Pharmacologic Activation of the Innate Immune System to Prevent Respiratory Viral Infections

Guanjun Cheng[1], Liang-Chuan S. Wang[1], Zvi G. Fridlender[1], Guang-Shing Cheng[2], Bei Chen[3], Nilam S. Mangalmurti[1], Vassiliki Saloura[1], Zaifang Yu[1], Veena Kapoor[1], Krystyna Mozdzanowska[2], Edmund Moon[1], Jing Sun[1], James L. Kreindler[4], Noam A. Cohen[3], Andrew J. Caton[2], Jan Erikson[2], and Steven M. Albelda[1]

[1]Thoracic Oncology Research Laboratory, University of Pennsylvania, Philadelphia; [2]The Wistar Institute, Philadelphia; [3]Department of Otorhinolaryngology-Head and Neck Surgery, University of Pennsylvania, Philadelphia; and [4]Department of Pediatrics, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania

Drugs that can rapidly inhibit respiratory infection from influenza or other respiratory pathogens are needed. One approach is to engage primary innate immune defenses against viral infection, such as activating the IFN pathway. In this study, we report that a small, cell-permeable compound called 5,6-di-methylxanthenone-4-acetic acid (DMXAA) can induce protection against vesicular stomatitis virus *in vitro* and H1N1 influenza A virus *in vitro* and *in vivo* through innate immune activation. Using the mouse C10 bronchial epithelial cell line and primary cultures of nasal epithelial cells, we demonstrate DMXAA activates the IFN regulatory factor-3 pathway leading to production of IFN-β and subsequent high-level induction of IFN-β–dependent proteins, such as myxovirus resistance 1 (Mx1) and 2′,5′-oligoadenylate synthetase 1 (OAS1). Mice treated with DMXAA intranasally elevate mRNA/protein expression of Mx1 and OAS1 in the nasal mucosa, trachea, and lung. When challenged intranasally with a lethal dose of H1N1 influenza A virus, DMXAA reduced viral titers in the lungs and protected 80% of mice from death, even when given at 24 hours before infection. These data show that agents, like DMXAA, that can directly activate innate immune pathways, such as the IFN regulatory factor-3/IFN-β system, in respiratory epithelial cells can be used to protect from influenza pneumonia and potentially in other respiratory viral infections. Development of this approach in humans could be valuable for protecting health care professionals and "first responders" in the early stages of viral pandemics or bioterror attacks.

Keywords: innate immunity; interferon; influenza; pneumonia; bronchial epithelium

Influenza virus can cause many different illness patterns, from mild common-cold symptoms to life-threatening pneumonia and secondary bacterial infections. The influenza pandemic of 1918 occurred suddenly and killed over 50 million people worldwide (1). Concerns about an avian influenza pandemic have been present for a number of years. The 2009 H1N1 influenza A pandemic initially raised similar concerns, but, fortunately, the pathogenicity of this virus was relatively low. However, this outbreak did illustrate that constraining a new strain of influenza to one geographic area will likely be impossible.

(*Received in original form July 6, 2010 and in final form November 3, 2010*)

This work was supported by grants NCI PO1 CA 66726 abd 5-T32 HL07748 (L.-C., S.W., and G.-S.C.)

Correspondence and requests for reprints should be addressed to Liang-Chuan S. Wang, Thoracic Oncology Research Laboratory, 1015F ARC, University of Pennsylvania, 3615 Civic Center Boulevard, Philadelphia, PA 19104-4318. E-mail: wanli@mail.med.upenn.edu

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 45. pp 480–488, 2011
Originally Published in Press as DOI: 10.1165/rcmb.2010-0288OC on December 10, 2010
Internet address: www.atsjournals.org

CLINICAL RELEVANCE

A small, cell-permeable compound called 5,6-di-methylxanthenone-4-acetic acid was able to induce production of IFN-β and IFN-β–dependent proteins and protect epithelial cells *in vitro* from virally induced cell death and to protect mice from a lethal dose of H1N1 influenza A virus. Development of this approach in humans could be especially valuable for protecting health care professionals and "first responders" in the early stages of viral pandemics or bioterror attacks.

We have two primary weapons against influenza. The first line of defense is antiviral drugs. Four antiviral drugs—zanamivir, oseltamivir, amantadine, and rimantadine— have been approved by the FDA in the United States to treat acute, uncomplicated influenza. Although extremely valuable, their widespread use will likely be limited by concerns of side effects, patient compliance, and, most worrisome, the presence or development of drug resistance (2). For example, the emergence of oseltamivir-resistant H1N1 viruses became common in this last outbreak (3). Our second line of defense is the development of appropriate vaccines; however, the recent H1N1 pandemic made it clear how difficult it will be to generate a vaccine quickly enough and in sufficient quantities for use during the initial stages of a pandemic. The need for new antiinfluenza treatments or prophylactic strategies that can be used rapidly is obvious.

Respiratory tract mucosal epithelial cells, which form the majority of the cells lining the epithelial tract and lungs, are the primary targets of respiratory viruses. These cells are usually resistant to viral infection due to a complex defense system involving physical barriers (i.e., mucus), innate immune responses of the epithelial cells and resident leukocytes, and ultimately, the development of acquired immune responses (antibodies and T cells). One of the most important early defenses against influenza or other viral infections is the release of a variety of immunostimulatory cytokines and chemokines by epithelial cells, macrophages, and neutrophils, the most important being IFN-β and IFN-α (4). These Type 1 IFNs trigger the up-regulation of a cascade of IFN-responsive genes (such as 2′,5′-oligoadenylate synthetase 1 [OAS1] and myxovirus resistance 1 [Mx1]) that protect cells from viral replication (5–7). The hypothesis of this study is that activation of these antiviral innate immune pathways by a pharmacologic agent would result in resistance to viral infection in a very rapid and nonspecific fashion, potentially allowing for at least temporary protection from any newly arising strain of influenza or other viral respiratory pathogen (such as the SARS virus).

Our candidate drug is 5,6-di-methylxanthenone-4-acetic acid (DMXAA), a small molecule of the flavanoid/xanthone class

that has strong antitumor activity through its ability to activate dendritic cells and macrophages (and to a lesser extent lymphocytes) via release of a variety of immunostimulatory cytokines and chemokines (8, 9). In the context of antitumor biology, it has been established that DMXAA can activate multiple innate immune pathways. These include activation of NF-κB through an undefined pathway that does not require the Toll-like receptor (TLR) adaptor molecule MyD88 (9–11) and through activation of the intracellular pathogen recognition receptors, nucleotide-binding oligomerization domain (NOD)1 and NOD2, that then signal via the downstream adaptor serine/threonine kinase rip-like interacting caspase-like apoptosis-regulatory protein kinase (12). DMXAA also activates the TANK-binding kinase 1 (TBK1)/IRF3 pathway in macrophages, which results in secretion of high levels of IFN-β (11). We also have evidence that DMXAA can activate mitogen-activated protein kinase pathways (13). Despite much research in leukocytes and endothelial cells, the effects of DMXAA have never been studied in epithelial cells. Given its ability to induce cytokines, we tested the hypothesis that DMXAA could also activate the IRF3/Type I IFN pathway in respiratory epithelial cells. After seeing strong activation in these cells, we evaluated the idea that DMXAA could be used as an antigen-independent approach to protect against viral pathogens. This was tested in cultured and primary mouse respiratory epithelial cell lines using vesicular stomatitis virus (VSV), in a mouse respiratory epithelial cell line, and in an animal model using a mouse-adapted H1N1 influenza A virus. These studies identify DMXAA as a prototype for a cell-permeable, non–bacterially derived compound that activates the IRF3/Type I IFN pathway in respiratory epithelial cells *in vitro* and *in vivo* and has the ability to prevent viral infection in an antigen-independent fashion.

METHODS

Cell Culture and Reagents

C10 immortalized mouse bronchial epithelial cells were grown as described (14). The cells were regularly tested and maintained negative for *Mycoplasma* spp. DMXAA was purchased from Sigma (St. Louis, MO). The vehicle for DMXAA was distilled, deionized water (12) unless otherwise mentioned.

