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A TLR3 ligand that exhibits potent inhibition of influenza virus replication and has strong adjuvant activity has the potential for dual applications in an influenza pandemic

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ABSTRACT

The appearance and spread of the H5N1 highly pathogenic avian influenza (HPAI) raise concern of a possible pandemic. Current preventive measures include the development of a pre-pandemic influenza vaccine and stockpiling of neuraminidase inhibitors. However, their benefits can be significantly reduced by mutations in the hemagglutinin or neuraminidase resulting in antigenic changes and the appearance of drug-resistance, respectively. Drugs that target the innate immune system to achieve a 'heightened antiviral' state represent another class of antiviral agents that could contribute to the control and treatment of influenza infection. In this study, PIKA (a stabilized dsRNA) provides broad-spectrum prophylaxis against a number of influenza A viruses. In addition, when PIKA was admixed with influenza vaccine preparations, including a formalin-inactivated whole-virion H5 vaccine, significant adjuvanting effect leading to accelerated viral clearance was observed in a murine model. These biological effects appear to be mediated by the ability of PIKA to promote the maturation of dendritic cells, including up-regulation of co-stimulatory molecules, such as CD80 and CD86, and the induction of various cytokines and chemokines. Toll-like receptor 3 (TLR3) was shown to recognize PIKA in a concentration-dependent manner. The potency and versatility in its activities make PIKA an attractive candidate for use in an influenza pandemic.

1. Introduction

The most effective tool for controlling an influenza pandemic is a suitable vaccine. For optimal effectiveness, the pandemic vaccine needs to be based on the causative strain and therefore, vaccine cannot be produced until the pandemic strain is identified. This criterion poses serious constraints in the capability of vaccine manufacturers to prepare pandemic vaccines in advance for stockpiles. In addition, a number of studies have shown that the hemagglutinin (HA) molecules of avian H5 viruses are poorly immunogenic [1,2], where up to 90 μg of antigen (6 times the normal dose of human influenza virus HA) was required to elicit potentially protective responses in a substantial number of subjects [2]. This need for a larger antigen dose further reduces the availability of the 'limited' pandemic vaccines. In an attempt to increase the immunogenicity of the antigen, adjuvants like alum and MF-59 have been incorporated into vaccine formulations. Although alum may enhance the magnitude of the antibody response to a high dose of antigen, it does not have significant antigen-sparing effect [3,4]. Other adjuvants, such as MF59 or a proprietary oil emulsion adjuvant, have been tested and data indicate that the inclusion of these adjuvants in the vaccine preparation would significantly increase antibody titers [5,6].

Given the likelihood that a suitable vaccine will not be available before the onset of a pandemic, initial protection in a pandemic may have to rely on antiviral treatment and prophylaxis. Currently available anti-influenza drugs include neuraminidase inhibitors (oseltamivir and zanamivir) or ion-channel blockers (adamantanes). The success of these drugs, both for prophylaxis or therapeutics, is based on the susceptibility of the viruses to these drugs. Recently reported studies, however, have shown that a number of H5 isolates are resistant to these drugs [7–9]. These drugs or classes of drugs alone may thus be insufficient in protecting the human population from an influenza pandemic. An alternative, antigen-independent approach that could slow down the transmission of the virus and provide more time for vaccine production is thus highly desirable.

The toll-like receptors (TLRs) are a collection of receptors that detect conserved molecular components encoded by microorganisms (reviewed in Refs. [10,11]) and stimulation of these receptors by specific ligands leads to the activation of the innate immune cells, such as dendritic cells (DC), macrophages and NK cells [12,13],

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through a variety of signaling pathways, such as NF-κB transcription factors, c-Jun NH2 terminal kinase (Jnk), mitogen-activated protein kinases (MAPKs), resulting in quantitative and qualitative changes in immunological functions, including antigen presentation. In addition, cytokines, such as IFN-α, TNF-α and IL-12p40, are produced *in vivo* shortly after TLR activation [13,14]. The production of these cytokines results in antiviral effects observed in a number of infection models. For example, administration of synthetic lipid A analogs (TLR4 agonists) protects mice against an influenza challenge [15]. A possible explanation for this anti-influenza effect could be related to the production of type 1 interferons, which induce the activation of IFN-stimulated genes including Mx proteins, protein kinase R and 2′5′ oligoadenylate synthetase [16]. The induction of these proteins increases the resistance to the replication of the influenza virus [17–19].

The present study was designed to investigate the efficacy of PIKA, which is a synthetic stabilized form of double stranded RNA, as a stand-alone agent for its antiviral effect and to evaluate the efficacy of PIKA as an adjuvant when co-administered with inactivated influenza vaccines, including an H5N1 vaccine. Our results show that PIKA has potent anti-influenza effect when administered 6 h prior to or immediately after influenza virus challenge in mice. The antiviral effect is not strain-specific. When used as an immuno-adjuvant, PIKA was able to achieve significant antigen-sparing effect. When incubated with immature DC, PIKA altered the expression pattern of a number of immune genes. In addition, PIKA was capable of interacting with both human and mouse TLR3, leading to the production of NF-κB. Thus, in the face of a potential influenza pandemic, PIKA could be a candidate as a prophylactic drug or could be used as an adjuvant to increase the population coverage when pandemic vaccines become available.

2. Materials and methods

2.1. Mice

Specific pathogen-free (SPF) male BALB/c mice (6–8 weeks old) were purchased from the Centre for Animal Resources (CARE), National University of Singapore. The animal protocol used in this study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of DSO National Laboratories, Singapore.

2.2. Viruses

A/Puerto Rico/8/34 (H1N1) and the reassortant virus Mem71, which bears the HA of A/Memphis/1/71 (H3N2) and the neuraminidase (NA) of A/Bellamy/42 (H1N1) are gifts from Professor Lorena Brown, The University of Melbourne. A/WS/33 (VR-1520) is from the American Type Culture Collection (ATCC). The reverse-engineered H5N1 influenza virus was made based on the method described by Hoffmann et al. [20], using an expression plasmid provided by Dr Brendon Hanson (DSO National Laboratories, Singapore). The HA sequence of A/Indonesia/CDC625L/2006 (CY014465, a clade 2, subclade 1 virus which is closely related to A/Indonesia/5/05) was downloaded from GenBank and was synthesized by Geneart AG (Regensburg, Germany). The viruses were grown in the allantoic cavity of 10-day-old embryonated chicken eggs for 2 days at 37 °C. Allantoic fluids were pooled and divided into aliquots, and stored at −80 °C until use.

2.3. Vaccine

The 2006–2007 South Hemisphere seasonal influenza vaccine, Fluvax, was from CSL Ptd Ltd. (Victoria, Australia). Virions of the reverse-engineered H5N1 virus were concentrated by ultra-centrifugation and resuspended in PBS. Formalin-inactivated purified whole-virion vaccine was prepared by inactivating the virions with 0.5% formalin for 3 days at 37 °C followed by dialysis in PBS. The total protein concentration in the vaccine was measured by Coomassie Plus (Pierce Biotechnology, Inc., IL, USA). The amount of HA content was estimated to be 30% of the total protein content.

2.4. Adjuvants

Complete and Incomplete Freund's adjuvant (CFA and IFA) were obtained from Sigma–Aldrich (St Louis, USA). PIKA was obtained from NewBiomed PIKA Pte Ltd. (Singapore).

