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Elucidating the characteristics of *Mx1* and resistance to influenza A virus subtype H1N1 in the newly developed KWM/Hym mice

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Abstract

Background: Inbred mice have several advantages, including genetic similarity to humans, a well-established gene manipulation system, and strong tolerance to inbreeding. However, inbred mice derived from a limited genetic pool have a small genetic diversity. Thus, the development of new inbred strains from wild mice is needed to overcome this limitation. Hence, in this study, we used a new strain of inbred mice called KWM/Hym. We sequenced the *Mx1* gene to elucidate the genetic diversities of KWM/Hym mice and observed the biological alterations of the Mx1 protein upon influenza A infection.

Results: The *Mx1* gene in KWM/Hym mice had 2, 4, and 38 nucleotide substitutions compared to those in the *Mx1* gene in A2G, CAST/EiJ, and *Mus spretus* mice, respectively. Moreover, the Mx1 protein in KWM/Hym mice had 2 and 25 amino acid substitutions compared to those in the Mx1 protein in CAST/EiJ and *M. spretus* mice, respectively. To elucidate the function of the Mx1 protein, we inoculated the influenza A virus (A/WSN/1933) in KWM/Hym mice. Nine days after infection, all infected KWM/Hym mice survived without any weight loss. Four days after infection, the lungs of the infected KWM/Hym mice showed mild alveolitis and loss of bronchiolar epithelium; however, the pulmonary viral titers of the infected KWM/Hym mice were significantly lower than that in the infected BALB/c mice ($2.17 \times$ plaque-forming units mL^{-1}).

Conclusions: Our results demonstrate that the KWM/Hym mice are resistant to influenza A virus infection. Further, these mice can be used as a model organism to understand the mechanism of influenza A virus susceptibility.

Keywords: Influenza A (H1N1) virus, *Mx1* gene, KWM/Hym mice, Alveolitis

Background

The type A influenza virus causes seasonal infections, resulting in illness and death every year. However, mutations in the influenza A virus led to the emergence of the novel H1N1 virus, which caused respiratory diseases in humans and was responsible for the 2009 swine-flu pandemic [1]. The emergence of new influenza virus strains highlights the need to better understand the pathogenic mechanisms of influenza virus-host interactions since the genetic variation of hosts affects the infection of the influenza A virus subtype H1N1 [2].

Mx genes exist in almost all vertebrate genomes and serve as a defense against RNA viruses. Mx proteins are evolutionarily conserved in vertebrates, suggesting that they are critical for antiviral defense across species [3]. The interferon-induced GTP-binding protein, Mx1, is one such antiviral protein that restricts influenza viruses

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in humans and mice, although the effect depends on the virus strain. The mouse *Mx1* (myxovirus resistance protein 1, Mx dynamin-like GTPase 1, and interferon-inducible protein P78) gene encodes the Mx1 protein, an interferon-inducible nuclear protein, that selectively inhibits influenza A and Thogoto viral multiplication [4].

The house mouse is classified into four subspecies (*M. m. domesticus, M. m. musculus, M. m. castaneus,* and *M. m. bacterianus*) based on their biochemical markers, mitochondrial DNA, and other genetic characters [5–7]. The genetic background of the host is critical for susceptibility to influenza A virus infections in mice [8, 9]. Most laboratory inbred mouse (*M. m. domesticus*) strains, including BALB/c, carry nonfunctional *Mx1* alleles because of deletions or nonsense mutations and consequently exhibit high virus susceptibility [10]. The A2G mice have a wild type *Mx1* allele and resistance against influenza and Togota viruses [10, 11]. Interestingly, several wild-derived strains, including M. spretus, PWK/PhJ, and NZO/HILtJ carry a wild-type *Mx1* allele and are highly resistant to the influenza virus [12]. Interestingly, several wild-derived strains, including SPRET/Ei, PWK/PhJ, and NZO/HILtJ carry a wild-type *Mx1* allele and are highly resistant to the influenza virus. However, wild-derived CAST/EiJ mice carrying two amino acid changes in the Mx1 restriction factor exhibit high susceptibility to influenza A [13, 14]. The wild mice captured in South Korea have mixed genetic components of *M. m. molossinus* and *M. m. musculus* [15, 16]. KWM/Hym mice, a new strain developed from the wild mice captured from the Chuncheon city in Korea, show a 96.4% similarity with PWK/Phj in single nucleotide polymorphism analysis [16]. However, the sequence of the *Mx1* gene, the functional features of Mx1 protein, and whether Mx1 can provide resistance to influenza A virus in the KWM/Hym mice have not been reported yet. Hence, in this study, we investigated the influenza A (H1N1) virus susceptibility of KWM/Hym mice to determine if the strain will be useful for studying influenza A virus infection.