Mice

Female BALB/c and C57/B6 mice were purchased from Charles River Laboratories (Wilmington, MA). Animals were housed in the animal facility at the Wistar Institute (Philadelphia, PA). The Animal Use Committees of the Wistar Institute and University of Pennsylvania approved all protocols in compliance with the care and the use of animals (8, 9).

Isolation and Air–Liquid Interface Culture of Nasal Epithelial Cells

Murine nasal septal air–liquid interface cultures were prepared as previously described (15, 16). Briefly, cultures were grown under submersion for the first 3 days and transitioned to apical air exposure on Day 4 by removing the apical medium. Cells were then fed by the basolateral compartment.

Viruses

Stocks of VSV with a green fluorescent protein (GFP) insert (VSV-GFP) were a gift from the Core Viral Facility of Mayo Clinic (17). H1N1 influenza A (PR8) virus was propagated in the allantoic cavity of embryonated chicken eggs, and virus titers were determined as previously described (18). Virus was purified using a sucrose gradient.

Mouse Model of Viral Infection and DMXAA Administration

Virus challenge was performed by intranasal inoculation of 200 $TCID_{50}$ of H1N1 influenza A (PR8) virus to the nasal epithelia in anesthetized mice via direct instillation in 50 μl saline. Mice were administered 23 mg/kg DMXAA intranasally or intraperitoneally. Mouse body weight was measured daily (19–21). Mice were killed when their weight fell more than 25% of starting weight.

Virus Titer

Naive BALB/c mice were treated with 23 mg/kg of DMXAA intranasally at the same time as H1N1 influenza A (PR8) virus instillation. The untreated group was given H1N1 influenza A (PR8) virus only. Lung tissues were harvested 5 days after virus infection. Lung viral titers of H1N1 influenza A (PR8) virus–infected mice were determined by endpoint dilution assay in MDCK cells and expressed as $TCID_{50}$ (19–21).

Immunoblots

Protein samples were prepared by homogenizing mouse nasal and tracheal mucosa or cells in lysis buffer. Samples were run on 4 to 12% NuPAGE Novex Tris Acetate Mini Gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes (12, 22). Proteins were detected using rabbit polyclonal anti-OAS1, and mouse monoclonal anti–protein kinase R (PKR) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

Protein Studies for Cytokine/Chemokine Levels

The amount of IFN-β, IFN-γ–induced protein 10 kDa (IP10)/CXCL10, IL-6, and MCP-1/CCL2 secreted by cells activated by DMXAA was quantified using ELISA kits to detect murine IFN-β (VeriKine Mouse IFN Beta ELISA Kit, PBL Biomedical Laboratories), IP10 (DuoSet Mouse CXCL10/IP10/CRG-2; R&D Systems, Inc., Minneapolis, MN), and IL-6 (BD OptEIA Mouse IL-6 ELISA Set; BD Biosciences Pharmingen, San Diego, CA) according to the manufacturers' instructions.

RNA Isolation and Real-time, RT-PCR

Total RNA was isolated from cells or nasal epithelium with RNeasy Mini Kit (Qiagen, Valencia, CA), and cDNA was synthesized. Gene expression was checked using an Applied Biosystems StepOne Real-Time PCR System (Carlsbad, CA) following the manufacturer's protocol as previously described (12). Primer sequences can be obtained from the authors on request.

MTT Assays

To perform MTT [3–(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays, cells were plated in quadruplicate on 96-well plates and were infected with different multiplicities of infection (MOI) of the VSV-GFP viruses. Viability was assessed at 48 hours by developing the reaction assay according to the manufacturer's instruction (Promega, Madison, WI).

Immunoperoxidase Staining

Lung tissues were harvested after intranasal administration of DMXAA (23 mg/kg), embedded in OCT compound and frozen. The OCT blocks were cut into 5-μm sections. After incubation with the primary antimouse antibodies anti-OAS1 or anti-PKR (Santa Cruz Biotechnology), biotinylated-secondary antibodies were added to the sections. The slides were incubated with ABC-peroxidase (Vector Elite, Burlingame, CA) as previously described (17).

Statistical Analysis

Unless otherwise noted, data comparing differences between two groups were assessed using unpaired Student's t test. Comparisons with more than two groups were done using one-way ANOVA with appropriate *post hoc* testing. Log-rank comparisons of survival distribution were performed by Kaplan-Meier analysis using Epi Info Version 3.5.1 (Centers for Disease Control and Prevention, Atlanta, GA). Differences were considered significant when $P < 0.05$. Data are presented as mean ± SEM. Results from one individual experiment with three to five replicates of each condition are shown. Each study was repeated at least twice with similar results.

RESULTS

DMXAA Activates the IFN-β Signaling Pathway in C10 Mouse Respiratory Epithelial Cells

To determine the ability of DMXAA to activate IFN-β–mediated antiviral signaling pathways in mouse bronchial epithelial cells, C10 cells (immortalized, but nontransformed mouse bronchial epithelial cells) were exposed to DMXAA for 3 to 6 hours. The mRNA expression profiles were evaluated using real-time RT-PCR. The IFN-β and the IFN-inducible antiviral genes OAS1 and Mx1 (23) were significantly up-regulated (24- to 99-fold) by DMXAA ($P < 0.01$) (Figure 1A). Marked and significant ($P < 0.01$) increases in inflammatory cytokines and chemokines were also noted, including increases in IP10 (CXCL10), MCP-1 (CCL2), IL-6, and RANTES (CCL5) (Figure 1A).

The concentrations of IFN-β, IP10, and IL-6 proteins in the supernatants were evaluated by ELISA and found to be significantly increased ($P < 0.01$) (Figure 1B). Using immunoblotting, we also demonstrated that C10 cells stimulated by DMXAA expressed increased protein levels of the IFN-responsive gene OAS1 for up to 72 hours (Figure 1C).

DMXAA Prevents VSV-Induced Cell Death in C10 Mouse Respiratory Epithelial Cells

Given this activity, we assessed the protective effect of DMXAA against virus infection of C10 cells. We postulated that a useful model would be VSV. This virus replicates rapidly in epithelial cells and induces cell death, but infection is markedly inhibited by treatment with type 1 IFN (24). We first validated these findings in C10 cells by pretreating them with a range of concentrations of mouse IFN-β for 24 hours and then exposing the cells to varying MOI of VSV. The number of viable cells was measured 48 hours later. Figure 2A shows that VSV kills 70 to 80% of the C10 cells at even the lowest MOI of 0.01 viral particles per cell. However, even very minimal amounts of IFN-β (1 IU/ml = 5 pg/ml) can protect the cells from death.

After the system was validated, C10 cells were treated with DMXAA at various time points before or after VSV exposure. The ability of DMXAA to prevent cell death was determined using the MTT assay 48 hours after the addition of virus. Marked protection was observed when DMXAA was given at the same time, 2, 6, or 24 h before VSV infection (Figure 2B).