2.5. Immunization protocol

For intranasal (i.n.) immunization, groups of mice were anesthetized with an intraperitoneal (i.p.) injection of ketamine and xylaxine followed by i.n. administration of immunogens in 50 μL. For immunization by the subcutaneous (s.c.) route, the immunogens were administered in 100 μL at the base of the tail.

2.6. Administration of PIKA

In selected experiments, 100 μg of PIKA was given to anesthetized mice i.n. in a volume of 50 μL. When given by the s.c. or i.p. route, 100 μg of PIKA was given in 100 μL volume per dose. PIKA contains dsRNA that is greater than 100 base pairs in length.

2.7. Viral challenge

Three weeks after final vaccination, mice were challenged i.n. with $10^{1.7}$–$10^{4.5}$ PFU of various strains of influenza virus in a volume of 50 μL. Mice were sacrificed at various time-points and pulmonary viral titers were determined using previously described methods [21].

2.8. Serum collection and ELISA

Mice were anesthetized by ketamine/xylaxine injection and blood samples were collected from the orbital plexus. Sera were collected and stored at −20 °C until analysis. The titer of immunogen-specific antibodies were quantified by ELISA, as previously described [22]. Avidity ELISA was carried out using previously described method [23].

2.9. Transfection

HEK293 cells were maintained in OptiMem (Invitrogen) with 5% fetal calf serum and were transfected in flat-bottomed 96-well plates as previously described [24]. Each sample was tested in triplicate. Six hours after the stimulation, luciferase and β-galactosidase activity in each sample was measured by commercial kits (Promega Corporation, WI, USA). The relative stimulation of NF-κB was calculated by normalizing luciferase activity with β-galactosidase activity and was expressed using the readings from unstimulated wells as a baseline.

2.10. Quantitative real-time polymerase chain reaction

The level of expression of various cytokine genes was determined using a LightCycler (Roche, IN, USA) using primers described in Giulietti et al. [25].

2.11. Microarray

Samples containing 1 μg of total RNA from the abovementioned DC experiment were used for microarray analysis. PIQOR immunology microarrays (Miltenyi Biotech, Gladbach, Germany) were used. Samples were amplified and labeled with different fluorochromes according to manufacturer's instructions. Samples from PIKA-stimulated DC and unstimulated DC were labeled with Cy5 and Cy3, respectively. After hybridization, the slides were scanned using the ImaGene software (Biodiscovery). Each gene was printed in quadruplicate on the array. Net signal intensity, data normalization and calculation of the Cy5/Cy3 ratios were performed by Miltenyi Biotech using the PIQOR analyzer software.

2.12. Cytokine analysis

One million D1 cells were stimulated with 100 μg of PIKA or 1 μg of LPS (0111:B4, Sigma–Aldrich) overnight. The supernatants were collected and the cytokine protein levels measured using the Bioplex Protein Array system (Bio-rad, Hercules, CA), in duplicates and against a standard curve according to manufacturer's instructions.

2.13. Statistical analysis

The significance of any difference between any two different groups was assessed by the Mann–Whitney test using Prism 4 (GraphPad Software, CA, USA). Reported p values < 0.05 are considered significantly different.

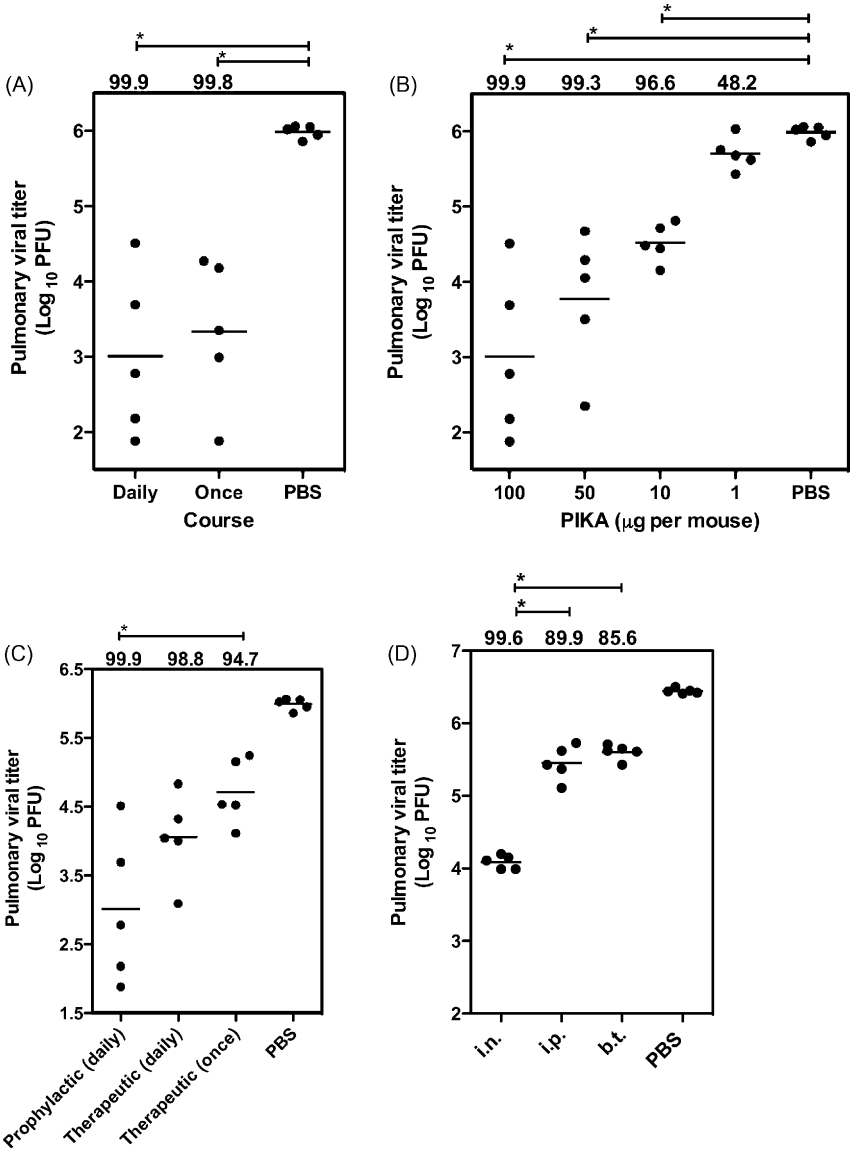

Fig. 1. Administration of PIKA reduces pulmonary viral titer in mice challenged with influenza virus. Groups of five mice each were given 100 μg of PIKA in PBS intranasally 6 h before being challenged with 50 PFU of PR8 virus intranasally. (A) One group of mice received PIKA treatment intranasally at 24 and 48 h post-infection and the rest of the mice were left untreated. On day 3 post-infection, the mice were sacrificed and titers of influenza virus in lung homogenates were determined by plaque assay in MDCK cells. Closed circles represent the lung virus titer of individual mice and the line represents the geometric mean of the group. The percent reduction in mean viral titer relative to the control group treated with PBS is shown above each column of data. The '*' symbol indicates when the difference between the two groups was statistically significant ($p < 0.05$). (B) Mice were given PIKA intranasally at different concentrations 6 h before intranasal challenge with 50 PFU of PR8 virus. The mice received treatment intranasally at 24 and 48 h post-infection. The pulmonary viral titers were determined on day 3 post-infection. (C) Mice were given 100 μg PIKA intranasally 6 h prior to (prophylactic group) or at the time of infection (therapeutic group). The daily groups received the PIKA treatment at 24 and 48 h post-infection while the rest were left untreated. Pulmonary viral titers were determined on day 3 post-infection using MDCK plaque assay. (D) Mice were given 100 μg of PIKA by the indicated route 6 h prior to viral challenge and were treated at 24 and 48 h post-infection. Pulmonary viral titers were determined on day 3 post-infection using MDCK plaque assay.