Results

The Mx1 protein sequence of KWM/Hym mice differed at two sites from Mx1 of CAST/EiJ

We sequenced the *Mx1* gene of KWM/Hym mice to determine the functional difference between the *Mx1* gene of the KWM/Hym and CAST/EiJ mice. As shown in Fig. 1, the *Mx1* gene in KWM/Hym mice had four nucleotide substitutions compared to the *Mx1* gene in CAST/EiJ mice (derived from *M. m. castaneus*). The mutations were as follows; G to A at position 247, C to A at position

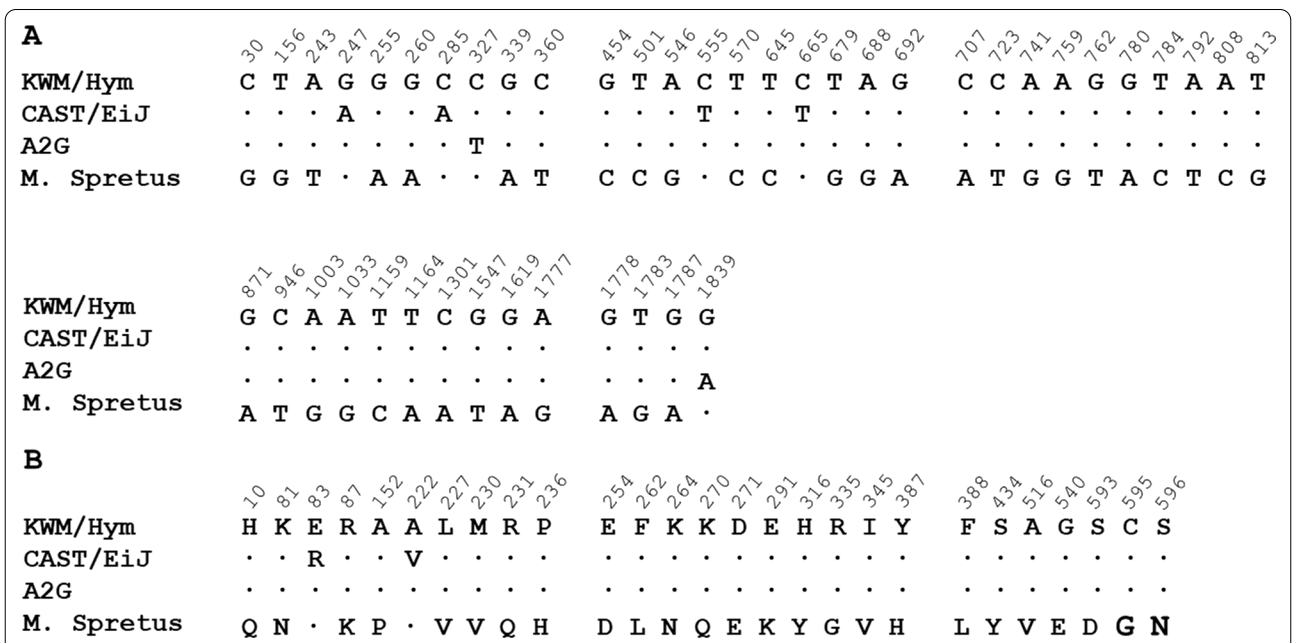

Fig. 1 The alignment of nucleotide sequences and amino acids in Mx1 of KWM/Hym mice. **A** The sequence of the *Mx1* gene in KWM/Hym has variation at two positions (327T>C and 1839 A>G) compared to that in A2G mice (*M. m. domesticus*). The sequence of the *Mx1* gene in KWM/Hym has variation with 4 and 38 sites compared to those in CAST/EiJ (*M. m. castaneus*) and *M. spretus* mice, respectively. **B** The KWM/Hym mice have two missense mutations (G83R, A222V) in the G domain of Mx1 protein compared to the protein in CAST/EiJ mice. The Mx1 protein in KWM/Hym compared to the protein in *M. Spretus* has 25 missense mutations, with 13 sites in the G domain, eight in the stalk domain, two in the loop L4 domain, and two in the bundle-signaling element domain. The amino acid sequences were aligned using the T-Coffee Multiple Sequence Alignment Program (https://www.ebi.ac.uk/Tools/msa/tcoffee/)

