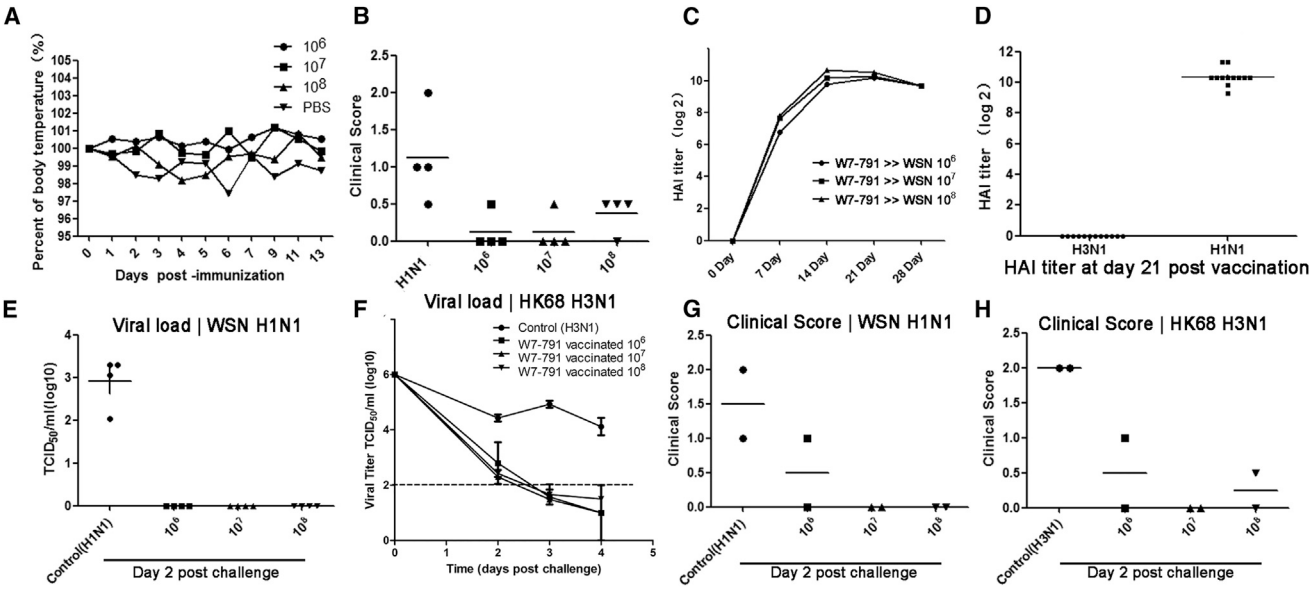

Figure 5. A Single Dose of W7-791 Elicits Heterologous Protection in the Ferret Model

(A) Temperature curve of ferrets inoculated i.n. with 10^6, 10^7, or 10^8 $TCID_{50}$ of W7-791 or PBS.

(B) Clinical scores of the ferrets 2 and 3 days post-inoculation with indicated doses of W7-791 or 10^6 $TCID_{50}$ of WSN. Clinical signs were evaluated as previously described (Reuman et al., 1989).

(C) HAI assay showing the increase of anti-W7-791 HA antibody titer from W7-791 inoculated ferrets after vaccination.

(D) HAI assay showing the titer of anti-H1HA or H3HA antibody 21 days post-inoculation.

(E–H) The viral titers (E and F) and the clinical scores (G and H) of the immunized and non-immunized ferrets challenged with 10^6 $TCID_{50}$ of WSN or HK68/H3. Data are mean body weight ± SEM.

within 2 dpi, compared to the unvaccinated group (control WSN or HK68/H3, respectively) (Figures 5E, 5F, and S5B). The vaccinated ferrets displayed an undetectable WSN or HK68/H3 viral load at 2 or 4 dpi, respectively. The vaccinated ferrets also showed improved clinical symptoms compared to non-vaccinated ferrets (Figures 5G and 5H). These data suggest that a single dose of W7-791 is safe in ferrets and can also elicit heterologous subtypic protection.

