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Attenuation of the influenza virus by microRNA response element in vivo and protective efficacy against 2009 pandemic H1N1 virus in mice

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SUMMARY

Background: The 2009 influenza pandemics underscored the need for effective vaccines to block the spread of influenza virus infection. Most live attenuated vaccines utilize cold-adapted, temperature-sensitive virus. An alternative to live attenuated virus is presented here, based on microRNA-induced gene silencing.
Methods: In this study, miR-let-7b target sequences were inserted into the H1N1 genome to engineer a recombinant virus – miRT-H1N1. Female BALB/c mice were vaccinated intranasally with the miRT-H1N1 and challenged with a lethal dose of homologous virus.
Results: This miRT-H1N1 virus was attenuated in mice, while it exhibited wild-type characteristics in chicken embryos. Mice vaccinated intranasally with the miRT-H1N1 responded with robust immunity that protected the vaccinated mice from a lethal challenge with the wild-type 2009 pandemic H1N1 virus.
Conclusions: These results indicate that the influenza virus containing microRNA response elements (MREs) is attenuated in vivo and can be used to design a live attenuated vaccine.

1. Introduction

Influenza virus infection represents a serious threat to public health. Each year, it affects a significant portion of the population worldwide. The spread of a novel 2009 influenza A H1N1 virus infection reminded us of public vulnerability to new viral strains. The development of a new generation of vaccines is urgently needed.[1–3]

Of all the vaccination strategies available, vaccination using live attenuated virus is the most effective method of immunization because the vaccinated virus is able to infect and replicate even though the replication efficiency is compromised. Consequently, it effectively stimulates all immune responses and offers robust protection against viral infection.[4] Most live attenuated vaccines utilize virus that possesses a cold-adapted, temperature-sensitive phenotype. Inducing this temperature-sensitive phenotype is time-consuming.[5] Virus can be attenuated by other mechanisms. For instance, microRNA (miRNA) regulates viral replication through repression of translation.[6] The engineering of a tissue-specific miRNA response element (MRE) in viral mRNAs has been used recently to restrict viral tissue tropism, including that of coxsackievirus, vesicular stomatitis virus, poliovirus, and dengue virus.[7–10] Incorporation of miRNA target sequences into the nucleoprotein (NP) segment,[11,12] or into the hemagglutinin (HA) segment,[13] has been reported to result in attenuated influenza A virus.

It was reasoned that such technology could be used to generate live attenuated influenza virus for vaccine production. The lungs are the main target organ of influenza viral infection. Let-7b expression is abundant in pulmonary tissues, as shown by the Miranda online database (http://www.microrna.org/) and other references.[14] In a previous study, the miR-let-7b target sequence was inserted into a 2009 pandemic H1N1 virus genome (A/Nanjing/NJU-108/2009) to engineer a recombinant virus – miRT-H1N1. It was demonstrated that this recombinant virus was unable to infect human bronchial epithelial cells (HBE cells) that express a high level of corresponding miRNA-let-7b.[15] Upon production of miRNA-let-7b, the viral replication was restricted and the virus became attenuated.

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The aim of the present study was to determine whether the miRT-H1N1 virus produces an attenuated phenotype in vivo. The viral replication efficiency, elicited immune responses, and protective efficacy were investigated in a mouse model immunized with the MRE-containing influenza A virus.

2. Materials and methods

2.1. Viruses

The influenza A H1N1 virus A/Nanjing/108/2009 (WT-H1N1) was initially isolated from a Chinese patient in 2009.[16] The genomic sequences were deposited in GenBank (accession numbers **JQ173100** through **JQ173107**). The genomic segments of WT-H1N1 were separately cloned into a plasmid, pDP2000, resulting in various cDNA constructs of PB2, PB1, PA, HA, NP, NA, M, and NS, respectively. A plasmid encoding a mutated PB1 segment was constructed by incorporating MREs of let-7b into the open reading frame (site of 83–107 bp).[15] Three amino acids were changed in the let-7b target sequence. The mutated virus (miRT-H1N1) was constructed by plasmid-based reverse genetics. A control virus (scbl-H1N1) was designed, containing additional mutations in addition to the three mutated amino acids engineered in miRT-H1N1. The control virus was used to distinguish phenotypic differences in the presence or absence of miRNA binding. The viruses generated through transfection were then expanded through infection of specific-pathogen free (SPF) chicken embryos (Qian YuanHao Biological Co. Ltd, Nanjing, China). Virus containing allantoic fluid was collected, aliquoted, and stored at −80 °C until use. The method developed by Reed and Muench was used to determine the 50% tissue culture infectious dose ($TCID_{50}$) for each virus.[17] All procedures involving handling of the WT and mutant viruses were performed in a biosafety level 3+ animal facility certified by the Academy of Military Medical Sciences (AMMS).

