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Administration of a dual toll-like receptor 7 and toll-like receptor 8 agonist protects against influenza in rats

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Abstract

Toll-like receptors (TLR) detect conserved molecular patterns expressed by pathogens. Detection of the "molecular signature" for RNA viruses including influenza has been attributed to TLR3, TLR7, and TLR8. In the present study, compound 3M-011 was shown to be a synthetic human TLR7/8 agonist and cytokine inducer. 3M-011 was investigated as a stand-alone immune response modifier in a rat model of human influenza. Intranasal (IN) administration of 3M-011 significantly inhibited H3N2 influenza viral replication in the nasal cavity when administered from 72 h before IN viral inoculation to 6 h after inoculation. Viral inhibition correlated with the ability of the TLR7/8 agonist to stimulate type I interferon (IFN) and other cytokines such as tumor necrosis factor-α, interleukin-12, and IFN-γ from rat peripheral blood mononuclear cells. Prophylactic administration of TLR7/8 agonist also suppressed influenza viral titers in the lung, which corresponded with local IFN production. The activity of the TLR7/8 agonist resulted in greater inhibition of viral titers compared to rat recombinant IFN-α administered in a comparable dosing regimen. These studies indicate that TLR7/8 agonists may have prophylactic and therapeutic benefits in the treatment of respiratory viral infections, such as influenza, when administered prior to or shortly after viral inoculation.

Keywords: Toll-like receptor; Influenza; Innate immunity; Interferon; Cytokine

1. Introduction

Influenza A viruses cause annual epidemics and occasional pandemics of acute respiratory disease. Although usually self-limiting, influenza infection can pose a serious risk to infants, the elderly, and individuals with underlying chronic diseases (Ferson et al., 1991; Nicholson et al., 1993; Izurieta et al., 2000; Tan et al., 2003; Ellis et al., 2003). In the United States alone, influenza is responsible for over 100,000 hospitalizations and 36,000 excess deaths per year (Brammer et al., 2002; Thompson et al., 2003). Vaccination can prevent up to 70% of laboratory-confirmed influenza in healthy adults, assuming that the viral strains used to make the vaccine actually resemble those that are currently circulating in the community (Edwards et al., 1994; Wilde et al., 1999; Harper et al., 2005). Since new antigenic strains of influenza continue to emerge such as H5N1 and rapid generation of effective vaccines is difficult, antigen-independent prophylaxis may provide an alternative approach to enhance host resistance against infection.

One of the earliest responses to influenza and other viruses is the production of type 1 interferons (IFN), such as IFN-α, by the innate immune system (Choudhary et al., 2001). Interactions of type 1 interferons with their receptor induce a non-specific antiviral state characterized by the activation of IFN-stimulated genes including Mx proteins, protein kinase R, and 2′5′ oligoadenylate synthetase (reviewed by Pestka et al., 2004). Although the use of recombinant IFN-α as a prophylactic measure against certain respiratory viral infections has been evaluated, the practical utility of this approach has yet to be demonstrated (Hayden and Gwaltney, 1984; Gwaltney, 1992; Loutfy et al., 2003).

An alternative strategy may be to stimulate the innate immune response through the activation of toll-like receptor (TLR) signaling pathways. Mammalian TLRs constitute a family of pattern recognition receptors which detect conserved molecular components encoded by microorganisms (reviewed by Medzhitov and Janeway, 2000; Akira et al., 2006). Adminis-

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tration of TLR4 agonists composed of synthetic lipid A analogs to mice by an intranasal (IN)/intrapulmonary route 48 h prior to challenge with influenza provided strong protection against the virus (Cluff et al., 2005). Immune response modifiers (IRM) including resiquimod (TLR7/8 agonist) and imiquimod (TLR7 agonist) have also exhibited potent antiviral activities in a number of animal models such as Herpes simplex virus-2 in guinea pigs, Banzi virus in mice; Rift Valley Fever in mice, cytomegalovirus in mice and guinea pigs, parainfluenza virus in Brown-Norway rats, and West Nile virus in mice (Miller et al., 1985; Harrison et al., 1988; Bernstein et al., 2001; Kende et al., 1988; Johnson et al., 1998; Chen et al., 1988; Stokes et al., 1998; reviewed by Miller et al., 1999; Morrey et al., 2004).

In contrast to most antiviral drugs, IRMs do not directly target viral replication in infected cells (Miller et al., 1999). Rather, antiviral effects have been attributed to enhanced production of mediators such as IFN-α through stimulation of TLR-dependent signaling pathways (Hemmi et al., 2002). Consistent with this hypothesis, the protective effect of the TLR7 agonist, imiquimod, on mouse models of Rift Valley Fever virus and Banzi virus was reversed upon administration of neutralizing murine anti-type I IFN antibody (Kende et al., 1988). However, the benefits afforded by IRM treatment may not be fully explained by IFN-α induction alone. For example, administration of antibody against tumor necrosis factor (TNF)-α blocked the ability of the TLR7/8 agonist, resiquimod, to improve the survival of mice infected with cytomegalovirus (Johnson et al., 1998). Cell-mediated immunity may likewise contribute to the observed antiviral activity of IRMs. In particular, the sustained therapeutic effect of imiquimod or resiquimod against recurrent HSV-2 infection in the guinea pig was linked to the generation of antigen-specific memory T-cells (Harrison et al., 1994).

The present study was undertaken to investigate the synthetic TLR7/8 agonist, 3M-011, as a stand-alone agent in a rat model of influenza infection. The rat influenza host resistance model was chosen because virus shedding and immunological recovery in rats is similar to humans and the model has been well established (Burleson, 1995). In addition, rats were used to assess safety of 3M-011, thus providing a measure of the therapeutic window in the same species. We report that a single IN dose of 3M-011 significantly increased viral clearance in the upper airways and lungs of rats when administered from 72 h before to 6 h after challenge with H3N2 influenza virus. The observed decrease in influenza virus replication correlated with the ability of the TLR7/8 agonist to enhance production of type I IFN as well as tumor necrosis factor-α, and interleukin-12 from rat peripheral blood mononuclear cells. Thus, TLR7/8-mediated activation of the innate immune response may facilitate the elimination of inhaled pathogens, such as influenza, when administered prior to or shortly after viral challenge.