DISCUSSION

We have taken the approach of combining genome-wide mutagenesis and in vivo growth profile screening for the rapid and high-throughput development of LAIVs. As proof of principle, we used this method to uncover a promising LAIV, W7-791, that is over 100-fold more attenuated than the parent H1N1 virus, and is even tolerated in neonatal mice with no observable lung pathology. This strain could also confer complete cross-protection against heterologous H3 and H5 subtype influenza strains in mice. We further demonstrated that the insertion could be broadly applicable to attenuate other clades of influenza virus. The knockout or modified influenza M2 has been previously described as a potential LAIV candidate (Hatta et al., 2011; Watanabe et al., 2009). This present study explored the untapped potential of the M protein in generating LAIVs. The quadrivalent LAIV (FluMist) that was administered in the United States over the previous three seasons was shown to be ineffective compared to the inactivated vaccine. Our own comparison between the 2015-2016 FluMist formulation and W7-791 showed that our mutant H1 strain could confer greater protection in mice against challenge with an H3 virus than FluMist, which itself contains an H3 strain component. One possible explanation is that the primary method of protection in this case was not via H3-specific epitopes that were otherwise present in the quadrivalent LAIV, but rather through conserved epitopes still present in our H1 mutant strain. It is also plausible that W7-791, which is derived from a mouse-adapted strain, stimulated the immune system more efficiently through active replication in mice, and whether it can outperform FluMist in primates remains to be investigated. Although the reason for the failure of the FluMist over the last three seasons is under investigation, it was observed that this failure came at a time when the formulation was changed to having four viral components. While the inactive quadrivalent vaccine formulation is still effective, it is possible that including too many live influenza strains in the vaccine causes growth competition and reduced infectivity. Therefore, our strain may simply have been more effective because it was a single-component vaccine. The failure of FluMist is unfortunate, as live attenuated vaccines induce cell-mediated responses that are thought to target the more conserved parts of the virus, giving them the greatest potential for inducing broad protection against a multitude of strains. This would not only bypass the need to adjust our vaccines each year to account for antigenic drift variants, but could also protect us from highly pathogenic avian strains and novel reassortment viruses that could become the next influenza pandemic. W7-791 has shown great promise in its ability to confer protection against lethal heterologous influenza H1, H3, and H5 strains in mice (including

neonates) and ferrets. We demonstrated that even a single dose of W7-791 in mice could induce protection, without the need for prime-boost strategies or adjuvants. However, we acknowledge that the current extent of our LAIV characterization is limited. Future studies could include transferring the insertion to a master donor strain used for LAIVs, and expanding the range of the challenge doses to further evaluate the potential of this candidate vaccine. It also remains to be seen whether W7-791 will be successful in animals that have been previously exposed to influenza virus. In short, the development of a better influenza vaccine will require much more investigation, and the present work is only an initial step.

Our study also advances our understanding of the roles of antibody and T cell responses in influenza infection. Furthermore, we hypothesize that our strategy of screening for a growth phenotype that permits limited replication in the host and can elicit both cell-mediated and humoral immune responses without causing illness may be applicable to finding attenuated vaccines for other viruses. It would also be interesting to compare how the innate and adaptive immune systems communicate in response to influenza infection, an LAIV, or an inactivated vaccine.

This work presents an LAIV development strategy that can rapidly generate and screen entire libraries of viral clones for attenuation. We associated a particular viral growth profile in mice with the ability to elicit both cell-mediated and humoral immune responses without causing significant disease. From a cluster of viruses showing this ideal level of attenuation, we identified a promising LAIV candidate. This approach could potentially be implemented to discover attenuated mutants for other RNA and DNA viruses. It would require no working knowledge of specific genes or their functions in the virus, but could be used to establish a comprehensive profiling of the entire genome in vitro and in vivo. This system would also be ideal for expediting the design of live attenuated vaccines against less understood or emergent viruses.

