Toll-like Receptor 7 Rapidly Relaxes Human Airways

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Rationale: Toll-like receptors (TLRs) 7 and 8 detect respiratory virus single-stranded RNA and trigger an innate immune response. We recently described rapid TLR7-mediated bronchodilation in guinea pigs.

Objectives: To characterize TLR7 expression and TLR7-induced airway relaxation in humans and in eosinophilic airway inflammation in guinea pigs. To evaluate the relaxant effects of other TLRs.

Methods: Human airway smooth muscle strips were contracted with methacholine *in vitro*, and responses to TLR7 and TLR8 agonists were assessed. TLR7-mediated nitric oxide production was measured using a fluorescent indicator, and TLR7 expression was characterized using immunofluorescence. TLR7 signaling was also evaluated in ovalbumin-challenged guinea pigs.

Measurements and Main Results: The TLR7 agonist imiquimod (R837) caused rapid dose-dependent relaxation of methacholine-contracted human airways *in vitro*. This was blocked by the TLR7 antagonist IRS661 and by inhibiting nitric oxide production but not by inhibiting prostaglandin production. TLR7 activation markedly increased fluorescence of a nitric oxide detector. TLR7 was expressed on airway nerves, but not airway smooth muscle, implicating airway nerves as the source of TLR7-induced nitric oxide production. TLR7-mediated relaxation persisted in inflamed guinea pig airways *in vivo*. The TLR8 agonists polyridylic acid and polyadenylic acid also relaxed human airways, and this was not blocked by the TLR7 antagonist or by blocking nitric oxide or prostaglandin production. No other TLRs relaxed the airways.

Conclusions: TLR7 is expressed on airway nerves and mediates relaxation of human and animal airways through nitric oxide production. TLR7-mediated bronchodilation may be a new therapeutic strategy in asthma.

Keywords: Toll-like receptor 7; imiquimod; nitric oxide; asthma; nerve

Toll-like receptor (TLR) 7 is a pattern recognition receptor in the lungs (1–3) that detects single-stranded RNA genomes, common to many respiratory viruses (4). Respiratory viruses cause airway hyperreactivity (an abnormal tendency to contract) (5, 6) and asthma exacerbations (7). A role for TLR7 in the development of virus-induced airway hyperreactivity has been proposed, because TLR7 activation causes a proinflammatory type 1 T-helper cell (Th1) immune response (8) and because TLR7 polymorphisms have been associated with asthma (9). In patients with

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Toll-like receptor (TLR) 7 detects viral RNA and triggers an innate immune response. We describe TLR7 expression on airway nerves and a rapid TLR7-mediated bronchodilator effect in human airways.

What This Study Adds to the Field

TLR7 is expressed on human airway nerves and mediates rapid relaxation of human airways via the production of nitric oxide. This is the first description of TLR7 on airway nerves and represents a novel mechanism for TLR7 signaling.

asthma, however, TLR7 responses were reduced (10). Furthermore, in mouse models of allergic airway inflammation, the TLR7 imidazoquinoline agonists imiquimod (R837) and resiquimod (R848) reduced airway hyperreactivity, airway eosinophilia, goblet cell hyperplasia, and smooth muscle hypertrophy (11–14), whereas TLR7 knockout mice were prone to the development of asthma-like airway pathology after pneumovirus infection (15). These studies suggest that TLR7 may be protective against the development of allergic type 2 T-helper cell airway inflammation, possibly by altering the Th1 versus Th2 immune balance (16). Similarly, a TLR7 agonist is currently in human trials for treatment of allergic rhinitis (17). The sum of TLR7's effects on airway function and inflammation, however, remains unresolved.

Although TLR7 activation classically triggers transcription of antiviral interferons and proinflammatory cytokines via nuclear factor- κ B (8), we recently found a novel TLR7 pathway that causes rapid bronchodilation in guinea pigs (18). This effect was distinct from TLR7's known transcriptional pathways. Multiple TLR7 agonists caused near-complete relaxation of precontracted airways *in vitro* and *in vivo* on the order of seconds to minutes. This effect was reversible after washout, indicating that relaxation was not due to cellular toxicity. TLR7 signaling in this time frame had not previously been assessed.