285, C to T at position 555, C to T at position 665. The sequences of *Mx1* gene had two nucleotide substitutions between the A2G and KWM/Hym mice(T to C at position 327 and A to G at position 1,829). Further, the *Mx1* gene in KWM/Hym mice had 38 nucleotide substitutions compared to the *Mx1* gene of *M. spretus* (Fig. 1A).

The Mx1 protein in KWM/Hym had two amino acid changes compared to the CAST/EiJ mice; G83R (glycine to arginine) and A222V (alanine to valine). The position 83 is located in the G domain of the Mx1 protein and is moderately conserved in vertebrates. The position 222 is also located in the G domain of the Mx1 protein and is highly conserved (Fig. 1B). These two nonsynonymous changes of the Mx1 protein in KWM/Hym mice can cause functional alteration of the Mx1 protein. However, despite two nucleotide substitutions of the *Mx1* gene between A2G and KWM/Hym mice, there are no amino acid substitutions between their Mx1 proteins. Further, there are 25 amino acid substitutions between KWM/Hym and *M. spretus*, indicating that the Mx1 protein of *M. spretus* has an altered function regarding the infection by the pathogenic influenza A virus.

All KWM/Hym mice survived without weight loss after influenza A virus infection

To quantify the effect of host genetics on virus susceptibility, BALB/c and KWM/Hym mice were intranasally inoculated with influenza A virus [A/WSN/1933 (mouse-adapted H1N1)]. All infected KWM/Hym mice survived without weight loss until the experiment ceased, nine days after infection (Fig. 2A). In contrast, the infected BALB/c mice showed a significant weight loss compared to the KWM/Hym mice ($p < 0.001$ for days 4–7 and $p < 0.01$ for day 8). The BALB/c mice exhibited 0% survival by day 9 after infection (Fig. 2B).

Influenza A virus mildly damages lung tissue in KWM/Hym mice

Excessive inflammation can cause severe lung lesions during influenza A infection. To evaluate the histopathological changes in the lungs of A/WSN/1933-infected mice, the lungs of each group at day 4 post-infection were examined. The lungs of A/WSN/1933-infected BALB/c mice showed severe alveolitis and fragmentation of alveolar walls along with the presence of lymphocytes, neutrophils, and plasma cells. In contrast, the A/WSN/1933-infected KWM/Hym mice showed mild alveolitis and loss of bronchiolar epithelium. No lesions were observed in the lungs of the PBS-treated mice (Fig. 3).

Influenza A virus does not replicate in the lungs of KWM/Hym mice

To investigate whether the survival rate and histopathological changes in the A/WSN/1933-infected mice were involved in the influenza A virus replication in the lungs, we checked the pulmonary viral titer. To measure virus titers in the lungs of the infected mice, we performed a plaque assay using MDCK cells. The pulmonary viral titers in the infected BALB/c mice ($2.17 \times$ pfu mL^{-1}) were significantly higher than the titers in the infected

