

A New Era of Targeting the Ancient Gatekeepers of the Immune System: Toll-Like Agonists in the Treatment of Allergic Rhinitis and Asthma

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Key Words

Toll-like receptors · CD14 · Hygiene hypothesis · Innate immunity · Immunotherapy · Adjuvants · CpG oligodeoxynucleotide · Immunostimulatory sequence oligonucleotides · Asthma · Allergic rhinitis

Abstract

Toll-like receptors (TLR) belong to a large family of pattern recognition receptors known as the ancient 'gatekeepers' of the immune system. TLRs are located at the first line of defense against invading pathogens as well as aeroallergens, making them interesting targets to modulate the natural history of respiratory allergy. Agonists of TLRs have been widely employed in therapeutic or prophylactic preparations useful for asthma/allergic rhinitis (AR) patients. MPL[®] (a TLR4 agonist) and the CpG oligodeoxynucleotide of 1018 ISS, a TLR9 agonist, show strong immunogenicity effects that make them appropriate adjuvants for allergy vaccines. Targeting the TLRs can enhance the efficacy of specific allergen immunotherapy, currently the only available 'curative' treatment for respiratory allergies. In addition, intranasal administration of AZD8848 (a TLR7 agonist) and VTX-1463 (a TLR8

agonist) as stand-alone therapeutics have revealed efficacy in the relief of the symptoms of AR patients. No anaphylaxis has been so far reported with such compounds targeting TLRs, with the most common adverse effects being transient and local irritation (e.g. redness, swelling and pruritus). Many other compounds that target TLRs have been found to suppress airway inflammation, eosinophilia and airway hyper-responsiveness in various animal models of allergic inflammation. Indeed, in the future a wide variability of TLR agonists and even antagonists that exhibit anti-asthma/AR effects are likely to emerge.

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Introduction

Atopic asthma and allergic rhinitis (AR) represent two closely related allergic diseases of the respiratory system [1]. The prevalence of these two diseases has increased over the past decades and, with respect to the costs, side effects and failures of conventional long-term symptomatic treatments or low compliance with allergen avoidance, many efforts have been made to find novel thera-

peutics [2]. So far, specific allergen immunotherapy (SIT) represents the only 'etiologic' treatment that directs towards the basis of atopic respiratory disease and influences the natural history of AR and asthma [3]. To develop safer and more efficacious allergy vaccines or novel stand-alone therapeutics, innate immunity stands out as a major target since it is the first line of defense [4, 5]. Innate immunity enables initial recognition of invading pathogens and presents them to the immune system to elicit an appropriate adaptive immune response. Pattern recognition receptors (PRRs) represent ancient and conserved structures of the innate immune system with endosomal, cell-membrane bound and soluble members that discriminate self from non-self [6]. They recognize patterns that are common among invading pathogens and orchestrate subsequent immune responses [7]. Toll-like receptors (TLR), C-type lectin receptors, nucleotide-binding oligomerization domain-like receptors and retinoic acid-inducible gene-1-like receptors are all members of the growing family of PRRs expressed in airway cells [8, 9].

TLRs exert dual roles in allergic diseases; activation of some TLRs offer sensitizations and breaking of the tolerance [10–12], while activation of some members of this family, particularly early in life, may promote tolerance to innocuous aeroallergens. The hygiene hypothesis suggests that a reduced microbial burden in early childhood increases the susceptibility to allergic disease via deficient maturation of the immune system [13]. Recently, the PASTURE study (Protection against Allergy: Study in Rural Environments) has shown that early-life exposure to a livestock farming environment, as well as drinking raw milk in the first year of life, reduces the chance of the allergic phenotype emerging by an overall increase in PRR gene expression [14]. On the other hand, Holt et al. [3] introduced the 2-Hit model of asthma in which atopy together with viral respiratory infections such as rhinovirus, respiratory syncytial viruses (RSV) and other respiratory viruses synergistically increase the risk of asthma in the presence of allergen exposure [3, 15, 16]. This shows that not all infections and not all farming environments are protective. Interestingly, many triggers of asthma, such as environmental allergens and air pollutants, act in part via modulation in PRR expression and function [17–20]. Furthermore, the generation of reactive oxygen species is increased in asthma and AR, with oxidative stress being one factor that causes exacerbation of disease. Interestingly, transcriptional factors activated through TLR signaling (e.g. NF- κ B) are redox sensitive and several anti-oxidant agents have been demonstrated to inhibit asth-

ma symptoms by reduction of reactive oxygen species generation [21–24]. In addition, the consumption of probiotics, which has been proposed to prevent allergic diseases, might work via modulation of TLRs [25–27].

TLRs are pivotal actors in shaping the effective and healthy adaptive immunity with the development of immune deviation from the T-helper (Th) 2 to Th1 phenotype and maturation of T-reg cells [28, 29]. Interestingly, the expression and function of TLRs [30–34] were demonstrated to be different in patients with asthma/AR as compared to healthy subjects. This difference in immune defense may be one of the possible reasons for the increased susceptibility to respiratory infections displayed by these patients. On the other hand, it has also been shown that impaired function of TLRs may be reversible through appropriate immunotherapy [35] or other medications used frequently for patients with allergy [36]. This observation offers real promise for immunotherapeutic approaches to restore the protective immune response in the airways of allergic subjects [37]. In this review, we summarize the results of studies on in vivo specific targeting of TLRs using animal models of allergic asthma, clinical trials I–IV and registries of clinical trials focused on specific targeting of TLRs in humans. The highest level of evidence provided by each study has been determined according to Oxford Centre for Evidence-Based Medicine (OCEBM) 2011, and thereby the highest level of evidence for efficacy of each compound has been extracted (Box 1). The results are categorized under appropriate subheadings. Overall, our review strongly suggests that TLR targeting may arrest the disease progression of an allergic response either by induction of tolerance to allergens (e.g. SIT) and/or by redirecting the immune response away from the airways.

Cell Surface TLRs

Cell surface TLRs have an active role in the identification of structural components at the cell surface of invading pathogens. Wide varieties of gram-positive and negative bacteria, as well as some viruses such as RSV [38, 39], known as risk factors for triggering the allergic airway disease, are recognized by these TLRs. To expand signaling, Toll/interleukin (IL)-1 receptor (TIR) domain-containing adaptors are employed and, with the mediation of TIR domain-containing adaptor protein (TIRAP) and myeloid differentiation primary response gene 88 (MyD88), a cascade is triggered that finally leads to the translocation of interferon regulatory factors 3/7, API

Box 1. Search strategy

To find relevant articles the databases of MEDLINE, Scopus and EMBASE were searched with keywords of 'TLR1-10', 'airway', 'asthma', 'allergy', 'rhinitis', 'CpG', 'AIC', 'QbG10', 'AZD1419', 'AEV0675', 'SAR21609', 'IMO2134', 'VTX-1463', 'R848', 'R837', 'AZD8848', 'polyI:C', 'Resveratrol', 'ssDNA-ODN', 'Capsazepinoids', 'MALP-2', 'Pam3Cys', 'Pam3CSK4', 'Zymosan', 'Opr 1', 'LP40', 'MPL', 'PGA', 'CRX-675', 'E5564' and 'flagellin'. Furthermore, hand searching was performed by checking all reference lists of articles provided by the electronic search. No time limitation or language restriction was used and whenever the required data of publication was inaccessible, we contacted the corresponding author to provide it. It is of note to say that no preference was given to include studies and all in vivo studies that employed PRR agonists or antagonists in airway hyper-responsiveness, whether on animal models or human subjects, were included in this review. Duplicated studies provided by searching the databases of MEDLINE, Scopus and EMBASE were excluded and preference was given to more recent and comprehensive in vitro studies. The highest level of evidence found in all possible sources from experimental studies to phase I-IV clinical trials in human subjects has been diligently summarized in this review. In accordance with 'OCEBM 2011 Levels of Evidence' and study design, the level of evidence provided by each study has been determined. According to OCEBM-2011, mechanism-based reasoning studies provide level 1, case series, case-control studies, or historically controlled studies provide level 2, non-randomized cohort studies provide level 3, randomized trials provide level 2 and systematic review and meta-analysis of randomized trials provide level 1 of evidence.

