

# Toll-Like Receptor–2/6 and Toll-Like Receptor–9 Agonists Suppress Viral Replication but Not Airway Hyperreactivity in Guinea Pigs

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Respiratory virus infections cause airway hyperreactivity (AHR). Preventative strategies for virus-induced AHR remain limited. Toll-like receptors (TLRs) have been suggested as a therapeutic target because of their central role in triggering antiviral immune responses. Previous studies showed that concurrent treatment with TLR2/6 and TLR9 agonists reduced lethality and the microbial burden in murine models of bacterial and viral pneumonia. This study investigated the effects of TLR2/6 and TLR9 agonist pretreatment on parainfluenza virus pneumonia and virus-induced AHR in guinea pigs *in vivo*. Synthetic TLR2/6 lipopeptide agonist Pam<sub>2</sub>CSK<sub>4</sub> and Class C oligodeoxynucleotide TLR9 agonist ODN2395, administered in combination 24 hours before virus infection, significantly reduced viral replication in the lung. Despite a fivefold reduction in viral titers, concurrent TLR2/6 and TLR9 agonist pretreatment did not prevent virus-induced AHR or virus-induced inhibitory M2 muscarinic receptor dysfunction. Interestingly, the TLR agonists independently caused non-M2-dependent AHR. These data confirm the therapeutic antiviral potential of TLR agonists, while suggesting that virus inhibition may be insufficient to prevent virus-induced airway pathophysiology. Furthermore, TLR agonists independently cause AHR, albeit through a distinctly different mechanism from that of parainfluenza virus.

**Keywords:** Toll-like receptor; airway hyperreactivity; muscarinic receptor; parainfluenza virus

Respiratory virus infections commonly cause exacerbations of asthma (1–3) and chronic obstructive pulmonary disease (COPD) (4). In healthy lungs, viruses also cause airway hyperreactivity (AHR; an abnormal tendency of the airways to constrict) (5, 6). Therapies for the prevention of respiratory virus infections are limited to influenza virus (7–10). Currently, no therapies target the respiratory viruses most commonly implicated in asthma and COPD exacerbations, including rhinoviruses and coronaviruses (2), or the prevention of virus-induced AHR.

Respiratory viruses cause AHR by altering the parasympathetic control of bronchoconstriction. Acetylcholine (ACh) released from the parasympathetic vagus nerves activates M3 muscarinic receptors on airway smooth muscle, causing contraction. ACh also activates presynaptic inhibitory M2 muscarinic receptors on the nerves, limiting further ACh release (11). In virus

## CLINICAL RELEVANCE

Toll-like receptor (TLR) 2/6 and 9 agonists attenuated parainfluenza virus in the lung. TLR agonists failed to suppress virus-induced airway hyperreactivity (AHR), and may independently induce AHR. Thus, the antiviral therapeutic potential of TLR 2/6 and 9 agonists may be limited by their adverse effects on airway physiology.

infection, inhibitory M2 receptors are dysfunctional, and this auto-feedback mechanism is lost (12). As a result, vagus nerves release more ACh onto airway smooth muscle, potentiating bronchoconstriction (13). Histamine, administered intravenously, causes bronchoconstriction indirectly via activation of the vagus neuronal reflex, and directly through the activation of histamine receptors at the level of airway smooth muscle. Both neuronal and airway smooth muscle physiology can be measured *in vivo*, using intravenous histamine before and after vagotomy (14). Neuronal inhibitory M2 receptor function and smooth muscle M3 receptor function can be assessed with the M2-selective inhibitor gallamine and intravenous acetylcholine, respectively (13).

Respiratory viruses are detected by Toll-like receptors (TLRs), which are highly conserved pattern-recognition receptors on respiratory epithelia (15, 16), smooth muscle (17), and inflammatory cells (18–21). Ten functional human TLRs have been identified (22). The detection by TLRs of molecular motifs on invading microorganisms triggers immune responses, including the production of interferons, cytokines, and antimicrobial factors via the activation of NF- $\kappa$ B (23). Defective TLR signaling and TLR polymorphisms have been associated with increased susceptibility to infection (24–27).

Because of the central role for TLRs in microbial detection and immune responses, they have garnered interest as therapeutic targets. Recently, concurrent treatment with the synthetic TLR2/6 lipopeptide agonist Pam<sub>2</sub>CSK<sub>4</sub> (Pam2) and the Class C oligodeoxynucleotide TLR9 agonist ODN2395 (ODN) was found to reduce lethality and the microbial burden in murine models of bacterial and viral pneumonia (28, 29). Treatment with the individual TLR agonists separately conferred little protection. Furthermore, the Class C oligodeoxynucleotide TLR9 agonists were superior to Class A or B oligodeoxynucleotides, possibly as a result of the Class C induction of both interferons and NF- $\kappa$ B–related cytokines, compared with interferons (Class A) or NF- $\kappa$ B–related cytokines (Class B) alone (29). In other settings, a TLR9 agonist has been used as a vaccine adjuvant to boost immunity against respiratory syncytial virus (30). Treatment with TLR2/6 agonists or TLR9 agonists has also been shown to reduce allergen-mediated AHR via the suppression of allergic Type 2 T-helper (Th2) cell inflammation (31–33). Despite this body of work, the potential of TLRs as therapeutic targets for the prevention of virus-induced AHR remains unknown.