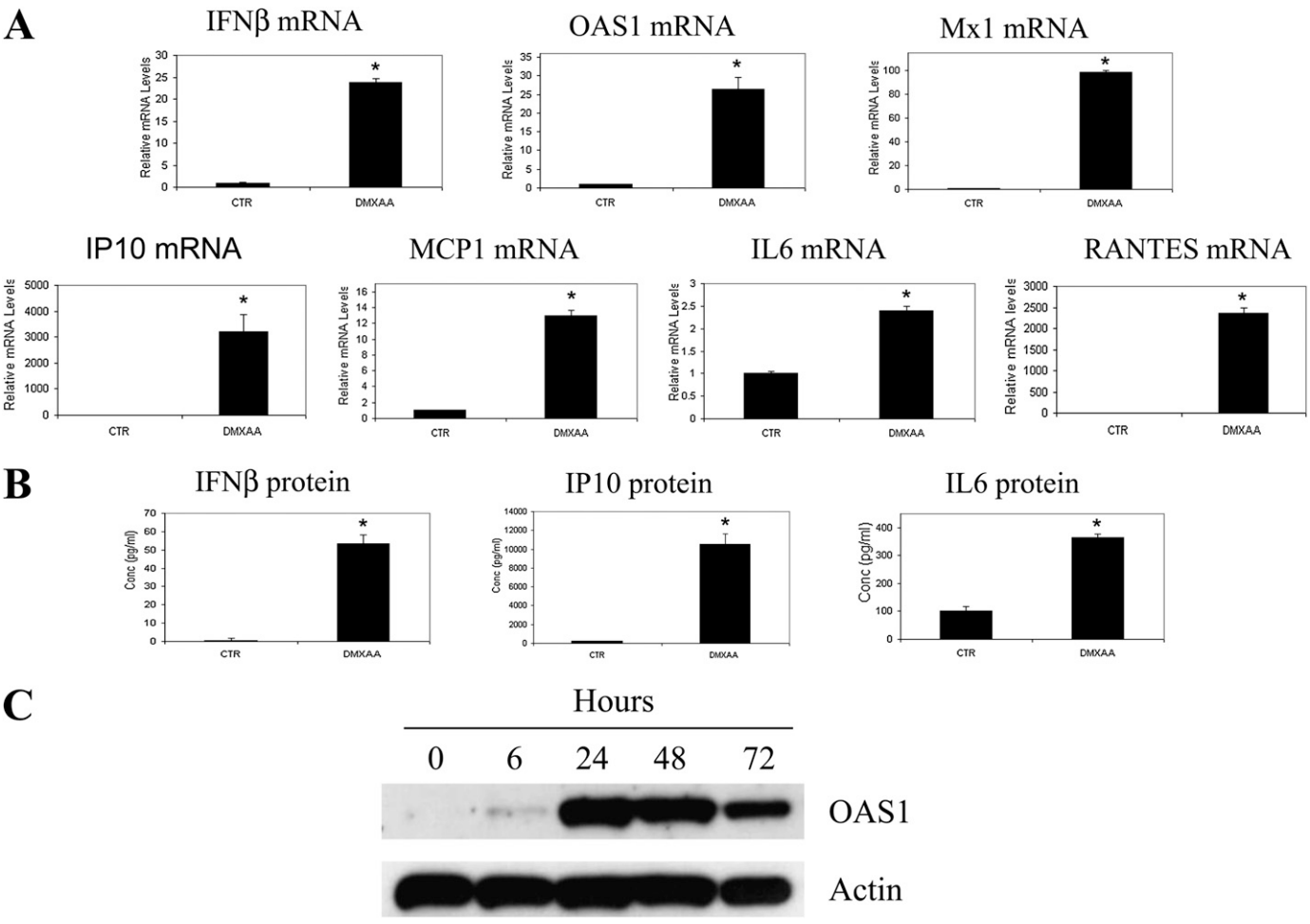

Figure 1. 5,6-Di-methylxanthenone-4-acetic acid (DMXAA) induces IFN-β and other chemokine/cytokines in C10 mouse epithelial cells. Cells were incubated with or without DMXAA (100 μg/ml) for 3 to 6 hours. (*A*) Total RNA was isolated, and the relative IFN-β, 2′,5′-oligoadenylate synthetase 1 (OAS1), myxovirus resistance 1 (Mx1), IFN-γ–induced protein 10 kDa (IP10), MCP-1, IL-6, and RANTES mRNA levels were measured by real-time RT-PCR. (*$P < 0.01$, CTR versus DMXAA groups). (*B*) Induction of IFN-β, IP-10, and IL-6 protein was determined from supernatants by ELISA. The results are presented as mean ± SEM (*$P < 0.01$, CTR versus DMXAA groups) from one of three representative experiments with similar results. (*C*) Cells were incubated with DMXAA (100 μg/ml) at indicated time points and lysed, and OAS1 protein levels were analyzed by immunoblot using an anti-OAS1 antibody. Expression levels of β-actin show equal loading of the lanes. CTR denotes for the untreated control.

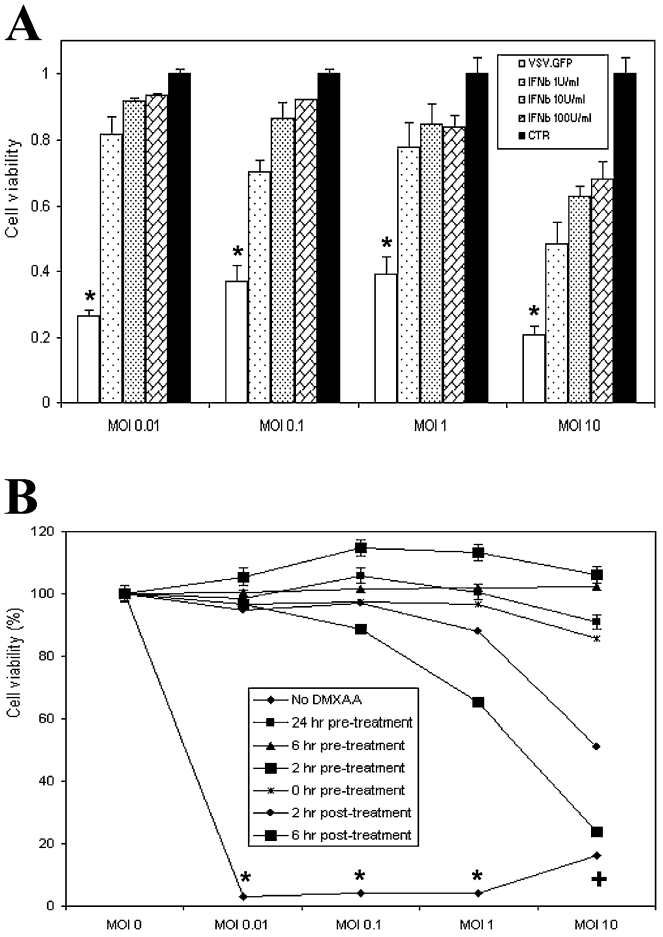

Figure 2. DMXAA protects C10 mouse epithelial cells from vesicular stomatitis virus (VSV)-induced cell death. (*A*) Cells were plated in 96-well plates and pretreated with the indicated doses of mouse IFN-β for 24 hours and exposed to varying multiplicities of infection (MOI) of VSV-green fluorescent protein (GFP) for 48 hours. The percentage of viable cells was assessed using MTT assay (*$P < 0.01$, VSV/GFP versus IFN-β or CTR groups). (*B*) Cells were treated with DMXAA (100 μg/ml) before or after being incubated with different MOI of the VSV-GFP viruses. An MTT assay was performed at 48 hours after infection, and the percentage of viable cells were determined. Each sample was run in quadruplicate (*$P <$ 0.01, CTR versus DMXAA groups at 0.01, 0.1, and 1 MOI; $^{+}P < 0.01$, CTR versus DMXAA groups except 6 h after treatment at 10 MOI).

Some protection was still detected even when DMXAA was given 2 and 6 hours after VSV infection.

We next evaluated effects of the PR8 strain, a mouse-adapted human influenza virus, on C10 cells. When mice were exposed to MOI of up to 1, we saw no cytopathic effects; however, using RT-PCR, we detected evidence of viral replication (*see* Figure E1A in the online supplement). The addition of DMXAA 2 hours before or at the same time as the addition of PR8 reduced the amount of viral RNA detected by approximately 50% (Figure E1B).

DMXAA Activates the IFN-β Signaling Pathway in Primary Cultured Normal Mouse Nasal Respiratory Epithelial Cells

To investigate whether DMXAA could effectively activate IFN-β signaling pathways in primary respiratory cells, mouse nasal epithelial cells were isolated in primary culture and grown on air–liquid interfaces. To validate the purity of the primary cultured mouse nasal epithelial cells, we performed real-time RT-PCR with primers against an epithelial cell biomarker (cytokeratin 19), a leukocyte biomarker (CD45), and an endothelial cell biomarker (CD31/PECAM-1). The data are plotted as the cycle number where mRNA was detected. In our system, detectable messages are usually seen at channel numbers less than 35 cycles. Figure 3A shows very high enrichment for epithelial cells (23.5 cycles for cytokeratin 19), with barely detectable expression of leukocytes (35 cycles for CD45) or endothelial cells (41.6 cycles for CD31/PECAM-1).