3. Results

3.1. The ability of PIKA to reduce pulmonary viral titer in mice

In this study, the murine influenza model was employed to demonstrate the antiviral properties of PIKA. In this model, anesthetized mice were challenged with influenza virus i.n. This mimics a total respiratory tract infection and the pulmonary viral titer reaches its peak on day 2 post-infection (pi) and remains at a high level till day 6 pi (data not shown). Using this model, groups of five mice were given 100 μg of PIKA i.n. 6 h prior to challenge with 50 plaque forming unit (PFU) of Influenza/A/PR/8/34. The pulmonary viral titer in the lungs was determined on day 3 pi. As shown in Fig. 1A, mice that received PIKA treatment at 24 and 48 h pi had the lowest pulmonary viral titer at day 3 and this was significantly lower than the titer in control mice that received PBS ($p = 0.0079$). In addition, significant reductions in pulmonary viral titers were also observed in mice that received only one dose of PIKA at 6 h prior to infection. The difference in the pulmonary viral titer between the group that received daily treatment and the group that received only one dose of PIKA was not statistically significant ($p = 0.75$).

After demonstrating that PIKA has an inhibitory effect on influenza viral replication *in vivo*, it was of interest to determine the optimal conditions for the treatment. To examine this, mice were given different concentrations of PIKA i.n. and were challenged with 50 PFU of PR8 virus i.n. The mice were treated at 24 and 48 h and were sacrificed on day 3. A dose-dependent response was observed (Fig. 1B), in which mice given 100 μg of PIKA daily had the lowest mean viral titer among all the groups. An inhibitory effect of PIKA on viral replication was detected at 10 μg per dose but not at 1 μg per dose ($p = 0.0556$). To determine whether PIKA could be used as a treatment option at the time of infection, mice were given PIKA immediately after infection with 50 PFU of PR8 virus. As shown in Fig. 1C, this treatment protocol was effective in reducing the pulmonary viral titer and the titer was not significantly different from the prophylaxis group that received PIKA 6 h prior to infection ($p = 0.1508$). However, it is apparent that daily treatment is preferable because the group that received only a single dose had a significantly higher titer of virus in the lungs compared to those that received daily doses ($p = 0.0159$). Intranasal administration of PIKA produced the most significant anti-influenza effect compared to s.c. or i.p. administration of the drug (Fig. 1D) though the pulmonary viral titers in the treated mice were still significantly lower than the titers of the PBS control group ($p = 0.0079$).

3.2. PIKA is effective in reducing pulmonary viral titers of several influenza A virus subtypes administered at different challenge doses

After demonstrating the effectiveness of PIKA in inhibiting the replication of PR8 virus *in vivo*, we sought to determine whether similar inhibition would be observed with different influenza viruses. Groups of five mice were given PIKA treatment and were challenged with 50 PFU of PR8 (H1N1), Mem71 (H3N1) and A/WS/33 (H1N1), as previously described. On day 3 pi, the viral titer of the PIKA-treated groups were significantly lower than the titers of the PBS group, regardless of the subtype of the challenge viruses (Fig. 2A–C, $p = 0.0079$, 0.0159, 0.0119) respectively. In addition, mice were also challenged with higher doses of virus, 500 or 5000 PFU (5 and 50 LD_{50} respectively) of PR8. As shown in Fig. 3A and B, the PIKA-treated mice had lower pulmonary titers than the PBS control group at these higher challenge doses ($p = 0.0079$). In addition, when challenged with $10^{4.5}$ PFU of Mem 71, there was a 99% reduction in viral titer in PIKA-treated mice compared with the PBS control group (Fig. 3C). To demonstrate the therapeutic potential of the treatment, groups of five mice were challenged with 50 PFU of PR8 i.n. and received PIKA treatment 24 or 48 h pi. On day 5 pi, as shown in Fig. 3D, significant reduction in pulmonary viral titers was observed in PIKA-treated mice ($p = 0.0079$, 0.0273) compared with the PBS-treated group.

Therefore, in summary, we demonstrated that PIKA has the ability to inhibit the replication of several strains of influenza *in vivo*. Although intranasal administration of the drug before the establishment of an infection provided the best protection, substantial viral reduction could still be achieved if the drug were given in the course of an established infection.

3.3. PIKA can act as a potent adjuvant by enhancing the immunogenicity of the seasonal influenza vaccine

Since PIKA is an effective adjuvant for hepatitis B vaccine [26], we examined whether PIKA could enhance the immunogenicity of influenza vaccine. We sought to demonstrate that the enhancement of the humoral responses would lead to significant viral reduction in replication of challenge virus. Previous studies have showed that

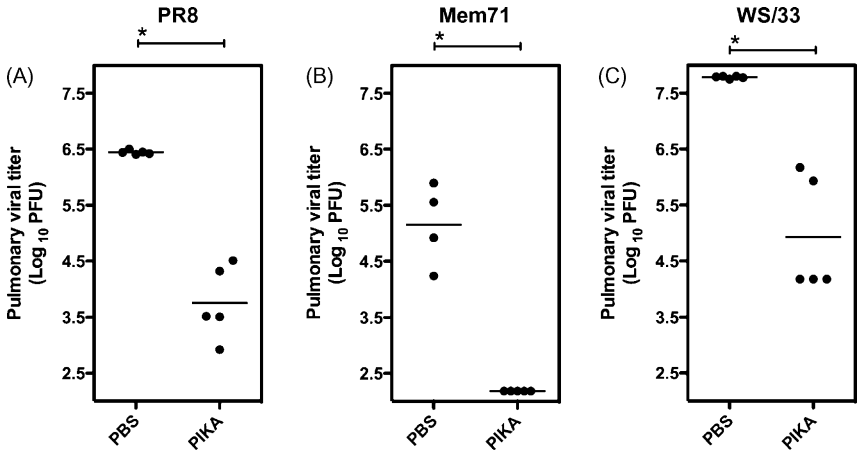

Fig. 2. PIKA is effective in reducing pulmonary viral titers of different influenza A virus subtypes. Groups of five mice each were given 100 μg of PIKA intranasally 6 h prior to challenge with 50 PFU of PR8 (H1N1) (A), Mem 71 (H3N1) (B) or WS/33 (H1N1) (C). Subsequently, mice received PIKA treatment at 24 and 48 h post-challenge. On day 3 post-infection, the mice were sacrificed and titers of influenza virus in lung homogenates were determined using in MDCK cells. Closed circles represent the lung virus titer of an individual mouse and the line represents the geometric mean titer of the group. The '*' symbol indicates that the difference between the two groups was statistically significant ($p < 0.05$).

