Intranasal Administration of the TLR2 Agonist Pam2Cys Provides Rapid Protection against Influenza in Mice

Amabel C. L. Tan,† Edin J. Mifsud,† Weiguang Zeng,† Kathryn Edenborough,† Jodie McVernon,† Lorena E. Brown,‡ and David C. Jackson*†

†Department of Microbiology & Immunology, The University of Melbourne, Parkville 3010, Victoria, Australia
‡Melbourne School of Population Health, The University of Melbourne, Parkville 3010, Victoria, Australia

ABSTRACT: The protective role played by the innate immune system during early stages of infection suggests that compounds which stimulate innate responses could be used as antimicrobial or antiviral agents. In this study, we demonstrate that the Toll-like receptor-2 agonist Pam2Cys, when administered intranasally, triggers a cascade of inflammatory and innate immune signals, acting as an immunostimulant by attracting neutrophils and macrophages and inducing secretion of IL-2, IL-6, IL-10, IFN-γ, MCP-1 and TNF-α. These changes provide increased resistance against influenza A virus challenge and also reduce the potential for transmission of infection. Pam2Cys treatment also reduced weight loss and lethality associated with virulent influenza virus infection in a Toll-like receptor-2-dependent manner. Treatment did not affect the animals’ ability to generate an adaptive immune response, measured by the induction of functional influenza A virus-specific CD8+ T cells following exposure to virus. Because this compound demonstrates efficacy against distinct strains of influenza, it could be a candidate for development as an agent against influenza and possibly other respiratory pathogens.

KEYWORDS: antigen-independent immunity, Pam2Cys, Toll-like receptor 2, influenza A virus, innate immunity

INTRODUCTION

Vaccination provides one of the most effective means of protection against infectious diseases. The effects of vaccination, however, are not immediate, and the recently inoculated population is vulnerable to infection during the period that antibodies and immune cells are being formed. This lag phase can range from several days to weeks depending on a vaccine’s efficacy and the vaccination regime.

The innate immune system forms a first line, and immediate, defense against pathogens including those that infect and replicate in cells lining the respiratory tract such as influenza A virus (IAV). IAV causes up to 1 billion infections and 300,000–500,000 deaths during seasonal influenza epidemics, and enormous costs to human health occur during pandemic outbreaks. In the early stages of IAV infection, local cellular innate mediators, particularly macrophages,1 neutrophils2–3 and NK cells4 as well as soluble factors including IL-12 and IFN-γ,5,6 play crucial roles in limiting viral replication and disease severity. The broad and robust activity of innate immune response suggests that agents which stimulate the innate immune systems could be at limiting respiratory infections while the adaptive immune response is evoked.

Their macrophage activating lipopeptide-2 (MALP2) is an agonist for the Toll-like receptor 2 (TLR2) and6 was originally isolated from Mycoplasma fermentans.8 Dipalmitoyl-S-glyceryl cysteine (Pam2Cys) is a synthetic analogue of the lipid component of MALP-2 that we have shown to be an agonist for TLRs.9,10 Our laboratory has exploited the dendritic cell-activating features of Pam2Cys in the design of vaccine candidates.10–12

In addition to dendritic cells, TLR2 signaling is known to be important in activation of other cells of the innate immune system.13 Changes in the lung, similar to those induced by IAV infection, have also been reported for MALP-2; these changes include the induction of proinflammatory chemokines and cytokines and infiltration of various cell types.14–16 It was these observations that prompted us to examine the effects of Pam2Cys on the lung and susceptibility to IAV exposure.

In this study we show that intranasal administration of Pam2Cys enhances viral clearance when animals are subsequently challenged with different IAV and reduces the impact of disease and death against a virulent strain of the virus. Although a number of studies have shown that TLR agonists can protect against mild and virulent IAV,17–20 we also show that mice treated with Pam2Cys and subsequently challenged with IAV demonstrate a reduced rate of viral transmission to naive animals.