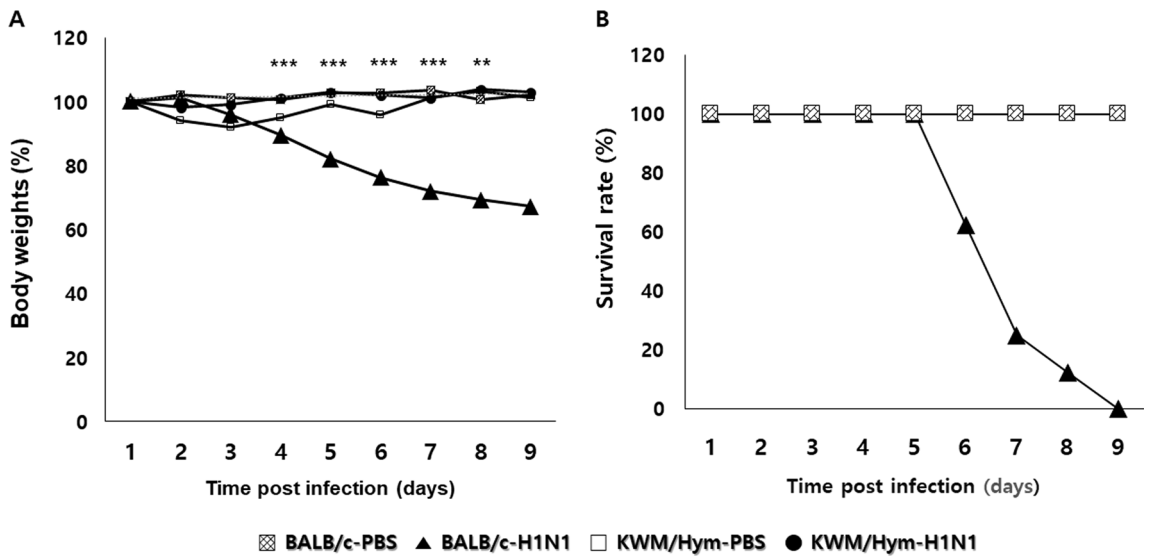

Fig. 2 Effect of influenza A virus infection on inbred BALB/c and KWM/Hym mice. The BALB/c and KWM/Hym female mice were challenged intranasally with mouse-adapted A/WSN/1933 virus (H1N1, 1×10^5 pfu/mouse). The body weight (**A**) and the survival rate (**B**) were monitored for nine days (N = 6/group)

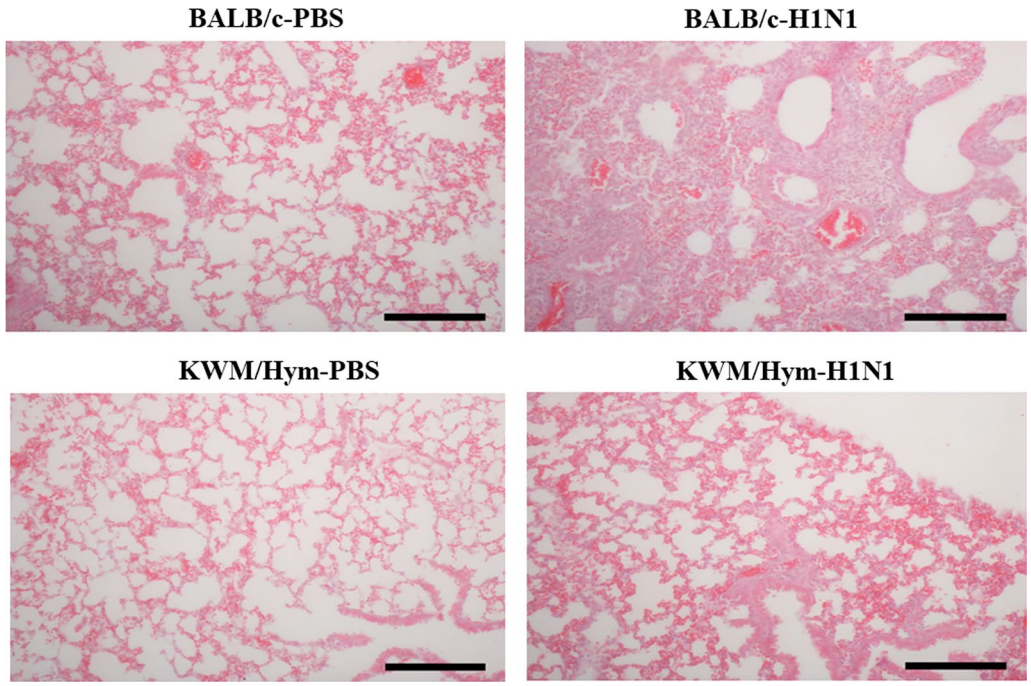

Fig. 3 Histopathological lesions in the lung of inbred BALB/c and KWM/Hym mice. The BALB/c and KWM/Hym female mice (N = 6/group) were challenged intranasally with mouse-adapted A/WSN/1933 virus (H1N1, 1×10^5 pfu/mouse). Four days after infection, the lungs were collected, and hematoxylin and eosin staining was performed. The infected KWM/Hym mice showed mild alveolitis and loss of bronchiolar epithelium, significantly less than that of the infected BALB/c mice (scale bars = 100 μm)