2. Materials and methods

2.1. Compounds

3M-011 is a member of the imidazoquinoline family of IRMs of which imiquimod and resiquimod are most familiar (Stanley, 2002; Gibson et al., 2002). The chemical name of 3M-011 is N-{2-[4-amino-2-(ethoxymethyl)-1H-imidazo[4,5-*c*]quinolin-1-yl]-1,1-dimethylethyl}methanesulfonamide. The compound was synthesized by 3M Pharmaceuticals and formulated either in a polyethyleneglycol (PEG)-400/lactate, buffered aqueous solution adjusted to pH 5 or a caryboxymethyl cellulose (CMC)/tartrate buffered aqueous solution adjusted to pH 5. All concentrations and doses were based on the molecular weight of the freebase form of the compound (MW = 391.5). Dosing solutions of 3M-011 were prepared as aqueous solutions at pH 4.5 and administered IN to rats. Instillation of 50 μl of 0.00375, 0.0375, and 0.375% 3M-011 solutions were calculated to deliver 0.01, 0.1, and 1.0 mg/kg, respectively.

Rat recombinant interferon-alpha (rIFN-α) was purchased from Serotec (Cat. #PRP13, Raleigh, NC) and Endogen (Cat. #RR2030U, Pierce Biotechnology, Rockford, IL) for data reported in Fig. 3 and from PBL Biomedical Laboratories (Cat. #13100-1, Piscataway, NJ) for data reported in Fig. 4. CpG 2059 was purchased from MWG Biotech (Highpoint, NC). Imiquimod was prepared as previously described (Gerster et al., 2005).

2.2. In vitro human TLR7, TLR8, and TLR9 activation

The 293 human embryonic kidney cell line (HEK293, Cat. #CRL-1573) was obtained from American Type Culture Collection (ATCC) (Manassas, VA) and was transfected with mammalian expression vectors encoding either human TLR7, TLR8, or TLR9, and a nuclear transcription factor kappa B (NF-κB)-luciferase reporter construct, as previously described (Gorden et al., 2005). Twenty-four hours post-transfection, 4×10^4 HEK293 cells were cultured in 96-well flat-bottom plates or 7.5×10^4 cells in 24-well flat-bottom plates. After 2 h, the cells were stimulated for 6 h (TLR7 assay) or 24 h (TLR8 and TLR9 assays) with varying concentrations of CpG (TLR9 agonist), imiquimod (TLR7 agonist), 3M-011, or with medium containing vehicle (0.5% DMSO). The cells were lysed and analyzed for luciferase production by determining the relative luciferase units (RLU). Results were expressed as the fold induction calculated from the compound mean value divided by the vehicle mean value from the same experiment. A two-fold or greater increase in luciferase production relative to the vehicle control was considered biologically significant.

2.3. Rat-adapted influenza virus

Human influenza virus, influenza A/Port Chalmers/1/73 (H3N2) from ATCC (VR-810), was adapted to rats through a series of 10 successive passages through infected lungs of F344 rats, following the method previously outlined (Burleson, 1995). Virus propagated in the allantoic fluid of 10-day-old embryonated chicken eggs was used as a stock virus. For the first passage, a 0.2 ml suspension of the stock virus solution was administered by IN instillation to rats. Lungs were harvested 24 h post-inoculation. The lungs were homogenized, centrifuged, and the supernatant analyzed for virus by using the Madin-Darby canine kidney (MDCK, ATCC) plaque assay (Burleson, 1995).

The homogenate showing the highest viral titer and causing the most advanced lung consolidation was chosen for the next passage. This process was continued until a plateau in viral titer was observed. MDCK cells were used to measure infectious virus to determine viral clearance. Dilutions of lung homogenates or nasal lavage fluids were added to monolayers of MDCK cells and covered with an agar overlay. Following incubation for 36–48 h to allow plaque development, the cell monolayers were fixed with buffered formalin and stained with crystal violet. Viral plaques were counted visually to determine infectious virus titer.

2.4. In vivo studies

Female Fischer rats (CDF) (F344/DuCrl) rats (approximately 6–8 weeks old, 100–125 g) were used to monitor the protective response against instilled influenza virus. The rats were purchased from Charles River (Raleigh, NC) and acclimated until the week prior to study. Animals were maintained on approximately 12-h day and 12-h night cycles. Animal room temperature and relative humidity were monitored and documented in study records. The rats were provided with Purina 5001 Rodent Chow and had access to tap water *ad libitum*. Animals were housed at the North Carolina State University (NCSU) College of Veterinary Medicine, an AALAC-accredited institution. Animal studies were conducted by Burleson Research Technologies, Inc. (BRT), Morrisville, NC. All protocols were reviewed and approved by the Institutional Animal Care and Use Committee at NCSU.

IN instillation was used to deliver aqueous drug formulations. Animals were lightly anesthetized with isoflurane and subsequently dosed with either drug or vehicle. A solution of vehicle or drug was instilled in a drop by drop manner (each drop was cleared from the nasal passage before the next drop was administered) to each nare (25 μl/nare) for a total of 50 μl/animal. Animals were then placed back into the cage and became fully alert within 5 min.

The pretreatment duration varied with the particular experimental protocol used, i.e., 0, 4, 24, or 48 h before viral challenge, or 6 h after viral challenge. Rats were infected IN with a non-lethal, rat-adapted influenza virus (RAIV) with 2×10^5 plaque forming units. The animals of the appropriate groups (6–10 animals/time point-there were some exceptions as pointed out in the figures) were necropsied after asphyxiation with CO_2. One group of naïve (unrelated, uninfected) rats was necropsied for baseline values.

Body weight and lung weights were obtained. Lungs were either homogenized immediately or frozen (−70 °C). Lung homogenates were prepared at 10% (w/v) to measure IFN-α and infectious virus to determine viral clearance. Serum IFN-α levels were also determined. To obtain nasal lavage samples, animals were euthanized, placed on their back, and a specially designed nasal lavage tool was placed in the posterior pharynx. A syringe was filled with the appropriate volume of Hanks' Balanced Salt Solution and connected to the nasal lavage tool via polyethylene tubing prior to nasal lavage. The nasal cavity was then flushed with 2 ml of solution. Samples were collected and stored at <−70 °C for viral quantification or at <−20 °C for antibody or cytokine analysis.

