H5N1 influenza A virus with K193E and G225E double mutations in haemagglutinin is attenuated and immunogenic in mice

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Live-attenuated influenza vaccines (LAIVs) are now available for the prevention of influenza, with LAIV strains generally derived from serial passage in cultures or by reverse genetics (RG). The receptor-binding domain (RBD) in haemagglutinin (HA) of influenza virus is responsible for viral binding to the avian-type 2,3-α-linked or human-type 2,6-α-linked sialic acid receptor; however, the virulence determinants in the RBD of H5N1 virus remain largely unknown. In the present study, serial passage of H5N1 virus A/Vietnam/1194/2004 in Madin–Darby canine kidney cells resulted in the generation of adapted variants with large-plaque morphology, and genomic sequencing of selected variants revealed two specific amino acid substitutions (K193E and G225E) in the RBD. RG was used to generate H5N1 viruses containing either single or double substitutions in HA. The RG virus containing K193E and G225E mutations (rVN-K193E/G225E) demonstrated large-plaque morphology, enhanced replication and genetic stability after serial passage, without changing the receptor-binding preference. Importantly, in vivo virulence assessment demonstrated that rVN-K193E/G225E was significantly attenuated in mice. Microneutralization and haemagglutination inhibition assays demonstrated that immunization with rVN-K193E/G225E efficiently induced a robust antibody response against WT H5N1 virus in mice. Taken together, our experiments demonstrated that K193E and G225E mutations synergistically attenuated H5N1 virus without enhancing the receptor-binding avidity, and that the RG virus rVN-K193E/G225E represents a potential H5N1 LAIV strategy that deserves further development. These findings identify the RBD as a novel attenuation target for live vaccine development and highlight the complexity of RBD interactions.

INTRODUCTION

The outbreaks caused by H5N1 viruses in poultry and the occasional cases of human infection have reminded people of past influenza pandemics (Heneberg, 1959; Taubenberger & Morens, 2006). The current human mortality rate for infection with highly pathogenic avian influenza (HPAI) H5N1 is ~53.2% in cases confirmed by the World Health Organization (WHO, 2015). Although the neuraminidase (NA) inhibitors oseltamivir and zanamivir are valuable for the treatment and prevention of H5N1 infections, oseltamivir-resistant H5N1 viruses have been identified from oseltamivir-treated patients (Kiso et al., 2004). Vaccination is well recognized as the most effective method of controlling an emergent influenza virus in the community.

Live-attenuated influenza vaccines (LAIVs) are now available in the USA since 2003. Most LAIVs are derived from serial passage in cultures and are manufactured by propagating the virus in embryonated chicken eggs. The yield of LAIVs is limited by inefficient replication in cells in vitro. Great efforts have been made to select high-yield strains of LAIVs (Hu et al., 2015). Recently, various strategies have been utilized to rationally generate LAIV candidates against H5N1 viruses by using standard eight-plasmid-based reverse genetics (RG) technology. Multiple genes, including NS1, PB2, NS1 and haemagglutinin (HA), were selected as attenuation targets. H5N1 RG viruses derived from A/Viet Nam/1203/04

Three supplementary figures and two supplementary tables are available with the online Supplementary Material.
(VN1203) have been characterized as potential LAIV candidates (Steel et al., 2009). Immunization with H5N1 RG virus containing an 11 aa deletion of M2 protein at the C terminus conferred full protection from lethal WT H5N1 virus challenge in mice (Watanabe et al., 2008). Reassortants possessing a modified H5 HA based on the cold-adapted H2N2 backbone have been proven to provide broad cross-protection in mice and ferrets (Suguitan et al., 2006). Considering the fact that inactivated H5N1 vaccines are generally poorly immunogenic in humans (Leroux-Roels et al., 2007), novel LAIV candidates against H5N1 viruses are urgently required.

