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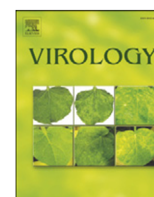

Activation of the innate immune system provides broad-spectrum protection against influenza A viruses with pandemic potential in mice

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ABSTRACT

The efficacy of a stabilized chemical analog of double-stranded ribonucleic acid (RNA), PIKA, as prophylaxis against infection with 5 different influenza A virus subtypes, including the 2009 swine-origin pandemic H1N1 virus, was evaluated in mice. Intranasal treatment with PIKA resulted in a significant reduction of viral replication in the respiratory tract. The inhibitory effect was mediated by rapid infiltration of immune cells into the lungs, and production of inflammatory cytokines. While TLR3 is important for the optimal production of these inflammatory cytokines, inhibition of viral replication was still observed in $TLR3^{-/-}$ mice. In addition, a significant synergistic effect in inhibiting H5N1 virus replication was observed when PIKA was coadministered with oseltamivir. The broad-spectrum protection provided by PIKA makes it an attractive option for prophylaxis from infection with influenza A viruses.

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Introduction

Influenza is an acute respiratory disease associated with significant morbidity and mortality worldwide, particularly among the elderly and young children (Izurieta et al., 2000; Neuzil et al., 2000; Nicholson et al., 2003). The newly emerged swine-origin H1N1 virus has caused the first influenza pandemic of this century (Centers for Disease Control and Prevention, 2009b). The enzootic of highly pathogenic H5N1 influenza A viruses in poultry in Asia with sporadic transmission to humans also raises concerns of a possible pandemic (Beigel et al., 2005; Lipatov et al., 2004). Vaccination is the most effective tool for controlling an influenza pandemic, but it is estimated that the global suppliers of vaccines can only produce ~2.5 billion doses within the first 12 months following receipt of a new vaccine strain and will take 4 years to meet the global demand (International Federation of Pharmaceutical Manufacturers & Associations, 24 February 2009). While antiinfluenza drugs, such as neuraminidase inhibitors (oseltamivir and zanamivir) or ion channel blockers (adamantanes) have been used for treatment and prophylaxis (Hayden et al., 1999; Treanor et al., 2000), drug resistance occurs and the appearance of drug resistant viruses before or during a pandemic would severely affect the efficacy of treatment strategies (de Jong et al., 2005; Hayden, 2006a, b; Hurt et al., 2007; Kiso et al., 2004; Le et al., 2005; McKimm-Breschkin et al., 2007). Therefore, additional antiviral strategies are needed.

In contrast to the adaptive immune system, the innate immune system provides broad-spectrum defense that can be activated immediately or within hours after infection. It is believed that these antiviral responses play a critical role in limiting the replication and dissemination of pathogens, providing time for adaptive immune effectors to develop. For example, different innate cell types, such as neutrophils (Fujisawa, 2008) and macrophages (Herold et al., 2008), contribute to the control of influenza A virus replication *in vivo* and plasmacytoid dendritic cells (pDCs) produce large amounts of type 1 interferon after exposure to influenza (Jego et al., 2003; Thitithanyanont et al., 2007).

We were interested in targeting innate immune mechanisms because they may be less strain-specific than adaptive immune mechanisms. We have previously demonstrated that the administration of a stabilized chemical analog of double-stranded (ds) RNA (PIKA) was able to inhibit the replication of three mouse-adapted laboratory strains (H1N1 and H3N1) of influenza A virus in mice (Lau et al., 2009). In the current study, we extended these observations to influenza A viruses with pandemic potential and variable virulence, including three clinical isolates from humans, an H5N1 virus, an H9N2 virus, and a swine-origin H1N1 virus from the 2009 pandemic. Using a number of assays, we also showed that a heightened antiviral state was achieved in the respiratory tract shortly after administration of the drug; several chemokines were produced and different cell types infiltrated into the lungs. This study demonstrates the feasibility of harnessing innate immunity to provide broad-spectrum protection against multiple influenza A virus subtypes, an attractive approach as a first line of defense in the event of an influenza pandemic. We also evaluated a combination of PIKA and oseltamivir for the prevention of H5N1 virus infection in mice.

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Results

Inhibition of viral replication in the respiratory tract of mice after PIKA administration

We previously showed that after intranasal (i.n.) administration of PIKA, pulmonary viral titers of three different laboratory strains of influenza virus (A/Puerto Rico/8/34 (H1N1), A/WS/33 (H1N1), and a reassortant of A/Memphis/1/71 (H3N1)) were substantially reduced compared to a PBS control group (Lau et al., 2009). In this study, we proceeded to evaluate the efficacy of this strategy against five wild-type (*wt*) influenza A viruses with pandemic potential. Groups of five lightly anesthetized 8- to 10-week-old female BALB/c mice were given 100 μg of PIKA or PBS, in a volume of 50 μL i.n. 6 hours before infection with four avian influenza viruses, namely A/teal/HK/W312/97 (H6N1), A/rhea/NC/93 (H7N1), A/HK/1073/99 (H9N2,) and A/Vietnam/1203/2004 (H5N1), and with an isolate from the 2009 H1N1 pandemic A/California/07/2009 (H1N1). Apart from the H5N1 virus, for which the challenge dose was set at 15 $TCID_{50}$ because it is exceptionally virulent in mice, other viruses were administered at a dose of 50 $TCID_{50}$. Mice continued to receive daily treatment with 100 μg of PIKA i.n. for 2 days before they were sacrificed on day 3 post-infection (p.i.), and virus titers in nasal turbinates (NT) and lungs were determined. As shown in Figs. 1A–E, the viruses replicated to high titers in the lungs of mice treated with PBS. Lung virus titers were statistically lower in mice that were treated daily with PIKA than in the PBS control group ($p<0.05$, Mann–Whitney test). The efficacy of PIKA against the H6N1 virus could only be assessed in the lower respiratory tract because the virus did not replicate to detectable levels in the NT of mice. For the other viruses, 5/5 (H7N1), 3/5 (H9N2), 2/5 (H5N1), and 2/5 (H1N1) of the PBS-treated mice had virus detected in the NT. Except for the H7N1 experiment in which one mouse in the PIKA-treated group had virus detected in the NT, none of the PIKA-treated mice in other challenge groups had virus detected in the NT, demonstrating the effectiveness of the treatment in inhibiting viral replication in the upper respiratory tract. In summary, the results suggest that PIKA prophylaxis can effectively inhibit the replication of several subtypes of *wt* influenza A viruses including the newly emerged H1N1 pandemic virus, leading to a substantial reduction of viral titers in the respiratory tract.