On the other hand, since a large amount of data relating to this topic may be unpublished and therefore not indexed in searched databases, we also searched the International Clinical Trials Registry Platform (ICTRP) of the World Health Organization (WHO) with the same keywords to find registries that used agonists or antagonists of PRRs in human subjects with airway hyper-responsiveness (last update of December 2012). ICTRP provides a monthly update of registries from the following registry systems: (1) Australian New Zealand Clinical Trials Registry (ANZCTR), (2) Chinese Clinical Trial Register (ChiCTR), (3) Clinical Research Information Service (CRiS), Republic of Korea, (4) ClinicalTrials.gov, national library of the USA, (5) Clinical Trials Registry – India (CTRI), (6) Cuban Public Registry of Clinical Trials (RPCEC), (7) EU Clinical Trials Register (EU-CTR), (8) German Clinical Trials Register (DRKS), (9) Iranian Registry of Clinical Trials (IRCT) ISRCTN.org, (10) Japan Primary Registries Network (JPRN), (11) Pan African Clinical Trial Registry (PACTR), (12) Sri Lanka Clinical Trials Registry (SLCTR), and The Netherlands National Trial Register (NTR). Finally, after pooling all eligible studies whether published or unpublished, duplicated results were identified and excluded.

With respect to the heterogeneity of the studies presented in this review in terms of study subjects (animal models or human subjects), variability in reporting of the results for upper or lower respiratory tract and wide spectrum of TLRs, a meta-analysis of various studies has not been performed. Finally, results of eligible studies have been explained in categorized subheadings.

and NF- κ B to the nucleus [40]. These transcriptional factors enhance type 1 interferon, inflammatory cytokine and chemokine expression, as well as the induction of MUC gene expression (fig. 1) [23, 41–44]. Targeting of these TLRs is based mainly on adjuvants of allergy vaccine, as seen in Pollinex[®] Quattro [6]. Other therapeutic preparations with agonists or antagonists of cell surface TLRs are also described below.

TLR1, 2, 6 and 10

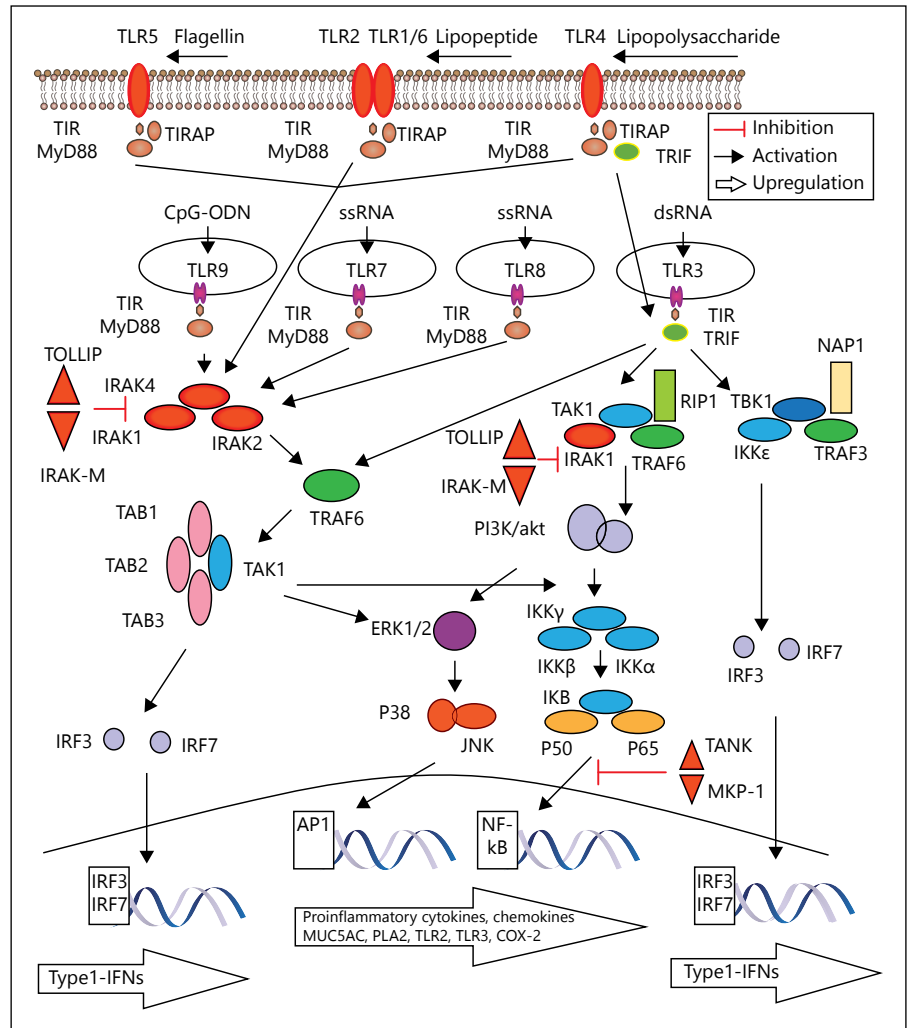
TLR2 agonists may act as either allergenic or anti-allergic agents based on the treatment schedule and materials. Pam3Cys and Pam3CSK4 (Pam3-Cys-Ser-Lys4) are synthetic triacylated lipoproteins (TLR2/1 agonists) in which Pam3Cys was shown to promote a Th2-biased response and airway inflammation [45], while Pam3CSK4 reduced Th2 cytokine release, airway hyper-reactivity (AHR), IgE levels, airway inflammation and nasal symptoms in murine models of asthma and AR [46–48]. In addition to the structural differences in the amino acid tail, these opposite

effects may originate from the schedule of administration; protective effects of Pam3CSK4 have been reported when administered after sensitization with a dose of 100 μ g per mouse [46, 47], while Pam3Cys was shown to be allergenic when administered in combination with ovalbumin with a dose of 50 μ g per mouse to induce sensitization [45]. Interestingly, in vitro co-stimulation of peripheral blood mononuclear cells (PBMCs) obtained from atopic asthmatics with Pam3CSK4 either during SIT [49] or outside of SIT [50] resulted in expanding the CD8+CD25+Foxp3+ T-reg population, suppression of CD4+ proliferation and dampening of the Th2 cytokine production. These findings suggest that Pam3CSK4 may be a good adjuvant for SIT. Certain TLR2 agonists that have shown anti-asthmatic effects are described below.