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This work investigated the effects of simultaneous pretreatment with TLR2/6 agonist Pam2 and TLR9 agonist ODN, administered 24 hours before infection, on parainfluenza virus replication and virus-induced AHR in a guinea model of viral pneumonia *in vivo*. The neural control of airway function in guinea pigs is similar to that in humans, making it a relevant model to investigate mechanisms of virus-induced AHR. We hypothesized that Pam2/ODN pretreatment would attenuate parainfluenza viral titers and alleviate virus-induced AHR. We found that Pam2/ODN significantly reduced viral replication in the guinea pig lung. Despite a fivefold reduction in viral titers, pretreatment did not protect against virus-induced AHR or M2 muscarinic dysfunction. Interestingly, Pam2/ODN caused AHR independent of virus infection, which was not attributable to M2 receptor dysfunction, suggesting that TLR2/6 and TLR9 agonists cause AHR in a different manner from parainfluenza virus in guinea pigs.

## MATERIALS AND METHODS

### Animals

Pathogen-free female Hartley guinea pigs (300–400 g; Charles River Breeding Laboratories, Wilmington, MA) were handled in accordance with National Institutes of Health guidelines. Our protocols were approved by the Institutional Animal Care and Use Committee at Oregon Health and Science University.

### Study Protocol

Guinea pigs were pretreated with TLR2/6 agonist Pam2 (4 nmol) and TLR9 agonist ODN (1 nmol), or PBS vehicle, delivered as an aerosol into the trachea (referred to as tracheal) with a Penn-Century Microsprayer (Penn-Century, Wyndmoor, PA), or in solution into the right nostril (referred to as nasal), 24 hours before infection. Guinea pigs were infected with  $10^6$  tissue culture infectious dose/ml parainfluenza (Sendai) virus or PBS instilled intranasally, as previously described (12). Airway function was assessed *in vivo* 4 days after virus infection. After the functional experiments, guinea pigs received a lethal dose of anesthesia, and were exsanguinated from the abdominal aorta. Bronchoalveolar lavage (BAL) and peripheral blood leukocyte counts, along with lung viral content, were assessed after the guinea pigs had been killed.

### Measurements of Airway Physiology

Guinea pigs were anesthetized with urethane (1.9 g/kg, administered intraperitoneally) and paralyzed with succinylcholine (10  $\mu$ g/kg  $\cdot$  min, administered intravenously), and their jugular veins and right carotid arteries were cannulated. Animals were tracheotomized and ventilated through a tracheal cannula with a rodent respirator (2.5 ml volume, 100 breaths/min; Harvard Apparatus, Inc., South Natick, MA). Peak pulmonary pressures ( $P_{\text{peak}}$ ; mm H<sub>2</sub>O) during each inspiration were measured at the trachea, using a BD DTXplus pressure transducer (Viggo-Spectramed, Oxnard, CA). Increases in  $P_{\text{peak}}$  reflect changes in airflow resistance attributable to changes in airway caliber (34). Bronchoconstriction

(measured as an increase in  $P_{\text{peak}}$  over baseline) was induced by histamine (1–5 mg/kg, intravenous) before and after vagotomy, and by acetylcholine (1–10  $\mu$ g/kg, intravenous) after vagotomy.

### Studies of M2 Muscarinic Receptor Function

Bronchoconstrictions were induced by electrical stimulation of the vagus nerves. The vagus nerves were ligated, attached to platinum electrodes, and stimulated at 40-second intervals (8 V, 15 Hz, 2-ms duration, 3 s on, 40 s off). The M2 muscarinic receptor antagonist gallamine (0.1–10 mg/kg, intravenous) was injected after every fourth period of vagal stimulation. The effect of gallamine on vagally induced bronchoconstriction was measured as the ratio of bronchoconstriction in the presence of gallamine to bronchoconstriction in the absence of gallamine.

### Virus Isolation and Titration

Viral titers were assessed by real-time RT-PCR from homogenized lung samples, as described in the online supplement.

### Drugs and Reagents

Histamine, gallamine, acetylcholine, succinylcholine, and urethane were purchased from Sigma-Aldrich (St. Louis, MO). Pam2 and ODN were obtained from Invivogen (San Diego, CA).

### Statistical Analysis

Data are expressed as means  $\pm$  SEMs. Histamine-induced, gallamine-induced, and acetylcholine-induced responses were analyzed using two-way ANOVA for repeated measures. Baseline data and leukocyte counts were analyzed using one-way ANOVA. Viral titers were compared using the Student *t* test. All statistical analyses were performed using Prism (GraphPad Software, La Jolla, CA). *P* < 0.05 was considered significant.