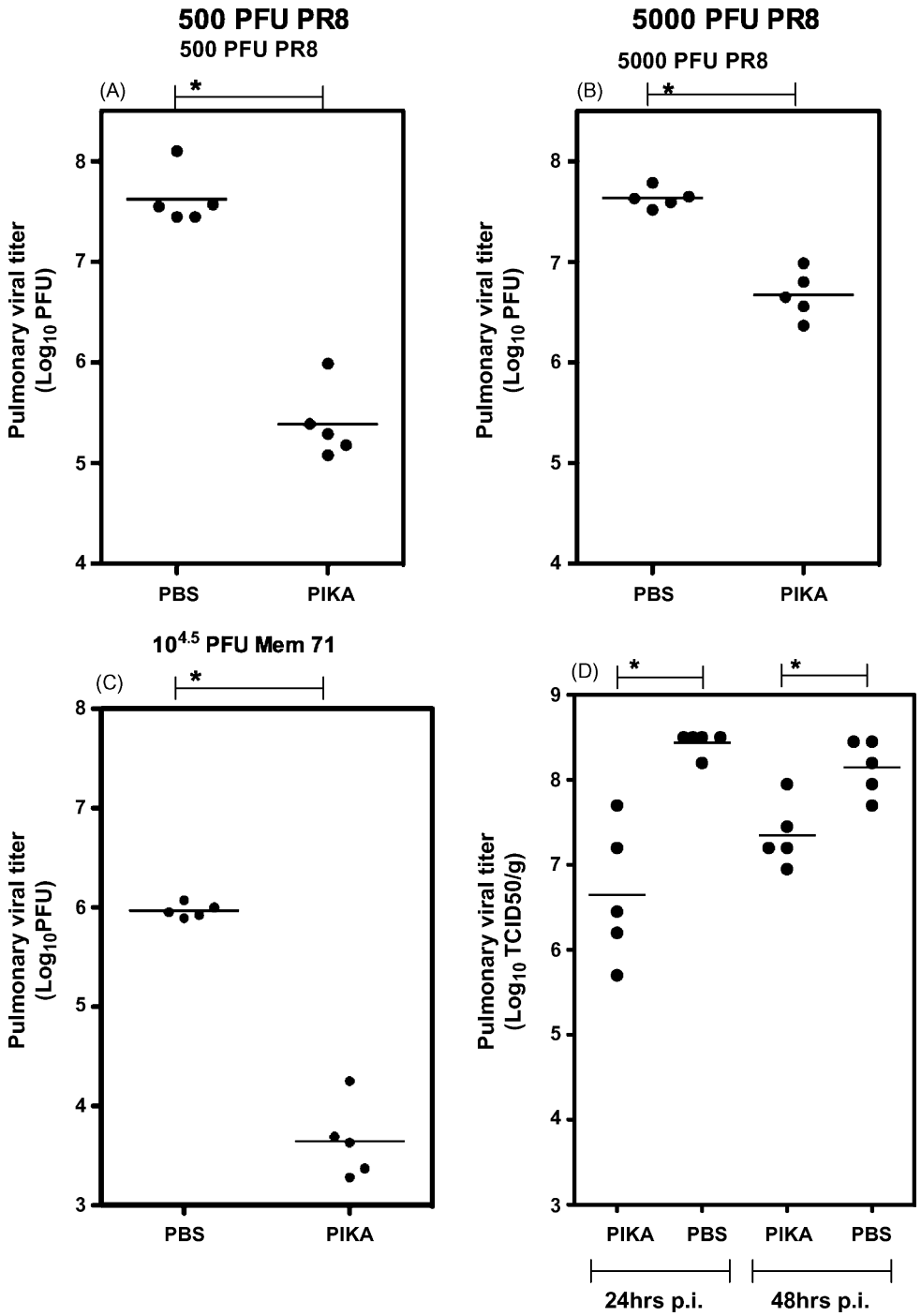

Fig. 3. Intranasal PIKA administration results in a substantial reduction in pulmonary viral titer even when mice are challenged with a high dose of infectious virus or after the establishment of an infection. Groups of five mice each were given 100 μg of PIKA intranasally 6 h prior to challenge with (A) 500 PFU or (B) 5000 PFU of PR8 virus or (C) $10^{4.5}$ PFU of Mem71 virus. Mice received PIKA treatment intranasally at 24 and 48 h post-challenge. On day 3 post-infection, the mice were sacrificed and titers of influenza virus in lung homogenates were determined in MDCK cells. Closed circles represent the lung virus titer of an individual mouse and the line represents the geometric mean titer of the group. (D) Groups of five mice were challenged with 50 PFU of PR8 as previously described and 100 μg of PIKA were given 24 or 48 h post-infection and continued to receive daily treatment. On day 5 post-infection, the titers of influenza virus in lung homogenates were determined. The '*' symbol indicates that the difference between the two groups was statistically significant ($p < 0.05$).

when administrated at between 1.5 and 5 μg per dose, different forms of influenza vaccine could induce robust antibody responses without any external adjuvant [27–29] with protection against viral challenge [27]. As shown in Fig. 4A, mice immunized with a single dose of the trivalent vaccine at 1.5 μg s.c. had robust antibody responses which were comparable to those induced when the vaccine was admixed with CFA. When the vaccine dose was reduced by 10-fold (0.15 μg), there was about 10-fold reduction in the magnitude of the antibody responses. To mimic a vaccine shortage situation, a dose of 0.015 μg, (~100-fold reduction from the optimal dose) was selected and we evaluated whether the inclusion of PIKA could compensate for the reduction in antigen concentration. Groups of five mice were vaccinated with a suboptimal dose of Fluvax vaccine by the s.c. or i.n. route. Three weeks after priming, sera were collected from the mice before they were boosted. Sera were collected from the mice 3 weeks after boost. As shown

