A Live Attenuated H1N1 M1 Mutant Provides Broad Cross-Protection against Influenza A Viruses, Including Highly Pathogenic A/Vietnam/1203/2004, in Mice

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The emergence of novel influenza A H1N1 and highly pathogenic avian influenza (HPAI) H5N1 viruses underscores the urgency of developing efficient vaccines against an imminent pandemic. M(NLS-88R) (H1N1), an A/WSN/33 mutant with modifications in the multibasic motif 101RKLKR105 of the matrix (M1) protein and its adjacent region, was generated by reverse genetics. The M(NLS-88R) mutant had in vitro growth characteristics similar to those of wild-type A/WSN/33 (wt-WSN), but it was attenuated in mice. Vaccination with M(NLS-88R) not only fully protected mice from lethal homologous challenges but also prevented mortality caused by antigenically distinct H3N2 and H5N1 viruses. M(NLS-88R)-induced homologous protection was mainly antibody dependent, but cellular immunity was also beneficial in protecting against sublethal wt-WSN infection. Adoptive transfer studies indicated that both humoral and cellular immune responses were crucial for M(NLS-88R)-induced heterologous protection. Our study suggests an alternative approach to attenuate wt influenza viruses for the development of a pandemic vaccine with broad cross-protection.

The continuous global spread of highly pathogenic avian influenza (HPAI) H5N1 viruses in avian species and humans poses a significant threat to public health. The recent outbreak of highly contagious novel influenza A H1N1 virus in Mexico and North America and its quick spread through direct transmission between humans deepen the fear of an imminent pandemic outbreak [1]. Under these circumstances, vaccines that can confer protection against viruses within the same subtype (subtype-specific immunity) or between different subtypes (heterosubtypic immunity) would be highly desirable. Inactivated influenza vaccines delivered intramuscularly can efficiently elicit neutralizing antibodies that are, however, generally restricted to circulating strains or viruses that are antigenically closely related. Unlike inactivated influenza vaccines, live attenuated vaccines are administered intranasally and are capable of inducing mucosal immunity and preventing virus entry and subsequent replication in the respiratory tract [2, 3]. In addition to inducing neutralizing antibodies against circulating strains, live attenuated vaccines can also elicit viral epitope-specific cytotoxic CD8+ T lymphocytes and confer heterosubtypic immunity [4–6].

We have long been interested in the roles of the viral M1 gene in the pathogenesis and growth of influenza viruses, especially the multibasic motif 101RKLKR105 of M1 protein. The RKLKR motif is involved in the...
nuclear localization signal (NLS) and in viral RNA and ribonucleoprotein (RNP) binding [7, 8]. Using wild-type A/WSN/33 (wt-WSN, H1N1) as a model, we previously have shown that double point mutations in the RKLKR motif (R101S-R105S) impair viral replication in vitro and result in temperature sensitivity (ts) and attenuation [9, 10]. In the present study, we demonstrated that an additional mutation (G88R) in the adjacent region of the RKLKR domain could restore viral replication to a level similar to that of wt-WSN in vitro but that it still retained the attenuation phenotype in mice. The vaccine potential of the new WSN M1 mutant, designated “M(NLS-88R),” was further explored.

**MATERIALS AND METHODS**

**Viruses.** The full-length matrix (M) complementary DNA of mouse-adapted-wt-WSN (H1N1) was cloned, and 3 point mutations (G88R, R101S, and R105S) in the RKLKR motif and its adjacent region of M gene were incorporated into the plasmid pPol I-WSN-M (a gift from Y. Kawaoka, University of Wisconsin–Madison, Madison), as described elsewhere [9, 10]. The pPol I-WSN-M expressing the mutated M gene was then mixed with the plasmids expressing the other 7 viral genes of wt-WSN (0.5 µg/plasmid), including pHW181-PB2, pHW182-PB1, pHW183-PA, pHW184-HA, pHW185-NP, pHW186-NA, and pHW188-NS (gifts from R. Webster, St. Jude Children’s Research Hospital, Memphis, Tennessee), followed by incubation with TransIT LT-1 (Panvera) to form transfection complex. The plasmid–transfection complex mixture was delivered into cocultured 293T and Madin-Darby canine kidney (MDCK) cells [11]. Six h later, the plasmid–transfection mixture was replaced by fresh Opti-MEM I medium (Invitrogen) containing 0.5 µg/mL L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)–trypsin. The rescued M1 mutant was designated “M(NLS-88R)” (H1N1) (Table 1). wt-WSN was similarly derived by reverse genetics. mu-rWSN (H1N1), a cold-adapted (ca) WSN mutant that has been reported elsewhere [12], contained the 5 key mutations in the PB1 (K391E, E581G, and 661A) and nucleoprotein (NP) (D34G) genes. Unless otherwise specified, groups of 4- to 6-week-old female BALB/c mice (Taconic Farm) were inoculated intranasally with M(NLS-88R) at 5×10⁴ pfu per 50 µL of PBS per mouse under light isoflurane anesthesia. Body weight was monitored for up to 2 weeks after infection. A separate set of mice that were infected with M(NLS-88R) at 5×10⁴ pfu per 50 µL of PBS per mouse were euthanized on days 2, 4, and 7 after infection. Lungs and brains were harvested, homogenized, and then titrated on 100% confluent MDCK cells by plaque assay. Mice infected with wt-WSN given at the same dose served as a control.