Influenza HA glycoprotein is the principal determinant of humoral immunity, and mediates influenza virus binding to host receptors and fusion between virus and host endosomal membranes. HA is synthesized as a precursor polypeptide, HA0, and folds into a homotrimer spike, which is subsequently cleaved into HA1 and HA2 by host proteases. The receptor-binding domain (RBD) is located at the N-terminal HA1 region, covering aa 112–263, and forms a shallow pocket insert into the globular head domain. The HA receptors include 2,3- and 2,6-α-sialic acids (SAs) on the cell surface glycoproteins, which determine the host specificity for infection. Avian influenza viruses preferentially bind 2,3-α-SA, whilst human isolates predominantly bind 2,6-α-SA (Shi et al., 2014). Extensive mutagenesis analysis of the RBD revealed the mechanism of interspecies transmission for H5N1 virus, which helps predict mutant HA-containing variants with human-to-human transmissibility. Results from two human-adapted H1 isolates indicated that the substitutions E190D and G225D completely changed the binding specificity to human-type 2,6-α-SA (Xu et al., 2012; Zhang et al., 2013). The human-adapted HA glycoproteins from H2 and H3 subtypes both differ from their avian-specific HA counterparts by the introduction of two amino acid substitutions, Q226L and G228S (Matrosovich et al., 2000; Pappas et al., 2010). Amongst H5 viruses, the amino acid substitutions that caused a change in receptor-binding preference and stability of the HA protein have been well demonstrated as: N158D, N224K, Q226L and T318I amongst strains from Vietnam, and H110Y, T160A, Q226L and G228S amongst strains from Indonesia (Herfst et al., 2012; Imai & Kawaoka, 2012).

In the current study, the H5N1 A/Vietnam/1194/2004 virus (VN1194), which was isolated from a human case of HPAI A/H5N1 infection, was serially propagated in Madin-Darby canine kidney (MDCK) cells, and multiple adaptive mutations, including K193E and G225E, in the RBD of HA were identified from variants with large-plaque morphology. Furthermore, in vitro and in vivo characterization of the H5N1 RG viruses with K193E and G225E mutations showed that together they significantly attenuated H5N1 virus without changing the receptor-binding preference and immunogenicity.

RESULTS

Adaptation of VN1194 in MDCK cells induces RBD mutations

To generate H5N1 variants, VN1194 virus was serially passed in MDCK cells at 33 °C. As shown in Fig. 1(a), the viruses at passage 1 showed a homogeneous small-plaque morphology in MDCK cells, whilst the proportion of large plaques increased gradually during the passage history and became predominant at passage 7. This observation indicated that these viruses with large-plaque phenotype may have acquired some amino acid change(s). Next, a panel of large-plaque clones were isolated from passages 5 and 7 by plaque purification. Viral genome sequencing of these MDCK adaptive variants revealed that amino acid substitutions were found in both surface proteins (HA and NA) and the inner gene nucleoprotein (NP) (Table 1). Amongst these mutations, G225 and K193 were within the RBD domain. Multiple sequence alignment showed that G225 was highly conserved in all natural H5N1 isolates recorded in GenBank (Fig. S1, available in the online Supplementary Material), with no variants ever identified. K193 was not so conserved and some isolates containing R193 were observed.

To predict the structural effects of K193E and G225E mutations, three-dimensional models of the HA monomer containing the corresponding mutations were generated using PyMol software based on the solved crystal structure of HA of VN1194. As shown in Fig. 1(b), K193 was located in the 190 helix and G225 was located within the 220 loop, both adjacent to the identified critical residues for receptor preference alteration of H5N1 viruses, Q196 (Watanabe et al., 2011) and Q226 (Shi et al., 2014). Whether these novel mutations in the RBD of H5N1 viruses had any effects on receptor-binding remains to be determined.

Characterization of RBD mutant RG viruses

To generate RG H5N1 virus with RBD mutations, either single or double mutation(s) were introduced into the HA of VN1194 using standard RG techniques. All three RG viruses were successfully recovered in 293T cells, and named as rVN-K193E, rVN-G225E and rVN-K193E/G225E. All rescued viruses were confirmed by whole-genome sequencing and no additional mutations were introduced. Plaque-forming assays in MDCK cells showed that both rVN-G225E and rVN-K193E/G225E viruses formed larger plaques as compared with the parental VN1194 virus (Table 2). Interestingly, RG virus rVN-K193E retained the small-plaque morphology.