## RESULTS

### Baseline Physiologic Characteristics

Baseline  $P_{\text{peak}}$  (a measure of baseline airway resistance) before the pharmacologic experiments was significantly increased by virus infection, compared with control guinea pigs (Table 1). Pretreatment with Pam2/ODN partly attenuated virus-induced elevations in baseline bronchoconstriction. Pam2/ODN pretreatment did not affect baseline bronchoconstriction in the absence of virus infection. No significant differences were evident in baseline heart rate, systolic blood pressure, diastolic blood pressure, or weight between groups.

### Effect of TLR2/6 and TLR9 Agonist Pretreatment on Parainfluenza Virus Replication

TLR2/6 agonist Pam2 and TLR9 agonist ODN, administered simultaneously 24 hours before infection, reduced parainfluenza virus replication in the lungs (Figure 1). This antiviral effect

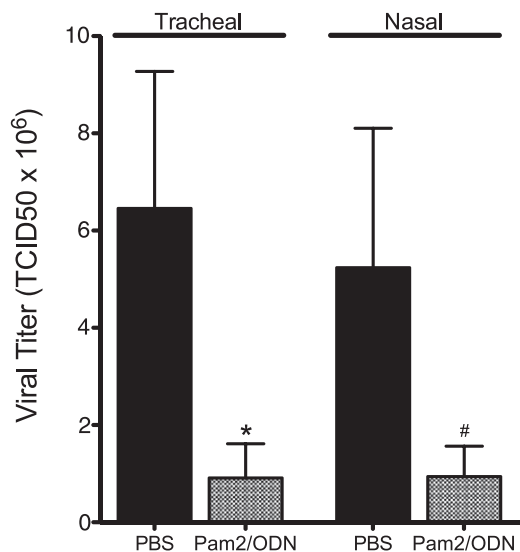
TABLE 1. BASELINE PHYSIOLOGIC CHARACTERISTICS

	Tracheal				Nasal			
	Control	Pam2/ODN	Virus	Pam2/ODN + Virus	Control	Pam2/ODN	Virus	Pam2/ODN + Virus
$P_{\text{peak}}$	90.0 (2.9)	99.0 (1.0)	159.6 (13.8)*	133.5 (11.7)	82.5 (4.4)	80.0 (7.3)	115.0 (19.1)	101.7 (6.0)
HR	278.8 (1.3)	298.8 (5.2)	297.7 (8.3)	306.7 (4.3)	273.3 (14.4)	291.7 (11.8)	288.3 (11.7)	300.8 (12.9)
SBP	47.8 (1.4)	47.5 (2.9)	51.4 (3.0)	52.3 (3.1)	42.0 (2.9)	49.9 (2.8)	46.3 (2.0)	51.3 (3.6)
DBP	24.0 (2.0)	22.5 (1.0)	25.2 (2.4)	27.0 (2.1)	19.8 (1.5)	26.3 (2.8)	29.0 (2.3)	27.3 (3.0)
Weight	0.371 (0.0004)	0.381 (0.013)	0.345 (0.007)	0.3734 (0.018)	0.371 (0.022)	0.377 (0.026)	0.356 (0.019)	0.354 (0.007)

Definition of abbreviations: DBP, diastolic blood pressure; HR, heart rate; ODN, ODN2395; Pam2, Pam<sub>2</sub>CSK<sub>4</sub>;  $P_{\text{peak}}$ , Peak pulmonary pressure; SBP, systolic blood pressure.

Baseline data represent the means  $\pm$  SEMs before pharmacologic manipulation.

\**P* < 0.05, compared with control samples.