We then exposed the nasal epithelial cells to DMXAA (100 μg/ml) at various time points. Similar to the C10 mouse bronchial epithelial cells, DMXAA induced a marked ($P <$ 0.01) up-regulation of mRNA for IFN-β, OAS1, Mx1, IP10, MCP-1, and IL-6 at 6 hours with significant but lower increases at 18 hours (Figure 3B). Consistent with the mRNA data, the concentrations of IFN-β, IP10, and IL-6 proteins in the supernatants (evaluated by ELISA) showed significant ($P < 0.01$) increases at 6 and 18 hours after DMXAA exposure (Figure 3C).

Intraperitoneal and Intranasal Administration of DMXAA Activates the IFN-β Signaling Pathway in Normal Mouse Respiratory Epithelial Cells *In Vivo*

To extend the *in vitro* results, C57BL/6 mice were given 23 mg/kg of DMXAA intraperitoneally. At various time points, mice were killed, the nasal epithelia were removed, and protein or mRNA was extracted. Real-time RT-PCR showed that the samples were highly enriched for epithelial cells (Figure 4A).

Similar to the cultured epithelial cells, DMXAA induced rapid up-regulation of mRNA for IFN-β and IFN-β–responsive genes in this *in vivo* system. We noted marked and significant increases ($P < 0.01$) in expression of IFN-β, OAS1, Mx1, IP10, MCP-1, and IL-6 mRNA expression, with peak levels in most of these at 2 hours (Figure 4B). We also demonstrated that DMXAA up-regulated OAS1 protein expression in mouse nasal epithelium when analyzed by immunoblot at 24 hours after intraperitoneal administration (Figure 4C).

To determine if DMXAA administered intranasally could also activate IFN-β signaling pathways in normal mouse respiratory epithelial cells (including the lung) *in vivo* and to check any strain-specific responses, BALB/c mice were given 23 mg/kg of DMXAA intranasally. At various time points, mice were killed, and the nasal or tracheal epithelium or lungs removed. Protein was extracted, and immunoblots or immunostaining were performed. DMXAA up-regulated OAS1 expression in nasal and tracheal epithelium when analyzed by immunoblot 24 hours after intranasal administration (Figure 5A). DMXAA also up-regulated the IFN-responsive OAS1 and PKR proteins in mouse bronchial epithelial cells and alveoli detected by immunostaining sampled 48 hours after intranasal DMXAA administration (Figure 5B). To further define DMXAA-induced inflammation, bronchoalveolar lavage was performed 6 and 24 hours after intranasal DMXAA (*see* online supplement for details). Endotoxin was instilled as a positive control. In contrast to an instillation of endotoxin, DMXAA resulted in only a mild inflammatory response in the lung, with minimally elevated polymorphonuclear cells (Figure E2) and slight changes in some BAL cytokine levels (Table E1).

Taken together, these data show that DMXAA activates IFN-β signaling pathways in mouse respiratory epithelial cells *in vitro* and *in vivo* and prevents VSV-induced cell death in C10 mouse bronchial epithelial cells.

Efficacy of DMXAA against H1N1 Influenza A Infection in the Lung

To examine the protective effect of intranasal or intraperitoneal administration of DMXAA against an actual respiratory in-

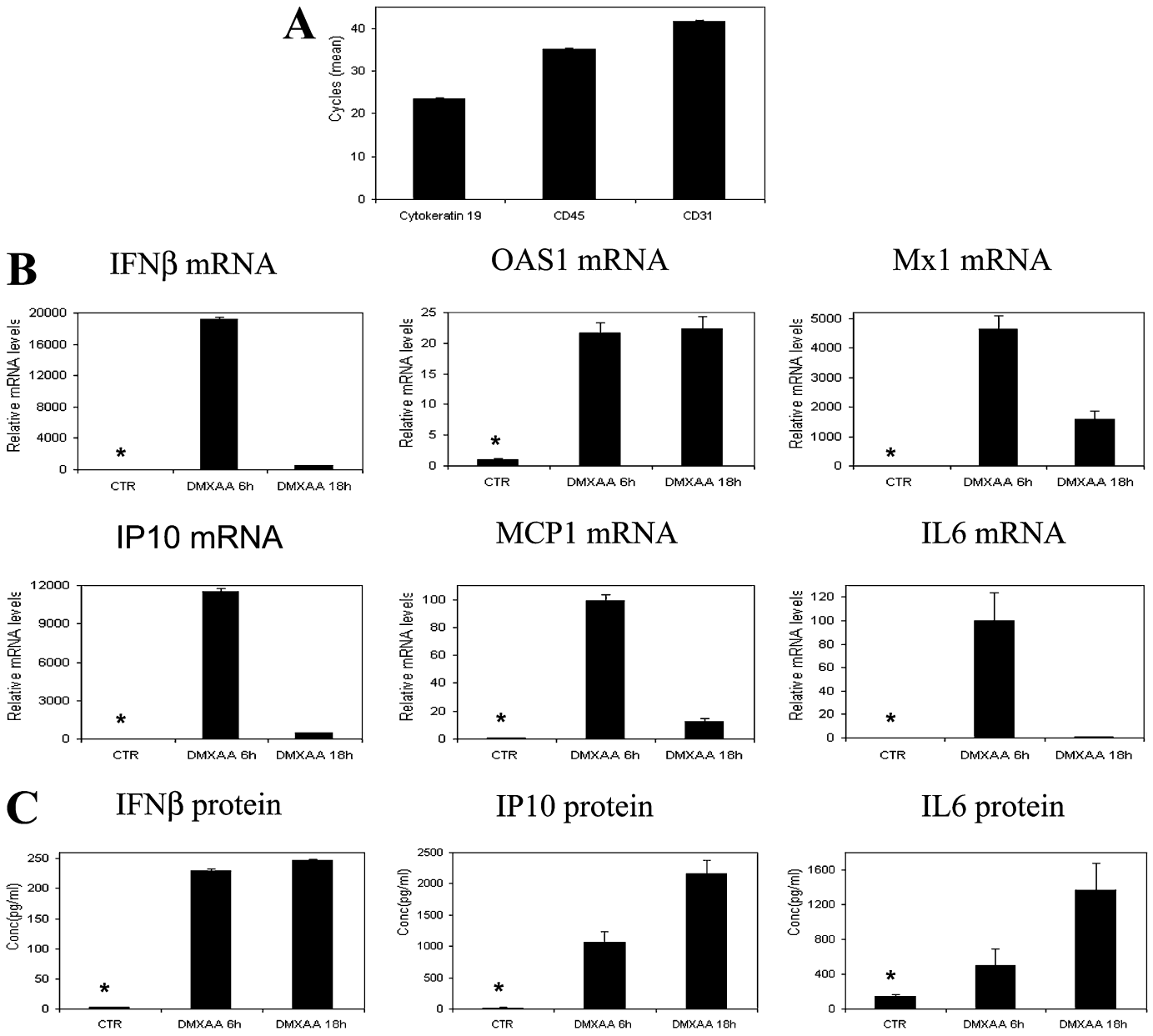

Figure 3. DMXAA activates the IFN pathway in primary cultures of normal mouse respiratory epithelial cells. (*A*) Mouse nasal epithelial cells isolated in primary culture and grown on air–liquid interfaces were validated by real-time RT-PCR with primers against an epithelial cell biomarker (cytokeratin 19), a leukocyte marker (CD45), and an endothelial cell marker (CD31) (PECAM). (*B*) Cells were incubated with or without DMXAA (100 μg/ml) at the indicated time points. The total RNA was isolated, and the relative IFN-β, OAS1, MX1, IP-10, MCP1, and IL-6 mRNA levels were measured by real-time RT-PCR (*$P < 0.01$, CTR versus DMXAA groups). (*C*) Induction of IFN-β, IP-10, and IL-6 protein was determined from supernatants by ELISA. The results are presented as mean ± SEM (*$P < 0.01$, CTR versus DMXAA groups) from one of three representative experiments with similar results.

fection with H1N1 influenza A, we used the PR8 strain, a mouse-adapted human influenza virus that induces pneumonia and death with initial infections of only 200 $TCID_{50}$ (25). Mice were infected intranasally with 200 $TCID_{50}$ of H1N1 influenza A and treated with 23 mg/kg DMXXA intranasally or intraperitoneally at various time points before, at the same time, or after viral infection. Within 8 to 10 days of H1N1 influenza A virus challenge, mice in the "virus alone" group showed marked body weight loss (> 25%) (Figure 6). BALB/c mice pretreated with intraperitoneal DMXAA administration 2 and 24 hours before infection showed less weight loss, but 4 of 5 mice in each group were killed, and survival was not significantly increased (Figure 6A). However, effects were enhanced when DMXAA was given intranasally. Simultaneous intranasal administration of DMXAA or DMXAA given 2 and 24 hours before virus instillation markedly increased survival ($P < 0.01$ versus control) (Figure 6B; BALB/c mice and 6D-C57/Bl6 mice). Because protection was seen when DMXAA was given 24 hours before viral instillation, direct killing of virus by the DMXAA does not explain our effects. No protection was observed with intranasal DMXAA given 24 hours after viral instillation (Figures 6B and 6D).