The growth properties of each mutant virus were next compared with VN1194 in MDCK and A549 cells at various temperatures. The results showed that rVN-K193E and rVN-G225E had generally similar growth properties and peak titres to VN1194 in both cell lines (Fig. 2).
However, the double-mutant rVN-K193E/G225E virus gave higher yields in MDCK cells at 37 °C, and in A549 cells at 33 and 37 °C.

To determine the impact of K193E and G225E mutation on viral receptor-binding properties, direct binding assays were performed as described previously (Matrosovich et al., 2000). As expected, VN1194 showed binding specificity to avian type 2,3-α-SA. All three RG viruses also showed the same preferential binding to 2,3-α-SA as VN1194. Compared with rVN1194 virus, rVN-K193E virus showed enhanced receptor-binding avidity to the synthetic sialylglycopolymer 3′SLN-PAA, but deceased avidity to 6′SLN-PAA (Fig. 3). The rVN-G225E virus showed enhanced binding avidity to both SLN-PAAs as compared with WT VN1194. Meanwhile, rVN-K193E/G225E showed similar binding avidity to 3′SLN-PAA as VN1194, but decreased binding avidity to 6′SLN-PAA.

The genetic stability of these RG viruses was also tested in MDCK cells after serial passage, and viral genome sequencing of the passaged viruses (Fig. S2) showed that both

### Table 1. Summary of mutants of VN1194 variants in all segments.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Passage</th>
<th>PB2</th>
<th>PB1</th>
<th>PA</th>
<th>HA*</th>
<th>NP†</th>
<th>NA†</th>
<th>M</th>
<th>NS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque A</td>
<td>5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>K193E</td>
<td>–</td>
<td>Y256C</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Plaque B</td>
<td>5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Plaque C</td>
<td>7</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>G225E</td>
<td>N109I/P318A</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Plaque D</td>
<td>7</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>K193E</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Plaque E</td>
<td>7</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Plaque F</td>
<td>7</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*H3 numbering.
†H5 numbering.
K193E and G225E substitutions were retained throughout the passage history. Plaque assays in MDCK cells showed that all the passaged viruses retained the same plaque morphology (Fig. S3), which was in agreement with the sequencing results. Together, these results identified a unique genetically stable H5N1 RG virus, rVN-K193E/G225E, with high-yield from cells, but with unaltered receptor-binding preference.

H5N1 virus virulence in mice correlates with virulence in humans, so in vivo virulence of three H5N1 RG viruses was further assayed in adult female BALB/c mice as described previously (Li et al., 2011). As expected, VN1194 was virulent in BALB/c mice and all mice infected with 10 p.f.u. VN1194 died within 7 days. The mean survival time (MST) was calculated to be 7.9 days. Notably, the mouse LD50 (MLD50) of rVN-K193E and rVN-G225E showed a nearly 20-fold increase compared with VN1194, indicating the mutations significantly attenuated H5N1 virus (Table 2). The K193E and G225E mutations showed a synergistic attenuating effect; the MLD50 of the double mutant VN-K193E/G225E was >1000 p.f.u. Results from mouse body weight loss curves also supported the attenuation phenotype of rVN-K193E/G225E (Fig. 4a). Furthermore, viral loads in mouse lungs at 3 and 6 days post-infection demonstrated that the replication of rVN-K193E/G225E was highly restricted as compared with the WT VN1194 virus. Also, both RG viruses with single mutations showed decreased viral titres at 6 days post-infection (Fig. 4b). Together, these in vivo data confirmed that the RG virus rVN-K193E/G225E was significantly attenuated in mice.