Administration of PIKA protects mice from lethality following challenge with A/Puerto Rico/8/34

To determine the significance of the reduction in pulmonary virus titer observed in our previous study (Lau et al., 2009) and in Fig. 1, groups of five mice were treated with PIKA as previously described, were challenged with 4 LD_{50} of A/Puerto Rico/8/34 and monitored for 2 weeks. As shown in Figs. 2A and B, mice that received three doses of PIKA were completely protected from lethal challenge with no significant weight loss and 100% survival, whereas all of the mice that received PBS treatment demonstrated significant weight loss and succumbed to infection. In addition, virus-specific antibodies were detected in the sera of the PIKA-treated mice on day 14 post-infection (Fig. 2C).

Coadministration of PIKA with oseltamivir provides synergistic protection against challenge with VN04 (H5N1) in mice

Although oseltamivir phosphate (Tamiflu) has been used widely in treating infections with highly pathogenic H5N1 influenza A viruses in humans, many patients still succumb to infection (Beigel et al., 2005), and therefore, more effective treatment is needed. We proceeded to determine the benefit of coadministering PIKA with oseltamivir for protection against H5N1 virus infection in mice. Groups of five mice were given 100 μg of PIKA i.n. and 10 mg/kg/day of oseltamivir by oral gavage 6 hours before challenge with 15 $TCID_{50}$ of VN04 (H5N1, ~6 MLD_{50}) *wt* virus i.n. The mice continued to receive daily treatment with PIKA and two doses of oseltamivir treatment daily. The dose of oseltamivir used was selected based on a study by Yen et al. (2005) that demonstrated that a 5-day regimen of 10 mg/kg/day protected 50% of mice infected with 5MLD_{50} of VN04

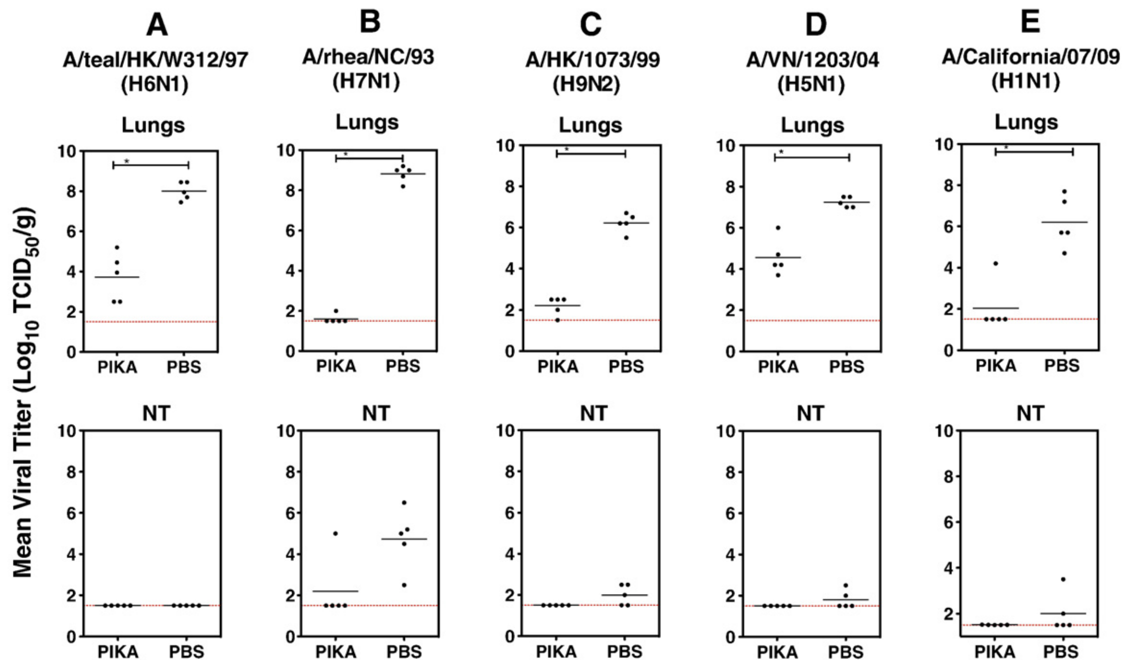

Fig. 1. Administration of PIKA inhibited replication of influenza viruses in the respiratory tract of mice. Groups of five mice each were given 100 μg of PIKA in PBS intranasally 6 h before challenge with the indicated viruses. The mice continued to receive PIKA treatment once a day for two additional days. Lungs and NT were harvested on day 3 post-infection. Virus titers in the lungs and NT were determined in MDCK cells. The lower limit of detection was $10^{1.5}$ $TCID_{50}$ per gram of tissue. The horizontal bars represent the geometric mean of the group. The '*' symbol indicates that the difference between the groups was statistically significant ($p<0.05$).