KWM/Hym mice ($p < 0.0005$). No plaques were observed in PBS-treated KWM/Hym and PBS-treated BALB/c mice (Fig. 4).

Discussion

In this study, we sequenced the *Mx1* gene and investigated the susceptibility of the KWM/Hym mice to influenza A virus infection. Sequencing revealed that the KWM/Hym mice carry the wild-type *Mx1* gene. The *Mx1* gene of KWM/Hym mice had 2, 4, and 38 nucleotide substitutions compared to those in the A2G, CAST/EiJ, and *M. spretus* mice, respectively (Fig. 1A). The Mx1 protein in KWM/Hym had two missense mutations compared to the Mx1 protein in CAST/EiJ mice (Fig. 1B). These two missense mutations (G83R, A222V) were located in the G domain of the protein. Further, the Mx1 protein in KWM/Hym had 25 missense mutations compared to the Mx1 protein in *M. spretus*, with 13 sites in the G domain, eight in the stalk domain, two in the loop L4 domain, and two in the bundle-signaling element domain (Fig. 1B). The *Mx1* gene on mouse chromosome 16 has a critical role in influenza A virus infection [17]. The *Mx1* gene is induced by interferon α and β following viral infection. Most inbred mouse strains have alterations in the *Mx1* gene and are susceptible to the influenza A virus [10]. However, some inbred strains (NJL, *Mus musculus*; SPR, *M. spretus*) originating from wild mice have a normal *Mx1* gene and show resistance against the influenza A virus [18, 19]. The loop L4 domain is critical for the binding of Mx1 to influenza A virus [12].

The two positions 83 and 222 on the G domain of Mx1 protein, where we observed amino acid substitutions in the KWM/Hym mice compared to the CAST/EiJ mice, are conserved among vertebrate Mx1 proteins. This suggests that these positions might be important for biological function. To prove our hypothesis, we investigated the resistibility of the KWM/Hym mice to the influenza A virus. As speculated, all influenza A virus-infected KWM/Hym mice survived without weight loss (Fig. 2). The lungs of infected KWM/Hym mice showed mild alveolitis and loss of bronchiolar epithelium on day 4 after influenza A virus infection (Fig. 3). However, the virus was not detected in the lungs of the infected KWM/Hym mice (Fig. 4). These results indicate that KWM/Hym mice have a normal functioning *Mx1* gene and are resistant to infection with the influenza A virus.

An immune response occurs in mice intranasally infected with the influenza A virus [20]. Influenza A virus-reactive IgM and IgG levels in the serum of the influenza A-infected mice decrease and increase, respectively, 5–14 days post-infection [21]. Hence, to confirm the resistance of KWM/Hym mice to the influenza A