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MATERIALS AND METHODS

Pegylated Pam2Cys (Figure 1) was synthesized in house using Fmoc chemistry. A glycine residue was coupled to the solid support (Rapp Polymere, Tuebingen, Germany) followed by addition of O-(N-(Fmoc-2-aminoethyl))-O’-(2-carboxyethyl)-undecaethyleneglycol (Fmoc-PEG11-OH, Merck Ltd., Kilsyth, Australia). Following addition of two serine residues, the Pam2Cys lipid moiety was attached at the N-terminal position using procedures described elsewhere. Peg-Pam2Cys was cleaved from the solid support21 and purified by reverse-phase high-performance liquid chromatography using a C4 VYDAC column (10 mm × 250 mm; Alltech, NSW, Australia) installed in a Waters HPLC system (Waters Millipore, Milford, MA, USA). The purity of Peg-Pam2Cys, determined by HPLC using a VYDAC C8 column (4.6 mm × 250 mm), was found to be greater than 95%. The authenticity of the product was determined by mass analysis using an Agilent 1100 Series LC/MSD ion-trap mass spectrometer (Agilent, Palo Alto, CA, USA) with a mass value of 1502.2 Da (calculated mass value, 1502.1 Da).

Animals. Male and female C57BL/6, BALB/c and transgenic mice (detailed below) aged 6–12 weeks were used in this study. TLR2-deficient mice (TLR2−/−) on a C57BL/6 background22 were provided by Dr. Shizuo Akira, Osaka University, Japan. C57BL/6 mice deficient in type 1 interferon receptor (IFNAR−/−) were obtained from Professor Paul Hertzog, Monash Institute of Medical Research, Victoria, Australia. Mice were bred and maintained in the Animal House Facility, Department of Microbiology and Immunology, University of Melbourne. All animal experimentation was conducted in accordance with institutional regulations following review and approval by the University of Melbourne Animal Ethics Committee.

Administration of Peg-Pam2Cys. Mice were anesthetized by isoflurane inhalation and Peg-Pam2Cys (20 nmol in 50 μL of saline) or saline alone then administered by intranasal (i.n.) instillation.

Challenge with Influenza A Virus. For mild IAV infection, mice received 10^{5.5} plaque-forming units (pfu) of the H3N1 virus, Mem which is a genetic reassortant of A/Memphis/1/71 (H3N2) × A/Bellamy/42 (H1N1) via the i.n. route while anesthetized with isoflurane. For upper respiratory tract infection (URT) with H3N2 virus, 10^{5.5} pfu of A/Udorn/307/72 virus was administered dropwise onto the nares of mice in a 10 μL volume without anesthesia. On day 5 following Mem challenge or day 4 following Udorn challenge, lungs were harvested into 1.5 or 3 mL of RPMI 1640 medium (Gibco, USA) for determination of viral titers by plaque formation in MDCK cells. Challenge with a virulent strain of IAV was carried out using 200 pfu of the H1N1 virus A/Puerto Rico/8/34 (PR8, Mount Sinai) administered by the intranasal route under anesthesia. Infection with PR8 induces a symptomatic infection characterized by significant weight loss. Mice were monitored daily for signs of weight loss and clinical symptoms of infection and euthanized at a predetermined humane endpoint.

Contact Transmission Study. “Donor” mice, BALB/c mice (n = 2), received 10^{4.5} pfu of H3N2 Udorn virus in 50 μL of saline administered i.n. and under anesthesia. Twenty four hours later, donor mice were cohoused with naive recipient mice (n = 3) for 24 h, after which donor mice were removed and viral titers were assessed in the nasal turbinates, trachea and lungs. Three and a half days following exposure, recipient mice were killed and viral titers in various tissues were assessed.

Preparation of Lung Cells. Following CO2 asphyxiation of mice, lungs were perfused with 10 mL of PBS and single cell suspensions prepared as described previously. To obtain bronchoalveolar lavage (BAL) fluid, mouse tracheas were cannulated with a blunt-end syringe and the air space was flushed with three separate 1 mL washes of RPMI 1640 and a final 1 mL rinse of RPMI 1640. The supernatant from the BAL washes was stored at −70 °C for later cytokine analysis. Viable cells were counted using a hemocytometer and trypan blue dye exclusion.

Characterization of the Pulmonary Cytokine Environment. Cytokine levels in the BAL fluid supernatant were determined using a BD Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer’s instructions with the exception that 2 μL of each capture bead was used for each 50 μL BAL sample. Samples were analyzed using a Becton Dickinson FACSCalibur flow cytometer and data analyzed using FlowJo software (Tree Star, Inc., USA).