**Table 2. MLD50 of RG viruses with mutations in HA**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Plaque diameter (mm)</th>
<th>MLD50*</th>
<th>MST (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rVN1194</td>
<td>0.61 ± 0.20</td>
<td>2.65</td>
<td>7.9</td>
</tr>
<tr>
<td>rVN-K193E</td>
<td>0.56 ± 0.10</td>
<td>50.12</td>
<td>&gt;13</td>
</tr>
<tr>
<td>rVN-G225E</td>
<td>1.32 ± 0.21</td>
<td>48.08</td>
<td>&gt;13</td>
</tr>
<tr>
<td>rVN-K193E/G225E</td>
<td>1.46 ± 0.14</td>
<td>&gt;1000</td>
<td>&gt;13</td>
</tr>
</tbody>
</table>

*Expressed as the p.f.u. to give 1 MLD50. MLD50 values were determined by inoculating groups of BALB/c mice with various doses of the indicated viruses.

**Fig. 2. Growth kinetics of H5N1 RG viruses.** Viral titres were determined by standard plaque assay in MDCK cells at the indicated time points. Mean ± SD titres were calculated from triplicate independent experiments. Data were analysed using two-way ANOVA, and statistically significant differences between the results of rVN-K193E/G225E and rVN1194 virus are represented by asterisks: *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

rVN-K193E/G225E is attenuated in mice

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rVN-K193E/G225E induces protective antibodies against WT H5N1 virus

To investigate the immunogenicity of rVN-K193E/G225E, groups of BALB/c mice were immunized subcutaneously with inactivated rVN-K193E/G225E. Subsequently, neutralization and haemagglutination inhibition (HAI) antibodies against WT H5N1 and each of the RG viruses were tested. As shown in Fig. 5, high-titre neutralization and HAI antibodies were both induced in mice immunized with rVN-K193E/G225E, and there was no significant difference in the geometric mean titre (GMT) against WT VN1194 and rVN-K193E/G225E. It should also be noted that the immune sera showed decreased GMT against rVN-K193E or rVN-G225E. These results suggested the double-mutant rVN-K193E/G225E could induce a considerable humoral immunity response against WT VN1194 virus.

DISCUSSION

Investigation of the genetic basis for the virulence of the HPAI H5N1 virus has identified several key factors in different segments, such as E627K at PB2, N66S at PB1-F2, the PDZ domain in NS1 and the multibasic HA cleavage motif (Conenello et al., 2007; Garten et al., 2009; Zheng et al., 2010; Russell et al., 2012). However, the RBD was rarely reported to associate with virulence of H5N1. In this study, we focused on the influence of RBD mutations on H5N1 influenza virulence. We identified the adaptive mutations K193E and G225E in the VN1194
HA protein after serial passage in MDCK cells, which was associated with an attenuated phenotype of H5N1 virus in mice.

The HA protein of WT H5N1 virus prefers the avian type of SAs rather than the human type of SAs (Gambaryan et al., 1997; Matrosovich et al., 2000; Nobusawa et al., 1991; Rogers & Paulson, 1983), and K193 or R193 of H5 HA is associated with a stronger 2,3-x-SA binding affinity than 2,6-x-SA (Tharakaraman et al., 2013), which is in agreement with our findings (Fig. 3). However, recent experiments have demonstrated that the K193R mutation of H5N1 exhibited increased binding to 2,6-x-SA (Imai & Kawaoka, 2012; Stevens et al., 2006; Wang et al., 2010). The K193E mutation in HA is rare in nature, and our results show that K193E increased the avidity to 2,3-x-SA, but decreased the avidity to 2,6-x-SA. Based on a cold-adapted H5N1 virus of A/Vietnam/1203/2004 (H5N1), G225E was shown to decrease binding avidity to 2,6-x-SA using an enzymically modified chicken red blood cells assay (Wang et al., 2010), again in agreement with our results. Although the G225E single mutation increased the viral binding avidity to both kinds of SAs, the binding avidity of rVN-K193E/G225E was almost the same as that of rVN-K193E. Structural modelling of the mutated HA–receptor complex indicated that G225E changes the conformation between the 220 loop and 130 loop, which enhanced the HA binding avidity to 2,3-x-SA (Fig. 1c). However, the 190 helix was slightly shrunk after K193E, which had little influence on receptor-binding avidity. It should be noted that the H5N1 RG virus rVN-K193E/G225E that contained double mutations remained an avian virus; no change in the binding preference compared with the WT H5N1 virus was observed.