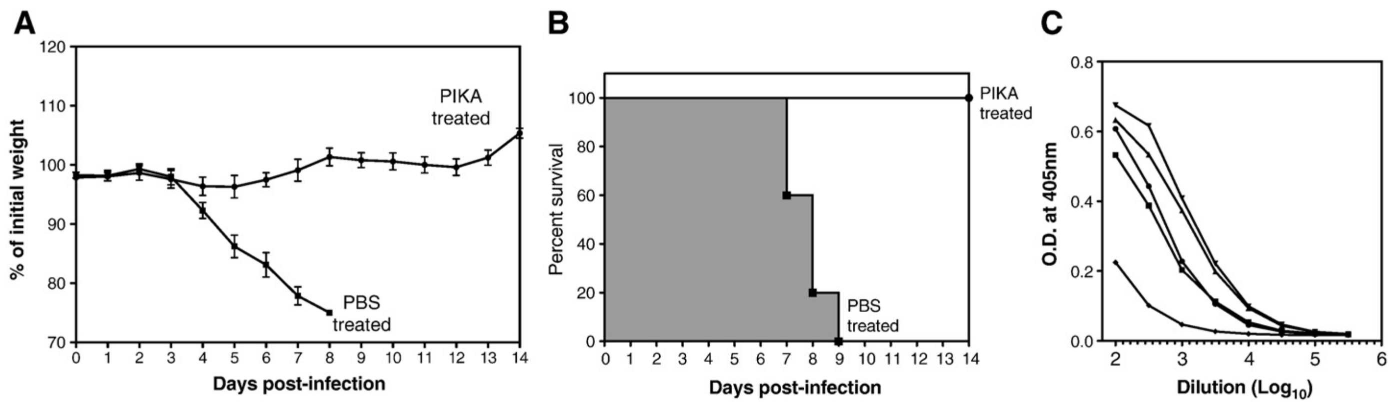

Fig. 2. Administration of PIKA protected mice from lethal challenge and elicited influenza-specific antibody responses. (A and B) Groups of five mice each were given PIKA or PBS as previously described and challenged with 4 LD_{50} of A/Puerto Rico/8/34 intranasally. The mice continued to receive PIKA treatment once a day for two additional days. Weight loss and survival rate was monitored for 2 weeks. (C) Serum samples were collected from PIKA-treated mice on day 14 post-infection, and influenza-specific antibody titers were determined by ELISA.

(H5N1) *wt* virus. Three days after infection, the level of viral replication in various organs was determined. This experimental design allowed us to determine the virologic benefit of the combined therapy over oseltamivir treatment alone. We had also intended to evaluate whether the combination of PIKA and oseltamivir would offer a survival benefit, but mice that received a 5-day course of oseltamivir showed 60%–100% mortality on day 6 compared with 0%–20% mortality in a group that received water alone by gavage on the same schedule, regardless of whether they were infected with an influenza virus. We infer that the filler material present in commercial oseltamivir might contribute to the increased death rate observed in mice because the dose of the drug is similar to that reported (Yen et al. (2005).

As shown in Fig. 3A, challenging mice with 15 $TCID_{50}$ of VN04 (H5N1) *wt* virus did not lead to significant viral replication in the NT on day 3 post-infection, even in mock-treated mice, and therefore, the difference in the level of protection between the combined or oseltamivir alone regimen did not reach statistical significance. However, none of the mice that received the combined treatment had detectable levels of virus, while some mice that received oseltamivir alone or mock-treated had virus detected in the NT (1/5 and 2/4, respectively). In the lungs, there was a significant reduction in pulmonary viral titers in mice that received the combined treatment compared to the groups that received oseltamivir alone or mock treatment (Fig. 3B; $p<0.05$, Mann–Whitney test). Three out of five mice that received the combined treatment had no detectable virus in their lungs on day 3 post-infection, and the average viral titer was lower than the group that received oseltamivir alone (Fig. 3) or PIKA alone (Fig. 1D). These results suggest that targeting different antiviral mechanisms is a feasible approach in achieving a synergistic virologic effect in combating H5N1 virus infection.

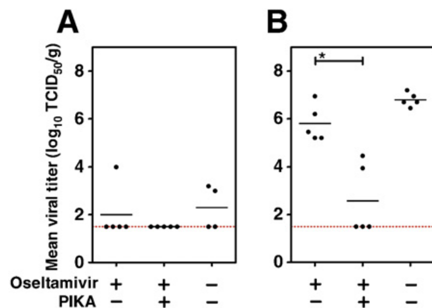

Fig. 3. Synergistic antiviral effect achieved against H5N1 infection in mice by coadministering PIKA with oseltamivir. (A and B) Groups of five mice were given either oseltamivir alone by oral gavage or together with 100 µg of PIKA i.n. 6 hours before challenge with 15 $TCID_{50}$ of VN04 *wt*. Mice continued to receive treatment for 2 additional days. NT and lungs were harvested for viral titration on day 3 post-infection. Virus titers in the NT (A) and lungs (B) were determined in MDCK cells. The lower limit of detection was $10^{1.5}$ $TCID_{50}$ per gram of tissue. The horizontal bars represent the geometric mean of the group. The '*' symbol indicates that the difference between the groups was statistically significant ($p<0.05$).