Characterization of Lung Cells. Lung cells (5 × 10^6) were stained with combinations of the following anti-mouse antibodies: FITC labeled anti-CD11b, PerCP-Cy5.5 anti-GR-1 (Ly-6G and Ly-6C), PE-labeled anti-CD11c, APC anti-F4/80, FITC anti-IA/IE MHC Class 2, PerCP-Cy5.5 anti-CD8, PE anti-CD4 (BD Pharmingen, San Diego, CA, USA). Pulmonary cell subsets were classified as follows: neutrophils, CD11bhi GR1hi CD11c− F4/80−; alveolar macrophages, CD11bhi GR1int CD11c− F4/80−; CD11blow GR1int CD11c+ and autofluorescent; dendritic cells, CD11c+ MHC Class 2hi GR1int; monocytes and interstitial macrophages, CD11bhi GR1int CD11c+ F4/80−; CD8+ T cells, CD8+ ; CD4+ T cells, CD4+.25–27 Samples were analyzed using a Becton Dickinson FACSCalibur flow cytometer and data analyzed using FlowJo software (Tree Star, Inc, USA).

Characterization of CD8+ T cells. Tetramer Staining. The H-2Db monomer complexed to either peptide PA224−233 (SSLENFRAYV) or peptide NP366−374 (ASNENMETM) (assembled by the Monomer Facility, Department of Microbiology and Immunology, The University of Melbourne) was used to generate tetramers with streptavidin-phycocyanin (PE) (Molecular Probes). Lymphocytes were treated with a 1/100 dilution (1 μg/mL) of tetramer in FACS wash (1% FCS and 5 mM EDTA in PBS) for 60 min at room temperature, washed twice in sort buffer (0.1% BSA in PBS) and then

**Figure 1.** Chemical structure of pegylated Pam2Cys (PEG-Pam2Cys). PEG-Pam2Cys consists of a single Pam2Cys molecule coupled to undecaethyleneglycol (polyethylene glycol, PEG) through two serines (Ser). A glycine residue was attached to a Rink linker at the C-terminus of the structure.
In its native form Pam2Cys is insoluble in aqueous solution. To obtain a soluble version we conjugated Pam2Cys to polyethylene glycol to generate pegylated Pam2Cys (PEG-Pam2Cys) (Figure 1). PEG-Pam2Cys is a stable compound that is readily soluble in saline and can be administered intranasally to mice enabling deposition in the pulmonary tract.

Figure 2. Intranasal administration of PEG-Pam2Cys induces inflammatory and Th1 cytokines and expansion of cell populations in the lung. (A) Schematic of the treatment and challenge schedule. C57BL/6 mice were inoculated intranasally with 20 nmol of PEG-Pam2Cys or 50 μL of saline, and organs were harvested and assessed 72 h and 7 days later. (B) Concentration of cytokines in the BAL fluid and (C) pulmonary cell populations. The dotted line in panel C separates cell populations indicated by the left or right axes. (D) Cytokine concentrations and (E) cell numbers obtained from TLR2−/− mice treated with PEG-Pam2Cys or saline. Bars represent the mean response of each group (n = 3), and error bars indicate the SD. *P < 0.05 vs the saline/naive groups, one-way ANOVA (post hoc Tukey’s multiple comparison test). The results were confirmed in an independently conducted repeat experiment.

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**RESULTS**

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The TLR2 Agonist Pam2Cys Enhances Innate Immunity in the Lungs. To examine the effect of PEG-Pam2Cys on the respiratory environment, C57BL/6 mice were administered intranasally (i.n.) with 20 nmol of PEG-Pam2Cys and the concentration of Th1 and inflammation-associated cytokines in the BAL fluid was determined 72 h and 7 days after administration (Figure 2A). PEG-Pam2Cys pretreatment induced significant increases in IL-6, IL-10, MCP-1, IFN-γ, and TNF-α levels at 72 h post treatment, levels which by day 7 had returned to preadministration concentrations (Figure 2B). It has been reported that TLR2 agonists can induce IFN-α in the context of viral infection, but in our system, no change in IFN-α could be detected from BAL fluid samples of Pam2Cys-treated mice (data not shown). Furthermore, when exposed in vitro to 10 nmol of PEG-Pam2Cys for 24 h, the supernatants of cells isolated from lungs of mice treated with PEG-Pam2Cys had significantly increased levels of IL-6, IL-10, MCP, IFN-γ and TNF-α, but not IFN-α (data not shown).