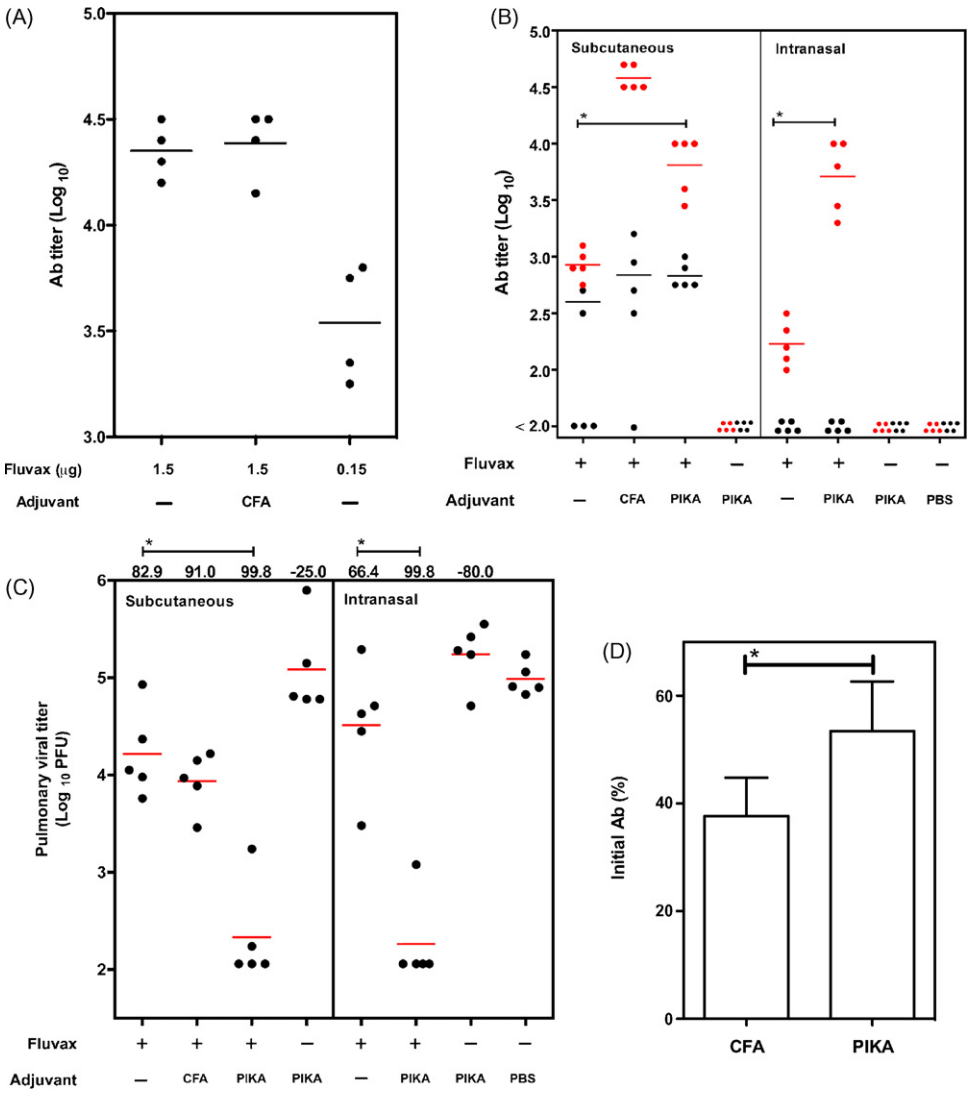

Fig. 4. PIKA acts as a potent adjuvant by enhancing the immunogenicity of the seasonal influenza vaccine. (A) Groups of four mice were vaccinated with either 50 or 5 μL of Fluvax influenza vaccine (containing 1.5 or 0.15 μg of HA from each subtype) in PBS or in CFA by the subcutaneous route. On day 21, sera were collected and the antibody titer of the sera was determined by an ELISA assay, using Fluvax as the coating antigen. (B) Groups of five mice were vaccinated with 0.5 μL of Fluvax influenza vaccine (containing 15 ng of HA from each subtype) in PBS, either by the subcutaneous route at the base of the tail or by the intranasal route. Some groups received the vaccine with additional adjuvant as indicated in the X-axis. For those that received PIKA as an adjuvant, 100 μg of PIKA was admixed with the vaccine prior to administration. On day 21 post-vaccination, sera were collected and the mice received another boost by the same route and sera were collected on day 42. The antibody titer of the sera was determined by an ELISA assay, using Fluvax as the coating antigen. Each dot represents the antibody titer of an individual mouse. The primary and secondary antibody titers are represented by the black and red dots respectively and the lines represent the geometric mean antibody titer of each group. The '*' symbol indicates that the difference between two groups is statistically significant ($p < 0.05$). (C) To determine the level of protection mediated by the antibody response, the mice were challenged with 50 PFU of PR8 intranasally 3 weeks after the boosting and the pulmonary viral titer was determined on day 5 post-infection. Closed circles represent the lung virus titer of an individual mouse and the line represents the geometric mean titer of the group of mice. The percent reduction in mean viral titer relative to the PBS control group is shown above each column of data. The '*' symbol indicates that the difference between the two groups was statistically significant ($p < 0.05$). (D) Serum samples were diluted 1:2000 and the avidity of the antibodies was tested in a urea-displacement ELISA. The O.D. of the urea-treated plates was expressed as a percent of the untreated plates. Data are represented as the mean and standard derivation of five mice.

in Fig. 4B, only two out of the five mice that received the vaccine without an adjuvant by the s.c. route had detectable amounts of anti-HA antibody in the primary response. For the CFA group, four mice had anti-HA antibody whereas all animals in the vaccine with PIKA group had detectable levels of anti-HA antibody. In the secondary responses, although all mice given vaccine alone developed anti-HA antibody, the antibody titers were significantly lower than those that were given vaccine with PIKA ($p = 0.0119$). As for intranasal delivery of vaccine, none of the vaccine protocols was able to elicit detectable anti-HA responses after one dose. When two doses of vaccine were given with PIKA, the vaccine recipient group showed a low titer of anti-HA response. The response was substantially improved by the addition of PIKA to the vaccine ($p = 0.0079$). Interestingly, the titer of the sera attained by two doses administered i.n. was comparable to titers achieved by two doses given by s.c. injection ($p = 0.5762$).

In order to demonstrate that the enhanced antibody responses can lead to improved efficacy, vaccinated mice were challenged with 50 PFU of PR8 i.n. and the pulmonary viral titers were determined 5 days after challenge. As shown in Fig. 4C, mice given two doses of the vaccine showed a 83% reduction in viral titer compared to the PBS control group ($p = 0.0556$). Although the CFA group had the highest titer of anti-HA responses (Fig. 4A), the group only showed a 91% reduction in viral titer, which was not statistically different from the group that received unadjuvanted vaccine ($p = 0.4206$). On the other hand, the group given vaccine with PIKA