2.2. Experimental infection of mice

Six-week-old female BALB/c mice (at the Laboratory Animal Center of Jinling Hospital, Nanjing, China) were placed under chloral hydrate anesthesia and infected intranasally with 10^5 $TCID_{50}$ of WT-H1N1, miRT-H1N1, or scbl-H1N1 virus in 50 μl phosphate-buffered saline (PBS). Animals were weighed daily and their mortality was monitored for 14 days after infection. Four mice per group were euthanized on days 3 and 5 post-infection and lung tissues were collected for viral titration. Tissue homogenate was clarified by low-speed centrifugation, and titrated in 96-well culture plates with a seeding monolayer of Madin–Darby canine kidney (MDCK) cells.[18] Titers were expressed as the $TCID_{50}$/g $\log_{10}$. MLD_{50}, the dose required to kill 50% of mice, was determined using previously described methods.[19]

2.3. Evaluation of immunogenicity in mice

Six-week-old female BALB/c mice were primed with 50 μl of PBS (mock-immunized group) or 10^5 $TCID_{50}$ of miRT-H1N1 virus intranasally on day 0, and boosted with the same dose on day 28. Blood samples were collected for antibody detection on days 28 and 42 post-vaccination. Meanwhile, six mice per group were euthanized 2 weeks after the second vaccination. Trachea–lung and nasal washes were harvested for the detection of mucosal sIgA.

IgG antibodies in serum and of IgA in trachea–lung and nasal washes were detected by ELISA.[20] Briefly, plates were first coated with purified H1N1 virus. The HA-coated plates were incubated with test samples, and the bound antibodies were detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin. Wells with an optical density (OD) value >0.2 at 405 nm were considered positive.

A microneutralization (MN) assay was used to determine neutralizing antibody titers in serum samples.[21] Heat-inactivated serum was serially diluted two-fold starting from a 1:20 dilution. Neutralizing antibody titers were expressed using the reciprocal of the highest dilution at which the infectivity of 100 $TCID_{50}$ of the WT-H1N1 virus was completely neutralized. An absence of viral cytopathic effect at day 4 was considered complete neutralization.

A hemagglutination inhibition (HI) assay was performed after the serum was treated with receptor-destroying enzyme (RDE; Denka Seiken Co. Ltd, Tokyo, Japan) and incubated with 0.5% chicken red blood cells (CRBCs).[22] Serum viruses were diluted with PBS to contain four agglutinating units.

For the detection of cytokines, single spleen cell suspensions were prepared and stimulated with purified H1N1 virus at a concentration of 5×10^6 cells/ml on day 28 post-vaccination.[23] Culture supernatants were harvested for the detection of interleukin 4 (IL-4) and interferon gamma (IFN-γ) by ELISA after 3 days of culture.

2.4. Analysis of efficacy against WT-H1N1 challenge

Mice were challenged intranasally with 100 LD_{50} of WT-H1N1 virus at 42 days post-vaccination. Mock-infected control mice only received 50 μl PBS. Challenged mice were monitored and their symptoms, body weight, and survival were recorded for 14 days. Three mice per group were euthanized and the lung tissues were harvested on day 5 post-challenge. The paraffin-embedded tissues were sliced for hematoxylin–eosin (HE) staining. The histopathological score (HPS) was determined by a pathologist who was blinded to the sample IDs. The HPS system, consisting of a numerical score ranging from 0 to 26, has been described by Cimolai et al.[24]

RNA was extracted from lung tissues using the RNeasy Kit (Qiagen). Quantitative RT-PCR was performed with a 7300 Real Time PCR System (Applied Biosystems). IL-6, IFN-β, and tumor necrosis factor alpha (TNF-α) mRNA levels of gene expression were normalized to levels of β-actin for each sample. The difference in signal was determined using the ΔΔCt cycle threshold calculation.

2.5. Statistical analysis

All quantitative data were calculated as the mean ± standard deviation (SD). The antibody titers for each group were plotted and error bars extended to 95% confidence upper limits. Analysis of variance (ANOVA) and/or the Student's *t*-test were used for comparisons. A *p*-value of <0.05 was considered statistically significant.

3. Results

3.1. Generation of mutant miRT-H1N1 virus and infection of chicken embryos

A 2009 pandemic H1N1 mutant virus (miRT-H1N1) carrying miR-let-7b target sequences was generated successfully. Ten-day-old embryonated chicken eggs were inoculated with WT-H1N1, miRT-H1N1, or scbl-H1N1 virus to amplify them. Allantoic fluid was harvested on day 2 after infection and titers were determined for each of the three strains. The results showed that miRT-H1N1 virus replicated as well as WT-H1N1 virus (Figure 1).