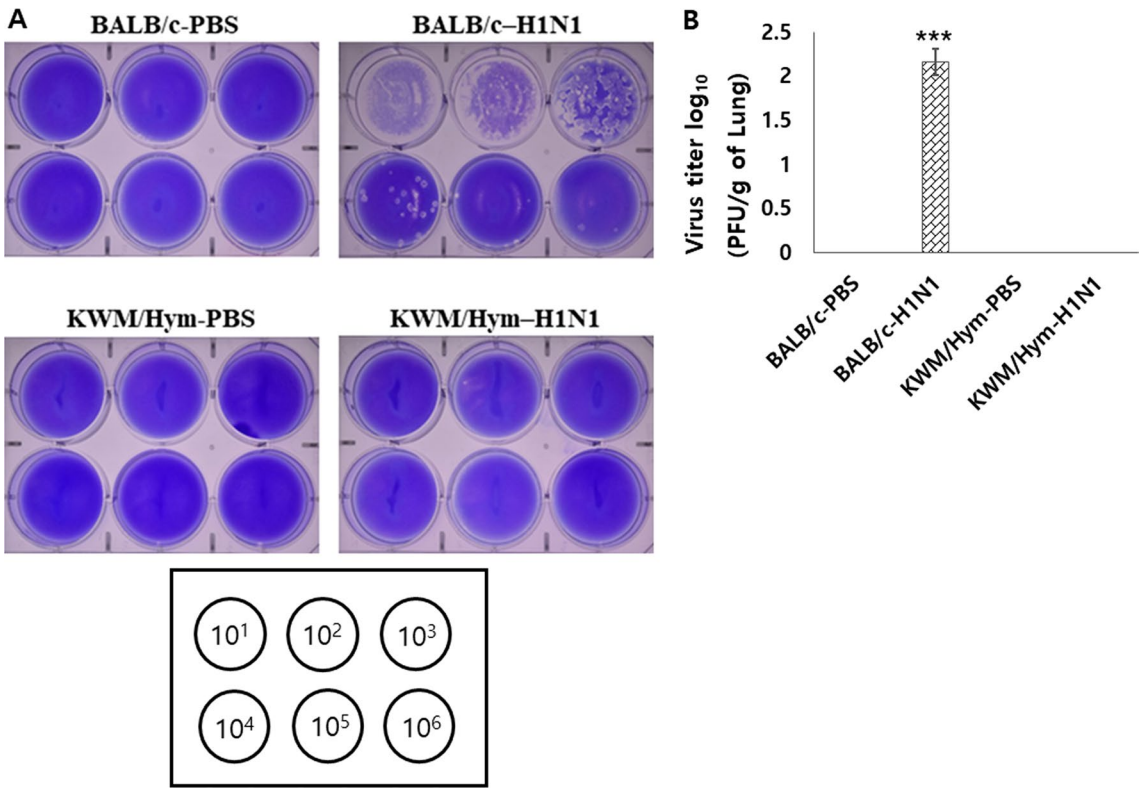

Fig. 4 Influenza A virus infection in the lungs of inbred BALB/c and KWM/Hym mice. BALB/c and KWM/Hym female mice were challenged intranasally with mouse-adapted A/WSN/1933 virus (H1N1, 1×10^5 pfu/mouse). Four days after infection, the lungs were collected and homogenized using a Tissue Lyser II. Virus titers were determined using a plaque assay in lung tissue homogenates. The experiment was performed in triplicates using the Madin-Darby Canine Kidney cells (N = 3/group). ***$p < 0.0005$

virus, the involvement of immune responses, such as the serum levels of influenza A virus-reactive IgM and IgG, should be measured 5 to 14 days post-infection. The pathological changes in the lungs of influenza A virus-infected KWM/Hym mice might be restored at a later stage of influenza A virus infection. Since the *Mx1* gene conveys resistance to infection with the Thogoto virus [22], it is interesting to consider whether KWM/Hym mice also have resistance to Thogoto virus.

Conclusions

Our results indicate that KWM/Hym mice might be useful animals for studying the mechanism of influenza A virus infection. Further, this mouse model may overcome the limitations of the current inbred mouse models that have impaired *Mx1* gene function.

Methods

Animals

Eight-week-old female BALB/c (inbred) and KWM/Hym mice were used in this study. BALB/c mice were purchased from DBL (Eumseong, Korea). KWM/Hym mice were generated from Korean wild mice. The mice were maintained in biosafety level (BSL)-2 conditions at 22 ± 2 °C, 55 ± 10% relative humidity, and a 12 h light and 12 h dark cycle. Normal rodent pellet diet (Cargill Agri Purina, Seongnam, Korea) and water were provided ad libitum. For infection, 1×10^5 plaque-forming units (pfu) virus/mouse were inoculated intranasally. Body weight was measured once a day during the experimental period. All animal experimentation, including animal care, was conducted in accordance with the regulations of the Institutional Animal Care and Use Committees of Hallym University (Hallym-2018–56).