EXPERIMENTAL PROCEDURES

Cell Culture

HEK293T cells were cultured in DMEM supplemented with 5% heat-inactivated fetal bovine serum (FBS). MDCK cells were maintained in DMEM containing 5% FBS, penicillin/streptomycin (100 U/mL and 50 μg/mL, respectively), and 1 mM sodium pyruvate at 37°C with 5% CO_2.

Generation of M Gene Segment Mutant Plasmid Library and Functional Profiling

To create the mutant plasmid library of the M gene segment of influenza A virus A/WSN/1933, a 15-nt sequence (5′-NNNNNTGCGGCCGCA-3′; N = duplicated 5 nucleotides from target DNA) was randomly inserted by Mu-transposon-mediated mutagenesis (MGS kit, Finnzymes) according to the manufacturer's instructions. The M gene mixed mutant pool was transformed into *E. coli* DH10B by electroporation at 2.0 kV, 200 Ω, 25 μF (ElectroMax DH10B, Invitrogen). The mutant M gene plasmid and seven remaining WT plasmids were transfected concomitantly into HEK293T cells for virus generation. Three days after transfection, the supernatant was collected and transferred to MDCK cells for propagation. Virus was collected after 48 hr, then either stored or used for further propagation for up to four passages. RNA was isolated with the TRIzol reagent (Invitrogen) after each generation. RT-PCR was carried out with the iScript cDNA Synthesis kit (Bio-Rad) to create cDNA. Three gene-specific forward primers approximately 400 bp apart in the M-gene segment (5′-AGCAAAAGCAGGTAGATATT-3′, 5′-GGGGCCAAAGAAATAGCACT-3′, and 5′-TCCTAGCTCCAGTGCTGGTC-3′) and a Vic-labeled insertion-specific mini-primer (5′-TGCGGCCGCA-3′) were used to amplify fragments containing the 15 nt insert using KOD Hot-Start polymerase (Novagen). The PCR conditions were set to 95°C for 10 min (1 cycle); 95°C for 45 s, 52°C for 30 s, and 72°C for 90 s (30 cycles); and 72°C for 10 min (1 cycle). The fluorescent-labeled PCR products were analyzed in duplicate with a Liz-500 size standard (Applied Biosystems) using a 96-capillary genotyper (3730xl DNA Analyzer, Applied Biosystems) at the UCLA GenoSeq Core facility. Sequencing data were analyzed for clarity using ABI software, with the following criteria: (1) all data passed the standard default detection level; (2) the first 70 bp were removed due to non-specific background noise; (3) all data were aligned to the nearest base pair in the influenza A WSN matrix gene; and (4) all genotyping experimental data were normalized with WT WSN-infected cells, non-transfected cells, and a different gene library as controls. This eliminated non-specific data from the PCR, primers, and the DNA Analyzer. For infection in vivo, the mutant virus pool was titered, concentrated by ultra-centrifugation and re-titered, and used for mouse injection. Two dpi, the lungs were harvested, homogenized, and resuspended in TRIzol for RNA isolation, followed by the same procedures as described above. PBS or WSN-infected mice served as controls.

Virus Strains

We used the influenza A/WSN/1933 reverse genetics system to generate seasonal A/H1N1 virus (Hoffmann et al., 2000). This strain is a mouse-adapted influenza virus and has been used as the parental strain to generate potential LAIVs using transposon mutagenesis. The eight plasmids containing the cDNA of A/WSN/33 (gift from Dr. Yuying Liang at Emory University) were transfected into HEK293T cells using TransIT LT-1 (Panvera) by the manufacturer's protocol. The virus was serially passaged three times in MDCK cells to a final titer of $10^{7.4}$ PFU/mL. Influenza virus A/Puerto Rico/8/1934 (seasonal A/H1N1 virus) was a gift from Dr. Yuying Liang. The virus was serially passaged three times in MDCK cells to a titer of $10^{7.5}$ PFU/mL. The MLD_{50} of both strains was determined in C57BL/6 mice.