3.2. The miRT-H1N1 virus was attenuated in mice

First, the respective MLD_{50} was determined for WT-H1N1, scbl-H1N1, and miRT-H1N1 viruses in mice. The MLD_{50} of WT-H1N1

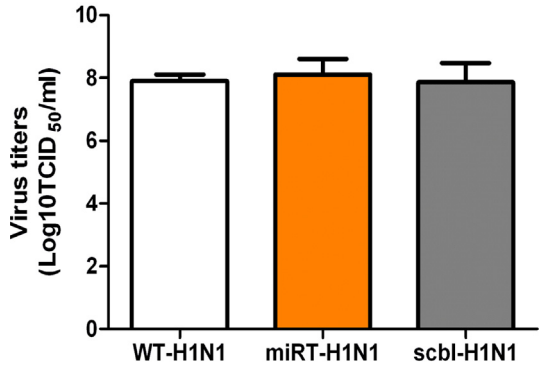

Figure 1. Growth properties of the recombinant viruses in chicken embryos. Viral titers of WT-H1N1 and MRE-containing H1N1 influenza A virus in infected 10-day-old embryonated chicken eggs. The viral titer was determined by $TCID_{50}$ using MDCK cells.

virus was $10^{5.3}$ $TCID_{50}$. The control virus scbl-H1N1 displayed a similar MLD_{50} value to wild-type, as expected ($10^{5.38}$ $TCID_{50}$). In contrast, the miRT-H1N1 virus was not lethal against any mice at this dose, resulting in increased doses (MLD_{50} $>10^7$ $TCID_{50}$).

Next, the virus replication level in mice was determined. WT-H1N1 and scbl-H1N1 replication produced high viral titers in lungs of infected animals ($10^{5.37}$ $TCID_{50}$/g and $10^{5.49}$ $TCID_{50}$/g on day 3 post-inoculation, respectively). The replication of miRT-H1N1, by contrast, was restricted to $10^{3.50}$ $TCID_{50}$/g. All viruses were detected at relatively low titers in the respiratory system on day 5 post-inoculation. The miRT-H1N1 virus grew more slowly than WT-H1N1 and scbl-H1N1 in lungs (Table 1).

RNA sequences of miRT-H1N1 virus isolated from the lungs of infected mice were also analyzed. Sequence analysis showed no mutations in the miR-let-7b target region (data not shown).

3.3. Antibody responses to miRT-H1N1 immunization in mice

All mice immunized with miRT-H1N1 virus showed appreciable neutralizing activity against the homologous WT-H1N1 virus (Figure 2B). Similarly, the levels of HI antibodies were higher in mice immunized with miRT-H1N1 virus than those treated with the PBS control (Figure 2A). The geometric mean titer (GMT) of HI antibody against the WT-H1N1 virus was 403 at 28 days after the first immunization, which increased sharply to 2032 at 2 weeks after the second immunization (42 days after the first immunization) (Figure 2A).

The IgG antibody response against the WT-H1N1 virus in the vaccinated mice was analyzed next. Similarly, the IgG antibody titers in serum were correspondingly increased with the changes in HI titers. Mice developed robust IgG antibodies (GMT 905; 28 days post-first immunization). A much stronger IgG antibody response (GMT 2873) appeared after the booster (42 days post-immunization) (Figure 2C). Furthermore, immunization with the miRT-H1N1 virus induced higher levels of sIgA in trachea–lung (GMT 64) and nasal washes (GMT 16) compared with the control group (Figure 3).

Additionally, stronger IL-4 and IFN-γ production was detected in spleen cells isolated from mice immunized with miRT-H1N1 virus compared with mock-immunized controls (Figure 4).

Table 1
Replication of viruses in mice

Virus	Infection dose ($TCID_{50}$/mouse)	Virus titer (mean $\log_{10}$ $TCID_{50}$/g ± SD)[a]	
		Day 3 post-inoculation	Day 5 post-inoculation
		Lungs	Lungs
WT-H1N1	10^5	5.37 ± 0.22^b	3.71 ± 0.24^d
scbl-H1N1	10^5	5.49 ± 0.33^c	3.49 ± 0.29^e
miRT-H1N1	10^5	$3.50 \pm 0.1^{b,c}$	$1.5 \pm 0.2^{d,e}$

$TCID_{50}$, 50% tissue culture infectious dose; SD, standard deviation.

[a] Mice were infected with 10^5 $TCID_{50}$ of WT-H1N1, scbl-H1N1, or miRT-H1N1 virus. Tissue samples were collected from four mice per group on the indicated days post-inoculation. Virus titers were determined in MDCK cells.

[b,c,d,e] Viral titer differences between two groups were tested for statistical significance using ANOVA ($p < 0.05$).

3.4. Protective efficacy of miRT-H1N1 virus immunization in mice

All animals vaccinated with miRT-H1N1 were fully protected against the lethal H1N1 infection with 100 MLD_{50} of WT-H1N1 virus, as demonstrated by the absence of signs of disease and limited weight loss after challenge compared with the mock-vaccinated animals (Figure 5, A and B). In contrast, mock-vaccinated mice succumbed to challenge within 8 days post-challenge. Symptoms included rough fur, quietness, and loss of appetite and weight in all of the control mice.

Virus titers were titrated in the lungs of the challenged mice on days 3 and 5 post-challenge (Table 2). All samples in the mock-immunized control group showed high viral titers on both days. The miRT-H1N1 virus-immunized mice group showed significantly lower or undetectable levels of WT virus in the lungs at 3 and 5 days post-challenge than the mock-vaccinated mice (Table 2).