Administration of PIKA led to changes in the cellular composition and chemokine production in the lungs of mice

To establish the mechanism(s) responsible for the reduction in viral titers achieved by PIKA, mice that were given one or three doses of PIKA were sacrficed at various time points to examine the lungs for changes in cellularity. Single-cell suspensions were made as previously described (Lau et al., 2006), and different cell populations were identified using cellular markers conjugated with fluorochromes (See Supplemental Figure 1 for the gating strategy). Using this approach, we were able to identify ~70% of the cell population in the lungs (data not shown). As shown in Fig. 4A, there was a two-fold increase in neutrophils ($CD11b^+$ $CD11c^-$, $Ly6G^+$, $Ly6C^+$) within 24 hours of a single dose of PIKA, reaching a peak on day 2 post-administration. The number of neutrophils in the lungs of mice after three doses of PIKA was significantly greater than in mice that received a single dose of the drug ($p<0.05$, unpaired t test). There was an infiltration of interstitial macrophages ($CD11b^{Hi}$, $CD11c^{Lo}$) in the lungs that peaked on day 1 post-inoculation and was maintained at an elevated level for the next 72 hours (Fig. 4B). On the other hand, the infiltration of pDCs ($CD11c^+$, $Gr\text{-}1^+$, $B220^+$; Nakano et al., 2001) into the lungs was more gradual, continuing to increase for 72 hours after a single dose of PIKA (Fig. 4C). Administration of three consecutive doses of PIKA led to a significant increase in both interstitial macrophages and pDCs ($p<0.05$, unpaired t test). However, there was a reduction in the number of alveolar macrophages ($CD11c^{Hi}$, $CD11b^{Lo}$) within 24 hours of PIKA treatment that was maintained for 72 hours (Fig. 4D). There were no significant changes in the numbers of natural killer cells, NKT cells, B lymphocytes, and $CD4^+$ and $CD8^+$ T lymphocytes in the lungs (data not shown).

Since cytokines and chemokines are responsible for cellular migration, we examined the levels of different cytokines and chemokines in the lungs at different time points after a single dose of PIKA. The lungs were homogenized and clarified homogenates were analyzed in duplicate with the Bio-plex Protein Array system to determine the protein levels of cytokines and chemokines. As shown in Fig. 5A, an early response of TNF-α was seen, with an increase 6 hours after administration of PIKA and return to baseline by 48 hours. The production of other cytokines such as IFN-γ, Rantes, MCP-1, and MIP-1b, increased 6 hours following administration of

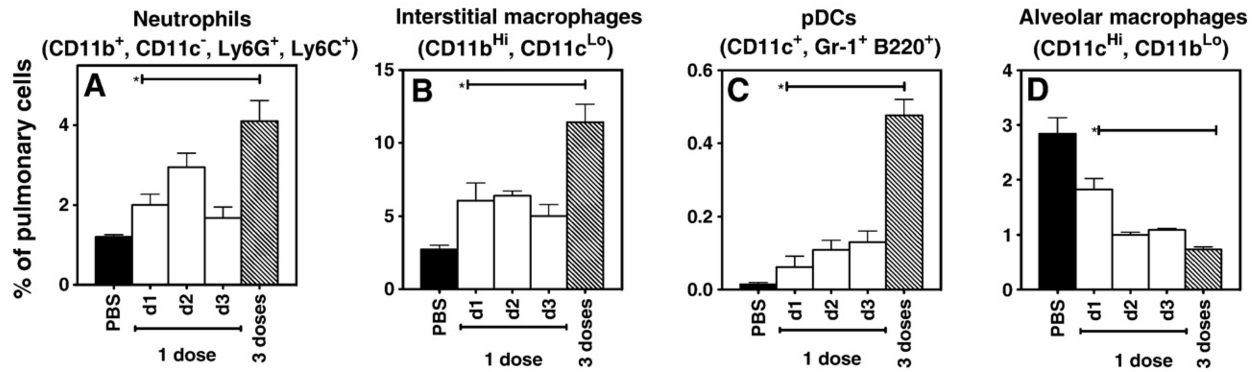

Fig. 4. Administration of PIKA changed the cellular composition of the lungs. Groups of three mice were given 100 μg of PIKA i.n. in PBS and sacrificed 1, 2, or 3 days later. An additional group received 100 μg of PIKA i.n. once a day for 3 consecutive days, and mice were sacrificed 24 hours after the last inoculation. Single-cell suspensions were prepared, and the cells were stained with the indicated cellular makers. Eighty thousand events were acquired for each sample. The bars and error bars represent the mean and standard errors of the groups. The '*' symbol indicates that the difference between the groups was statistically significant ($p<0.05$).

PIKA and was sustained for 48 hours (Fig. 5B; data not shown). An increase in production of KC was observed at 24 and 48 hours following PIKA administration (Fig. 5C). In addition, there was an increase in the production of IFN-β in lungs 24 hours after administration of PIKA (Fig. 5D). In summary, these data suggest that intranasal administration of PIKA leads to the activation of innate immunity in the respiratory tract, leading to infiltration of the lungs by immune cells and the production of cytokines and chemokines that are associated with inhibition of viral replication in the respiratory tract of mice.