In this study, we investigated TLR7 expression and TLR7-mediated relaxation of human airways. We report that the TLR7 agonist R837 caused rapid dose-dependent relaxation of precontracted human airways *in vitro* that was blocked by a TLR7 antagonist and by inhibiting nitric oxide production but not by inhibiting prostaglandin production. We confirmed TLR7 induces nitric oxide production in the airways using a nitric oxide-sensing fluorescent probe. We found that TLR7 was not expressed on airway smooth muscle but was present on airway nerves throughout airway smooth muscle, suggesting airway nerves are the source of TLR7-mediated nitric oxide production. We evaluated TLR7's rapid effects in the setting of antigen-induced eosinophilic inflammation, as eosinophils are frequently found in allergic asthma. Eosinophilic airway inflammation did

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not alter TLR7-mediated airway dilation. We also investigated the relaxant effects of other TLRs. Stimulating TLRs 1 to 6 or TLR9 did not relax airway smooth muscle, but TLR8 (which also detects viral single-stranded RNA) did. In contrast to TLR7, TLR8 agonist-mediated bronchodilation was not through nitric oxide. These data are the first to characterize TLR7's expression in the airways and to identify TLR7 on airway nerves in particular. Novel rapid TLR7-mediated bronchodilation in human airways may represent a therapeutic target for TLR7 agonists already in clinical trials for their anti-allergic inflammation properties (17). Some of the results of these studies have been previously reported in the form of an abstract (19).

METHODS

Experimental Animal and Human Airways

Female Hartley guinea pigs (300–400 g; Charles River Labs, Wilmington, MA) and female C57Bl/6 mice (Jackson Labs, Bar Harbor, ME) aged 6 to 8 weeks were handled in accordance with the U.S. Animal Welfare Act. Protocols were approved by the Institutional Animal Care and Use Committee. Human airways were obtained from the Pacific Northwest Tissue Bank. The Research Integrity Office waived institutional review board approval.

Measurement of Airway Contraction and Relaxation *In Vitro*

Organ bath experiments are described in detail in the online supplement. Smooth muscle strips (0.5 cm × 0.5 cm) from human trachea and primary bronchi and guinea pig tracheal segments (three cartilaginous rings long) were suspended in oxygenated Krebs solution. Tension was measured with an isometric force transducer (Grass Instrument, Quincy, MA). Airways were contracted with methacholine (MCh; 3 μ M) and treated with TLR agonists every 10 minutes. Some airways were pretreated for 30 minutes with either the TLR7 antagonist IRS661 (100 μ M), which is specific for TLR7 without antagonism of other TLRs (20), the cyclooxygenase inhibitor indomethacin (1 μ M), which is selective for cyclooxygenase 1 > 2, or the nonspecific nitric oxide synthase inhibitor *N*-methyl-L-arginine acetate salt (L-NMMA) (100 μ M) that blocks all three isozymes of nitric oxide synthase. Airway segments were evenly distributed based on their anatomic location among the treatment groups. Data were normalized to MCh-contracted phosphate-buffered saline vehicle-treated controls.

Detection of Airway Nitric Oxide Production

Copper-conjugated fluorescein (Strem Chemicals, Newburyport, MA) was used to measure nitric oxide (21) in mouse tracheas as described in the online supplement. Mouse tracheas were used due to their ideal tissue thickness for whole-mount microscopy.

TLR7 Expression in Human and Murine Airways

Airway whole mounts were immunostained as described in the online supplement.

In Vivo Guinea Pig Physiology

Guinea pig *in vivo* experiments are described in the online supplement (22). Reproducible bronchoconstrictions were induced by electrical stimulation of the vagus nerves (8 V, 15 Hz, 2 ms duration, 3 s on, 40 s off) in anesthetized, paralyzed, and ventilated guinea pigs. Bronchoconstrictions were measured as increases in peak inspiratory pressure during electrical stimulation. Lung compliance was not independently measured; however, the contribution of lung compliance to rapid changes in airway pressure using this technique are presumed negligible based on prior work (23). R837 (0.003–3 mg/kg, intravenous) was given after every four electrical stimulations. Some animals were treated with indomethacin (1 mg/kg, intravenous) or *N*-nitro-L-arginine methyl ester hydrochloride (L-NAME) (30 mg/kg, intravenous) 30 minutes before R837.

Sensitization and Challenge

Guinea pigs were sensitized to ovalbumin by intraperitoneal injection (Days 1, 3, and 5) and challenged by inhalation (Day 21; see online supplement). Airway physiology was measured 24 hours after the final ovalbumin exposure.