The lung histopathology of mice challenged with WT-H1N1 virus was analyzed at 5 days post-challenge. A severe pneumonia pathology, with alveolar collapse, inflammatory infiltrates, necrotizing bronchitis, and extensive hemorrhagic lesions, was detected in the lungs of the mock-vaccinated group (Figure 6A). By contrast, only mild histopathological alterations were detected in the lungs of miRT-H1N1 vaccinated mice. The HPS was significantly lower in miRT-H1N1 vaccinated mice than in the control mice (Figure 6B).

3.5. miRT-H1N1 immunization reduced the inflammatory response upon influenza challenge

There is innate immunity against influenza virus infection of the mucosa. The levels of IFN-β and the pro-inflammatory cytokines IL-6 and TNF-α were therefore examined in the lung tissue following H1N1 challenge, as markers of the innate immune response. Lower levels of IFN-β, IL-6, and TNF-α were detected on days 3 and 5 following challenge in the miRT-H1N1 vaccinated group compared with the PBS control groups (Figure 7). The lower inflammation was accompanied by lower viral titers and reduced pathology in the miRT-H1N1 vaccinated mice.

4. Discussion

Approximately 14 700 people in more than 209 countries died as a result of the 2009 influenza A (H1N1) virus pandemic infection.[25] Such a widespread infection affecting a large number of the population reminds us of the importance of effective vaccination of the mass population. Unfortunately, the current vaccine production scheme impedes such efforts. For instance, it is time-consuming to prepare live attenuated virus through the cold-adaption process. An innovative process to introduce attenuated virus is urgently needed.

A pandemic H1N1 2009 virus (A/Nanjing/NJU-108/2009) was isolated from the swab sample of an outpatient febrile child at Nanjing Children's Hospital, Nanjing, China during the pandemic in 2009. A reassorted live attenuated influenza vaccine (LAIV) for H1N1 (miRT-H1N1) was generated after MREs of microRNA-let-7b were inserted into the polymerase (P) gene PB1 segment of

