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The Therapeutic Potential of Toll-like Receptor 7 Stimulation in Asthma

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Abstract

Asthma is an inflammatory disorder of the airways frequently characterized by an excessive Th2 adaptive immune response. Activation of Toll-like receptor (TLR)-7, a single-stranded viral RNA receptor that is highly expressed in the airways, triggers a rapid innate immune response and favors a subsequent Th1 response. Because of this role in pulmonary immunoregulation, TLR7 has gained considerable interest as a therapeutic target in asthma. Synthetic TLR7 ligands, including the imidazoquinolines imiquimod (R837) and resiquimod (R848), and 8-hydroxyadenine derivatives have been developed for other clinical indications. TLR7 activation prevents ovalbumin-induced airway hyperreactivity, eosinophilic inflammation, goblet cell hyperplasia and airway remodeling in murine models of asthma. TLR7 activation also inhibits viral replication in the lung and prevents virus-induced airway hyperreactivity. Furthermore, it has recently been shown that stimulating TLR7 rapidly relaxes airway smooth muscle, dilating the airways. This bronchodilating effect, which occurs in seconds to minutes and depends on rapid production of nitric oxide, indicates that TLR7 can signal via previously unrecognized pathways. The effects of decreasing the allergic Th2 response, acting as an immediate bronchodilator, and promoting an antiviral immune environment, make TLR7 an attractive drug target. We examine the current understanding of TLR7 as a therapeutic target and its translation to asthma treatment in humans.

Keywords

asthma; Toll-like Receptor 7; inflammation; bronchodilator

Introduction

Asthma is characterized by bronchial wall inflammation, variable airflow obstruction and airway hyperresponsiveness (an abnormal tendency to constrict, AHR). Chronically, asthma

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leads to maladaptive airway remodeling including smooth muscle proliferation, goblet cell hyperplasia, and excessive mucus production. In many cases, asthma's pathogenesis is due to an excessive type-2 T-helper (Th2) cell adaptive immune response in the lungs [1]. Epidemiologic data suggest that this imbalance between Th2 and type-1 T-helper (Th1) cell immune responses may be the result of reduced childhood microbial exposure [2]. Microbes are detected by a set of specific pattern recognition receptors of the innate immune system, termed Toll-like Receptors (TLRs), which are activated by highly conserved molecular motifs on invading microorganisms. Ten distinct TLRs have been identified in humans. TLRs signal through NF- κ B to produce many pro-inflammatory cytokines, chemokines, adhesion molecules, and enzymes including the inducible form of nitric oxide synthase. In the case of TLR3, TLR7, and TLR8, Th1-directed responses are produced including type 1 interferons (IFN) [3]. A lack of TLR activation and Th1 responses during critical periods of immune system maturation may allow Th2 adaptive immunity to predominate [4].

TLR7 is of particular relevance to asthma. TLR7 is expressed in bronchial epithelial cells [5], airway smooth muscle [6] and several immune cell types, including lung plasmacytoid dendritic cells, natural killer cells (NKT), B lymphocytes and eosinophils [7–10]. TLR7 is located intracellularly in the endosome and binds to single stranded viral RNA, a molecular motif common to many respiratory viruses [11, 12]. The detection of respiratory viruses by TLR7 activates Th1 antiviral responses, promoting viral clearance. TLR7, however, is not alone in this function. TLR8 is highly homologous to TLR7 and also binds single stranded viral RNA. Additionally, TLR3 binds double-stranded RNA and TLR9 binds unmethylated viral DNA [3].

Respiratory viruses are a frequent cause of asthma exacerbations [13, 14]. Virus clearance relies on a TLR-mediated Th1 response, which may be inhibited in the asthmatic Th2 microenvironment [15]. Robust Th1 cytokine induction was associated with protection from exacerbation, whereas Th2 cytokines correlated with increased disease severity [16]. Certain viruses have evolved to inhibit TLR7 signaling as a means for enhancing infectivity [17]. Furthermore, TLR7 polymorphisms have been associated with asthma in humans [18, 19]. Taken together, these data support a role for TLR7 in asthma.