In the current study, RG viruses containing a single mutation of either K193E or G225E barely changed viral growth activity in different cell lines at different temperatures, but double mutations K193E/G225E in HA led to a substantial viral replication advantage in vitro. A number of critical amino acid residues in RBD of H5N1 viruses have been evidenced to influence viral replication. H5N1 virus with a single H196Q mutation in HA produced a substantial growth advantage in human airway epithelial cells (Watanabe et al., 2011). K193S combined with E190D, Q226L and G228S substitutions resulted in weaker viral replication in human Calu-3 bronchial epithelial cells than WT H5N1 virus (Maines et al., 2011). The replication of H5N1 RG virus carrying a G225E substitution in HA remained unchanged in the nasal turbinates and lungs of ferrets (Wang et al., 2010), which was in agreement with our in vitro results (Fig. 2). The biological effects of K193E/G225E double mutations are not yet fully understood; more work is needed to reveal the mechanism behind this phenomenon.

Our virulence tests in mice demonstrated that either K193E or G225E significantly attenuated H5N1 virus and double mutations had synergic effects. Several virulence determinants of H5N1 viruses have been reported. H5N1 RG viruses carrying multiple mutations E190D/K193S/Q226L/G228S, E187G/E190D/K193S/Q226L/G228S, E190D/K193S/Q226L/G228S or K193R/Q226L/G228S showed decreased replication in ferrets compared with WT H5N1 virus (Maines et al., 2011). Based on the RG of A/duck/Egypt/D1Br12/2007,
H196Q, which was near K193E, was suggested to enhance virulence in BALB/c mice (Watanabe et al., 2011). The introduction of K193E/G225E mutations in the RBD results in significant attenuation in vivo and thus K193E/G225E substitution can be deemed as a potential attenuation marker or target for H5N1 viruses. This attenuation strategy might provide a new path to create safer H5N1 strains with a non-virulent phenotype. Additionally, other known virulence and intra-species transmission determinants of H5N1 viruses, e.g. the polybasic site in HA and E627 in PB2, should be carefully removed or modified for safety concerns. It should be noted that H5N1 LAIV would not be used until the H5N1 virus has become widespread amongst humans. For any potential LAIV, specific concerns about the possibility of reassortment with natural influenza strains are valid.

Amino acid substitutions in the RBD would probably result in antigenic change. Several positions in HA adjacent to RBD, e.g. N193S and D193N, have been reported amongst H3N2 viruses (Koel et al., 2013). In our experiments, coinfection with rVN-K193E/G225E induced both neutralization and HAI antibodies against WT H5N1 virus, and there was no significant difference between the GMT against rVN-K193E/G225E and WT VN1194. However, compared with WT H5N1 virus, both neutralization and HAI titres against RG viruses with a K193E or G225E mutation decreased (Fig. 5b). Thus, these data suggested that rVN-K193E/G225E retained the immunogenicity of the WT H5N1 virus.

In summary, combining natural passaging and RG, we revealed that K193E and G225E mutations in HA synergistically attenuate H5N1 virus, and the resulting double mutant rVN-K193E/G225E replicates potently in various cell cultures and induces robust neutralization antibodies against WT H5N1 virus. This live-attenuated H5N1 strain rVN-K193E/G225E shows no change in receptor-binding preference and is genetically stable after serial passage. These properties make rVN-K193E/G225E a potential LAIV candidate against H5N1 that deserves further development.

METHODS

Cell and viruses. Madin–Darby canine kidney (MDCK) cells, human embryonic kidney cells expressing SV40 large T-antigen (293T) and human A549 lung epithelial cells were cultured in Dulbecco’s modified Eagle medium (DMEM; Gibco) supplemented with 10% FBS (Gibco) containing 10 mM HEPES and non-essential amino acids at 37 °C in a CO₂ incubator. The H5N1 VN1194 influenza virus was propagated once for 48 h in the allantoic cavities of 11-day-old chicken embryoembryonated eggs and virus titre was assayed by standard plaque assay on MDCK cells (Dinh et al., 2011). All experiments involving live H5N1 virus were performed in a Biosafety Level 3 facility at Beijing Institute of Microbiology and Epidemiology.

