Pulmonary surfactant lipids inhibit infections with the pandemic H1N1 influenza virus in several animal models

Mari Numata1, James R. Mitchell1, Jennifer L. Tipper3, Jeffrey D. Brand5, John E. Trombley5, Yoji Nagashima4, Pitchaimani Kandasamy1, Hong Wei Chu1, Kevin S. Harrod5, and Dennis R. Voelker11

From the 1Department of Medicine, Program in Cell Biology, National Jewish Health, Denver, Colorado 80206, the 4Department of Surgical Pathology, Tokyo Women’s Medical University Hospital, Tokyo 1628666, Japan, and the 5Department of Anesthesiology and Perioperative Medicine, School of Medicine, University of Alabama, Birmingham, Alabama 35294

Edited by Karen G. Fleming

The influenza A (H1N1)pdm09 outbreak in 2009 exemplified the problems accompanying the emergence of novel influenza A virus (IAV) strains and their unanticipated virulence in populations with no pre-existing immunity. Neuraminidase inhibitors (NAIs) are currently the drugs of choice for intervention against IAV outbreaks, but there are concerns that NAI-resistant viruses can transmit to high-risk populations. These issues highlight the need for new approaches that address the annual influenza burden. In this study, we examined whether palmitoyl-oleoyl-phosphatidylglycerol (POPG) and phosphatidylinositol (PI) effectively antagonize (H1N1)pdm09 infection. POPG and PI markedly suppressed cytopathic effects and attenuated viral gene expression in (H1N1)pdm09-infected Madin-Darby canine kidney cells. POPG and PI bound to (H1N1)pdm09 with high affinity and disrupted viral spread from infected to noninfected cells in tissue culture and also reduced (H1N1)pdm09 propagation by a factor of 102 after viral infection was established in vitro. In a mouse infection model of (H1N1)pdm09, POPG and PI significantly reduced lung inflammation and viral burden. Of note, when mice were challenged with a typically lethal dose of 1000 plaque-forming units of (H1N1)pdm09, survival after 10 days was 100% (14 of 14 mice) with the POPG treatment compared with 0% (0 of 14 mice) without this treatment. POPG also significantly reduced inflammatory infiltrates and the viral burden induced by (H1N1)pdm09 infection in a ferret model. These findings indicate that anionic phospholipids potently and efficiently disrupt influenza infections in animal models.

The pandemic influenza A (H1N1)pdm09 virus outbreak in 2009–2010 highlights the problems of the emergence of novel influenza A virus strains. The (H1N1)pdm09 is a triple reassortant virus with genetic heritage derived from mammalian, avian, and porcine influenza A sources (1–3) that contribute to its virulence. The (H1N1)pdm09 strain continues to reappear during the annual flu season as one of multiple predominant strains (4–6). It is noteworthy that neuraminidase inhibitor-resistant variants of this virus appeared (7–11) along with other novel variant viruses (12). These issues demonstrate the need for new approaches to the annual influenza burden.

Pulmonary surfactant is a complex secreted at the air–tissue interface of the alveoli, consisting of ~90% lipids and 10% proteins, which regulates biophysical and immunological properties of the lung (13, 14). Approximately 10% of the pulmonary surfactant lipid complex consists of the phosphatidylglycerol (PG) class, and palmitoyl-oleoyl-phosphatidylglycerol (POPG) is the major molecular species (15). Pulmonary surfactant contains the only significant enrichment of secreted PG in any mammalian tissue, and these levels exceed 3 mg/ml in the extracellular alveolar fluid (14, 16). We previously reported that POPG and PI markedly inhibit respiratory syncytial virus (RSV) infection by binding the virus with high affinity and preventing attachment to epithelial cell surfaces (17, 18). POPG has potent activities against laboratory strains of IAV (e.g. H3N2, H1N1-PR8) in vitro and in vivo (18, 19). Examination of POPG turnover in mice after intranasal administration demonstrates a short bronchoalveolar lifetime (t1/2 ~45 min) (18). However, measurement of supplemental PG turnover in human neonates indicates a half-life as long as 30 h (20), suggesting an extended therapeutic window for application in humans. A second minor, anionic pulmonary surfactant phospholipid, PI, is also an effective inhibitor of RSV infection (17). PI has a longer extracellular half-life (~6 h) in mice, suggesting that PI might have greater efficacy than POPG for application as a human therapeutic.

This work was supported by National Institute of Health Grants GM118819, RHL144396A, AI125357, HL132821, HL073907, and AI111475; Flight Research Grant RHL144396A, AI125357, HL132821, HL073907, and AI111475; National Emphysema Foundation 2019. M. N. and D. R. V. are the co-inventors of the patent is owned by National Jewish Health, Denver, CO. All of the aforementioned grants and patents are focused upon mechanisms of antiviral and anti-inflammatory actions of phospholipids. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

This article contains Figs. S1–S5.

1 To whom correspondence should be addressed: Dept. of Medicine, National Jewish Health, 1400 Jackson St., Denver, CO 80206. Tel.: 303-398-1300; Fax: 303-398-1806; E-mail: voelkerd@njhealth.org.