Influenza virus A/Victoria/3/75 (seasonal A/H3N2 virus), A/Wisconsin/65/05 (seasonal A/H3N2 virus), and A/Hongkong/68 (seasonal A/H3N1 virus) were gifts from Dr. Ioanna Skountzou at Emory University. These viruses were amplified using MDCK cells for two to three passages to a final titer of $10^{5.5}$ PFU/mL, $10^{5.4}$ PFU/mL, and 10^{7} PFU/mL, respectively. The MLD_{50} was determined in C57BL/6 and BALB/c mice.

Influenza virus A/Cambodia/P0322095/05 (highly pathogenic avian influenza H5N1 virus) was originally isolated from human patients at the Pasteur Institute in Cambodia (Buchy et al., 2007). Virus was propagated in MDCK cells and virus-containing supernatants were pooled, clarified by centrifugation, and stored at −80°C. The $TCID_{50}$ and the MLD_{50} of the viruses were determined in MDCK cells and in BALB/c mice, respectively, and were calculated as described previously (Ding et al., 2011).

Virus Titrations

The concentration of infectious viruses was determined by plaque assay and end-point titrations. Plaque assays were performed in MDCK cells and calculated as PFU/μL of supernatant. The viral samples were serially diluted in dilution buffer (PBS with 10% BSA, $CaCl_2$, 1% DEAE-dextran, and $MgCl_2$). Diluents were added to a monolayer of MDCK cells in 6-well plates for 1 hr at 37°C, and then covered with growth medium containing 1% low-melting agarose and TPCK-treated trypsin (0.7 μg/mL). Infected cells were stained after 48 hr (1% crystal violet, 20% ethanol, in PBS) to visualize the plaques. Virus titrations were performed by end-point titration in MDCK cells. MDCK cells were inoculated with 10-fold serial dilutions of the virus, then washed with PBS once 1 hr after inoculation, and cultured in DMEM for 48 hr to visualize cell viability. The viral titer was determined by luminescence assay or by plaque assay. To measure the growth of individual mutants in vitro (Figure S2B), an influenza virus-responsive *Gaussia* luciferase (gLuc) reporter system was used. Briefly, the gLuc coding region was inserted in the reverse-sense orientation between a human RNA polymerase I promoter and a murine RNA polymerase I terminator. The gLuc coding sequence was flanked by the UTRs from the PA segment of influenza virus A/WSN/33 strain so that gLuc expression is dependent on influenza virus infection. The gLuc reporter was transfected into HEK293Ts for 24 hr before the supernatants containing mutant or WT influenza

viruses were added. Upon active infection, gLuc is released into the supernatant and can be quantified with *Renilla* luciferase substrate (Promega).

Animals

Adult Mice

Female C57BL/6 mice, 6–8 weeks old, were purchased from the Jackson Laboratory. All animals were housed in pathogen-free conditions within the UCLA animal facilities.

Neonatal Mice

Fifteen-day-old BALB/c mice (Vital River Beijing) weighing 6–9 g were inoculated i.n. with PBS, 10^4 $TCID_{50}$ of WSN virus, or dilutions of W7-791. For the dose-dependent experiment, mice were inoculated i.n. with 10^6, 10^7, and 10^8 $TCID_{50}$ of W7-791. Sixteen days post-treatment, mice were challenged i.n. with a lethal dose (10^5 or 10^6 $TCID_{50}$/mouse) of WSN or (10^6 or 10^7 $TCID_{50}$/mouse) A/Hong Kong/68 H3N1 (HK68/H3) in a 30 μL volume. Randomly selected mice from each group were sacrificed for pathological examinations of the lung at 4 and 6 dpi. Then the lungs were homogenized to measure viral titer using end-point-dilution assays.