Intranasal administration of PEG-Pam2Cys also resulted in significant increases in the total lung populations of neutrophils, interstitial and alveolar macrophages and lymphocytes in C57BL/6 mice 72 h after treatment (Figure 2C). We also detected increased levels of γδ T cells and activated IFN-γ producing NK cells at this time point (Figure 2C). By day 7 following treatment, most of the elevated levels of innate cell populations returned to levels observed in the group treated with saline. The lymphocyte populations (B and T cells), however, remained elevated. Similar changes in cytokine levels and cellular numbers were also detected in BALB/c mice treated with Pam2Cys (data not shown).
In order to verify that TLR2 is necessary for the cytokine and cellular changes elicited by PEG-Pam2Cys, we examined its effects in TLR2\(^{-/-}\) knockout mice. We found that PEG-Pam2Cys failed to induce increases in cytokine levels in these animals (Figure 2D). At 72 h following administration of Pam2Cys to wild type C57BL/6 mice, neutrophil and macrophage counts were dramatically increased but there were no significant increases in the proportions of these cell subsets (Figure 2E) or the total number of lung cells in TLR2\(^{-/-}\) mice (data not shown) confirming the dependence of TLR2-signaling for the Pam2Cys-mediated effects on the lung.

**Pam2Cys Elicits in Vivo Antiviral Activity against Different Strains of IAV.** To determine if these pulmonary changes and enhancement of innate immune mediators could affect susceptibility to respiratory IAV infection, C57BL/6 mice received PEG-Pam2Cys (20 nmol) 24 h, 72 h, and 7 days prior to intranasal challenge (under anesthesia) with 10⁴.5 pfu of H3N1Mem virus (Figure 3A). This virus causes a mild self-resolving infection in mice. The results (Figure 3B) demonstrate that pretreatment with PEG-Pam2Cys significantly reduces viral load in the lungs and this reduction is evident for up to 7 days after administration.

In order to assess whether PEG-Pam2Cys has the ability to reduce both upper and lower respiratory tract infections, BALB/c mice were treated with PEG-Pam2Cys and challenged 72 h later via the URT with 10⁴.5 pfu of Udorn virus. The results (Figure 3C) show that viral titers in the trachea and lung were approximately 100-fold lower in PEG-Pam2Cys treated animals than in saline-treated animals and, in seven of the eleven Pam2Cys-treated mice, no virus could be detected from the lung. There was, however, no difference in the viral load isolated from the nasal turbinates. These data suggest that although intranasal Pam2Cys-treatment does not prevent establishment of infection in the nasal turbinates, it can significantly reduce the progression of virus, and the extent of viral burden in the trachea and lungs.

**Pam2Cys Prophylaxis is Effective against Challenge with Virulent IAV.** Having shown that Pam2Cys pretreatment can reduce viral burden following challenge with mild IAV virus strains (Figure 3), we determined whether the antiviral activity of Pam2Cys was effective against the virulent PR8 (H1N1) strain of IAV.

Saline-treated mice when challenged with PR8 virus experienced substantial weight loss (Figure 4A) and developed clinical symptoms of infection. By day 8 all but one of the saline-treated mice were culled having reached the defined humane end point (Figure 4B). In contrast, mice treated with Pam2Cys either 72 h or 7 days before virus challenge experienced substantially lower weight losses which were significant on days 7 and 8 postchallenge, and all but one of the Pam2Cys-treated mice survived infection (Figure 4B). Protection against challenge with the H1N1 virus was shown to be TLR2-dependent because Pam2Cys failed to provide protection to TLR2\(^{-/-}\) mice (Figures 4C and 4D).

Treatment of IFNAR\(^{-/-}\) mice, which lack receptors for the type 1 interferons IFN-α and IFN-β, with Pam2Cys completely protected these animals against death associated with PR8 infection. These results (Figures 4E and 4F) confirm that the mode of action of PEG-Pam2Cys is independent of type 1 interferon signaling.

To confirm that it is local and not systemic effects that are associated with protection, we delivered PEG-Pam2Cys via two different routes, subcutaneous (s.c.) in the base of tail and intravenously (i.v.), and then challenged the mice 72 h later with PR8 virus. Subcutaneous and intravenous delivery of Pam2Cys each failed to protect mice against weight loss and the clinical symptoms associated with PR8 challenge (Figures 5A and 5B). These results demonstrate that the local immunostimulatory effects of Pam2Cys on the respiratory
Pam2Cys groups (unpaired Student’s t test). These results are combined from at least two independent conducted experiments. 