2 The abbreviations used are: (H1N1)pdm09, Influenza A/California/07/2009; pH1N1-IAV, Influenza A/California/07/2009; PG, phosphatidylglycerol; POPG, palmitoyl-oleoyl-phosphatidylglycerol; PI, phosphatidylinositol; POPC, palmitoyl-oleoyl-phosphatidylcholine; BALF, bronchoalveolar lavage fluid; DMEM, Dulbecco’s modified Eagle’s medium; NA, neuraminidase inhibitor; IAV, influenza A virus; MDCK, Madin-Darby canine kidney cell; RSV, respiratory syncytial virus; m.o.i., multiplicity of infection; HRP, horseradish peroxidase; MP, matrix protein; NA, neuraminidase protein; pfu, plaque-forming unit; IFN, interferon; BALF, bronchoalveolar lavage fluid.
antiviral (17). The purpose of this study was to examine interactions between anionic surfactant phospholipids and (H1N1)pdm09 to determine the following: 1) whether POPG or PI could antagonize this virus infection in vitro; 2) the mechanism of antiviral action; 3) the in vivo antiviral activities of the lipids in mice; 4) the ability to prevent lethal infection by (H1N1)pdm09 in mice; and 5) the antiviral activity in ferrets, which are considered an optimal model to mimic human IAV infection (21, 22). Our findings demonstrate that POPG and PI exert overlapping antiviral functions in the lungs and suggest that these molecules could be useful in short-term prophylaxis and treatment of (H1N1)pdm09 infection. Our data suggest that both POPG and PI are novel antiviral agents with strong potential for application in humans.

**Results**

**POPG and PI inhibit (H1N1)pdm09 propagation in MDCK cells and prevent cytopathic effects induced by the virus in vitro**

We examined the effects of POPG and PI upon MDCK cells challenged with (H1N1)pdm09. Cells were infected at an m.o.i. = 0.5 and after 24 h were harvested and probed for the expression of viral proteins by immunoblotting. Both POPG (200 μg/ml) and PI (200 μg/ml) significantly reduced viral NA and
M protein (MP) expression (Fig. 1, A–C). POPG reduced NA expression by 51% and MP expression by 73%, whereas PI reduced both NA and M protein expression greater than 95%. In contrast, the control lipid, POPC, failed to alter the expression of either protein. The protective effects of POPG and PI were also evident by H&E staining, as shown in Fig. S1. Moreover, cells treated with 1000 μg/ml POPG, PI, and PC showed no cytotoxic effects assessed by 3[H]leucine incorporation into total proteins (Fig. S2, A and B) of either MDCK or human A549 epithelial cells. These data demonstrate that POPG and PI can provide significant protective effects from (H1N1)pdm09.

**POPG and PI bind to (H1N1)pdm09 with high affinity and prevent attachment of virus to the plasma membrane of epithelial cells**

We examined whether POPG and PI could interact directly with viral particles and disrupt viral infection. The attachment of viral particles to phospholipid solid phases was quantified by ELISA, using goat anti-influenza A antibodies. The results presented in Fig. 2 demonstrate that (H1N1)pdm09 binds to lipid solid phases containing POPG and PI in a high-affinity and concentration-dependent saturable interaction. Maximum virus attachment is greater for the POPG-coated wells compared with the PI-coated wells. In contrast to the interactions with POPG and PI, the virus fails to bind to lipid solid phases composed of PC or to control microtiter wells treated with ethanol without lipid.

In Figs. 3 and 4, we determined the effects of adding phospholipids to monolayers of MDCK cells and monolayers of A549 cells, respectively, when the cultures were exposed to viral particles (m.o.i. = 20) at 0 °C to enable attachment, but not endocytosis. Figs. 3A and 4A show representative blots for MDCK cells and A549 cells, respectively. Figs. 3B and 4B show the corresponding quantitative data for relative recovery of MP, and variation from three different experiments. For MDCK cells, virus attachment to the cell surface was inhibited ~70% by both lipids. For the human cell line, A549, virus attachment was reduced 95% by POPG and 70% by PI. We also examined whether POPG or PI has direct toxicities to MDCK cells or A549 cells (Fig. S2). These data clearly demonstrate that POPG and PI do not have any significant cell toxicity. Further data supporting this latter conclusion comes from experiments examining IL-8 production in response to viral challenge (Fig. S3). Likewise, high doses of PG and PI (1000 μg/ml) are well tolerated (Figs. S1 and S4). We also determined that POPG or PI does not have direct viricidal effects upon (H1N1)pdm09 (Fig. S5).

**POPG and PI inhibit spread of an established viral infection in vitro**

To examine POPG and PI effects against (H1N1)pdm09 virus propagation after infections were established, MDCK cells were grown in 24-well plates and inoculated with (H1N1)pdm09 at m.o.i. = 5 × 10⁻³ for 2 h in DMEM either with or without phospholipids. Subsequently, MDCK cells infected with (H1N1)pdm09 were incubated in DMEM with or
without phospholipids (either as simultaneous treatments or post-infection treatments at 1 mg/ml POPG, PI, or POPC, respectively). After 24 h, the total contents of the wells were harvested in culture media and subjected to plaque assays (17).