**Immunogenicity of M(NLS-88R).** Unless otherwise specified, groups of 4- to 6-week-old female BALB/c mice were inoculated intranasally with a single dose of M(NLS-88R) at 5×10⁴ pfu per 50 µL of PBS per mouse. Serum samples were collected by tail or orbital plexus bleeding at 4 weeks after infection or immediately before challenges, and they were stored at −20°C until use. Aliquots of hyperimmune serum were pretreated with receptor-degrading enzyme (Denka-Seiken) before hemagglutination inhibition (HAI) assay was performed using 4 hemagglutinin (HA) units of virus and 0.5% turkey red blood cells, for H1N1 and H3N2 viruses, or 1% horse red blood cells, for H5N1 virus [16].

**Table 1. Point Mutations in the Amino Acid Sequences of the WSN Mutants Used in the Present Study**

<table>
<thead>
<tr>
<th>Gene, position</th>
<th>wt-WSN</th>
<th>R101S-R105S</th>
<th>M(NLS-88R)</th>
<th>mu-rWSN</th>
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<td>105</td>
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NOTE. Using wild-type A/WSN/33 (wt-WSN) as the background, R101S-R105S [9, 10], M(NLS-88R) (present study), and mu-rWSN [12] were generated by reverse genetics with different point mutations in corresponding internal genes. -, Amino acid on this position remains unchanged as wt-WSN.

or 50% egg infectious dose (EID₅₀), as described elsewhere [14, 15].

In vitro and in vivo characterization of M(NLS-88R). The ts phenotype of wt-WSN, M(NLS-88R), and R101S-R105S was determined by comparing viral replication in MDCK cells at 33°C versus 39°C. Viruses that had ≥2 logs of reduction from viral titers at 39°C to those at 33°C were considered to be of the ts phenotype.

Four- to 6-week-old specific-pathogen–free female BALB/c mice (Taconic Farm) were inoculated intranasally with M(NLS-88R) at a dose range of 10⁴–10⁶ plaque-forming units (pfu) per 50 µL of phosphate-buffered saline (PBS) per mouse under light isoflurane anesthesia. Body weight was monitored for up to 2 weeks after infection. A separate set of mice that were infected with M(NLS-88R) at 5×10⁴ pfu per 50 µL of PBS per mouse were euthanized on days 2, 4, and 7 after infection. Lungs and brains were harvested, homogenized, and then titrated on 100% confluent MDCK cells by plaque assay. Mice infected with wt-WSN given at the same dose served as a control.
Figure 1. In vitro and in vivo characterization of M(NLS-88R). The temperature-sensitive (ts) phenotype (A) and replication kinetics (multiplicity of infection, 0.01) (B) were determined by plaque assay. Four-week-old mice were infected intranasally with wild-type A/WSN/33 (wt-WSN) or M(NLS-88R) at 5 x 10⁶ plaque-forming units (pfu)/50 µL of phosphate-buffered saline/mouse. Body weight (BW) was monitored for up to 2 weeks after infection (C). Survival is expressed in parentheses as the no. of survivors/total no. of mice assessed. Entire lungs from groups of 3 mice were harvested on days 2, 4, and 7 after infection. Pulmonary viral replication was determined by plaque assay and expressed as the mean number of plaque-forming units per gram ± standard error (3 mice per group per time point) (D). *P < .05 and ***P < .001, by unpaired Student’s t test performed on log-transformed data. The lower detection limit is denoted by the dashed horizontal line.