Ferrets

Healthy young adult outbred female ferrets (Mustela putorius furo; between 4 and 5 months of age) were purchased from a commercial breeder (Wuxi) and confirmed to be seronegative by HAI assay to A/WSN/1933 (H1N1), A/Victoria/3/75 (H3N2), HK68 (H3N1), and W7-791(H1N1). A minimum of three independently housed ferrets were inoculated i.n. with 0.5 mL (0.25 mL per nostril) of 10^6, 10^7, or 10^8 $TCID_{50}$ of W7-791 or PBS. Anesthesia was performed on the quadriceps muscles of the left hind leg with a total volume of 0.02 mL Lumianning (Hua Mu Animal Care). Serum samples were collected at days 0, 7, 14, 21, and 28 post-immunization for HAI studies. Nasal washes were collected 0–7 days after immunization. Four weeks after immunization, the ferrets were challenged i.n. with 10^6 $TCID_{50}$ of WSN (H1N1) or HK68 (H3N1). Weights and temperatures were monitored daily for 7 days after inoculation. Nasal washes were collected 0–7 days after the challenge. Clinical signs were evaluated 3 days prior to vaccination, then 9, 11, 13, and 15 dpi, and 2 days prior to challenge and 1–7 dpi. The clinical signs were scored as previously described (Reuman et al., 1989). All animal studies were performed according to the guidelines of the UCLA Animal Research Committee.

Mouse Immunization and Challenge

Female C57BL/6 and BALB/c mice were randomly divided into groups of five or six mice. Groups were inoculated i.n. or intratracheally with either PBS or W7-791 in a volume of 50 μL. Intratracheal injection was performed by anesthetizing mice intraperitoneally with a ketamine/xylazine mixture, then surgically exposing the trachea for direct injection of 30 μL of solution with a sterile 27G needle (Shahangian et al., 2009). Four weeks after immunization, all mice were challenged i.n. or intratracheally with an influenza strain in a 50 μL volume: A/WSN/1933 (H1N1) at 4 MLD_{50}, A/Puerto Rico/8/1934 (H1N1) at 4 MLD_{50}, A/Cambodia/P0322095/05 (HPAI-H5N1) at 2 MLD_{50}, or A/Victoria/3/75 (H3N2) at 2 MLD_{50}. Mice were monitored and recorded daily for signs of illness, such as lethargy, ruffled hair, and weight loss. When mice lost 30% or more of their original weight, they were euthanized and counted as dead. For the adoptive transfer experiment, female C57BL/6 mice were randomly divided into two sets of vaccinated or unvaccinated groups. Unvaccinated mice were sham immunized, whereas the vaccinated group received a single dose of W7-791 at 10^6 PFU/mouse. One set from each group was used to harvest cells for the transfer experiment 4 weeks post-vaccination, while the other set was used as a vaccinated, but not transferred, control. Total CD4+ and CD8+ T cells were isolated from the spleens of the vaccinated and the unvaccinated mice using the Mouse Pan T Cell Isolation Kit and MS columns (Miltenyi Biotec). On the same day, the cells from the same group were pooled, and $\sim 10^{6.3}$ T cells/mouse were injected via the retro-orbital route to a new set of naive female C57BL/6 mice. Likewise, sera were isolated from either the vaccinated or unvaccinated groups and matching groups were pooled, then 100 μL/mouse of serum was administered retro-orbitally to a new set of naive female C57BL/6 mice. The mice in all groups were challenged i.n. at 24 hr post-adoptive transfer with 2 MLD_{50} of WSN or 2 MLD_{50} of HK68/H3.

In Vivo Challenge Using HPAI Virus H5N1

All animal protocols were approved by the Institutional Animal Care and Use Committee at the Pasteur Institute of Cambodia. Female BALB/c mice (*Mus musculus*) at the age of 6–8 weeks were purchased from Charles River Laboratories and housed in microisolator cages ventilated under negative pressure with HEPA-filtered air and a 12/12 hr light/dark cycle. Virus challenge studies were conducted in BSL3 facilities at the Pasteur Institute of Cambodia. Before each inoculation or euthanasia procedure, the mice were anesthetized by intraperitoneal (i.p.) injection of pentobarbital sodium (75 mg/kg; Sigma).

Ethical Statement

All animal experiments were carried out at biosafety level 3 (BSL3) containment facilities complying with the Ethics Committee regulations of the Institut Pasteur, in accordance with EC directive 86/609/CEE and were approved by the Animal Ethics Committee of the Institut Pasteur in Cambodia (permit number VD100820). Before each inoculation or euthanasia procedure, the mice were anesthetized by i.p. injection of pentobarbital sodium, and all efforts were made to minimize suffering.