Macrophage-Activating Lipopeptide-2

Macrophage-activating lipopeptide-2 or MALP-2 (evidence from mechanism based studies, level 5) is a TLR2/6 agonist obtained from *Mycoplasma fermentans*. MALP-2 can induce CD80 (B7-1), CD86 (B7-2), major histocompatibility complex I and II and CD40 expression in B cells

Fig. 1. a Cell surface TLRs include TLR1, 2, 6, 10, 4 and 5. Their ligands are structures in the cell wall of invading pathogens as well as allergens like HDMs and short ragweed. Intracellular endosomal membrane-bound TLRs include TLR3, TLR7, TLR8 and TLR9. They recognize genetic materials of viruses and bacteria. MyD88, TIRAP and TRIF are the main adaptor molecules. Interferon regulatory factor 3/7 (IRF3/7), P50 and P65, nuclear factor- κ B (NF- κ B) and AP1 transcription factors enhance expression of proinflammatory cytokines, chemokines and type 1 IFNs. TLR4 uses both of the MyD88-dependent and TRIF-dependent pathways. Several inhibitors regulate the signaling cascade of PRRs at different steps and include: IL-1 receptor-associated kinase-M (IRAK-M), suppressor of cytokine signaling (SOCS), MAPK phosphatase-1 (MKP-1), Toll-interacting protein (TOLLIP) and TRAF family member-associated NF- κ B (TANK).



and dendritic cells (DCs) [51, 52]. Furthermore, MALP-2 has the ability to increase the release of interferon- γ (IFN- γ) from DCs in response to allergens [53]. Mucosal delivery of MALP-2 via the intratracheal route resulted in a marked decrease in AHR, eosinophilia and Th2 cytokines in a murine model of asthma [54]. In addition, MALP-2 promoted airway neutrophilia and the production of the IL-12 p70 subunit [54]. Interestingly, it has been shown that MALP-2 is able to activate neutrophils to produce chemokines and stimulate T cell, B cell and natural killer cell accumulation in the lung of treated mice, with more potency in male and adult subjects [55]. Bisacyloxypropylcysteine polyethylene glycol (BPPcysMPEG) is a derivate of MALP-2 that is also capable of stimulating TLR2/6 and also has the ability to abrogate Th2 response and airway eosinophilia in murine asthma models [56]. BPPcysMPEG exerts its immunomodulatory effects by in-

ducing production of IL-1 β , chemokine (C-C motif) ligand 4 (CCL4; macrophage inflammatory protein-1 β) and IL-10 in viable precision-cut slices of lung tissues in vitro [57]. Both systemic and local delivery of BPPcysMPEG with allergens leads to the maturation of DCs and induction of appropriate adaptive immune response against the allergen in sensitized mice [58]. In mice sensitized to house dust mites (HDMs), BPPcysMPEG also reduced eosinophilia and Th2 cytokine production and enhanced TNF- α and IFN- γ generation accompanied by the induction of a Th1 response in bronchoalveolar lavage fluid and mediastinal lymph nodes [59].

Lipoprotein 1

Lipoprotein 1 (Opr1; evidence from mechanism-based studies, level 5) is a TLR2/4 agonist obtained from *Pseudomonas aeruginosa*, and can prime DCs and T cells to

produce IL-12 and IFN- γ , respectively. Additionally, its co-administration with ovalbumin abolished the production of IL-4, IL-13 and airway eosinophilia in a murine asthma model [60]. Lipopeptide-CGP40774 (LP40; evidence from mechanism-based studies, level 5) is a similar TLR2 agonist capable of shifting the immune response toward a Th1 profile, reducing IgE production, AHR and airway inflammation, and increasing the T-reg response [25, 61]. Additionally, it has been shown that LP40 can suppress allergic airway dysfunction and accompanying airway eosinophilia in mice [25, 61].

TLR4/MD2/CD14

Targeting of TLR4 to modify asthma/AR is based on the activation of TLR4 as an adjuvant in allergy vaccines to induce tolerance, and the inhibition of expression and inflammatory function of TLR4 with a TLR4 antagonist. Since TLR4 is upregulated in patients with asthma/AR, its targeting via allergy vaccines seems to work efficiently; however, it should be done with caution to preserve the safety of patients.

Adjuvants of Allergy Vaccines

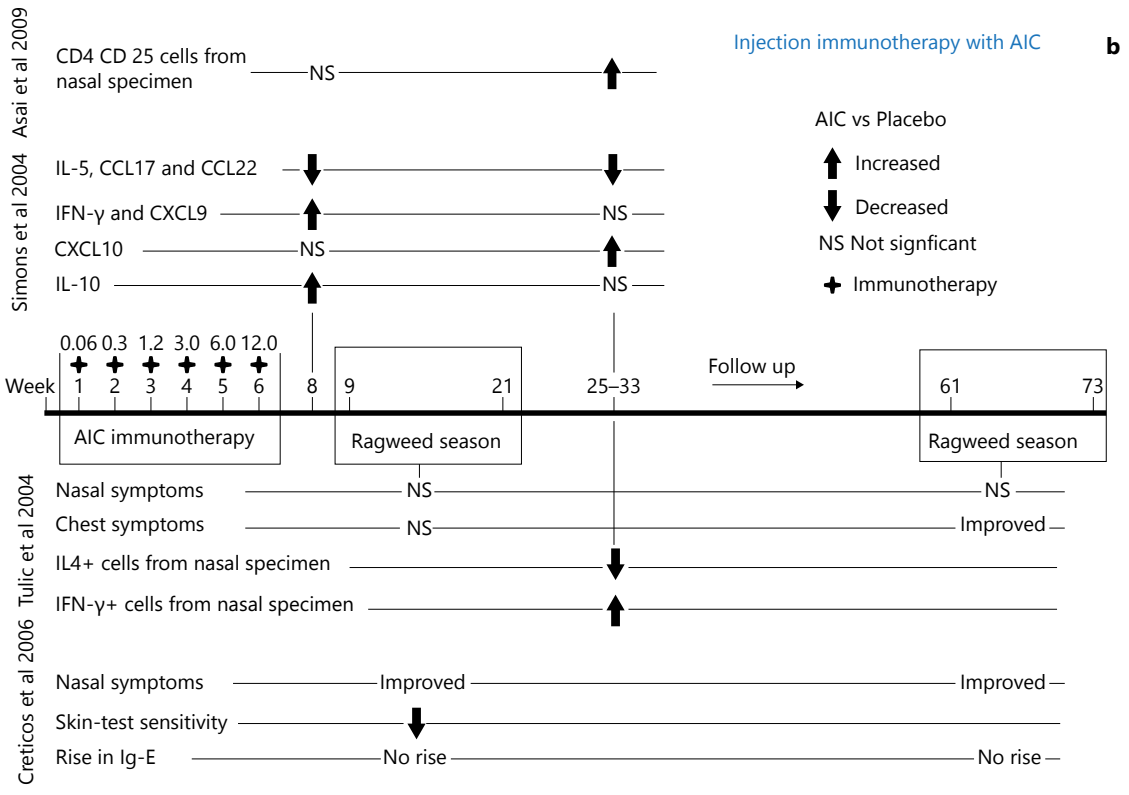
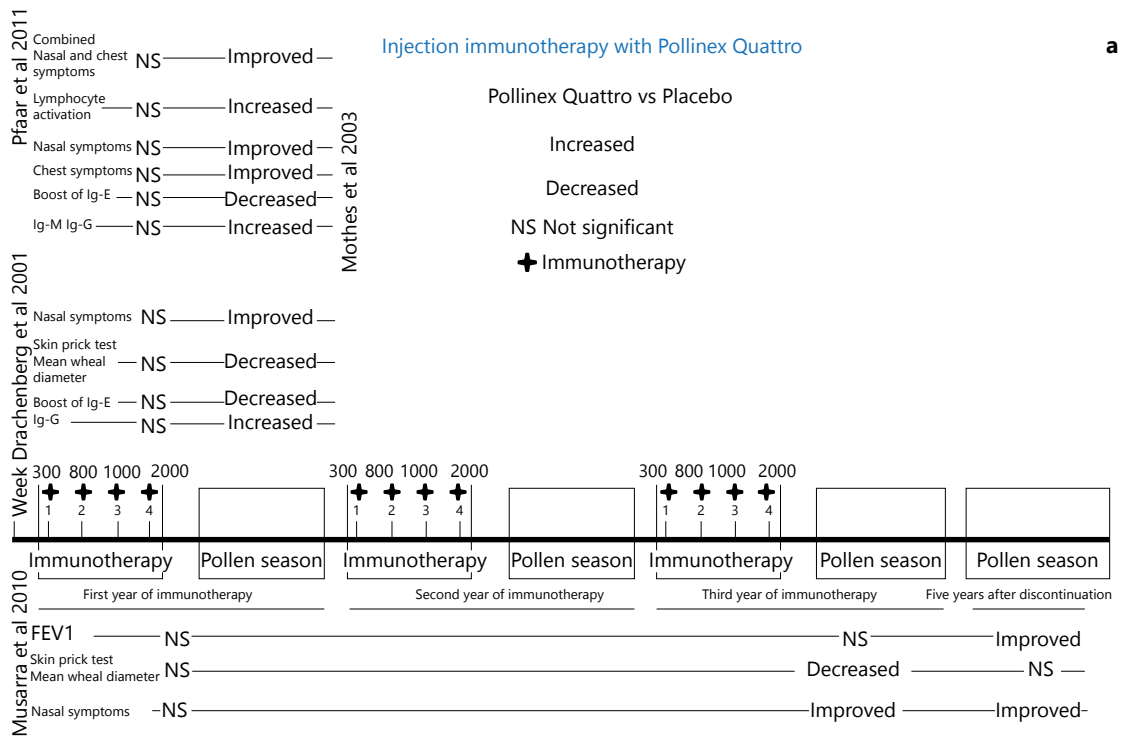
MPL[®] (monophosphoryl lipid A), a TLR4 agonist, is a detoxified derivative from *Salmonella minnesota* that is used in the Pollinex Quattro allergy vaccine (Allergy Therapeutics, Worthing, UK; phase IV clinical trial for AR, phase I/II for asthma). Pollinex Quattro is a glutaraldehyde modified L-tyrosine adsorbed pollen allergen (formulations containing grass, flower and tree pollens are available) with MPL that can be administered via subcutaneous or sublingual routes. The dose of MPL administered orally can be 15 times higher than that used in subcutaneous injection therapy. This TLR4 agonist