2.5. Type I IFN titer assay

A cytopathic effect-reduction IFN bioassay system was used to determine rat type I IFN titer levels in nasal lavage samples using the LMS-C2 cell line according to methods previously described (Burleson and Burleson, 1995). Assays were performed at BRT. Lung homogenates and serum samples were also analyzed in some cases. Type I IFN levels were expressed as U/ml. Type I IFN levels that were three times the lower limit of sensitivity (1 U/ml) were considered to be biologically relevant.

2.6. Cytokine production by immune cells

Human mononuclear cells that were subjected to apheresis were obtained from healthy volunteers at 3M Pharmaceuticals (St. Paul, MN) or Memorial Blood Centers (Minneapolis, MN). Human peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Ficoll-Paque PLUS® (Amersham Biosciences/GE Healthcare, Piscataway, NJ) as recommended by the manufacturer. The isolated PBMC were resuspended in RPMI 1640 (Protide Pharmaceuticals, Inc., St. Paul, MN) with 10% heat-inactivated fetal bovine serum, FBS (Biosource, Int., Camarillo, CA) and 1% penicillin/streptomycin, 100× (Biosource) and cultured in 96-well BD Falcon™ cell culture plates (BD Biosciences, Bedford, MA) at 2×10^6 cells/ml in 0.25 ml and treated with various concentrations of 3M-011. After 18–24 h of incubation at 37 °C with 5% CO_2, the cell-free supernatants were collected and stored at −20 °C until cytokine levels were determined by sandwich immunoassays. Human IFN-α was measured by ELISA (Cat. #41105 PBL). Human TNF-α and IL-12p40/70 were measured by ORIGEN (IGEN) assay (BioVeris Corp., Gaithersburg, MD). The lower limits of sensitivity of the human IFN-α, TNF-α, and IL-12p40/70 assays were 20, 24, and 48 pg/ml, respectively. Values that were at least three-fold over the lower limit of sensitivity for each assay were considered biologically relevant and were defined as the minimal effective dose.

Rat blood was obtained by cardiac puncture and collected in EDTA-treated vacutainer tubes (Becton Dickenson, Franklin Lakes, NJ). Blood was then diluted 1:1 with PBS and layered on a density gradient (Lympholyte R) specific for rat blood (Accurate Chemical Company, Westbury, NY). After centrifugation at $600 \times g$ for 20 min at room temperature, the interface containing mononuclear cells was carefully removed and washed twice with PBS. Rat PBMC were resuspended to a final concentration of 2×10^6 cells/ml in RPMI complete containing 5×10^{-5} M 2-mercaptoethanol (Life Technologies, Rockville, MD) in 48-well plates (Costar) in 500 μl. The cells were incubated for 18–24 h at 37 °C with 5% CO_2 in the presence of various concentrations of TLR agonist. Culture supernatants were collected and stored at −20 °C until they were assayed for cytokine concentrations. Rat IFN was assayed at BRT by bioassay as described above. ELISAs were used to measure rat TNF-α (Cat. #550734, BD Biosciences Pharmingen, San Jose, CA) and IL-12p40/70

(Cat. #KRC0121, Biosource). Values that were at least three-fold over mean background (vehicle control) levels were considered biologically relevant and were defined as the minimal effective dose.

2.7. Statistical analysis

For data analysis, treatment groups were compared by using a one-way ANOVA followed by individual *t*-test comparisons between vehicle treated-virally infected controls and drug treated-virally infected groups. The Dunnett multiple comparison method was used to compare treatment groups with the appropriate vehicle control group following significant results from the one-way ANOVA. This adjustment preserved the overall type I error at 0.05.

3. Results

3.1. 3M-011 induces NF-κB activation through TLR7 and TLR8

3M-011 was tested for TLR agonistic activity in genetic reconstitution experiments using HEK293 cells transfected with human TLR7, TLR8, or human TLR9 genes (Fig. 1). Transfected HEK293 cells were stimulated with 3M-011 (0.03–100 μM), imiquimod (0.1–100 μM), CpG (0.03–100 μM), or vehicle. 3M-011 activated NF-κB in cells expressing TLR7 at concentrations equal to or greater than 0.1 μM with concentrations of 0.3 μM and above inducing the highest level of activation (Fig. 1, Panel A). As expected, imiquimod at 10–100 μM activated NF-κB in cells expressing TLR7. 3M-011 at 1–100 μM also activated cells expressing TLR8 with concentration of 10–100 μM being most effective, whereas imiquimod failed to activate TLR8 at concentrations up to 100 μM (Fig. 1, Panel B). 3M-011 did not activate TLR9 transfected cells at concentrations up to 100 μM, whereas concentrations of CpG greater then 1 μM resulted in TLR9 activation (Fig. 1, Panel C). The results demonstrate that 3M-011 is a selective dual human TLR7/8 agonist.

3.2. Cytokine production by human and rat blood cells stimulated with 3M-011

The ability of 3M-011 to stimulate cytokine production was investigated using PBMCs isolated from humans or rats. As shown in Fig. 2, human and rat PBMC produced IFN, TNF-α, and IL-12p40/70 following stimulation with 3M-011. The lowest median concentrations of 3M-011 needed to induce at least a three-fold increase in cytokine levels from human PBMC were 0.04, 0.37, and 0.37 μM for IFN-α, TNF-α, and IL-12, respectively. The lowest median concentrations of 3M-011 needed to elicit at least a three-fold increase in cytokine levels from rat PBMC were 0.04, 0.12, and 0.12 μM for type I IFN, TNF-α, and IL-12, respectively. These data indicate that human and rat PBMC are quantitatively similar in their response to 3M-011 and that the rat is a sensitive species for the evaluation of the effects of this TLR7/8 agonist.