Serial passage of VN1194. VN1194 virus was serially passaged eight times in MDCK cells at 33 °C to select variants that exhibited large-plaque morphology. Briefly, MDCK cells were infected with 10³ PFU VN1194 virus in 1.5 ml DMEM (Invitrogen) containing 2% FBS in 12-well plates at 33 °C in a CO₂ incubator. The supernatant of infected cells was collected when the cytopathic effect (CPE) reached 80–90%. The initial stock of VN1194 virus was referred as passage 0, and the subsequently passaged viruses were referred to as passages 1–7. The supernatants of passages 1, 3, 5 and 7 were collected and used for in the plaque-forming assay. The plaques with large morphology at passages 5–7 (eight plaques per passage) were isolated. A random panel of variants was selected for plaque purification as described previously (Wang et al., 2010). The MDCK-adapted mutants were subsequently cloned by plaque purification in monolayer cells of MDCK. Viral RNAs were extracted by a RNA Mini kit (Life Technologies). The viral RNAs were reverse transcribed using the Uni-12 primer (Hoffmann et al., 2001) followed by PCR using Pyrobest (TaKaRa). The primers are shown in Table S1. The cDNA of each segment was cloned into pEASY (Transgen) vectors for DNA sequencing.

Generation of VN1194 RG viruses. VN1194 RG viruses with specific mutations in HA were rescued by RG techniques as described in our previous study (Li et al., 2011). Briefly, HAs containing K193E, G225E and K193E/G225E were generated by PCR-based site-directed mutagenesis using primers (Table S2) in the VN1194 HA background. All constructs were confirmed by complete sequencing to ensure the absence of unwanted mutations. The mutant HA genes were cloned into pHW2000 plasmids. 293T cells were transfected with the eight-plasmid (mutant HA combined with VN1194 backbone) mixture using Lipofectamine 2000 (Invitrogen) at 37 °C. The supernatant of the transfected cells was collected 48 h post-transfection and inoculated into MDCK cells with infection medium. The rescued viruses were propagated in MDCK cells and each segment of the rescued viruses was sequenced accordingly. The plaque sizes of RG viruses were measured by micrometer callipers after staining with crystal violet (Murakami et al., 2008) and at least 10 plaques for each virus were counted for statistical analysis.

The genetic stability of RG viruses was evaluated by sequence analysis and plaque-forming morphology. The RG viruses were serial passaged eight times in MDCK cells and viruses of each passage were sequenced. The plaque-forming morphologies of viruses from each passage were detected on MDCK cells.

Growth curves of viruses in MDCK and A549 cells. The growth curves of VN1194 and RG viruses were assessed by inoculation of MDCK cells at m.o.i. 0.001 and A549 at m.o.i. 0.01 in 24-well plates. Cells were incubated at 33, 37 and 39 °C for 1 h, followed by washing three times with PBS. Next, the cells were cultured with 1 ml DMEM (2% FBS) per well at 33, 37 and 39 °C. The supernatants were sampled at 12, 24 and 48 h after inoculation and titrated by plaque-forming assay on MDCK cells. The growth curves were evaluated in three independent experiments.

Haemagglutination assay. All viruses were diluted (twofold) serially in V-bottom 96-well plates and 50 µl washed chicken red blood cells (1%, v/v) were incubated with 50 µl virus dilutions. The plates were kept at room temperature for 60 min (Gulati et al., 2005). The reciprocal of the highest dilution which showed the haemagglutination of the chicken red blood cells was calculated as the HA titre.