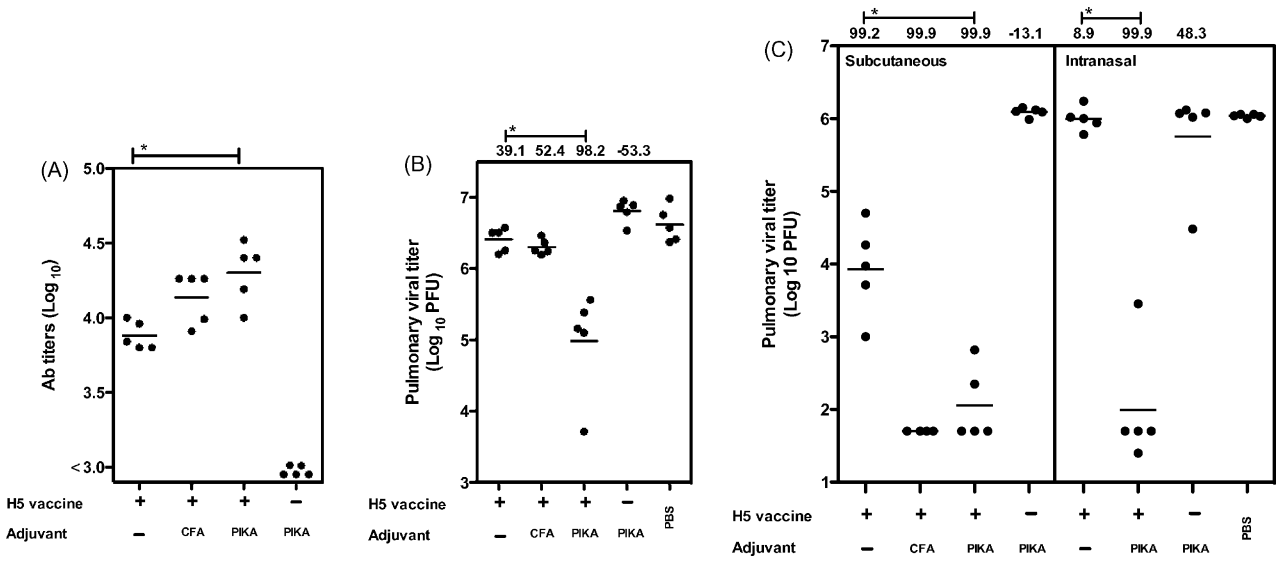

Fig. 5. PIKA enhances the immunogenicity of formalin-inactivated whole H5 virion vaccine. (A) Groups of five mice were vaccinated with a formalin-inactivated H5 vaccine subcutaneously at the base of the tail (300 ng of total protein) with or without PIKA as an adjuvant. On day 21, sera were collected and the antibody titers against H5 virus were determined by ELISA. (B) The mice were challenged intranasally with 50 PFU of a reverse-engineered H5 influenza virus intranasally to evaluate the degree of protection. The pulmonary viral titer was determined in lung homogenates obtained on day 5 post-infection. (C) Groups of five mice were vaccinated as described in (B) and were boosted on day 21 post-vaccination. On day 42 post-vaccination, the mice were challenged with 50 PFU of the reverse-engineered H5 influenza virus intranasally. The pulmonary viral titer was determined as previously described. The percent reduction in mean viral titer relative to the group treated with PBS is shown above each column of data. The '*' symbol indicates that the difference between the two groups was statistically significant ($p < 0.05$).

showed the most potent viral reduction, with 99.8% reduction in viral titer compared to the PBS control group, and this difference was statistically significant ($p = 0.0079$). The group that received PIKA alone served as control to ensure that the observed reduction in pulmonary viral titer was due to the anti-HA antibody responses, instead of the direct inhibitory effects of PIKA on viral replication shown in the Fig. 1. The enhancement in viral clearance was also observed in the group that received the vaccine by the i.n. route (Fig. 4C). Mice that received either vaccine alone or vaccine with PIKA showed a 66.4% and 99.8% reduction, respectively, and the difference between the 2 groups was statistically significant ($p = 0.0079$). It is interesting to note that, although the group received the vaccine admixed with CFA had the highest antibody titers (Fig. 4B), however, the mice had higher pulmonary viral titers compared with mice received the vaccine administrated with PIKA (Fig. 4C). To investigate this discordance between the two parameters, the relative avidity of the antibodies of the two groups against the vaccine antigen was examined in a urea-displacement ELISA. As shown in Fig. 4D, the group received the vaccine with PIKA had antibodies with higher avidity to the surface glycoprotein of the virus as significantly more antibodies remained bound to the HA when exposed to 6 M urea as compared to those induced with CFA as an adjuvant ($p = 0.0317$).

3.4. PIKA enhances the immunogenicity of formalin-inactivated whole H5 virion vaccine

There is an accumulating body of data showing that H5N1 virus vaccines are generally poorly immunogenic. We therefore proceeded to examine whether PIKA was an effective adjuvant for an H5N1 vaccine. Groups of five mice were inoculated by the s.c. route with a formalin-inactivated H5 vaccine, with or without an adjuvant, and 3 weeks later, sera were collected to determine the antibody titer against the virus. As shown in Fig. 5A, the whole-virion vaccine was capable of inducing a measurable titer of antibody after one dose. However, with the inclusion of PIKA, there was a ~3-fold increase in antibody titer ($p = 0.0157$), and this was comparable to the titer achieved in the group that received the vaccine with CFA. When challenged with the homologous virus i.n., however, this group of mice was the only group which showed significant reduction in pulmonary titer on day 5 pi (Fig. 5B, $p = 0.0079$). The efficacy was further boosted by a second dose of H5 vaccine with PIKA. Mice in this group showed a further 1000-fold reduction in viral titer (Fig. 5C). In addition, the vaccine-PIKA formulation was efficacious in reducing viral replication even when the vaccine was delivered intranasally. In contrast, in the absence of PIKA, the vaccine administered by the i.n. route failed to induce a significant reduction in viral titer compared with the PBS control ($p = 0.2492$).

In summary, we have demonstrated that the inclusion of PIKA in two different formations of influenza vaccine can achieve substantial antigen-sparing with robust humoral immune responses, leading to potent pulmonary viral titer reduction *in vivo*.

3.5. PIKA is able to interact with TLR3 to activate dendritic cells

In an attempt to elucidate the biological basis for the inhibition of viral replication and the adjuvant effects of PIKA, we sought to identify the receptor(s) that might be involved in these processes and to examine how cells of the innate immune system respond to PIKA stimulation. Using a human TLR3-expressing plasmid, we showed that PIKA is capable of interacting specifically with TLR3 in a dose-dependent manner (Fig. 6). Similar results were obtained when the experiment was carried out with mouse-TLR3-expressing plasmid (data not shown).

Next, we investigated the outcome of stimulating DC *in vitro* with PIKA given that DCs are the most potent antigen presenting cells (APC), responsible for antigen capture and presentation to naïve T lymphocytes. Using primary immature DC culture, D1 cells, we examined how DC responded to PIKA stimulation *in vitro*. PIKA was added into DC cultures for 16 h following which total RNA was extracted from the cells. The expression level of more than 1000 immunologically related genes between PIKA-stimulated and unstimulated samples were compared using microarray technology. As shown in Fig. 7A, PIKA-stimulated DCs up-regulated expression of a number of cellular activation markers, such as CD80 and CD86 compared to unstimulated cells.

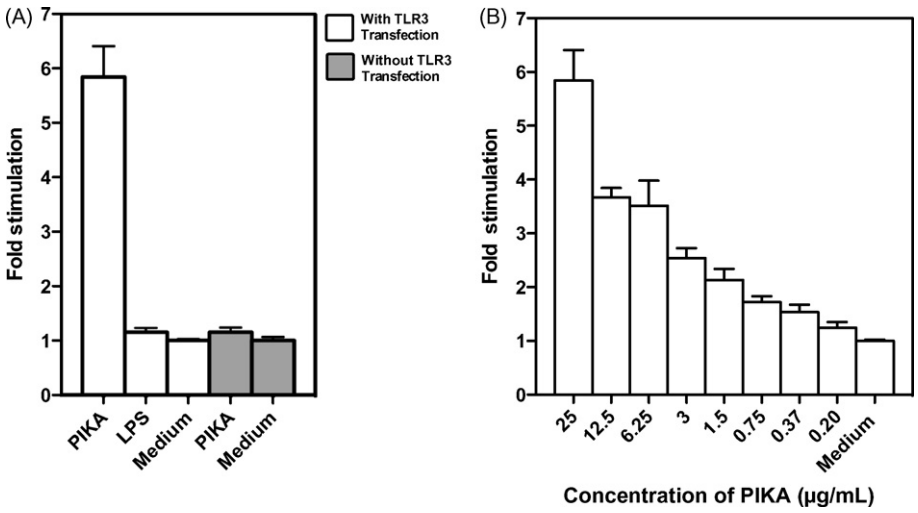

Fig. 6. PIKA interacts with human TLR3. HEK293 cells were transfected with an NF-κB-luciferase reporter gene, with or without co-transfection of a human TLR3-expressing plasmid. Twenty-four hours after transfection, the cells were stimulated with 25 μg per mL of PIKA, 1 μg per mL of LPS or medium alone (A) or 2-fold serially diluted PIKA (B). Six hours after stimulation, the cells were lysed and luciferase activity was determined. The data were normalized with respect to β-galactosidase activity and expressed relative to unstimulated samples. The bars and error bars represent the mean and standard deviation of triplicate samples and are representative of three independent experiments.