As an additional way to evaluate the effect of DMXAA, lungs were removed from animals 5 days after instillation of

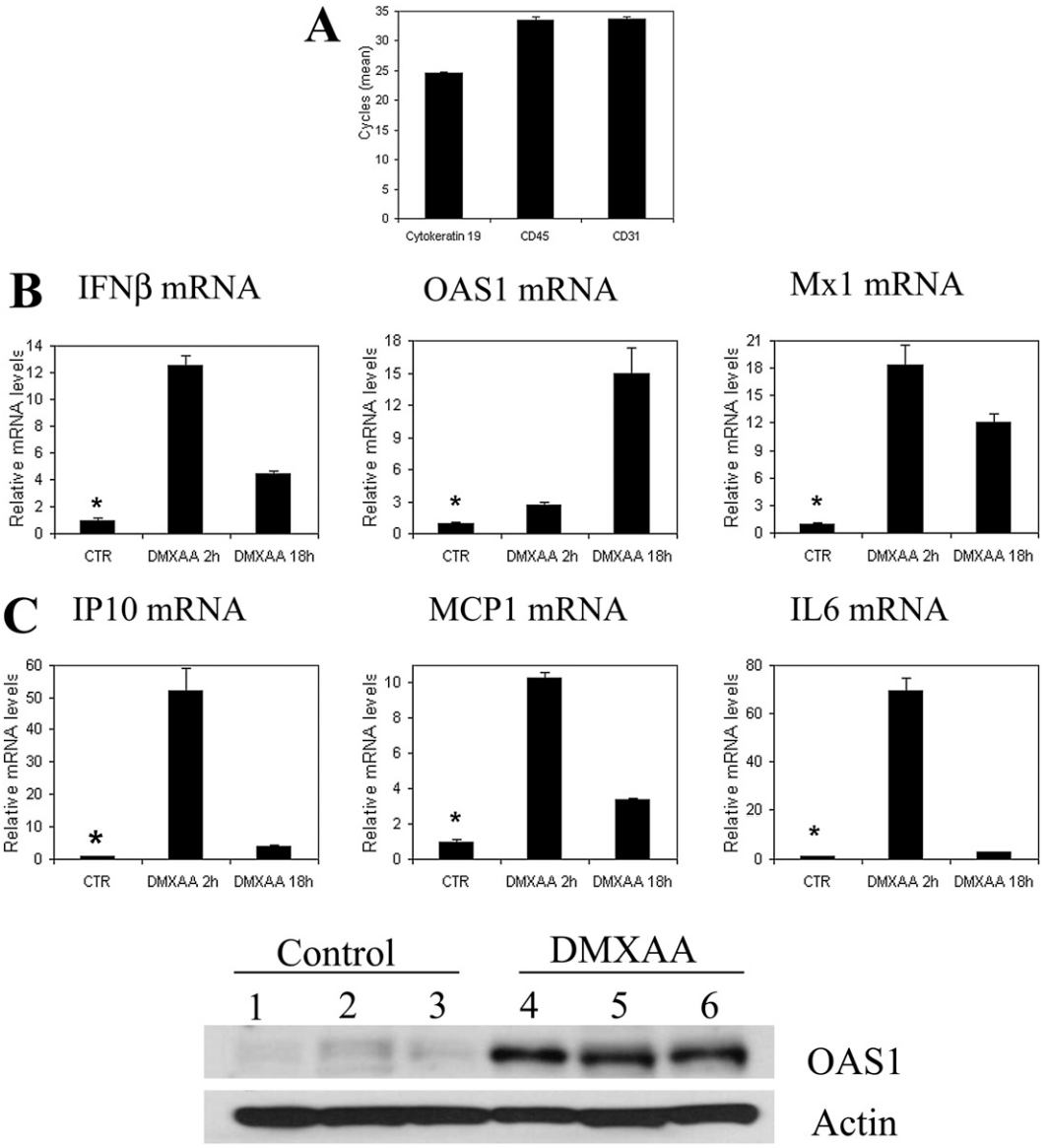

Figure 4. DMXAA activates the IFN pathway in normal mouse respiratory epithelial cells *in vivo* after intraperitoneal injection. (*A*) C57/B6 mice (3–5 per group) were given 23 mg/kg of DMXAA intraperitoneally and killed at various time points. The nasal epithelia were removed, and epithelial enrichment was validated by real-time RT-PCR with primers against an epithelial cell biomarker (cytokeratin 19), a leukocyte marker (CD45), and endothelial cell marker (CD31). (*B*) The relative IFN-β, OAS1, MX1, IP-10, MCP1, and IL-6 mRNA levels of mouse nasal epithelium were measured by real-time RT-PCR. The results are presented as mean ± SEM (*$P <$ 0.01, CTR versus DMXAA groups) from one of three representative experiments with similar results. (*C*) Mouse nasal epithelium was lysed 24 hours after intraperitoneal administration of DMXAA, and OAS1 protein levels were analyzed by immunoblot using an anti-OAS1 antibody. Expression levels of β-actin show equal loading of the lanes.

virus, and the viral titer was determined. The viral titers in lungs of DMXAA intranasally treated mice were approximately 8-fold lower ($P < 0.001$) than those of mice with virus challenge alone (Figure 6C).

DISCUSSION

Viral infection of leukocytes or respiratory epithelium activates a variety of pattern recognition-receptors (PRR), such as the TLRs or the NOD receptors that induce the production of IRF3, NFκB, and other cell activation pathways (26). Virally induced secretion of IFN-β (largely mediated through IRF3) is especially important because this molecule acts in a paracrine fashion on neighboring cells to up-regulate a group of key antiviral proteins (such as OAS1, MX1, and PKR) that prevent subsequent infection and spread of the virus.

Most viruses have developed sophisticated (and different) mechanisms to try to prevent IRF3/IFN pathway activation (27, 28). VSV, for example, encodes a Matrix protein that inhibits host cell gene expression by targeting a nucleoporin and blocking nuclear export (28). Hence, any mRNAs induced by activation of the IRF pathway after viral replication is initiated will not lead to production of IFN or its downstream antiviral effector genes. The NS1 protein of H1N1 influenza virus has also been implicated in a number of regulatory functions during influenza virus infection, including binding of the poly(A) tails of mRNAs (to inhibit their nuclear export) (29, 30), inhibiting host mRNA polyadenylation, contributing to the virus-induced shutoff of host protein synthesis (31), and inhibiting pre-mRNA splicing (29, 31–34). In addition, binding of NS1 to dsRNA prevents *in vitro* activation of PKR (35).