Lung Homogenization

After animals were sacrificed, lungs were perfused by injecting 1 mL PBS containing 5 mM EDTA into the right ventricle. Whole lungs were removed and the lymph nodes were dissected away. The lungs were homogenized with 1 mL PBS containing a proteinase inhibitor cocktail (Roche Applied Science), and virus titers in lungs were evaluated by plaque assay. After homogenates were centrifuged at 10,000 × *g* for 10 min, the supernatant was collected for genotyping.

Sequence Comparisons

Influenza A Matrix 1 and Matrix 2 protein sequences from ~300 previously reported strains from 1918 to 2014 were compared and aligned using the NCBI influenza database (https://www.ncbi.nlm.nih.gov/genomes/FLU/aboutdatabase.html).

Structure Analysis

Conserved and viable mutations in the M gene were mapped onto the crystal structure of the monomeric M1 gene (PDB: 2Z16) and the tetrameric M2 gene (PDB: 2L0J), which were obtained from PDB. The structure labeling was performed using PyMOL v.1.0.

In Vitro Assays

Cell Viability Assay

Cell viability was measured by CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) according to the manufacturer's instructions.

HAI Assay

Viruses A/WSN/1933, A/Puerto Rico/8/1934, A/Wisconsin/65/05, and A/Hong Kong/68 were diluted to 4 HA units and incubated with an equal volume of serially diluted sera for 30 min at room temperature. An equal volume of 1% chicken red blood cells was added to the wells and incubation continued on a gently rocking plate for 30 min at room temperature. Button formation was scored as evidence of HAI. Assays were performed in triplicate.

Microneutralization Assay

MDCK cells (5×10^5 cells per well) were seeded onto a 12-well culture plate in complete DMEM overnight. To test the neutralization activity of immune sera, serial 3-fold dilutions of sera were incubated with $10^{6.5}$ PFU/mL, $10^{4.4}$ PFU/mL, and $10^{4.2}$ PFU/mL of viruses A/WSN/1933, A/Hongkong/68, and A/Puerto Rico/8/1934 at the final volume of 100 μL at room temperature for 1 hr. After the incubation, the mixture was added onto a monolayer of MDCK cells and was incubated for 1 hr at 37°C and then covered with growth medium containing 1% low-melting-point agarose and TPCK-treated trypsin (0.7 μg/mL). Infected cells were stained after 48 hr (1% crystal violet, 20% ethanol, in PBS) to visualize the plaques. Assays were performed in triplicate.

Pseudovirus Neutralization Assay

H5N1 pseudotype virus expressing the H5HA derived from A/Cambodia/P0322095/05 (GenBank: ADM95463), the N1NA (GenBank: AY555151) derived from A/Thailand/1(KAN-1)/2004, and a luciferase reporter gene were used in this experiment. The ferret sera were diluted in 2-fold serial dilutions from 1/20 to 1/1,280 and the mouse sera were diluted from 1/10 to 1/1,280. Sera from mice immunized by injection of H5HA DNA (GenBank: AAS65615) from A/Thailand/1(KAN-1)/2004 were used as a positive control. IC_{50} values were defined as the dilution of a given immune serum that resulted in 50% reduction of RLA. The assay was performed in triplicate.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2017.02.007.

AUTHOR CONTRIBUTIONS

Conceptualization, G.C.; Methodology, L.W., S.-Y.L., and H.-W.C.; Investigation, L.W., S.-Y.L., H.-W.C., J.X., M.C., T.Z., F.Z., Y.E.W., N.Q., and G.W.; Writing – Original Draft, S.-Y.L. and L.W.; Writing – Review & Editing, L.W., M.C., and N.Q.; Resources, X.T., Z.H., L.L., W.Y., D.J.S., and Y.L.; Supervision, T.J., R.M., B.R.B., Q.L., J.C.D., P.Z., F.X.-F.Q., and G.C.

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