A number of genes that have important immunological functions were also found to be up-regulated after PIKA treatment, including IRF-7 (interferon regulatory factor 7), IL-1, IL-15, IL-6 and IL-12 receptor. Many chemokines, such as MIP (macrophage inflammatory protein), Rantes, MCP-2 (macrophage chemoattractant protein) and IP-10 (interferon gamma inducible protein) were also found to have increased expression. Quantitative real-time PCR was used to confirm these findings. Using cDNA from unstimulated DC as the baseline, many genes were up-regulated in a dose-dependent manner (Fig. 7B). While certain genes, such as iNos and IL-12, were up-regulated to a comparable level in LPS-stimulated DC, other genes, such as CD40L, MIP3 and IFN-γ, were up-regulated more readily by PIKA stimulation. To demonstrate that the observed changes in gene expression lead to changes in these cytokines/chemokines, the level of these proteins in the culture supernatants was measured. Consistent with the up-regulation of cytokine/chemokines genes, there was an increase in the production of these cytokines/chemokines in the supernatant from PIKA-stimulated DC compared with those from the unstimulated DC (Fig. 7C).

4. Discussion

It has been 11 years since the first case of human infection with a highly pathogenic H5N1 virus was identified in Hong Kong [30] and the virus has now spread to many regions, including Southeast Asia, Western China [31], Africa [32], Turkey [33] and Siberia [34]. Most human cases to date involve close contact with infected poultry and human-to-human transmission remains limited [35]. Nonetheless, the lack of anti-H5 immunity in humans, together with the continuous evolution of the virus in close proximity with the human population, threatens a pandemic. Should the virus acquire the property of efficient transmissibility between humans, in combination with the high case-fatality rate resulting from infection, this virus has the potential of causing an influenza pandemic with damage on a scale similar to that achieved by the 1918 'Spanish' flu pandemic. Therefore, there is an urgent need to develop effective prophylaxis and therapeutic strategies in preparation for a possible pandemic.

In the current study, using a murine influenza model, we have evaluated the potential of PIKA for prophylaxis and treatment of influenza infection as well as an adjuvant for influenza vaccine. Our data demonstrate that administration of PIKA intranasally prior to or shortly after an influenza infection can inhibit influenza replication, leading to a significant reduction in pulmonary viral titers. This effect is unlikely to be due to a direct antiviral activity, because pre-incubating influenza virus with PIKA did not inhibit virus infectivity for embryonated chicken eggs (Lynn Tang, unpublished data). Instead, PIKA appears to act by stimulating the innate immune system (Fig. 7), resulting in the production of several chemokines, cytokines and interferons and these, in turn, mediate the observed antiviral activity. Myxovirus resistance (Mx) proteins, protein kinase R and 2'5' oligoadenylate synthetase are some of the products that have been reported to be up-regulated after type 1 interferon production (reviewed in Ref. [16]), achieving an antiviral state in the host [36].

The fact that PIKA administration results in the stimulation of several antiviral proteins in the host, it decreases the likelihood that viruses will develop resistance through mutation. Furthermore, as PIKA does not target a specific component of the virion for its antiviral activity, it is likely to be effective against multiple strains and subtypes of influenza viruses as demonstrated in this study. This broad-spectrum activity is not limited to influenza A viruses only. A number of studies have shown that TLR-ligands are capable of inhibiting a wide range of viruses, including herpes simplex virus-2 [37], cytomegalovirus [38], parainfluenza [39], West Nile virus [40], severe acute respiratory syndrome coronavirus (SARs-CoV) [41] and influenza virus [42,43]. The abovementioned advantages make this novel antiviral approach a compelling alternative to traditional antiviral drugs and vaccines especially since avian influenza viruses have rapidly developed resistance to antiviral drugs [44] and some mutant viruses can maintain drug-resistance without losing virulence [45]. In addition, Thitithanyanont et al. showed that stimulating DC with Poly(I:C) conferred the cells with resistance to the cytopathic effects of H5N1 virus which might be important for the induction of virus-specific immune responses during the infection [46].

Apart from being an effective agent for prophylaxis and treatment, PIKA can also act as an effective adjuvant. Using two different forms of influenza vaccine (split subunit vaccine and whole inactivated vaccine), the magnitude of the humoral responses elicited by the combination with PIKA was comparable to those formulated in Freund's adjuvant. Our observation is consistent with the report by Shen et al. [26] which showed that administering

(A)

Cell activation marker	Fold increase / variation
CD14	7.27 / 3 %
CD25	2.11 / 3 %
CD69	16.10 / 64 %
CD72	4.12 / 20 %
CD80	13.08 / 8 %
CD86	2.35 / 2 %
CD205	2.00 / 22 %

Interleukins and their receptors	
Interferon regulatory factor 7 (IRF-7)	123.41 / 17 %
Interleukin-1 alpha precursor, (IL-1a)	19.06 / 33 %
Interleukin-12 beta chain precursor (IL-12b)	5.70 / 5 %
Interleukin-15 precursor (IL-15)	3.21 / 3 %
Interleukin-6 precursor (IL-6)	12.54 / 34 %
Interleukin-12 receptor beta-2 chain precursor	2.78 / 7 %

Chemokines and their receptors	
CCL3,macrophage inflammatory protein 1-alpha	3.89 / 3 %
CCL4,macrophage inflammatory protein 1-beta	5.73 / 6 %
CCL5, Rantes	8.23 / 4 %
MCP-2 precursors	3.19 / 21 %
CXCL10, IP-10	13.05 / 13 %
C-X-C chemokine receptor type 4 (CXC-R4)	2.46 / 7 %
CCL22	3.19 / 1 %