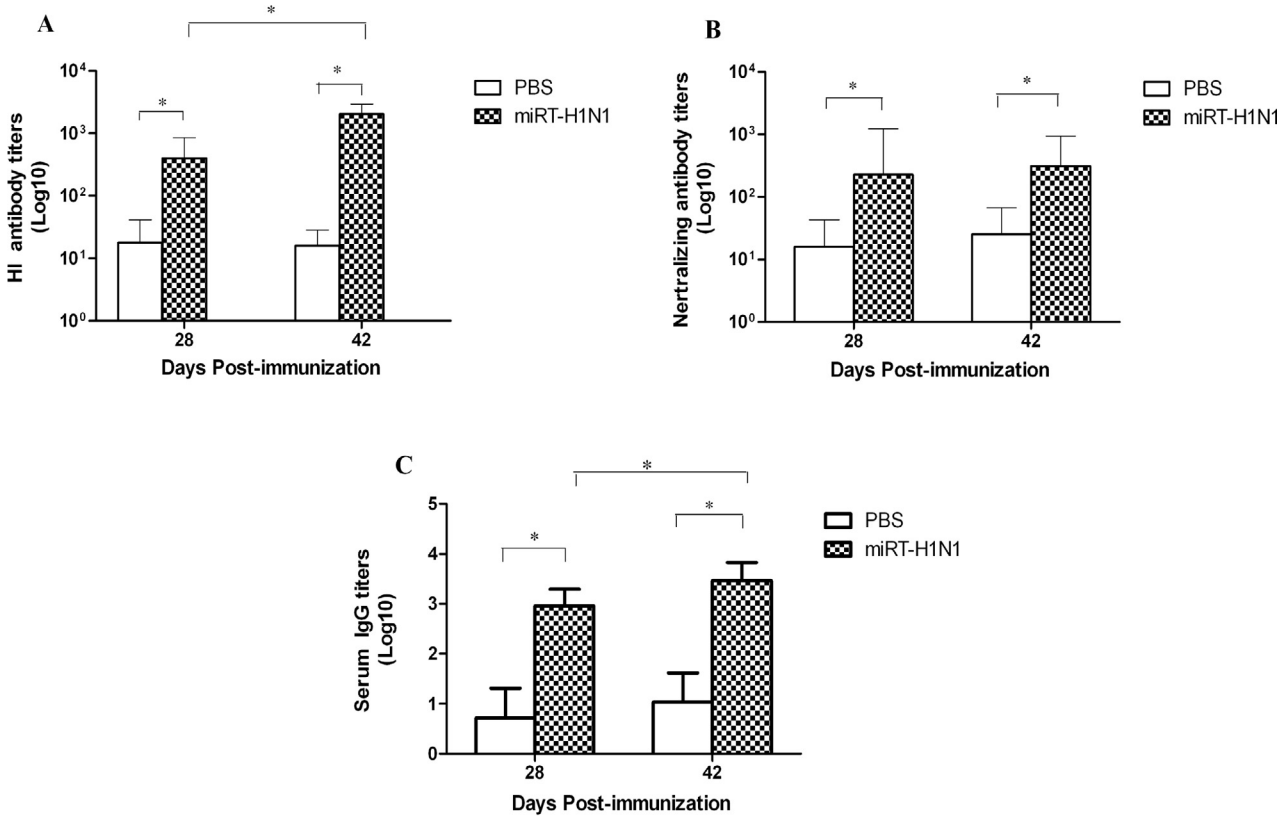

Figure 2. Humoral antibody responses in mice immunized with miRT-H1N1 virus. Serum samples were collected 4 weeks after the first immunization (on day 28 post-immunization) and 2 weeks after the second immunization (on day 42 post-vaccination). Serum antibody titers were determined by hemagglutination inhibition (HI) and microneutralization assays. The anti-influenza IgG levels in sera were measured by ELISA. (A) HI, and (B) neutralizing antibody responses to the miRT-H1N1 viruses after vaccination with10^5 $TCID_{50}$ of the miRT-H1N1 viruses. (C) IgG levels in sera. (*$p < 0.05$, compared with the negative PBS control group; $n = 6$ in each group.)

A/Nanjing/NJU-108/2009. In the present study, the protective efficacy of immunization with this mutant virus, which was attenuated via miR-let-7b mechanism, was investigated through infection in mice. MicroRNA-let-7b is expressed abundantly in lung tissue and airway epithelial cells, but sparsely in HEK293 cells and chicken embryos.[26,27] Previous data from our laboratory demonstrated that the replication of miRT-H1N1 virus was suppressed in HBE cells expressing high levels of miR-let-7b, while it maintained wild-type characteristics in HEK293 cells expressing low levels of miR-let-7b. Further, it was found that the growth of miRT-H1N1 virus was comparable to WT-H1N1 virus in MDCK cells or embryonated hen eggs in which let-7b is inefficiently expressed (Figure 1). These results demonstrate that attenuation of this virus is let-7b-dependent.

Whether attenuation of miRT-H1N1 virus could also be introduced in vivo was investigated in the present study. It is well known that several species of laboratory animals, including mice and ferrets, are susceptible to influenza virus infection and may serve as animal models. The study of pathogenesis and evaluation of efficacy of influenza vaccines are frequently carried out in mice, particularly the BALB/c strain,[28] with lungs expressing high levels of let-7b. BALB/c mice were selected for this study.

Consistent with previously published studies,[29,30] the present results showed that wild-type A/Nanjing/NJU-108/2009

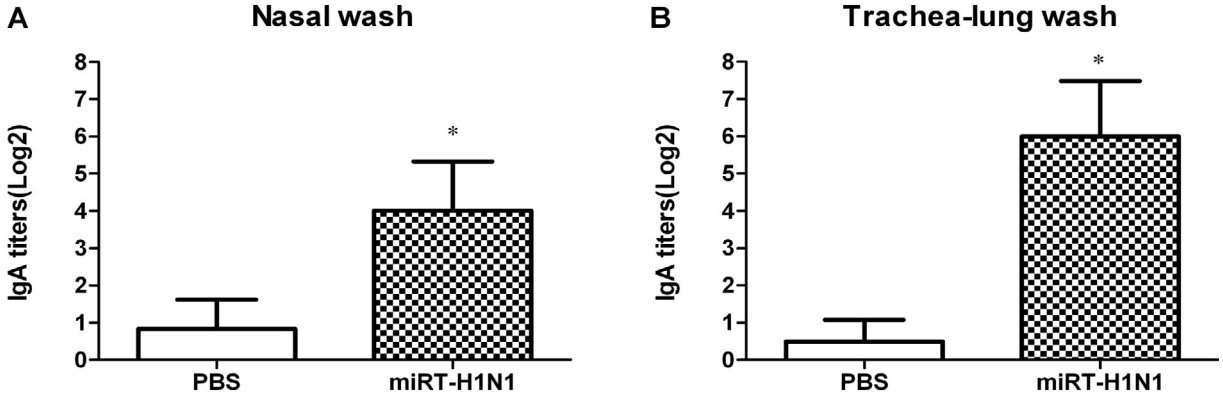

Figure 3. Mucosal antibody responses in mice immunized with miRT-H1N1 virus. Trachea–lung and nasal washes were collected 2 weeks after the second vaccination. (A) sIgA levels in nasal washes, (B) sIgA levels in trachea–lung washes. (*$p < 0.05$, compared with the negative PBS control group; $n = 6$ in each group.)