Asthma therapies target the two cornerstones of the asthma phenotype: airway inflammation and excessive bronchoconstriction. Oral or inhaled glucocorticoids reduce airway inflammation and serve as chronic, controller therapies. Inhaled beta-agonists reverse bronchoconstriction in both short-acting rescue, or long-acting controller medications [20]. Adjunctive therapies may include muscarinic antagonists [21], leukotriene inhibitors [20] or IgE antibody immunomodulators [22]. Despite this approach, asthma accounts for approximately \$50 billion in United States direct health care costs [23] and its prevalence is rising [24]. Novel asthma therapies are needed to address these deficiencies in asthma management.

Recently, TLR7 has gained considerable interest as a therapeutic target in the treatment of a variety of diseases [25, 26, 27, 28]. Several synthetic TLR7 agonists have been developed including the imidazoquinolines imiquimod (R837) and resiquimod (R848) [29], guanine nucleoside analogs [30] and the 2-substituted-8-hydroxyadenine derivative SA-2 [31]. The

potent immunomodulatory properties of TLR7 make it particularly appealing for allergic diseases. For example, a phase 1 clinical trial is currently being conducted using a TLR7 agonist for the treatment of allergic rhinitis [32]. Asthma is also frequently an allergic disease. TLR7 presents a new focus for drug development. The purpose of this manuscript is to review the current understanding of TLR7 as a therapeutic target in asthma, including its newly identified role as a bronchodilator.

TLR7 attenuates airway inflammation

Airway inflammation is central to asthma pathogenesis, and leads to bronchoconstriction and AHR. Originally identified on autopsies of patients with fatal asthma, inflammation has been identified even in mild disease [33]. Exposure to inhaled allergens in sensitized individuals results in production of Th2 cytokines including IL-4, IL-5 and IL-13. These cytokines contribute to the recruitment of eosinophils, mast cells and lymphocytes to the airways, which subsequently induce airway edema and bronchoconstriction [1]. Suppressing this allergic cascade is a central focus for asthma therapy.

TLR7 stimulation prevents Th2-mediated airway inflammation in a variety of animal models of asthma [34]. Administration of R848 or R837 acutely reduces IL-4, IL-5, IL-13 and eosinophilic airway inflammation in ovalbumin sensitized and challenged animals (Table 1). This effect occurs with substitute adenine-2 and 8-oxoadenine derivatives as well, confirming that TLR7 suppresses Th2-directed inflammation in response to a wide variety of TLR7 agonists. Attenuation of airway inflammation occurs whether TLR7 agonists are given at the time of ovalbumin sensitization or challenge, suggesting TLR7 stimulation may be effective against both disease development and exacerbation. TLR7 stimulation reduces levels of eotaxin, a potent eosinophil chemoattractant in the lung [49], which may contribute to the reduction in eosinophilic infiltration in this model. TLR7 stimulation also reduces IgE [38]. The effect of TLR7 signaling may further depend on the IL-10-mediated inhibition of IL-17 and IL-13 production [52]. Indeed, IL-10 and IL-12 are thought to play a central role in the TLR7 response, as both are required for full protective effects of TLR7 signaling [42].

The suppression of inflammation by TLR7 agonists persists in chronic models of experimental asthma. TLR7 stimulation attenuates leukocyte infiltration into the airways up to 40 days after TLR7 agonist treatment, despite repeated ovalbumin challenge [50]. Similarly, R848 suppresses leukocyte recruitment, as well as IL-5 and IL-13 production at 2- and 4-weeks after treatment [45]. These data support the use of TLR7 stimulation in suppression of Th2-directed airway inflammation.