Given this ability of IRF3/IFN to prevent viral infection, activation of this pathway as a potentially "antigen-independent" way of controlling disease has been investigated. As early as the 1960s, type I IFNs were reported to be antivirals (36). Early reports of success using nasal spray preparations of IFN-α by Russian drug companies for prophylaxis and treatment of influenza attracted the interest of Western scientists into this arena (37). Subsequent studies were conducted that found that the reported beneficial effects were minimal in relationship to the considerable amount of side effects associated with the treatment (38). The limited efficacy of direct application of IFN was likely related a number of issues, including: (*1*) dose-limiting toxicities, such as cognitive dysfunction, dyspnea, fatigue, nausea, and

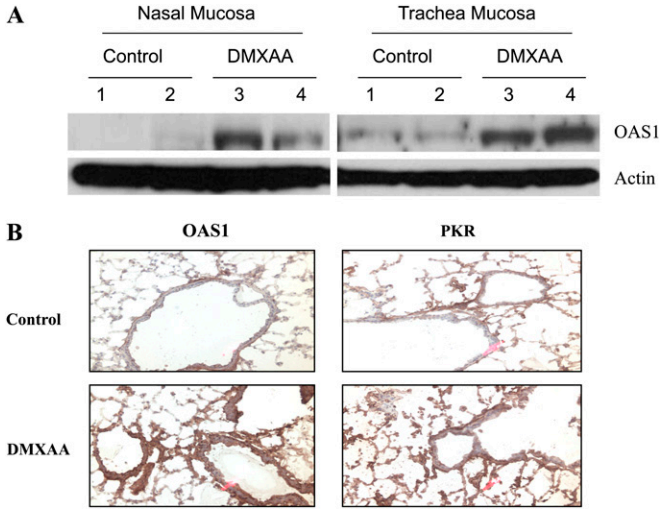

Figure 5. DMXAA activates the IFN pathway in normal mouse respiratory epithelial cells *in vivo* after intranasal administration. (*A*) BALB/c mice (3–5 per group) given 23 mg/kg of DMXAA intranasally and killed after 24 hours. The nasal epithelium and tracheal mucosa were removed and lysed, and OAS1 protein levels were analyzed by immunoblot using an anti-OAS1. Expression levels of β-actin show equal loading of the lanes. (*B*) Representative photomicrographs of lung sections sampled 48 hours after control and DMXAA administration were immunostained for OAS1 and protein kinase R (PKR).

vomiting (39); (*2*) a very short half-life due to high renal clearance (38); and (*3*) potential generation of neutralizing antibodies. However, given recent advances in recombinant IFN production that can prolong the half-life of IFN and better nebulization technology, a reinvestigation of this approach may be in order (40).

An alternative strategy to harness the innate immune system is to use agents that mimic viral infection and activate the innate immunity through PRR. There are four known TLRs (TLR-3, -7, -8, and -9) that recognize different forms of "foreign" nucleic acids and induce type I IFN to activate antiviral gene function (38). Compared with using recombinant IFN protein, agonists that activate specific TLRs have been shown to be less toxic, to induce longer IFN exposure, and to be easier to administer and more effective (41). A TLR-7 agonist, imiquimod, was approved by the FDA in 1997 for the treatment of external genital warts (42). Similarly, polyinosine–polycytidylic (poly I:C) is a TLR-3 agonist has been studied for the treatment of influenza infection (43). Although a number of studies reported that polyinosine–polycytidylic can be used for prophylaxis against influenza infection, two major limitations of using nuclear acid–based antiviral agents are their susceptibility to nuclease degradation *in vivo* and the fact that these molecules are not cell permeable. The latter point is a critical issue because the TLR3 receptor is intracellular (44). Attempts of overcome these obstacles have included chemical modifications to the nucleic acid backbones and sugars and packaging with liposomes. Unfortunately, these approaches have been shown to increase toxicity (43, 44).

The strategy of activating pattern recognition receptors has recently been applied using an aerosolized lysate of nontypeable haemophilus influenzae (21). Inhalation of this lysate induced profound inflammation in the lungs with an accompanying increase in a variety of IFNs and cytokines, yet it strongly protected mice against subsequent infection with bacterial, fungal, and viral (including influenza) pathogens. You and

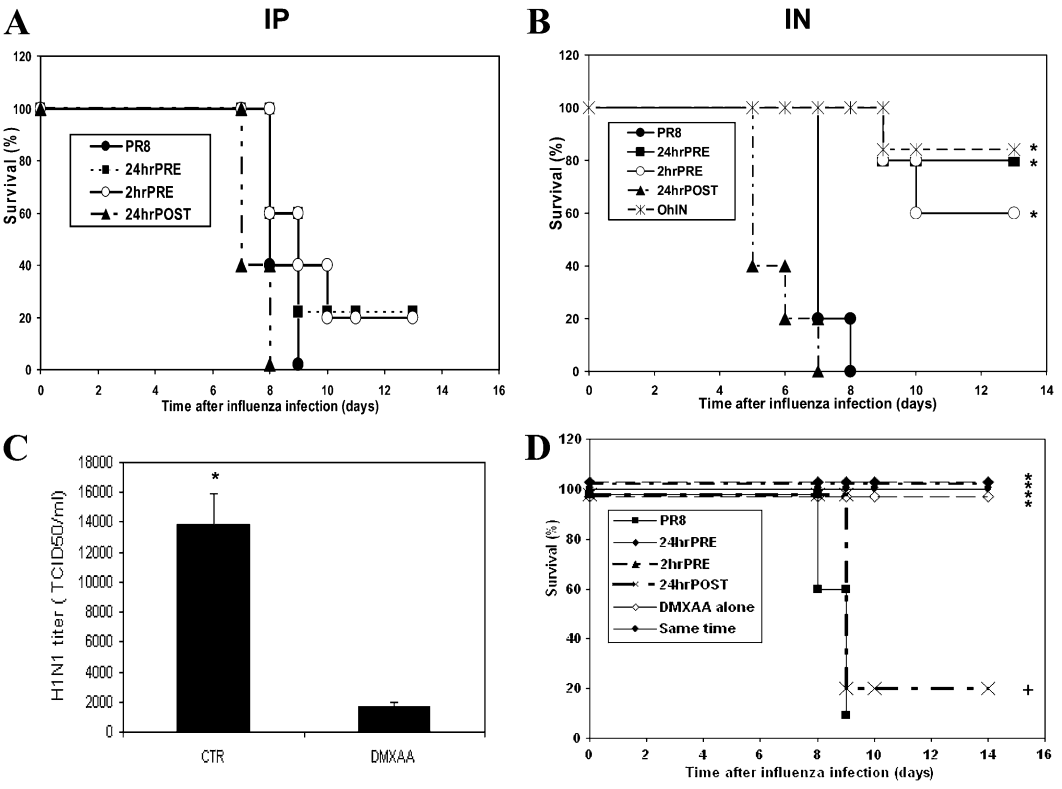

Figure 6. Intranasal or intraperitoneal administration of DMXAA protects against H1N1 influenza challenge. BALB/c mice were infected with H1N1 influenza A (PR8) virus and treated with DMXAA at various time points. (*A*) Survival after intraperitoneal (IP) DMXAA. (*B*) Survival after intranasal (IN) DMXAA (*$P <$ 0.05, CTR versus DMXAA groups). (*C*) BALB/c mice were treated with saline (control) or DMXAA administered intranasally and challenged with H1N1 influenza A (PR8) virus. The viral titers in lungs of mice were measured. The results are presented as mean ± SEM from one of three representative experiments with similar results (*$P < 0.01$, CTR versus DMXAA groups). (*D*) C57/B6 mice (five per group) were infected with H1N1 influenza A (PR8) virus and treated with DMXAA intranasally at various time points. Survival after IN DMXAA (*$P < 0.01$, PR8 CTR versus DMXAA groups except 24 hrPost group; $^{+}P > 0.05$, PR8 CTR versus DMXAA 24 hrPost group).

colleagues (45) suggested that this effect could be reproduced with lower toxicity by administration of two specific TLRs (but not by single TLR agonists). Thus, this strategy could be promising if the initial induction of lung inflammation could be minimized.

The data presented in this report suggest that there are other agents, like DMXAA, that have the ability to activate antiviral pathways after systemic or intranasal instillation without inducing strong lung inflammatory responses. In this study, we showed that DMXAA directly stimulated IFN-β production in respiratory epithelial culture (Figures 1A, 1B, 3A, and 4B) as well as inducing the downstream IFN-stimulated antiviral genes PKR and OAS-1 (Figures 1A, 1C, and 4C). This activation of the IFN pathway by DMXAA correlated with protection of C10 bronchial epithelium from VSV-mediated cytopathic effects in culture (Figure 2A). We also showed activation of the IFN pathway after administering DMXAA to mice intranasally or intraperitoneally. There was a significant increase in OAS1 protein in epithelial cells along the respiratory tract, including the nasal mucosa, the trachea mucosa, and the lung epithelium (Figure 5). Although intraperitoneal administration of DMXAA showed suboptimal protection against influenza virus challenge (Figure 6A), intranasal administration was able to prolong the survival of mice (Figures 6B and 6D) and to decrease the viral titer (Figure 6C) while inducing only minor amounts of inflammation, as assessed by changes in the bronchoalveolar lavage fluid.