elsewhere, with some modifications [6]. In brief, serum samples serially diluted in 10-fold increments were incubated with 100 pfu of live wt virus in equal volumes at room temperature for 60 min. The remaining infectivity of the virus-serum mixtures was detected by plaque assay and expressed as a percentage of plaque reduction from the normal serum-treated (1:50 dilution) virus mixtures. H5N1-specific neutralizing antibody titers were assessed by microneutralization assay. In brief, hyperimmune serum samples were serially diluted in 2-fold increments starting at a 1:10 dilution and were incubated with an equal volume of 1 hundred 50% tissue culture infectious doses (TCID₅₀) of HPAI A/Vietnam/1203/2004 at room temperature for 60 min. The virus-serum mixtures were then incubated with 10⁴ MDCK cells/well in 96-well plates at 33°C overnight. The residual infectivity of H5N1 virus was measured using an NP-specific mouse monoclonal antibody, followed by fluorescein isothiocyanate–labeled secondary antibody.

Protection against homologous and heterologous challenges. Animal infection studies using mouse-adapted influenza viruses or human H5N1 vaccine strains were performed in a biosafety level–2 facility at CBER/FDA, in accordance with the protocols approved by the animal care and use committee of the CBER/FDA. Mice immunized with M(NLS-88R) were challenged intranasally with wt-WSN (H1N1), FM (H1N1), A/Philippines/2/82/X-79 (H3N2), or the human H5N1 vaccine strain A/Vietnam/1203/2004/X-PR8 at 4 weeks or 2–6 months later. Morbidity (as measured by weight loss) and mortality were monitored daily for up to 2 weeks after infection. Lungs from challenged mice were harvested on day 3 after infection, homogenized, and titrated on MDCK cells by plaque assay. In a separate experiment, mice immunized with M(NLS-88R) were depleted of CD8 or CD4 T cells by intraperitoneal injection of 2 doses of anti-CD8 (Clone 53-6.7; eBioscience) or anti-CD4 (Clone RM4-5; eBioscience) at 0.5 mg/dose one day before and one day after wt-WSN challenge, as described elsewhere [5]. The depletion efficiency was confirmed by flow cytometry. Mice injected with isotype-matched rat immunoglobulin (Ig) G2a (eBioscience) served as control subjects.

The challenge experiment with HPAI H5N1 A/Vietnam/1203/2004 was conducted under biosafety level–3 containment at the Centers for Disease Control and Prevention (Atlanta, Georgia), including enhancements required by the United States Department of Agriculture and the Select Agent Program. Naïve or M(NLS-88R)-immunized mice (6 mice/group) were inoculated intranasally with 2 hundred 50% mouse lethal doses (MLD₅₀) of HPAI A/Vietnam/1203/2004 virus, and they were observed daily...
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Figure 2. Immunogenicity of M(NLS-88R) in mice. BALB/c mice were inoculated intranasally with 5 × 10^6 plaque-forming units (pfu) of M(NLS-88R) (H1N1) per 50 μL of phosphate-buffered saline per mouse. Naive mice served as negative controls. Serum hemagglutination inhibition (HAI) titers against wild-type A/WSN/33 (wt-WSN, H1N1) were determined 4 weeks after immunization with M(NLS-88R) (8 mice/group) (A). wt-WSN–specific neutralizing activity of M(NLS-88R)-induced hyperimmune serum was measured by plaque reduction after neutralization (8 mice/group) (B). Four weeks later, immunized mice were challenged intranasally with wt-WSN (5 × 10^6 pfu/mouse) or A/FM/1/47-MA (FM, H1N1; 5 × 10^6 pfu/mouse), and lung virus titers were determined by plaque assay performed on day 3 after infection (3 mice/group) (C). The lower detection limit is denoted by the dashed horizontal line. ** and *** , by unpaired Student’s t test performed on log-transformed data.

for 14 days for morbidity and mortality. Four mice from each group were euthanized on day 6 after infection. Lungs, noses, and brains were collected, and virus titers were determined by EID_{50}, as described elsewhere [3].