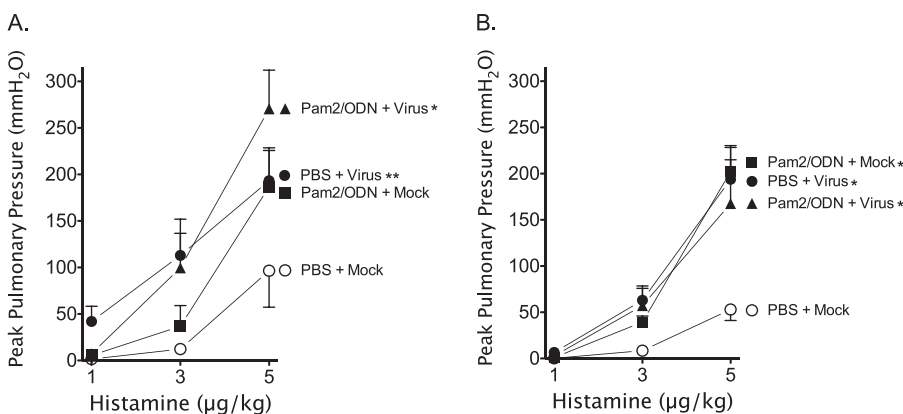


**Figure 1.** Pam<sub>2</sub>CSK<sub>4</sub> (TLR2/6) and ODN2395 (TLR9) synergistically inhibit parainfluenza virus replication *in vivo*. Guinea pigs were pretreated simultaneously with Pam<sub>2</sub>CSK<sub>4</sub> (4 nmol) and ODN2395 (1 nmol), or with PBS vehicle, administered as an aerosol directly into the trachea (Tracheal), or as a nasal solution (Nasal), 24 hours before parainfluenza virus infection. Parainfluenza RNA from lung homogenates was quantified by RT-PCR, 4 days after infection. Both tracheal and nasal Pam2/ODN pretreatment inhibited virus replication, although the effect of nasal Pam2/ODN fell short of statistical significance (tracheal, Pam2/ODN,  $n = 12$ ; PBS,  $n = 11$ ; \* $P < 0.05$ ; nasal, Pam2/ODN,  $n = 6$ ; PBS,  $n = 10$ ; # $P = 0.08$ ). Viral titers were normalized to 18S RNA and transformed to tissue culture infectious dose (TCID) 50 titers using a parainfluenza standard curve quantified by rhesus monkey kidney cell titration. Data shown represent the means  $\pm$  standard errors of the mean.

was profound, resulting in an 80% reduction in parainfluenza virus mRNA 4 days after infection. This treatment effect was present with both tracheal and nasal deliveries of TLR agonists.

#### Effect of TLR2/6 and TLR9 Agonists on Virus-Induced Vagal-Reflex Airway Hyperreactivity

Histamine-induced bronchoconstriction (1–5  $\mu\text{g}/\text{kg}$ , intravenous) before vagotomy assesses the neuronal control of airway tone by activating efferent parasympathetic vagus nerves. Both virus infection and Pam2/ODN pretreatment independent of virus infection



**Figure 2.** Parainfluenza virus and Pam<sub>2</sub>CSK<sub>4</sub> (TLR2/6)/ODN2395 (TLR9) cause vagal-reflex airway hyperreactivity. Guinea pigs were pretreated simultaneously with Pam<sub>2</sub>CSK<sub>4</sub> (4 nmol) and ODN2395 (1 nmol), or with PBS vehicle, administered as an aerosol directly into the trachea (Tracheal), or as a nasal solution (Nasal), 24 hours before parainfluenza virus or mock infection. Histamine (1–5  $\mu\text{g}/\text{kg}$ , intravenous) induced dose-dependent bronchoconstrictions, measured as increases in peak pulmonary pressures ( $P_{\text{peak}}$ ), by activating the vagal-reflex innervation of the airways. Parainfluenza infection (PBS + Virus) and Pam<sub>2</sub>CSK<sub>4</sub>/ODN2395 pretreatment (Pam2/ODN + Mock) independently, and concomitantly (Pam2/ODN + Virus), potentiated vagal-reflex bronchoconstriction. (A) Aerosolized pretreatment administered into the trachea ( $n = 4$  per group). (B) Nasal solution pretreatment ( $n = 5$  per group). Data shown represent the means  $\pm$  standard errors of the mean. \* $P < 0.05$ , compared with PBS + Mock control group. \*\* $P = 0.053$ , compared with PBS + Mock control group.

potentiated histamine-induced vagal-reflex bronchoconstriction in guinea pigs (Figures 2A and 2B). Despite the significant reduction in parainfluenza virus replication in the lung attributable to Pam2/ODN pretreatment, no attenuation of AHR was evident in virus-infected guinea pigs.

#### Effect of TLR2/6 and TLR9 Agonists on Virus-Induced Non-Neuronal Airway Hyperreactivity

Histamine-induced bronchoconstriction (1–5  $\mu\text{g}/\text{kg}$ , intravenous) after vagotomy measures airway responsiveness without vagal-reflex input. Histamine-induced bronchoconstriction after vagotomy was potentiated by virus infection, and by Pam2/ODN pretreatment independent of virus infection (Figures 3A and 3B). Pam2/ODN pretreatment did not attenuate non-neuronal AHR in virus-infected guinea pigs, despite significant reductions in parainfluenza virus replication in the lungs.

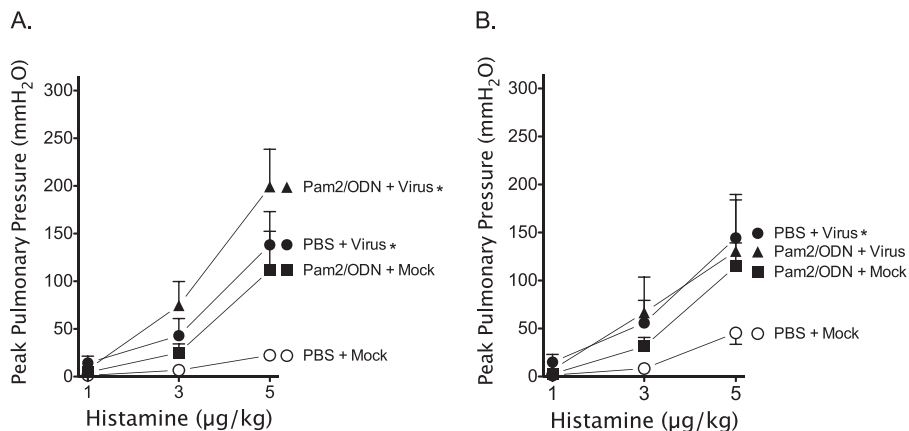
#### Effect of TLR2/6 and TLR9 Agonists on Virus-Induced M2 Muscarinic Receptor Dysfunction