Figure 4. Pam2Cys administration protects against virulent IAV infection in a TLR2-dependent manner. C57BL/6 mice (n = 10–13/group) were inoculated intranasally with 20 nmol of PEG-Pam2Cys or 50 μL of saline 72 h or 7 days prior to challenge with 200 pfu of H1N1 PR8 virus. Mice were monitored daily following challenge for signs of illness and weight loss. Mice with >20% weight loss with signs of illness were killed, having reached the humane end point. (A) The mean body weight of mice is shown as a percentage (%) of the original weight at the time of challenge. (B) The percentage of surviving mice is shown in panels C and E, and survival is shown in panels D and F. For panels A, C and E, the symbols indicate the mean weight of the group and error bars indicate the SEM. In panels A and E, *P < 0.05 between the saline and PEG-Pam2Cys treatment groups (unpaired Student’s t test). In panels B and F, #P < 0.01 vs saline group (Chi-square test). These results are combined from at least two independently conducted experiments.

The Use of Pam2Cys as a Prophylactic Antiviral Agent Permits Development of IAV-Specific CD8+ T Cell Responses. Our findings have demonstrated that Pam2Cys prophylaxis significantly reduces the impact of virulent influenza infection although it is apparent from our studies with mild strains of IAV, that viral infection is established albeit at significantly lower titer. It was of interest to us to determine whether mice that receive PEG-Pam2Cys are able to develop IAV-specific immunity during the lower grade infection.

In order to determine this, we performed an experiment using Mem virus which is not lethal and therefore enables assessment of CD8+ T cell responses following resolution of infection. Groups of mice treated with saline or PEG-Pam2Cys were challenged with 10^4.5 pfu of Mem, and 6 weeks later the presence of IAV-specific CD8+ T cells was examined.

IAV infection in C57BL/6 mice is characterized by immunodominant CD8+ T cell responses that are directed against the H-D b restricted epitopes in the nucleoprotein (NP366–374) and acid polymerase subunit of the viral RNA polymerase (PA224–233). We used tetramers to both of these epitopes to identify NP and PA-specific cells and determined the quality of the CD8 T cell responses by measuring the expression of the activation (CD43) and memory (CD27) markers as well as cytokine production.

In saline-treated mice that have experienced infection with the Mem virus, both NP366–374 and PA224–233 specific responses are evident by tetramer staining (Figure 6A and 6B). The tetramer positive cells were predominantly CD27hi, and expressed memory-like (CD27 hiCD43 hi) phenotypes (Figure 6B) that are associated with high recall capacity in the lungs. PEG-Pam2Cys pretreated groups possessed similar memory phenotypes in the IAV-specific CD8+ T cell population to those observed in animals treated with saline (Figure 6A).

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The functional quality of lung-resident CD8+ T cells was determined by ICS assay. PA and NP-specific CD8+ T cells produced either IFN-γ, INF-γ, and TNF-α, INF-γ, and IL-2 or IFN-γ, TNF-α and IL-2 following in vitro exposure to peptide. These functional CD8+ T cell populations could be identified in both saline-treated and PEG-Pam2Cys-treated animals (Figures 6C and 6D). The trend for lower numbers of lung-resident CD8+ T cells was again seen in the PEG-Pam2Cys treated group.

Pam2Cys Prophylaxis Reduces the Rate of Viral Transmission. The results that we have so far reported have demonstrated that Pam2Cys protects mice against influenza challenge and could therefore be developed as a potential antiviral agent against IAV. Optimally, such an agent would provide individual benefits and population level benefits if it could reduce transmission of virus.

In order to determine whether Pam2Cys treatment can reduce the spread and transmission of virus, we utilized a mouse model of contact transmission (Edenborough, K., Gilbertson, B., Brown, L.E., manuscript submitted). In this context are necessary for providing protection against lethal viral challenge.
The results show that prophylactic treatment of donor mice with PEG-Pam2Cys at 24 h, 72 h, 5 days or 7 days prior to viral challenge reduced viral loads an average of 10–100-fold compared to mice treated with saline (Figure 7B, upper panel). The establishment of infection in all of the recipient mice cohoused with saline-treated mice confirmed the capacity for transmission to occur between donor and cohoused recipients (Figure 7B, lower panel). Although mice receiving PEG-Pam2Cys 5 days or 7 days prior to challenge were able to transmit virus to recipient mice, animals that had received PEG-Pam2Cys 24 or 72 h prior to virus challenge were unable to transmit infection to cohoused recipient mice. These findings demonstrate that Pam2Cys pretreatment not only can reduce viral loads in treated mice but also reduces the potential to spread infection.