Major histocompatibility class I tetramer staining and flow cytometry. Phycoerythrin (PE)–labeled NP_{147–155}–specific major histocompatibility class I tetramer was produced by the National Institutes of Health Tetramer Core Facility and Beckman-Coulter. Lungs harvested from M(NLS-88R)-infected mice on day 7 after infection were minced and digested in the presence of collagenase type II (300 U/mL) and deoxyribonuclease I at 37°C for 1 h. Single suspended lung cells were then stained with PE-labeled NP_{147–155}–specific major histocompatibility class I tetramer and counterstained with fluorescein isothiocyanate–labeled CD8 (Clontech) and PE–cyanine dye 5 (Cy5)–labeled CD3 (BD Biosciences). The acquisition was completed in a FACSCanto II cytometer (BD Biosciences), and the data were analyzed using FlowJo software (Tree Star).

Adoptive transfer experiments. Hyperimmune serum samples obtained from M(NLS-88R)-immunized mice were pooled and injected intravenously into naive BALB/c mice at 100 μL/mouse. Single-splenocyte suspensions free of red blood cells were prepared from mice immunized with a single dose of M(NLS-88R) (H1N1), and 2–4 × 10^7 cells/mouse were adoptively transferred intravenously into naive BALB/c hosts. Twenty-four h later, the recipient mice were challenged with wt-WSN (H1N1) or A/Philippines/2/82/X-79 (H3N2). Mortality was monitored for up to 28 days after infection. In some experiments, a separate set of naive mice were included that received the same volume or number of mu-rWSN (H1N1; 5 × 10^4 pfu given intranasally per mouse)–induced hyperimmune serum or primed splenocytes, to exclude the possibility of nonspecific heterologous protection against A/Philippines/2/82/X-79 associated with transfer process.

Statistical analysis. Comparisons between experimental groups were evaluated using unpaired Student’s t test with a 2-tailed P value. Statistical significance was defined by P < .05.

RESULTS

In vitro and in vivo characterization of M(NLS-88R). Using wt-WSN as the parent strain, we previously have shown that substitution of arginine with serine at amino acid positions 101 and 105 of the RKLKR motif reduced viral replication in MDCK
Figure 3. Protection against homologous virus challenges. Mice were immunized intranasally with M(NLS-88R) (H1N1) at $5 \times 10^4$ plaque-forming units (pfu)/50 μL of phosphate-buffered saline (PBS)/mouse, and serum and spleens were harvested and pooled at 2 months after infection. Immunized mice were then challenged intranasally with wild-type A/WSN/33 (wt-WSN, H1N1) at pfu/50 μL of PBS/mouse (A) or A/FM/1/47-MA (FM, H1N1) at pfu/50 μL of PBS/mouse (B) at 4 weeks after infection. Naïve mice were transferred intravenously with M(NLS-88R)-specific hyperimmune serum (100 μL/mouse) or M(NLS-88R)-primed splenocytes (2 × 10^7 cells/mouse) at 24 h before lethal challenges with wt-WSN at 5 × 10^5 pfu/mouse (C) or at 1 × 10^5 pfu/mouse (one 50% mouse lethal dose) (D). Body weight (BW) and mortality were monitored for at least 2 weeks after infection. The nos. in parentheses denote the no. of survivors/total no. of mice in each group.

cells and resulted in ts in vitro and attenuation in vivo [10]. However, the rescue process of this R101S-R105S double mutant was quite tricky, and the rescued R101S-R105S was unstable, with some phenotypes altered after multiple passages in embryonated eggs (data not shown). Hence, we introduced a third mutation at position 88, with an arginine substituted for a glycine, which was a compensatory mutation that emerged during the attempts of rescuing R10S-R105S; the resultant triple mutant was designated “M(NLS-88R)” (Table 1). Unlike an early passage of the double mutant R101S-R105S that had >2 logs of reduction in viral titers when the temperature increased from 33°C to 39°C, the M(NLS-88R) replicated equally efficiently at both temperatures (Figure 1A), indicating that this new mutant was not ts. M(NLS-88R) also demonstrated an improved replication potential, compared with the double mutant R101S-R105S, with a growth curve similar to that of wt-WSN in vitro (Figure 1B). Moreover, M(NLS-88R) remained genetically and phenotypically stable after >15 passages in MDCK cells and/or embryonated eggs (data not shown).