Gallamine (0.1–10 mg/kg, intravenous) increases vagally mediated bronchoconstriction by blocking inhibitory presynaptic vagal M2 muscarinic receptors, thereby increasing acetylcholine release at the neuromuscular junction. Lack of an increase in vagally mediated bronchoconstriction after gallamine administration indicates M2 receptor dysfunction. Virus infection caused M2 muscarinic receptor dysfunction in both vehicle control and Pam2/ODN-pretreated guinea pigs compared with mock-infected control guinea pigs (Figure 4A). Interestingly, although TLR agonists potentiated vagal-reflex and non-neuronal bronchoconstriction in mock-infected animals, they did not cause M2 receptor dysfunction.

Inhibitory M2 muscarinic receptors are also present on cardiac muscle, and reduce heart rate in response to vagal stimulation. Gallamine (0.1–10 mg/kg, intravenous) inhibited vagally mediated bradycardia in all treatment groups (Figure 4B), indicating that neither the virus nor the TLR agonists cause dysfunction of cardiac inhibitory M2 receptors.

#### Effect of TLR2/6 and TLR9 Agonists on M3 Muscarinic Receptor Function

Intravenous acetylcholine causes bronchoconstriction by activating airway smooth muscle M3 muscarinic receptors. No differences in acetylcholine-induced bronchoconstriction (1–10  $\mu\text{g}/\text{kg}$ , intravenous) were evident between groups (Figure 5). This



**Figure 3.** Parainfluenza virus and Pam<sub>2</sub>CSK<sub>4</sub> (TLR2/6)/ODN2395 (TLR9) cause non-neuronal airway hyperreactivity. Guinea pigs were pretreated simultaneously with Pam<sub>2</sub>CSK<sub>4</sub> (4 nmol) and ODN2395 (1 nmol), or with PBS vehicle, administered as an aerosol directly into the trachea (Tracheal), or as a nasal solution (Nasal), 24 hours before parainfluenza virus or mock infection. Histamine (1–5 µg/kg, intravenous) induced dose-dependent bronchoconstrictions, measured as increases in P<sub>peak</sub>, after vagotomy by activating histamine receptors at the level of the airways. Parainfluenza virus infection (PBS + Virus) and Pam<sub>2</sub>CSK<sub>4</sub>/ODN2395 pretreatment (Pam2/ODN + Mock) independently, and concomitantly (Pam2/ODN + Virus), potentiated bronchoconstriction. (A) Aerosol pretreatment administered directly into the trachea (*n* = 4 per group). (B) Nasal solution pretreatment (*n* = 5 per group). Data shown represent the means ± standard errors of the mean. \**P* < 0.05, compared with PBS + Mock control group.

demonstrates that airway smooth muscle M3 muscarinic receptors are not affected by virus infection or by TLR 2/6 and TLR9 agonists.