TLR3 is dispensable in mediating the antiviral response to PIKA in mice

Using a plasmid expression system, we previously showed that PIKA interacts with TLR3 (Lau et al., 2009). Using the same expression system, we showed that, in addition to TLR3, PIKA can also be recognized by melanoma differentiation-associated gene 5 (MDA-5) and retinoic acid-inducible gene I (RIG-I) (Fig. 6A). Since TLR3 has been identified as a receptor for *ds*RNA with abundant expression in the lungs (Alexopoulou et al., 2001), mice with a targeted defect in the TLR3 gene were used to determine the role of TLR3 in mediating the antiviral effect of PIKA. TLR3$^{-/-}$ and TLR3$^{+/+}$ mice were given 100 μg of PIKA or PBS intranasally, and the concentrations of various cytokines in lung homogenates were determined. As shown in Fig. 6B, compared with TLR3$^{+/+}$ mice, the TLR3$^{-/-}$ mice showed a general trend of diminished cytokine production. The level of reduction varied between cytokines, with no increase of TNF-α and IFN-γ detected in TLR3$^{-/-}$ mice after PIKA stimulation. For other cytokines such as MIP-1b, Rantes, and MCP-1, there was an increase in production in TLR3$^{-/-}$ mice in response to PIKA treatment; however,

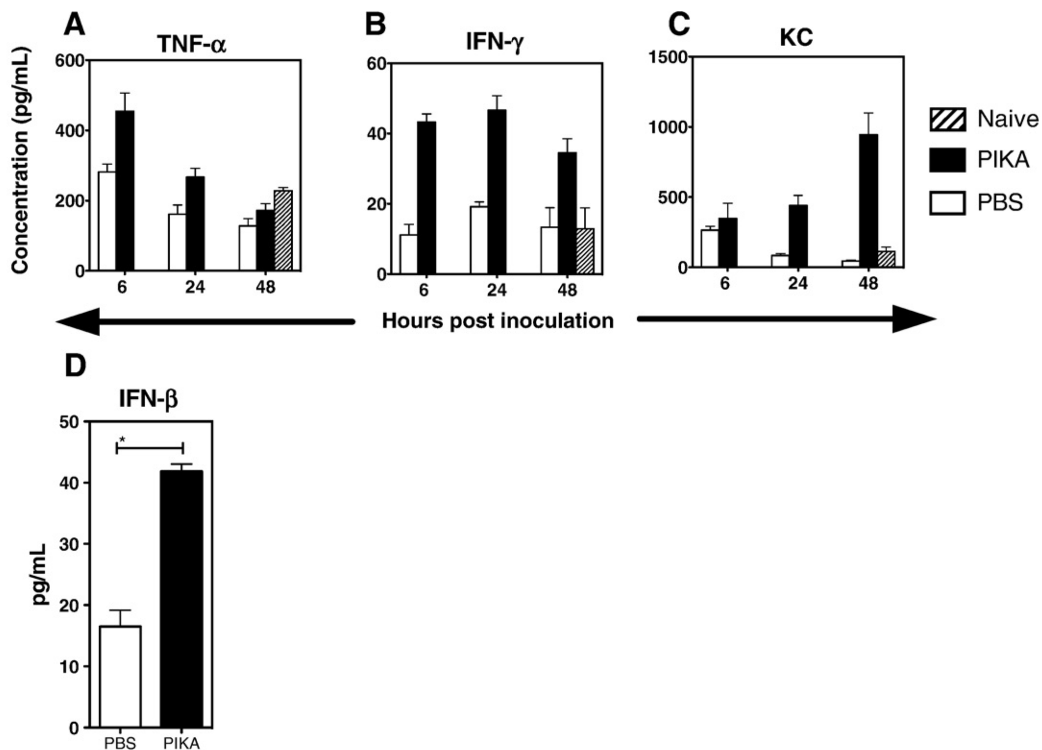

Fig. 5. Administration of PIKA induced production of cytokines in the lungs. Groups of three mice each were given 100 μg of PIKA i.n. in PBS and were sacrificed at the indicated time. The lungs were stored at −80 °C till all samples were collected and homogenized in 1 mL of RPMI-1640 media. (A–C) To measure the level of different cytokines/chemokines in the samples, 50 μL of the clarified samples was tested in duplicate using the Bio-plex Protein Array system. (D) For IFN-β, 100 μL of the clarified samples were tested using an ELISA kit. The bars and error bars represent the mean and standard error of the groups.