3.3. Cytokine production in rats following intranasal instillation of 3M-011

Serum type I IFN levels were measured in rats 2, 4, 6, and 24 h after a single intranasal (IN) dose of 1.0 mg/kg 3M-011 (Table 1). In naïve rats, type I IFN levels in the serum, nasal lavage fluid, and in the lung homogenate were all less than or equal to 1.0 (U/ml). Animals receiving 3M-011 had significantly elevated levels of type I IFN in the serum, nasal lavage fluid, and lung homogenate with peak titers occurring at 2 h

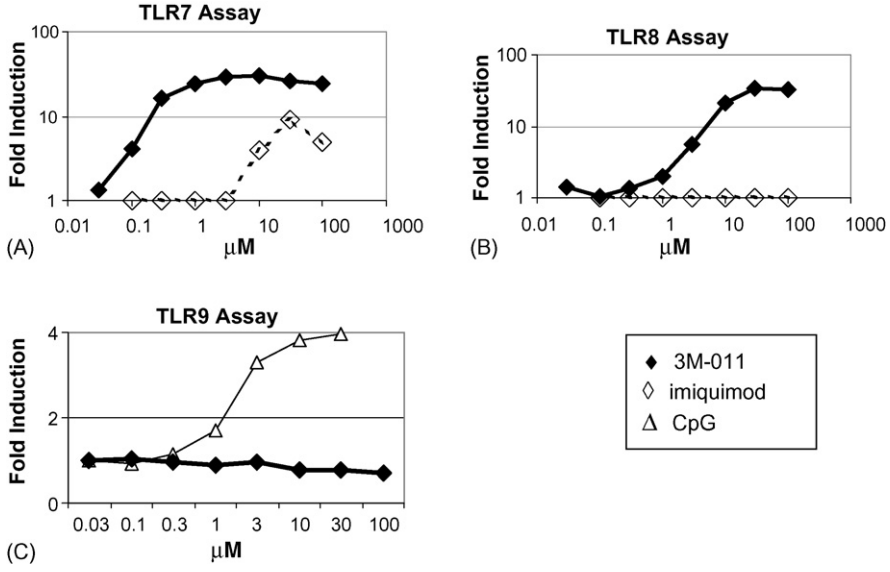

Fig. 1. 3M-011 is a dual TLR7 and TLR8 agonist. HEK293 cells transfected with a luciferase reporter construct and either human TLR7 (Panel A), TLR8 (Panel B), or TLR9 (Panel C) were stimulated with various concentrations of 3M-011, imiquimod, CpG, or vehicle (5% DMSO). Relative luciferase units (RLU) were measured and results are expressed as the fold induction of 3M-011 (closed diamonds), imiquimod (open diamonds), or CpG (open triangles) relative to the vehicle control calculated from the compound mean value ($n = 3$) divided by the vehicle mean value ($n = 3$) from the same experiment.

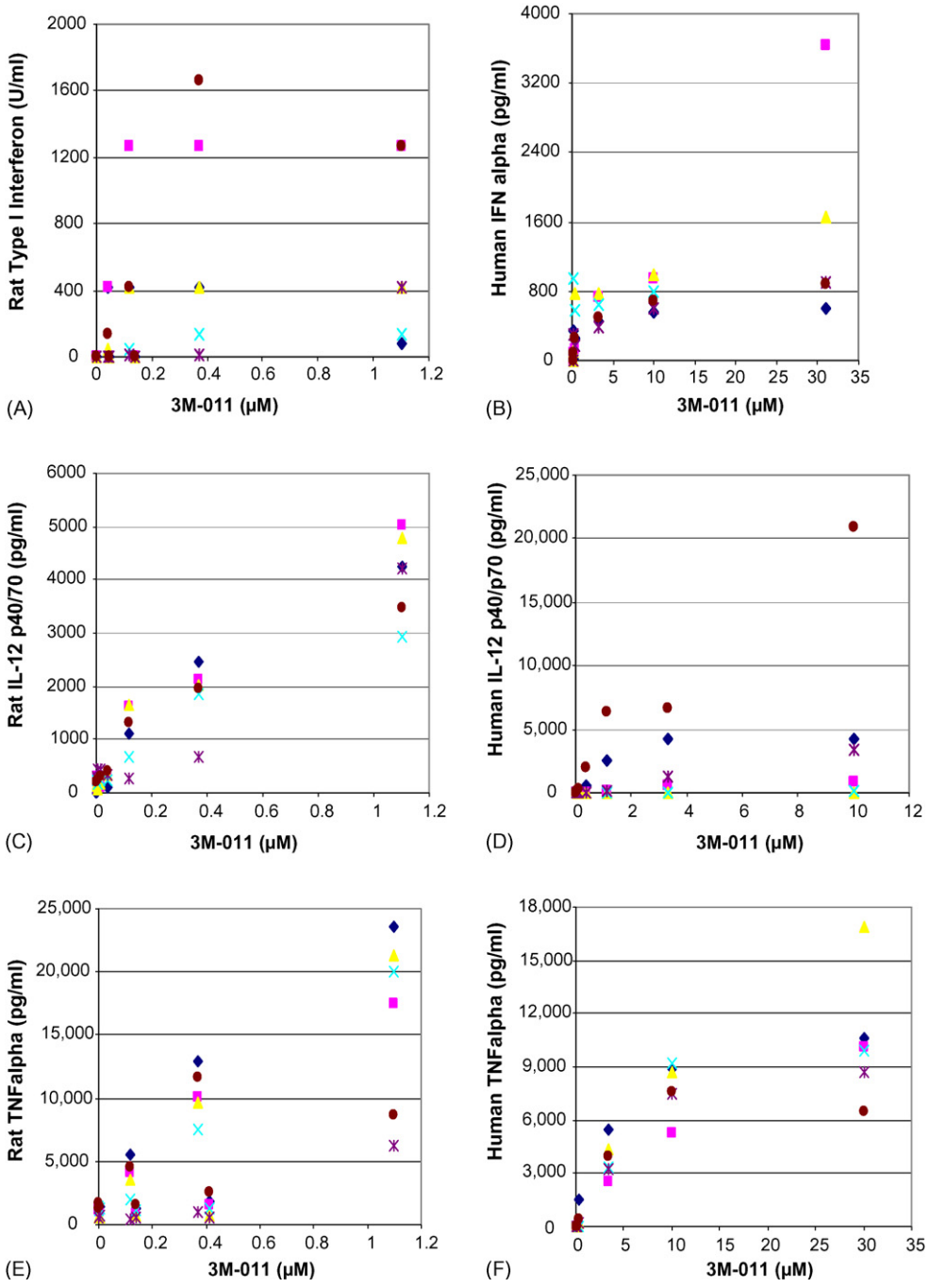

Fig. 2. The TLR7/8 agonist stimulates human and rat peripheral blood mononuclear cells to produce IFN, TNF-α, and IL-12 p40/70. Rat type I IFN (Panel A) was measured by bioassay (U/ml). Individual animal responses are shown. Human IFN-α (Panel B) was measured by ELISA (pg/ml). Individual subject responses are shown. Rat TNF-α (Panel D) and rat IL-12 p40/70 (Panel F) were measured by ELISA and were expressed in pg/ml. Human TNF-α (Panel C) and human IL-12 p40/70 (Panel E) were measured by IGEN and were expressed in pg/ml.

post dose. Significant increases in IFN levels were also seen in serum at 4 and 6 h and in lung at 4 h after 3M-011 instillation (Table 1). Serum levels of TNF-α and IL-12 also significantly increased during the first 6 h following intranasal delivery of 1 mg/kg 3M-011 (Table 2). An IN dose of 0.01 or 0.1 mg/kg 3M-011 significantly elevated serum levels of type I IFN but not TNF-α or IL-12 (Table 2). Thus, intranasal administration of 3M-011 elevated serum cytokine levels in a dose-dependent and cytokine-dependent manner.