has been evaluated in children over 6 years of age with similar safety and efficacy as compared with adult patients [62]. Pre-seasonal ultra-short SIT with Pollinex Quattro can alter the course of respiratory allergy in either the upper or lower respiratory tract [63] and its beneficial effects have been shown to be sustained for over 5 years [63, 64]. Interestingly, it prevents new sensitizations and asthma development after the cessation of treatment [64]. Along with clinical improvements, the immune profile of patients will be changed through immunotherapy with MPL serving as a strong inducer of the Th1 response [65] (fig. 2a). In post-marketing open clinical trials conducted by Rosewich et al. [62, 66], the symptom scores and medication requirements decreased during the 3 years following the first course of injection and were augmented with subsequent injections. In their experience, clinical improvements were associated with T-reg induction and shifting from IgE to blocking IgG production. Similarly, a pre-seasonal ultra-short course of Pollinex Quattro led to a decrease in lung inflammation in asthmatics in terms of oxidative stress markers such as 8-isoprostane in exhaled breath condensate [63]. No life-threatening side effects were reported and transient local reactions were seen only with the dose of 2,000 standard units (SU)/ml (fig. 3). No toxicity with the safety margin used in the clinic was detected in experimental studies [67]. Polymite[®] (evidence from mechanism-based studies, level 5) is a glutaraldehyde-modified L-tyrosine adsorbed mite (*Dermatophagoides pteronyssinus* and *D. farina*) protein extract with MPL. The formula of 10:50:20 mg/ml for protein:MPL:tyrosine is used in the Polymite vaccine. Subcutaneous injection of Polymite resulted in minimal side effects or toxicity with a low-dose injection (0.1–1 ml/week for 13 weeks) in rodents [68]. To the best of our knowledge no study has tested its efficacy to date.

Fig. 2. Changes to the immune profile following immunotherapy with two well-studied vaccines containing TLR 4/9 agonists. Pollinex Quattro contains MPL, a TLR4 agonist, and AIC contains 1018 ISS, a TLR9 agonist versus placebo. **a** Pollinex Quattro schedule is as follows: weekly injection of up-dosing vaccine with 300–2,000 SU, 2–8 weeks prior to the pollen season. It is safe and efficient in abolishing symptoms of AR/asthma-sensitized patients to pollens. It also shifts the Th2 response toward Th1/T-reg and IgE toward IgG production. Of note, IgG production is differed based on pollen since Timothy and rPhl p 5 allergen-containing vaccines induced greater values of IgG than other pollens. The allergen-specific lymphocyte proliferation response is increased with the maximum quantities prior to the last injection of Pollinex Quattro

[64, 136, 139]. **b** The AIC schedule is as follows: weekly injection of up-dosing AIC with 0.06–12.0 μ g (Amb a 1: 1018 ISS molar ratio of 1: 4). Tulic et al. [129], Asai et al. [126] and Creticos et al. [127] started their studies before the ragweed season of 2001, and Tulic et al. [129] and Creticos et al. [127] followed their participants until the ragweed season of 2002. Only assessed immunologic variables with at least one significant change between active versus placebo have been stated ($p < 0.05$; significant). Nasal symptoms included rhinorrhea, pruritus, sneezing and nasal congestion, and the chest symptoms included tightness, wheezing, cough and shortness of breath. Cytokines were measured using supernatant culture of fresh PBMCs from participants exposed with 100 μ g/ml ragweed [126–129].

(For figure see next page.)