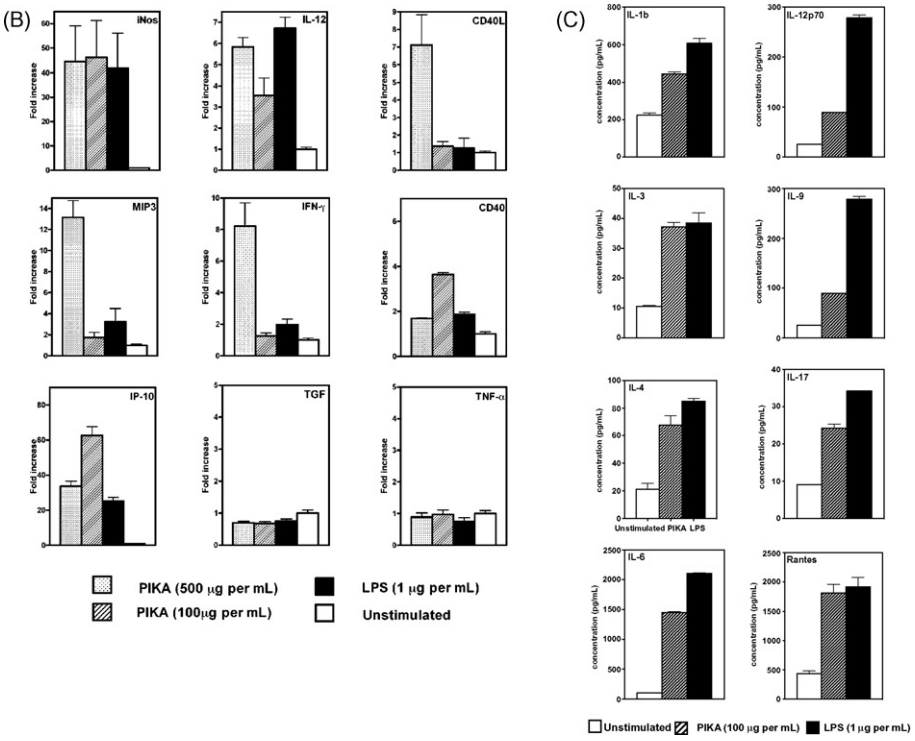

Fig. 7. PIKA induces maturation of dendritic cells, with expression of a wide range of immunological genes. Immature murine dendritic cells, D1 cells, were incubated with 500 μg per mL of PIKA or were unstimulated overnight. (A) Total RNA was harvested from the cells and converted into cDNA. The cDNAs derived from PIKA-stimulated DC and unstimulated DC were amplified and labeled with Cy5 and Cy3 dye respectively. The samples were hybridized overnight to a microarray chip and fluorescence signals were measured by an array scanner. Each gene target was printed 4 times on the array and the normalized mean Cy5/Cy3 ratio and coefficient of variation (cv) of the four replicates were determined by the software. (B) RNA was harvested from the DCs and converted into cDNA. The expression level of each cytokine gene was determined by quantitative real-time PCR. The expression level was normalized with beta-actin, a house-keeping gene, and data were expressed relative to unstimulated samples. The bars and error bars represent the mean and standard deviation of triplicate samples and are representative of two independent experiments. (C) Supernatants were harvested from DCs stimulated overnight with either 100 μg of PIKA, 1 μg of LPS or unstimulated. Fifty microliters of the supernatant was used to test for the presence of various cytokines/chemokines in the supernatants using the Bioplex Protein Array system and were measured in duplicate. The bars and error bars represent the mean and standard error.

PIKA with hepatitis B vaccine boosted the humoral response. We explored the concept further by demonstrating that a substantial antigen-sparing effect could be achieved by incorporating PIKA into vaccines. Using the commercial split vaccine, the antigen-sparing effect was about 100-fold (Fig. 4A and B). Furthermore, we demonstrated that the augmented humoral response led to a functional reduction in viral titer (Fig. 4C). Our data also showed that successful immunization could be achieved by a non-parenteral route. With two doses, the magnitude of the response achieved by intranasal immunization was comparable to that achieved by subcutaneous immunization and was associated with a similar level of viral clearance. Our finding is consistent with the report from

Ichinohe et al. [29] in which they showed that another TLR3 ligand, Ampligen, was capable of enhancing the immunogenicity of a trivalent inactivated influenza vaccine when administrated intranasally. In their study, the enhancement effect was demonstrated using a higher dose of the trivalent vaccine (1 μg) and in this current study, we extended the observation further by showing that similar enhancement effect can be achieved even when the concentration of the immunogen was reduced to 0.045 μg (22-fold lower). The antigen-sparing effect of PIKA might be an important strategy for maximizing vaccine coverage when vaccine supplies are limited. It is of interest to note that although the group that received the influenza vaccine with PIKA did not have the highest antibody titer, it had the lowest pulmonary viral titer (a 10-fold reduction compared with the CFA adjuvant vaccine group). The higher avidity of the antibodies induced by the vaccine with PIKA might allow more effective inhibition of viral replication *in vivo* which translated into lower pulmonary viral titers as observed in this study. This suggests that in addition to augmenting the amount of antibody produced, PIKA may also have an impact on the quality of the humoral responses, such as affinity maturation [47,48].

Because PIKA is a stabilized form of double stranded (ds) RNA that lacks direct anti-influenza activity, we evaluated its effect on the innate immune system in search of the mechanism underlying the observed activity. dsRNA was shown to be a ligand of TLR3 [49] and using a transient transfection system, we showed that PIKA maintained its TLR3 binding property. Incubation of PIKA with primary immature murine DC *in vitro* resulted in maturation of dendritic cells, with marked up-regulation of co-stimulatory molecules, such as CD80 and CD86 and cytokines with potent immunostimulatory functions such as IL-12 and IL-15. Our observation is consistent with those observed by Shen et al. with BMDC [26]. The maturation process enables DC to act as a potent antigen presenting cells for effective activation of naïve lymphocytes [13,50], which in turn, leads to a more robust immune response. In addition, a study conducted by Perera et al. [51] showed that a vaccinia virus expressing IL-15 induced superior cellular and humoral immune responses compared to the parental strain. Therefore, the induction of IL-15 by PIKA could also have resulted in a more efficient induction of $CD4^+$ T cell responses, translating to more robust humoral responses.

Lung tissue has recently been shown to constitutively express TLR3 *in vivo* in mice [52] and intranasal administration of PIKA could directly stimulate these TLR-3 expressing cells, which is perhaps why PIKA worked most effectively when administrated intranasally (Fig. 1D). We believe that PIKA will have similar antiviral activity in humans, because we showed that human TLR-3 can recognize PIKA (Fig. 6) and a number of studies have demonstrated the expression of TLR-3 in various respiratory cell types, such as nasal epithelial cells [53], airway smooth muscle cells [54] and airway epithelial cells [55]. In addition, ds RNA was found to be the most effective TLR ligand in activating airway epithelial cells in producing IL-8 [55]. Although our experimental data are limited to TLR-3, dsRNA can also be recognized by RNA helicase, RIG-1 and MDA5, and can induce NF-κB activation with type-1 IFNs [56]. It is possible that PIKA stimulates several pathways *in vivo* to achieve its antiviral and adjuvant activities. The knockout-mice, such as TLR3 k/o, MDA5 k/o and RIG-1 k/o mice, will be useful in shedding more light on the contribution of these receptors to the activity of PIKA.

In summary, although Poly(I:C) has been shown to be an effective mucosal adjuvant, its use in clinical trials was associated with side-effects that has limited its usage [57]. In a recent toxicity trial, PIKA showed no significant toxicity in mice and a phase 1 clinical study showed that the use of PIKA as an adjuvant was well-tolerated in humans without significant side-effects (Peter Brazier, unpublished data). The safety profile, together with the long shelf-life, makes PIKA an attractive complement to currently licensed antiviral drugs. In the early phase of an influenza pandemic, when a well-matched vaccine is unlikely to be available, PIKA can be used as an antiviral drug for temporary protection of the susceptible population, slowing down the rate of viral transmission and allowing more time for vaccine production. Furthermore, global influenza vaccine production is likely to lag well behind the demand for pandemic influenza vaccine, so the inclusion of an effective antigen-sparing adjuvant such as PIKA can ensure that limited supplies of vaccine achieve maximum coverage.

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