Interestingly, intranasal administration of M(NLS-88R) mutant at a dose range of 10^-1 to 10^5 pfu/mouse resulted in no morbidity or mortality (Figure 1C and data not shown), indicating an attenuated phenotype with an MLD_50 >1 × 10^6 pfu/mouse. Conversely, wt-WSN at a dose of $5 \times 10^4$ pfu/mouse caused a mortality rate of 100% among infected mice by day 7 after infection (Figure 1C). The attenuation of M(NLS-88R) in mice was further confirmed by significant reductions in lung viral replication, compared with wt-WSN (Figure 1D). By day 7 after infection, M(NLS-88R) was completely cleared from the respiratory tract (with viral titers below the detection limit), whereas wt-WSN virus still sustained a significantly high level of replication in mouse lungs at the same time (Figure 1D). Systemic spread of virus to the brain was not detected with either M(NLS-88R) or wt-WSN (data not shown), indicating that replication of these viruses was restricted to the murine respiratory tract.

Immunogenicity of M(NLS-88R). A single intranasal administration of M(NLS-88R) elicited a strong antibody response against the wt-WSN virus 4 weeks later, with a geometric mean HAI titer >380 (Figure 2A). Even at 1:5000 dilution, the M(NLS-88R)-induced hyperimmune serum samples still yielded >40% plaque reduction than did naive mouse serum-treated control, exhibiting strong neutralizing activity toward wt-WSN (Figure 2B). Moreover, M(NLS-88R) immunization completely prevented pulmonary replication of wt-WSN in challenged mice on day 3 after infection (below the limit of
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Protection against homologous subtype (H1N1) challenges. A single dose of M(NLS-88R) not only fully protected mice from morbidity or mortality after a lethal wt-WSN challenge (Figure 3A) but also fully protected mice from lethal infection with an antigenically distant FM H1N1 virus (Figure 3B). Depletion of the CD4 or CD8 T cell population had no effect on the body weight and survival of M(NLS-88R)-primed mice after a lethal wt-WSN challenge (data not shown), suggesting that humoral response but not T cell immunity may be responsible for M(NLS-88R)-induced homologous protection. To confirm this finding, we conducted transfer studies using M(NLS-88R)-induced hyperimmune serum and splenocytes. As shown in Figure 3C, passive transfer of M(NLS-88R)-induced hyperimmune serum samples protected the recipient mice from lethal challenge with wt-WSN, except for transient morbidity that occurred on day 3 after infection. However, the mice receiving M(NLS-88R)-primed splenocytes showed significant morbidity similar to that among naive mice, but they eventually all survived (Figure 3D); this outcome indicated that cell-mediated immunity was still beneficial, but apparently it had a relatively limited role in homologous protection.

Protection against heterologous viruses, including HPAI A/Vietnam/1203/2004. We sought to determine whether priming with M(NLS-88R) (H1N1) could protect mice from heterologous challenges. After the challenge of A/Philippines/2/82/X-79, an H3N2 reassortant between A/Philippines/2/82 and A/PR/8/34 (a vaccine strain used during the 1983–1984 flu season), all naive mice died (Figure 4A). However, M(NLS-88R)-immunized mice all survived, despite experiencing transient weight loss within the first 6 days (Figure 4A). M(NLS-88R)-primed mice also exhibited significantly reduced pulmonary replication of A/Philippines/2/82/X-79 (H3N2) on day 3 after infection, compared with naive mice (P<.01) (Figure 4B).

A/Vietnam/1203/2004/X-PR8 (H5N1) is a human vaccine strain that contains a multibasic amino acid motif–deleted H5 HA and wt N1 genes from the HPAI A/Vietnam/1203/2004 and 6 internal genes from PR8. This reassortant is attenuated in ferrets and chickens but is lethal to mice at high infectious doses. As shown in Figure 4C, all M(NLS-88R)-vaccinated mice, but not naive mice, survived a lethal challenge with this reassortant virus 2 months after initial immunization. When chal-
Figure 5. Protection against challenge with highly pathogenic avian influenza (HPAI) H5N1 A/Vietnam/1203/2004. Mice immunized intranasally with M(NLS-88R) (H1N1) at plaque-forming units (pfu)/50 μL of phosphate-buffered saline/mouse were challenged intranasally 3 months later with 2 hundred 50% mouse lethal dose (MLD50) of highly pathogenic avian influenza (HPAI) A/Vietnam/1203/2004. Virus titers in lungs, brains, and noses on day 6 after challenge with HPAI A/Vietnam/1203/2004 were determined by titration in eggs (4 mice/group) (A). Body weight (BW) and survival were monitored for 2 weeks after infection (6 mice/group) (B). The detection limit was (lung) or 50% egg infectious dose (EID50) per milliliter (nose and brain). The nos. in parentheses denote the no. of survivors/total no. of mice in each group. ** , by unpaired Student’s t test performed on log-transformed data.