3.4. Characterization of viral titers and type I IFN response in rats after influenza challenge

The initial rat experiment examined the magnitude and duration of viral replication as well as type I IFN production at 24, 48, and 72 h following influenza challenge. This served to determine infection parameter time points for the following antiviral experiments. Results are shown in Table 3. As expected, IN instillation of rat-adapted H3N2 influenza virus

Table 1
Effects of a single intranasal instillation of a TLR7/8 agonist on serum, nasal cavity, and lung type I IFN levels at 2, 4, 6, and 24 h after administration in rats[a]

Treatment group ($n = 10$)	Time (h)	Mean ± S.E.		
		Serum IFN (U/ml)	Nasal IFN (U/ml)	Lung IFN (U/ml)
None (naïve)	0	<1	<1	<1
Vehicle	2	<1	<1	<1
3M-011	2	3536 ± 253*	62 ± 41*	131 ± 9*
3M-011	4	1347 ± 293*	<1	16 ± 0*
3M-011	6	117 ± 16*	<1	6 ± 1
3M-011	24	2 ± 1	<1	<1

[a] Rats were dosed IN with either vehicle or 3M-011 at 1.0 mg/kg. 3M-011 was formulated as described. Serum, nasal cavity, or lung fluids were collected at the indicated times post dosing, and type I IFN levels were determined by bioassay.
* $p \leq 0.05$ vs. appropriate vehicle control group.

Table 2
Serum type I IFN, TNF-α, and IL-12 levels following a single intranasal instillation of 3M-011 to rats[a]

Treatment group	Dose (mg/kg)	Mean ± S.E.		
		Type I IFN[b] (U/ml)	TNF-α[c] (pg/ml)	IL-12[c] (pg/ml)
Vehicle control	0	<1	55 ± 12	1329 ± 157
3M-011	0.01	421 ± 0.0*	38 ± 3	1027 ± 134
3M-011	0.1	2353 ± 590*	43 ± 10	1253 ± 139
3M-011	1.0	401 ± 20*	283 ± 41*	3042 ± 523*

[a] Rats were dosed IN with vehicle or 3M-011 at 0.01, 0.1, or 1.0 mg/kg. Serum samples were collected at various times after instillation. Each data point represents the mean ± S.E. of the amount of cytokine measured. Samples of 10 rats were used for each time point.
[b] Results from serum collected at 6 h after instillation.
[c] Results from serum collected at 1.5 h after instillation.
* $p \leq 0.05$ vs. appropriate vehicle control group.

was non-lethal and resolved by approximately 72 h (Lebrec and Burleson, 1994). There were no significant weight changes in the animals following the influenza inoculation (data not shown). Maximal increases in viral titer occurred in approximately the first 24 h after inoculation, as did increases in lung type I IFN levels (>500 U/ml). Maximal increases in viral titer detected in nasal fluids also peaked at 24 h. Low levels of IFN were detected at 48 and 72 h in nasal lavage fluid. These data demonstrate the magnitude and the kinetics of influenza virus-induced type I IFN in the rat lung and nose.

3.5. Suppression of lung viral titers in rats treated with TLR7/8 agonist or rIFN prior to influenza challenge

The effects of a single or two high doses of the TLR7/8 agonist (3M-011 at 1 mg/kg) or rIFN-α (10^5 U/kg) on the titer of influenza virus were determined (Fig. 3). Rats were dosed IN with vehicle, 3M-011, or recombinant rat IFN-α either immediately prior (1×) to viral challenge or 24 h and immediately before virus challenge (2×). 3M-011 caused a dramatic reduction (≥2 logs) in lung viral titer, either as a single or two IN doses at both 24 h (Fig. 3A) and 48 h (Fig. 3B) after virus challenge. In contrast, a single or two IN instillations of rIFN-α did not substantially reduce the lung viral titer at this dose (i.e., <1 log reduction).

3.6. Suppression of nasal viral titers in rats treated with TLR7/8 agonist or rIFN prior to influenza challenge

Additional studies were conducted in the rat influenza model to investigate whether pretreatment with 3M-011 blocks virus replication in the nasal cavity. IN administration of 3M-011

Table 3
Effects of a single intranasal instillation of vehicle on nasal cavity and lung homogenate influenza viral titers and type I IFN levels at 24, 48, and 72 h after administration in rats[a,b]

Treatment group ($n = 10$)	Time post exposure (h)	Mean log lung viral titer ± S.E.	Mean log nasal viral titer ± S.E.	Mean lung type I interferon ± S.E. (U/ml)	Mean nasal type I interferon ± S.E. (U/ml)
Vehicle	24	6.4 ± 0.49	5.59 ± 0.08	519 ± 203	2 ± 1
Vehicle	48	5.04 ± 0.81	3.64 ± 0.50[c]	271 ± 120	26 ± 8[c]
Vehicle	72	1.36 ± 1.07[c]	0.3 ± 0.3	69 ± 23[c]	4 ± 1

[a] Rats were dosed IN with vehicle. Nasal cavity fluids or lung homogeneates were collected at the indicated times post dosing, and IFN levels were determined by bioassay.
[b] Each rat was inoculated IN with non-lethal, rat-adapted H3N2 influenza virus with 2×10^5 plaque forming units in a volume of 50 μl.
[c] $N = 9$ for these treatment groups.