Solid-phase binding assay. Receptor-binding avidities of influenza viruses to 2,3-α- or 2,6-α-linked receptor analogues were measured using an adapted solid-phase binding assay as previously described (Matrosovich et al., 2000). In brief, viruses were concentrated by high-speed centrifugation through a 25% sucrose cushion. 1.5 ml of synthetic biotin-labelled sialylglycoplyomers, 3’SLN-PAA and 6’SLN-PAA, that contained the Neu5Ac2,3-α-2,6-Gal and Neu5Ac2,6-α-2,6-Gal moieties, respectively, were from Glycotech. A total of 1 µl resuspended virus with a titre of 128 haemagglutination units in PBS was adsorbed onto a 96-well ELISA plate (Costar) at 4 °C overnight. After addition of 3’SLN and 6’SLN at different concentrations in the presence of NA inhibitor xanamivir (Sigma), the plate was incubated
for 2 h at room temperature. Then, samples were washed and incubated with streptavidin-conjugated HRP for 1 h at 37 °C. The substrate solution (0.01% 3,3′,5,5′-tetramethylbenzidine and 0.004% hydrogen peroxide in citrate-phosphate buffer) was added. The reaction was stopped by 2 M H2SO4 and the absorbance measured.

**Mouse studies.** All animal experiments were approved by and performed according to the institution’s Experimental Animal Ethics and Welfare Committee. Groups of 6-week-old female BALB/c mice were anaesthetized with 1% napental and inoculated intranasally with 10-fold dilutions of each mutant virus and VN1194. The MLD50 values were calculated using the method described previously (Muench & Reed, 1938) and expressed as p.f.u. Mice were monitored daily for 2 weeks and body weight was recorded every day. Mice intranasally infected with 10 p.f.u. H5N1 virus were terminated on days 3 and 6 post-infection, and the entire mouse lung was removed and homogenized in 1 ml PBS; TCID50 ml−1 titres were determined by the standard method.

**Mouse immunization.** Six-week-old female BALB/c mice were immunized subcutaneously with various doses in 50 μl (107 p.f.u. per mouse) formalin-inactivated rV-K193E/G225E in Freund’s incomplete adjuvant (Sigma) at weeks 0 and 3 as previously described (Prabakaran et al., 2010). Mice injected with PBS were used as a control group. Two weeks after the second immunization, terminal bleeds were collected, and subjected to microneutralization and HAI assays.

**Microneutralization test.** The microneutralization test was performed following a previously described protocol (Suguitan et al., 2010). In brief, MDCK cells were cultured in 96-well culture plates to form a monolayer at 37 °C. Heat-inactivated (56 °C for 30 min) serum samples were serially twofold diluted with cell culture medium and mixed at a ratio of 1:1 with 100 TCID50 H5N1 virus and incubated at 37 °C for 1 h. The mixtures were transferred to MDCK cells in triplicate wells. The neutralizing titres of mouse antiserum were defined as the reciprocal dilutions that prevented CPE completely (Mayrhofer et al., 2009).

**HAI assay.** The standard HAI assay was performed as previously described (Chen et al., 2012). In brief, heat-inactivated mouse serum was treated with receptor-destroying enzyme at 37 °C overnight. Then, 25 μl twofold serially diluted serum samples were added into 96-well V-bottom microplates. Four haemagglutination units of the indicated viruses (25 μl) were added into the serum, mixed and incubated at room temperature for 30 min. Next, 25 μl chicken red blood cells (1%) was added to each well and incubated for 45 min. The reciprocal of the highest serum dilution that inhibited virus haemagglutination was defined as the HAI titre. GMTs of the HAI assay were calculated from samples of three mice.

**Homology modelling of HA protein structure.** The solved crystal structure of H5N1 VN1194 virus HA (Protein Data Bank ID: 2IBX) with human receptor was used as a template. HA tertiary structures of glycoprotein monomers of RG viruses were simulated and generated as Protein Data Bank files using the 3D-JIGSaw homologous database server (Contreras-Moreira & Bates, 2002; Zhou et al., 2011). All of the HA structure was extracted by PyMol 1.3 and used as an HA monomer (Ekier et al., 2009). Molecular docking calculations were performed using AutoDock 4.2 (Tran-To Su et al., 2013).

**Statistical analysis.** Data from the solid-phase assay, growth kinetics and viral titres in mouse lungs were analysed using a two-way ANOVA method and corresponding P values were calculated. The results of microneutralization and HAI assays were analysed using Student’s t-test. All statistical analyses were performed using GraphPad Prism.

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**REFERENCES**