#### Effects of TLR2/6 and TLR9 Agonists and Virus on Bronchoalveolar Lavage and Peripheral Leukocyte Counts

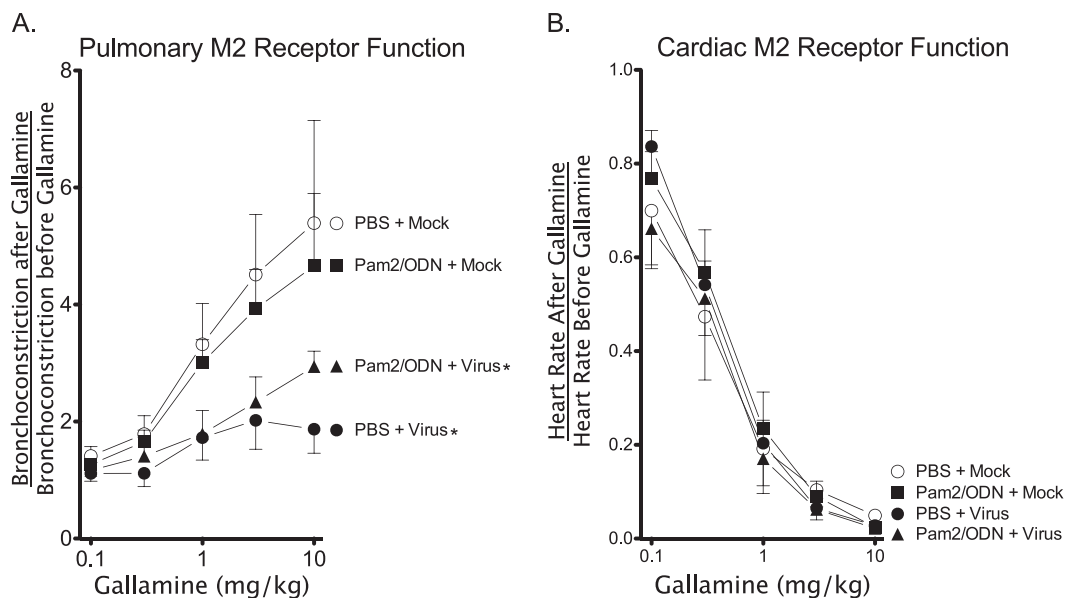
Virus infection and pretreatment with Pam2/ODN increased total BAL leukocytes (Figures 6A and 6B). In both cases, the increase in total leukocytes was largely attributable to increases in macrophages and neutrophils. Treatment with aerosol PBS vehicle, but not nasal PBS vehicle, increased total BAL leukocytes, independent of virus infection or TLR agonists. No differences in eosinophils or lymphocytes were detected between the groups in either the aerosol or nasal treatment cohort.

Virus infection reduced peripheral blood total leukocyte counts (Figures 6C and 6D). Specifically, virus infection decreased lymphocytes and neutrophils. In mock-infected animals, Pam2/ODN

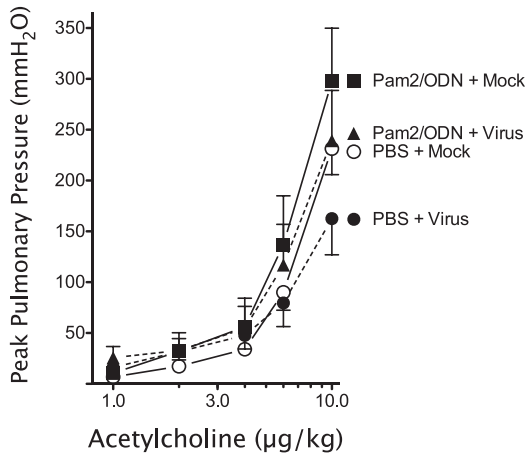
pretreatment partly decreased neutrophils, although this effect did not reach statistical significance. No changes in peripheral eosinophils or monocytes were evident.

#### DISCUSSION

Virus infections of the respiratory tract are a major cause of asthma and COPD exacerbations. Therapeutic strategies for the prevention of virus infection and virus-induced AHR remain limited (7–10). The experiments described in this report were designed to assess the effects of TLR2/6 and TLR9 agonists on parainfluenza virus replication and virus-induced AHR in the guinea pig lung. Pam2 (TLR2/6) and ODN (TLR9) were chosen because of previous work demonstrating their synergistic antimicrobial effects in mice (28, 29). Our data indicate that simultaneous pretreatment with TLR2/6 and TLR9 agonists, administered as an aerosol directly into the trachea or as a nasal solution, decreases viral replication in guinea pig lungs. This work was the first to



**Figure 4.** Parainfluenza infection, but not Pam<sub>2</sub>CSK<sub>4</sub> (TLR2/6)/ODN2395 (TLR9), causes pulmonary M2 muscarinic receptor dysfunction. Guinea pigs were pretreated with Pam<sub>2</sub>CSK<sub>4</sub> (4 nmol) and ODN2395 (1 nmol) in combination, or with PBS vehicle, administered as a nasal solution 24 hours before parainfluenza virus or mock infection. Transient bronchoconstrictions were produced by electrical stimulation of the vagus nerves (8 V, 15 Hz, 2-ms duration, 3 seconds on, 40 seconds off). Gallamine (1–10 mg/kg, intravenous), an M2 muscarinic receptor antagonist, was administered after every fourth bronchoconstriction. (A) Gallamine potentiated vagally induced bronchoconstriction by inhibiting M2 muscarinic receptor function in control (PBS + Mock) and in mock-infected Pam<sub>2</sub>CSK<sub>4</sub>/ODN2395 pretreated guinea pigs (Pam2/ODN + Mock), but not in virus-infected guinea pigs (PBS + Virus). Pam<sub>2</sub>CSK<sub>4</sub>/ODN2395 pretreatment did not prevent virus-induced M2 receptor dysfunction (Pam2/ODN + Virus; *n* = 5 per group). (B) Gallamine blocked vagally induced decreases in heart rate by inhibiting cardiac myocyte M2 receptors. Gallamine blocked decreases in heart rate similarly in all groups (*n* = 5 per group). Data shown represent the means ± standard errors of the mean. \**P* < 0.05, compared with PBS + Mock control group.