Lung Lavage

Guinea pig lungs were lavaged as described in the online supplement.

Reagents

TLR agonists were from Invivogen (San Diego, CA), and the TLR7 antagonist IRS661 and 21-mer phosphorothioated RNAs PolyU and PolyA were from Invitrogen (Carlsbad, CA). Ovalbumin, L-NAME, L-NMMA, and indomethacin were from Sigma-Aldrich (St. Louis, MO).

Statistics

Dose responses were analyzed using two-way analysis of variance for repeated measures with Bonferroni *post hoc* test (GraphPad Software, La Jolla, CA). Physiologic baselines, cell counts, and mean fluorescence were analyzed using one-way analysis of variance with Bonferroni *post hoc* test. Error bars represent the SEM.

RESULTS

TLR7 Acutely Relaxes Human Airway Smooth Muscle

The TLR7 agonist R837 (10–300 μ M) dose-dependently relaxed human airway smooth muscle strips precontracted with MCh (Figure 1A; $P < 0.005$ compared with vehicle-treated controls).

Figure 1. Toll-like receptor (TLR) 7 agonist relaxes human airway smooth muscle. (A) The TLR7 agonist R837 (10–300 μ M, $n = 9$) relaxed precontracted human airway smooth muscle in dose-dependent fashion. The TLR7 antagonist IRS661 (100 μ M, $n = 5$) blocked R837-mediated smooth muscle relaxation. (B) The nitric oxide synthase inhibitor *N*-methyl-L-arginine acetate salt (L-NMMA) (100 μ M, $n = 4$) blocked R837-mediated relaxation. (C) The cyclooxygenase inhibitor indomethacin (1 μ M, $n = 4$) did not block R837-mediated relaxation. The same R837 data are graphed against each inhibitor. Data shown are means $\pm$ SEM that have been normalized to MCh-contracted TLR agonist vehicle-treated controls. * $P < 0.05$. MCh = methacholine.

Relaxation occurred over seconds to minutes (see Figure E1 in the online supplement) and was blocked by the TLR7 antagonist IRS661 (100 μ M; Figure 1A). IRS661 did not alter baseline airway smooth muscle tone or contractility (Figure E1).

TLR7-Dependent Bronchorelaxation Is Mediated by Nitric Oxide

TLR7-mediated relaxation of precontracted human airway smooth muscle strips was blocked by the nitric oxide synthase inhibitor L-NMMA (100 μ M; Figure 1B), indicating that TLR7's effect is mediated via nitric oxide production. In contrast, the cyclooxygenase inhibitor indomethacin (1 μ M) had no effect on TLR7-mediated bronchorelaxation (Figure 1C). Neither L-NMMA nor indomethacin altered baseline airway smooth muscle tone or contractility to MCh.

TLR8 Agonists PolyU and PolyA Cause TLR7-Independent Relaxation of Human Airway Smooth Muscle

Both TLR8 agonists 21-mer PolyU and PolyA (10–300 μ g/ml) dose-dependently relaxed human airway smooth muscle strips precontracted with MCh (Figure 2A; $P < 0.05$ compared with vehicle-treated controls). Although the TLR7 antagonist IRS661 (100 μ M) and nitric oxide synthase inhibitor L-NMMA (100 μ M) each blocked TLR7 agonist-induced relaxation, neither blocked relaxation induced by the TLR8 agonist PolyU (Figures 2B and 2C). The cyclooxygenase inhibitor indomethacin (1 μ M; Figure 2D) also had no effect on PolyU-mediated relaxation. These data

Figure 2. 21-mer PolyRNA causes Toll-like receptor (TLR) 7-independent human airway smooth muscle relaxation. (A) The 21-mer single-stranded RNAs PolyU and PolyA (10–300 μ g/ml, $n = 4$ per group) caused dose-dependent relaxation that was significantly different from MCh-contracted TLR agonist vehicle-treated control experiments ($P < 0.05$). Neither the TLR7 antagonist IRS661 (B, 100 μ M, $n = 4$), the nitric oxide synthase inhibitor *N*-methyl-L-arginine acetate salt (L-NMMA) (C, 100 μ M, $n = 4$), nor the cyclooxygenase inhibitor indomethacin (D, 1 μ M, $n = 3$) blocked PolyU-mediated bronchorelaxation. The same PolyU data are graphed against each inhibitor. Data shown are means $\pm$ SEM that have been normalized to MCh-contracted TLR agonist vehicle-treated controls. MCh = methacholine.

demonstrate that PolyU relaxes human airway smooth muscle independent of TLR7, nitric oxide production, or the cyclooxygenase pathway, likely via TLR8.