The data shown in Fig. 5 demonstrate that simultaneous treatment of MDCK cells with phospholipids at 1 mg/ml at the time of infection reduced plaque numbers by 72% with POPG and by a factor of $10^{2}$ with PI. The treatment with POPG, or PI at 2 h after (H1N1)pdm09 infection, still reduced the plaque numbers by 80% with POPG, and by a factor of 50 with PI. These in vitro data demonstrate that POPG and PI can prevent amplification of viral infection after viral adsorption and internalization (19) were established.

**POPG and PI inhibit (H1N1)pdm09 infection in mice**

Next, we examined the efficacy of POPG and PI as antiviral agents in vivo in mice. Mice were challenged with 100 pfu of (H1N1)pdm09, administered intranasally in either the absence or presence of 3 mg of POPG or 0.6 mg of PI (17). The infections progressed for 6 days, and then the animals were euthanized; the lungs were lavaged with 1 ml of PBS and then processed for plaque assays and lung histology (16, 18, 19). Analyses of the lavage and tissue are shown in Fig. 6. The total cells in lavage were increased ~9-fold by the viral infection, but the inclusion of POPG reduced the cellular infiltrates to levels near that of uninfected animals (Fig. 6, A). The virus-induced increase in cell numbers in lavage was characterized by a large influx of neutrophils and lymphocytes, and this response was inhibited ~90% by POPG (Fig. 6, B and C). In Fig. 6D, virally-infected mice expressed up to 3 ng/ml IFN-γ in BALF and that was reduced ~95% by POPG. The viral burden (Fig. 7) measured by plaque assay was reduced by a factor of $10^2$ with POPG treatment.

Fig. 8, A and B, provides histological assessment of the viral infection in the absence and presence of POPG (16–19, 23). The treatment of mice with virus and POPG (pH1N1 + PG) significantly ($p < 0.01$) reduces the histopathology score and
inflammatory cellular infiltrations in the lung compared with the pH1N1-IAV group.

We also examined the effects of PI upon viral infection in mice (Figs. 9 and 10). Fig. 9A demonstrates that PI significantly \((p < 0.001)\) reduces the total cellular infiltrates in BALF induced by viral infection by ~75%. In Fig. 9, B and C, the numbers of neutrophils and lymphocytes, respectively, in the lavage are also significantly \((p < 0.05)\) reduced by PI. IFN-\(\gamma\) levels were reduced by ~90% \((p < 0.01)\) with PI treatment as shown in Fig. 9D, and the tissue burden of the virus determined by plaque assays was also reduced by more than 90% \((p < 0.001)\) (Fig. 10). Histopathology score (Fig. 11A) demonstrates a modest but significant \((p < 1 \times 10^{-6})\) effect of PI upon virus-elicited inflammatory cell infiltration of the lungs (Figs. 11, A and B).

**POPG protects against lethal infection by (H1N1)pdm09 in mice**

We utilized a lethal infection model for (H1N1)pdm09 by challenging mice with 1000 pfu of infectious particles administered intranasally, either without or with 3 mg of POPG. The outline of the experimental design is shown in Fig. 12A, and 3 mg of POPG were added at the time of the initial infection. The mortality/weight threshold data are shown in Fig. 12B, and the changes in weights of the animals over the course of the experiment are shown in Fig. 12C. Animals were removed from the study when they died or were scored as moribund, once the weight loss exceed 20% of starting body weight, as dictated by IACUC protocol at National Jewish Health. As shown in Fig. 12B, on day 6 the animals infected with virus but not treated with lipid began to die, and this entire population either succumbed or exceeded the weight loss threshold by day 10. Within the (H1N1)pdm09 group, seven mice died during these experiments before reaching 20% body weight loss. In contrast, all animals treated with POPG and virus survived until the end of the study on day 12. Animal weights throughout the study are shown in Fig. 12C, and they demonstrate that mice infected with virus began to lose weight on day 2, and this trend continued progressively until all animals either died or crossed the 20% weight loss removal threshold by day 10.

**POPG inhibits pH1N1-IAV infection in ferrets**

In additional studies, we examined the efficacy of POPG against pH1N1-IAV infection in ferrets. Ferrets share similarities with humans in lung physiology, cellular receptor distribution, and clinical symptoms of infection by influenza (21, 22, 24). In our experiments, we infected ferrets using an intranasal inoculum of \(1 \times 10^6\) pfu in a 0.5-ml volume. In Fig. 13A, total cell numbers in BALF at day 4 were similar in the pH1N1-IAV + POPG group and the CONL group but significantly different from the pH1N1-IAV group by a factor of ~2 \((p < 0.05)\). Inflammatory cell infiltrates in the lung were reduced ~60% by POPG treatment (Fig. 13B).

To examine the effect of POPG upon pH1N1-IAV propagation in ferrets, we analyzed samples from three different compartments (nasal swab, pharyngeal swab, and BALF). Results shown in Fig. 14A demonstrate POPG reduced plaque numbers ~70% compared with the (H1N1)pdm09 group \((p < 0.05)\) in nasal swab samples. In pharyngeal swab samples (Fig. 14B) and BALF samples (Fig. 14C), plaque numbers were reduced by 80% with POPG treatments \((p < 0.01)\). The appearance of plaques from BALF is shown in Fig. 14D. The data shown in Fig. 14 demonstrate POPG efficacy against pH1N1-IAV propagation in ferrets was potent and consistent. POPG treatment attenuated virus-induced lung damage (Fig. 15, A and B) and significantly reduced the lung histopathology score (Fig. 15A, \(p < 0.02\)) (16–19, 23). In summary, POPG significantly attenuated pH1N1-IAV infection in vivo in ferrets.