DISCUSSION

The RKLKR motif of M1 protein is involved in both RNA and RNP binding and nuclear localization signal of influenza viruses [7, 8]. In addition to 2 previously reported mutations (R101S and R105S) in the RKLKR motif, a third mutation (G88R) was introduced into the nearby region in the present study; this not only alleviated the rescue process, it also improved the stability of M(NLS-88R)-immunized mice was delayed until 6 months after initial immunization, M(NLS-88R)-immunized mice were still protected from the lethality of A/Vietnam/1203/2004 (Figure 4D). These results suggested that immunization with M(NLS-88R) induced strong and long-lasting heterologous protection against influenza A viruses. A similar protective effect was observed in M(NLS-88R)-vaccinated mice after a lethal challenge with another PR8 reassortant virus bearing modified H5 HA and wt N1 gene derived from the clade 2 HPAI H5N1 A/Indonesia/05/2005 (data not shown). More importantly, when challenged with HPAI A/Vietnam/1203/2004, a clinical isolate recovered from a fatal human case, M(NLS-88R)-immunized mice had significantly reduced viral titers in lungs, brains, and noses, compared with naive mice (Figure 5A), and they were completely protected from the lethality of HPAI A/Vietnam/1203/2004 without obvious body weight loss (Figure 5B).

Because live attenuated vaccines are able to elicit CD8+ T cells against conserved epitopes that are shared by different influenza A strains [5], we assessed epitope-specific CD8+ T cell responses in M(NLS-88R)-immunized mice. As shown in Figure 6A, NP147–155 epitope–specific CD8+ T cells were readily detected in murine lungs by day 7 after infection with M(NLS-88R) (H1N1) (Figure 6A). Adoptive transfer of M(NLS-88R)-primed splenocytes resulted in a survival rate of ~50% after lethal challenge with A/Philippines/2/82/X-79 (H3N2), whereas 90% of naive mice died (Figure 6B). mu-rWSN (H1N1), a ca WSN mutant at a single intranasal inoculation of 5 × 10^4 pfu/mouse, provided complete homologous protection against wt WSN (H1N1) but no cross-protection against A/Philippines/2/82/X-79 (H3N2) (data not shown). We hence included a set of naive mice transferred with mu-rWSN–primed splenocytes as an internal control, to exclude the possibility of nonspecific protection associated with transfer process. Unlike the mice receiving M(NLS-88R)-primed splenocytes, adoptive transfer of the same number of mu-rWSN–primed splenocytes protected ~20% mice from the lethality of A/Philippines/2/82/X-79 (Figure 6B), indicating that the transferred partial heterologous protection observed was specific for M(NLS-88R)-primed splenocytes. Surprisingly, the passive transfer of M(NLS-88R)-induced hyperimmune serum also resulted in ~50% of mice surviving lethal infection with A/Philippines/2/82/X-79 (Figure 6C), although no antibodies cross-reacting with A/Philippines/2/82/X-79 (H3N2) or A/Vietnam/1203/2004/X-PR8 (H5N1) were detected in M(NLS-88R)-specific hyperimmune serum by HAI or microneutralization assays (data not shown). Similar to passive transfer of mu-rWSN–primed splenocytes, passive transfer of mu-rWSN (H1N1)–specific hyperimmune serum yielded no better cross-protection against A/Philippines/2/82/X-79 (H3N2) than did serum from naive control mice (Figure 6C), suggesting that M(NLS-88R)-specific hyperimmune serum was indeed responsible for the partial cross-protection transferred to the recipient mice.
Figure 6. Protective mechanisms of M(NLS-88R) immunization against heterologous influenza virus challenge. Murine lungs were harvested on day 7 after M(NLS-88R) (H1N1) immunization (plaque-forming units/H11003 pfu/50 mL of phosphate-buffered saline/mouse) to assess NP 147–155-specific CD8⁺ T cells by flow cytometry. Representative dot plots of lung NP147–155-specific CD8⁺ T cells were shown (A). Naive mice were intravenously transferred with M(NLS-88R) (H1N1)– or mu-rWSN (H1N1)–primed splenocytes (cells/mouse) (B), or M(NLS-88R- or mu-rWSN–specific hyperimmune serum (100 µL/mouse) (C) at 24 h before lethal challenges with A/Philippines/2/82/X-79 (H3N2; pfu/mouse) (9–10 mice/group). PE, phycoerythrin.