TLR7 Causes Nitric Oxide Production in Mouse Airways

Isolated whole-mount mouse trachealis muscle loaded with a nitric oxide sensor (copper-conjugated fluorescein derivative; 1 μ M) (21) had dramatically increased nitric oxide fluorescence after R837 exposure (30–300 μ M; Figures 3A and 3B). R837-induced fluorescence was blocked by the TLR7 antagonist IRS661 (100 μ M) and by the nitric oxide synthase inhibitor L-NMMA (100 μ M), indicating that R837 causes nitric oxide production by stimulating TLR7.

TLR7 Is Expressed on Human and Mouse Airway Nerves but Not Airway Smooth Muscle

Staining human and mouse airways with antibodies for TLR7 (green) and the pan-neuronal marker PGP9.5 (red) demonstrated TLR7 on a specific subpopulation of airway nerves and airway parasympathetic ganglia (Figure 4). TLR7 was not detected on human or mouse airway smooth muscle. In humans, TLR7 was expressed on large bundles of axons (Figures 4A and 4B) and on individual axons innervating airway smooth muscle alongside TLR7-negative axons (Figures 4C and 4D). Similarly, in mice, TLR7 was expressed on nerve axons and on certain nerve cell bodies (Figures 4F and 4G) as well as individual axons innervating airway smooth muscle (Figures 4H and 4I). IgG primary controls immunostained with PGP 9.5 and 4',6-diamidino-2-phenylindole, but with nonspecific IgG substituted for the TLR7 primary antibody, showed minor punctate nonspecific fluorescein binding (Figure 4E). Airways immunostained for PGP 9.5 and 4',6-diamidino-2-phenylindole but omitting the TLR7 primary antibody showed no fluorescein binding (Figure 4J).

TLR7 and -8 Agonist-mediated Airway Relaxation Is Unique among TLRs 1 to 9

We tested the role of other TLRs in mediating relaxation using guinea pig airways *in vitro* because guinea pig is pharmacologically similar to humans and spares the use of limited human tissues. As in human airways, R837 (10–300 μ M) and PolyU (10–300 μ g/ml) relaxed guinea pig airway segments precontracted with MCh *in vitro* (Figure 5; $P < 0.05$ compared with vehicle-treated controls). In contrast, agonists for TLR2/1 (Pam₃CSK₄), TLR2/6 (Pam₂CSK₄), TLR3 (Poly I:C), TLR4 (LPS), TLR5 (flagellin), and TLR9 (ODN2395) did not induce airway relaxation.

TLR7-mediated Bronchorelaxation Is Not Altered by Antigen Sensitization and Challenge

TLR7-mediated bronchorelaxation was investigated in guinea pigs with antigen-induced eosinophilic inflammation *in vivo*. R837 inhibited vagally mediated bronchoconstrictions equally in nonsensitized, ovalbumin-sensitized, and ovalbumin-sensitized and -challenged guinea pigs (Figure 6A; 0.003–3 mg/kg, intravenous), indicating that TLR7-mediated relaxation is not affected by eosinophilic inflammation. Eosinophilia in the lungs was confirmed by bronchoalveolar lavage (Figure 6B). There were no differences in baseline peak inspiratory pressures or hemodynamics between the groups (Table 1).

TLR7-Dependent Bronchorelaxation in Guinea Pigs *In Vivo* Is Mediated by Nitric Oxide

R837-mediated (0.003–0.1 mg/kg, intravenous) bronchorelaxation was completely inhibited by the nitric oxide synthase

Figure 3. Toll-like receptor (TLR) 7 stimulates nitric oxide production in the airway. (A) R837 (30 μ M, $n = 4$; 100 μ M, $n = 3$; 300 μ M, $n = 7$) significantly increased fluorescence of murine airway smooth muscle loaded with a nitric oxide-detecting fluorescent indicator (* $P < 0.05$ compared with vehicle control; *** $P < 0.001$ compared with all other treatment groups except 100 μ M R837). The TLR7 antagonist IRS661 (100 μ M, $n = 5$) and the nitric oxide synthase inhibitor *N*-methyl-L-arginine acetate salt (L-NMMA) (100 μ M, $n = 5$) blocked R837-induced increases in fluorescence. IRS661 ($n = 5$) and L-NMMA ($n = 5$) did not independently affect fluorescence compared with vehicle controls ($n = 5$). The mean of 10 nonoverlapping images was obtained per experimental replicate. (B) Representative images obtained with Zeiss laser scanning confocal microscope (dimensions 134 $\times$ 134 μ m; magnification = 63 $\times$; numerical aperture = 1.4).