Conversely, Th1 cytokines were frequently, although not uniformly upregulated by TLR7 stimulation. The imidazoquinolines R837 and R848 increase IFN- γ and IL-6, while the synthetic adenine TLR7 agonist SA-2 decreases IFN- γ while modulating T cell regulation through IFN- α , IL-27 and IL-10 [52]. Differences in the observed Th1 cytokine levels may represent differences in the timing of treatment and sample collection protocols, as Th1-mediated type-1 IFN production peaked at 2 hours and had largely resolved by 6 hours after treatment [53]. Unique regulation of TLR7 signaling via different TLR7 ligands however cannot be ruled out. The concept of selective regulation of the complex TLR7 signaling

cascade is not without precedent. Biffen et al. recently demonstrated preferential IFN production over NF- κ B activation using a rapidly metabolized adenine derivative antedrug [50]. Similarly, TLR7 stimulation with an imidazoquinoline 3M-011 results in differential expression of Th1-directed cytokines in a dose-dependent manner. At low concentrations, 3M-011 produces only type 1 IFN. Higher doses additionally produce TNF- α and IL-12 [53]. These data provide evidence for functional selectivity of TLR7 signaling at the level of receptor occupancy. Alternatively, these Th1 differences may be due to differences in off-target effects of TLR agonists including potential dual activation of TLR8. R837 is believed to activate only TLR7, while R848 activates both TLR7 and TLR8 in humans, but was thought to activate only TLR7 in mice [54]. Recent data however suggest that TLR8 is indeed functional in mice when stimulated by imidazoquinolines in the presence of oligodeoxynucleotides [55]. The effects of TLR7-TLR8's functional redundancy on Th1 cytokine production and TLR7 agonist off target effects remain a focus of investigation.

Downstream TLR7 signaling affects specific leukocyte populations that are implicated in mediating suppression of asthmatic airway inflammation. Invariant NKT (iNKT) cells are believed to confer protection against asthma at disease onset [56, 57]. TLR7 stimulation alters gene expression and recruits iNKT cells to the lungs [43]. Reversal of ovalbumin-induced allergic inflammation is seen after adoptive transfer of splenocytes from iNKT-positive R848-treated wildtype mice, but not from R848-treated iNKT-deficient donors [47]. Similarly, Foxp3-expressing regulatory T (Treg) cells have been implicated in the prevention of airway inflammation. Depleting Treg cells or blocking the Treg cell mediator TGF- β reverses TLR7-mediated suppression of lung inflammation [49]. In contrast, depletion of cytotoxic CD8⁺ T cells has no effect on acute TLR7-mediated prevention of airway inflammation [43]. However, CD8⁺ T cells may play a role in late suppression of Th2 responses in an IFN- γ -dependent manner [45].

TLR7 promotes Th1 responses in human immune cells

The effects of TLR7 signaling are not limited to animal models of Th2-directed inflammation. Human peripheral mononuclear cells decrease IgE production in response to R848 in an IFN- γ -dependent manner *in vitro* [58]. R848 inhibits IgE synthesis in favor of IgA in human B lymphocytes [59]. Furthermore, Th2 cells are reduced and Th1 cells are increased after treatment with SA-2 and R848 [60]. Increases in regulatory and Th1-directed cytokines are also observed, including IL-10, IL-12, IFN- γ , TNF- α and IFN- α [61]. These data confirm the potent immunomodulatory effects of TLR7 stimulation in altering the Th2/Th1 balance and demonstrate these effects are conserved in humans.

TLR7 reverses airway hyperresponsiveness

Airway hyperresponsiveness (AHR) may result from Th2-mediated airway inflammation in asthma, and manifests clinically as wheezing, shortness of breath and cough. AHR is well characterized in allergen challenged animal models of asthma. Bronchoconstriction in response to inhaled methacholine is potentiated by ovalbumin sensitization and challenge [62].

TLR7 agonists, given either intranasally [39, 45], intraperitoneally [40, 48, 49, 52] or via inhalation [35, 36, 37] attenuate ovalbumin-induced AHR. TLR7 stimulation ameliorates AHR when animals are treated either 24-hours before or 30-minutes after antigen challenge, suggesting a therapeutic role of TLR agonists for both chronic disease progression and acute exacerbations. Prevention of AHR was dependent on iNKT cells and IFN- γ production [47]. Intracellular signaling through NF- κ B, p38 MAP kinase and ERK have all been implicated as mediators of this response *in vitro* [6], although *in vivo* data regarding these pathways are lacking.