Variant analysis of the *Mx1* gene

Genomic DNA was extracted from the liver of KWM/Hym mice by InstaGene Matrix (Bio-Rad, USA). The primer sequences were as follows: 1F, 5′-GAGTTCTTA AGAACGTCAGAAGG-3′ 1R, 5′-GATACACCAGGT TCCGCATC-3′, 2F, 5′-CAGGAGGTGGACCCTGAA G-3′, 2R, 5′-CGGATCAGGTTTTCAGCTTCC-3′, 3F, v-TGGTCCAAATGCCCTTCGTA-3′, 3R, 5′-AAAGCC ACATAGCTAGCCTGG-3′. PCR was carried out with

Dr. MAX DNA Polymerase (Doctor protein INC, Korea) and subjected to the following conditions: initial denaturation at 95 °C for 5 min, 35 cycles (95 °C for 30 s, 55–60 °C for 30 s, 72 °C for 60 s), final elongation at 72 °C for 7 min. The PCR products were purified using the Multiscreen filter plate (EMD Millipore Corporation, Billerica, USA). The purified PCR products were sequenced by ABI PRISM 3730XL Analyzer with BigDye (R) Terminator v3.1 cycle Sequencing Kit (Applied Biosystems, Foster City, USA). Variant Reporter Software Version 1.1 (Applied Biosystems) was used to detect variants with three reference sequences (CAST/EiJ: KX774216, A2G: AH0020046, *M. spretus*: KT591117.1). All variants of the *Mx1* gene were confirmed from three individual mice. The nucleotide and amino acid sequences were aligned using the T-Coffee Multiple Sequence Alignment Program (https://www.ebi.ac.uk/Tools/msa/tcoffee/).

Influenza A virus culture

Influenza A virus [A/WSN/1933 (mouse-adapted H1N1)] was obtained by inoculation in specific-pathogen-free embryonated eggs or infection of the Madin-Darby Canine Kidney (MDCK) cell line [23]. MDCK cells were purchased from American Type Culture Collection (ATCC, Manassas, USA) and maintained in minimum essential media with 10% fetal bovine serum, 100 μg/mL streptomycin, and 100 U/mL penicillin. Virus preparation and experiments were performed under BSL-2 conditions.

Plaque assay for titration of virus

A plaque assay was performed using the procedures of Gautam et al. [21] with modifications. Lungs, harvested four days after intranasal infection, were homogenized using a Tissue Lyser II (Qiagen, Hilden, Germany). The lysates were centrifuged at 13,000 rpm for 5 min at 4 °C and the supernatants were collected. The tenfold serially diluted supernatants were plated onto MDCK-monolayer containing six-well plates, which were previously washed with PBS. The plates were then allowed to stand at room temperature for 1 h, with shaking at 15–20 min intervals. The supernatant was then discarded, and the plates were overlaid with Dulbecco's Modified Eagle Medium/F12 agar (2 mM glutamine, 4% bovine serum albumin, 10 mM HEPES, 2.5% sodium bicarbonate, 50 mg/mL diethylaminoethyl dextran, 1 μg/mL L-tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.6% immunodiffusion-grade agar). After the agar layer solidified, the plates were incubated at 37 °C for 72 h in a 5% CO_2 atmosphere. Following incubation, plates were stained with 0.1% crystal violet and inspected for plaques 1 h later.

Histopathological analysis of lungs

Collected lung sections were fixed in 10% neutral buffered formalin, routinely processed, and embedded in paraffin. Sections were cut to 5 μm sections and stained with hematoxylin and eosin. Histopathological alterations of the lung were examined under an inverted microscope and the images were analyzed using an Axiovision Rel. 4.7 software (Carl Zeiss, Oberkochen, Germany).

Statistical analysis

Results are presented as the mean ± standard deviation. The statistical significance of differences between the two samples was evaluated using a Student's *t*-test; P-values less than 0.05 were considered statistically significant.

Abbreviations

ATCC: American Type Culture Collection; BSL: Biosafety level; MDCK: Madin-Darby Canine Kidney; Mx1: Myxovirus resistance protein 1; pfu: Plaque forming units.

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Not applicable.

Author contributions

HN, BK, AG, YK, EP, and JL performed experiment. HK, JK and JG designed experiments, analyzed data, and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and analyzed in this study are available from the corresponding author upon reasonable request.

Declarations

Competing interests

The authors declare that there is no financial competing interests to publish these results.

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