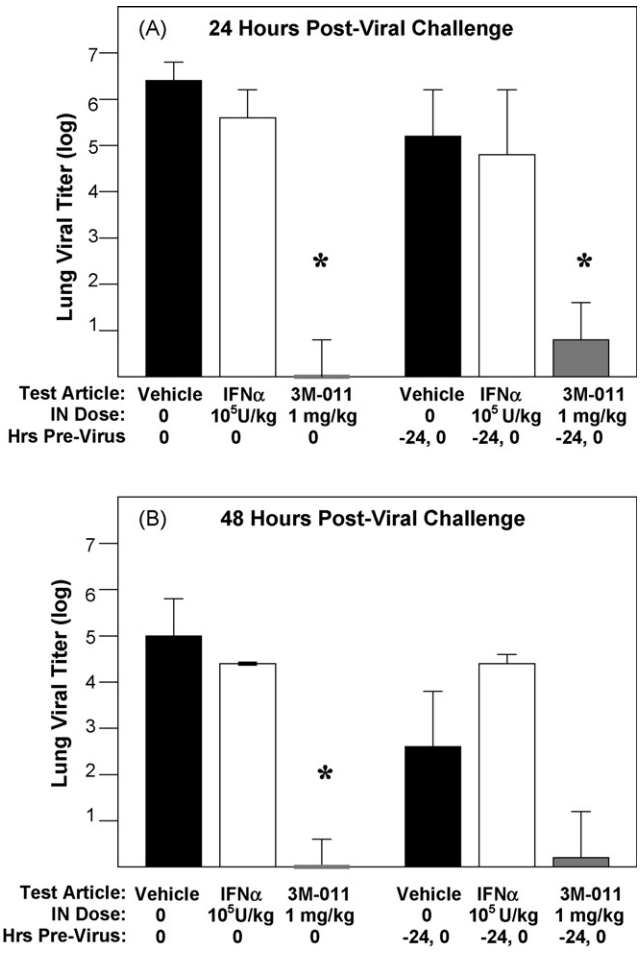

Fig. 3. Effects of TLR7/8 agonist or rat rIFN-α on lung viral titers when administered prior to intranasal influenza inoculation. Results following a single or two IN instillations of test articles on lung viral titers 24 h (Panel A) or 48 h (Panel B) after IN inoculation of rats with influenza virus are shown. Animals were dosed IN with vehicle, 3M-011 (PEG/lactate formulation), or rat rIFN-α either immediately before (1×) or 24 h and immediately prior (2×) to virus inoculation. Each rat was inoculated IN with non-lethal, rat-adapted H3N2 influenza virus with 2×10^5 plaque forming units. Bars represent the lung viral titer (mean ± S.E.) for 8–10 rats for the vehicle and 3M-011 groups and 6 rats for the rIFN-α group. Asterisk (*) indicates $p < 0.05$ when compared to vehicle.

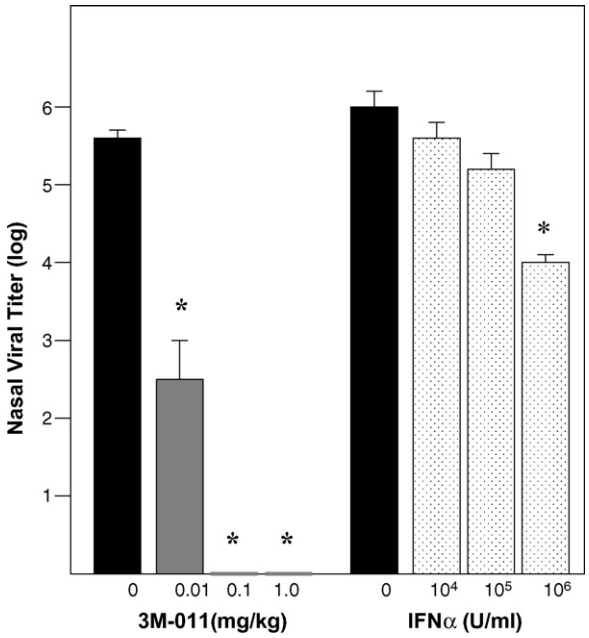

Fig. 4. Concentration dependent effects of a TLR7/8 agonist or rat rIFN-α on nasal viral titers when administered prior to intranasal influenza inoculation. A single IN instillation of vehicle, 3M-011 (0.01, 0.1, and 1 mg/kg) in a CMC/tartrate formulation or rat rIFN-α (10^4, 10^5, and 10^6 U/ml) was given 4 h before IN inoculation of rats with influenza virus. Viral titers were measured 24 h after virus inoculation and data are presented as the mean ± S.E. of the nasal viral titer for 8–10 rats. Asterisk (*) indicates $p < 0.05$ when compared to vehicle.

at 0.01, 0.1, and 1.0 mg/kg produced statistically significant reductions in nasal viral titers compared to IN administration of vehicle when given prior to influenza challenge with the 0.1 and 1.0 mg/kg doses being most effective (Fig. 4). The minimum effective dose of 3M-011 needed to significantly decrease nasal viral titers was not determined.

Similar studies were conducted in rats using a single IN dose of either rIFN-α (at 10^4, 10^5, or 10^6 U/ml) or vehicle given 4 h before H3N2 influenza virus challenge. Fig. 4 shows that only the highest dose of rIFN-α achieved a statistically significant decrease in nasal viral titers. However, at this IN dose of rIFN-α (i.e., 10^6 U/ml) a severe pulmonary distress response was observed, resulting in the death of four out of nine rats. In comparison, no behavioral or clinical side-effects were observed in rats treated with vehicle or any dose of 3M-011 tested.

The kinetics of protection against influenza infection for 3M-011 was investigated using a single IN dose of 3M-011 (1 mg/kg) given 24, 48, or 72 h prior to H3N2 influenza inoculation. As illustrated in Fig. 5A, nasal viral titers were significantly reduced even when the TLR7/8 agonist, 3M-011, was administered 72 h prior to virus challenge. The maximal time period that 3M-011 could be administered before influenza challenge and still confer protection was not determined.

3.7. Suppression of nasal viral titers in rats treated with TLR7/8 agonist shortly after influenza challenge

To investigate if therapeutic administration of 3M-011 could block the observed increase in nasal viral titers, the TLR7/8 agonist was given 6 h after IN instillation of virus. As shown in Fig. 5B, administration of 3M-011 6 h post-inoculation produced statistically significant inhibition of nasal viral titers compared to IN administration of vehicle following influenza challenge. Viral replication was completely suppressed in rats that received a single IN dose of 3M-011 at 0.1 or 1.0 mg/kg 6 h after influenza virus inoculation. The maximal time period that 3M-011 could be administered after influenza challenge and still block nasal viral replication was not determined.