inhibitor L-NAME (Figure 6C; 30 mg/kg, intravenous) in non-sensitized guinea pigs *in vivo*, indicating that in this dose range R837's effect was mediated via nitric oxide production. Higher doses of R837 (0.3–3 mg/kg, intravenous) were not inhibited by L-NAME, indicating a non-nitric oxide-dependent mechanism at high doses in this species. The cyclooxygenase inhibitor indomethacin (1 mg/kg, intravenous) had no effect on R837-mediated bronchorelaxation *in vivo* (Figure 6D).

TLR7 Does Not Relax Vascular Smooth Muscle

R837 (0.003–3 mg/kg intravenous) did not affect heart rate (Figure 6E) or mean arterial blood pressure (Figure 6F) in guinea pigs *in vivo*. Baseline physiologic characteristics are given in Table 1.

DISCUSSION

TLR7 binds respiratory virus single-stranded RNA and via transcription triggers production of antiviral interferons and proinflammatory cytokines (8). Here we report a novel TLR7 pathway that rapidly and potently relaxes precontracted human airways through the production of nitric oxide. The speed of relaxation is on the order of seconds to minutes, which is not consistent with TLR7's known transcriptional effects. TLR7-mediated relaxation of human airways did not involve a cyclooxygenase

pathway, which differs from our report in guinea pig airways *in vitro*, in which R837 caused relaxation through both a TLR7-dependent nitric oxide pathway and TLR7-independent cyclooxygenase pathway (18). These findings represent a fundamentally unique TLR7 signaling mechanism in human airways.

TLR7 has not previously been linked to nitric oxide production, which raises the possibility that TLR7 regulates airway functions beyond relaxation. Nitric oxide is generated from L-arginine by nitric oxide synthases and serves as a cell-signaling molecule in many physiologic processes (24). In the lungs, nitric oxide affects epithelial cell ciliary beat frequency (25) and trans-epithelial anion transport (26), which facilitate airway clearance. Nitric oxide affects parasympathetic nerve function, which controls bronchoconstriction (27). Furthermore, nitric oxide has direct antiviral effects (28, 29) and can influence immune signaling (30). It is possible that TLR7 regulates many airway functions via its control of nitric oxide production.

Although TLR7's production of nitric oxide is novel, nitric oxide's ability to relax smooth muscle is well characterized. Nitric oxide causes relaxation via reduction in intracellular calcium by a cGMP-dependent pathway (24). This effect is not specific to particular contractile agonists and therefore dilates airways irrespective of the mediators of bronchoconstriction. Our prior work in guinea pigs found that TLR7 relaxed airways precontracted with a variety of agonists, including histamine and potassium chloride (18). In this study, airway contractions

Figure 4. Toll-like receptor (TLR) 7 is expressed on a subpopulation of human and murine airway nerves but not airway smooth muscle. Whole mounts of human and murine trachea and primary bronchi were immunostained with antibodies to TLR7 (green), the pan-neuronal marker PGP 9.5 (red), and the nuclear stain 4',6-diamidino-2-phenylindole (DAPI) (blue). Both human (A–D) and murine (F–I) airways demonstrated colocalization of TLR7 and PGP 9.5 indicating the presence of TLR7 on specific airway nerves and nerve cell ganglia. TLR7 was not expressed on airway smooth muscle in either species. (A, B) TLR7 expression on airway nerve bundles. (C, D) TLR7-positive nerves innervating airway smooth muscle alongside TLR7-negative nerve axons. (F) Specific nerve cell bodies expressing TLR7. (G) TLR7-negative nerve cell body alongside TLR7-positive axons. (H, I) TLR7-positive nerve axons innervating airway smooth muscle. (E) IgG primary control immunostained with PGP 9.5 and DAPI, but substituting nonspecific IgG for the TLR7 primary antibody, showed minor punctate nonspecific fluorescein binding. (J) No primary antibody control immunostained with PGP 9.5 and DAPI, but omitting the TLR7 primary antibody showed no fluorescein binding. Images were obtained with a Zeiss laser scanning confocal microscope at 20× and 63× magnification.

which is a common inflammatory cell type in asthma. As a result, excessive acetylcholine is released at the neuromuscular junction, causing increased M3 activation and airway hyperreactivity (31). Therefore, our experiments focused on a particularly pertinent dysfunctional pathway in allergic airway inflammation.