**Discussion**

Pulmonary surfactant is a complex of lipids and proteins that plays a central role in regulating innate immunity in the lung (13, 14, 25). The phospholipid content of surfactant is remarkably high, with concentrations estimated to be 35–50 mg/ml (14, 25). One minor lipid component of pulmonary surfactant consists of the (PG) class, for which the POPG molecular species is the most abundant (25). The function of PG in surfactant has long been enigmatic, but our recent work now identifies this lipid as a critical regulator of both inflammatory and infective processes in the lung (16, 26–28).

We previously reported that POPG exerts potent anti-inflammatory and antiviral activity against both RSV and influenza A viruses (H3N2-IAV and H1N1-PR8-IAV) (16, 18, 19). Another minor anionic pulmonary surfactant phospholipid, PI, shares some structural similarity with POPG (17), and it also has antiviral effects against RSV and has a longer half-life in the mouse lung (17). Multiple studies from our group have shown the biological role of minor anionic surfactant phospholipid classes (PG and PI) is to act as endogenous suppressors of innate immunity, mainly by blocking activation of multiple Toll-like receptors (26, 28, 29). Work shown in this paper adds to the accumulating evidence that both POPG and PI have sig-
significant and direct antiviral effects against multiple respiratory
viruses (e.g. RSV and influenza A viruses) (16–19).

As stated above, the pH1N1-IAV outbreak in 2009–2010
highlights the problems of the emergence of novel influenza A
virus strains, for which there is little pre-existing immunity. The pH1N1-IAV is a triple reassortant virus (1–3) that caused
major health problems (30), during its 1st year of circulation in
the United States. The virus disproportionately affected young
healthy people compared with seasonal human influenza (31,
32). One pathological feature of this virus is that it suppresses
innate immunity (33). In the most recent (2018–2019) influ-
enza season, the pH1N1-IAV strain has been a persistent con-
tributor to infections in healthy individuals (4–6).

Vaccination is the primary strategy against influenza. How-
ever, mismatch between flu vaccine strains and circulating
strains remains a recurrent problem that results in increased
influenza-associated hospitalization and mortality, especially
in the elderly and children (4). NAIs are currently the drugs of
choice for pharmacological intervention against IAV out-
breaks. During the 2018–2019 influenza season, a new anti-flu
medicine (Baloxavir marboxil (Xofluza)) that acts by disrupting
viral replication became available (34, 35). However, 2.2–9.7%
of viral isolates analyzed during the Xofluza study showed
reduced sensitivity to the Baloxavir compound. Although pre-
liminary, such findings suggest that development of resistance
to Xofluza may be unexpectedly high (34). Based on our previ-
ous reports and data in this report, both POPG and PI are

Figure 6. POPG inhibits inflammatory cell infiltrates and IFN-γ production in response to (H1N1)pdm09 infection in mice. A, mice (5–8/group) were either sham-infected (CONL) or treated with (H1N1)pdm09 at 10^6 pfu/mouse, delivered intranasally (pH1N1), or virus + 3 mg of POPG (pH1N1 + PG) or only 3 mg of PG (PG), as indicated. The addition of lipids was in 50 µl of an aqueous suspension of unilamellar liposomes. The infections progressed for 6 days. B shows quantification of neutrophil populations recovered in lavage. C shows quantification of lymphocyte populations in lavage. D shows IFN-γ levels recovered in cell-free lavage. Values are means ± S.D. Significance: §§ indicates p < 0.001. Data are from three independent experiments.

Figure 7. POPG reduces the viral burden resulting from (H1N1)pdm09 infections. Mice (5–8/group) were treated with (H1N1)pdm09 at 10^6 pfu/ mouse, delivered intranasally (pH1N1), or virus + 3 mg of POPG (pH1N1 + PG). After 6 days, mice were euthanized, and the lungs were harvested, homoge-

Figure 7. POPG reduces the viral burden resulting from (H1N1)pdm09 infections. Mice (5–8/group) were treated with (H1N1)pdm09 at 10^6 pfu/mouse, delivered intranasally (pH1N1), or virus + 3 mg of POPG (pH1N1 + PG). After 6 days, mice were euthanized, and the lungs were harvested, homoge-

nized, and then processed for viral plaque assays. Data are from three inde-

pendent experiments. Values are expressed as means ± S.D. Significance: §§ indicates p < 0.001.
Figure 8. PG reduces negative histopathology associated with (H1N1)pdm09 infections. A, mice were treated with virus and lipids as described for Fig. 6. At 6 days after infection, animals were euthanized by asphyxiation with CO₂, and the right lungs were harvested, fixed, sectioned, and stained with H&E solution. The sections were scored for histopathology in a blinded fashion, and the results are shown in A. B shows representative sections for uninfected (CONL), virally infected (pH1N1), virally infected and lipid-treated (pH1N1 + PG), and lipid alone (PG) treated mice. Data shown are from three independent experiments, with 5–8 mice per group in each experiment. Data are means ± S.D. Significance: §§ indicates $p < 0.001$ when compared with virus treatment alone.