TLR7 prevents airway remodeling

Chronic asthma results in airway remodeling including smooth muscle proliferation, goblet cell hyperplasia, and fibrosis. Over time, humans with asthma may develop irreversible airflow obstruction. One goal of treatment is preventing these changes [63]. In mouse models of chronic asthma, airway remodeling occurs following repeated ovalbumin challenges. TLR7 stimulation prevents airway remodeling. R848 administration given 24 hours before antigen challenge prevents airway smooth muscle and goblet cell hyperplasia [41]. R837 administered by inhalation similarly prevents goblet cell hyperplasia and collagen deposition in a chronic sensitization model [37]. Attenuation of airway remodeling is present as early as 24 hours following antigen challenge, and persists up to four weeks after TLR7 stimulation. These data suggest TLR7 may prevent chronic irreversible airway remodeling associated with asthma.

TLR7 mediates acute bronchodilation

The effects of TLR7 stimulation described above all likely involve changes in gene expression. More recently, Kaufman et al. demonstrated TLR7 stimulation with R837 in guinea pigs results in immediate bronchodilation within seconds *in vivo* and *in vitro* [64]. The TLR7 antagonist IRS661 or nitric oxide synthase inhibitor L-NMMA partially inhibited R837-mediated bronchodilation. The magnitude of inhibition by IRS661 and L-NMMA was not additive, confirming R837 bronchodilates via production of nitric oxide in a TLR7-dependent manner. In addition, R837-induced bronchoconstriction was partially inhibited by the cyclooxygenase inhibitor indomethacin and the large conductance, calcium-gated potassium channel blocker paxilline. The magnitude of R837 inhibition was greatest when blocking the TLR7-nitric oxide pathway and the cyclooxygenase-potassium channel pathway concurrently, suggesting R837 works via a TLR7-dependent-nitric oxide pathway in parallel with a TLR7-independent pathway involving prostanoid production and potassium channels. While the mechanism of the R837-mediated TLR7-independent effect remains unresolved, it may be through TLR8, which is highly homologous to TLR7 and similarly binds single stranded viral RNA. Given that R837 acts through TLRs responsible for binding respiratory virus RNA, it is possible that this bronchodilatory mechanism protects against airway obstruction during early respiratory virus infection.

This previously unrecognized role for TLR7 represents a major change in our understanding of TLR7 signaling in the lungs. The acute bronchodilating effect is fundamentally different from TLR7-mediated reversal of AHR. In those models, a hyperresponsive

bronchoconstrictor response due to allergen challenge is reduced to the response in the non-challenged control animals. In contrast, acute TLR7 relaxant effects inhibit normal physiologic bronchoconstriction in response to acetylcholine or vagal stimulation in control nonsensitized and ovalbumin sensitized animals. This effect occurs in seconds, and acts at the level of the airway smooth muscle, independent of airway epithelium. These characteristics suggest TLR7 has relevance as a rescue therapy in asthma for reversal of acute bronchoconstriction. Alternatively, a long acting TLR7 agonist might serve as a maintenance bronchodilator.

TLR7 stimulation for treatment of respiratory virus infection

Respiratory viruses are responsible for the majority of asthma attacks [13]. Despite this burden of disease, there are few therapeutic options. Vaccines are the mainstay of prevention for respiratory virus infection, but are limited principally to influenza virus. Several studies found reduced asthma exacerbations following vaccination, however others do not replicate this finding [65]. Furthermore, no vaccine exists for rhinovirus, the most common cause of virus-induced asthma exacerbations, due in part to its multiple serotypes [14]. New methods for prevention and treatment of respiratory virus infection are needed.

Effective vaccines require robust Th1 cellular responses that propagate antibody generation via antigen presentation to CD8⁺ T cells by dendritic cells [66]. To boost Th1-mediated responses, an ovalbumin vaccine was conjugated to an imidazoquinoline molecule. TLR7 agonist-ovalbumin conjugation increases dendritic cell vaccine uptake and T cell responses. Maximum effect depends on IL-12 and type 1 IFN production [67]. Similar approaches are being investigated in phase II trials for directing host immune responses against malignant gliomas [68].