**Discussion**

The innate immune response has been shown to play a vital role in early control of IAV infection. This led us to question whether pulmonary innate immune enhancement with the Toll-like 2 agonist Pam2Cys could be exploited to reduce the impact of infection with respiratory pathogens. The construction of Pam2Cys into a soluble compound through pegylation provided us a compound which could be delivered into the lungs by intranasal administration for the purpose of inducing local immune response in the immediate site of respiratory infection. In this study we demonstrate that intranasal administration of PEG-Pam2Cys induces significant changes in the cellular and cytokine environment of the lung and that these changes correlate with an increased resistance to subsequent challenge with influenza A virus and the effects are TLR2-dependent.

An important feature of the PEG-Pam2Cys-mediated prophylactic effect is that it does not totally prevent infection and permits generation of memory and functional IAV-specific CD8+ T cell responses. A trend for lower responses in the lung-specific population (but not the spleen) was detected, and we speculate that this could be due to the effects of PEG-Pam2Cys on reducing viral burden and hence antigen load in the lungs. The relevance of these findings is that during PEG-Pam2Cys prophylaxis the adaptive immune response is intact enabling generation of CD8+ T cells. CD8+ T cells have been shown to provide protection against different strains of IAV (reviewed in ref 32) and therefore could be particularly important when one considers the potential for viruses to recirculate in the unvaccinated or immunologically naive population. This finding also speaks to the concern that use of an antiviral such as oseltamivir (Tamiflu) can hinder the generation of IAV immunity that would otherwise provide protection against reinfection.

During IAV infection, effectors of the innate immune system contribute to protection as well as exacerbation of disease, with excessive cytokine and macrophage levels associated with the pathology observed following severe H5N1 and H1N1 virus infection. In our hands, we found that elevated levels of cytokines such as TNF-α and IL-6 in PEG-Pam2Cys treated mice actually predicted an improved outcome following IAV challenge. Studies in mice deficient in TNF-α, IL-6 or MIP-α have shown that these mice are not resistant to H5N1 challenge, suggesting that at least in the murine model of infection, these cytokines are not the sole contributors to disease severity.
In animals treated with PEG-Pam2Cys 7 days prior to challenge and despite no elevation in cytokine levels or lung cell counts, we observed protection against PR8 challenge. It will be interesting to determine whether the cellular compartment is responsible for protection, or if Pam2Cys administration could somehow prime or modify the immune response for improved responsiveness to infection as a result of innate imprinting.41 Preliminary toxicological studies we have conducted showed no significant alterations in liver and kidney weights following treatment with PEG-Pam2Cys indicating that treatment does not lead to an overt and acute systemic toxicity (Tan et al., unpublished results). Although we have observed the changes to the lung to be transient, we are, however, now extending our studies to examine the acute and long-term effects of Pam2Cys dosing on lung airway function and respiration.

This study has demonstrated proof of concept studies, that Pam2Cys is an immunostimulant that reduces the impact of IAV infection. Our preliminary results also show a similar protective effect against respiratory syncytial virus and with the bacterium Legionella pneumophila (Tan et al., unpublished results). Because these immunomodulatory agents work in an antigen-independent manner, they could be viable candidates for development as broad-spectrum treatment options against viral and/or bacterial respiratory infections. The anti-IAV activity exhibited by PEG-Pam2Cys, together with its ability to restrict viral transmission, demonstrates the potential of these agents as mitigation strategies during influenza epidemics and pandemics.

■ AUTHOR INFORMATION

Corresponding Author
*Department of Microbiology & Immunology, The University of Melbourne, Parkville 3010, Victoria, Australia. E-mail: davidcj@unimelb.edu.au. Tel: 61-3-8344-9940. Fax: 61-3-8344-9941.

Notes
The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

IAV, influenza A virus; PEG, pegylated; TLR, Toll-like receptor

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