4. Discussion

Influenza infection triggers an array of host immune responses that effectively limit the replication and spread of viral infection. Both arms of the adaptive immune system, i.e., neutralization of free virus by antibody and termination of intracellular virus replication by antiviral cytotoxic T-cells, effectively

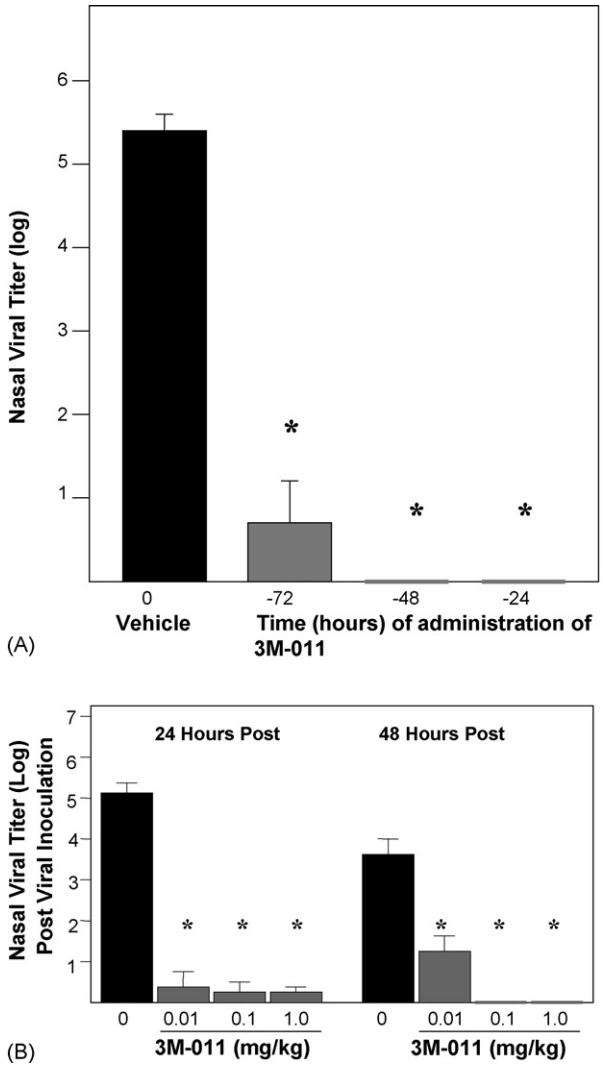

Fig. 5. TLR7/8 agonists inhibit influenza virus infection when given (A) before virus inoculation or (B) post-intranasal influenza challenge. (A) A single IN instillation of a PEG/lactate formulation of 3M-011 (1.0 mg/kg) was given 24, 48, or 72 h prior to virus inoculation. Twenty-four hours after virus inoculation nasal viral titer were measured and results are presented as the mean ± S.E. for 8–10 rats. Asterisk (*) indicates $p<0.05$ when compared to vehicle. (B) A single intranasal instillation of an aqueous CMC/tartrate formulation of 3M-011 (0.01, 0.1, and 1 mg/kg) was administered 6 h after IN influenza virus inoculation. Nasal viral titers were measured 24 and 48 h after intranasal viral inoculation. Results are presented as the mean viral titer ± S.E. for 8–10 rats per group. Asterisk (*) indicates $p < 0.05$ when compared to vehicle.

contribute to virus elimination and recovery from illness. Since the adaptive immune response typically is not fast enough in naïve individuals to eliminate invading pathogens, the innate immune response confers rapid, early protection against viral pathogens. Innate immune receptors known as TLR detect conserved molecular "signatures" expressed by viruses and bacteria (Takeda et al., 2003). Recognition of the "molecular signature" for influenza (i.e., single-stranded and double-stranded RNA) has been attributed to TLR7, TLR8, TLR3, and retinoic acid-inducible protein I (Diebold et al., 2004; Heil et al., 2004; Lund et al., 2004; Matikainen et al., 2006). However, the influenza virus has developed a number of mechanisms to evade destruction by the immune system (Oh and Eichelberger, 2000; Garcia-Sastre, 2002; Grandvaux et al., 2002).

In common with influenza, small IRMs also activate the innate immune receptors, TLR7 and TLR8 (Gorden et al., 2005). 3M-011 belongs to the imidazoquinoline family of IRMs of which imiquimod and resiquimod are most familiar (Stanley, 2002; Gupta et al., 2004). Engagement of TLR7/8 triggers MyD88-dependent intracellular signaling pathways and subsequent activation of NF-κB, a transcription factor that stimulates production of pro- and anti-inflammatory cytokines (e.g., TNF-α, IFN-β, IL-6, IL-8, and IL-12) and also activate MyD88-dependent signaling pathways coupled to the production of IFN-α (Miller et al., 1999; Hemmi et al., 2002; Gibson et al., 2002). Although the specific molecular mechanism of action for 3M-011 has not been fully elucidated, 3M-011 functions as a mixed TLR7/8 agonist as demonstrated by the activation of reporter systems in cells containing the human TLR7 or TLR8 genes.

The infection of mice and rats with influenza virus has become the prototypic animal model for the characterization of viral upper respiratory infections. Burleson and coworkers have previously reported that the rat-adapted influenza virus infection in rats resembles human influenza virus infection, both with respect to time course and morphogenesis of the respiratory tract lesions (Burleson, 1995, 2000; Burleson et al., 1995; Ibanes et al., 1996). In the present study, a single IN dose of TLR7/8 agonist (0.01, 0.1, or 1 mg/kg 3M-011) significantly reduced nasal viral titers when given up to 72 h before or 6 h post challenge with H3N2 influenza virus. IFN-α is known to establish an antiviral state that lasts several days via induction of Mx protein, 2′5′ oligoadenylate synthetase and possibly other antiviral factors (Pestka et al., 2004). Marked inhibition of lung viral titers was also observed using the TLR7/8 agonist and administration of a second dose of the agonist had no additional effect on viral inhibition. Although a dose-dependent effect was observed, the lowest prophylactic or therapeutic IN dose of 3M-011 that significantly reduced nasal viral load in the rat influenza model was not determined in this study.