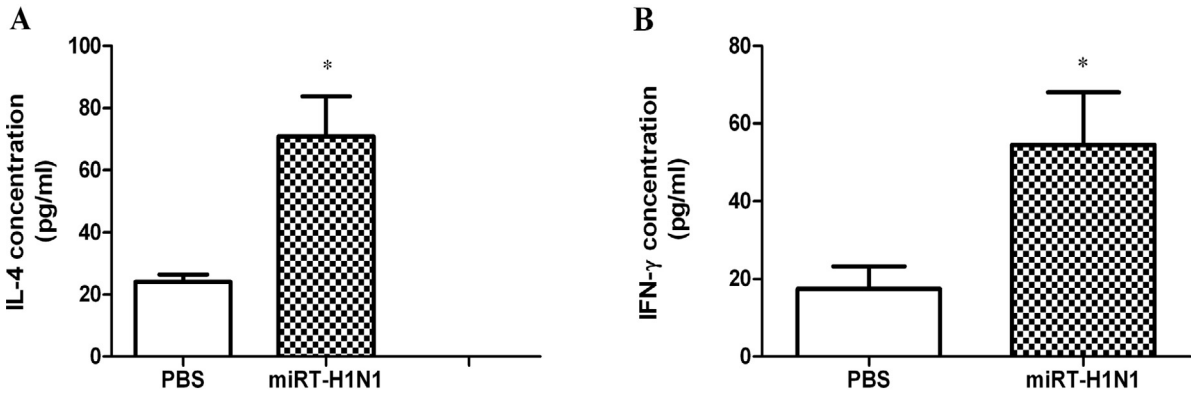

Figure 4. Cytokine levels in lung alveolar fluid of vaccinated mice as determined by ELISA. Mice were primed on day 0 and then boosted on day 28 with PBS or miRT-H1N1 virus in a 50-μl volume, and the single spleen cell suspensions were stimulated with recombinant HA (rHA) on day 14 post-boost. Culture supernatants were harvested after 3 days and cytokines were detected. (A) Interleukin 4 (IL-4) concentration, (B) interferon gamma (IFN-γ). (*$p < 0.05$, compared with the negative PBS control group; $n = 3$ in each group.)

(WT-H1N1) replicates efficiently, leading to high viral titers and severe pathological changes in the lungs of infected mice (Table 1). However, the replication of miRT-H1N1 virus was compromised and the miRT-H1N1 virus was attenuated and not lethal in all mice. It was also found that attenuation was a result of interference mediated by the endogenous miRNA-let-7b, which binds to MREs carried by miRT-H1N1, resulting in suppression of miRNA repression.

Although MRE-based attenuation is a rational approach to the development of live attenuated vaccines, reversion of live attenuated vaccine virus into a virulent phenotype is still a potential risk. Virus escape from miRNA-mediated suppression has been described, which can occur through deletions or mutations within the region of the miRNA target sequence.[31–33] However, no escape mutants were observed in vivo in the present study. These results demonstrate the genomic stability of miRT-H1N1 virus. To prevent reversion and enhance safety, multiple miRNAs targeting conserved viral sequences need to be combined.[13,34]

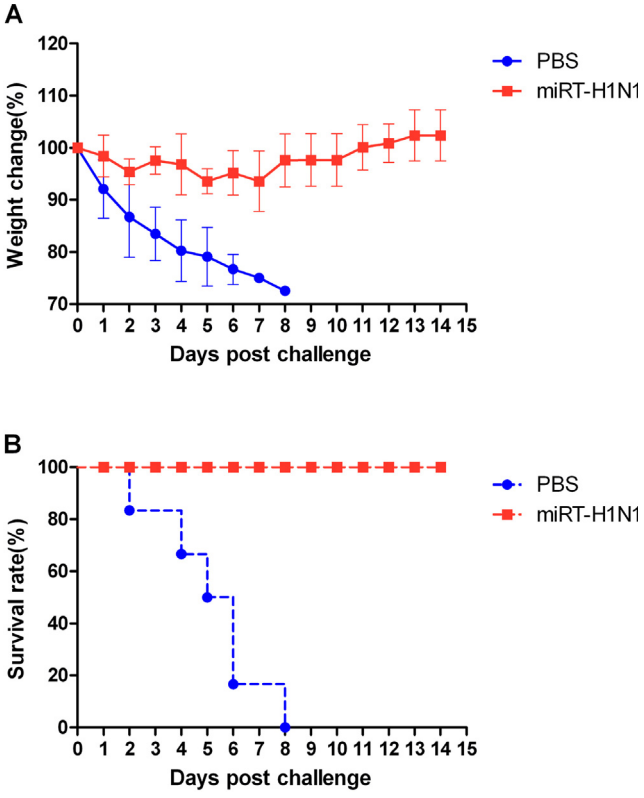

Figure 5. Protection of miRT-H1N1 virus-immunized mice from homologous H1N1 virus challenge. Mice were immunized with miRT-H1N1 virus, or PBS as a negative control. They were then challenged with 100 LD_{50} of WT-H1N1 virus on day 42 post-immunization. (A) Weight change caused by WT-H1N1 virus challenge, (B) mouse survival rate following the challenge with WT-H1N1 virus ($n = 6$ in each group).