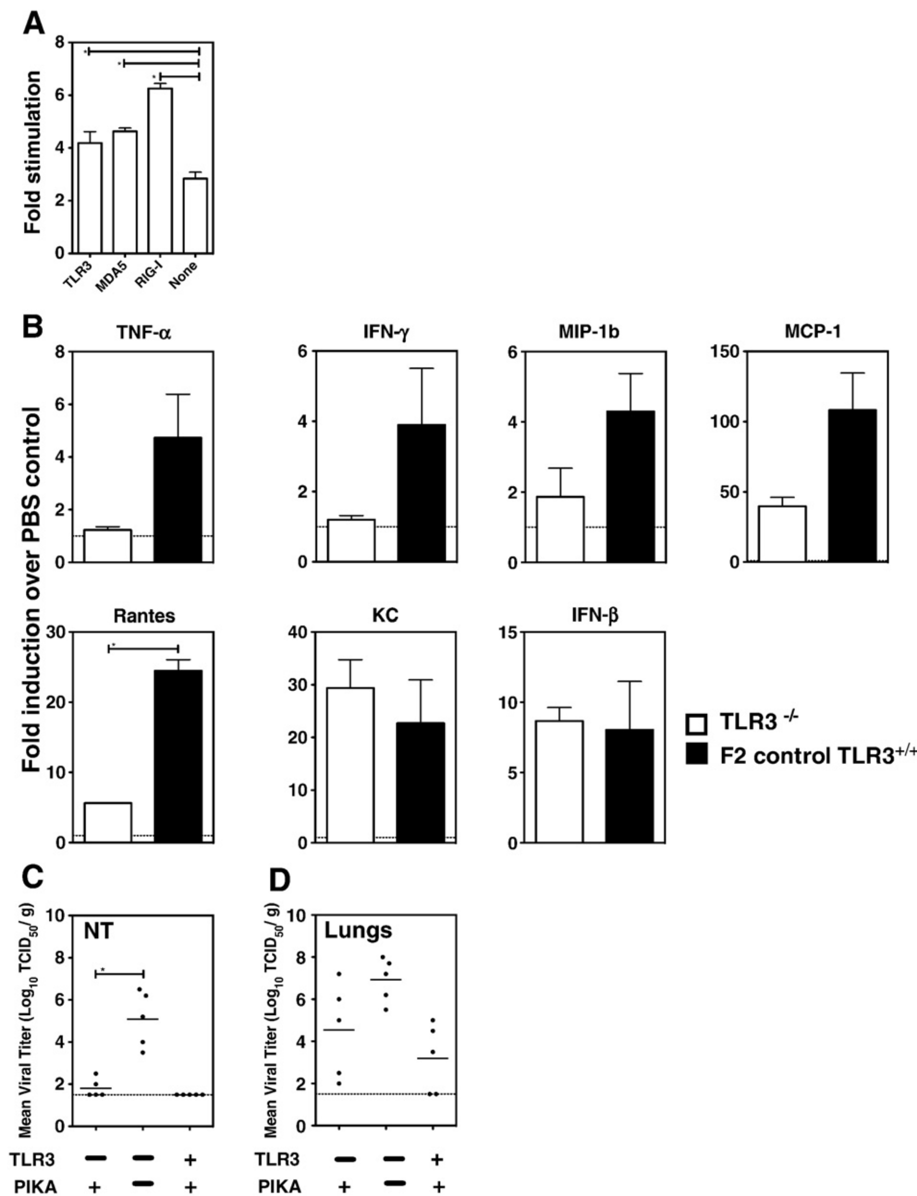

Fig. 6. TLR3 is dispensable in mediating the antiviral responses of PIKA in mice. (A) Human embryonic kidney (HEK 293 cells) were transfected with an NF-κB-luciferase reporter gene and a β-galactosidase-expressing plasmid with or without co-transfection of the indicated TLR3, MDA-5, or RIG-I receptor-expressing plasmids. Twenty-four hours after transfection, the cells were stimulated with 50 ng of PIKA in plain medium for 6 hours before the cells were lysed, and luciferase activity in the lysates was determined. The data were normalized to β-galactosidase activity and expressed as fold increase relative to expression in cells that were transfected with respective receptor-expressing plasmids without PIKA stimulation. The bar and error bars represent the mean and standard error of eight replicates and are representative of two independent experiments. (B) Groups of three $TLR3^{-/-}$ or $TLR3^{+/+}$ mice received PIKA or PBS intranasally and were sacrificed 24 hours later. Lung homogenates were prepared, and the concentration of various cytokines was determined as previously described. Data are expressed as the fold increase in PIKA-treated mice over PBS-treated mice. Groups of five $TLR3^{-/-}$ or $TLR3^{+/+}$ mice received PIKA and were challenged with the H7N1 virus as previously described. Virus titers in the NT (C) and lungs (D) were determined in MDCK cells. The horizontal bars represent the geometric mean of the group. The '*' symbol indicates that the difference between the groups was statistically significant ($p<0.05$).

the fold increase was lower than those observed with $TLR3^{+/+}$ mice, with the difference for Rantes reaching statistical significance ($p<0.05$). The production of KC and IFN-β was comparable between the two strains of mice. The results suggest that TLR3 is important for optimal cytokine production in response to PIKA. To determine the impact of the diminished cytokine responses on the antiviral effect of PIKA, $TLR3^{-/-}$ mice were treated with PIKA or PBS and were challenged with 50 $TCID_{50}$ of the H7N1 virus. This virus was chosen because it was able to replicate to high titer in both the upper and lower respiratory tracts of mice (Fig. 1B), allowing us to determine the importance of TLR3 in both areas. As shown in Fig. 6C, the virus replicated to high titer in the NT in the $TLR3^{-/-}$ mice treated with PBS, and the titer was significantly higher than in mice treated with PIKA ($p<0.05$, Mann–Whitney test). The virus titers in the lungs of the $TLR3^{-/-}$ mice were variable (Fig. 6D), and the mean titer in the two groups of mice was not statistically different. The results suggest that the production of cytokines induced by PIKA in the absence of TLR3 signaling was still sufficient to inhibit viral replication *in vivo*.

Discussion

We have demonstrated the efficacy of PIKA against different influenza A virus subtypes with pandemic potential that have caused infections in humans, including the newly emerged 2009 H1N1 pandemic virus (Butt et al., 2005; Centers for Disease Control and Prevention, 2009b; de Jong et al., 2005; Peiris et al., 1999; Subbarao et al., 1998). Although it may seem obvious that a drug that induces an interferon-induced antiviral state would prevent infection by

influenza viruses, a demonstration of the efficacy of this approach against viruses of varied virulence is important, and the virologic benefit of combining PIKA prophylaxis with oseltamivir is novel. We have also explored the mechanism underlying the antiviral effects of PIKA.