The ability of 3M-011 to suppress viral titers in the rat influenza challenge model is likely indirect, i.e., via the production of cytokines and subsequent cytokine-induced expression of antiviral gene products, since no direct anti-influenza virus activity is seen in vitro (unpublished results). For example, IN administration of 3M-011 may induce non-specific protection from viral replication via the production of IFN-α. In this study, IFN-α levels measured in local (nasal cavity and lung) as well as systemic (serum) compartments increased significantly 2 h after IN dosing with 3M-011 (1 mg/kg). In consideration that cytokines other than IFN-α may have contributed to the observed antiviral activity, it is interesting to note that significantly higher levels of TNF-α and IL-12 were also detected in the serum of rats dosed IN with 3M-011. Likewise, sub-micromolar concentrations of 3M-011 were shown to enhance in vitro production of TNF-α, IL-12, and IFN-α by human and rat antigen-presenting cells. Indeed, previous reports have documented the antiviral actions of TNF-α, IL-12, and IFN-γ (Karupiah et al., 1993, 1998; Bhardwaj et al., 1996; Arulanandam et al., 1999, 2000; Seo and

Webster, 2002). Some of these cytokines could also contribute to several days of antiviral activity following 3M-011 administration. The possibility of reduced cytokine responses following multiple IN doses of 3M-011 was not examined in these studies. In separate studies, rats administered 3M-011 intravenously three times per week for 4 weeks produced serum IFN and TNF levels after the last dose. Further studies are needed to determine the optimal treatment regimen for maintaining a prolonged antiviral response. The molecular mediators conferring the antiviral effects are currently under investigation.

The observation that prophylactic administration of either a single or two doses of rat rIFN-α was not as effective as prophylactic administration of 3M-011 further suggests that IFN-α alone may not be sufficient to effectively block the increase in viral replication following influenza challenge. Although plasma IFN levels were not measured in this study, adverse effects may be due to either local or systemic exposure. A severe pulmonary distress response occurred at the highest IN dose of rIFN-α (10^6 U/ml) required to achieve a statistically significant decrease in nasal viral titers. Previous studies have demonstrated the transmucosal absorption of human recombinant interferon-alpha B/D hybrid in rat and rabbit (Bayley et al., 1995). Bioavailabilities in the lung were 6.8% (rat) and 2.9% in the nasal cavity (rabbit). In comparison, no behavioral or clinical side-effects were observed in rats treated with vehicle or any dose of 3M-011 tested. IN instillation of the TLR7/8 agonist produced significant transient (peak responses at 4 h) increases in type I IFN production in both local (lung homogenate and nasal fluid) and in the systemic (serum) compartment. Interestingly, imiquimod cream massaged into the nares of non-human primates was shown to produce IFN-α and TNF-α protein in post-nasal fluids (Clejan et al., 2005). These findings suggest the formulation and route of administration may affect the therapeutic utility of TLR agonists and recombinant cytokines.

Apart from TLR7/8 agonists, TLR3, TLR4, and TLR9 agonists also have been investigated as stand-alone immunomodulators in murine models of influenza infection. Liposomal poly ICLC (synthetic double-stranded polyriboinosinic-polyribocytidylic acid stabilized with poly-L-lysine:carboxymethyl cellulose), a TLR3 agonist, administered via either intramuscular or IN routes provided complete protection against a lethal respiratory challenge of influenza A virus in mice that persisted for up to 3 weeks post-drug treatment (Wong et al., 2005). Poly ICLC pretreatment also protected mice against a respiratory challenge of western equine encephalitis (WEE) virus, at a level comparable to inactivated WEE vaccine (Wong et al., 2005). Pretreatment with a single, IN dose of structurally-related TLR4 agonists (lipid A mimetics) was reported to significantly increase the percentage of mice surviving 21 days after challenge with a lethal dose of influenza. The TLR4 agonist or vehicle was administered 48 h prior to influenza virus inoculation (Cluff et al., 2005). Likewise, a single, IN dose of a TLR9 agonist (CpG) provided marginal protection against clinical signs of influenza infection when administered 5 days before virus challenge (Wong et al., 2005). TLR9 agonists alone and in combination with vaccines have also been delivered via different routes (oral, intrarectal, and IN) to elicit immune responses to antigens including influenza and also show direct antiviral protection as well (McCluskie and Davis, 2001; Hayashi et al., 2005; Wong et al., 2005). For example, a single, intranasal dose of CpG provided partial protection against clinical signs of influenza infection when administered 5 days before virus challenge; whereas CpG incorporated into liposomes demonstrated 100% protection against influenza challenge (Wong et al., 2005).

Despite similarities in the types of mediators that are produced by PBMC upon activation with different TLR agonists, the tissue expression of specific TLR in vivo is quite different. TLR4 appears to have broader tissue expression compared to TLR7, TLR8, or TLR9 (reviewed by Takeda et al., 2003). TLR4 expression has been reported on hematopoietic cells such as monocytes, macrophages, neutrophils, B cells, myeloid dendritic cells, mast cells, platelets; renal, corneal, and gut epithelium; human dermal endothelium; vascular smooth and skeletal muscle; and cerebral tissues (Takeda et al., 2003; Okayama, 2005; Rivest, 2003; Cognasse et al., 2005; Yang et al., 2005). TLR7 expression and/or activity has been detected in plasmacytoid dendritic cells and B cells (Takeda et al., 2003). In contrast, TLR8 expression has been detected on monocytes, myeloid dendritic cells, neutrophils, mast cells, and potentially T regulatory cells (Takeda et al., 2003; Radsak et al., 2004; Okayama, 2005; Peng et al., 2005). It should be noted that although rodent cells do not appear to have a functional TLR8, broader tissue expression of TLR7 by rodent monocytic and dendritic cells results in a phenotype that mimics the phenotype exhibited by human TLR8 expressing cells (J.P. Vasilakos, personal observation). Finally, TLR9 expression in humans appears restricted to B cells and pDC (Takeda et al., 2003). As a result of differences in tissue expression, different TLR agonists are likely to be distinguished by the breadth of their pharmacological effects.

The results of the present study indicate that TLR7/8 agonists may provide benefit for populations at risk for influenza infection. The advantage of a small molecule (less than 400 kDa) to act locally and induce an antiviral state without causing adverse systemic effects may be feasible with intranasal IRM formulations. The fact that the antiviral response is non-specific suggests that these agents may be useful in preventing or limiting the morbidity and mortality associated with natural (pandemic or epidemic) or intentional (bioterrorism) exposures to a wide array of infectious agents.

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