TLR7 stimulation also improves early host anti-viral responses independent of priming immune responses to vaccines. In rats, oral R837 treatment 1 day prior and 3 days after parainfluenza type 1 virus infection significantly reduces respiratory virus replication. This effect was seen in conjunction with elevated levels of IFNs [69]. The anti-viral effect of TLR7 signaling has subsequently been demonstrated against H3N2 influenza virus infections [53], as well as several other non-respiratory virus models [70, 71, 72]. Interestingly, R837 treatment also attenuates AHR in virus-infected animals [69]. Considering the strong association of respiratory virus infections with asthma exacerbations, the ability of TLR7 to reduce a broad range of viral infections in addition to preventing AHR and airway remodeling makes it a particularly interesting target for asthma therapy.

Significance of TLR7-TLR8 homology and functional overlap

TLR7 and TLR8 are highly homologous and phylogenetically similar. Both are endosomally located and have overlapping ligand specificity. Single-stranded RNA activates both TLR7 and TLR8 in humans, but only activated TLR7 in mice [54]. This led to the belief that TLR8 was nonfunctional in certain species. Recently, this has been refuted. TLR8 activation in mice occurs with imidazoquinoline stimulation, however this requires co-stimulation with an oligodeoxynucleotide [55]. Despite these data, TLR8 remains one of the least studied mammalian TLRs and the significance of TLR7-TLR8 functional redundancy remains

largely unknown. In TLR8 knockout mice, TLR7 activation was augmented suggesting TLR8 may regulate TLR7 function or that TLR7 compensates for the loss of TLR8 [73]. While most TLRs homodimerize upon activation, some heterodimerize, namely TLR2/1 and TLR2/6. It remains unknown whether TLR7-TLR8 heterodimerize or are activated in parallel by their natural ligand single-stranded RNA. When cotransfected into HEK293 cells, physical interactions between TLR 7, 8, and 9 suggests they cross regulate each other's function [74]. Whether targeting TLR7 preferentially over TLR8 or targeting both simultaneously confers therapeutic benefit in allergic disease needs to be established. The redundancy of these receptors remains unresolved, but may hold important implications for drug development.

TLR7 Side Effects

There is a growing body of data supporting a role for TLR7 in asthma therapy, but this must be tempered by justifiable concerns regarding the predictable side effects from induction of systemic Th1 cytokines. TLR7 agonists have been used in human studies for the treatment of hepatitis C virus and cancer, with side effects ranging from fevers and myalgias to sepsis [25, 75]. To avoid these systemic effects of TLR7 administration, Biffen et al. synthesized an antedrug ester of an adenine derivative that is rapidly metabolized in the blood by butyrylcholinesterase to a significantly less potent metabolite [50]. In this manner, the effects of TLR7 stimulation may be limited to the lungs. The lungs provide the ideal route for delivery by inhalation for such a drug. This compound highlights the potential for development of TLR-directed pulmonary therapies with limited systemic side effect profiles.

Conclusions

TLR7 agonists attenuate Th2-mediated airway inflammation, AHR and chronic airway remodeling. Recently identified TLR7-mediated bronchodilation underscores its diverse role in pulmonary function and immunoregulation, and further increases its attractiveness as a drug target in asthma. Novel approaches for designing TLR7-directed agonists hold great promise for combining TLR7-mediated acute bronchodilation and prophylactic anti-inflammatory treatment while limiting systemic side effects.