While immunoprophylaxis with poly IC has been described using mouse-adapted influenza strains (Saravolac et al., 2001; Wong et al., 1995, 1999), the mechanism responsible for the antiviral effects has not been studied in detail. Our data show that a number of immune effectors were activated following intranasal administration of PIKA. The initial wave of cytokine production occurred 6 hours following administration of PIKA included cytokines TNF-α and IFN-γ that have antiviral properties (Seo and Webster, 2002). Inflammatory cytokines, such as Rantes (CCL5) (Dorner et al., 2002) and monocyte chemotactic protein-1 (MCP-1), were produced in response to PIKA and led to the recruitment of effector cells such as neutrophils, macrophages, and pDCs into the lungs within 24 hours of administration of a single dose of PIKA. Fujisawa et al. (1987) have demonstrated the important role of polymorphonuclear leukocytes (PMN) in the early response to influenza virus infection, and Hashimoto et al. (2007) demonstrated that neutrophils and macrophages were capable of phagocytosing influenza-induced apoptotic cells *in vivo*, thereby disrupting the infectious cycle of the virus. Several investigators have used monoclonal antibodies to deplete neutrophils and/or macrophages *in vivo* and found an increase in viral titers associated with severe clinical disease (Tate et al., 2008; Tumpey et al., 2005). pDCs have the ability to produce large amounts of type 1 IFNs after exposure to influenza virus (Jego et al., 2003; Thitithanyanont et al., 2007,) and the release of type 1 IFNs can lead to the activation of IFN-stimulated genes with antiviral functions, such as Mx proteins, protein kinase R, and 2′5′ oligoadenylate synthetase (reviewed in Samuel, 2001). Since it is more difficult for pathogens to simultaneously develop resistance to two or more pathways than to a single pathway, the induction of antiviral effects by multiple pathways reduces the likelihood of viruses becoming resistant to PIKA. In addition, we demonstrated that the synergism achieved by coadministering PIKA with oseltamivir was sufficient to inhibit viral replication of the H5N1 virus (Figs. 1D and 3). Because the strategy of activating generic antiviral mechanisms by targeting the innate immune system is effective against a number of viruses, including herpes simplex virus type-2 (Bernstein et al., 2001; Harrison et al., 1994), cytomegalovirus (Chen et al., 1988), parainfluenza (Stokes et al., 1998), West Nile virus (Morrey et al., 2004), and influenza virus (Wong et al., 2005, 2007), we believe PIKA may have the potential to inhibit the growth of other viral pathogens.

While the antiviral properties of dsRNA compounds, such as poly ICLC, have been well documented, they are often associated with side effects. In a study by Wong et al. (1999), mice showed ~11% weight loss and hypothermia after receiving two doses of poly ICLC (~20 μg per dose). In contrast, PIKA displays antiviral properties in mice without causing significant weight loss. Although TLR3 has been identified as a receptor for *ds*RNA and cells from TLR $3^{-/-}$ mice showed reduced responses to poly I:C *in vitro* (Alexopoulou et al., 2001; De Miranda et al., 2009), our experiment in $TLR3^{-/-}$ mice suggested that other receptors might also be involved in mediating the antiviral activity of PIKA. Arimura (1975) and Kato et al. (2008) demonstrated that the interferon-inducing activity and recognition by RIG-I and MDA-5 of Poly I:C are dependent on the length of the poly I:C molecules. PIKA contains *ds*RNA greater than 100 bp in size (Peter Brazier, unpublished data), its receptor usage *in vivo* might be different from those used by poly I:C due to different size distribution. In addition, while Gitlin et al. (2006) reported that MDA-5, rather than TLR3, played an important role in mediating the type 1 interferon response to poly I:C *in vivo*, others showed that both MDA-5 and TLR3 are required for optimal biological activity of poly I:C (Kumar et al., 2008; Trumpfheller et al., 2008). The contradictory results could be because poly I:C was administered by different routes in these studies. We were unable to determine the relative importance of RIG-I and MDA-5 on the antiviral effect of PIKA *in vivo*, and we are currently investigating how PIKA can access MDA-5 and RIG-I, which are cytosolic sensors.

In an influenza challenge experiment using $TLR3^{-/-}$ and wild-type mice, Le Goffic et al. (2006) demonstrated that TLR3 activation in the context of an influenza virus infection resulted in an increase in cytokine production and the number of inflammatory cells in the lungs, resulting in immunopathology and reduced survival. The sequence of events in our experiments was different: mice were pretreated with PIKA that induced TLR3 activation and cytokine production. When the mice were subsequently infected with influenza virus, there was reduced viral replication, less inflammation and improved survival. In our lethal challenge experiment, the mice pretreated with PIKA showed no significant weight loss after infection, an observation that is markedly different from Le Goffic's study. We speculate that timing of TLR3 activation relative to influenza virus infection determines the outcome.

Wong et al. (2009) reported recently that administration of dsRNA condensed with poly-L-lysine and carboxymethylcellulose improved the survival of mice challenged with a lethal dose of A/chicken/Henan/5/2004 (a clade 2 H5N1 virus). In addition, Tuvim et al. (2009) showed that induction of local pulmonary inflammation led to protection against influenza infection in mice. Our findings further extend these observations by demonstrating the breadth of protection that PIKA can provide against a range of influenza viruses with pandemic potential. Furthermore, the transient inhibition of viral replication mediated by PIKA allows mice to be primed and develop influenza-specific antibody responses that can provide long-term protection from reinfection. A number of studies have shown that intranasal administration of exogenous type I interferon protects mice, ferrets, and guinea pigs from influenza virus infection (Kugel et al., 2009; Tumpey et al., 2007; Van Hoeven et al., 2009). Activation of the innate immune system using synthetic compounds to induce endogenous type I interferon production might be more cost-effective than exogenous type I interferon administration.