Passive transfer of M(NLS-88R)-specific hyperimmune serum completely protected the recipient mice against lethal wt-WSN infection, clearly suggesting that an optimal antibody response is crucial for homologous protection. We further demonstrated that cell-mediated immunity could provide moderate protection against homologous viruses, although it is unable to prevent infection as efficiently as an antigenically matched antibody response. This finding suggests that preexisting cellular immunity could be beneficial in “buying” time for the host to mount a sufficient humoral response against influenza infections, even if preselected vaccine strains do not exactly match the circulating viruses. In this regard, live attenuated vaccines may be advantageous over inactivated vaccines, because the former are likely to be better at eliciting cellular immunity.

In addition, infection with M(NLS-88R) or wt-WSN yielded no detectable antibodies against H3N2 or H5N1 viruses, by HAI or microneutralization assay, or cross-reactive antibodies to recombinant H3 HA or H5 HA, by enzyme-linked immunosorbent assay (ELISA) (data not shown). However, M(NLS-88R)-primed mice were still protected from subsequent lethal challenges with heterologous influenza A viruses, which was partially attributed to M(NLS-88R)-induced humoral response. This could be the result of insensitivity of the current detection methods to measure the minute portion of crucial antibodies masked by nonspecific immunoglobulin (Ig) G or serum proteins or the fact that mechanisms other than HA- or NA-specific of the resultant mutant M(NLS-88R). More importantly, the additional mutation at position 88 (G88R) of the M1 protein restored viral replication to the wt level in vitro, suggesting that the RKLKR motif of M1 protein and its adjacent region are both involved in controlling viral replication. However, the M(NLS-88R) mutant still exhibited significantly reduced pathogenesis in vivo and was attenuated in mice even at high infectious doses (eg, 10⁶ pfu/mouse). An H5N1 mutant with an M2 cytoplasmic tail deletion was also reported to have reduced pathogenesis in mice, despite a growth rate similar to that of the wt strain in vitro [17]. These results suggest that in vitro replication apparently does not always correlate with viral lethality in vivo. In addition, M(NLS-88R)-immunized mice were protected from lethal infections with both homologous and heterologous influenza A viruses, including HPAI A/Vietnam/1203/2004 (H5N1), after a single vaccination dose. All of these findings suggest that modifying the RKLKR motif and its adjacent region of the viral M1 gene could be a potential approach to attenuating wt influenza A viruses for vaccine development. However, modifying only the M1 gene is insufficient to warrant the safe application of resultant live attenuated vaccine candidates in humans. This can be improved by incorporating additional mutations into the other internal genes [13, 16], such as NP, PB1, and PB2, that are currently under investigation.
IgG or IgA have not yet been identified. Early studies suggest that antibodies to conserved viral internal proteins, such as NP or M1, do not play a significant role in protection in vivo [18–21]. However, a recent study reported that NP-specific non-neutralizing antibodies could significantly reduce morbidity, lower viral titers, and transfer protection to naive mice [22]. A separate study conducted by the same group also suggested that nonneutralizing antibodies conferred heterosubtypic protection only in the presence of memory T cells [23]. The transferred heterosubtypic protection provided by M(NLS-88R)-specific hyperimmune serum is also unlikely because of M2-specific nonneutralizing antibodies, because M2 is a small transmembrane protein existing in a minimal quantity in viral particles, and because natural infection induces a poor antibody response to M2 [7, 23–25]. Epitope-specific CD8+ T cells can facilitate viral clearance and contribute to heterosubtypic protection against H5N1 viruses [29, 30]. Vaccination with M(NLS-88R) also yielded a considerable level of NP147–155-specific CD8+ T cells, but adoptive transfer of M(NLS-88R)-induced splenocytes cells was insufficient to fully protect recipient mice against the heterologous challenge. This is perhaps not surprising, because B cells—but not T cells—are vital for heterosubtypic protection, and memory T cells are less efficient in providing cross-protection in the absence of B cells [23, 30].

Taken together, our results suggest that modifying the RKLKR motif of the M1 gene and its adjacent region could be a potential approach to attenuating influenza viruses for vaccine development with broad cross-protection.

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References


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