Strong immunity against influenza virus infection can be generated with either inactivated or live attenuated influenza vaccine. There are several advantages of live attenuated vaccine over inactivated vaccine. Intranasal administration of live attenuated influenza virus vaccines is accompanied by robust mucosal immunity and cellular responses, which offer effective and longer-lasting protection.[35,36]

In this study, it was successfully shown that immunization with two doses of miRT-H1N1 live attenuated vaccine induced robust humoral and mucosal immune responses. The immunized mice produced higher specific IgG and IgA levels and were fully protected against homologous WT-H1N1 virus challenge (Figures 2 and 3). Mucosal immunity consisting of mucosal IgA, which binds and neutralizes incoming viruses, is critical to protection against early influenza infection.[37] Immunization with live attenuated influenza virus induces cell-mediated immunity, which interacts with B cells, possibly via helper T cells or via cytotoxic T cells. Therefore, cell-mediated immunity further contributes to a stronger mucosal antibody response.[38] This is supported by the appreciable production of IFN-γ and IL-4 in spleen cells (Figure 4), a confirmation that the miRT-H1N1 virus was able to elicit both Th-1 and Th-2 responses. An impact on innate immunity was also detected. The expression of inflammatory cytokines was reduced in the lungs of

Table 2
Viral replication in immunized mice after challenge

Virus	Virus titer (mean $\log_{10}$ $TCID_{50}$/g ± SD)[a]	
	Day 3 post-challenge	Day 5 post-challenge
	Lungs	Lungs
PBS	5.13 ± 0.47[b]	3.8 ± 0.5
miRT-H1N1	1.53 ± 0.68[b]	ND

$TCID_{50}$, 50% tissue culture infectious dose; SD, standard deviation; PBS, phosphate-buffered saline; ND, not detected; LD_{50}, dose required to kill 50% of mice.

[a] Three BALB/c mice per group were immunized intranasally with 10^5 $TCID_{50}$ of the miRT-H1N1 virus or PBS (50 μl per mouse). The immunized mice were challenged intranasally with 100 LD_{50} WT-H1N1 virus at 4 weeks post-immunization.

[b] Viral titer differences between two groups were tested for statistical significance using the Student's *t*-test ($p < 0.05$).

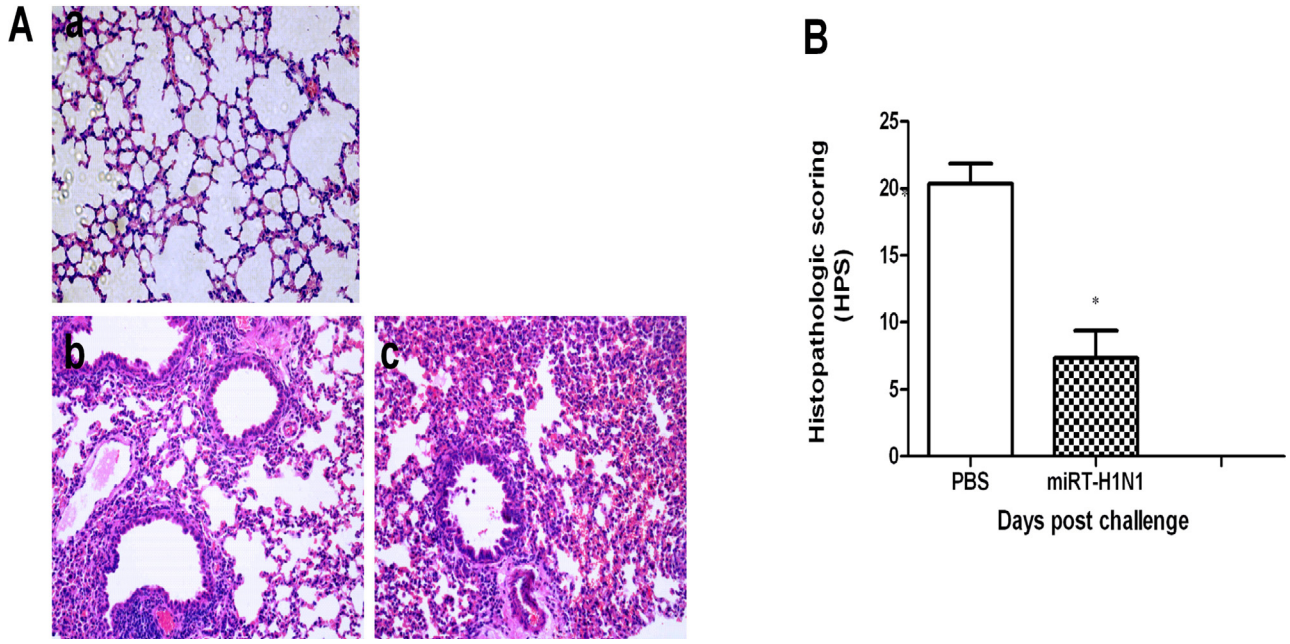

Figure 6. Histopathology of infected lungs on day 5 post-challenge. (A) Pathological findings in mice: (a) lungs of control mice showing normal bronchioles and alveoli (HE ×200); (b) lungs from mice vaccinated with miRT-H1N1 displaying mild histopathological lesions, including inflammatory infiltration and mild hemorrhage (with alveoli and airways indistinguishable from those of control mice) (HE ×200); (c) mock-vaccinated mice showing a severe histopathology characterized by extensive hemorrhagic lesions, necrotizing bronchitis, inflammatory infiltration, and alveolar collapse (HE ×200). (B) HPS of lung sections at 5 days post-challenge ($^*p < 0.05$, compared with the negative PBS control group; $n = 3$ in each group).