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Abbreviations

AHR	airway hyperresponsiveness
CD8⁺	cluster of differentiation 8 cell
ERK	extracellular signal-regulated kinase
IFN	interferon

IgA	immunoglobulin A
IgE	immunoglobulin E
IL	interleukin
NF-κB	nuclear factor- κ B
NKT	natural killer T cell
p38 MAP Kinase	p38 mitogen activated protein kinase
R837	imiquimod
R848	resiquimod
SA-2	2-substituted-8-hydroxyadenine
TGF-β	transforming growth factor β
Th1	type-1 T-helper cell
Th2	type-2 T-helper cell
TLR	toll-like receptor
TNF-α	tumor necrosis factor α
Treg	regulatory T cell

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Figure 1

Figure 1. TLR7-mediated inhibition of allergen-induced airway inflammation

TLR7 stimulation induces Th1-specific cytokines, T regulatory cell (Treg) and invariant natural killer cell (iNKT) recruitment to the lungs. In contrast, allergens detected by antigen presenting cells (APC) induce Th2 inflammation and eosinophil recruitment that results in airway hyperresponsiveness, airway smooth muscle (ASM) proliferation and goblet cell hyperplasia. TLR7-Th1 immune activation prevents allergen-induced Th2-mediated airway inflammation.

Figure 2. TLR7 rapidly bronchodilates

TLR7 agonist R837 relaxes airway smooth muscle through a TLR7-dependent pathway via production of nitric oxide, and a TLR7-independent pathway, perhaps through TLR8, that involves cyclooxygenase (COX) and large-conductance, calcium-gated potassium channels (BK_{Ca}). The TLR7 antagonist IRS661, nitric oxide synthase (NOS) inhibitor L-NMMA, COX inhibitor indomethacin, and potassium-channel blocker paxilline partially inhibit R837-mediated airway smooth muscle relaxation.

Table 1

Studies of TLR7 agonist effects on airway inflammation.

Species/model	Inflammation		Airway Remodeling	AHR	Route	Author
	Th1	Th2				
R-837 (Imiquimod)						
Rat/ovalbumin-sensitization	↑	↓	↓	↓	Aerosol	Bian et al., 2006 [35]
Mouse/ovalbumin-sensitization	↑	↓	n/a	↓	Aerosol	Jin et al., 2006 [36]
Mouse/ovalbumin-sensitization	n/a	↓	↓	↓	Aerosol	Du et al., 2009 [37]
Rat/ovalbumin-sensitization	↑	↓	n/a	n/a	IP	Meng et al., 2011 [38]
R-848 (Resiquimod)						
Mouse/ovalbumin-sensitization	↑	↓	↓	↓	IN	Quarcoo et al., 2004 [39]
Mouse/ovalbumin-sensitization	↓	↓	n/a	↓	IP	Moisan et al., 2006 [40]
Rat/ovalbumin-sensitization	↓	↓	↓	n/a	IP	Camateros et al., 2007 [41]
Mouse/ovalbumin-sensitization	↑	↓	↓	↓	IP, SC, IV	Sel et al., 2007 [42]
Mouse/ovalbumin-sensitization	n/a	↓	↓	n/a	IP	Camateros et al., 2009 [43]
Mouse/ovalbumin-sensitization	n/a	↓	n/a	↓	IP	Aumeunier et al., 2010 [44]
Mouse/ovalbumin-sensitization	↑	↓	↓	↓	IN	Xirakia et al., 2010 [45]
Mouse/ovalbumin-sensitization	←→	↓	↓	↓	IT	Duechs et al., 2011 [46]
Mouse/ovalbumin-sensitization	↑	↓	n/a	↓	IP, Cell Transfer	Grela et al., 2011 [47]
Mouse/ovalbumin-sensitization	n/a	↓	n/a	↓	IP	Marino et al., 2011 [48]
Mouse/ovalbumin-sensitization	↑	↓	n/a	↓	IP	Van et al., 2011 [49]
AZ12441970						
Mouse/ovalbumin-sensitization	←→	↓	n/a	n/a	IT	Biffen et al., 2011 [50]
Substitute Adenine-2						
Mouse/ovalbumin-sensitization	←→	↓	↓	n/a	IT, IP	Vulciaggio et al., 2009 [51]
Mouse/ovalbumin-sensitization	↓↑	↓	↓	↓	IP	Vulciaggio et al., 2011 [52]

n/a: not assessed; IN: intranasal; IP: intraperitoneal; IT: intratracheal; IV: intravenous; SC: subcutaneous