Figure 9. PI protects mice from (H1N1)pdm09 infection. A, mice (5–8/group), were either sham-infected (CONL) or treated with (H1N1)pdm09 at $10^2$ pfu/mouse, delivered intranasally (pH1N1), or virus + 0.6 mg of PI (pH1N1 + PI) or only 0.6 mg of PI (PI), as indicated. Lipids were added as aqueous suspensions of small unilamellar liposomes in a volume of 50 μl along with virus. B, inflammatory neutrophilic infiltrates recovered from lung lavage. C, lymphocytic infiltrates recovered from lavage after (H1N1)pdm09 infection. D, IFN-γ levels recovered from cell free lavage. Significance: §§ indicates $p < 0.001$; * indicates $p < 0.05$. Data are from three independent experiments.
potent against pH1N1-IAV, and other subtypes of influenza, in addition to RSV in vitro and in vivo (16–19). The actions of POPG and PI in tissue culture studies provide strong evidence that the lipids prevent the successful transmission of virus between infected and uninfected cells in close contact, which occurs in sheets of epithela lining the respiratory tree. This observation raises the possibility that administration of these lipids to individuals with an established and active infection may significantly reduce the successful transmission of infectious particles to other members of the same household. This feature of the lipids may be especially useful in households with individuals who have underlying chronic lung diseases such as asthma and chronic obstructive pulmonary disease (14). In summary, we propose that POPG and PI will have the potential for application as novel antiviral agents, with little risk of toxicity.

Materials and methods

Cell culture, viruses, virus quantification, and liposome preparation

MDCK cells and A549 cells were obtained from American Tissue Culture Collection (ATCC) (19). Influenza A/California/07/2009, (H1N1)pdm09, was purified as described previously (36). Viral titers and growth were measured using quantitative plaque assays (19). Phospholipids (Avanti) were prepared as unilamellar liposomes as described previously (16–19). To assess the cytopathic effects of (H1N1)pdm09 by phospholipids (POPG and PI) or the control lipid POPC (16–19), virus was added to MDCK cells at a m.o.i. = 2 in either the presence or absence of 0.2–1 mg/ml phospholipids for 24 h. The cultures were fixed in 10% buffered formalin overnight, and hematoxylin and eosin staining was performed with infected and uninfected MDCK cells (19).

Pulmonary surfactant phospholipids inhibit influenza

Pulmonary surfactant lipid treatments and viral infection in vitro

To examine the efficacy of PI and POPG against (H1N1)pdm09 infection, MDCK cells were pretreated with POPG or PI (200 μg/ml) for 30 min and subsequently infected with (H1N1)pdm09 at a m.o.i. = 0.5. Total cell lysates were processed after 24 h, and immunoblotting was performed for MP, NA, and β-actin. Separated proteins were blotted onto nitrocellulose and probed with polyclonal goat anti-(H1N1)pdm09 (1:500) (Millipore Sigma, AB1074) (19). Quantification of MP and NA protein expression was performed using immunoblotting and Image analysis with NIH Image J-1.34 software (National Institutes of Health, Bethesda, MD). The efficacy of POPG and PI against (H1N1)pdm09 was assessed by quantitative plaque assays (16, 19).

Phospholipid interaction with (H1N1)pdm09 and inhibition of virus attachment to epithelial cells

To examine the direct interaction of POPG and PI with (H1N1)pdm09, virus attachment to phospholipid solid phases was quantified as described previously (21). We also examined (H1N1)pdm09 attachment to MDCK and A549 cells by incubating monolayers with viral particles at a m.o.i. = 20 (18, 19) at 0 °C to prevent endocytosis. Cell extracts were subjected to electrophoresis (19), and MP protein expression was analyzed as described above.

POPG and PI inhibition of (H1N1)pdm09 infection in mice

6-Week-old BALB/c female mice were obtained from The Jackson Laboratory. Mice (5–8 per experimental group) were anesthetized using isoflurane inhalation and subjected to intranasal inoculations in a volume of 50 μl. The inoculations consisted of six conditions as follows: 1) sham infection containing only PBS; 2) (H1N1)pdm09 infection (100 pfu/mouse); 3) (H1N1)pdm09 (100 pfu/mouse) + 3 mg of POPG; 4) (H1N1)pdm09 + 600 μg of PI; 5) POPG alone (3 mg of POPG in PBS); and 6) PI alone (600 μg of PI in PBS). After 6 days, the mice were euthanized by CO2 asphyxiation. Each lung was subjected to a 1-ml lavage with saline (19). Following lavage and dissection, the right lungs were homogenized and used for plaque assays (16, 19). Right lung segments were used for histopathology scoring (16–19). Lavage fluid was used to determine total cell numbers, cell populations, and quantifying IFN-γ (16–19). All studies with mice were performed under protocols approved by the National Jewish Health, Institutional Animal Care and Use Committee (IACUC).