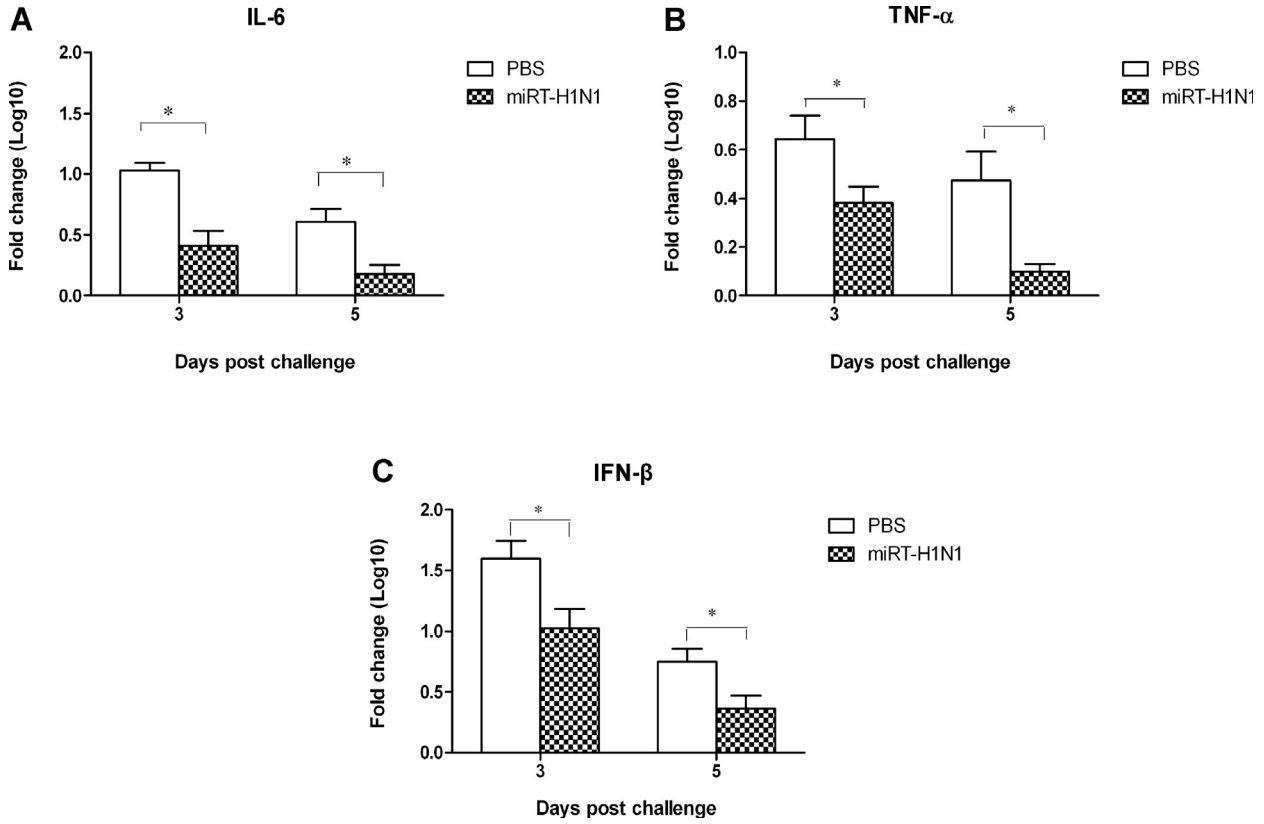

Figure 7. miRT-H1N1 virus vaccination reduces inflammatory cytokines in the lungs following influenza challenge. Mice were administered miRT-H1N1 virus or PBS. On day 42 post-vaccination, mice were challenged intranasally with 100 LD_{50} WT-H1N1 virus. On days 3 to 5 post-vaccination, lungs were harvested; cDNA was prepared and real-time PCR was performed for (A) IL-6, (B) TNF-α, and (C) IFN-β ($^*p < 0.05$, $n = 6$ in each group).

miRT-H1N1 vaccinated mice compared with the unvaccinated group after WT-H1N1 virus challenge. The immune response to influenza A infection includes an initial innate as well as adaptive immune response, with T cell infiltrates in the lungs.[39] Thus, the present results indicate that both innate and adaptive responses were generated with the miRT-H1N1 vaccination.

It was found that the miRT-H1N1 virus replication is let-7b-dependent. This feature allows efficient virus production in chicken embryos and direct use as live vaccine, which can be attenuated once the infection is established in vivo.

In summary, the miRT-H1N1 elicited robust immunity in mice and protected the immunized mice from infection with homologous H1N1 viruses. These results prove the principle that MRE-containing influenza attenuated virus (miRT-H1N1) can be used as a live attenuated vaccine against pandemic viruses. Further studies including investigations of cross-protective immunity and comparisons of efficacy with currently approved live vaccines in mice, are warranted.

Acknowledgements

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Ethical approval: This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animal research was approved by the Animal Care and Use Committee at Jingling Hospital under university-approved standards.

Conflict of interest: No conflict of interest to declare.

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