These activities of DMXAA are potentially important for a number of reasons. First, the drug appears to be highly permeable, unlike agents such as poly I:C, and thus does not require liposomal encapsulation for administration. Second, DMXAA has been shown to simultaneously activate multiple antiviral pathways in macrophages. These pathways include (*1*) NFκB activation (12), (*2*) IFN-β through TBK1/IRF3 activation (11), (*3*) the NOD pathway (12), and (*4*) the MAP kinase pathways (p38, ERK, and Jun) (13). A recent study by Sabbah and colleagues (46) showed that NOD2 can also function as a cytoplasmic viral PRR by triggering activation of IRF3 and production of IFN-β. Our data indicate that the same pathways seen in macrophages are activated in epithelium. Third, DMXAA does not appear to induce strong inflammatory responses in the lungs (unlike bacterial lysates) and was well tolerated by the mice. In fact, DMXAA is in Phase III clinical trials for use in lung cancer and has not induced serious toxicities. One major unanswered question is how a small organic compound, like DMXAA, is able to trigger such a broad response. Despite many years of work in multiple labs, the "DMXAA receptor" has yet to be discovered. However, it is known that DMXAA does not require MyD88 or the RIG-I–like receptors (9, 11). Further research into this question is ongoing.

There are a number of caveats to this study. DMXAA had a relatively narrow window of therapeutic efficacy and worked best when administered close to the time of viral infection; treating mice with DMXAA 24 hours after infection was not effective. This may not be surprising considering the range of virally produced proteins that inactivate IFN responses. The value of agents like DMXAA would thus appear to be primarily in prophylaxis rather than therapy (i.e., to be used when a known exposure would be likely to occur). Another limitation of DMXAA is that there appears to be a refractory period that lasts 3 to 5 days (data not shown). This suggests that daily administration may not be effective. Finally, the effects of DMXAA in human (versus murine) bronchial epithelial cells are attenuated. We studied human immortalized (but not transformed) bronchial epithelial cells and primary cultures of human tracheal epithelial cells and found lower IFN responses after DMXAA administration (Figure E3) accompanied by minimal anti-VSV protection (data not shown). This species difference has been previously observed in human leukocytes (47, 48). For these reasons, it is unlikely that DMXAA itself will be useful clinically in patients; however, related compounds with higher activity in human cells may be valuable.

In summary, this study provides additional "proof of principal" data that pharmacologic activation of the IRF3/type I IFN pathway is an attractive strategy in the development of prophylactic antiviral therapies. This can be achieved in mice in a nontoxic and efficient manner by a small, cell-permeable, xanthone-like molecule (DMXAA) that activates multiple antiviral pathways by an undiscovered mechanism. Understanding the molecular targets of DMXAA could be important because this would likely aid development of related compounds with high activity in humans. Even if the effects are relatively short lived, the prophylactic use of a DMXAA-like drug or other types of IRF-3 activators (such as TLR agonists) could be highly useful for the protection of "front-line" health professionals or military professionals who might need to enter environments where exposure to pandemic viruses or viral bioterror agents could be expected.

Author Disclosure: J.K. has received an industry-sponsored grant from Bionorica. N.C. has served as a served as a consultant for Medtronic ENT, has served as an expert witness for World Trade Center Captive Insurance Company, Inc., has received industry-sponsored grants from Entent Care Inc. and Medtronic ENT, and has received royalities from NeilMed Pharmaceuticals. SA has received industry-sponsored grants from Biogen Idec and Centocor. Z.F. has received lecture fees from Astra-Zeneca and GlaxoSmithKline. None of the other authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Note added in proof: After acceptance of this manuscript, Shirey *et al.* (Shirey KA, Nhu QM, Yim KC, Roberts ZJ, Teijaro JR, Farber DL, Blanco JC, Vogel SN. The anti-tumor agent, 5,6-dimethylxanthenone-4-acetic acid [DMXAA], induces IFN-beta-mediated antiviral activity in vitro and in vivo. *J Leukoc Biol* 2011;89: 351–357) showed very similar findings illustrating that DMXXA could protect mice from H1N1 PR8 influenza infection through induction of interferon-beta.

References

1. Ahmed R, Oldstone MB, Palese P. Protective immunity and susceptibility to infectious diseases: lessons from the 1918 influenza pandemic. *Nat Immunol* 2007;8:1188–1193.
2. Sugrue RJ, Tan BH, Yeo DS, Sutejo R. Antiviral drugs for the control of pandemic influenza virus. *Ann Acad Med Singapore* 2008;37:518–524.
3. Baz M, Abed Y, Papenburg J, Bouhy X, Hamelin ME, Boivin G. Emergence of oseltamivir-resistant pandemic H1N1 virus during prophylaxis. *N Engl J Med* 2009;361:2296–2297.
4. Shahangian A, Chow EK, Tian X, Kang JR, Ghaffari A, Liu SY, Belperio JA, Cheng G, Deng JC, Type I. IFNs mediate development of post-influenza bacterial pneumonia in mice. *J Clin Invest* 2009;119:1910–1920.
5. Garofalo R, Mei F, Espejo R, Ye G, Haeberle H, Baron S, Ogra PL, Reyes VE. Respiratory syncytial virus infection of human respiratory epithelial cells up-regulates class I MHC expression through the induction of IFN-beta and IL-1 alpha. *J Immunol* 1996;157:2506–2513.
6. Kuenzel S, Till A, Winkler M, Hasler R, Lipinski S, Jung S, Grotzinger J, Fickenscher H, Schreiber S, Rosenstiel P. The nucleotide-binding oligomerization domain-like receptor NLRC5 is involved in IFN-dependent antiviral immune responses. *J Immunol* 2010;184:1990–2000.
7. Wang J, Oberley-Deegan R, Wang S, Nikrad M, Funk CJ, Hartshorn KL, Mason RJ. Differentiated human alveolar type II cells secrete antiviral IL-29 (IFN-lambda 1) in response to influenza A infection. *J Immunol* 2009;182:1296–1304.
8. Jassar AS, Suzuki E, Kapoor V, Sun J, Silverberg MB, Cheung L, Burdick MD, Strieter RM, Ching LM, Kaiser LR, *et al.* Activation of tumor-associated macrophages by the vascular disrupting agent 5,6-dimethylxanthenone-4-acetic acid induces an effective CD8+ T-cell-mediated antitumor immune response in murine models of lung cancer and mesothelioma. *Cancer Res* 2005;65:11752–11761.
9. Wallace A, LaRosa DF, Kapoor V, Sun J, Cheng G, Jassar A, Blouin A, Ching LM, Albelda SM. The vascular disrupting agent, DMXAA,