In the present study, we found that TLR7 was strongly expressed by airway nerves but not by airway smooth muscle. To our knowledge, TLR7 has not previously been described on airway nerves, whereas an absence of TLR7 expression by human airway smooth muscle has only been assessed in primary cell culture (32). TLR7's pattern of expression suggests that airway nerves, but not airway smooth muscle, are the source of TLR7-mediated nitric oxide production. Nonadrenergic noncholinergic inhibitory (NANCI) airway nerves are known to produce nitric oxide and relax the airways (33), but it remains unknown if NANCI nerves are the TLR7-positive nerves identified in this work. Alternatively, neuronal TLR7 may cause nitric oxide production by airway smooth muscle via an intermediate cell. Although airway epithelium also expresses TLR7 (3), we have previously shown that epithelium is not necessary for TLR7's relaxant effect (18).

TLR7's expression by airway nerves also suggests that airway nerves have a role in the detection and response to respiratory viruses. The presence of TLRs on central and peripheral nerves has only recently been established, and their function remains uncertain. In Schwann cells, TLR7 may be involved in immune surveillance (34), whereas dorsal root ganglia exposed to the dual TLR7 and -8 agonist resiquimod (R848) had increased calcium flux, suggesting that TLR7 regulates sensory nerve function (35). TLR7 may have a similar effect on airway nerve signaling during respiratory virus infection. Furthermore, in the peripheral nervous system, TLR expression varied by nerve subtype (34), which is consistent with our finding that not all airway nerves express TLR7.

The presence of TLR7 on airway nerves implicates neuronal nitric oxide synthase as the particular isozyme involved in TLR7's effect. Neuronal nitric oxide synthase is constitutively expressed and rapidly activated by calcium to produce nitric oxide (36). Our observation that TLR7's effects occurs within minutes *in vitro* and within seconds *in vivo* is consistent with neuronal nitric oxide synthase's kinetics. Although the nitric oxide synthase inhibitors used in these experiments were not specific for a particular nitric oxide synthase isozyme, endothelial nitric oxide synthase is located in airway epithelium (37), which is not involved in TLR7's effect, whereas inducible nitric oxide synthase is transcriptionally

were induced with either methacholine *in vitro* or by parasympathetic nerve stimulation *in vivo*. Both methods activate post-junctional M3 muscarinic receptors (G_q -class G-coupled protein receptors) on airway smooth muscle. Parasympathetic nerves become dysfunctional in the setting of eosinophilic inflammation,

Figure 5. Toll-like receptor (TLR) 7 and 8 agonist-mediated airway relaxation is unique among TLRs 1 to 9. Isolated guinea pig airways in an organ bath were precontracted with methacholine (MCh) (3 μM) and treated with TLR1–9 agonists. Only TLR7 agonist R837 (10–300 μM, $n = 7$) and PolyU (10–300 μg/ml, $n = 4$) caused airway relaxation. The TLR2/1 agonist Pam₃CSK₄ (0.01–1 μM, $n = 4$), TLR2/6 agonist Pam₂CSK₄ (0.01–1 μM, $n = 3$), TLR3 agonist Poly I:C (3–300 μg/ml, $n = 7$), TLR4 agonist LPS (0.01–1 μg/ml, $n = 6$), TLR5 agonist flagellin (0.01–1 μg/ml, $n = 4$), and TLR9 agonist ODN2395 (0.03–3 μM, $n = 4$) did not cause airway relaxation. Data shown are means $\pm$ SEM. * $P < 0.05$ and *** $P < 0.001$ compared with vehicle-treated controls.

regulated (38) and is therefore unlikely to be involved in the immediate effect of TLR7 stimulation.