Since it remains uncertain which influenza strain(s) will eventually emerge as a pandemic virus, drugs that target the innate immune system and provide broad-spectrum antiviral activity have the potential to be used as a first line of defense. Our findings are promising and provide support for further evaluation of PIKA for prophylaxis. Areas for further study include an assessment of the risk of immunologic tolerance resulting from stimulation of the innate immune system (Trinchieri and Sher, 2007), the risk of secondary bacterial infection that is a common complication of influenza virus infection (Centers for Disease Control and Prevention, 2009a; Morens et al., 2008; Sun and Metzger, 2008), and studies to establish the feasibility of using PIKA prophylaxis in humans including the timing and duration of treatment.

Materials and methods

Mice

In all mouse experiments, 6- to 8-week-old female BALB/c mice (Taconic Farms, Inc., Germantown, NY) were used. The B6;129 S1-$Tlr3^{tm1Flv}$/J (stock number 005217) and B6129SF2/J (stock number 101045) were purchased from The Jackson Laboratory (Bar Harbor, ME). The animal protocols were approved by the National Institutes of Health's Animal Care and Use Committee, and the experiments were conducted at the NIH.

Viruses

Viruses used in this study were kindly provided by Dr Robert G. Webster, St. Jude Children's Research Hospital, Memphis, TN; Dr

Alexander Klimov, Influenza Division, Centers for Disease Control and Prevention, Atlanta, GA; and Dr David Swayne, Southeast Poultry Research Laboratory, USDA, Athens, GA. Virus stocks were propagated in the allantoic cavity of 9-day-old embryonated specific pathogen-free hen's eggs (Charles River Laboratories, Wilmington, MA) at 37 °C. Clarified allantoic fluids were aliquoted and stored at −80 °C.

Administration of PIKA and oseltamivir phosphate

PIKA was obtained from NewBiomed PIKA Pte Ltd. (Singapore). The endotoxin level of the lot of PIKA used in this study was determined by an independent accredited laboratory and was <1 endotoxin unit/mg of PIKA. Mice were lightly anesthetized with 4% isoflurane and inoculated intranasally with 100 μg of PIKA in 50 μL of sterile PBS. Control mice received 50 μL of sterile PBS. Oseltamivir phosphate (Tamiflu, 75 mg; Roche, NJ) was obtained from a local pharmacy. The contents of the 75-mg capsules were emptied from the shell and dissolved in water. Mice were given oseltamivir by oral gavage twice daily at 10 mg/kg/day in a volume of 200 μL for 3 days. Control mice received sterile water on the same schedule.

Viral titration assay

Lungs and nasal turbinates were harvested, weighed, and homogenized in L-15 medium to prepare a 10% wt./vol. tissue homogenate. The materials were clarified by low-speed centrifugation, and viral titers of the samples were determined using MDCK cells as previously described and expressed as $\log_{10}$ $TCID_{50}$/g of tissue (Gillim-Ross et al., 2008).

Cytokine analysis using Bio-plex and ELISA

The concentration of various cytokines or chemokines in clarified lung homogenates (10% wt./vol. in L15 media) harvested at specified time points were measured by the Bio-plex Protein Array system (Bio-rad, Hercules, CA). The assay was performed according to the manufacturer's instructions, and the results were analyzed using the Bio-Plex manager software. One hundred microliters of lung homogenate was used to measure IFN-β using a mouse IFN-β ELISA kit (PBL Biomedical Laboratories, Piscataway, NJ).

Preparation of single-cell suspensions from lungs

Single-cell suspensions were prepared from lungs as previously described (Lau et al., 2006). In brief, the lungs were harvested, finely minced, and digested in RPMI-1640 medium containing 2 mg/mL of collagenase A (Roche Diagnostics GmbH, Mannheim, Germany) for 30 min at 37 °C. The collagenase A-treated tissues were then homogenized by pressing against sieves (40 nm; BD Falcon) with plastic plungers. Red blood cells were removed from the samples by hypotonic shock with 0.15 M ammonium chloride in 17 mM Tris–HCl for 5 min and were resuspended in PBS with 1% fetal calf serum (FCS) for flow cytometry analysis.

FACS analysis

Monoclonal antibodies (mAbs) against mouse CD3 (PerCP), CD4 (PE or ALPC), CD8 (PE or ALPC), CD11b (PerCP), CD11c (ALPC), Ly-6G (PE), Ly-6G/C (PE), CD14 (FITC), CD19 (PE), and B220 (PerCP) were purchased from BD PharMingen (San Diego, CA). Cell suspensions were treated with Mouse Fc Block™ (BD PharMingen, CA) on ice for 15 min before staining with various combinations of MAb for 30 min on ice. Cells were washed twice with PBS with 1% FCS before analysis on a FACSCalibur (BD Biosciences). A total of 80,000 events were acquired for each lung sample. The data were analyzed by FlowJo (Tree Star, Inc. Ashland, OR).

NF-κB reporter gene assay

The mouse RIG-I and MDA-5 expressing plasmids were from InvivoGen (San Diego, CA) and human embryonic kidney (HEK) 293 cells were transfected with the plasmids and the assay was performed as previously described (Lau et al., 2009).

ELISA

ELISAs were performed with serum samples as described previously (Deliyannis et al., 1998) using BPL-inactivated A/Puerto Rico/8/34 as the coating antigen. The optical density of each well was measured at 405 nm using the SpectraMax M5 microplate reader (Molecular Devices. Sunnyvale, CA).

Statistical analysis

The significance of differences between different groups was assessed by the Mann–Whitney test using Prism 5 (GraphPad Software, Inc. San Diego, CA). In selected experiments, where the sample size of each group was less than 5, the unpaired t test was used. Reported p values <0.05 are considered significantly different.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2010.07.008.

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