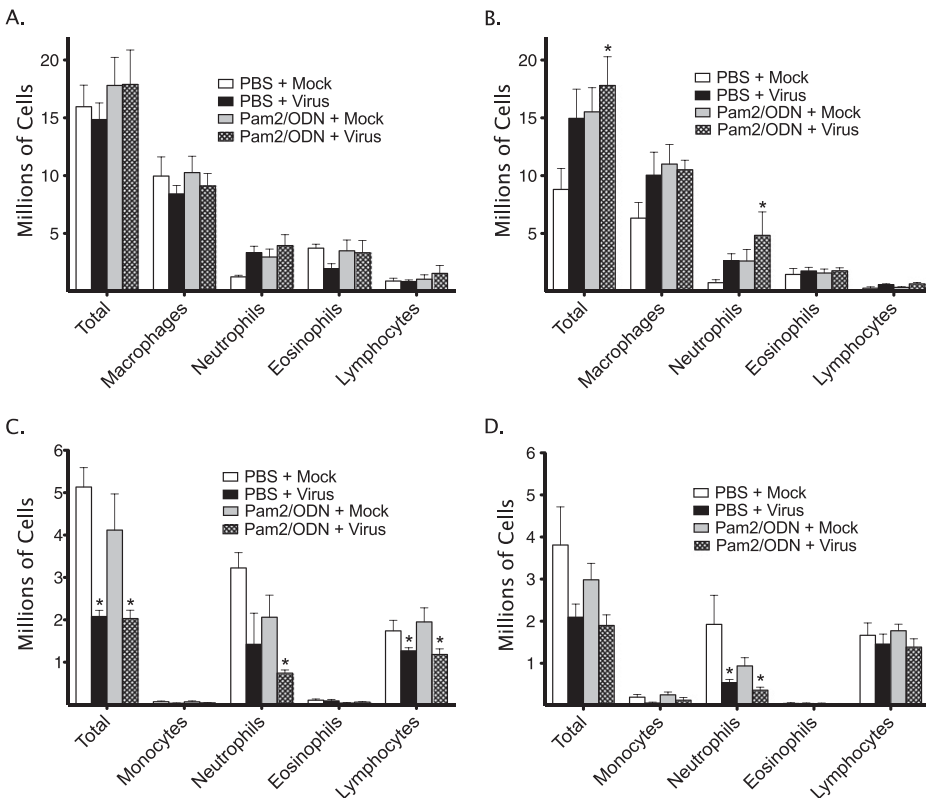


**Figure 5.** Pulmonary M3 muscarinic receptor function is unaffected by parainfluenza infection or Pam<sub>2</sub>CSK<sub>4</sub> (TLR2/6)/ODN2395 (TLR9) pretreatment. Acetylcholine (1–10 µg/kg, intravenous) caused bronchoconstriction, measured as increases in  $P_{\text{peak}}$  by activating airway smooth muscle M3 muscarinic receptors. Acetylcholine-induced bronchoconstriction was similar between groups ( $n = 5$  per group). Data shown represent the means  $\pm$  standard errors of the mean.

establish the potent TLR2/6 and TLR9 agonist antiviral effect in this animal model. Moreover, we show that TLR2/6 and TLR9 agonists exert an antiviral effect that is conserved across species, and we demonstrate two therapeutic delivery methods (nasal and inhalational) applicable to future drug development in humans. Furthermore, guinea pig airway nerve function and anatomy resemble those in humans, making it more applicable than a murine model for investigating *in vivo* dynamic airway responsiveness to a variety of stimuli (35).

TLRs are central to immune responses against invading microbes. The TLR agonists used in these experiments targeted both a virus-sensing TLR (TLR9) and a bacterial-sensing TLR (TLR2/6) (23). Interestingly, the synergistic antimicrobial effect of TLR2/6 and TLR9 agonists was lost when these agonists were administered individually in mice (28, 29). This effect was dependent on the classic TLR–MyD88 signaling pathway, but was not dependent on the presence of leukocytes, suggesting that airway epithelial cells are capable of inducing the TLR agonist response (36, 37). Furthermore, the synergistic effect of TLR9 agonists with TLR2/6 agonists was greatest with Class C oligodeoxynucleotides compared with Class A or B oligodeoxynucleotides, possibly because of the induction by Class C of interferons and the transcription of cytokines via NF- $\kappa$ B, compared with either interferons (Class A) or NF- $\kappa$ B-related cytokines (Class B) alone (38). Determining the relative contributions of these specific pathways to the effects of Pam2/ODN was beyond the scope of this study. However, the available evidence suggests that Pam2/ODN pretreatment synergistically triggers TLR2/6 and TLR9 to promote an antiviral milieu capable of inhibiting viral replication at the onset of infection.