POPG inhibition of lethal (H1N1)pdm09 challenge in mice

We also conducted experiments applying a lethal challenge to mice with (H1N1)pdm09 using an inoculation containing 1000 pfu of virus. Mice were anesthetized using isoflurane inhalation and subjected to intranasal inoculations in a total volume of 50 μl. The groups in this experiment consisted of the following: 1) sham infection containing only PBS; 2) (H1N1)pdm09 infection (1000 pfu/mouse); 3) (H1N1)pdm09 (1000 pfu) + 3 mg of POPG; and 4) 3 mg of POPG. Mice remained in the protocol until they died or lost >20% body weight, after which they were euthanized by CO2 asphyxiation, as approved by the IACUC at National Jewish Health.
POPG inhibition of (H1N1)pdm09 challenge in ferrets

12-Week-old castrated male ferrets (Triple F Farms, Sayre, PA) were examined for the protective effects of POPG against viral infection. The animals were anesthetized by isoflurane inhalation and subjected to intranasal inoculations of 500 pfu.

The groups in this experiment consisted of the following: 1) sham infection, administered as only PBS; 2) (H1N1)pdm09 infection (1 × 10^6 pfu/ferret); 3) (H1N1)pdm09 (1 × 10^6 pfu) + 5 mg of POPG; and 4) 5 mg of POPG. Infections were allowed to progress for 4 days, after which nasal and pharyngeal swabs...
were taken, and the ferrets were euthanized with 0.05 mg/kg dexdormitor, followed by 5 mg/kg ketamine injected intramuscularly (21). Tracheas were cannulated to perform lung lavage with 10 ml of saline instilled three times, and lavages were collected for analysis of total cells, cell populations, and viral burden as measured by plaque assays. All experiments performed with ferrets were conducted using protocols approved by the University of Alabama, Birmingham, Animal Care and Use Committee.

**Statistical analysis**

All results are shown as means ± S.D. One-way analysis of variance was used for statistical analysis to assess significant differences among all groups. Differences among groups were
considered significant at $p < 0.05$. Routinely, comparisons were made between virus-infected cells or animals and those either sham-infected or those receiving virus and phospholipid antagonists, or those receiving virus and a control lipid (POPC).

**Author contributions**—M. N., K. S. H., and D. R. V. conceptualization; M. N., K. S. H., and D. R. V. resources; M. N., J. R. M., J. L. T., Y. N., H. W. C., and D. R. V. data curation; M. N., H. W. C., and D. R. V. writing—original draft; M. N., J. R. M., J. L. T., J. D. B., J. E. T., P. K., and D. R. V. methodology; M. N. writing—review; M. N., H. W. C., K. S. H., and D. R. V. visualization; M. N., H. W. C., K. S. H., and D. R. V. formal analysis; M. N., J. R. M., J. L. T., J. D. B., J. E. T., Y. N., P. K., H. W. C., K. S. H., and D. R. V. methodology; M. N. writing—original draft; M. N., J. R. M., P. K., and D. R. V. conceptualization; M. N., K. S. H., and D. R. V. supervision; M. N., K. S. H., and D. R. V. funding acquisition; M. N., J. R. M., J. L. T., J. D. B., J. E. T., P. K., H. W. C., K. S. H., and D. R. V. project administration; M. N., K. S. H., and D. R. V. writing—review and editing.

**References**

1. Shinde, V., Bridges, C. B., Uyeki, T. M., Shu, B., Balish, A., Xu, X., Lindstrom, S., Gubareva, L. V., Deyde, V., Garten, R. J., Harris, M., Gerber, S., Vagasky, S., Smith, F., Pascoe, N., et al. (2009) Triple-reassortant swine influenza A (H1) in humans in the United States, 2005–2009. *N. Engl. J. Med.* 360, 2616–2625 CrossRef Medline

2. Garten, R. J., Davis, C. T., Russell, C. A., Shu, B., Lindstrom, S., Balish, A., Sessions, W. M., Xu, X., Sképner, E., Deyde, V., Okomo-Adhiambo, M., Gubareva, L., Barnes, J., Smith, C. B., Emery, S. L., et al. (2009) Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. *Science* 325, 197–201 CrossRef Medline

3. Centers for Disease Control and Prevention (CDC). (2012) Update: Influenza activity–United States, October 2, 2011–February 11, 2012. *MMWR Morb. Mortal. Wkly. Rep.* 61, 123–128 CrossRef Medline

4. Epperson, S., Blanton, L., Kniss, K., Mustaquim, D., Steffens, C., Wallis, T., Dhara, R., Leon, M., Perez, A., Chaves, S. S., Elal, A. A., Gubareva, L., Xu, X., Villanueva, J., Bresee, J., et al. (2014) Influenza activity–United States, 2013–14 season and composition of the 2014–15 influenza vaccines. *MMWR Morb. Mortal. Wkly. Rep.* 63, 483–490 CrossRef Medline

5. Blanton, L., Dugan, V. G., Abd Elal, A. I., Alabi, N., Barnes, J., Brammer, L., Budd, A. P., Burns, E., Cummings, C. N., Garg, S., Garten, R., Gubareva, L., Kniss, K., Kramer, N., O’Halloran, A., et al. (2019) Update: Influenza activity–United States, September 30, 2018–February 2, 2019. *MMWR Morb. Mortal. Wkly. Rep.* 68, 125–134 CrossRef Medline