Because the 21-mer single-stranded PolyU and PolyA RNA relax guinea pig airways (18), we tested them here as relaxants of contracted human airways. Both PolyU and PolyA relaxed

human airways *in vitro*, although unlike in guinea pigs, their effect was not mediated by TLR7, or by nitric oxide or prostaglandins. This TLR7-independent effect in human airways is novel. In guinea pigs, PolyU exclusively activated TLR7 (18). In humans, however, uridylic acid and adenylic acid-rich motifs

Figure 6. Antigen sensitization and challenge does not affect Toll-like receptor (TLR) 7-mediated bronchodilation. (A) R837 (0.003–3 mg/kg, intravenous) inhibited vagally mediated bronchoconstriction in nonsensitized ($n = 5$), ovalbumin-sensitized ($n = 6$), and ovalbumin-sensitized and -challenged ($n = 6$) guinea pigs *in vivo*. (B) Bronchoalveolar lavage demonstrated an eosinophil-predominant inflammatory cell influx in the sensitized and challenged group. * $P < 0.05$ compared with nonsensitized animals. (C) Bronchodilation induced by R837 at doses up to 0.1 mg/kg were blocked by the nitric oxide synthase inhibitor *N*-nitro-L-arginine methyl ester hydrochloride (L-NAME) (30 mg/kg, intravenous; $n = 5$; * $P < 0.05$ compared with vehicle-treated control animals). (D) R837-mediated relaxation was not blocked by the cyclooxygenase inhibitor indomethacin (1 mg/kg, intravenous) in nonsensitized control guinea pigs *in vivo* ($n = 6$). R837 had no effect on heart rate (E) or mean arterial blood pressure (F). Data shown are means $\pm$ SEM.

preferentially activate TLR8 and not TLR7 (39). Although less is known about TLR8, it is highly homologous to TLR7 and similarly recognizes single-stranded RNA. Furthermore, species-specific variation in PolyU-mediated TLR8 activation has previously been described in immune cells (40, 41). PolyU activated TLR8 in human and bovine leukocytes but failed to activate TLR8 on immune cells in mice and rats (39). Our findings are consistent with these data. TLR8 remains incompletely understood, in part due to a lack of TLR8-specific inhibitors and potential cross-regulation of TLR7 by TLR8 in certain species (42,

43). Our data suggest that PolyU and PolyA cause TLR8-mediated bronchorelaxation in human airways, although the specific mechanism remains a focus for further investigation.

Our data indicate that TLR7 and TLR8 may have a rapid salutary relaxant effect in the airways. This effect contradicts the airway hyperreactivity and bronchospasm caused by respiratory viruses (5, 44). However, respiratory virus infections activate many immune receptors in the airway, including other TLRs (TLR3, TLR9), as well as retinoic acid-inducible gene-I (RIG-I)-like receptors and nucleotide-binding oligomerization domain

TABLE 1. BASELINE PHYSIOLOGIC CHARACTERISTICS BEFORE R837 ADMINISTRATION

Treatment Group	Ppi (mm H ₂ O)	HR (beats/min)	SBP (mm Hg)	DBP (mm Hg)
Nonsensitized ($n = 5$)	129.0 $\pm$ 6.4	280.0 $\pm$ 12.4	43.0 $\pm$ 4.0	27.0 $\pm$ 1.0
Nonsensitized + L-NAME ($n = 5$)	115.0 $\pm$ 11.2	275.0 $\pm$ 15.2	65.0 $\pm$ 4.2*	50.6 $\pm$ 4.5*
Nonsensitized + indomethacin ($n = 6$)	128.3 $\pm$ 14.2	268.3 $\pm$ 11.8	42.3 $\pm$ 3.1	24.2 $\pm$ 2.6
Sensitized ($n = 6$)	111.4 $\pm$ 8.6	282.9 $\pm$ 6.2	43.0 $\pm$ 2.5	23.4 $\pm$ 1.7
Sensitized + challenged ($n = 6$)	120.0 $\pm$ 4.7	278.3 $\pm$ 6.7	38.5 $\pm$ 3.2	17.0 $\pm$ 1.8

Values shown are means $\pm$ SEM. DBP = diastolic blood pressure; HR = heart rate; Ppi = peak inspiratory pressure; SBP = systolic blood pressure.

* $P < 0.05$ compared with all other group means.