Despite significant reductions in parainfluenza virus replication *in vivo* attributable to TLR2/6 and TLR9 agonist pretreatment, no improvement in viral-induced vagal-reflex AHR was evident. This lack of improvement in AHR may be attributable to an incomplete inhibition of virus replication *in vivo*. This is surprising, however, because previous work has demonstrated improvements in virus-induced airway dysfunction with the suppression of viral titers (39). Interestingly, the TLR agonists caused AHR even in the absence of virus infection, which may counteract the positive effects of the suppression of viral replication. This work is the first to establish that TLR2/6 and TLR9 agonists cause AHR in the nonallergic lung. This finding stands in contrast with studies of allergen-sensitized mice, in which



**Figure 6.** Parainfluenza infection and Pam<sub>2</sub>CSK<sub>4</sub> (TLR2/6)/ODN2395 (TLR9) cause pulmonary and systemic inflammatory cell changes. Guinea pigs were pretreated with Pam<sub>2</sub>CSK<sub>4</sub> (4 nmol) and ODN2395 (1 nmol) in combination, or with PBS vehicle, administered as an aerosol directly into the trachea (Tracheal), or as a nasal solution (Nasal), 24 hours before parainfluenza virus or mock infection. Bronchoalveolar (BAL) and peripheral blood total and differential leukocyte counts were evaluated 4 days after virus infection. (A) Tracheal pretreatment BAL counts ( $n = 6$  per group). (B) Nasal pretreatment BAL counts ( $n = 5$  per group). (C) Tracheal pretreatment peripheral leukocyte counts ( $n = 6$  per group). (D) Nasal pretreatment peripheral leukocyte counts ( $n = 5$  per group). Data shown represent the means  $\pm$  standard errors of the mean. \* $P < 0.05$ , compared with PBS + Mock control group.

individual TLR agonists did not cause AHR (31–33), although the combination of TLR agonists used in the present study was not specifically tested. Despite TLR2/6- and TLR9-mediated reduction of respiratory virus replication, it remains unclear whether this technique may provide net benefit in virus-induced exacerbations of lung disease.

Virus infection and pretreatment with TLR2/6 and TLR9 agonists also induced AHR in vagotomized guinea pigs. Vagotomy removes the efferent vagal-nerve input to the airways, allowing airway smooth muscle responsiveness to histamine to predominate. The TLR agonists did not prevent nonreflex virus-induced AHR, despite significant reductions in viral titers. The link between TLR activation and non-neuronal histamine-induced AHR has not previously been reported, and suggests that TLR agonists and virus infection cause AHR through both neuronal and non-neuronal mechanisms.

TLR agonists and virus infection produced significant changes in BAL and peripheral blood leukocyte counts. The nasal delivery of TLR agonists with or without virus infection produced a doubling of total BAL leukocytes, attributable to significant increases in macrophages and neutrophils. Conversely, nasal TLR agonists caused a decline in systemic neutrophils, but no difference in total leukocytes. Tracheal aerosol TLR2/6 and TLR9 pretreatment also caused an increase in total BAL leukocytes and neutrophils, and a decline in peripheral blood neutrophils. Surprisingly, aerosolized PBS vehicle also increased total BAL leukocytes in control guinea pigs. Despite this leukocyte influx, aerosolized PBS vehicle did not increase bronchoconstriction. This suggests that TLR agonist-mediated AHR is not caused by leukocyte influx into the airway lumen, or that leukocytes require activation by virus or TLR agonists to produce AHR.

Similar to previous work (12), virus infection caused the dysfunction of vagal presynaptic inhibitory M2 muscarinic receptors, but not cardiac M2 receptors. Despite significant reductions in viral titers, pretreatment with TLR2/6 and TLR9 agonists did not prevent virus-induced vagal M2 dysfunction. Interestingly, TLR2/6 and TLR9 pretreatment caused AHR in the absence of virus infection, but did not cause M2 receptor dysfunction. Thus, TLRs produce AHR in a different manner from virus infection. Our data indicate this is not attributable to functional changes in airway smooth muscle M3 muscarinic receptors, because no differences were seen in the airway smooth muscle response to intravenous acetylcholine. Similarly, the potentiation of histamine-induced vagal-reflex and non-neuronal bronchoconstriction by virus and TLR agonists is not likely attributable to changes in the intrinsic contractility of the smooth muscle, because such changes would likely affect the responses to all agonists.

Our results are the first to demonstrate the antiviral effects of TLR2/6 and TLR9 agonist pretreatment on parainfluenza virus infection in guinea pigs, using two therapeutically relevant delivery techniques. Despite significant reductions in virus, the TLR agonists did not protect against virus-induced AHR or virus-induced M2 receptor dysfunction. Interestingly, TLR2/6 and TLR9 agonists produced AHR in both ligand delivery models independent of virus infection, but without dysfunction of the prejunctional inhibitory M2 muscarinic receptors. This suggests that TLR2/6 and TLR9 stimulation can be therapeutically useful for their antiviral effect, but may be limited by the physiological effects of the TLR agonists themselves in the airways.

**Author disclosures** are available with the text of this article at [www.atsjournals.org](http://www.atsjournals.org).

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