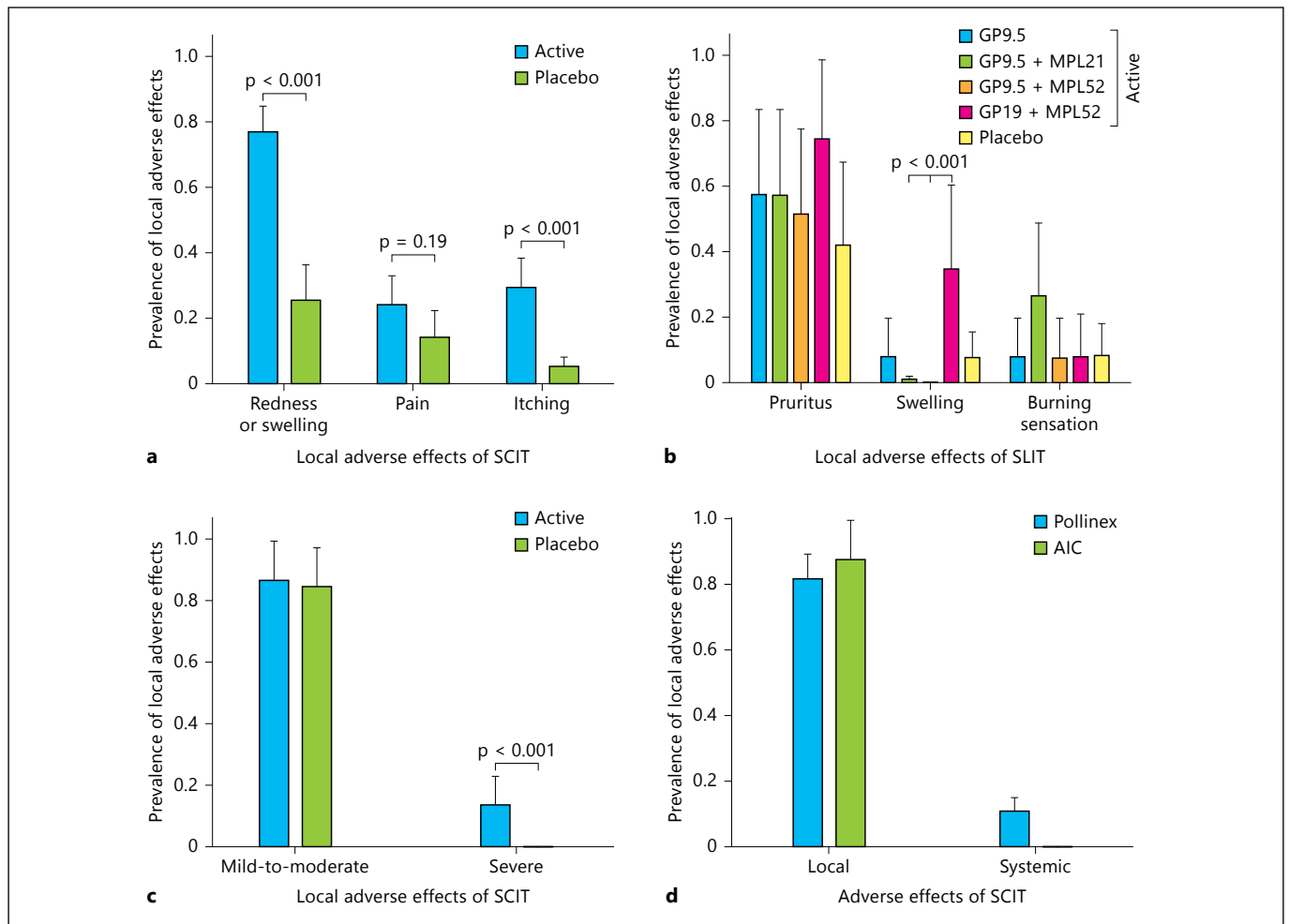


Fig. 3. Safety profile of two well-studied vaccines containing TLR4/9 agonists. The side effects of allergy vaccines of Pollinex Quattro, which contains MPL, a TLR4 agonist, and AIC, which contains 1018 ISS, a TLR9 agonist. The Pollinex Quattro with grass pollens and the same preparation with tree pollens elicited slightly higher local side effects [137]. **a** The most common local adverse effects of subcutaneous immunotherapy (SCIT) with Pollinex Quattro as the active group include: redness or swelling (61/81, 75%), pain (19/81, 23%) and itching (23/81, 28%) [139]. **b** The most common local adverse effects of sublingual immunotherapy (SLIT) with Pollinex Quattro as the active group include: pruritus (26/48, 54%), swelling (4/48, 9%) and burning sensation (5/48, 10%). The materials employed in active treatment included grass major allergen *Phleum pratense* (Phl p) and MPL as follows: GP9.5: 9.45 µg of Phl p 1; GP9.5 + MPL21: 9.45 µg of Phl p 1 + 21 µg of MPL; GP9.5 + MPL52 9.45 µg of Phl p 1 + 52.5 µg of MPL, and GP19 + MPL52: 19.04 µg of Phl p 1 + 52.5

µg of MPL. Placebo was the buffered glycerine solution [136]. **c** The local adverse effects of SCIT immunotherapy with AIC were categorized as mild-to-moderate versus severe by Tulic et al. [129], and none of them required any medication. **d** Total local and systemic side effects of immunotherapy with Pollinex Quattro or AIC obtained from the aforementioned studies. Systemic side effects included rhinoconjunctivitis, joint pain, headaches, nausea, hot flushes, chills, hypotension and tachycardia. The probability of a systemic reaction by injection of Pollinex Quattro was 8.6%, while it was 10.7% with AIC. No anaphylactic shock was reported due to either Pollinex Quattro or AIC administration. Error bars have been used to present the prevalence of adverse effects. Comparisons were made using χ^2 test with SPSS version 16.0 (SPSS Inc., Chicago, Ill., USA). Probabilities of less than 0.05 were considered significant and shown as p for corresponding comparisons.

Monotherapy with TLR4 Agonist

All data are from mechanism-based studies, level 5 of evidence except CRX675 (phase I/II clinical trial for AR, level 2), which is an aqueous MPL compound, the intranasal administration of which led to the reduction of na-

sal symptoms of AR patients only at a dose of 100 µg [69]. A similar compound of lipid-A exerted anti-asthmatic effects in a murine model of asthma as monotherapy. In this model, it was able to reduce total serum IgE levels, AHR, airway eosinophilia and IL-13 production [48].

Poly- γ -glutamic acid (γ -PGA) is a TLR4 agonist obtained from the cell wall of bacilli which induces expression of the co-stimulatory molecules, CD80, CD86 and CD40 on DCs, and also upregulates IL-12 [70]. Its administration to a murine model of allergic-type lung inflammation led to the suppression of the Th2 cytokines, AHR, airway inflammation and eosinophilia [70]. ER803022 is another synthetic TLR4 agonist that showed anti-asthmatic effects in murine models. It inhibited AHR, airway eosinophilia and Th2 cytokine production on TLR-dependent MyD88 activation and IL-12/IFN- γ production, rather than a TIRAP-inducing interferon- β (TRIF)-dependent cascade [71]. UT12, an agonistic antibody against TLR4, can suppress airway-allergic disease through the abrogating production of Th2 and Th17 cytokines without the induction of T-reg or Th1 cytokines [72]. On the other hand, E5564 is a TLR4 antagonist that was safe and efficient in reducing IL-6 generation, AHR, airway inflammation, neutrophilia and eosinophilia in allergen-sensitized and challenged mice [73].

TLR5

TLR5, another cell surface TLR, recognizes flagellin and triggers the MyD88-dependent signaling cascade. Its expression has been shown in monocyte-derived cells [30], neutrophils [74], eosinophils [74], airway epithelial cells [75] and airway smooth muscle cells (ASMCs) [76]. Some claim that its expression and function is not different between asthmatics and non-asthmatics [30, 74], while others believe that its expression in the airways is decreased in asthmatics [77]. Indoor allergens such as those from HDMs are thought to promote allergic respiratory disease, a Th2-biased response and IgE production in part due to inappropriate TLR5 activation [17]. On the other hand, TLR5 is also implicated in the defense against many pathogens of the respiratory system such as *Pseudomonas aeruginosa* [75], *Yersinia pestis* [78], *Streptococcus pneumoniae* [79] and influenza virus [80] in such a way that the addition of flagellin to the formulation of vaccines against the aforementioned pathogens enhanced the efficacy of immunization. Interestingly, when co-administered with the allergens, flagellin was shown to reduce AHR, inflammatory cell influx to airways and Th2 type cytokine production in an ovalbumin murine model of allergic airway inflammation [81] via the induction of T-reg responses [82]. Accordingly, TLR5 looks like a double-

edged sword; on one hand, it can be the criminal in producing an exaggerated and inappropriate response to innocuous aeroallergens in allergic patients, while on the other hand, TLR5 ligands are strong adjuvants either in allergy vaccines or vaccines against infectious agents (evidence from mechanism-based studies, level 5).