directly activates dendritic cells through a MyD88-independent mechanism and generates antitumor cytotoxic T lymphocytes. *Cancer Res* 2007;67:7011–7019.
10. Wang LC, Woon ST, Baguley BC, Ching LM. Inhibition of DMXAA-induced tumor necrosis factor production in murine splenocyte cultures by NF-kappaB inhibitors. *Oncol Res* 2006;16:1–14.
11. Roberts ZJ, Goutagny N, Perera PY, Kato H, Kumar H, Kawai T, Akira S, Savan R, van Echo D, Fitzgerald KA, *et al.* The chemotherapeutic agent DMXAA potently and specifically activates the TBK1-IRF-3 signaling axis. *J Exp Med* 2007;204:1559–1569.
12. Cheng G, Sun J, Fridlender ZG, Wang LC, Ching LM, Albelda SM. Activation of the nucleotide oligomerization domain signaling pathway by the non-bacterially derived xanthone drug 5′6-dimethylxanthenone-4-acetic acid (vadimezan). *J Biol Chem* 2010;285:10553–10562.
13. Sun S, Wang LC, Fridlender ZG, Kapoor V, Cheng G, Ching LM, Albelda SM. Activated protein kinases by 5,6-dimethylxanthenone-4-acetic acid (DMXAA) plays an important role in macrophage stimulation. *Biochem Pharm* (In press)
14. Smith GJ, Le Mesurier SM, de Montfort ML, Lykke AW. Development and characterization of type 2 pneumocyte-related cell lines from normal adult mouse lung. *Pathology* 1984;16:401–405.
15. Woodworth BA, Antunes MB, Bhargave G, Palmer JN, Cohen NA. Murine tracheal and nasal septal epithelium for air-liquid interface cultures: a comparative study. *Am J Rhinol* 2007;21:533–537.
16. Antunes MB, Woodworth BA, Bhargave G, Xiong G, Aguilar JL, Ratner AJ, Kreindler JL, Rubenstein RC, Cohen NA. Murine nasal septa for respiratory epithelial air-liquid interface cultures. *Biotechniques* 2007;43:195–196, 198, 200.
17. Saloura V, Wang LC, Fridlender ZG, Sun J, Cheng G, Kapoor V, Sterman DH, Harty RN, Okumura A, Barber GN, *et al.* Evaluation of an attenuated vesicular stomatitis virus vector expressing interferon-beta for use in malignant pleural mesothelioma: heterogeneity in interferon responsiveness defines potential efficacy. *Hum Gene Ther* 2010;21:51–64.
18. Larkin J III, Rankin AL, Picca CC, Riley MP, Jenks SA, Sant AJ, Caton AJ. CD4+CD25+ regulatory T cell repertoire formation shaped by differential presentation of peptides from a self-antigen. *J Immunol* 2008;180:2149–2157.
19. Sun K, Metzger DW. Inhibition of pulmonary antibacterial defense by interferon-gamma during recovery from influenza infection. *Nat Med* 2008;14:558–564.
20. Sun J, Madan R, Karp CL, Braciale TJ. Effector T cells control lung inflammation during acute influenza virus infection by producing IL-10. *Nat Med* 2009;15:277–284.
21. Tuvim MJ, Evans SE, Clement CG, Dickey BF, Gilbert BE. Augmented lung inflammation protects against influenza A pneumonia. *PLoS ONE* 2009;4:e4176.
22. Kimoto M, Iwai S, Maeda T, Yura Y, Fernley RT, Ogawa Y. Carbonic anhydrase VI in the mouse nasal gland. *J Histochem Cytochem* 2004;52:1057–1062.
23. Perry AK, Chen G, Zheng D, Tang H, Cheng G. The host type I interferon response to viral and bacterial infections. *Cell Res* 2005;15:407–422.
24. Basu M, Maitra RK, Xiang Y, Meng X, Banerjee AK, Bose S. Inhibition of vesicular stomatitis virus infection in epithelial cells by alpha interferon-induced soluble secreted proteins. *J Gen Virol* 2006;87:2653–2662.
25. Nhu QM, Shirey K, Teijaro JR, Farber DL, Netzel-Arnett S, Antalis TM, Fasano A, Vogel SN. Novel signaling interactions between proteinase-activated receptor 2 and toll-like receptors in vitro and in vivo. *Mucosal Immunol* 2010;3:29–39.
26. Evans SE, Xu Y, Tuvim MJ, Dickey BF. Inducible innate resistance of lung epithelium to infection. *Annu Rev Physiol* 2010;72:413–435.
27. Sherry B. Rotavirus and reovirus modulation of the interferon response. *J Interferon Cytokine Res* 2009;29:559–567.
28. von Kobbe C, van Deursen JM, Rodrigues JP, Sitterlin D, Bachi A, Wu X, Wilm M, Carmo-Fonseca M, Izaurralde E. Vesicular stomatitis virus matrix protein inhibits host cell gene expression by targeting the nucleoporin Nup98. *Mol Cell* 2000;6:1243–1252.
29. Fortes P, Beloso A, Ortin J. Influenza virus NS1 protein inhibits pre-mRNA splicing and blocks mRNA nucleocytoplasmic transport. *EMBO J* 1994;13:704–712.
30. Qiu Y, Krug RM. The influenza virus NS1 protein is a poly(A)-binding protein that inhibits nuclear export of mRNAs containing poly(A). *J Virol* 1994;68:2425–2432.
31. Nemeroff ME, Barabino SM, Li Y, Keller W, Krug RM. Influenza virus NS1 protein interacts with the cellular 30 kDa subunit of CPSF and inhibits 3′end formation of cellular pre-mRNAs. *Mol Cell* 1998;1:991–1000.
32. Lu Y, Qian XY, Krug RM. The influenza virus NS1 protein: a novel inhibitor of pre-mRNA splicing. *Genes Dev* 1994;8:1817–1828.
33. Qiu Y, Nemeroff M, Krug RM. The influenza virus NS1 protein binds to a specific region in human U6 snRNA and inhibits U6–U2 and U6–U4 snRNA interactions during splicing. *RNA* 1995;1:304–316.
34. Wang W, Krug RM. U6atac snRNA, the highly divergent counterpart of U6 snRNA, is the specific target that mediates inhibition of AT-AC splicing by the influenza virus NS1 protein. *RNA* 1998;4:55–64.
35. Lu Y, Wambach M, Katze MG, Krug RM. Binding of the influenza virus NS1 protein to double-stranded RNA inhibits the activation of the protein kinase that phosphorylates the eIF-2 translation initiation factor. *Virology* 1995;214:222–228.
36. Friedman RM. Role of interferon in viral interference. *Nature* 1964;201:848–849.
37. Beilharz MW, Cummins JM, Bennett AL. Protection from lethal influenza virus challenge by oral type 1 interferon. *Biochem Biophys Res Commun* 2007;355:740–744.
38. Clarke CJ, Trapani JA, Johnstone RW. Mechanisms of interferon mediated anti-viral resistance. *Curr Drug Targets Immune Endocr Metabol Disord* 2001;1:117–130.
39. Homewood J, Watson M, Richards SM, Halsey J, Shepherd PC, Adult Leukaemia Working Party. Treatment of CML using IFN-alpha: impact on quality of life. *Hematol J* 2003;4:253–262.
40. Boxall CB, Dowall S, Plank J, Graham V, Bruce C, Monk PD. Interferon-beta inhibits infection of a lung epithelial cell line with novel variant H1N1 (swine flu) [abstract]. *Am J Respir Crit Care Med* 2010;181:A2634.
41. Urosevic M, Fujii K, Calmels B, Laine E, Kobert N, Acres B, Dummer R. Type I IFN innate immune response to adenovirus-mediated IFN-gamma gene transfer contributes to the regression of cutaneous lymphomas. *J Clin Invest* 2007;117:2834–2846.
42. Fahey LM, Raff AB, Da Silva DM, Kast WM. Reversal of human papillomavirus-specific T cell immune suppression through TLR agonist reatment of Langerhans cells exposed to human papillomavirus type 16. *J Immunol* 2009;182:2919–2928.
43. Wong JP, Christopher ME, Viswanathan S, Karpoff N, Dai X, Das D, Sun LQ, Wang M, Salazar AM. Activation of toll-like receptor signaling pathway for protection against influenza virus infection. *Vaccine* 2009;27:3481–3483.
44. Wong JP, Nagata LP, Christopher ME, Salazar AM, Dale RM. Prophylaxis of acute respiratory virus infections using nucleic acid-based drugs. *Vaccine* 2005;23:2266–2268.
45. You D, Gilbert BE, Duggan JM, Tuvim MJ, Dickey BF, Evans SE. Induced resistance to influenza A infection by cooperative TLR2/6 and TLR9 activation [abstract]. *Am J Respir Crit Care Med* 2010;181:A2629.
46. Sabbah A, Chang TH, Harnack R, Frohlich V, Tominaga K, Dube PH, Xiang Y, Bose S. Activation of innate immune antiviral responses by Nod2. *Nat Immunol* 2009;10:1073–1080.
47. Philpott M, Joseph WR, Crosier KE, Baguley BC, Ching LM. Production of tumour necrosis factor- alpha by cultured human peripheral blood leucocytes in response to the anti-tumour agent 5,6-dimethylxanthenone-4-acetic acid (NSC 640488). *Br J Cancer* 1997;76:1586–1591.
48. Wang LC, Thomsen L, Sutherland R, Reddy C, Tijono S, Chen CJ, Dunbar PR, Ching LM. Neutrophil influx and chemokine production during the early phases of the anti-tumor response to the vascular disrupting agent DMXAA (ASA404). *Neoplasia* 2009;11:793–803.