6. Budd, A. P., Abd Elal, A. I., Alabi, N., Barnes, J., Blanton, L., Brammer, L., Burns, E., Cummings, C. N., Dugan, V. G., Garg, S., Garten, R., Grohskopf, L. A., Gubareva, L., Kniss, K., Kramer, N., et al. (2018) Influenza activity–United States, September 30–December 1, 2018. *MMWR Morb. Mortal. Wkly. Rep.* 67, 1369–1371 CrossRef Medline

7. Dharan, N. J., Gubareva, L. V., Meyer, J. I., Okomo-Adhiambo, M., McClinton, R. C., Marshall, S. A., St George, K., Epperson, S., Brammer, L., Klimov, A. I., Bresee, J. S., Fry, A. M., and Oseltamivir-Resistance Working Group. (2009) Infections with oseltamivir-resistant influenza A(H1N1) virus in the United States. *JAMA* 301, 1034–1041 CrossRef Medline

8. Hayden, F. G. (2006) Antiviral resistance in influenza viruses—implications for management and pandemic response. *N. Engl. J. Med.* 354, 785–788 CrossRef Medline

9. Moscona, A. (2005) Neuraminidase inhibitors for influenza. *N. Engl. J. Med.* 353, 1363–1373 CrossRef Medline

10. Weinstock, D. M., and Zucctti, G. (2009) The evolution of influenza resistance and treatment. *JAMA* 301, 1066–1069 CrossRef Medline

11. Gooskens, J., Jonges, M., Claas, E. C., Meijer, A., van den Broek, P. J., and Kroes, A. M. (2009) Morbidity and mortality associated with nosocomial transmission of oseltamivir-resistant influenzae A (H1N1) virus. *JAMA* 301, 1042–1046 CrossRef Medline

12. Lloren, K. K. S., Kwon, J. J., Choi, W. S., Jeong, J. H., Jeong Ahn, S., Ki Choi, Y., Baek, Y. H., and Song, M. S. (2019) In vitro and in vivo characterization of novel neuraminidase substitutions in influenza A(H1N1)pdm09 identified using laninamivir-mediated in vitro selection. *J. Virol.* 93, e01825–18 CrossRef Medline

13. Wright, J. R. (2005) Immunoregulatory functions of surfactant proteins. *Nat. Rev. Immunol.* 5, 58–68 CrossRef Medline

14. Numata, M., and Voelker D. R. (2010) Asthma and Infections. In Lung Biology in Health and Disease. Chapter 10: Pulmonary Surfactant. Innate Immunology.
Immunity and Asthma (Martin RI, Sutherland ER, eds.), pp. 145–165, Informa Healthcare, New York, NY.

15. Wright, S. M., Hocket, P. M., Enhorning, G., Strong, P., Reid, K. B., Holgate, S. T., Dijkmanovic, R., and Postle, A. D. (2000) Altered airway surfactant phospholipid composition and reduced lung function in asthma. J. Appl. Physiol. 89, 1283–1292 CrossRef Medline

16. Numata, M., Chu, H. W., Dakhama, A., and Voelker, D. R. (2010) Pulmonary surfactant phosphatidylglycerol inhibits respiratory syncytial virus-induced inflammation and infection. Proc. Natl. Acad. Sci. U.S.A. 107, 320–325 CrossRef Medline

17. Numata, M., Kandasamy, P., Nagashima, Y., Posey, J., Hartshorn, K., Numata, M., Kandasamy, P., Nagashima, Y., Fickes, R., Murphy, R. C., and Voelker, D. R. (2010) Pulmonary surfactant phosphatidylglycerol provides short-term prophylaxis against respiratory syncytial virus infection. J. Lipid Res. 51, 2133–2143 CrossRef Medline

18. Numata, M., Kandasamy, P., Nagashima, Y., Moore, M. L., Berry, K. Z., Chan, M., Kandasamy, P., Peebles, R. S., Jr., Murphy, R. C., and Voelker, D. R. (2013) Phosphatidylglycerol suppresses influenza A virus infection. Am. J. Respir. Cell Mol. Biol. 46, 479–487 CrossRef Medline

19. Numata, M., Kandasamy, P., Nagashima, Y., Possey, J., Hartshorn, K., Woodland, D., and Voelker, D. R. (2012) Phosphatidylglycerol suppresses influenza A virus infection. J. Lipid Res. 53, 578–587 CrossRef Medline

20. Hallman, M., Merritt, T. A., and Bry, K. (1994) The fate of exogenous surfactant in neonates with respiratory distress syndrome. Clin. Pharmacokinet. 26, 215–232 CrossRef Medline

21. Koster, F., Gouveia, K., Zhou, Y., Lowery, K., Russell, R., MacInnes, H., Pollock, Z., Layton, R. C., Cromwell, J., Toleno, D., Pyle, J., Zabelewicz, M., Harrod, K., Sampath, R., Hofstadler, S., et al. (2012) Exhaled aerosol transmission of pandemic and seasonal H1N1 influenza viruses in the ferret. PLoS One 7, e33118 CrossRef Medline