Intracellular TLRs

Intracellular TLRs recognize the genomic material of invading pathogens and their most important role is to trigger appropriate antiviral responses. TLR3, 7, 8 and 9 are intracellular TLRs and their signaling cascade is depicted in figure 1 [19, 23, 41, 42]. Viral infections, especially with rhinovirus, account for the most common causes of asthma exacerbation in children and cause more severe forms of wheezing episodes in allergic subjects [83]. As described below, boosting the immune response through targeting these TLRs may promise a etiologic treatment for patients with respiratory allergy in the shape of an allergy vaccine (evidence from a phase II clinical trial) or lead to more effective drugs in viral-induced asthma exacerbations, such as imidazoquinoline compounds (evidence from mechanism based studies, level 5).

TLR3: Sensor of Double-Stranded RNA

TLR3 activation mimics a viral infection that alters the immune response and airway microenvironment toward inflammation [84, 85], AHR [86] and bronchospasm [87]. TLR3 agonists such as polyinosinic-polycytidylic acid (polyI:C) at a low dose are used to create in vitro and animal models of viral-induced asthma [86, 88]. Accordingly, the employment of TLR3 inhibitors seems to be beneficial for the treatment of respiratory allergies, and there is one report regarding the usefulness of polyI:C, a TLR3 agonist, in the suppression of murine allergic airway inflammation [89].

PolyI:C

Despite all the inflammatory effects of polyI:C (evidence from mechanism-based studies, level 5), Sel et al. [89] found that systemic administration of 200 μ g/200 μ l of polyI:C reduced AHR, inflammation and IgE production in murine models of asthma via the induction of IL-10 and IL-12 production. Interestingly, the dose of polyI:C

used in this study (200 µg/200 µl) [89] was similar to what others (1 mg/1 ml) [86] employed to induce an asthma phenotype.

Capsazepinoids

Analogues to capsazepine (in vitro results) have been recently shown to act as a TLR3 inhibitor. They suppress the production of inflammatory cytokines including TNF-α, IL-8 and TSLP (thymic stromal lymphopoeitin) by airway epithelial cells collected from asthmatics [90]. Additionally, they have revealed bronchodilator effects by relaxation of ASMCS. Indeed, these compounds are believed to reverse AHR induced by polyI:C [90].

Single-Stranded DNA Oligonucleotides

Single-stranded DNA oligonucleotides (ssDNA-ODNs; evidence from mechanism-based studies, level 5) inhibit not only binding of the polyI:C to TLR3, but also production of proinflammatory cytokines triggered by TLR3 activation in PBMCs [91]. Intranasal administration of ssDNA-ODNs prevented Th2-type lung inflammation in a non-human primate model and suppressed inflammatory cell infiltration into the respiratory tract in monkeys treated with polyI:C [91].

Resveratrol

Resveratrol or trans-3,4,5-trihydroxystilbene (evidence from mechanism-based studies, level 5) is a herbal extract that is abundant in grapes. It showed protective effects on asthma development following RSV infection by preventing upregulation of TLR3 expression and inhibition of its adaptor molecule TRIF [24].

TLR7 and TLR8: Sensors of Single-Stranded Viral RNA

Imidazoquinoline is a common structure among TLR7/8 agonists; however, other classes, such as poly(RNA) molecules, also exist [92]. The employment of lipid carriers, phosphorothioate modification of RNA backbone or addition of arabinonucleotides prevents nuclease degradation and increase the stability of the product [93]. Systemic administration of TLR7/8 agonists resulted in fatal side effects, including peripheral leukocyte depletion due to systemic activation of endothelia that express vascular adhesion molecules [94] and progressive lymphoid destruction mimicking HIV-mediated immunopathology [95], perhaps due to the TNF-α storm [96]

induced by this particular type of TLR7 agonist. Taken together, it will be important to choose the proper TLR7/8 ligand, its dose and route of administration to develop a safe and efficient protocol for the treatment of patients with allergic asthma, which of course should be done under the close supervision of physicians.

Imiquimod

Imiquimod or R837: (Aldara[®], 3M pharmaceuticals, mechanism-based studies, level 5 of evidence) is an FDA-approved drug used for treating skin basal cell carcinoma, actinic keratosis and external genital warts with many suggested off-label benefits in other disease, such as melanoma [6]. Interestingly, it has been recently demonstrated that topical administration of this TLR7 agonist mediates systemic changes and modulates the composition of the cell/cytokine milieu of the respiratory system [97]. Imiquimod treatment of mice led to a reduction in alveolar macrophages, B cells and TNF-α production, along with an increase in DC and natural killer accumulation in the lung tissue and IL-10 production. Similar cellular changes in blood were also observed but without an overt inflammatory response [97]. Imiquimod also showed bronchodilator effects in both murine [98] and porcine [92] models of AHR, possibly through induction of nitric oxide, prostaglandins and large-conductance calcium-activated potassium channels, but not adenosine receptors [92]. As imiquimod can also promote antiviral defense and protect against virus-induced airway dysfunction [99], it seems an interesting drug for viral-induced asthma exacerbations.

Resiquimod

Resiquimod or R848 or S28463 (3M Pharmaceuticals, Maplewood, Minn., USA; mechanism-based studies, level 5 of evidence) is a TLR7/8 agonist, the systemic [25, 89, 100, 101] or intranasal [96] application of which has been shown to suppress AHR and airway remodeling in murine models of asthma with similar dose responses [96, 100–103]. Resiquimod administration suppressed acute asthma with a shifting of the immune response toward Th1 and type-1 IFN production, a reduction in both Th1 and Th2 cytokine levels in the lungs of rats and mice, and a reduction in lung eosinophilia, goblet cell hyperplasia and total IgE levels [96, 100–103]. Despite consistency regarding suppression the of Th2 response, some studies report that it can suppress Th1 responses in vivo in allergic asthma in mice and rats [101]. Interestingly, resiquimod treatment resulted in the promotion of long-lasting

protection from experimental asthma by IFN- γ production and induction of memory CD8+ T cells [96]. Similar to imiquimod, resiquimod with concomitant antiviral and anti-asthmatic effects can be used for viral-induced asthma exacerbations [104].

SA2 and PolyUs

9-Benzyl-2-Butoxy-8-Hydroxy Adenine, also called SA2, has been shown to reduce Th17/Th2 family cytokines, neutrophilia and AHR, along with enhanced IL-10 production in murine models of allergic-type lung inflammation [105]. Distinct from this, PolyUs (21-mer single-stranded phosphorothioate polyuridylic acid), a synthetic ssRNA, has been reported to exert bronchodilator effects in a porcine model of asthma through a TLR7-dependent mechanism with induction of nitric oxide production [92] (mechanism-based studies, level 5 of evidence).

AZD8848

AZD8848 (phase II clinical trial for AR) is a TLR7 agonist that was shown to be safe and efficacious in reducing nasal symptoms in seasonal AR patients. AZD8848 led to an increase in IL-1Ra production and a decrease in mast cell tryptase and α 2-macroglobulin in the nasal lavage of AR patients, suggestive of a reduction in plasma exudation and mast cell activity [106]. The side effects were dose dependent and included influenza-like symptoms, epistaxis, pharyngeal pain, pyrexia, rhinorrhea, nasal blockage or ulcers, nasopharyngitis, malaise and myalgia [106].