TABLE 2. HUMAN DONOR CHARACTERISTICS

#	Age (yr)	Sex	Race	Tobacco Use	Cause of Death	Comorbidities
1	71	F	White	No	Head trauma	—
2	55	F	White	N.G.	CVA	—
3	54	M	Hispanic	No	CVA	Hypertension
4	52	M	White	No	CVA	—
5	44	M	White	Yes	CVA	IVDU
6	47	F	White	No	PEA	Acute respiratory illness
7	50	F	N.G.	No	CVA	Obesity
8	24	M	Hispanic	N.G.	Head trauma	IVDU
9	25	F	White	No	Head trauma	Alcohol abuse
10	40	F	White	No	Anoxia	Hypertension
11	60	F	White	N.G.	Head trauma	—
12	48	M	Asian	No	CVA	Hypertension
13	52	M	N.G.	N.G.	N.G.	—
14	45	F	White	No	MI	—
15	49	F	White	N.G.	CVA	—
16	65	F	White	Yes	Anoxia	—
17	55	F	White	Yes	CVA	Alcohol abuse

Comorbidities were included when available. No specific lung conditions were available at time of donation. CVA = cerebral vascular accident; F = female; IVDU = intravenous drug use; M = male; MI = myocardial infarction; N.G. = not given; PEA = pulseless electrical activity.

(NOD)-like receptors (8). Our model did not investigate the pleiotropic effects of viruses in the airway but instead focused on the early events after isolated TLR7 activation. The rapid effects of TLR7 activation described in this work have not previously been investigated in humans.

To determine if other TLRs besides TLR7 and TLR8 cause airway relaxation, we tested agonists for TLRs 1 to 9 in guinea pigs airways *in vitro*. We found that no other TLR causes rapid airway relaxation, despite the similarities in many of the TLRs' signaling pathways. For example, TLR9 recognizes viral unmethylated DNA, is located on the endosome alongside TLR7 and TLR8, and signals through the adaptor protein MyD88, like TLR7 and TLR8 (45). TLR2/1 and TLR2/6 are heterodimerized receptors, whereas TLR4 and TLR5 are homodimers, but all of these receptor complexes signal through MyD88 (8). TLR3 does not signal through MyD88 but is located on the endosome and detects viral double-stranded RNA (46). Despite these similarities, no other TLRs caused airway relaxation, which reinforces the novelty of the rapid TLR7 and TLR8 effects.

Given the association between TLR7 and asthma (9, 10), we investigated TLR7 signaling in an antigen sensitization and challenge model in guinea pigs. R837 inhibited vagally mediated airway contractions in guinea pigs *in vivo* through production of nitric oxide. Similar to our previous work in guinea pigs, however, R837 also had significant off-target effects at higher doses (18). Importantly, R837's bronchodilating effect was not altered by antigen sensitization alone or by sensitization and challenge in guinea pigs. This suggests TLR7's function may be preserved in the setting of eosinophilic inflammation, common in allergic asthma. Furthermore, despite TLR7's profound bronchodilatory effect in the airways, TLR7 stimulation had no effect on blood pressure (mean arterial pressure) or heart rate. TLR7 agonists are unlikely to have cardiovascular effects in humans, either, given that human systemic and pulmonary vascular endothelium does not express TLR7 (47, 48). A lack of systemic effects is advantageous for designing TLR7-targeted therapies for the airways.

Although our study demonstrates TLR7-mediated nitric oxide production and airway relaxation across multiple mammalian species, the heterogeneity of our human subjects was constrained by the available donor pool (Table 2). Human donors were mostly white and more often women. Minimal clinical characteristics (e.g., the presence or absence of respiratory disease) were given at the time of donation. Our donors, however, were from all adult ages. Given the similarities between TLR7's

effect in humans and the other mammalian species tested, we do not believe our human donor characteristics limit the validity or interpretation of the data.

This study is the first to identify TLR7 on airway nerves and the first to demonstrate a rapid and potent TLR7-mediated relaxation of human airways. We define a TLR7-independent pathway mediated by the TLR8 agonists PolyU and PolyA, and we have established that TLR7's function is not altered by eosinophilic inflammation. These findings suggest TLR7 may have a role as a therapeutic target, particularly in allergic asthma. A TLR7 agonist capable of inducing airway relaxation while suppressing Th2 immune responses has the potential to address both components of the allergic asthma phenotype: bronchoconstriction and allergic inflammation. Furthermore, neuronal TLR7 expression is compelling evidence that airway nerves have a role in the antiviral immune response and raises the possibility that TLR7 alters airway nerve function during a respiratory virus infection.

Author disclosures are available with the text of this article at www.atsjournals.org.

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