22. Belser, J. A., Barclay, W., Barr, I., Fouchier, R. A. M., Matsuyama, R., Nishiura, H., Peiris, M., Russell, C. J., Subbarao, K., Zhu, H., and Yen, H. L. (2018) Ferrets as models for influenza virus transmission studies and pandemic risk assessments. Emerg. Infect. Dis. 24, 965–971 CrossRef Medline

23. Cimolai, N., Taylor, G. P., Mah, D., and Morrison, B. J. (1992) Definition and application of a histopathological scoring scheme for an animal model of acute Mycoplasma pneumoniae pulmonary infection. Microbiol. Immunol. 36, 465–478 CrossRef Medline

24. Herfst, S., Schrauwen, E. J., Linster, M., Chutinimitkul, S., de Wit, E., Munster, V. I., Sorrell, E. M., Bestebroer, T. M., Burke, D. F., Smith, D. I., Rimmelzwaan, G. F., Osterhaus, A. D., and Fouchier, R. A. (2012) Airborne transmission of influenza A/H5N1 virus between ferrets. Science 336, 1534–1541 CrossRef Medline

25. Numata, M., Kandasamy, P., and Voelker, D. R. (2012) Anionic pulmonary surfactant lipid regulation of innate immunity. Expert Rev. Respir. Med. 6, 243–246 CrossRef Medline

26. Kuronuma, K., Mitsuzawa, H., Takeda, K., Nishitani, C., Chan, E. D., Kuroki, Y., Nakamura, M., and Voelker, D. R. (2009) Anionic pulmonary surfactant phospholipids inhibit inflammatory responses from alveolar macrophages and U937 cells by binding the lipopolysaccharide-interacting proteins CD14 and MD-2. J. Biol. Chem. 284, 25488–25500 CrossRef Medline

27. Kandasamy, P., Numata, M., Berry, K. Z., Fickes, R., Leslie, C. C., Murphy, R. C., and Voelker, D. R. (2016) Structural analogs of pulmonary surfactant phosphatidylglycerol inhibit toll-like receptor 2 and 4 signaling. J. Lipid Res. 57, 993–1005 CrossRef Medline

28. Muller, M., Brandenburg, K., Dedrick, R., Schromm, A. C., and Seydel, U. (2005) Phospholipids inhibit lipopolysaccharide (LPS)-induced cell activation: a role for LPS-binding protein. J. Immunol. 174, 1091–1096 CrossRef Medline

29. Bitar, D., Mailles, A., Herida, M., Vaux, S., and Lévy-Bruhl, D. (2010) Severe hospitalised 2009 pandemic influenza A(H1N1) cases in France, 1 July–15 November 2009. Euro Surveill. 15, 19463 CrossRef Medline

30. Kandasamy, P., Numata, M., Berry, K. Z., Fickes, R., Leslie, C. C., Murphy, R. C., and Voelker, D. R. (2010) Pulmonary surfactant phosphatidylglycerol inhibits Mycoplasma pneumoniae-stimulated eicosanoid production from human and mouse macrophages. J. Biol. Chem. 286, 7841–7853 CrossRef Medline

31. Heo, Y. A. (2018) Baloxavir: first global approval. Drugs 78, 693–697 CrossRef Medline

32. Hayden, F. G., Sugaya, N., Hirotsu, N., Lee, N., de Jong, M. D., Hurt, A. C., Ishida, T., Sekino, H., Yamada, K., Pyle, J., Zabelewicz, M., Harrod, K., Sampath, R., Hofstadler, S., et al. (2018) Exhaled aerosol transmission of pandemic and seasonal H1N1 influenza viruses in the ferret. PLoS One 7, e33118 CrossRef Medline

33. Zeng, H., Pappas, C., Katz, J. M., and Tumpey, T. M. (2011) The 2009 pandemic H1N1 and triple-reassortant swine H1N1 influenza viruses replicate efficiently but elicit an attenuated inflammatory response in polarized human bronchial epithelial cells. J. Virol. 85, 686–696 CrossRef Medline

34. Fuhrman, C., Bonmarin, I., Paty, A. C., Duport, N., Chiron, E., Lucas, E., Bitar, D., Mailles, A., Herida, M., Vaux, S., and Lévy-Bruhl, D. (2010) Severe hospitalised 2009 pandemic influenza A(H1N1) cases in France, 1 July–15 November 2009. Euro Surveill. 15, 19463 CrossRef Medline

35. Zeng, H., Pappas, C., Katz, J. M., and Tumpey, T. M. (2011) The 2009 pandemic H1N1 and triple-reassortant swine H1N1 influenza viruses replicate efficiently but elicit an attenuated inflammatory response in polarized human bronchial epithelial cells. J. Virol. 85, 686–696 CrossRef Medline

36. Clay, C. C., Reader, J. R., Gerriets, J. E., Wang, T. T., Harrod, K. S., and Miller, L. A. (2014) Enhanced viral replication and modulated innate immune responses in infant airway epithelium following H1N1 influenza infection. J. Virol. 88, 7412–7425 CrossRef Medline

Pulmonary surfactant phospholipids inhibit influenza