GSK2245035

GSK2245035 (phase II clinical trial for respiratory allergy) is a highly selective TLR7 agonist suggested to be beneficial in respiratory allergy via intranasal administration. Phase I of the pharmacodynamics, safety and efficacy assessment has been completed, but so far no result has been published (NCT01480271; March 2012). Participants are now being recruited for phase II (NCT01607372; June 2012).

VTX-1463

VTX-1463 or VTX-378 (phase II clinical trial for AR) is a TLR8 agonist currently in clinical development for AR patients; its safety and efficacy has been tested with promising results. It led to a reduction in nasal symptoms in grass pollen-sensitized AR patients after a course of 5-dose intranasal treatment without any reported side effects. However, detailed results have not yet been pub-

lished – only one trial has been published as an abstract [107], along with a comprehensive review by Horak [108].

TLR9: Sensor of Bacterial DNA

A decade of experimental employment of TLR9 agonists in animal models of asthma was promising [109, 110]. Synthetic TLR9 agonists reduced airway eosinophilia, IL-4, IL-5 and IgE production, and enhanced IL-10 levels in bronchoalveolar lavage fluid of murine models of asthma [102, 109, 111]. Furthermore, they inhibited airway remodeling by reducing airway collagen deposition, metalloproteinase activity and angiogenesis [112–115]. CpG-ODNs enhance the ability of the immune system to combat invading pathogens via increased expression of structures that are necessary for efficient antigen presentation [116]. They showed efficacy in reducing respiratory allergy in the context of sensitization to either pollen or fungal allergens [117] via either the subcutaneous [118] or mucosal [119] routes of administration. TLR9 ligands act as both prophylactic [109] and therapeutic [120] agents in new-onset or established [121] asthma/AR [122, 123] with long-lasting effects. Furthermore, CpG-ODN treatment was shown to be efficacious in this regard in both the aged murine models of AHR and the neonates [119] of a maternal transmitted model of asthma. However, mice with pre-existing severe allergic airway inflammation did not benefit from CpG-ODN administration as neither the Th2 immune response nor AHR were reduced following treatment [124]. Similarly, children with severe asthma showed a reduced response to TLR9 agonists as compared with mild asthmatics or healthy controls in terms of IL-12 or IFN production [125].

Allergen-TLR9-L ISS Conjugate

Therapeutic administration of allergen-TLR9-L ISS conjugate (AIC; Dynavax, phase II clinical trial) in sensitized mice to ragweed led to inhibition of airway inflammation [115]. Results with AIC also look promising in the clinic (fig. 2b) [126–129]. AIC administration resulted in a reduction of Th2 responses and an increase in Th1 and T-reg cytokines [128]. Along with an increased Th1/Th2 ratio, AIC led to an antibody switching from IgE dominant to neutralizing IgG production [129]. It is of note that after AIC treatment a transient increase in IgE production is possible [127]. Despite the promising results with AIC in the clinic, AIC failed to reduce nasal symptoms of AR patients in initial clinical trials [129] and there

Table 1. TLR agonists/antagonists which may be beneficial for AHR

Targeted TLR	Compound	Level of evidence	TH2/TH1 response	DC activation	Bronchodilator of ASMCs or reversing remodeling
TLR2 complexes	MALP-2 [140]	level 5	↓	yes	no
TLR2 complexes	BPPcysMPEG [56, 58, 59]	level 5	↓	yes	no
TLR2 complexes and TL4	Lipoprotein1 (Opr1) [60]	level 5	↓	yes	no
TLR2 complexes	Lipopeptide-CGP40774 (LP40)	level 5	↓	yes	yes
TLR4	MPL [62, 65, 66]	level 2	↓	yes	no
TLR4	CRX675 [140]	level 2	↓	yes	yes
TLR4	γ-PGA [70]	level 5	↓	yes	no
TLR4	ER803022 [71]	level 5	↓	yes	no
TLR4	UT12 [72]	level 5	↓	no	no
TLR4	E5564 [73]	level 5	↓	no	no
TLR3	ssDNA-ODN [91]	level 5	no	no	no
TLR3	Resveratrol [24]	level 5	no	no	no
TLR7/8	Imiquimod [92]	level 5	↓	yes	yes
TLR7	Resiquimod [96]	level 5	↓	yes	yes
TLR7	SA2 [105]	level 5	↓	no	no
TLR7	PolyUs [92]	level 5	↓	no	yes
TLR7	AZD8848 [106]	level 2	↓	no	no
TLR8	VTX-1463 [108]	level 2	↓	no	no
TLR9	1018ISS [126–129]	level 2	↓	yes	yes
TLR9	QbG10 [132]	level 2	↓	yes	yes

The highest level of evidence for efficacy of each compound for AHR is presented.

remains controversy regarding the onset and duration of its efficacy [127, 129]. Furthermore, it failed to reduce asthma symptoms in treated children [130]. No significant local or systemic side effects related to AIC have so far been reported (fig. 3) [126–129]. Finally, administration of 1018 ISS revealed no significant improvement in the symptoms of asthmatics as a stand-alone therapy [131].

QbG10

QbG10, an A-type CpG-ODN (phase II clinical trial), has been tested in HDM-sensitized AR patients as either monotherapy [132] or in an allergy vaccine formulation [133]. It manifests the ability to reduce symptoms, concomitant medication needs and increase the quality of life of AR patients [132]. Assessment of QbG10 in mild-to-moderate and moderate-to-severe asthma is now subject to ongoing clinical trials (NCT00890734 and NCT01673672, respectively). The side effects reported to be due to QbG10 administration include erythema, lymphadenopathy, influenza-like symptoms, pyrexia, fatigue and headache [132]. Recently, several new TLR9 agonists have been introduced for the treatment of patients with AR/asthma, including AZD1419 (Dynavax),

AEV0675 (Coley Pharmaceuticals), SAR21609 (Coley Pharmaceuticals), and IMO2134 (Idera Pharmaceuticals) [134]. However, to our knowledge there is no publication regarding their efficacy or safety in AHR patients.

Conclusions and Future Directions

Since almost all the TLRs are positioned to play a sentinel role in the development of respiratory allergy, they provide major therapeutic targets to modulate the natural course of allergic disease. So far, agonists rather than antagonists of TLRs have been widely employed in therapeutic or prophylactic preparations useful for asthma/AR patients. MPL (a TLR4 agonist) and 1018 ISS (a TLR9 agonist) show strong immunogenicity effects that make them appropriate adjuvants for allergy vaccines (fig. 2). Although patients should be informed regarding possible side effects, it seems from experience to date that these are mostly local and transient (fig. 3). TLR4 and TLR9 agonists in the shape of allergy vaccines are the most promising compounds in this family to be used in the clinic. Intranasal administration of AZD8848 (TLR7 agonist) and VTX-1463 (TLR8 agonist) have revealed efficacy in the

Table 2. Clinical efficacy of targeting TLRs

Targeting	Study	Design	Level of evidence	Compound	Dose of compound	Route	Participants (active:placebo)	Age, years	Symptom score (active vs. placebo)	Medication score (active vs. placebo)
TLR4 agonist	DuBuske et al. [135] 2011	Single-center RCTDB	Level 2	Pollinex Quattro	300–2,000 SU/ml ×4 ultra-short course	SCIT	AR patients sensitized to grass pollens, 514:514	NA	Significantly reduced	Significantly reduced
TLR4 agonist	Pfaar et al. [136] 2011	Single-center RCTDB	Level 2	Pollinex Quattro rPhl p + MPL	9.45 or 19.04 µg Phl p + 21 or 52.5 µg MPL/day for 8 weeks	SLIT	AR patients sensitized to grass pollen, 64:16	35.9 (18–64)	NA (combined symptom and medication score was reduced)	NA (combined symptom and medication score was reduced)
TLR4 agonist	Musarra et al. [64] 2010	Single-center open clinical trial	Level 2	Pollinex Quattro	300–2,000 SU/ml ×4 ultra-short course	SCIT	AR/asthma-sensitized patients to grass pollen, 29:28	33.7 (10–59)	Significantly reduced as assessed by VAS	NA
TLR4 agonist	Rosewich et al. [66] 2010	Post-marketing multi-center open trial (cohort)	Level 3	Pollinex Quattro	300–2,000 SU/ml ×4 ultra-short course	SCIT	AR/asthma patients sensitized to grass pollen, 34 active	10.2 (6–18)	Significantly reduced	Significantly reduced
TLR4 agonist	Rosewich et al. [62] 2010	Post-marketing multi-center open trial (cohort)	Level 3	Pollinex Quattro	300–2,000 SU/ml ×4 ultra-short course	SCIT	AR/asthma patients sensitized to grass pollen, 421 active	13.2 (6–18)	Significantly reduced	Significantly reduced
TLR4 agonist	Casale et al. [69] 2006	Single-center RCTDB	Level 2	CRX-675	2–20–100–200 µg/patient	Intranasal	AR patients sensitized to ragweed, 48:16	31.5 (19–50)	Improved only with 100 µg/safety assessment	NA
TLR4 agonist	Von Baehr et al. [65] 2005	Single-center RCT	Level 2	Pollinex Quattro	300–2,000 SU/ml ×4 ultra-short course	SCIT	AR/asthma patients sensitized to grass/tree pollens, 21:14	31.4 (18–61)	NA	NA
TLR4 agonist	Drachenberg et al. [137] 2003	Multi-center open trial	Level 2	Pollinex Quattro	300–2,000 SU/ml ×4 ultra-short course	SCIT	AR/asthma patients sensitized to grass pollen, 90 (all active)	12.5 (6–17)	Significantly reduced	Significantly reduced
TLR4 agonist	Mothes et al. [138] 2003	Single-center RCTDB	Level 2	Pollinex Quattro	300–2,000 SU/ml ×4 ultra-short course	SCIT	AR patients sensitized to pollens, 11:9	NA	Significantly reduced	Significantly reduced
TLR4 agonist	Drachenberg et al. [139] 2001	Multi-center RCTDB	Level 2	Pollinex Quattro	300–2,000 SU/ml ×4 ultra-short course	SCIT	AR/asthma patients sensitized to grass pollen, 90 (active), 81:60	28.2 (18–60)	Significantly reduced	NS
TLR7 agonist	Greiff et al. [106] 2012	Single-center RCTDB NCT00770003	Level 2	AZD8848	60 µg/week ×5	Intranasal	Grass pollen-sensitized AR patients, 34:34	27 (18–46)	Significantly reduced	Significantly reduced

Table 2. (continued)

Targeting	Study	Design	Level of evidence	Compound	Dose of compound	Route	Participants (active:placebo)	Age, years	Symptom score (active vs. placebo)	Medication score (active vs. placebo)
TLR8 agonist	Horak et al. [107] 2011 (abstract)	Single-center RCTDB	Level 2	VTX-1463	0.25, 0.50, 0.75 and 1.0, or 62.5 µg/week ×4	Intranasal	Grass pollen-sensitized AR patients, 80:NA	NA	Significantly reduced	Significantly reduced
TLR9 agonist	Klimek et al. [132] 2011	Multi-center RCTDB	Level 2	CYT003-QbG10	0.5 or 1 mg/week ×6	SCIT	HDM-sensitized AR patients, 99:35	31.2 (18–64)	Significantly reduced	Significantly reduced
TLR9 agonist	Senti et al. [133] 2009	Single-center open-label	Level 3	QbG10	300 µg/week ×6	SCIT	HDM-sensitized AR patients, 20:0	34.0 (18–56)	Significantly reduced	Significantly reduced
TLR9 agonist	Creticos et al. [127] 2006	Single-center RCTDB	Level 2	Amb a1-1018 ISS (AIC)	0.06–12 µg/week ×6	SCIT	Ragweed-sensitized AR patients, 14:11	39.4 (23–60)	Significantly reduced	Significantly reduced
TLR9 agonist	Nayak et al. [130] 2006 (abstract)	Single-center RCT	Level 2	Amb a1-1018 ISS (AIC)	0.06–12 µg/week ×6	SCIT	Ragweed-sensitized asthmatic patients, 18:6	NA (6–17)	NS	NS
TLR9 agonist	Gauvreau et al. [131] 2006	Single-center RCTDB	Level 2	1018 ISS	36 mg/week ×4	Inhalation	Atopic asthmatics, 21:19	24.8 (18–55)	NS	NS
TLR9 agonist	Tulic et al. [129] 2004	Single-center RCTSB	Level 2	Amb a1-1018 ISS (AIC)	0.06–12 µg/week ×6	SCIT	Ragweed-sensitized AR patients, 28:29	39.9 (27–55)	Significantly reduced (second year)	Significantly reduced

The level of evidence provided by each study has been determined according to OCEBM 2011 Levels of Evidence with respect to study design. Any significant ($p < 0.05$) improvement in allergic airway symptoms compared with baseline or placebo was considered an improvement. Mothes et al. [138] and Nayak et al. [130] did not mention the exact age of participants in the full text of their articles. The full text of the study of DuBuske et al. [135] was not available and no response was received regarding the missing data from

the corresponding author. NS = Not significant (active vs. placebo), symptom and medication scores were usually recorded using diary cards; RCTDB = randomized double-blind clinical trial; RCTSB = randomized single-blind clinical trial; RCT = randomized clinical trial without blindness; Phl p = *Phleum pratense*; NA = data was not available/applicable; VAS = visual analogue scale.

relief of symptoms in AR patients. Many other drugs that target TLRs were found to suppress airway inflammation, eosinophilia and AHR at least in animal model systems. Indeed, most of our knowledge regarding TLR agonists/antagonists rose from experimental studies (table 1). The future for a wide variability of PRR agonists and antagonists in the manipulation of the allergic disease pathways holds considerable promise.

However, many questions remain to be answered. There is poor evidence regarding the usefulness of targeting TLRs in allergic children since most clinical trials so far have been conducted on adult participants (table 2); specifically, how much targeting of the immature immune system of a child is safe and efficacious? The answer is of utmost importance since the prevalence of atopic asthma is higher in children and in these subjects

the risk of persistence of asthma into adult life is greater than in non-atopic patients. Furthermore, despite the promising experiments with ISS in dampening established AHR and airway remodeling, SIT is usually employed in mild-to-moderate asthma; therefore, it remains to be established if targeting of TLRs could be successfully applied to severe asthma. Finally, timing, dosage, patient selection and many other questions regarding the best formulation to be utilized in targeting the TLRs need to be answered since inappropriate administration may precipitate exaggerated immune responses.

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